PUROMYCIN INHIBITION OF PROTEIN SYNTHESIS

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I. INTRODUCTION

The antibiotic puromycin is produced by the actinomycete, *Streptomyces alboniger* (77). The structure of this antibiotic has been determined (96) and confirmed by total synthesis (10). Puromycin consists of an aminonucleoside linked to the amino acid *p*-methoxyphenylalanine. Inspection of the structure shows that it can be regarded as an analogue of adenosyl-phenylalanine, specifically of the terminal group of the phenylalanyl-sRNA (fig. 1). The antibiotic appears to be readily absorbed by all tissues, and it is probably rapidly changed in the body; it is excreted by the kidneys in altered form (86). Puromycin has a broad range of biological activities, including an inhibitory effect upon the growth of bacteria, protozoa, parasitic worms, algae, plants, and cells of mammalian origin. The exceedingly wide variety of plant and animal material showing this inhibition is explained by the fact that this antibiotic interferes with protein synthesis, a major biochemical process that is similar in all organisms thus far investigated. Puromycin has been used as a tool for studying protein synthesis in a number of systems.

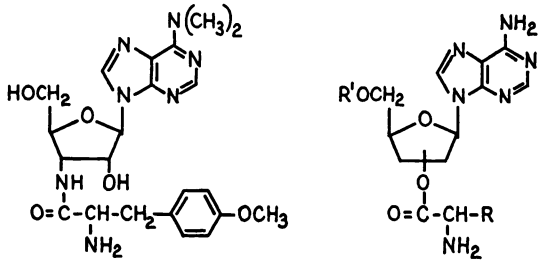

FIG. 1. Structures of puromycin and the amino acid-bearing end of transfer RNA. R represents the remainder of the amino acid residue, R′ represents the remainder of the RNA polymer.

II. MECHANISM OF INHIBITION OF PROTEIN SYNTHESIS BY PUROMYCIN

A. Current concept of protein synthesis

In the biosynthesis of protein, we deal not only with the formation of peptide bonds, but also with the replication of an amino acid sequence specific for a particular protein (69). It is now assumed that the base sequence of deoxynucleic acid (DNA) specifies the base sequence of a particular ribonucleic acid (RNA), and that RNA in turn controls the amino acid sequence of protein through the action of messenger RNA (mRNA), which carries information for the synthesis of specific proteins from DNA to ribosomes. The mechanism of the initial activation of amino acids for the peptide bond formation in proteins is relatively well established. This activation is catalyzed by a series of amino acid-specific enzymes with specific transfer RNA or soluble RNA (sRNA) molecules as the acceptor and carrier of each activated amino acid. The initial step is an enzymatic reaction between the carboxyl group of the amino acid and adenosine triphosphate (ATP) to yield amino acyl adenylate and pyrophosphate. The amino acyl moiety is then transferred by the same enzyme to the 2′- or 3′-hydroxyl group of the adenosine terminal of sRNA to form the ester. When the amino acid becomes fixed to its specific sRNA, the amino acid specificity of the unit is then transferred to sRNA, presumably by pairing with a complementary sequence in messenger RNA, which is a direct copy of genetic DNA. The formation of a polypeptide chain then occurs in the ribosomes by a process that requires guanosine triphosphate (GTP), two soluble enzymes, magnesium and potassium ions, and a complex structure consisting of ribonucleoprotein particles attached at intervals along the messenger (mRNA) molecule. Many of the details of this process remain to be elucidated.

B. General effect of puromycin upon protein synthesis

That puromycin blocks protein synthesis was firmly established in 1959 by Yarmolinsky and de la Haba (101), and its suppression of protein synthesis *in vivo* was first demonstrated by Gorski *et al.* (37). The action of puromycin was

found by Yarmolinsky and de la Haba (101) to occur in the transfer of the amino acids in a cell-free system from sRNA into protein. The activation of the amino acids and their attachment to sRNA were unaffected by the antibiotic. The antibiotic appears to exert its effect at a level involving the sRNA-ribosomal complex (2), where it could substitute for amino acyl sRNA and become attached by its amino group to the incomplete polypeptide chain. The carboxyl group of puromycin would not be available for additional peptide bond formation; accordingly, the polypeptide chain could not grow further. Relatively short chains, each carrying a puromycin molecule, would then be released. This concept is shown in the following diagram:

amino acyl sRNA	amino acyl sRNA plus puromycin
↓	↓
polypeptide chains	polypeptide chains with terminal puromycin
↓	↓
released from ribosome	released from ribosome
↓	↓
small polypeptides	small polypeptides with terminal puromycin
↓	
proteins	

