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ACTIONS OF 2-METHYL-1,4-NAPHTHOQUINONE IN *BACILLUS CEREUS**

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Abstract—*Bacillus cereus* was used to study the biochemical basis of the antibacterial action of 2-methyl-1,4-naphthoquinone (menadione). At 2·5 μM, menadione doubled the generation time of logarithmic phase cultures of *B. cereus*. The effect of the drug on the incorporation of labeled precursors for macromolecular synthesis was compared at equivalent turbidities. Incorporation of [^{14}C]adenine and [^{14}C]uracil into RNA was immediately inhibited. In contrast, incorporation of [^{14}C]adenine into DNA, [^{14}C]-L-lysine into protein, and [^{14}C]diaminopimelic acid into cell wall was depressed only in accordance with decreased cell mass. RNA content of inhibited cells was also decreased when measured by the colorimetric orcinol method. Sucrose density gradient analysis of cell-free extracts from control and drug-treated cells labeled with [^{14}C]-uracil showed that the drug effect was not specific for any particular RNA-containing fraction in that the incorporation of [^{14}C]uracil was decreased in all macromolecular components, including ribosomes, ribosomal subunits and soluble RNA. Menadione produced an immediate dose-dependent decrease in ATP content of inhibited cells. Although other possibilities cannot be excluded, the growth-inhibitory effect of menadione in *B. cereus* might be due to decreased ATP levels causing a "shift-down" response in which growth retardation is characterized by a selective inhibition of RNA compared to DNA, protein or cell wall synthesis. Menadione also decreased the acid-soluble sulfhydryl content of inhibited cells. However, the mechanisms of this depletion as well as the relationship of this effect to inhibition of RNA synthesis and growth is unclear. Reduced glutathione, but not glutathione disulfide prevented the growth-inhibitory effect of menadione. Thiodione, a naphthoquinone thioether formed by reaction of reduced glutathione and menadione, did not inhibit the growth of *B. cereus*.

THE ANTIBACTERIAL activities of naphthoquinones are well known, although their mode of action is unclear. Comprehensive studies on the effects of a simple naphthoquinone such as 2-methyl-1,4-naphthoquinone (menadione) on various biochemical parameters in growing bacterial cells are relatively uncommon.[1]

The gram-positive aerobe, *Bacillus cereus*, has been used as a model system in this laboratory in attempting to elucidate the biochemical basis of action of several carcinostatic drugs[2,3] and the phenothiazine tranquilizer, chlorpromazine.[4] Gram-positive bacteria are usually more sensitive to the growth-inhibitory effects of menadione and other naphthoquinones than are gram-negative bacteria.[1] Therefore, the *B. cereus* system seemed particularly suitable for a study of the antibacterial action of menadione.

The results show that menadione specifically inhibits RNA synthesis in exponentially growing *B. cereus* cells, while DNA, protein, and cell wall synthesis were depressed only in accordance with decreased cell mass. Although the basis for the

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inhibition of RNA synthesis remains unclear, it may be related to the decreased ATP and acid-soluble sulfhydryl (SH) content observed in menadione-inhibited cells. In addition, the growth inhibition by menadione could be prevented by simultaneous addition of reduced glutathione (GSH).

A preliminary report of the growth-inhibitory effects of menadione bisulfite on *B. cereus* has already appeared.[5]

MATERIALS AND METHODS

Bacterial growth and medial. B. cereus 569H was grown on a casamino acids (Difco)-salts medium, pH 7·0, in a New Brunswick gyratory shaker at 37°. Growth was monitored turbidimetrically as absorbance at 540 mμ (A_{540}) in a Beckman spectrophotometer, model DU.

Synthesis of cellular macromolecules. Macromolecular biosynthesis was measured according to the membrane filtration method.[6,7] Menadione was dissolved in a minimum amount of ethanol and diluted to volume in water. The concentration of drug was checked by measuring absorbance at 245 mμ.[8] Radioactively labeled compounds were added to a suspension of logarithmic phase *B. cereus* cells at A_{540} 0·10, and the suspension was immediately divided into control and drug-treated cultures. Approximately 1 ml of menadione solution was added per 100 ml of the latter culture, and control culture received an equivalent volume of ethanol in water. These supplementations had no effect on the pH of the bacterial medium. For most radioactive compounds, 0·01–0·1 μc was added per ml of medium. Cultures were sampled periodically to measure turbidity, and a 2-ml aliquot of the bacterial suspension was added to 2 ml of 10% trichloroacetic acid or 1 N KOH prior to membrane (B-6 Bac-T-Flex, Schleicher & Schuell Company, Keene, N. H.) filtration. The filters were then air-dried and glued on to aluminium planchets for counting in a Nuclear-Chicago windowless gas-flow counter.

