**387.** *The Properties of Cobalamins with Sulphur-containing Ligands*

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Cobalamins with sulphur-containing ligands have been examined, and the physical and chemical properties of the compounds have been shown to be intermediate between those of cyanocobalamin (vitamin B_{12}) and methylcobalamin. Photolysis of the sulphur-containing cobalamins is described as well as reactions with mineral acids, cyanide, chloramine-T, and alkylating agents.

THE X-ray crystallographic elucidation [1] of the structure of the vitamin B_{12} coenzyme (partial structure I; R = 5′-deoxyadenosyl) was soon followed by a partial synthesis [2,3] of the alkylcobalamins, including the coenzyme, from hydroxocobalamin, vitamin B_{12b} (I; R = H_2O) by reduction to hydridocobalamin, B_{12s} (I; R = H) and subsequent reaction with suitable alkylating agents. Intermediate between hydroxo- and hydridocobalamin is B_{12r},[4] a bivalent cobalt complex [5,6] which does not form alkylcobalamins with alkylating agents, e.g., methyl and ethyl iodides, methyl toluene-p-sulphonate, 2′,3′-isopropylidene-5′-toluene-p-sulphonyladenosine, under a variety of experimental conditions, e.g., in vacuo or in an atmosphere of nitrogen or hydrogen at temperatures of up to 100°. Some of these observations have been confirmed recently by Williams et al.,[7] who also modified their earlier views [8] on the nature of B_{12r}. There are many striking differences between the properties of cyanocobalamin, vitamin B_{12} (I; R = CN) and hydroxocobalamin on the one hand, and the alkylcobalamins,[9] including the coenzymes,[10] on the other, especially the ease of protonation and photolysis of the alkylcobalamins. The physical and chemical properties of any cobalamin (I) are affected substantially by the nature of the group R and in order to permit further generalisation, cobalamins with sulphur-containing ligands

(I)

(II)
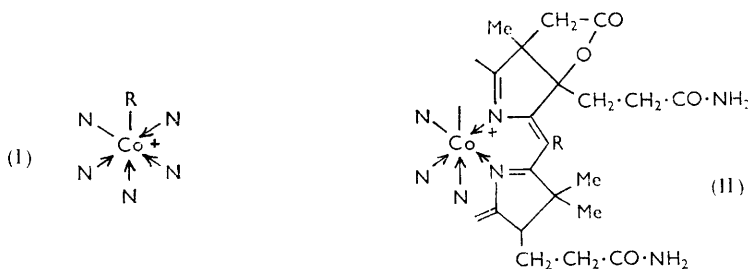
have been examined, and compared with those containing cobalt–carbon bonds. The preparations of some cobalamins bearing sulphur-containing ligands, e.g., sulphato-,[11] thiocyanato-,[12] sulphito-,[8,13,14] and toluene-p-sulphonyl-cobalamins [15] have already been

[1] Lenhert and Hodgkin, *Nature*, 1961, **192**, 937; " Vitamin B_{12} und Intrinsic Factor," F. Enke, Stuttgart, 1962, p. 82.

[2] Smith, Mervyn, Johnson, and Shaw, *Nature*, 1962, **194**, 1175; *J.*, 1963, 4146.

[3] Bernhauer, Müller, and Müller, *Biochem. Z.*, 1962, **336**, 102; Müller and Müller, *ibid.*, p. 299.

[4] Diehl et al., *Iowa State J. Sci.*, 1952, **26**, 555; *J. Amer. Chem. Soc.*, 1954, **76**, 4345.

[5] Hogenkamp, Barker, and Mason, *Arch. Biochem. Biophys.*, 1963, **100**, 353.

[6] Dolphin, Johnson, Rodrigo, and Shaw, *Pure and Appl. Chem.*, 1963, **7**, 539.

[7] Hill, Pratt, and Williams, *Chem. and Ind.*, 1964, 197.

[8] Hill, Pratt, and Williams, *J. Theor. Biol.*, 1962, **3**, 423.

[9] Dolphin, Johnson, and Rodrigo, *J.*, 1964, 3186.

[10] Johnson and Shaw, *J.*, 1962, 4608.

[11] Kaczka, Wolf, Kuehl, and Folkers, *J. Amer. Chem. Soc.*, 1951, **73**, 3569.

[12] Buhs, Newstead, and Trenner, *Science*, 1951, **113**, 625.

[13] Dolphin, Johnson, and Shaw, *Nature*, 1963, **199**, 170.

[14] Smith, Ball, and Ireland, *Biochem. J.*, 1952, **52**, 395; Fricke, U.S.P. 2,721,162 (*Chem. Abs.*, 1956, **50**, 2129); Bernhauer, Wagner, et al., *Biochem. Z.*, 1962, **335**, 443; 1963, **337**, 366.

