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Inhibition of Cytochrome Systems of Heart Muscle and Certain Bacteria by the Antagonists of Dihydrostreptomycin: 2-Alkyl-4-Hydroxyquinoline N-Oxides

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Antagonism of streptomycin activity by culture filtrates of *Pseudomonas aeruginosa* was first reported by Sureau, Arquie, Boyer & Saviard (1948). The factor responsible for the antagonism was isolated (Lightbown, 1954) and was shown by Cornforth & James (1954, 1956) to be a mixture of 2-alkyl-4-hydroxyquinoline N-oxides. The present paper describes an investigation of some of the biological properties of these compounds; part of this work has already been briefly reported (Lightbown & Jackson, 1954; Jackson & Lightbown, 1955).

METHODS AND MATERIALS

Assay of dihydrostreptomycin antagonist activity. A modified cup-plate diffusion method (Lightbown, 1954) was employed. Cups containing the solutions of the quinoline N-oxides were placed on plates of deep-seeded nutrient agar containing a concentration of dihydrostreptomycin five times that sufficient to prevent growth of the test organism in the absence of the antagonist. After incubation, sharply defined zones of growth appeared around the cups and the comparative potencies of the samples could be calculated from the diameters of these zones, as in a normal antibiotic cup-plate assay. With large assay plates accommodating 36 cups arranged in a 6 × 6 Latin square fiducial limits of ±5 to 10% were regularly obtained for potency ratios derived from a single plate.

Bacterial strains. *Bacillus subtilis* I.C.I. strain (N.C.T.C. 8236), *Bacillus pumilus*, Mill Hill strain (N.C.T.C. 8241) and typical laboratory strains of *Staphylococcus aureus*, *Escherichia coli* and *Proteus vulgaris* were used in experiments.

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Bacterial growth curves. Nutrient broth (200 ml.) in a 2 l. conical flask was inoculated and incubated, with shaking, for 3 hr. Samples (10 ml.) were then transferred to separate 250 ml. conical flasks. These smaller flasks had special side arms constructed to fit the carrier of the absorptiometer, so that rapid measurements of optical density could be made by tipping the contents of the flask into the side arm. Aeration of all cultures was effected by shaking at 180 oscillations/min., amplitude of stroke 1 in. Optical-density measurements were made at 650 mμ.

Bacterial suspensions and cell-free preparations. Bacteria were grown in nutrient broth in shaken culture or on nutrient agar for 18 hr. at 36°. The cells were collected and washed three times with distilled water by centrifuging. Dry weights were determined by heating at 110° to constant weight, and opacity/dry-weight relationships were measured with a photoelectric absorptiometer.

Cell-free preparations were prepared by shaking bacterial suspensions (density 10–20 mg./ml.) with ballotini beads, size 14, in a Mickle disintegrator. Shaking was continued for 40 min., or until microscopical examination showed the presence of only occasional whole cells. Unbroken cells and large fragments were removed by centrifuging at 800 g in an angle head for 15 min. and the supernatant fluids collected.

Heart-muscle succinic-oxidase preparations were prepared from fresh pig heart by the method of Slater (1949a), except that the minced and washed muscle was homogenized in a high-speed blender for 2 min., instead of grinding with sand. Fat-free dry weights of these preparations were determined by the method of Slater (1949a).

Spectroscopic examination of the different preparations was carried out with either a Zeiss or Beck low-dispersion micro-spectroscope and a 150 c.p. Pointolite.

