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INHIBITION OF MAMMALIAN PROTEIN SYNTHESIS BY ANTIBIOTICS[1]

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TABLE OF CONTENTS

I. INTRODUCTION

In 1953 Gale and Folkes (75) first demonstrated that chloramphenicol inhibited protein synthesis in bacteria. Subsequently, it has been shown that chloramphenicol interferes with the final stages of assembly of amino acids in peptide synthesis (216). As other antibiotics have become available, their mechanism of action with respect to the various steps involved in protein synthesis have also been investigated. It has become apparent that most anti-

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biotics, with the exception of those that act on cell wall assembly, inhibit protein synthesis in bacterial systems, and it is commonly accepted that the bacteriostatic and bacteriocidal effects of these antibiotics are related to their inhibitory effects on protein synthesis.

Because of technical difficulties, it has been considerably easier to study the effects of antibiotics in bacterial systems than in mammalian systems. As a result, a great deal more data are available concerning the effect of these agents on microbial than on mammalian protein synthesis. However, enough studies have been performed in mammalian systems to confirm that most antibiotics also have a significant effect on mammalian protein synthesis. Although there are many similarities in the mechanism of action of antibiotics on protein synthesis in both systems, there are some striking dissimilarities. The purpose of this report is to review the effects of the various antibiotics now available on mammalian protein synthesis.

Protein synthesis is the end result of three major processes: 1) deoxyribonucleic acid (DNA) synthesis, or replication; 2) DNA-dependent ribonucleic acid (RNA) synthesis, or transcription; and 3) RNA-dependent protein synthesis or translation. An antibiotic that inhibits any one of these processes will inhibit protein synthesis. The antibiotics that primarily inhibit translation are those which have been found to be most useful clinically. The agents that inhibit protein synthesis by a primary inhibition of DNA or RNA synthesis have not been found to be clinically useful as antibiotics because of their toxicity. Despite their limited clinical application, the agents that inhibit transcription have been included in this review since they have been extremely useful in studying the steps involved in mammalian protein synthesis.

II. ANTIBIOTICS THAT AFFECT TRANSCRIPTION

Inhibition of protein synthesis in mammalian cells occurs when chemical or physical agents interfere with the synthesis of RNA. The inhibition is most readily apparent when the synthesis of messenger RNA (mRNA) is interrupted but also may follow a defect in the production of ribosomal RNA (rRNA) or transfer RNA (sRNA). Several antibiotics are believed to exert their effect principally through interference with RNA production.

The basic steps involved in the synthesis of RNA, which has a nucleotide sequence complementary to that of nuclear DNA, appear to be similar in bacterial and mammalian cells. Helical DNA serves as a template, and the nucleotides are assembled in a complementary sequence by an enzyme, DNA-dependent RNA polymerase. The biochemistry of these reactions has been reviewed by several authors (52, 112, 135, 183). Antibiotics that alter the structure of the template DNA or inhibit the polymerase enzyme system will interfere with the synthesis of mRNA, and consequently with cellular protein synthesis.

A. Antibiotics making complexes with DNA

The actinomycins are polypeptide antibiotics highly toxic to mammalian cells and tissues as well as to bacteria. The formation of complexes between these

antibiotics and DNA has been clearly shown *in vivo* and *in vitro* in a variety of mammalian and bacterial DNA preparations. The diverse effects of the actinomycins, particularly actinomycin D(C_1) on protein synthesis in mammalian systems may be adequately explained as a result of the complex-formation with DNA and subsequent interference with RNA synthesis. Extensive reviews have appeared outlining the biological action of the actinomycins (89, 163, 164, 166).

The formation of the complex between DNA and actinomycin appears to be quite specific and requires the presence of guanine-containing helical deoxyribonucleotides. Polynucleotides not containing guanine (dAT, dAdT, dIdC) do not form complexes with actinomycin (164). Single-stranded, native DNA has less capacity to bind actinomycin (165), and the denaturation of DNA by heat or acid with loss of the ordered helical structure is accompanied by dissociation of the DNA-actinomycin complexes (82). A model for the structure of the actinomycin-DNA complex that would explain this specificity in binding has been presented and discussed by Reich *et al.* in a recent review (165).

The replication of most RNA viruses in mammalian cells is unaffected by actinomycin, while the cellular DNA-dependent RNA replication is extremely sensitive, with resulting inhibition of protein synthesis. This is evidence that actinomycin complexes only with DNA. DNA-actinomycin complexes formed *in vitro* do not function as an accurate template for RNA polymerase. In contrast, artificial polynucleotides (dAT, dAdT), which do not form complexes, function in an unimpaired manner (89). These studies have been useful in evaluating the function of DNA-dependent RNA polymerase. Actinomycin is now widely used as a tool to block messenger RNA production in mammalian and bacterial cells (1, 7, 134, 201) and so to separate direct effects on protein synthesis from indirect effects on DNA synthesis or translation. As an example, extensive studies have been performed with actinomycin in mammalian systems *in vivo* and *in vitro* to elucidate the mechanism of protein synthesis induced by the action of hormones on target cells (170). These studies have demonstrated that actinomycin interferes with the function of many of these hormones by inhibiting mRNA production.

Chromomycins are produced by a strain of *Streptomyces guseus* as a complex of closely related cancerostatic variants. The variant present in greatest amount, and that which has been studied extensively, is chromomycin A_3. It is inhibitory to the growth of Gram-positive bacteria (121, 204), is rapidly lethal to mice after intraperitoneal or intravenous injection (35, 121), and inhibits the growth of HeLa cells in culture (171, 193).

Wakisaka *et al.* (211) have demonstrated a selective inhibition of RNA synthesis by chromomycin A_3 in cultures of mammalian cells. The fact that chromomycin A_3 complexed with template DNA (98, 122, 123) suggested that this inhibition might be mediated through interference with the function of the DNA-dependent RNA polymerase system. Bivalent cations (Mg^{++}, Mn^{++}, Zn^{++}, and Co^{++}) were shown to interact with chromomycin A_3 and to have an important role in initiating and maintaining the complex of this antibiotic with template DNA (213). A similar interaction of bivalent cations with the antibiotic oliovmycin occurs (25, 26).

Olivomycin I is the principal component of a complex of antibiotics recovered from the culture medium of *Streptomyces olivoreticuli*. Its chemistry has been extensively investigated and a structural formula proposed (14–16). Its mechanism of action appears to resemble that of chromomycin in its ability to complex with template DNA. Olivomycin, like chromomycin, forms complexes with bivalent metal ions (25), and this metal-antibiotic complex seems important in binding to DNA (213). Experiments *in vitro* by Gause *et al.* have shown that this antibiotic strongly inhibits DNA-dependent RNA synthesis from ribonucleoside triphosphates (80, 81). Studies with cultured mammalian cells (80, 200, 229, 230) have also demonstrated a selective inhibition of RNA synthesis.

An antibiotic similar to chromomycin and olivomycin has been isolated from another *Streptomyces* and identified as mithramycin (162). This antibiotic can be separated from the others by chromatography (128). Studies on the mechanism of action of mithramycin suggest that its behavior is identical to that of chromomycin and olivomycin (123, 213). In the presence of a bivalent cation, usually Mg^{++}, mithramycin forms a complex with template DNA. This DNA-metal-antibiotic complex interferes with the transcription of DNA by DNA-dependent RNA polymerase.

Nogalamycin, an antibiotic isolated from *Streptomyces nogalater* var. *nogalater* (19), has marked cytotoxic effects on cultured mammalian cells. It binds to DNA and appears to require a specific sequence of deoxyadenine and deoxythymidine residues for complex formation (21, 213). In this respect it differs from actinomycin, which requires the presence of deoxyguanine for binding. The transcription of template DNA to RNA is inhibited by nogalamycin, but the replication of RNA viruses, in which RNA functions as the template for further RNA synthesis, is unaffected (213). Comparison of the inhibition by nogalamycin of protein synthesis by bacteria and mammalian cells (19) suggests that the stability of mammalian messenger RNA explains the lower sensitivity of mammalian cells to this compound. Experiments involving the induction of tryptophan pyrollase in rat liver by hydrocortisone *in vivo* (93) strongly suggest that nogalamycin inhibits the synthesis of messenger RNA.

Daunomycin and cinerubin are two closely related antibiotics produced by *Streptomyces* species. These antibiotics contain anthracycline groups (29). They strongly inhibit the growth of mammalian cells *in vivo* (42, 50) and experimental tumors *in vitro* (51, 54). The principal cytological observation has been the rapid development of chromosomal aberrations and "breaks," and a drop in the number of mitotic cells (48).

