MECHANISMS OF ANTIBIOTIC ACTION

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I. INTRODUCTION

The word "antibiotic" has, during recent years, become restricted in use to mean a substance, produced by a living organism, which inhibits the growth or activity of another living organism. An antibiotic therefore displays selective toxicity. In the least selective sense, the antibiotic does no harm to the organism that synthesises it but may be toxic to all other types of cell. At the other end of the scale, an antibiotic may differentiate between ranges of organisms and, if its selectivity is such that it can be used to inhibit the growth of a pathogenic organism within a host while having no harmful effect on that host, then that antibiotic has medical and economic value. The number of antibiotics described during the last twenty years runs into hundreds but the number whose selective action is such that they are of clinical use forms a very small proportion of the whole, and it is mainly on these few that research into modes of action has tended to centre. There are three main reasons for this concentration on the selective agents. The history of chemotherapy shows that, apart from the dis-

covery of antibiotics, successful drugs have resulted from chemical modification of toxic substances and the antibiotics provide new series of chemical structures to be modified, structures which already possess selective toxicity. Secondly, the rational development of chemotherapy in the future must be based upon a knowledge of comparative biochemistry; identification of the sites of action of selective antibiotics can point the way to components and reactions which differ in host and parasite and these, in turn, can be further exploited as a basis for differential inhibition. Thirdly, a very useful by-product of research in this field is the gain of knowledge of biochemical systems whose impairment results in cessation of growth or metabolism.

Information important to the biochemist, microbiologist, chemist and pharmacologist can be obtained from studies of the mechanism and consequences of the selective action of antibiotics. This review will be restricted to discussion of investigations that have been carried out with a few of the more selective agents. The review will not deal with problems of the discovery, isolation, biosynthesis, chemistry or chemical modification of the antibiotics mentioned and will be further restricted to those substances which are antibacterial in their action.

In the present state of our knowledge we can say that the general nature of the mode of action of certain antibiotics is known but in no case can a final answer be given to the question, "With what specific component or enzyme in the sensitive cell does the antibiotic combine or react to produce the toxic effect?" Research of the last decade has shown that selective antibiotics can be divided into three main groups according to their mode of action: 1) those whose action results in impairment of the structure, synthesis, or both, of the bacterial cell wall; 2) those whose action results in impairment of function, synthesis, or both, of the protoplast membrane, and 3) those whose action results in impairment of the synthesis of protein, or nucleic acid, or both. In each group the way has first been shown by detailed investigations of the action of one antibiotic or group of antibiotics, and the observation that other antibiotics produce similar effects in sensitive cells has then indicated that they produce the same type of lesion. It is certainly not safe as yet to allot antibiotics to these three groups without reservation and the plan adopted here will be to discuss mechanisms of action under these three general headings but to concentrate in each case on one or two antibiotics, the reason for whose classification is relatively well established. In this way the general signs and consequences of the type of lesion can be set forth so as to allow mention of other antibiotics which appear to act in the same general way. The wider problem of the interrelationships of the three types of action is largely for the future to determine; it seems probable, for example, that impairment of wall synthesis results from interaction of an antibiotic with a component of the underlying membrane, and there is current controversy (see under Streptomycin, section IV B) whether inhibition of protein synthesis can result in impairment of membrane function, or *vice versa*. Our grouping of antibiotics is therefore probably not based on fundamentally different mechanisms but provides a classification of convenience at a time when an article such at this must essentially be of a descriptive nature.

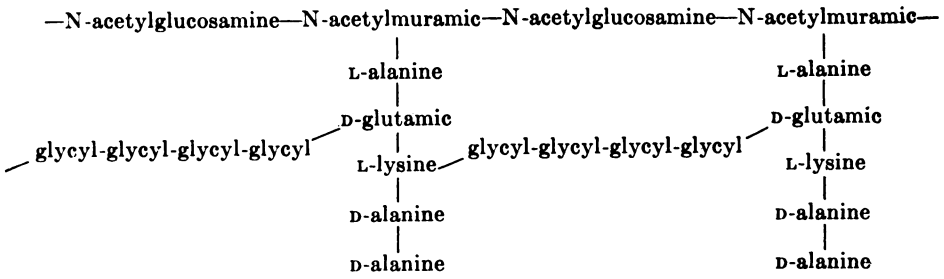
II. ANTIBIOTICS WHOSE ACTION RESULTS IN IMPAIRMENT OF THE STRUCTURE OR SYNTHESIS OF THE BACTERIAL CELL WALL

Nature of the bacterial cell wall. Studies of the bacterial cell wall as a separate organelle stem from the discovery of Dawson (56) that shaking thick suspensions of bacteria with glass ballotini results in separation of the outer envelope and disintegration of internal structures so that the "walls" can be centrifuged out of the broken debris. Various modifications of this method have been used to prepare clean walls from a wide variety of bacteria and a considerable knowledge not only of their fine structure but also of their chemical composition has now been built up. The subject has recently been reviewed by Salton (199, 200) and Rogers (190, 194). If the "wall" is defined as the anatomical structure prepared by such methods, then there are marked differences between walls obtained from Gram-negative and from Gram-positive bacteria. The former contain a higher lipid content (10 to 20% of the dry weight) and yield on hydrolysis a complete mixture of the amino acids normally found in proteins, whereas the latter contain little lipid (2 to 5%) and yield on hydrolysis only four or five amino acids; all types of wall contain a variety of sugars and amino sugars. It will be shown below that the bacterial membrane contains a high proportion of lipoprotein and it is possible that whereas the Gram-positive "wall" separates as a mucopeptide-containing fragment distinct from the lipoprotein material of the membrane, the "wall" obtained from Gram-negative cells includes membrane material. Studies of the impairment of wall synthesis by antibiotics were initially carried out on Gram-positive organisms where the wall can be defined as the outermost separable layer (other than the capsule) which is distinct from the underlying lipoprotein membrane and it is this structure, devoid as far as is known of enzyme activity, which is concerned in the discussion in this section.

Hydrolysis of walls separated from Gram-positive cells yields a mixture of sugars, amino sugars, polyols, and amino acids of which glutamic acid, alanine, lysine or 2,6-diaminopimelic acid, glycine, serine and aspartic acid are most commonly found (52, 199, 200, 242). The walls of those Gram-positive organisms which have been subjected to detailed investigation all contain glucosamine, N-acetylmuramic acid, D-glutamic acid, and DL-alanine. It is believed that these components form a common mucopeptide ground-substance consisting of a backbone of hexosamine units with peptide side-chains. The structure of this mucopeptide has been deduced from three main lines of investigation. First, the walls of certain organisms (*Micrococcus lysodeikticus*, certain other Micrococci, *Bacillus megaterium*) are digested by the action of lysozyme and studies of the products of partial and complete digestion have indicated that the lysozyme substrate contains a $1 \rightarrow 4$ β-glucoside link between N-acetylglucosamine and N-acetylmuramic acid (34, 199) (see p. 487). Second, investigation of the products of partial and complete acid hydrolysis of walls from *M. lysodeikticus* can be pieced together with the work on lysozyme digestion to show that the backbone of the mucopeptide probably consists of alternating units of N-acetylglucosamine and N-acetylmuramic acid (90, 179, 213). Third, treatment of Staphylococci

with penicillin (see below) results in accumulation within the cells of uridine diphosphate derivatives of a muramic acid peptide with an empirical amino acid composition related to that of the cell wall (178, 215); the sequence of the amino acids in the nucleotide derivative has been worked out (216, 217) and may be assumed to be the same as that of the similar unit in the cell wall. This mucopeptide forms 95% of the wall of *M. lysodeikticus* (179), about 65% of the wall of *Staphylococcus aureus* but only 1 to 10% of the wall separated from *Escherichia coli* (143).

The most complex of the uridine diphosphate muramic acid peptides isolated from *Staph. aureus* by Park (175) contained lysine, D-glutamic acid and DL-alanine; the walls of *Staph. aureus* and *M. lysodeikticus* contain 4 glycine residues for each D-glutamic acid. Perkins and Rogers (179) have isolated a fragment from *M. lysodeikticus* wall and shown that it contains glycine linked to glucosamine. Ghuysen (90) obtained a larger fragment by lysozyme digestion and showed the presence of N-acetylglucosamine and N-acetylmuramic acid, together with alanine, glutamic acid, lysine and glycine in the relative quantities 2:1:1:1; a dimer of this fragment was also isolated in which the two peptide chains were linked through the ϵ-NH_2 of one of the lysine residues. Strominger (213) has suggested that there may be polyglycyl bridges between peptide chains in the mucopeptide such that the D-glutamyl residue of one chain is linked to the lysyl of the adjacent chain to give a mucopeptide molecule of the following structure:



Mandelstam *et al.* (146) have recently described a uridine diphospho-acetyl muramyl hexapeptide from penicillin-treated *Streptococcus faecalis*; this contains the usual pentapeptide structure isolated from *Staph. aureus* plus an additional L-alanyl residue linked to the ϵ-NH_2 group of the lysine residue. The details of the molecular organisation of the mucopeptide ground-substance have yet to be confirmed but it is believed to be the material which imparts the strength and rigidity to the wall.

A further series of polymers in bacterial cell walls has been discovered by Baddiley and his co-workers, and named the teichoic acids (7, 8, 14, 16). There are two types of teichoic acid: in one, the backbone is formed of ribitolphosphate residues; in the other, the polymerising unit is glycerophosphate. In both types, side chains of amino acids and sugars have been found. All teichoic acids so far

described contain D-alanyl residues linked through O-ester bonds to the free hydroxyl of glycerol or ribitol and also the sugar residues. A variety of sugar residues has been described and differs with the organism studied: D-glucopyranosyl residues have been found in *Lactobacillus arabinosus*; glucose and N-acetylgalactosamine in *Staph. aureus*. It is interesting that both mucopeptide and teichoic acid polymers contain D-alanine, and Strominger (213) has suggested that the amino acid could form a bridge between the mucopeptide and teichoic acid. Baddiley (16) has pointed out, however, that alanine can be readily removed from teichoic acid without detaching the teichoic acid polymer from the wall and that this cannot be reconciled with the alanine-bridge concept.

Function of the bacterial cell wall. The bacterial cell is able to assimilate from the external medium soluble substances, such as amino acids, purines, pyrimidines, nucleotides and phosphate and other ions, and concentrate them within the cell so that the concentration gradient across the cell surface may be as high as 400 to 500 times (43, 74). Consequently a high osmotic pressure is set up within the cell. Mitchell and Moyle (153) measured the osmotic pressure in Staphylococci and found that it ranged from 20 to 25 atmospheres while in a rod-shaped organism such as *Esch. coli*, the value, though less, was still of the order of 5 atmospheres. The osmotic barrier lies in the bacterial membrane which is itself a fragile structure and quite unable to withstand unbalanced pressures of this or far smaller values. This is clearly demonstrated when cells such as *M. lysodeikticus* or *B. megaterium* are treated with lysozyme; if the process takes place in water or normal hypotonic media, complete lysis ensues but if the osmotic pressure within the cells is balanced by addition to the medium of a non-penetrating solute, such as sucrose or sodium chloride, then the cell wall is digested and intact spherical forms, called protoplasts, are released (147, 233). Such protoplasts are highly sensitive to osmotic lysis but possess all the biosynthetic and metabolic activities of the cell (147). Intact cells can be washed without damage in water, whereas the protoplasts are lysed immediately on suspension in water; the difference between the two cellular forms is due to the protection afforded to the membrane by the rigid cell wall of the intact organism. Impairment of the cell wall consequently results in the production of osmotically sensitive organisms which are unable to survive in a normal hypotonic medium or habitat.

Penicillin. Exploratory studies: Early investigations of the effects of growing sensitive bacteria in the presence of penicillin showed three things: 1) lethal concentrations of penicillin had no effect on the respiratory and metabolic processes of non-growing cells (38, 88, 89, 105, 106, 107); 2) growth of cells in growth-inhibitory concentrations of penicillin led to the formation, within a time approximately equal to one generation time, of non-viable cells in which respiratory and other properties were progressively impaired until lysis eventually took place (38, 105); if the growth process was inhibited by bacteriostatic substances or omission of an essential nutrient, then penicillin was without effect; 3) growth of cells in limiting or sublethal concentrations of penicillin led to the formation of abnormal, swollen, large and twisted forms (61, 89). Duguid (61) suggested that the production of these abnormal forms was consistent with "a

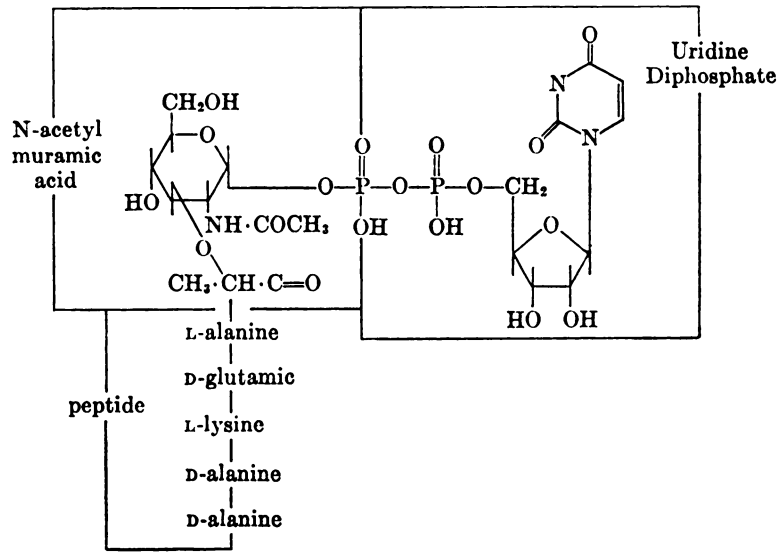
specific interference with the formation of the outer supporting cell wall whilst otherwise allowing growth to proceed until the organism finally bursts its defective envelope and so undergoes lysis."

Early biochemical investigations followed two main lines: attempts to discover metabolic systems in non-growing cells which would be inhibited by penicillin, and attempts to trace the metabolic changes arising in cells which were growing in the presence of penicillin with the object of identifying the processes first affected in the development of the non-viable culture. Little success was obtained in the search for penicillin-sensitive enzyme systems, although it was found that the breakdown of ribonucleic acid and of nucleosides could be inhibited by concentrations of penicillin several orders above those required to prevent growth of the organisms concerned (92, 129). Gale and Folkes (80, 82) discovered that the incorporation of certain amino acids into the trichloroacetic acid-insoluble fraction of *Staph. aureus* was inhibited to the extent of about 60% by growth-inhibitory concentrations of penicillin; the inhibition was unusual in that only certain amino acids were affected and that the level of inhibition reached a plateau value which was different for each amino acid. It later became apparent that the amino acids concerned (glycine, glutamic acid, lysine and alanine) were those occurring in the mucopeptide of the wall and that incorporation into the wall material was occurring under the conditions of the experiments (75, 95, 100, 144, 145). Creaser (51) found that, although β-galactosidase of *Staph. aureus* is not inhibited by penicillin, induction of the enzyme in cells incubated in a medium containing amino acids and glucose was decreased by concentrations of the order of 1 mg antibiotic/ml. The synthesis of other enzymes and of cellular protein in general was not affected but the synthesis of ribonucleic acid (RNA) under induction conditions was partially inhibited by penicillin (83). Strominger (212) also found a partial inhibition of RNA synthesis in the staphylococcus, while Thatcher and Roberts (219), studying the incorporation of ^{15}N in *Bacillus subtilis*, observed changes in the turnover of the nucleic acid fraction indicating an interference by penicillin in RNA metabolism.