C. Effects of puromycin in purified cell-free systems

1. General action upon amino acid transfer. Two considerations directed the attention of Yarmolinsky and de la Haba (101) to the effect of puromycin upon the amino acid transfer from sRNA into protein: first, the observation of Creaser (22) that induced β-galactosidase synthesis in *Staphylococcus aureus* was completely inhibited by low levels of puromycin (2×10^{-5} M), and second, the structural resemblance between the antibiotic and that proposed for the amino acid-bearing end of sRNA as shown in figure 1. Although the bond between the amino acid and the remainder of the molecule differs in the two compounds, the peptide bond to some extent resembles the ester bond in its chemical activity. Yarmolinsky and de la Haba (101) found inhibition of protein synthesis with puromycin, not only of the incorporation of C^{14}-leucine into protein in a cell-free preparation from rat liver, but also of the transfer of C^{14}-leucine from C^{14}-leucyl sRNA to protein in this same system; inhibition was significant with puromycin at a concentration of 8×10^{-7} M and 98% inhibition occurred at 4×10^{-4} M. The antibiotic had no effect upon ATP synthesis, amino acid activation, or formation of amino acyl sRNA. In the presence of puromycin, the transfer of the amino acyl residues from sRNA to the microsomes was thus strongly reduced, but the activation of amino acids by pH-5 enzymes (enzymes extracted from rat liver at pH 5) and their attachment to sRNA were unaffected. This inhibition of the transfer of the amino acids from sRNA into protein of the rat-liver microsomes by puromycin was confirmed by von der Decken and Hultin (24) using C^{14}-valyl sRNA. Incubation with the antibiotic at a concentration of 2×10^{-3} M led to 85% inhibition of the transfer reaction. Nathans and Lipmann (66) also confirmed this work with a cell-free system of ribosomes of *Escherichia coli*.

Puromycin, at a concentration of 10^{-4} M, inhibited by more than 98% the transfer of C^{14}-leucine to protein. A high magnesium concentration was required. The antibiotic acted directly on the ribosomes, irreversibly and independently either of transfer factor or of guanosine triphosphate (GTP). Additional experiments, however, indicated that ribosomes that could no longer transfer amino acids to protein were still active in enzyme deacylation. This suggested that puromycin still leaves intact a partial reaction in the amino acyl transfer to protein and blocks only the final reaction in the sequence by preventing the condensation of the activated amino acids to peptide linkages in the ribosomes.

2. Structural requirements for inhibition of amino acid transfer. Nathans and Neidle (67) examined the effect of analogues and isomers of puromycin in the *E. coli* ribosomal system in order to define the structural requirements for inhibition that would help in understanding its mode of action. Their major conclusions with regard to the structural specificity of puromycin follow: (1) both the aminonucleoside and amino acid are required; (2) the aminonucleoside may be substituted at the 3′ position; (3) the amino acid must be of L- configuration; (4) aromatic amino acid derivatives are the most potent; and (5) compounds with a dipeptide side chain are inactive. Great differences in the activity of puromycin-like compounds with different amino acids were noted. Substitution with glycine or proline led to analogues with no activity, whereas substitution with leucine gave slight activity, and with phenylalanine or tyrosine, marked activity. The authors suggested that the greater potency of the latter could reflect a tighter binding of the aromatic amino acyl derivative to the inhibited site.

3. Release of soluble peptides in amino acid transfer. The final steps in protein synthesis were studied by Nathans *et al.* (68) using bacterial ribosomes of *E. coli*. Comparisons with a puromycin-inhibited system indicated that sRNA cycled between the activating enzyme, where it was charged, and the ribosome, where it discharged its amino acids into peptide bonds; sRNA was apparently not degraded during the protein-synthesizing reaction.

The release of soluble peptides was investigated by Arlinghaus *et al.* (6) in their studies on the effect of puromycin upon polypeptide synthesis by rabbit reticulocyte ribosomes. The antibiotic (6×10^{-5} M) not only inhibited the formation of polypeptides but caused a loss of total C^{14}-phenylalanine from the ribosomes. The radioactive material which was lost was acid-soluble but was not free amino acid.

Parallel with, or probably intimately associated with, the inhibition of protein synthesis, puromycin has an effect on the amino acyl–sRNA–ribosomal system by inducing the formation of acid- or alcohol-soluble peptides of relatively low molecular weight from the amino acids originally attached to sRNA. This direct effect of the antibiotic on the ribosome resulting in the release of soluble C^{14}-protein was first reported by Morris and Schweet (62) in their studies with leucine and rabbit reticulocytes. This discharge was nonenzymatic and occurred at 10^{-3} M puromycin in the presence of excess magnesium chloride. Hultin (50) using rat-liver ribosomes and C^{14}-valine also reported that the release of labeled protein was stimulated by the antibiotic (10^{-3} M). Further work

