STUDIES ON THE MODE OF ACTION OF NOVOBIOCIN[1]

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Novobiocin (Streptonivicin) is a new broad-therapy antibiotic recently announced by Smith *et al.* (1956). Chemically it is an acid containing a nine carbon sugar attached glycosidically to the seven position of 3 - [4 - hydroxy - 3 - (3 - methyl - 2 - butenyl) - benzamido] - 4,7 - dihydroxy - 8 - methyl coumarin (Hoeksema *et al.*, 1956; Hinman *et al.*, 1956). Some *in vitro* studies are presented here which may have some bearing on the mode of action of this antibiotic. These include the effect of $MgSO_4$ and other metals on antibiotic activity, the induction of filamentation, and the effect of the antibiotic on the synthesis of nucleic acid.

METHODS

Cultural methods. The majority of the work was performed with *Escherichia coli* strain ATCC 26 and *Staphylococcus aureus* strain ATCC 6538. The cultures were grown in brain-heart infusion medium (Difco) from 5 to 6 hr at 37 C. A 0.05-ml aliquot of these cultures was used to inoculate 500-ml shake flasks containing 100-ml medium or culture tubes containing 10 ml of medium. Media used included brain heart infusion and the synthetic medium of Loveless *et al.* (1954) which contains $Na_2HPO_4 \cdot 12H_2O$, 15 g; KH_2PO_4, 3.0 g; NH_4Cl, 1.0 g; NaCl, 0.5 g; $MgSO_4$, 0.2 g; glucose (autoclaved separately), 4 g in 1 L of deionized water.

Antibiotics. The novobiocin used was the monosodium salt prepared in these laboratories. Other antibiotics used were potassium penicillin G, erythromycin-free base, neomycin sulfate, and tetracycline hydrochloride, all obtained from the Production Division of The Upjohn Company.

Analytical methods. The cells grown overnight in shake flasks at 37 C were cooled after removing a 10-ml aliquot for turbidity and dry weight measurements. To the remaining 90 ml were added 9 g of trichloroacetic acid. The suspension was centrifuged and the precipitate washed with cold 10 per cent trichloroacetic acid and then extracted with hot 10 per cent trichloroacetic acid at 100 C for 20 min. This extract was diluted with distilled water and assayed for ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) by the orcinol and diphenylamine methods, respectively (Dische, 1955). Samples of commercial RNA and DNA from Nutritional Biochemicals Co. were used as standards.

Turbidity readings were made on a Lumetron Photoelectric Colorimeter at 650 mμ and were correlated with dry weight determinations. The effect of antibiotics on growth was evaluated visually and turbidimetrically. The per cent inhibition figures were calculated from turbidity readings. However, the minimum inhibitory concentration was determined as the lowest concentration of antibiotic giving complete inhibition by visual examination.

RESULTS

Effect of metals on antibiotic activity. It was found that the minimum inhibitory concentration of novobiocin against *E. coli* was considerably lower in brain-heart infusion than in the synthetic medium, even though growth was about equal in both media. Each ingredient from the synthetic medium was added singly to brain-heart infusion and it was found that only $MgSO_4$ caused an increase in the minimum inhibitory concentration. Different concentrations of $MgSO_4$ and different metal salts were then tested. Two- to eight-fold reversal of novobiocin inhibition of *E. coli* was found with the divalent cations of $MgSO_4$, $CaCl_2$, $BaCl_2$ and $SrCl_2$. The effect was most marked with $MgSO_4$. The following metal salts, when tested at non-toxic concentrations, were ineffective: $MnSO_4$, $FeCl_3$, $FeSO_4$, $ZnCl_2$, $CoCl_2$, $CuCl_2$, $HgCl_2$, $AlCl_3$, $CdCl_2$, $SnCl_2$, $CeCl_2$, $UO_2(NO_3)_2$.

Since novobiocin inhibits a wide variety of microorganisms, the experiments with $MgSO_4$

[1] The trademark of The Upjohn Company for novobiocin is Albamycin.

