Table I—Peak Heights of Metoprolol–Hydrochlorothiazide Standard Solutions

	Concentration in Simulated Gastric Fluid, mg/L									
	10:5		20:10		60:30		80:40		100:50	
	MET[a]	HCT[b]	MET	HCT	MET	HCT	MET	HCT	MET	HCT
	6.3	4.2	17.1	8.4	58.7	24.8	75.0	32.0	98.0	41.0
	6.3	4.1	17.2	9.1	58.1	32.5	75.0	32.0	98.0	42.0
	6.3	4.2	17.0	8.6	56.5	23.7	74.2	31.2	98.0	41.0
	6.1	4.0	17.0	8.2	56.5	24.3	75.7	31.6	96.5	40.5
Mean	6.3	4.1	17.1	8.6	57.5	24.1	75.0	31.7	97.6	41.1
SD	0.1	0.1	0.1	0.4	1.1	0.6	0.6	0.4	0.8	0.6

[a] MET, metoprolol. [b] HCT, hydrochlorothiazide.

The advantage of a rapid screening technique for monitoring individual release rates of the active components of a multicomponent product is shown in Fig. 3. The composite release from the 100:25- and 100:50-mg formulations appear similar; however, the hydrochlorothiazide is the faster component in Fig. 3B (100:25 mg) and the slower component in Fig. 3C (100:50 mg).

A typical chromatogram is shown in Fig. 4, where six analyses were completed. Peak height measurements for a series of standards are listed in Table I. Linear regression analysis on the mean peak height values of metoprolol and hydrochlorothiazide *versus* concentration yielded correlation coefficients of 0.9996 and 0.9994, respectively.

The intent of these experiments was to demonstrate the utility of a novel approach to analyzing the dissolution rate profile of combination products. For this article, we selected an example of a dual-entity product which was manufactured in several strengths. The ease of operation and facility of this technique is demonstrated in the reduced time required for quantitative analysis of each of the two components of this combination product. The progress being made in HPLC techniques and equipment should allow this technique to be developed for other combination products and for single-entity products which have excipients that interfere in normal UV analyses.

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Some Effects of 1-(2,4-Dichlorophenyl)-4-dimethylaminomethyl-1-nonen-3-one Hydrochloride on *Escherichia coli* GK-19

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Received December 27, 1982, from the **Department of Applied Microbiology and Food Science, College of Agriculture*, ‡*Department of Microbiology, College of Medicine*, and §*College of Pharmacy, University of Saskatchewan, Saskatoon, Saskatchewan S7N 0W0, Canada.* Accepted for publication April 26, 1983.

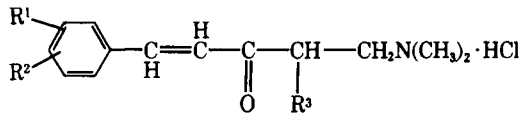
Abstract □ 1-(2,4-Dichlorophenyl)-4-dimethylaminomethyl-1-nonen-3-one hydrochloride (Id) was shown to inhibit the growth of *Escherichia coli* GK-19 at a concentration of 50 μg/mL in a medium of pH 6.5. Maximal antibacterial activity was found during the logarithmic growth phases rather than at the early stationary phase. Electron microscopy revealed that Id caused lysis, and inhibition of respiration and retardation of RNA and protein syntheses occurred in the bacteria with this compound at 50 μg/mL.

Keyphrases □ 1-(2,4-Dichlorophenyl)-4-dimethylaminomethyl-1-nonen-3-one hydrochloride—antibacterial activity in *Escherichia coli*, effects of concentration, temperature, and medium pH □ Antibacterial activity—1-(2,4-dichlorophenyl)-4-dimethylaminomethyl-1-nonen-3-one hydrochloride against *Escherichia coli*, effects of concentration, temperature, and medium pH

Previous work from these laboratories showed that a number of Mannich bases derived from conjugated styryl ketones had antibacterial properties (1–3). The aim of the present investigation was twofold. First, it was hoped to unravel some of the factors affecting the action of a representative Mannich base on bacterial growth, and second, to discern the site or sites of action of the compound toward the bacteria.

EXPERIMENTAL

Synthesis of Compounds—Compound Ia was prepared by the literature method (4); Ib, d–f were synthesized by a previously described procedure (5), as was Ic (6).