by Bosch and Bloemendal (14) with rat-liver microsomes also confirmed these early results. These observations constituted the first evidence that soluble protein could be freed from ribosomes in the absence of incorporation and without ribosomal breakdown (84). Morris *et al.* (63) studied the release of C^{14}-labeled material by puromycin (7×10^{-4} M) using leucine and valine and considered it to result from the displacement of incomplete globin chains and earlier intermediates from the ribosome. This mechanism implies a reversibility of puromycin inhibition, in contrast to an irreversible process such as ribosomal breakdown or removal of an essential component from the ribosome. Nathans and Neidle (67) in their studies on the ribosomal system of *E. coli* with C^{14}-labeled phenylalanine and leucine found that the amount of alcohol-soluble compounds formed in the presence of the antibiotic (10^{-3} and 10^{-4} M) correlated reasonably well with the degree of protein inhibition. They concluded that enzymatic formation of small peptides from amino acyl-sRNA is intimately related to the mechanism by which puromycin inhibits protein synthesis; they suggested that the antibiotic may interact with the peptide bond-forming enzyme.

4. Attachment to released peptides in amino acid transfer. Nathans *et al.* (68) proposed that puromycin, by substituting for the next incoming amino acyl-sRNA, leads to the splitting of this compound from the template of peptides with possible linkage through the terminal carboxyl group to the amino acid of puromycin. After incubation of the complete *E. coli* system with C^{14}-leucyl-sRNA and puromycin (5×10^{-4} M), chromatography of the alcohol-soluble fraction revealed radioactive material other than free leucine.

Allen and Zamecnik (2) and Allen and Schweet (3) carried out extensive investigations on the effect of puromycin on rabbit reticulocyte ribosomes with C^{14}-valine and presented direct evidence that puromycin was attached to the end of the chain of released peptides. In their opinion, the original proposal of Yarmolinsky and de la Haba (101) that puromycin acts as an analog of amino acyl-sRNA remains the most intriguing approach to its mode of action upon the ribosomal system. The antibiotic might inhibit protein synthesis by displacing the attached sRNA residues at the growing C-terminal end that bind the chain to the ribosome and then releasing the freed incomplete peptide chain. The mode of binding of puromycin to the released polypeptides suggested by Allen and Zamecnik (2) involves a nucleophilic substitution of the amino group to the *p*-methoxyphenylalanyl moiety of puromycin on the C-terminal acyl group of the growing polypeptide chain with displacement of the sRNA to which the peptide chain is considered to be esterified. In their studies, the lowest concentration of puromycin for maximum C^{14}-ribosomal protein binding (2×10^{-4} M) was similar to the lowest level at which maximum inhibition of protein synthesis or maximum release of protein from the ribosome occurred.

III. INHIBITION OF PROTEIN SYNTHESIS IN CELL-FREE PREPARATIONS

Although Gale and Folkes (34) found that incubation with puromycin (10^{-3} to 10^{-5} M) inhibited the incorporation of glycine (25%) into the protein fraction of disrupted cells of *S. aureus* and used this suppression to study the de-

pendence of protein synthesis upon nucleic acid, a major difficulty in all studies on protein synthesis *in vitro* has been the necessity for preparing fresh enzyme extracts, since techniques were not available for their stabilization and storage. Matthaei and Nirenberg (60) developed a procedure for obtaining a stable cell-free system from *E. coli*. This system incorporated C^{14}-valine into protein at a rapid rate, and the incorporation was markedly inhibited by puromycin (2×10^{-5} M). The characteristics of amino acid incorporation by these extracts were strongly suggestive of protein synthesis *de novo*. Nirenberg and Matthaei (72) reported that this incorporation was stimulated by exogenous RNA and that synthetic polyuridylic acid stimulated the synthesis of a "protein" containing only one amino acid, phenylalanine. The incorporation of this amino acid was strongly inhibited by puromycin (2×10^{-4} M). Using a different technique of preparing extracts from frozen, washed cells of *E. coli*, Tissières and Watson (94) found 94% inhibition of the incorporation of twenty C^{14}-amino acids into protein when preparations were incubated with puromycin (8×10^{-5} M). The antibiotic had no effect upon the breakdown of mRNA.

Guarino *et al.* (42) inhibited the incorporation of amino acids into protein by cell-free extracts of *E. coli* with puromycin, using the technique of Matthaei and Nirenberg (60). The level of antibiotic necessary for half-maximal inhibition during a 10-minute incubation period was 2.2×10^{-6} M. There was significant inhibition of C^{14}-label into protein but not into sRNA; these results confirmed that the inhibition of protein synthesis by puromycin involved the transfer of label from sRNA to ribosomal protein. In these experiments, sRNA, charged with labeled leucine, was used as the source of amino acids for protein synthesis; accordingly, the systems responsible for the activation and transfer of the amino acids to sRNA were bypassed.

