ANTIBACTERIAL CHEMOTHERAPY[1,2]

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Introduction

This article departs from the usual pattern of the *Annual Review of Pharmacology*, which ordinarily undertakes a more-or-less exhaustive survey of a specified field for a stated period. We have chosen, instead, to select a limited number of topics in which interest is active. Primarily, however, these will be areas which show recent progress toward the elucidation of a substance's locus and mechanism of action.

A definition of locus of action in the biochemical sense may be quite different from that of mechanism of action in the chemotherapeutic sense. The latter, ideally, defines, at the molecular level, the basis for the selective toxicity of the drug. Some of the antibiotics, e.g., penicillin, have as their targets structures that are absent from the host, and their differential effects require little comment. Some, however, affect processes that are common to parasite and host, e.g., streptomycin; and, while significant progress in the definition of their biochemical loci of action has been made, the task remains to provide an adequate understanding of their selectivities.

Exciting progress has been made in the identification of the biochemical processes affected by a number of these agents. It appears that streptomycin and possibly other antibiotics attach to the ribosome. The demonstration that this can lead to predictable misreading of "the code" has important implications for molecular biology. This area provides another example of the interaction of the empirical and the theoretical, the practical application and the fundamental, that is advantageous to both.

A considerable array of new antibiotics has been left unreviewed. The reader is referred to *Antimicrobial Agents and Chemotherapy* for 1961, 1962, and 1963 (1, 2, 3) and the *Proceedings of the Third International Congress of Chemotherapy* (4) for introductions to these. Similarly, a new synthetic antibacterial, nalidixic acid (5), has been passed over for the reason that little or nothing has been reported about its mode of action.

Inhibitors of Cell-Wall and Cell-Membrane Synthesis and Structure

The penicillins (mode of action).—The bulk of the evidence accumulated in the past dozen years on the locus of action of penicillin in bacteria has

[1] Survey of the literature for this review was completed in August, 1964.

[2] The following abbreviations are used: Ala (alanine); Glu (glutamic acid); Lys (lysine); Gly (glycine); UDP (uridine diphosphate); Leu (leucine); *p*-AB (*p*-aminobenzoic acid); Phe (phenylalanine); Thr (threonine); Dab (α, γ-diaminobutyric acid); Ipel (isopelargonic acid); Poly U (polyuridylic acid); NAc. Mur. (N-acetylmuramic acid); and NAc. Gm (N-acetylglucosamine).

dealt with inhibition of the formation of cell-wall components. The development of this view has aided and has been stimulated by progress in understanding the nature and composition of bacterial cell walls [see Perkins (6) for a comprehensive review]. The mutual interaction in these fields has been of great benefit to both. This area of investigation was suggested initially by Park's report which identified three uracil-containing nucleotides isolated from cells of *Staphylococcus aureus* grown in the presence of penicillin (7). The largest of these nucleotides (the "Park nucleotide") contains one molecule each of muramic acid, lysine, and glutamate and three moles of alanine per mole of uridine diphosphate. The amino acids and muramic acid were later found in the cell wall of *S. aureus* in the same proportions, leading Park & Strominger (8) to propose that the Park nucleotide might be a precursor of the normal mucopeptide component of the cell walls. The mechanism of action of penicillin, thus, was postulated as an interference with the synthesis of this mucopeptide by inhibition of the transfer of the muramyl peptide from the uracil nucleotide to acceptors in the cell wall.

It was thought that the inhibition of the formation of the mucopeptide component of cell walls would cause the cell walls to lose their rigidity. Such an alteration in the cell wall previously had been observed by Lederberg (9), who studied the action of penicillin on growing cells of *Escherichia coli*. When grown in the presence of penicillin and high concentrations of sucrose, the cells lost their rod shape and became spherical; when the sucrose concentration was then lowered, the cells burst, suggesting a loss of rigidity of the cell wall structure.

Convincing evidence of the role of the Park nucleotide as a precursor of mucopeptide has been forthcoming recently. The biosynthesis of this nucleotide from UDP-N-acetyl muramic acid and the appropriate amino acids, by enzymes isolated from *S. aureus*, has been demonstrated (10, 11). The amino acids apparently were added in a stepwise manner, concluding with the addition of the dipeptide, D-alanyl-D-alanine. The final product was UDP-N-acetyl muramyl-L-alanyl-D-glutamyl-L-lysyl D-alanyl-D-alanine, the Park nucleotide. The incorporation of the muramyl peptide into mucopeptide (Figure 1) by a cell-free system isolated from *S. aureus* has now been reported by Chatterjee & Park (12). A particulate fraction was obtained from the cells consisting of membrane material and ribosomes, which apparently catalyzed the polymerization of UDP-muramyl peptide with UDP-N-acetyl glucosamine and the formation of polyglycine crosslinks (Figure 1). Further fractionation of the particulate portion revealed that the ribosomes are not essential but only stimulatory to the action of the membrane material in the incorporation of the Park nucleotide into mucopeptide, while they are essential to the incorporation of glycine. The incorporation of glycine also required a nondialysable supernatant fraction and was inhibited by ribonuclease, suggesting that the polyglycine crosslinks of the mucopeptide may be formed by the usual protein-synthetic mechanisms of the cell. This demonstration of the utilization of the Park nucleotide in the synthesis of mucopep-

tide has supplied evidence of the physiological importance of the product which accumulates in cells during penicillin inhibition, and it is gratifying that this nucleotide now has been shown to be a precursor of a vital component of the cells.

Inhibition of mucopeptide synthesis in whole cells by penicillin was first reported by Park in 1958 (13). Penicillin inhibited the formation of mucopeptide in a system consisting of cells of *S. aureus*, glucose, uracil, salts, and the cell-wall amino acids. More recently, this type of system has been used to examine the relationship between the concentration of drug required to inhibit mucopeptide synthesis with the concentration required to inhibit growth. These concentrations agreed within a two- to threefold range in