Reagents. Cytochrome c was obtained from L. Light and Co. Ltd.; the methylene blue was the 'biologically tested' grade obtained from British Drug Houses Ltd. The p-

Table 1. *Maximum solubilities ($\mu\text{g./ml.}$) of 2-alkyl-4-hydroxyquinoline N-oxides in 0.35M phosphate buffers at 22°*

Compound	pH 7.0	pH 9.0
2-n-Heptyl-4-hydroxyquinoline N-oxide	3.5	60
2-n-Nonyl-4-hydroxyquinoline N-oxide	0.3	5.7
2-n-Undecyl-4-hydroxyquinoline N-oxide	—	0.27

Table 2. *Relative activity of various quinoline derivatives in antagonizing dihydrostreptomycin activity against B. pumilus as determined by a modified cup-plate assay*

Derivative	Relative activity
Natural crystalline antagonist	100
2-Methyl-4-hydroxyquinoline N-oxide	<0.1
2-n-Heptyl-4-hydroxyquinoline N-oxide	33
2-n-Nonyl-4-hydroxyquinoline N-oxide	206
2-n-Undecyl-4-hydroxyquinoline N-oxide	66
Mixture of substituted quinoline N-oxides in proportions present in natural antagonist, i.e. 63.7% heptyl, 33.7% nonyl, 2.6% undecyl	110
2-n-Heptyl-3-bromo-4-hydroxyquinoline N-oxide	420
2-n-Heptyl-3-carboxy-4-hydroxyquinoline N-oxide	0
6-n-Heptyl-4-hydroxyquinoline N-oxide	0
2-Methyl-4-hydroxyquinoline	0
2-n-Heptyl-4-hydroxyquinoline	0
2-n-Octyl-4-hydroxyquinoline	0
2-n-Nonyl-4-hydroxyquinoline	0

phenylenediamine used was a recrystallized sample kindly prepared by Dr D. C. Nicholson.

Substituted quinoline N-oxides. These compounds were prepared by Dr Cornforth as described (Cornforth & James, 1956). Some difficulty was experienced owing to the very low solubility of the compounds in water. Approximate solubilities of some compounds are shown in Table 1. Stock solutions of 2-heptyl-4-hydroxyquinoline N-oxide (heptyl N-oxide) were prepared in 0.01N-NaOH at a concentration of 50–100 $\mu\text{g./ml.}$ and of the 2-nonyl-4-hydroxyquinoline N-oxide (nonyl N-oxide) in 0.001N-NaOH at a concentration of 5 $\mu\text{g./ml.}$ For assay of dihydrostreptomycin antagonist activity, dilutions were prepared in potassium phosphate buffer, pH 9.0, 0.035M, at concentrations less than the maximum solubility at pH 7.2, the pH value of the assay medium. Solutions of the nonyl compound in 0.001N-NaOH kept at room temperature decreased in strength, probably owing to adsorption on to the glass container. The concentrations of all solutions were checked immediately before use by measurement of the density at the ultraviolet absorption maximum at 346 m μ . (Cornforth & James, 1956). Because of the low solubility of the nonyl compound, the heptyl compound has been used in most experiments.

Antimycin A was kindly supplied by Dr D. E. Green and was used as a solution in ethanol.

Manometric experiments. Oxygen consumption was measured by the conventional Warburg technique at a temperature of 36°. Equilibration was for 12 min. at a shaking rate of 80 oscillations/min. and the rate was increased to 130 oscillations/min. for the experiments. The total volume of reagents in the flask was 2.9 ml., and when bacteria were used 0.1 ml. of 20% KOH was placed in the centre well, together with a folded filter paper. The inhibitor, when used, was placed in the reaction

vessel; the substrate was added from the side arm after equilibration.

Determinations of succinic-dehydrogenase activity. The Thunberg tube technique was as described by Umbreit, Burris & Stauffer (1949), except that the contents of the tubes were: 0.5 ml. of enzyme preparations or bacterial suspension; 0.5 ml. of 1:4000 methylene blue; 2.0 ml. of 0.1M phosphate buffer, pH 7.0; 0.5 ml. of 0.02M sodium succinate in the hollow stopper. The time for 95% reduction of the methylene blue was determined at 37°.

RESULTS

Dihydrostreptomycin-antagonist activity

Table 2 shows that the ability of the natural mixture of substituted quinoline N-oxides isolated from culture filtrates of *Ps. aeruginosa* to prevent the inhibition of growth of *B. pumilus* by dihydrostreptomycin could be reproduced completely by a mixture of the synthetic compounds. Variations in length of the 2-alkyl side chain affected this antagonist activity of the individual N-oxides which was maximal with a 2-n-nonyl substitution. Introduction of bromine into the 3-position of the heptyl compound greatly increased its activity, whereas the corresponding 3-carboxy compound was inactive.