Techniques have also recently been developed by Florini (33) for the study of amino acid incorporation into protein by cell-free preparations from rat skeletal muscle; the transfer of C^{14}-leucine in these systems was inhibited by puromycin.

The reactions governing the incorporation of C^{14}-amino acids (alanine, glycine, and leucine) into the proteins of isolated thymus cell nuclei (which retain their soluble proteins and nucleoproteins) were studied by Allfrey *et al.* (4). Inhibition of protein synthesis in this system required considerably more puromycin than in liver cytoplasm. The nuclear uptake was reduced by about 50% with 2×10^{-5} M, and at 8×10^{-5} M the incorporation of the C^{14}-amino acids was only 15% of that observed in the absence of the antibiotic. The authors proposed that most of the incorporation of amino acid into protein by the cell nucleus proceeded through an amino acyl-RNA intermediate, as is the case with the cytoplasm.

Rabinovitz and Fisher (79) reported that protein synthesis by rabbit reticulocytes was also affected by puromycin. With the reticulocytes, the labeling of all cell proteins was immediately inhibited by puromycin at 22°; at 37° incorporation into total soluble protein and hemoglobin was blocked, but a small increase in the labeling of ribosomal protein was observed. Differences in protein synthesis between reticulocyte ribosomes and cell-free bacterial systems were noted by

Arlinghaus and Schweet (5), and it was suggested that different sites were involved in the two types of peptide bond synthesis. The ribosomes were able to incorporate C^{14}-phenylalanine under conditions in which their synthesis of hemoglobin was greatly reduced (65%) by incubation with puromycin (6×10^{-4} M). Further studies with reticulocyte ribosomes and C^{14}-valine by Arlinghaus *et al.* (7) suggest that a ribosomal intermediate occurred that was not yet bound into the peptide chain of globin. Later studies by Arlinghaus *et al.* (6) showed that the transfer from amino acyl-sRNA to protein in rabbit reticulocytes involved two steps and that puromycin at a concentration of 6×10^{-5} M interfered only with the second of these reactions. A labile, ribosome-bound C^{14}-phenylalanine was postulated as an intermediate in the transfer of C^{14}-phenylalanine from C^{14}-phenylalanyl-RNA to polyphenylalanine.

Sisakyan *et al.* (87) found that puromycin inhibits the incorporation of C^{14}-amino acids in lipoids and proteins of isolated chloroplasts of the alga *Chlorella vulgaris*. Introduction into the incubation mixture of 1.3×10^{-4} mole puromycin per 4 mg protein brought about 75% inhibition. When the source of radioactivity was lipoids that had previously incorporated amino acids, the antibiotic exerted only partial inhibition (25%) even at a 2.5-fold greater concentration. These results may indicate that peptide chains that have developed cannot be released under the influence of puromycin, while incomplete polypeptide chains may be readily freed by the antibiotic which appears to inhibit not only their formation in lipoid compounds but also the further development of the protein molecule. Morphogenesis in the tips of anucleate fragments of another alga, *Acetabularia mediterranea*, was reported by Brachet (15) to be distorted in the presence of puromycin at concentrations of 2 to 6×10^{-5} M. This effect may be caused by an interruption in the orderly synthesis of the proteins which accumulate in the algal tips during normal growth. Investigations by Birnstiel and Hyde (12) with peas show that the incorporation of C^{14}-amino acids into protein was inhibited partially (50%) by puromycin (2×10^{-4} M) in the nucleus-free preparations of isolated nucleoli.

IV. EFFECTS OF PUROMYCIN IN INTACT CELLS OR WHOLE ANIMALS

The possibility that nucleic acid metabolism is involved in the mode of action of puromycin was first proposed by Agosin and von Brand (1) as a result of studies *in vivo* in which adenine sulfate antagonized the chemotherapeutic and metabolic effects of puromycin upon trypanosomes. They proposed that purine, rather than carbohydrate metabolism, is involved since adenine was known to protect *Lactobacillus casei* against the action of purine antagonists (25). Hewitt *et al.* (45) found the inhibitory activity of puromycin with *Trypanosoma equiperdum* infections in mice to be partially reversed by a number of purines, and this action suggested that the antibiotic might interfere with the purine metabolism of the parasite. Bortle and Oleson (13) noted that guanylic acid was a competitive antagonist for puromycin in the protozoan, *Tetrahymena pyriformis*. Inhibition of growth (50%) occurred at an antibiotic concentration of 4×10^{-6} M. Since this inhibition could be reversed by guanylic acid, puromycin was

thought to block nucleic acid synthesis. A similar observation was made with *Lactobacillus plantarum* (51). In studies with the synthesis of purine compounds by *Trypanosoma cruzi*, Fernandes and Castellani (29) showed that preformed bases were used to build up the purine nucleotides and polynucleotides and that incubation with puromycin (2.8×10^{-4} M) did not inhibit this utilization; accordingly, the antibiotic did not therefore interfere with the early stages of nucleic acid synthesis *in vivo*.