Luciferase assay for adenosine triphosphate. The ATP content of *B. cereus* cells was determined as previously described.[9] Duplicate 1-ml samples taken from treated or control suspensions of bacteria were added to 4 ml of boiling water. The samples were then boiled for 10 min, placed in ice to cool, and stored overnight in a freezer. To 50 mg of desiccated firefly tails (Sigma FLE-50) was added 5 ml of distilled water to make a solution of final concentration 50 mM KH_2AsO_4 and 20 mM $MgSO_4$, pH 7·4. The mixture was shaken vigorously for 2 min and centrifuged at 14,500 *g* for 35 min in a Sorvall refrigerated centrifuge. The supernatant was decanted and stored in a freezer overnight before use. Only minimal loss of enzyme activity occurred on freezing and thawing of the enzyme solution. The measurement of light intensity was made in a Beckman DPM-100 liquid scintillation counter with coincidence circuit disconnected. Light intensity was measured on a variable discriminator module set from 3 to 100 units. The assay vial, which contained 3·8 ml of 6 mM KH_2AsO_4 pH 7·4 (prepared by dilution of stock Sigma FFAS-100 arsenate buffer, 0·1 M), and 0·2 ml of enzyme solution, gave a background reading of 2000 counts/min/0·1 min. Bacterial solution, or ATP, 0·2 ml, was added rapidly; the mixture was swirled vigorously, then placed in the counter and counted repetitively at 0·1-min intervals, the third interval being recorded as a measure of light intensity for each sample. The ATP present in the bacterial extract was calculated from a standard curve prepared at the same time from the same enzyme solution. Light intensity

was proportional to ATP in the range 2–20 mμmoles. Menadione, 0·1 mμmole, when added to the assay vial did not affect light output due to standard ATP or bacterial extract. All assays were carried out in a darkened room under indirect incandescent light.

Acid-soluble sulfhydryl content of B. cereus. A logarithmic culture of *B. cereus*, 1 l. at A_{540} 0·10, was divided in half and menadione, to give a final concentration of 2·5 μM, was added to one flask. When the culture reached A_{540} 0·15, the cells were collected by centrifugation at 4°, washed with cold water, and recentrifuged. The pellet was extracted at 4° with 0·4 ml of 0·1 M disodium ethylenediamine tetraacetic acid, pH 7·0, plus 2·6 ml of 5% trichloroacetic acid. The suspension was centrifuged and a 2·0-ml aliquot of the supernatant was used to measure acid-soluble SH by a modification[10] of a colorimetric method using 5,5′-dithiobis(2-nitro)benzoic acid.[11] A standard curve was prepared using GSH.

Sucrose density gradient analysis. To a suspension of log phase *B. cereus* cells, 2-[^{14}C]uracil (0·02 μc/ml) was added at A_{540} 0·10, and the suspension was immediately divided into control and menadione, 2·5 μM cultures. Aliquots from both cultures were removed at A_{540} 0·175, and bacterial cells were centrifuged at 10,000 *g* for 10 min at 4°, washed with 10 mM Tris buffer, pH 7·8, containing 10 mM magnesium acetate and 60 mM KCl (TMK buffer). The cells were resuspended in 0·3 ml of TMK buffer and sonicated for 5 min at 0° in an MSE model 60W ultrasonic disintegrator, followed by centrifugation for 30 min at 10,000 *g* at 4°. The extracts were dialyzed at 4° for 18 hr against 1000 vol. of the TMK buffer. The dialyzed extract was then applied onto 5 ml of a linear (5–20%) sucrose–TMK gradient prepared by use of a Buchler gradient machine, and the tubes were centrifuged at 39,000 rev/min for 110 min in an SW 50L rotor. Gradients were analyzed using an ISCO model 180 automatic density gradient fractionator (Instrumentation Specialties Company, Inc., Lincoln, Neb.) equipped with an ISCO model 222 single-beam ultraviolet analyzer connected to and recorded by an ISCO model 170 servographic recorder. Fractions of four drops (counted by a Gilford drop counter and equal to about 0·16 ml) were collected in vials, 10 ml of Bray's[12] solution was added to each vial, and radioactivity was counted in the Beckman DPM-100 liquid scintillation counter.

Colorimetric assay for ribonucleic acid. RNA content of *B. cereus* was measured as previously described.[13] Duplicate aliquots of bacterial cultures, 5 ml were removed at intervals and added to 0·5 ml of 50% trichloroacetic acid. The resulting suspension was centrifuged, supernatant decanted and the remaining precipitate extracted with 3 ml of 5% trichloroacetic acid at 90° for 15 min. To a 1·5-ml aliquot of the supernatant was added 1·5 ml of orcinol reagent (1 g purified orcinol in 100 ml of concentrated HCl containing 0·5 g $FeCl_3$); the mixture was boiled for 20 min and read at 660 mμ in the spectrophotometer. A standard curve was prepared using yeast RNA.

