Biochemical Pharmacology, 1961, Vol. 7, pp. 221–225. Pergamon Press Ltd., Printed in Great Britain.

THE REDUCTION OF CYTOCHROME *C* BY REDUCED VITAMIN K_1*

WALTER D. WOSILAIT

Department of Pharmacology, State University of New York,
Downstate Medical Center, Brooklyn 3, New York

(*Received* 21 *January* 1961)

Abstract—The mitochondrial fraction of rat liver catalyzes the reduction of cytochrome *c* by reduced vitamin K_1. This activity, which has been partially solubilized, is inhibited to only a slight extent by the anticoagulants. The reduction of cytochrome *c* is accompanied by the oxidation of reduced vitamin K_1.

INTRODUCTION

ALTHOUGH previous work with vitamin K has been largely concerned with its role in blood clotting, recent studies have indicated that it may be involved in electron transport. For example, the reduction of menadione (2-methyl-1:4-naphthoquinone) and vitamin K_1 (2-methyl-3-phytyl-1:4-naphthoquinone) by the reduced pyridine nucleotides is catalyzed by an anticoagulant-sensitive enzyme designated pyridine nucleotide-vitamin K_1 reductase.[1, 2] In order to determine whether the reduced form of vitamin K_1 (vitamin K_1H_2) might in turn reduce a cytochrome such as cytochrome *c*, cell fractions of rat liver were tested for such catalytic activity. The mitochondrial fraction catalyzed the reduction of cytochrome *c* by vitamin K_1H_2 and an attempt to dissociate this activity from the mitochondria was partially successful. The present report is concerned with some of the properties of this fraction, which is tentatively designated vitamin K_1H_2–cytochrome *c* reductase.

METHODS

The standard assay system contained the following components: buffer of pH 6·2 containing citrate and ethylenediaminetetra-acetic acid (EDTA) (60 and 6 μmoles, respectively), 10 μmoles of neutralized KCN, 0·087 μmole of vitamin K_1H_2, 0·038 μmoles of horse heart ferri-cytochrome *c* (Sigma Chemical Co), water, and enzyme to a final volume of 1·0 ml. The reactions, which were started by the final addition of enzyme, were measured by following the increase in absorbance at 550 mμ, which is characteristic of the reduction of the alpha band of cytochrome *c*, in a Beckman model DU spectrophotometer at about 25 °C. The increase in absorbance was measured at 30-sec intervals for the first 2 min and at 1-min intervals thereafter for 5 min. Relative activities were based on the net change in absorbance observed over the 5-min period, after corrections were made for the non-enzymic contribution. In most of these studies from 50 to 150 μg of partially purified enzyme were used under conditions in which enzyme was the limiting factor.

* This research was supported, in part, by a grant (H3055) from the U.S. Public Health Service.

Vitamin K_1 was solubilized as described previously[2] and reduced as follows: 4 μmoles of solubilized vitamin K_1 in 12 ml of water were reduced with 200 μmoles of freshly dissolved potassium borohydride (KBH_4). The mixture was allowed to stand for 10 min at room temperature, after which the excess KBH_4 was destroyed by adjusting the pH to 1·9 with 1 N HCl and allowing the mixture to stand for 30 min at 0 °C before use. The reduced samples were kept at 0 °C and were protected from light with aluminium foil during use. Since there was a gradual oxidation of the reduced vitamin, these samples were used for no more than 6 hr. The ultraviolet spectrum of the chemically reduced material was similar to that of enzymically reduced vitamin K_1,[2] and after reoxidation the material migrated in the same manner as vitamin K_1, as shown by paper chromatography. The spectral changes observed after chemical reduction were similar to those observed after enzymic reduction, hence the chemically reduced substance is designated vitamin K_1H_2, but since other changes may have occurred, which were not detectable by spectrophotometric or chromatographic procedures, the designation must be tentative. Solvent controls containing BRIJ-35 (polyoxyethylene lauryl alcohol), without vitamin K_1, were treated in the same way.