The anthracycline antibiotics form complexes with DNA. Spectral alterations and alterations in sedimentation velocity in a density gradient occur when daunomycin or cinerubin are added to solutions of DNA (33, 122). Alterations in the viscosity of the DNA solutions also occur (33). The formation of the antibiotic-DNA complex stabilizes the DNA in a helical configuration, inferred from the increased temperature of thermal transition or "melting" (123). Although the presence of guanine is not an absolute requirement for binding, as in the case of actinomycin, complex formation increases with rising guanine and cytosine

content of the DNA (123). Increasing ionic strength, especially of bivalent cations such as Mn^{++} or Mg^{++}, decreases complex formation (33). Complex formation between daunomycin and DNA inhibits RNA synthesis in bacteria (12) and mammalian cell cultures (49). This effect can be accounted for by an inhibition of the DNA-dependent RNA polymerase reaction (213).

Pluramycin A is the most active component of a group of antibiotics isolated from cultures of *Streptomyces pluricolorescens*. Together with its antibacterial activity, it inhibits the growth of cultured mammalian tumor cells (154, 195). Pluramycin stabilizes DNA to thermal denaturation and shifts the thermal transition curve (198). *In vitro*, it interferes with the DNA-dependent RNA polymerase reaction (198).

B. Novobiocin and colicins

Two antibiotics with dissimilar mechanisms of action indirectly affect DNA-directed RNA synthesis. Novobiocin is an antibiotic that binds bivalent cations, especially magnesium. As a consequence of cellular magnesium deficiency, RNA synthesis is markedly reduced. The mechanism of action of this antibiotic has been extensively reviewed (28).

The colicins are polypeptide antibiotics that interfere with cellular oxidative phosphorylation, and consequently with all cellular macromolecular synthesis (157). The action of these antibiotics is also an example of an indirect effect on transcription within the cell.

C. Clinical applications

None of the agents that affect transcription has found clinical application as antibiotics because of their extreme toxicity. Actinomycin has been used in the chemotherapy of embryonal cell tumors, choriocarcinoma, and Wilms tumor with some success either alone or in combination with other chemotherapeutic agents. Mithramycin has found very limited use in the therapy of embryonal cell carcinoma. Recently daunomycin has been employed in the therapy of acute leukemia, but experience in its use is limited. None of the other antibiotics that inhibit transcription has been used clinically to an appreciable extent.

III. ANTIBIOTICS THAT AFFECT TRANSLATION

During the past 10 years the mechanism for translating the genetic message into protein has been studied extensively in bacteria and, to a lesser extent, in mammals with both cell-free systems and intact cells. Although the basic mechanisms for translation appear to be similar for bacterial and mammalian cells, occasional differences have been observed in the inhibitory effect of certain antibiotics on the two species. This has led to the assumption that certain agents may inhibit bacterial protein synthesis without affecting mammalian protein synthesis. However, it has become apparent that several antibiotics previously thought to affect only bacterial systems may, under certain specific conditions, inhibit mammalian systems and therefore may threaten the host as well as the invading bacteria.

The general process of translating the genetic code into protein can be considered to involve four major reactions: 1) formation of aminoacyl-sRNA; 2) interaction between ribosome, mRNA, and aminoacyl-sRNA to form a ternary complex; 3) synthesis of peptide bonds; and 4) translocation of the ribosome along the mRNA strand. Formation of aminoacyl-sRNA involves interactions between a specific amino acid, a specific enzyme, and a specific sRNA molecule. Once the amino acid has been attached, the coding specificity resides in the RNA molecule, which appears to contain a trinucleotide sequence that is complementary to a trinucleotide sequence on the mRNA (6, 143, 173, 180). Since there are few antibiotics known that interfere with aminoacylation of sRNA, this discussion has been confined to the latter three reactions.

According to the above scheme, ribosomes bind to the mRNA strand much like beads on a string, starting at the initial mRNA codeword and moving to the terminal codeword, where they are released together with the completed peptide chain. During this process aminoacyl-sRNA molecules are shuttled on and off the ribosome, giving up the appropriate amino acids to the growing peptide chain. As the first ribosome moves down the mRNA strand, other ribosomes attach and move forward until the entire strand is filled to give the multicomponent assembly known as the polyribosome. This complex is formed by attachment of a ribosome to a site at the 5′-phosphate end of the mRNA strand, followed by binding of two sRNA molecules.

As the ribosomes move along the messenger RNA, the growing peptide chain remains attached through its ester link with one of the sRNA molecules, which in turn is noncovalently bound to the ribosome. Although the mechanisms and requirements for translation have not been completely defined, it is thought that certain antibiotics may specifically inhibit ribosomal movement without affecting any other reaction involved in protein synthesis (156).

Differences in enzymatic requirement, as well as in the size of individual ribosomes, have been observed in mammalian and bacterial cells. Vazquez and Monro (208) have shown that the effect of various antibiotics in different systems seems to correlate with ribosomal size. Such differences in enzymatic requirements and structure may account for the differences in the effects of some antibiotics on mammalian and bacterial protein synthesis.

In the following discussion, antibiotics affecting the translation step in protein synthesis have been placed in three general categories. Group I includes those agents which inhibit the formation of the mRNA-ribosome-sRNA complex, Group II includes agents that inhibit the peptide synthetase reaction, and Group III covers agents that interfere with the translocation of the ribosome along the mRNA strand.

A. Group I agents

These antibiotics exert their effect while the cell is depositing newly formed messenger RNA on the ribosomes to form polyribosomes. Once this complex is formed, the mammalian cell is no longer susceptible to inhibition by the antibiotic. In contrast, the complex in bacterial cells appears to remain sensitive to

these antibiotics even after it is formed, although the site of action appears to be the same in both systems.

1. Chloramphenicol. Chloramphenicol inhibits microbial protein synthesis in a wide variety of bacteria without directly affecting energy-yielding processes, cell permeability, or cell wall synthesis (27). Inhibition occurs both in intact bacteria and in cell-free systems, and it is assumed that the bacteriostatic activity of the drug is due to inhibition of protein synthesis. Although the mechanism by which chloramphenicol inhibits microbial protein synthesis has been investigated intensively, it is still not understood. There have been several conflicting observations that are difficult to reconcile. These have been reviewed in detail by Weisberger (216). The preponderance of evidence now available indicates that in bacteria chloramphenicol acts on a ribosomal site at a stage after the binding of mRNA and during peptide synthesis to prevent the final condensation of amino acids and the growth of nascent polypeptide chains (109, 205).

Although chloramphenicol readily inhibits protein synthesis in microbial systems, protein synthesis in mammalian systems is usually markedly resistant to inhibition by chloramphenicol. The difference in sensitivity to chloramphenicol is most apparent in cell-free systems, in which cellular permeability is not a factor. Nirenberg and Matthei (153) were able to obtain almost complete inhibition of protein synthesis in *Escherichia coli* cell-free systems with 0.15 μmoles of chloramphenicol/ml reaction mixture. In contrast, Von Ehrenstein and Lipmann (210) obtained comparatively little inhibition of protein synthesis in mammalian systems with 10 μmoles of chloramphenicol/ml reaction mixture. Similar observations have been made in cell-free systems by Wang (212) with thymus preparations, by Allen and Schweet (1a) with reticulocyte preparations, and by Rendi (167) with rat liver ribosomes. Protein synthesis in intact cells is also resistant to inhibition by chloramphenicol. Borsook *et al.* (24) were unable to inhibit amino acid incorporation by reticulocytes *in vitro* except with amounts of chloramphenicol that greatly exceed therapeutic concentrations. The failure of chloramphenicol to inhibit protein synthesis by intact tumor cells was noted by LePage (133). The resistance of mammalian protein synthesis to inhibition by chloramphenicol is so remarkable that Von Ehrenstein and Lipmann (210) suggested the possibility that the difference in sensitivity might be due to the presence in bacteria of an extra, drug-sensitive step involved in the transfer of amino acids to ribosomes.

Despite the seeming resistance of mammalian systems to inhibition by chloramphenicol, a number of subsequent observations indicated that maturing or proliferating mammalian cells were uniquely susceptible to inhibition by chloramphenicol. Saidi *et al.* (169) demonstrated that when chloramphenicol is administered to patients whose hematologic status is characterized by the presence of immature or proliferating erythrocytes, there is a uniform increase in erythropoietic suppression. Ambrose and Coons (3) demonstrated that newly induced antibody synthesis by lymph node fragments in tissue culture is suppressed by low concentrations of the drug and suggested that chloramphenicol might act by interfering with the function of mRNA formed in response to antigenic stimu-

lation. The inhibition of protein synthesis in human bone marrow cultures exposed to prolonged low levels of chloramphenicol and the inhibition of protein synthesis by chick fibroblasts in tissue culture (4) may also be manifestations of the increased susceptibility of proliferating or maturing cells to the antibiotic. These observations suggest that mammalian cells might be susceptible to inhibition by chloramphenicol only during phases when there is a rapid turnover of mRNA associated with newly induced protein synthesis or when numerous cells are being committed to the rapid synthesis of protein.

