Biochemical Effects of Novobiocin on

Staphylococcus aureus

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ABSTRACT

Wishnow, Rodney M. (Washington University School of Medicine, St. Louis, Mo.), Jack L. Strominger, Claire H. Birge, and Robert H. Threnn. Biochemical effects of novobiocin on Staphylococcus aureus. J. Bacteriol. 89:1117-1123. 1965.—Novobiocin induced accumulation in Staphylococcus aureus of uridine nucleotide precursors of the cell-wall glycopeptide, of a cytidine nucleotide precursor of the telchoic acid in the cell wall, and of a large number of other ultraviolet-absorbing materials. These substances accumulated promptly on addition of the antibiotic at levels near the growth-inhibitory concentration. Isotopic experiments showed that, in addition to inhibiting cell-wall synthesis, novobiocin also inhibited both protein and nucleic acid synthesis. The lack of selectivity in the action of novobiocin is similar to that previously observed with gentian violet, and is quite different from the selective inhibition of cell-wall synthesis observed with penicillin or with d-cycloserine. Although inhibition of cell-wall synthesis is not excluded as one of the primary lethal effects of novobiocin, it is also possible that all of the observed phenomena are secondary to some other primary metabolic lesion.

In recent years, many attempts have been made to elucidate the mechanism of action of various antibiotics. A striking feature of this work has been the increasing recognition of the importance of the cell wall and membrane as targets for cytotoxic agents. Novobiocin has an antibacterial spectrum similar to that of erythromycin, and has proven useful in the treatment of resistant staphylococcal infections. It has a complex structure consisting of a substituted phenol, a substituted coumarin, and the sugar moiety, noviose (the carbamate ester of a derivative of L-rhamnose). It has been reported briefly that novobiocin causes accumulation of cell-wall precursors in Staphylococcus aureus (Strominger and Threnn, 1959). Details of novobiocin-induced nucleotide accumulation and its specificity are reported in this paper.

Uridine nucleotide accumulation. Measurement of accumulation of derivatives of uridine diphosphate (UDP)-acetylmuramic acid, which are cell-wall precursors, was carried out as described previously (Strominger, 1957) with 100-ml cultures. As a matter of convenience, extraction of cells with 1 ml of water in a boiling-water bath for 10 min is now preferred to extraction with cold trichloroacetic acid. Acetylamino sugar-containing nucleotides in the hot-water extract were measured colorimetrically.

Accumulated nucleotides were recovered from the extracts by charcoal adsorption and elution, as described by Strominger (1962a). On a small scale (100-ml cultures), the nucleotide eluates were subjected to two-dimensional paper chromatography in solvent A (isobutyric acid-0.5 N NH4OH, 5:3) and solvent B [ethanol-N x ammonium acetate (pH 7), 7.5:3]. Nucleotides were then visualized by printing the chromatograms in ultraviolet light with reflex document copying paper.

On a larger scale (9 liters) nucleotides in trichloroacetic acid extracts were separated on Dowex 1-chloride, essentially as described by Strominger (1957), and then recovered from the column eluates by charcoal adsorption and elution and purified by paper chromatography in solvents A and B.

Measurement of cell-wall, protein, and nucleic acid synthesis with isotopes. Experiments to determine cell-wall, protein, and nucleic acid synthesis were carried out as described previously (Nathen-
Observation of accumulated nucleotides by two-dimensional paper chromatography. A 100-ml amount of culture at half-maximal growth was treated with novobiocin (50 μg/ml) for 90 min. The cells were collected by centrifugation, washed, and extracted with hot water. Cold trichloroacetic acid (final concentration, 5%) was added, and the insoluble material was removed by centrifugation. Nucleotides were adsorbed onto charcoal and recovered by elution with ammoniacal ethyl alcohol. About 90% of the ultraviolet-absorbing material was recovered by this procedure, and was subjected to two-dimensional paper chromatography (Fig. 3). The pattern of nucleotides obtained can be contrasted with the nucleotide accumulation observed with “gentian violet” (G. T. Gurr, Ltd., lot 635, 100 μg/ml), penicillin (200 μg/ml), and β-cyclodextrin (Oxamycin, 100 μg/ml) prepared at the same time and in a similar manner (Fig. 4 to 6). [As previously reported (Strominger, 1959), most of bottles of gentian violet do not induce uridine nucleotide accumulation in S. aureus. The phenomenon appears to be due to an impurity in the particular lot of dye employed; hence, “gentian violet” is written in quotation marks.] The amount of ultraviolet-absorbing compounds present in the extract of a control culture (Fig. 7) is too small to be seen by this technique.