[15] Dr. E. Lester Smith, private communication.

described. It has also been reported [16,17] that glutathione and certain other thiols, including sodium hydrogen sulphide, will react with hydroxocobalamin to form violet- and then brown-coloured complexes and that in presence of oxygen the thiol is oxidised to the disulphide.[18] Treatment of the cobalamine–sulphide complexes with methyl iodide gives methylcobalamin.[16,17] These reactions are relevant to biosynthetic studies on the vitamin B_{12} coenzyme [19] which have shown that a reduced flavin ($FADH_2$), a thiol (*e.g.*, glutathione or 2-mercaptoethanol), as well as adenosine triphosphate (ATP) and manganese ions were required to effect the conversion of hydroxocobalamin to the coenzyme. The nature of the products obtained from the action of thiols on hydroxocobalamin has not been established fully as they are stable only in solution and in the presence of excess of thiol but the behaviour of the violet thiol complexes on electrophoresis leads us to regard them as complexes (I; R = S-alkyl) containing cobalt–sulphur bonds.

All the sulphur-containing cobalamins mentioned above contain cobalt–sulphur bonds except the sulphato complex (I; R = $O \cdot SO_2 \cdot OH$) which is similar to hydroxo- and cyano-cobalamin in its behaviour. Infrared evidence is adduced to support the assigned structures, for example the presence of two strong bands at 983 and 1150 cm.$^{-1}$ in the spectrum of sulphitocobalamin are associated with the cobalt–sulphur linked sulphito-group in another series of cobalt complexes.[20]

The ultraviolet and visible spectra of the cobalamins with sulphur-containing ligands form an interesting series with the intensity of absorption at *ca.* 350 mμ and the absorption maxima located between those of cyano- and hydroxo-cobalamin on the one hand and the alkylcobalamins on the other. The spectrum of sulphatocobalamin, which contains a cobalt–oxygen bond is, as expected, similar to that of hydroxocobalamin.

The principle cobalamin absorption bands
($\varepsilon \times 10^{-4}$ in parenthesis)

Cobalamin (I)

Cyano-		361(2·75)	550(0·85)
Hydroxo-		351(2·02)	530(0·76)
Sulphato-	274(1·71)	352(2·10)	525(0·76)
Thiocyanato- (in 0·1N-KCNS)	315(0·80)	358(1·78)	542(0·74)
Sulphito-	315(1·35)	365(1·40)	515—540(0·76)
Tosyl-	335(1·61)	368(1·62)	520—545(0·74)
Glutathione complex	333(1·47)	375(1·27)	530—560(0·71)
Methyl-	343(1·20)	375(1·04)	522(0·74)

Sulphito- and toluene-*p*-sulphonyl-cobalamins as well as the sulphide complexes, but not sulphato- or thiocyanato-cobalamins, show the typical spectrum of yellow protonated forms in dilute acid solution; in concentrated acid they are hydrolysed to hydroxocobalamin.

Reaction with Cyanide.—It has been shown [9] that many of the simple alkylcobalamins do not react with aqueous potassium cyanide in the dark. However under similar conditions both the B_{12} coenzyme and 2-ethoxycarbonylethylcobalamin (I; R = $CH_2 \cdot CH_2 \cdot CO_2Et$) react with cyanide to form the dicyano-complex.[21] All the cobalamins with sulphur-containing ligands reacted rapidly with aqueous potassium cyanide even in the absence of both air and light to give quantitative yields of the dicyano-compound.

Reaction with Acids.—The B_{12} coenzyme and its alkyl analogues are protonated by cold dilute mineral acid with a consequent red to yellow colour change.[8,22] On the other hand, hydroxo- and cyano-cobalamin require, for example, concentrated sulphuric acid to

[16] Dolphin and Johnson, *Proc. Chem. Soc.*, 1963, 311.
[17] Wagner and Bernhauer, Conference on Vitamin B_{12} Coenzyme, New York, 1963; *Annals New York Acad. Sci.*, 1964, **112**, 580.
[18] Peel, *Biochem. J.*, 1963, **88**, 296; Aronovitch and Grossowicz, *Biochem. and Biophys. Res. Comm.*, 1962, **8**, 416.
[19] Brady, Castanera, and Barker, *J. Biol. Chem.*, 1962, **237**, 2325; Weissbach, Redfield, and Peterkovsky, *ibid.*, p. 3217.
[20] Baldwin, *J.*, 1961, 3123.
[21] Wijmenga, Veer, and Lens, *Biochim. Biophys. Acta*, 1950, **6**, 229.
[22] Ladd, Hogenkamp, and Barker, *J. Biol. Chem.*, 1961, **236**, 2114.

bring about a similar colour change.[8]   The sulphur-containing cobalamins fall into the same two groups in this respect.   Thus thiocyanato- and sulphato-cobalamins require concentrated sulphuric acid to produce yellow protonated forms, whereas sulphito- and toluene-*p*-sulphonyl-cobalamins and all the violet complexes derived from thiols, underwent a reversible red to yellow colour shift with dilute acids.   This effect is controlled by the electronic properties of the ligand R in the cobalamin (I), those of methyl contrasting with those of cyano, and those of an alkylthiol contrasting with those of thiocyanato in the same manner.