As a result of these observations, the effect of chloramphenicol on mammalian protein synthesis has been studied in cell-free systems designed to simulate the sequence of events in cells being committed to rapid protein synthesis. Weisberger *et al.* (220) demonstrated that when stimulatory RNA is added to reticulocyte ribosomes in a cell-free system, chloramphenicol inhibits protein synthesis induced by the added RNA but has almost no inhibitory effect on unstimulated or endogenous ribosomal protein synthesis. Almost complete inhibition was obtained with the same concentrations of chloramphenicol which are effective in bacterial systems. Since the inhibitory effect of the drug could not be overcome by adding excessive amounts of stimulatory RNA, direct inactivation of RNA by the antibiotic is unlikely. Armentrout and Weisberger (8) showed that the inhibitory effect of chloramphenicol was markedly affected by magnesium ion concentration. Zelkowitz *et al.* (231) have not been able to confirm an inhibitory effect of chloramphenicol in similar systems. The reason for this discrepancy has not been elucidated.

When reticulocyte ribosomes were stimulated with a synthetic template, polyuridylic acid (poly U), inhibition by chloramphenicol was also obtained (217), but the effect was not as marked as with natural template RNA. Inhibition was observed only when small amounts of poly U were employed and only in the presence of low magnesium ion concentration. Furthermore, the inhibition could be overcome by adding more poly U to the reaction mixture. These results indicate that the effect of chloramphenicol on synthetic template-ribosomal interactions may not be the same as that occurring with natural template RNA and suggest that interpretations based on results obtained with synthetic templates may not be valid.

Evidence has been obtained that the inhibitory effect of chloramphenicol on RNA-stimulated protein synthesis is related to an effect of the drug on ribosomal-RNA interaction. It has been suggested that the antibiotic inhibits the function of messenger RNA by blocking its attachment to ribosomes rather than by directly inactivating the template activity of RNA (217, 220). In support of this binding of ^{14}C-poly U to reticulocyte ribosomes is partially inhibited by chloramphenicol (224). Jardetzky (116) made a similar observation with *E. coli*. Additional supportive evidence for the view that the antibiotic acts by inhibiting the attachment of mRNA to ribosomes was obtained by Murthy (145) in studies with rat brain and rat liver ribosomes. Zelkowitz *et al.* (231), however, were unable to demonstrate any inhibition of binding of poly U to reticulocyte ribosomes in the presence of chloramphenicol. Armentrout and Weisberger (9)

have since shown that chloramphenicol interferes with polyribosome formation but does not inhibit the binding of messenger ribonucleoprotein to reticulocyte monoribosomes.

The observations of Talal and Exum (196) are unique in that they have obtained data indicating that protein synthesis by a certain class of ribosomes can be directly inhibited by chloramphenicol. These investigators separated rat spleen cell ribosomes into those bound to endoplasmic reticulum and those free in the cytoplasm. Cell-free protein synthesis by bound ribosomes was inhibited by 10^{-4} M chloramphenicol, whereas protein synthesis by free ribosomes was resistant to chloramphenicol. They suggested that bound ribosomes might represent an earlier stage of ribosomal development and that their sensitivity to chloramphenicol might be related to the immaturity of the ribosomes. Further study of this phenomenon is needed since it is difficult to reconcile these observations with the numerous examples of resistance of endogenous mammalian protein synthesis to chloramphenicol.

In addition to the effect of chloramphenicol on cell-free protein synthesis by ribosomes, the antibiotic has also been shown to affect protein synthesis by mitochondria. Farese (55) found that chloramphenicol inhibits adrenocorticotropin (ACTH)-stimulated production of corticosterone, and this effect has been correlated with inhibition of amino acid incorporation into protein in a mitochondrial cell-free system derived from rat adrenal gland (77). In similar studies with systems derived from heart and liver, low levels of chloramphenicol inhibited mitochondrial protein synthesis (124, 167). Frikin and Linane (61) found that low levels of chloramphenicol (10 to 40 μg/ml) inhibited the synthesis of cytochromes while high levels of the drug (100 to 150 μg/ml) inhibited mitochondrial respiration. Martelo *et al.* (137) found 50 to 80% inhibition of protein synthesis by bone marrow mitochondria with low levels of chloramphenicol.

A considerable amount of attention has been focused on the question of whether protein synthesis by mitochondria is an inherent property of the particle or whether it is the result of contamination of the mitochondrial preparations with either ribosomes or bacteria (159). The possibility of a major bacterial contribution to the incorporation activity measured in mitochondrial preparations is difficult to exclude. Accordingly, the significance of observations of the effect of chloramphenicol on mitochondrial protein synthesis remains uncertain until the absence of bacterial contamination has been demonstrated.

Although the data indicate that chloramphenicol may have an inhibitory effect on mitochondrial protein synthesis as well as on RNA-stimulated ribosomal protein synthesis, it is difficult to reconcile the mitochondrial effects of the drug with observations in whole cells. Inhibition of mitochondrial protein synthesis *per se* does not, for example, explain the resistance of most mammalian cells to inhibition by chloramphenicol and the marked susceptibility of cells being newly committed to protein synthesis. The immunosuppressive effect of chloramphenicol, both *in vitro* and *in vivo*, is best explained by the drug interfering with the function of messenger RNA formed in response to antigenic stimulation and is in keeping with the observations in cell-free systems that chloramphenicol inter-

feres with RNA-ribosomal interactions. Furthermore, Godchaux and Herbert (85) found no significant alteration in cellular adenosinetriphosphate levels in reticulocytes and in bone marrow cells exposed to chloramphenicol; this finding indicates that the effect of the drug on mitochondrial function in intact cells is minimal. Nevertheless, some observers consider that the effects of chloramphenicol on mitochondrial protein syntheses are of primary importance in mammalian systems and doubt the validity of observations with respect to protein synthesis by ribosomes (231).

The ability of chloramphenicol to inhibit protein synthesis by intact mammalian cells is particularly well shown by studying the effect of the drug on antibody synthesis. Such suppression was shown by Ambrose and Coons (3) with cultures of lymph node fragments and by Svehag (191, 192) with spleen cells. Suppression of the primary immune response by chloramphenicol has been obtained in mice but required large amounts of the drug (32, 43). Weisberger *et al.* (218), however, uniformly obtained suppression of the primary immune response in rabbits when therapeutic blood levels of chloramphenicol were maintained. Although little or no primary immune response was detected in these animals, they subsequently exhibited a normal anamnestic response after a second antigenic stimulus. The prompt development of a normal anamnestic response in such animals indicated that cells were prepared for antibody synthesis during the period when chloramphenicol suppressed the synthesis of primary antibody. This observation is in accord with the hypothesis that the drug inhibits *de novo* mammalian protein synthesis by blocking the function of messenger RNA without directly affecting DNA or RNA synthesis.

The effect of chloramphenicol on the cellular aspects of the immune response has been examined by Schoenberg *et al.* (172). The absence of intracellular antibody, as well as suppression of circulating antibody, was demonstrated in rabbits receiving a variety of antigens combined with complete Freund's adjuvant. A delayed appearance and slower rate of maturation of antibody-producing cells of the spleen was observed in animals receiving chloramphenicol. However, the fact that an immune response developed promptly when the antibiotic was discontinued indicates that immunologically competent cells were prepared.

The immune mechanisms involved in homograft rejection in rabbits are also suppressed by chloramphenicol (218) and this results in significant prolongation of skin transplant survival. Chloramphenicol also suppresses the immune nephritis induced in rabbits by the injection of avian antirabbit kidney serum (219). Rabbits receiving chloramphenicol failed to develop measurable antikidney antibody or significant damage of renal glomeruli so long as the drug was administered. When the antibiotic was discontinued, some of the animals developed a mild nephritis, presumably because of the persistence of heterologous nephrotoxic antigen.

Various analogs prepared by substitution of the NO_2 group of the nitrobenzene moiety of chloramphenicol have recently been shown by Weisberger *et al.* (218) in this laboratory to suppress antibody synthesis in rabbits. The methylsulfonyl analog of chloramphenicol has been studied more extensively than other analogs

and has been shown to suppress circulating antibody even when administration of the drug was begun 3 days after antigen was injected and to prolong the survival of skin homografts. On a weight basis, the methylsulfonyl analog is as potent as chloramphenicol in cell-free systems but is 4 to 5 times more potent than chloramphenicol *in vivo*. Analogs of chloramphenicol that maintain the steric configuration of the molecule inhibit mammalian protein synthesis as well as bacterial protein synthesis. Alteration of the propanediol moiety of chloramphenicol results in loss of antibiotic activity, and such compounds no longer inhibit protein synthesis. These observations are probably related to the findings of Jardetzky (115) with respect to the structural resemblance of chloramphenicol in solution to pyrimidine nucleotides.