Asanuma (9) reported that puromycin inhibited the synthesis of protein in *Pseudomonas fluorescens* but did not alter the synthesis of RNA. He also described the effect of Mg^{++} upon the action of the antibiotic and reported that the concentration of puromycin necessary to inhibit the growth of this microorganism was 10^{-4} M in the presence of 20 μg/ml Mg^{++}, while it was six times as great at 300 μg/ml Mg^{++}. The incorporation of amino acid into proteins by ribosomes of *E. coli in vitro* at low concentrations of Mg^{++} has also been found to be markedly lower than its incorporation at higher levels (95). Takeda *et al.* (91) in their studies with *P. fluorescens* confirmed the effect of Mg^{++} upon bacterial growth in the presence of puromycin. These authors also upheld the observation that the antibiotic completely inhibited protein synthesis without affecting RNA synthesis, as evidenced by the incorporation of P^{32}-orthophosphate into cellular RNA during exposure to the antibiotic. This ineffectiveness of puromycin in RNA synthesis in bacteria is in agreement with the findings of Heymann *et al.* (46) using pancreatic RNA preparations. The addition of puromycin to *E. coli* inhibited amino acid incorporation into acid-insoluble products, and sub-bacteriostatic levels of the antibiotic were found by Culbreth and Esposito (23) to lead to the production of cells which appeared to have higher-than-usual ratios of RNA to protein. These effects varied with the concentration of Mg^{++}; at lower Mg^{++} levels, less puromycin was required. While a puromycin-resistant strain did not respond in this manner, a cell-free preparation from resistant cells did.

Gorski *et al.* (37) studied the effect of puromycin upon certain tissues of the ovariectomized rat, including liver, heart, kidney, and thymus; they found reduced protein synthesis in all instances. The synthesis of RNA, however, was inhibited at 0, 1, 2, and 3 hours after the administration of puromycin (15 mg intraperitoneally) only in the thymus, and the synthesis of phospholipid was stimulated only in the liver and uterus.

Robinson and Seakins (80) showed that puromycin induces fatty liver formation in the rat with a concomitant fall in plasma lipid concentration. Female rats were injected intraperitoneally with a total of 20 mg puromycin (2 mg every 1.5 hours). In rats given the antibiotic, the mean liver triglyceride level was 290 % of normal, while the mean plasma phosphatide, cholesterol, and esterified fatty acid concentrations were, respectively, 29, 21, and 37 % of normal. There was, however, no significant difference between the mean specific activities of the liver proteins in the control and test groups. This failure to show reduced activity after puromycin contrasts with the results of Gorski *et al.* (37) and presumably reflects the lower dosage of the antibiotic.

Preliminary experiments with puromycin as an inhibitor of protein synthesis by cells in culture were carried out in human carcinoma cells (HeLa) by Rueckert and Mueller (81). They suggested that protein synthesis might play a role in preparing a portion of the cell population for DNA synthesis. The effect of puromycin was further studied in HeLa cells by Mueller *et al.* (65). The antibiotic at a concentration of 2×10^{-5} M inhibited the incorporation of C^{14}-leucine by 90%, and at 5×10^{-5} M blocked all incorporation. This inhibition of protein synthesis could be reversed by the replacement of puromycin-containing medium with puromycin-free medium. Concentrations of the antibiotic which inhibited protein synthesis prevented the acceleration of DNA synthesis without inhibiting the established rate of DNA synthesis. These investigators proposed that the DNA in HeLa cells exists in two physiological states: a fraction competent for replication and a fraction that becomes competent through a puromycin-sensitive process presumed to involve synthesis of protein.

Tamaoki and Mueller (92) studied the effect of puromycin on RNA metabolism in HeLa cells and showed that the synthesis of a RNA fraction of relatively high molecular weight continued in the presence of the antibiotic (4×10^{-5} M) and that it blocked the introduction of C^{14}-guanine into other classes of RNA. It was suggested that in the synthesis of ribosomal RNA the nucleotides are first incorporated into a polymeric precursor A that is converted by a puromycin-sensitive process to precursor B which can then be converted to the ribosomal RNA. The site of protein synthesis in HeLa cells has been investigated by Zimmerman (102), and his data are consistent with the hypothesis that the RNA proteins of relatively high molecular weight in these cells are synthesized on the polysomes. In these studies, incubation with 10^{-4} M puromycin blocked the incorporation of H^3-leucine.