Similar results were obtained with *Staph. aureus*, except for the 3-bromo derivative of heptyl N-oxide, which was only twice as active as the parent compound instead of approximately thirteen times as active, as with *B. pumilus*.

Fig. 1. Effect of heptyl *N*-oxide and dihydrostreptomycin on growth of *B. subtilis* in nutrient broth. The following were added at 4½ hr.: flask I, 40 μg. of dihydrostreptomycin sulphate/ml.; II, 0.15 μg. of heptyl *N*-oxide/ml.; III, 40 μg. of dihydrostreptomycin sulphate and 0.15 μg. of heptyl *N*-oxide/ml.; IV, control. Optical-density readings were taken every 30 min. Mean values for the four flasks are plotted from 0 to 5 hr., and separate values from 5 to 10 hr. Curves of II and III were identical. *A* indicates the point at which dihydrostreptomycin and heptyl *N*-oxide were added.

The dihydrostreptomycin-antagonist effect of heptyl *N*-oxide is shown in Fig. 1. Logarithmic growth of *B. subtilis* was completely inhibited by 40 μg. of dihydrostreptomycin/ml. after an interval of approximately 30 min. This inhibition was reduced by the simultaneous addition of 0.15 μg. of heptyl *N*-oxide/ml. to the slight inhibition produced by the heptyl *N*-oxide itself. Growth of *B. subtilis* on the surface of nutrient agar was completely inhibited during 24 hr. incubation by the presence of 3 μg. of heptyl *N*-oxide/ml. A concentration of 10 μg. of heptyl *N*-oxide/ml. had no effect on the viability of *Staph. aureus* under the same conditions, although the size of the bacterial colonies was reduced by 75%. No significant antagonism of dihydrostreptomycin activity could be demonstrated with *E. coli*.

Effect on bacterial respiration

Oxygen uptake by washed suspensions of *Staph. aureus* in the presence of succinate was inhibited by heptyl *N*-oxide (Fig. 2).

Fig. 2. Inhibition by heptyl *N*-oxide of oxygen uptake by *Staph. aureus* with succinate as substrate. Flask contents: 4 mg. dry wt. of *Staph. aureus*; 0.4 ml. of 0.1M sodium succinate; 0.2 ml. of 0.01N-NaOH (●); or 0.2 ml. of 0.01N-NaOH containing heptyl *N*-oxide to give a final concentration of 0.35 μg./ml. (O). Endogenous respiration (x). Final phosphate concentration 0.07M, pH 7.3.

The Q_{O_2} (μl. of O_2 /mg. dry wt./hr.) of *B. pumilus* with succinate was lower than that of *Staph. aureus* but the sensitivity to the inhibitor was about the same. Both these organisms showed high endogenous respiration rates which were not reduced by the inhibitor.

In contrast to these results with *Staph. aureus* and *B. pumilus* the O_2 uptakes of whole cell suspensions of *E. coli* and *Pr. vulgaris* incubated with succinate were unaffected by concentrations of heptyl *N*-oxide up to 6 μg./ml. Both these organisms actively oxidized succinate and had strikingly lower endogenous respiratory activity than the two Gram-positive organisms tested.

These effects on succinate oxidation directed attention to possible effects of the inhibitor on the cytochrome system.

Effects on cytochrome oxidation and reduction

Direct visual spectroscopy, by the technique of Keilin (1925), showed that oxidation and reduction of the cytochrome pigments were affected. With the heart-muscle succinic-oxidase system and added succinate it was seen that 0.5 μg. of heptyl *N*-oxide/ml. greatly delayed reduction of cytochromes *a* and *c* but not of cytochrome *b*, the band of which appeared to be slightly intensified.

Washed suspensions of *B. subtilis* showed absorption bands corresponding fairly closely in position to the *a*, *b* and *c* bands of heart muscle, and, with succinate as substrate, reduction of the

oxidized *a* and *c* components was inhibited by heptyl *N*-oxide but reduction of the oxidized *b* component was unaffected.

