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Nalidixic Acid: an Antibacterial Paradox

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Nalidixic acid was found to be most bactericidal against various species of gram-negative bacteria at 50 to 200 μg/ml. With all species tested, increases in the concentration of nalidixic acid above this range reduced, rather than increased, its bactericidal effect so that, at levels in the region of 400 μg/ml, the drug was relatively bacteriostatic. Therefore, the mode of action of nalidixic acid at various concentrations was investigated. It was found that at the most bactericidal concentration deoxyribonucleic acid synthesis, but no ribonucleic acid (RNA) or protein synthesis, was inhibited. However at higher concentrations, where the drug is least bactericidal, both RNA and protein synthesis were found to be inhibited. Results are presented which suggest that the protein synthesis inhibition is a secondary manifestation of the ability of the drug to inhibit RNA synthesis, and that of RNA synthesis is most likely the second target site for the action of the drug when bacteria are exposed to it in high concentrations. The clinical implications of these findings are discussed.

Nalidixic acid (NAL) is unusual among antibacterial agents in extensive clinical use because it is a specific inhibitor of bacterial deoxyribonucleic acid (DNA) synthesis (1, 10). Although the primary effect of NAL appears to by upon an, as yet unknown, aspect of DNA polymerization (3, 19), this inhibition does not alone constitute the bactericidal effect of the drug. It has been shown that a bactericidal effect can only occur in the presence of competent ribonucleic acid (RNA) and protein synthesis (8) which has been interpreted as representing lethal unbalanced growth (2; W. H. Dietz, T. M. Cook, and W. A. Goss, Bacteriol. Proc., p. 82, 1966)

NAL is also unusual among antibacterial agents in respect of the dose-response pattern elicited by NAL-susceptible organisms. Winshell and Rosenkranz (26) showed that when NAL was used in vitro to treat *Escherichia coli* B, the drug was most lethal at a concentration of 20 μg/ml, above which concentration NAL became decreasingly lethal. This apparently anomalous dose-response pattern was investigated by Winshell (Ph.D. thesis, Columbia Univ., New York, 1967), who tested the possibility that NAL might be enzymatically reduced to an inactive form in vivo as happens with streptonigrin (25) or mitomycin C (15). However, although chemically reduced NAL was found to lack any lethal activity, no evidence was found to suggest that such a reduction occurred in vivo (Winshell, Ph.D. thesis, 1967).

We investigated the dose-response patterns of NAL on several susceptible strains of gram-negative bacteria. For all organisms tested there was a concentration of NAL that was most bactericidal, and any concentration exceeding this level resulted in a dramatic reduction in the lethality of the drug. Having established that the dose-response pattern is neither strain nor species specific, we elucidate a mechanism that explains the paradoxical dose-response behavior of *E. coli* K-12 to NAL.

MATERIALS AND METHODS

Bacterial strains. The bacterial strains are shown in Table 1.

Materials. Nutrient broth no. 2 and MacConkey agar were supplied by Oxoid Ltd. Davis and Mingioli liquid medium was prepared as described before (7). NAL was given by the Sterling-Winthrop Group (United Kingdom); rifampin was supplied as the pure base by Lepetit (United Kingdom); and chloramphenicol was given by Parke-Davis (United Kingdom). [^{3}H]thymidine, [^{3}H]uridine, and [^{3}H]leucine were purchased from the Radiochemical Centre, Amersham (United Kingdom).

Effects of NAL on bacterial survival. Cultures were grown overnight in nutrient broth and diluted with fresh sterile broth to give approximately 2×10^8 viable bacteria per ml. Aliquots (0.1 ml) of the diluted cultures were inoculated into sterile tubes containing various concentrations of NAL in 4.9-ml amounts of nutrient broth and incubated at 37 C for 3 h. After incubation the samples were washed by centrifugation at 3,000 × *g* for 15 min and resuspended to 5 ml with fresh nutrient broth. Viable counts were done

TABLE 1. *Bacterial strains*

Strain and genotype	Source and/or references
Escherichia coli K-12	
KL16—Hfr *str*^{s} *nal*^{s}	R. Sternglanz (13)
J5-3—F$^-$ *met*$^-$ *pro*$^-$ *nal*^{s}	N. Datta (4)
J62-2—F$^-$ *pro his*$^-$ *try nal*^{s}*rif*^{r}	N. Datta (6)
χ908—F$^-$ *str*^{s} *dna* B(ts) *nal*^{s}	R. Curtiss III (9)
Other genera	
Klebsiella aerogenes 415	J. M. T. Hamilton-Miller (11)
Proteus mirabilis	NCTC 5887
Shigella sonnei	NCTC 8220
Salmonella typhimurium LT2	Authors' laboratory

using the techniques of Miles and Misra (18) on MacConkey agar plates which were incubated overnight at 37 C.

Measurement of DNA, RNA, and protein synthesis. Synthesis of DNA was measured by following the incorporation of [^{3}H]thymidine as described by Schubach et al. (23). Bacteria were incubated at 37 C in Davis and Mingioli medium containing [^{3}H]thymidine (0.1 μCi/ml final concentration) plus 50 μg of deoxyadenosine per ml in the presence of NAL concentrations ranging from 0 to 400 μg/ml. At the end of the incubation period 1-ml portions of the cultures were chilled and treated with 0.1 ml of ice-cold 50% trichloroacetic acid containing 1 mg of unlabeled thymidine/ml. After precipitation at 4 C the treated cultures were filtered through Whatman GF/C filters and washed successively with 1 volume of 1% trichloroacetic acid, boiling distilled water (3 volumes), 5% NaCl (1 volume), and 2 volumes of 5% acetic acid. The filters were then dried and the radioactivity was determined by scintillation counting.

