The Reduction of Vitamin K₁ by an Enzyme from Dog Liver*

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(Received for publication, November 12, 1959)

Most of the research concerning the biological role of vitamin K₁ (2-methyl-3-phytyl-1,4-naphthoquinone) has been concerned with its function in blood clotting (1); however, substances which possess vitamin K activity are found in organisms such as bacteria and higher plants (1, 2) which have no blood-clotting system. The role of the vitamin in these organisms has not been established but may be related to one of the unique properties of naphthoquinones, viz., the ease with which they undergo oxidation and reduction. The possible participation of a naphthoquinone in electron transport was suggested by a previous study (3, 4), in which an enzyme from Escherichia coli was described which catalyzed the transfer of electrons from the reduced pyridine nucleotides to menadione (2-methyl-1,4-naphthoquinone) and was accordingly designated pyridine nucleotide-menadione reductase. This activity was also found in mammalian and plant tissues. Vitamin K₁, tested as an emulsion, could not substitute for menadione as an electron acceptor. Whether this was due to the unsuitable nature of the emulsion or due to the specificity of the enzyme was not determined.

In order to determine whether vitamin K₁ could serve as an electron carrier, various agents were used to solubilize it for testing with a menadione reductase from dog liver. Of the agents tried, BRIJ35 (polyoxyethylene lauryl alcohol) served well in solubilizing vitamin K₁ so that it could replace menadione in the assay system. The present report is concerned with the purification and properties of an enzyme from dog liver which catalyzed the reduction of vitamin K₁.

EXPERIMENTAL

Materials

Cofactors and Other Reagents—DPN and TPN were obtained from the Pabst Laboratories; deamino DPN from the Nutritional Biochemicals Corporation. The pyridine nucleotides were reduced enzymatically as previously reported (4); chemically reduced TPNH was prepared with the use of the procedure described elsewhere (5). FAD and FMN¹ were obtained from the Sigma Chemical Company; d-α-tocopherol, d-α-tocoquinone, naphthoquinones, and nitrophenols from Eastman; vitamin K₁ from Nutritional Biochemicals and from Merck Sharp and Dohme; DEAE-cellulose from Brown and Company; BRIJ-35 (Lot 230) from the Atlas Powder Company. Coenzyme Q₁₀ was kindly provided by Dr. Karl Folkers of Merck Sharp and Dohme. Glass-distilled water was used in the preparation of all reagents.

Vitamin K₁ was solubilized as follows: 0.1 ml of vitamin K₁ was suspended in 10 ml of an aqueous solution of BRIJ-35 (0.25 g per ml), diluted with 60 ml of water, and heated in a tightly stopped 100-ml volumetric flask at 105 to 115° for about 16 hours. Any droplets of vitamin K₁ were removed by filtration. The filtrate, designated solubilized vitamin K₁, was stored at room temperature and protected from light. The concentration of vitamin K₁ in the filtrate was determined by the procedure of Irreverre and Sullivan (6) and provided the characteristic color of 2,3-alkyl-disubstituted naphthoquinones. The ultraviolet spectrum of solubilized vitamin K₁, even after storage for a few weeks, was essentially the same as that of a fresh solution of vitamin K₁ in ethanol. With the use of the chromatographic procedures described below, solubilized vitamin K₁ migrated on the same ultraviolet spectrum, and provided the same Rf values as untreated vitamin K₁. The vitamin did not appear to have been degraded by solubilization. Solutions of menadione were prepared for use by dissolving 5.4 mg of menadione in 1.0 ml of acetone and then rapidly diluting to 100 ml with distilled water.

Chromatography—The solvent systems described by Green and Dam (7) were used for the paper chromatography of vitamin K₁ and the solubilized preparations. In the present study, descending paper chromatograms were carried out on Schleicher and Schuell Paper 2499 at 25° to 27° for 16 to 20 hours. With the isopropanol-acetic acid-water system the Rf values were approximately 0.28 and with the n-propanol-acetic acid-water system approximately 0.76.

Cell Fractions—Bat and dog liver homogenates were prepared in 10 volumes of 0.25 M sucrose containing 1 × 10⁻⁴ M ethylenediaminetetraacetic, pH 7.4, with a Teflon homogenizer at 0°. Cell fractions were obtained by differential centrifugation essentially according to Schneider and Hogeboom (8).