Ia: $R^1 = R^2 = R^3 = H$
Ib: $R^1 = R^2 = H$; $R^3 = (CH_2)_4CH_3$
Ic: R^1 = 3-OH; $R^2 = H$; $R^3 = (CH_2)_4CH_3$
Id: R^1 = 2-Cl; R^2 = 4-Cl; $R^3 = (CH_2)_4CH_3$
Ie: R^1 = 2-Cl; R^2 = 6-Cl; $R^3 = (CH_2)_4CH_3$
If: R^1 = 3-Cl; R^2 = 4-Cl; $R^3 = (CH_2)_4CH_3$

Measurement of Bacterial Growth—In this study, an isolate of *Escherichia coli* K-12 strain designated GK-19 (7) was employed. The bacteria were grown in a minimal salts medium (8) to which casamino acids (0.5% w/v) and thiamine (7.5 μg/mL) had been added. Adjustment of the pH was made using Sörensen's buffer solutions (9). The cultures were grown in Erlenmeyer flasks

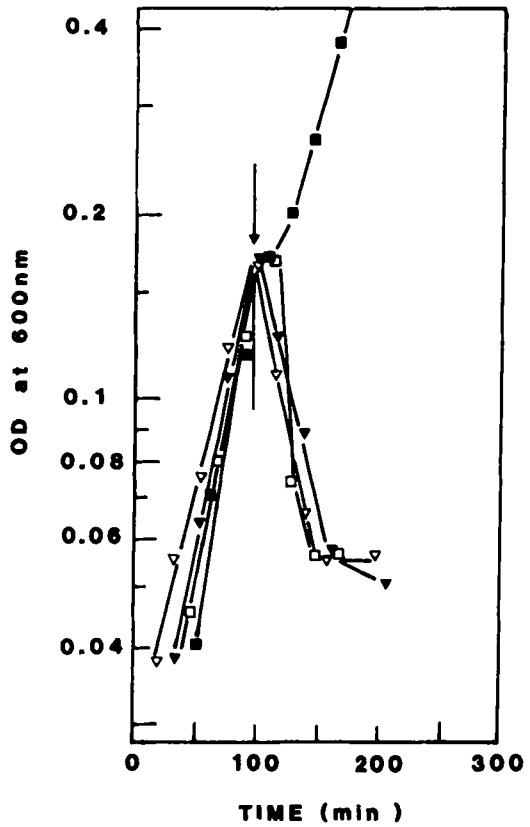


Figure 1—*Effect of Id (50 μg/mL) on the growth of* E. coli *GK-19 at pH 7.0 (■), 6.5 (□), 6.0 (▼), and 5.0 (▽). Compound Id was added (arrow) after exponential growth of the bacteria had commenced.*

at 37°C (unless otherwise stated) with a culture-to-flask volume of 1:5 or 1:10, using a gyratory shaker incubator[1]. The growth of the cultures was monitored by determining the absorbancy at 600[2] and 610[3] nm. The determination of cell viability was made by the appropriate dilutions of the culture being placed onto nutrient agar[4] and incubated at 37°C for 24–36 h. Colony-forming units were determined by plating. The compounds were dissolved in water prior to the addition of the medium unless otherwise indicated.

Electron Microscopy—A pellet of bacterial cells was prepared[5] by centrifugation (9650×*g* for 3 min), and the cells were fixed in a solution of glutaraldehyde (2.5% w/v) in 0.1 M cacodylate buffer, pH 7.4. After this process (105 min), the cells were embedded in a resin[6], and ultrathin sections were prepared[7] and stained with uranyl acetate solution in methanol (1% w/v) followed by lead citrate solution (2.66% w/v). Negative staining was accomplished by placing the cells prior to the fixation process, on 200-mesh grids followed by treatment with phosphotungstic acid solution (3% w/v). The samples obtained by these procedures were examined by electron microscopy[8].

Measurement of Respiration—Compound Id was added to *E. coli* GK-19 at log phase in standard biological oxidation-demand bottles attached to a polarographic dissolved-oxygen electrode[9]. The addition and mixing process was complete within 30 s and oxygen consumption was measured[10] every 2 min at 22–23°C.

Effect of Id on DNA and RNA Syntheses—Compound Id (50 μg/mL) was added to a culture of *E. coli* GK-19 at an optical density of 0.44, and the extent of incorporation of [^{3}H]thymidine (1 μCi/mL of culture) and [^{3}H]uracil (0.1 μCi/mL of culture) into DNA and RNA, respectively, was made by the batch method described previously (8) using a scintillation counter[11]. The experiments examining the rates of DNA and RNA syntheses were undertaken once and in triplicate (±5%), respectively.

[1] New Brunswick Scientific Co., Edison, N.J.
[2] Spectronic 20 spectrophotometer; Bausch and Lomb, Rochester, N.Y.
[3] Horizon 5965-50 spectrophotometer; Horizon Ecology Co., Chicago, Ill.
[4] Difco, Detroit, Mich.
[5] Microfuge B; Beckman, Palo Alto, Calif.
[6] Araldite epon mixture.
[7] LKB III ultratome; Stockholm, Sweden.
[8] Hitachi H412 electron microscope.
[9] Model 97-08; Orion Research Inc., Cambridge, Mass.
[10] Model 301; Orion Research Inc., Cambridge, Mass.
[11] Model LS-9000 scintillation counter; Beckman, Palo Alto, Calif.