Photolysis.—The behaviour of the sulphur-containing cobalamins on photolysis showed that there were again two types and that the subdivision was the same as had been observed in the protonation experiments.   Solutions of sulphato- and thiocyanato-cobalamins proved to be stable to prolonged photolysis both in the presence and absence of oxygen. In the presence of oxygen, sulphito- and toluene-*p*-sulphonyl-cobalamins underwent photolysis to yield hydroxocobalamin at a rather slower rate than the coenzyme.   However, unlike the coenzyme, but similar to the alkylcobalamins,[9] both sulphito- and toluene-*p*-sulphonyl-cobalamins are stable to photolysis in the complete absence of oxygen.   Solutions of the thiol complexes for photolytic study had to be prepared in the presence of excess of thiol and most of this work has been carried out on aqueous solutions of the glutathione complex.   In the absence of light, the solution was relatively stable in air and only about 5% of the chromophore was destroyed by secondary reactions after 70 hr. (estimated spectroscopically after reaction with cyanide and comparison with a standard solution). In the presence of light (200w tungsten bulb at 20 cm. distance), the main product was hydroxocobalamin but about 40% of the chromophore had been destroyed after 70 hr. In the absence of oxygen (10^{-6} mm.), photolysis caused a slow change in the spectrum of the solution until after 70 hr. the product contained approximately equal amounts of vitamin B_{12r} and the thiol complex.   When air was admitted to the product, the spectrum changed to that of the thiol complex.   It was estimated that about 10% of the chromophore was destroyed during this sequence.   When cysteine was substituted for glutathione in the last experiment a larger proportion of vitamin B_{12r} was obtained, and when 2-mercaptoethanol or thioglycollic acid were used, the conversion into vitamin B_{12r} occurred after only 10 min.   After longer periods decomposition of the chromophore took place and after 6 hr. it was irreversibly decomposed, possibly by addition of the thiol to one or more of the chromophoric double bonds but this has not yet been established with certainty.

Hydrogenolysis.—Although the simple alkylcobalamins are stable in presence of sodium borohydride or zinc and acetic acid, methylcobalamin (and presumably its homologues) can be hydrogenolysed in presence of a platinum catalyst to vitamin B_{12r} and methane, a reaction which is related to the microbiological production of methane.[23]   The sulphur-containing cobalamins are more susceptible to reduction and conversion into vitamin B_{12r} occurs with the sulphito-, sulphato-, and thiocyanato-cobalamins as well as with the thiol complexes with either sodium borohydride or with zinc and acetic acid.   In the case of the thiol complexes, reduction to vitamin B_{12r} occurs and on re-oxidation to vitamin B_{12b} complexing with the thiols again takes place.

*Reaction with Chloramine-*T.—The reaction of cyano- or hydroxo-cobalamin with chloramine-T initially gives the so-called B_{12} lactone (partial structure II; R = H) [24] and further reaction causes substitution into the chromophore, possibly at C_{10}, to yield the chlorolactone (II; R = Cl).   Barton and Beckwith [25] have recently suggested a free-radical mechanism for the cyclisation of saturated amides to lactones by the action of halogens in the presence of light but these experimental conditions are quite different from those used in the formation of the B_{12} lactone where neither light nor lead tetra-acetate is required

[23] Blaycock and Stadtmann, *Biochem. Biophys. Res. Comm.*, 1963, **11**, 34.
[24] Bonnett, Cannon, Clark, Johnson, Parker, Smith, and Todd, *J.*, 1957, 1158.
[25] Barton and Beckwith, *Proc. Chem. Soc.*, 1963, 335.

for the cyclisation.   Different mechanisms [cf. 24] may be involved in these reactions.   In the case of the alkylcobalamins, the substitution reaction occurs before the cyclisation,[9] but it has now been shown that each type of sulphur-containing cobalamin reacts with chloramine-T in a different manner.   Thus, sulphatocobalamin resembled cyano- and hydroxo-cobalamins in that the first equivalent of reagent caused mainly cyclisation (but with a little substitution).   The second equivalent of reagent then completed the cyclisation and caused substitution.   Treatment of the product with cyanide gave the same dicyano-chloro-lactone as had been obtained by the action of excess of cyanide on the chloro-lactone derived from cyanocobalamin.   In a preliminary Communication,[13] we have reported that the behaviour of sulphitocobalamin with chloramine-T parallels that of the alkylcobalamins in that the first equivalent of the reagent formed the chloro-sulphito-cobalamin and the second equivalent yielded the corresponding lactone.   This product was again converted into the dicyano-chloro-lactone with excess of cyanide.   On the other hand, little reaction occurred with thiocyanatocobalamin with one equivalent of chloramine-T and more than 70% of the unchanged cobalamin could be recovered.   Reaction with larger quantities of the reagent caused some cyclisation but also resulted in the loss of the thiocyanato-ligand.