Assay Procedures—Enzymatic activity was measured at 22 to 25° in a Beckman model DU spectrophotometer (light path 1.0 cm) by following the oxidation of reduced pyridine nucleotides at 340 μM. Since vitamin K₁ and menadione absorbed at 340 μM they were added to the check cell which included all additions.

The average molecular weight of BRIJ-35 was reported to be about 1215, while the micellar molecular weight at 25° was 48,800 (personal communication, Dr. Paul Becher, Atlas Powder Company). On the basis of chemical determinations for vitamin K₁ in these preparations, this corresponded to about 1 molecule of vitamin K₁ per BRIJ-35 micelle, assuming the heat treatment caused no change in the micellar molecular weight.

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* This research was supported in part by a grant (H4056) from the United States Public Health Service.

¹ FMN is flavin mononucleotide.
but DPNH and enzyme. After addition of the enzyme, the decrease in absorbancy was measured at 15- to 30-second intervals (Fig. 1) for the first 3 minutes. Corrections were made for the fractions which oxidized DPNH in the absence of vitamin K₁ or menadione.

Vitamin K₁ Reductase Assay—This system contained 150 μmoles of imidazole buffer, pH 7.4, 0.9 μmole of solubilized vitamin K₁ (about 0.3 ml), 0.005 μmole of FAD, 0.3 μmole of DPNH, enzyme and water to a final volume of 3.0 ml. In most of these studies an amount of enzyme causing a change in absorbancy of 0.080 to 0.100 per minute was used; in this range, activity was approximately proportional to enzyme concentration (Fig. 1). A unit of enzyme is defined as the amount of enzyme which causes the oxidation of 1 μmole of DPNH per minute as calculated from the change between the 15 and 45 second readings. Specific activity is expressed as units per mg of protein. Protein concentration was determined by the procedure of Lowry et al. (9) in the crude fractions and by ultraviolet absorption (10) in the more highly purified fractions.

Menadione Reductase Assay—This system contained 185 μmoles of Tris buffer, pH 8.2, 0.3 μmole of menadione, 0.3 μmole of DPNH, 0.005 μmole of FAD, water and enzyme to a final volume of 3.0 ml. Activity, units, and specific activities were determined as above.

**RESULTS**

Preparation of Homogenate—Medium sized dogs of various breeds were killed with pentobarbital and bled by severing the neck arteries. The livers, weighing 200 to 400 g, were perfused with cold 0.9% NaCl solution, removed from the animal, and chilled.

All steps of the purification procedure summarized in Table I were carried out at about 0°C. The liver was cut into small pieces and homogenized in a Waring Blender for 2.5 minutes with 2 volumes of distilled water (Fraction I). The pH of the homogenate was adjusted to 6.9, centrifuged, and the sediment discarded. Unless otherwise indicated all centrifugations were at about 25,000 × g for 30 minutes. The supernatant fluid contained a variable but appreciable amount of DPNH oxidase which was reduced to a low level by adjusting the pH of the sample to 6.0 and storing it for at least 1 week at −17°C before carrying out the next step.

Butanol Fractionation—The precipitate formed on thawing the preparation was removed by centrifugation. An equal volume of n-butanol was added to the preparation over a period of 15 minutes, and after an additional 15 minutes, the mixture was centrifuged. The aqueous phase was allowed to stand overnight and the sediment which formed was removed by centrifugation for 15 minutes at 2,000 × g. The supernatant fluid was designated Fraction II.

Acetone Fractionation—Acetone was added to a final concentration of 30% and the mixture allowed to stand 10 minutes, after which, it was centrifuged at 2,000 × g for 15 minutes. The precipitate was discarded and the acetone concentration in the supernatant fluid was increased to 50% and after 10 minutes was recentrifuged. The supernatant fluid was discarded, the precipitate was suspended in 150 ml of 0.01 M potassium phosphate, pH 7.4, and transferred as a suspension to a dialyzing casing and dialyzed with mechanical agitation for 4 hours against 2 liters of the suspending media, after which, the sample was prepared for column chromatography by dialysis overnight against 2 liters of 0.01 M Tris buffer, pH 8.9.