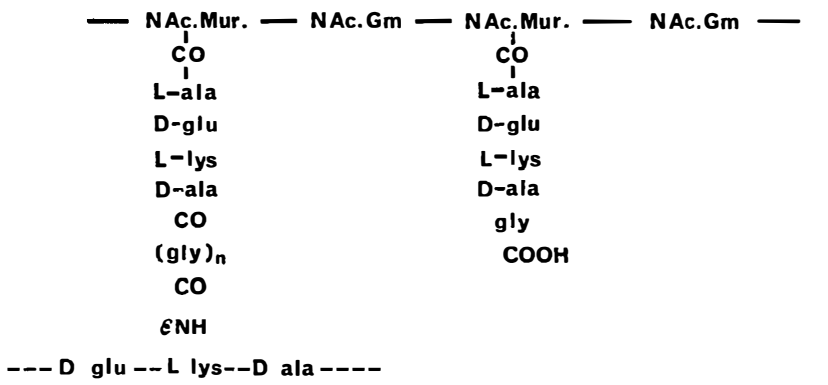
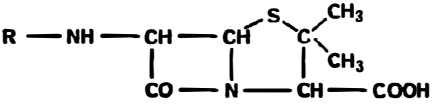


FIG. 1. Proposed structure of mucopeptide [after Perkins (6)].

several penicillin G-sensitive and -resistant (nonpenicillinase-producing) strains of *S. aureus* studied (14) and in a strain of *Bacillus subtilis* (15). The higher concentrations required to inhibit growth of the resistant strains were reflected in the higher concentrations needed to inhibit mucopeptide formation by these strains. The penicillin nucleus, 6-aminopenicillinic acid, and two of the semisynthetic penicillins, methicillin and propicillin (Figure 2) also were tested against *S. aureus*. The order of effectiveness of these drugs on mucopeptide formation was paralleled by their order of effectiveness on growth. Methicillin was equally inhibitory towards penicillin G-sensitive and -resistant organisms, indicating the presence of alternate pathways or different binding to the sensitive and resistant target enzymes.

Although penicillin G is much less active towards gram-negative than gram-positive bacteria, its site of action is apparently the same in both groups. This was suggested by the original observation on the induction of protoplast formation in *E. coli* by penicillin (9). More specifically, Rogers & Mandelstam (16) have reported an inhibition of the synthesis of mucopeptide in whole cells of *E. coli*. Although the levels of inhibitor were much higher



Generic Name	R Group
6-aminopenicillanic acid	H—
Penicillin G (Benzyl penicillin)	CH_2-CO-
Penicillin V	$O-CH_2-CO-$
Phenethicillin	$O-CH(CH_3)-CO-$
Propicillin	$O-CH(C_2H_5)-CO-$
Phenbenicillin	$O-CH(C_6H_5)-CO-$
Methicillin	$CO-$ (2,6-dimethoxyphenyl)
Oxacillin	$CO-$ (5-methyl-3-phenylisoxazol-4-yl)
Cloxacillin	$CO-$ (2-chloro-6-methyl-3-phenylisoxazol-4-yl)
Nafcillin	$CO-$ (2-ethoxy-1-naphthyl)
Diphenicillin	$CO-$ (2-phenylphenyl)
Ampicillin	$CH(NH_2)-CO-$

FIG. 2. The penicillins.

than were required for similar inhibitions using gram-positive bacteria, the same close relationship between the inhibitory concentration and the concentration required for interference with mucopeptide formation was observed. The broad-spectrum semisynthetic penicillin, ampicillin (Figure 2), was more active than penicillin G, as was predicted from its lower inhibitory concentration.

The hypothesis that penicillin inhibits cell-wall formation by its interference with the transfer of muramyl peptide from the Park nucleotide to cell-wall acceptors has been strengthened by work on the cell-free mucopeptide synthesizing system referred to above (12). When the active particulate fraction was isolated from cells grown in the presence of penicillin, the amount of mucopeptide subsequently synthesized during incubation *in vitro* was greatly reduced. Since the introduction of an inhibitor of protein synthesis (chloramphenicol) into the growth medium produced a similar inhibition, the authors suggested that only newly synthesized enzyme was active. The antibiotics had no effect when added directly to the reaction mixtures, which agrees well with the long-standing observation that penicillin kills susceptible bacteria only during active growth (17).

Thus, it appears that penicillin blocks the utilization of UDP-muramyl peptide for the biosynthesis of mucopeptide, possibly by acting as an antimetabolite of N-acetylmuramic acid. Collins & Richmond (18) compared solid models of penicillin and N-acetylmuramic acid and pointed out three areas of similar length, direction, position in space, and strength of hydrogen bonding. They suggested that penicillin could bind tightly to the active site of an enzyme which deals with N-acetylmuramic acid. The recent work on the mucopeptide synthesizing system of *S. aureus* (12) suggests that the binding site involved may be on the polymerizing enzyme.

The penicillins (clinical aspects).—The recent development of methods of obtaining the nucleus of the penicillin molecule, 6-aminopenicillinic acid has paved the way for the synthesis of a number of penicillins with unnatural side chains (Figure 2). Some of these modified penicillins have been found to have certain clinical advantages over penicillin G, and are now widely used. The bacteriological and clinical aspects of these semisynthetic penicillins have been extensively reviewed in the past few years (19, 20, 21, 22, 23) and will be mentioned here only briefly.

These modified penicillins are usually placed in one of three groups, according to the particular advantage over penicillin G which is most evident in each. The first group comprises the acid-resistant penicillins. These are α-phenoxyalkyl derivatives which possess the same antibacterial spectrum as penicillin G but are acid resistant, and, therefore, may be administered orally. These include phenoxymethyl penicillin (penicillin V), phenethicillin, propicillin, and phenbenicillin (Figure 2). They differ to some degree in antibacterial activity, but their better absorption from the gastrointestinal tract makes them preferable to penicillin G for use in susceptible infections (19, 23).

Probably the most important group of semisynthetic penicillins are those resistant to attack by penicillinase: methicillin, oxacillin, cloxacillin, nafcillin, and diphenicillin (Figure 2). These substances are less active than penicillin G against penicillin G-sensitive organisms but are considerably more active against penicillinase-producing strains (19). Methicillin was the first of this group to be introduced and has been widely used. The incidence of methicillin-resistant staphylococci is low (24, 25), but, nevertheless, can be a problem, for methicillin-resistant strains have become endemic in some hospitals (24). Oxacillin, cloxacillin, and nafcillin have an advantage over methicillin, since, in addition to their resistance to penicillinase, they are acid resistant and may be given orally. Recent clinical trials with nafcillin indicate that it is as active as methicillin (26, 27) and penicillin G (26) in infections susceptible to these drugs. If extensive clinical studies confirm the finding that nafcillin has the efficacies of both methicillin and penicillin G, and resistance does not become a problem (26), nafcillin may become the drug of choice in the treatment of both penicillin G-sensitive and penicillinase-producing resistant organisms.