In man, chloramphenicol and its analogs also have an immunosuppressive effect, even when employed in doses commonly used to treat clinical infections. Chloramphenicol suppresses the anamnestic response to tetanus toxoid (44). The methylsulfonyl analog of chloramphenicol has been studied as an immunosuppressive agent in lupus nephritis by Svec *et al.* (190). After a single course of this drug (2 g daily for 16 days), 4 of 6 patients exhibited a rise in serum complement, a fall in anti-DNA and antinuclear factor titers, and a disappearance of glomerular-bound *gamma*-globulin. These immunosuppressive effects were associated with stabilization of kidney function. The most surprising observation in this study is that sustained remissions have persisted from 1 to 3 years, and it is suggested that the drug may have blocked an autoimmune mechanism or possibly induced a form of immune tolerance.

In summary, chloramphenicol has a strong inhibitory effect on mammalian protein synthesis but only in circumstances of rapid cell turnover or when cells are being committed to newly induced protein synthesis. Although chloramphenicol is reported to affect mitochondrial protein synthesis in mammalian systems, the inhibitory effect of the drug is best explained by an interference with the function of mRNA. Chloramphenicol acts only during the earliest stages of mRNA-ribosomal interactions and, unlike bacteria, once the polyribosomal complex is formed, mammalian cells are no longer accessible to inhibition by chloramphenicol.

2. Tetracycline family. The effect of this group of antibiotics on mammalian protein synthesis both *in vivo* and *in vitro* has been extensively investigated by several groups. Nikolov and Ilkov (150) have shown that in rabbits chlortetracycline inhibited the incorporation of ^{35}S-labeled methionine into the proteins of liver, gastric mucosa, and spleen. Nikolov *et al.* (151) showed that tetracycline, oxytetracycline, and chlortetracycline inhibited antibody formation in spleen cells, but that chlortetracycline was more potent than the other two. In a recent study, Greenberger (94) demonstrated that the incorporation of ^{14}C-leucine into the protein of rat jejunal slices was inhibited by 29% when the rats had been treated with tetracycline intraperitoneally. Yeh and Shils (226) found inhibition of amino acid incorporation into the proteins of a large number of rat tissues, regardless of whether the antibiotic was given intragastrically, intramuscularly, or intravenously.

In contrast, Franklin (67) found no effect on ^{14}C-leucine incorporation into rat liver proteins or on the induction of tryptophan pyrollase, when chlortetracycline was given as a single injection or fed to the animals. Since he had previously found (66) that chlortetracycline inhibited ^{14}C-leucine incorporation into protein in a cell-free system from rat liver, Franklin concluded that the drug did not reach the active site because of cell impermeability. It is also possible that in the liver chlortetracycline is inactivated so rapidly that the organ is protected from the effect seen in other tissues.

In a discussion of the metabolic effects of tetracyclines, Shils (176) noted that occasionally tetracycline therapy in patients is followed by increased urinary loss of nitrogen, which he interpreted as a manifestation of a general inhibition of protein synthesis. These findings may be related to the tendency of tetracycline to decompose spontaneously to anhydro- and epianhydrotetracycline. These breakdown products cause a reversible lesion of the upper nephron. The possibility that these materials are present in aged tetracycline should be considered when tetracycline has any of the above effects, particularly when the experiment involves whole animals.

The effect of tetracycline on mammalian protein synthesis has been examined in cell-free systems. Rendi and Ochoa (168) reported that oxytetracycline had no effect on the transfer of ^{14}C-leucine from aminoacyl-sRNA to protein in a cell-free system from rat liver. In contrast, Franklin (65) demonstrated inhibition by chlortetracycline of overall protein synthesis in a cell-free system of rat liver and demonstrated that the sensitive step was the "transfer reaction", *e.g.*, the transfer of amino acids from aminoacyl-sRNA into protein. In a subsequent paper, Franklin (66) compared the effect of the same concentration of chlortetracycline, oxytetracycline, tetracycline, and chloramphenicol on the "transfer reaction." Chlortetracycline gave 78% inhibition, while oxytetracycline and tetracycline gave only 27%, and chloramphenicol had no effect. He suggested that Rendi and Ochoa had probably used too small a concentration of the antibiotic. In a later report Franklin (68) used sRNA labeled with ^{14}C-cytosine or ^{14}C-uracil to study the "energy dependent" binding of sRNA to rat liver ribosomes but failed to find an inhibition of this reaction by any of the tetracyclines. He suggested that the high level of binding in the absence of energy might have obscured an inhibitory effect.

In similar studies with a cell-free system from rabbit reticulocytes, Weisberger *et al.* (220) found that tetracycline at a concentration of 0.01 μmoles/ml caused a 75% inhibition of the protein synthesis directed by added mRNA but had no effect on the synthesis that occurred in the absence of added mRNA. This finding suggested that cells may be susceptible to tetracycline inhibition only during periods of rapid protein synthesis.

More recently Clark and Chang (36), using the millipore technique (152), showed that chlortetracycline inhibited poly U-directed enzymatic binding of ^{14}C-phenylalanyl-sRNA to reticulocyte ribosomes, without an effect on the binding of poly U to the ribosomes. They also found that chlortetracycline did not alter the puromycin-induced release of peptides from ribosomes. This indi-

cated that chlortetracycline did not inhibit the formation of peptide bonds. Similar results have been obtained with tetracycline.

These studies indicate that the tetracyclines can inhibit mammalian protein synthesis at the translational level, although the concentrations required are slightly higher than those needed to show the same effect in bacteria. They appear to act by inhibiting the binding of amino-acyl-sRNA to the mRNA-ribosome complex. The mechanism of the increased inhibitory effect by the chlorinated tetracyclines requires further investigation.

3. Aminoglycoside family. This group of agents includes streptomycin, dihydrostreptomycin, kanamycin, hydroxystreptomycin, mannosidostreptomycin, neomycin, and paromomycin I (catenulin), which share the streptamine nucleus in common; spectinomycin and kasugamycin, which share the actinamine nucleus; and gentamicin and hygromycin B, for which the structure is incompletely known (114). Of these agents, only streptomycin, dihydrostreptomycin, neomycin, and kanamycin have been evaluated for an effect on mammalian protein synthesis.

The mechanism of action of streptomycin in bacteria has been the subject of extensive investigation. Davies *et al.* (45) demonstrated that streptomycin caused a significant misreading of the genetic code, which they believed to be due to an alteration of the bacterial ribosome. They also examined the effect of streptomycin in mammalian cell-free systems. They demonstrated inhibition of endogenous protein synthesis but were unable to find significant miscoding when mammalian ribosomes were stimulated by poly U. In similar studies with cell-free systems derived from rat liver and rabbit reticulocytes, a low level of misreading of poly U occurred in the presence of streptomycin, and this misreading was almost completely eliminated by high concentrations of sRNA (71, 72, 214, 215). There was also a slightly greater misreading in the presence of high levels of magnesium and in the absence of streptomycin. However, under both conditions the amount of miscoding was much smaller than that seen with *E. coli* ribosomes. Stavy (187) also found no effect on coding by streptomycin.

Streptomycin has also been shown to inhibit mammalian protein synthesis in intact cells. Moskowitz and Kelker (141) found that streptomycin killed chick fibroblasts in tissue culture, but that dihydrostreptomycin had no effect at the same concentration. They suggested that mammalian cells might be impermeable to dihydrostreptomycin. These authors also found (142) that the sensitivity of strain-L mouse fibroblasts to streptomycin varied with changes in the composition of the culture medium. The addition of cysteine to the culture medium caused the cells to become susceptible to streptomycin. They speculated that streptomycin might have caused a misreading of the code so that a lethal protein, or lethal amount of useless protein, was produced.

Studies by Krueger (126, 127) showed that streptomycin, dihydrostreptomycin, neomycin, and kanamycin inhibited the synthesis of neutralizing antibody against phage MS-2 in cultures of spleen and lymph node cells from immunized rabbits. Streptomycin did not inhibit total antibody synthesis, but the antibody formed in the presence of streptomycin could combine only with the incomplete MS-2 phage. It also did not cross-react with other phage particles to the same

extent as antibody produced in the absence of streptomycin. This indicated that streptomycin acted to change the antigenicity of the phage or that it interacted with the ribosomes to cause a misreading of the mRNA code (90). The investigators preferred the latter hypothesis since streptomycin did not alter the ability of normal antibody to combine with the phage, and no binding of ^{3}H-dihydrostreptomycin to MS-2 phage could be demonstrated.