Preparation of S-(2-methyl-1,4-naphthoquinonyl-3-)glutathione (thiodione). Thiodione was prepared as previously described by reaction of GSH with menadione and recrystallized from 50% aqueous ethanol.[14] Paper chromatography (descending) on Whatman No. 1 in isopropanol–formic acid–water (70:10:10, v/v) gave one spot (R_f 0·79) which was ultraviolet absorbing, and ninhydrin and chloroplatinate-positive.[15]

Chemicals. 8-[^{14}C]Adenine, 8-[^{14}C]guanine and 2-[^{14}C]uracil were obtained from New England Nuclear, Boston, Mass.; 1,7-[^{14}C]diaminopimelic acid from Interna-

tional Chemical and Nuclear Corp., Irvine, Calif.; and uniformly labeled [^{14}C]-L-lysine from Nuclear-Chicago, Chicago, Ill. Menadione was obtained from Nutritional Biochemicals, Cleveland, Ohio. Firefly lantern extract, stock 0·1 M arsenate buffer, pH 7·4, GSH and GSSG were obtained from Sigma Chemical Company, St. Louis, Mo.; yeast RNA from Worthington Biochemical Company, Freehold, N.J.; and disodium ATP from Calbiochem, Los angeles, Calif. Other chemicals were from commercially available sources.

RESULTS

Effect on growth. The addition of menadione at 1·0 and 2·5 μM to exponentially growing cultures of *B. cereus* at A_{540} 0·10 resulted in an instantaneous retardation of growth which was dose-dependent (Fig. 1). As can be seen, 2·5 μM menadione produced an approximate 50 per cent inhibition in growth rate; therefore, this concentration was used in all further studies. Recovery from growth inhibition due to 2·5 μM menadione occurred after approximately one doubling in the presence of the drug and was a consistent observation. If menadione-inhibited cells were harvested, washed, and resuspended in fresh medium, they resumed growth at a normal rate without any lag period.

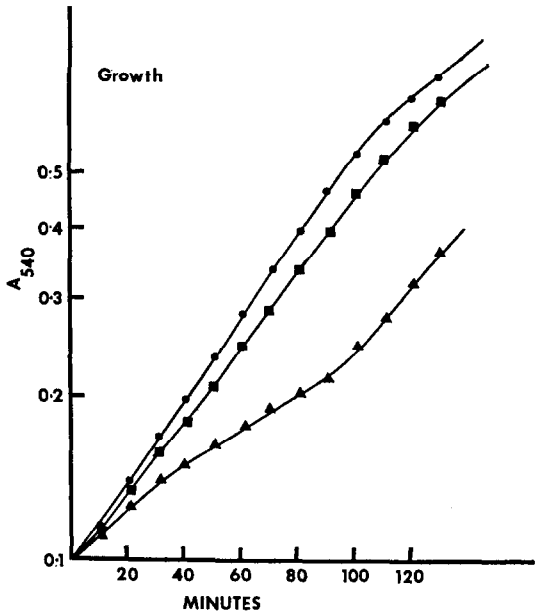

FIG. 1. Effect of menadione on growth of *B. cereus*. Growth was measured turbidimetrically in a Beckman model DU spectrophotometer. The absorbance at 540 mμ (A_{540}) was plotted against time. Menadione added at time 0 at A_{540} 0·10 at concentrations: ●—●, 0; ■—■, 1 μM; ▲—▲, 2·5 μM.

Effect on macromolecular synthesis. In an attempt to localize the site of action of menadione, the effect of the drug on the incorporation of various labelled precursors for the synthesis of macromolecules in *B. cereus* was examined by the membrane filtration method.[6,7] The incorporation into appropriate cell fractions was plotted

in relation to increased cell mass in order to detect differences produced by the drug rather than those due to delayed growth.

Protein synthesis. Protein synthesis was followed by measuring the incorporation of [^{14}C]L-lysine into trichloroacetic acid-insoluble material. When incorporation of [^{14}C]L-lysine (counts per minute) was plotted against change in cell mass (ΔA_{540}), the points obtained from control and drug-treated cells fell along the same line, indicating that 2·5 μM menadione did not preferentially affect protein synthesis.

Cell wall synthesis. Menadione, 2·5 μM, did not specifically inhibit the incorporation of [^{14}C]diaminopimelic acid, a bacterial cell well precursor,[6,7] into trichloroacetic acid-washed cells in that, when incorporation of [^{14}C]diaminopimelic acid (counts per minute) was plotted against increase in cell mass (ΔA_{540}), the lines drawn through points obtained from control and drug-treated cells were congruent. Therefore, the drug was not specifically inhibiting bacterial cell wall synthesis.

