Edetate Disodium-Mediated Chloramphenicol Resistance in *Pseudomonas cepacia*

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Abstract □ The presence of edetate disodium decreased the susceptibility of a particular strain of *Pseudomonas cepacia* to chloramphenicol. The mechanism of this edetate disodium effect, which may be unique to this strain, remains obscure. Tests showed no enzymatic destruction by the microorganism of the chloramphenicol nor any chemical complexation of the antibiotic by the salt. The possibility does exist that edetate disodium alters the cell envelope or cytoplasmic membrane so as to block the transport of chloramphenicol to its site of action within the cell. This possibility is now under investigation.

Keyphrases □ Edetate disodium—mediated chloramphenicol resistance in *Pseudomonas cepacia* □ *Pseudomonas cepacia*—edetate disodium-mediated resistance □ Chloramphenicol—edetate disodium-mediated resistance in *Pseudomonas cepacia*

A house isolate (Strain 2487[1]) of *Pseudomonas cepacia* lost its normal susceptibility to chloramphenicol in the presence of edetate disodium. This was considered unusual since edetate disodium has been shown to reverse antibiotic resistance in some species of *Pseudomonas* (1), and chloramphenicol is one of the few antibiotics effective against *P. cepacia*. This report presents the results of an investigation of this phenomenon.

EXPERIMENTAL

Cultures of Strain 2487 and, for comparison, other house isolates or strains[2] were maintained on agar slants[3], incubated at 35°, and subcultured weekly. The desired concentration of sterile-filtered edetate disodium[4] was added to assay plates, either under aseptic conditions or just prior to autoclaving the media. The plates were seeded by swabbing over the agar surface in three planes 10^{-2} dilutions of cultures of the organisms as grown for 24 hr in broth[5] at 35°. Susceptibility determinations were done with disks[6] impregnated, respectively, with chloramphenicol (30 μg), colistin (10 μg), sulfisoxazole (50 μg), gentamicin (10 μg), and polymyxin B (50 μg), and applied to the surfaces of the seeded agar. These plates, in turn, were incubated for 24–48 hr at 35°, the longer period being necessary in some instances because high concentrations (0.1%) of edetate disodium slowed the growth of *P. cepacia*.

RESULTS AND DISCUSSION

The effect of edetate disodium on the activity against Strain 2487 of various antibiotics was mixed (Table I): Susceptibility to gentamicin was increased; susceptibility to chloramphenicol was decreased; and the activity of the other three agents was not altered.

The observed degree of resistance of Strain 2487 to chloramphenicol was related directly to the concentration of the salt. A linear response was obtained over a range of 0.0–0.1% of edetate disodium in the assay medium. This suggested the possibility that edetate disodium triggers the release of a substance from *P. cepacia* cells which destroys chloramphenicol or modifies the antibiotic to render it ineffective against the organism.

Accordingly, an experiment was carried out following the procedure of Miyamura (2) to determine whether edetate disodium promoted the release of a cell wall-bound enzyme such as chloramphenicol acetyltransferase which would inactivate the antibiotic, as is known to occur in other organisms (3).

Results (Table II) indicate there is present in *P. cepacia* no enzymatic inactivating mechanism for chloramphenicol. Apparently, this is not so in the case of *P. aeruginosa*; ~25–50% of the chloramphenicol was lost in the cultures of this species. This suggests, as previously reported (4), some uptake or destruction of the antibiotic.

Another possibility, that edetate disodium chelates chloramphenicol to make it unavailable to act upon *P. cepacia*, does not seem likely. In fact, additional sensitivity tests showed that edetate disodium actually enhanced the action of the antibiotic against *P. aeruginosa* and *Escherichia coli* (Table III). The data in Table III further suggest that this phenomenon may not be a uniform characteristic in *P. cepacia*. Strain 25416[2] failed to grow in the presence of edetate disodium, while another wild strain (Strain C) was essentially resistant to chloramphenicol.

In the apparent absence of enzymatic destruction or chemical complexation of chloramphenicol, the actual mechanism of the edetate disodium effect remains obscure. The possibility exists that edetate disodium chemically alters the cell envelope or cytoplasmic membrane so that transport of chloramphenicol to its point of action within the cell is blocked. More direct evidence might be obtained with the use of labeled chloramphenicol, and this is a subject for further investigation.

Table I—Sensitivity Pattern of *P. cepacia*[a]

Antibiotic	Agar[b]	Agar[b], +0.1% Edetate Disodium
Chloramphenicol	Susceptible	Resistant
Colistin	Resistant	Resistant
Sufisoxazole	Susceptible	Susceptible
Gentamicin	Resistant	Susceptible
Polymyxin B	Resistant	Resistant

[a] Vick Strain 2487. [b] Eugon agar, Becton Dickinson and Co.

Table II—Recovery of Chloramphenicol Following Incubation

Organism	Chloramphenicol, μg/ml	Recovery, %
P. cepacia[a]	25.0	100.0
P. cepacia[a], with medium containing 0.1% edetate disodium	24.5	98.0
P. aeruginosa[b]	18.5	74.0
P. aeruginosa[c]	14.5	58.0
Medium Control	23.0	92.0
Medium plus Edetate Disodium Control	23.5	94.0

[a] Vick Strain 2487. [b] Ps 28. [c] ATCC 15442.

Table III—Effect of Edetate Disodium on Chloramphenicol Susceptibility Pattern of Gram-negative Bacteria

	Chloramphenicol	
Organism	Agar[a], mm	Agar[a] +0.1% Edetate Disodium, mm
P. cepacia[b]	12.0[c]	0.0
P. cepacia[d]	18.0	8.6
P. cepacia[e]	5.4	No growth
P. cepacia[f]	<1.0	0.0
P. aeruginosa[g]	2.0	12.1
E. coli[h]	16.6	31.5

[a] Eugon agar, Becton Dickinson and Co. [b] Vick Strain 2487. [c] Diameter of inhibition zone surrounding disk. [d] American Type Culture Collection (ATCC) 17759. [e] ATCC 25416. [f] Vick Strain C. [g] ATCC 9027. [h] ATCC 8739.

[1] Richardson-Vicks, Inc.
[2] American Type Culture Collection (ATCC).
[3] Eugon Agar slants, Becton Dickinson and Co.
[4] Sequestrene, Ciba-Geigy Corp.
[5] Eugon broth, Becton Dickinson and Co.
[6] Bacto-Sensitivity Discs, Difco Labs.

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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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