A new type of reaction was observed with toluene-*p*-sulphonylcobalamin and chloramine-T.   With one equivalent of the reagent the products were hydroxocobalamin and toluene-*p*-sulphonyl chloride.   Larger quantities of chloramine-T reacted with hydroxocobalamin to form first the corresponding lactone (II; R = H) and then the chloro-lactone (II; R = Cl).   When less than one equivalent of chloramine-T was used, the yield of hydroxocobalamin was proportional to the amount of chlorinating agent and no lactonisation or chlorination of toluene-*p*-sulphonyl cobalamin was observed.

Reaction with Alkylating Agents.—No reaction was observed with sulphato-, sulphito-, toluene-*p*-sulphonyl, or thiocyanato-cobalamins and either alkyl halides or 5'-toluene-*p*-sulphonyl-2',3'-isopropylideneadenosine.   However when any of the cobalamin thiol complexes mentioned above, including the glutathione complex,[cf. 17,26] was treated with methyl iodide in the absence of air and light, small amounts of methylcobalamin were formed, and similar reactions were observed with ethyl iodide.   When sodium sulphide or sodium hydrogen sulphide was treated with hydroxocobalamin in aqueous solution and methyl iodide, a rapid reaction occurred and methylcobalamin was obtained in high yield, even though there was no apparent colour change, although the spectrum changed from that of hydroxocobalamin to that of methylcobalamin.   Addition of sodium sulphide to aqueous hydroxocobalamin caused the formation of a brown solution and addition of methyl iodide caused the formation of methylcobalamin.   The rate of methylation was increased about threefold if the brown intermediate was allowed to form as a preliminary stage.   Although, as stated above, vitamin B_{12r} alone does not react with methyl iodide to form methylcobalamin, if sodium sulphide is added to a solution of vitamin B_{12r}, the resulting solution has a spectrum identical to that of the brown solution formed from sodium sulphide and hydroxocobalamin, and in each case the product gives a high yield of methylcobalamin with methyl iodide.   Measurement of reaction rates showed that the formation of the brown intermediate from hydroxocobalamin and sodium sulphide was a relatively slow reaction but the subsequent methylation of the brown intermediate was very fast.   In view of these experiments, we regard the reactive brown intermediate as a thiol complex of cobalt in a reduced valency state, probably bivalent, but in the presence of excess of methyl iodide the reduction step does not appear to occur, and the intermediate may then be the thiol complex of tervalent cobalt.   However, when hydroxocobalamin, sodium sulphide, and ethyl iodide are mixed, the formation of the brown intermediate is clearly observed.   In this case, the formation of ethylcobalamin from the brown thiol intermediate with ethyl iodide is only a little faster than its formation from a mixture of hydroxocobalamin, sodium sulphide, and ethyl iodide.   Alkylation reactions were also carried out

[26] Bernhauer, Müller, and Wagner, *Angew. Chem. Internat. Edn.,* 1964, **3,** 200.

with the n-propyl halides and chloroacetic acid although the rate of reaction decreased with increasing size of the alkyl group and no B_{12} coenzyme derivative could be obtained by alkylation of the thiol complex with 5'-toluene-*p*-sulphonyl-2',3'-isopropylideneadenosine.

EXPERIMENTAL

Ultraviolet and visible absorption spectra were measured on aqueous solutions in Perkin-Elmer 137 and Unicam S.P. 500 spectrophotometers. Infrared spectra were determined on KBr discs. The principal chromatographic solvent systems used were: solvent I, butan-2-ol–water–25% ammonium hydroxide, 50 : 36 : 14; solvent II, butan-1-ol–ethanol–water, 50 : 15 : 35; solvent III, butan-1-ol–propan-2-ol–water, 10 : 7 : 10.

Sulphitocobalamin.—Hydroxocobalamin (100 mg.) was dissolved in water (10 ml.) and sodium sulphite (100 mg.) was added. The mixture was kept in the dark for 5 min. and then purified through phenol and the resulting solution chromatographed on a column (30 × 2 cm.) of carboxymethylcellulose. The fast running fraction was collected and reduced in volume to 0·5 ml., when acetone was added dropwise until a faint turbidity was produced. The solution was kept in the dark at room temperature for 6 days when the red needles of sulphitocobalamin were separated, washed with acetone, and dried (83 mg.). The infrared spectrum (KBr disc) contained max. at 983 and 1150 cm.$^{-1}$ which were not present in the spectrum of hydroxocobalamin. Light absorption: max. at 266, 278, 315, 365, 421, and 515—540 (broad) mμ (ε, 19,000, 20,000, 13,300, 14,000, 4800 and 7600, respectively), and in N-hydrochloric acid: max. at 265, 276, 286, 320, 425, and 460 mμ (ε, 22,200, 19,400, 16,900, 14,700, 7400, and 7000, respectively).