Gale and Taylor (88) found that the ability of *Staph. aureus* to accumulate glutamic acid was not affected in non-growing suspensions of washed cells but that this ability was steadily impaired when cells were grown in the presence of penicillin; after growth under such conditions for 90 minutes cells were no longer able to take up glutamic acid from the medium although this amino acid continued to be withdrawn from the internal "pool" for synthetic purposes. The effect of penicillin in damaging amino acid transport or "permease" (42) systems was later extended to other amino acids (33, 97) and the onset of the damage preceded any obvious effect on respiration, fermentation, protein synthesis, loss of soluble cell contents, or lysis. Mitchell and Moyle (150) observed a disturbance of nucleic acid metabolism in *Staph. aureus* growing in the presence of penicillin and found that this was accompanied by an accumulation of extractable nucleotide within the cells; the nature of this material was not followed up but it was presumably related to the nucleotides isolated by Park (see below). Mitchell and Moyle (151, 152) also found that the RNA fraction of *Staph. aureus* contained

non-nucleotide phospho-compounds and reported that the formation of these "XP" substances was inhibited by penicillin and that the inhibition was effective from the moment of addition of penicillin to the growth medium. "XP" was tentatively identified as a glycerophosphoprotein and, in the light of more recent knowledge, it seems probable that "XP" was closely related to glycerol-teichoic acid.

Disturbance of wall synthesis: The observations which first led to the finding that penicillin inhibits the synthesis of mucopeptide were made by Park and Johnson (177) and Park (175). *Staph. aureus* growing in the presence of penicillin was found to accumulate new labile phosphate-containing materials which were then identified as uridine diphosphate derivatives of muramic acid and muramic acid peptide. The most complex of these "Park nucleotides" isolated has the structure (175, 213, 216, 217):



No function could be attributed to these compounds when they were first isolated in 1951. They were unusual amongst biological products in that they contained three "new" residues: the lactyl ether of N-acetylglucosamine [later named N-acetylmuramic acid (211)] and the "unnatural" D-isomers of alanine and glutamic acid. By 1957 the chemistry of the walls of Gram-positive bacteria had been worked out to an extent that enabled Park and Strominger (178, 215) to point out that the uridine nucleotide complex and the cell wall of *Staph. aureus* possessed N-acetylmuramic acid, D-glutamic acid, DL-alanine, and L-lysine in the same relative proportions. Park and Strominger (178) then suggested that the nucleotide accumulated as a result of impairment of cell wall synthesis by penicillin and that the lethal action of the latter could be attributed to the formation of osmotically sensitive forms of the cells during growth in

hypotonic media. Further weight has been lent to this suggestion by the isolation of nucleotide derivatives containing aspartic acid and glycine from organisms whose cell walls contained these amino acids (117) while Reynolds (personal communication) has now isolated a nucleotide containing 2,6-diaminopimelic acid from *B. megaterium* treated with vancomycin (see below). It was already known that protoplasts of lysozyme-sensitive organisms could be stabilised by addition of sufficient sucrose to the suspending medium. Lederberg (134, 135) and others (40, 95, 147) were then able to show that growth of *Esch. coli* in the presence of penicillin, salts and 10% sucrose gave rise to osmotically sensitive, swollen and distorted forms closely resembling protoplasts. Lederberg further demonstrated that removal of the penicillin was followed in a small proportion of cases by reversion of the swollen forms and growth of normal bacilli. The notion that penicillin acts by inhibiting the synthesis of wall substance was thus established.

Many of the phenomena described in earlier work could now be explained. Penicillin specifically inhibits the synthesis of wall substance but is without effect on preformed walls; consequently, it does not act on non-growing cells. When growth takes place, protein synthesis proceeds normally and the protoplast increases in size; new wall substance is required to protect and contain the growing protoplast but the presence of penicillin prevents the synthesis of that substance with the result that osmotically sensitive forms are produced. At the stage of attenuation of the existing wall, gross morphological changes would be expected and the whole process would eventually lead to lysis of the cell. When staphylococcal cells are incubated with glucose and a mixture of the four "wall amino acids" (see p. 484), mucopeptide synthesis takes place and a direct inhibition by penicillin of the incorporation of these amino acids into the wall fraction can be demonstrated (75, 176, 190). If the incubation conditions are suitable for protein synthesis to occur, then the incorporation of these four amino acids into the trichloroacetic acid-insoluble fraction of the cells is only partially inhibited by penicillin as the antibiotic has no effect on the synthesis of the cytoplasmic protein. The synthesis of uracil by *Staph. aureus* may be limiting since growth under anaerobic conditions is dependent on an exogenous supply of uracil (188); in the presence of penicillin, uridine diphosphate derivatives of muramic acid accumulate within the cell and this might well lead to a disturbance of RNA metabolism (219) and even a partial inhibition of RNA synthesis (212). The question arises whether the effects on amino acid accumulation and β-galactosidase synthesis can also be related to damage to the cell wall. Hancock (97) has shown that these effects can be obviated if experiments are carried out with cells growing in a medium containing 1 to 2 M NaCl or other non-penetrating solute. This suggests that the effects of penicillin on amino acid permease mechanisms and β-galactosidase synthesis are due to osmotic damage to the membrane rather than a direct action on the mechanisms themselves. Finally, the accumulation of the "Park nucleotides" indicates that penicillin affects the synthesis of the mucopeptide portion of the cell wall and so explains the selective action of the antibiotic since the muramic acid-containing mucopeptide appears to be a component unique to bacteria.

Effect of penicillin on Gram-negative cells: The evidence that penicillin inhibits the synthesis of cell wall material has been derived mainly from studies with Gram-positive bacteria. Gram-negative bacteria are, in general, markedly less sensitive to inhibition by penicillin but are nevertheless killed by the antibiotic in sufficient concentration. The cell wall preparation obtained from an organism such as *Escherichia coli* is chemically far more complex than that obtained from, *e.g.*, *Staphylococcus aureus* but a mucopeptide similar to that from staphylococcal walls is present although it may form as little as 1 to 2% of the dry weight of the wall preparation (143). The mucopeptide of Gram-negative cells may also differ in that the peptide contains diaminopimelic acid in place of lysine (143). Pardee and his colleagues (181, 222) were unable to demonstrate any inhibition by penicillin of incorporation of glucose-carbon into the cell wall of *Esch. coli* but found, instead, that the first effect of the antibiotic was to cause a release from the cells of material absorbing in the 260 mμ-band (purine and pyrimidine derivatives). This was followed after a few minutes by loss of crypticity (the condition in which an impermeable surface structure prevents intracellular enzymes from acting on external substrates, see p. 497), activation of ribonuclease and eventual cessation of synthesis of macromolecules. Synthesis of specific protein was necessary before these other effects of penicillin became evident and it was suggested that penicillin was responsible for the induction or activation of a lytic enzyme. Meadow (149) also found a release of 260 mμ-absorbing material immediately after the addition of penicillin to *Esch. coli* and was unable to demonstrate any inhibition of incorporation of glucose, lysine or diaminopimelic acid within the first 30 minutes of exposure to the antibiotic. The work of Lederberg (134, 135), described above, showed that penicillin causes the formation of osmotically sensitive forms of *Esch. coli* and Weidel *et al.* (235) proposed that the mucopeptide layer of the cell wall must undergo continuous hydrolysis and resynthesis during growth and that the action of penicillin could be attributed to activation of the hydrolytic mechanism. Nathenson and Strominger (159) found that benzyl penicillin inhibits the incorporation of radioactivity from tritium-labelled 2,6-diaminopimelic acid, while Rogers and Mandelstam (192), in a recent study, found that both benzylpenicillin and 6-[D(−)-α-aminophenylacetamido]penicillanic acid (one of the "new penicillins") inhibit the incorporation of ^{14}C-glucose into bound diaminopimelic acid of *Esch. coli*. Since in *Esch. coli*, bound diaminopimelic acid is found only in the cell wall they deduced that the antibiotic inhibits the synthesis of wall mucopeptide in this organism as in staphylococci. They suggested that the fractionation used by Trucco and Pardee (222) was such that effects of penicillin on mucopeptide synthesis would have been difficult to detect, while the failure of Meadow (149) to observe inhibition of diaminopimelic acid incorporation could have been due to the absence of sucrose from the suspension medium.

Site of action of penicillin: If we accept that there is sufficient evidence now to show that the effects of penicillin are explicable in terms of a lesion in the mechanism of cell wall synthesis, we can look next for the location in the cell of this mechanism and the site of its inhibition. Isolated cell walls appear to have no enzymatic or synthetic activities and this would be in accord with the absence of

true protein from the walls of Gram-positive bacteria. It would follow that the wall substance must be synthesised within the cell or within the protoplast membrane. Crathorn and Hunter (49) have suggested that the mucopeptide is synthesised by the protoplast membrane of *B. megaterium*. Ito and Strominger (118) have obtained synthesis of the nucleotide–muramic acid–peptide (p. 487) by cell-free extracts of *Staph. aureus*. No evidence has yet been obtained to indicate the site of synthesis of teichoic acids. A series of investigations [reviewed by Cooper (47)] has been made to identify the site within the cell which will fix radio-penicillin. In *Staph. aureus* penicillin is fixed by lipoprotein-containing material which probably originates in the protoplast membrane. If the assumption is made that penicillin is fixed by the component whose activity it inhibits, there is *a priori* evidence that the sensitive site of wall synthesis lies in the protoplast membrane.

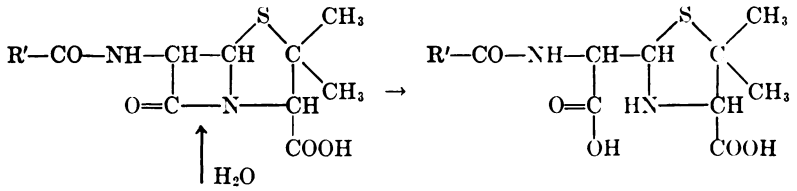
Landman and Ginoza (133) have summarised the evidence that the membrane is the primary site of penicillin action in Salmonella and have pointed out that destruction of nascent septa (the growing points from which septa form within the cell before division) can be seen in low concentrations of penicillin before any effect on the synthesis of the wall itself is evident. They found also that filamentous growth, an event that indicates inhibition of cell division, can be obtained before the formation of protoplasts or spheroplasts (25) takes place. It may be, therefore, that penicillin has a more specific action—for example, on the processes involved in septa initiation and synthesis—than a general one on the processes involved in mucopeptide synthesis as such.

Nature of the chemical reaction inhibited by penicillin: The inhibition of wall synthesis in the Staphylococcus is accompanied by the accumulation within the cells of the "Park nucleotides" and the suggestion has been made that these nucleotides are precursors of mucopeptide. The suggestion is supported by the finding (193, 221) that 5-fluorouracil also inhibits wall synthesis in *Staph. aureus* and is accompanied by the accumulation of 5-fluorouridine diphosphate analogues of the muramic acid peptides. Direct demonstration of the incorporation of the muramic acid peptide moiety from the nucleotide into the wall mucopeptide has yet to be obtained and, until this has been unequivocally demonstrated, there is a possibility that the nucleotides represent a side reaction, or trapping of the muramic peptide, rather than direct precursors of wall mucopeptide. Mandelstam and Rogers (145) have obtained some evidence for the formation of an abnormal mucopeptide during the growth in the presence of penicillin. Studies by Roberts, Wylie and Johnson (189, 243) of the incorporation of labelled glucose into cell wall material during growth of *Esch. coli* (243) and *B. subtilis* (189) have shown that the labelling of glucosamine and diaminopimelic acid residues is inhibited to a greater extent than the labelling of other wall components. It can be deduced that penicillin inhibits a process involved in the insertion of N-acetylmuramic acid into the mucopeptide polymer and Collins and Richmond (46) have pointed out that penicillin is a stereo-analogue of N-acetylmuramic acid.

However, it is possible that inhibition of wall synthesis in some manner unrelated to the muramic acid structure could likewise result in accumulation of

muramic nucleotides. Baddiley and his co-workers (15) and Saukkonen (201) have observed, for example, that teichoic acid synthesis is inhibited when growth is limited by the presence of antibiotics and Saukkonen (201) has demonstrated the accumulation of cytidine diphosphate ribitol in cells inhibited by penicillin. Consequently a parallel situation arises in mucopeptide and teichoic acid synthesis and it is not outside the bounds of possibility that an as yet unsuspected activity of penicillin is the cause of both inhibitions.

Resistance to penicillin: Resistant mutants of organisms normally sensitive to penicillin can be selected by growth of cultures in limiting antibiotic concentrations and highly resistant strains of staphylococci have recently attained dangerous predominance in hospital infections. The most common type of resistance is that due to the presence of a penicillinase which attacks and inactivates the penicillin structure as follows:



The β-lactam thiazolidine nucleus is common to all penicillins and any molecule with this nucleus is potentially a substrate for the penicillinase and an inducer of its formation (180). The penicillin in common use has a benzyl-group in position R′ but other substituents have been inserted by controlled biosynthesis and the resulting penicillins have proved to be effective antibiotics (239, 240). Of the various penicillins obtained by the biosynthetic method, none was markedly more effective as a clinical agent than benzylpenicillin or markedly less susceptible to destruction by penicillinase. The recent preparation of 6-aminopenicillanic acid both from fermentation liquors (18, 19) and by enzymic breakdown of benzylpenicillin (17) has made possible the synthesis of a new series of penicillins with new types of substitution in the R′ position (37, 59, 195, 196). Some of these new compounds have an antibacterial activity approaching that of benzylpenicillin but are much less susceptible to breakdown by penicillinase. Substitution, for example, of dimethoxybenzene in position R to give "methicillin" (6-(2′:6′-dimethoxybenzamido)penicillanic acid) reduces the affinity of the enzyme to such an extent that the rate of hydrolysis of bactericidal concentrations is negligible. Table 1 shows some values for the affinity and rates of hydrolysis by penicillinase of some of the new penicillins. The relative amounts of the various penicillins which are required to inhibit mucopeptide synthesis are of the same order as the amounts required to stop growth (191). Despite the slow rates of hydrolysis of the new molecules, they are all inducers of the formation of penicillinase. Hence, exposure of a culture to, *e.g.*, methicillin will induce penicillinase in organisms that will then attack benzylpenicillin at maximal rate.