RESULTS

Uridine nucleotide accumulation as a function of time and novobiocin concentration. Novobiocin induced the accumulation in S. aureus Copenhagen of UDP-acetylamino sugar compounds, which are cell-wall precursors. The lowest concentration of novobiocin which induced uridine nucleotide accumulation was 1.5 μg/ml. Maximal accumulation occurred at 15 μg/ml (Fig. 1). Measurement of antibiotic sensitivity by tube dilution with an inoculum of 1% of a fully grown culture showed that the minimal growth-inhibitory concentration of novobiocin was about 4 μg/ml; total inhibition occurred at 16 μg/ml.

Nucleotide accumulation began promptly after the addition of novobiocin. The time for half-maximal accumulation was about 5 min. The maximal accumulation of nucleotide was reached in 60 min, and amounted to 17 μmoles per liter of culture (Fig. 2).
Fig. 3. Nucleotides accumulated in cells treated for 90 min with novobiocin (50 μg/ml). (A) Two-dimensional chromatogram of the cell extract photographed in ultraviolet light. The first dimension (solvent A) was run in isobutyric acid-1 N NH₄OH (5:3), and the second dimension (solvent B), in ethanol-1 M ammonium acetate, pH 7 (7.5:3). (B) Schematic drawing of A. The numbers are the same as those used in Table 1.

Fig. 4. Nucleotides accumulated in cells treated for 90 min with "gentian violet" (100 μg/ml). Compounds 1, 2, 3, 5, 6, 8, 9, 10, 11, and 13 were the same as those found in novobiocin-treated cells. Compound 4 in this case was a mixture of UDP-acetylglucosamine, UDP-acetylglucosamine-pyruvate enol ether, and UDP-acetylmuramic acid. Compound 7 (UDP-acetylmuramyl-L-ala-D-glu-D-ala-D-ala) and compound 12 (nicotinamide adenine dinucleotide phosphate) were absent. Small amounts of several unidentified compounds not present in novobiocin-treated cells can be seen.

Fig. 5. Nucleotides accumulated in cells treated for 90 min with penicillin. The major materials present are compound 4 (a mixture of UDP-acetylmuramic acid and UDP-acetylmuramyl-L-ala in this case) and compound 7 (UDP-acetylmuramyl-L-ala-D-glu-L-lys-D-ala-D-ala). No cytidine nucleotides accumulate, and compound 5 contains only UMP. Small amounts of compounds 1, 2, 5, and 8 are also seen.
Novobiocin, like "gentian violet," induced massive accumulation of a large number of different ultraviolet-absorbing materials. The patterns observed with these two substances were very similar. In contrast, a much more specific accumulation of only a few compounds occurred with penicillin and p-cycloserine.

Analysis of nucleotides from novobiocin-treated cells. The ultraviolet-absorbing compounds were eluted from the chromatogram with water. The base present was identified by its ultraviolet-absorption spectrum in acid and alkali. Nicotinamide adenine dinucleotide and nicotinamide adenine dinucleotide phosphate were identified in part from the secondary absorption maximum of these compounds at 320 nm in 0.1 M NaCN. Phosphate, acetylamino sugar, and amino acid analyses were also carried out. By these techniques, as well as by the position on the two-dimensional chromatogram, a provisional identification of some of the compounds was obtained (Table 1). Two of the ultraviolet-absorbing areas contained UDP-acetylamino sugar compounds.

One of these areas contained mainly UDP-acetylmuramic acid. This area would also have contained UDP-acetylmuramyl-1-ala. This latter compound is present in penicillin-treated and cycloserine-treated cells but is absent from both novobiocin-treated and "gentian violet"-treated cells. The other area contained a peptide derivative of UDP-acetylmuramic acid and is described below.