Toluene-p-sulphonylcobalamin.—Hydroxocobalamin (100 mg.) was dissolved in water (10 ml.) in an atmosphere of nitrogen. Sodium borohydride (50 mg.) was added in one portion while maintaining the inert atmosphere and was followed by air free dilute acetic acid (2 ml. of N) when the cobalamin solution had turned grey-green. Toluene-*p*-sulphonyl chloride was then added and the mixture kept in the dark for 5 min. It was then purified through phenol, and the resulting aqueous solution chromatographed on carboxymethylcellulose when the fast running fraction was collected, reduced in volume to 0·5 ml., and crystallised from aqueous acetone as in the previous experiment. The deep red needles (73 mg.; 66%) were separated, washed, and dried (Found: C, 55·8; H, 6·35; N, 12·45; S, 2·6. $C_{69}H_{95}CoN_{13}O_{16}PS$ requires C, 55·9; H, 6·45; N, 12·3; S, 2·15%). Light absorption: max. at 267, 281, 335, 368, 406, 426, and 520—545 (broad) mμ (ε 10,100, 15,600, 16,100, 16,200, 4400, 4000, and 7400, respectively), with an inflection at 293 mμ (ε 13,000), and in N-hydrochloric acid: max. at 265, 276, 286, 324, 421, and 512 mμ (ε 17,000, 16,000, 14,300, 15,100, 10,100, and 4300, respectively).

Thiocyanatocobalamin.[cf. 12]—Potassium thiocyanate (15 mg.) was added to a solution of hydroxocobalamin (100 mg.) in water (10 ml.). Acetone (100 ml.) was added and the mixture kept for 30 min. The solid product was separated, redissolved in water (1·5 ml.), acetone was added until a faint turbidity was produced, and the mixture kept overnight. The thiocyanato-complex (91 mg.; 88%) was separated, washed with acetone, and dried *in vacuo*. The infrared spectrum contained a band at 2105 cm.$^{-1}$ (SCN). Light absorption: max. at 261, 272, 288, 315, 352, 411, 503, and 529 mμ (ε 18,000, 18,200, 15,100, 8000, 20,200, 3400, 6800, and 7500) but in 0·1N-potassium thiocyanate solution the spectrum changed to max. at 266, 280, 287, 315, 358, 420, and 542 mμ (ε 18,000, 17,900, 17,300, 8200, 17,800, 3200, and 7400, respectively), indicating that the thiocyanate complex was appreciably hydrolysed in aqueous solution.

Sulphatocobalamin.—Prepared (64%) from hydroxocobalamin by the method of Kaczka *et al.*[11] The infrared spectrum contained bands at 618, 913, 1021, 1047, and 1120 cm.$^{-1}$ which are not present in the spectrum of hydroxocobalamin and are associated with the sulphate group.[27] Light absorption: max. at 274, 352, 410, and 525 mμ (ε 17,100, 21,000, 3300, and 7600, respectively[cf. 11]).

Reaction of Hydroxocobalamin with Thiols.—The thiol (200 mg.) was added to a solution of hydroxocobalamin (50 mg.) in water (10 ml.) in an atmosphere of nitrogen. The colour of the solution immediately turned from orange-red to violet. Attempted purification by the phenol extraction process led to decomposition with the formation of some hydroxocobalamin and consequently reactions of the thiol complexes were carried out in solutions with excess of the thiol present. Violet complexes were obtained by reaction with the following thiols: glutathione, 2-mercaptoethanol, thioglycollic acid, cysteine, homocysteine, ethylthiol, ω-mercaptotoluene, sodium hydrogen sulphide. In electrophoresis at pH 2·5 on Whatman No. 1

[27] Nakamoto, Fujita, Tanaka, and Kobayashi, *J. Amer. Chem. Soc.*, 1957, **79**, 4904.

paper at 10 v./cm. for 3 hr., the following movements were obtained (values in parentheses indicate migration in mm. towards the cathode): cyanocobalamin (1·0), hydroxocobalamin (29·0), glutathione complex (2·0), and at pH 11·0 for 4 hr.: cyanocobalamin (1·0), lactone of cyanocobalamin [24] (16·0), hydroxocobalamin (1·0), glutathione complex (30·0). A solution of the violet cobalamin-glutathione complex showed max. at 280, 285, 291, 333, 375, 408, 428, 535, and 562 mμ (ε 18,900, 22,300, 22,400, 14,700, 12,700, 3600, 4000, 7100, and 7100, respectively), and in 3N-hydrochloric acid: max. at 277, 287, 335, 432, and 507 mμ (ε 24,000, 22,700, 17,300, 6100, and 6700, respectively). A solution of the violet cobalamin-cysteine complex showed max. at 280, 285, 290, 335, 374, 430, 532, and 560 mμ (ε 19,000, 22,000, 22,000, 14,000, 12,500, 4000, 7000, and 7000, respectively).