Weinstein *et al.* (214, 215), studying cell-free protein synthesis in systems derived from rat liver and rabbit reticulocytes, found that both systems were markedly inhibited by neomycin at 25 μg/ml and by steptomycin at 10^{-4} M, irrespective of the type of mRNA used. Clark and Chang (36) showed that neomycin inhibited poly-U-directed polyphenylalanine synthesis in a rabbit reticulocyte cell-free system and also inhibited endogenous protein synthesis. However, at low concentrations (0.25 μmoles/ml), there was nearly a two-fold stimulation of the poly-U-directed enzymatic binding of ^{14}C-phenylalanyl-sRNA to the ribosomes. Also, concentrations of the antibiotic that partially inhibited amino acid incorporation did not inhibit the puromycin-induced release of peptides from prelabeled ribosomes, whereas at concentrations that stimulated sRNA binding, there was an inhibition of the puromycin effect. This finding suggested that neomycin altered the attachment of aminoacyl-sRNA to the ribosome, and that when this alteration was maximal peptide synthesis could no longer occur. This would also suggest the possibility that the primary effect may be to cause a conformational change in the structure of the ribosome so that it could bind more sRNA but could not continue down the mRNA strand.

It is apparent that these antibiotics have inhibitory effects on mammalian protein synthesis, but that there are significant differences from bacterial systems which remain to be resolved.

4. Macrolide family. The macrolides share a large lactone ring of 12 to 22 carbon atoms and have one or more sugars attached, which may or may not be nitrogenous. In the strictest sense any antibiotic that contains a lactone ring can be termed a macrolide, but this would include the streptogramins, and these have been placed in a separate category since there appears to be a functional difference between the two groups (206, 207).

Erythromycin and, to a lesser extent, oleandomycin have been studied in bacterial systems (96). This group of antibiotics contains angolamycin, erythromycin, leucomycin A_1, macrocin, methymycin, narbomycin, neomethymycin, niddamycin, oleandomycin, picromycin, relomycin, spiramycin I, II, and III, neospiramycin III, forocidin III, and tylosin. There have been few studies on the effect of macrolide antibiotics on mammalian systems. Weisberger *et al.* (220) found that erythromycin at a concentration of 0.01 mM completely inhibited the protein synthesis stimulated by added mRNA in a reticulocyte cell-free system (see also 208).

5. Streptogramin family. None of these antibiotics has been studied for its effect on mammalian protein synthesis. They are included here because of their close resemblance to the macrolides in structure and action. In bacterial cell-free systems some of the streptogramins appear to inhibit protein synthesis by acting on the 50 S ribosomal subunit to inhibit the binding of aminoacyl-sRNA. Others

inhibit protein synthesis at a point after aminoacyl-sRNA formation and before formation of the complete polypeptide, but the mechanism is as yet unknown. A more detailed account of these effects is presented in the review by Vazquez (207, 208). It would appear likely that some of these antibiotics might interfere with mammalian protein synthesis.

6. Edeine. Edeine is a basic polypeptide of unknown structure which has been shown to inhibit mammalian neoplastic cells as well as several strains of bacteria (17). Its effect on the translation process in protein synthesis has been studied only in bacteria, where it inhibits the formation of the ternary complex between mRNA, poly U, and ribosomes (105). It also increases the number of 70 S ribosomes at a magnesium concentration that usually results in the presence of only 50 S and 30 S subunits (131). Although edeine has an inhibitory effect on translation, this property probably does not account for its antimicrobial activity, since DNA synthesis in the intact cell is more sensitive to inhibition by the antibiotic than RNA or protein synthesis (129, 130).

7. Pactamycin. Pactamycin is an antitumor antibiotic that causes 50% inhibition of growth in KB (human epidermoid carcinoma) cells in tissue culture (18, 20). Protein synthesis was shown to be more sensitive to inhibition than either DNA or RNA synthesis. Similar results were also obtained with HeLa cells (227).

The mechanism of action of pactamycin was studied in mammalian cell-free systems by Felicetti *et al.* (58) and Colombo *et al.* (40). When mammalian ribosomes were treated with pactamycin and subsequently studied in the cell-free system, they were found to be inactive in protein synthesis. The activating enzymes and sRNA fraction obtained from these cells were fully active. Examination of the polysome patterns from reticulocytes incubated with pactamycin revealed that there was no effect on the polysomes that were already present, but that no new polysomes were formed. These findings indicated that pactamycin acted on the ribosome to inhibit protein synthesis at a point before formation of the peptide bond, possibly at the time when mRNA, ribosomes, and sRNA were forming a ternary complex.

8. Lincomycin. Lincomycin is another antibiotic that inhibits bacterial protein synthesis but as yet has not been shown to affect mammalian protein synthesis. Baglioni demonstrated that the initial rate of protein synthesis in a cell-free system from rabbit reticulocytes was unaffected by lincomycin (11). It should be pointed out that the effect of this agent has not been studied on mammalian systems that are initiating protein synthesis and it seems possible that it could have an action similar to that of chloramphenicol.

B. Group II agents

The antibiotics in this category have the common ability to inhibit the translation process by interacting with the enzyme responsible for peptide bond formation. The mechanism of action of these antibiotics cannot always be clearly separated from that of those antibiotics that interrupt translation by affecting ribosomal movement. Both processes may be required for the formation of the peptide bond.

All the agents that we have placed in this group share a common chemical

structure and can be classified as aminoacylnucleosides. A recent review by Fox *et al.* (74) gives an excellent discussion of the chemical structure and functions of all of the nucleoside antibiotics. They have suggested that the agents in this group inhibited protein synthesis by acting as analogs of aminoacyl-sRNA, and that the structure of such analogs probably required an aminoacyl group on one end of the nucleoside and a second basic center (pK near 7) at the other end.

Although this "common structural" hypothesis is consistent with most of our knowledge of these agents, some differences in effect have been found between certain ones, and it has not been determined that they all act in the same manner. No distinction has been made between antibiotics investigated in bacterial or mammalian systems since all the antibiotics that have been used in both systems have had identical effects. These antibiotics are not useful clinically because of their marked toxicity. Their major use has been in dissecting the steps of protein synthesis.

1. Purine-containing group.

a. Puromycin. Puromycin, the most intensively studied agent in this group, is a substituted aminoacylnucleoside, made up of three components: 6-dimethylaminopurine (dimethyladenine), 3-deoxy-3-amino-D-ribose, and *p*-methoxy-l-phenylalanine (148). It has been shown to completely inhibit the growth of *E. coli* at a concentration of 0.4 mM, *Sarcina lutea* at 0.004 mM (158), HeLa cells at 0.1 mM (222), and mice at 1.1 mM/kg (174). Wheelock (222) found complete inhibition of protein synthesis in HeLa cells at a concentration of 10^{-4} M puromycin, with complete restoration of proten synthess in 30 min after removal of the drug.

An inhibition of RNA synthesis in HeLa cells was also reported (108, 197), but this effect lagged behind the inhibition of protein synthesis and was probably a secondary effect. In the presence of puromycin, HeLa cells continued to synthesize DNA but not at the expected rate (144). This is consistent with the known dependence of bacterial DNA synthesis on protein synthesis (136) and it is likely that any effect of puromycin on mammalian DNA synthesis is secondary to inhibition of protein synthesis. Some of the effects of puromycin on the intact cell may be due to hydrolytic cleavage to the aminonucleoside, which is known to inhibit RNA synthesis in animal cells (56, 57, 188). A renal lesion resembling the nephrotic syndrome in man has been induced in rats by the administration of puromycin or the aminonucleoside (70). Since the aminonucleoside does not inhibit protein synthesis (47, 149), and since it is more potent than puromycin (23) in inducing the renal lesion, it is probable that this effect is due to changes in RNA or nucleotide metabolism.

Since puromycin is an analog of adenosine, the possibility that it might directly interfere with nucleotide metabolism was investigated. Puromycin inhibited the incorporation of ^{14}C-glycine into guanine by ascites tumor cells (69). The mechanism of this effect was unclear, since Buchanan (30) and Hutchings (113) showed that puromycin had no effect on the enzymes responsible for purine biosynthesis.

Studies by Hofert *et al.* (106, 107) showed that puromycin had a striking

glycogenolytic effect in mice, which was not correlated with inhibition of protein synthesis. They suggested that this effect might be due to inhibition of breakdown of adenosine-3′,5′phosphoric acid (cyclic AMP), which would act to stimulate glycogen phosphorylase. The same mechanism could be invoked to explain the action of puromycin and its aminonucleoside on hormone regulation of fatty acid release by adipose tissue (125).