As in the case of "gentian violet"-inhibited cells, cytidine diphosphate (CDP)-ribitol also accumulated in the presence of novobiocin. A small amount of CDP-glycerol was also found.

Detailed analysis of the peptide-containing UDP-acetylamino sugar compound. Analysis of the peptide-containing UDP-acetylamino sugar compound was carried out to compare this nucleotide with the peptide-containing compound obtained from S. aureus strain H treated with penicillin. The samples employed were obtained from large-scale preparations on Dowex 1-chloride. By the methods employed, the two nucleotides were identical (Table 2), and are represented by the structure UDP-acetylmuramyl-1-ala-d-glu-L-lys-d-ala-d-ala.

Effects of novobiocin on the incorporation of C14-lysine and P32-inorganic phosphate into cell wall, protein, and nucleic acid. To investigate the specificity of the effect of novobiocin on cell-wall synthesis, the incorporation of C14-lysine and P32-inorganic phosphate into cell wall and into the protein and nucleic acid fraction was measured (Tables 3 and 4). At a novobiocin concentration of 15 μg/ml, the incorporation of C14-lysine into cell walls was inhibited by 72%. The incorporation of label into the protein and nucleic acid fraction, however, was also inhibited by 44%. At a novobiocin concentration of 100 μg/ml, slightly greater effects were observed.
TABLE 1. Identification of compounds from novobiocin-treated Staphylococcus aureus

<table>
<thead>
<tr>
<th>Compound no. a</th>
<th>Base</th>
<th>Ratio of organic phosphate to base b</th>
<th>Ratio of acetyl-amino sugar to base c</th>
<th>Other data</th>
<th>Provisional identification d</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>A</td>
<td>0.9</td>
<td></td>
<td>Secondary absorbancy maximum at 325 μg in 0.1 M KCl</td>
<td>AMP</td>
</tr>
<tr>
<td>2</td>
<td>C</td>
<td>1.3</td>
<td></td>
<td>--</td>
<td>CMP</td>
</tr>
<tr>
<td>3</td>
<td>A</td>
<td>1.9</td>
<td></td>
<td>--</td>
<td>NAD</td>
</tr>
<tr>
<td>4</td>
<td>U + C</td>
<td>2.1</td>
<td>1.1</td>
<td>No amino acids after acid hydrolysis e</td>
<td>UDP-acetyluramic acid</td>
</tr>
<tr>
<td>5</td>
<td>C</td>
<td>1.4</td>
<td>0</td>
<td>Glycerol after enzymatic hydrolysis e</td>
<td>UMP + CDP-glycerol</td>
</tr>
<tr>
<td>6</td>
<td>C</td>
<td>2.0</td>
<td></td>
<td>Ribitol after enzymatic hydrolysis e</td>
<td>CDP-ribitol</td>
</tr>
<tr>
<td>7</td>
<td>U</td>
<td>2.1</td>
<td>1.2</td>
<td>Amino acids present, see Table 9 e</td>
<td>UDP-acetyluramyl-alaglu-lys-ala-ala</td>
</tr>
<tr>
<td>8</td>
<td>G</td>
<td>0.9</td>
<td></td>
<td>--</td>
<td>GMP</td>
</tr>
<tr>
<td>9</td>
<td>U</td>
<td>2.7</td>
<td>0</td>
<td>--</td>
<td>UDP</td>
</tr>
<tr>
<td>10</td>
<td>C</td>
<td>2.0</td>
<td></td>
<td>--</td>
<td>CDP</td>
</tr>
<tr>
<td>11</td>
<td>A</td>
<td>2.1</td>
<td></td>
<td>--</td>
<td>ADP</td>
</tr>
<tr>
<td>12</td>
<td>A</td>
<td>2.8</td>
<td></td>
<td>Secondary absorbancy maximum at 325 μg in 0.1 M KCl</td>
<td>NADP</td>
</tr>
<tr>
<td>13</td>
<td>G</td>
<td>1.8</td>
<td></td>
<td>--</td>
<td>GDP</td>
</tr>
</tbody>
</table>

a Numbers of the compounds refer to those in Fig. 3.