**Purification on DEAE-Cellulose Column**—The dialyzed enzyme (Fraction III) was chromatographed on a column of DEAE-cellulose (5 cm in diameter and about 40 cm in length after packing under pressure, 10 p.s.i.) which had been equilibrated with 0.01 M Tris buffer, pH 8.9. The enzyme sample, usually 60 to 80 ml, was applied to the column at a rate of about 70 ml per hour and followed by a wash with 200 ml of the equilibrating fluid. The enzyme was eluted with the use of a gradient eluting system with an apparatus similar to that described by Busch et al. (11). The mixing chamber contained 150 ml of 0.01 M Tris, pH 8.9 and the reservoir contained 0.1 M Tris, pH 8.9, containing 0.3% NaCl. Fractions (10 to 14 ml) were collected on a fraction collector at a rate of about 70 ml per hour. The protein content of the eluate was measured by ultraviolet absorption and enzyme activity by the standard vitamin K₁ reductase assay. The first protein usually appeared in the effluent after about 300 ml, but the vitamin K₁ reductase activity was usually associated with the second protein peak. During purification the yield and increases in specific activity of vitamin K₁ reductase and menadione reductase activity ran parallel. During the above purification, DPNH oxidase and DPNH cytochrome c reductase (12) were removed so that little if any of these were found in the final preparations which were highly purified.

**TABLE I**

**Summary of purification of vitamin K₁ reductase from dog liver**

| Fraction No. | Volume | Units | Total protein | Specific activity *
<table>
<thead>
<tr>
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<tbody>
<tr>
<td></td>
<td>mg</td>
<td>mg</td>
<td>units/mg protein</td>
<td></td>
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<tr>
<td>I. Homogenate</td>
<td>840</td>
<td>1622</td>
<td>62,160</td>
<td>0.026</td>
</tr>
<tr>
<td>II. Butanol fraction</td>
<td>350</td>
<td>550</td>
<td>5,180</td>
<td>0.13</td>
</tr>
<tr>
<td>III. 30-50% acetone</td>
<td>65</td>
<td>270</td>
<td>1,406</td>
<td>0.18</td>
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<tr>
<td>IV. Column eluate</td>
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<tr>
<td>Pooled tubes 44-49</td>
<td>70</td>
<td>92</td>
<td>36</td>
<td>2.5</td>
</tr>
<tr>
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<td>42</td>
<td>140</td>
<td>22</td>
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<tr>
<td>Pooled tubes 53-54</td>
<td>28</td>
<td>44</td>
<td>15</td>
<td>2.9</td>
</tr>
</tbody>
</table>

* 280 g of liver.
† μmoles of DPNH oxidized per minute.
also appeared to be free of xanthine oxidase (13) and alkaline phosphatase. The column was regenerated with 4 liters of 0.1 M Tris containing 0.9% NaCl.

Stability of Enzyme—The enzyme, as collected from the column, was quite stable at 0-4°, and was generally stored at this temperature. Exposure of the enzyme to a temperature of 57° or higher for 5 minutes resulted in a decrease in activity of more than 90% (Fig. 1). The enzyme was stable to dialysis against 0.01 M potassium phosphate buffer, pH 7.5, and 0.01 M Tris buffer, pH 8.2 and 8.9, for 16 hours or more at 30°.

Intramembrane Distribution—The intracellular distribution of the enzyme was determined in both rat and dog liver. BRIJ-35, in the concentrations used in the solubilized vitamin K₁ preparations, was found to have deleterious effects on mitochondria, so menadione was used as an electron acceptor in the distribution studies. These reactions were carried out in 0.1 M Tris, pH 7.4 instead of at pH 8.2. In a typical experiment, the activities, expressed as ᵐoles of DPNH oxidized per minute per g of rat liver, were: homogenate, 9; twice-washed mitochondrial fraction, 1; microsomal fraction, 1.2; soluble fraction, 6. Since activity was found in each fraction, each fraction was also tested for sensitivity to Dicumarol at 10⁻² m, and the soluble fraction was the only one inhibited to any extent. Similar results were obtained with dog liver. An analysis of these fractions for K₃H₂ (reduced menadione) oxidase, with the use of the procedure described by Colpa-Boonstra and Slater (14), showed more than 85% of the activity of the homogenate to be associated with the mitochondrial fraction.