Synthesis of RNA was measured by estimating the incorporation of [^{3}H]uridine into RNA by the method of Cohen and Ennis (5). Bacteria were incubated in Davis and Mingioli medium containing [^{3}H]uridine (0.1 μCi/ml final concentration) in the presence of concentrations of NAL ranging from 0 to 400 μg/ml. At the end of the incubation period the cultures were divided into two portions of 1 ml each. Each portion was mixed with an equal volume of ice-cold 10% trichloroacetic acid and held at 4 C for 1 h. One portion from each culture (the untreated sample) was then filtered through a Whatman GF/C filter disk and washed three times with cold 5% trichloroacetic acid containing 1 mg of unlabeled uridine/ml. NaOH (0.8 N) was added to the second portion and incubated at 37 C for 2 h. The mixture was then neutralized with HCl and chilled, and trichloroacetic acid was added to 5%. After standing for 15 min at 4 C the precipitate was collected, filtered through a Whatman GF/C filter disk, and washed as before with the untreated sample. The radioactivity of the untreated sample gives the amount of precurser incorporated into both nucleic acids, and the value from the alkali-treated sample gives that in DNA alone. Subtraction yields the amount of isotope incorporated into RNA.

Protein synthesis was measured by incorporation of [^{3}H]leucine into hot trichloroacetic acid-insoluble material as described by Roozen et al. (22). The precipitates were collected on GF/C disks as above.

All radioactivity measurements were made by immersing the disks, after drying, in Bray solution and counting with a Packard model 574 liquid scintillation spectrometer.

RESULTS

Dose-response studies. The survival of *E. coli* K-12 strains J5-3, J62-2, KL16, and χ908 after 3 h of treatment with various concentrations of NAL is shown in Fig. 1. It can be seen that all four strains behaved similarly in that 50 to 100 μg of NAL per ml was most lethal, and further increases in the concentration of NAL progressively reduced its bactericidal effect. Thus, results similar to those of Winshell and Rosenkranz (26) were obtained. Similar dose-response patterns were obtained for other NAL-susceptible species of gram-negative bacteria, irrespective of whether they were urinary tract pathogens (Fig. 2) or enteropathogenic bacteria (Fig. 3). Thus, the results show that the dose-response pattern elicited by NAL was neither strain nor species specific.

It may have been that the survivors from cultures treated with high concentrations of NAL were NAL-resistant organisms. However, when survivors from such concentrations (>200 μg/ml) were subcultured in nutrient broth and retreated with NAL, similar dose-response patterns were again obtained. In addition, when survivors from NAL (>200 μg/ml) were tested directly for NAL resistance on solid media (i.e., without subculture), they were found to be as susceptible as the original bacteria before exposure to NAL. These results indicate that the effects of high levels of NAL did not select resistant mutants, and thus the drug had a bacteriostatic rather than a bactericidal effect at high concentrations.

The effect of varying the period of exposure of *E. coli* strain KL16 to NAL was investigated. It was found (Fig. 4) that extending the incubation time with NAL, as expected, did decrease the percentage of viable cells surviving treatment. Nevertheless, the most lethal concentration of NAL was still 50 to 100 μg/ml despite prolonging the period of exposure to the drug.

Requirements for the lethal effect of NAL. It has been suggested that in NAL-susceptible bacteria the drug is bactericidal only when RNA and protein synthesis proceed (8), whereas DNA synthesis is inhibited (18). This was confirmed