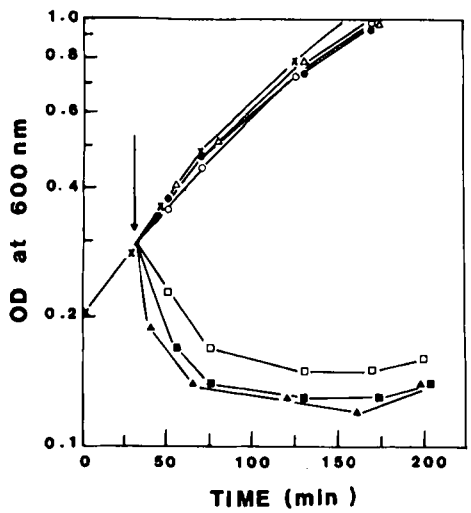


Figure 2—*Effect of Ia–f (100 μg/mL) on the growth of* E. coli *GK-19. The arrow indicates the time of addition of Ia (○), Ib (●), Ic (△), Id (▲), Ie (□), and If (■); (X) untreated controls.*

RESULTS AND DISCUSSION

Six candidate antibacterial agents I were considered in which the substitution pattern of the aromatic ring varied, while the *n*-pentyl group present in Ib–f was absent in Ia. A laboratory strain of *E. coli* K-12, designated as strain GK-19, was used as the test organism.

The initial experiments were designed to examine some of the factors affecting the bacterial growth by a representative Mannich base. Since Mannich bases are known to be unstable under alkaline conditions (5, 10), one of the derivatives (Id) was evaluated against *E. coli* GK-19 at neutral pH and in media which was mildly acidic (Fig. 1). At pH 7.0, a transient reduction of the growth rate of the bacteria was observed after the addition of Id, but afterward virtually normal growth resumed. Under acidic conditions (pH values of 6.5, 6.0, and 5.0) minimum absorbancy was noted after 1 h. Changes in pH can affect the ratio of un-ionized and ionized molecules (11), and it is conceivable that lowering the pH increases the percentage of Id existing in the ionic form, which may be the bioactive species.

The six Mannich bases were also examined at pH 6.5 for their effect on bacterial growth (Fig. 2). Compounds Ia–c were virtually bereft of antibacterial activity, while the dichlorinated derivatives (Id–f) caused a marked decrease in culture turbidity. Mannich base Id was selected for further studies. Reduction of the concentration of Id showed that the antibacterial effect was detectable at the lowest concentration examined, *i.e.*, 5 μg/mL (Fig. 3), while at 35 μg/mL substantial antibacterial activity was observed. However, this effect reversed after ~1 h, and colony-forming ability returned. At 35 μg/mL, Id was bacteriostatic (Fig. 3A) and at 50 μg/mL it was bactericidal (Fig. 3A and B). Figure 3B shows that at 50 μg/mL, the bactericidal activity is >99.9%.

Compound Id was dissolved in three different solvents to observe the effect of solvent on the antibacterial activity of Id (Fig. 4). When Id was dissolved in water and ethanol, minimal absorbancy was attained more rapidly than when a solution of Id in dimethyl sulfoxide was administered to a culture of the microorganisms. Gradual recovery occurred with solutions of Id in dimethyl sulfoxide and ethanol, but not when water was used.

The effect of the antibacterial effect of Id on *E. coli* GK-19 at different temperatures is seen in Fig. 5. At 6°C, little bacterial growth is observed, in contrast to that seen at 23°C, 37°C, and 40°C. At 23°C, addition of Id virtually abolished growth, but it was only after 1 h that the lytic effect was noted. At 37°C and 40°C, lysis began on addition of Id. This observation is of interest insofar that a reduction in the respiration-inhibitory properties of some related Mannich bases on mitochondrial function was noted at 20°C compared with 37°C (12) and, hence, the present study is germane to recent reports in changes of biochemical function with variation in temperature (13–15). In addition, the fatty acid composition of a yeast membrane varied with temperature (16). In summary, unless otherwise indicated subsequent experimentation utilized compound Id at a concentration of 50 μg/mL in a medium of pH 6.5 and a temperature of 37°C.