Reaction of the Sulphur-containing Cobalamins with Potassium Cyanide.—(i) *In the absence of light and air.* An aqueous solution of the cobalamin was frozen and degassed in the dark in an apparatus consisting of a silica cell attached to two bulbs, one containing the cobalamin solution and the other solid potassium cyanide. The pressure above the frozen solution was reduced to 10^{-6} mm., and the sample then sealed off from the pumping system. The solution was warmed to room temperature and mixed with the potassium cyanide, all operations being carried out with exclusion of light. The spectrum of the solution was measured at intervals and after 30 min. all the cobalamins, *i.e.*, sulphito-, sulphato-, toluene-*p*-sulphonyl-, thiocyanato- and the complexes prepared from hydroxocobalamin and glutathione and cysteine, had been converted into the dicyanocobalamin [21] (max. at 367, 540, and 580 mμ).

(ii) *In the absence of light.* The above experiments were repeated in the presence of air and once again all the sulphur-containing cobalamins were rapidly converted into the dicyano-cobalamin (spectrum) after 30 min.

*Reactions with Chloramine-*T.*—(a) Sulphatocobalamin.* (i) A solution of sulphatocobalamin (60 mg.) in water (10 ml.) containing N-sulphuric acid (6 drops) was mixed with a solution of chloramine-T (12 mg.; 1 equiv.) in water (1 ml.) and the mixture kept for 2 hr. Acetone (200 ml.) was added to the product, and the solution kept at room temperature for 5 hr. and then at −2° for 18 hr. The red crystalline product (45 mg.) was separated, washed, and dried. The infrared spectrum contained bands at 618, 913, 931, 1022, 1048, 1120, and 1778 cm.$^{-1}$. Examination of the product by electrophoresis showed that it consisted of a mixture of unchanged starting material and a lactone in the ratio 1 : 2.

(ii) A solution of sulphatocobalamin (60 mg.) as above was mixed with a solution of chloramine-T (35 mg.; 3 equiv.) in water (1 ml.). The mixture was kept for 3 hr., then acetone (250 ml.) was added, and the solution kept at −2° for a further 23 hr. The resulting purple amorphous solid (45 mg.) was collected by filtration, washed with acetone, and dried. The infrared spectrum contained bands at 618, 913, 931, 1021, 1047, and 1778 cm.$^{-1}$ which is consistent with the chloro-lactone of sulphatocobalamin. Light absorption: 356, 542, and 554 mμ (ε 18,250, 6920, and 7050, respectively).

Addition of excess of aqueous potassium cyanide to an aqueous solution of the product, and purification through phenol gave a derivative which appeared homogeneous by paper chromatographic examination, and by comparison with an authentic specimen, was shown to be the chloro-lactone of cyanocobalamin.

(b) *Thiocyanatocobalamin.* A solution of thiocyanatocobalamin (65 mg.) in water (3 ml.) containing 3 drops of acetic acid was mixed with a solution of chloramine-T (37 mg.; 3 equiv.) in water (1 ml.) and kept for 16 hr. Acetone was added until a faint turbidity was obtained and the solution kept at −2° for 24 hr. The red amorphous product (40 mg.) was separated, washed, and dried. The infrared spectrum showed that some lactonisation had occurred but that the thiocyanato-group had been lost.

A similar reaction with equimolecular quantities of the reactants gave only unchanged starting material.

(c) *Toluene-*p-*sulphonylcobalamin.* (i) A solution of toluene-*p*-sulphonylcobalamin (100 mg.) in water (10 ml.) was covered with ether (10 ml.) and mixed with a solution of chloramine-T (20 mg.) in water (1 ml.) to which had been added N-hydrochloric acid (3 drops). The mixture was shaken in the dark for 15 min., the ether layer separated, and the aqueous layer extracted with ether (2 × 10 ml.). Chromatography of the aqueous layer showed that only hydroxocobalamin was present. The combined ethereal layers were washed with water (10 ml.) then saturated sodium chloride solution (10 ml.) and dried, and the volume reduced to 0·5 ml. The product was examined by gas–liquid chromatography in a Perkin-Elmer model 800

apparatus (GC 22 support, 80—100 mesh, with 15% DC 710 silicone oil; 12 ft. × 1/8 in. column) at 174° with nitrogen as the carrier gas at 33 ml./min. and an injection temperature of 243°. Under these conditions only one compound, other than ether, was detected having a retention time of 9 min. and being indistinguishable from an authentic specimen of toluene-*p*-sulphonyl chloride.

