Biochemical Pharmacology, Vol. 17, pp. 1923–1929. Pergamon Press. 1968. Printed in Great Britain

# STUDIES ON THE MODE OF ACTION OF NOVOBIOCIN

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(*Received* 12 *February* 1968; *accepted* 25 *April* 1968)

**Abstract**—Novobiocin (NB) reduced lysis and prevented spheroplast formation in penicillin-treated *E. coli*. It caused a slow lysis of preformed spheroplasts when these were suspended in an environment conducive to their growth, and prevented reversion of spheroplasts to rods. It did not lyse "resting" protoplasts of *Bacillus megaterium*, but inhibited the growth of protoplasts, and brought about slow lysis of these forms.

NOVOBIOCIN (NB) has been reported to exert a variety of effects on bacteria.[1–11] However, it is unlikely that inhibition of cell wall synthesis[1–6] is of primary importance, since NB prevents growth of both protoplasts[12, 13] and L-forms.[14–16] It is also unlikely that NB causes a magnesium deficiency intracellularly by forming a complex with $Mg^{2+}$ ions, as recent findings disagree with the results previously obtained.[17, 18]

This study investigates the effects of NB on spheroplasts and protoplasts, in order to obtain more information as to the site of action of the antibiotic. The results of experiments carried out on 'normal' bacterial cells treated with NB in the presence of benzylpenicillin are also given.

## EXPERIMENTAL

*Organisms. E. coli* NCTC 9001 or *Staphylococcus aureus* Oxford (NCTC 6571) was grown for 18 hr in 40 ml nutrient broth at 37°. The medium was centrifuged, the deposit washed twice with sterile water, and finally resuspended in sterile water to give c. $10^8$ viable cells/ml.

*Bacillus megaterium* NCTC 6005 was grown for 18 hr at 37° in 100 ml nutrient broth in a shaking incubator operating at 70 opm; a washed suspension was used as described later.

*Chemicals.* Sodium succinate was of Laboratory Reagent grade. All other chemicals were of Analytical Reagent quality.

*Antibiotics.* Novobiocin monosodium, B.P., was purchased from Merck, Sharp & Dohme, Ltd., Hoddesdon, Benzylpenicillin, B.P., from Glaxo, Ltd., Greenford. Polymyxin B sulphate (PM) and Mitomycin C (MC) were gifts from Wellcome Research Laboratories, London, and Dales Pharmaceuticals, Ltd., London, respectively.

Egg-white lysozyme was purchased from British Drug Houses, Ltd., London, and penicillinase ("Neutrapen") from Riker Laboratories, Ltd., Loughborough.

*Culture media.* Medium A (nutrient broth no. 2, Oxoid Laboratories, Ltd., London) with or without added magnesium sulphate ($10^{-2}$M) was used for *E. coli*; for spheroplasts, medium A was supplemented with sucrose (0·33 M) and magnesium sulphate,

$MgSO_4 . 7H_2O$ ($10^{-2}$M) (medium B). When required, media A and B were solidified by adding 1% w/v Ionagar (Oxoid).

For the growth of *S. aureus* and *B. megaterium*, medium C (nutrient broth no. 1, Oxoid) was used.

A medium (medium D) of the following final composition was used for the growth of protoplasts of *B. megaterium*: peptone (Oxoid) 0·5 g, yeast extract (Difco) 0·5 g, glucose 0·5 g, sodium succinate 8 g, magnesium sulphate 0·25 g, 0·013 M phosphate buffer, pH 7·2, sucrose 16·7 g, per 100 ml.

*NB + penicillin action on* E. coli *and* S. aureus. 0·5 ml of a washed suspension was added to 10 ml of medium A or C in nephelos flasks in a shaking incubator operating at 100 oscillations/min at 37°. When the cultures were in the logarithmic phase, benzylpenicillin was added; NB was added simultaneously or at intervals thereafter. Subsequent incubation was at 37°, and turbidity was measured with the EEL nephelometer (Evans Electroselenium, Ltd., Harlow).