Ampicillin (Figure 2) is representative of a group of broad-spectrum penicillins—those with significant activity against gram-negative as well as gram-positive bacteria (19). Ampicillin is acid resistant, but not penicillinase resistant; it is readily hydrolyzed by the penicillinases of both gram-positive and gram-negative bacteria (28, 29). Several workers have suggested that natural and acquired resistance to various penicillins, including ampicillin, may be attributed to the penicillinase activity of the resistant organisms (29, 30).

Those semisynthetic penicillins which are hydrolyzed slowly, or not at all, appear to be capable of acting as inhibitors of penicillinase (31). The possibility of improving the activity, *in vitro*, of ampicillin against penicillinase-producing bacteria by the simultaneous administration of subinhibitory concentrations of penicillinase-resistant derivatives has been tested (28, 32). The minimum effective concentrations of ampicillin (and even penicillin G) against certain gram-negative bacteria were lowered by the presence of methicillin or cloxacillin, but the degree of synergism was not sufficient to be clinically useful (28). Ampicillin has been used for the treatment of gram-negative infections with moderate success (19). Perhaps its greatest significance lies in its being the first of a class of broad-spectrum penicillins. Its activity against gram-negative bacteria has provided the basis for optimism that other, more powerful derivatives might be found which would increase the armamentarium of agents useful in infections with these organisms.

Cephalothin.—The cephalosporins are a group of antibiotics closely related in structure to penicillin, derived from a species of cephalosporium (see refs. 19 and 23 for recent reviews). The first clinically important one is cephalothin (Figure 3), a semisynthetic compound prepared from 7-aminocephalosporanic acid (34). Cephalothin is a broad-spectrum antibiotic, with significant activity *in vitro* against both gram-positive and gram-negative

bacteria (35, 36). Significantly, it is unaffected by penicillinase, although it and its congeners are inducers of the enzyme (33, 37, 38); and, consequently, it is effective against penicillinase-producing strains of *S. aureus* (36). However, many bacteria have been found to produce an enzyme, called cephalosporinase, which hydrolyzes the β-lactam ring of the cephalosporins (37). The enzyme has penicillinase activity, but purified penicillinase has no effect on cephalothin, and cephalosporinase is much more active against cephalothin than against penicillin (27, 38, 39).

Results from clinical trials of cephalothin indicate that this drug is a useful antibiotic. A compilation of the data from 351 cases recently treated with cephalothin revealed satisfactory responses in 76 percent of the patients, equivocal results in 7 percent, and failure in 17 percent (40–44). The drug can be used with even greater success than indicated by these figures, for many of the failures were in patients with infections caused by organisms insensitive to the drug *in vitro*. Cephalothin is completely inactive against *Pseudomonas* (40, 42, 44), and the activity for gram-negative bacteria is

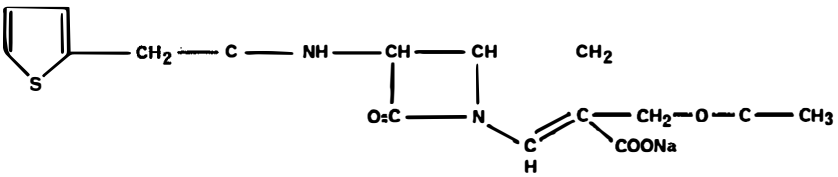


FIG. 3. Cephalothin.

lower than for gram-positive (40, 43). Successful clinical results were obtained against many serious gram-positive infections, as well as certain gram-negative urinary infections (40–43, 45). This drug appears to be particularly useful for the treatment of penicillin-susceptible infections in patients with allergic responses to penicillin, for no cross sensitivity has been noted in these patients (40, 42, 44).

The mode of action of cephalothin is thought to be similar to that of penicillin, since the morphological changes which occurred in cephalothin-treated cells were similar to those observed with penicillin (46). More directly, Chang & Weinstein (46) found that cephalothin inhibited the incorporation of C^{14}-lysine into cell wall mucopeptide without affecting its incorporation into cell protein. The accumulation of N-acetylglucosamine, a mucopeptide precursor, also was noted. Further work is required to determine whether the specific site of interference is the same for cephalothin and penicillin.

OTHER INHIBITORS OF CELL-WALL FORMATION

A number of antibiotics, in addition to penicillin and the cephalosporins, have been demonstrated to inhibit cell wall formation. These include D-

cycloserine, bacitracin, vancomycin, novobiocin, and the ristocetins. They are less important, clinically, than the penicillins, and, except for cycloserine, less is known about their loci of action.

Vancomycin was demonstrated to inhibit the incorporation of labeled cell-wall amino acids into cell-wall material (mucopeptide); the drug had no effect on the incorporation of these amino acids into cell protein (47, 48, 49). An accumulation of compounds containing N-acetylamino sugars was observed when the cells were treated with concentrations of the drug near the minimum growth-inhibitory level (47); one of these compounds was a nucleotide with the same molar proportions of components as the Park nucleotide. Later it was shown that the Park nucleotide does, indeed, accumulate in *S. aureus* cells inhibited by vancomycin (12). Chatterjee & Park (12) reported that vancomycin inhibited mucopeptide synthesis by a cell-free system when the cellular fraction was taken from cells grown in its presence, but, as had been found with penicillin, vancomycin added *in vitro* to preparations from untreated cells was ineffective.

Not all of the effects of vancomycin can be explained by a penicillin-like locus of action; in fact, a lack of cross resistance between the two drugs has been reported (47). Shockman & Lampen (50) demonstrated inhibition of the growth of protoplasts at approximately the same concentration of drug required to inhibit the growth of normal cells. It has recently been reported that sublytic concentrations of vancomycin caused the leakage of intracellular metabolites from *B. subtilis* (49). The simultaneous administration of magnesium and drug reduced the growth and mucopeptide-inhibitory effects but did not prevent lysis or leakage of metabolites (49).

Bacitracin inhibited mucopeptide synthesis in whole cells (13), as well as in the cell-free system discussed above (12), and caused the accumulation of uridine-containing nucleotides (13). However, like vancomycin, bacitracin also caused lesions to other components of the cell. This drug inhibited the growth of protoplasts (50) and has been reported to suppress induced enzyme formation (51).

Novobiocin has been shown to cause the accumulation of uridine nucleotides (52) similar to the Park nucleotides found during penicillin inhibition. This effect was also reported for restocetin A and B (and partial acid hydrolysis products of them) along with inhibition of the incorporation of labeled amino acids into mucopeptide (53). Cell lysis and accumulation of amino sugars were noted during growth inhibition by 5-fluorouracil (54). Brockman & Chambers (54) felt that there was suggestive evidence for the formation of a 5-fluorouracil analogue of the Park nucleotide.