b A = adenosine; C = cytosine; G = guanosine; U = uracil. The base present was determined from its characteristic ultraviolet-absorption spectrum between 220 and 310 μg in 0.01 N HCl and 0.05 N NaOH, measured in a Cary recording spectrophotometer, model 14. The spectrum of compound 5 could not be identified. This compound (0.02 μmole) was hydrolyzed in 40% perchloric acid at 100 C for 2 hr, and then chromatographed in isopropanol-HCl (Markham and Smith, 1952). Two ultraviolet-absorbing areas were seen in the positions of uracil and cytosine. Ultraviolet-absorption spectra of these two compounds (measured after elution from the paper) were identical to those of uracil and cytosine.

c Organic phosphate was measured by the method of Lowry et al. (1951).

d Acetyl amino sugar was measured, after hydrolysis in 0.1 N HCl, by the modified Morgan-Elson reaction (Ressig, Strominger, and Leloir, 1955; Strominger, 1957).

* In addition to the data presented, all of the compounds mentioned were in the same position on the two-dimensional chromatograms as authentic materials. AMP = adenosine monophosphate; CMP = cytidine monophosphate; UMP = uridine monophosphate; GMP = guanosine monophosphate; ADP = adenosine diphosphate; NAD = nicotinamide adenine dinucleotide phosphate.

* Qualitative elution of amino acids was carried out by hydrolyzing 0.1 to 0.2 μmole in 6 N HCl at 100 C overnight. After removal of HCl in vacuo, the sample was subjected to paper chromatography in n-butanol-acetic acid-water (3:1:1). The paper was developed with ninhydrin reagent.

e Samples (0.1 μmole) were hydrolyzed by snake venom phosphodiesterase (Worthington Biochemicals Corp., Freehold, N.J.) and Escherichia coli phosphomonoesterase (Sigma Chemical Co., St. Louis, Mo.). The polyol present in a sample of the hydrolysate was then identified by thin-layer chromatography followed by detection with the periodate-Schiff reagent as described by Saukkonen et al. (1964). We are grateful to J. J. Saukkonen for providing us with this procedure prior to publication.

Incorporation of P32-inorganic phosphate into cell wall at a novobiocin concentration of 15 μg/ml was inhibited by 31%. At this concentration, the incorporation of P32-inorganic phosphate into cell protein and nucleic acid was inhibited by 75%. Similar effects were observed at a novobiocin concentration of 100 μg/ml.

DISCUSSION

It is apparent that novobiocin induces accumulation of the uridine nucleotide precursors of the glycopeptide polymer in the cell wall of S. aureus as well as of CDP-ribitol, a precursor of the teichoic acid polymer in the cell wall. Like "gen- tian violet," however, a large number of other ultraviolet-absorbing compounds accumulate, most of which have not been completely identified. This massive accumulation induced by these two substances stands in contrast to the more selective accumulation of the uridine nucleotide precursors of the glycopeptide which is induced by penicillin or by d-cycloserine (Strominger, 1962b). Isotope experiments, moreover, clearly demonstrate that the effect of novobiocin is not
TABLE 2. Analyses of nucleotides from novobiocin-treated Staphylococcus aureus strain Copenhagen and penicillin-treated S. aureus strain H

<table>
<thead>
<tr>
<th>Substance measured</th>
<th>Compound from novobiocin-treated strain Copenhagen</th>
<th>Compound from penicillin-treated strain H</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphate</td>
<td>2.00</td>
<td>2.10</td>
</tr>
<tr>
<td>Acetylaminosugar</td>
<td>1.08</td>
<td>1.02</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>1.08</td>
<td>0.99</td>
</tr>
<tr>
<td>Lysine</td>
<td>1.06</td>
<td>1.00</td>
</tr>
<tr>
<td>Alanine</td>
<td>3.10</td>
<td>2.51</td>
</tr>
<tr>
<td>D-Alanine</td>
<td>2.10</td>
<td>1.86</td>
</tr>
<tr>
<td>L-Alanine</td>
<td>0.99</td>
<td>0.95</td>
</tr>
</tbody>
</table>

* Results are expressed as micromoles per micromole of uridine.

