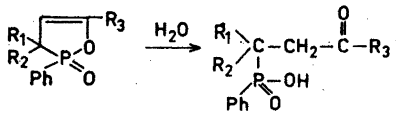

(IV, V) (VII, VIII)

IV and VII: $R_1 = H$, $R_2 = R_3 = Ph$
V and VIII: $R_1 = R_2 = R_3 = CH_3$

Experimental. 2-Oxo-2,3,5-triphenyl-1,2-oxaphospholine (IV). Phenylphosphine dichloride (15.9 g), benzalacetophenone (20 g), and acetic anhydride (10.2 g) were stirred for 24 h at room temperature. The white, solid reaction product was filtered off and washed three times with sodium-dried benzene. After four recrystallizations from anhydrous benzene-ether 17.5 g (53 %) of white needles was obtained, m.p. 162–163°C. (Found: C 75.74; H 4.99; *E* 331.4. Calc. for $C_{21}H_{17}O_2P$: C 75.8; H 5.10; *E* 331.4.)

2-Oxo-2-phenyl-3,3,5-trimethyl-1,2-oxo-phospholine (V). Mesityloxide (4.9 g), phenylphosphine dichloride (8.9 g), and acetic anhydride (5.1 g) were refluxed for 24 h, and the product was distilled *in vacuo* to give 2.6 g (24 %), b.p. 170–172°/10 mm, $n_D^{20} = 1.5449$. (Found: C 65.32; H 6.55; *E* 220.9. Calc. for $C_{12}H_{15}O_2P$: C 65.0; H 6.75; *E* 222.0.)

The *keto-phosphinic acids* VI and VII were obtained after refluxing the cyclic compounds IV and V with water for 20 h, and evaporation of the water.

1-Phenyl-1-(2,4-diphenyl-propanone-4)-phosphinic acid (VII). Recrystallized four times from ethanol-water, m.p. 243–244°C. (Found: C 71.84; H 5.28; *E* 348.6. Calc. for $C_{21}H_{19}O_3P$: C 71.90; H 5.42; *E* 350.)

1-Phenyl-1-(2,2,4-trimethyl-propanone-4)-phosphinic acid (VIII). Recrystallised four times from ethanol-water, m.p. 93.5°C. (Found: C 59.72; H 6.58; *E* 237.2. Calc. for. $C_{12}H_{16}O_2P$: C 60.00; H 6.70; *E* 239.0.)

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Isolation of Methylcobalamin from Liver

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It has been established by Toohey and Barker [1] that the major cobamide in mammalian liver is B_{12} coenzyme, 5′-deoxyadenosyl cobamide. Apart from this cobamide the same authors found mainly hydroxocobalamin. There is, however, another cobamide occurring in liver which was mentioned briefly in a report by Lindstrand.[2] The isolation procedure of others has been modified to permit characterization of this third cobamide with a reasonable degree of certainty of its structure and the procedure is described in detail in this paper.

Material and methods. Frozen calf liver was obtained from a slaughter-house. Methylcobalamin was synthesized as described by Lester Smith *et al.*[3] Ionic exchange resins were washed with 0.5 M NaOH or 0.5 M NaCl and 0.5 M HCl and finally converted to the desired form with the appropriate solution.

Absorbance measurements for ultraviolet and visible light were carried out in microcuvettes in a Unicam SP 500 spectrophotometer.

Preliminary studies of B_{12} in liver, were made according to a slightly modified bioautographical technique.[4,5]

Isolation procedure. All operations were carried out in the dark, or in dim light. All glassware was covered with black cloth and metal foil. Because of the large volumes used in this study, 100 kg of calf liver was thawed, minced, and the total amount divided into

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two 50 kg portions which were treated identically in the extraction procedure. Each portion was suspended in 25 l of ethanol at room temperature and this suspension was added to 175 l of hot (80°C) ethanol. The suspensions were maintained at 80°C with continuous stirring for 45 min. The suspensions were then allowed to cool down overnight, after which they were filtered, and the filtrates were combined. The entire extract was evaporated under reduced pressure to a volume of 17 l. This solution was then extracted three times with 10 l of ethyl ether to remove lipids. It was found nesessary to centrifuge the material after each extraction to break up the emulsion which formed. The lipid free, largely aqueous heavier layer was filtered through 100 g of Dicalite and 100 g of Celite 512. The pH was adjusted to 6.0 with 10 % NaOH, and 1 kg of Dowex 50-Na^+ was added. The solution was stirred for one hour and filtered. The pH was readjusted to 9.0 with 10 % NaOH and 1 kg of Dowex 2-OH^- was added. The solution was stirred for 1.5 h, followed by filtration and adjustment of the pH to 7.0 with dilute H_2SO_4.

The cobamides were extracted five times with 3 l of phenol each time and were displaced back into water with acetone and ether. Ether dissolved in the water was removed by evaporation and the aqueous solution was then filtered through a thin layer of Celite. The pH was adjusted to 5.0 and the solution was poured onto a Dowex 50 W-Na^+ column (30.5 + 2.6). The column was washed with distilled water. The aqueous eluate was passed on to an Amberlite CG 50/11 column (26.5 × 1.7) and washed with 100 ml of distilled water. The aqueous eluate was discarded. The cobamide analogue was eluted with 1000 ml of 0.5 N NaAc and extracted with phenol, displaced back into water with ether and acetone, and pH was adjusted to 10.0. This aqueous solution was passed through Dowex 2-OH^- (20 × 2.5) and the phenol, ether, acetone procedure was repeated. The aqueous solution was then evaporated to dryness. The residue was dissolved in 4 ml of butanol-isopropanol-water (10/7/10) and placed on top of a Sephadex G 25 (54 × 3.2) column pre-equilibrated with butanol-isopropanol-water, (10/7/10). The chromatogram was developed with the same solvent system. The eluate was collected in 15 ml fractions. Tubes number 20, 21, and 22 were combined and designated fraction I and tubes 36, 37, 38 combined and designated fraction II. Both fractions were shaken with an equal amount of ether and the water phases separated. The ether was removed and the solutions were passed through Permutit "DE-Acidite" FF (SRA 65 strongly basic) at neutral pH. Finally both fractions were lyophilized, dissolved in a minimum volume of water, and ten times this volume of acetone was added. The solutions were then left for crystallization in a sealed tube.