*Spheroplasts of* E. coli. Sufficient of a washed suspension of *E. coli* (to give c. 3–4 $\times$ $10^7$ total cells/ml) was included in 10 ml of medium B containing 250 units/ml benzylpenicillin. Routinely, spheroplasts present after incubation for 270 min at 37° were used. These were centrifuged, the deposit resuspended in medium B, or in a solution, buffered at pH 7·2, containing 0·33 M sucrose and $10^{-2}$ M magnesium sulphate, and the process repeated. The effect of NB on spheroplast formation and on preformed spheroplasts was studied.

*Protoplasts of* B. megaterium. Optimum protoplast formation occurred in a medium containing 0·5 M sucrose, 40 $\mu$g/ml lysozyme, 0·013 M phosphate buffer, pH 7·2, and 0·24 mg dry wt/ml of washed suspension. Protoplast production was complete after 60 min at 20°; magnesium sulphate, $5 \times 10^{-3}$ M, was then added.

The effects of antibiotics on these non-growing protoplasts at 20°, and on the growth of protoplasts in medium D at 37°, were studied.

*Viable counts.* For rod forms, serial dilution was made in 9-ml. vol of water, followed by plating into medium A containing Ionagar. For spheroplasts, serial dilution was made in 9-ml vol. of 0·33 M sucrose containing $10^{-2}$ M magnesium sulphate, followed by plating into medium B containing Ionagar. In each case, the first dilution tube contained sufficient penicillinase to neutralise the penicillin carried over. All plates were incubated for 48 hr at 37° and the colonies counted.

*Total counts.* These were made, under a phase-contrast microscope ($\times$ 400) with either a Helber chamber or a haemocytometer slide.

## RESULTS

The minimum inhibitory concentration (m.i.c.) of NB against *E. coli*, *S. aureus* and *B. megaterium* was 500, 4 and 5 $\mu$g/ml, respectively. Other antibiotics were used at their respective m.i.c. level.

When a mixture of NB and penicillin was added to logarithmically-growing *E. coli* in medium A at 37°, the lytic action of penicillin appeared to be reduced in both rate and extent (Fig. 1). Similar results were obtained when $10^{-2}$ M magnesium sulphate was included in the medium. A similar effect of NB on the lysis induced in penicillin-treated *S. aureus* in medium C was also found.

When a mixture of NB and penicillin was added to logarithmically-growing *E. coli* rods in medium B, lysis was again observed (Fig. 2). Viable counts made at intervals

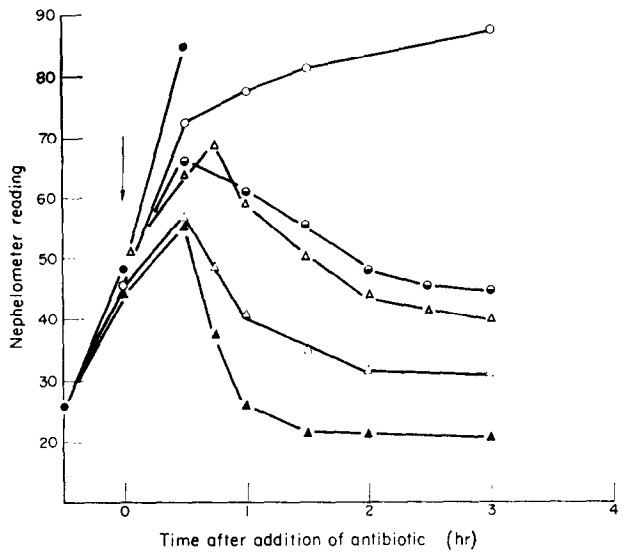


FIG. 1. Effect of NB on penicillin-induced lysis of *E. coli* in medium A. Penicillin (to give 250 units/ml) added as indicated by ↓. NB (to give 1000 μg/ml) added simultaneously (◒—◒), 5 min later (Δ—Δ) or 15 min later (△—△). NB-treated *E. coli* (penicillin absent), ○—○. Control (no antibiotic), ●—●. Penicillin-treated *E. coli* (NB absent), ▲—▲.