Deoxyribonucleic acid synthesis. The synthesis of DNA was followed by measuring the incorporation of [^{14}C]adenine into KOH-insoluble material; this procedure normally measures incorporation of purines and pyrimidines into DNA exclusively.[6,7] Menadione, 2·5 μM, did not specifically affect the incorporation of [^{14}C]adenine into DNA in that, when incorporation (counts per minute) was plotted against change in cell mass (ΔA_{540}), the lines drawn through points obtained from control and drug-treated cells were congruent. Similar results were obtained with [^{14}C]uracil, [^{14}C]guanine, [^{14}C]formate, and [^{32}P]-inorganic phosphate, indicating that DNA synthesis was not specifically depressed by menadione.

Ribonucleic acid synthesis. The synthesis of RNA as measured by incorporation of [^{14}C]adenine (Fig. 2) and [^{14}C]uracil (Fig. 3) was immediately and specifically

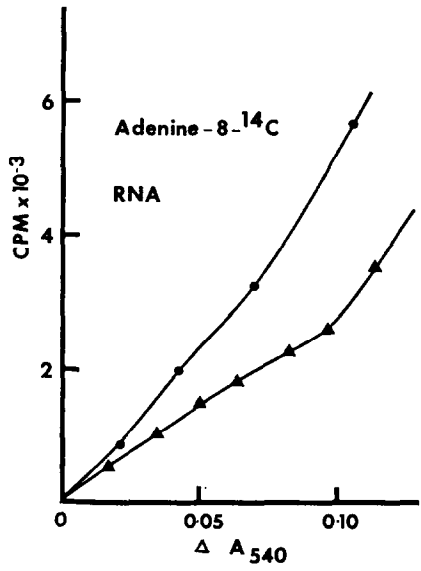

FIG. 2. Effect of menadione on incorporation of 8-[^{14}C]adenine into RNA. Cells were grown in the presence and absence of drug, and comparisons were made for similar increases in cell mass (ΔA_{540}). Incorporation is represented as the difference in total counts per minute between trichloroacetic acid-washed cells (RNA plus DNA) and KOH-insoluble material (DNA) present in 2 ml of bacterial suspension. Concentrations of drug: ●—●, 0; ▲—▲, 2·5 μM.

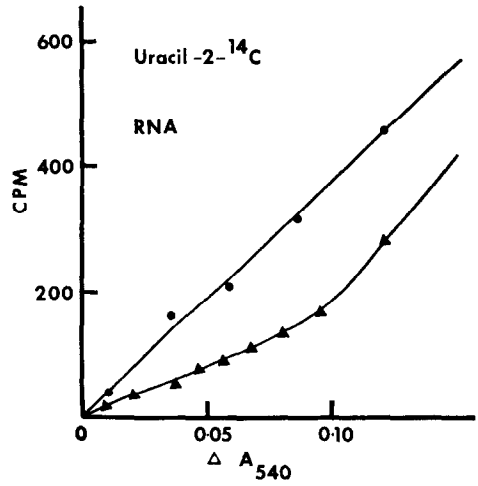

FIG. 3. Effect of menadione on incorporation of 2-[^{14}C]uracil into RNA. Concentrations of drug: ●—●, 0; ▲—▲, 2·5 μM.

inhibited by 2·5 μM menadione. These results were obtained consistently in three other experiments with [^{14}C]adenine and three other experiments using [^{14}C]uracil. Although not shown, the incorporation into RNA of [^{14}C]guanine, [^{14}C]hypoxanthine, [^{32}P]-inorganic phosphate, and [^{14}C]formate was also inhibited by 2·5 μM menadione. Menadione, 2·5 μM, decreased the incorporation of these various precursors into RNA by about 50 per cent compared to control cells grown to the same turbidity. As can be seen (Figs. 2 and 3), inhibition, by 2·5 μM menadione of incorporation of [^{14}C]adenine and [^{14}C]uracil into RNA was relieved after approximately one doubling of cell mass that is, at approximately the same point in time when the growth rate returned to normal (see Fig. 1).

Ribonucleic acid content. As shown in Fig. 4, the RNA content, as measured by the colorimetric orcinol method, was lower in menadione-treated cells. Three other experiments were consistent with the observation shown in Fig. 4. These experiments are in agreement with the results obtained using isotopically labeled precursors, and indicate that the effect of the drug involves specific inhibition of RNA biosynthesis.

