THE FORMS OF VITAMIN B_{12} IN FOODSTUFFS AND THE OCCURRENCE OF ARTEFACTUAL SULPHITOCOBALAMIN.

BY

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SUMMARY.

Extraction yields of cobalamins were examined with tissues containing both incorporated and added radioactive vitamin B_{12} (^{57}Co.vit.B_{12}) and pronase used to increase the yields from ca. 65 - 75% by its proteolysis of the vitamin B_{12} binders. Pronase itself was however found to be a source of vitamin B_{12} and thus its use was discontinued.

The extracted cobalamins were separated by silica gel thin-layer chromatography and the chromatograms developed by the standard bioautography assay, using an Escherichia coli mutant, which was vitamin B_{12} dependent for growth.

An unknown cobalamin which was encountered during these assays was examined by ion-exchange chromatography and light-sensitivity and identified as artefactual sulphito B_{12}, produced from hydroxo B_{12}.

To prevent this conversion pH control of the extraction using a bicarbonate buffer was attempted but met with little success, because of the ease of formation of sulphito B_{12} and the inability to maintain the buffered conditions throughout the extraction.

Pre-conversion of the hydroxo B_{12} to ammonia B_{12} with an ammonia buffer system was successful in

eliminating the artefact, and moreover produced a cobalamin which was indistinguishable from hydroxo B_{12} on chromatography in ammonia containing solvent systems.

The light sensitivities of the 'naturally' occurring cobalamins were estimated under controlled light exposure conditions to establish the most suitable method for preparation of tissues and isolation of cobalamins.

The forms of vitamin B_{12} in items of diet were found by the chromatographic and bioautographic analysis of tissue extracts and a degree of quantitation of results introduced by examination of a series of extract diluents. Distinct variations were evident in the forms of vitamin B_{12} isolated in the three categories of foodstuffs examined, namely dairy produce, meat & poultry (muscle tissue) and fish.

INTRODUCTION.

The purpose of this introduction is not to present a lengthy, detailed and critical review of some of the thousands of papers on vitamin B_{12} which have been published in the last twenty seven years, but rather to outline briefly those aspects of the developing knowledge of vitamin B_{12}, which are relevant to the subject matter of this thesis. For this reason a greater dependency has been placed on monographs and review articles rather than original papers dealing with a relatively restricted aspect, unless this has been considered desirable.

The 'history' of vitamin B_{12} began in 1948 when the first reports of its isolation in pure crystalline form were published almost simultaneously by two groups of industrial chemists, one working in the United States (Rickes et al., 1948) and the other in the United Kingdom (Smith & Parker, 1948). The train of events which led to the discovery however, goes back to the clinical descriptions of disease states by Combe in 1824, Addison in 1855, and by Biermer in 1872 (Chanarin, 1969) and although these reports preceded the descriptions by Ehrlich in 1880 (Chanarin, 1969) of the characteristic haematological feature of megaloblastosis, they are now regarded as the earliest descriptions of pernicious anaemia.

Pernicious anaemia remained an invariably fatal disease until 1926, when Minot & Murphy showed that ingestion of about half a pound of raw or lightly cooked liver each day was followed by a rapid and sustained improvement in health with restoration of peripheral blood values to normal. From then, the hunt was on for the factor, or factors, in liver which were responsible for this therapeutic effect.

Some advances were made with methods of extraction and concentration of the active substance from liver, and these had a practical benefit in the form of liver extracts which were effective in the treatment of pernicious anaemia when given parenterally, but progress was slow for several reasons. The very low concentration of the factor in tissues, the lack of knowledge of any chemical characteristics that could be exploited in extraction procedures, the relatively crude methods then available for extraction and more importantly for isolation of the factor, and the total dependency on cases of pernicious anaemia in relapse for estimation of therapeutic effect of any extract were all reasons which delayed the isolation of the factor until 1948, when it was christened vitamin B_{12} by the American group.

Knowledge of the chemistry of the newly isolated vitamin was built up rapidly and the structure elucidated using X-ray crystallography by Hodgkin (1957). The accepted formula became, $C_{63}H_{88}O_{14}N_{14}$

P Co. xH_2O, the material being water soluble (ca. 1.2%, w/v) and crystallising as dark red needles or prisms. The molecular weight of the non-hydrated vitamin was 1355, but this varied depending on the degree of hydration of the crystals (Fantes et al.,1949). The molecule could be divided into two major portions, a planar group, corrin ring, and a nucleotide, the former containing a central cobalt atom linked to four reduced pyrrole rings forming a macro ring and the latter being 5,6-dimethylbenzimidazole (Fig.1). The central cobalt atom in an octahedral environment, was coordinately linked to one of the nitrogen atoms of the benzimidazole and carried a cyanide group on the axial ligand, position six.

At the very early stage in the isolation of vitamin B_{12} it was appreciated that there were a number of compounds which had chromatographic features closely related to those of vitamin B_{12} itself and the nature of these compounds and others which resulted from analytical procedures such as, hydrolysis, oxidation, reduction and halogenation of vitamin B_{12} were studied in detail. It became clear that the relationship between many of the compounds depended in large measure on the nature of the ligand occupying the sixth position on the cobalt atom, and a system of nomenclature was based on these observations, the term cobalamin being introduced for the vitamin B_{12} molecule devoid of the cyano group and

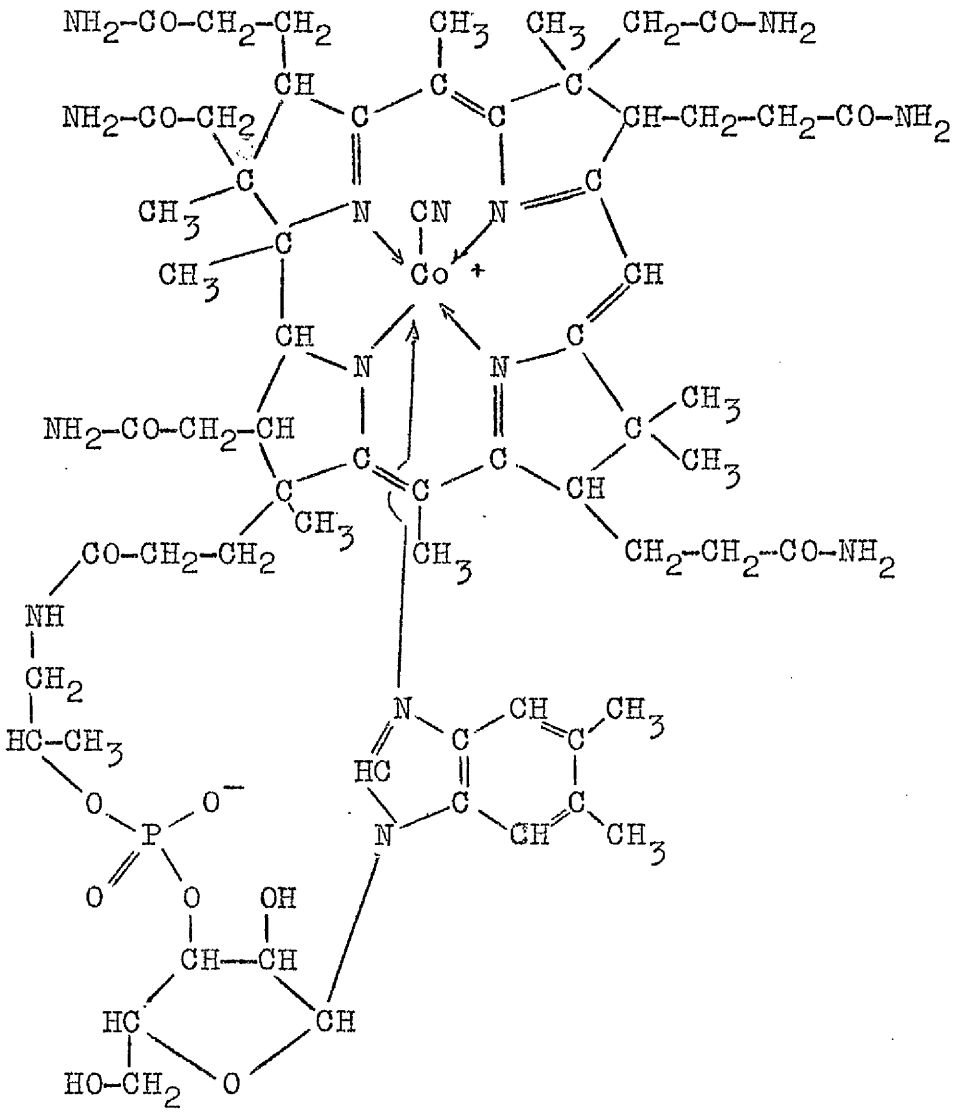


Fig. 1 The Structure of Vitamin B_{12}.

prefixed by the nature of the ligand occupying the sixth position, when it was known; thus vitamin B_{12} also became known by the systematic name of cyanocobalamin.

In addition to the cobalamins a large number of analogues of vitamin B_{12} were synthesised, these differing in a more fundamental way from vitamin B_{12} than the cobalamins, usually in regard to the nucleotide base. Some of the cobalamins and analogues which had been synthesised in the laboratory were found to have the same biological activity as cyanocobalamin in systems using organisms such as E.coli, L.leichmanii (Fantes & O'Callaghan, 1956), E.gracilis (Ford et al., 1953) and Ochromonas (Blumberger et al. 1957) and some had biological activity apparently equal to that of cyanocobalamin when given to patients in relapse.

These findings suggested that pernicious anaemia could not be regarded as a consequence of cyanocobalamin deficiency unless the body possessed some mechanism for the conversion of the biologically active cobalamins and analogues to cyanocobalamin and for some time the significance of the various cobalamins and analogues in relation to human metabolism remained in doubt, the problem being compounded by the discovery of a large number of analogues resulting from guided biosynthesis and also occurring naturally in materials as diverse as sewage sludge and the fore stomach of ruminants.

The problem remained unsolved largely because the small amounts of vitamin B_{12} in human tissues and the difficulty in extracting, isolating and identifying the native cobalamins, until 1958 when a major development came from an unexpected source. In that year, Barker and his colleagues reported the isolation of a coenzyme form of pseudovitamin B_{12} (αadenylcobamide), which was specific for the isomerisation of glutamate to βmethyl-aspartate in Clostridium tetanomorphum. Further studies led to the isolation of the coenzyme form of vitamin B_{12} from propionic acid bacteria (Barker et al., 1960.), a cobalamin which was readily photolysed to hydroxo B_{12}.

Subsequent work showed that the ligand occupying the sixth position on the cobalt atom of coenzyme B_{12} was an organic moiety, adenosine minus the 5' hydroxo group and the cobalamin then came to be known as 5' deoxyadenosyl-cobalamin. Although the significance of light in the interconversion of cobalamins in vitro had been appreciated by early workers, the extreme importance of light in the isolation and identification of cobalamins from biological material had not been fully appreciated and in retrospect it seemed possible that the cyano-cobalamin isolated in 1948 from animal livers could have been an artefact in the sense that it was derived from coenzyme B_{12}, converted by light and exposure to cyanide during the extraction process. This possibility was examined by Barker and his group, and showed that with

appropriate precautions against photolysis and exposure to cyanide the coenzyme could be detected in significant amounts in the livers of animals and of man.

The next stage in the knowledge of the forms of vitamin B_{12} in human biology came in 1963 when Lindstrand, using precautions against photolysis identified cyanocobalamin, hydroxocobalamin and coenzyme B_{12} in human plasma and also an additional compound which he named the 'fourth factor' and which like coenzyme B_{12} was very light sensitive. Further studies showed that this compound had the same photosensitivity, chromatographic properties and ultra-violet absorption spectrum as methylcobalamin, which had not previously been isolated from human material but which had been produced by synthesis from hydroxocobalamin in vitro many years before by Smith and his colleagues.

A considerable amount of further work on the forms of cobalamins in human material has been reported in recent years by Linnell and colleagues, who have shown that cyanocobalamin exists only in trace amounts, if at all, in human tissues and that forms which can be extracted and identified are coenzyme B_{12}, hydroxo-cobalamin and methylcobalamin.

Long before the isolation of vitamin B_{12} but only a few years after the discovery of the value of liver therapy, Castle and his colleagues suggested that the development of pernicious anaemia was a consequence of

failure by the stomach to secrete a substance which he called intrinsic factor, which in normal circumstances combined or acted upon a substance in meat which he called extrinsic factor, to yield a haemopoietic factor which was essential for normal haemopoiesis. (Smith, 1965).

With the availability of radioactive vitamin B_{12}, and methods of measuring the absorption of radioactive vitamin B_{12}, came the opportunity to test this theory and it soon became clear that the function of intrinsic factor, although this substance had not been isolated in pure form, was to promote the absorption of small oral doses of vitamin B_{12} and that a characteristic feature of pernicious anaemia was a failure to absorb significant fractions of small oral doses of vitamin B_{12} unless these were given with a source of intrinsic factor either in the form of a suitable preparation of human gastric juice or gastric mucosa. For the most part, absorption studies of vitamin B_{12} were performed with radioactive cyanocobalamin, partly because this was the form in which vitamin B_{12} was originally isolated and partly because cyanocobalamin was the most chemically stable of the cobalamins and was therefore the most suitable for presentation for routine diagnostic purposes.

The results of absorption tests using other cobalamins had shown however that at the same dose level of crystalline compounds, the amount of cyanocobalamin which was absorbed could be significantly greater or significantly less than that of several other cobalamins

(Adams et al., 1971) and it followed from this that absorption studies with cyanocobalamin, while apparently perfectly adequate for routine clinical purposes, might give misleading results when considered in terms of physiology. This consideration was subject to provisos regarding comparison between the absorption of cobalamins in crystalline form usually given on a fasting stomach and the absorption of food bound cobalamins taken in circumstances where some stimulation of gastric secretion would be natural. Regard must be taken of possible changes in the structure of cobalamin molecules during absorption from the ileal lumen. There is considerable delay between the presentation of the intrinsic factor-vitamin B_{12} complex to the ileal absorbing site and the appearance of vitamin B_{12} in peripheral blood, suggesting that there may be some metabolic process before absorption possibly involving removal of the ligand occupying the sixth position on the cobalt atom and substitution with another ligand. (Linnell et al., 1971)

Consideration of the physiology of nutrition with vitamin B_{12} required knowledge of the form of vitamin B_{12} in foods and it was surprising to find that as late as 1972, when so much work had been done on the metabolism of vitamin B_{12} in man, that practically nothing had been reported about the forms of vitamin B_{12} in foods and relatively little even about the mass of vitamin B_{12} in

foods. The former subject was therefore chosen for study.

Methods for the identification of the forms of vitamin B_{12} in human tissues had been described by several authors and it seemed likely that with some relatively minor modifications these methods could be used to study the forms of vitamin B_{12} in foodstuffs. At an early stage of this project however, it became evident that the extraction and identification of forms of vitamin B_{12} in foods was not as simple a matter as might have been hoped for.

The material in this thesis therefore contains as much on the technology of the extraction and identification of forms of vitamin B_{12} as it does on the forms of vitamin B_{12} in foods.

TERMINOLOGY.

Mention has already been made of terminology. Formal systems of nomenclature for vitamin B_{12} and its host of derivatives and analogues have existed since 1957 when the Commission on the Nomenclature of Organic Chemistry of the International Union of Pure and Applied Chemistry adopted the so called 'Hamburg' system and revisions have since been published, the most recent in 1974. The systems have been based largely on consideration of the overall cobalamin structure as being composed of a

corrin ring system, plus ribose and phosphate termed cobamide coupled to an organic benzimidazole moiety. Such methods of terminology inevitably lead to the construction of long and unwieldy systematic names in order to uniquely define each cobalamin. For example, the compound which is commonly referred to as cyano-cobalamin is formally named,

α-(5,6-dimethylbenzimidazolyl) cobamide cyanide.

The use of a precise terminology such as the I.U.P.A.C. forms would clearly be unsuitable in the general narrative text of this thesis because of their constant re-iteration and therefore they were avoided on the grounds of ease of reading and economy of space. Retention of the suffix -cobalamin to describe the whole molecule except the ligand attached to the cobalt atom in the sixth position in the octahedral co-ordination and hence produce abbreviated versions such as cyano-cobalamin and hydroxocobalamin would also lead to extended and cumbersome sentences. In common with other workers, the author has therefore adopted a wholly unofficial terminology for bench work and it has seemed justifiable for reasons given above to adopt this terminology (Table 1) in the text. Permissive names such as cyanocobalamin, hydroxocobalamin, aquocobalamin, adenosylcobalamin (coenzyme vitamin B_{12}), methylcobalamin and sulphitocobalamin have been abbreviated to cyano B_{12}, hydroxo B_{12}, aquo B_{12}, coenzyme B_{12}, methyl B_{12} and

SYSTEMATIC NAME.	SEMI-SYSTEMATIC NAME.	ADOPTED ABBREVIATED NAME.
α-(5:6-dimethylbenzimidazolyl)		
-aquocobamide chloride (etc.)	Aquocobalamin	$AquoB_{12}$
-hydroxocobamide	Hydroxocobalamin	$HydroxoB_{12}$
-amminecobamide chloride	Ammoniacobalamin	$AmmoniaB_{12}$
-cobamide cyanide	Cyanocobalamin	$CyanoB_{12}$
-5'-deoxyadenosylcobamide	5'-deoxyadenosyl-cobalamin	$CoenzymeB_{12}$
-cobamide sulphite	Sulphitocobalamin	$SulphitoB_{12}$
-methylcobamide	Methylcobalamin	$MethylB_{12}$

Table 1 Systematic and Adopted Terminology of Cobalamin Nomenclature.

sulphito B_{12} respectively.

The term cobalamin has been retained as a collective form when reference is made to a mixture of cobalamins, the individual components of which are either known or unknown. With diagrams the need is even greater for abbreviation, such that the permissive names were further reduced to CNB_{12}, OHB_{12}, H_2OB_{12}, MeB_{12} and SO_3B_{12} and the term $AdoB_{12}$ used for coenzyme B_{12} instead of CoB_{12} to avoid any confusion which may arise between this form and $^{57}CoB_{12}$, the abbreviation used to describe the radioactive vitamin.

In certain circumstances which will be detailed later in this thesis, techniques were employed which converted aquocobalamin to hydroxocobalamin and ammoniacobalamin so that differentiation between these compounds was necessarily imprecise. In these circumstances compounds are all termed 'hydroxocobalamin' unless the identity of ammoniacobalamin is specifically required to enhance the interpretation of results. Similarly the distinction between aquocobalamin and hydroxocobalamin, the proportions of which are dependent on their acid/base equilibrium are generally discounted and the term hydroxocobalamin used for both. Both cobalamins exhibit identical behaviour on partition chromatography but if overall molecular charge becomes significant in respect of protonation of the hydroxocobalamin, eg. in ion-exchange chromatography, then reference is made to aquocobalamin.

CHAPTER 1

EXPERIMENTAL PROCEDURE.

Preparation of Tissues: & Extraction.

Tables of estimates of cobalamin mass in foods as obtained by microbiological assays were consulted in order to give some guide to the quantity of tissue required to yield a final extract which would contain an adequate concentration of cobalamins for identification. An appropriate mass of tissue devoid of fat and inedible material was weighed and removed to a darkroom, illuminated by two Ilford 'Safelights', fitted with 15 watt Osram bulbs and a dark brown glass filter, F904, and all further work carried out in the darkroom.

The tissue was cut into small pieces and added to ten times its weight of distilled water. The material was homogenised for two minutes in an M.S.E. Ato-mixer at half speed setting and the contents with washings homogenised for two minutes using a Silverson mixer emulsifier (No.15045) with an axial flow head at position five on the speed control. The homogenate was further homogenised for two minutes with a microhead attachment, at the end of which time the tissue was present as a very fine precipitate. The homogenate was then divided into aliquots, each containing a suitable quantity of tissue for analysis of cobalamin content. The samples were stored at -20°C in 100 ml. polystyrene screw-capped bottles wrapped in aluminium foil.

After thawing the homogenate was rehomogenised using the Silverson machine and microhead for two minutes in view of evidence (Adams et al 1972) that the availability of

cobalamins for Euglena gracilis at least could be reduced by repeated freezing and thawing.

Extraction and Concentration of Cobalamins.

The thawed rehomogenised sample was poured into a three-neck flask (250 ml.) set up in an 'Electrothermal' heating mantle with energy regulator and fitted with an ether condenser, thermometer and mechanical stirrer. 100 mls. of ethanol (B.D.H. 'AnalaR' grade) were added to the flask and the contents refluxed with constant stirring at ca. 82°C for thirty minutes to precipitate the protein present. The solution was cooled by immersion in a water bath (20°C) and the protein allowed to settle. The supernatant was filtered through Whatman 54 filter paper under reduced pressure of 1.3×10^4 N/m^2, (100 mm.Hg) and the protein precipitate washed with ethanol, dried, weighed when required and discarded. The filtrate was rotary evaporated to ca. 20 mls. on a 'Wright' thin film rotary evaporator at 2.7×10^3 N/m^2 pressure (20 mm.Hg), the temperature of the water bath being raised gradually from 20°C to 40°C to give a controlled evaporation without bumping and resultant loss of material in the distillate.

The aqueous solution was run into an equal volume of 'AnalaR' phenol:chloroform (1:1, w/v) acidified with one drop of dilute hydrochloric acid (10^{-3} mol./l.) and shaken fairly vigorously for several minutes to extract the

cobalamins into the phenol phase while leaving behind other water soluble material. Occasionally at this stage the two phases coalesced to form an emulsion which took several hours to separate under gravity and in these circumstances the emulsion was centrifuged at 3000 r.p.m. for 15 minutes in foiled centrifuge tubes to regenerate the initial aqueous and phenol layers. The upper milky-white aqueous layer was removed, added to a further volume of phenol/chloroform and shaken as before. The lower phenol layer was retained and pooled with the phenol phase obtained after the separation of the second extraction emulsion. The phenol solution was then shaken with a one-fifth volume of distilled water in a separating funnel to complete the removal of water soluble salts.

After separation the upper aqueous layer was discarded and the lower phenol phase run into another separating funnel (250 mls.) containing an organic phase of two volumes of diethyl ether and a half volume of acetone, together with one volume of distilled water. The separating funnel was shaken vigorously for ten minutes with constant valve opening to release the volatile solvents. After separation by gravity, the upper organic phase (ether/acetone/phenol/chloroform) was discarded and the lower aqueous phase shaken with a half volume of diethyl ether to remove any dissolved phenol from the distilled water.

The cobalamins present had now been back-extracted into the aqueous phase after breakdown of the phenol solvation by the diethyl ether. The aqueous phase was rotary evaporated to a suitable volume, ca. 1 ml., in a 250 ml. round bottom flask at a temperature of 40°C and pressure of $2.7 \times 10^{3} N/m^{2}$ (20 mm.Hg.). If a more concentrated extract was required the solution was pipetted into a 10 ml. round bottom flask, in which, with care the volume of the solution could be reduced to a limit of 40 µls. without loss of product on the flask surface.

The concentrate was removed by pipette (0.1 ml.) from the flask and stored at -20°C in foiled polystyrene tubes. With good yields, that is of more than 60% and the multiple concentrating stages in the extraction procedures, the mass of cobalamins present in the final extract was usually in the region of 5 - 100 ngms. of vitamin B_{12}.

SECTION 2

Separation of Extracted Cobalamins, & Identification.

The separation of the cobalamins in the extract was effected by thin-layer chromatography using Eastman 'Chromagram' silica gel sheets 6061 (20 x 20 cms.), each composed of a silica layer 0.25 mm. thick, bound to a polyvinyl base. All sheets were stored in a desiccating cabinet to prevent the surface adsorption of moisture.

2 - 6 µls. of the extract were applied, using an E-mil micropipette (2 µls.), at a distance of 30 mms. from the base of a silica sheet and a 'Camag' spotting guide used to ensure that multiple samples were applied equidistantly from each other and separated sufficiently to prevent overlap of growth zones on bioautography. In addition to the sample under investigation, standards of cyano B_{12}, coenzyme B_{12}, hydroxo B_{12} and methyl B_{12}, prepared from B.D.H. crystalline cobalamin material and purified by passage through CM cellulose and DEAE cellulose ion exchangers, were also applied and the chromatogram dried in an airstream provided by a Pifco electric fan. Heat was avoided in the drying process in case of losses of the pico quantities of cobalamins applied.

After drying the chromatogram was suspended in a 20 cm. 'Camag' chromatographic developing tank, containing butan-2-ol, N.propanol, water and ammonia (7:4:3:1, v/v) and the chromatogram allowed to run for 6½ - 7 hours, by

which time the solvent had travelled the length of the sheet. On removal from the tank the solvent front was marked and the chromatogram again air dried. Thereafter all procedures were performed in the daylight.

Identification of the separated Cobalamins by Bioautography.

The first stage in the bioautographic visualisation of the cobalamins separated by t.l.c., involved the preparation of plates and culture of the growth organism, a vitamin B_{12} dependent E.coli mutant NCIB-9270, a running culture of which was maintained on blood agar plates in the Bacteriology Department. The organism was sub-cultured in 'Serva' lactalbumin, peptone water (1%, w/v.) and incubated at 37°C for six to seven hours prior to use as the inoculum.

3 litres of salt growth medium containing:-

KH_2PO_4	28.0 gms.
K_2HPO_4	12.0 gms.
$(NH_4)_2SO_4$	4.0 gms.
Sodium citrate	2.0 gms.
$MgSO_4.7H_2O$	0.4 gms.
NaCl	0.2 gms.

were prepared in sterile distilled water and adjusted to pH 7, if necessary, with dilute sodium hydroxide (10^{-3}mol./l.) or hydrochloric acid (10^{-3}mol./l.).

300 ml. aliquots of the medium were added to 500 ml. saline infusion bottles, each containing three grams of 'Davis' Japanese agar. The bottles were stoppered, capped and sterilised in a hot air oven at 140°C for 20 minutes. On removal, while cooling, they were inverted several times until the contents gelled and stored at room temperature (20°C) until required.

The plates for bioautography, obtained from the Bacteriology Dep't., were composed of a glass base 22 cm. square and surrounding metal framework 1 cm.high. The plates were washed with detergent and tap water and after drying the glass surface was wiped with ethanol and flamed with a Bunsen burner. Accurate positioning of the plate before pouring the medium was achieved by use of levelling screws and spirit levels, to ensure that the plate was on a horizontal plane.

A bottle of prepared medium was melted in the hot air oven at 100°C, and the stopper pierced by a syringe needle to allow degassing of the gel. The melted agar was poured into a clean 600 ml. beaker, contained in an evaporating basin of cold water to hasten the cooling, and stirred. As the temperature of the agar fell, 0.9 gms. of glucose and 0.045 gms. of 2,3,5 triphenyl-tetrazolium chloride were added, the latter to produce red zones at the sites of E.coli growth on the bioautogram and thereby aid their visualisation (Ford &

Holdsworth, 1953). At 43°C the inoculum was added and the seeded agar poured onto the bioautography plate.

When the prepared plate had cooled the chromatogram was carefully laid onto the gel so that the silica surface of the chromatogram and the agar surface came into close contact without intervention of air bubbles. The agar gel and chromatogram were then carefully turned out of the plate by inversion onto the metal lid and incubated agar side uppermost at 37 - 39°C for 16 to 18 hours in an incubator. After removal the plates were stored at 4°C to minimise further growth before a detailed record of growth zones was made.

SECTION 3

Semi-Quantitation of Results by Stepwise Dilution.

Two methods have been described for quantitation of the cobalamins separated by t.l.c. and identified by bioautography. One method utilises a scoring system for the diameter of each growth zone based on visual comparison of growth areas and the other the use of a scanning densitometer. (Linnell et al., 1969 & 1971)

In the absence of a scanning densitometer the former method was used in the work reported here with a modification aimed at improving the system. This modification involved examination of each sample in its original extracted form and also at various dilutions, these being prepared with distilled water. The cobalamin which could be identified on bioautography in neat extract state and also in dilutions of 1:2, 1:4, 1:8, 1:16 and 1:32 would plainly be one which quantitatively was greater than one which appeared in neat and 1:2 dilutions only.

In addition, where possible each neat extract was diluted and subsequently assayed using L.leichmanii to give total cobalamin content. From this value and from the presence or absence of growth in the extract and dilutions of the unknown it was felt that the ratios of individual cobalamins present could be found with rather greater accuracy than by visual scoring alone.

To affirm the validity of the previous quantitation method the growth responses of equal concentrations of individual cobalamins and as a consequence their limit of detection on bioautography had to be shown to be identical.

MATERIALS & METHODS.

Crystalline coenzyme B_{12}, cyano B_{12}, hydroxo B_{12} and methyl B_{12} were obtained from B.D.H. and examined for purity by passage, where appropriate, through CM cellulose and DEAE cellulose. Ammonia B_{12} was prepared by the addition of hydroxo B_{12} (5 mgms.) to an ammonia solution (0.1 mol./l.). Solutions of ammonia B_{12}, coenzyme B_{12}, cyano B_{12}, hydroxo B_{12} and methyl B_{12} were prepared at a conc. of 7.5×10^{-8} mol./l. in distilled water and reduced to 3.0×10^{-10} mol./l. by a series of stepwise 1:1 dilutions. 2µls. of each dilution were applied to a silica gel chromatogram and after drying developed by bioautography. The growth areas were scored by the method of Linnell (1969) and the results recorded (Table 2).

DISCUSSION.

Trace impurity in the coenzyme B_{12} and hydroxo B_{12} was demonstrated by the retention of a small amount of material in a thin red band at the top of the DEAE cellulose column in each case. No significant retention of material occurred on either column however, and the

standards were therefore prepared without purification.

COBALAMIN.	Applied CONCENTRATION(X 10^{10} mol./l.).								
	750	375	188	94	47	23.5	11.7	5.8	2.9
NH_3B_{12} :	+++	+++	+++	+++	++	++	+	+	
pgs.B_{12}:	202	101	50.5	25.3	12.6	6.3	3.2	1.6	0.8
$AdoB_{12}$:	+++	+++	+++	++	++	++	+	+	
pgs.B_{12}:	236.8	118.4	59.2	29.6	14.8	7.4	3.7	1.9	0.9
CNB_{12} :	+++	+++	+++	+++	++	++	++	+	+
pgs.B_{12}:	203.4	101.7	50.9	25.4	12.7	6.4	3.2	1.6	0.8
OHB_{12} :	+++	+++	+++	++	++	++	+	+	
pgs.B_{12}:	202	101	50.5	25.3	12.6	6.3	3.2	1.6	0.8
MeB_{12} :	+++	+++	+++	+++	++	++	+	+	
pgs.B_{12}:	201.6	100.8	50.4	25.2	12.6	6.3	3.2	1.6	0.8

Table 2 Growth Responses and Limits of Detection of the Standard Cobalamins on Bioautography.

The growth areas obtained indicated that the growth responses and therefore the concentrations at the visual detection limit for coenzyme B_{12}, ammonia B_{12}, hydroxo B_{12} and methyl B_{12} were equivalent. A trace growth response at the lowest dilution was found for cyano B_{12}, but this was not thought important since the occurrence of cyano B_{12} was likely to be negligible in tissue

extracts. Semi-quantitation by the dilution method was therefore a valid technique and was employed in the assay of tissue extracts.

Ammonia B_{12} was examined at this stage because, during chromatography all the hydroxo B_{12} present is converted to this form by the ammonia in the solvent system.

CHAPTER 2.

EXTRACTION YIELDS.