The majority of studies of the effects of puromycin have been concerned with its primary effect on the translation step in protein synthesis. Originally Yarmolinsky and DeLa Haba (225) noted the similarity between the 3′end of aminoacyl-sRNA and puromycin. On the basis of this structural similarity these investigators have suggested that puromycin might act as an analog of aminoacyl-sRNA. Subsequent work, based on this hypothesis, showed that there was a requirement for the amino acid side chain and the aminonucleoside, and also that the amino group of the amino acid must be unsubstituted (148). Nathans and Neidle (149) showed that substitution of the amino acid moiety with other amino acids reduced the inhibitory effect. In contrast, Takanami (194) found that ribonuclease-generated fragments of aminoacyl-sRNA, containing the esterified adenosine end of the molecule, acted like puromycin but showed no amino acid specificity. Although the amount of inhibition depended on the amino acid present in puromycin, the inhibitory effect had no direct relationship to the mRNA code (76, 149). There is no explanation for this specific amino acid requirement.

Further studies of the inhibitory effect of puromycin on protein synthesis demonstrated that it did not affect aminoacyl-sRNA formation (148, 225) or the formation of the ternary complex between mRNA, ribosomes, and sRNA (36, 186). However, in both mammalian and bacterial systems, puromycin exposure led to the release of incomplete peptide chains from ribosomes. With ascites tumor cells, puromycin decreased the amount of protein associated with the ribosomes while increasing the amount of soluble protein (161). Reticulocyte ribosomes labeled with ^{14}C-amino acids also released their protein into the postribosomal supernatant when incubated with puromycin (2, 140). In the phage RNA-directed synthesis of coat protein by *E. coli* extracts, puromycin caused the release of incomplete proteins of varying lengths, which could be identified by their peptide pattern after tryptic digestion (147). When the amount of radioactive amino acids incorporated into specific peptides in the presence of puromycin was compared with controls, it was found that the radioactivity in the puromycin-released peptides decreased in proportion to their distance from the N-terminal amino acid. This finding indicated that puromycin could interrupt the growth of one peptide chain at any point during its synthesis.

Using the polyuridylate-directed synthesis of polyphenylalanine in an *E. coli* system, Gilbert found that, in the absence of puromycin, polyphenylalanine was synthesized and remained attached to the ribosomes through the ester link to sRNA (83). In the presence of puromycin and a soluble cell fraction, polyphenylalanine chains were released from the ribosomes and were no longer attached to sRNA. This fact suggested that puromycin promoted a hydrolytic cleavage of

the peptidyl-sRNA bond. However, when radioactive puromycin was incubated with reticulocyte ribosomes, each globin chain released contained one N-terminal valine molecule and one molecule of radioactive puromycin (2). Also, the amino group of puromycin was no longer free. Further studies in intact bacterial cells revealed that the entire molecule of puromycin was linked through a peptide bond at the C-terminal end of the released polypeptides (146).

Using the polyadenylate-directed synthesis of ^{3}H-polysine in the presence of radioactive puromycin, Smith *et al.* (185) showed the attachment of puromycin to a series of lysine peptides, each of which contained one molecule of puromycin. On treatment of dilysyl-puromycin with trypsin, dilysine was released. This confirmed that puromycin was linked to the C-terminal position *via* a peptide bond. These findings suggested that puromycin acted as an analog of aminoacyl-sRNA to accept the growing peptide chain and thereby caused its release from the ribosome. In the polyadenylate-directed system, puromycin could never be found attached to a single molecule of lysine; and this suggested that puromycin could act as an analog for the acceptor sRNA but not for the donor sRNA.

The evidence that puromycin acted by attaching to the carboxyl end of the growing peptide chain *via* a peptide bond led to its use as a model for studying peptide-bond formation in protein synthesis and also as a tool to differentiate the points at which various agents act on the translation process.

The possibility that puromycin could react with aminoacyl-sRNA only in one of the two ribosomal binding sites led to further investigations in both mammalian and bacterial systems. In a bacterial cell-free system Traut and Monro (203), studying the puromycin-induced release of polyphenylalanine, found that the reaction was partially dependent on the postribosomal supernatant and guanosinetriphosphate (GTP). In the absence of these two factors only 40% of the polyphenylalanine formed was released by puromycin. Traut and Monro suggested that puromycin could react only with the peptidyl-sRNA present in the second, or donor site on the ribosome, and that enzymes and GTP were required for movement of the sRNA from the first, or acceptor site to the donor site. Studies by Heintz *et al.* (102) with a system from reticulocytes with partially purified enzymes for binding and for peptide bond synthesis suggested that puromycin could not react with peptidyl-sRNA unless it was in the correct binding site.

In a more detailed report (103) a polyuridylate-directed, cell-free system from rabbit reticulocytes was used to study the various requirements for phenylalanyl-puromycin formation. It was suggested that in the absence of binding enzyme and GTP, aminoacyl-sRNA was bound to a ribosomal site (donor site), which would not allow interaction with puromycin, and that in the presence of binding enzyme and GTP the aminoacyl-sRNA was moved into the reactive site. The most interesting aspect of these studies involved the stimulation by GTP of phenylalanyl-puromycin formation with the nonenzymatically bound phenylalanyl-sRNA. These authors and other investigators (102, 185) have previously noted that a single molecule of phenylalanine did not react with puromycin under conditions that allowed formation of diphenylalanyl-puromycin. In these experi-

ments, this reaction could apparently occur, although the reasons for a change were not explained.

Gottesman (92) studied the effect of puromycin on polyadenylate- and polyuridylate-directed synthesis of polylysine and polyphenylalanine. Lysyl-sRNA and phenylalanyl-sRNA, bound to bacterial ribosomes, would not react with puromycin, but polylysyl-, polyphenylalanyl-, and N-acetyl-phenylalanyl-sRNA did react. He concluded that the enzyme responsible for peptide-bond formation could react only with an amino acid that was substituted on the amino group. However, comparison with mammalian systems may not be valid, since they have not been shown to require a substituted aminoacyl-sRNA for initiation of protein synthesis.

The use of puromycin as a research tool was also exploited by Skogerson and Moldave (179, 180), who presented evidence from a rat liver cell-free system that there are five reactions involved in the formation of aminoacyl puromycin. Despite apparent discrepancies between this rat liver-cell free system and the reticulocyte cell-free system, it is apparent that both have marked similarities, especially in the studies of peptide synthetase.

A secondary effect of puromycin resulting in polyribosome breakdown to monomers has been noted by several investigators. When intact reticulocytes were incubated in the presence of puromycin, there was a progressive loss of large polyribosomes associated with an increase in 80 S ribosomes (31, 97). Similar findings have been made on rat liver polyribosomes from whole animals treated with puromycin (209) and in cell-free preparations from rat liver (155) and reticulocytes (31, 223). Subsequent observations have shown that this puromycin-induced breakdown of polyribosomes can be distinguished from the release of peptide chains. When polyribosomes from reticulocytes were incubated with puromycin in the absence of an energy source, the peptide chains were released but the polyribosomes remained intact; but with puromycin and an energy source, polyribosome disaggregation occurred (223). In the presence of cycloheximide, another inhibitor of protein synthesis, puromycin caused chain release without appreciable polyribosome breakdown (62). These findings suggest that the breakdown of polyribosomes is the result of a continuation of protein synthesis and not of instability caused by release of peptide chains. This possibility was explored by examining the peptides formed in intact reticulocytes after removing them from a short exposure to puromycin (95, 223). The resulting polypeptide fragments contained the C-terminal half of a polypeptide chain. This evidence supported the concept that peptide chain elongation is an orderly linear process starting at the N-terminus and progressing to the C-terminus of the polypeptide chain.

In summary, puromycin has no clinical application because of its toxicity. It has been used primarily as an experimental tool in studying the steps involved in protein synthesis. In general, its mechanism of action is the same in both bacterial and mammalian systems. Two effects of puromycin have been found in whole cells and in cell-free systems. First, puromycin is incorporated at the C-terminal end of the growing polypeptide chain and causes the release of the

incomplete chain from the polyribosomes. Second, puromycin causes the dissociation of polyribosomes to monoribosomes when active protein synthesis is taking place.

b. Homocitrullylaminoadenosine and lysylaminoadenosine. These two agents, with a structure very similar to that of puromycin (74), have not yet been examined for their effect on mammalian protein synthesis but are included for the sake of completeness. From the studies of Guarino *et al.* (95) it seems likely that homocitrullylaminoadenosine may well have an action similar to that of puromycin. This antibiotic inhibited protein synthesis by altering the sRNA-ribosomal complex and had no effect on aminoacyl-sRNA formation. A similar mode of action was suggested for lysylaminoadenosine.

c. Nucleocidin. This antibiotic, like puromycin in containing an adenosine nucleoside moiety, is the first instance of a sulfamate in ester linkage in a naturally occurring compound (62, 74). Although it does not contain an amino acid, the sulfamate moiety is in the position of the amino acid substituent, and this may possibly account for its inhibitory effect on protein synthesis. This agent is a broad spectrum antibiotic (199) which has a remarkable activity against trypanosomes (104). The inhibitory effect of this agent on mammalian protein synthesis, although incompletely defined, appears to be unique. In mammalian systems its effect was primarily on protein synthesis. It had no effect on oxidative phosphorylation (132) and no effect on RNA synthesis in cell-free preparations from rat liver nuclei (64).