The strain of *Staph. aureus* showed absorption bands at approximately 625, 605 and 560 $m\mu$, which would correspond to cytochromes a_2 , *a* and b_1 respectively (Smith, 1954*a*). The inhibitor delayed reduction of the a_2 and *a* components, but not that of the b_1 component.

Heptyl *N*-oxide had no effect on the oxidation or reduction of the cytochrome components (a_2 , *a* and b_1) present in whole-cell suspensions of *E. coli* and *Pr. vulgaris*.

Analysis of effects on heart-muscle succinic-oxidase system

In order to elucidate further the site of action of heptyl *N*-oxide, its effects on the different steps between succinic dehydrogenase and cytochrome

Fig. 3. Inhibition by heptyl *N*-oxide of oxygen uptake by heart-muscle succinic-oxidase preparation with succinate as substrate. Flask contents: 1.8 mg. of heart-muscle succinic-oxidase preparation; 0.2 ml. of $5 \times 10^{-4} M$ cytochrome *c*; 0.4 ml. of $0.4 M$ sodium succinate; 0.2 ml. of $0.01 N$ -NaOH (●); or 0.2 ml. of $0.01 N$ -NaOH containing heptyl *N*-oxide to give a final concentration of $0.35 \mu g./ml.$ (○); $0.7 \mu g./l.$ (▲); $6.9 \mu g./ml.$ (×). Final phosphate concentration $0.14 M$, pH 7.3.

oxidase were examined. Fig. 3 shows that oxygen uptake by the complete system with succinate as substrate was inhibited. Oxidation of *p*-phenylenediamine, however, was not affected by $6.9 \mu g.$ of heptyl *N*-oxide/ml., whether or not cytochrome *c* was added.

It has been shown that oxidation of *p*-phenylenediamine is brought about by cytochrome oxidase, cytochrome *a* and cytochrome *c* (Slater, 1948). The failure of heptyl *N*-oxide to inhibit oxidation of this compound suggested that its site of action was between succinate and cytochrome *c*. The possibility that the inhibition of the oxidation of cytochrome *b*, already observed, was due to an effect on succinic dehydrogenase or cytochrome *b* itself was investigated by means of the Thunberg technique. No increase in reduction time of methylene blue was produced by the addition of $10 \mu g.$ of heptyl *N*-oxide/ml. to the reaction mixture. It seemed possible that the methylene blue might have inactivated the *N*-oxide, thus preventing an effect on succinic dehydrogenase. The antagonist activity of the *N*-oxide with *B. pumilus* as test organism was unaffected, however, by the addition of methylene blue in the same proportion as in the Thunberg tubes. It was unlikely, therefore, that any modification of the *N*-oxide molecule had occurred, and it would appear that succinic dehydrogenase is not affected by this inhibitor.

When spectroscopic examinations were made on heart-muscle preparation to which had been added successively succinate, heptyl *N*-oxide and oxaloacetate or malonate, it was found that the strong cytochrome *b* band was partially oxidized by the addition of methylene blue. The addition of 2:6-dichlorophenolindophenol (dichloroindophenol) in place of methylene blue to a similar preparation brought about a more rapid and complete disappearance of the band of reduced cytochrome *b*, coincidentally with the reduction of the dye. When this was repeated without added oxaloacetate or malonate the band of reduced cytochrome *b* decreased in intensity but did not completely disappear. Aeration of this preparation resulted in the disappearance of the bands of reduced

Fig. 4. Electron transport in heart-muscle succinic-oxidase system, showing possible site of action of heptyl *N*-oxide. X represents unknown factor (Slater, 1949*b*; Potter & Reif, 1952). The arrows indicate direction of electron transfer. The interrupted line indicates the possible site of action.

