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An Improved Method for the Partial Synthesis and Purification of 5′-Deoxyadenosylcobalamin*

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ABSTRACT: Difficulties encountered in the preparation and purification of 5′-deoxyadenosylcobalamin by published methods have led to the development of a rapid method for the preparation and purification of this cobamide in good yield.

5′-Deoxyadenosylcobalamin is prepared by reduction of cyanocobalamin (vitamin B_{12}) to hydridocobalamin (B_{12s}) and reaction of the latter with a solution of crude 5′-tosyl-2′,3′-isopropylideneadenosine. After removal of the solvent by evaporation under reduced pressure, the isopropylidene group is removed in dilute acid. Gel filtration of the products on G-15 Sephadex separates the 5′-deoxyadenosylcobalamin from impurities of lower molecular weight and partially separates it from hydroxocobalamin (B_{12a}) which is formed during the preparation. The 5′-deoxyadenosylcobalamin is finally crystallized from aqueous acetone.

Supplies of 5′-deoxyadenosylcobalamin are necessary for further investigation of several enzymic reactions in which the cobamide has recently been shown to participate (Weissbach and Dickerman, 1965). The substance is not commercially available so that its preparation from natural sources or by a convenient chemical synthesis is of importance. However, extraction of 5′-deoxyadenosylcobalamin from natural sources is lengthy (*e.g.*, Barker *et al.*, 1960) and, in our experience, published chemical syntheses suffer from a number of difficulties. A modified method of synthesis is described in which these difficulties are avoided.

The partial chemical synthesis of 5′-deoxyadenosylcobalamin was first reported by Johnson *et al.* (1963) and Bernhauer *et al.* (1962). They reduced hydroxocobalamin (B_{12a}), prepared from cyanocobalamin (B_{12}), with sodium borohydride (Johnson *et al.*, 1963) or zinc in 10% ammonium chloride solution (Bern-

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hauer *et al.*, 1962). The fully reduced product, "hydridocobalamin" (B_{12s}), was then treated with freshly prepared 5′-tosyl-2′,3′-isopropylideneadenosine, and the product, 5′-deoxy-2′,3′-isopropylideneadenosylcobalamin, was freed from salts by extraction into phenol. Hydroxocobalamin was removed from the aqueous solution by chromatography on CM-cellulose, and cyanocobalamin was removed by chromatography on DEAE-cellulose. After freeze drying the solution, the residue was redissolved and crystallized from aqueous acetone (Johnson *et al.*, 1963). Treatment of the crystalline cobamide with 0.1 N HCl for 15 min at 90° (Johnson *et al.*, 1963) hydrolyzed off the isopropylidene group and 5′-deoxyadenosylcobalamin in the resulting solution was purified by the method used for the isopropylidene derivative.

We found this method to be time consuming and tedious and in these respects a recently published method (Hogenkamp and Pailes, 1966) shows little difference from earlier procedures. We have devised an appreciably shorter method by eliminating unnecessary steps. We also found that the literature methods of preparation gave variable and often low yields, and that the purification procedures were ineffective. The method reported here has repeatedly given good yields of a product of high purity. Published methods of paper chromatography of the cobamides were found unsatisfactory for examination of the products and alternative chromatographic, electrophoretic, and enzymic methods are described which were used in conjunction with spectrophotometry.

Methods

Crystalline cyanocobalamin was obtained from Glaxo, sodium borohydride from Fluka, Switzerland, and 2′,3′-isopropylideneadenosine from Cyclo Chemical Corp., Los Angeles. G-15 Sephadex (Pharmacia Fine Chemicals, Uppsala, Sweden) was prepared for use by repeated suspension in a large volume of water at pH 6.

5′-Tosyl-2′,3′-isopropylideneadenosine was prepared by reaction of 2′,3′-isopropylideneadenosine (0.65 g, recrystallized from boiling methanol) with tosyl chloride (0.43 g) in pyridine (6.5 ml) according to the method of Weygand and Trauth (1951). The amount of tosyl chloride may be increased to 1 g with little difference in the course of the synthesis and subsequent purification. The crude reaction mixture was used in the subsequent reaction without further treatment.