Sucrose density gradient analysis. Since menadione produced a specific inhibition in RNA synthesis of log phase *B. cereus* cells, it was of interest to see what effect the drug had on particular macromolecular components containing RNA. Therefore, we examined the effect of menadione on incorporation of a labeled precursor into ribosomes, ribosomal subunits, and soluble RNA. Figure 5 shows the results of a sucrose density gradient centrifugation of cell-free extracts obtained from control and 2·5 μM menadione-treated cells which had been grown in the presence of [^{14}C]uracil. Both radioactivity (counts per minute) and ultraviolet absorption (A_{254}) from control and menadione-treated systems showed the expected 70S and 4S peaks with smaller 50S and 30S peaks, so that no major qualitative differences between control and menadione-treated suspensions were seen. The major quantitative difference was in the marked decrease in specific activity (counts per minute/A_{254}) of ribosomes, ribosomal subunits, and soluble RNA from menadione-treated cells compared to

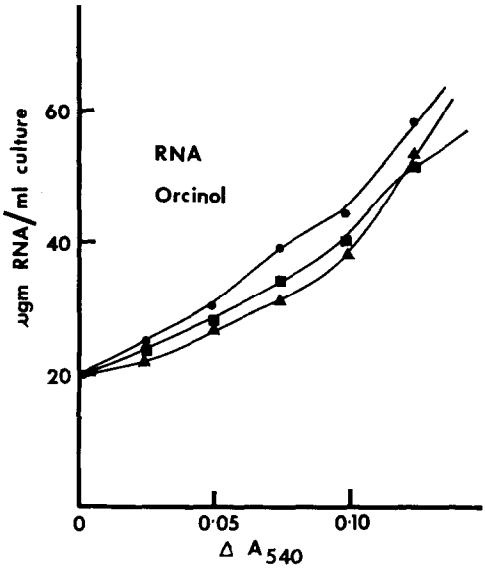

FIG. 4. Effect of menadione on total RNA of *B. cereus*. Duplicate 5·0 ml samples were removed from cultures and assayed for ribose by the orcinol test as described under Methods. Concentrations of drug: ●—●, 0; ■—■, 1·0 µM; ▲—▲, 2·5 µM.

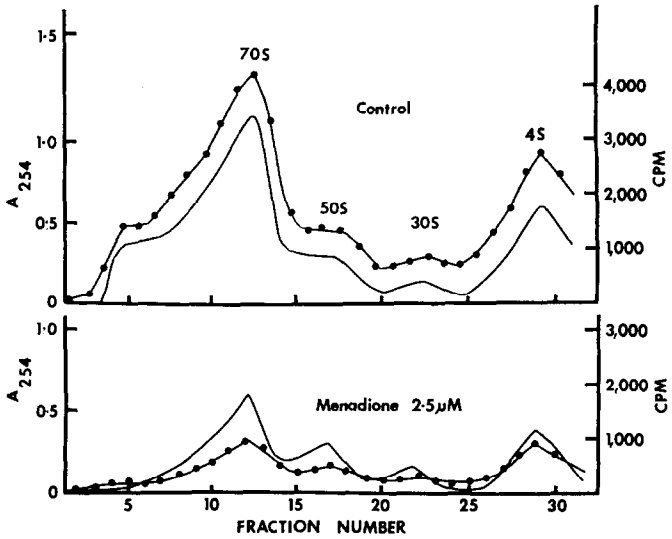

FIG. 5. Sucrose density gradient analysis of extracts from control and 2·5 µM menadione-treated *B. cereus* labeled with 2-[^{14}C] uracil. To a log phase culture at A_{540} 0·10 2-[^{14}C]uracil was added and the suspension divided into control and menadione (2·5 µM) cultures. At A_{540}: 0·175, aliquots from both cultures were removed, the cells harvested, washed, disrupted by sonication, and the cell-free extract was dialyzed overnight against TMK buffer. The dialyzed extract was applied to 5 ml of 5–20% sucrose–TMK gradient and centrifuged at 39,000 rev/min for 110 min. Gradients were analyzed for radioactivity and A_{254} as described under Methods. Symbols: ●—●, counts per minute; —, A_{254}.

control cultures. It appears that the drug effect was not specific for any particular macromolecular component in that the incorporation of [^{14}C]uracil was decreased in all RNA-containing fractions. In addition, in menadione-treated cultures there appeared to be an increase in 30S and 50S peaks relative to the 70S peaks, as measured by A_{254}, indicating that some accumulation of these ribosomal subunits may occur in menadione-treated cells. Similar results, as described above, were obtained when [^{14}C]adenine was used as an RNA precursor.

Effect on adenosine triphosphate levels. Menadione can uncouple oxidative phosphorylation in bacterial cell-free extracts[16] and in mammalian liver mitochondrial preparations.[17] Uncoupling of oxidative phosphorylation has been suggested as a possible mechanism to explain the antibacterial action of menadione and related quinones.[1] Therefore, we examined the effect of menadione on ATP levels of log phase *B. cereus* cells. Bacterial ATP was determined by the luciferase assay using a liquid scintillation counter to measure light output. As shown in Fig. 6, menadione, 1·0 and 2·5 μM, produced an immediate dose-dependent decrease in the ATP content of inhibited cells. As can be seen, the menadione-induced depression of ATP levels was reversible. ATP could not be detected in the cell-free medium obtained after centrifugation of cell suspensions from inhibited cultures, indicating that the decrease in ATP was not accompanied by leakage of ATP out of the cells.