In Ehrlich ascites tumor cells, Rabinovitz and Fisher (78, 79) reported that incubation with puromycin (5×10^{-5} M) inhibited by 90% the incorporation of C^{14}-amino acids (valine, leucine, lysine, and phenylalanine) into ribosomal protein at a concentration too low to inhibit labeling of total cell protein. Under these conditions a stimulation of the labeling of soluble protein took place because of elimination of the lag phase. The soluble protein labeled in the presence of puromycin was not, however, the same as that formed in its absence. These results indicate that the antibiotic did not prevent the release of preformed labeled protein at the ribosomal site but brought about its continuous premature release. The transfer of C^{14}-isoleucine into the proteins of Ehrlich and Krebs ascites carcinoma cells was studied by Gotto *et al.* (39), and this incorporation was also sensitive to puromycin.

V. SYSTEMS IN WHICH PUROMYCIN HAS BEEN USED AS A TOOL FOR STUDYING PROTEIN SYNTHESIS

In recent years it has become evident that puromycin is a powerful tool for studies on the relationship between protein synthesis and other physiological processes in many systems including those of the higher animals and plants, as well as of microorganisms, Ehrlich ascites tumor cells, HeLa cells, and viruses.

A. In vitro

1. Hormone. Puromycin has been used *in vitro* to test whether changes in hormonal activity require protein synthesis. Ferguson (26) reported that puromycin (10^{-3} M) abolished the stimulatory effect of ACTH or cyclic 3′,5′-adenylic acid on steroid output by rat adrenal fragments as measured by the release of steroids into the medium. The action of the antibiotic was completely reversible if it was present only throughout the preincubation period and was omitted during incubation with ACTH or cyclic 3′,5′-adenylic acid. The action of adenosine 3′,5′-monophosphate, which mimics the effect of ACTH, was also blocked by puromycin. The stimulation of steroid synthesis by a triphosphopyridine nucleotide-generating system, however, was not eliminated by puromycin. Analysis of the steroid content of adrenal tissue indicated that the antibiotic blocked the synthesis of steroid in response to ACTH, and not merely the release of formed steroid. The data are consistent with the view that protein synthesis is necessary for ACTH responsiveness. This concept was also proposed by Ferguson (27, 28) who found that puromycin inhibited completely *in vitro* both the incorporation of C^{14}-leucine into adrenal protein and the steroidogenic response of the adrenal to ACTH.

In studies by Hall and Eik-Nes (43) with slices of rabbit testis, the response to interstitial cell-stimulating hormone (ICSH) as evidenced by steroid biosynthesis was inhibited by puromycin (2×10^{-4} M). When animals were pretreated *in vivo* with human chorionic gonadotropin (HCG) or ICSH, however, the antibiotic (2×10^{-4} M) had no effect *in vitro* upon the incorporation of C^{14}-acetate into testosterone. This pretreatment stimulated the incorporation of C^{14}-valine and tryptophane into protein. This latter incorporation was markedly inhibited both *in vitro* and *in vivo* by puromycin (2×10^{-4} M).

The marked dependence on androgens of the protein biosynthetic mechanism has also been studied with puromycin by Kochakian *et al.* (55). Addition of the antibiotic to kidney tissue homogenate equally inhibited the rate of incorporation of C^{14}-leucine into the cytoplasmic protein of normal or castrated mice or those treated with testosterone propionate. An inhibition of 60% was obtained at a concentration of 2×10^{-5} M puromycin, and 80% at concentrations of 0.2 and 2×10^{-3} M.

Field *et al.* (30) reported that the effect of thyroid-stimulating hormones on glucose oxidation in thyroid slices of dogs was not abolished by a concentration of puromycin (1.5×10^{-4} M) which inhibited 98% of the incorporation of C^{14}-leucine into protein. This result indicates that the hormone did not act by a mechanism that directly required the synthesis of new protein. Seed and Goldberg (85) in their studies on the effect of puromycin on the formation of the large protein thyroglobulin by lamb thyroid slices suggest the possible involvement of a subunit precursor protein from which thyroglobulin could be derived without further protein synthesis.

2. Enzyme. Puromycin has been used *in vitro* to test whether changes in the levels of various enzymes require protein synthesis. The increases in both tyro-

sine-α-ketoglutarate transaminase and tryptophane pyrrolase activities induced by hydrocortisone in the isolated, perfused rat liver were investigated with puromycin by Goldstein *et al.* (36). Five additions of puromycin, each of 15 mg at hourly intervals, to 100 ml of blood perfusing the liver not only prevented the hydrocortisone-induced rise but decreased the base level of both activities without changing hepatic lactate dehydrogenase action, bile flow, or protein content. The results indicate that the antibiotic interfered with the continuing formation of active enzymes. No necessary connection, however, was established between this interference and the inhibition of protein synthesis. McGeachin and Potter (61) found that puromycin (1.4×10^{-4} M) also prevented amylase production by isolated rat liver perfused with oxygenated, heparinized rat blood. On the basis of studies with this system, Arnold and Rutter (8) suggested that a part of the metabolic activity of the liver cell is specifically directed toward the synthesis of serum amylase which appears to proceed by a mechanism similar to that for serum proteins. Puromycin both *in vivo* (at a dose of 15 mg) and *in vitro* (20 mg/10 g liver) specifically abolished the incorporation of C^{14}-leucine into proteins; the accumulation of amylase in the medium, however, was reduced only 75% under these circumstances.