A further question posed was the effect of Id on the age of the culture of

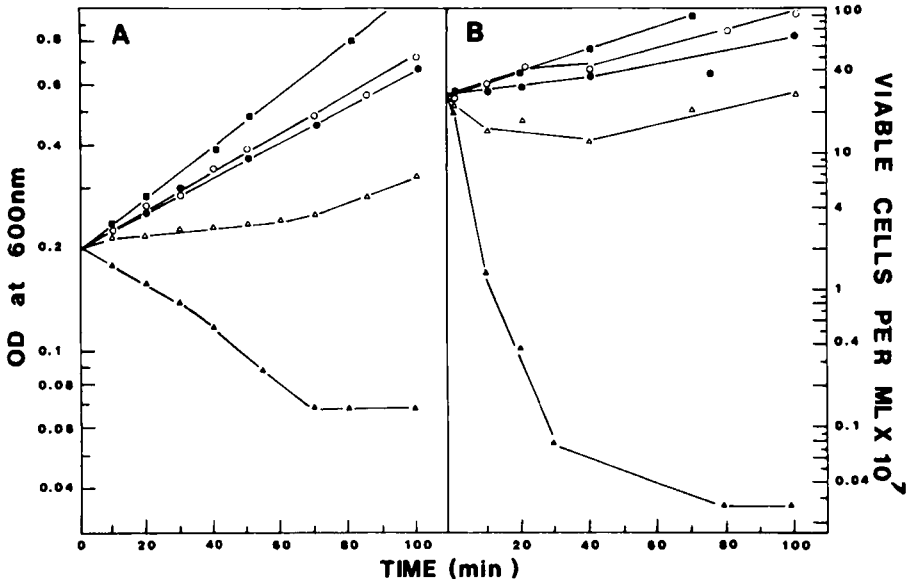


Figure 3—*Effect of Id at concentrations of 5 (○), 20 (●), 35 (△), and 50 (▲) μg/mL on the growth of* E. coli *GK-19, (■) untreated controls. Optical density (A) and colony-forming ability (B) were measured at different intervals.*

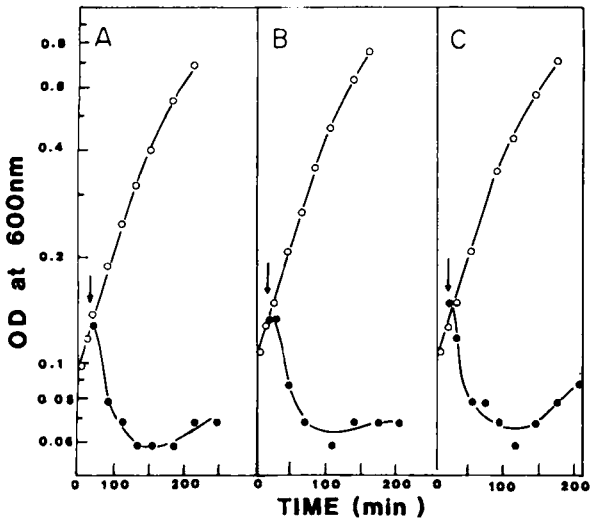


Figure 4—*Effect of the addition (arrow) of Id (50 μg/mL) dissolved in dimethyl sulfoxide (A), water (B), and ethanol (C) on the growth of treated (●) and untreated (○) cultures of* E. coli *GK-19.*

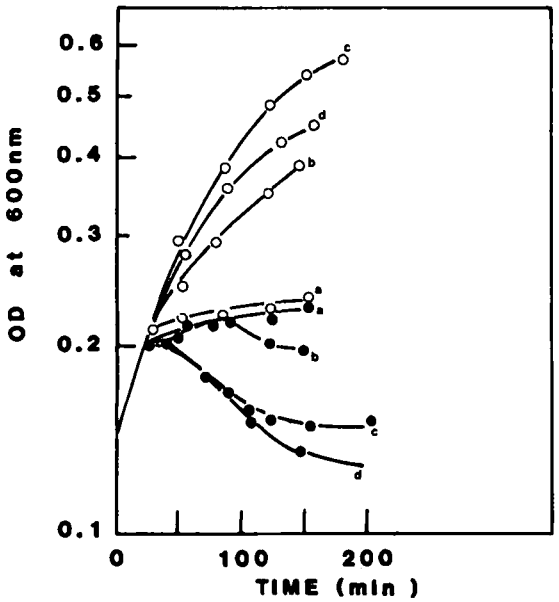


Figure 5—*Effect of the addition of Id (50 μg/mL) on treated (●) and untreated (○) cultures of* E. coli *GK-19 at 6°C (a), 23°C (b), 37°C (c), and 40°C (d).*

E. coli GK-19. Figure 6 shows that after a short lag phase of ~0.5 h, the bacteria entered their logarithmic (log) growth phase, which continued for 5 h. Compound Id was added to culture aliquots at times corresponding to early log, mid log, late log, and early stationary phases; antibacterial activity was demonstrated on all four occasions, although greater activity was seen during the logarithmic growth phases. However, the larger number of cells present in the early stationary phase could be an explanation for the reduced antimicrobial activity of Id at this point in time. To examine such a possibility, a culture of *E. coli* GK-19 was grown to early-mid log phase and concentrated fivefold by centrifugation; two quantities of Id (50 μg/mL) were added, separated by a 40-min period, and then the turbidity of the culture was followed for a further 90 min. No different effect was observed to that found for the early stationary phase shown in Fig. 6. In addition, the difference in the culture growth phase sensitivity to Id could be due to changes in the growth media, *i.e.*, the concentration of certain nutrients could have diminished by the time the bacteria had entered the stationary phase or, alternatively, new compounds could have formed and be present in the media. To test this possibility, cells at the mid log phase were harvested by centrifugation and resuspended in both freshly prepared media and also in media from which bacteria at the early stationary phase had been removed. The bacteria in both media responded to Id in an identical manner and, hence, the differential