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TABLE 1

Effect of $MgSO_4$ on action of novobiocin against different organisms in brain heart infusion

Organism	Minimum Inhibitory Concentration	
	Brain-heart	Plus 0.05% $MgSO_4$
	μg/ml	μg/ml
Escherichia coli strain ATCC 26	64	500
Salmonella typhosa strain ATCC 167	250	1000
Aerobacter aerogenes strain ATCC 8308	250	1000
Proteus vulgaris strain ATCC 8427	64	250
Klebsiella pneumoniae strain ATCC 10031	8	32
Staphylococcus albus strain ATCC 151	4	2
Staphylococcus aureus strain ATCC 6538	1	0.5
Streptococcus pyogenes strain Upjohn 147	2	2
Bacillus subtilis strain Upjohn 28	4	4

TABLE 2

Effect of $MgSO_4$ on activity of antibiotics against Escherichia coli in brain-heart infusion

Antibiotic	Minimum Inhibitory Concentration	
	Brain-heart	Plus 0.05% $MgSO_4$
	μg/ml	μg/ml
Novobiocin	64	500
Penicillin	125	125
Erythromycin	16	32
Neomycin	25	25
Tetracycline	1.5	3

were extended. These data are presented in table 1. It can be seen that the activity of novobiocin is reversed by $MgSO_4$ against gram negative bacteria, but not against gram positive bacteria.

To see how general the effect of $MgSO_4$ is, four other antibiotics were tested against *E. coli*. In table 2 it can be seen that a marked effect was found only with novobiocin.

Filamentation. Smith *et al.* (1956) noticed that subinhibitory concentrations of novobiocin caused filamentation of certain bacteria. Consequently a survey of this phenomenon was made with a number of bacteria. Filamentation in broth tubes was determined by microscopic examination of crystal violet-stained smears after overnight incubation at 37 C. It was rated in arbitrary units from 0 (no filamentation) to 5 (best filamentation). The results of this survey are shown in table 3. It can be seen that novobiocin causes filamentation of varying degrees in all of the gram negative bacteria tested, but in none of the gram positive bacteria.

Filamentation with *E. coli* was quite marked and the microscope field resembled a tangled mass of threads which were many times the normal length. Cell wall staining by the methods of Webb (1954) and Robinow (1945) indicated that there were no cross walls, even in the longest filaments.

TABLE 3

Effect of novobiocin on filamentation of various bacteria in brain-heart infusion

Organism	Filamentation*	μg/ml†
Escherichia coli strain ATCC 26	5	16
Salmonella typhosa strain ATCC 167	4	32, 16
Salmonella schottmuelleri strain ATCC 9149	2	125
Proteus vulgaris strain ATCC 8427	1	32
Klebsiella pneumoniae strain ATCC 10031	2	1
Aerobacter aerogenes strain ATCC 8308	3	64, 32, 16
Serratia marcescens strain ATCC 60	4	4
Pseudomonas aeruginosa strain ATCC 9027	1	500
Shigella dispar strain Upjohn 139	3	64
Staphylococcus albus strain ATCC 151	0	—
Staphylococcus aureus strain ATCC 6538	0	—
Streptococcus pyogenes strain Upjohn 147	0	—
Bacillus subtilis strain Upjohn 28	0	—

* Filamentation rated from 5 (best) to 0 (none).

† Concentration listed is that which gave optimum filamentation.

TABLE 4

Effect of novobiocin on ribose nucleic acid and desoxyribose nucleic acid synthesis

Organism	Per cent Inhibited	Mg RNA/ 100 Mg Dry Weight	Mg DNA/ 100 Mg Dry Weight
Escherichia coli	0	10.0	4.3
	17	8.0	1.7
	69	10.7	2.6
	80	9.3	2.3
Staphylococcus aureus	0	5.3	0.87
	50	5.5	0.58