Investigations with rat-liver mitochondria by Neubert and Lehninger (71) showed that the ATP-induced reversal of the swelling caused by glutathione and thyroxine was not inhibited by puromycin at a concentration of 10^{-3} M.

That puromycin could interfere with the formation of enzymes was confirmed by the studies of Garren and Howell (35) on the role of mRNA in the induction of enzymes by steroid hormone in mammalian liver *in vitro*. A rapid loss in the activity of the enzymes tryptophane pyrrolase and tyrosine transaminase was noted after incubation with the antibiotic.

Using puromycin (10^{-4} M) as an inhibitor, Cohen *et al.* (20, 21) obtained evidence to support the postulate that oxytocin and acetylcholine increase glucose oxidation in mammary gland slices from lactating rats by selectively affecting protein synthesis. Phosphate interfered with inhibition by puromycin of the incorporation of C^{14}-leucine into protein. Investigations by Burkhalter (18) on the acetylcholinesterase activity of chick embryo intestine cultured *in vitro* provide evidence that increases of the activity of this enzyme may be brought about by changes in protein synthesis caused by puromycin. The addition of 5×10^{-5} M puromycin inhibited enzymatic cleavage of acetylthiocholine, virtually abolished cholinesterase activity, and caused a 30% decrease in protein content. At a concentration of 5×10^{-6} M the antibiotic permitted cholinesterase activity while it blocked the hydrolysis of acetylthiocholine induced by acetylcholine. These actions were not considered to be directly due to enzyme inhibition since the addition of puromycin to the enzyme assay system was without effect.

B. In vivo

Puromycin has been used *in vivo* as a tool for the detection of protein synthesis in hormonal and enzymatic systems, cellular division, viral replication, spore germination, vitamin production, and antibiotic production.

1. Hormone. Mueller *et al.* (64) reported that protein synthesis in estrogen-induced responses of the uterus of the ovariectomized rat was suppressed by puromycin (15 mg intraperitoneally, four times, at 0, 1, 2, and 3 hours), but inhibition of the synthesis of phospholipid or RNA did not occur. The antibiotic also prevented the early acceleration of the synthesis of phospholipid and RNA by estradiol and prevented the hormone-induced imbibition of water by the uterus. The expression of early estrogen action was thus dependent upon a puromycin-sensitive process, *e.g.*, protein synthesis.

The work of Mueller *et al.* (64) was repeated by Hamilton (44), except that he used lower doses of estradiol and, in addition, two other naturally occurring estrogens, estriol and estrone. Minimum doses required for uterine response in ovariectomized rats were established *in vivo*, and combined treatments of estrogen at these doses and puromycin confirmed the results found by Mueller *et al.* These results support the theory that protein synthesis is mandatory for early estrogen action in the uterus and that selective control of enzymatic synthesis of protein is a key aspect of the inceptive action of estrogen on the metabolic apparatus of uterine tissue.

Data obtained by Noteboom and Gorski (73, 74) with immature female rats suggest that early effects on RNA synthesis induced by estrogens are dependent upon protein synthesis while estrogen itself has no apparent control over the total synthesis of protein. Estrogen appeared to stimulate selectively the synthesis of specific proteins essential to the estrogen response. When incorporation of C^{14}-glycine and H^3-leucine into protein was reduced by puromycin (5 mg at 0 and 1 hour) at 2 hours to a level only 20 % of that of the controls, the estrogen-stimulated increase in labeled RNA in both nuclear and cytoplasmic fractions was completely inhibited.

The inhibition by puromycin of cortisone-induced elevation of tryptophane pyrrolase and tyrosine-α-ketoglutarate transaminase activities, as well as the substrate-induced elevation of the latter, were investigated in male, bilaterally adrenalectomized rats by Greengard *et al.* (41). This technique was employed in order to establish whether the antibiotic interfered with the direct or indirect action of tryptophane. Puromycin administered intraperitoneally in a dose of 3.5 mg per 100 g body weight interfered with both types of induction mechanism.