Effect of pH—The effect of pH on vitamin K₁ reductase activity was determined by substituting other buffers (0.1 m) for imidazole in the reaction mixture. In the pH range of 7 to 8, activity increased with pH using either imidazole, Tris, or glycylglycine, but at any given pH in this range the rate was greatest in imidazole. In the pH 8 to 9 range, the activity in Tris, glycylglycine, or glutamate continued to increase with pH. Between pH 9 and 10 the activity in glutamate buffer increased with pH up to 9.7 above which it decreased. The activity observed in glutamate at pH 9.7 was twice that in imidazole at pH 7.4; nevertheless, the latter was used in the standard assay.

Reduced Pyridine Nucleotides as Electron Donors—DPNH, de-amino DPNH, and TPNH served as electron donors in the vitamin K₁ reductase system as well as the menadione reductase system. The maximum rates were similar with DPNH and TPNH; however, the rate diminished with higher concentrations of TPNH (Fig. 2). This diminution in rate occurred with either chemically or enzymatically reduced TPNH. At low concentrations of DPNH and TPNH, the reactions were carried near completion in one minute so accurate initial rates could not be measured in the present assay system. Samples tested at various stages of purification showed the same ratio of activity with DPNH and TPNH, with either solubilized vitamin K₁ or menadione as an electron acceptor.

Effect of Flavins—Fig. 3 shows the activity of the purified enzyme as a function of FAD and FMN concentration. An estimate of the dissociation constants from the curves indicates that one-half maximal stimulation was obtained at about 7 × 10⁻⁸ m and 5 × 10⁻⁸ m for FAD and FMN, respectively. The maximal rates observed with saturating amounts of flavin nucleotides were about the same and were observed only with the more highly purified enzyme preparations. Menadione reductase activity was affected similarly by FAD and FMN. In dialysis experiments FAD was found to be more tightly bound by the enzyme than FMN. Preincubation of the purified enzyme with either FMN or FAD, followed by dialysis at pH 8.2 for as long as 2 weeks, resulted in the FMN-treated sample, but not the FAD-treated sample, requiring the addition of flavin nucleotide to the vitamin K₁ reductase assay for full activity.

Electron Acceptors—The effect of vitamin K₁ concentration is shown in Fig. 4. The dissociation constant of the vitamin K₁ enzyme complex as estimated from the curve is 5 × 10⁻³ m. This does not necessarily correspond to that which might occur in vivo because the detergent micelle used to suspend the vitamin may have altered this significantly. Other materials tested which appeared to serve as electron acceptors in place of vitamin K₁ were menadione, 1,4-naphthoquinone, benzoquinone, and co-enzyme Q₁₀ (solubilized with BRIJ-35). Among the inactive electron acceptors were vitamin K₁, diphasphate, menadione diphasphate, vitamin K₁ oxide, d-a-tocopherol, and d-a-tocoquinone (the last three substances were solubilized with BRIJ-35).

Identification of Products and Stoichiometry of Reaction—In experiments in which vitamin K₁ was limiting and DPNH was in
excess, the DPNH was completely oxidised presumably because of the aerobic reoxidation of reduced vitamin K$_1$ which resulted in the regeneration of electron acceptor. Since reduced vitamin K$_1$ is auto-oxidizable, the stoichiometry as well as the spectrum of the reduced vitamin were determined under anaerobic conditions. The time course of such a reaction is presented in Fig. 5 and the spectral changes are presented in Fig. 6. The reaction was initiated by tipping in the enzyme from the side arm of the evacuated cuvette, and vitamin K$_1$ reduction was followed by measuring the decrease in absorption in the experimental cuvette at 275 nm against an appropriate check cell whereas the oxidation of DPNH was measured at 340 nm against a different check cell. Although the reaction was essentially complete at 20 minutes it was kept under vacuum for an additional 30 minutes to make certain the reaction was at equilibrium. On the basis of change in absorbancy at 340 nm, 0.16 pmole of DPNH had been oxidized at this time. Since vitamin K$_1$ exhibits a slight increase in absorbancy at 340 nm upon reduction, a correction was obtained for this by measuring the change in absorbancy of an identical sample treated with a few crystals of KBrO$_3$. The increase in absorbancy amounted to 0.090 which corresponded to an apparent deficit of 0.043 pmole of DPNH oxidized. The correction of 0.04 pmole applied to 0.16 pmole indicated 0.20 pmole of DPNH oxidized in the presence of 0.22 pmole of vitamin K$_1$. In this experiment the spectrum of the reduced product was obtained after 50 minutes (Fig. 6) after which air was admitted and the spectrum of the oxidized product was restored (Fig. 6). As shown in Fig. 5 the reoxidation of reduced vitamin K$_1$ was rapid. Interference from the reduced form appeared to be negligible in the standard assay. Similar results were obtained when menadione was used as an electron acceptor in place of vitamin K$_1$.