(ii) Similar experiments were conducted with 0·5 and 0·25 mole of chloramine-T per mole of toleuene-*p*-sulphonylcobalamin. The only cobalamin detected in the products was hydroxocobalamin other than unchanged toluene-*p*-sulphonylcobalamin.

(iii) A solution of toluene-*p*-sulphonylcobalamin (33 mg.) in water (10 ml.) was treated with chloramine-T (20 mg.; 3 equiv.) in water (2 ml.) to which had been added N-hydrochloric acid (10 drops). After the solution had been kept in the dark for 18 hr., the product was purified through phenol and then chromatographed on carboxymethylcellulose. No unchanged toluene-sulphonylcobalamin remained and the product was eluted with 0·1N-hydrochloric acid, then treated with excess of aqueous potassium cyanide and again purified through phenol. Chromatography on paper using each of solvents I, II, and III showed that the product consisted of 60% of the lactone of cyanocobalamin [24] and 40% of the chlorolactone of cyanocobalamin.[24] Similar results were obtained when sodium hypochlorite was used in place of chloramine-T.

Aerobic Photolysis.—(*a*) *Thiocyanato- and sulphato-cobalamins.* Solutions of these cobalamins (2 mg.) were prepared in water (10 ml.) and samples in a silica cell were photolysed for 12 hr. at a distance of 10 cm. from a Phillips M.L.U. 300w-bulb. In both cases the cobalamins were unchanged.

(*b*) *Sulphitocobalamin.* A sample of a solution of sulphitocobalamin (2 mg.) in water (10 ml.) was placed in a silica cell and photolysed by a 100w-bulb at a distance of 20 cm. The spectrum of the product was measured at intervals and the final product shown by chromatography in solvents I, II, and III to be hydroxocobalamin. Isosbestic points were observed at 248, 338, 359, 455, and 498 mμ.

(*c*) *Toluene*-p-*sulphonylcobalamin.* This was carried out as for the thiocyanato- and sulphato-cobalamins (above). The photolysis was slow (*ca.* 36 hr.), and chromatography of the final photolysed product showed that hydroxocobalamin was the only cobalamin present.

(*d*) *The glutathione and cysteine complexes.* Hydroxocobalamin (0·4 mg.) and glutathione (3·2 mg.) were dissolved in water (10 ml.) and a sample of the solution placed in a 1 cm. silica cell and photolysed by a 200w tungsten lamp at a distance of 20 cm. After 70 hr. the ultraviolet and visible spectrum (max. at 275, 351, and 525 mμ) showed that only hydroxocobalamin was present. Excess of potassium cyanide was added to the solution and a comparison of the resulting spectrum with that of the original solution with excess of potassium cyanide showed that about 40% of the chromophore had been destroyed. The experiment was repeated with exclusion of light and in this case after 70 hr. only about 5% of the chromophore was destroyed.

Similar reactions were carried out with cysteine in place of glutathione and essentially similar results were obtained.

Anærobic Photolyses.—(*a*) *Sulphitocobalamin.* A solution of sulphitocobalamin (2·0 mg.) in water (10 ml.) was frozen with complete exclusion of light and the pressure above the solid reduced to 10⁻⁶ mm. The solid was then sealed off from the pumping system and allowed to melt at room temperature. The solution was photolysed in daylight for 60 hr. but no change in the spectrum was observed indicating that a solution of sulphitocobalamin is stable under these conditions.

(*b*) *Toluene*-p-*sulphonylcobalamin.* A similar experiment with toluene-*p*-sulphonylcobalamin showed that it was stable in daylight for at least 60 hr. under anærobic conditions.

(*c*) *The glutathione complex.* Hydroxocobalamin (0·4 mg.) and glutathione (3·2 mg.) were dissolved in water (10 ml.), a sample of the solution was frozen, and the pressure reduced to 10⁻⁶ mm. in the usual manner. The solution was warmed to room temperature and photolysed in a 1-cm. silica cell by means of a 200w tungsten lamp at a distance of 20 cm. The ultraviolet and visible spectrum of the solution slowly changed and after 70 hr., it showed the presence of a mixture of vitamin B_{12r} and the violet glutathione complex ($\lambda_{max.}$ 290, 313, 333, 375, and 475 mμ). Air was now admitted to this solution, and after shaking in the dark the spectrum was again measured ($\lambda_{max.}$ 333, 375, 428, 535, and 562 mμ) showing that the vitamin B_{12r} had been oxidised and reacted with more glutathione to re-form the violet complex. Addition of excess of potassium cyanide to the product gave dicyanocobalamin ($\lambda_{max.}$ 367, 540, and 580 mμ) and comparison of the intensity of absorption with that of a blank solution prepared similarly but omitting the

photolysis step showed that about 10% of the chromophore had been destroyed during the photolysis.