Results. Fraction I did not crystallize though it formed an amorphous precipitate (0.5 mg). Fraction II gave a crystalline product (8 mg). Both products were washed with a small volume of acetone followed by ethyl ether. The products were identified by means of spectrophotometry, electrophoresis, and chromatography.

The crystalline fraction II was identified as 5′-deoxyadenosyl cobamide.

Fraction I was chromatographed in *sec*-butanol-glacial acetic acid-water (100/3/50) and butanol-isopropanol-water (10/7/10) on Whatman No. 1 paper by the descending method. Both systems gave the same R_F values as synthetic methylcobalamin.

Paper electrophoresis at pH 7.0 and 3.0 was done and in both cases the cobamide preparation and synthetic methylcobalamin behaved as neutral compounds.

The spectrum of the cobamide preparation in water solution is in good agreement with the spectrum of methylcobalamin made by partial synthesis (Fig. 1). Both the extracted material and synthetic methylcobalamin changed colour from red to yellow on acidification of the aqueous solution and both gave spectra typical for hydroxocabalamin (Fig. 2) after they were exposed to light.

Discussion. The yield of methylcobalamin is small and without a doubt 5′-deoxyadenosyl cobamide is the pre-

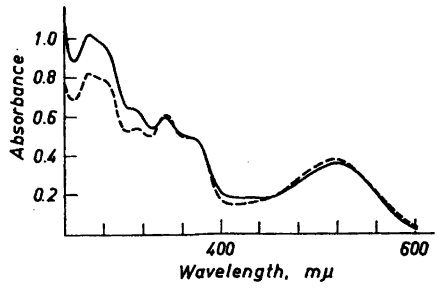

Fig. 1. Absorption spectra of methylcobalamin from liver (———); partially synthesized methylcobalamin (– – – –); in water.

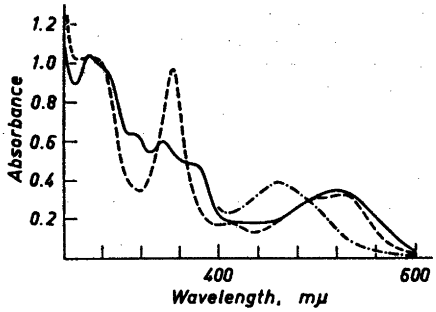

Fig. 2 Absorption spectra of methylcobalamin, from liver, in water (———); the same solution after acidification (– • – •); then light treatment (– – – –).

dominant form in alcoholic extracts of liver. Let us assume that during the isolation procedure proportionate amounts of the coenzyme and methylcobalamin have been lost and that 50 % of extracted B_{12} was the coenzyme (Toohey *et al.* found that 50–80 % was coenzyme). This means that approximately 2–5 % of total B_{12} is methylcobalamin if frozen liver is used as a source. However, we have reasons to believe that this figure is much too low for intact liver. Specimens of body-warm liver and liver frozen prior to extraction, were extracted in alcohol and subjected to chromato-bioautographical examination for comparison of the proportions of different cobamides obtained by the different methods (Fig. 3). Frozen liver gave only minimal amounts, while warm liver, on the other hand gave good amounts of methylcobalamin. The amounts of hydroxocobalamin varied but it could be said that the yield was less from warm liver. No cyanocobalamin could be detected in either case. In all instances 5′-deoxyadenosyl cobamide was obtained in good yield.

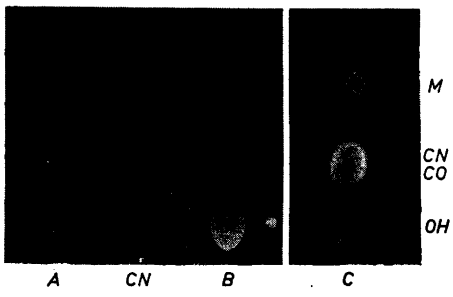

Fig. 3. Photograph of bioautography plates. A & B Liver, frozen prior to extraction. C Bodywarm liwer extracted immediately in ethanol. CN = Cyanocobalamin used as reference subtance. M = Methylcobalamin. CO = 5-deoxyadenosyl cobamide coenzyme. OH = Hydroxocobalamin.

It is known that the 5′-deoxyadenosyl form and methylcobalamin are decomposed to hydroxocobalamin. Methylcobalamin is undoubtedly broken down very rapidly. However, the coenzyme does not seem to be as susceptible to degradation as methylcobalamin. The hydroxocobalamin, found by Toohey and Barker,[1] thought to be derived from the coenzyme by chemical decomposition cannot be entirely correct. Part of the hydroxocobalamin has most likely been produced from methylcobalamin. This experiment does not eliminate the possibility that hydroxocobalamin also occurs in liver. In view of what has been found above it is obvious that it is preferable to use fresh, warm liver for isolation of methylcobalamin.

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