SECTION 1

Extraction Yields. - $^{57}Co.B_{12}$.

It was obviously desirable to have some estimate of the yield of vitamin B_{12} from extraction procedures, and even more informative would be estimates of the yields at each stage of an extraction. In extractions of vitamin B_{12} from tissues however, such estimates were not easy to obtain.

One method was the measurement of vitamin B_{12} in the original tissue homogenate and then at various stages in the procedure, due allowance being made for variables such as concentration.

Estimates of vitamin B_{12} obtained by microbiological assays however, were only accurate within ± 20% (Ross et al., 1956.) and a rather higher standard of accuracy was desirable. Such assays in any case might be adversely affected by the presence of phenol, a bacterial growth inhibitor, used in the extraction procedure. The addition of radioactive cyano B_{12} ($^{57}Co.B_{12}$) to the tissue and estimates of yields by radioactivity measurements at various stages had obvious advantages provided that the molecular structure of the vitamin remained intact during extraction. The drawback to this method however, was that measurements of radioactive vitamin B_{12} added to tissue homogenates would not necessarily reflect the situation in which vitamin B_{12} was actually incorporated intracellularly in the tissues and possibly changed chemically by the

binding involved and not merely bound to the tissue surface. For this reason the only tissues in which yields could be estimated with any accuracy would be those in which radioactive vitamin B_{12} had been incorporated naturally.

Fortunately human liver tissue became available from a patient who had been given 0.08µgms; 15µCi of ^{57}Co vitamin B_{12} for an investigational purpose irrelevant to this enquiry. 92days had elapsed between the administration of the dose and the death of the patient from natural causes and from the available evidence it seemed likely that the administered radioactive cyano B_{12} would then have become part of the body pool of vitamin B_{12} and undergone a normal chemical transformation. Investigations were therefore carried out on the yields at various stages in the extraction procedure using this tissue. Measurements of radioactivity were performed on an I.D.L. type 663 scintillation counter, with a sodium iodide thallium activated crystal and a photomultiplier tube. The crystal dimensions were 5.5 cms. diameter and 6.9 cms. deep forming a well of diameter 2.75 cms. and depth 5.5 cms., in which samples were counted by the 'all in' method. The crystal and photomultiplier tube were shielded by 10 cms. of lead and connected to a scaler Model 1700. All samples were conted for 100 secs. and appropriate background corrections made in every case.

Initial studies were made using 1 gm. and 5 gms. of

tissue in 10 mls. and 50 mls. of homogenate respectively.

From the results obtained as shown in table 3 , it was clear that there were considerable losses in the extraction process, these being largest ca. 20% at the phenol extraction stage. A loss of 7% of activity was also recorded in the organic phase in each case and final yields of activity of 47% and 43% respectively left about 20% of the total activity unaccounted for in each extraction. Since the activity of all the discarded phases was measured this activity must have been lost in the protein precipitate.

In view of these findings, the extraction procedure was modified in two respects - extension of the ethanol reflux period to one hour and duplication of the phenol extraction - in an attempt to decrease the losses in the protein precipitate and aqueous phase respectively. Six further extractions were performed using the same tissues as were examined previously and with the possibility that there had been considerable loss of activity in the protein precipitate, these were dried and the activity of each measured.

From the results in table 4 , it was clear that the new extraction conditions, together with a more vigorous mixing of the phenol and aqueous phases considerably reduced losses. These were reduced to an average of 5% in the protein precipitate and a mere 7% in the aqueous phase from the phenol extraction.

PHASE.	EXTRACT.	ACTIVITY. (c.p.s.)	PERCENTAGE YIELD/LOSS.
Homogenate.	1	31.7	100%
	2	296.9	100%
Initial Aqueous.	1	8.9	28.0% Loss.
	2	57.0	19.2% Loss.
Phenol.	1	17.8	56.0% Yield.
	2	144.3	48.6% Yield.
Phenol/Ether/ Acetone.	1	2.2	7.0% Loss.
	2	21.6	7.2% Loss.
Final Aqueous concentrate.	1	14.9	47.0% Yield.
	2	127.7	43.0% Yield.

Table 3 Initial Yields of $^{57}Co.B_{12}$ from Human Liver.

PHASE.	EXTRACTS. (%'AGE. OF TOTAL ACTIVITY)						AVERAGE %'AGE. YIELD/LOSS.
	1	2	3	4	5	6	
Homogenate.	100	100	100	100	100	100	
Aq. Ethanol filtrate.	88.2	91.9	99.4	95.3	94.3	95.9	94.2% Yield.
Protein precipitate.	5.0	5.5	5.8	5.0	4.1	5.0	5.1% Loss.
Aq. Ethanol concentrate.	82.9	90.7	98.5	99.4	94.8	94.9	93.5% Yield.
Aq. Ethanol distillate.	0.0	0.0	0.0	0.9	0.0	0.0	0.2% Loss.
Phenol.	82.5	89.5	89.5	84.8	73.0	76.3	82.6% Yield.
Initial Aqueous.	1.8	5.8	4.4	7.3	19.0	3.9	7.0% Loss.
Phenol/Ether Acetone.	1.4	6.7	0.2	4.0	6.1	4.0	3.7% Loss.
Final Aqueous.	71.8	74.3	88.2	83.8	60.3	72.9	75.2% Yield.

Table 4 Yields of $^{57}Co.B_{12}$ from Human Liver using the Revised Extraction Procedure.

The final yields of activity ranged between 60.3% and 88.2% with an average of 75.2% and were clearly very dependent on extraction conditions to such an extent that vigorous shaking of the aqueous and phenol phases reduced activity losses by about 15%.

It seemed likely that the yields from tissues which had been subjected to cooking might well be lower because of denaturation of the protein binders during the cooking process and therefore studies were carried out on tissues which were pressure-cooked for 20 mins. at 120°C. Again six identical extractions were performed using 50 ml. homogenates containing 5 gms. of cooked tissue.

From the results in table 5 , it was evident that an increase in the loss of activity in the protein precipitate had occurred with average losses of 10.1%. This together with increased losses of 15.7% in the aqueous phase from the phenol extraction led to final activity yields between 60.8% and 70.4% with an average of 66.3%. Cooking of the tissue thus made more difficult the freeing of incorporated vitamin B_{12}.

The studies on extraction yields using tissues containing incorporated radioactive vitamin B_{12} had perforce to be limited to the one tissue which had become available. It had been planned to extend this study to eggs containing the incorporated radioactive

PHASE.	EXTRACTS. (%'AGE. OF TOTAL ACTIVITY)						AVERAGE %'AGE. YIELD/LOSS.
	1	2	3	4	5	6	
Homogenate.	100	100	100	100	100	100	
Aq. Ethanol filtrate.	95.0	93.3	96.8	94.4	93.0	93.1	94.3% Yield.
Protein precipitate.	12.5	8.1	9.4	8.8	9.3	12.7	10.1% Loss.
Aq. Ethanol concentrate.	94.8	93.7	94.9	97.9	91.6	93.0	94.3% Yield.
Phenol.	77.1	74.8	71.2	71.8	71.8	68.7	72.6% Yield.
Initial Aqueous.	11.7	12.6	17.9	14.1	19.8	18.2	15.7% Loss.
Phenol/Ether Acetone.	6.0	0.8	10.6	3.0	3.5	4.4	4.7% Loss.
Final Aqueous.	68.4	70.4	64.2	70.4	63.5	60.8	66.3% Yield.

Table 5 Yields of $^{57}Co.B_{12}$ from Human Liver tissue Cooked for 20 mins.

vitamin B_{12}, a method for this having been described (Schade & Schilling, 1967), but circumstances made it impossible to proceed with this project. Under the circumstances therefore the studies were limited to those in which $^{57}Co.CNB_{12}$ was added to eggs in vitro. It was appreciated from the onset of these studies that the added radioactive cyano B_{12} might simply be loosely surface bound to protein and that in such circumstances the yields might be misleadingly high when compared to yields of vitamin B_{12} incorporated into the egg protein, which might well be considerably lower.

It was also felt however, that if trapping of vitamin B_{12} in denatured protein was a factor in the occurrence of low yields, this would be found in extraction yields of vitamin B_{12} from eggs to which radioactive cyano B_{12} had been added in vitro prior to denaturation of the protein.

$^{57}Co.CNB_{12}$, (0.5 ml., 0.05μCi, 15 ngms.B_{12}) was injected into the yolk of two raw eggs, both gently shaken and weighed. One egg was boiled for three minutes at 100°C and both eggs were then reweighed in the dark after removal of shell and membrane. The tissues were homogenised, divided into aliquots each containing 10 gms. of tissue and subjected to the usual extraction process, modified only by the extension of the duration and repetition of the ethanol reflux.

With the uncooked egg, large losses of ~25% of vitamin B_{12} activity occurred in the protein precipitate despite repetition and extension of duration of reflux. In addition the fine protein precipitate which was not removed by the filtration process led to losses in excess of 10% (table 6), due to its retention in the aqueous phase during the phenol extraction.

The loss of vitamin B_{12} activity in the protein precipitate in the extraction from the boiled egg homogenate was similar to that for the uncooked egg, at about 23% (table 7). In this case overall losses in the aqueous phase at the phenol extraction and the organic phase were on average about 5% higher than for the uncooked egg homogenate at ca. 15% and 10% respectively, and led to a low average yield of 39%.

Added radioactive vitamin B_{12} appeared to be tightly bound up in the egg protein and as with liver the cooking process reduced the activity yield by about 10%. The low recoveries of radioactive cyano B_{12} added to eggs in vitro were in keeping with the results reported by Adams et al. (1972.), who found that the mean recoveries of cyano B_{12} added to egg homogenate were as low as 37%, the measurements being made by microbiological assay.

PHASE.	EXTRACT.	ACTIVITY (c.p.s.)	PERCENTAGE YIELD/LOSS.
Homogenate.	1	121.6	100%
	2	117.2	100%
Protein precipitate.	1	31.8	26.2% Loss.
	2	26.2	22.4% Loss.
Initial Aqueous.	1	20.7	17.0% Loss.
	2	8.8	7.5% Loss.
Phenol/Ether Acetone.	1	4.9	4.0% Loss.
	2	4.6	3.9% Loss.
Final Aqueous.	1	48.3	39.7% Yield.
	2	67.9	58.0% Yield.

Table 6 Yields of $^{57}Co.B_{12}$ from Raw Egg Homogenates.

PHASE.	EXTRACT.	ACTIVITY (c.p.s.).	PERCENTAGE YIELD/LOSS.
Homogenate.	1	134.4	100%
	2	127.4	100%
	3	117.3	100%
Protein precipitate.	1	30.9	23.0% Loss.
	2	33.0	25.9% Loss.
	3	24.3	20.7% Loss.
Initial Aqueous.	1	15.7	11.7% Loss.
	2	20.3	15.9% Loss.
	3	24.9	21.2% Loss.
Phenol/Ether Acetone.	1	10.5	7.3% Loss.
	2	8.9	7.0% Loss.
	3	16.9	14.4% Loss.
Final Aqueous.	1	58.8	43.7% Yield.
	2	46.9	36.8% Yield.
	3	43.0	36.7% Yield.

Table 7 Yields of $^{57}Co.B_{12}$ from Boiled Egg Homogenates.

SECTION 2

Use of Pronase in the Extraction Procedure.

To reduce the loss of vitamin B_{12} in the protein precipitate it was decided to use pronase, a non-specific proteolytic enzyme, to break down the denatured protein in the cooked tissue homogenates into its constituent amino acids and thereby release any hitherto bound cobalamins. It was expected that losses in the protein precipitate in excess of 10% for liver tissue and 20% for cooked and raw egg tissue might be appreciably reduced by such a pronase treatment.

MATERIALS & METHODS.

A brisket (boiled) homogenate was prepared and divided into aliquots each containing 4 gms. of tissue. Pronase 'B' grade, derived from Streptomyces griseus was obtained from Calbiochem and a 0.4% (w/w) solution prepared in phosphate buffer at pH 7.6.

30 mls. of the pronase solution were added to 40 mls. of brisket homogenate in a foiled 250 ml. conical flask, shaken for 10 mins. and the solution divided in two 60 ml. aliquots, one incubated for 3 hrs. and the other for 18 hrs. at 37°C. The cobalamin extraction procedure was carried out on each solution with additional measurement of the weight of the protein precipitate to determine the extent of proteolysis in each case. In addition a control extraction was carried out on another

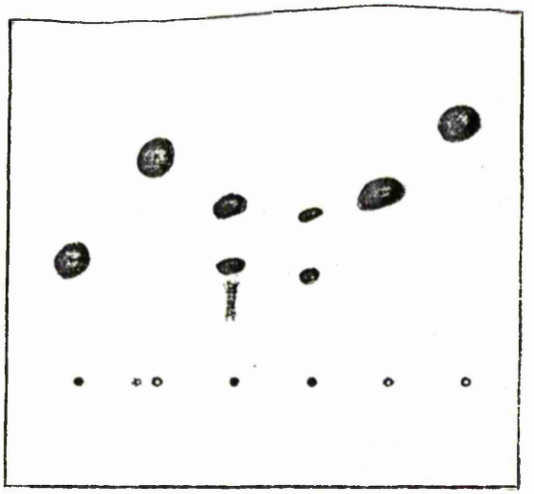
40 mls. of brisket homogenate and the cobalamin yield compared with those from the pronase incubated samples. Chromatograms of the extracts were run and developed by the standard bioautographic technique.

RESULTS & DISCUSSION.

The control extract on bioautography, fig. 2 , yielded small growth zones corresponding to coenzyme B_{12} and hydroxo B_{12}. The extracts from the incubated samples, fig's. 3 & 4 , gave rise to proportionally much larger growth zones implying an increased cobalamin extraction efficiency with the use of pronase. Comparison of bioautograms however showed anomalous results in that, with apparently greater extraction efficiency only one cobalamin, coenzyme B_{12} was detected in the incubation extracts, while hydroxo B_{12} was also found in the control extract.

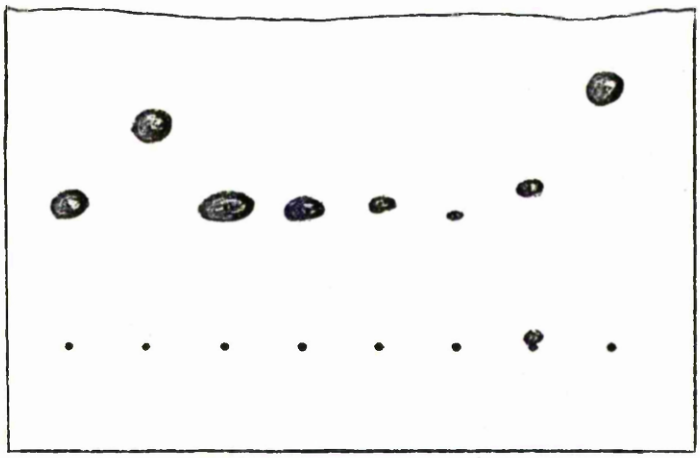
The yield of protein precipitates from the 3 hour and 18 hour incubations of 0.24 gms. and 0.065 gms. respectively implied that proteolysis in the latter extraction was virtually complete with the proteins reduced in the homogenate to their amino acid constituents, which being water soluble were not removed on filtration.

The increase in yield of cobalamins was reflected in fig. 4 , where a growth response could be detected for the 18 hr. incubation extract at an applied level of ~4 pgs.B_{12}. To avoid the extension of an already long



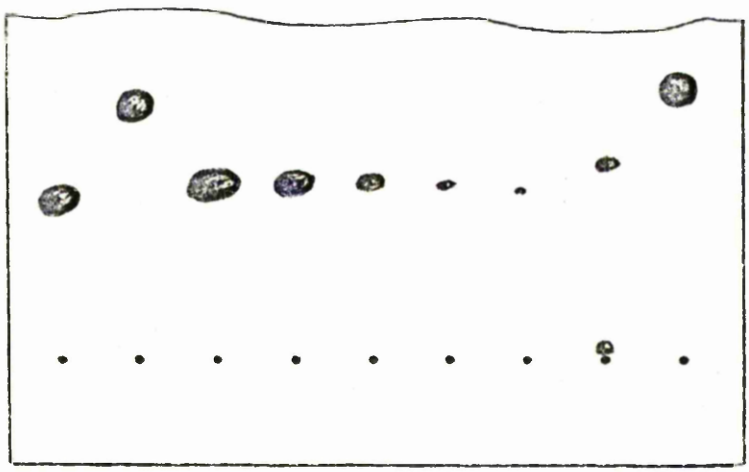
B_{12} Form :	Ado	CN	EXTRACT	'OH'	Me
pgs. B_{12} :	100	100	<184 <92	100	100

Fig. 2 Brisket, boiled, extract, - Bioautogram.



B_{12} Form :	Ado CN	E X T R A C T	'OH' Me
pgs. B_{12} :	100 100	<102<63 <34 <17	100 100

Fig. 3 Brisket, boiled, extract, - Bioautogram.
3 hr. incubation in 0.4% (w/w) Pronase.



B_{12} Form :	Ado CN	E X T R A C T	'OH' Me
pgs. B_{12} :	100 100	<63 <34 <17 <3.5<4.3	100 100

Fig. 4 Brisket, boiled, extract, - Bioautogram.
18 hr. incubation in 0.4% (w/w) Pronase.

duration extraction procedure from one to two days with an 18 hr. incubation it was decided to use the pronase at a higher concentration and again incubate the homogenates for 3 hours.

MATERIALS & METHODS.

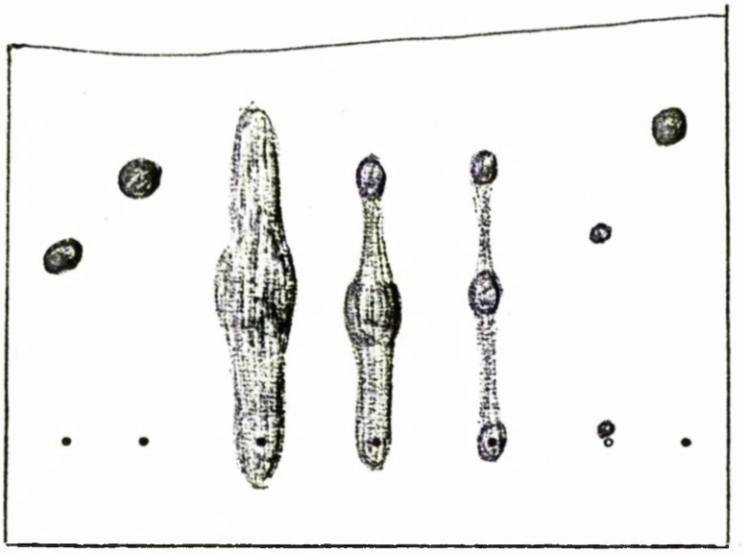
As with the previous extraction, except a 7% (w/w) pronase solution was prepared and incubated with the brisket homogenate for 3 hrs. at 37°C. After extraction of cobalamins the chromatogram was run and developed by bioautography.

RESULTS & DISCUSSION.

From the bioautogram, fig. 5 , it was apparent that if the vitamin B_{12} content of brisket of 12 µgms.B_{12}/kg. tissue as quoted by Adams et al. (1972.) was accurate, then excessive growth occurred on the bioautogram when using high concentration 7% (w/w) pronase, implying that the pronase itself contained vitamin B_{12} as an impurity.

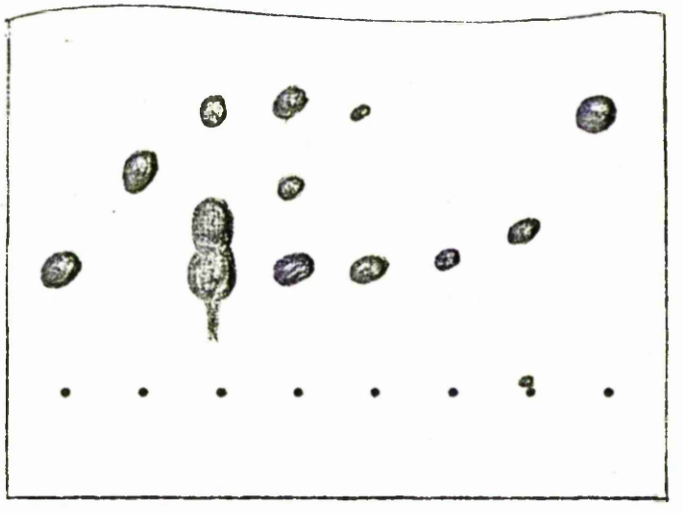
An extraction was therefore carried out on a 0.02% (w/v) pronase solution prepared in 40 mls. of phosphate buffer, pH 7.6. The final extract was again analysed by chromatography and bioautography.

Pronase was found to be rich in cobalamins, see fig. 6 , containing predominantly coenzyme B_{12} together with smaller amounts of cyano B_{12} and methyl B_{12}. From serial dilutions and comparison with the growth areas of the standards, pronase 'B' grade was estimated to



B_{12} Form :	Ado CN	E X T R A C T			'OH'	Me
pgs. B_{12} :	100 100	<159	<53	<26.5	100	100

Fig. 5 Brisket, boiled, extract, - Bioautogram.
3 hr. incubation in 7% (w/w) Pronase.



B_{12} Form :	Ado CN	E X T R A C T			'OH'	Me
pgs. B_{12} :	100 100				100	100
Dilution		1	1/2	1/4 1/8		

Fig. 6 Pronase extract in Phosphate Buffer, pH 7.4.

contain 1.25 µgms.B_{12}/gm. Pronase used at the recommended enzymic concentration of 0.2 - 0.4% (w/w) was clearly unsuitable in aiding extraction of cobalamins from tissues which contained less than ~10 µgms.B_{12}/kg. and therefore it could seldom be employed.

The presence of cobalamins in pronase undoubtedly arose from the fact that it was derived from the bacterium Streptomyces griseus which synthesises vitamin B_{12} itself. This finding explained why only the coenzyme B_{12} growth zone was present in fig. 3 , since the cobalamins present in the brisket were obscured on the bioautogram by those isolated from the pronase.

CHAPTER 3

AN UNKNOWN COBALAMIN

SECTION 1

An Unknown Cobalamin. - Properties.

During the investigation into extraction yields it became apparent from the bioautographic growth zones in figs. 2 & 6 , that the bulk of the standard hydroxo B_{12} growth occurred at a position between coenzyme B_{12} and cyano B_{12}, with an $R_f \simeq 0.2$ and did not correspond to any of the other standard cobalamins. A second growth area at the origin was also obtained for hydroxo B_{12} on bioautograms and was attributed to ammonia B_{12}, through conversion of the hydroxo B_{12} present by the ammonia in the chromatographic solvent system,

$$\left[OH.B_{12}\right] \xrightarrow{NH_3\ (l)} \left[NH_3.B_{12}\right]^+ + OH^- \quad ;$$

The growth area at $R_f \simeq 0.2$ might be due to aquo B_{12}, which in strong acid solution (pH < 0) was known to be stable with respect to ammonia B_{12} (Pratt, 1964.). However, since the relative concentrations of hydroxo B_{12} and aquo B_{12} are governed by the simple acid/base equilibrium,

$$\left[OH.B_{12}\right] \underset{OH^-}{\overset{H^+}{\rightleftharpoons}} \left[H_2OB_{12}\right]^+ \quad ;$$

then any samples maintained at a constant pH should contain concentrations of hydroxo B_{12} and aquo B_{12} in a similar ratio, determined by the equilibrium constant k, where

$$k = \frac{\left[H_2O.B_{12}\right]^+}{\left[H\right]^+\left[OH.B_{12}\right]} \quad ;$$

In an attempt to confirm this theory, tissue extractions were carried out, the cobalamins isolated at a known pH and the overall charge of the unknown cobalamin determined by its behaviour towards ion-exchange celluloses. In addition the light stability of the unknown was established.

MATERIALS & METHODS.

The standard cobalamins (coenzyme B_{12}, cyano B_{12}, hydroxo B_{12} and methyl B_{12}) prepared in distilled water were run on a silica gel chromatogram in the solvent system without ammonia - butan-2-ol : n. propanol : water (7 : 4 : 3, v/v) - and the position of the hydroxo B_{12} on the resultant bioautogram noted.

A gigot chop homogenate was prepared and divided into aliquots each containing 4 gms. of tissue. The cobalamins were extracted from the tissue and finally isolated in freshly prepared distilled water at pH 6.4. At the same time a standard hydroxo B_{12} solution was prepared in the distilled water, a t.l.c. of the standards and chop extract run and the corresponding bioautogram developed.

Two Quickfit chromatography columns (10 x 1 cm.), each containing a sintered glass disc support, were connected via flexible plastic tubing to two 100 ml. separating funnel reservoirs. One of the columns was packed with Whatman DE 11 activated DEAE cellulose ($-\overset{+}{N}HR_2$) and the

reservoir filled with distilled water, acidified with dilute HCl(10^{-3} mol./l.). The other column was packed with Whatman CM 11, activated CM cellulose ($-CO_2^-$), the reservoir filled with distilled water and the flow rate through each column adjusted to ca. 40 mls./hr.

Another gigot chop homogenate containing 4 gms. of tissue was put through the extraction procedure and the final aqueous extract containing the cobalamins rotary evaporated to 0.5 mls. 0.4 mls. of this were diluted to 1.0 ml with distilled water and added by pipette to the CM cellulose column. After 30 mins. the 20 mls. of eluant which had been collected were rotary evaporated to 0.5 mls., 0.25 mls. of which were further diluted to 1.0 ml. with distilled water and added to the DEAE cellulose column. The 20 mls. of eluant obtained initially from the column were rotary evaporated to 0.3 mls. and stored for assay with the remainder of the concentrated eluant from the CM cellulose column. A chromatogram of the chop extract and the column eluants was run and developed by bioautography.

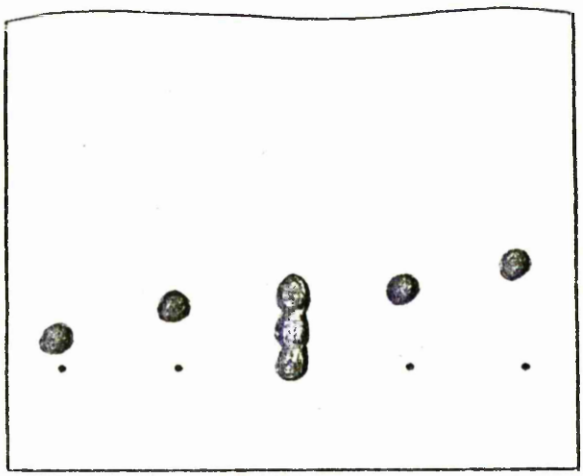
To determine the light stability of the unknown material the gigot chop extract was again examined by chromatography and bioautography in the usual way, another sample spotted onto a silica gel sheet and after drying exposed to daylight for the 6½ hr. duration of chromatographic separation.

RESULTS & DISCUSSION.

Bioautogram, fig. 7 , showed that omission of ammonia from the solvent system led to very poor separation of the cobalamin standards on silica gel. The hydroxo B_{12} was found to have travelled from the origin, but it remained uncertain if its final position lay between that of coenzyme B_{12} and cyano B_{12} or not.

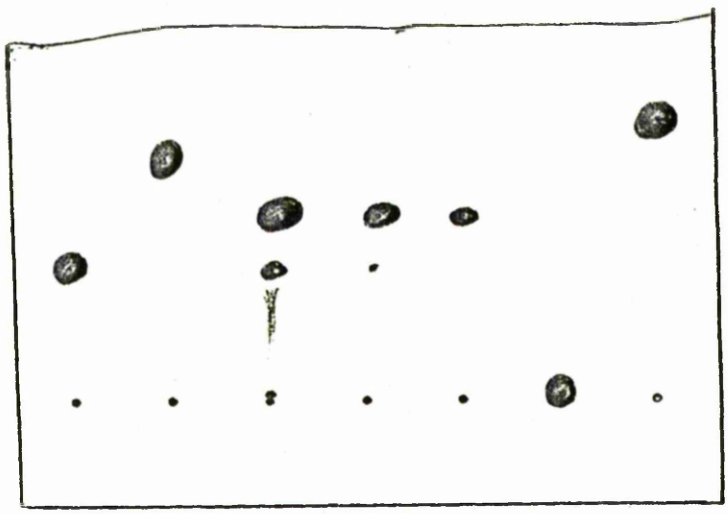
If our theory that the two growth zones for the hydroxo B_{12} standard were due to 'hydroxo B_{12}' (as ammonia B_{12}) and aquo B_{12} was correct, then the growth pattern and distribution obtained for the hydroxo B_{12} standard should be similar to that for any hydroxo B_{12} isolated from the chop extract at the same pH. From the bioautogram, fig. 8 , this was clearly not the case, since the freshly prepared hydroxo B_{12} standard at pH 6.4 gave rise to only one growth zone at the origin, while the gigot chop extract at the same pH produced a large growth area at the position between coenzyme B_{12} and cyano B_{12} with only trace growth at the origin. These results violated the OHB_{12}/H_2OB_{12} equilibrium and implied that this major growth zone at $R_f \simeq 0.2$ was not due to aquo B_{12}. It seemed more likely that the hydroxo B_{12} present in the chop extract had been chemically converted, perhaps via aquo B_{12} to another microbiologically active cobalamin.

Bioautogram, fig. 9 , illustrated again that the second gigot chop extract contained predominantly the



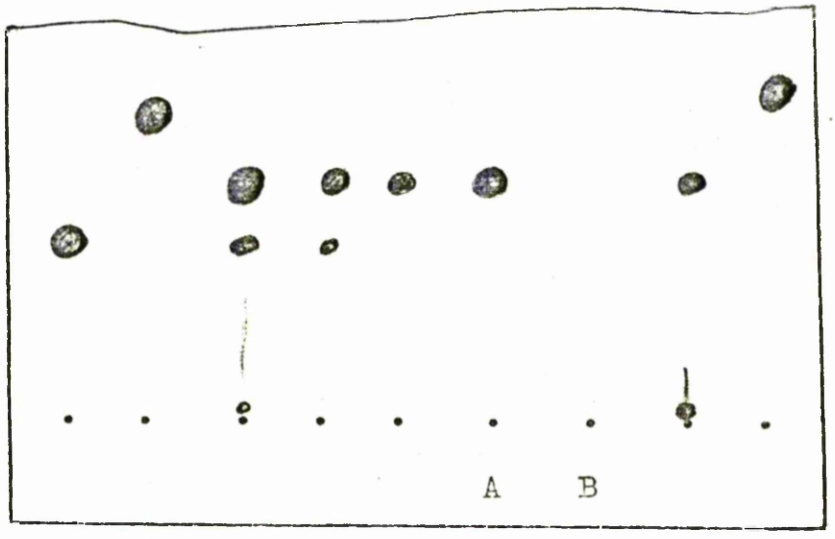
B_{12}.Form :	Ado	CN STANDARDS	OH	Me
pgs.B_{12} :	100	100 5 x 25	100	100

Fig. 7 Bicautogram of the Standard Cobalamins, Coenzyme B_{12}, Cyano B_{12}, Hydroxo B_{12} and MethylB_{12} Separated by Thin-Layer Chromatography in the Solvent System without Ammonia.



B_{12}.Form :	Ado	CN	E X T R A C T			OH	Me
pgs.B_{12}	100	100	<96	<48	<24	100	100

Fig. 8 Bioautogram of Gigot Chop (Stewed) in Distilled Water, pH 6.4 and Standard Cobalamins also at pH 6.4.