RESULTS AND DISCUSSION

In intracellular distribution studies, 85 per cent or more of the vitamin K_1H_2–cytochrome *c* reductase activity of the homogenate was associated with the mitochondrial fraction, while the soluble and microsomal fractions contained little or no activity. As a routine procedure, the enzyme was prepared, by a procedure described elsewhere,[3] from twice-washed rat liver mitochondria which were isolated in 0·25M sucrose containing 10^{-3} M EDTA of pH 7·4. The mitochondria were diluted with sucrose–EDTA to a concentration of about 20 mg of protein per ml and stored at −17 °C until the time of use. After thawing, the samples were centrifuged for 15 min at 15,000 **g**. The collected sediment, which contained the activity, was suspended in sucrose–EDTA and brought to one-fourth the original volume. Partial dissociation of the activity was attained by adding a solution of 10% Lubrol WX (Arnold Hoffman & Co, Inc.) to the sample to make a 2% solution, and the mixture was allowed to stand for 30 min, after which it was centrifuged as before. The sediment was discarded and the supernatant fluid was then centrifuged for 1 hr at 100,000 **g**. The collected sediment, which contained most of the activity, was suspended in one-fourth the previous volume of sucrose–EDTA and diluted with an equal volume of 0·1 M imidazole buffer pH 7·4 for storage at −17 °C. Such preparations were used for most of these studies.

The time course of reduction of cytochrome *c* in a typical reaction is illustrated in Fig. 1 by the increase in absorbance at 550 mμ. Cyanide was required in the system for reduction to be observed, presumably because of the presence of cytochrome oxidase in the preparation. Similar results were obtained under anaerobic conditions. Heating the non-dialysable reductase for 10 min at 100 °C resulted in a loss of 80 per cent or more of its activity. Other proteins, such as bovine serum albumin, crystalline yeast hexokinase, phosvitin, and ovalbumin at similar concentrations, did not appear to catalyze this reaction. Chemically reduced menadione also served as an electron donor in this system; however, the rate of non-enzymic reduction of cytochrome *c* by reduced menadione was greater than with reduced vitamin K_1. Hydroquinone also served in place of vitamin K_1H_2; reduced coenzyme Q_{10} has not yet been tried.

Definitive specificity studies will be carried out when more pure enzyme preparations become available.

The oxidation of vitamin K_1H_2, during the reduction of cytochrome *c*, was followed by observing the spectral changes in the 250 to 290 mμ region of the ultraviolet, where the absorbance by the oxidized and reduced forms of the vitamin are different. However, to follow such changes it was necessary to modify the standard assay system to suboptimal conditions which resulted in a greater non-enzymic contribution to the

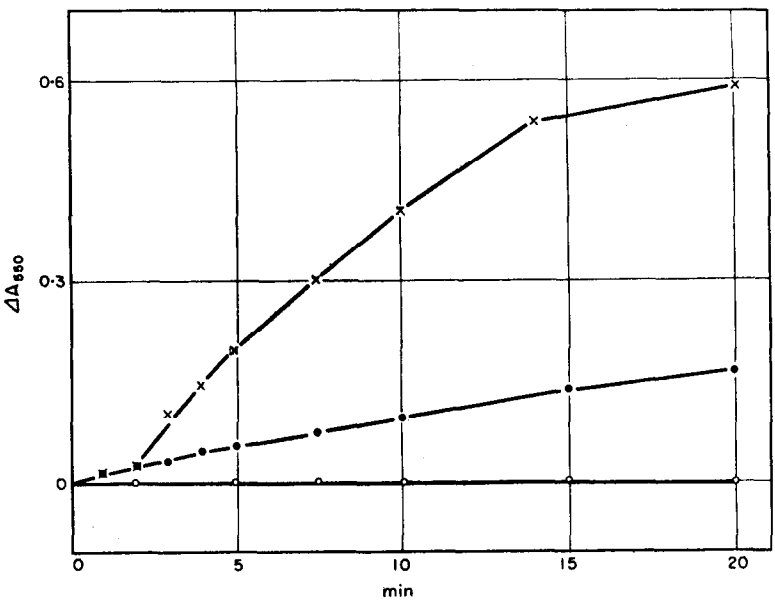

FIG. 1. Reduction of cytochrome *c* by vitamin K_1H_2. The standard assay was carried out as described in the text. At 2 min, 270 μg of enzyme was added to the complete system (×——×), and also to the control, in which BRIJ-35 without vitamin K_1 was used (○——○). Enzyme was omitted from sample (●——●) in order to follow the non-enzymic reduction of cytochrome *c*.