Although little is known about the actual site and mechanism of action of the cell-wall inhibitors discussed above, the recent report (12) on the nature of the cell-free mucopeptide-synthesizing system leads to some interesting speculations. Many of the effects of these antibiotics could be explained if one assumes that they affect the polymerizing enzyme during the final stages of its formation, resulting in a nonfunctional enzyme (perhaps even a drug-

enzyme complex). This is suggested by the observations that only newly synthesized enzyme is active in the formation of mucopeptide and that it was necessary for the antibiotics to be present during the growth of the cells (i.e. during wall formation, protein synthesis, and enzyme formation) to permit the demonstration of inhibition.

The only antibiotic inhibitor of cell wall synthesis with a locus of action that is well understood at the present time is D-cycloserine, an analog of D-alanine (Figure 4). Its effects on wall synthesis are indicated by the formation of protoplasts and the accumulation of an amino sugar compound when *E. coli* is grown in its presence (55). The accumulated compound was shown to

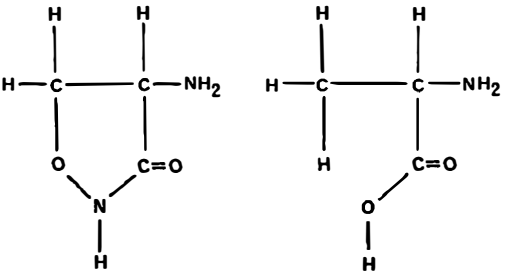


FIG. 4. Comparative structures of D-cycloserine (left) and D-alanine (right).

be identical to the Park nucleotide, except for the omission of the terminal D-alanyl-D-alanine (56). The reversal of cycloserine inhibition of growth in *S. faecalis* by D-alanine (57), the demonstration of the competitive inhibition of the enzymes alanine racemase (catalyses the interconversion of D- and L-alanine) (58), and D-alanyl-D-alanine synthetase (catalyses the synthesis of the dipeptide from D-alanine) (11, 59) have documented the role of cycloserine as an analog of D-alanine. Neuhaus & Lynch (59) recently reported kinetic studies on D-alanyl-D-alanine synthetase from *S. faecalis*. They concluded that the enzyme has two binding sites for D-alanine and that D-cycloserine is a competitive inhibitor at both sites. Modification of the cycloserine molecule resulted in different effects at the two sites, indicating different requirements for binding at each. Thus, interference by cycloserine with cell-wall formation is caused by inhibition of the formation of D-alanyl-D-alanine at three different sites. As a consequence of these blocks, one of the immediate precursors of mucopeptide, the Park nucleotide, remains incomplete, and mucopeptide synthesis is stopped.

THE POLYMYXINS

The polymyxins are a group of polypeptide antibiotics (Figure 5) which are bactericidal to most gram-negative bacteria, including *Pseudomonas*. They act by disorganizing the bacterial cell membrane, causing the loss of

low-molecular-weight metabolites from the cells and the disruption of cytoplasmic components (60). This was first indicated by the work of Newton on the release of 260 μ-absorbing material from polymyxin-treated cells (61) and on the penetration of a fluorescent dye into such cells (62). The detergent-like lytic action of polymyxin B has recently been demonstrated in *Vibrio cholerae* (63) and *Leptospirae canicola* (64). It has been suggested that the polymyxins attach to anionic binding sites on the membrane, for this action is prevented by the addition of cations such as Mg^{++} (60, 63, 65, 66).

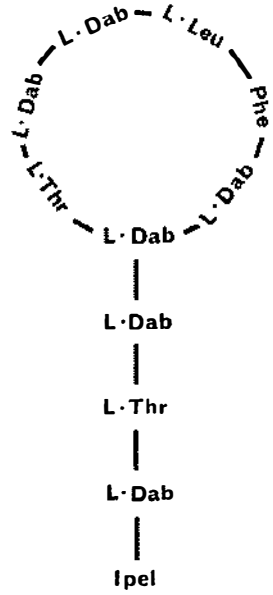


FIG. 5. Polymyxin B.

Much of the recent literature on the polymyxins has been concerned with comparisons between polymyxin B and colistin. Colistin has been found to be a member of the polymyxin group, being identical in structure and biological activity to polymyxin E (67, 68). The mode of action is apparently the same as that of polymyxin B (69, 70), as is the antibacterial spectrum (71). The injectable form, colistin methanesulfonate, is less toxic than the free polymyxin but also has lower antibacterial activity (71). The problem of toxicity is still an important one, for, as was pointed out by Petersdorf & Plorde (72), when colistin methanesulfonate is given in doses sufficient to treat systemic infections, 30 to 50 percent of the cases show toxic effects. However, since no other drug is available for the treatment of *Pseudomonas* infections, the polymyxins must be used. Successful results have been obtained from their use in *Pseudomonas* infections of the urinary tract (72) and the cornea (73).

in burn patients (74), and in systemic infections (72). The toxicity of the polymyxins might be lowered and their efficacy increased by their use in combination with sulfonamides. The synergism of these two types of agent has been demonstrated *in vitro* (75) and in preliminary trials in man (76). The polymyxin is believed to affect the cell membrane, allowing more of the sulfonamide to enter the cell (75).

Inhibitors of Protein Synthesis

Streptomycin.—Although a comprehensive theory of the action of streptomycin (Figure 6) has still to be advanced, the major events following its

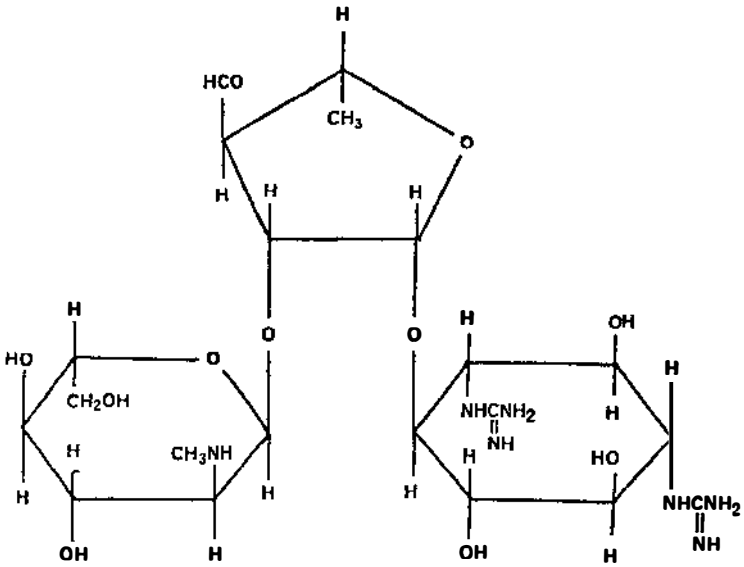


Fig. 6. Streptomycin.

administration to living cells have been described and the reader is referred to the excellent review of Davis & Feingold (77) and the recent article of Hurwitz (78). The assignment of physiological significance to the observations still awaits completion after 20 years of vigorous investigation. Some hypotheses and suggestions that have been advanced recently are described below.