TABLE 1

Penicillins and penicillinase (170, 180)

Potencies relative to benzylpenicillin = 100. Absolute values for benzylpenicillin are given at the head of each column.

	Inhibition of Growth in Sensitive Staphylococci (0.03–0.1 μM)	Staphylococcal Penicillinase	
		V_{max} (1 μmol/unit/hr)	Affinity (K_m = 2.5 μM)
Benzylpenicillin (6-(phenylacetamido)penicillanic acid)	100	100	100
Phenoxymethyl-penicillin (6-(phenoxyacetamido)penicillanic acid)	50	120	66
Phenoxyethyl-penicillin (6-(phenoxypropionamido)penicillanic acid)	50	90	25
D-α-Phenoxypropyl-penicillin (D-6-(α-phenoxybutyramido)penicillanic acid)	25	65	19
L-α-Phenoxypropyl-penicillin (L-6-(α-phenoxybutyramido)penicillanic acid)	6.25	140	5
6-Aminopenicillanic acid	0.03	11	0.3
2,6-Dimethoxyphenyl-penicillin (6-(2′:6′-dimethoxybenzamido)penicillanic acid)	1	3.3	0.009
Aminocarboxybutyl-penicillin (Cephalosporin N)	0.75	54	5
Cephalosporin C	0.01	0.18	<1

Resistant mutants are found which do not destroy penicillin and the basis of their resistance is not yet understood. Gale and Rodwell (86) found that cultures of *Staph. aureus* "trained" to grow in high concentrations of penicillin were correspondingly less susceptible to the effect of penicillin on glutamic acid accumulation and that, as high levels of resistance (greater than 1000 units/ml) were reached, variants were selected which were able to synthesise their amino acid requirements from glucose and ammonium salts. A curious, and as yet controversial, finding in this type of work has been the appearance of pleomorphic Gram-negative cells in cultures of *Staph. aureus* subcultured in high concentrations of penicillin. The first description of such organisms was by Klimek and co-workers (20, 127); similar results were obtained by Gale and Rodwell (86) and, more recently, by workers in four other laboratories (28, 41, 50, 148, 195). Growth of the Gram-negative organism obtained by Gale and Rodwell (85, 86) was not dependent on the addition of amino acids but required the same growth factors (thiamine and nicotinic acid) as the staphylococcus; the organism was strictly aerobic and had the same range and order of activity of amino acid deaminases as the initial staphylococcus. Cultures made from single colony isolates, on cultivation in the absence of penicillin, gave rise, in a proportion of cases, to normal Gram-positive staphylococci with penicillin-resistance intermediate between that of the initial strain and that of the Gram-negative organism. Reversion to staphylococci was also reported in other investigations.

Attempts to repeat the isolation of Gram-negative organisms from staphylococcal cultures have failed in the hands of two further groups (104, 172), while Briggs *et al.* (29) were unable to repeat their findings when they returned to the problem after an interval of a year or so. Hilson and Elek (104) emphasised their belief that the Gram-negative organisms are contaminants picked up in the course of the transfers involved in the training procedure. It is certainly possible that the six groups of workers all isolated contaminants, that these contaminants had similar properties and that the reported reversions to staphylococci were further contaminants; on the other hand, it may be that Gram-negative forms of a primitive character can be selected from the variants arising from staphylococci growing in the presence of high concentrations of penicillin but that experimental conditions which ensure consistent isolation have not yet been discovered.

Other antibiotics affecting cell wall (mucopeptide) synthesis. Effects of cell wall damage: The work with penicillin has shown that impairment of cell wall mucopeptide synthesis can give rise to the following results: (A) Growing cultures are killed in the presence of the antibiotic but the drug is without effect on the viability of non-growing cells. (B) Growth of sensitive organisms in the presence of the antibiotic and a non-penetrating solute at a concentration sufficient to balance the internal pressure of the organism, gives rise to the development of osmotically sensitive forms or spheroplasts (25). (C) Growth of *Staph. aureus* in the presence of the antibiotic leads to accumulation within the cells of "Park nucleotides." (D) Incubation of cells with the antibiotic under conditions in which mucopeptide synthesis can otherwise take place leads to inhibition of the incorporation of the amino acids of the mucopeptide into the wall fraction. (E) Growth of cells in the presence of the antibiotic results in progressive damage to the membrane. This damage is reflected in *progressive* impairment of the mechanisms required for amino acid accumulation, crypticity, and ability to retain cell components of small molecular weight (*i.e.*, cells begin to leak free amino acids, purines, pyrimidines, phosphates, *etc.*). These effects are not obtained in non-growing cells and are prevented in growing cultures by the addition of an inhibitor such as chloramphenicol.

It is recognised that these effects refer particularly to damage of mucopeptide cell walls and that, in organisms in which mucopeptide is only a minor component of the cell wall, there might well be other mechanisms which would result in ineffective wall production. In such cases one might expect that effects A, B and E could be demonstrated, whereas C and D would not necessarily appear. The antibiotics listed below all appear to affect mucopeptide synthesis and to have some properties in common with penicillin in their mode of action.

Cephalosporins: The chemistry and mode of action of the cephalosporins have recently been reviewed in these Reviews by Abraham (1) and will consequently be dealt with briefly here. Cephalosporin N is a substituted penicillin and cephalosporin C a penicillin-like antibiotic possessing a β-lactam dihydrothiazine ring in place of the β-lactam thiazolidine ring of the penicillins. Of the effects listed above, B, C and D have been observed with cephalosporin C and suitable organisms. Cephalosporin C is an inducer of penicillinase but is effective against

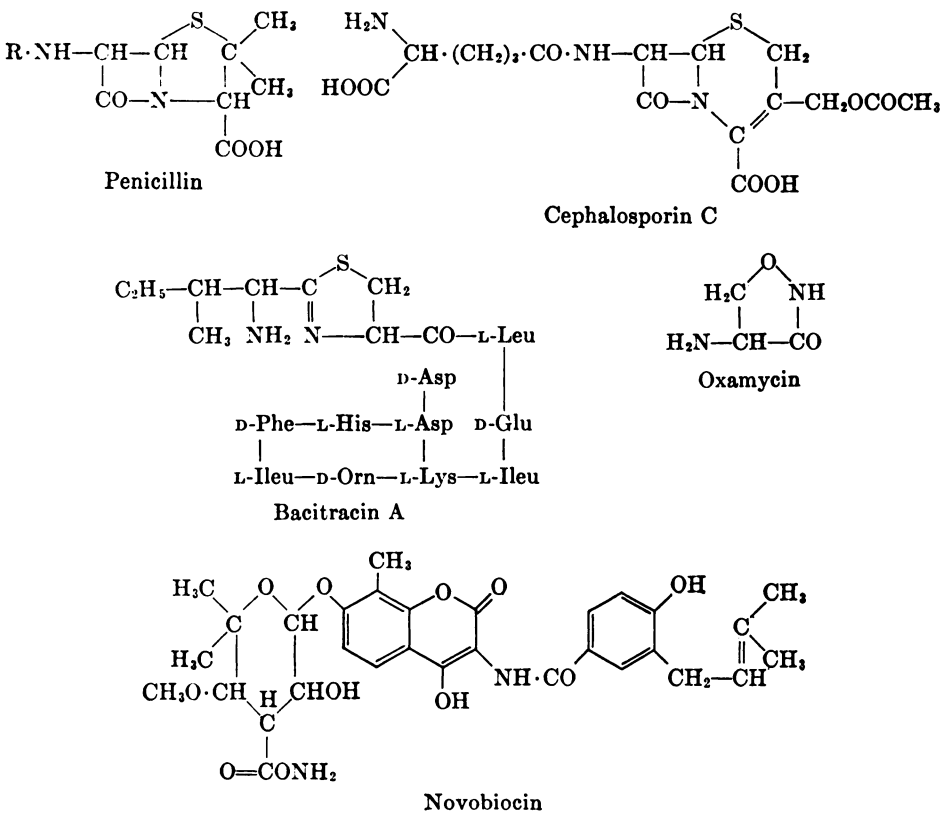


FIG. 1. Antibiotics affecting cell wall mucopeptide synthesis.
The structure shown for penicillin is a general one in which R represents one of the many possible substituted groups.

penicillinase-producing strains, since its rate of hydrolysis by the enzyme is negligible.

Bacitracins: The bacitracins are a group of peptide antibiotics containing a thiazoline ring structure (48). Paine (171) found a similarity in the activities of bacitracin and penicillin; both exhibited cross-resistance. Effect C was shown by Abraham and Newton (2) and effect D by Park (176). Reynolds (187) has shown that bacitracin and penicillin compete for fixation sites in *Staph. aureus.*

Novobiocin: Strominger and Threnn (216) obtained nucleotide derivatives of muramic acid in staphylococci treated with novobiocin (effect C). Brock and Brock (32) found that cells incubated in a growth medium with novobiocin showed a progressive leakage of internal constituents, while mutants of *Esch. coli* which were cryptic towards β-galactosidase (see p. 497) developed β-galactosidase activity without increase in enzyme content. These effects were abolished by the further addition of chloramphenicol and the authors suggested that the findings would be consistent with an action of the antibiotic on membrane synthesis. However, the results are essentially those of effect E and it would seem

that they could equally well be due to progressive osmotic damage of the membrane following impairment of wall mucopeptide synthesis.

Vancomycin (146a): The work of Reynolds (186) supported by that of Jordan (122) has shown that vancomycin is similar to penicillin in that cells treated with the antibiotic show effects A to E. Vancomycin is, however, effective against penicillin-resistant Gram-positive organisms and displays differences in its antibacterial spectrum. Other differences are now being found: Hancock (97) demonstrated that the effects of penicillin on β-galactosidase induction and amino acid transport in *Staph. aureus* could be annulled by the presence of 1.2 M NaCl in the suspension medium; this has been confirmed by Reynolds (186) who found, however, that the similar effects produced by vancomycin are not affected by the presence of salt.

Oxamycin (Cycloserine): Oxamycin will produce effects B, C and D in sensitive organisms (40, 176). Ito and Strominger (118, 119, 213) have demonstrated that the peptide chain of the "Park nucleotide" (p. 487) is assembled by the stepwise addition of amino acid residues one at a time until the structure:

Uridine diphosphate-N-acetyl muramic acid-L-alanyl-D-glutamyl-L-lysine

has been built up. The terminal D-alanyl-D-alanine is then added as the dipeptide. In the presence of oxamycin, the principal nucleotide to accumulate is that terminating in lysine (shown above). Whereas penicillin can be regarded as an analogue of N-acetylmuramic acid (46), oxamycin is an analogue of D-alanine and its inhibitory action can be antagonised by this amino acid (206, 218). Strominger *et al.* (214) found that oxamycin inhibits both alanine racemase and also the enzyme responsible for the synthesis of D-alanyl-D-alanine. It would appear from this that oxamycin inhibits the enzymes concerned in D-alanine metabolism and so prevents the synthesis of the peptide sequence necessary for completion of the wall mucopeptide. It also appears that we are closer to a complete description of the mode of action of this antibiotic than of any other.

Sites of action: The various antibiotics listed above all give rise to similar effects in sensitive cells. A complex such as the cell wall must offer a number of different sites, interference at any of which would give rise to the over-all result of cell wall impairment. It is of interest therefore to know whether these antibiotics act at the same or different sites. Penicillin is fixed by sensitive cells and, although it does not follow that the site of fixation is the site of biochemical inhibition, it is possible to determine whether the antibiotics compete for the same acceptors as those involved in penicillin fixation. Reynolds (187, and personal communication) has studied this competition in *Staph. aureus* and *B. megaterium*; S^{35}-labelled phenoxymethyl penicillin (6-(phenoxyacetamido)penicillanic acid) is fixed very rapidly by both organisms at 0° or 37° and the fixation is depressed in the presence of bacitracin or cephalosporin C but not of novobiocin, vancomycin or oxamycin. Similarly, pretreatment of sensitive cells with bacitracin or cephalosporin C prevents the subsequent fixation of penicillin; pretreatment with novobiocin, vancomycin or oxamycin has no effect on the subsequent fixation of penicillin. It would appear, therefore, that penicillin, cephalosporin C and baci-

tracin are fixed by the same, or closely related, acceptors in the cell but that novobiocin, vancomycin and oxamycin react with different acceptors. This is in accord with the biochemical evidence that penicillin and oxamycin inhibit mucopeptide synthesis at different stages. Shockman and Lampen (206a) have compared the effects of antibiotics on the growth of protoplasts and intact cells of *Strep. faecalis* and found that, whereas penicillin and oxamycin are without effect on protoplasts, novobiocin, bacitracin and vancomycin are equally effective against either protoplasts or intact cells and cannot, therefore, act solely on cell wall synthesis.

III. ANTIBIOTICS WHOSE ACTION RESULTS IN IMPAIRMENT OF THE FUNCTION OF THE PROTOPLAST MEMBRANE

Nature of the protoplast membrane. Investigations of the chemistry of the membrane have so far been undertaken in the few organisms in which the cell wall can be readily removed by lysozyme or other methods (153) with the release of the protoplasts. The protoplasts can then be broken by osmotic lysis and the membrane fraction centrifuged out of the broken material. Data are available for the composition of the membrane of *M. lysodeikticus*, *B. megaterium* and *Staph. aureus*, where the membrane preparation contains a high proportion of lipid (25 to 30% of the dry weight), some 50% protein and the remainder, carbohydrate or glycolipid (91, 153, 234). Mitchell and Moyle have pointed out that the composition of the lipoprotein part of the membrane is consistent with a structure consisting of a mono-layer of protein and a mono-layer of lipid. The lipid fractions of *M. lysodeikticus*, *Staph. aureus* and certain Clostridia have been investigated by MacFarlane (139, 140, 141) and that of *B. megaterium* by Yudkin (245). The greater part of the lipid is present as phospholipid, 70 to 80% of the fatty acid residues having 15-carbon branched chains (*iso-* and ante*iso-*). The main sugar in *M. lysodeikticus* is mannose (partly as mannosylglyceride) while *Staph. aureus* contains β-glucosylglyceride. In a recent note, MacFarlane (141) reported the identification of lipo-amino acid complexes in bacterial membranes.