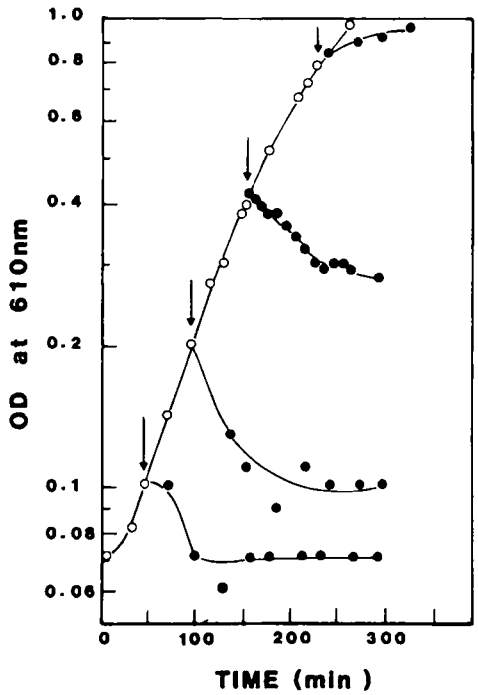


Figure 6—*Effect of multiple additions (arrows) of Id (50 μg/mL) on treated (●) and untreated (○) cultures of* E. coli *GK-19.*

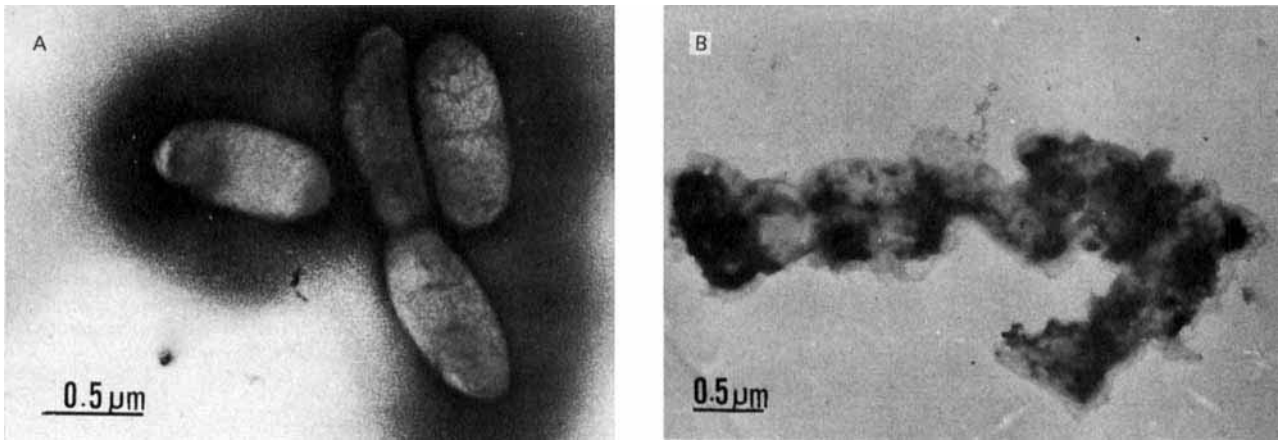


Figure 7—*Electron micrographs of negatively stained cells of* E. coli *GK-19 before (A) and after (B) treatment with Id (50 μg/mL).*