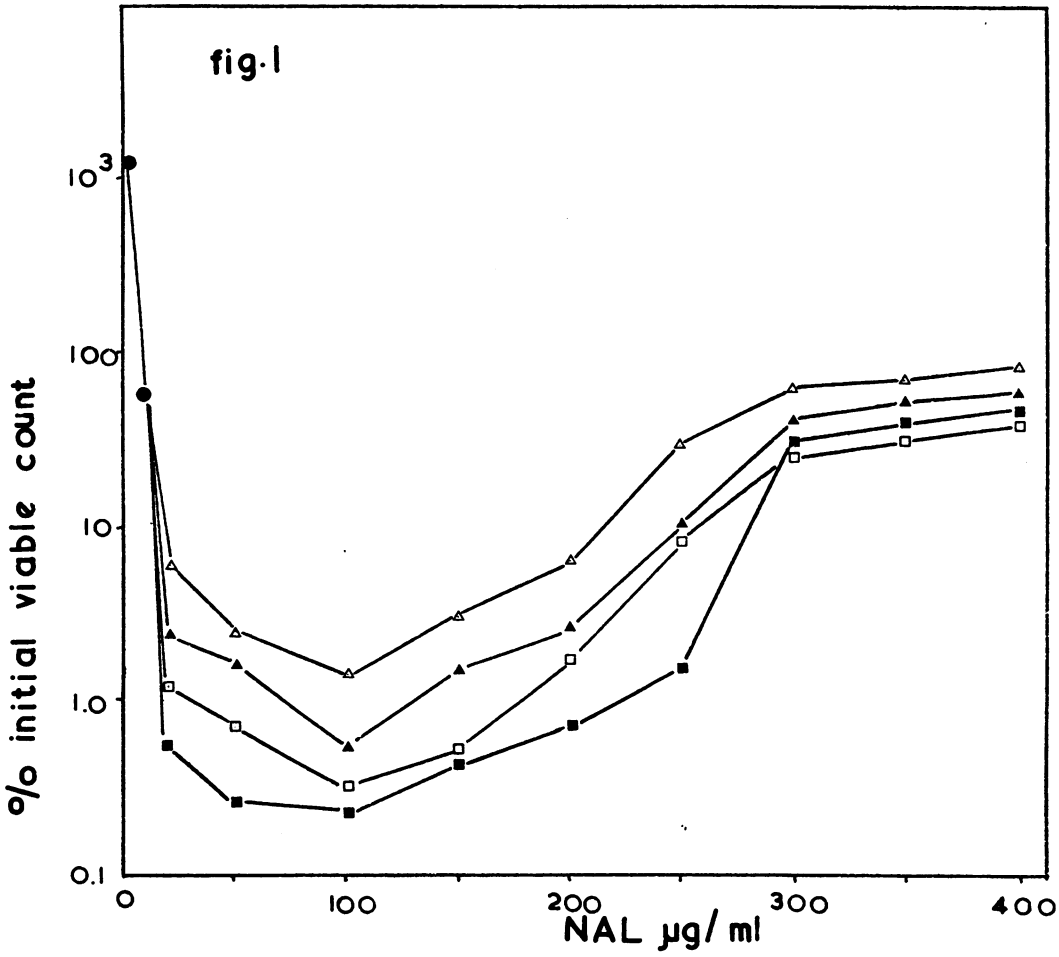

FIG. 1. *Four NAL-susceptible strains of E. coli K-12 diluted to approximately 10^7 bacteria per ml in nutrient broth containing various concentrations of NAL. After 3 h of incubation at 37 C the cultures were washed and viable counts were estimated by plating on MacConkey agar. Symbols: △, strain χ908; ▲, strain J62-2; □, strain J53; and ■, strain KL16.*

by testing the effect of the addition of chloramphenicol during treatment of *E. coli* K-12 strain J5-3 with the most lethal concentration of NAL. The results (Fig. 5a) show that inhibition of protein synthesis by chloramphenicol immediately rescued bacteria from the bactericidal effect of NAL. Similarly, when RNA synthesis was blocked with rifampin the lethal effect was also abolished (Fig. 5b). However, it has been suggested that the NAL target in bacteria is common to DNA and RNA synthesis (20). Hence, these results with rifampin could be interpreted to mean that rifampin could be interfering with the action of NAL on DNA synthesis. However, as rifampin rescue did not occur in a mutant known to possess a rifampin-resistant RNA polymerase (Fig. 5c), this possibility can be discounted. Thus rifampin rescue would seem to result from its inhibitory action on RNA synthesis.

From these results it appears that continued RNA and protein synthesis is essential for the lethal effect of NAL and supports the previous suggestions (2; W. H. Dietz et al., Bacteriol. Proc., p. 82, 1966) that the lethal event results from unbalanced growth of the cell in the absence of DNA synthesis.

Effects of NAL on nucleic acid and protein synthesis. From these results it seemed that the dose-response pattern of NAL could be explained if the drug exerted a second inhibitory action on RNA, or protein, synthesis at concentrations exceeding those necessary to inhibit DNA synthesis. Indeed Goss et al. (10)

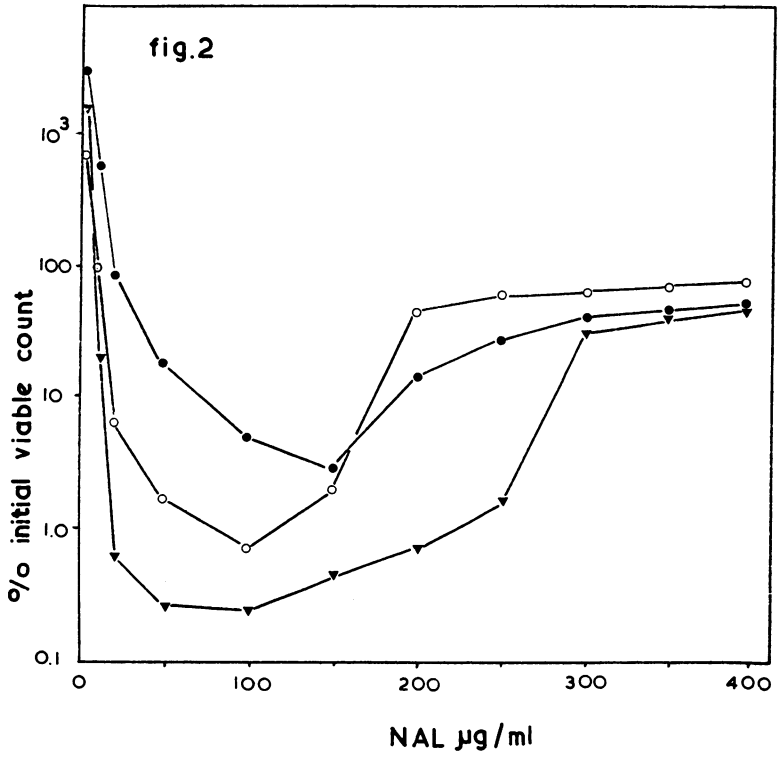
fig.2
10^3
100
10
1.0
0.1
% initial viable count
0
100
200
300
400
NAL µg/ml