Functions of the membrane. Since bacterial cells contain substances of small molecular weight (*e.g.*, amino acids, nucleotides, inorganic ions) at concentrations 400 to 500 times their concentration in the external medium, it follows that there must be an osmotic barrier somewhere in the surface of the cell. Removal of the cell wall itself does not release the internal solutes; the volume impenetrable to glutamic acid is less than the volume of the intact cell but equal to that of the protoplast (153); and damage to the membrane is followed by release of the concentrated internal solutes. It follows that the membrane contains or poses an osmotic barrier to free diffusion between the internal and external media. The membrane is not, however, simply a passive barrier. Many essential metabolites (*e.g.*, glutamic acid, K ions, purines, and pyrimidines) are unable to diffuse through the membrane but if the cells are provided with a source of energy, these substances are rapidly transported by an active process across the membrane and concentrated within the cell. The active transport of sugars and amino acids is brought about by the mediation of specific protein components of the membrane;

these components have been called "permeases" by Cohen and Monod (42). Permeases display a specificity towards the substance they transport; the permease which transports valine, for example, does not transport phenylalanine or galactosides. The formation of permeases is gene-controlled and transport-deficient mutants occur; thus an organism may be unable to form galactoside permease while its ability to produce β-galactosidase is unimpaired. Such an organism is unable to metabolise lactose although it possesses β-galactosidase; it is called a "cryptic mutant." If the membrane of such a mutant is damaged to an extent that allows lactose to enter the cell, then the cell will apparently gain the ability to attack the lactose although there has been no change in its content of β-galactosidase. Such a cell is said to have lost its crypticity and the process can be taken as an indication that the membrane has changed or lost some of its properties. Mitchell and Moyle (153) have claimed that enzyme systems of the electron transport mechanism are located within the membrane, while Butler and co-workers (35) have maintained that the membrane is the site of cell wall synthesis and also plays a role in protein synthesis. It is clear that the membrane is a highly organised cellular structure and impairment of its function will necessarily have major repercussions on the metabolism and viability of the cell.

Types of membrane damage: From the brief summary given above, it would appear that the membrane has three major functions: 1) as an osmotic barrier, 2) as an organelle effecting concentration of nutrients and metabolites within the cell, and 3) as a site for the biosynthesis of other cell components. An antibiotic could affect any or all of these directly or by impairing the synthesis of the membrane itself. There is definite evidence that certain antibiotics have a direct effect on the function of the membrane as an osmotic barrier while a number of suggestions, based on less definite evidence, have been put forward that other antibiotics, mentioned below, inhibit the proper synthesis of the membrane in sensitive cells.

Tyrocidin and gramicidin S. Some of the earliest investigations on the mode of action of an antibiotic were those of Hotchkiss (109, 110) on the toxic peptides, called tyrocidin and gramicidin, produced by *B. brevis*. Hotchkiss found that these two substances were very different in their effects on the respiration of staphylococci; tyrocidin produced a rapid decrease (not necessarily complete inhibition) of respiration whereas gramicidin gave rise to a transient stimulation of oxygen uptake associated with cessation of phosphate uptake. The action of tyrocidin was accompanied by massive leakage of nitrogenous and phosphorus-containing substances from the cells into the medium. Gale and Taylor (87) showed that staphylococci and streptococci possess high concentrations of free amino acids within the cells and that treatment with tyrocidin results in a rapid release of these amino acids (but not proteins) into the external medium. It was later shown that tyrocidin is similar to other surface-active agents and that such agents destroy the osmotic barrier so that amino acids, phosphates, purines, pyrimidines, phosphate esters, *etc.* leak across the membrane into the surrounding medium. This results in dilution of essential metabolites to the extent

that enzyme activities and biosynthetic processes are reduced to negligible proportions. Gramicidin, on the other hand, is different in its mode of action and acts as an uncoupler of oxidative phosphorylation (109). Gramicidin S (27), however, is related to tyrocidin and again destroys the permeability properties of the membrane.

The substances whose release is most readily measured are the purine and pyrimidine derivatives with absorption in the ultraviolet at 260 mμ. The simplest and most direct test for membrane damage is therefore the release of substances absorbing at 260 mμ (45, 198). With tyrocidin or the cationic detergents that have been investigated (87, 110), there is a direct relationship, for concentrations producing less than 99% killing, between the amount of agent added and the amount of soluble contents released and also between the amount of agent added and the number of cells rendered non-viable. Release of soluble contents begins immediately when cells and antibiotic come together and thus differs from the gradual onset of leakiness that occurs during growth in the presence of penicillin, *etc.* The latter type of damage is prevented by inhibition of growth or by the presence of antibiotics such as chloramphenicol; the action of the surface-active agents such as tyrocidin is independent of growth and is not affected by the presence of chloramphenicol.

Polymyxin. The polymyxins form a group of cyclic peptides produced by *Bacillus polymyxa* and their mode of action has been reviewed by Newton (166). Polymyxins fall into the group of antibiotics affecting cell membranes as shown by the release of soluble contents from treated cells. Gram-negative organisms are in general more sensitive than are Gram-positive organisms and polymyxin is the antibiotic of choice for treatment of skin wounds, burns, grafts, *etc.*, where pseudomonad infection is probable (120).

Newton found that, for concentrations of polymyxin which gave less than 99% killing of sensitive cells, there was a linear relationship between the amount of antibiotic added and the proportion of cells killed, which was itself related to the amount of 260 mμ-absorbing material released. With many organisms tested, the amount of 260 mμ-absorbing material corresponded to the amount of free purine, *etc.*, held within the "pool" of the intact cells but with *Pseudomonas aeruginosa*, incubation with polymyxin led to autolytic breakdown of nucleoprotein within

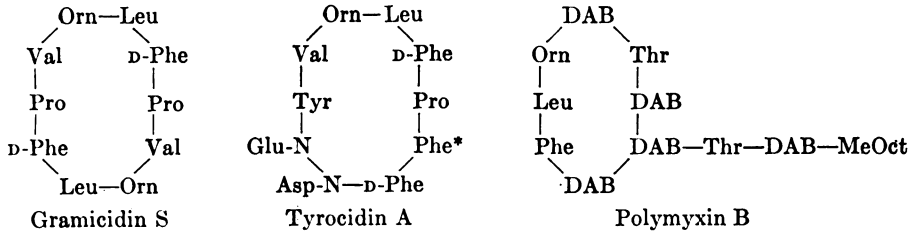


FIG. 2. Antibiotics affecting membrane permeability.

Orn = ornithine, Pro = proline, Asp-N = asparagine, Leu = leucine, Tyr = tyrosine, Thr = threonine, Val = valine, Try = tryptophane, DAB = diaminobutyric, Phe = phenylalanine, Glu-N = glutamine, MeOct = methyloctanoate. Tyrocidin B has Try instead of Phe*.

the cells so that the amount of 260 mμ-absorbing material released was eventually much greater than that initially free in the pool. Electron microscope examination of pseudomonads showed that growth-limiting concentrations of polymyxin released most of the electron-dense material from within the cells, leaving ghosts. Higher concentrations apparently inhibited the autolytic processes and cells were obtained that, although grossly damaged, were electron-dense. Alteration in the permeability of the cell membrane was shown by use of the dye, N-tolyl-α-naphthylamine-8-sulphonic acid, which fluoresces when in contact with protein. Little fluorescence was obtained when intact cells were treated with the dye but the addition of polymyxin allowed the dye to penetrate into the cells where it fluoresced brilliantly in contact with the cytoplasmic proteins.

Resistant cells absorb less antibiotic than sensitive cells (69, 164) and, if cells are broken after treatment with polymyxin, the antibiotic is found in combination with small particles of lipoprotein. Newton (165) traced the location of the antibiotic in the cell by tagging polymyxin with a fluorescent marker, 1-dimethylamino-naphthalene-5-sulphonic chloride, and was able to demonstrate combination between the fluorescent antibiotic and the protoplast membrane of *B. megaterium*. Fixation of the antibiotic by the cells is antagonised by cations and the relative efficiency of a series of cations in competing with the antibiotic corresponds to the affinity of those cations for phosphate groups (163). Newton therefore suggested that polymyxin reacts with polyphosphate groups (phospholipid ?) in the membrane and so brings about disorientation of the lipoprotein structure. Few and Schulman (67, 68) investigated the surface properties of the polymyxins and also their interaction with bacterial lipids; results were again consistent with an interaction between the antibiotic and ionised phosphate groups in the lipids.

The mode of action suggested by these investigations is that polymyxin brings about a disorientation of the lipoprotein membrane so that this no longer fulfills its function as an osmotic barrier. However, the action may be more complex than this, especially in cells where the difference between "wall" and "membrane" is less marked than in some Gram-positive bacteria. Thus Warren, Gray and Yurcheno (232) have found that *Neisseria catarrhalis* can be rendered sensitive to lysozyme by pretreatment with polymyxin while Tuttle and Gest (224) have found that treatment of *Rhodospirillum rubrum* with 250 μg polymyxin/ml results in the formation of protoplast-like bodies. In the presence of 60 μg/ml the organism grew in bacillary instead of spirillum form; treatment of cells with both lysozyme and polymyxin gave "ghosts." In these cases it would appear that polymyxin has some effect on the wall of the cell or, at least, in rendering the wall-membrane complex susceptible to breakdown by other processes.

Nature of the interaction with membranes. Cyclic peptides of bacterial origin (27) are surface-active; the antibacterial activity of surface-active agents has been reviewed by Newton (167). It is generally believed that such substances owe their effects to the presence of lipophilic and lipophobic groups separated within the molecule and their ability to become orientated between lipid and protein films. The polymyxins are cyclic peptides with a methyl-octanoate side-chain (55, 237); hydrolysis of tyrocidin and gramicidin S releases amino acids

only. Unusual amino acids are found in all these antibiotics: L-diaminobutyric acid in polymyxin, ornithine and D-phenylalanine in tyrocidin and gramicidin S. Harris and Work (102) synthesised pentapeptides with the amino acid composition of gramicidin S but found these to have negligible biological activity. Erlanger and co-workers (65, 66) synthesised a decapeptide with the same sequence as gramicidin S and found that it was bactericidal to *Staph. aureus* and *Esch. coli* but with an activity only 10% of that of gramicidin S itself. Substitution of the δ-amino group of ornithine removed the bactericidal action of the decapeptide. Erlanger and Goode (65) suggested that the greater activity of the cyclic structure might be due to its lesser susceptibility to enzymatic destruction. More recently Schwyzer (205) has pointed out that gramicidin S exists in the form of an anti-parallel pleated sheet with separation of the lipophilic valyl and leucyl residues on one side from the lipophobic groups on the other so that the antibiotic activity may lie in this orientation within the molecule. In the Gram-negative cell there is closer contact between the wall mucopeptide and lipoprotein than there is in the gram-positive cell, and it may be that the anatomical relationship depends upon phosphate bonds of the type which react with polymyxin; this would explain the effect of this antibiotic upon the wall and the membrane in certain cases.

Selective action: Newton (166) pointed out that cell wall preparations from polymyxin-sensitive cells take up more of the antibiotic than preparations from resistant cells and suggested that the difference in sensitivity lies in chemical or structural differences, or both, in the walls. Analysis of typical wall preparations from sensitive and resistant cells showed that the main difference lay in the lipid-phosphorus so that the lipid-P/lipid-N ratio for sensitive cells was about twice that for resistant ones. It may be therefore that sensitivity to polymyxin is determined by the phospholipid fraction but confirmation awaits more detailed knowledge of the chemistry of this fraction in bacterial cells.

Signs of membrane damage: The investigations outlined above show that damage to the membrane can give rise to the following results: (A) Loss of the permeability barrier as shown by rapid diffusion out of the cells of small molecular weight constituents such as amino acids, inorganic ions, purines and pyrimidines ("260 mμ-absorbing substances"), *etc.*, and loss of crypticity. (B) These permeability changes begin immediately cells and antibiotic come into contact and are not dependent on growth or metabolism and are not prevented by chloramphenicol. (C) For concentrations of the antibiotic producing less than 99% killing there is a stoichiometric relationship between the amount of antibiotic and the number of cells killed and also the amount of soluble "pool" contents leaking out of the cells.

Interference with membrane synthesis. In contrast to the immediate effect of surface-active antibiotics on the osmotic barrier, antibiotics which inhibit the synthesis of the wall (albeit by combining with a membrane component responsible for that synthesis) produce a progressive change in permeability and decrease in crypticity. If leakage of soluble contents is the sole criterion, then the difference between the two types of actions lies in 1) the time taken for the defect to develop, and 2) whether growth or the presence of an inhibitor of protein

synthesis, such as chloramphenicol, has an effect on the development of the defect. It has been suggested that signs similar to those attributed to osmotic damage following upon failure of wall synthesis could also be produced by interference with the synthesis of membrane itself. Thus Brock and Brock (32) observed that in sensitive cells novobiocin causes progressively increasing leakage of cell contents and progressive loss of crypticity in β-galactosidase-cryptic mutants. They suggested that these effects could be ascribed to an impairment of membrane synthesis by novobiocin. Chloramphenicol annulled these effects.

Investigations with streptomycin (see also below) led Davis *et al.* (9, 10, 60, 197) to a similar conclusion concerning its mode of action. They observed that suspension of *Esch. coli* under growth conditions in the presence of streptomycin led to a progressively increasing release of 260 mμ-absorbing materials from the cells, increasing loss of crypticity, progressive impairment of the ability to take up amino acids from the medium, and an increase in the potassium ion flux. These effects were annulled if chloramphenicol was added at the same time as streptomycin. The amount of "nucleotide" leaking from the cells was eventually much greater than that in the pool at the beginning of the experiment but there was no decrease in the pool nucleotide during the early stages of the incubation. The concentration of nucleotide in the pool is maintained partly by an increase in synthetic activity and partly by breakdown of preformed RNA under the influence of streptomycin (60a). Investigation of the uptake of labelled streptomycin by the cells showed that this took place in two stages: first a very rapid uptake, apparently due to fixation of the antibiotic by primary acceptors and, second, a larger uptake apparently due to the formation of complexes between streptomycin and intracellular acceptors such as the nucleic acids. The secondary uptake could be prevented by the presence of chloramphenicol, or simulated by toluene treatment of the cells. These results indicate that streptomycin has an effect on the permeability barrier of the cell but whether this is direct or secondary remains to be decided. Davis *et al.* (9, 10, 60, 60a, 197) suggested that the effects could be due to impairment of membrane formation but it is evident that some of the results are similar to those which might be expected to follow from damage to wall synthesis. Yudkin (245), who has devised a method for studying the synthesis of membrane in growing protoplast (*B. megaterium*) preparations, found that streptomycin affects the incorporation of amino acids and glycerol into the membrane fraction to the same extent as into the trichloroacetic acid-precipitable fraction of the whole protoplasts.