cytochromes *a* and *c*, and the *b* band became fainter. After cessation of aeration the three bands reappeared or intensified, but the time for return of the *a* and *c* bands was much less than when heptyl *N*-oxide was present without dichloroindophenol. Thus the dichloroindophenol had apparently partially overcome the inhibition due to the heptyl *N*-oxide. Results in all respects similar to these were obtained with antimycin A in place of heptyl *N*-oxide. In contrast to the behaviour of reduced cytochrome *b* in the inhibited system containing either of the two dyes was the finding that the heptyl *N*-oxide inhibited auto-oxidation of cytochrome *b*. Keilin & Hartree (1939) described the auto-oxidation of cytochrome *b* in heart-muscle succinic-oxidase preparation inhibited with oxaloacetate and cyanide. In this system aeration caused the cytochrome *b* band to disappear and, using the same technique, we have found that the heptyl *N*-oxide prevented the disappearance of the reduced cytochrome *b* band even after prolonged aeration; similar results were obtained with antimycin A.

The ability of dichloroindophenol to counteract the inhibitory effect of heptyl *N*-oxide in heart-muscle succinic-oxidase system has been confirmed manometrically. Results were obtained resembling those of Potter & Reif (1952), who examined the effect of certain dyes on the antimycin A-inhibited heart-muscle system.

Effects on cell-free preparations of Escherichia coli and Proteus vulgaris

The failure of heptyl *N*-oxide to antagonize significantly the activity of dihydrostreptomycin against *E. coli* and the lack of inhibition of growth, oxygen consumption and cytochrome reduction with both *E. coli* and *Pr. vulgaris* suggested the possibility that the *N*-oxide did not penetrate into the cell.

It was found that when washed whole suspensions of *B. subtilis* or *Staph. aureus* were suspended in a solution of heptyl *N*-oxide in phosphate buffer at pH 7.0 and then centrifuged, 100 and 80 % respectively of the antagonist activity (antidihydrostreptomycin activity) was lost from the supernatant solution. By extracting the centrifuged cells with 0.01 *N*-NaOH at 100°, 10–15 % of the activity could be recovered.

Similar experiments performed with *E. coli* and *Pr. vulgaris* showed that the whole of the activity remained in the supernatant fluid after centrifuging. When cell-free preparations of *E. coli* and *Pr. vulgaris* were used, however, 90 % of the activity of the added heptyl *N*-oxide was removed by centrifuging at 17 000 *g*. All of this activity could be recovered from the insoluble deposit by extraction with 0.01 *N*-NaOH at 100°.

Direct spectroscopic examination of the cell-free preparations of *E. coli* and *Pr. vulgaris* showed absorption bands at 625 and 560 *mμ*, corresponding to cytochromes *a*₂ and *b*₁ as in the whole cells, but the *a*₂ band was rather faint. Whereas, however, in whole cells the oxidation and reduction of these cytochrome components with added succinate was unaffected by the addition of heptyl *N*-oxide, in the cell-free preparations reduction of both oxidized cytochromes *a*₂ and *b*₁ was inhibited. This inhibition of reduction of oxidized cytochrome *b*₁ is in direct contrast to the behaviour of the cytochrome *b* components in heart-muscle succinoxidase, the *b* component in *B. subtilis* and the *b*₁ in *Staph. aureus*.

Manometric experiments showed that oxygen uptake by cell-free preparations of *E. coli* with succinate as substrate was inhibited by the heptyl *N*-oxide (Fig. 5). Similar results were obtained with *Pr. vulgaris*. The inhibition was less than that produced by comparable concentrations of the inhibitor with whole-cell suspensions of *Staph. aureus*. There was, however, a suggestion that the '*b*₁' component of the Gram-negative organisms tested was complex. Although aeration of suspensions, in the presence of inhibitor, caused the disappearance of the main part of the absorption at 560 *mμ*, a faint band persisted in spite of vigorous aeration.

Fig. 5. Inhibition by heptyl *N*-oxide of oxygen uptake by *E. coli* cell-free preparation with succinate as substrate. Flask contents: *E. coli* preparation, 0.5 mg. dry wt.; 0.4 ml. of 0.1 *M* sodium succinate; 0.2 ml. of 0.01 *N*-NaOH (●); or 0.2 ml. of 0.01 *N*-NaOH containing heptyl *N*-oxide to give a final concentration of 0.69 *μg*/ml. (○); 6.9 *μg*/ml. (×). Final phosphate concentration, 0.14 *M*, pH 7.3.