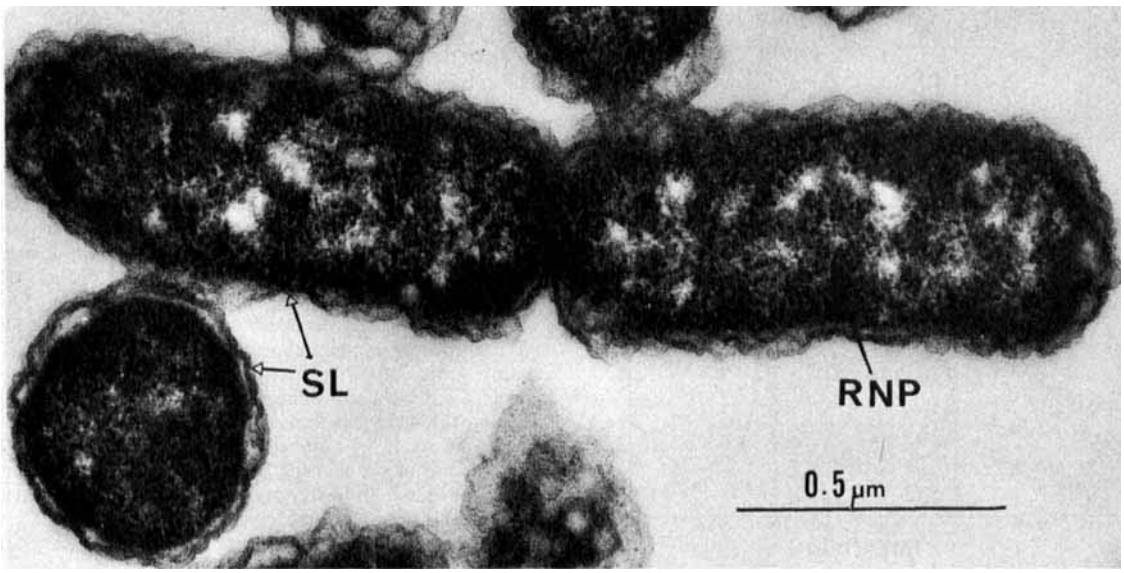


Figure 8—*Electron micrograph of thin sections of* E. coli *GK-19 displaying surface layers (SL) and ribonucleoprotein (RNP).*

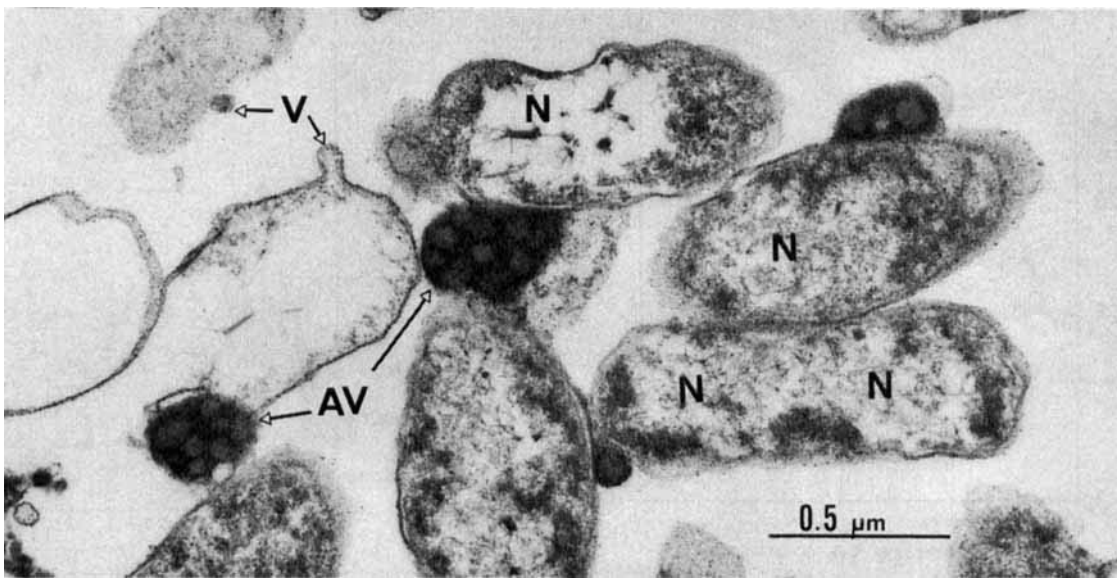


Figure 9—*Electron micrograph of thin sections of* E. coli *GK-19 after treatment with Id (50 μg/mL) displaying nuclear condensation (N), vesicle formation (V), and aggregated vesicles (AV).*

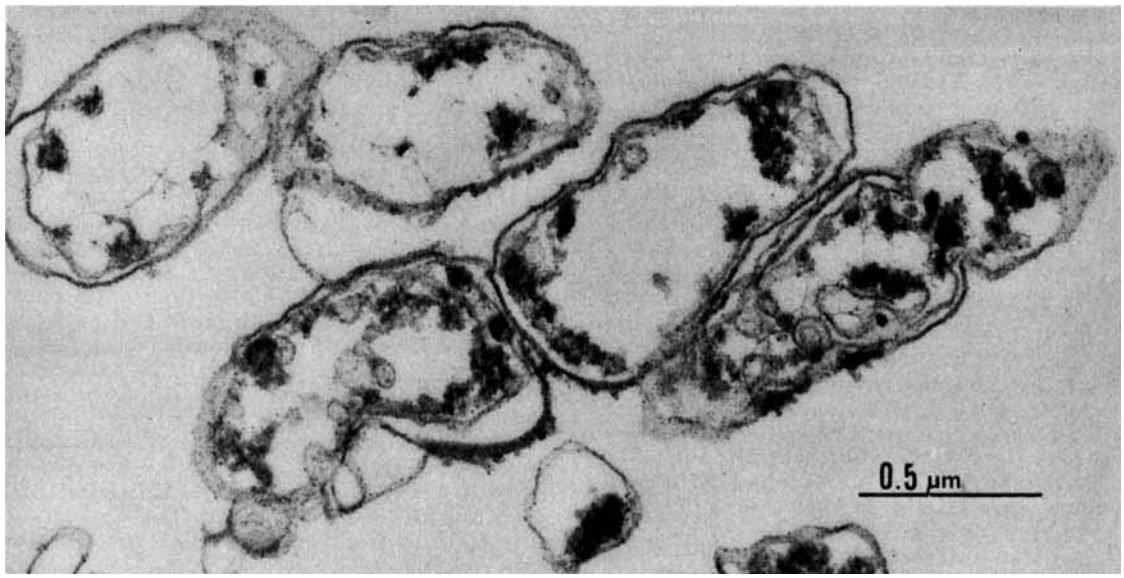


Figure 10—*Electron micrograph of thin sections of lysed cells of* E. coli *GK-19 caused by treatment of Id (50 μg/mL).*