Hurwitz and Rosano (113, 114, 115) have also investigated the uptake of labelled streptomycin by *Esch. coli* and confirmed that there are two stages in the process. The total amount of antibiotic accumulated by the cells can be separated into three fractions: that adsorbed by surface structures, that present in the cells at the time when loss of viability begins, and that accumulated by dead cells. The amount present in the cells when viability is first affected, presumably the fraction having the lethal effect, accounts for no more than 10% of the total. These workers also confirmed that chloramphenicol added at the same time as streptomycin prevents the lethal effect of the latter but if the cells are exposed to streptomycin before the addition of chloramphenicol, then

streptomycin accumulation continues and the cells are killed in the presence of the chloramphenicol. There is, therefore, an initial chloramphenicol-sensitive phase which precedes killing by streptomycin. The duration of this phase depends upon the time of exposure and the concentration of streptomycin present before the addition of chloramphenicol. The authors deduced that there must be a period of streptomycin-induced protein synthesis before the antibiotic can exert its killing effect, and that this process relates to a change in the properties of the membrane. The kinetics of the process are consistent with the idea that a streptomycin-transporting system must be synthesised by the membrane before the antibiotic can penetrate and kill the cell, but do not support the suggestion that the cells die simply as a result of the formation of a leaky membrane. The further point was made that the selectivity of streptomycin could be explained if host cells were unable to carry out the streptomycin-initiated synthesis of protein. Plotz and Davis (179a) were unable to confirm the occurrence of killing by streptomycin in the presence of chloramphenicol. They pointed out that the experiments of Hurwitz and Rosano were carried out in an unbuffered, N-free medium in which the cells were dying at an appreciable rate in the absence of antibiotic. They suggested that it is not possible, on the available evidence, to decide whether the lethal action occurs at the membrane or in the cell. Opposed to the "membrane theories" of streptomycin action are those discussed below (section IV B), which indicate that the antibiotic inhibits the synthetic function of bacterial ribosomes. The various effects may well stem from one common action and, if so, tracing the connection between inhibition of ribosome function and loss or alteration of membrane properties should yield data of importance to our understanding of bacterial physiology.

Streptomycin differs from other antibiotics discussed in this review in the ease with which dependent and resistant mutants of otherwise sensitive strains are obtained; a number of workers have studied streptomycin-dependence in the belief that the nature of that dependence should throw light on the mode of action of the antibiotic on sensitive strains. Landman and Burchard (132) have studied the division process in dependent Salmonellae and found that dependent cells cannot form septa in the absence of streptomycin with the result that growth leads to the formation of long filaments. Addition of streptomycin is followed by the onset of synchronous normal division. If growth of the dependent cells takes place in the absence of streptomycin but on soft serum agar in the presence of penicillin, then L forms (globular bodies containing granules that produce minute colonies on serum agar, similar to those formed by the pleuropneumonia organisms) develop and these require only 5% of the streptomycin needed by bacillary forms for growth. Streptomycin is also required for maintenance of the heritable septation system since, under conditions of streptomycin starvation, 95% of the survivors are converted to stable L forms. The authors suggested that, since the requirement for streptomycin is largely eliminated by by-passing septation, the primary requirement for streptomycin must be concerned with the septation process. They concluded that the site of action of streptomycin lies in those parts of the membrane where septation originates.

IV. ANTIBIOTICS WHOSE ACTION RESULTS IN INHIBITION OF PROTEIN OR NUCLEIC ACID SYNTHESIS, OR BOTH

A. Mechanism of protein and nucleic acid synthesis

It is impossible, in a review of this nature, to present an adequate summary of the evidence leading to the present concept of the mechanism of protein and nucleic acid synthesis. There have been over 500 papers on the subject within the last four years and the literature is now in a phase of exponential growth following the fundamental advances made in 1961–62. The literature up to mid-1961 has been reviewed elsewhere by the present author (77) and references will be given in this section only to certain papers published since that time. Although many (if not most) of the details remain to be filled in, the general scheme of protein and nucleic acid syntheses can now be outlined and some, at least, of the main reactions indicated.

It has long been thought that the control of protein and nucleic acid synthesis lies in the deoxyribonucleic acid (DNA) of the chromosome; consequently DNA can be taken as the starting point of the whole process. DNA is a macromolecule in which the units are deoxyribonucleotides joined by 3′-5′ phosphoester links; four deoxyribonucleotides (nucleotides of A, adenine; G, guanine; C, cytosine, and T, thymine) are involved and the sequence of the nucleotide bases along the polymer is believed to determine the amino acid sequence of proteins synthesised within the same cell. Native DNA consists of two complementary polynucleotide chains wound as a double helix and held together by hydrogen bonds between adenine or guanine residues of one chain and thymine or cytosine, respectively, of the other. When replication takes place, the two strands separate and each forms a primer or template for the synthesis of a further complementary chain. Synthesis of the new polynucleotide is brought about by an enzyme, DNA polymerase (22, 128), which requires the triphosphates of the four deoxyribonucleotides and the primer:

$$\left.\begin{matrix} \text{n dATP} \\ \text{n dGTP} \\ \text{n dCTP} \\ \text{n dTTP} \end{matrix}\right\} + \text{DNA} = \overline{\text{DNA}}/\text{DNA} + (\text{PP})_n \qquad (1)$$

where dATP = deoxyadenosinetriphosphate, *etc.*, PP = pyrophosphate, DNA = polynucleotide primer and $\overline{\text{DNA}}$ = newly synthesised polynucleotide.

DNA can also act as primer for the ribonucleic acid (RNA) polymerase which, in the presence of the triphosphates of the four ribonucleotides, synthesises a polyribonucleotide in which the base sequence is determined by the base sequence of the DNA primer (with substitution of uracil for thymine) (39, 238):

$$\left.\begin{matrix} \text{n ATP} \\ \text{n GTP} \\ \text{n CTP} \\ \text{n UTP} \end{matrix}\right\} + \text{DNA} = \text{RNA} + (\text{PP})_n + \text{DNA} \qquad (2)$$

The polyribonucleotide synthesised by this DNA-dependent RNA polymerase

has a base sequence modelled by that of the DNA and is believed to be the "template" or "messenger RNA" which passes to the site of protein synthesis in the ribosome and so determines the specificity of the protein synthesised in that ribosome (26, 94, 121). The nature of the correspondence between the base sequence of the messenger RNA and the amino acid sequence of the protein synthesised is currently the subject of very active research. It is believed that combinations of bases in the RNA [probably triplets (3)] "code" specific amino acids and that the polynucleotide provides the information tape controlling the sequence of amino acids linked by the ribosomal mechanism. The messenger RNA appears in some (but possibly not all) cases to be unstable so that it breaks down after its use in the ribosome and the ribosome has then to be activated again by a further supply of new messenger RNA. The unstable messenger RNA forms a small proportion of the total RNA of the cell; the remainder falls into two types differing in molecular size. The larger proportion has a molecular weight of 10^6 or more and is associated with protein to form the ribonucleoprotein particles or ribosomes which act as the site of protein synthesis. The other type of RNA has a molecular weight of *ca.* 10^5, is not sedimented from the cell sap at 100,000 g for 4 hours and is consequently called the soluble RNA (sRNA).

Ultracentrifugation of cell extracts reveals that the ribonucleoprotein particles are present in a number of sizes or degrees of aggregation; the degree of aggregation and the distribution of sizes is determined (*in vitro*) by the magnesium ion concentration in the suspending medium. At Mg concentrations of the order 0.01 M, most of the ribonucleoprotein sediments with a coefficient of 100 or 80 Svedberg units. As the Mg concentration is decreased, the 100S particles dissociate to 70S, then the 70S particles dissociate to a mixture of 50 and 30S particles. Ultracentrifuge and electron microscope studies have shown that each 70S particle dissociates to one 50S and one 30S particle while parallel investigations on the ability of these particles to incorporate amino acids have shown that protein synthesis takes place in 70S particles (or larger aggregates thereof) but not in the smaller particles. When 70S particles are suspended in low concentrations of Mg, the bulk of the ribosomes dissociate but a small proportion remains unaffected and, again, it appears that it is these stable or "active ribosomes" (colloquially the "stuck 70s") that are the sites of protein synthesis. Whether or not it is possession of messenger RNA which renders the active ribosomes non-dissociable is a matter for present study. The protein of the ribosomal particles consists of a common basic protein together with a small proportion (probably less than 1 %) of specific proteins in the course of synthesis by the ribosomes. The enzymatic synthesis of ribosomal RNA (as opposed to messenger RNA) has not yet been worked out and it is thought at present that the bulk of the ribosomal RNA, like the bulk of the ribosomal protein, may be nonspecific (see, however, p. 506).

sRNA consists of a mixture of polyribonucleotides, each with a chain length of 100 to 200 nucleotide residues. Some of these molecules, designated "transfer RNA," will combine with specific amino acids to form an amino acyl—sRNA in which the amino acid is ester-linked to the ribose of the terminal nucleotide. Be-

fore a sRNA molecule can combine with an amino acid, it must possess the specific terminal sequence cytidyl—cytidyl—adenylate; there is specificity between the transfer RNA and the amino acid it will accept and such specificity must lie in the base sequence somewhere in the polynucleotide at a stage removed from the essential, but common, terminal —C—C—A. Incubation of sRNA in the absence of nucleoside triphosphates results in the removal of the terminal —C—C—A and the sRNA can no longer act as amino acid acceptor. The terminal grouping can be restored, and the sRNA reactivated as transfer RNA, by incubation in the presence of ATP and CTP and enzymes bringing about terminal addition:

$$\text{sRNA} + \text{CTP} + \text{ATP} = \text{sRNA—CMP—CMP—AMP} \quad (3)$$

A third enzyme, polyribonucleotide phosphorylase, brings about polynucleotide synthesis when incubated in the presence of nucleoside diphosphates, singly or in mixtures, and an oligonucleotide primer. With a single nucleoside diphosphate, such as ADP, the enzyme brings about end-addition of nucleotide residues to the primer:

$$(\text{XNA})_y + \text{nADP} = (\text{XNA})_y\text{—AMP—AMP—AMP—} + \text{nP} \quad (4)$$

If a mixture of the diphosphates is supplied, the product is a mixed polymer and the residues are believed to be incorporated into the product in approximately the same proportions as those in the incubation mixture. No determination of sequence has been shown with this enzyme and its function in the cell is at present a matter for debate. The reaction is readily reversed by the presence of inorganic phosphate and one suggestion is that the role of the enzyme is to destroy (by phosphorolysis) excess messenger RNA after its use in the ribosome.

Other nucleic acid polymerases are being discovered in cell extracts and recent reports include a RNA-dependent RNA polymerase (156) and an enzyme which produces a product containing both ribo- and deoxyribonucleotide residues.

Amino acids are prepared for protein synthesis by a series of well-characterised steps. First, each amino acid is "activated" by conversion to a reactive amino acid adenylate in the presence of ATP and a specific "activating enzyme":

$$\alpha + \text{ATP} = \alpha\text{-AMP} + \text{PP} \quad (5)$$

where α = amino acid residue and α-AMP = amino acid adenylate. The amino acid adenylate then reacts with its specific transfer RNA to form the amino acyl—sRNA; this reaction is probably brought about by the same enzyme as that involved in the adenylate formation and is consequently called the amino acid:sRNA ligase:

$$\alpha\text{-AMP} + \text{sRNA} = \alpha\text{-sRNA} + \text{AMP} \quad (6)$$

The mixture of amino acyl—sRNA molecules then passes to the ribosomes where, under the influence of messenger RNA, the amino acid residues are assembled in specific sequence and peptide bonds are formed. The sRNA moieties

are released and recycled. The details of this part of the process are not yet known. Guanosine triphosphate (GTP) is necessary for this step in the synthesis and one molecule of GTP is broken down for each molecule of amino acid added to the polypeptide being synthesised. Starting from a point where the ribosome already contains a partially assembled polypeptide chain PEP, it appears that the amino acyl—sRNA (corresponding to the right sequence as ordered by the messenger RNA) joins the end of that peptide to give PEP—α^1-sRNA^1. The next amino acid in the sequence is then incorporated in the form of its amino—acyl—sRNA, releasing the sRNA of the previous end group:

$$\text{PEP—}\alpha^1\text{-sRNA}^1 + \alpha^2\text{-sRNA}^2 \xrightarrow[\text{messenger RNA}]{\text{GTP}} \text{PEP—}\alpha^1\text{-}\alpha^2\text{-sRNA}^2 + \text{sRNA}^1 \qquad (7)$$

until, in this manner, the complete specific sequence is assembled and the polypeptide ready to be released as the finished protein.

A corollary of the scheme set out above is that, if the messenger or template RNA is removed, protein synthesis and amino acid incorporation cease. If the template is replaced by a different one, then amino acid incorporation will start again but the sequence in which the amino acids are assembled will be different. An interesting way of imitating or controlling the overall mechanism was discovered by Nirenberg and Matthaei (168) when they found that cell-free bacterial ribosome systems could be activated by addition of specific RNA preparations. In the course of their work, they included synthetic polyuridylic acid (*i.e.*, the polymer prepared by incubating polyribonucleotide phosphorylase with UTP) as a control template and found that it promoted a startling incorporation of phenylalanine. No other amino acid gave an effect approaching that of phenylalanine, the product had the properties of polyphenylalanine, and the intermediate formation of phenylalanyl—sRNA was demonstrated. This experiment not only provided a method for testing synthetic polymers as sources of amino acid codes but has also provided an experimental procedure which can be used to test the activation of ribosomes by "synthetic messengers" and the possible interference by drugs in this system.

Figure 3 outlines the mechanism in diagrammatic form and also indicates various points at which inhibitors could act in order to produce the overall result of cessation of protein or nucleic acid synthesis. The reactions set out above are undoubtedly complex; reaction 6 applies to some 20 different enzymes, each specific for one amino acid, and an antibiotic need inhibit only one of these in order to stop protein synthesis. Apart from enzyme inhibition, an antibiotic could combine with one of the carriers (sRNA, ribosomes, ribosome precursors, *etc.*) or primers or substitute, as an analogue, for an amino acid, purine or pyrimidine. Reaction 7 is a particularly complex one: not only does it involve the breakdown of GTP in an as-yet-unexplained process but it will take place only in 70S ribosomes which have been activated by messenger RNA. Consequently, reaction 7 could fail for many reasons including: 1) damage to, or alteration of,

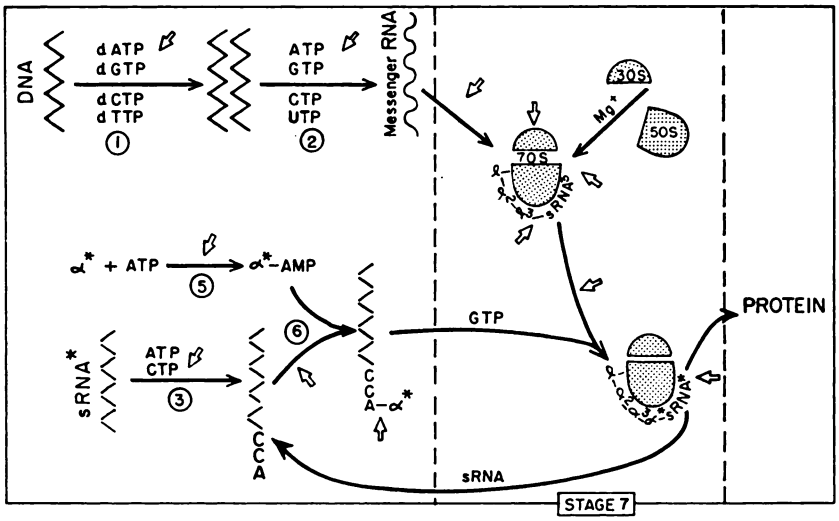


FIG. 3. Mechanism of protein and amino acid synthesis.