A suggested mechanism for the action of streptomycin assigns primary significance to damage to the cell membrane. On exposure to streptomycin, cells of *E. coli* excrete considerable quantities of material absorbing at 260 μ. This has been identified as adenosine- and guanosine-5′-phosphates (79). On the other hand, the excretion of the nucleoside-2′- or -3′-phosphates did not occur (80).

Uptake of the drug appears to be dependent on the continued synthesis of cell wall (81). Environmental conditions that prevented growth and bactericidal action also decreased the uptake of the drug (82). The antibiotic is apparently assimilated into a bound form that is not readily displaced by washing in the presence or absence of streptomycin (83). However, lysis of the protoplasts of *Bacillus* megaterium, previously treated with streptomycin-C^{14}, resulted in the recovery of 99 percent of the drug.

After studying the nature of the uptake of streptomycin-C^{14} in *E. coli*, Anand, Davis & Armitage suggested that the primary site of action of the drug was the cell membrane (84). They observed a short initial adsorption, presumably on the surface, followed by a second and longer period of uptake. The secondary uptake was presumably a result of damage inflicted during or after the first phase of uptake. This theory helped to explain the observation that other drugs which damage the permeability barrier of the cell also promoted the entry of streptomycin and showed action synergistic with it. The membrane as a site of action for streptomycin also was indicated by the studies of Landman & Burchard on a streptomycin-dependent *Salmonella* (85). In the absence of streptomycin, these organisms cannot form septa and grow into long filaments but do produce L-form colonies when grown on soft agar containing streptomycin. It was concluded by the authors that, "The site of activity of streptomycin is at the bacillary membrane." In this regard, streptomycin has also been shown to cause the lysis of protoplasts of *S. flexneri* (86).

Recently, Tzagoloff & Umbreit have suggested that the killing effect of streptomycin is not a result of the excretion of nucleotides, since in strains of *E. coli* streptomycin killed cells without causing nucleotide excretion (87). Furthermore, certain strains were killed before nucleotide excretion had begun.

During the period of the investigations discussed above, it was becoming increasingly clear that streptomycin possessed the ability to interfere with protein synthesis. Fitzgerald, Bernheim & Fitzgerald (88) reported that the formation of an adaptive enzyme of a certain *Mycobacterium* was inhibited by streptomycin. Later work showed that in resting cells of a streptomycin-sensitive strain of this *Mycobacterium friburgensis* streptomycin inhibited the incorporation of C^{14}-DL-tyrosine into protein. No such effect was noted when a streptomycin-resistant strain was employed (89). In addition, Flaks, Cox & White have shown that streptomycin interferes with protein synthesis *in vitro* (90). Using a system of the type employed by Nirenberg (91), they found streptomycin at a concentration of $10^{-5}M$ to suppress the incorporation of phenylalanine into protein by 95 percent. For maximum inhibition, it was necessary to add the streptomycin before the addition of the polyuridylic acid "messenger".

Much recent research with the primary site of action of streptomycin involves the interaction of the antibiotic with the subcellular ribosomal particles. In 1961, Spotts & Stanier (92) proposed that streptomycin sensi-

tivity, resistance, and dependence all resulted from alterations in the structure of ribosomes that affect their ability to combine with messenger RNA. Dependence was seen as a genetic modification of ribosomes which prevented the proper attachment of the RNA. Streptomycin counteracted this defect and resulted in the production of a functional ribosome-RNA complex. Evidence that the action of streptomycin was indeed localized at the ribosome was presented by Speyer, Lengyel & Basilio (93). In a cell-free system containing ribosomes of a streptomycin-sensitive strain of *E. coli*, streptomycin decreased phenylalanine-C^{14} uptake unto protein. The drug did not inhibit when the system contained ribosomes of a resistant strain together with supernatant solution of either resistant or sensitive cells. Protein synthesis is also inhibited by streptomycin, in like manner, in a cell-free system of *Diplococcus pneumonia* (94). Experiments, in which subcellular fractions from streptomycin sensitive and resistant organisms were combined, showed that both sensitivity and resistance are properties of the ribosome. However, no differences between sensitive and resistant ribosomes could be detected by immuno-electrophoresis. Streptomycin appears to prevent the dissociation of the 70 S ribosomes into 30 S and 50 S particles in the presence of low magnesium-ion concentrations, and resistant ribosomes do not show this effect.

Davies (95) has now shown that streptomycin interferes with the function of the messenger-RNA-ribosome complex and not with its formation. Labeled polyuridylic acid and extracts of *E. coli* were studied in a sucrose gradient in the presence of streptomycin. The attachment of polyuridylic acid to the 100 to 200 S ribosomes was not prevented, but the incorporation of phenylalanine by this fraction was lowered by 40 percent in the presence of the drug. A series of reconstitution experiments showed that the antibiotic was attached to the 30 S ribosomal subunit. It was suggested that streptomycin may compete with magnesium ion for the messenger-RNA binding site on the 30 S ribosome. Van Kippenberg, Veldstra & Bosch (96) also have found that streptomycin does not prevent the attachment of messenger RNA, since addition of the antibiotic after incubation of the complete system for 10 min still reduces phenylalanine incorporation. Cox, White & Flaks (97) also have shown that streptomycin does not inhibit the binding of messenger RNA to the ribosomes and have related this to earlier observations that streptomycin is lethal to cells only when they are engaged in active protein synthesis. As early as 1951, Jawetz (98) demonstrated that chlortetracycline (Aureomycin), oxytetracycline (Terramycin), and the inhibitor of protein synthesis, chloramphenicol, also interfere with the action of streptomycin. Cox, White & Flaks (97) stated that the rate of killing by streptomycin depends on the capacity of the cells for protein synthesis at the time the drug is added. It is, thus, possible that a ribosomal site, presumably on the 30 S subunit, must be cleared of protein before new streptomycin attachments can take place. Streptomycin might then "jam" the ribosomal site, inactivating the ribosome and its messenger RNA and prevent the action of all other ribo-

somes that might subsequently become attached to the polyribosome complex. On the basis of their results, these authors have proposed that the original hypothesis of Spotts & Stanier (92) be modified to state that "a streptomycin-sensitive ribosome in the presence of streptomycin does attach messenger RNA." In fact, just such an attachment may be the basis of the lethality of streptomycin.