For methods of measurement, see Table 1.

A sample (0.2 to 0.5 μmol) was hydrolysed in 6 N HCl at 100°C overnight, and then subjected to chromatography on Dowex 50 with HCl as the eluent, as used previously (Strominger, Park, and Thompson, 1959).

Measured on samples eluted from the Dowex 50 column. The specificity of β-amino acid oxidase and L-alanine-α-ketoglutarate transaminase was employed. The pyruvate formed in each case was measured with reduced nicotinamide adenine dinucleotide and lactic dehydrogenase.

TABLE 3. Incorporation of C14-lysine into cell wall and into cell protein and nucleic acid in Staphylococcus aureus

<table>
<thead>
<tr>
<th>Flask condition</th>
<th>Cell wall</th>
<th>Inhibition</th>
<th>Cell protein and nucleic acid</th>
<th>Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>10,800</td>
<td>%</td>
<td>1,970</td>
<td>%</td>
</tr>
<tr>
<td>Novobiocin, 15 μg/ml</td>
<td>3,450</td>
<td>72%</td>
<td>1,100</td>
<td>44%</td>
</tr>
<tr>
<td>Novobiocin, 100 μg/ml</td>
<td>1,050</td>
<td>90%</td>
<td>970</td>
<td>51%</td>
</tr>
</tbody>
</table>

* Results are expressed as counts per minute per milligram of protein. For details of the method employed, see Nathenson and Strominger (1961).

restricted to its effect on cell-wall synthesis, but includes inhibition of both protein and nucleic acid synthesis. Again, these data are strikingly similar to data obtained with "gentian violet" (Nathenson and Strominger, 1961) and are in contrast to the selective effect of penicillin or D-cycloserine on cell-wall synthesis.

The effect of novobiocin on cell-wall synthesis is, however, a prompt effect of addition of the antibiotic, and occurs at concentrations which are similar to the minimal growth-inhibitory concentration of this antibiotic. At the present time it is impossible, therefore, to state whether inhibition of cell-wall synthesis is the primary lethal effect, whether it is one of several lethal effects, or whether it is secondary to some other primary action of novobiocin. Other investigators working with Escherichia coli have suggested that it causes damage to the membranes of growing cells or that all of its effects may be due to intracellular magnesium binding (Brock and Brock, 1959; Brock, 1962a, b). More recently, it has been suggested that novobiocin damages membrane integrity by inducing an intracellular magnesium deficiency, magnesium ions being necessary to stabilize cell membranes (Brock, 1962b). Novobiocin is a coumarin derivative, and it has been shown, also, to inhibit oxidative phosphorylation in Mycobacterium phlei (Weber and Rosso, 1963). A vitamin K compound is involved in electron transport in this organism, so that inhibition of both oxidative phosphorylation and of cell growth by novobiocin was reversed by vitamin K. It is difficult to find a common denominator between these results and the data reported here. The synthesis of the accumulated nucleotides in S. aureus requires both divalent cation and a large amount of adenosine triphosphate. It is, of course, not certain that the effect of novobiocin on different bacteria is due to the same mechanism of action.

Other investigators (Shockman and Lampen, 1962) have shown that novobiocin inhibits growth of protoplasts of Streptococcus faecalis at the same concentration at which it inhibits intact cells. Growth of these protoplasts was not inhibited by penicillin or by D-cycloserine. Baci- tracin, vancomycin, and rifosti, which have all been implicated as inhibitors of cell-wall synthesis, caused inhibition of protoplast growth as well as of cells, although the concentration of...
ristocetin required to inhibit protoplasts was five times that required to inhibit cells. Isotopic experiments with ristocetin suggest that its effect on cell-wall synthesis is a specific one (Wallas and Strominger, 1963). The inhibition of protoplast growth by novobiocin is in agreement with the large number of effects of this antibiotic in S. aureus. Moreover, it explains the failure of this antibiotic to induce spheroplast formation in Escherichia coli (unpublished data), since continued growth of the protoplast after cell-wall synthesis ceases is a requirement for emergence of the spherical form of E. coli.

Acknowledgments

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Literature Cited