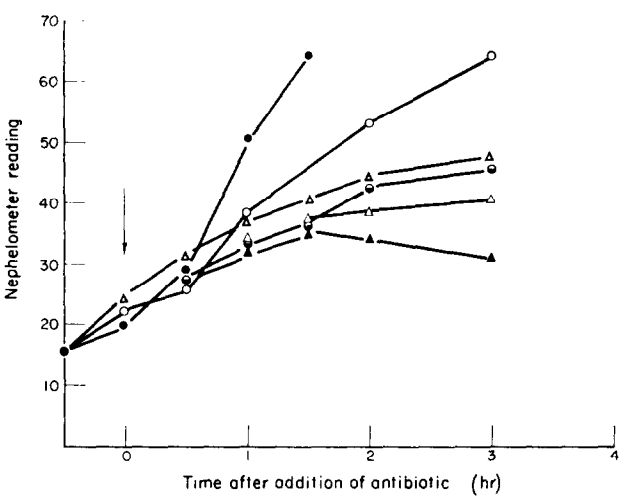


FIG. 2. Effect of NB on the turbidity of penicillin-treated *E. coli* in medium B. Symbols, as for Fig. 1.

during this experiment are given in Table 1. These results indicate (a) that NB and penicillin have an enhanced effect when used in combination: this does not conform to the classical definition of 'synergism', however, as no attempt was made to use each antibiotic at one-half of its effective concentration; (b) that, when stabilised, penicillin-induced spheroplasts can revert to the rod form; (c) that NB does not induce osmotically fragile forms in medium B; (d) that NB in combination with

TABLE 1. BACTERICIDAL EFFECT OF NB (1000 $\mu$g/ml), PENICILLIN (250 units/ml) AND A MIXTURE ON *E. COLI* IN MEDIUM B

| Hr after addition of antibiotic | Antibiotic | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | None | | NB | | Penicillin | | NB + Penicillin | |
| | a | b | a | b | a | b | a | b |
| 0 | $4{\cdot}5 \times 10^8$ | $6 \times 10^8$ | $7{\cdot}8 \times 10^8$ | $8 \times 10^8$ | $5{\cdot}8 \times 10^8$ | $7 \times 10^8$ | $5{\cdot}7 \times 10^8$ | $6 \times 10^8$ |
| 2 | $1{\cdot}2 \times 10^9$ | $1{\cdot}3 \times 10^9$ | $3{\cdot}8 \times 10^8$ | $4 \times 10^8$ | $1{\cdot}1 \times 10^7$ | $1{\cdot}46 \times 10^8$ | $4{\cdot}6 \times 10^6$ | $6 \times 10^6$ |
| 4 | $2 \times 10^9$ | $2{\cdot}2 \times 10^9$ | $1 \times 10^7$ | $1{\cdot}2 \times 10^7$ | $2{\cdot}5 \times 10^6$ | $1{\cdot}5 \times 10^8$ | $1{\cdot}35 \times 10^6$ | $1{\cdot}6 \times 10^6$ |

Figures are no. of viable cells/ml.
a, cells serially diluted in water and plated into nutrient agar.
b, cells serially diluted in sucrose + $Mg^{2+}$ solution, and plated into sucrose. $Mg^{2+}$ agar.

penicillin is either preventing the production of osmotically fragile spheres by the latter, or is preventing reversion of any such spheres which might have been formed. Experiments in which preformed spheroplasts were treated with NB in penicillin-free medium B indicated that NB prevented reversion of spheroplasts to rods. Similar results were obtained with MC.

Further evidence in support of these conclusions was obtained when *E. coli* rods were inoculated into medium B containing no antibiotic (control), NB, penicillin and a NB + penicillin mixture; in the absence of any antibiotic, the total counts increased, NB used alone prevented an increase in total numbers (but caused no lysis), penicillin used alone induced spheroplasts, and the total number of organisms in the NB + penicillin system was reduced by c. 43 per cent. Thus, NB induces some lysis in *E. coli* cells when used in conjunction with penicillin.

