REFERENCES

(1) R. Weiser, A. W. Asscher, and J. Wimpenny, *Nature (London)*, **219**, 1365 (1968).

(2) S. Miyamura, *J. Pharm. Sci.*, **53**, 604 (1964).

(3) W. V. Shaw, *Trans. Assoc. Am. Physicians*, **84**, 190 (1971).

(4) J. M. Ingram and M. Moustafa-Hassan, *Can. J. Microbiol.*, **21**, 1185 (1975).

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Detection of Phytonadione in Vegetable Oil

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Abstract □ A simple detection test for phytonadione (vitamin K_1) in vegetable oil is presented. A saturated sodium ethoxide solution was used to saponify vegetable oil and react with the freed phytonadione to form a blue compound. The specificity and mechanism of the colored compound formation is discussed.

Keyphrases □ Phytonadione—detection in vegetable oil □ Vegetable oil—detection of phytonadione □ Vitamins—detection of phytonadione in vegetable oil

Although many methods are available in the literature for the determination of phytonadione in standard solutions, pharmaceuticals, and infant formulas, no simple detection procedure for phytonadione determination in oil has been reported. Detection of phytonadione in oil is of use since vegetable oil solutions of phytonadione are used in the manufacture of multivitamin preparations and elemental diets (1, 2). The present report discusses the chemical (noninstrumental) detection of phytonadione in vegetable oil and aspects of the specificity and mechanism of the reaction.

Reviews for the determination of phytonadione by gas chromatography (3), fluorometric analysis (4), and thin-layer and paper chromatography (5) are available. Circular TLC (6), thin layer silica gel impregnated with silver nitrate or paraffin (7), and more recently UV derivatization (8) and electrochemical detection (9) have been employed for phytonadione analyses. Any of the described phytonadione determinations can be used if the phytonadione is contained in hexane, petroleum ether, acetone, or ethanol standard or sample solutions. Few determinations of phytonadione in vegetable oil samples have, however, been reported.

Phytonadione in vegetable oil presents complex sample-handling problems. The lipophilic nature of phytonadione prevents easy extraction and its alkaline sensitivity prohibits saponification of the vegetable oil without destroying the vitamin (10, 11). Phytonadione is photosensitive (12). Determination of phytonadione in vegetable oil has only been accomplished after time-consuming and complex sample preparation such as reduction by Raney's nickel catalyst (13) or enzymatic hydrolysis (11), conditions not desirable for a simple detection procedure.

Quality control laboratories, and others with similar time and financial limitations, may not find the time-consuming and complex methods presented thus far satisfactory for the detection (presence or absence) of phytonadione. In nonlipid media, some simple chemical detection tests have been reported. Phytonadione may be detected visually in ethanolic solution by reaction with sodium diethyldithiocarbamate (15), or 2,4-dinitrophenylhydrazine (16). No color tests have been reported for phytonadione in vegetable oil solutions.

A procedure has been developed in which detection of phytonadione in vegetable oil is based on the blue complex formed by sodium ethoxide. The procedure differs from that described previously (14) insofar as a saturated (3.4 *N*) sodium ethoxide solution saponifies the lipid medium. The sensitivity of the test is increased by the white background provided by saponification of the vegetable oil. The saponification results in the extraction of some solvent-dissociated phytonadione, which then reacts with sodium ethoxide.

EXPERIMENTAL

Reagents—All chemicals were analytical reagent grade and were used without further purification.

Procedure—One milliliter of a safflower oil solution of phytonadione was added to 1 ml of a saturated (3.4 *N*) solution of sodium hydroxide in ethanol. After the saponification process, a solid blue mass remained. Standards (in ethanol) and samples ranged in concentration from 50–5000 μg phytonadione/ml. In samples containing ≥500 μg/ml, an intensely blue-colored solution was formed almost immediately. The color then faded to gray or brown over a 10-min period. A blank of safflower oil treated with the alkaline alcoholic solution yielded a white soap. Cotton and peanut oil samples yielded identical results. Ascorbyl palmitate and polysorbate 80 did not interfere. The procedure was then applied to 1,4-naphthoquinone, menadione, and the bromination product of phytonadione.

The standards and samples were then tested with sodium diethyldithiocarbamate and 2,4-dinitrophenylhydrazine reagents using the reported procedures (15, 16).

RESULTS AND DISCUSSION

The standard solutions reacted with sodium ethoxide, sodium diethyldithiocarbamate, and 2,4-dinitrophenylhydrazine to form the colors reported in the literature. The sensitivity was found to be satisfactory to repetitively detect (6 trials) 50-μg/ml phytonadione/ml standard with sodium ethoxide and 10-μg/ml standard with sodium diethyldithiocarbamate. The 2,4-dinitrophenylhydrazine was formed an average of 50% of the trials at the 100-μg/ml level. No further investigation of this procedure was attempted. Neither the sodium ethoxide nor diethyldithiocarbamate procedure detected phytonadione in samples.

Detection of phytonadione in vegetable oil then was attempted by

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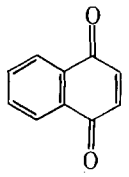

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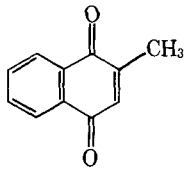

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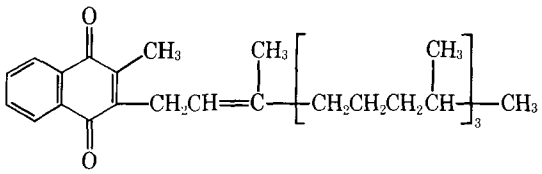

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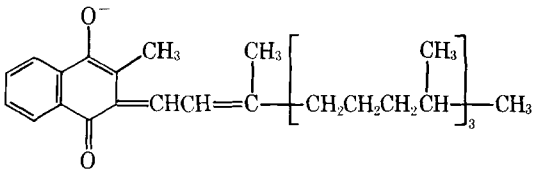

IV

saponification of the oil with sodium hydroxide. Potassium hydroxide did not consistently saponify the vegetable oil at room temperature. In the absence of degradation or dissolution in the saponified matrix or remaining lipid material, reaction with sodium ethoxide or sodium diethyldithiocarbamate should be possible. The sodium diethyldithiocarbamate color test was negative on all sample solutions. The limit of detectability was found to be 50 ppm for the sodium ethoxide color reaction. Of the reactions mentioned, only the sodium ethoxide reaction gave a positive test in vegetable oil, and then, only when the vegetable oil was saponified.