Table 3. *Relative inhibitory activities of various substituted quinoline N-oxides on oxygen uptake by heart-muscle succinic-oxidase system*

Compound	Approximate relative activities
2- <i>n</i> -Heptyl-4-hydroxyquinoline <i>N</i> -oxide	1
2- <i>n</i> -Heptyl-3-bromo-4-hydroxyquinoline <i>N</i> -oxide	2.5
2- <i>n</i> -Nonyl-4-hydroxyquinoline <i>N</i> -oxide	3.0
2- <i>n</i> -Undecyl-4-hydroxyquinoline <i>N</i> -oxide	3.0
6- <i>n</i> -Heptyl-4-hydroxyquinoline <i>N</i> -oxide	0

Effect of other quinoline N-oxide derivatives on heart-muscle succinic-oxidase system

The inhibitory activities of certain other derivatives on the heart-muscle succinic-oxidase system, when compared manometrically with that of heptyl *N*-oxide, are shown in Table 3.

DISCUSSION

It is clear from the results presented that both the heart-muscle and bacterial cytochrome systems are sensitive to the inhibitory action of micromolar concentrations of the heptyl *N*-oxide. In the heart muscle the site of action is between succinic dehydrogenase and cytochrome *c*. The presence of an unknown factor, in addition to cytochrome *b*, participating in electron transport between the dehydrogenase and cytochrome *c* in this system has been postulated by several workers, e.g. Straub's S.C. factor (1942) and Slater's factor (1949*b*). The evidence for such a factor is circumstantial and based on inactivation studies, which show that the succinic-oxidase system can be inactivated without inactivating either cytochrome oxidase, cytochromes *c* and *a* or succinic dehydrogenase and cytochrome *b*. As has been stressed by Keilin & Hartree (1949) and Slater (1949*c*), such inactivation could be due either to specific interference with an unknown intermediate between cytochromes *c* and *b* or to some change in the spatial relationship of the different components within the insoluble system which they form. The latter explanation has been claimed by Keilin & Hartree (1949) and Slater (1949*c*) to account for the evidence for the existence of the S.C. factor. More recently, however, Widner, Clark, Neufeld & Stotz (1954) have produced further evidence for the existence of the S.C. factor which they claim to have concentrated, although they have not been able to separate the factor from succinic dehydrogenase and cytochrome *b*. The step between cytochromes *b* and *c* in heart-muscle succinic oxidase has been shown by Potter & Reif (1952) to be sensitive to antimycin A, at very low concentrations which do not affect electron transport on either side of the *b* to *c* step. This inhibition, which is very specific, has been assumed to be due to combination with a factor

between cytochromes *b* and *c* rather than to a disturbance of spatial relationship which would be expected to affect cytochrome-oxidase and succinic-dehydrogenase activities. So far, however, since no factor has been isolated and characterized, direct evidence cannot be obtained.

The effects of the alkyl-substituted quinoline *N*-oxides on heart-muscle succinic oxidase resemble those of antimycin A very closely and suggest that the same site of action is involved in this system. Whereas, however, the substituted quinoline *N*-oxides are as active in inhibiting certain bacterial cytochrome systems, antimycin A is inactive. It has been suggested (Smith, 1954*b*) that the failure of antimycin A to inhibit cytochrome pathways in bacteria may be due either to failure to penetrate the cell or to an absence of the 'Slater factor' from the bacteria. Even with cell-free preparations of *E. coli* and *Staph. aureus* we have been unable to demonstrate inhibition of cytochrome or succinate oxidation by 0.7 µg. of antimycin A/ml.; so it seems improbable that lack of penetration into the cell accounts for the absence of sensitivity.