B_{12}.Form :	Ado	CN	-CHOP EXTRACT-			ELUANTS	OH	Me
pgs.B_{12} :	50	50	<96	<48	<24		50	50

Fig. 9 Bioautogram of Gigot Chop Extract and Eluants from passage through CM cellulose (A) and DEAE cellulose (B).

unknown cobalamin together with a trace of coenzyme B_{12} and hydroxo B_{12}, both of which were removed by passage through CM cellulose, while the unknown material was collected in the first 20 mls. of eluant. This material was however removed by DEAE cellulose and no trace of it appeared in the first 40 mls. of eluant implying that it was acidic in character.

Retention of the compound on DEAE cellulose as opposed to mere retardation of its passage through the cellulose indicated that the cobalamin was not neutral in character but carrying an overall negative charge; as it was derived solely from hydroxo B_{12} (or aquo B_{12}) this inferred that the unknown was produced by reaction with a divalent acid anion, ie :-

$$\left[OH.B_{12}\right] + A^{2-} \longrightarrow \left[A.B_{12}\right]^{-} + OH^{-} ;$$

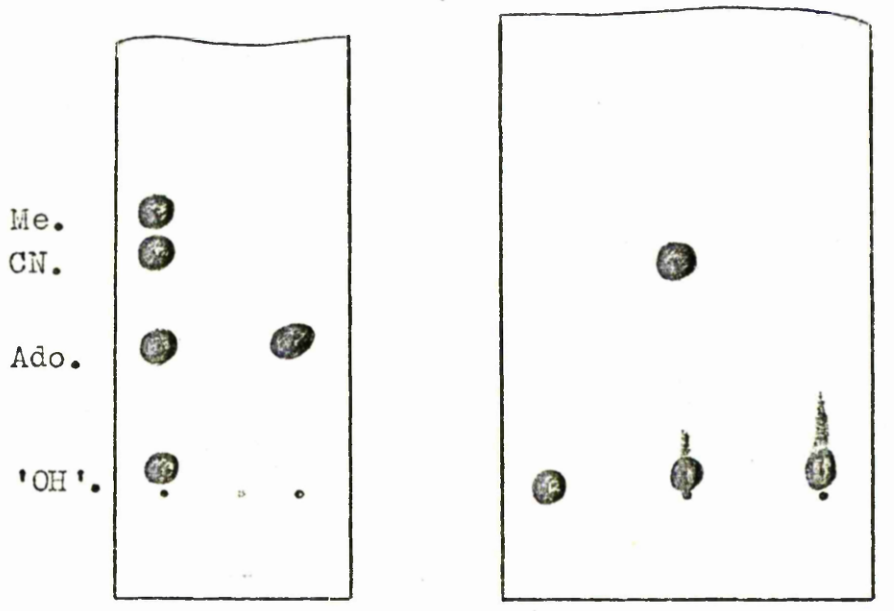
where A^{2-} is the divalent acid anion. The acidic cobalamin so produced $\left[A.B_{12}\right]^{-}$, would then be readily retained on DEAE cellulose :-

$$\downarrow \left[A.B_{12}\right]^{-} \left|\, {}^{+}NHR_2-\right.$$
$$\left[A.B_{12}\right]^{-} \left|\, {}^{+}NHR_2-\right.$$
$$OH^{-} \left|\, {}^{+}NHR_2-\right.$$

From bioautogram, fig 10 b, the unknown material was shown to be very light-sensitive being completely converted during chromatography to a compound with an R_f identical to that of 'hydroxo B_{12}' in this system.

NON LIGHT-EXPOSED.

LIGHT-EXPOSED.



B_{12} Form:	STANDARDS	CHOP EXTR.	'OH' STANDARDS Ado/CN/Me.	CHOP. EXTR.
pgs. B_{12} :	4 x 25	48	100 3 x 33	48

Figs. 10 a & 10b Bioautograms of Light-Exposed and non Light-Exposed Gigot Chop Extracts respectively.

SECTION 2

Identification of the Unknown Cobalamin.

The fact that the unknown material was acidic, probably derived from nucleophilic attack on the cobalt ion by the divalent acid anion, readily converted to hydroxo B_{12} in the dark, led us to the consideration that the material was sulphito B_{12}, formed as a consequence of contamination of the distilled water, which was stored in carboys and used to receive and homogenise tissues. Such contamination might have arisen as a result of dissolution in the water of atmospheric sulphur dioxide, which is very soluble in water (11·3 mgms./ml.), with chemical equilibria involved being best represented by the following equations :-

1) $SO_{2(g)} + x.H_2O \rightleftharpoons SO_2.xH_2O$ (hydrated sulphur dioxide)

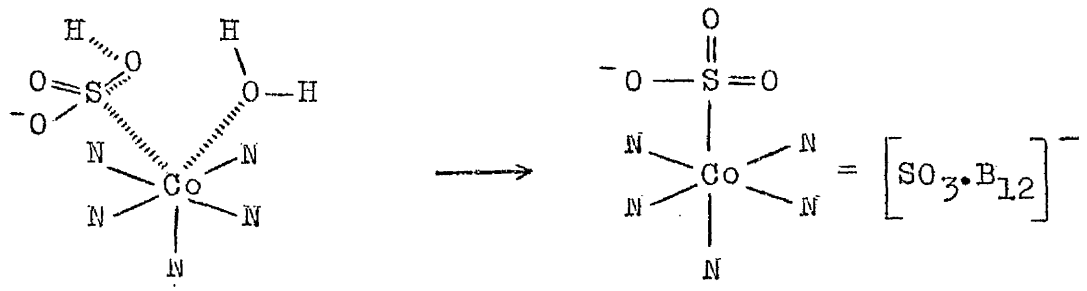
2) $SO_2.xH_2O \rightleftharpoons HSO_3^-(aq.) + H_3O^+ + (x-2).H_2O$

In the acidic solution so produced hydroxo B_{12} present in an extract would become protonated to the aquo B_{12} form and their acid/base equilibrium would lie to the right hand side :-

3) $\left[OH.B_{12}\right] \rightleftharpoons \left[H_2O.B_{12}\right]^+$

The aquo B_{12} would then react with the bisulphite ions of equation 2, thus :-

$$\left[H_2O.B_{12}\right]^+ + HSO_3^- \longrightarrow$$



and produce sulphito B_{12} by removal of the coordinated water molecule. Whether a seven co-ordinate intermediate as depicted or a five co-ordinate intermediate would be formed by a bimolecular or monomolecular substitution respectively was uncertain, although steric hindrance might be encountered in the former. The reaction appeared to be very favourable, although its rate was clearly dependent on the availability of bisulphite ions. During the time this work was done the atmospheric concentration of sulphur dioxide at a local authority atmospheric pollution station near the laboratory lay in the range $10 - 600 \times 10^{-9}$ kg. SO_2/m^3. and when allowances were made for time of exposure to contamination, solubility of sulphur dioxide in water and the quantities of cobalamins present, these concentrations were in keeping with the proposed mechanism.

Absolute identification of the major cobalamin encountered in the chop extract and hydroxo B_{12} standards remained impossible by any direct method such

as spectroscopy due to the extremely low concentrations of the unknown involved ($< 7.5 \times 10^{-8}$ mol./l.). If, however a cobalamin prepared at high concentration ($> 7.5 \times 10^{-4}$ mol./l.) and therefore readily identified by absorption spectroscopy exhibited similar chemical and microbiological characteristics to that of the unknown at low concentration, then the prepared cobalamin could reasonably be considered identical to the unknown. Cosequently sulphito B_{12} samples were prepared by the reaction of sodium dithionite (method A) and sodium metabisulphite (method B), with hydroxo B_{12}.

MATERIALS & METHODS.

A). 5 mls. of a solution of hydroxo B_{12} (7.4×10^{-5} mol./l.) in distilled water were added to 5 mls. of sodium dithionite, $Na_2S_2O_4$, (10^{-3} mol./l.) in a foiled 20 ml. universal glass bottle and the solution magnetically stirred for 20 mins. at room temperature ($20^{\circ}C$). Sodium dithionite was used in large excess to ensure complete conversion to sulphito B_{12}, and the final solution diluted one thousand-fold to a conc. $\leq 3.7 \times 10^{-8}$ mol./l. suitable for subsequent microbiological assay.

B). 5 mls. of an aqueous solution of hydroxo B_{12} (7.6×10^{-3} mol./l.) were added to 5 mls. of sodium metabisulphite, $Na_2S_2O_5$, (10^{-2} mol./l.) and the solution stirred as before in the dark for 20 mins. at room temperature. The solution was then added to a 25 ml.

separating funnel and passed through a CM cellulose ion exchange column (10 x 1 cm.) at a rate of 40 mls./hr. to remove any unconverted hydroxo B_{12}. The sulphito B_{12} was eluted with distilled water (50 mls.) and a thin red band of unconverted hydroxo B_{12} remained bound to the cellulose at the top of the column. 80% of the eluant (60 mls.) containing the sulphito B_{12} was added to a 250 ml. round bottom flask and evaporated to dryness at 40°C and $2.7 \times 10^{3} N/m^{2}$, (20 mm. Hg) pressure and the remaining 20% of eluant (15 mls.) passed through a DEAE cellulose ion-exchange column.

The dark red crystalline material obtained from rotary evaporation was recrystallised from aqueous acetone, dried in an oven at 120°C for 15 mins. and then weighed. The weight of the product was 28.7 mgms., which represented a yield of 66.56% assuming both starting material and product were in the non-hydrated form during weighing.

10 mgms. of the crystalline material were analysed on a Pye 'Unicam' Infrared Spectrometer at the medium scan speed and the spectrum obtained, fig. 11 , compared with those of the other standard cobalamins, figs. 12, 13, 14 & 15. For resolution of the strong absorptions at 980 cm^{-1} and 1100 cm^{-1} attributed to sulphito B_{12} by Dolphin et al., (1963), KBr discs were used since they provided optimum transmission of infra-red radiation at the lower frequencies. All samples were prepared at a

conc. of 1 mg./300mg., scanned between 4000 cm^{-1} and 625 cm^{-1} and as the cobalamins were examined in the crystalline state they were found not to be light sensitive during analysis (Smith, 1965, p. 48).

The rest of the prepared material was dissolved in distilled water (3.7 mls.) to give a sulphito B_{12} solution of conc. 3.5×10^{-3} mol./l. (5 mgms./ml.) suitable for visible t.l.c. 2µls. of this sulphito B_{12} solution were applied to a silica gel chromatogram together with visible amounts of the cobalamin standards (20 µgms.) and run in the standard solvent system. 1ml. of the solution was also diluted one hundred thousand-fold to give a final solution of conc. 3.5×10^{-8} mol./l. (50 pgs.B_{12}/µl.) and both sulphito B_{12} solutions prepared from sodium dithionite and sodium metabisulphite run on a silica gel chromatogram and developed by bioautography.

RESULTS & DISCUSSION.

The sulphito B_{12} prepared from the reaction of hydroxo B_{12} and sodium metabisulphite was as expected completely retained on DEAE cellulose and on visible t.l.c. appeared at a position between coenzyme B_{12} and cyano B_{12} identical to that adopted by the unknown cobalamin at low concentration on bioautograms, fig.16

The infra-red spectra of the standard cobalamins were found to be very similar to each other and also to the prepared 'sulphito B_{12}', which was inevitable since the cobalamins examined had almost identical molecular structures except for the variation in the one axial

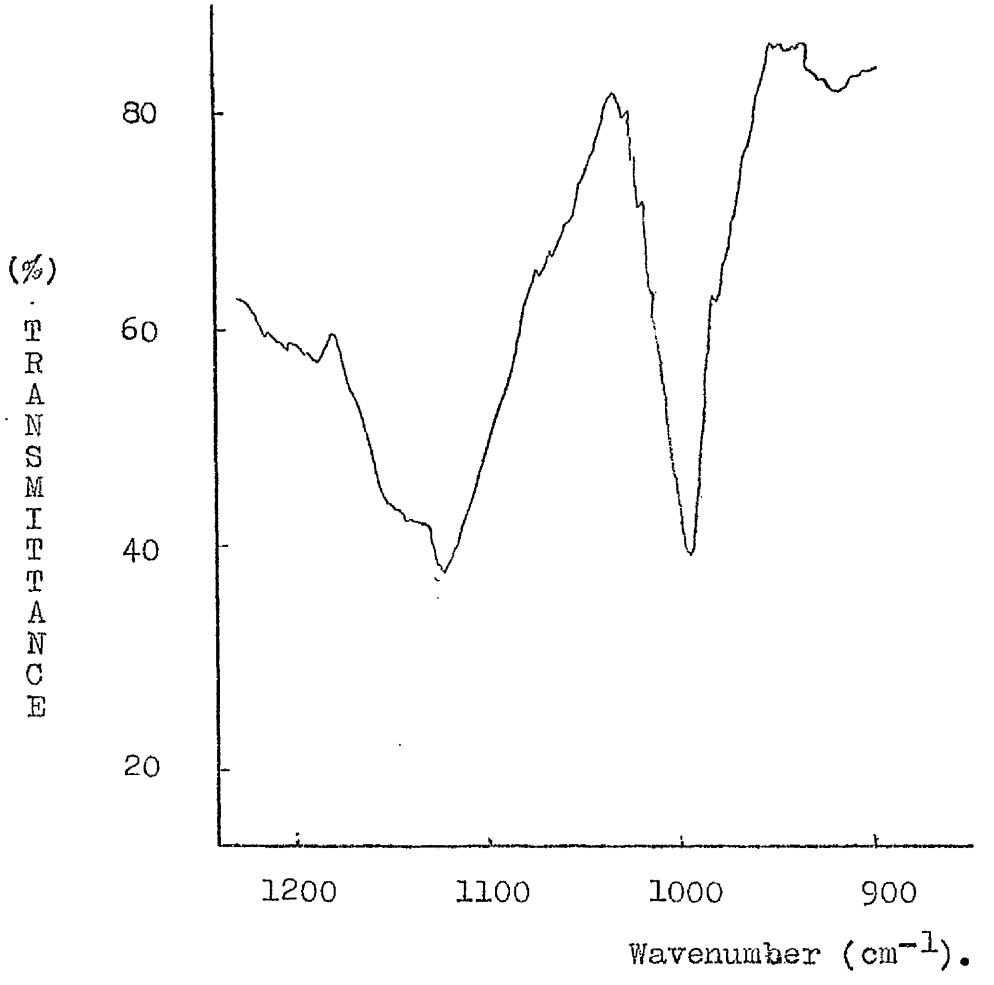


Fig. 11 KBr Disc Infra-red Spectrum of Prepared 'Sulphito B_{12}'.

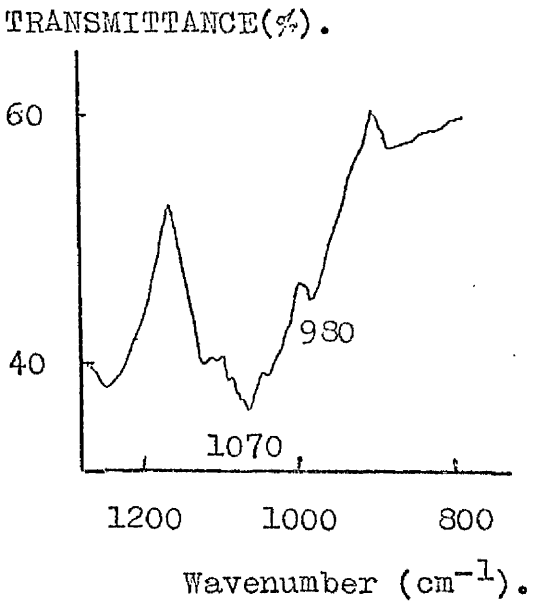


Fig. 12 KBr Disc I.R. Spectrum of Ado.B_{12}.

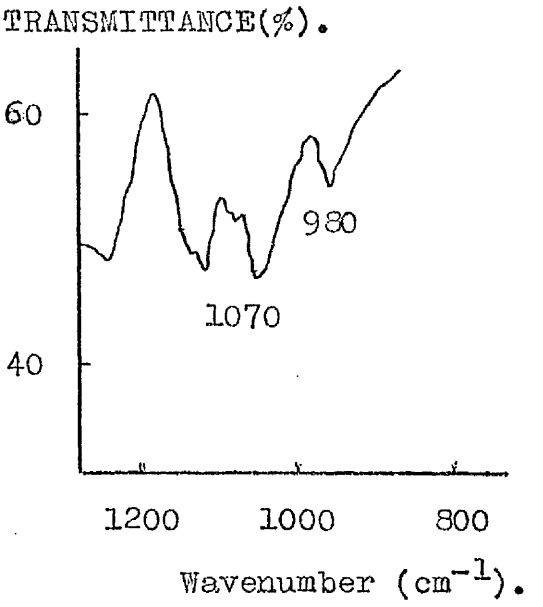


Fig. 13 KBr Disc I.R. Spectrum of CN.B_{12}.

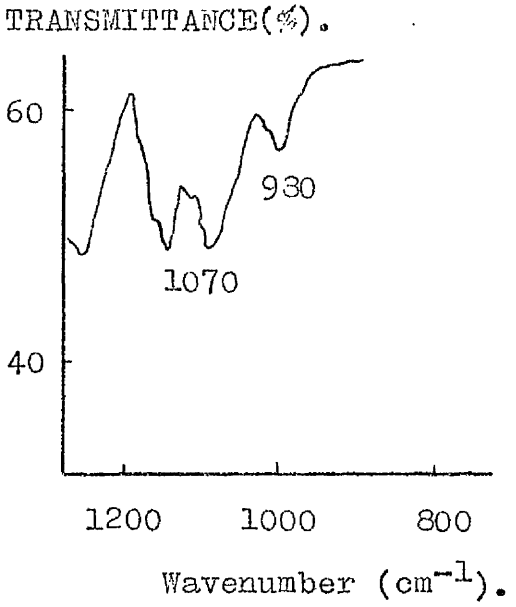


Fig. 14 KBr Disc I.R. Spectrum of OH.B_{12}.

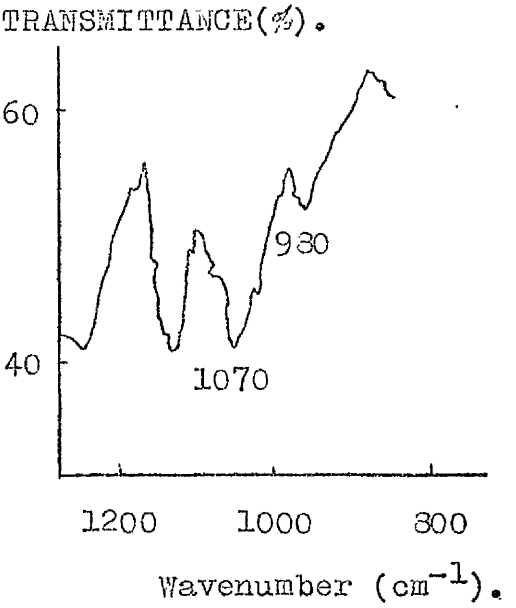


Fig. 15 KBr Disc I.R. Spectrum of Me.B_{12}.

ligand substituent at the sixth position, bound to the central cobalt atom. The infra-red absorptions at 980 cm^{-1} and 1100 cm^{-1} which were said to characterise sulphito B_{12} were not found to be unique to that cobalamin alone and indeed an absorption peak centred at 980 cm^{-1} was present on all the spectra, although at at a much weaker intensity in the standard cobalamins. These cobalamins also yielded a broad absorption at about 1070 cm^{-1} which again tended to question the significance of the absorption at 1100 cm^{-1} obtained for the prepared 'sulphito B_{12}' in attempts to establish the exact structure of that cobalamin.

Sufficient evidence for absolute identification of the prepared cobalamin could not be derived from infra-red spectroscopy because the 'fingerprint' region of the spectrum below ca. 1500 cm^{-1} could not, in itself, provide enough information to enable molecular structure to be established if no other unique absorptions were present at lower wavelengths <6500 nms. (>1500 cm^{-1}), which was the situation with the prepared cobalamin.

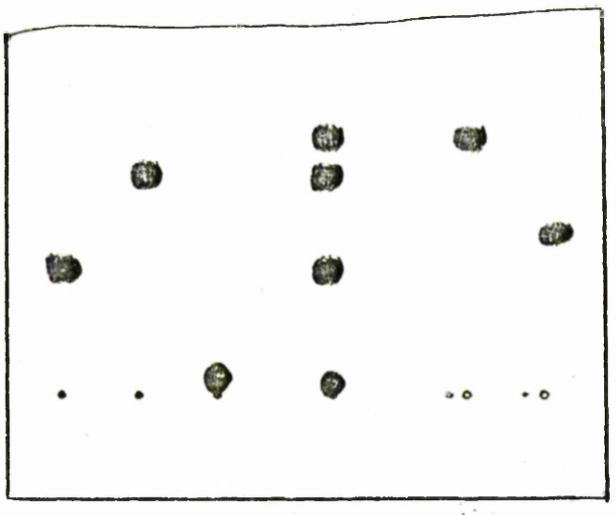
Infra-red characterisation of cyano B_{12} was possible from the unique weak vibrational mode at 2130 cm^{-1} of the cyanide residue, whereas the hydroxyl group vibration of hydroxo B_{12} at ca. 3400 cm^{-1} was masked by a broad absorption band between 3100 and 3500 cm^{-1} due to the hydroxyl group absorptions of the co-ordinated water molecules which prohibited structural

identification. The molecular vibrations of the 5'-deoxy-adenosyl and methyl groups of coenzyme B_{12} and methyl B_{12} were similarly swamped by the absorptions from the rest of the molecule and were thus valueless in defining structure.

Both 'sulphito B_{12}' solutions prepared from sodium dithionite and sodium metabisulphite at low concentration (10^{-8} mol./l.) on bioautography (fig.17) appeared at the position between coenzyme B_{12} and cyano B_{12} similar to that for the unknown cobalamin. The identical behaviour of these prepared cobalamins at both high and low concentrations on ion-exchange column chromatography on cellulose and thin-layer chromatography on silica implied that no conversion of the prepared 'sulphito B_{12}' was occurring in solution at low concentration and that any properties defined using visible amounts of the prepared cobalamin would also apply to the unknown cobalamin. The structure of the former however could not be established from infra-red spectroscopy.

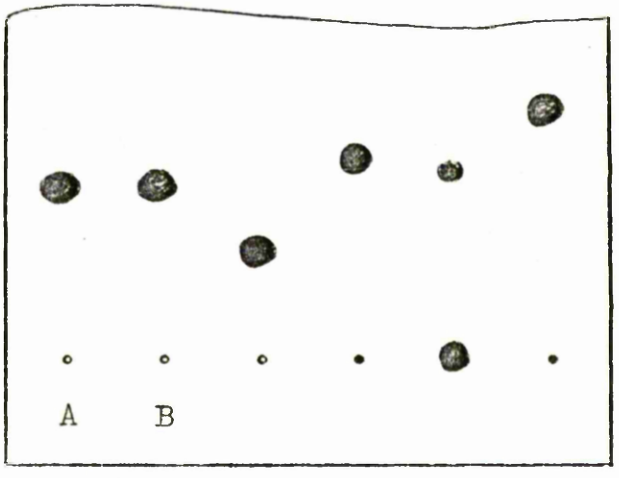
Ultra-Violet Absorption Spectroscopy.

Ultra-violet absorption spectroscopy was employed in a further attempt to characterise the prepared 'sulphito B_{12}'. This technique was not adopted initially although UV spectroscopic data had been published for cyano B_{12} (Brink et al., 1949), coenzyme B12 (Barker, 1960.), methyl B_{12} (Smith et al., 1962.) and sulphito B_{12} (Smith, 1965.), because it was felt that the



B_{12}.Form :	Ado	CN	'OH'	STANDARDS	Me	SO_3
ugms.B_{12} :	20	20	20	4 x 10	20	10

Fig. 16 'Visible' Thin-Layer Chromatogram of Sulphito B_{12} from Sodium Metabisulphite.



B_{12}.Form:	SO_3	SO_3	Ado	CN	'OH'	Me
pgs.B_{12} :	100	100	100	100	100	100

Fig. 17 Bioautogram of Sulphito B_{12} from both Sodium Dithionite (A) and Sodium Metabisulphite (B).

absorption maxima obtained for the latter might be somewhat suspect since UV spectroscopy necessarily involved light exposure of the cobalamin in aqueous solution both during preparation and scanning and at this time the light sensitivity of sulphito B_{12} was yet to be established. Light sensitivity work (pp.131-150) later showed that the light stability of sulphito B_{12} was of the same order as that of methyl B_{12} and coenzyme B_{12} and thus minimal conversion of sulphito B_{12} would occur during UV analysis provided that it was examined immediately after light exposure, which was the case.

MATERIALS & METHODS.

The standard cobalamins together with the 'sulphito B_{12}' were prepared in distilled water at concentrations between $3.2 - 3.7 \times 10^{-5}$ mol./l. (50 µgms./ml.), suitable for UV spectroscopic analysis, and 3 mls. of each solution pipetted in turn into one of five 1 cm. 'Sarstedt' plastic cuvettes. On exposure to daylight each of the cuvettes was placed immediately in the spectrophotometer and scanned between 700 and 325 nms. All spectra were run on a 'Unicam' SP 800 UV Spectrophotometer at the fast scan speed. (figs.18, 19, 20, 21, 22).

RESULTS & DISCUSSION.

The prepared 'sulphito B_{12}' gave rise to two

absorption maxima with λmax. at 363 nms. and 525 nms., fig. 18 , corresponding to those found for sulphito B_{12} by Smith (1965, p.35) and supporting the view that the prepared cobalamin was sulphito B_{12}. Thus this prepared sulphito B_{12} exhibited the same ion-exchange, chromatographic and bioautographic properties as the unidentified cobalamin present in the hydroxo B_{12} standard and gigot chop extracts, strongly suggesting that it too was sulphito B_{12} produced by the chemical conversion of hydroxo B_{12} in the dark.

At this stage it appeared that any sulphito B_{12} present in extracts had arisen solely by conversion of hydroxo B_{12} as none of the other cobalamins exhibited any trace of sulphito B_{12}. It would therefore have been possible to proceed with an unaltered extraction technique although careful analysis of subsequent bioautograms would then be necessary to obtain an accurate interpretation of cobalamins present. If steps were not taken to prevent the formation of artefactual sulphito B_{12} however, it would not be possible to determine whether any sulphito B_{12} on a bioautogram had been present originally in the tissue being analysed or whether it had arisen as an artefact, and therefore measures to prevent conversion of hydroxo B_{12} seemed desirable.

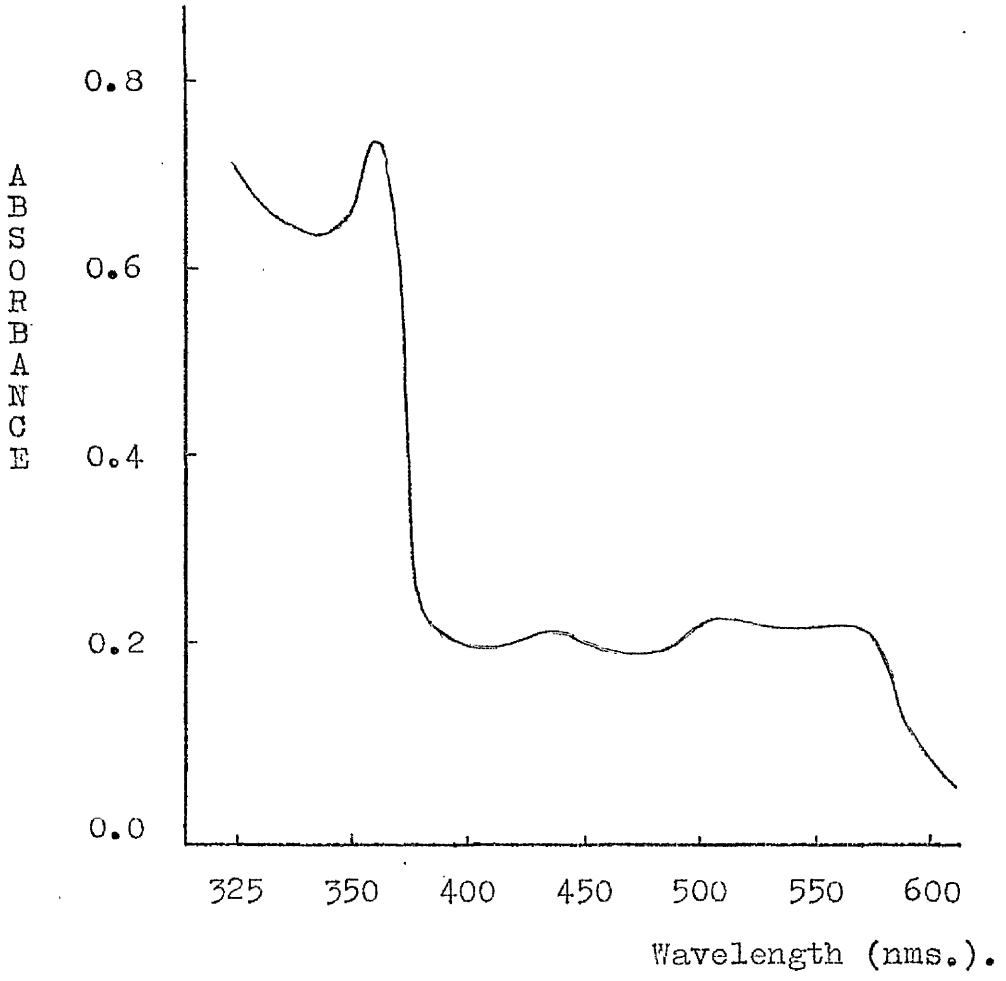


Fig. 18 Ultra-Violet Absorption Spectrum of Prepared 'Sulphito B_{12}'.

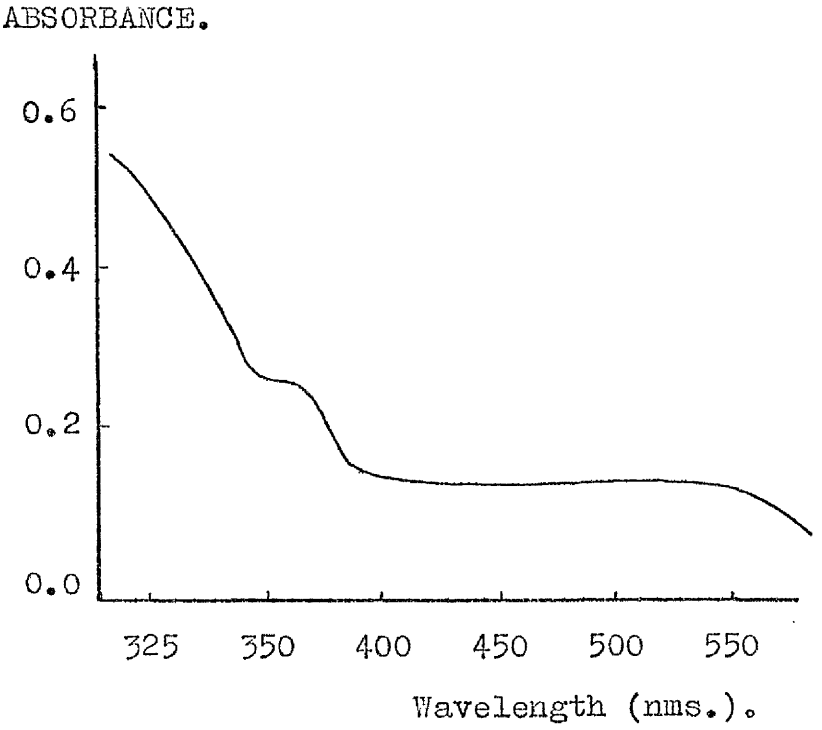


Fig. 19 Ultra-Violet Absorption Spectrum of Coenzyme B_{12}.