Reduction of solubilized vitamin K$_1$ by KBrO$_3$ produced similar spectral changes with an increase in absorbance in the 245 nm region and a decrease in absorbance in the 275 nm region. When examined in a Beckman DK 2 recording spectrophotometer, vitamin K$_1$ in ethanol exhibited similar changes in spectrum within two minutes upon reduction with KBrO$_3$, however, further changes occurred and after 30 to 60 minutes both peaks absorbed less than that of oxidized vitamin K$_1$.

With the use of a procedure similar to that described above but with DPNH in stoichiometric excess, the amount of DPNH oxidized corresponded to the amount of vitamin K$_1$ present. In one experiment, for example, with 0.20, 0.30, and 0.40 pmole of vitamin K$_1$, and 0.6 μm DPNH, 0.21, 0.31, and 0.41 pmole, respectively, of DPNH were oxidized when corrected for the change

3 Using anaerobic silica cells of the Thunberg type, Pyrocell Manufacturing Company, New York.
in absorbancy at 340 m\(\mu\) resulting from the reduction of vitamin K\(_1\). Oxidized DPN was identified as described previously (4).

Effects of Metals and Inhibitors—A number of materials were tested for possible effects on the purified enzyme system. Ions such as Mg\(^{++}\), Fe\(^{++}\), Fe\(^{+++}\), Mn\(^{++}\), Zn\(^{++}\), at concentrations of \(10^{-2}\) to \(10^{-4}\) M showed little or no effect on activity. Orthophenanthroline, \(o, o'-\)dipyridyl, and S-hydroxyquinoline at \(10^{-4}\) M caused an inhibition of activity of approximately 50\% which was reversible by dialysis.

Iodoacetic acid, iodacetamide, iodosobenzoic acid, and \(p\)-chloromercuribenzoate at concentrations of \(10^{-3}\) M had no effect on vitamin K\(_1\) reductase activity; thyroxine and triiodothyronine caused an inhibitory potency with biological potency was not made, because these materials are only very slightly soluble at neutral pH values, they could not be tested over the same range of concentrations. The inhibitory effect of the materials tested on vitamin K\(_1\) reductase is presented in Table II. An attempt to correlate inhibitory potency with biological potency was not made, because these substances are probably bound to tissue proteins to different extents, and metabolized and excreted at different rates, all of which would influence biological potency. Nevertheless it may be noted that derivatives of different series of compounds such as coumarin derivatives, phenindione derivatives and salicylate which possess anticoagulant activity also inhibit vitamin K\(_1\) reductase. Furthermore, Dicoumarol, an effective anticoagulant, was found to be an effective inhibitor of vitamin K\(_1\) reductase while salicyclic acid, a weak anticoagulant, was a less effective inhibitor of vitamin K\(_1\) reductase.

A number of nitrophenol derivatives were tested on the activity of vitamin K\(_1\) reductase. At a final concentration of \(10^{-4}\) M the following caused little or no inhibition of activity: 2-amino-4-nitrophenol, \(p\)-nitrophenol, and \(o\)-nitrophenol. At the same concentration, the following agents produced the indicated percentage inhibition: 2,4-dinitrophenol, 39\%; 2,4-dinitroaniline, 33\%; 2,4-dinitromephtanal, 90\%.