FIG. 2

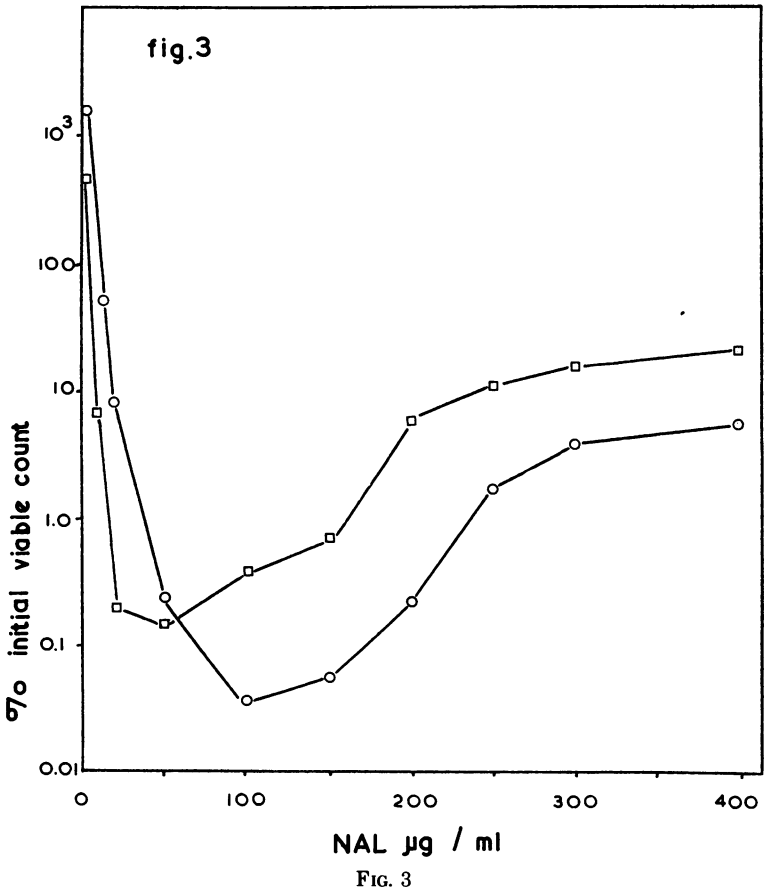
fig.3
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FIG. 3

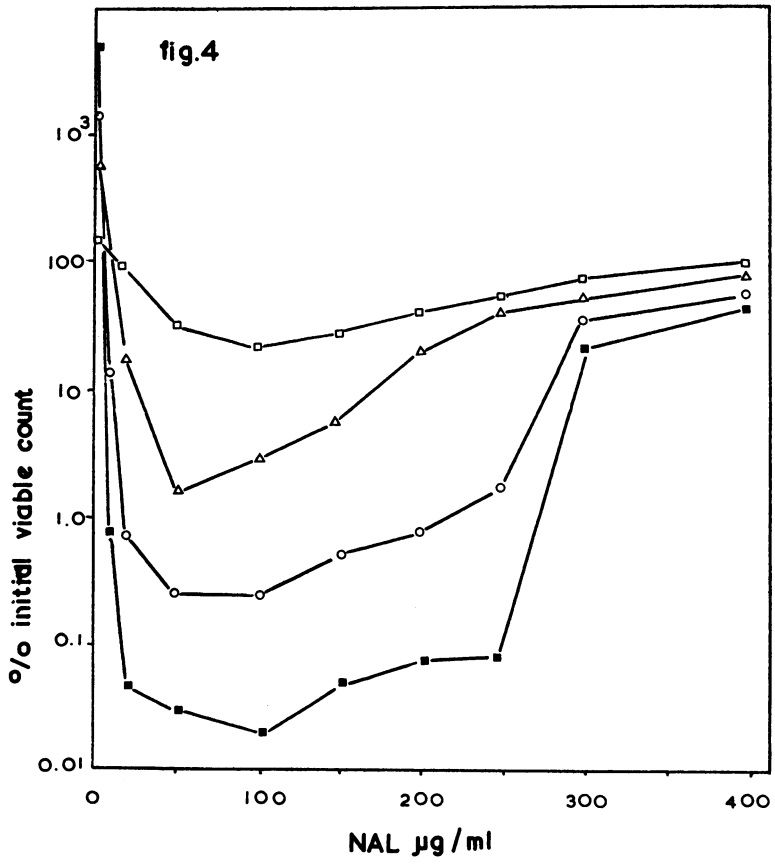


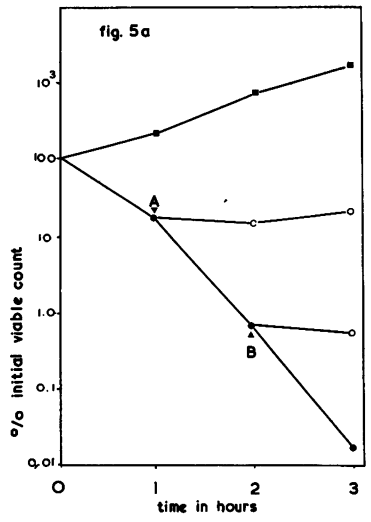
FIG. 4. *E. coli K-12 strain KL16 was diluted to approximately 10^7 bacteria per ml in nutrient broth containing various concentrations of NAL. After various periods of incubation samples were withdrawn and washed, and the viable counts were estimated by plating on MacConkey agar. Symbols:* □, *1.5 h;* △, *2 h;* ○, *3 h; and* ■, *4 h of incubation at 37 C.*