overall reaction. As illustrated in Fig. 2, the spectrum between 240 and 290 mμ changed from one typical of vitamin K_1H_2 (0 min) to one typical of vitamin K_1 (20 min) during the reduction of cytochrome *c*. Moreover, there was an increase in absorbancy between 290 and 340 mμ which may be related to the reduction of cytochrome *c*; however, there is some uncertainty concerning this point.[4] It may be pointed out that when beef heart and different horse heart cytochrome *c* preparations were used, similar spectral changes were observed. The spectral changes which occurred in the absence of enzyme were similar, but the rate was much reduced. The non-enzymic contribution in the experiment illustrated was greater than usual, for reasons given above, and in this experiment this contribution was estimated to be about 40 per cent of the overall change. A comparison of the changes observed at 272 mμ with those observed at 550 mμ showed about a 1:1 relationship between the amount of vitamin K_1H_2 oxidized and the amount of cytochrome *c* reduced. This is greater than expected, and might be due, in part, to the fact that these reactions were carried out under aerobic conditions. Experiments are in progress to clarify this point.

The enzyme was inhibited about 50 per cent by bishydroxycoumarin (Dicumarol), acenocoumarol (Sintrom), cumachlor, and phenindione, at a concentration of 10^{-4} M, and about 20 per cent by ethyl biscoumacetate (Tromexan) and warfarin at the same concentration. The possibility that Dicumarol might accept electrons from vitamin K_1H_2 and compete with cytochrome *c*, thus producing an apparent inhibition, was considered, but on the basis of spectral studies carried out, this did not appear to take place under the standard assay conditions. In general, vitamin K_1H_2–cytochrome *c* reductase was less sensitive to the anticoagulants than was the pyridine nucleotide–vitamin K_1 reductase.[2] Antimycin A caused about 85 per cent inhibition at a concentration of 0·1 μg per ml. 2:4-Dinitrophenol caused about 40 per cent inhibition at 10^{-4} M, while *p*-nitrophenol and 2:4-dinitroaniline had little or no effect. Metal binders such as α:α'-dipyridyl, orthophenanthroline and EDTA, at concentrations of 10^{-4} M, did not appear to influence the activity.