In studies with a cell-free system from rat liver, nucleocidin was no more inhibitory on a molar basis than puromycin (63, 64). There was a 5 to 10 min lag before the effect of nucleocidin became evident. This lag could be eliminated by a prior incubation of the crude activating enzymes and sRNA. If partially purified transfer enzymes were used instead of the crude fraction, no inhibitory effect of nucleocidin was observed. If ribosomes were reisolated after incubation with nucleocidin and the crude enzyme fraction, their ability to synthesize protein was inhibited, but this condition could be reversed by incubation with the fresh crude enzyme, sRNA fraction. These results led to the conclusion that nucleocidin, ribosomes, and a soluble component from the crude fraction were required for demonstration of an inhibitory effect. In other experiments nucleocidin had no effect on mRNA binding, aminoacyl-sRNA binding, or movement of the ribosomes down the mRNA strand. In addition, nucleocidin did not release nascent peptide chains from the polyribosomes, nor did it inhibit the puromycin-induced release of peptide chains. On the basis of these findings, it is apparent that, although this antibiotic seems to fulfill the structural requirements (74) for analogs of aminoacyl-sRNA, it does not act at the same point as puromycin.

2. Pyrimidine-containing group.

a. Gougerotin. This is a broad spectrum antibiotic of only limited clinical value because of its weak antibiotic activity and its toxicity to mammalian systems (37). The currently accepted structure is 1-(cytosinyl)-4-sarcosyl-D-serylamino-1,4-dideoxy-B-D-galactopyranuronamide (73).

The interest in this agent stemmed from the resemblance of the originally proposed, but incorrect, structure to puromycin (95). Clark and Gunther (38) found that gougerotin inhibited protein synthesis in an *E. coli* cell-free system without any effect on aminoacyl-sRNA formation. In subsequent investigations with cell-free systems from mouse liver (177) and rabbit reticulocytes (34, 36), it was possible to localize the inhibitor effect of gougerotin to the transfer of amino acids from sRNA into protein.

The similarity in structure between gougerotin and puromycin prompted investigators to examine its ability to release peptide chains from prelabeled ribosomes (34, 36). Although it was found that gougerotin did not release peptide chains, the possibility remained that it might act at the same site as puromycin and therefore should inhibit the effect of puromycin. This was confirmed by demonstrating the inhibition by gougerotin of the puromycin-generated release of peptide chains from ribosomes (36). Gougerotin is a strict competitive inhibitor of puromycin and has no effect on any other reactions involved in protein synthesis, such as sRNA binding, mRNA binding, or the final GTP-dependent release of completed globin chains from rabbit reticulocytes ribosomes (36, 104).

b. Blasticidin S. This antibiotic is a pyrimidine similar to gougerotin in that they both contain a cytosine aglycone, at least one amino acid, and a 4-amino hexose in the D configuration (74). This antibiotic has been used to control fungus infection of rice plants due to *Piricularia oryzae* and has not been used clinically (139). Experiments have not yet been performed in mammalian cell-free systems.

In cell-free extracts of *P. oryzae*, blasticidin S inhibited amino acid incorporation into protein without affecting aminoacyl-sRNA formation (110). This effect, as well as the similarity in structure to gougerotin, suggested that it also might act as an analog of aminoacyl-sRNA to inhibit the formation of peptide bonds. However, a difference in the amount of inhibition was observed when the effects of puromycin and blasticidin S were compared in cell-free extracts of *P. oryzae* and *Pellicularia sasakii* (111, 139). A concentration of blasticidin S that completely inhibited protein synthesis in the *P. oryzae* system had no effect on the *P. sasakii* system. In contrast, a concentration of puromycin that completely inhibited protein synthesis in the *P. sasakii* cell-free system had no effect on the *P. oryzae* cell-free system. This result indicated that, despite the similarity in structure of these two agents, the enzyme systems from two closely related organisms reacted to them in an opposite manner. This finding also suggested that the common structural hypothesis advanced by Fox *et al.* (74) may not be universal.

c. Amicetin, bamicetin, and plicacetin. (The latter two are synonyms for allomycin, and sacromycin.) These three antibiotics are very similar in structure and contain cytosine linked to a dipeptide and a disaccharide (74). Although their configuration is slightly different from that of the other cytosine antibiotics, Fox pointed out that the requirements for the "common structural hypothesis" may still be met (74). Although amicetin is the only one of these three agents which has been investigated for an effect on protein synthesis, it is probable that they will have a similar mode of action.

Early investigations of the effect of amicetin on neoplastic cells showed that it partially inhibited growth of the KB strain of human epidermoid cancer cells (184). The mechanism of action of amicetin was investigated in a cell-free system from *E. coli* (22). Amicetin inhibited neither the formation of aminoacyl-sRNA nor the binding of aminoacyl-sRNA to the ribosomes, but it did inhibit the transfer of amino acids from sRNA into protein. The mechanism of this inhibition is as yet unclear, but it does appear that amicetin acts at a point in the translation process after the binding of aminoacyl-sRNA to the ribosome.

C. Group III Agents

Placing this small group of antibiotics in a separate category is based on exclusion, rather than any specific evidence that they share a common mechanism of action. They do not appear to affect the formation of the ternary complex between mRNA, sRNA, and ribosomes, and they have not been found to be competitive inhibitors of the peptidyl-puromycin reaction. Although these antibiotics are presumed to interfere with the movement of the ribosomes along the messenger RNA strand, the mechanisms of this inhibition, as well as the nature of the ribosomal movement, are not yet clear. As the role of ribosomal movement in protein synthesis is more clearly defined, a more precise classification of these antibiotics will be possible.

1. Glutarimide family. This is a large group of structurally related antibiotics that have in common the β (2-hydroxyethyl) glutarimide moiety attached to a cyclic or acyclic ketone. The chemical structure and general biological properties of these agents have recently been reviewed by Sisler and Siegel (178). The antibiotics included in this group are cycloheximide (actidione, naramycin A), stereoisomers of cycloheximide such as naramycin B, isocycloheximide, neocycloheximide, and α-epi-isocycloheximide, acetoxycycloheximide, streptovitacins A, B, C_2, and D, streptimidone, inactone, actiphenol, protomycin, fermicidin, and niromycins A and B. Only two of these antibiotics, cycloheximide and acetoxycycloheximide, have been studied in any depth, and they appear to have a similar effect on mammalian protein synthesis. In addition to their effects on protein synthesis, these agents also have an effect on steroid hormone secretion.

Cycloheximide was originally found to inhibit liver and tumor cell protein synthesis in intact animals (227, 228). It also inhibits antibody formation in mice (41), as well as protein synthesis and the estrogen response in the uterus of rats (91). Cycloheximide had no effect on respiration or glycolysis in concentrations that inhibited the growth of mammalian cells in tissue culture (10) or in oxidative phosphorylation by isolated rat liver mitochondria (132).

In a cell-free system from rat liver, Ennis and Lubin (53) showed that both cycloheximide and acetoxycycloheximide inhibited polypeptide synthesis, directed by either polyuridylate or natural mRNA. They found no effect on aminoacyl-sRNA formation at concentrations of the antibiotics sufficient to inhibit protein synthesis. Streptovitacin A, streptimidone, cycloheximide, and acetoxycycloheximide also inhibited polypeptide synthesis in a cell-free system derived from cells of mouse Adenocarcinoma 755 (13).

Wettstein *et al.* (221) showed that cycloheximide inhibited the breakdown of polyribosomes to monosomes that accompanies protein synthesis. Similar studies with a rabbit reticulocyte cell-free system showed that an increase in polyribosomes, with a decrease in monosomes, occurred during incubation with cycloheximide (223). In the intact reticulocyte, Colombo *et al.* (39) found that cycloheximide inhibited protein synthesis without causing release of polypeptide chains from the polyribosome. These workers also found that cycloheximide reduced the amount of peptide released by puromycin during esposure to both agents. They suggested that cycloheximide inhibited the attachment of puromycin to the peptide chain and directly inhibited peptide synthetase. Williamson and Schweet (223) were unable to confirm these findings, but these investigators did not examine the early phase of the reaction. In rabbit reticulocytes pretreated with sodium fluoride (NaF) to dissociate polyribosomes to monosomes, cycloheximide prevented the usual reassembly of polyribosomes that occurs after removal of NaF (39). These experiments suggest that cycloheximide interferes with protein synthesis by preventing movement of the ribosomes along the mRNA chain.