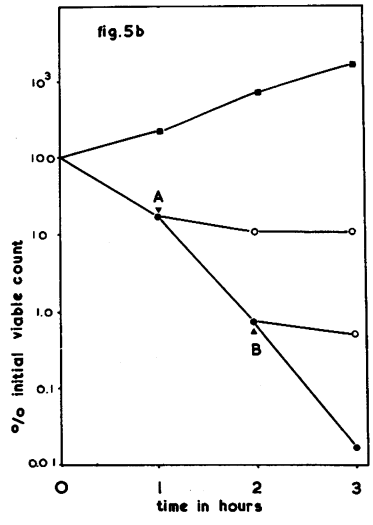
have shown that NAL at low levels (0.1 µg/ml) inhibits DNA synthesis by 66% but does not inhibit RNA synthesis. At much higher concentrations they found that NAL inhibits both DNA and RNA synthesis. However, their results do not indicate whether the effect of high concentrations of NAL on RNA synthesis is a direct effect or is a consequence of the inhibition of DNA synthesis.

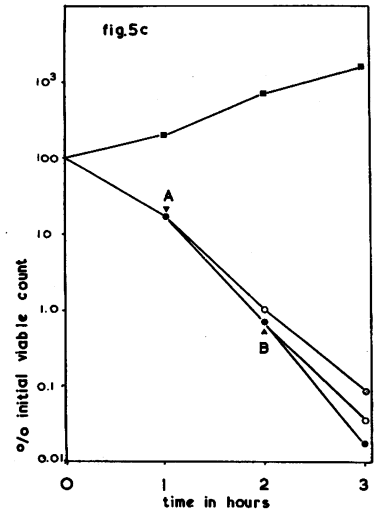
To investigate the effects of NAL, *E. coli* strain KL16 was treated with various concentrations of NAL for 2 h and the incorporation of [^{3}H]thymidine into DNA and [^{3}H]uridine into RNA and DNA during this period was estimated. The results (Fig. 6) are plotted by using an ascending scale for DNA synthesis and a descending scale for RNA synthesis. It can be seen that DNA synthesis and RNA synthesis were progressively inhibited by increasing the concentration of NAL. However, DNA synthesis was more susceptible to inhibition by low concentrations of the drug so that a 60% inhibition of DNA synthesis occurred at 50 µg/ml, whereas at this concentration of NAL RNA synthesis was inhibited by only 20%. The two inhibition curves crossed at 100 µg of NAL/ml and the fact that this concentration was the most lethal concentration of NAL for this strain (see Fig. 1)

FIG. 2. *Three uropathogenic species of gram-negative bacteria were tested for susceptibility to NAL using the experimental conditions described previously (see Fig. 1). Symbols:* ○, *Klebsiella aerogenes 415;* ●, *Proteus mirabilis NCTC 5887; and* ▼, *E. coli KL16 (data taken from Fig. 1).*

FIG. 3. *Two enteropathogenic species of gram-negative bacteria were tested for susceptibility to NAL using the experimental conditions described previously (see Fig. 1). Symbols:* □, *Shigella sonnei NCTC 8220; and* ○, *Salmonella typhimurium LT2.*







may, or may not, be coincidental. In addition, it can be seen that the inhibition of DNA synthesis by NAL is confirmed by the Cohen and Ennis method (5) for determining the incorporation of [^{3}H]uridine into DNA.

The effect of NAL upon protein synthesis was then investigated in strain KL16 by carrying out a similar labeling procedure using [^{3}H]leucine and the method of Roozen et al. (22). The results of this experiment are shown in Fig. 7, which contains for comparative purposes the RNA synthesis results from Fig. 6. It can be seen that protein synthesis followed closely the pattern of RNA synthesis in that low concentrations of NAL had little effect, but at high levels protein synthesis was inhibited by the drug.

Having thus established that high levels of NAL can inhibit both RNA and protein synthesis, the following question arises: are these direct inhibitory effects and if so which synthesis does it inhibit first, or are they merely inhibited as a consequence of DNA synthesis inhibition? To resolve this, the effects of NAL upon RNA and protein synthesis in the absence of any action upon DNA synthesis were investigated.

A temperature-susceptible *dnaB* mutant of *E. coli* (strain χ908) was used. This strain has been shown to cease DNA synthesis immediately upon transfer from the permissive temperature (30 C) to the restrictive temperature (42 C) (9). However, the viability of strain χ908 at the restrictive temperature was relatively short-lived (90 min before the viable count declined significantly) and so we had to adopt a slightly different experimental procedure to that used with strain KL16.

A single concentration of NAL was used (100 μg/ml), which has been shown to inhibit significantly both RNA and protein synthesis in strain KL16 (Fig. 7.).