sensitivity of Id at various growth stages was not due to changes in content of the media occurring during the growth of the bacteria.

The second phase of the investigation consisted of attempts to delineate the site(s) of action of Id on *E. coli* GK-19. An initial question posed was whether there was retention of the integrity of the cell membrane and cellular constituents after treatment with Id or whether a lytic process occurred, in which case rupture of the cell membrane and leakage of intracellular material would take place. Figure 7A is an electron micrograph of *E. coli* GK-19 cells taken from a culture of cells in the mid log growth phase, while Fig. 7B shows the effect of Id on these cells. After addition of Id, there are distortions of the cell shapes, loss of surface layer, and altered stainability compared with the untreated cells as noted in Fig. 7. Thin sections of some of the untreated cells revealed the cell membrane and the cytoplasmic regions containing ribonucleoprotein complexes (Fig. 8); regions of nuclear material are shown as lighter areas. After treatment with Id, there is the appearance of condensation of nuclear material, disorganization of the cell membrane, disappearance of the ribonucleoprotein structures, and appearance of pockets of small rounded vesicles inside cells, as well as in aggregate forms outside the cells (Fig. 9). Lysed cells of *E. coli* GK-19 after treatment with Id showed an increased loss of cellular constituents (Fig. 10). Hence, the Mannich base Id exerts a lytic effect on these bacteria at 50 μg/mL.

It is conceivable that Id could exert antibacterial activity in addition to lysis. In other words, while the lysis was in progress, Id could also act, for example, on the respiration apparatus and the macromolecular synthetic pathways of the bacteria. Figure 11 shows the effect of the addition of various concentrations of Id on oxygen consumption; at 50 μg/mL, there is complete inhibition of respiration. A plot of the rate of oxygen consumption at the end of 20 min *versus* concentration of Id (on linear paper) is shown as an inset in Fig. 11.