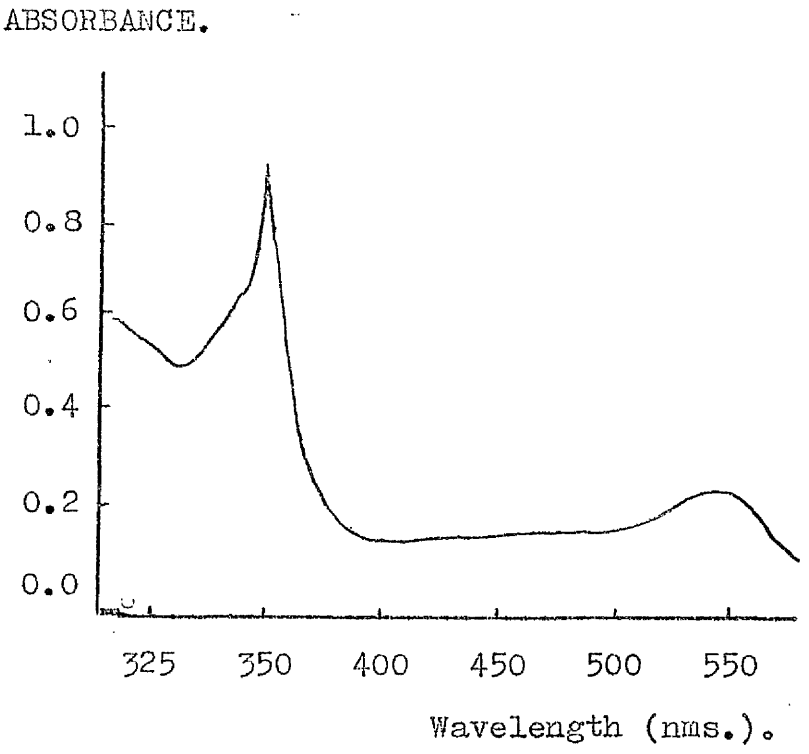


Fig. 20 Ultra-Violet Absorption Spectrum of Cyano B_{12}.

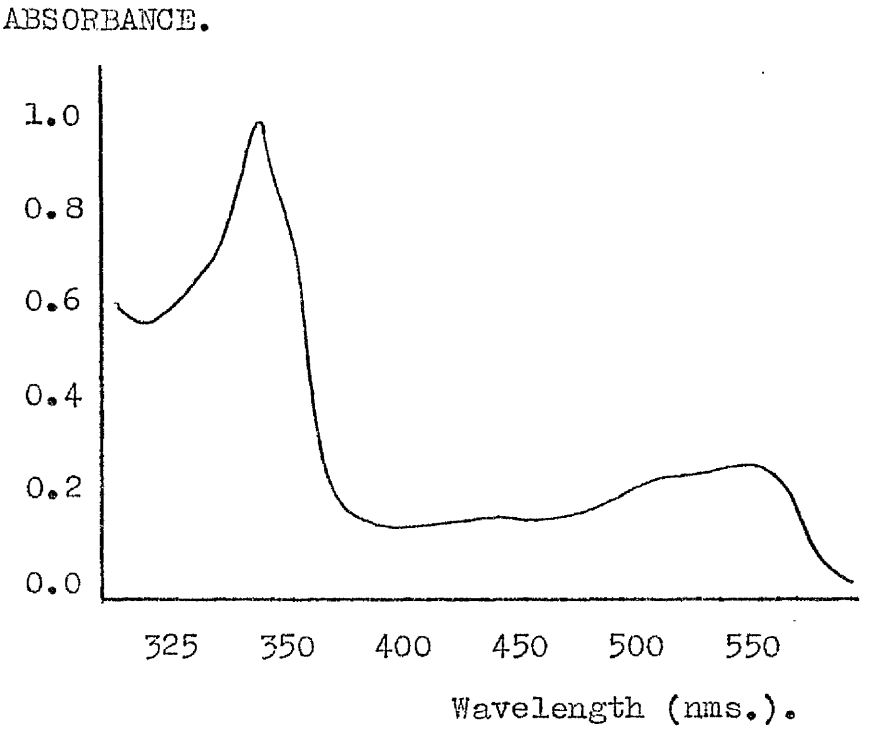


Fig.21 Ultra-Violet Absorption Spectrum of Hydroxo B_{12}.

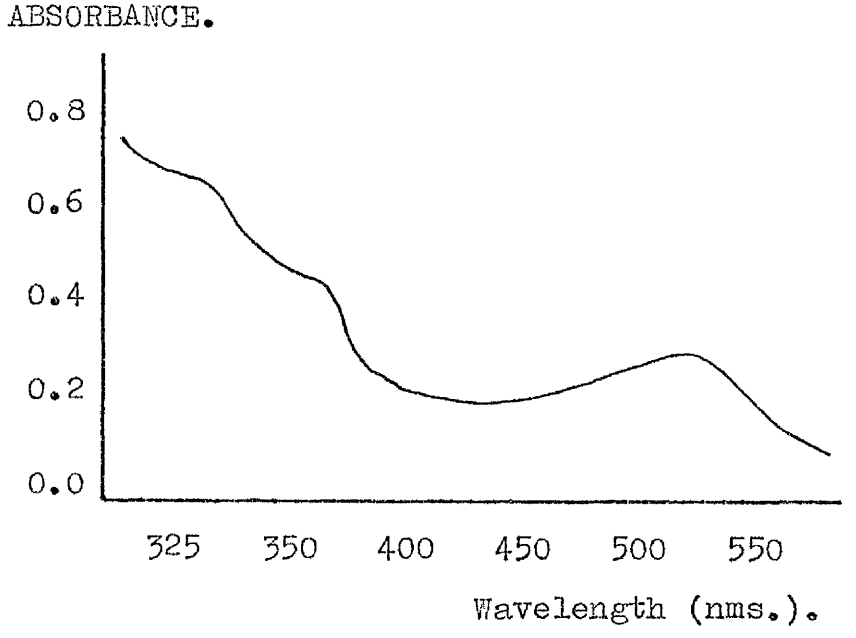


Fig.22 Ultra-Violet Absorption Spectrum of Methyl B_{12}.

CHAPTER 4

BICARBONATE BUFFER

SECTION 1

pH CONTROL.

As the formation of sulphito B_{12} from hydroxo B_{12} involved the conversion of a basic cobalamin to an acidic cobalamin, the effect of the pH on such a reaction was clearly critical. Studies on the effect of pH on the conversion of hydroxo B_{12} to sulphito B_{12} were therefore undertaken.

It was recognised from the outset that, although maintenance of a constant pH was possible in all the aqueous phases during cobalamin extractions by the use of a buffer system, pH control would be lost whenever the organic solvents were employed. Another difficulty with such a technique lay in the fact that most tissue extracts of cobalamins were required in a small final aqueous volume (~ 1 ml.) to yield an extract of sufficient cobalamin concentration for bioautography, and the replacement of these aqueous phases with an effective buffering solution might lead to precipitation of buffer salts in the final rotary evaporation step.

Buffer systems were chosen which were likely to be inert with respect to reactivity with the standard cobalamins so far encountered, namely coenzyme B_{12}, cyano B_{12}, hydroxo B_{12}, methyl B_{12} and sulphito B_{12} and an extraction carried out on a standard hydroxo B_{12} solution prepared in the alkaline buffer.

MATERIALS & METHODS.

To determine the effect of pH on the conversion, hydroxo B_{12} was added to buffer solutions and the forms of vitamin B_{12} present after six days determined.

Buffer systems used were :-

An acetic acid/NaOH buffer at pH 4.8, by the addition of 73.8 mls. of acetic acid (mol./l.) to 50 mls. of NaOH (mol./l.) and dilution to one litre. It was noted at this stage that the acetate group might not be inert with respect to the standard cobalamins and could react either through the alkyl or acyl group to produce carboxymethyl-cobalamin ($Co{-}{-}CH_2COOH$) or acetylcobalamin ($CH_3.CO{-}{-}Co$) respectively, both of which on photolysis yield acetic acid (Johnson et al., 1963a; Bernhauer and Irion, 1964b).

A phosphate buffer at pH 7.0 by the addition of 38.2 mls. of KH_2PO_4 (0.5 mol./l.) to 25.8 mls. of NaOH (0.5 mol./l.) and diluted to one litre.

A bicarbonate buffer at pH 9.6 by the addition of 33 mls. of $NaHCO_3$ (mol./l.) to 8.44 mls. of NaOH (mol./l.) and dilution to one litre.

2 mgms. of hydroxo B_{12} were added to 10 mls. of each buffer and a series of stepwise dilutions carried out to yield hydroxo B_{12} solutions of concentration 36 pgs.B_{12}/µl.

Chromatograms of these solutions together with standards prepared in distilled water were run immediately after preparation and after storage in foiled, air-tight

glass bottles for six days and the resultant bioautograms developed.

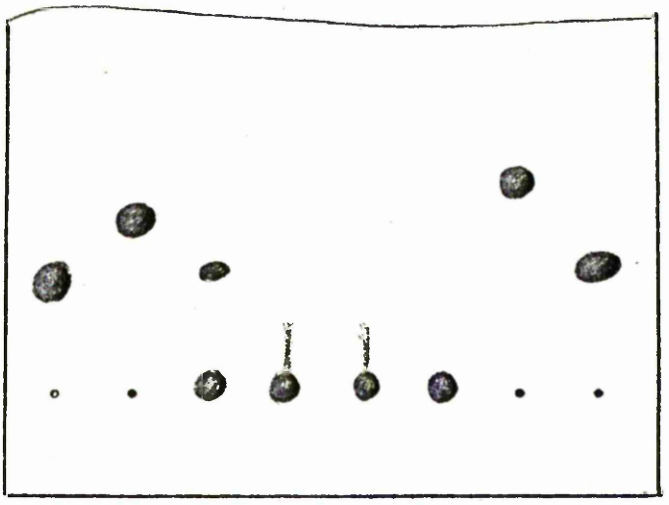
2 mls. of another hydroxo B_{12} solution (28 pgsB_{12}/µl.) prepared in the bicarbonate buffer were diluted to 50 mls. with the buffer before addition to ethanol (100 mls.) and refluxed at 82°C for 30 mins. After extraction by the standard procedure the hydroxo B_{12} was back-extracted from the phenol phase into 50 mls. of the bicarbonate buffer and concentrated by rotary evaporation.

Solutions of the standard cobalamins prepared in the bicarbonate buffer, pH 9.6, were run on a silica gel chromatogram with standards prepared in distilled water. The chromatogram was developed and the growth zones obtained from bioautography compared.

RESULTS & DISCUSSION.

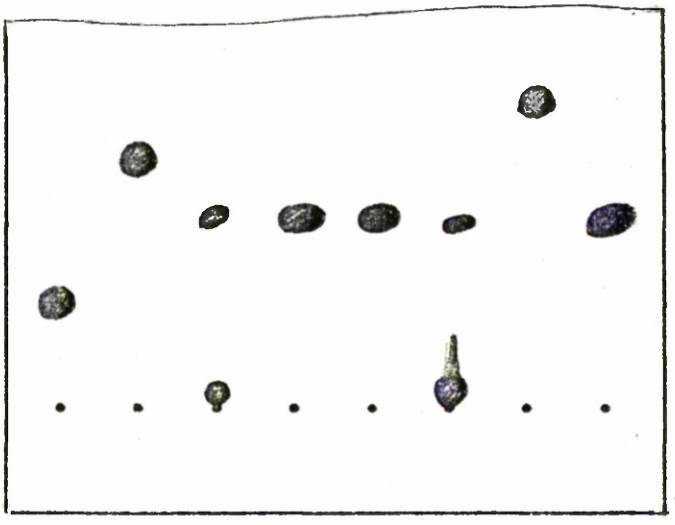
It could be seen from bioautogram, fig. 23 , that even when applied directly to a silica gel sheet some conversion of the hydroxo B_{12} standards at pH 4.8 and 7.0 was evident from the degree of tailing on their bioautographic growth zones. As the pH of the solvent medium fell below 7.0 as expected the rate of conversion of hydroxo B_{12} to sulphito B_{12} increased, until after six days, (fig. 24) both hydroxo B_{12} solutions prepared in phosphate buffer, pH 7.0 and acetate buffer, pH 4.8 were completely converted to sulphito B_{12}.

The hydroxo B_{12} solution prepared in bicarbonate



pH :			4.8	7.0	9.6			
B_{12}.Form:	Ado	CN	OH	OH	OH	OH	Me	SO_3
pgs.B_{12} :	70	70	70	72	72	72	70	70

Fig. 23 Bioautogram of Hydroxo B_{12} Samples Prepared at Different pH Values and Examined Immediately After Preparation.



pH :			4.8	7.0	9.6			
B_{12}.Form:	Ado	CN	OH	OH	OH	OH	Me	SO_3
pgs.B_{12} :	70	70	70	72	72	72	70	70

Fig. 24 Bioautogram Of Hydroxo B_{12} Samples Examined After Storage for 6 Days.

buffer at pH 9.6 also yielded a sulphito B_{12} growth zone after six days despite storage in an airtight container implying that the buffer did not completely neutralise the bisulphite ions produced on dissolution of atmospheric sulphur dioxide;

$$SO_2 \cdot xH_2O \longrightarrow HSO_3^- \begin{cases} \xrightarrow{OH.B_{12}} SO_3 \cdot B_{12}^{-1-} \\ \xrightarrow[NaHCO_3]{} NaHSO_3 + HCO_3^- \end{cases}$$

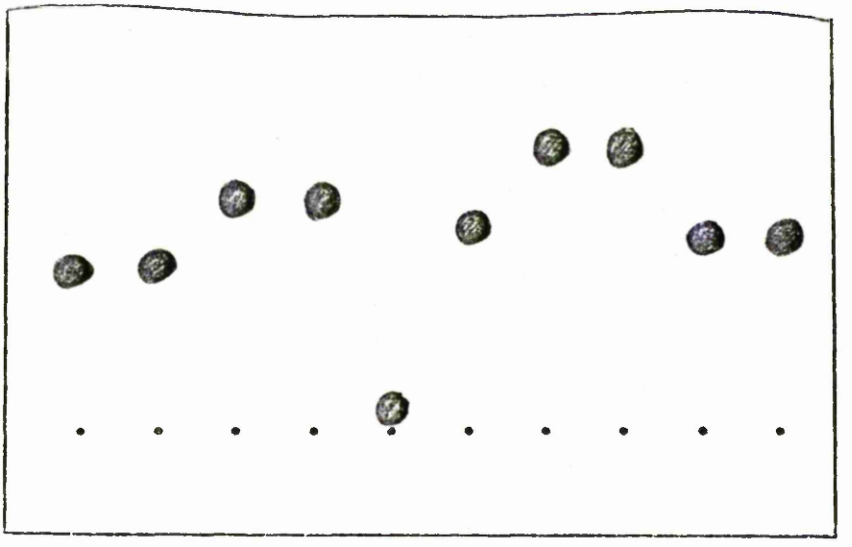
beforeconversion of part of the hydroxo B_{12} present.

These findings were in agreement with the observations that a hydroxo B_{12} standard prepared in freshly distilled water, pH 6.8 ± 0.2 gave rise initially to only one growth zone at the origin on bioautograms, whereas after storage a second zone corresponding to sulphito B_{12} was obtained, until eventually total conversion to sulphito B_{12} occurred.

From fig. 25 , it was apparent that the bicarbonate buffer had no effect on the other standard cobalamins, which were found at their normal positions on the bioautogram.

As expected during the extraction of the standard hydroxo B_{12}, precipitation of the buffer salt, $NaHCO_3$ occurred after a fifty-fold concentration of the final buffer phase had reduced the volume to ca. 1 ml. This precipitation made difficult isolation of a suitable final extract and modification of this technique was essential if it was to be of practical value.

The bicarbonate buffer, pH 9.6 however, seemed to be effective in the short term (<6 days) prevention of formation of artefactual Sulphito B_{12} and its usage was further investigated.



pH :	9.6	7.0	9.6	7.0	9.6	7.0	9.6	7.0	9.6	7.0
B_{12}.Form:	Ado	Ado	CN	CN	OH	'OH'	Me	Me	SO_3	SO_3
pgs.B_{12} :	70	100	70	100	70	100	70	100	70	100

Fig. 25 Bioautogram of the Standard Cobalamins in Bicarbonate Buffer at pH 9.6.

SECTION 2

Ultrafiltration.

To prevent the salt precipitation in the final aqueous extracts, it was decided to employ ultrafiltration in a concentration/desalting stage.

By using an appropriate membrane it seemed possible to ultrafilter the final cobalamin extracts in bicarbonate buffer (I = 0.01), with retention of the cobalamins present and passage of the low molecular weight salts (M.W. <500) through the membrane into the ultrafiltrate.

In order to obtain an estimate of the desalting efficiency of the ultrafiltration process, the conductivities (C) of the solutions, concentrate and ultrafiltrate, had to be related to their corresponding ionic strengths (I) by a standard curve of resistance (1/C) against ionic strength.

To estimate the ionic strength contribution to the final aqueous extract of the salts isolated in tissue extracts, an extraction was carried out on a standard skeletal muscle tissue received, homogenised and extracted in distilled water. A solution of ionic strength and cobalamin content ($\simeq$50 ngms.B_{12}) comparable with that of a final aqueous phase from a standard tissue extract was produced, to obtain the combined desalting and cobalamin retention efficiencies of the ultrafiltration process.

Measurements of the cobalamin retention were made using radioactive $^{57}Co.CNB_{12}$ with an activity which yielded a count rate in excess of 100 c.p.s.

MATERIALS & METHODS.

A range of salt solutions of ionic strengths, I = 10^{-1}.... 10^{-6} was prepared and the conductivity of each measured on a 'Griffin' Conductance Bridge between standard platinum electrodes at 20 ± 1°C. A calibration curve of log (1/C) against -log I was constucted, fig.26 from the results obtained in the following table:-

Ionic Strength		Resistance	
(I)	-log I	(1/C)(ohms) :	log (1/C)
10^{-1}	1	0.17×10^{3}	2.23
10^{-2}	2	0.14×10^{4}	3.15
10^{-3}	3	0.11×10^{5}	4.04
10^{-4}	4	0.11×10^{6}	5.04
10^{-5}	5	0.67×10^{6}	5.83
10^{-6}	6	0.25×10^{7}	6.40

Table 8 Resistances of NaCl Solutions Measured at 19°C.

A gigot chop homogenate containing 5 gms. of tissue was extracted in distilled water and the conductivity of the final aqueous phase (50 mls.) measured. The reading was made at 20°C, corrected for the distilled water conductivity and the corresponding equivalent salt concentration derived from the previous calibration curve, fig. 26 .

A range of bicarbonate buffer solutions of varying ionic strengths, $I = 10^{-1} \ldots 10^{-3}$, was prepared and the conductivities of each measured as before to yield a standard calibration curve of log (1/C) against -log I, fig. 27 , from the results obtained in the following table:-

Ionic Strength.		Resistance.	
(I)	-log I	(1/C)(ohms)	log (1/C)
10^{-1}	1	0.28×10^{3}	2.45
5×10^{-2}	1.30	0.50×10^{3}	2.70
10^{-2}	2	0.23×10^{4}	3.36
5×10^{-3}	2.30	0.40×10^{4}	3.60
10^{-3}	3	0.20×10^{5}	4.30

Table 9 Resistances of Bicarbonate Buffer Solutions Measured at 20°C.

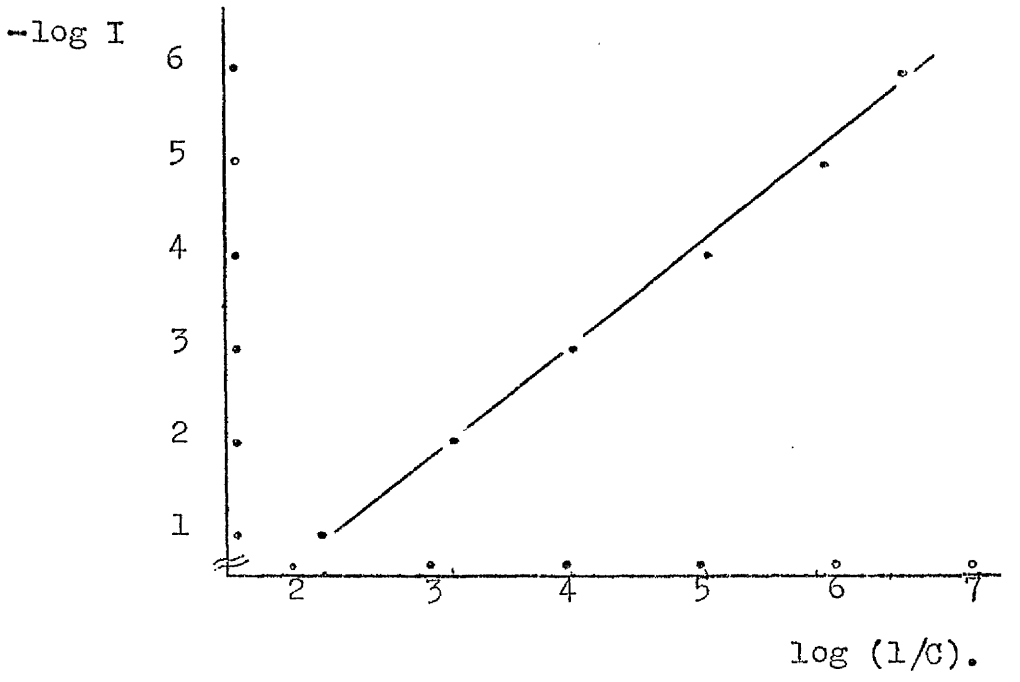


Fig. 26 Calibration Curve of log (1/C) against -log I for a series of Salt (NaCl) Solutions.

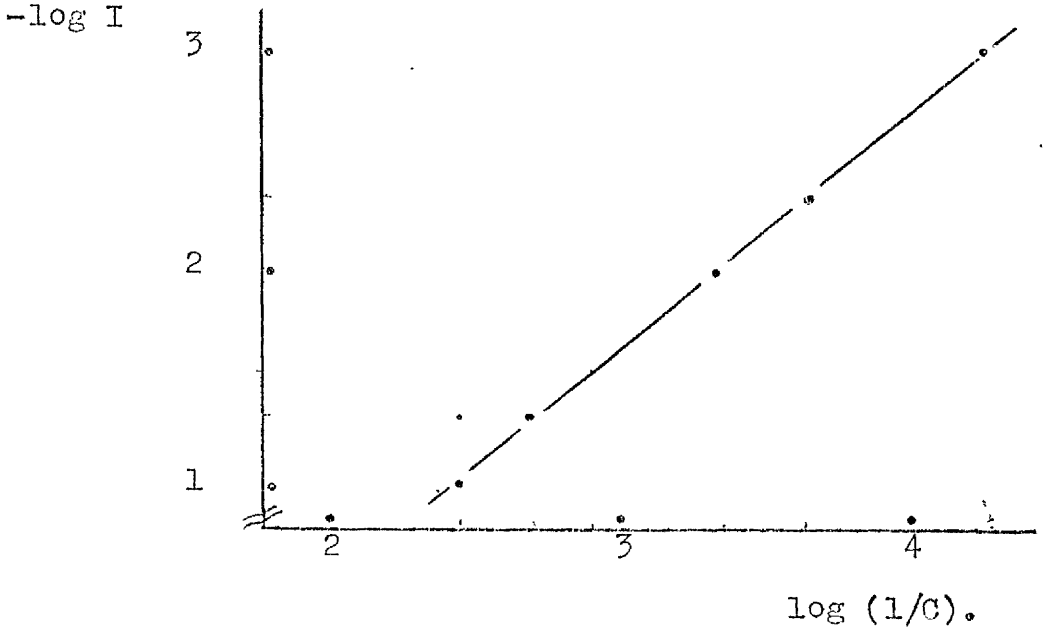


Fig. 27 Calibration Curve of log (1/C) against -log I for a series of Bicarbonate Buffer Solutions.

5 mls. of a radioactive solution, 10 ngms.CNB_{12}/ 8 n.Ci/ml., were added to 50 mls. of bicarbonate buffer and the activity and conductivity of the solution measured. The activity was measured using the well-type scintillation system described on p.41 , except that the scaler Model 1700 was replaced by a 'Nuclear Enterprise' Counter Ratemeter Model MS 310 and all samples counted for 100 secs.

The solution was transferred to an 'Amicon' Model 52 ultrafiltration cell and using a UM-05 membrane an ultrafiltration was carried out under a nitrogen pressure of ~5×10^5 N/m^2 (~70 p.s.i.) at a rate of 20 mls./hr. The ultrafiltrate was collected until the volume of the solution had been reduced to ca. 5 mls., when the pressure was released and radioactivity and conductivity measurements made on both the concentrate and ultrafiltrate. Using the conductivity results, the corresponding ionic strengths were obtained from the calibration curve, fig 27 .

RESULTS & DISCUSSION.

The conductivity of the gigot chop extract final aqueous phase of 4.2×10^{-5} siemen represented an ionic strength, $I = 3.2 \times 10^{-4}$ and thus such an ionic strength contribution to the overall conductivity of the bicarbonate buffer, where I = 0.01, was negligible and could be discounted.

The efficiency of the ultrafiltration process was found from the conductivity and radioactivity results (table 10), which showed that a ten-fold reduction in volume of the original bicarbonate buffer solution (55.0 mls. to 5.5 mls.), was accompanied by a two-fold increase in the ionic strength of the concentrate and thus desalting by ultrafiltration was very efficient and much faster than by dialysis. From the activities however, a significant loss of 20% of added $^{57}Co.CNB_{12}$ was recorded in the ultrafiltrate with 80% retention of the cobalamin in the concentrate.

Despite this loss of cobalamins the ultrafiltration step was incorporated into future extractions using the bicarbonate buffer, to allow for the reduction of the final aqueous phase to a volume $\leqslant 1$ ml. without salt precipitation.

PHASE.	CONDUCTIVITY ≡ I (siemen)		ACTIVITY (c.p.s.)	%'AGE OF TOTAL ACTIVITY.
Initial Sol'n.	2.0×10^{-3}	0.050	263.1	100
Ultrafiltrate	1.3×10^{-3}	0.032	54.0	20.5
Concentrate	4.5×10^{-3}	0.112	214.0	81.3

Table 10 Conductivity and Radioactivity Measurements of an Ultrafiltered Bicarbonate Buffer Solution Containing $^{57}Co.CNB_{12}$.

SECTION 3

Alkaline Hydrolysis.

To determine whether a combination of high temperature, as experienced during ethanol reflux (>80°C) and alkaline pH would have a serious destructive effect on the cobalamins in the bicarbonate buffer, pH 9.6, these were studied at 85°C.

Studies of the alkaline hydrolysis of vitamin B_{12} had been reported by Armitage et al., (1953) and Bonnett et al., (1957) and cold dilute alkali appeared to give the same red acid products as mild acid hydrolysis. Brief boiling with alkali in the presence of air however, gave a neutral crystallisable red substance which was microbiologically inactive. The structure of the compound was elucidated mainly by X-ray crystallography and appeared to be 'dehydrovitamin B_{12}', with a lactam ring arising from the acetamide chain fused to ring B, as shown in the following diagram:-

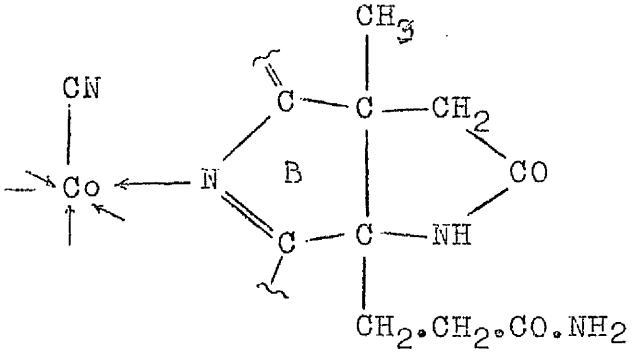


Fig. 28 Lactam from Cyano B_{12} - 'Dehydrovitamin B_{12}'.

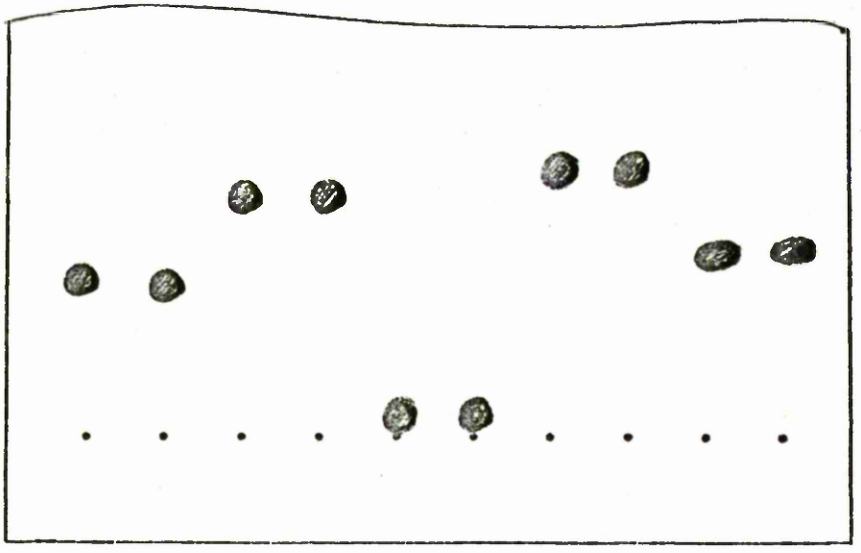
Cyano B_{12} and hydroxo B_{12} are both about 90% inactivated by one hour at 100°C at pH 8 (Frost et al., 1952 and Hartley et al., 1950) and indeed heating in strongly alkaline solution has been used as a means of quantitatively destroying vitamin B_{12} for 'blank' determinations in microbiological assays.

The 'dehydrovitamin B_{12}' formed in hot alkali being microbiologically inactive is therefore not detected on bioautography. This analytical technique is at best semi-quantitative, but if hydrolysis was significant (>25%), then it was felt that losses would be readily detected visibly, by the resultant reduction in E. coli growth responses.

5 mls. of each of the standard cobalamin solutions (35 pgs.B_{12}/µl.) prepared in the bicarbonate buffer were heated at 85°C in 20 ml. foiled, autoclavable glass bottles for 30 mins. A chromatogram of these sterilised solutions together with standards (35 pgs.B_{12}/µl.) stored at room temperature was run and developed by bioautography.

Visual interpretation of the bioautogram, fig 29 , showed no apparent difference between the growth areas for the sterilised and non-sterilised cobalamins. Although at this time no quantitative assay was available which could differentiate between the dehydrovitamin and the other cobalamins, it was evident that inactivation of

cyano B_{12} and hydroxo B_{12} by lactam ring formation, if occurring, was much less extensive at 85°C than previously found at 100°C.



Temp.(°C)	:	-	85	-	35	-	35	-	35	-	85
B_{12}.Form	:	Ado	Ado	CN	CN	OH	OH	Me	Me	SO_3	SO_3
pgs.B_{12}	:	70	70	70	70	70	70	70	70	70	70

Fig. 29 Bioautogram of the Standard Cobalamins Sterilised at 85°C for 30 mins. in Bicarbonate Buffer, pH 9.6.