FIG. 5. *(a) Samples of E. coli K-12 strain J53 were diluted to approximately 10^7 bacteria per ml in nutrient broth with and without 100 μg of NAL/ml and incubation was continued at 37 C. After 1 h (A) or 2 h (B) of incubation, chloramphenicol was added to 20 μg/ml and incubation was continued. After washing, the viable counts of all samples were estimated at hourly intervals. Symbols: ■, control (no drug); ●, 100 μg of NAL per ml; and ○, 100 μg of NAL per ml plus 20 μg of chloramphenicol per ml at the times indicated. (b) As for (a) except that the effects of the addition of rifampin to 100 μg/ml after 1 (A) and 2 h (B) of incubation in the presence of 100 μg of NAL per ml were tested. Symbols: ■, control (no drug); ●, 100 μg of NAL per ml; and ○, 100 μg of NAL per ml and 100 μg of rifampin per ml at the times indicated. (c) As for (b) except that a rifampin-resistant mutant of E. coli (strain J62-2) was used.*

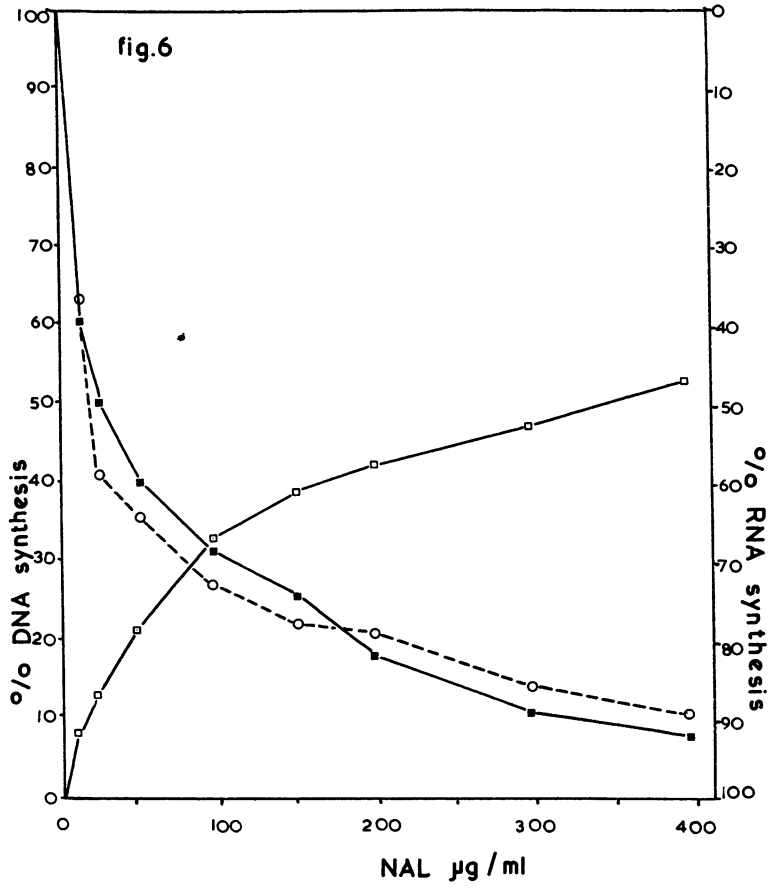


FIG. 6. *E. coli K-12 strain KL16 was incubated for 2 h at 37 C in the presence of various concentrations of NAL and [^3H]uridine or [^3H]thymidine. The incorporation of the label from uridine into DNA (O) and into RNA (□) was followed as described. The incorporation of labeled thymidine into DNA was also followed (■). The radioactive counts were corrected for background and for quenching. The results are plotted as percentages of the respective incorporations observed in untreated control cultures.*

The integrity of RNA and protein synthesis at various times after addition of the drug was then measured at the restrictive temperature in strain χ908. It was found that NAL markedly inhibited the RNA synthesis in strain χ908 at the restrictive temperature, i.e., in the absence of DNA synthesis (Fig. 8). The percentage of inhibition caused by 100 µg of NAL/ml in strain χ908 compared well with the inhibition given by this drug concentration in strain KL16, in which DNA synthesis was not artificially blocked. Thus it would seem that RNA synthesis can be inhibited whether or not DNA synthesis is occurring and it would seem unlikely that RNA synthesis inhibition occurs as a consequence of the effects of NAL on DNA synthesis. However, the possibility still remains that the inhibition of RNA synthesis results from a primary inhibition of protein synthesis. Therefore another experiment was carried out using strain χ908. DNA synthesis was blocked by carrying out the study at 42 C, and protein synthesis was also blocked by the addition of 20 µg of chloramphenicol/ml. Even though the additional presence of chloramphenicol reduced the level of RNA synthesis to approximately 20 to 25% of that of the previous experiment, NAL still exerted a 20% inhibition of RNA synthesis (Fig. 9). Hence it would seem that NAL inhibits RNA synthesis directly and not as a consequence of the inhibition of DNA or of protein synthesis.