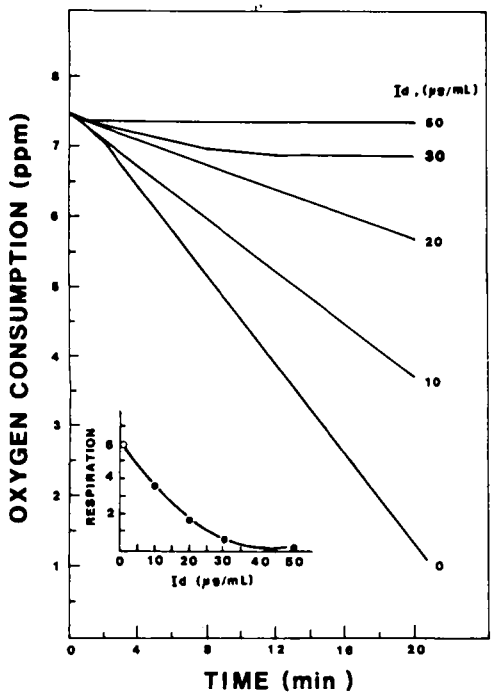


Figure 11—*Effect of Id on oxygen consumption in* E. coli *GK-19.*

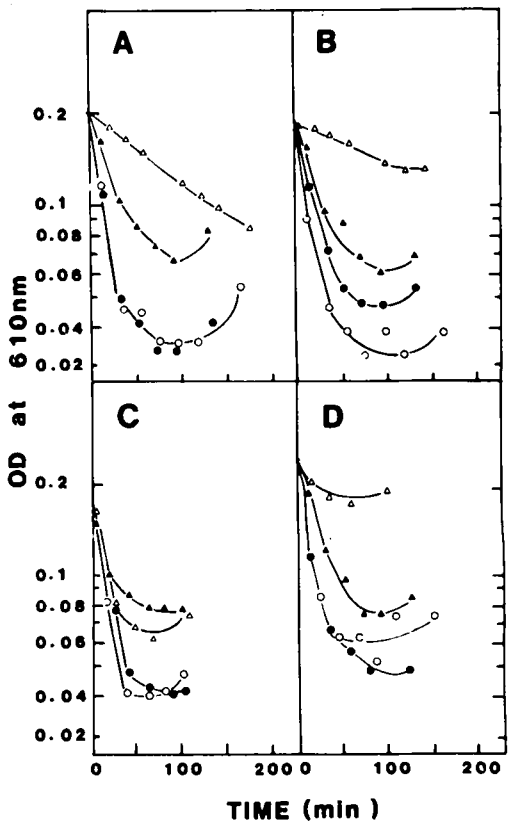


Figure 12—*Effect of the sensitivity of* E. coli *GK-19 to Id (50 μg/mL) by treatment with metabolic inhibitors. Key: (A) potassium cyanide (1950 μg/mL); (B) sodium azide (1950 μg/mL); (C) 2,4-dinitrophenol (18.4 mg/mL); (D) chloramphenicol (150 μg/mL). Compound Id was added simultaneously (●), 5 (○), 30 (△), or 120 (▲) min after the addition of the metabolic inhibitor.*

Table I—Effect of Id (50 μg/mL) on the Incorporation of [^{3}H]Thymidine and [^{3}H]Uracil into the DNA and RNA of *E. coli* GK-19.

Time, min	Counts per Minute of [^{3}H]-Thymidine[a]		Counts per Minute of [^{3}H]-Uracil[a]	
	Control	Treated	Control	Treated
5	100	100	100	100
10	102	100	294	193
20	106	99	650	224
30	108	97	650	237
40	112	96	650	255

[a] Figures in the table are relative to the counts per minute of labeled precursor in control experiments at the end of 5 min.

In addition, treatment of *E. coli* GK-19 with potassium cyanide, sodium azide, and 2,4-dinitrophenol, which are known inhibitors of respiration and energy production, was undertaken; Id was added simultaneously or at varying times after the addition of the inhibitors. The results are portrayed in Fig. 12A-C. With pretreatment times of 30 and 120 min, the antibacterial effect of Id is markedly reduced, which further indicates that cellular respiration or energy, in part at least, is likely required for the lytic effect of Id on *E. coli* to be manifested.

A similar study with chloramphenicol, which is an inhibitor of protein synthesis, showed that protein synthesis is required for the lysis of cells (Fig. 12D). It is concluded that in order for Id to exert its maximum lytic effect, normal respiration, energy production, and protein synthesis are required. Because of the cellular physiological functions that were affected by Id, it was suspected that macromolecular synthesis, in general, could also be affected. The conviction that protein synthesis was a site of action of this Mannich base was strengthened by its inhibition of the biosynthesis of the enzyme β-galactosidase. In *E. coli* GK-19, it is possible to induce the synthesis of this enzyme within 2-3 min with isopropylthiogalactoside (17), but addition of Id to a culture of this microorganism abolished the ability of the cells to synthesize this enzyme.

The effect of Id on the synthesis of DNA and RNA was measured using radioactively labeled thymidine and uracil, respectively, and the results are shown in Table I. The data indicate that while the synthesis of DNA is virtually unaffected, the inhibition of RNA synthesis commences after 5 min and is reduced by 66% after 20 min.

In conclusion, this study has unearthed some of the factors which affect the antibacterial activity of 1-(2,4-dichlorophenyl)-4-dimethylaminomethyl-1-nonen-3-one hydrochloride (Id) against *E. coli* GK-19. In addition, the bioactivity of this compound is due, at least in part, to its causation of lysis, and adverse effects on respiration, as well as protein and RNA biosynthesis, take place.

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Determination of Amphetamine, Norephedrine, and Their Phenolic Metabolites in Rat Brain by Gas Chromatography

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Abstract □ A specific analytical procedure for the quantitation of amphetamine (I), norephedrine (III), and their amphoteric metabolites, *p*-hydroxyamphetamine (II) and *p*-hydroxynorephedrine (IV), in biological samples using electron-capture gas chromatography (GC-EC) is described. The procedure utilizes the ion-pairing reagent, bis(2-ethylhexyl)phosphoric acid, which frees the amines from most contaminants and permits the efficient extraction of the amphoteric compounds (as acetates) from the aqueous solution. Amines I and III and acetylated amines II and IV were perfluoroacetylated prior to GC-EC analysis. Metabolism of I, II, and III in the rat brain was studied. Results indicate that both *in vivo* and *in vitro* amines I and III are *p*-hydroxylated to II and IV, respectively, and II is β-hydroxylated to give IV. Norephedrine (III) was not detected as a rat brain metabolite of amphetamine (I).

Keyphrases □ Amphetamine—rat brain metabolism, norephedrine, phenolic metabolites, gas chromatography □ Norephedrine—rat brain metabolites, amphetamine, phenolic metabolites, gas chromatography □ Metabolites, phenolic—amphetamine and norephedrine, rat brain metabolism, gas chromatography

When amphetamine (I) is administered systemically to rats, appreciable amounts of I and trace quantities of *p*-hydroxyamphetamine (II) and *p*-hydroxynorephedrine (IV) are detected in the brain tissue. In attempts to associate these metabolites with some of the pharmacological actions of I, their presence in various brain regions and other tissues have been

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