In addition to the results here reported, it is also known that the heptyl *N*-oxide inhibits oxidation of reduced Co I, both by the heart-muscle system and cell-free bacterial extracts, but that the diaphorase activity of the heart-muscle preparation is unaffected (Jackson & Lightbown, 1956). This further emphasizes the similarity of the action of the heptyl *N*-oxide and of antimycin A in the heart-muscle system, in which oxidation of reduced Co I has been claimed to involve an unknown factor (Slater, 1950) and is inhibited by antimycin A (Potter & Reif, 1954). The inhibition of such oxidation by the heptyl *N*-oxide in heart muscle, and in the *E. coli* and *Staph. aureus* preparations again suggests that some similar sensitive step is present in all and that it is concerned with the same processes in the different systems.

It seems unlikely that the mechanism of inhibition will be explained until the affinity of isolated individual components of the cytochrome system for inhibitors such as the substituted quinoline *N*-oxides or antimycin A can be tested. If the inhibition is due to surface-active interference with the spatial relationships of the components of the pathway, the surface activity must be highly

specific, disturbing only one link in the chain in heart-muscle succinic oxidase and producing a similar effect in bacteria without affecting the viability of these cells. Such a mode of action of the inhibitor would necessitate only the adjacent cytochromes at the sensitive step being separated, without the inhibitor combining with the sites active in electron transport. In order to do this the inhibitor must combine specifically with either cytochrome *c* or *b*, in the heart-muscle preparation, or with an intermediate factor if it exists. That the substituted quinoline *N*-oxides and antimycin A are highly active against heart-muscle succinic oxidase in the presence of excess of added cytochrome *c* would suggest that cytochrome *c* is not the site of action. It is more difficult to exclude the possibility that the site of action is cytochrome *b*. Until cytochrome *b* is isolated in a functionally active form, only spectroscopic evidence can be obtained which is difficult to evaluate. However, in the inhibited succinic-oxidase system the cytochrome *b* can still be readily reduced by the dehydrogenase and oxidized by dichloroindophenol which, if it is a single component, would suggest that it is unaltered. It is claimed by Green & Beinert (1955) that the cytochrome *b* component contains several haem groups, and it would appear possible that the dyes may be linked with a group different from that linking the cytochrome oxidase and cytochromes *a* and *c*. In the absence of further evidence, the most plausible explanation of our results seems to be that the site of action is an unknown component in the electron-transport pathway between cytochromes *b* and *c*. If such a factor is the site of action it must be auto-oxidizable and responsible for the apparent auto-oxidation of cytochrome *b*, since this is inhibited by the heptyl *N*-oxide and antimycin A. This effect on auto-oxidation of cytochrome *b* is not observed with other inhibitors such as urethane (Keilin & Hartree, 1939) and would suggest a specific interference with oxidation rather than a physical effect on spatial relationship.

Two explanations of the failure of antimycin A to affect the bacterial systems seem possible. There might be a common step sensitive to heptyl *N*-oxide and insensitive to antimycin A in both the heart-muscle and bacterial systems, but if this is so it is necessary to postulate a second step which is antimycin-sensitive between cytochromes *b* and *c* in the heart-muscle system. It is perhaps more likely that the two systems possess functionally similar components sensitive to heptyl *N*-oxide but differing slightly in structure. The differences in the spectroscopic absorption maxima of the reduced cytochromes in the bacteria and heart muscle imply differences in chemical structure, although the pigments appear to be functionally

similar. The structure of antimycin A is not known, but its molecular weight is at least twice that of the heptyl *N*-oxide (Ahmad, 1949). This difference is not large, but it is conceivable that differences in spatial relationship in the organized bacterial-cytochrome systems might prevent access of the large antimycin A molecule to the sensitive site.