DISCUSSION

Susceptible bacteria were shown to behave anomalously in that low concentrations of NAL were more bactericidal than high concentrations. This paradox has been resolved by finding that competent RNA and protein synthesis,

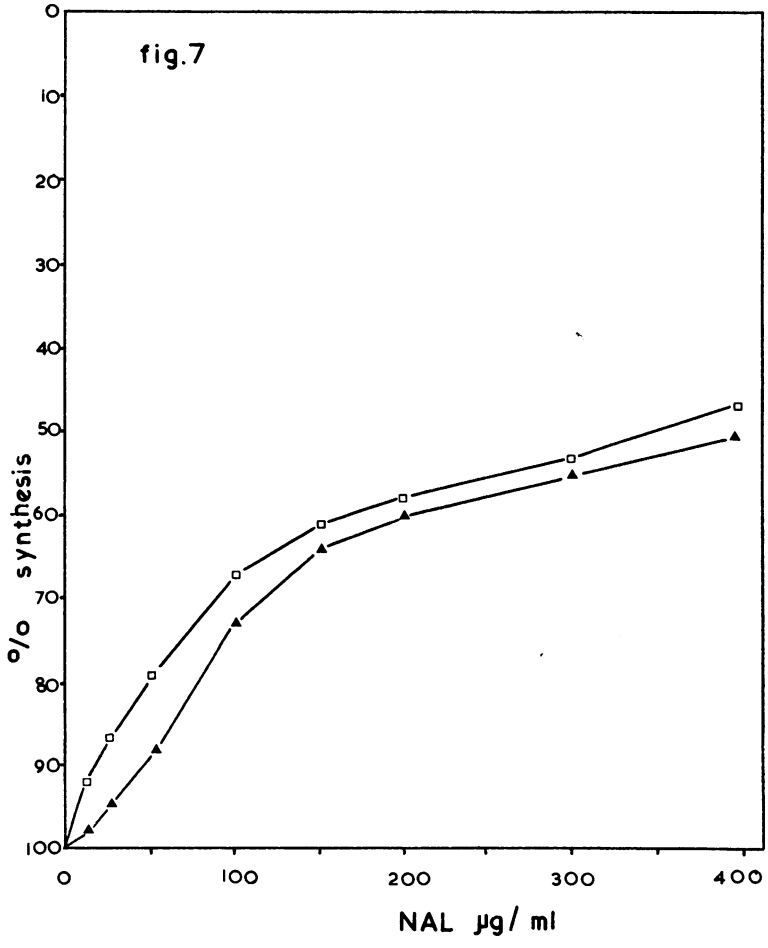


FIG. 7. *E. coli K-12 strain KL16 was incubated for 2 h at 37 C in the presence of various concentrations of NAL and [^{3}H]uridine or [^{3}H]leucine. The incorporation of the label from uridine into RNA (□) and of leucine into protein (▲) was followed as described. Other details are as shown in the legend to Fig. 6.*

which are essential for the lethal action of the drug, are both inhibited by it in high concentrations. Hence NAL effectively antagonizes its own lethal action, resulting in a bacteriostatic, rather than a bactericidal, effect when excessive amounts are used.

Evidence is presented suggesting that NAL inhibits not only DNA synthesis but also RNA synthesis and these are independent effects of the drug. It is possible to deduce the action of NAL on RNA synthesis in more detail. It has been shown previously that inhibition of protein synthesis in a stringent bacterial strain (24) represses ribosomal RNA and transfer RNA synthesis, but has little effect upon messenger RNA synthesis (12). As 100 µg of NAL/ml can inhibit the RNA synthesis of strain χ908 at the restrictive temperature in the presence of chloramphenicol, it would seem that the second target for NAL in the bacterial cell is messenger RNA synthesis. This is borne out by the findings of Javor (16), who showed that RNA synthesis is inhibited by NAL during amino acid starvation, and of Puga and Tessman (20), who showed that NAL inhibited messenger RNA synthesis in phage S13. Inhibition of an RNA synthesis would also seem to explain why we found the inhibition of protein synthesis to follow so closely the inhibition of RNA synthesis.

It has been shown that NAL induces the formation of single-strand nicks in DNA (14) and that this ultimately leads to irreversible degradation of the DNA into acid-soluble fragments (21). Thus the question that remains is: why should the inhibition of messenger RNA