The addition of $MgSO_4$ to brain-heart infusion medium had no effect on the degree of filamentation of *E. coli*, although the optimum concentration of novobiocin for filamentation was higher. A number of tests were run with *E. coli* in graded concentrations of novobiocin in which filamentation and growth were determined simultaneously. In 37 different experiments in various media, maximum filamentation occurred on the average when the organism was 65 per cent inhibited. There was no correlation between concentration of novobiocin and degree of filamentation in various media. Whenever a tube in a particuar series showed around 65 per cent inhibition, this tube showed maximum filamentation. When 2-fold differences in concentration of antibiotic were used, there were times when in certain media (especially synthetic), the dose-response curve was such that there was no tube which had around 65 per cent inhibition. In these series filamentation was poor. However, when a more closely graded series of concentrations was run under these conditions, it was possible to get a tube with about 65 per cent inhibition and in this tube filamentation was good. Thus there is no evidence that the composition of the medium has any influence on filamentation other than to alter the dose-response curve and make it difficult to achieve 65 per cent inhibition. This is an important consideration in any study of the influence of conditions on filamentation.

Nucleic acid synthesis. Analyses for RNA and DNA were made on partially inhibited cultures. Table 4 lists these values for *E. coli* and *S. aureus*. With *E. coli* there was some filamentation in the inhibited flask cultures, although it was less than in similarly inhibited unshaken tube cultures. No filamentation occurred with *S. aureus*. It is apparent that the amount of RNA was essentially unchanged in the partially inhibited cells as compared to the control cells. There was a decrease in DNA synthesis in both cases, but this may not be significant.

DISCUSSION

Two lines of evidence in this paper argue for a different mode of action of novobiocin against gram negative than against gram positive bacteria. In gram negative but not in gram positive bacteria, novobiocin causes filamentation and inhibition is reversed by $MgSO_4$. Other antibiotics which cause filamentation, such as penicillin (Gardner, 1940), do so in both types of organisms. Oxytetracycline inhibition is reversed by Mg^{++} in both types of organisms (Weinberg, 1955). Polymyxin inhibition is reversed by $MgSO_4$ against *S. aureus*, but not against *E. coli* (Rhodes *et al.*, 1953). This is the reverse of the novobiocin situation.

The work of Webb (1953) on the effect of magnesium on cell division in bacteria is interesting in this respect. He found an excess of magnesium to cause filamentation in gram negative and gram positive bacteria whereas a magnesium deficiency caused filamentation only in the latter. Also, Webb reports that the magnesium requirements for growth of gram positive bacteria are about ten times those of gram negative bacteria. Since the gram positive complex is magnesium ribonucleate (Henry and Stacey, 1943), this higher requirement is not surprising.

A number of chemicals have been reported (Loveless *et al.*, 1954) to cause filamentation in *E. coli* and their action was attributed to their ability to produce carbonium ions in solution. The 4-hydroxy-3-(3-methyl-2-butenyl) benzamide portion of the novobiocin molecule would be expected to produce carbonium ions in solution, but 4-hydroxy-3-(3-methyl-2-butenyl) benzoic acid by itself does not cause filamentation or growth inhibition of *E. coli* even at 2000 μg/ml.

A number of antibiotics have been tested for inhibition of nucleic acid synthesis in bacteria and so far there is no evidence for this (Wisseman *et al.*, 1954; Gale and Folkes, 1953). Novobiocin has no effect on RNA synthesis and little or no

effect on DNA synthesis. Thus it will probably have to join the growing list of antibiotics which do not alter nucleic acid synthesis.

The simplest explanation for the magnesium reversibility of novobiocin is that the magnesium complexes the antibiotic and renders it inactive. There is no chemical evidence for this. Also, since magnesium does not reverse inhibition against gram positive bacteria, it seems unlikely that it is inactivating novobiocin. Thus a biochemical explanation for this phenomenon will have to be sought.

SUMMARY

It has been shown that $MgSO_4$ will reverse the activity of novobiocin against gram negative but not against gram positive organisms. Also, novobiocin causes filamentation in gram negative but not in gram positive bacteria. There is no evidence that the antibiotic affects RNA synthesis even in filamentous cells. DNA synthesis may be decreased. The reversal of inhibition by $MgSO_4$ will have to be given a biochemical rather than a chemical explanation.

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