Table II

<table>
<thead>
<tr>
<th>Substance</th>
<th>Inhibition Final concentration</th>
</tr>
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<tr>
<td></td>
<td>(10^{-4}) M</td>
</tr>
<tr>
<td>Dicoumarol</td>
<td>%</td>
</tr>
<tr>
<td>Tromexan</td>
<td>38</td>
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<tr>
<td>Warfarin</td>
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<tr>
<td>Sintrin</td>
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</tr>
<tr>
<td>Cumarchlor</td>
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</tr>
<tr>
<td>Phenindionide</td>
<td>10</td>
</tr>
<tr>
<td>Salicylate</td>
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</table>

Discussion

Menadione reductase activity was previously reported to be present in a number of animal tissues, plants, and bacteria (4). At that time, a suitable procedure for the solubilization of vitamin K\(_1\) was not available, so it was not known whether the vitamin was included within the specificity of the enzyme from these different sources; however, on the basis of the present studies, it is included within the specificity of the enzyme from dog and rat liver. It may be noted that Martius and Mäki (15) have reported the presence of a phylloquinin-reductase obtained from beef liver, which has some similarities. Since benzoquinones and naphthoquinones readily undergo oxidation and reduction, it seemed possible that this enzyme might be involved in electron transport. With respect to menadione, it was reported to stimulate the respiration of various tissues, cf. Green et al. (16) for references, and also yeast (17). This might be due to the reduction of menadione by a menadione reductase with the electrons subsequently being transferred to a system such as K\(_2\)H\(_2\) oxidase (14) or directly to oxygen. In the case of dog and rat liver, assuming that the activity observed in homogenates is close to that observed in vivo, there is sufficient enzyme present for it to contribute appreciably to respiration. It should be pointed out that the vitamin K\(_1\) used in these studies was in a micelle that might bear only slight resemblances to the natural micelle and the activity in vivo may be quite different from that observed in vitro. Since the enzyme from liver was inhibited by various anticoagulants, studies were carried out to determine the effect of these inhibitors on the respiration of rat liver slices, and their relative potency paralleled their inhibitory action on vitamin K\(_1\) reductase. 4 Since other processes such as oxidative phosphorylation are also affected by these agents, the depression may be in part a secondary effect and cannot be attributed solely to the action of these agents on a system such as vitamin K\(_1\) reductase.

In addition, the inhibition of the enzyme by anticoagulants also aided in distinguishing this enzyme from others, e.g. the activity associated with the mitochondrial fraction and xanthine oxidase which was reported to reduce menadione (18).

Although the presence of vitamin K\(_1\) per se has not been clearly established in mammalian tissue (19), factors possessing antihemorrhagic activity were found (1). Furthermore, in a study of beef liver, Green and Dam (20), using a bioassay, found vitamin K\(_1\) activity associated mainly with the mitochondrial fraction. With the use of a different approach, Martius (21) administered \(C^{14}\)-labeled menadione to chickens and found a labeled factor associated with the mitochondria which appeared to be different from menadione, and suggested that menadione may be a precursor of vitamin K\(_1\). Such a factor might be closely related to vitamin K\(_1\) in structure, e.g. 2-methyl-3(geranyl-geranyl)-1,4-naphthoquinone later reported by Martius and Esee (22). In addition to naphthoquinones, benzoquinone and coenzyme Q\(_10\) were found to serve as electron acceptors indicating the possibility that a substance of this sort might be the natural electron acceptor. Investigations are in progress to determine the nature of the electron acceptor in dog liver. When this is known, a more appropriate name can be assigned to the enzyme than that presently used and a better understanding of its biological role can be considered.

4 Unpublished experiments.
SUMMARY

The purification and properties of an enzyme from dog liver which catalyzed the reduction of vitamin K₁ (2-methyl-3-phytyl-1,4-naphthoquinone) by reduced di- or triphosphopyridine nucleotides is described. The vitamin K₁ used as an electron acceptor was solubilized with BRIJ-35 (polyoxyethylene lauryl alcohol). Menadione, coenzyme Q₁₀, and benzoquinone also served as electron acceptors. Flavin adenine dinucleotide and flavin mononucleotide increased the rate of reaction whereas dicumarol and other anticoagulants decreased the rate. On the basis of spectrophotometric evidence, the reduction of vitamin K₁ appeared to take place in the naphthoquinone nucleus.

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