SECTION 4

Buffer Efficiency.

To test the efficiency of the bicarbonate buffer, pH 9.6, in preventing the conversion of hydroxo B_{12} to sulphito B_{12}, extractions were carried out on a hydroxo B_{12} standard, a braised steak homogenate and a sample of bottled, whole milk which should contain hydroxo B_{12} through its light-exposure prior to addition to the bicarbonate buffer. The use of skeletal muscle tissue and milk ensured that the buffer was tested in the varying conditions in which protein bound vitamin B_{12} was found in foodstuffs and which would later be encountered in tissue extractions.

MATERIALS & METHODS.

2 mls. of a hydroxo B_{12} solution (28 pgs.B_{12}/µl.) prepared in the bicarbonate buffer were added to a further 50 mls. of buffer, refluxed in ethanol (100 mls.) and extracted by the standard procedure. The final aqueous buffer phase (50 mls.) was rotary evaporated to 20 mls., reduced to 5 mls. by ultrafiltration using the UM-05 membrane and further evaporated to 1 ml.

A braised steak homogenate prepared in bicarbonate buffer was divided into aliquots each containing 5 gms. of tissue. The cobalamins were extracted and a final aqueous buffer extract (50 mls.) rotary evaporated to 15 mls. and reduced to 3 mls. by ultrafiltration as

before. This was again evaporated to ca. 1 ml. in a 10 ml. round-bottom flask and stored in a foiled container at -20°C.

8 mls. of bottled, whole milk, collected without light precautions, were added to 40 mls. of bicarbonate buffer and extracted by the standard procedure. The final buffer phase (50 mls.) was ultrafiltered to 10 mls. and reduced to 1 ml. by evaporation. The hydroxo B_{12}, braised steak and milk extracts were run with cobalamin standards on a silica gel chromatogram and developed by bioautography.

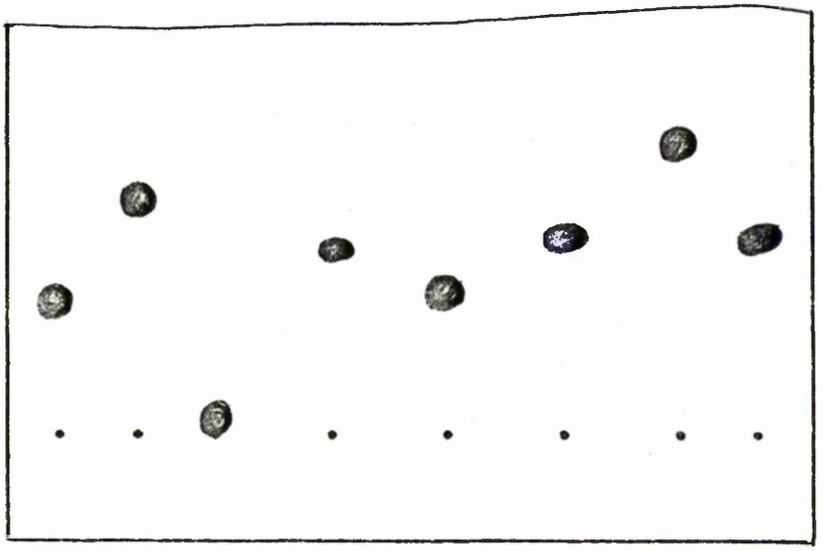
RESULTS & DISCUSSION.

With the insertion of the ultrafiltration step, on no occasion did rotary evaporation to ca. 1 ml. lead to buffer salt precipitation.

The bioautogram, fig 30 , however, demonstrated the inability of the bicarbonate buffer to prevent the conversion of the hydroxo B_{12} to sulphito B_{12}, as total conversion of the hydroxo B_{12} standard occurred during the extraction presumably when regulation of an alkaline pH was lost in the organic solvents.

The braised steak extract yielded only coenzyme B_{12} and thus no information of artefactual sulphito B_{12} was possible.

Like the hydroxo B_{12} extract, the whole milk extract contained only sulphito B_{12} implying that maintenance of an alkaline pH, by the use of the bicarbonate buffer in



pH :	9.6	9.6	9.6	9.6	9.6	9.6	9.6	9.6
B_{12}.Form:	Ado	CN	OH	OH-Extr.	STEAK	MILK	Me	SO_3
pgs.B_{12} :	70	70	70	44	100	96	70	70

Fig.30 Bioautogram of Hydroxo B_{12}, Braised Steak and Bottled, Whole Milk Extracts Obtained from Extractions Using the Bicarbonate Buffer, pH 9.6, in the Aqueous Phases.

all the aqueous phases in the extraction, was insufficient to prevent the conversion of hydroxo B_{12} to sulphito B_{12}. This finding did not however exclude the presence of 'genuine' sulphito B_{12} in milk. Although light-exposed the high fat content of the milk could have reduced the light penetration and thereby the conversion of light sensitive cobalamins present, such as sulphito B_{12} to hydroxo B_{12}.

SECTION 5

Stage of Hydroxo B_{12} to Sulphito B_{12} Conversion.

In order to determine the step in the extraction procedure at which the conversion of hydroxo B_{12} to sulphito B_{12} took place it was decided to withdraw samples for chromatography at various intervals in the extraction and determine the extent of conversion at these stages by subsequent bioautographic analysis. Such knowledge would permit modification of the extraction to reduce or prevent conversion.

MATERIALS & METHODS.

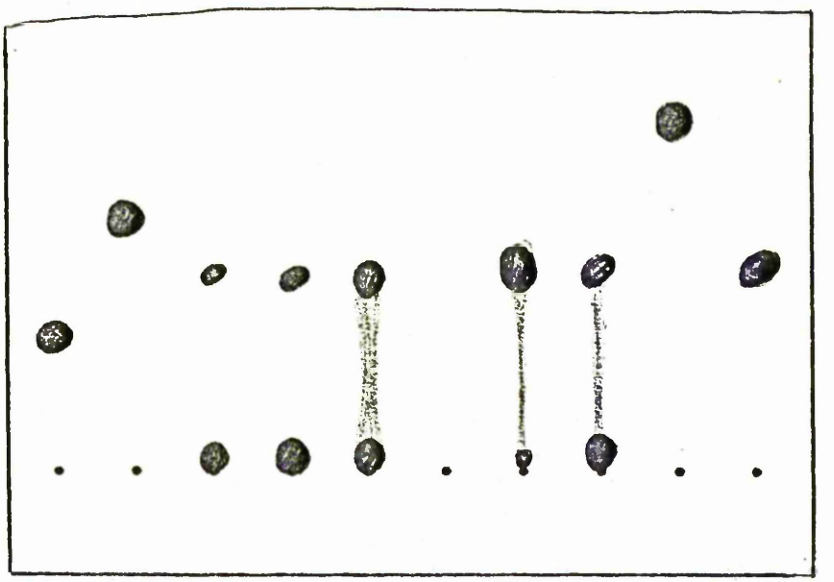
10 mls. of a hydroxo B_{12} solution (100 pgs.B_{12}/µl.) freshly prepared in bicarbonate buffer, pH 9.6 and stored overnight for 16 hrs., were added to a further 40 mls. of buffer and refluxed in ethanol (100 mls.) at 82°C for 30 mins. The solution was cooled, filtered and rotary evaporated to 25 mls. at 40°C and 5.3×10^3 N/m^2 pressure (40 mmHg).

2.5 mls. of this extract were removed, 2 µls. spotted directly onto a silica gel chromatogram and the remainder stored in a foiled container. The rest of the aqueous phase (22.5 mls.) was shaken with phenol/chloroform (2 x 25 mls.) to extract the cobalamins present and 4 µls. of this phenol phase spotted onto the chromatogram. The cobalamins were then back-extracted into an aqueous bicarbonate buffer phase (50 mls.) which after shaking

with ether (20 mls.) was rotary evaporated to ca. 12 mls. 2 µls. of this phase were spotted onto the chromatogram with a further 2 µls. of the initial hydroxo B_{12} solution now sampled 22 hours after preparation. As before the chromatogram was run and developed by bioautography.

RESULTS & DISCUSSION.

From the bioautogram, fig.31 , it was evident that some degree of conversion of hydroxoB_{12} to sulphito B_{12} had occurred in the starting material after storage for 16 hours. This material did however contain predominantly hydroxo B_{12} prior to its use, but as the extraction proceeded the percentage of hydroxo B_{12} present decreased with a corresponding increase in sulphito B_{12}. This was presumed to be mainly due to the inability to maintain an alkaline pH in the system during extraction into the organic phenol/chloroform phase, in which the phenol ($pK_a 10^{-10}$) was responsible for the pH $\simeq$ 3.0 attained. This acidic pH would lead to the protonation of hydroxo B_{12} to aquo B_{12} and the fast reaction with any sulphite ion present to form sulphito B_{12}. The aqueous ethanol extract was found to contain about equal proportions of hydroxo B_{12} and sulphito B_{12}, while the final aqueous phase was composed almost entirely of sulphito B_{12}. The phenol extract applied to the chromatogram did not give rise to any bacterial growth presumably as a result of the bacteriostatic



Extract :				1	2	3	4	5		
B_{12}.Form:	Ado	CN	OH						Me	SO_3
pgs.B_{12} :	50	50	50	100	<71	<74	<148	100	50	50

Fig.31 Determination of the Stages of Maximum Conversion of Hydroxo B_{12} to Sulphito B_{12} - Bioautogram of Extracts from Several Phases.

EXTRACTS.

1. Hydroxo B_{12} Standard ; 16 hrs. after preparation.
2. Aqueous Ethanol Concentrate - 2 µls. applied.
3. Phenol Phase - 4 µls. applied.
4. Final Aqueous Phase - 2 µls. applied.
5. Hydroxo B_{12} Standard ; 24 hrs. after preparation.

effect of phenol.

The extent and ease of occurrence of artefactual sulphito B_{12} throughout the previous procedure ruled out modification of the extraction technique in an attempt to prevent conversion. The use of the bicarbonate buffer was therefore discontinued and further studies in the field of prevention of artefact formation by pH control were not undertaken.

CHAPTER 5

AMMONIA BUFFER.

SECTION 1

Preconversion of Hydroxo B_{12}.

The affinity of aquo B_{12} for the sulphite ligand was very strong and in the dark a ligand exchange or substitution reaction occurred,

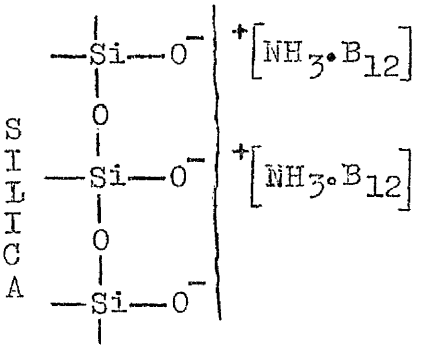
$$\left[H_2O.B_{12}\right]^+ + SO_3^{2-} \overset{K}{\rightleftharpoons} \left[SO_3.B_{12}\right]^- \quad ;$$

where K, the stoichiometric equilibrium constant is 2.2×10^7 and $\log K = 7.3$ (Firth et al., 1969) for

$$K = \frac{\left[SO_3.B_{12}\right]^-}{\left[H_2O.B_{12}\right]^+ \left[SO_3\right]^{2-}} \quad ;$$

The constant K is expressed in units of $(mol./l.)^{-1}$ and we found that the above equilibrium was attained almost instantaneously. This consideration suggested that a more rewarding approach to the prevention of artefactual sulphito B_{12} formation than control of pH would be stabilisation of hydroxo B_{12} by its conversion in tissues to a form which would be resistant to attack by acid anions. The possibility that this could be achieved arose from the knowledge that in the chromatographic solvent system containing ammonia, hydroxo B_{12} was converted to ammonia B_{12} (Bilkus and Mervyn, 1971, p.20) and that the equilibrium constant for the ligand substitution, K was 10^7 and $\log K = 7$ (Hayward et al., 1971) and therefore formation of ammonia B_{12} was very favourable.

Consideration of the chemistry of ammonia B_{12} showed that it would be stable in weak acid (pH~>3) and only rendered unstable with respect to aquo B_{12} and the ammonium ion at pH <0 (Pratt, 1964.) an acid strength greatly exceeding those encountered in the cobalamin extraction procedure. The basicity of ammonia B_{12} was greater than that of hydroxo B_{12}, pK 6.9 - 7.8 (Buhs et al., 1951 and Hayward et al., 1965) and as such it did not travel on the acidic silica gel during chromatography;



A theoretical objection to the principle of converting hydroxo B_{12} to ammonia B_{12} was that any ammonia B_{12} in foodstuffs could not be identified as such but would be submerged in the sum of native ammonia B_{12} and ammonia B_{12} converted from hydroxo B_{12}. This objection was accepted in the knowledge that when a chromatographic solvent system containing ammonia was employed the conversion occurred as a matter of course.

If conversion of hydroxo B_{12} to ammonia B_{12} occurred with an ammonia treatment then prevention of formation of sulphito B_{12} would not only be assured but further

use of the ammonia in the extraction procedure would not be necessary and no salt precipitation problems would be encountered during the final rotary evaporation step as had occurred with the bicarbonate buffer. For these reasons it was decided to employ an ammonia buffer to receive and homogenise the tissues being examined.

MATERIALS & METHODS.

An ammonia/ ammonium chloride buffer, pH 9.6 was prepared by the addition of 139 mls. of ammonia solution (mol./l.) to 50 mls. of HCl (mol./l.) and the volume made up to 1 litre. To determine the effect of the ammonia buffer on the standard cobalamins (coenzyme B_{12}, cyano B_{12}, hydroxo B_{12}, methyl B_{12} and sulphito B_{12}), each was prepared in the buffer at a conc. of 35 pgs./µl. At the same time a hydroxo B_{12} solution which had been previously prepared in bicarbonate buffer, pH 9.6, was exposed to daylight for 40 mins. before use to ensure that any artefactual sulphito B_{12} present was reconverted to hydroxo B_{12}.

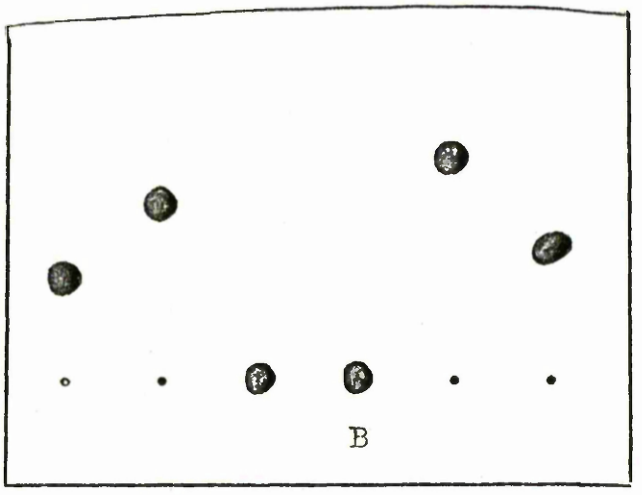
Two identical silica gel chromatograms were spotted with 2 µls. of each of the above standards, one chromatogram developed in the standard ammonia containing solvent system and the other chromatogram in the solvent system without ammonia. Although it had been previously found that the latter system did not clearly separate the cobalamins (see fig. 7), its use was essential to establish the relative positions of the hydroxo B_{12} prepared in the bicarbonate and in the ammonia buffers and hence show whether conversion of hydroxo B12 to ammonia B_{12} had occurred in the latter. The bioautograms were developed by the standard procedure.

RESULTS & DISCUSSION.

From the bioautogram, fig.32 , developed from the chromatogram run in the ammonia containing solvent system, both the hydroxo B_{12} standards prepared in the ammonia and in the bicarbonate buffers gave rise to a single growth zone at the origin. The other cobalamins prepared in the ammonia buffer also appeared at their standard positions on the bioautogram.

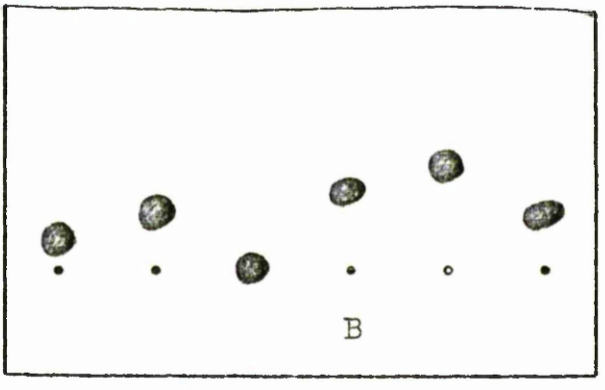
The chromatogram run in the solvent system without ammonia gave poor separation of the cobalamins, but it was evident from the bioautogram, fig.33 , that the hydroxo B_{12} prepared in the ammonia buffer gave rise to a single growth zone at the origin, while the hydroxo B_{12} standard in the bicarbonate buffer travelled on the silica to a position between cyano B_{12} and methyl B_{12}. Again the positions and areas of growth zones for the standard cobalamins prepared in the ammonia buffer implied that they were unaffected either qualitatively or quantitatively by this treatment.

These findings indicated that the hydroxo B_{12} prepared in the ammonia buffer was indeed converted to ammonia B_{12}, which remained at the point of application during chromatography on the acidic silica surface, while hydroxo B_{12} itself as shown by the standard prepared in the bicarbonate buffer, travelled from the origin during chromatography.



B_{12}.Form:	Ado	CN	OH	OH	Me	SO_3
pgs.B_{12} :	70	70	70	70	70	70

Fig. 32 Bioautogram of the Standard Cobalamins Prepared in Ammonia Buffer, pH 9.6 and Separated in the Ammonia Containing Solvent System; Together with OH.B_{12} (B) Freshly Prepared in Bicarbonate Buffer, pH 9.6.



B_{12}.Form:	Ado	CN	OH	OH	Me	SO_3
pgs.B_{12} :	70	70	70	70	70	70

Fig. 33 As with Fig. 32 Except the Cobalamins were Separated in the Solvent System Without Ammonia.

The ammonia buffer, pH 9.6, appeared to be efficient at protecting hydroxo B_{12} from attack by sulphite ion by converting it to ammonia B_{12} and since it had no effect on the other cobalamins its use was further investigated.

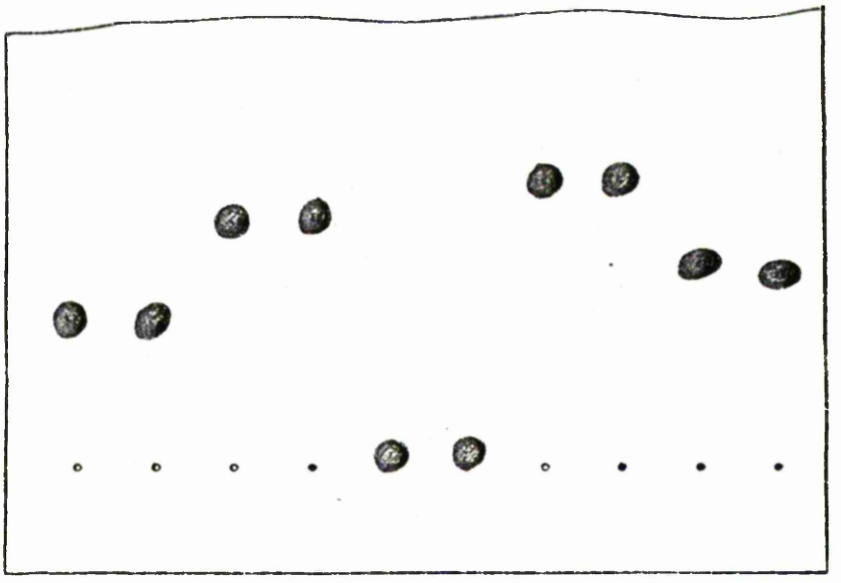
SECTION 2

Alkaline Hydrolysis.

As with the bicarbonate buffer of pH 9.6, determination of the effect on the cobalamins of high temperature and alkaline pH, using the ammonia buffer was studied. If a combination of temperature and pH conditions led to the formation of the 'dehydrovitamin B_{12}' during ethanol reflux at 80+°C in the ammonia buffer at pH 9.6, then the resultant loss of microbiological activity might be detectible on bioautography.

5 mls. of each of the standard cobalamins prepared in the ammonia buffer were heated in 20 ml. autoclavable glass bottles at 85°C for 30 mins. A chromatogram of these sterilised solutions together with standards stored at room temperature was run and developed by bioautography.

As found with the bicarbonate buffer, pH 9.6, (fig.29) the bioautogram obtained (fig.34) showed no apparent difference between the growth zones for the sterilised and non-sterilised cobalamins in ammonia buffer.



Temp.(°C) :	35	-	35	-	35	-	35	-	35	-
B_{12}.Form. :	Ado	Ado	CN	CN	OH	OH	Me	Me	SO_3	SO_3
pgs.B_{12} :	70	70	70	70	70	70	70	70	70	70

Fig.34 Bioautogram of the Standard Cobalamins in Ammonia Buffer, pH 9.6 : Sterilised at 35°C for 30 mins.

SECTION 3

Ammonia Buffer Efficiency.

It was now necessary to establish whether the ammonia buffer could convert the hydroxo B_{12} released from tissues during ethanol reflux and whether once formed the ammonia B_{12} could be extracted efficiently and without conversion.

Bottled, whole milk previously found to contain only sulphito B_{12} (fig. 30) despite constant exposure to light, was used in extractions with the ammonia buffer to establish whether the sulphito B_{12} had occurred as an artefact. Extractions were carried out on aliquots of whole milk which had been added to ammonia buffer either directly to the buffer in daylight or after removal to the darkroom for several minutes, an interval which might allow for some conversion of hydroxo B_{12} present to sulphito B_{12}.

MATERIALS & METHODS.

4 mls. of a hydroxo B_{12} solution (18 pgs. $B_{12}/\mu l.$) prepared in ammonia buffer were added to 20 mls. of the buffer and refluxed in ethanol (100 mls.) at 80°C for 30 mins. The subsequent aqueous phases were prepared from a deionised water source, pH 6.2 ± 0.2, and the final aqueous extract stored at -20°C.

After removal to the darkroom for ca. 5 mins., 10 mls. of bottled, whole milk were added to 40 mls. of ammonia

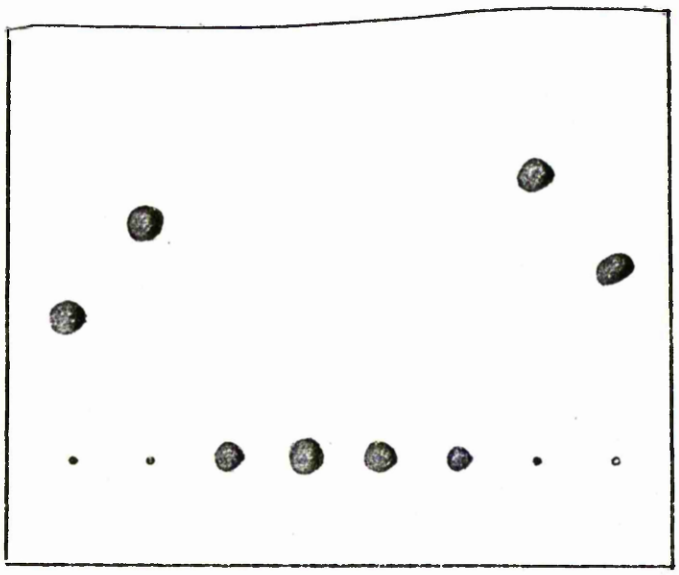
buffer and shaken for 20 mins. in a foiled container to aid the conversion of hydroxo B_{12} present to ammonia B_{12} before reflux. The cobalamin extraction was then carried out, again using the deionised water source.

A further 10 mls. of bottled, whole milk were added in daylight to 40 mls. of ammonia buffer and shaken in a foiled container for 20 mins. before removal to the darkroom. After extraction of the cobalamins, chromatograms of the milk and ammonia B_{12} extracts were run and developed by bioautography.

RESULTS & DISCUSSION.

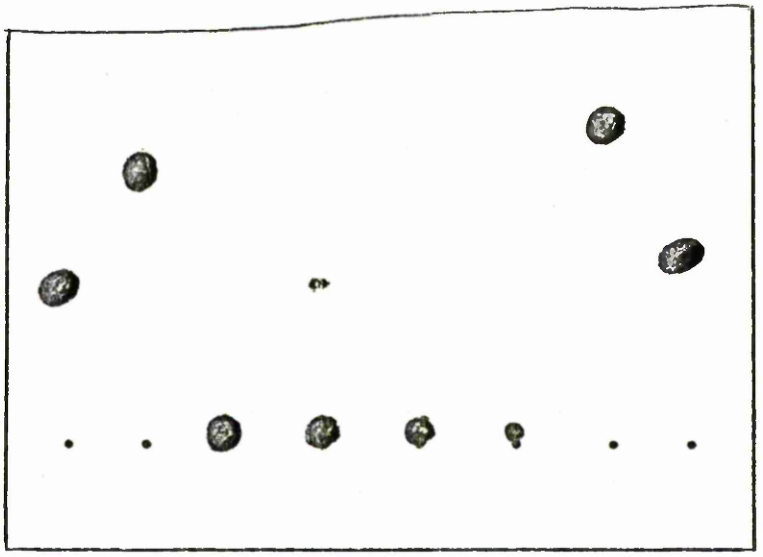
Conversion of the ammonia B_{12} did not occur (fig.35) and areas of growth zones implied that conversion of hydroxo B_{12} to ammonia B_{12} produced a cobalamin which was efficiently extracted and resistant to attack by sulphite ion. In ammonia B_{12} we have produced a cobalamin somewhat more basic than hydroxo B_{12}, however this change in charge distribution about the central cobalt atom had no effect on the solubility and isolation of the ammonia B_{12}.

The appearance of growth zones corresponding to sulphito B_{12} in the extract of whole milk added to the ammonia in the dark (fig.37) seemed to cast doubt on the value of the buffer in the prevention of occurrence of artefactual sulphito B_{12}, as bottled, whole milk exposed for hours to daylight might have been expected to



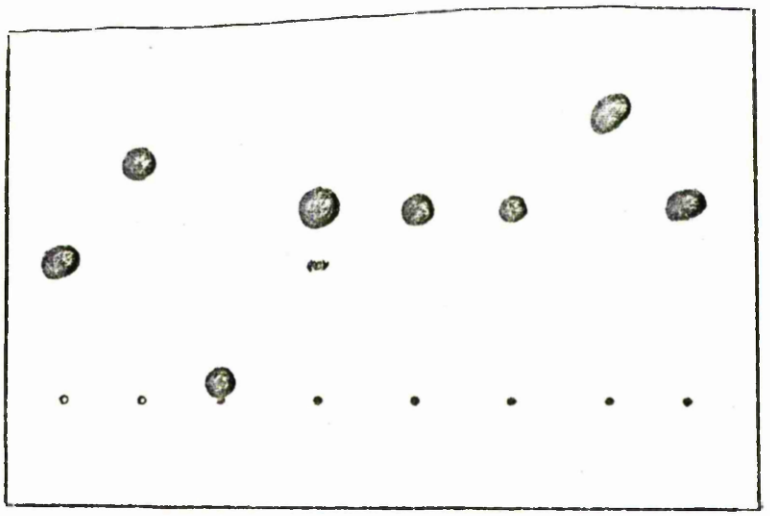
B_{12}.Form:	Ado	CN	OH	'OH' EXTRACT	Me	SO_3
pgs.B_{12} :	36	36	36	<131 <65 <33	36	36

Fig. 35 Bioautogram of 'Hydroxo B_{12}' Standard Extracted After Preparation in Ammonia Buffer, pH 9.6.



B_{12}.Form :	Ado	CN	OH	MILK EXTRACTS			Me	SO_3
pgs.B_{12} :	100	100	100	<133	<67	<34	100	100

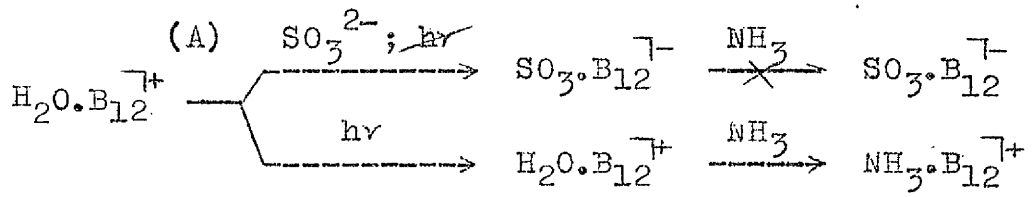
Fig. 36 Bioautogram of Extract of Bottled, Whole Milk, Added to Ammonia Buffer in Daylight.



B_{12}.Form :	Ado	CN	OH	MILK EXTRACTS			Me	SO_3
pgs.B_{12} :	36	36	36	<200	<100	<50	36	36

Fig. 37 Bioautogram of Extract of Bottled, Whole Milk, Added to Ammonia Buffer in the Dark.

contain hydroxo B_{12}. However consideration of the conditions of preparation of the initial milk solution in ammonia buffer and of the kinetics of the ligand substitution involved suggested an explanation for the resultant isolation of sulphito B_{12}. In this extraction, the milk was measured into a flask in the darkroom before being added to the ammonia buffer, allowing sufficient time to elapse for the 'dark' mediated conversion of hydroxo B_{12} to sulphito B_{12} in the presence of sulphite ion :-



Reaction (A) appeared to be virtually instantaneous, which indeed would be the case if the sulphite ion concentration was not a limiting factor on the reaction rate, which was thought to be first-order depending only on the concentration of the initial cobalt complex, ie.

$$\text{Rate} = k \left[H_2O \cdot B_{12}\right]^{+} \quad ;$$

In milk the sulphite ion nucleophile appeared to be derived from indigenous inorganic sulphite ($>10^{-8}$ mol/l) rather than the limited supply provided by dissolution of atmospheric sulphur dioxide.

Bottled, whole milk added to ammonia buffer in the daylight was found to contain only hydroxo B_{12} (fig.36)

showing that in these conditions the buffer converted the hydroxo B_{12} in the milk to ammonia B_{12}, resistant to conversion to sulphito B_{12}. This established conclusively that the vitamin B_{12} present in bottled milk was 'naturally' in the hydroxo B_{12} form when exposed to daylight but reverted to sulphito B_{12} in the dark.

As the cobalamins present in foodstuffs at the time of ingestion were to be considered, addition of the tissue to the ammonia buffer in the daylight before homogenisation would be expected to give the best estimation of cobalamin content, because several workers (Taylor and Weissbach, 1968 & Pailes and Hogenkamp, 1968) have observed that organo-corrinoids can be stabilised towards photolysis when protein bound. This method would also prevent formation of any artefactual sulphito B_{12} by the 'dark' mediated reaction prior to extraction of the cobalamins.

All food homogenates were henceforth prepared in ammonia buffer, pH 9.6 and any sulphito B_{12} isolated regarded as having been present originally in the tissue being examined.