A similar reaction with the cysteine–hydroxocobalamin complex gave a higher yield of vitamin B_{12r} after photolysis ($\lambda_{max.}$ 313 and 475 mμ with inflections at 333, 375, and 545 mμ).

Alkylation of the Cobalamin Thiol Complexes.—(i) Glutathione, 2-mercaptoethanol, thioglycollic acid, cysteine, homocysteine, ethylthiol, and ω-mercaptotoluene were each (2 ml. of 1·0M-solution) treated with hydroxocobalamin (2 ml. of 0·01M-solution) in the presence of a pH 2·5 buffer solution (2 ml.). Methyl iodide (1 ml. of a 1M-ethanolic solution) was added to the resulting purple solution in an atmosphere of nitrogen and the mixture then stoppered and kept in the dark for 24 hr. The resulting solution was reduced in volume and chromatographed on Whatman 3 MM paper, n-butanol–ethanol–water (50 : 15 : 35) being used as solvent. The strip having the same R_F value as authentic methylcobalamin [2] in each case was cut out and eluted with water. The spectrum of each of the resulting solutions was identical with that of methylcobalamin and the identity was also proved by chromatography: R_F of products from thiol complexes and methyl iodide in solvent I, 0·54; II, 0·44; III, 0·58; Methylcobalamin, I, 0·54; II, 0·44; III, 0·58; Cyanocobalamin, I, 0·43; II, 0·28; III, 0·45. By electrophoresis at pH 2·5 on Whatman No. 1 paper at a potential gradient of 10 v/cm., the cobalamin produced by methylation of the thiols as well as authentic methylcobalamin, moved 21 mm. towards the cathode, cyanocobalamin 1 mm. towards the cathode, and hydroxocobalamin 37 mm. towards the cathode.

Reaction of Hydroxocobalamin with Sodium Sulphide and Methyl Iodide.—Recrystallised sodium sulphide (200 mg.) was added to a solution of hydroxocobalamin (100 mg.) in water (20 ml.). The resultant purple solution turned brown after 4 min. Light was excluded from the reaction vessel and a solution of methyl iodide (50 mg.) in ethanol (2 ml.) added and well mixed. After 5 min. the cobalamins were extracted through phenol and chromatographed on a column of carboxymethylcellulose (15 × 1 cm.). The main red band was collected and the volume of the fraction reduced to 0·5 ml. Acetone was added until a faint turbidity was observed and then kept in the dark for 12 hr.; red crystals (92 mg.; 92%) separated, and these were washed and dried. Light absorption: max. at 268, 343, 375, and 525 mμ (ε 22,700, 12,000, 9560, and 7600, respectively), corresponding to that of authentic methylcobalamin. The chromatographic and electrophoretic behaviour of the product corresponded exactly with that of methylcobalamin (see above).

In other experiments with the sulphide complex of hydroxocobalamin, methyl toluene-*p*-sulphonate was used as the alkylating reagent and also gave methylcobalamin identified as above. 1-Chloro-, 1-bromo-, and 1-iodo-propane all gave n-propylcobalamin, identical with the product obtained by the action of 1-iodopropane on vitamin B_{12s}: R_F in solvent I, 0·67; solvent II, 0·50; and solvent III, 0·61. Similarly chloroacetic acid and the sulphide complex of hydroxocobalamin gave carboxymethylcobalamin, identical with an authentic specimen: R_F in solvent I, 0·35; solvent II, 0·28; and solvent III, 0·46.

Reaction of Hydroxocobalamin with Sodium Hydrogen Sulphide and Ethyl Iodide.—Sodium (50 mg.) was dissolved in ethanol (100 ml.) and a portion of this solution (20 ml.) was saturated with hydrogen sulphide. Hydroxocobalamin (100 mg.) was added, light was excluded from the reaction vessel, and a solution of ethyl iodide (50 mg.) in ethanol (2 ml.) was added. The ethanol was removed at room temperature, the residue extracted through phenol, and the cobalamin product (89 mg.; 89%) purified by chromatography and crystallisation as in the previous experiment. Light absorption: max. at 268, 343, 375, and 522 mμ (ε 22,700, 12,000, 10,400, and 7400, respectively), corresponding to that of authentic ethylcobalamin. The identity of the product was confirmed by chromatography: R_F of product from sulphide reaction in solvent I, 0·59; II, 0·46; III, 0·59; Ethylcobalamin, I, 0·59; II, 0·46; III, 0·59; Cyanocobalamin, I, 0·43; II, 0·28; III, 0·45. By electrophoresis at pH 2·5 on Whatman No. 1 paper at a potential gradient of 10 v/cm., the reaction product as well as authentic ethylcobalamin, moved 23 mm. towards the cathode, cyanocobalamin 1 mm. towards the cathode, and hydroxocobalamin 37 mm. towards the cathode.

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