In subsequent experiments, spheroplasts were centrifuged and resuspended in medium B or in sucrose. $Mg^{2+}$. buffer, containing penicillin, and NB or MC added. Total counts were made during incubation at 4° and 37° (Table 2). With penicillin only (i.e. control tubes), the spheroplast counts remained constant in all cases. NB caused a decrease in spheroplast numbers in medium B at 37° but not at 4°, and not at 37° in sucrose. $Mg^{2+}$. buffer. Similar results were obtained with MC. Since PM is

TABLE 2. EFFECT OF NB (1000 $\mu$g/ml), PM (100 units/ml) AND MC (1 $\mu$g/ml) ON TOTAL COUNTS OF SPHEROPLASTS* OF *E. COLI*

| Hr after antibiotic added | No. of spheroplasts/ml in medium B | | | |
|---|---|---|---|---|
| | Control | Spheroplasts treated with | | |
| | | NB | MC | PM |
| 0 | $3{\cdot}3 \times 10^7$ | $3{\cdot}9 \times 10^7$ | $3{\cdot}0 \times 10^7$ | $2{\cdot}8 \times 10^7$ |
| 1 | $3{\cdot}1 \times 10^7$ | $2{\cdot}5 \times 10^7$ | $2{\cdot}3 \times 10^7$ | $< 1 \times 10^5$ |
| 2 | — | $1{\cdot}4 \times 10^7$ | $1{\cdot}6 \times 10^7$ | |
| 3 | $3{\cdot}5 \times 10^7$ | $1{\cdot}1 \times 10^7$ | $1{\cdot}0 \times 10^7$ | |

* Penicillin-induced spheroplasts were centrifuged, and resuspended in penicillin-containing media (except in experiments in which PM was added, where penicillin was absent). NB, MC or PM was added, except in controls, and the tubes incubated.

incompatible with penicillin, the centrifuged spheroplasts obtained above were resuspended in medium B, and PM added: this lysed virtually all the spheroplasts within 1 hr at 37°. Table 3 shows that NB is bactericidal to spheroplasts and to rods in medium B, but not to spheroplasts in sucrose. $Mg^{2+}$. buffer. Thus, spheroplasts are affected by NB only when they are in an environment conducive to their growth.

The effect of NB on protoplasts of *B. megaterium* was next studied. NB induced a slow lysis of growing protoplasts at 37° (Fig. 3), but was ineffective against non-growing protoplasts over a 4-hr period at 20°.

TABLE 3. BACTERICIDAL EFFECT OF NB (500 μg/ml) ON RODS AND ON PREFORMED SPHEROPLASTS OF *E. COLI*

| Min after addition of NB | Spheroplasts | | | | Rods | |
|---|---|---|---|---|---|---|
| | Control | | NB | | Control | NB |
| | a | b | a | b | a | a |
| 0 | $2{\cdot}4 \times 10^6$ | $1{\cdot}9 \times 10^6$ | $2{\cdot}4 \times 10^6$ | $1{\cdot}9 \times 10^6$ | $2{\cdot}25 \times 10^7$ | $2{\cdot}25 \times 10^7$ |
| 45 | $3{\cdot}6 \times 10^6$ | $2{\cdot}1 \times 10^6$ | $1{\cdot}0 \times 10^6$ | $1{\cdot}58 \times 10^6$ | $6{\cdot}1 \times 10^7$ | $1{\cdot}8 \times 10^7$ |
| 90 | $7{\cdot}2 \times 10^6$ | $2{\cdot}52 \times 10^6$ | $4{\cdot}1 \times 10^5$ | $2{\cdot}07 \times 10^6$ | $1{\cdot}6 \times 10^8$ | $1{\cdot}2 \times 10^7$ |

Rods or penicillin-induced spheroplasts were centrifuged and resuspended in a medium containing no penicillin, and NB added (except to controls). a, spheroplasts suspended in medium B; b, spheroplasts suspended in sucrose. $Mg^{2+}$. buffer. Plating medium, medium B solidified with agar. Figures are no. of cells/ml.