Possible sites of interference by antibiotics are marked by arrows. Numbers refer to reactions noted in text (pp. 503 and 505), where the abbreviations are explained.

messenger RNA; 2) failure of messenger RNA to react with and activate the ribosome; 3) breakdown or dissociation of the ribosome; 4) inhibition of the actual peptide bond forming system; 5) loss of the terminal sRNA or the peptide in the ribosomal mechanism; 6) hydrolysis of the amino acyl—sRNA before peptide bond formation; 7) destruction of GTP. Reaction 7 is clearly highly complex, is better referred to as "stage 7" and, as will be seen below, is the site of action of a number of antibiotics. Perhaps the detailed study of their action will provide some of the answers to the nature of the overall reaction.

Inhibition of many ancillary reactions—synthesis of an amino acid, purine or pyrimidine, ATP, mono- or triphosphates; transport of amino acids across membranes, *etc.*—will also give the end-result of cessation of protein synthesis. Inhibition of such reactions can, however, be studied in separate experimental systems and eliminated. In this section we are concerned with antibiotics which have negligible effects on such ancillary reactions and whose site of action lies within the scope of figure 3.

B. Antibiotics whose primary effect is inhibition of protein synthesis

Chloramphenicol. Exploratory studies: Before it can be stated that an antibiotic acts at some site within the mechanism concerned in protein synthesis, the possibility that it prevents one of the ancillary reactions must be eliminated. In the case of chloramphenicol (Chloromycetin) it was early established that, whereas protein synthesis was abolished at growth-inhibitory concentrations, processes such as fermentation, respiration and accumulation of amino acids within the "pool" were unaffected unless the antibiotic concentration was several orders higher than that required to prevent growth (84). Many other

processes and reactions have been investigated and found to be insensitive to the antibiotic; detailed references can be found in a number of reviews (31, 76, 77, 208). From these various studies it is known that chloramphenicol, at the lowest concentration to inhibit growth, gives complete inhibition of protein synthesis, partial inhibition of DNA synthesis, and no inhibition of RNA synthesis; has no effect on the synthesis of wall mucopeptide and does not alter the properties of the cell membrane. This antibiotic therefore has a site of action coming within the scope of figure 3.

Effect on RNA synthesis: Gale and Folkes (80) first demonstrated an increase in the rate of synthesis of RNA in the staphylococcus in the presence of concentrations of chloramphenicol which limit protein synthesis. Gros and Gros (93) and Pardee and Prestidge (174) reported that RNA synthesis in the presence of chloramphenicol required the presence of the full complement of amino acids necessary for protein synthesis although protein synthesis was in fact inhibited. Pardee and co-workers (173) then turned their attention to the nature of the RNA formed under these conditions. They investigated, by zone electrophoresis, the behaviour of nucleoprotein extracts from *Esch. coli* grown in the presence and absence of the antibiotic. Extracts from normal cells contained two major components of differing electrophoretic mobility; the fast-moving fraction was of smaller molecular weight, had lower affinity for protein and possessed a base composition different from that of the slower-moving fraction. In extracts from cells grown in the presence of chloramphenicol, newly synthesised RNA moved with a mobility equal to that of the faster-moving normal component although its base composition resembled that of the slower-moving normal component. If radio-phosphate was added to the growth medium, it was incorporated into all fractions when growth took place in the absence of antibiotic, but when chloramphenicol was present, only into the fast-moving fraction. The presence of a new peak of small sedimentation constant in the ultracentrifugal pattern was shown in extracts from the cells grown in antibiotic. Similar results were obtained for extracts of *Staph. aureus* by Gale (75); in normal cells, ^{14}C-glutamic acid was incorporated into all the electrophoretic fractions but when chloramphenicol was present during the incubation, the amino acid became incorporated into the fast-moving component only; the binding of the amino acid to this fast-moving component was labile and readily reversible. Dagley and Sykes (54) investigated the ultracentrifugal pattern of ribonucleoprotein extracted from *Esch. coli* under a variety of growth conditions, and confirmed the presence of fractions with small S values in extracts from chloramphenicol-grown but not from normal cells. Further data were supplied by Nomura and Watson (169) who showed the presence in extracts from normal cells of aggregates with sedimentation values of 30 and 50S; extracts from cells grown in chloramphenicol showed a new component at 15S, no material at 30S and a greatly reduced component at 50S. The 15S "chloramphenicol particles" consisted of 75% RNA and 25% protein and were unstable in high concentrations of salt. Studies of their behaviour in low ion concentrations suggested that they consisted of loosely coiled polyelectrolytes resembling nucleic acid rather than ribosomes (130). Dissociation of the par-

ticles showed that they contained 16S and 23S RNA with composition resembling that of ribosomal RNA. The "chloramphenicol particles" therefore resemble normal ribosome precursors. The studies of Britten *et al.* (30) on the formation of ribosomes suggest that ribonucleic acid structures of sedimentation 14S ("eosomes") are normal precursors of ribosomes and that aggregation to larger particles is dependent upon the formation of nucleoprotein; consequently the accumulation of 14 to 16S particles when growth takes place in the presence of chloramphenicol may be a simple reflection of the inhibition of protein synthesis.

Neidhardt and Gros (162) found a further difference in that the RNA formed in *Esch. coli* during growth in the presence of chloramphenicol was unstable once the antibiotic was removed from the medium. After breakdown of the unstable RNA had taken place, addition of chloramphenicol to the medium was followed by resynthesis of RNA. Horowitz *et al.* (108) confirmed this instability but showed that the RNA is not stabilised by the antibiotic but that, instead, the presence of chloramphenicol stimulates RNA synthesis to the extent that net synthesis takes place only while the antibiotic is present. These various investigations established that the RNA formed in the presence of chloramphenicol is of small molecular weight (sedimentation *ca.* 15S), is in a condition of rapid turnover in the cell, and is readily dissociated from protein.

Site of inhibition of protein synthesis: Of the various reactions set out above, chloramphenicol has no significant inhibitory effect on: 1) DNA-dependent DNA polymerase (62); 2) DNA-dependent RNA polymerase (231); 3) polyribonucleotide phosphorylase (78); 4) amino acid activation (58); 5) formation of amino acyl—sRNA (131). Lacks and Gros (131) studied the fixation of amino acids by the sRNA fraction of *Esch. coli* and found that neither the fixation nor the release of amino acyl residues was affected by chloramphenicol although the rate of turnover of amino acyl—sRNA was reduced owing to inhibition of protein synthesis. The site of inhibition is therefore reduced by elimination to the complex stage 7 of p. 507, involving the transfer of amino acids from sRNA to the peptide-bond forming site in the ribosome. Chloramphenicol inhibits the incorporation of amino acids into protein by *in vitro* systems containing ribosomes from *Esch. coli* (220). Nathans and Lipmann (158) found that 60 μg chloramphenicol/ml gave 65% inhibition of the transfer of amino acyl residues from sRNA to ribosomes in an *Esch. coli* system; this confirms that the site of inhibition is properly allocated to stage 7.

The question that must now be settled relates to the specific nature of the interference at stage 7 and the nature of the RNA which accumulates when chloramphenicol is present. One of the events included in stage 7 is the activation of nonspecific ribosomes by messenger RNA. The properties of messenger RNA that can so far be described are (a) sedimentation constant of approximately 15S, (b) rapid turnover in cells, and (c) base composition correlated with that of the DNA which determined its synthesis. If an antibiotic combines with messenger RNA and so prevents it from reacting with the ribosome, or combines with the ribosome and prevents its activation by messenger RNA, then protein synthesis will cease and presumably something akin to messenger RNA may

accumulate within the cell. It would be an attractive hypothesis that chloramphenicol reacts in one of these ways and that the RNA accumulating in its presence is messenger RNA which cannot react with the ribosomes. The "chloramphenicol RNA" has a sedimentation constant of the right order and is known to be in a state of rapid turnover. Hahn and Wolfe (96) have endeavoured to supply the missing criterion by relating its base composition to that of the bacterial DNA in experiments with *B. cereus*. Analyses made previously of the base composition of the RNA formed in the presence of chloramphenicol have shown nothing unusual about that composition (23, 173); the RNA extracted from the cell must, however, be a mixture and Hahn and Wolfe made use of the fact that the "chloramphenicol RNA" is unstable. They therefore allowed the RNA to break down and estimated the base ratios of the material released during breakdown. They claimed that these ratios were closer to those of the DNA than to those of the RNA of the cell. For example, the guanine and adenine in the fragments were 20.8 ± 0.9 and 29.8 ± 0.5, respectively, while those for DNA were 17.8 and 32.2, and for RNA, 31.0 and 25.5, respectively. The values would not appear to substantiate the claim unequivocally. An alternative explanation, that the 16S RNA represents ribosomal precursor which cannot undergo further aggregation in the absence of protein synthesis, has been discussed above and is also in agreement with the work of Kurland *et al.* (130). A third possibility is that the "chloramphenicol RNA" contains both messenger RNA and ribosomal precursor. An answer to this particular dilemma awaits better methods of separating small molecular weight RNA into messenger, ribosome precursor and other fractions. Soluble RNA may also accumulate and it seems very probable that this is also coded in order to provide for the specificity of amino acid acceptors; sRNA separates in the ultracentrifuge with sedimentation of 5 to 7S; this does not enter into arguments based on the nature of the 14 to 16S material. Flaks *et al.* (72), in the course of their studies with streptomycin discussed below, have investigated the action of chloramphenicol on the *in vitro* ribosome system. They found that the antibiotic inhibits the stimulation of phenylalanine incorporation which is promoted by polyuridylic acid but is without effect on the control incorporation obtained in the absence of polyuridylic acid. The implication is, again, that chloramphenicol prevents reaction between the messenger and the ribosome but it is as yet too early in our understanding of the nature of stimulation by "synthetic messengers" such as polyuridylic acid for us to be certain what is the meaning of this type of experimental finding. Vazquez (private communication), working in the author's laboratory, has found that labelled chloramphenicol, added either to growing cultures or cell-free extracts of *Staph. aureus*, becomes associated with the ribosomal fraction. This association is prevented by the presence of streptogramin or erythromycin but not by puromycin or the tetracyclines (see below).

Ishihama *et al.* (116) have indicated yet another possibility for interference by chloramphenicol with messenger RNA. By the use of methylated albumin columns they have separated "messenger RNA" into four fractions which differ in sedimentation coefficient. Their fraction IV, with sedimentation coefficient

23 to 30S, has a specific ability, not possessed by the other fractions, to combine *in vitro* with 70S ribosomes to give RNA-ribosome complexes with sedimentation 80 to 85S. The four fractions are separated both from normal *Esch. coli* and from cells after infection with T2 phage. The addition of 100 μg chloramphenicol/ml to the T2-infected cell system, or 500 μg/ml to uninfected cells in exponential growth prevents the appearance of radioactivity in those eluates from the column which normally correspond to fraction IV; fractions II and III are not affected and appear to accumulate. The authors' interpretation was that activation of the ribosomes is due to the specific fraction IV of the messenger RNA and that inhibition of protein synthesis by chloramphenicol follows from specific inhibition of the synthesis of this fraction.

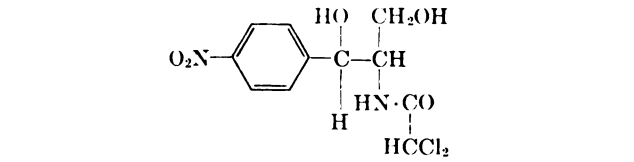
The early investigations of Woolley and others (21, 154, 223, 241) showed that the action of chloramphenicol can, under certain conditions, be partially antagonised by certain amino acids, especially phenylalanine. Woolley pointed out that the antibiotic can be regarded as an analogue of phenylalanine and the possibility that it can substitute for or displace this amino acid at some stage in the protein synthesising mechanism has not yet been ruled out. Yet another mode of action would be inhibition of the release or transfer of amino acyl residues from amino acyl—sRNA and there is no evidence at present to indicate whether this is the case or not. The somewhat similar example of puromycin is discussed below and a more definite answer provided there. In a series of investigations with cell-free enzymes, Smith (207, 208) claimed that chloramphenicol at bacteriostatic concentrations inhibited liver and bacterial esterases. Since the link between amino acids and sRNA is an ester bond, this might be a most important finding but no recent confirmation of the claim has been forthcoming.

An interesting and possibly important side-issue has concerned the requirement of bacteria for a full complement of amino acids before RNA synthesis can take place (24, 79, 93, 174). Gros and Gros (93) and Pardee and Prestidge (174) confirmed that this requirement is still shown for the synthesis of RNA in the presence of chloramphenicol but none of the cell-free systems so far described has shown a requirement for amino acids as cofactors for nucleic acid synthesis. Aronson and Spiegelman (12, 13) have studied the problem in protoplasts of *B. megaterium*. It has been known for some time that, although chloramphenicol gives 100% inhibition of enzyme synthesis, incorporation of amino acids into protein is seldom inhibited to an extent of more than 90 to 95%. Aronson and Spiegelman obtained evidence that the amino acid requirement in nucleic acid synthesis is related to the formation of a special type of protein, low in sulphur, which is kinetically related to, and associated with, RNA synthesis and that the formation of this protein fraction is not inhibited by bactericidal concentrations of chloramphenicol. However, high concentrations (400 μg/ml) prevent the synthesis of this protein fraction and, at such levels, RNA synthesis is no longer amino acid-dependent. They found, further, that "chloramphenicol RNA" is easily removed from particles in cell extracts and that amino acids are required for the synthesis of a special protein fraction which stabilises the RNA so formed. The amino acid dependence of RNA synthesis is controlled by a single genetic

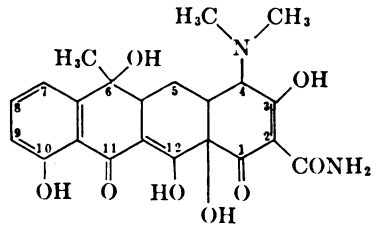
locus (210) and alteration of this locus results in a mutant which can synthesise RNA in the absence of protein synthesis. Neidhardt (161) has investigated the properties of such mutants and found that they show the usual protein/RNA correspondence during normal growth. However, if they are subcultured from a medium rich in amino acids to one devoid of added amino acids, the mutants continue to accumulate RNA whereas RNA synthesis is suppressed in wild type strains. The mutants display a lag period of 4 to 5 hours before resuming growth under such conditions. Neidhardt concluded that the amino acid dependence is essential for regulation of RNA synthesis under conditions of physiological stress. In a recent paper, Kellenberger *et al.* (123a) have shown that DNA synthesis may also be restored by chloramphenicol in amino acid-starved auxotrophs of *Esch. coli.*

Selective action: The selective toxicity of chloramphenicol is such that it can be used as an effective chemotherapeutic agent against both Gram-negative and Gram-positive bacterial infections in man. Yet the site of action appears to involve a fundamental stage in protein synthesis. During recent years, ribosomal preparations from a wide variety of animal, plant and microbial cells have been investigated and their ability to incorporate amino acids studied *in vitro.* Many investigations have included some test of the effect of chloramphenicol in the reconstituted systems and the various data have been collected by Gale (77). Although many of the studies lack detailed investigation of the effect of a range of concentration of chloramphenicol, it is nevertheless evident that amino acid incorporation in *in vitro* systems containing bacterial ribosomes is sensitive to chloramphenicol in concentrations of 10 to 100 μg/ml, while systems containing ribosomes from mammalian tissues are unaffected by concentrations much greater than this. Von Ehrenstein and Lipmann (228) noted that they have never observed a reliable effect of chloramphenicol, even at concentrations of 10 mM (3000 μg/ml), on a mammalian system and commented: "We are beginning to feel that the difference between the mammalian system and the microbial system must indicate a special, probably additional, feature in the microbial system which precedes the polymerisation of amino acids, the mechanism of which we presently presume to be the same in all cells." The matter may become clearer when we have learned more of the nature of messenger RNA and its relation to the ribosome. It seems possible that the rapid turnover of messenger RNA that has been described in phage-infected bacteria may be a property of that particular biological system rather than an essential property of messenger RNA itself. It has been suggested, for example, that, if messenger RNA is a component of the protein-synthesising system of reticulocytes, then it must be a more stable substance and associated more firmly with the ribosomes than in bacterial systems. An antibiotic which interfered with the function of messenger RNA might then be expected to have a greater effect on the more dynamic bacterial system.