Regeneration of G-15 Sephadex. Before reuse the column was washed with water until the effluent no longer showed absorption at 260–280 mμ and contained no chloride. After repeated use washing of the column with water was unsatisfactory as a preparation for further use and the following was found to be a more efficient procedure. The G-15 was suspended in a large excess of water, and when the particles had settled out, the supernatant liquid was removed by decantation. The Sephadex was washed again in this manner with water, methanol (300 ml/100 g of Sephadex), 0.01 N HCl (1 l./100 g of Sephadex), 0.01 N NaOH (1 l./100 g of Sephadex), and finally with water until the supernatant solution had pH 5–6. Before use the Sephadex was allowed to stand in a large excess of distilled water for 3–4 hr, the supernatant water being changed twice in this time.

Chromatography and Electrophoresis of Cobamides. Since 5′-deoxyadenosylcobalamin was not mentioned in previous reports of thin layer chromatography of vitamin B_{12} derivatives (Popova *et al.*, 1966; Tadayoshi and Kawasaki, 1964), thin layer chromatography of this cobamide was investigated. Chromatography was carried out on precoated silica gel sheets (Przybylowicz *et al.*, 1965) K3031, supplied by Eastman Kodak, Rochester, N. Y. The sheets were dried at 110° for 30 min before use and the chromatograms were run in two solvent systems: (1) *sec*-butyl alcohol–water–ammonia solution (25%) (100:36:14, v/v/v); (2) sodium tetraborate solution (saturated)–*sec*-butyl alcohol–ammonia solution (25%) (50:25:10, v/v/v). The latter solvent gave a better separation of the 5′-deoxy-2′,3′-isopropylidene adenosylcobalamin from cyanocobalamin.

The results with this method were better than those obtained by paper chromatography in any of a large number of solvent systems which were tested. Providing the sheets were dried before use, resolution was good and small amounts of material (0.4 μg) could be used.

Electrophoresis was performed on Whatman No. 3MM paper with the use of 0.01 M acetate buffer, pH 4.2, at 42 v/cm for periods of from 3 (the minimum time required for good separation of cyanocobalamin from the dicarboxylic and monocarboxylic acid analogs) to 7 hr for good separation of 5′-deoxyadenosylcobalamin and 5′-deoxy-2′,3′-isopropylideneadenosylcobalamin. Visible and ultraviolet spectra were recorded with a Cary 15 spectrophotometer.

Enzymic Assay of the Coenzyme. The method employed was a modification of the assay for ribonucleotide reductase of *Lactobacillus leichmannii* (Vitols and Blakley, 1965). The complete assay system contained, in a total volume of 0.5 ml, 0.2 M potassium phosphate (pH 7.3) 4 mM EDTA, 0.2 mM NADPH,[1] 7.2 μM thioredoxin, and 10 μg of thioredoxin reductase (each prepared from *Escherichia coli*) 3 mM GTP, 16 μg of ribonucleotide reductase, and 5′-deoxyadenosylcobalamin at concentrations between 0.25 and 2.32 μM. The reference cuvet contained the complete system less the 5′-deoxyadenosylcobalamin and the course of the enzymic reduction was followed spectrophotometrically by observing the oxidation of NADPH with a Cary 14 spectrophotometer at 340 mμ.

Results

Preliminary investigations into the published methods for preparing the cobamide showed that conversion of cyanocobalamin to hydroxocobalamin by photolytic

[1] Abbreviations used: NADPH, reduced nicotinamide-adenine dinucleotide phosphate; GTP, guanosine triphosphate.

or hydrogenation methods is tedious and unnecessary in the cobamide preparation, provided that hydrogen cyanide formed in the reduction of cyanocobalamin is removed by a nitrogen stream, reduced pressure, and warming. It was also discovered that the occurrence of variable yields in the preparation was caused by partial degradation of the 5′-tosyl-2′,3′-isopropylideneadenosine during its isolation as a solid according to the procedure of Clark *et al.* (1951). The crude reaction mixture from the tosylation of the isopropylideneadenosine is stable for 1 week at 0° in dim light, and gives good, reproducible yields in the cobamide synthesis.