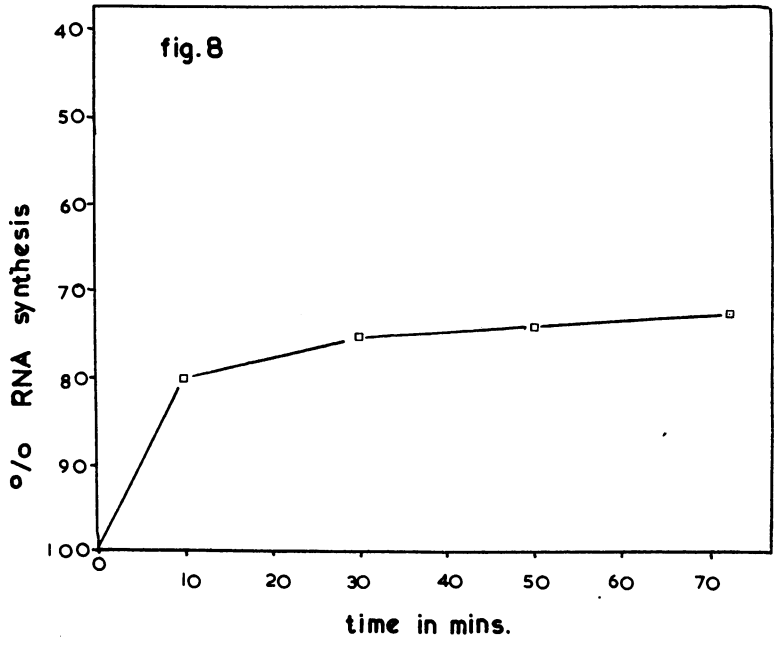


FIG. 8. *E. coli K-12 strain χ908, a dnaB temperature-susceptible mutant, was incubated at the restrictive temperature (42 C) in the presence and absence of 100 μg of NAL/ml and [³H]uridine. Samples were taken at intervals and the incorporation of uridine into RNA was followed as before (see Fig. 6). The results are plotted as percentages of the RNA incorporation observed in untreated controls. The DNA synthesis of these controls was also measured directly by [³H]uridine and [³H]thymidine incorporation. In both cases no significant DNA synthesis was observed. The level of RNA synthesis at the restrictive temperature was comparable to that observed at the permissive temperature, both in the absence of the drug.*

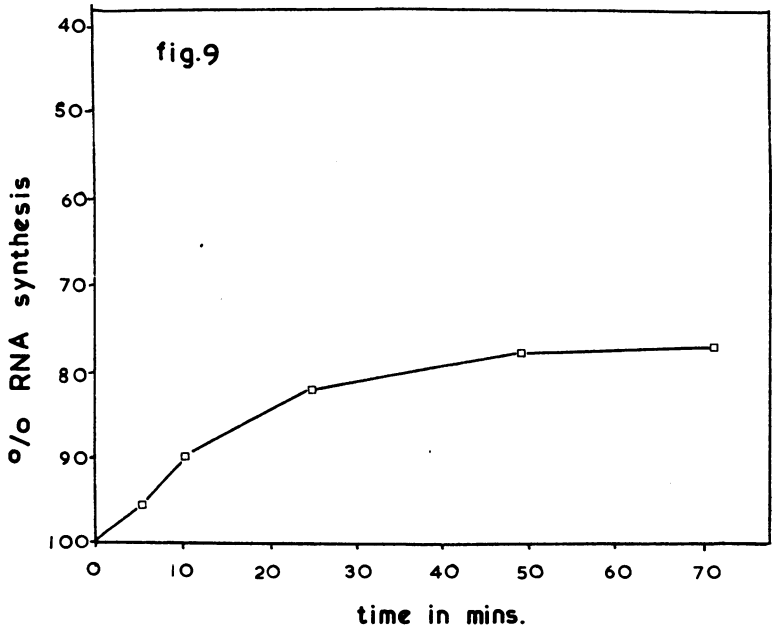


FIG. 9. *E. coli K-12 strain χ908 was incubated at 42 C, at which temperature DNA synthesis was found to be absent as judged by tests made on independent control cultures. In addition chloramphenicol was added to 20 μg/ml to prevent protein synthesis (also as shown by checks on independent control cultures). The effect of 100 μg of NAL/ml upon the incorporation of [³H]uridine into RNA under these conditions was measured at various time intervals and compared with the levels of incorporation observed in the absence of NAL. In the absence of NAL the level of RNA synthesis was found to be 23% of that obtained in Fig. 8, where DNA, but not protein synthesis, was blocked.*

synthesis by high concentrations of NAL dramatically reduce the bactericidal effect of the drug, even over extended periods of incubation? There are two possible explanations for this: one is that in NAL-susceptible cells the inhibition of RNA synthesis by high levels of NAL leads to the inhibition of DNA synthesis at a step prior to the normal primary target for low levels of the drug. Such an inhibition would remove the presence of any subsequent NAL-susceptible step. The second possibility is that inhibition of messenger RNA synthesis may prevent the synthesis of nucleases that degrade bacterial DNA from the nicks caused by NAL.

As a consequence of resolving the paradoxical dose-response effects of NAL upon susceptible bacteria by finding a second inhibitory action of the drug at high levels, another unusual feature in its character becomes apparent. If a NAL-susceptible strain were to undergo a mutation at a single, at present unknown, locus resulting in a NAL-resistant RNA target, the resultant strain would be more susceptible to high concentrations of the drug so that NAL would be bactericidal at all concentrations. Clearly therefore such a mutant would be impossible to select directly.

Clinical implications. As NAL is used extensively in the clinical treatment of urinary tract infections, the fact that it is bacteriostatic in high concentrations and bactericidal in low concentrations does have some important clinical implications.

The routine oral dose of NAL (1 g) gives rise to a urine level of active drug in the region of 200 to 350 μg/ml within 4 to 6 h in patients with normal renal function (17). Such urine levels are in excess of the most lethal concentrations of NAL for the susceptible organisms used in this study as representatives of the most common urinary tract pathogens. For example, about 60% of the inoculum of *E. coli* remains viable in 250 to 350 μg of NAL per ml after 3 h of exposure to the drug in vitro at 37 C. With *Klebsiella aerogenes* the situation was even worse as 80% survived these conditions. Because of the curious nature of the response of gram-negative organisms to NAL, this agent would merely act bacteriostatically when urinary tract infections are treated with excessive amounts of the drug. Such a situation could favor the emergence of resistant strains.

Up to a point the lower the level of NAL the greater its bactericidal effect. Hence, if the dosage were decreased, then not only would the risk of adverse reactions be minimized and the emergence of resistant strains be made less likely, but also the eradication of bacteriuria might be more rapid.

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