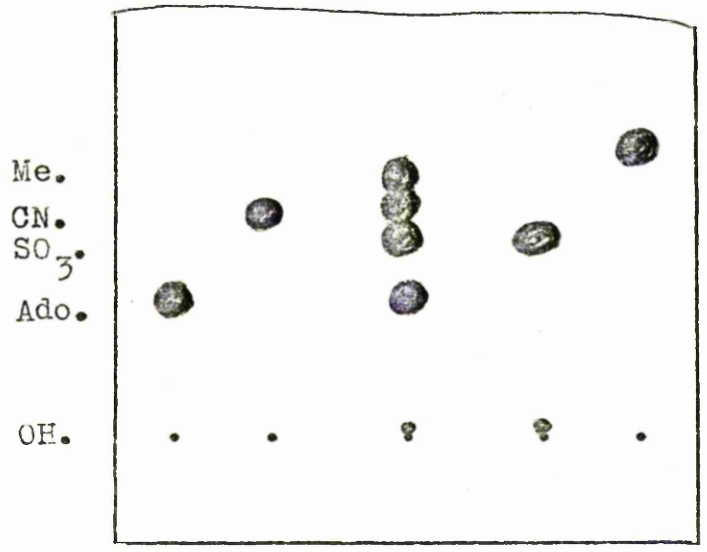
CHAPTER 6

SOLVENT SYSTEMS.

Solvent Systems.

A major difficulty in the form of congestion of growth zones on the bioautograms arose with the presence of sulphito B_{12}. Before its occurrence, coenzyme B_{12} and cyano B_{12} were well separated on chromatography and always gave clear, distinct growth zones when present. The appearance of sulphito B_{12} however, at a position between that of coenzyme B_{12} and cyano B_{12} led to overlap of growth areas and resultant obscuring of the individual cobalamins present. This was first encountered when a mixture of standards of coenzyme B_{12}, cyano B_{12}, hydroxo B_{12} and methyl B_{12} prepared in distilled water was applied to a silica gel chromatogram and run in the standard solvent system before development by bioautography. The hydroxo B_{12} on storage had undergone conversion to sulphito B_{12} and as a result made interpretation of the bioautogram (fig. 38) more difficult by merging of the sulphito B_{12} and cyano B_{12} growth zones.

In an attempt to improve the chromatographic separation and hence resolution of the growth zones a range of solvent systems was prepared for t.l.c. As the cobalamins are most soluble in the lower alcohols (Smith, 1965.) it was decided to employ methanol, ethanol, N.propanol and isopropanol in the solvent systems to increase the R_f values obtained with a view to improving the separation of cobalamins.



B_{12}.Form:	Ado	CN	Standards	'OH'	Me
pgs.B_{12} :	100	100	4 x 50	100	100

Fig. 38 Bioautogram of Cobalamin Standards, Ado.B_{12}, CN.B_{12}, OH.B_{12} and MeB_{12} together with Artefactual SO_3.B_{12} to Illustrate Congestion of Growth Zones.

MATERIALS & METHODS.

Four silica gel chromatograms (20 cm. square) were cut into 32 strips (20 x 2.5 cms.) and 2 µls. of a standard mixture of the five cobalamins were applied to each strip. Each strip was then added to one of the solvent systems (table 11) and the chromatograms run in 100 ml. measuring cylinders containing about 15 mls. of the solvent. The time taken for each run was noted and the chromatograms developed by bioautography.

RESULTS & DISCUSSION.

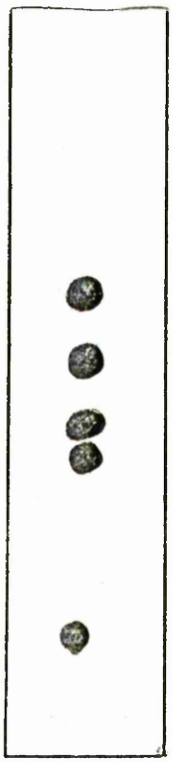
The best separation of cobalamins appeared to be afforded by the standard solvent system, butan-2-ol, n.propanol, water, ammonia (35%) (7:4:3:1, v/v) in fig.39 but good resolution of growth areas was also obtained when butan-2-ol was replaced by n.propanol to yield a solvent system of n.propanol, water, ammonia (10:3:1, v/v) as in fig. 40 . The substitution of a lower alcohol, n.propanol increased the R_f values obtained for all the cobalamins (except hydroxo B_{12} which remained anchored at the origin) and substantially reduced the chromatographic developing time from 6¼ hrs. to 5 hrs.

The separation of the sulphito B_{12}, cyano B_{12} and methyl B_{12} growth zones was clearer in the standard solvent system than in the n.propanol system, where a degree of overlap of the above cobalamins occurred. At this stage the subsequent use of the latter system was

not discounted as it produced greater R_f values, together with reasonable separation of all five cobalamins in a shorter time than the standard system.

The R_f values for the growth zones clearly separated in the other solvent systems were calculated (table 12) and illustrated that none of these systems succeeded in separating more than four of the five cobalamins present.

For these reasons the standard solvent system would subsequently be generally employed and overloading of chromatograms avoided, to ease identification of cobalamins on bioautograms by reducing the overlap of the sulphito B_{12} growth area with either of the coenzyme B_{12} or cyano B_{12} zones.



B_{12}.Form : STANDARDS

pgs.B_{12} : 5 x 25

Fig.39 Bioautogram of the Standard Cobalamins Separated by Chromatography in Butan-2-ol, N.propanol, water, ammonia (7:4:3:1, v/v).

STANDARDS

5 x 25

Fig.40 Bioautogram of the Standard Cobalamins Separated by Chromatography in N.propanol, water, ammonia (10:3:1, v/v).

SOLVENT: (mls.)	SOLVENT SYSTEMS. 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Methanol	10	8	6	4	2											
Ethanol		2	4	6	8	10	8	6	4	2						
N.Propanol							2	4	6	8	10	8	6	4	4	2
Isopropanol																
Sec.Butanol												2	4	6	7	8
Water	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3
Ammonia, 35%	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1

Table cont'd.

SOLVENT: (mls.)	SOLVENT SYSTEMS. 17	18	19	20	21	22	23	24	25	26	27	28	29	30	31
Methanol		1	2	3											
Ethanol					2	4	6	8	10						
N.Propanol		4	4	4							2	4	6	8	10
Isopropanol										10	8	6	4	2	
Sec.Butanol	10	7	7	7	8	6	4	2							
Water	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3
Ammonia, 35%	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1

Table 11 Thin-Layer Chromatography Solvent Systems.

SOLVENT SYSTEMS:	Rf Values.				
1	.02	.72			
2	.02	.74			
3	.02	.66			
4	.02	.62			
5	.02	.67			
6	.01	.60			
7	.01	.53	.64		
8	.01	.44	.56		
9	.01	.36	.48		
10	.01	.44			
11	.01	.36	.43		
12	.01	.28	.34		
13	.01	.27	.34		
14	.01	.20	.26	.31	
X 15	.01	.17	.21	.27	.33
16	.00	.12	.17	.22	
17	.00	.06	.11	.14	
18	.01	.24	.32		
19	.01	.26	.36		
20	.01	.28	.37		
21	.01	.26	.35	.39	
22	.01	.38	.45	.50	
23	.01	.41	.50	.56	
24	.01	.55	.59		
25	.02	.57			
26	.02	.30	.36	.40	
27	.02	.38	.45		
28	.01	.37	.43	.48	
29	.01	.33	.40	.46	
30	.01	.36	.42	.47	
X 31	.01	.39	.52	.54	.60

Table 12 Rf Values of the Cobalamin Growth Zones Separated by Chromatography in the Solvent Systems of Table 11.

CHAPTER 7

COBALAMIN LIGHT SENSITIVITIES.

Light Sensitivity of the Cobalamins.

All the cobalamins so far encountered were to some degree light sensitive being converted to hydroxo B_{12} in the presence of oxygen by either photoaquation, in which no change in the valency of the cobalt occurred:-

$$Co^{III}.X + H_2O \xrightarrow{h\nu} Co^{III}.OH_2 + X ;$$

where X could be CN or NH_3 (Cooley et al., 1951) or by photoreduction, in which homolytic cleavage occurred to give a cobalt (II) complex and a free radical, ie

$$Co^{III}.Y \xrightarrow{h\nu} Co^{II} + Y\cdot ;$$

(Brady and Barker, 1961).

where Y could be CH_3, 5'-deoxyadenosyl or SO_3.

Veer et al. (1950) were the first to report that cyano B_{12} was converted to hydroxo B_{12} by the action of light and this was later quantified by DeMerre & Wilson (1956) who stated that prolonged exposure to sunlight caused a loss of 10% of cyano B_{12} for each ½hour of exposure.

Methyl B_{12} extracted from light exposed plasma was found to be almost totally converted to hydroxo B_{12} after 2 mins. exposure (Linnell et al.,1969), whereas under controlled conditions of exposure using a 60-watt tungsten lamp source, methyl B_{12} was only 23.0% decomposed in a similar time interval (Müller and Müller

1962a).

The rate of light conversion of cobalamins also appeared to be markedly affected by pH, as coenzyme B_{12} was found to be extremely sensitive to light when a yellow acidified solution of the coenzyme turned red due to the formation of hydroxo B_{12} within one minute of exposure to air and sunlight (Hogenkamp & Barker, 1961), while Muller and Muller (1962a) found only a 15% decomposition of coenzyme B_{12} in aqueous solution under their controlled exposure conditions.

The ease of oxidation of sulphito B_{12} was reported by Smith et al., (1952a) but no effect of light noted and indeed to this time little information on the rate of light conversion of this cobalamin could be found. A similar dearth of information on the light sensitivity of all the cobalamins at alkaline pH 9.6 existed and warranted a systematic investigation of these cobalamins to light exposure if subsequent extractions were to be performed using the ammonia buffer.

In addition both types of photolytic reaction were reversible in the dark in respect of the cyano, methyl and sulphito ligands:-

$$CN.B_{12} + H_2O \underset{\text{dark}}{\overset{h\nu}{\rightleftharpoons}} H_2O.B_{12} + CN^- \quad ;$$

$$\& \quad Co.Y \underset{\text{dark}}{\overset{h\nu}{\rightleftharpoons}} Co^{II} + Y\cdot \quad ;$$

where Y could be CH_3 or SO_3^{2-}.

When considering such a study, two properties of the incident light beam had to be considered, namely the intensity and wavelength of the light source. The latter was not investigated but a standard intensity of exposure was maintained by irradiating all samples at a constant distance from a uniform white light in the 'dark'.

MATERIALS & METHODS.

To determine the effect of pH on light conversion, the light sensitive cobalamins coenzyme B_{12}, cyano B_{12}, methyl B_{12} and sulphito B_{12} were dissolved in both distilled water, pH 6.2 ± 0.2 and ammonia buffer, pH 9.6 and made up to concentrations between 25 and 50 pgsB_{12}/µl, (1.88 - 3.75 x 10^{-8} mol./l.). 2 mls. of each solution were added in turn to 1 cm. diameter polystyrene tubes, which were aligned at a distance of 20 cms. from a 15 watt 'Osram' white light bulb. During exposure of the methyl B_{12}, coenzyme B_{12}, sulphito B_{12} and cyano B_{12} for 30 mins., 1 hr., 2 hrs. and 6 days respectively, 0.2 ml. samples of each were removed at regular time intervals and stored in foiled tubes at -20°C for subsequent analysis.

In addition 2 µl. aliquots of the methyl B_{12}, sulphito B_{12} and cyano B_{12} solutions prepared in distilled water were similarly withdrawn and applied directly to silica gel chromatograms to prevent any reconversion of the hydroxo B_{12} formed by photolysis, to the starting material during storage in air in the dark.

A further cyano B_{12} solution in distilled water was exposed to daylight for 6 days (and nights) and 2 µl. samples removed as before and applied directly to a chromatogram. Chromatograms of all the light exposed samples were run and developed by bioautography.

RESULTS & DISCUSSION.

From the bioautograms obtained, figs. 41 - 51 , a table of approximate half-times, $t_{\frac{1}{2}}$, representing 50% decomposition of starting material, could be constructed for the photolytic reactions in both solvent systems.

COBALAMIN	$t_{\frac{1}{2}}$ *in* WATER.	$t_{\frac{1}{2}}$ *in* AMMONIA.
Cyano-	~6 days	~6 days
Sulphito-	~40 mins.	~40 mins.
5'-deoxyadenosyl-	10-20 mins.	~20 mins.
Methyl-	~15 mins.	~15 mins.

Table 13 Qualitative Analysis of the Rates of Photolysis of the Standard Cobalamins in Distilled Water, pH 6.2 and Ammonia Buffer, pH 9.6.

Coenzyme B_{12} prepared in ammonia buffer was found to be almost totally converted to hydroxo B_{12} after exposure for one hour, fig. 45 , whereas although coenzyme B_{12} prepared in distilled water underwent a similar rate of

light conversion it appeared on the bioautogram, fig. 44, at the position of sulphito B_{12} presumably as a result of conversion of hydroxo B_{12} to sulphito B_{12} in the dark. A similar pattern was obtained on exposure of methyl B_{12} in ammonia buffer, fig. 42, and in distilled water, fig. 43, except that conversion to hydroxo B_{12} was almost complete after 30 mins. and again the hydroxo B_{12} in the distilled water appeared to be largely converted to sulphito B_{12} in the dark.

Sulphito B_{12} in ammonia buffer was totally converted to hydroxo B_{12} after 2 hours light exposure, fig. 48, whereas with sulphito B_{12} in distilled water, fig. 47, it was again evident that storage of samples in the dark led to reconversion of hydroxo B_{12} to sulphito B_{12} and under these conditions the sulphito B_{12} appeared to be light stable (see p. 142).

In all cases the ammonia buffer appeared to have no effect on the rate of light conversion (see table 13), although it did prevent the formation of sulphito B_{12} in the dark presumably by converting the hydroxo B_{12} to the resistant ammonia B_{12} species.

The bioautograms obtained for light exposed cyano B_{12}, fig. 49, methyl B_{12}, fig. 41 and sulphito B_{12}, fig. 46, where the samples were applied directly to the chromatograms to avoid the 'dark' reactions, as expected were almost identical to those for light exposed samples in ammonia buffer.

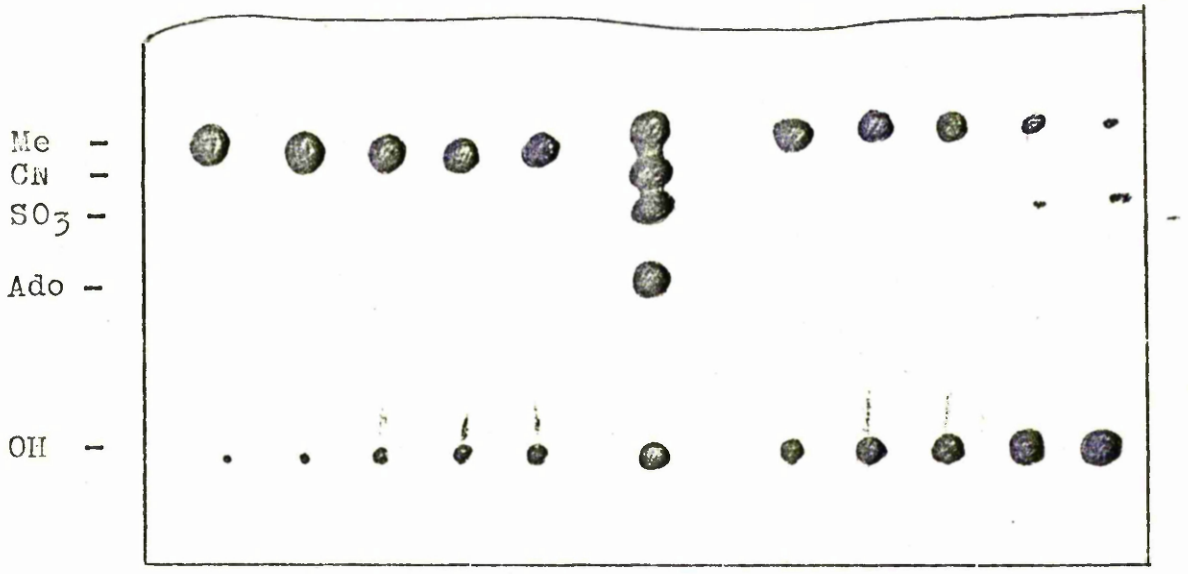


Fig. 41 Bioautogram of Light-Exposed Methyl B_{12} in Distilled Water, Samples Applied Directly.

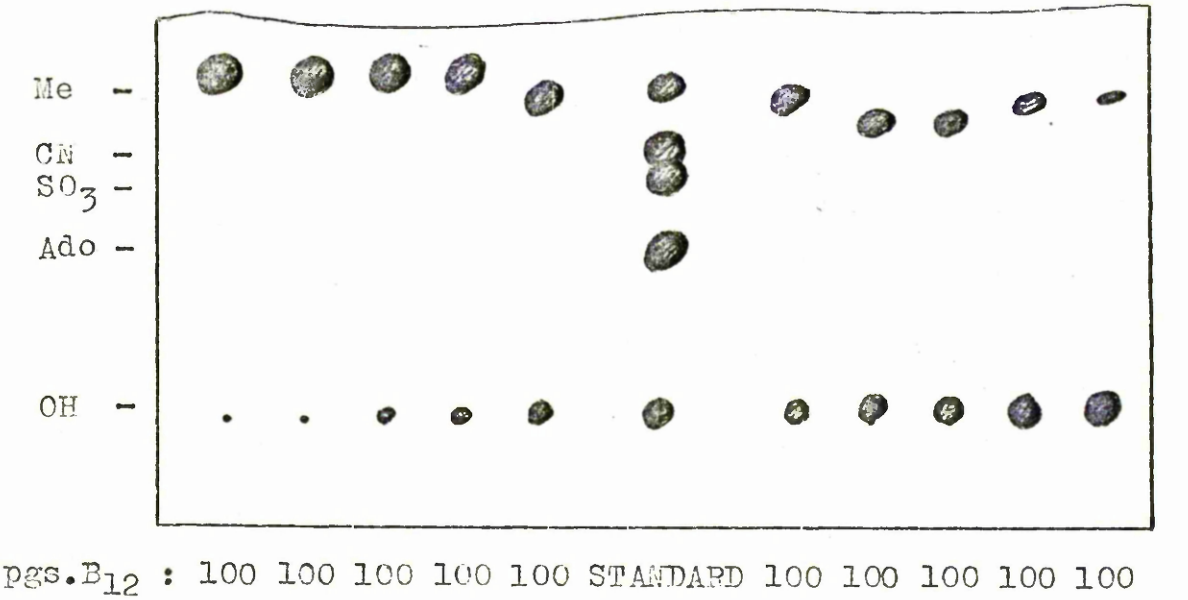
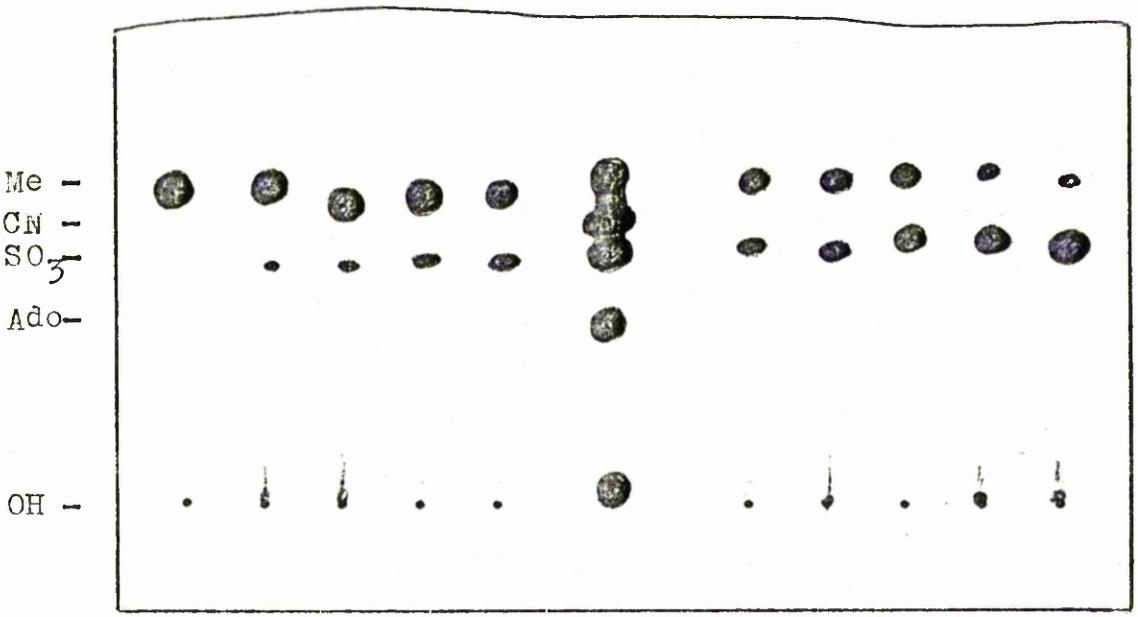


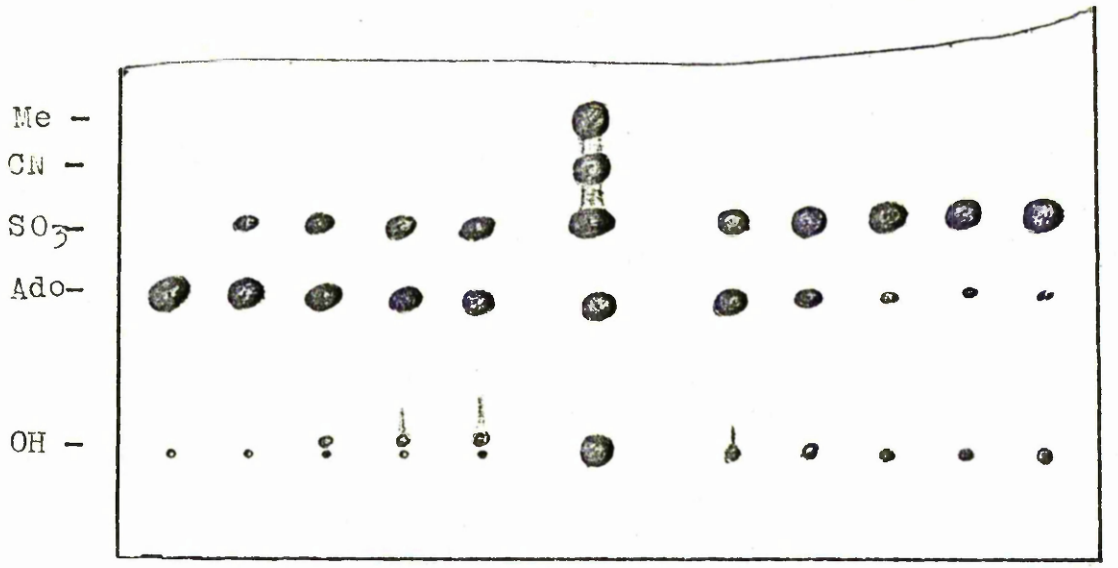
Fig. 42 Bioautogram of Light-Exposed Methyl B_{12} in Ammonia Buffer, pH 9.6.



pgs.B_{12} : 100 100 100 100 100 STANDARD 100 100 100 100 100

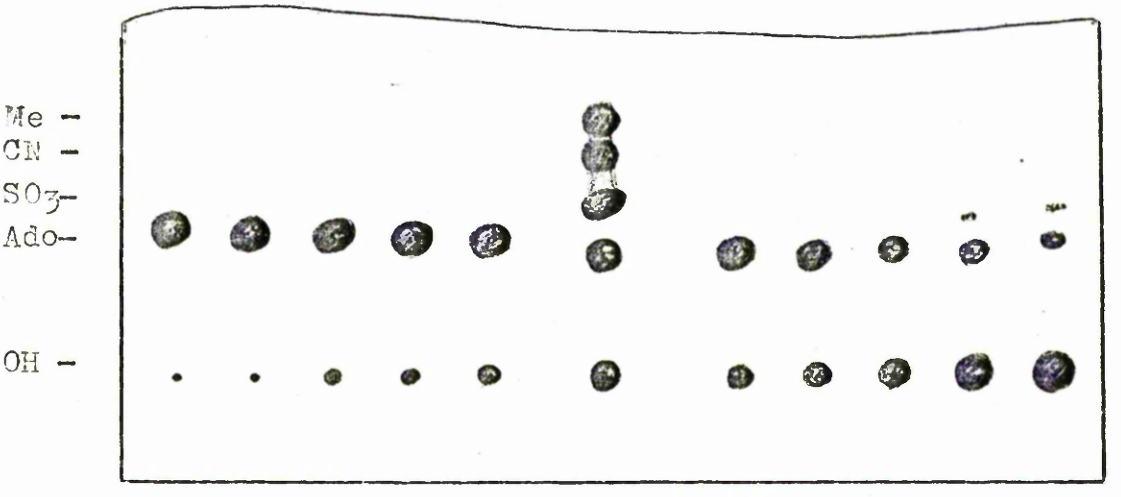
Exposure Time(min): 0 2 4 6 8 10 15 20 25 30

Fig. 43 Bioautogram of Light-Exposed Methyl B_{12} in Distilled Water; Samples Stored in the Dark Prior to Chromatography.



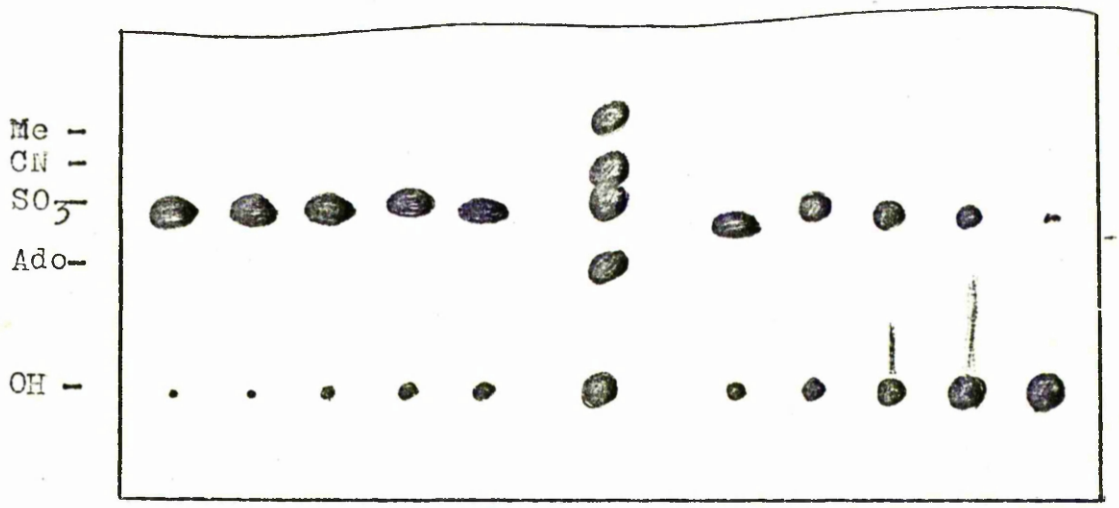
pgs. B_{12} :	100	100	100	100	100	STANDARD	100	100	100	100	100
Exposure: Time(min)	0	2	4	6	8		10	20	30	40	60

Fig. 44 Bioautogram of Light-Exposed Coenzyme B_{12} in Distilled Water.



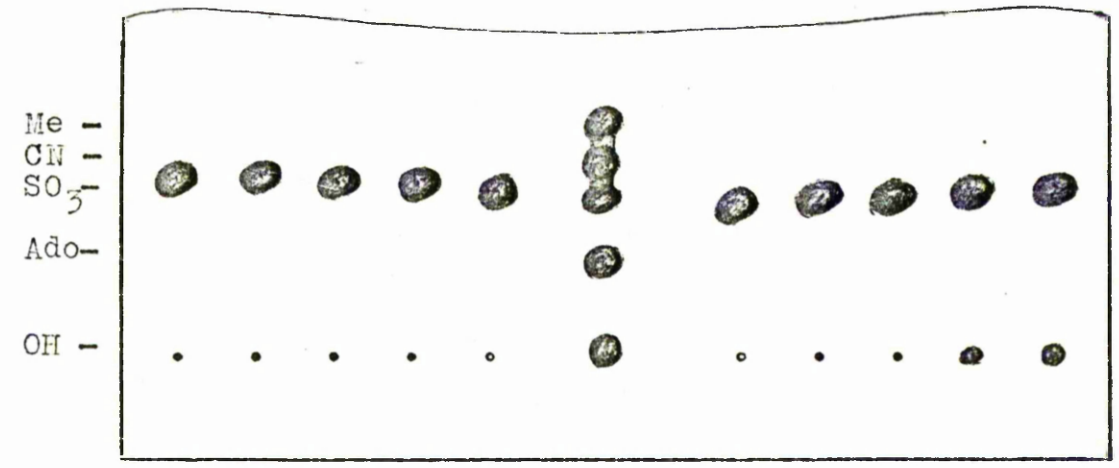
pgs. B_{12} :	72	72	72	72	72	STANDARD	72	72	72	72	72
Exposure: Time(min)	0	2	4	6	8		10	20	30	40	60

Fig. 45 Bioautogram of Light-Exposed Coenzyme B_{12} in Ammonia Buffer, pH 9.6.



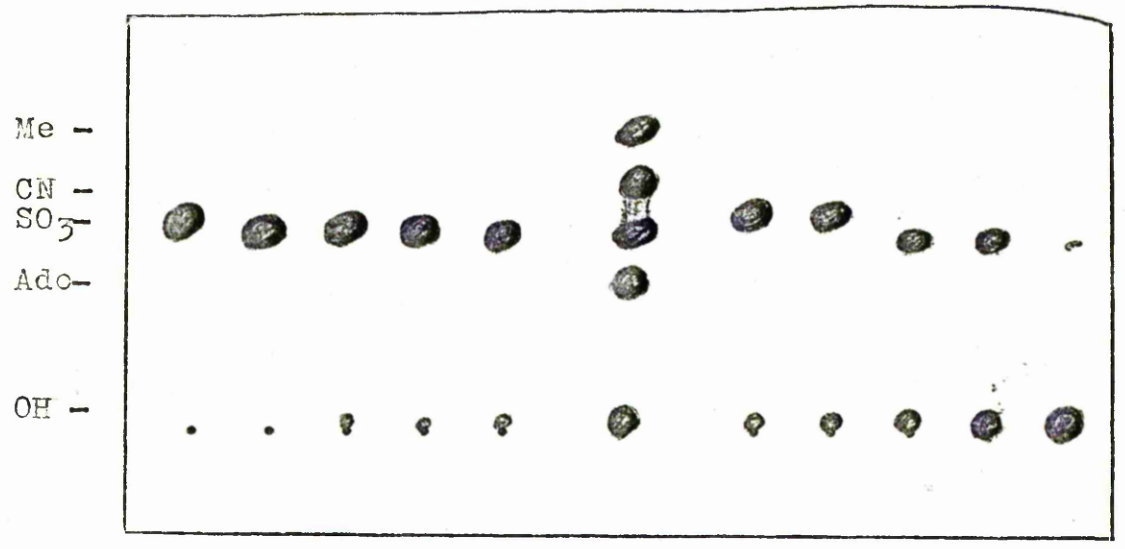
pgs.B_{12} :	100	100	100	100	100	STANDARD	100	100	100	100	100
Exposure: Time(min)	0	2	4	6	10		20	30	40	60	120

Fig. 46 Bioautogram of Light-Exposed Sulphito B_{12} in Distilled Water; Samples Applied Directly to Chromatograms After Exposure.



pgs.B_{12} :	100	100	100	100	100	STANDARD	100	100	100	100	100
Exposure: Time(min)	0	2	4	6	8		10	20	30	40	60

Fig. 47 Bioautogram of Light-Exposed Sulphito B_{12} in Distilled Water; Samples Stored in the Dark Prior to Chromatography.