Since the use of thiols for reducing hydroxocobalamin has been reported in the literature (Dolphin and Johnson, 1963, 1965; Wagner and Bernhauer, 1964), we attempted reaction of tosylisopropylideneadenosine with cyanocobalamin after reduction of the latter by NaHS and by glutathione. Yields of 5′-deoxy-2′,3′-isopropylideneadenosylcobalamin were found to be very low (as shown by chromatography) and substantial amounts of hydroxocobalamin were formed instead. It was found, however, that the methyl analog of the cobamide can be prepared in good yield using the thiol reduction technique, in agreement with reports in the literature (Dolphin and Johnson, 1963, 1965; Wagner and Bernhauser, 1964).

Purification procedures which have been previously used, were, in our experience, unsatisfactory. It was found, in particular, that phenol extraction did not remove low molecular weight reactants and their breakdown products and it has been reported that this procedure may destroy certain cobamides (Hogenkamp *et al.*, 1965). The procedure which is described in this report employs G-15 Sephadex and in one step desalts, removes low molecular weight impurities, and partially removes hydroxocobalamin. The following procedure was adopted for preparation of the cobamide.

Preparation of 5′-Deoxyadenosylcobalamin. Cyanocobalamin (250 mg, 156 μmoles) was dissolved in aqueous methanol (1:1, v/v, 5 ml) in a 100-ml round-bottomed, three-necked flask, which had rubber seals (serum stoppers) closing two of the necks. A vacuum line was connected to the third neck. Oxygen was removed from the solution by passage of oxygen-free nitrogen which had been saturated with methanol, through a glass capillary inserted through one of the rubber seals. After 15 min, solid sodium borohydride (100 mg) was quickly added through the third neck, the connection to the vacuum line being temporarily removed for this purpose. The connection to the vacuum line was quickly replaced and the reduction was allowed to proceed under a nitrogen stream until the solution was greyish in color (5–15 min) indicating the formation of hydridocobalamin. During the reduction the reaction vessel was warmed until the temperature reached about 60° and the gas pressure in the vessel was reduced in order to promote removal of hydrogen cyanide. The temperature was maintained at 60° for about 10 min and the vessel then cooled to room temperature.

All subsequent steps in the preparation were carried out in dim light or under a red safety light (Wratten 1A filter). Apparatus containing the light-sensitive cobamides was also covered with aluminium foil.

During the period of the reduction, oxygen was removed from the solution of the crude tosylisopropylideneadenosine in pyridine by passage of a stream of nitrogen. A sample (2.5 ml) of this solution was transferred by the use of a gas-tight hypodermic needle and syringe to the solution of hydridocobalamin. A stream of nitrogen was then passed through the reaction mixture at room temperature for 2 hr.

The solution was pipetted off from solids which precipitated during the reaction, the flask was washed out with 1–3 ml of methanol which was then withdrawn by pipet, and the solution and washings were evaporated to dryness on a rotary evaporator under reduced pressure with bath temperature at 40°. Pyridine and other volatile impurities were removed from the residue at a pressure of 0.01 mm over a 6-hr period.

The 5′-deoxy-2′,3′-isopropylideneadenosylcobalamin can be crystallized from aqueous acetone after purification by passage of the dissolved residue through G-15 Sephadex in the manner described in detail below for 5′-deoxyadenosylcobalamin.

Usually, however, the solid residue from the reaction mixture was treated directly with 0.1 N HCl (25 ml) and heated at 90° for 30 min to remove the isopropylidene group from the adenosine residue. The solution was then neutralized with 0.1 N NaOH and evaporated to dryness on the rotary evaporator with a bath temperature of 40°. The residue was extracted with methanol (1–4 ml), the methanolic solution was filtered, evaporated to dryness at 40° under reduced pressure, and the residue was taken up in the minimum volume of water (2–3 ml).

The deep red solution was applied to a G-15 Sephadex column (150 × 2.5 cm), which had been equilibrated with distilled water at pH 6. The sample was eluted with distilled water, at a flow rate of 40 ml/hr and the effluent was collected in approximately 5-ml fractions with an automatic fraction collector. 5′-Deoxyadenosylcobalamin was conveniently detected in the fractions by its maxima at 263 and 375 mμ. The first few colored fractions contained chiefly unreacted hydroxocobalamin and were discarded (Figure 1). On some occasions hydroxocobalamin separated completely from the coenzyme but often the discarded hydroxocobalamin fractions contained a small amount of deoxyadenosylcobalamin. The fractions containing 5′-deoxyadenosylcobalamin only were pooled and evaporated to small volume (2 ml) under reduced pressure (bath temperature 40°).