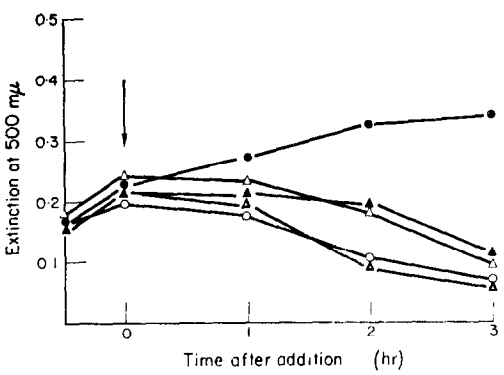


FIG. 3. Effect of NB (added as indicated) on the growth of protoplasts of *B. megaterium* in medium D. Concentrations of NB: absent, ●—●; 5 μg/ml, ○—○; 15 μg/ml, △—△; 25 μg/ml, Δ—Δ.

## DISCUSSION

The results presented indicate that NB interferes with membrane integrity in both a Gram-negative and a Gram-positive organism, and that this occurs only when the cell is able to grow but not necessarily divide. However, a direct physical effect cannot be entirely excluded, since it is conceivable that cell membranes can exist in different arrangements depending on whether the cell is growing or not; moreover, NB may not be adsorbed on to non-growing cells. However, the effect of NB on membranes is quite distinct from that of PM, which causes a very rapid lysis of spheroplasts,

although NB, itself, possesses surface-active properties.[19] PM is considered to have a direct effect on bacterial membranes.[20] If this effect of NB on bacterial membrane integrity is the primary action of the drug, there is a need to consider how this could give rise to its effects on deoxyribonucleic acid (DNA) synthesis;[10, 11] conversely, it must also be considered whether these other individual effects could give rise to membrane damage. It is highly probable that cell wall precursors are formed at, and transported across, the cytoplasmic membrane. Similarly, it has been shown[21] that the bacterial chromosome is attached to the membrane before replication occurs. Thus, damage to the membrane could result in the accumulation of wall precursors and the inhibition of nucleic acid synthesis. This would also explain the leakage of 260 mμ-absorbing material from *E. coli*,[7] although this event may be peculiar to certain strains of *E. coli*.[11] NB and MC, an antibiotic reported to inhibit DNA synthesis,[22] both induce lysis of spheroplasts, and this finding suggests that membrane damage is a consequence of inhibition of DNA synthesis. In contrast, recent results obtained with phenethyl alcohol[23] suggest that membrane damage results in an inhibition of DNA synthesis. Besides preventing chromosome replication, membrane damage could also have an indirect effect on DNA synthesis by causing the loss of essential smaller molecules from the bacterial cell.

Bazill[24] has recently proposed that an inhibition of bacterial cell wall synthesis alone could lead to inhibition of DNA synthesis and the formation of filamentous forms. It is, however, unlikely that NB acts in this way, since it not only interferes with cell wall synthesis[1-6] but also prevents the formation of spheroplasts by penicillin and the reversion of preformed spheroplasts to rods.

One puzzling feature of this investigation has been the finding that although NB used alone does not cause lysis of rods of *E. coli* (although it does induce filamentation[25]), NB + penicillin induces lysis even in the presence of an osmotic stabiliser. It could well be that adsorption and subsequent absorption, of NB is greater in the presence of penicillin; thus, when NB is allowed to treat spheroplasts, the effects on the membrane are magnified. Alternatively, the membrane could be more susceptible to NB action when the amount of mucopeptide in the rigid (R-) layer of the cell wall is reduced, so that an osmotic stabiliser would not fulfil every aspect of mucopeptide function within the cell.

Although the present study has not elucidated the primary effect of NB, membrane damage obviously plays an important role in the loss of viability of NB-treated bacteria, and may well be the primary cause. However, we feel that attention should be directed to a determination of the possible release of mature phage particles, as noted previously[26] with MC-treated strains of different bacilli.

*Acknowledgement*—We thank the Science Research Council for a grant to one of us (A.M.).

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