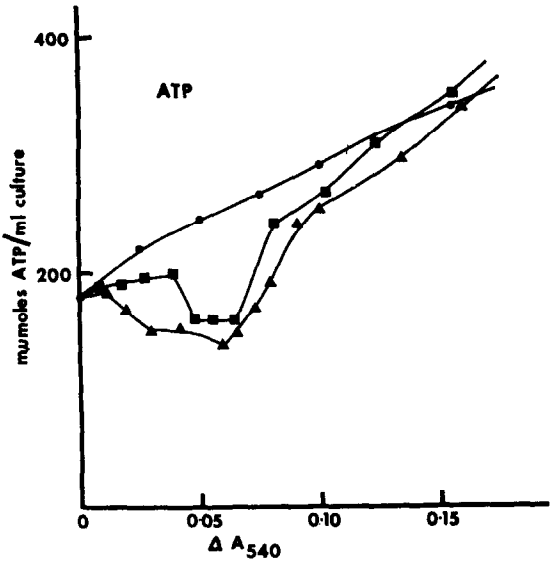

FIG. 6. Effect of menadione on ATP levels in *B. cereus*. At A_{540} 0·10, a log phase culture was divided into control and drug-treated suspensions. Cultures were sampled periodically for absorbance at 540 mμ, and duplicate 1·0-ml samples were added to 4 ml of boiling water and boiled for 10 min. Aliquots, 0·2 ml of the bacterial extract, were measured for ATP content by the luciferase assay using a Beckman DPM-100 liquid scintillation counter to measure luminescence intensity as described under Methods. The ATP content present in the bacterial extract was calculated from a standard curve prepared at the same time from the same luciferase solution. Concentrations of drug: ●—●, 0; ■—■, 1·0 μM; ▲—▲, 2·5 μM.

Effect on acid-soluble sulfhydryl content. Depletion of cellular SH groups has been proposed as one mechanism to account for the antibacterial action of naphthoquinones.[1] Therefore, we examined the effect of menadione on the content of acid-soluble SH compounds in log phase *B. cereus* cells. As can be seen (Table 1), at equivalent turbidities, menadione (2·5 μM) decreased the acid-soluble SH content of inhibited cells 42 per cent compared to control cells, as determined by a colorimetric method using 5,5′-dithiobis(2-nitro)benzoic acid.[10,11]

TABLE 1. EFFECT OF MENADIONE ON ACID-SOLUBLE SULFHYDRYL CONTENT OF *Bacillus cereus**

	Acid-soluble sulfhydryl (mμmoles SH/l. of culture at A_{540}: 0·15)
Control	168 ± 0·01 (100%)
Menadione (2·5 μM)	97 ± 0·07 (58%)†

* A logarithmic phase culture of *B. cereus*, 1 l., at A_{540} 0·10, was divided in half and menadione, 2·5 μM, was added to one flask. When the cultures reached A_{540} 0·15, they were harvested by centrifugation at 4°, washed with cold water by centrifugation, and the acid-soluble SH content was measured colorimetrically as described under Methods. The values represent the average ± S.E.M. of four experiments.

† $P < 0·01$ as determined by *t*-test using method of paired comparisons.

Reversal of growth-inhibitory effect of menadione by reduced glutathione. Sulfhydryl-containing compounds can prevent or reverse the antibacterial effects of menadione;[18] however, the mechanism is unknown. As can be seen (Fig. 7), when GSH (1 mM)

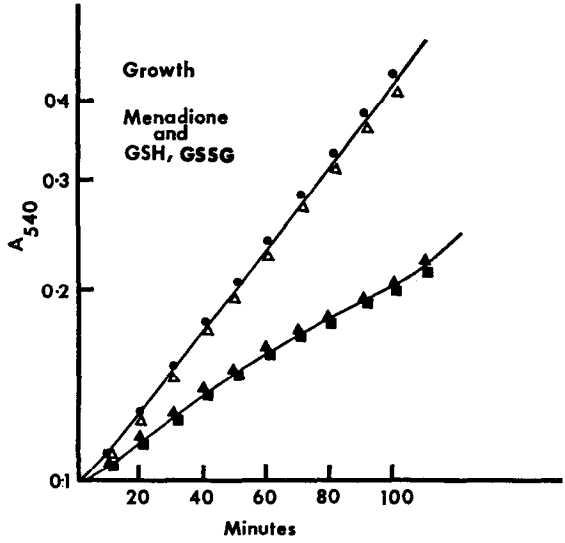

FIG. 7. Effect of GSH and GSSG on growth inhibitory action of menadione. Menadione and GSH or GSSG, as indicated, were added at 0 time at A_{540} 0·10 and growth was measured turbidimetrically. Concentrations of drug: ●—●, 0; ▲—▲, 2·5 μM menadione; △—△, 2·5 μM menadione and 1 mM GSH; ■—■ 2·5 μM menadione and 0·5 mM GSSG.