Davies, Gilbert & Gorini (99) recently have demonstrated that the effect of streptomycin may be more subtle than mere blockade of the translation of information along the ribosome complex. The effect of streptomycin on protein synthesis, *in vitro*, caused extensive misreading of the polynucleotide code. In the presence of streptomycin, the normal incorporation of phenylalanine directed by the polyuridylate template was decreased about 50 to 70 percent, while the incorporation of isoleucine, leucine, and serine were stimulated greatly. Kanamycin and neomycin produced similar effects. The effect is not specific for uridylic-acid-containing polymers, since polycytidylic-adenylic acid (2:1) also directed the incorporation of unexpected amino acids when streptomycin was present. It is, thus, possible that streptomycin may cause the production of misconstructed and nonfunctional protein, *in vivo*. The misreading of the code cannot be induced by streptomycin when streptomycin-resistant ribosomes are employed. Conversely, streptomycin-dependence may be the result of altered ribosomes which form only nonsense protein in the absence of the drug. In these cases, streptomycin may correct the perturbation of the decoding process that dependence introduced.

Lederberg, Cavalli-Sforza & Lederberg (100) recently have put forward an interpretation of certain similarities in the effects of streptomycin and enzyme repressors that is based on the possible effects of these agencies on ribosomal configuration. A strain of *E. coli*, K 12, which has lost the ability to synthesize galactose transferase, can partially regain its capacity to ferment galactose either through a second mutation or the presence of streptomycin. Both the mutation and the drug are believed to act by interfering with the effects of a suppressor gene. Genetic studies suggest that the locus of the mutation and of that for streptomycin-resistance are near together. In both instances, it is suggested that the effects are primarily on the ribosome.

Gorini (101) has recently reported the isolation of a conditional streptomycin-dependent strain which requires either a growth factor or streptomycin. "Over suppressible" strains contain a second suppressor mutation which, although it increases the level of the suppressible defective enzyme in the presence of the antibiotic, also decreases the levels of other enzymes in the cell and inhibits growth. One remarkable feature of this "oversuppression" is that cells are still able to grow slowly while as much as 50 to 80 percent of their protein is inactive. In this light, it is interesting to recall the observation of Flaks & Whiting (102) that newly synthesized protein accumulates in the cell membrane in the presence of lethal concentrations of streptomycin.

The recent measure of success in explaining streptomycin activity at the ribosomal level should not obscure the fact that a variety of streptomycin's effects are considerably less clear. Several examples can be given. Dubin,

Hancock & Davis (103) have reported that an early stimulation of RNA synthesis precedes the extensive late degradation of cellular RNA. Further investigations of these phenomena have attempted to explain how RNA production is stimulated by inhibition of protein synthesis, if the latter is the primary event in the action of streptomycin (104). It has been proposed that the antibiotic blocks the incorporation of amino acids from the transfer-RNA amino acid complex into the polypeptide chains of the protein. This would result in the maintenance of a high ratio of charged to uncharged RNA, and this might act as a control device to stimulate the production of all forms of cellular RNA. Stern & Cohen (105) have used an *E. coli* auxotroph to demonstrate that the drug stimulates the breakdown of a particular class of RNA in a manner which corresponds with the lethal action of the antibiotic. Death can take place even in the absence of DNA synthesis. Cohen & Lichtenstein (106) earlier had suggested, on the basis of observations on the precipitation of DNA-streptomycin complexes, that interference with DNA synthesis

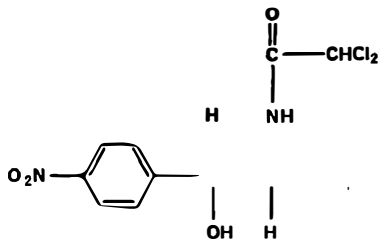


FIG. 7. Chloramphenicol.

might be a basis for the lethal effects of streptomycin. Stern & Cohen (105) are reluctant to abandon this position and feel that the more recent observations do not constitute definitive evidence against such an hypothesis. Rosenkranz (107) recently has made the interesting observation that streptomycin can no longer combine with DNA when its terminal phosphate groups have been removed by alkaline phosphatase. Streptomycin-dependent strains of *E. coli* contain high levels of alkaline phosphatase, even when grown in a medium which results in the repression of the enzyme in the wild strain. It was suggested that this synthesis of the enzyme might result from an altered permeability of the cells for inorganic phosphate.

Finally, Brock & Woolley have studied streptomycin as an antiviral agent (108). Both streptomycin-sensitive and -resistant phage of a strain which attacks *Streptococcus faecium* have been isolated. This suggests that the antibiotic attacks only the phage and not the host. The inhibition of phage growth can be reversed by some divalent cations, polyamines, and streptidine. It was suggested that streptomycin may inhibit either the injection of phage P9 by cross-linking the coiled DNA or by linking the DNA to its protein coat.

Chloramphenicol.—As early as 1953, chloramphenicol (Figure 7) was ob-

served by Gale & Folkes (109) to inhibit protein synthesis in suspensions of *Staphylococcus aureus*. This observation has since been confirmed both in intact cells (110) and in extracts of other bacteria (111).

In contrast, ribonucleic acid synthesis was observed to continue at an unaltered (112) or even accelerated rate (113) in the presence of chloramphenicol. Considerable attention has been given to this "chloramphenicol" RNA, particularly to its instability (114). Aronson & Speigelman (115) have suggested that this material is preribosomal RNA which can be converted by the cell to a stable form (ribosomes) in the presence of amino acids. This view was supported by their finding that a similar type of unstable ribonucleic acid occurs in the absence of chloramphenicol. Protein synthesis may be needed for normal ribosome production, since Dagley et al. (116) have shown that chloramphenicol-treated cells give rise to ribosomes with lower-than-normal protein to ribonucleic acid ratios.