Other antibiotics affecting protein synthesis (stage 7). Puromycin: Puromycin is related to chloramphenicol both in being an analogue of phenylalanine and in acting as an inhibitor in stage 7 (244). Inspection of its structure (fig. 4) shows



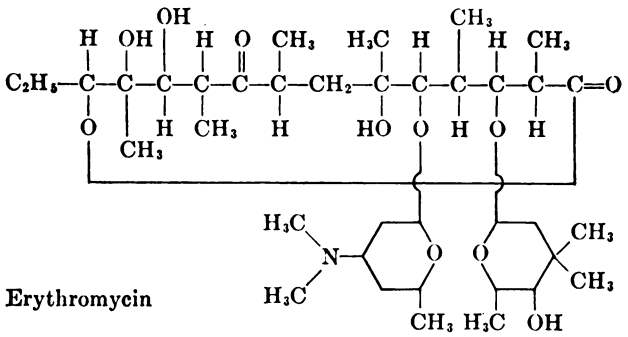
Chloramphenicol



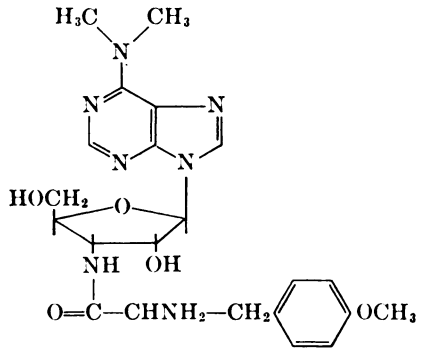
Aureomycin
= 7-chloro-tetracycline

Terramycin
= 5-hydroxy-tetracycline

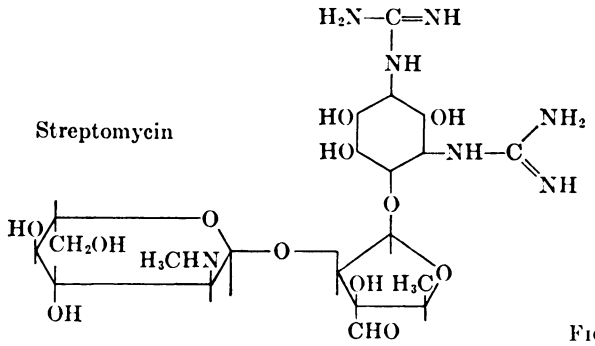
Tetracycline



Erythromycin



Puromycin



Streptomycin

FIG. 4. Antibiotics whose primary effect is inhibition of protein synthesis.

that it can be regarded as an analogue of adenosyl-phenylalanine, *i.e.*, of the terminal group of phenylalanyl—sRNA. Its inhibitory action appears to be related to the transfer of amino acyl residues from sRNA to peptide bonds in the ribosome (244). Nathans and Lipmann (158) found that 0.4 mM puromycin gave almost complete inhibition of the transfer of leucine from leucyl—sRNA to ribosomes from *Esch. coli* but that deacylation of the sRNA occurred during the experiment, the deacylation reaction requiring all the factors necessary for transfer of the amino acid to protein. They suggested that this means that the complete mechanism functions in the presence of puromycin but hydrolysis of the amino acyl—sRNA occurs instead of condensation of the amino acid residue with the peptide chain being formed in the ribosome. Evidence was obtained that puromycin acts upon the ribosome and that treated ribosomes are unable to synthesise peptide chains but deacylate amino acyl—sRNA instead. Arlinghams, Schweet and their colleagues (11, 155) have observed that puromycin inhibits haemoglobin synthesis by reticulocyte ribosomes and results in non-enzymic release of soluble, labelled protein from the ribosomes. They found that amino acids are incorporated into soluble material released in the presence of puromycin and that free amino acids are then liberated by the action of soluble enzymes on part of this released material. They suggested that puromycin attaches to a site in the ribosome and displaces the incompletely formed peptide chains as well as other ribosomal intermediates. Allen and Zamecnik (6) also observed rapid release of incomplete polypeptide chains from reticulocyte ribosomes in the presence of puromycin. The chains possessed the normal N-terminal valine. Labelled puromycin was found attached to the released polypeptide in the proportion of one residue of puromycin for each N-terminal valine. The authors deduced that puromycin displaced the sRNA which normally binds the peptide to the ribosome. Von Ehrenstein and Lipmann (228) pointed out that puromycin is effective in both bacterial and mammalian systems, whereas chloramphenicol is ineffective in the latter; they suggested that chloramphenicol blocks a reaction which precedes that affected by puromycin.

Tetracyclines: The results of treating staphylococci with tetracycline derivatives (chlortetracycline, Aureomycin; oxytetracycline, Terramycin—see fig. 4) are very similar to those obtained with chloramphenicol: inhibition of protein synthesis and stimulation of RNA synthesis at bactericidal concentrations (80). These antibiotics have no effect on ancillary reactions unless their concentration is increased to values 100 to 1000 times the minimum growth-inhibitory level. Park (176) observed that synthesis of wall mucopeptide is inhibited by Aureomycin at concentrations of 100 μg/ml, but not by concentrations which are effective in limiting growth (0.1 to 1 μg/ml). The RNA which accumulates in the presence of Aureomycin has an electrophoretic behaviour similar to that of the material formed in the presence of chloramphenicol (36). Albert (3, 4, 5) has pointed out that the tetracycline molecule is a highly effective chelating agent and has suggested that this property might be a factor in its antibiotic action. The suggestion receives support from the finding that the organic nitro-reductase of *Aerobacter* is inhibited by Aureomycin, and that this inhibition is released by Mn

ions and that the enzyme is a manganoflavoprotein (202, 203, 204). The enzyme is, however, inhibited by antibiotically inactive degradation products of Aureomycin and, although the enzyme extracted from Aureomycin-resistant cells was less sensitive to the antibiotic than the preparation from normal cells, it was equally sensitive to degradation products of Aureomycin. The relative insensitivity of the nitro-reductase from resistant cells was associated with a firmer binding of the metal to the enzyme complex. In the author's laboratory it has not been possible to demonstrate antagonism or reversal by manganese of the action of Aureomycin on protein synthesis in *Staph. aureus*. Nevertheless there seems a possibility that chelating agents might affect the integrity of active ribosomes which depend upon magnesium ions for maintaining the degree of aggregation necessary for activity.

Erythromycin: Preliminary investigations in the author's laboratory have shown that this antibiotic produces effects in *Staph. aureus* which are again similar to those obtained in the presence of chloramphenicol or the tetracyclines.

Streptogramin (39a): Vazquez (226) found that this antibiotic falls into the group producing effects on protein and nucleic acid synthesis similar to those produced by chloramphenicol. An interesting difference is that, whereas the action of chloramphenicol is bacteriostatic and reversible, streptogramin is lethal and its action on protein synthesis irreversible. Concentrations of chloramphenicol well below those necessary to prevent growth of staphylococci nevertheless protect the organism against the lethal action of bactericidal concentrations of streptogramin.

Streptomycin. Exploratory studies: It will have become evident to those who have read this review to this stage that the site of action of many antibiotics has been narrowed down to a limited metabolic field or to a limited functional structure. Streptomycin is unusual in that several quite different types of action have been postulated and controversy exists today concerning the major site of its inhibitory effect. Some of the earliest studies were those of Umbreit and his colleagues (225) who found an inhibition of pyruvate metabolism in sensitive cells that had been "aged" by exposure to acid conditions or by standing in washed suspension for some hours. Experiments indicated that a reaction involving pyruvic and oxaloacetic acids was sensitive to inhibition by the antibiotic and Umbreit postulated a respiratory cycle involving condensation of these two compounds to a new 7-carbon substance. The normal "condensing enzyme" which forms citric acid from pyruvic acid was not sensitive to streptomycin and citric acid formation was, if anything, increased in the presence of the antibiotic. Some evidence was obtained for the formation in *Esch. coli* of a phosphorylated compound corresponding in chromatographic properties with the "Rapoport compound" (182), 2-phospho-4-hydroxy-4-carboxy-adipic acid. Although no net changes in the amount of this material could be observed, investigation of the specific activity after labelling with phosphorus showed that its metabolism was inhibited by streptomycin. The identification of the labelled material was equivocal and quantitative aspects were difficult to reconcile with the major role proposed for the substance. This work was performed before the nature of the

bacterial cell wall was understood and if it were repeated today, attention might well be turned to the possibility of interference with the metabolism of 7-carbon constituents of the cell wall—especially since streptomycin contains N-methyl-L-glucosamine as part of its molecule. Fitzgerald and Bernheim (70, 71) found that streptomycin prevented the oxidation of aromatic compounds by avirulent strains of *M. tuberculosis* but tracked the effect down to an inhibition of the formation of inducible enzymes required for the oxidation process. This was the first indication that streptomycin affects protein synthesis. Creaser (51) observed inhibition of the induced formation of β-galactosidase in *Staph. aureus* but Gale and Folkes (74, 80) were unable to detect any significant inhibition of protein synthesis in washed staphylococci incubated in an appropriate medium.

Metabolic suppression: The inhibitory effects of streptomycin on pyruvate metabolism seem to be part of a wider suppression of metabolic activities. Hancock (98, 99) has studied such effects in *Staph. aureus*, *B. megaterium*, and *Esch. coli*. He found that the rate of killing is greater under aerobic than anaerobic conditions, and that streptomycin is not bactericidal in the absence of growth. The oxidation of glucose and other substrates is partially inhibited during inhibition of growth. The incorporation of various labelled precursors was studied in the major cell fractions and all the processes appeared to undergo partial inhibition to approximately the same extent. Lysates of protoplasts from streptomycin-inhibited *B. megaterium* showed oxidative activities some 20 to 45% of those of corresponding lysates from normal cells; the difference appeared to lie mainly in the sedimentable "ghost" fraction of lysed protoplasts. A similar general suppression of metabolism was observed by Kirk (126) in his investigations of the effect of streptomycin in bleaching *Euglena*.

Membrane damage: Reference has been made to the work of Davis *et al.* (9, 10, 60 197) which led those authors to suggest that streptomycin impaired the synthesis of membranes in growing cells. Hancock (98, 99) was unable to obtain evidence for a change in the permeability of the organisms he studied, and it is possible that damage to the oxidative mechanisms of membranes might produce secondary effects of the type described by Davis *et al.* The investigations of Landman and Burchard (132), outlined above, suggest that the site of action of streptomycin lies in the control of septation and, as such, in membrane function.

Protein synthesis and ribosome function: A new approach has been proposed by Spotts and Stanier (209) who pointed out that, since genetic analysis shows that streptomycin-sensitivity, -resistance, and -dependence are determined by multiple alleles of the same locus (57, 103, 137, 160), all the effects of streptomycin should be explicable on a single basis—interference with the synthesis of a single protein, or inhibition of function of a single component. They proceeded to study changes in the composition of a streptomycin-dependent strain of *Esch. coli* when grown under conditions of streptomycin deprivation. In high concentrations of the antibiotic, cells contained normal complements of protein, DNA and RNA but when growth took place under streptomycin starvation, the DNA and protein content decreased while RNA increased markedly. When cells were grown in excess streptomycin and then removed to media free from antibiotic, normal

growth continued until the "pool" streptomycin was exhausted when changes characteristic of growth under starvation conditions supervened. If streptomycin was then added, RNA synthesis ceased until the synthesis of DNA and protein had restored the normal relationship. Fractionation of the RNA from starved cells showed that the changes affected sRNA and ribosomal RNA to the same extent. The amount of enzyme in starved cells varied with the enzymes concerned, some being suppressed much more than others. A small amount of antibiotic, amounting to 250,000 molecules per cell, was firmly bound by the cells and could be removed only by treatment with hot perchloric acid. The suggestion was made that protein synthesis by dependent cells requires that streptomycin be bound by receptors, and that these receptors are the ribosomes. Ribosomes are activated by combination with messenger RNA and it was suggested that streptomycin is essential for combination of messenger and ribosome in dependent organisms. Deprivation would then affect the synthesis of individual proteins to the extent that attachment of the corresponding messenger molecules was dependent upon the presence of streptomycin. The excessive synthesis of RNA under conditions of deprivation was explained on a basis of the normal feedback control being thrown out of gear. Referring to the "unitary hypothesis" that sensitivity, dependence and resistance must be explained on the same basis, Spotts and Stanier (209) proposed that streptomycin prevents the attachment of messenger to ribosomes in sensitive cells, is unable to react with ribosomes in resistant cells, and is essential for messenger attachment in dependent cells. The three types of reaction to streptomycin are thus explained in terms of a mutation affecting the attachment of streptomycin to the ribosome. Evidence cited in favour of the explanation for sensitivity is that of Erdös and Ullmann (63, 64) that low concentrations of streptomycin inhibit the transfer of amino acyl residues from sRNA to the ribosome (stage 7) in extracts from sensitive cells but not from those of the resistant phenotype.