Polyribosomes derived from the livers of mice treated with cycloheximide were just as active in cell-free systems as untreated polyribosomes, when incubated with the enzymes and sRNA from untreated rats (202). Cycloheximide prevented the usual breakdown of liver polyribosomes induced by ethionine or puromycin and delayed the reassembly of polyribosomes that occurred after injection of methionine and adenosinetriphosphate into ethionine-treated animals. The polyribosomes isolated from the liver were lighter than those normally found. Recent studies in a partially purified cell-free system derived from rabbit reticulocytes suggested that cycloheximide inhibited polypeptide synthesis by interfering with the interaction between peptide synthetase and the ribosomes (179, 189). These results confirmed the earlier findings of Colombo *et al.* (39), which showed that cycloheximide inhibited the initial rate of puromycin-induced release of peptide chains. Since peptide synthetase may catalyze the translocation of the ribosome along the mRNA chain, cycloheximide may prevent ribosomal movement as originally proposed by Noll (156).

Recently Davis and Garren (46), studying the interactions of cycloheximide and ACTH, found a specific effect on one enzyme in the pathway from cholesterol to pregnenolone. Early studies of the inhibition of ACTH-stimulated secretion of corticosterone by cycloheximide suggested that this effect resulted from the inhibition of synthesis of a regulator protein with a rapid turnover time (78, 79). Subsequent studies localized the effect of cycloheximide to a specific inhibition of the ACTH-regulated transformation of cholesterol to pregnenolone (46). Since the effect of cycloheximide appears to be specific, while the enzymes involved were mixed-function oxidases with the same cofactor requirements, these workers suggested that cycloheximide inhibited the synthesis of an enzyme with a more rapid turnover rate than the others in this pathway.

A number of investigators (59, 60, 117–120) have observed effects of cycloheximide in the intact animal that appear to be mediated by adrenal and pituitary

hormones. The mechanisms by which cycloheximide produces these effects is unclear at present.

In summary, the glutarimides are a group of antibiotics which are not useful clinically because they are extremely toxic to mammalian cells but have almost no effects on bacteria (178). Cycloheximide, the most extensively studied of these antibiotics, appears to have two effects in mammalian systems. First, in the cell-free system, cycloheximide interferes with the interaction between the enzyme peptide synthetase and the ribosome and may inhibit the translocation of the ribosome along the mRNA strand. Second, in the intact animal cycloheximide alters the synthesis and action of a variety of steroid hormones.

2. Fucidin family. This group contains three structurally related steroid antibiotics known as helvolic acid, fusidic acid, and cephalosporin P_1. The chemical structures and biological properties of these agents have recently been reviewed by Harvey *et al.* (101).

The only studies on the mechanism of action of this group have been carried out with systems dervied from yeast or bacteria. Their primary mechanism of action is not clearly understood and appears to be inhibition of the translation process during protein synthesis. The possibility that they may affect mammalian systems is suggested by the finding that they inhibit cell-free protein synthesis in a system derived from a diploid yeast (99, 100). Cell-free studies with these agents showed that they inhibited polypeptide synthesis directed by either synthetic or endogenous mRNA (99, 100). They had no effect on aminoacyl-sRNA formation or on the formation of the ternary complex between sRNA, mRNA, and ribosomes. Inhibition by the fucidins could not be overcome by increasing the concentration of any of the components of the cell-free system but appeared to be completely reversible when the agent was removed by dialysis. The fact that the amount of inhibition by fucidin in the presence of streptomycin or chloramphenicol was additive suggested that its site of action differed from that of either of these other two antibiotics.

3. Sparsomycin. This antibiotic of unknown structure was found to have a marked cytotoxic effect on KB cells in tissue culture and has been evaluated *in vivo* as an antitumor agent (5, 160). Unfortunately, the antitumor effect was evident only at toxic, or nearly toxic levels.

The mode of action of sparsomycin has been examined extensively in bacteria and to a lesser degree in reticulocytes. Studies by Slechta, with intact bacterial cells as well as cell-free extracts, showed that the primary effect of sparsomycin was an inhibition of an early stage of protein synthesis (181, 182). Goldberg and Mitsugi (86–88), in a cell-free system from *E. coli*, showed that this antibiotic had an action similar to that of cycloheximide, acting as a strict competitive inhibitor of puromycin. However, these studies did not differentiate between effects on peptide synthetase and those on the ribosome.

Colombo *et al.* (40) and Baglioni (11), using intact rabbit reticulocytes and reticulocyte cell-free systems, showed that sparsomycin had not effect on protein synthesis in the whole cell. Apparently this was due to a failure of the antibiotic to penetrate the cell, since there was marked inhibition of protein synthesis in the

cell-free system with low levels of sparsomycin. Studies on the mechanism of action of this antibiotic suggested that it inhbited the terminal steps of translation, *i.e.*, it prevented the puromycin-induced release of peptide chains from the polyribosome. The data show that sparsomycin caused an increase in the number of large polyribosomes and a decrease in the number of monosomes. This is similar to the effect of cycloheximide (223).

4. Tenuazonic acid. This antiamoebic agent is the first tetramic acid isolated from natural sources and has been characterized as 3-acetyl-5-*sec*-butyl-tetramic acid (138, 175). Tenuazonic acid inhibited the growth of human adenocarcinoma propagated in the embryonated egg and, on the basis of molecular weight, was 20 times more potent that hadacidin or 6-mercaptopurine, 2 times more potent than azaserine, and $\frac{1}{20}$ as potent as triethylene melamine (84).

Studies in whole animals revealed that an intraperitoneal injection of 500 mg/kg of tenuazonic acid inhibited the incorporation of ^{14}C-glycine and ^{14}C-lysine into the proteins of liver, spleen, thymus, and intestinal mucosa (175). In suspensions of Ehrlich ascites cells, the incorporation of ^{14}C-labeled glycine, formate, lysine, phenylalanine, and valine into protein was inhibited by tenuazonic acid. This result suggested that tenuazonic acid acted on the general process of protein synthesis rather than affecting the incorporation of one single amino acid. In cell-free systems from ascites cells and rat liver, it was found that this antibiotic inhibited the incorporation of both leucine and valine into protein. When the effect of tenuazonic acid on the puromycin-induced release of peptides synthesized by *microsomes* was studied, a marked inhibition of this release was observed. No studies were done to determine if *purified ribosomes* also reacted similarly in the presence of puromycin and tenuazonic acid. It has been suggested that tenuazonic acid may interact with the endoplasmic reticulum to inhibit protein synthesis (175). This fact would be consistent with its lack of effect on cells that do not contain an endoplasmic reticulum (bacteria and yeast) and also with the loss of effect on removal of the reticulum from microsomes.

IV. CONCLUSION

Most clinically useful antibiotics, with the exception of those that act on cell wall assembly, act primarily by inhibiting bacterial protein synthesis. Many of these antibiotics are also inhibitors of mammalian protein synthesis and often do so in therapeutic concentration. Mammalian cells that are turning over rapidly or that are being committed to new protein synthesis are particularly susceptible to inhibition. Mammalian cells appear to be uniquely vulnerable during the period when new mRNA is being formed and deposited on ribosomes to direct induced protein synthesis. Therefore, the failure to demonstrate inhibition of protein synthesis in resting mammalian systems does not eliminate a possible inhibitory effect during phases of rapid protein synthesis. In view of the effects of these agents on mammalian protein synthesis, it is unlikely that any antibiotic will have sufficient specificity to inhibit protein synthesis in bacteria without affecting protein synthesis to some extent in the host. The toxicity that results from the clinical use of several antibiotics may be related to inhibition of protein

synthesis in host cells. Such inhibition of protein synthesis in the host cells may in some instances have potential clinical application as shown by the preliminary observations that chloramphenicol has a remarkable immunosuppressive effect.

Antibiotics have been shown to inhibit mammalian protein synthesis either during transcription or during translation. Agents that inhibit the transcription of DNA to RNA have not been useful as antibiotics in clinical practice because of their extreme toxicity. They are, however, used to some extent as chemotherapeutic agents in malignancy. The commonly used antibiotics in clinical practice are those that have been found to interfere with translation of information by mRNA-ribosomal complexes.

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