The specificity of the reaction of phytonadione with sodium ethoxide has not been reported. Some confusion concerning even the family of compounds that react to form a blue complex in dilute sodium ethoxide appears in the literature. It was noted (17), in part, that phytonadione dissolved in petroleum ether yielded a positive (blue) test with dilute sodium ethoxide, whereas the vitamin dissolved in oil gave a negative test. It was speculated that phytonadione was less stable in petroleum ether than in oil and that a reaction with an unspecified degradation product of phytonadione was actually responsible for the blue color. The selectivity of the reaction towards phytonadione analogs, therefore, has not been specified. It also is not clear whether the reaction occurs with a parent species or a degraded species.

In an attempt to examine the selectivity of the ethoxide reaction, 1,4-naphthoquinone (I) was subjected to the detection test. The absence of blue color indicated the 1,4-naphthoquinone nucleus was not sufficient for reaction. Menadione (vitamin K_3, II) did not give a positive test. Since phytonadione (III) differs only from menadione by a phytyl group, the phytyl group was found essential for the blue complex formation. Bromination of the double bond originating on the β-carbon of the phytyl group of phytonadione yielded a product that did not form a blue complex. The blue complex formed in the reaction with phytonadione was soluble in water, and it eluted before phytonadione on a reversed-phase LC column. The polar species formed was consistent with the structure of IV. The structure predicts that vitamin K_2 (which contains a polyprenyl group in place of the phytyl group) would form the same extended conjugation system. The vitamin K_2 family of compounds yield blue colors with ethoxide (16, 17). The shift from yellow (in phytonadione) to blue (IV) is consistent with increasing the conjugation of 1,4-naphthoquinone to the phytyl side chain to form an extended π or quasi-π system (18).

A previous report (17) has been cited (15, 16) as evidence that the reaction between phytonadione and ethoxide ion may actually be a reaction with a degradation product of phytonadione. It now appears that the reason ethoxide reacted with petroleum ether samples, but not with vegetable oil samples, may have been due to the lipophilic nature of phytonadione and solution conditions conducive to the formation of IV rather than with the selectivity of the reaction.

A simple detection method for phytonadione in vegetable oil has been developed in which an alkaline ethanolic solution simultaneously transforms the vitamin into a blue compound (consistent with IV) which can be easily visualized on a background of white, saponified vegetable oil. The reaction in standard ethanol solutions and vegetable oil samples is sensitive to phytonadione and vitamin K_2. The test is postulated to be specific to 1,4-naphthoquinones containing alkyl side chains with β-carbon double bonds which may form extended conjugation systems. The selectivity was found to be identical in ethanol standards and vegetable oil samples.

REFERENCES

(1) T. Takebe, K. Nakano, and R. Machida, *Japan* 77 50,251 (Cl. A61 K9/08), 23 Dec. 1977. Appl. 68.92,009, 17 Dec. 1968; through *Chem. Abst. Jpn.*, **88**, 177222s (1978).

(2) A. Osol, "Remington's Pharmaceutical Sciences," Mack Publishing, Easton, Pa., 1975, p. 951.

(3) A. J. Sheppard, A. R. Prosser, and W. D. Hubbard, *J. Am. Oil Chem. Soc.*, **49**, 619 (1972).

(4) A. T. R. Williams, *Lab. Equip. Dig.*, **14**(3), 67 (1976).

(5) H. Mayer and O. Isler, "The Vitamins," Vol. III, Academic, New York, N.Y., 1971, p. 418.

(6) M. H. Hashmi, F. Rafique Chughtai, and M. I. D. Chughtai, *Mikrochim. Acta*, **1**, 53 (1969).

(7) J. T. Matsehiner and J. M. Amelotti, *J. Lipid Res.*, **9**, 176 (1968).

(8) J. C. Vire and G. J. Patriarcho, *J. Pharm. Belg.*, **31**(2), 139 (1976).

(9) J. C. Vire and G. J. Patriarcho, *Analusis*, **6**(4), 155 (1978).

(10) O. Isler, *Angew. Chem.*, **71**, 7 (1959).

(11) S. A. Barnett, L. W. Frick, and H. M. Baine, *Anal. Chem.*, **52**, 610 (1980).

(12) J. P. Green and H. Dam, *Acta Chem. Scand.*, **8**, 1341 (1954).

(13) N. R. Trenner and F. A. Bacher, *J. Biol. Chem.*, **137**, 745 (1941).

(14) H. Dam, A. Geiger, J. Glavind, P. Karrer, W. Karrer, E. Rothschild, and H. Salomon, *Helv. Chim. Acta*, **22**, 310 (1939).

(15) F. Irreverre and M. X. Sullivan, *Science*, **94**, 497 (1941).

(16) A. Novelli, *ibid.*, **93**, 358 (1941).

(17) E. Fernholz and S. Ansbacher, *J. Am. Chem. Soc.*, **61**, 1613 (1939).

(18) C. N. R. Rao, "Ultraviolet and Visible Spectroscopy," Plenum, New York, N.Y. 1967, p. 174.

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