The concentrated aqueous solution was treated with acetone (five volumes), allowed to stand for 12 hr at 3°, any solid material was removed by centrifugation, and the cobamide was crystallized by seeding and the careful addition of a further two volumes of acetone. The crystals were washed twice with acetone and ether and air dried (Barker *et al.*, 1960, 1963;

Hogenkamp and Pailes, 1966). The alternative method of crystallization which has been used, namely, slow evaporation of a concentrated aqueous solution over concentrated sulfuric acid under reduced pressure in a desiccator at 3° (Barker *et al.*, 1960), was found to be less satisfactory.

Yields of 5′-Deoxyadenosylcobalamin. In the initial preparations the yields of crystalline coenzyme were determined from the weight of the dried product. However, this method is lengthy and inaccurate owing to the difficulty of estimating the hydration of the coenzyme crystals.

A better method employed in later preparations, is that of Barker *et al.* (1960) in which solutions of the coenzyme are converted to dicyanocobalamin, by treatment with 0.1 M potassium cyanide at room temperature for 1 hr and the concentration of the solutions is determined from the absorbance at 367 mμ, using the molar extinction coefficient of 30.4×10^3 for dicyanocobalamin.

Yields determined from dry weight were 96, 128, and 108 μmoles, corresponding to 62, 82, and 69% of theoretical (based on the use of 250 mg of cyanocobalamin, which was found to be equivalent to 156 μmoles by dicyanocobalamin estimation). Yields of the crystalline coenzyme, determined by the dicyanocobalamin method, were 120 and 107 μmoles, corresponding to 77 and 68% theoretical.

Properties and Purity of the Product. When the product was subjected to chromatography and electrophoresis as described in the Methods section, only one spot could be detected. Table I shows the results obtained on thin layer sheets with the synthesized 5′-deoxyadenosylcobalamin and some related compounds. The spectrum of the product showed no significant difference from those given in the literature for 5′-deoxyadenosylcobalamin. Hogenkamp and Pailes (1966) reported the presence, in their coenzyme preparations, of a red compound, with a spectrum identical with that of the 5′-deoxyadenosylcobalamin but inactive in some enzyme systems.

TABLE I: Thin Layer Chromatographic Results for the Coenzyme and Related Compounds.[a]

Compound	Spot Color	R_F Value: Solvent 1[b]	R_F Value: Solvent 2[b]
B_{12}	Purple	0.31	0.24
B_{12a}	Pink	0.03	0.04
Isopropylidene CoB_{12}	Red	0.38	0.33
Coenzyme B_{12}	Red-orange	0.18	0.20
Monocarboxylic acid from B_{12}	Purple	0.11	0.18
Dicarboxylic acid from B_{12}	Purple	0.05	0.06
Dicyanocobalamin	Purple	0.21	*c*

[a] Results were obtained with Eastman Kodak precoated silica gel sheets, K3031. [b] See Methods section. [c] Dicyanocobalamin was not run in this system.

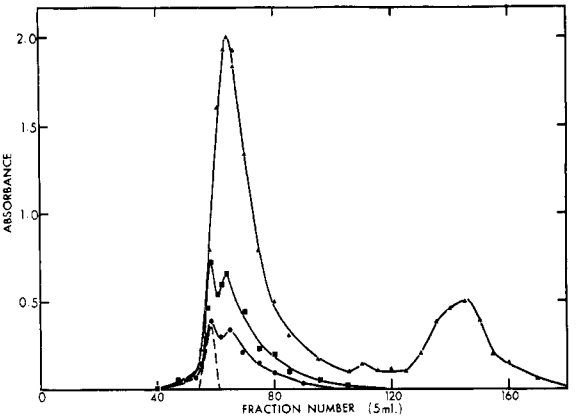

FIGURE 1: Gel filtration of hydrolysis products from crude 5′-deoxy-2′,3′-isopropylideneadenosylcobalamin on G-15 Sephadex (150 × 2.5 cm). ▲——▲, absorbance measurements at 263 mμ; ■——■, absorbance measurements at 350 mμ; ●——●, absorbance measurements at 375 mμ; – – – –, absorbance at 350 mμ minus calculated absorbance due to 5′-deoxyadenosylcobalamin at 350 mμ. This curve indicates the position of elution of hydroxocobalamin.