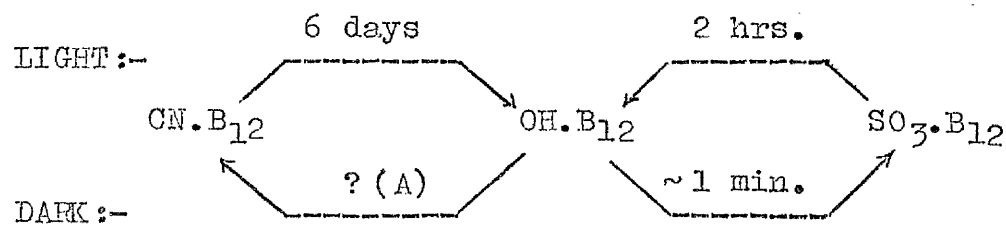
pgs.B_{12} :100 100 100 100 100 STANDARD 100 100 100 100 100

Exposure Time(min): 0 2 4 6 10 20 30 40 60 120

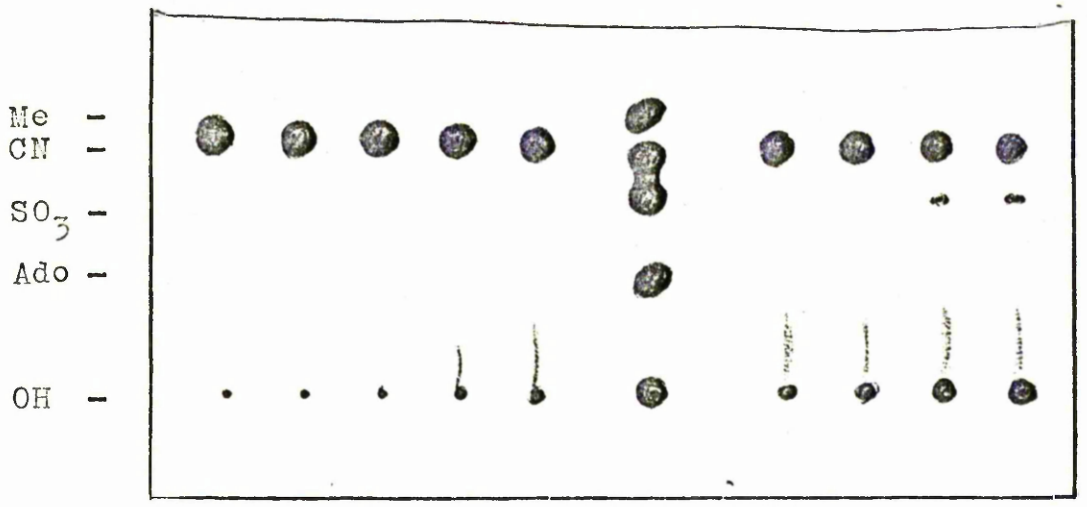
Fig. 48 Bioautogram of Light-Exposed Sulphito B_{12} in Ammonia Buffer, pH 9.6.

From the literature (Veer et al., 1950) it was expected that cyano B_{12} would be completely converted to hydroxo B_{12} in the first day of exposure to the light source. This did not occur however, and the cyano B_{12} solutions in both ammonia buffer, fig.51 and distilled water, fig.50 , remained predominantly intact during the first 8 hours of exposure, implying that the intensity of this light radiation was too weak for significant conversion of cyano B_{12} to hydroxo B_{12} in such a time interval. Further continuous, controlled light exposure showed in each case that cyano B_{12} was ca. 50% converted to hydroxo B_{12} after 6 days (see table 13).

Complications arose with storage of samples of light exposed methyl B_{12} and cyano B_{12} in distilled water, which like sulphito B_{12} were reformed from hydroxo B_{12} in the dark, making possible the following series of interconversions for cyano B_{12}:-

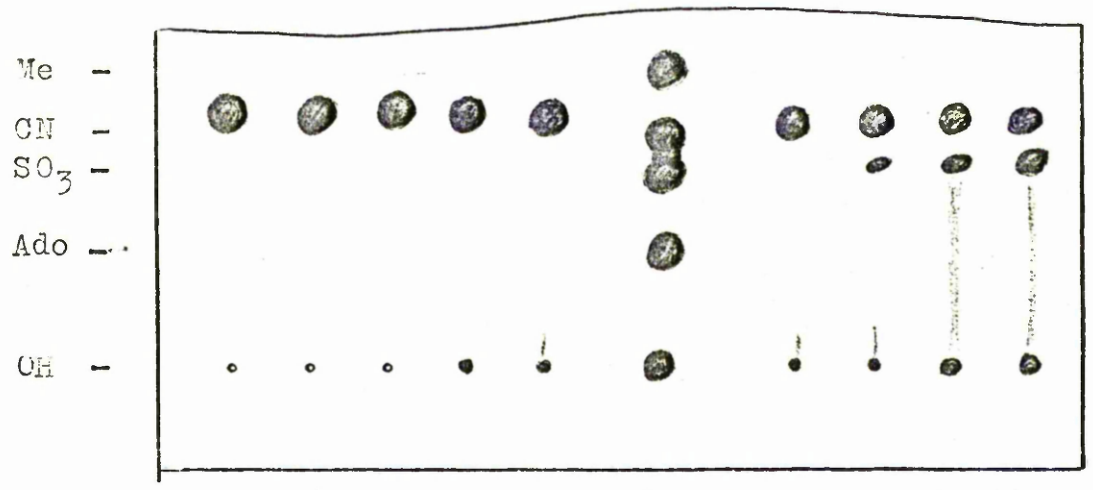


Lightproof storage of cyano B_{12} ultimately yielded after 6 days a solution composed of about equal proportions of cyano B_{12} and sulphito B_{12}. Comparison of these stored cyano B_{12} samples with those applied directly indicated that in distilled water the reconversion of



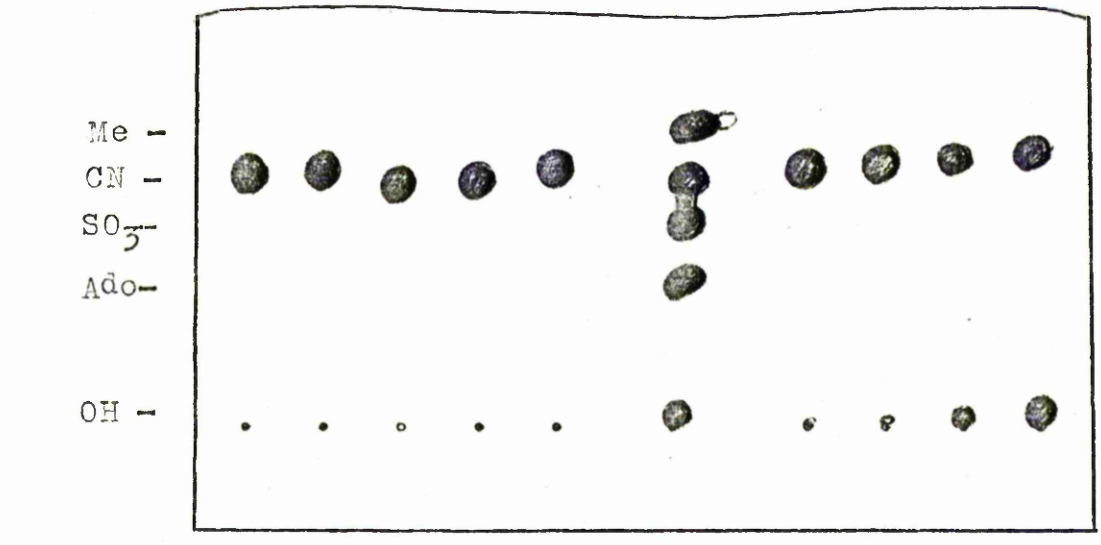
pgs.B_{12} :	100	100	100	100	100	STANDARD	100	100	100	100
Exposure: Time(hrs)	0	1	2	4	8		24	48	96	144

Fig. 49 Bioautogram of Light-Exposed Cyano B_{12} in Distilled Water; Samples Applied Directly to Chromatograms After Exposure.



pgs.B_{12} :	100	100	100	100	100	STANDARD	100	100	100	100
Exposure: Time(hrs)	0	1	2	4	8		24	48	96	144

Fig. 50 Bioautogram of Light-Exposed Cyano B_{12} in Distilled Water; Samples Stored in the Dark Prior to Chromatography.



pgs.B_{12} :	100	100	100	100	100	STANDARD	100	100	100	100
Exposure: Time(hrs)	0	1	2	4	8		24	48	96	144

Fig. 51 Bioautogram of Light-Exposed Cyano B_{12} in Ammonia Buffer, pH 9.6.

hydroxo B_{12} to cyano B_{12}, reaction A, was negligible compared with the dark mediated reaction of hydroxo B_{12} to sulphito B_{12}, which was virtually instantaneous in the presence of adequate sulphite ion.

The cyano B_{12} exposed to daylight and applied immediately to the chromatogram when sampled after 6 days (and nights) yielded a solution composed of cyano B_{12}, hydroxo B_{12} and sulphito B_{12} in about equal proportions. Such a distribution probably arose due to the removal of samples in the daylight, in which some of the sulphito B_{12} formed overnight would have been converted to hydroxo B_{12}.

From our exposure of the cobalamins to the standard white light environment it was clear that the light conversion rate was considerably slower than had been previously found in all instances where samples were exposed to daylight or sunlight (DeMerre & Wilson, 1956 and Linnell et al., 1969), in which the shorter wavelength ultra-violet light should increase the rate of cobalt-carbon and cobalt-sulphur bond cleavage.

It was found that the light conversion of the cobalamins increased in the order $CNB_{12} \ll SO_3B_{12} < AdoB_{12} < MeB_{12}$, with conversion times to hydroxo B_{12} of ~10 days $\gg$120 mins. > ~60 mins. > ~40 mins. respectively, after exposure to the 15 watt white light source at a distance of 20 cms.

These results implied that although great care would be required to eliminate light as far as possible during

subsequent extractions on foodstuffs, stringent photographic darkroom-type conditions would not be essential.

A parallel investigation into the light sensitivity of the cobalamins was also performed by Ultra-violet spectroscopy using a continuous scan method. It was however appreciated that conversion of samples would occur during preparation and scanning and because of this no attempt was made to control the incident light sources to which the samples would inevitably be exposed.

MATERIALS & METHODS.

0.05 mgm.B_{12}/ml. solutions of coenzyme B_{12} (3.17 x 10^{-5}mol./l.), hydroxo B_{12} (3.71 x 10^{-5}mol./l.), methyl B_{12} (3.72 x 10^{-5}mol./l.) and sulphito B_{12} (3.55 x 10^{-5} mol./l.) were prepared in distilled water and stored in foiled containers.

The solutions (2 mls.), contained in 1 cm. 'Sarstedt' plastic cuvettes were scanned at the fast speed on a 'Unicam' SP 800 UV Spectrometer between 700 and 325 nms.

Between analysis the solutions were exposed to strong sunlight and laboratory fluorescent lighting. In each case scans were performed at time intervals of 1, 10, 25 and 40 mins. (and where necessary 50, 60 and 80 mins.) until two consecutive spectra overlapped and coincided with that of hydroxo B_{12} with λmax. at 349 nms. (fig. 21).

The absorbance A, where

$$\varepsilon c l = A = \log \frac{I}{I_o} \quad ; \text{ \& where } c = \text{conc. (mol./l.).}$$

for coenzyme B_{12}, methyl B_{12} and sulphito B_{12} was noted at 349 nms. before exposure to light, at t_o. Similarly the absorbance values at 349 nms. after complete light conversion of the cobalamins at time t_c were recorded (Table 14). The absorbance values at t_o and t_c for each cobalamin were averaged to give the absorbance associated with 50% decomposition of that cobalamin at time, $t_{\frac{1}{2}}$.

From the absorption curves the run which yielded an absorbance at 349 nms. corresponding to the above $t_{\frac{1}{2}}$ absorbances was noted for each cobalamin, together with its light exposure time. Knowing these $t_{\frac{1}{2}}$ values would then allow us to calculate approximate rate constants, k for the photolysis of each cobalamin from the equation:-

$$k = \frac{0.693}{t_{\frac{1}{2}}} \quad ;$$

RESULTS & DISCUSSION.

From the spectra for coenzyme B_{12}, fig. 52 , methyl B_{12}, fig. 53 and sulphito B_{12}, fig. 54 the $t_{\frac{1}{2}}$ and hence the associated k values for the photolysis of these cobalamins were of the same order as previously found in the controlled exposure experiment, with increasing light sensitivities thus $SO_3B_{12} < AdoB_{12} < MeB_{12}$.

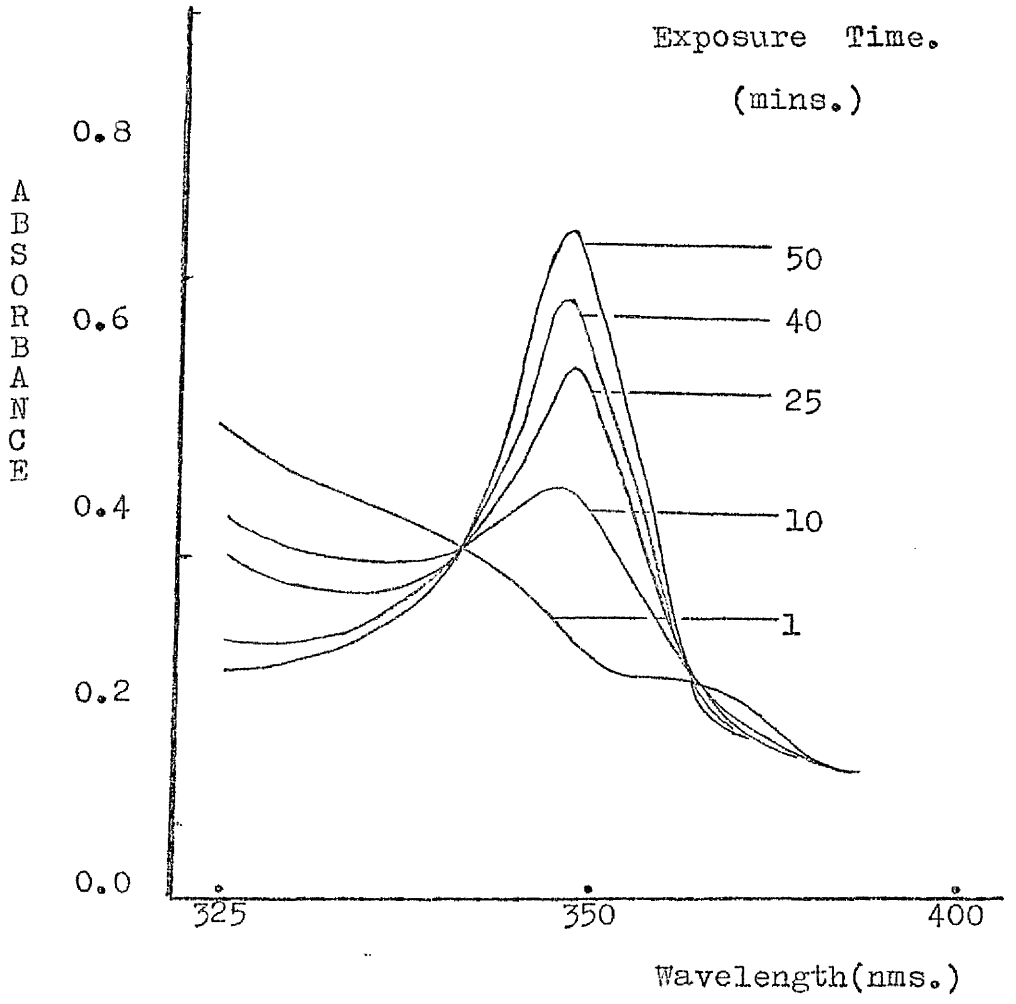


Fig. 52 Repeat Scan Ultra-Violet Spectra of Light Exposed Coenzyme B_{12} in Distilled Water.

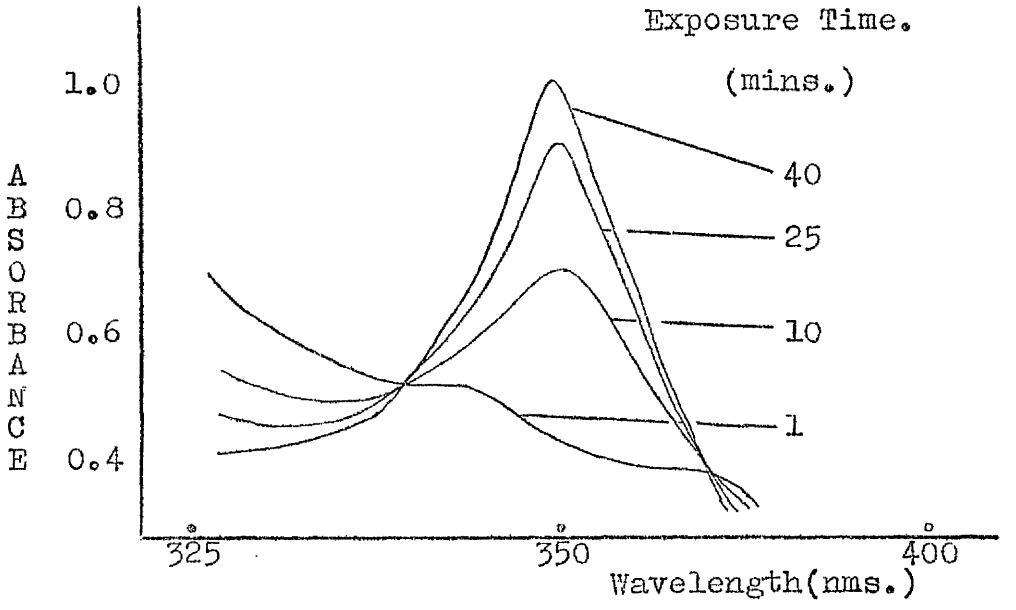


Fig. 53 Repeat Scan Ultra-Violet Spectra of Light Exposed Methyl B_{12} in Distilled Water.

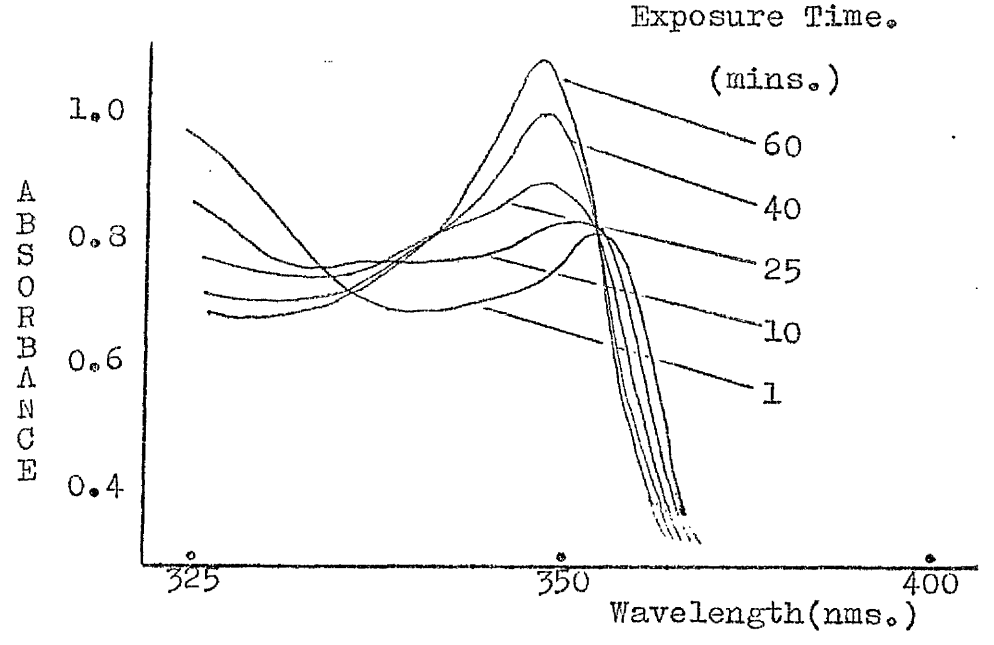


Fig. 54 Repeat Scan Ultra-Violet Spectra of Light Exposed Sulphito B_{12} in Distilled Water.

COBALAMIN	ABSORBANCE AT t_o	ABSORBANCE AT t_c	$t_o + t_c/2$	$t_{\frac{1}{2}}$ (secs)	$X10^{+2}k$ (sec^{-1})
coenzyme	0.41	0.58	0.495	~660	~0.105
methyl	0.56	0.91	0.735	~540	~0.128
sulphito	0.71	0.96	0.835	1500	0.046

Table 14 First-order Rate Constants for the Photolysis of Aqueous Solutions of Coenzyme B_{12}, Methyl B_{12} and Sulphito B_{12}

Although the same order of light sensitivity was obtained for coenzyme B_{12}, methyl B_{12} and sulphito B_{12} the corresponding conversion times of 50, 40 and 60 mins. showed an increase in the rate of conversion, presumably as a result of the exposure to the higher energy ultra-violet radiation. Knowledge of the intensity (I) and energy (or wavelength) of the incident light was clearly important in defining the light sensitivity of the cobalamins, since the number of molecules photolysed per quantum of radiation absorbed, the quantum yield Φ, varied, depending on the wavelength of the radiation (Pratt and Whitear, 1971).

CHAPTER 8

OCCURRENCE OF ARTEFACTUAL SULPHITO B_{12}

The Occurrence of Artefactual Sulphito B_{12}.

The formation of artefactual sulphito B_{12} from hydroxo B_{12} appeared to be such a facile process that its widespread occurrence seemed inevitable during extractions from biological tissues, most of which contained very low cobalamin concentrations of the order of~10 µgms.B_{12}/kg. tissue. To avoid such a conversion to sulphito B_{12}, without protecting the hydroxo B_{12} as the ammonia B_{12}, seemed to be extremely difficult unless all extractions were carried out under an inert nitrogen atmosphere, using distilled water at pH 7 and applying extracts to the chromatograms immediately on isolation. These conditions to prevent the formation of sulphite ion were clearly impractical for general use and to our knowledge, neither an inert gas atmosphere nor stringent pH control have previously been routinely employed during extractions and yet no reports of the occurrence of artefactual sulphito B_{12} were found.

This dearth of corroborative evidence for such an occurrence warranted an investigation into the commonly used extraction and development procedures in an attempt to clarify previously contradictory findings.

Our final extraction and chromatographic procedures were based largely on those of Mervyn et al., (1972), while the bioautographic technique was predominantly that of Linnell et al., (1969), with some minor modifications

in each case. An examination of the behaviour of the standard cobalamins using the precise procedures of these authors was undertaken to determine, if any, the effect of the modifications. Furthermore an investigation of the chromatographic systems of Lindstrand (1965) and Lindstrand and Stahlberg (1963) was performed.

MATERIALS & METHODS.

Solutions of the standard cobalamins (coenzyme B_{12}, cyano B_{12}, hydroxo B_{12}, methyl B_{12} and sulphito B_{12}) were prepared in ammonia buffer at a conc. of 50 pgs.$B_{12}/\mu l$. and 1 µl. of each run on a glass plate (20 cm. square) coated with a 3:1 mixture of Whatman CC 41 cellulose and Merck silica gel G. After separation of cobalamins by ascending chromatography in sec. butanol/ ammonia/ water (75: 2: 25, v/v), the plate was redeveloped at right angles to the direction of the first separation for 30 mins., in water saturated with benzyl alcohol (Linnell et al., 1970). The resultant bioautogram was prepared and the growth zones recorded, fig.55 .

1 ml. of a hydroxo B_{12} solution of conc. 50 pgs$B_{12}/\mu l$. in distilled water, pH 6.6 ± 0.2 was extracted as follows using the precise method of Mervyn et al., (1972).

After dilution to 15 mls. with distilled water and reflux in 50 mls. of ethanol (99.8%) for 30 mins. at 80°C, the solution was filtered through Whatman 54 filter paper at reduced pressure.

The reflux and filtering were repeated, the filtrate rotary evaporated to ca. 5 mls. at 40°C and added to a separating funnel with an equal volume of liquified phenol 'BP' and one drop of dilute HCl (10^{-3}mol./l.)to acidify. This mixture was shaken vigorously until a clear water layer was obtained and the lower phenol layer run off before addition to a one-fifth volume of distilled water to remove salts. This was repeated and the lower phenol layer added to an equal volume of distilled water, a one-half volume of acetone and two volumes of diethyl ether in a separating funnel, which was shaken vigorously with frequent valve opening to release volatile solvent. After repetition the lower aqueous phase was withdrawn, shaken with diethyl ether (2 x 5 mls.) to remove dissolved phenol, and rotary evaporated to ca. 1 ml. at 40°C. This extract was immediately applied with standards to a silica gel, Kodak 'chromagram' sheet 6061, by means of a 10 µl. Hamilton syringe and run in the butan-2-ol; n.propanol; water; ammonia (7:4:3:1, v/v) solvent system. The chromatogram was developed by bioautography.

2 µls. of each of the standard cobalamins prepared in ammonia buffer at a conc. of 5 µgms.B_{12}/µl.(ca. 10^{-3} mol./l.) were separated on paper chromatography using Whatman No. 2 grade filter paper in the Lindstrand (1965) solvent system, butan-2-ol; acetic acid; water, (100:3:50, v/v) and the Lindstrand & Stahlberg system (1963), n.butanol; isopropanol; water, (10:7:10, v/v).

The chromatograms were run for 6½ hrs. and the relative positions of the cobalamins noted.

RESULTS & DISCUSSION.

From fig. 55 , it was apparent that in the ammonia containing solvent system the hydroxo B_{12} was readily converted to ammonia B_{12} which did not travel (Rf 0.09) on the cellulose/ silica chromatograms. Sulphito B_{12} on this two-dimensional chromatography appeared at a position with R_f 0.73 (Table 15), previously attributed by Linnell to hydroxo B_{12} and this misinterpretation of bioautograms explained the lack of reports on artefactual sulphito B_{12} formation. It was however recognised at this time that the position of hydroxo B_{12}, as opposed to ammonia B_{12}, was yet to be established for this chromatographic system and this could not be achieved using the ammonia containing solvent system. None of the other cobalamins prepared in ammonia buffer displayed anomalous behaviour on separation, allowing for the clear separation of 'hydroxo' B_{12} and coenzyme B_{12} by one-dimensional chromatography in butan-2-ol, ammonia and water. This was not previously possible since coenzyme B_{12} and the sulphito B_{12} artefact from hydroxo B_{12} had the same R_f value in chromatography and were termed 'Co-OH'. (Linnell et al., 1969 & 1971).

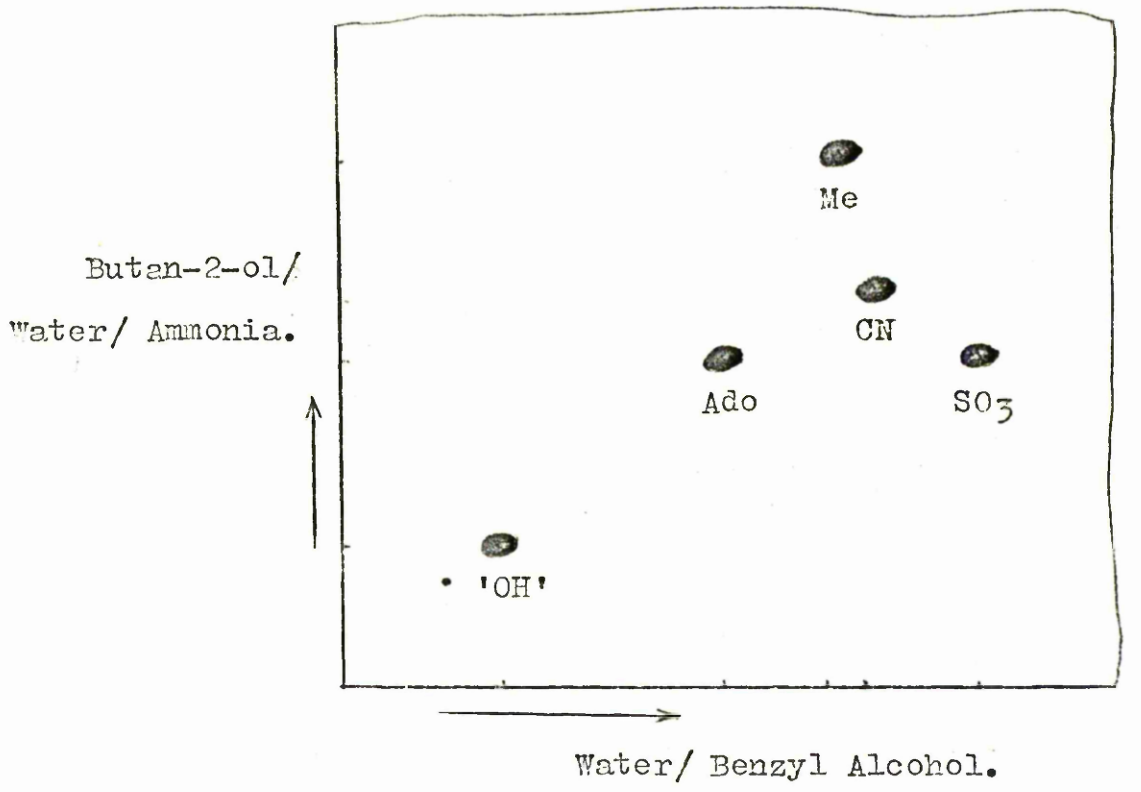
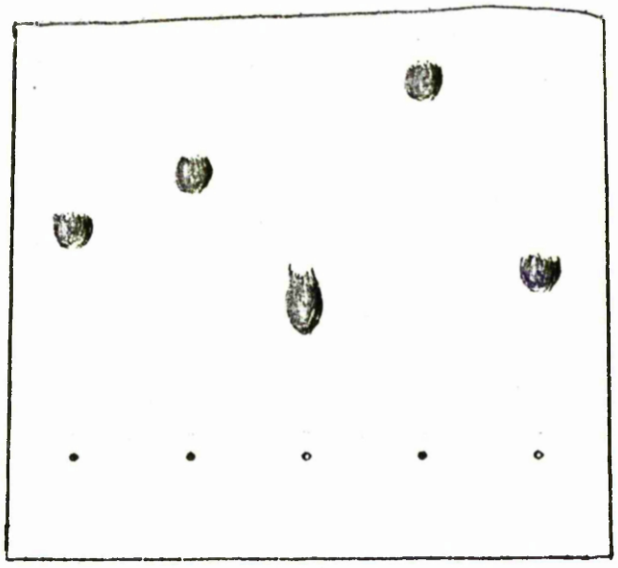


Fig. 55 Separation and Location of Standard Cobalamins in Ammonia Buffer by Two-Dimensional Chromatography and Bioautography.