was added simultaneously with 2·5 μM menadione it completely protected the bacteria against the growth-inhibitory effects of the drug; 0·5 mM glutathione disulfide (GSSG) was completely ineffective in this respect. Neither GSH (1 mM) nor GSSG (0·5 mM) had any effect on growth of control cultures of *B. cereus*. Although not shown, 1 mM D- or L-cysteine also prevented growth inhibition by 2·5 μM menadione, while 0·5 mM L-cystine was inactive. Although not shown, the inhibition in incorporation of [^{14}C]adenine into RNA by 2·5 μM menadione (as seen in Fig. 2) could be completely prevented by simultaneous addition of 1 mM GSH.

Effect of thiodione on B. cereus. The affinity of menadione for SH compounds is known.[19] One possible mechanism to explain the protection afforded by exogenous GSH against menadione is that the GSH reacts rapidly with menadione to form an inactive thioether adduct. The synthesis of thiodione by reaction of GSH with menadione has been reported.[14] This compound was prepared and its effect on growth of *B. cereus* examined. Thiodione, even at a concentration of 1mM, had no effect on growth of *B. cereus* in that when cell mass (A_{540} 0·1 to 0·4) was plotted against time (minutes) the lines drawn through points obtained from control and thiodione-treated cells were congruent.

DISCUSSION

The inhibition by menadione of the incorporation of [^{14}C]uracil and [^{14}C]-adenine into RNA of logarithmic phase culture *B. cereus* occurred shortly after addition of the drug and at concentrations which are similar to the minimal growth-inhibitory concentration of the drug. In addition, the RNA content of inhibited cells was also decreased when measured by the colorimetric orcinol method which assayed ribose RNA. Other isotope experiments demonstrated that the effect of menadione was specifically restricted to interference with RNA synthesis, since DNA, protein, and cell wall synthesis were only depressed in accordance with the decreased cell mass.

The inhibition of RNA synthesis involved both ribosomal and soluble RNA, indicating that menadione blocked RNA synthesis at a step prior to or during polymerization. The mechanism by which menadione inhibits RNA biosynthesis in *B. cereus* is unresolved. However, it appears likely that inhibition of RNA synthesis is casually related to the inhibition of growth, since drugs which block RNA synthesis in *B. cereus*, including salicylate,[20] thioguanine,[21] and quinacrine,[22] invariably inhibit cell growth.

The selective inhibition by menadione of RNA compared to DNA, protein, and cell wall synthesis resembles in some ways the phenomenon of a "shift-down". Under such circumstances, when a culture is shifted from an adequate to a poor nutrient medium, the decreased rate of growth is accompanied by a preferential inhibition of RNA, particularly ribosomal RNA synthesis compared to DNA or protein synthesis.[23] Similar effects, comparable to nutrient-induced "shift-downs", have been ascribed to several drugs known to interfere with oxidative phosphorylation. For example in *Escherichia coli*, azide and cyanide produce a preferential inhibition in RNA synthesis.[24] Comparable observations have been reported with 2,4-dinitrophenol in *E. coli*[25] and Ehrlich ascites cells.[26] In addition, levallorphan has been shown to produce a marked intracellular decrease of ATP in *E. coli*, possibly by an activation of adenosine triphosphatase (ATPase), which leads to a generalized inhibition of

macromolecular synthesis.[27] It has been postulated that such an effect might account for the selective inhibition of ribosomal RNA synthesis observed with levorphanol in *E. coli*[28,29] where in low concentrations of levorphanol produced a situation comparable to a "shift-down". As we have shown, menadione, a known uncoupler of bacterial oxidative phosphorylation,[16] caused an immediate decrease in ATP content of inhibited *B. cereus* cells. Therefore, it is possible that the decrease in ATP due to menadione produces a "shift-down" response in which growth retardation is characterized by a greater depression in RNA synthesis compared to DNA, protein, or cell wall synthesis.