A variety of hypotheses have been advanced to explain the effects of chloramphenicol. Woolley (117) originally proposed that chloramphenicol acted as a phenylalanine analogue, and Jardetsky (118) has recently suggested that the compound may be a pyrimidine ribonucleotide analogue. Chloramphenicol has also been shown to be an inhibitor of energy-linked processes in maize mitochondria with a site distinct from either dinitrophenol or oligomycin (119, 120).

It is likely, however, that the principal inhibitory activity of chloramphenicol is directed against protein synthesis. Convincing evidence has been accumulated from experiments with cell-free systems of *E. coli* which indicate that the specific site of action of the antibiotic is the transfer of the amino acid of aminoacyl-RNA to protein (121, 122, 123). C^{14}-chloramphenicol has been shown in *B. megaterium* and *S. aureus* to associate with the ribosomes (124) and particularly with the 50 S subunits (125). Attachment of the drug required potassium and is unaffected by the presence of messenger ribonucleic acid (poly U).

The fact that chloramphenicol is a useful chemotherapeutic agent implies a selective action against the microbial parasite rather than its human host. It has, indeed, been noted that protein synthesis is more easily inhibited in *E. coli* than in rabbit reticulocytes (126). The exact basis of this selectivity is not apparent. Chloramphenicol does penetrate into mammalian cells (127). Von Ehrenstein & Lyman have suggested that the differences may be due to a special additional feature of protein synthesis in bacteria (126). An alternative explanation may be found in the recent experiments of Vazquez (128) concerning the basis of chloramphenicol resistance and sensitivity. In bacteria, the activity of the antibiotic paralleled its uptake. Since this was also true in protoplast preparations, resistance may be a property of the cell membrane. Ribosomes from sensitive and resistant bacteria bound chloramphenicol equally. On the other hand, the observation that ribosomes of yeast, protozoa, mammalian, and plant cells cannot bind chloramphenicol may explain the insensitivity of these species to the drug.

It should be noted that additional mechanisms of resistance to chloramphenicol may also exist. Certain strains of bacteria produce an extracellular substance capable of inactivating the antibiotic (129). The ability to inactivate chloramphenicol is probably widespread among bacteria, although *Pseudomonads* may lack this ability (130).

It would be a mistake, however, to overemphasize the selectivity of chloramphenicol. Prolonged dosage of rabbits with the antibiotic results in alteration of lipid, protein, and carbohydrate metabolism (131). Anemia (132) and bone-marrow degeneration (133) have also been noted. Ambrose & Coons (134) have reported that the drug can inhibit the secondary immune response in immunized rabbits. Djordjevic & Szybalski (135) have shown a 65 percent inhibition of protein synthesis in human bone marrow, by the drug.

Several reviews of the properties of chloramphenicol are available. The reader is referred to the definitive article of Brock (136) as well as those of Davis & Feingold (77) and Feingold (65).

Tetracyclines.—Although the tetracyclines have been less thoroughly investigated than either streptomycin or chloramphenicol, they, too, are believed to be inhibitors of protein synthesis. The drugs are able to prevent the formation of adaptive enzymes in *E. coli* and apparently act at the same site as chloramphenicol (137). Exposure of *S. aureus* to the drugs caused a cessation of protein synthesis, but the inhibited cells continued to produce nucleic acid (138). However, studies with *Aerobacter aerogenes* suggest that a second locus of action, which is concerned with H^+ elimination, may operate under low oxygen tension (139) and manifest itself as an inhibition of the production of formate by resting cells (140).

The effect of tetracyclines on amino acid metabolism appears complex. In the presence of the antibiotic, the intracellular concentration of amino acids drops except for alanine and glutamic acids, while several other amino acids including phenylalanine and tyrosine completely disappear (141). These observations may be explained by the reports that tetracycline interferes with D-glutamic acid incorporation (142) as well as the production of 5-dehydroshikimic acid, a precursor of aromatic amino acids (143).

A rather interesting aspect of tetracycline is its ability to chelate metals, a property which is the basis of one assay of the drug (144). The activities of certain analogs of tetracycline are predictable on the basis of their abilities to bind with cupric, nickel, and zinc ions (145), and oxytetracycline toxicity can be reversed by salts of Fe^{+++}, Mg^{++}, Mn^{++}, and MoO_4^+ (146). It has been suggested that a metal bridge may be essential for the binding of the drug to the receptor site (145).

Several other effects of tetracycline are known, but their physiological significance is still in doubt. The drug has been observed to inhibit a mammalian catalase, specifically (147), and a flavin enzyme from *E. coli* that is responsible for the reduction of aromatic nitro groups (148). On the basis of structural considerations and experiments with *S. aureus*, Plakunov (149)

has recently advanced the theory that tetracyclines are analogues of vitamin K. A comparison of the spectrum of microbial susceptibility to tetracyclines shows fairly good coincidence with the spectrum of action of known vitamin K antagonists.

Literature concerned with the mechanism of tetracycline resistance is sparse, but some evidence is available which indicates that resistant organisms do not accumulate tetracycline as well as sensitive ones (150, 151).

An interesting and potentially useful application of tetracycline has recently been suggested by Hooser et al. (152). The addition of the antibiotic (20 μg per ml) to embryonic human tissue cultures showed no toxicity through eleven passages of the cells and resulted in the elimination of all pleuropneumonia-like organisms after two passages.

Several reviews are available concerning the metabolic aspects (153), development (154), use (155), and structural modification (156) of the tetracyclines.

Erythromycin.—Although erythromycin is the most popular of the macrolide antibiotics in clinical use, the drug has failed to stimulate any major interest among investigators concerning its mode of action. On the basis of experiments with cell-free extracts of *E. coli* (157) and *B. subtilis* (158), it is probable that the drug inhibits protein synthesis without interfering with DNA synthesis. Since the drug is unable to penetrate the cell wall of gram-negative bacteria, its action is restricted to gram-positive organisms and protoplasts (159).

Erythromycin-resistant strains of *B. subtilis* neither exclude nor degrade the drug (160). It has been suggested that " . . . an increased tolerance to the antibiotic-inhibited processes has occurred" (160).

Puromycin.—Lack of selective action has prevented the use of puromycin as a chemotherapeutic agent. The drug is commonly considered to be an inhibitor of protein synthesis on the basis of the experiments of Yarmolinsky & De la Haba (161), which showed that the antibiotic inhibited the transfer of RNA-C^{14}-leucine into microsomal protein. Gorski, Aizawa & Mueller demonstrated similar inhibitions *in vivo* (162). It has been regarded as a specific inhibitor of protein synthesis (162, 163); but, as is discussed below, this view may be subject to modification in view of recent observations on its effects on purine biosynthesis.