	SOLVENT SYSTEMS.	
COBALAMIN.	But-2-ol/Amm./H_2O :	H_2O/Bz.Alc.
5'-deoxyadenosyl-	0.18	0.39
cyano-	0.23	0.57
'hydroxo'- (ammonia-)	0.03	0.09
methyl-	0.35	0.52
sulphito-	0.18	0.73

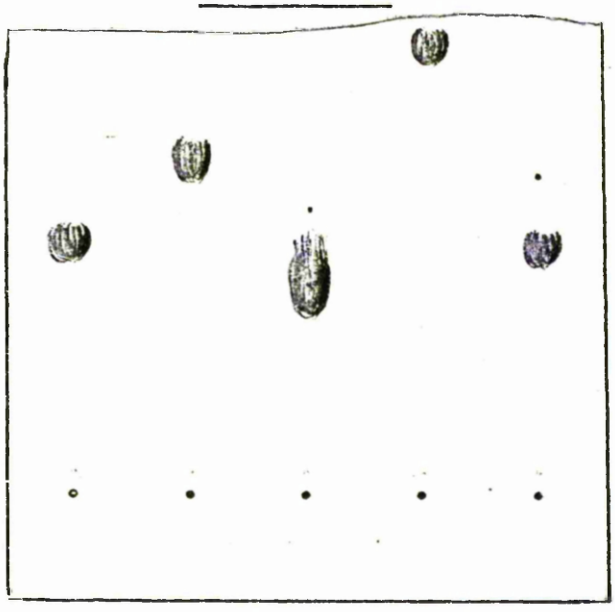
Table 15 R_f values for the above Standard Cobalamins Separated by Two-Dimensional Chromatography.

The hydroxo B_{12} standard extracted in distilled water, pH 6.6, by the method of Mervyn et al. (1972), gave rise to two growth zones in bioautography as expected, corresponding to hydroxo B_{12} and sulphito B_{12} the latter having arisen because no precautions were taken either to maintain an alkaline pH or to pre-convert the hydroxo B_{12} before extraction. These findings concur with those of Bilkus and Mervyn (1971), whose low concentration hydroxo B_{12} solutions ($\simeq 10^{-6}$ mol./l.) yielded two growth areas on bioautography and despite daily preparation of relatively high concentration working standards of 10 µgms.B_{12}/ml. (ca. 7×10^{-6} mol/l) considerable conversion of hydroxo B_{12} to sulphito B_{12} still appeared to occur in the solutions prepared in distilled water. One anomaly however remained in that while the author found sulphito B_{12} to travel to a position between coenzyme B_{12} and cyano B_{12} in this system, Bilkus and Mervyn (1971) reported that sulphito B_{12} ran between hydroxo B_{12} and coenzyme B_{12}, a position it was found to occupy however on paper chromatography in both solvent systems of Lindstrand (fig. 56) and Lindstrand and Stahlberg (fig. 57). Although no mention was made of sulphito B_{12} in their work its presence might explain both the second growth area which they obtained from the hydroxo B_{12} standard and attributed to cyano B_{12} contaminant, and the three growth zones isolated on



B_{12}.Form :	Ado	CN	OH	Me	SO_3
µgms.B_{12} :	10	10	10	10	10

Fig.56 Chromatogram of the Standard Cobalamins separated in Butan-2-ol, acetic acid, water (100:3:50, v/v).



B_{12}.Form :	Ado	CN	OH	Me	SO_3
µgms.B_{12} :	10	10	10	10	10

Fig.57 Chromatogram of the Standard Cobalamins separated in n.Butanol, isopropanol, water, (10:7:10, v/v).

bioautography on exposure of 'fourth factor', methyl B_{12} to light, attributed to cyano B_{12}, coenzyme B_{12} and hydroxo B_{12} respectively. It seemed more likely from our findings that, on exposure of the aqueous solution of methyl B_{12} to light, the hydroxo B_{12} which was formed would be converted to sulphito B_{12} in the dark and this together with tailing of the hydroxo B_{12} gave the appearance of cyano B_{12} and coenzyme B_{12} growth zones respectively.

From these considerations the author suggests that sulphito B_{12} has in fact occurred in previous work, but for a variety of reasons it has not been appreciated to be sulphito B_{12}. The presence of artefactual sulphito B_{12} derived from hydroxo B_{12} at low conc. ca. 10^{-8}mol/l. explained why 'hydroxo B_{12}' and coenzyme B_{12} could not be readily separated on cellulose at this concentration (Linnell et al., 1969) and yet at high concentration ca. 10^{-3}mol./l. such a separation was possible (Suomela, 1967) because the hydroxo B_{12} remained largely unconverted.

In conclusion it should be emphasised that any misinterpretation of sulphito B_{12} as hydroxo B_{12} does not appear to have ledd to any serious misunderstanding of the forms of vitamin B_{12} in tissues. Provided that a tissue does not contain natural sulphito B_{12} then the interpretation of artefactual sulphito B_{12} for hydroxo B_{12} is of little consequence because only hydroxo B_{12}

is converted to artefactual sulphito B_{12}. On the other hand it is reasonable to suggest that further work on the forms of vitamin B_{12} in tissues should take account of the possibility that sulphito B_{12} may be present in 'natural' form.

CHAPTER 9

THE FORMS OF VITAMIN B12 IN FOOD.

The Forms of Vitamin B_{12} in Food.

Although much is known about the forms of vitamin B_{12} found in human tissues, nothing is known about the forms of vitamin B_{12} which are ingested in diet and, indeed, the information on the vitamin B_{12} content of items of diet and of meals is relatively scanty. A good deal of the information presented by McCance & Widdowson (1969), Robinson (1966), Chanarin (1969) and Love (1970) is hampered by lack of information about some items of diet, mostly about the range of values likely to be encountered and by the fact that the bulk of the information relates to uncooked foodstuffs which does not allow for weight changes, losses or destruction of vitamin B_{12} or changes in the forms of vitamin B_{12} during cooking and other preparative processes. These changes may be of some importance because it has been established that the forms of vitamin B_{12} may be absorbed differentially, ie. that one cobalamin may be more readily absorbed than another at any one dose range (Adams et al., 1971). It therefore seemed of some importance to study the forms of vitamin B_{12} in food. This was done for the most part on foods after they had been prepared for consumption.

MATERIALS & METHODS.

The foods which were studied were those considered by McCance & Widdowson (1969) and by Adams et al., (1973), to have a relatively high vitamin B_{12} content, and the figures published by Adams et al. (1972.) were taken as a guide to the vitamin B_{12} content of each tissue. Most of the cooked items were prepared in the hospital kitchens, and others mostly processed foods were obtained by purchase in local stores. Inedible material and gravies were removed, and the tissues weighed in daylight before being homogenised in darkroom conditions.

In most cases two identical extractions were performed on the same tissue to determine the reproducibility of the technique by examination of the cobalamins isolated. Another extraction was sometimes performed on a different sample of the same tissue to establish the variation in cobalamin content between samples.

The tissues (5 gms. or 10 mls.) were extracted by the standard method (see pp.26-38) except that they were received in ammonia buffer (50 mls.). Knowing the initial cobalamin content enabled a final extract to be produced with a conc. of 10 - 100 pgs.$B_{12}/\mu l.$, suitable for chromatographic and bioautographic analysis. Where possible the final extract was diluted to yield a series of solutions of neat concentration and 1:2, 1:4, 1:8, 1:16 and 1:32 of the original. These solutions together with the standard cobalamins were run on silica gel chromatograms and

developed by bioautography. From the bioautograms the Rf values of all the growth zones were calculated and each cobalamin scored according to the number of times it appeared in the extract to find the approximate ratio of each cobalamin present.(figs.58 & 59).

In addition 0.1 ml. of each extract ($\simeq 10^3 - 10^4$ pgs.B_{12}) was diluted thirty-fold to a concentration of ca. 30 - 300 pgs.B_{12}/ml. and assayed for total cobalamin content by the standard Lactobacillus leichmanii method.

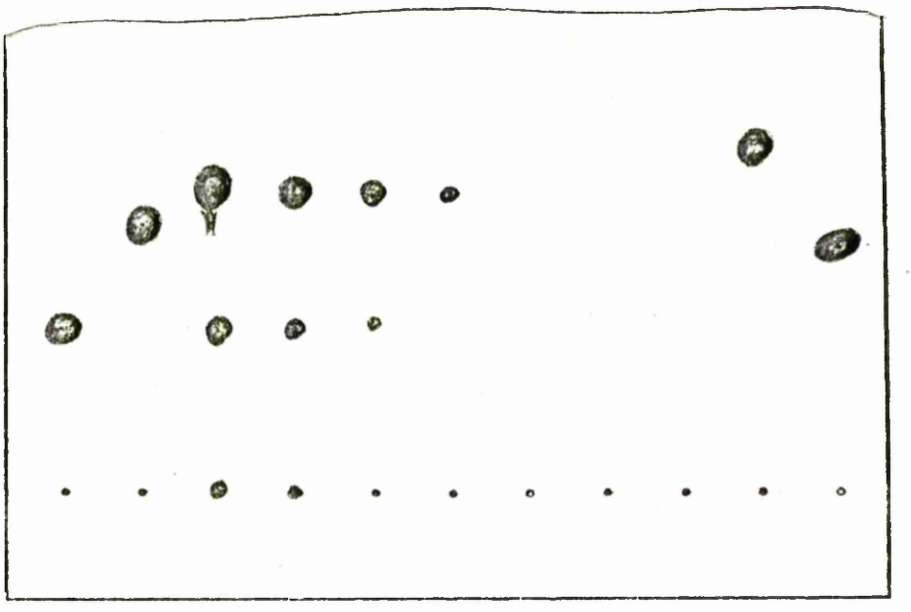
Knowing the total vitamin B_{12} present enabled the calculation of the amounts of the individual cobalamins from the ratios of each, found by dilution.

The items investigated could be divided into three specific categories, namely:-

Dairy Produce - Eggs, milk and cheese in both processed and raw forms (table 16).

Muscle Tissue - Beef in its natural and processed forms, bacon, gammon, chicken, oxtail etc. (table 17).

Fish - Haddock, salmon and sardines + liver and wheat germ (table 18).



B_{12}·Form :	Ado	CN	-	E	X	T	R	A	C	T	-	OH	Me	SO_3
pgs.B_{12} :	50	50	<266	<133	<66	<33	<16	<8				50	50	50
Dilution :			1	1:2	1:4	1:8	1:16	1:32						

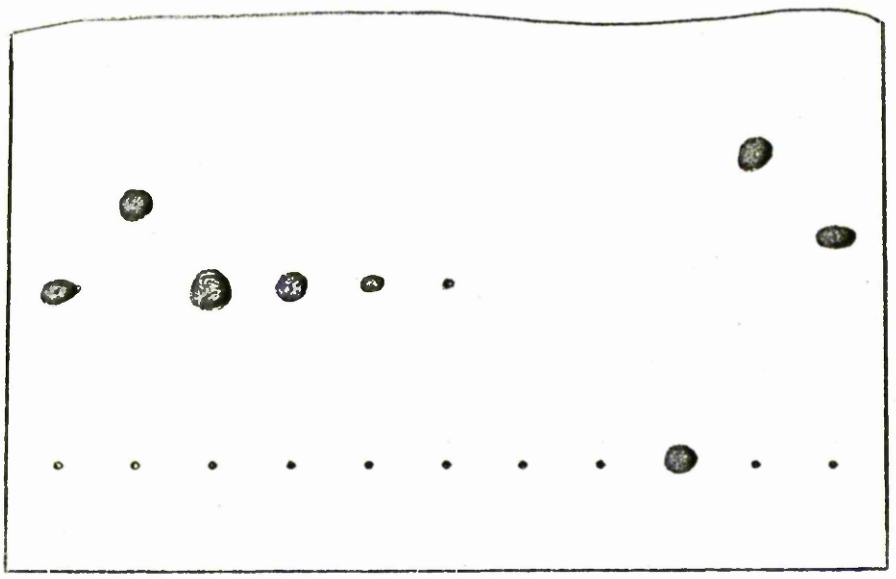
R_f's.

Me.B_{12} :	-	-	.28	.28	.28	.28	-	-	-	.31	-
CN.B_{12} :	-	.26	-	-	-	-	-	-	-	-	-
SO_3·B_{12} :	-	-	-	-	-	-	-	-	-	-	.23
Ado.B_{12} :	.17	-	.16	.16	.17	-	-	-	-	-	-
OH.B_{12} :	-	-	.00	.00	-	-	-	-	.00	-	-

COBALAMIN.

	Ado.	CN.	OH.	Me.	SO_3.
No. of Appear-ances in Extr.	3	0	2	4	0
%'ages :	28.6	0	14.3	57.2	0

Fig. 58 Bioautogram of Cheese Spread (Kraft) Extract in Ammonia Buffer & %'Ages of Cobalamins Present.



B_{12}.Form :	Ado	CN	-	E X T R A C T	-	OH	Me	SO_3
pgs.B_{12} :	50	50	<200	<100	<50	<25	<12	<6	50	50	50
Dilution :			1	1:2	1:4	1:8	1:16	1:32			

R_f's.

Me.B_{12} :	-	-	-	-	-	-	-	-	-	.28	-
CN.B_{12} :	-	.25	-	-	-	-	-	-	-	-	-
SO_3.B_{12} :	-	-	-	-	-	-	-	-	-	-	.22
Ado.B_{12} :	.15	-	.15	.16	.16	.16	-	-	-	-	-
OH.B_{12} :	-	-	-	-	-	-	-	-	.00	-	-

COBALAMIN.

	Ado.	CN.	OH.	Me.	SO_3.
No. of Appearances in Extract.	4	0	0	0	0
%'Ages. :	100	0	0	0	0

Fig.59 Bioautogram of Braised Beef Extract in Ammonia Buffer & %'Ages of Cobalamins Present.

Food & Method of Preparation	Sample.	Extr.	B_{12} Content.	COBALAMINS Ado.	CN.	OH.	Me.	SO_3
Butter	1	1	0.0	-	-	-	-	-
Cheese;								
Cheddar	1	1	3.2	1.8	-	1.8	3.7	0.9
	1	2	7.8	1.0	0.5	4.2	2.1	-
	2	1	10.1	1.0	-	8.1	1.0	-
Spread (Kraft)	1	1	3.2	0.9	-	0.5	1.8	-
	1	2	10.3	3.1	-	1.5	6.2	-
	2	1	7.6	2.5	-	-	5.1	-
Egg;								
Yolk, raw	1	1	6.3	1.3	-	2.5	2.5	-
	1	2	30.0	1.2		9.2	18.4	1.2
White, raw	1	1	0.39	0.26	0.13	-	-	-
	1	2	0.13	0.06	0.03	-	0.02	0.02
Milk;								
Whole, pasteurised	1	1	1.30	-	-	1.3	-	-
	1	2	0.72	-	-	0.7	-	-
	2	1	0.38	0.1	-	0.7	0.1	-
Whole, raw	1	1	2.6	0.7	-	1.2	0.7	-
	1	2	2.6	0.4	-	1.8	0.4	-
Evaporated (Carnation)	1	1	0.75	0.4	-	-	0.4	-
	1	2	0.62	0.3	-	-	0.3	-
	2	1	0.16	0.1	-	-	0.1	-
	2	2	0.31	0.4	-	-	0.4	-

Table 16 Forms of vitamin B_{12} and vitamin B12 content (µgms/kg or µgms/l.) for various items of Dairy Produce.

Food & Method of Preparation	Sample.	Extract.	B_{12} Content.	COBALAMINS. Ado.	CN.	OH.	Me.	SO_3.
Bacon, grilled.	1	1	1.5	0.7	-	0.4	0.4	-
	1	2	2.7	1.8	-	0.9	-	-
Beef;								
Rump, braised	1	1	18.2	12.1	-	6.1	-	-
	1	2	12.6	10.1	-	2.5	-	-
Corned, canned	1	1	5.0	-	-	4.0	-	1.0
(St.Michael)	1	2	9.7	-	-	7.8	-	1.9
	2	1	25.2	1.9	-	7.8	15.5	-
Spread,(Princes)	1	1	8.9	2.5	-	5.1	1.3	-
	1	2	7.6	3.8	-	3.8	-	-
Spread,(Shippams)	1	1	10.3	4.8	-	4.8	0.7	-
	1	2	8.7	4.1	-	4.1	0.5	-
Chicken;								
Casserole	1	1	5.1	2.5	-	2.6	-	-
	2	1	6.5	5.9	-	-	0.6	-
& Ham Roll	1	1	2.5	0.3	-	2.2	-	-
(Crosse & Blackwell)	1	2	9.0	1.0	-	8.0	-	-
Gammon,tinned	1	1	1.5	0.2	-	1.3	-	-
(Danoxa)	1	2	2.5	-	-	2.5	-	-
Tongue,ox,boiled	1	1	10.8	-	-	8.6	-	2.2
	1	2	9.4	-	-	7.5	-	1.9
Tail,ox,stewed	1	1	10.6	5.0	-	5.0	0.6	-
	1	2	8.4	3.2	-	3.2	0.4	1.6

Table 17 Forms of vitamin B_{12} and vitamin B_{12} content (μgms/kg or μgms/l) for various Muscle Tissues Prepared for Consumption.

Food & Method of Preparation	Sample.	Extr.	B_{12} Content.	COBALAMINS. Ado.	CN.	OH.	Me.	SO_3.
Haddock, boiled	1	1	9.6	5.9	0.4	2.9	0.4	-
	1	2	6.5	4.0	0.3	1.9	0.3	-
Salmon:								
canned (John West)	1	1	14.4	3.6	-	7.2	-	3.6
	1	2	7.9	2.0	-	3.9	-	2.0
	2	1	24.0	4.8	-	9.6	9.6	-
spread (Princes)	1	1	21.8	2.4	-	19.4	-	-
	1	2	8.6	2.6	-	5.3	0.7	-
	2	1	12.5	2.3	-	9.1	1.1	-
Sardines, canned	1	1	57.6	14.4	-	28.8	-	14.4
	1	2	63.2	21.1	-	21.1	-	21.1
('Joy to Eat')	2	1	42.0	16.8	-	16.8	-	8.4
Liver, lamb, raw	1	1	137.4	61.1	-	61.1	7.6	7.6
	1	2	93.6	44.0	-	44.0	5.6	-
Wheat Germ	1	1	0.34	-	0.28	0.56	[illegible]	-
(Kretschmer)	1	2	0.24	-	0.24	-	-	-

Table 18 Forms of vitamin B_{12} and vitamin B_{12} content (μgms/kg or μgms/l) for various Fish Preparations + Liver and Wheat Germ Extracts.

RESULTS & DISCUSSION.

The R_f values calculated for the growth zones from sample extracts on each bioautogram were compared with the R_f values of the standard cobalamins to identify the cobalamins present. With some samples of low dilution, overloading of bioautograms led to merging and hence poor resolution of growth areas, while at higher dilution better separation of cobalamins with resultant increased R_f values generally made identification possible.

The total vitamin B_{12} content (µgms./kg or µgms./l) of each tissue was calculated from the L. leichmanii assay results and apportioned to the individual cobalamins present by the semi-quantitative, serial dilution method (pp. 35-38).

Comparison of results of the cobalamins in muscle tissue (table 17) showed that tinned preparations generally contained a higher proportion of hydroxo B_{12} than their 'natural' cooked counterparts e.g., Braised beef contained 20 - 33% (av. 26%) of hydroxo B12, whereas tinned corned beef and beef spreads contained 31 - 80% (av. 64%) and 46 - 57% (av. 50%) hydroxo B_{12} respectively.

Similarly the 89% hydroxo B_{12} content of tinned chicken & ham roll greatly exceeded the hydroxo B_{12} levels found in ham (grilled) and chicken (casserole) of 0 - 50% (av. 28%). Whether this difference in distribution of cobalamins was due to the method of

preparation of the former or cooking of the latter remained uncertain although severe treatment would be required in both cases for conversion of any protein bound cobalamins.

Sulphito B_{12} (20%) appeared to be present as a genuine product in ox tongue (boiled).

Initial observations of the forms of vitamin B_{12} in dairy produce (table 16) demonstrated a clear difference from those in muscle tissue (table 17), with the presence of significant amounts of coenzyme B_{12} and methyl B_{12} in all the tissues examined (except light-exposed, pasteurised, whole milk). In contrast to the muscle tissues, processing of dairy produce appeared to have little effect on the 'natural' cobalamins present and in fact in most items an increase in the more unstable, light-sensitive cobalamins, coenzyme B_{12} and methyl B_{12} occurred in these preparations e.g. raw, whole milk contained 21% of both coenzyme B_{12} and methyl B_{12}, while cheese products, cheddar cheese and cheese spread (Krafts) contained an average of 18% and 29% of coenzyme B_{12} and 36% and 56% of methyl B_{12} respectively. This increase in 'natural' forms might however, be due to the bacterial activity involved in cheesemaking. Craft et al. (1971) found no methyl B_{12} in two samples of fresh cow's milk, which yielded a single spot on chromatography with the R_f of Co-OH B_{12}. The absence of methyl B_{12} might have arisen with light-exposure of samples which should have been eliminated by our direct collection of the raw

milk in ammonia buffer.

Egg yolk, raw, was found to contain mainly methyl B_{12} (av. 50%), whereas coenzyme B_{12} (av. 58%) predominated in egg white, raw, together with significant yields of cyano B_{12} (av. 29%), although in all the tissues examined on no occasion was cyano B_{12} present at a concentration in excess of 0.5 µgms/kg tissue.

As previously discussed (pp.117-121) sulphito B_{12} (95%) was found in pasteurised, whole milk added to the ammonia buffer in the dark, whereas addition in the daylight yielded hydroxo B_{12} (80 - 100%, av. 93%), coenzyme B_{12} (av. 3.5%) and methyl B12 (av. 3.5%). Comparison with raw, whole milk showed clearly that preparation and light exposure of the bottled, pasteurised whole milk had led to conversion of the methyl B_{12} and coenzyme B_{12} initially present. Pasteurisation of the raw milk also reduced the total cobalamin content from an average of 2.6 µgms/l. to 0.96 µgms/l.

Extracts of fish and fish preparations (table 18) exhibited similar cobalamin distributions to that of muscle tissues (meat & poultry) with hydroxo B_{12} (29 - 89%) and coenzyme B_{12} (11 - 61%) present in all extracts.

Sulphito B_{12} was most prominent in this group of tissues, being present in tinned preparations of salmon (0 - 25%, av 17%) and sardines (20 - 33%, av. 26%) and the high concentration in which it occurred in the latter

>8 µgms/kg implied that it was present naturally as opposed to artefactually. Haddock, boiled, was the only fish product found to contain cyano B_{12} although this was at a low level (4%).

Lamb's liver, raw, contained predominantly hydroxo B_{12} and coenzyme B_{12} (45 - 47%, av. 46%) with smaller amounts of methyl B_{12} (av. 5.5%) and sulphito B_{12} (av. 2.5%)

Wheat germ which had not previously been found to contain naturally occurring cobalamins, yielded a mixture of trace amounts of cyano B_{12} and hydroxo B_{12}, which had probably been added in the preparation.

In all the extractions from the same sample comparable results were obtained with only minor variations in the forms of vitamin B_{12} isolated. Significant differences in the latter were however encountered in several extractions on different samples of the same tissue. The second samples of tinned salmon and corned beef yielded 40% and 62% of methyl B_{12} respectively, a cobalamin which was absent in both the initial, duplicated extractions. Such major differences in cobalamins could have arisen in the preparation of the product but were thought more likely to have been as a result of light conversion of the methyl B_{12} in the initial extracts. These samples apart, a good correlation of results between extracts was obtained with again only minor differencies in the forms of

vitamin B_{12} isolated.

To illustrate the clearly defined variations between the forms of vitamin B_{12} in meat & poultry (table 17), dairy produce (table 16) and fish (table 18) a final table representing a summary of the results from each of these tables was compiled and from this an approximation of the daily intake of each cobalamin could be established.

Food.	No. of Tissues	No. of Extracts	Av. B_{12} Content:	Av. Content of Each Cobalamin			
				Ado.	CN.	OH.	Me.	SO_3.
Meat & Poultry.	10	21	8.4	2.9	0.0	4.2	0.95	0.41
% Age				33.8	0.0	50.0	11.3	4.9
Dairy Produce	8	20	4.7	0.78	0.03	1.68	2.17	0.11
% Age				16.2	0.7	35.2	45.8	2.2
Fish	4	11	24.4	7.3	0.06	11.45	1.1	4.5
% Age				30.0	0.2	47.0	4.5	18.4

Table 19 Average vitamin B_{12} content (µgms/kg or µgms/l) and Percentage of Each Cobalamin in the various Types of Foodstuff examined.

REFERENCES.

ADAMS, J.F., ROSS, S.K., MERVYN, L. & KING, P. (1971).
Scand. J. Gastroent. 6. 249-252.

ADAMS, J.F., McEWAN, F. & WILSON, A. (1972).
Br. J. Nutr. 29, 65-72.

ARMITAGE, J.B., CANNON, J.R., JOHNSON, A.W., PARKER, L.F.J.
SMITH, E.L., STAFFORD, W.H. & TODD, A.R. (1953).
J. Chem. Soc., 3349.

BARKER, H.A., WEISSBACH, H. & SMYTH, R.D. (1958).
Proc. Nat. Acad. Sci., 44, 1093.

BARKER, H.A., SMYTH, R.D., WEISSBACH, H., TOOHEY, J.I.,
LADD, J.N. & VOLCANI, D.E. (1960).
J. Biol. Chem. 235, 480-488.

BERNHAUER, K. & IRION, E. (1964).
Biochem. Zeit. 339, 530.

BILKUS, D.I. & MERVYN, L. (1971).
The Cobalamins 1st. ed. p. 20. (Armstein, H.P.V. &
Wrighton, R.J., eds.), Edinburgh and London, Churchill
Livingstone.

BLUMBERGER, K., PETRIDES, P. & BERNHAUER, K. (1957).
Vitamin B12 and Intrinsic Factor 1. Europaisches
Symposion, Hamburg (1956), p. 82. Ed. Heinrich, H.C.,
Stuttgart: Ferdinand Enke.

BRADY, R.O. & BARKER, H A. (1961).
Biochem. Biophys. Res. Comm. 4, 373.

BONNETT, R., CANNON, J.P., CLARK, V.M., JOHNSON, A.W., PARKER, L.F.J., SMITH, E.LESTER, & TODD, SIR ALEXANDER. (1957), J. Chem. Soc., 1158; ibid., 1148.

BRINK, N.G., WOLF, D.E., KACZKA, E., RICKES, E.L., KONIUSZY, F.R., WOOD, T.R. & FOIKERS, K. (1949). J. Amer. Chem. Soc. 71, 1354.

BUHS, R.P., NEWSTEAD, F.G. & TRENNER, N.R. (1951) Science 113, 625.

CHANARIN, I. (1969). The Megaloblastic Anaemias, Blackwell Scientific Publications, Oxford and Edinburgh.

COOLEY, G., ELLIS, B., PETROW, V., BEAVEN, G.H., HOLIDAY, E.R.&JOHNSON, F.A. (1951). J. Pharm. Pharmacol. 3, 271.

CRAFT, I.L., MATTHEWS, D.M. & LINNELL, J.C. (1971). J. Clin. Path., 24, 449-455.

DeMERRE, L.J. & WILSON, C. (1956). J. Am. Pharm. Ass., Scient. Edn., 45, 129.

DOLPHIN, D.H., JOHNSON, A.W. & SHAW, N. (1963). Nature 199, 170-171.

FANTES, K.H., & O'CALLAGHAN, C.H. (1956). Biochem. J., 63, 10P.

FANTES, K.H., PAGE, J.E., PARKER, L.F.J., & SMITH, E.L. (1949). Proc. Roy. Soc. B., 136, 592.

FIRTH, R.A., HILL, H.A.O., PRATT, J.M., THORP, R.G.& WILLIAMS, R.J.P. (1969).
J. Chem. Soc. (A), 381.

FORD, J.E., & HOLDSWORTH, E.S. (1953).
Biochem. J. 53, xxii.

FORD, J.E., HOLDSWORTH, E.S., KON, S.K., & PORTER, J.W.G. (1953.) Biochem. J., 59, 36.

FROST, D.V., LAPIDUS, M., PLAUT, K.A., SCHERFLING, E., & FRICKE, H.H. (1952). Science, 116, 3005.

HARTLEY, F., STROSS, P. & STUCKEY, R.E. (1950).
J. Pharm. Pharmacol., 2, 643.

HAYWARD, G.C., HILL, H.A.O., PRATT, J.M., VANSTON, N.J. & WILLIAMS, R.J.P. (1965).
J. Chem. Soc. 6435.

HAYWARD, G.C., HILL, H.A.O., PRATT, J.M. & WILLIAMS R.J.P. (1971). J. Chem. Soc. (A), 196.

HODGKIN, D.C., (1957). Vitamin B_{12} and Intrinsic Factor 1. Europaisches Symposion, Hamburg (1956), pp. 31-39. Ed. Heinrich, H.C., Stuttgart: Ferdinand Enke.

HOGENKAMP, H.P.C. & BARKER, H.A. (1961).
J. Biol. Chem. 236, 3097.

JOHNSON, A.W., MERVYN, L., SHAW, N. & SMITH, E. LESTER. (1963). J. Chem. Soc. 4146.

LINDSTRAND, K. (1965). Acta Chem. Scand. 7, 1762-1763.

LINDSTRAND, K. & STÅHLBERG, K.G., (1963). Acta Med. Scand., 174, 665-669.

LINNELL, J.C., MACKENZIE, H.M., WILSON, J.,& MATTHEWS,D.M. (1969)., J. Clin. Path., 22, 545-550.

LINNELL, J.C., HUSSEIN, H.A-A. & MATTHEWS, D.M. (1970). J. Clin. Path. 23, 820-321.

LINNELL, J.C., HOFTBRAND, A.V., PETERS, T.J. & MATTHEWS, D.M. (1971). Clinical Science, 40, 1-16.

LOVE, R.M. (1970). The Chemical Biology of Fishes 1st. ed. London and New York: Academic Press.

McCANCE, R.A. & WIDDOWSON, E.M. (1969). M.P.C. Spec. Rep. No. 297, The Composition of Foods, 3rd. Edn., H.M.S.O. London.

MERVYN, L., BIKUS, D.I. & TURNER, M.K. (1972). Personal Communication.

MULLER, O. & MULLER, G. (1962). Biochem. Zeit. 336, 299.

PAILES, W.H., & HOGENKAMP, H.P.C. (1968). Biochemistry. 7, 4160.

PRATT, J.M., (1964). J. Chem. Soc. 5154.

PRATT, J.M. & WHITTAR, B.R.D., (1971).
J. Chem. Soc. (A), 252.

RICKES, E.L., BRINK, N.G., KONIUSZY, F.R., WOOD, T.R.,
& FOLKERS, K. (1948). Science, 107, 396.

ROBINSON, F.A. (1966). The Vitamin Co-factors of
Enzyme Systems 1st. Ed. Oxford: Pergamon Press.

ROSS, G.I.M., HUTNER, S.H. & BACH, M.K. (1956).
Vitamin B_{12} and Intrinsic Factor 1. Europaisches
Symposion, Hamburg (1956), pp. 305-310. Ed. Heinrich, H.

SCHADE, S.G. & SCHILLING, R.F. (1967).
Am. J. Clin. Nutr., 20, 636-640.

SMITH, E.L., (1965). Vitamin B_{12}, 3rd. edn. Methuen &
Co. Ltd., London.

SMITH, E.L. & PARKER, L.F.J., (1948).
Biochem. J., 43, viii.

SMITH, E.L., BALL, S. & IRELAND, D.M., (1952).
Biochem. J., 52, 395-400.

SMITH, E.L., MERVYN, L., JOHNSON, A.W. & SHAW, N. (1962).
Nature, Lond. 194, 1175.

SUOMELA, H.,(1967). Scand. J. Clin. Lab. Invest. 95, 35.

TAYLOR, R. T. & WEISSBACH, H.,(1968).
Arch. Biochem. Biophys., 123, 109.

VEER, W.L.C., EDELHAUSEN, J.H., WIJMENGA, H.G., & LENS, J. (1950)., Biochim. Biophys. Acta 6, 225.