Effects of naphthoquinones on RNA synthesis have been reported in other systems. 2-Methyl-1,4-naphthoquinol-bis(disodium phosphate) (synkavite) has been shown to inhibit the uptake of formate and glycine into the RNA purines of Ehrlich ascites cells *in vivo* but not into DNA or acid-soluble purine nucleotides.[30] Synkavite, which is rapidly dephosphorylated enzymatically to give menadione,[31] was also found to reduce the ATP content of Ehrlich mouse ascites tumor cells *in vivo* and *in vitro*.[32] More recently it was reported that treatment with synkavite of Ehrlich ascites cells *in vitro* reduced the rate of synthesis of RNA and DNA from exogenous ribonucleosides and thymidine, respectively, by decreasing the rate of their incorporation into intracellular nucleoside triphosphates.[33] Increases in RNA content of a phylloquinone-requiring strain of *Fusiformis nigrescens* were produced when this anaerobe was grown in a phylloquinone-deficient medium. It was suggested that this was the result of a membrane-mediated effect of phylloquinone on RNA and possibly protein synthesis in this microorganism.[34] Two water-soluble derivatives of menadione inhibited RNA metabolism, respiration, and glycolysis of rabbit reticulocytes; phylloquinone was inactive in this system.[35]

It has been suggested that the antibacterial action of menadione is due, at least in part, to interference with essential SH-containing metabolites or enzymes.[1,36] Decreases in acid-soluble SH content of bacteria by menadione[37] and in mouse ascites and solid tumor cells *in vivo* by synkavite[38,39] and menadione bisulfite[39] have been reported. Our experiments shown that menadione produced a marked decrease in the acid-soluble SH content of log phase *B. cereus* cells. The acid-soluble SH pool of *B. cereus* consists almost exclusively of GSH.[21] The relationship between the decrease in acid-soluble SH produced by menadione in *B. cereus* and the growth-inhibitory effect of the drug is unclear.

A direct relationship between GSH and RNA synthesis has been suggested by the observation that in a relaxed strain of *E. coli* treated with diamide (which specifically oxidized GSH to GSSG) RNA synthesis is inhibited.[40] In addition, 4-nitrobenzofurazan, which is highly reactive with SH groups, inhibited the incorporation of 5-[^{3}H]uridine into sheep lymphocyte RNA *in vitro*. The effect of 4-nitrobenzofurazan could be abolished by preincubation for 10 min with GSH or other SH-containing compounds, including 2-mercaptoethanol, cysteine and its methyl ester, but not by cystine or alanine methyl ester.[41]

Other possibilities to account for the inhibition of RNA synthesis by menadione in *B. cereus* must be considered. DNA-dependent RNA polymerase from *Azotobacter vinelandii* has been shown to be sensitive to inhibition by *p*-chloromercuribenzoate; SH compounds are required for maximal activity.[42] Depletion by menadione of bacterial GSH or direct effects of the drug on SH-sensitive sites on RNA polymerase

of *B. cereus* might account for the inhibition in RNA synthesis which was observed. Evidence for the complexing of menadione with DNA by electron spin resonance techniques has been reported.[43] The possibility that menadione is binding to bacterial DNA and thereby interfering with the normal function of RNA polymerase cannot be excluded.

Menadione, phylloquinone and a variety of other oxidant compounds can provoke hemolysis of erythrocytes.[44] The hemolytic action of these compounds has been ascribed to effects which include depletion of reduced pyridine nucleotides and GSH as well as generation of hydrogen peroxide.[45,46]

The decrease in the acid-soluble SH content of *B. cereus* produced by menadione may be the result of one or more effects. At identical turbidities (A_{540} 0·15), menadione-inhibited cells contained 71 nmoles SH per liter less than control cells. Since 2·5 μmoles menadione is added per liter of culture, the direct reaction of menadione with endogenous GSH to form a thioether such as thiodione[14] could account for the total decrease in acid-soluble SH observed. However, menadione might also be functioning catalytically to interfere with the maintenance of normal SH levels.

GSH reductase (EC 1.6.4.2), which catalyzes an NADPH-dependent reduction of GSSG, has been found in a variety of microorganisms.[47] Menadione can act catalytically to oxidize bacterial NADPH,[16] and thereby could limit the availability of NADPH for generation of GSH by GSH reductase. In addition, oxidation of menadiol[16] could generate a peroxy free radical[48] or hydrogen peroxide,[49] which would in turn consume GSH either non-enzymatically or by enzymatic oxidation if glutathione peroxidase ($GSH:H_2O_2$ oxidoreductase; EC 1.11.1.9) were present in *B. cereus*.[50–52] Finally, direct inhibition of glutathione reductase by SH reagents has been reported.[53]

The growth-inhibitory action of menadione in *B. cereus* could be prevented by GSH. Since thiodione was inactive against *B. cereus*, one possible mechanism to explain the protective effect of exogenous GSH is that it rapidly inactivates menadione by formation of a thioether such as thiodione. The rapid reaction of menadione with GSH or other SH compounds to form thioethers has been described.[14,19] Furthermore, GSSG, which cannot react directly with menadione, failed to protect against menadione. Alternatively, if depletion by menadione of bacterial GSH is directly related to the growth-inhibitory action of the drug, then perhaps exogenous GSH is acting as a source of GSH to maintain normal bacterial levels of GSH, despite the presence of menadione. However, so far as we are aware, the utilization of exogenous GSH by *B. cereus* or other microorganisms has not been described.

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