In reticulocytes, puromycin promotes a rapid nonenzymatic release of soluble proteins from the ribosome (164, 165, 166). Since the amino acid composition of these polypeptides resembled that of hemoglobin, it was suggested that puromycin might act through interruption of the synthesis of hemoglobin at the ribosomal level. It would, thus, act as a spurious aminoacyl RNA to block the completion of the protein chains at various points and result in the separation of the incomplete peptide chain from the ribosome (167). This view was supported by the observation that C^{14}-labeled puromycin was covalently bound to the protein released from reticulocyte ribosomes (168), and one molecule of puromycin was bound for each polypeptide chain (i.e. for each N-terminal valine).

Puromycin has also been considered as a purine antagonist (169). Adenine and some adenine derivatives were able to reverse puromycin inhibition in *Trypanosoma equiperdum*. Kidder & Dewey (170) have recently noted that while purines and their derivatives do not reverse inhibition of *Tetrahymena pyriformis*, certain peptides do. They have suggested that puromycin interferes with the assimilation of free amino acids but not with the uptake of peptides which can then be hydrolyzed to amino acids.

Other recent work shows that puromycin inhibits the synthesis of HeLa cell ribosomal ribonucleic acid (but not messenger RNA) (171) and also the formation of infectious RNA in HeLa cells infected with polio or western-equine encephalitis virus (172). In Ehrlich ascites cells, the inhibition of synthesis of purines *de novo* was confined, in the main, to the guanine fraction of the newly synthesized purine, suggesting a block in the pathways lying between inosinic and guanylic acids (173).

Inhibitors of Folate Biosynthesis and Function

Sulfonamides.—The utility of sulfonamides has somewhat declined over the years; nevertheless, these substances maintain an important place in the therapeutic armamentarium (174). New sulfonamides continue to be introduced, primarily on the basis of pharmacological properties that differ from those of the earlier widely used sulfadiazines.

The role of sulfonamides as competitors of *p*-aminobenzoic acid (PAB) has been clarified by investigations of the biosynthesis of (dihydro) folate at the molecular level (175, 176, 177). In cell-free systems, the sulfonamides not only block the condensation of 2-amino-4-hydroxy-7,8-dihydropteridine-6-methanol with PAB, but appear to condense with it themselves, to form spurious folates (175). This is somewhat at variance with earlier observations with whole cells (178). Since some of the more active participants in the lethal synthesis are compounds which are relatively inactive with whole cells, attention is directed to factors, other than affinity for the dihydrofolate synthetase, such as ionization and transport across cell membranes. No major differences in antibacterial spectra exist among sulfonamides of recent introduction.

The pharmacological factors that affect the utility of a sulfonamide include absorption, side effects, time and route of clearance, protein binding, acetylation and affinity for erythrocytes. Acetylation results in inactivation, as well as excretory difficulties. Susceptibility to acetylation may vary widely with different derivatives. Absorption and side effects are largely unpredictable; newer derivatives are likely to have fewer known side effects than older derivatives. Since, in any case, the side effects are not inherent in the biochemical mode of action, there is no theoretical reason why an essentially 'nontoxic' sulfonamide should not be attainable.

Many of the newer sulfonamides have prolonged clearance times [e.g., sulfamethoxypyridazine (3-sulfanilamido-6-methoxypyridazine); sulfaphenazole (3-sulfanilamido-2-phenylpyrazole); sulfadimethoxine (2,4-dimethoxy-6-sulfanilamidopyrimidine); sulfamethoxydiazine (2-sulfanilamido-5-methoxy-

pyrimidine); and 4-sulfanilamido-5,6-dimethoxypyrimidine] that permit once-daily dosage. A few others, requiring dosage on a twice-daily schedule, also have been introduced [e.g., sulfasomizole (5-sulfanilamido-3-methylisothiazole); and sulfamethoxazole (5-methyl-3-sulfanilamidooxazole.]

It would be expected that the chemotherapeutic effectiveness of a sulfonamide would be related closely to the plasma concentration of free sulfonamide. This, apparently, is not always true (179). It has been asserted that protein-bound sulfonamide is not antibacterial (180); and the inference has been drawn that, since the long-acting sulfonamides, sulfamethoxypyridazine in particular, are highly bound to protein (181, 182), they should have low tissue concentrations and be relatively inactive against infections of the nervous systems (174). However, Madsen, Ovsthus & Boe (183) assert, in investigations of sulfadimethoxine and sulfamethoxypyridazine, that the antibacterial activity of plasma is closely correlated with the sulfonamide concentration, regardless of protein binding; Lapeyssonie et al. report that sulfamethoxypyridazine is highly effective in the therapy of cerebrospinal meningitis (184).

It is still problematical how much of the extended survival of the long-acting drugs is to be attributed to protein binding. An interdependence between protein binding and renal clearance seemed probable to Portwich, Buettner & Englehardt (185) with regard to both glomerular filtration and tubular reabsorption. In addition, a recirculation mechanism, involving biliary excretion and subsequent reabsorption from the gut, is suggested by the high hepatic excretion of sulfadimethoxine, demonstrated by Preziosi et al. (186).

Inhibitors of Dihydrofolate Reductase

Small molecular inhibitors of dihydrofolate reductase have chemotherapeutic activities in their own right and add a new dimension to the actions of sulfonamides. The basis for their selective effects was regarded, on the basis of structure-activity considerations as applied to a range of biological systems, as selective inhibition of the reductase of the parasite (187). This view recently has been confirmed at the molecular level (188). The selectivity may reach striking dimensions; for example, trimethoprim [2,4-diamino-5-(3,4,5-trimethoxybenzyl)pyrimidine] binds the dihydrofolate reductases of bacteria between 10^4 and 10^5 times as tightly as those of mammalian liver (188).

Since the locus of action of these inhibitors is in sequence to that of the sulfaonamides, substantial potentiative effects are shown when the two types of drug are used in combination (189).

The antimalarial, pyrimethamine, was the first of these drugs to be introduced commercially. Diaveridine [2,4-diamino-5-(3,4-dimethoxybenzyl) pyrimidine] has been issued as a coccidiostat (190) and trimethoprim as an antibacterial (191). The chemotherapeutic properties and mode of action of pyrimethamine have been reviewed (192), and the general principles governing selectivities of the reductase inhibitors have been discussed (176).

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