In order to determine the contamination by this product in our preparations, two syntheses, on the 250-mg scale, were carried out and the products were purified by G-15 Sephadex filtration. The red compound was isolated from each of the effluents by chromatography on Dowex 50W X2 (200–400 mesh) at pH 3, as described by Hogenkamp and Pailes (1966). In each case only 3–5 μmoles of the red compound was present, representing about 2.5% contamination in the final product.

The cobamide was exposed to visible light for 12 hr and the products were investigated by chromatography and spectrophotometry. These showed the main product to be hydroxocobalamin, in agreement with previous reports (Johnson *et al.*, 1963). On cleaving the cobamide with 0.1 M KCN, chromatography and spectrophotometry confirmed that the main product is dicyanocobalamin (Johnson *et al.*, 1963).

The cobamide was fully active in the enzyme assay described in the Methods section and gave an apparent K_m of 4.8×10^{-7} M. This is a sensitive test for impurities since the ribonucleotide reductase enzyme of *L. leichmannii* has a high specificity for 5′-deoxyadenosylcobalamin, only two other closely related cobamides being active and these to a much lesser extent than 5′-deoxyadenosylcobalamin (Blakley, 1966).

Discussion

The method described is quicker and more convenient

than previous methods, saving of time being affected by eliminating the conversion of cyanocobalamin to hydroxocobalamin, the isolation of tosylisopropylideneadenosine and of 5′-deoxy-2′,3′-isopropylideneadenosylcobalamin, and the tedious purification steps involving phenol extraction and ion-exchange columns. The modified procedure for the preparation and purification of 5′-deoxyadenosylcobalamin should prove valuable when applied to the study of analogs of the cobamide.

The purity of the product equals if not surpasses that of the compound as prepared by previously published methods. Strong evidence for an improved purity is the apparent K_m value of 4.8×10^{-7} M obtained in the assay system. Samples of 5′-deoxyadenosylcobalamin kindly supplied by Dr. E. Lester Smith and Dr. H. P. C. Hogenkamp showed K_m values in the range $7–8 \times 10^{-7}$ M. Determinations of K_m with different enzyme preparations and a single cobamide preparation showed little variation in K_m values so that the differences in K_m obtained in determinations made with the same enzyme preparation but different cobamide preparations are significant. It seems likely that the gel filtration is effective in removing impurities which are not removed by other methods, including crystallization of the cobamide.

The hydrolysis conditions which we have used for the removal of the 2′,3′-isopropylidene group from 5′-deoxy-2′,3′-isopropylideneadenosylcobalamin are as used by Johnson *et al.* (1963) except that for complete hydrolysis it was necessary to increase the hydrolysis period from 15 to 30 min. This is probably due to the fact that borate and residual pyridine in the crude deoxyisopropylideneadenosylcobalamin used in our method decreases the hydrogen ion concentration. Under the conditions adopted there was minimal formation of hydroxocobalamin and of the coenzyme-inactive red compound referred to by Hogenkamp and Pailes. The larger amount of this compound found by the latter authors is probably a result of the different hydrolysis conditions used by them. This explanation is consistent with the fact that although the contaminant has not been identified, it behaves in chromatography and electrophoresis like the deoxyadenosylcobalamin analog in which one side-chain amide group has been hydrolyzed. Additional evidence that the hydrolysis of the 5′-deoxy-2′,3′-isopropylideneadenosylcobalamin did not produce significant amounts of such mono- or dicarboxylic acids, was obtained by electrophoresis of the cobamide products at pH 4.2. The acids migrated to the anode and are clearly separated from deoxyadenosylcobalamin which migrated to the cathode. No material migrating in the same direction as the acids could be detected in the synthetic product by this technique. Chromatography also showed the product to be free of detectable quantities of the mono- and dicarboxylic acids. These facts together with the high activity of the product in the enzymic assay indicate that the conditions of hydrolysis are satisfactory.