The behaviour of the cytochrome *b*₁ components of *E. coli* and *Pr. vulgaris*, in the cell-free preparations treated with heptyl *N*-oxide differed strikingly from that of the *Staph. aureus* *b* component (usually also called *b*₁). Whereas reduction of the oxidized pigments was inhibited in the former cases, in the latter case oxidation of the pigment was prevented. This suggests that in spite of the similarity of the absorption bands of the reduced forms of these substances, the *b*₁ components of *E. coli* and *Pr. vulgaris* occupy a different level in the respiratory chain, relative to the heptyl *N*-oxide sensitive step, from that of the *Staph. aureus* *b*₁ component. This does not exclude the possibility that *E. coli* and *Pr. vulgaris* have a 'b' component which behaves similarly to that of *Staph. aureus*. The application of quantitative spectroscopic methods would be necessary to assess the significance of a portion of the *b*₁ band of *E. coli* and *Pr. vulgaris* preparations which persists in spite of vigorous aeration in the presence of heptyl *N*-oxide. The asymmetric nature of the *b*₁ band of *E. coli* and the possibility that it might be composed of two fused bands belonging to two distinct compounds has been mentioned by Keilin & Harpley (1941).

The mode of action of the substituted quinoline *N*-oxides in antagonizing the inhibitory action of dihydrostreptomycin is not at present clear. Concentrations of the heptyl *N*-oxide, which are effective against dihydrostreptomycin, are sufficient to produce marked inhibition of electron transport in the cytochrome systems of the organisms studied, but by themselves have relatively little effect on the growth rate in broth. The possibility that the inhibition of electron transport and antagonism towards dihydrostreptomycin are related is suggested by the parallelism between the two activities in the various alkyl *N*-oxides; maximum activity both for antagonism and inhibition of cytochrome electron transport was obtained with a 2-alkyl chain length of nine carbons. Removal of the oxygen atom from the nitrogen resulted in loss of both activities, and introduction of a bromine atom in the 3-position increased the cytochrome effect as well as antagonist activity. Loss of both activities also resulted when the heptyl side chain was moved from the 2- to the 6-position. It is perhaps not surprising, considering the association of the cytochrome inhibition and

antagonist activities, that antimycin A was found not to counteract the inhibitory action of dihydrostreptomycin on bacteria.

SUMMARY

1. 2-Alkyl-4-hydroxyquinoline *N*-oxides antagonized the inhibitory activity of dihydrostreptomycin against *Bacillus subtilis* and *Staphylococcus aureus* but not *Escherichia coli* or *Proteus vulgaris*.

2. The dihydrostreptomycin antagonist activity was affected by the length of the 2-alkyl chain, being maximal with a nonyl side chain. The nitrogen oxide group was essential for activity.

3. 2-*n*-Heptyl-4-hydroxyquinoline *N*-oxide (heptyl *N*-oxide) inhibited heart-muscle succinic-oxidase activity but not cytochrome oxidase or succinic dehydrogenase or reduction of cytochrome *b*.

4. Oxidation of reduced cytochrome *b* by cytochrome *c* in the heart-muscle preparation was inhibited by heptyl *N*-oxide. The inhibition was strikingly reduced by 2,6-dichlorophenolindophenol.

5. Heptyl *N*-oxide inhibited growth of *B. subtilis* and *Staph. aureus*. This inhibition probably occurred as a result of interference with terminal respiration, and was associated with effects on cytochrome oxidation and reduction comparable to those found with heart-muscle succinic oxidase.

6. Heptyl *N*-oxide had no effect on respiration of whole cells of *E. coli* and *Pr. vulgaris*, but inhibited respiration of their cell-free preparations. The failure to inhibit respiration of whole cells is probably due to non-penetration of the cell by heptyl *N*-oxide.

7. The effect of heptyl *N*-oxide on oxidation of cytochrome *b*₁ in *E. coli* and *Pr. vulgaris* suggests that these pigments are functionally different from the cytochrome *b* and *b*₁ of heart muscle and *Staph. aureus* respectively.

8. No qualitative difference was observed in the effects of heptyl *N*-oxide and antimycin A on the heart-muscle succinic-oxidase system. Antimycin A was, however, inactive against the bacterial-cytochrome systems tested, even in cell-free preparations.

9. The modifications of the quinoline *N*-oxide molecule which resulted in loss or reduction of dihydrostreptomycin-antagonist activity were accompanied by loss or reduction of the effect on cytochrome electron transport.

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