The messenger hypothesis is again an attractive one and offers a possible explanation of the various experimental findings cited. It is clearly not, in the present state of knowledge, an exclusive and mandatory explanation of these findings and in no way accounts for the membrane effects described by others. It would not require a great exercise of ingenuity to construct a parallel "unitary hypothesis" which invoked membrane function as its basis. As explained above, it appears that it may be possible to test the messenger hypothesis by use of the *in vitro* system using phenylalanine incorporation in *Esch. coli* ribosomes under the stimulation of polyuridylic acid as "synthetic messenger." Flaks *et al.* (72, 73) have studied the effect of streptomycin in this system. The "basal" incorporation of phenylalanine that occurs in the absence of added messenger is unaffected. Addition of polyuridylic acid gives a marked stimulation of incorporation and this stimulation is reduced by 95% if streptomycin is added prior to the polyuridylic acid. If ribosomes are pretreated with streptomycin, centrifuged out and resuspended in the presence of polyuridylic acid, the normal stimulation is not obtained so it would appear that streptomycin is bound by the ribosome in such a way that the latter can no longer be stimulated by polyuridylic acid. The

action of polyuridylic acid can be prevented by the presence of 1 or 2 molecules of streptomycin per ribosome. Chloramphenicol has been found to act in a similar manner, but whereas half maximal inhibition is obtained with 5×10^{-7} M streptomycin, the concentration of chloramphenicol required to produce a similar degree of inhibition is 10^{-3} M. Puromycin was found to inhibit phenylalanine incorporation whether the process was basal or stimulated by polyuridylic acid. Investigations were then extended to streptomycin-resistant and -dependent cells and the ribosomes extracted therefrom. Ribosomes from dependent and resistant cells were incubated in the presence of supernatants from the same cells and from the sensitive strain (73). The incorporation and its sensitivity to antibiotic were insignificantly affected by the source of the supernatant used, implying that the differences in the systems reside in the ribosomes. The ribosomes from resistant and dependent strains were not inhibited by 10^{-6} M streptomycin but were affected by a concentration of 10^{-3} M; consequently they were about 1000 times less sensitive to antibiotic than those from the sensitive cells. It was not possible to show any requirement for streptomycin by the ribosomes from dependent cells. Somewhat similar experiments have been carried out by Mager *et al.* (142) who confirmed that the site of streptomycin action lies in the ribosome. Ribosomes are known to contain spermidine and other polyamines as minor components (44) and it has been suggested that these substances may be concerned in the association of 30 and 50S particles to form the active 70S ribosomes. Mager *et al.* (142) found that polyamines at high concentrations have an inhibitory effect on amino acid incorporation by ribosomes but that they exert a partial relief of the inhibition produced by streptomycin; streptomycin and polyamines are mutually antagonistic in this respect. The authors therefore suggested that streptomycin and polyamines have a common site of action, so that both may affect the proper formation of active ribosomes. White and Flaks (236) found that the first demonstrable effect of streptomycin is inhibition of the formation of sRNA and ribosomal RNA in growing cells with the consequent accumulation of 30S RNA. The synthesis of messenger RNA is unaffected by the antibiotic and a fragment of messenger attaches to preformed ribosomes in the presence of streptomycin. Their explanation of the results is that streptomycin causes a fraudulent attachment of messenger with the production of non-functional ribosomes. Again it is clear that a proper understanding of the action of streptomycin and its physiological consequences awaits better knowledge of all the factors concerned in "stage 7."

C. Antibiotics whose action results in inhibition of nucleic acid synthesis

Actinomycin. The actinomycins are a series of polypeptide-containing pigmented antibiotics (227, 229), the first of which was reported by Waksman and Woodruff (230). The formula of actinomycin C is given in figure 5; the antibiotic is toxic to many organisms, Gram-positive bacteria being more sensitive than Gram-negative species. Kirk (125) found that addition of actinomycin D to an exponentially-growing culture of *Staph. aureus* resulted in immediate cessation of

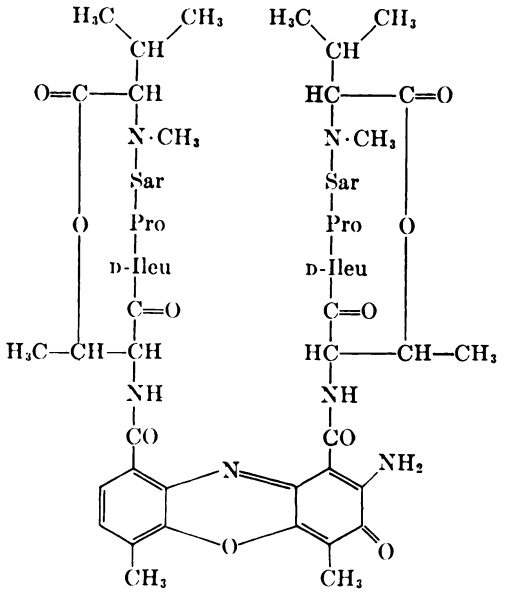


FIG. 5. Actinomycin C.

Sar = sarcosine, Pro = proline, D-Ileu = D-allo-*iso*leucine. In actinomycin D, D-allo-*iso*leucine is replaced by D-valine.

RNA synthesis; the syntheses of protein and DNA were also inhibited but only after a lag period of 15 to 20 min. The inhibition of DNA synthesis for a given concentration of antibiotic was less than that of either RNA or protein synthesis. Formation of wall mucopeptide was not affected at levels of antibiotic giving complete inhibition of protein synthesis. The action on RNA and protein synthesis was antagonised by DNA but not by RNA; DNA preparations from *Staph. aureus*, *B. megaterium*, Brucella, calf thymus, wheat germ, and herring roe were all antagonists. The formation of a complex between actinomycin and DNA was indicated by the following findings: addition of actinomycin at pH 7 to DNA reduced the UV absorption of the antibiotic and shifted the absorption maximum towards the red; on electrophoresis in starch, actinomycin moved with the same mobility as the DNA; addition of actinomycin to DNA solutions resulted in an increase in the viscosity of the latter. The complex was found to be freely dissociable; equilibrium dialysis indicated that 84% of the actinomycin was complexed in a solution containing 136 μg antibiotic and 600 μg DNA/ml. Actinomycinic acid, the dicarboxylic acid derived from actinomycin, was without antibacterial action and the addition of DNA had no effect on its absorption spectrum; bacterial RNA, heat-denatured DNA and heparin had no effect on the spectrum of actinomycin. Kersten *et al.* (124) found that the inhibition of growth of *Neurospora crassa* is antagonised by DNA, while the formation of an actinomycin-DNA complex has been confirmed by Rauen *et al.* (183) and by Kawamata and Imanishi (123). One explanation would be that the nature of the complex formed between actinomycin and DNA is similar to that between acridines and

DNA. From a study of the viscosity, sedimentation coefficient and X-ray diffraction patterns of DNA in the presence of acridines, Lerman (138) concluded that the dye molecule is intercalated between adjacent nucleotide pairs so extending and unwinding the deoxyribose backbone. In this connection it is interesting to find that actinomycin has inhibitory actions similar to those of proflavine (2,8-diaminoacridine) (138). However, Hamilton, Fuller and Reich (94a) have now investigated the X-ray diffraction patterns of the actinomycin-DNA complex and their results cannot be explained on the basis of intercalation of the actinomycin in the DNA helix. Goldberg and co-workers (91a, 91b) have found that the binding of actinomycin is dependent upon the presence of guanine residues and the amount bound increases with the guanine content of the DNA polynucleotide. Hamilton *et al.* (94a) suggest, from model studies, that the heterocyclic chromophore of actinomycin is hydrogen-bonded to the guanine residues of the DNA helix. The positions of the chromophore and cyclic peptides are then stabilised by tight packing in the small groove of the DNA helix and by interaction between the NH groups of the peptides and phosphate oxygen groups of the polynucleotide chain.

The antagonism between DNA and actinomycin raises the question whether the antibiotic produces its inhibitory effects by complexing with DNA *in vivo*. Kirk (125) found that actinomycin inhibits the transformation of *Haemophilus influenzae* from sensitivity to streptomycin-resistance when added to high concentrations of transforming DNA from a resistant strain. The antibiotic was ineffective at low concentrations of transforming DNA, presumably due to dissociation of the antibiotic-DNA complex in dilute solutions. The DNA-dependent polymerases are obviously of interest in this connection. Kirk (125) demonstrated that actinomycin inhibits the DNA-dependent DNA polymerase (see p. 503) and this has been confirmed and studied in greater detail by Hurwitz *et al.* (112) and Elliott (62). Elliott showed that there is a relationship between the amount of antibiotic present, the amount of primer added and the degree of inhibition of the polymerase. The decrease in DNA synthesis could not be explained by stimulation of DNAase activity in the preparation.

Harbers and Muller (101) studied the action of actinomycin on RNA synthesis in Ehrlich ascites tumour cells. They found that nucleotide synthesis was not affected, while the specific activity of nucleoside di- and tri-phosphates increased during RNA inhibition by actinomycin. They concluded that actinomycin must act at the polymerisation level. Radioactive actinomycin was incorporated by tumour cells, and more than 80% of the antibiotic taken up was located in the nuclei, 95% being associated with DNA. During a short incubation period, the antibiotic was found to inhibit the incorporation of uracil into nuclear RNA but not into cytoplasmic RNA and little effect could be shown on the incorporation of orotic acid into cytoplasmic RNA.

Proof that actinomycin inhibits the DNA-dependent synthesis of RNA has been obtained by Hurwitz *et al.* (112) working with the cell-free RNA polymerase. The inhibition can be reduced by increasing the concentration of DNA primer but not of the other components of the system. Reciprocal plots show that the

inhibition is competitive. The antibiotic inhibited the polymerase when this was primed with DNA from bone marrow, thymus, *M. lysodeikticus*, or bacteriophage $\phi\chi 174$ (single stranded DNA), but there was no inhibition of synthesis primed by "synthetic" polythymidylate. RNA polymerase catalyses an exchange reaction between nucleoside triphosphates and pyrophosphate (see reaction 2, p. 503) but this reaction was relatively insensitive to actinomycin. Hurwitz *et al.* confirmed in *B. cereus* the earlier finding of Kirk (125) in *Staph. aureus* that RNA synthesis is more sensitive than DNA or protein synthesis. They pointed out that, since RNA synthesis in the cells is completely inhibited by actinomycin, the inhibition cannot refer only to messenger RNA and, consequently, it is possible that the synthesis of sRNA and ribosomal RNA may also be DNA-dependent.

There is an interesting difference in the actinomycin sensitivity of the cell-free DNA polymerase and the DNA-dependent RNA polymerase: at 0.5 μM actinomycin, RNA polymerase is 80% inhibited while the DNA polymerase is virtually unaffected. Reciprocal plots of the velocity against substrate concentration give K_i (a measure of the association between the enzyme and the DNA-actinomycin complex) as 2.76×10^{-8} M for RNA polymerase and 2.7×10^{-7} M for DNA polymerase, so that the synthesis of DNA is approximately 10 times less sensitive than that of RNA. This difference in the sensitivity of the two systems suffices to explain the differential effects in intact cells described by both Kirk and Hurwitz. Hurwitz *et al.* (112) also demonstrated inhibition of the two polymerase systems by proflavine. In a recent note Reich, Goldberg, and Rabinowitz (185a) described investigations with a series of different actinomycins and showed that there is a strong correlation between the ability of the antibiotics to complex with DNA and their inhibitory action on the growth of *B. subtilis*, the division of HeLa cells, and the cell-free bacterial RNA polymerase.

Reich *et al.* (184, 185) have made the interesting observation that actinomycin D inhibits the multiplication of a DNA virus but not that of a ribonucleic acid virus in tissue culture. They reported that RNA synthesis can be uncoupled from both DNA and protein synthesis by the antibiotic until RNA synthesis in the host is completely suppressed. The multiplication of Mengovirus (a ribonucleic acid virus) is unaffected by a concentration of actinomycin which gives 99.9% inhibition of host RNA synthesis. This can perhaps be correlated with the finding of Hurwitz *et al.* (112) that the RNA-dependent RNA polymerase is unaffected by concentrations of actinomycin which inhibit DNA-dependent RNA polymerase. Nakata *et al.* (157) reported that phage T2 formation and phage protein synthesis are inhibited by actinomycin S at concentrations which do not affect phage DNA synthesis.

V. DISCUSSION

The great importance of antibiotics as clinical agents arises from the selective nature of their toxicity. Prior to the time of many of the investigations described in this review, little was known of the basis of selective toxicity although it was surmised that differential effects could arise from the presence of essential

components specific to the sensitive cells or metabolic processes peculiar to those cells. The last decade has seen many advances in our knowledge of the action of toxic substances and studies on antibiotics have contributed substantially to knowledge of the sites of action which enable drugs to differentiate between bacteria and mammals. The pin-pointing of mucopeptide synthesis as the basis of the action of penicillin is the outstanding gain in information and has turned the attention of many workers to the surface layers of cells in general as possible sites of selective attack. Whether there are such marked differences in the structures of cell membranes is still a matter for investigation but the action of polymyxin and the other antibiotics with surface active properties suggests that there are possibilities for further exploitation here. It is interesting that many of the clinically effective antibiotics have sites of action other than in the surface structures so that the substances discussed in section IV B act upon mechanisms "which we presently presume to be the same in all cells" (228). This has led von Ehrenstein and Lipmann (228) (see p. 512) to suggest that the bacterial mechanism must possess some feature different from that of mammalian cells. Differences may, however, be quantitative rather than qualitative. The bacterial cell possesses rates of synthesis and versatility of adaptation to change far greater than the mammalian cell and it may be that the faster turnover of components such as messenger RNA or transfer RNA in the bacterial system renders it more susceptible to inhibition by substances acting upon those components. The intensive research at present centred on this subject may provide an answer to this particular problem within a short time.

Biochemists are attracted by the antibiotics as tools for the unravelling of complex biosynthetic processes. This has been very noticeable in the case of actinomycin: its action in complexing with DNA was described in 1960 and each month now sees the publication of papers describing the use of actinomycin as a tool for the investigation of DNA synthesis, DNA-dependent syntheses, messenger RNA, and virus multiplication. Chloramphenicol and streptomycin should be of use in the biochemical dissection of what we have called "stage 7" in the mechanism of protein synthesis, while penicillin has already contributed to our knowledge of cell wall synthesis by showing up the existence of the nucleotide precursors. In this way every new antibiotic is potentially a new weapon in the biochemist's armoury.

Before the advent of antibiotics, chemists had achieved success in the field of chemotherapy by the modification of known toxic agents. New fields are now open to them in modification of the molecules of the antibiotics. One of the problems in the clinical use of these drugs has been the rapidity with which pathogenic organisms become resistant. One form of resistance involves the development of enzymes which alter the structure and so destroy the activity of the antibiotic. The outstanding example here has been penicillin-resistance due to penicillinase, and the development of the "new penicillins" has shown the important part that the chemist can play in this connection. In general, antibiotics have structures so complex that total synthesis, even where possible, would be uneconomic but experience with the new penicillins has shown what can be accomplished by modification of a part of the molecule of otherwise biosynthetic material.

The future should hold promise of new selective agents based upon biochemical differences between organisms. The chemistry of bacterial cell walls held the key to the action of penicillin. It may be possible to exploit other differences in the wall chemistry. Who knows what may come from a full knowledge of the comparative biochemistry of membranes, nuclei, ribosomes, lipids, proteins, enzymes and isozymes?

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