As noted above, it was not always possible to achieve complete removal of hydroxocobalamin from the required product by gel filtration. Nevertheless, by selection of appropriate fractions the bulk of the cobamide can be obtained free from hydroxocobalamin. When overlap of the product and hydroxocobalamin peaks is considerable, so that a large loss of product would be entailed if tubes containing hydroxocobalamin were discarded, deoxyadenosylcobalamin may be separated from the hydroxocobalamin in these fractions on a smaller G-15 Sephadex column or by one of the methods which have been published previously (Johnson *et al.*, 1963; Muller and Muller, 1963; Hogenkamp and Pailes, 1966).

5′-Deoxy-2′,3′-isopropylideneadenosylcobalamin was retarded slightly more than 5′-deoxyadenosylcobalamin (two or three fractions difference) on G-15 Sephadex but complete separation of the two was not possible. Occasionally a red band of material eluted very slowly from the Sephadex after the coenzyme had been eluted. This product was not identified but may perhaps be related to factor A or B.

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Physical Studies of Hemocyanins. III. Circular Dichroism and Absorption Spectra*

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ABSTRACT: Studies of the circular dichroism of *Octopus vulgaris* and *Loligo pealei* hemocyanins reveal that the absorption band in the visible spectrum is not simple, but involves at least three components, centered at 440, 570, and about 700 mμ. This has been confirmed by measurement of the absorption spectrum in glycol–water glass at very low temperatures. The set of bands now observed in the hemocyanin spectrum corresponds closely to the structures of the spectra of a number of proteins containing cupric copper. On this basis, mechanisms for oxygen binding by hemocyanin are discussed.

Although it has been known for many years that the oxygen binding by hemocyanin depends upon the presence of copper in the protein, the mode of attachment of this copper, or even its oxidation state, remains uncertain. It appears that the metal in the deoxygenated protein is cuprous, for only by the use of cuprous salts can active hemocyanin be regenerated from the apohemocyanin (Kubowitz, 1938), and chemical studies reveal that all of the copper, when released from the deoxygenated protein, is cuprous (Klotz and Klotz, 1955; Felsenfeld, 1960). Similar experiments indicated that about one-half of the copper is cupric in the oxygenated protein. However, these results have been criticized because of the possibility of oxidation of the copper by released oxygen or its reduction by reducing groups on the protein (see, for example, Manwell, 1964.) Finally, oxygenated hemocyanin does not display the electron paramagnetic resonance (epr) signal which would normally be expected if cupric copper were present (Nakamura and Mason, 1960; Manwell, 1964; D. L. Lloyd and K. E. Van Holde, unpublished). Thus, it is held by some that the metal is in the cuprous form in both oxygenated and deoxygenated hemocyanin (for example, Malmstrom and Nielands, 1964). In contrast to this, the spectral changes induced by oxygenation have been cited. The deoxygenated protein is colorless, and exhibits a spectrum in the ultraviolet region very similar to that of other proteins. When oxygen is added, a rather strong absorption band appears in the visible region, yielding a deep blue color reminiscent of that of many cupric complexes. A very intense band also appears in the near-ultraviolet region. Because of its intensity (extinction coefficients of about 10^4 have been commonly reported), the latter band has been attributed by many to a charge-transfer transition. Those who hold that the copper remains cuprous upon oxygenation have suggested that the absorption in the visible spectrum also corresponds to charge-transfer bands. In this region a clear distinction cannot be made upon the basis of intensity, for the extinction coefficients are in the neighborhood of 500 l. $(\text{g-atom})^{-1}$ cm^{-1}, which is somewhat high for cupric d–d transitions but low for typical charge-transfer bands.

Under these circumstances, any information which may lead to an understanding of the absorption spectrum is of potential value. Recent measurements of optical rotatory dispersion (ORD) (Foss, 1964; Cohen and Van Holde, 1964) have shown that both the band in the visible and that in the ultraviolet are optically active. However, the detailed analysis of these ORD spectra is complicated by the overlap of the Cotton effects, and the enormous contribution to the optical rotation from the peptide Cotton effects centered near 200 mμ. For this reason, it seemed that circular dichroism (CD) measurements, with inherently greater resolving power, might yield more information. Some preliminary measurements have been reported by Foss (1964). However, while the report of a negative

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