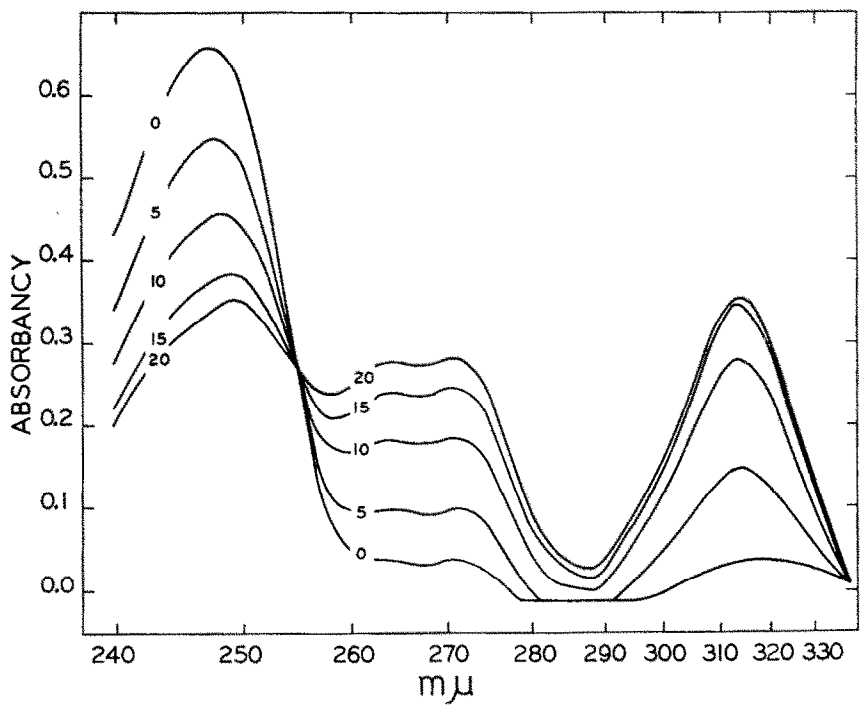

FIG. 2. Oxidation of vitamin K_1H_2 during reduction of cytochrome *c*. The standard assay system was increased to 3·0 ml and the concentration of vitamin K_1H_2 was reduced to 0·017 μmole per ml; thus, the spectral changes which took place could be followed in a Beckman model DK2 recording spectrophotometer.* The reference cell contained all components, including enzyme, except vitamin K_1H_2. The 0-min recording was started immediately after enzyme addition, while the others were started at the indicated times; hence, these changes indicate the general course of the reaction.

* Generously made available by Dr E. Muntwyler.

Although vitamin K_1 has not been unequivocally identified in mammalian tissue, Green and Dam reported that the mitochondrial fraction of beef liver contained a factor which behaved as vitamin K in the bioassay.[5] In addition, Martius reported that ^{14}C-labeled menadione was converted to a substance which was associated with

the mitochondria,[6] and the product was later reported to be 2-methyl-3-(geranyl-geranyl)-1:4-naphthoquinone in chicken and rat.[7] Another quinone, coenzyme Q, is also associated with the mitochondria, but, according to Lester *et al.*, members of this series did not elevate the prothrombin levels of vitamin K-deficient chicks; similarly, coenzyme Q_{10} did not restore the prothrombin level of rats treated with warfarin.[8] Since the present reaction has been studied with a particle which is undoubtedly complex, the possibility that vitamin K_1H_2 may be non-enzymatically reducing a component, such as vitamin K or coenzyme Q, or even some other unidentified factor associated with the particle, and in this fashion introducing electrons into the electron transport chain, cannot be excluded. Since the details of this reaction are not yet known and the reduction of cytochrome *c* is inhibited by Antimycin A, it is possible that a system similar to that described in heart by Ambe and Crane,[9] which involves coenzyme Q and is sensitive to Antimycin, may be present in the preparations used and involved in this reaction. As yet, neither the yield nor the purity of the preparations has been great enough to permit satisfactory analyses for substances such as vitamin K or coenzyme Q. Although complete solubilization of the factor in rat liver has not been attained, fractions were found in dog liver and spinach which did not sediment at 100,000 **g** yet catalyzed the reduction of cytochrome *c* by reduced vitamin K_1. The view that vitamin K may be involved in electron transport is supported by the results of previous studies, in which the reduction of vitamin K_1 was described,[2] and also by the results of the present investigation, in which the subsequent transfer of electrons to cytochrome *c* is described. Further studies are in progress in order to clarify the nature of these reactions and to determine the identity of the natural electron carrier, in order that the possible role of vitamin K in electron transport can be more accurately evaluated.

REFERENCES

1. W. D. Wosilait and A. Nason, *J. Biol. Chem.* **208**, 785 (1954).
2. W. D. Wosilait, *J. Biol. Chem.* **235**, 1196 (1960).
3. W. C. Schneider and G. H. Hogeboom, *J. Biol. Chem.* **183**, 123 (1950).
4. R. Lemberg, *Hematin Compounds and Bile Pigments*, p. 349. Interscience, New York (1949).
5. J. P. Green, E. Søndergaard and H. Dam, *Biochim. et Biophys. Acta* **19**, 182 (1956).
6. C. Martius, *Biochem. Z.* **327**, 407 (1956).
7. C. Martius and O. H. Esser, *Biochem. Z.* **331**, 1 (1958).
8. R. L. Lester, Y. Hatefi, C. Widmer and F. L. Crane, *Biochim. et Biophys. Acta* **33**, 169 (1959).
9. K. S. Ambe and F. L. Crane, *Biochim et Biophys. Acta* **43**, 30 (1960).