Studies with puromycin led Tata to suggest (93) that the calorigenic and growth-promoting actions of thyroid hormones are secondary manifestations of a primary reaction at the cellular level that influences the rate of protein synthesis, and that normal functioning of the protein synthetic mechanism is essential for thyroid hormones to elicit these responses. When puromycin was administered (intraperitoneally at a dosage level of 12 mg/100 g) to thyroidectomized rats, the calorigenic action of triiodothyronine was suppressed; but the antibiotic did not affect the basal metabolic rate or body weight of the thyroidectomized animals. Weiss and Sokoloff (99) suppressed not only protein synthesis (>90 %) but also the stimulation of protein synthesis by thyroxine (75 %) by giving puromycin to rats at a dosage of 20 mg intraperitoneally in two equally divided doses 45 minutes apart. Since the effect of thyroxine on oxidative

metabolism was being fully expressed up to the time it was acutely interrupted by the inhibition of protein synthesis by puromycin, a direct relationship was suggested between the thyroxine effects on protein synthesis and on oxidative metabolism. The antibiotic reversed the hypermetabolism induced in rats by prior administration of thyroxine and restored the oxygen consumption of the thyrotoxic rats to the euthyroid level by decreasing the metabolic rate (35%). Sokoloff *et al.* (88) suggested that the stimulation of protein synthesis by thyroxine is localized at the step involving the transfer of sRNA-bound amino acid to microsomal protein; this is the step that puromycin inhibits.

2. Enzyme. The early work of Creaser (22) on the effect of puromycin upon induced β-galactosidase synthesis in bacteria was confirmed by Sypherd and Strauss (90). The antibiotic interfered with the synthesis of the enzyme in cells of *E. coli*; when puromycin was added at a concentration causing a 40 to 50% reduction in growth rate, a 30% decrease in enzyme synthesis occurred. Nucleic acid synthesis was not inhibited under these conditions.

The effects of puromycin on the synthesis of DNA in regenerating rat liver *in vivo* and *in vitro* were compared by Gottlieb *et al.* (38). When puromycin was administered intraperitoneally to partially hepatectomized rats in 3 injections of 12.5 mg over an 8-hour period, it inhibited the incorporation of C^{14}-orotic acid into DNA of both 24- and 40-hour regenerating livers. *In vitro* the incorporation by the supernatant fraction of a homogenate of 24-hour regenerated liver was reduced to 10% when puromycin was injected 16 hours after hepatectomy; when injections were started 32 hours after hepatectomy, significant incorporation was observed with the 40-hour supernatant fraction. The authors suggest that the activities of the enzymes involved in the last steps of DNA synthesis are not rate-limiting and that protein synthesis is required prior to DNA synthesis.

On the basis of studies in the mouse in which puromycin induced glycogenolysis and inhibited protein synthesis, Hofert (47) and Hofert and Boutwell (48) suggested that the two processes operate independently. The antibiotic was injected in single or multiple doses at a level of 260 mg/kg. Administration of sodium pentobarbital blocked the glycogenolytic response without arresting the inhibition of the incorporation of C^{14}-glycine into protein.

Studies were undertaken by Manner and Gould (59) in the chick embryo to determine whether hydroxyproline could be derived from the proline pool despite inhibition of collagen synthesis. In this investigation puromycin had a pronounced effect on collagen formation. The antibiotic (1 mg) was injected intravenously together with C^{14}-proline into the chorioallantoic membrane of 12-day-old embryos. The incorporation of proline and hydroxyproline into both soluble and insoluble collagen was markedly inhibited (90%) 15 to 60 minutes after injection. The free hydroxyproline that accumulates under normal conditions, however, was inhibited considerably less (by only 7% in 15 minutes and by 50% in 50 minutes). This effect may possibly be attributed to the high general toxicity of puromycin at the level used, which was lethal within 24 hours.

Puromycin was found by Peck and Acs (75) to induce tyrosinase activity *in vivo* by intact neural retinal tissue of the vertebrate eye when added at a level

sufficient to inhibit protein synthesis. This activity is not exhibited ordinarily either *in vivo* or *in vitro* by the intact tissue but only by disaggregated cells. The mechanisms by which tryptophane pyrrolase activity is increased in the newborn and adult rat and in the guinea pig after the injection of L-tryptophane were investigated by Nemeth and de la Haba (70). Normal developmental increase in the newborn was blocked completely by four intraperitoneal injections of puromycin (15 mg each, at 0, 1, 2, and 3 hours), while adaptive increase in the adult was inhibited 50%. Under the conditions of the experiment over 98% of the incorporation of C^{14}-valine into total liver protein was blocked. These results lead to the conclusion that the normal increase probably depends entirely on the formation of new enzyme molecules from amino acid and that the adaptive increase is brought about partly by new enzyme formation and partly by the activation of pre-existing protein. Animals receiving puromycin had a greater amount of C^{14} in the amino acid pool and a reduced amount in the cytoplasmic sRNA, total RNA, and protein. These findings suggest that puromycin blocks the incorporation of C^{14}-valine into liver protein *in vivo* by inhibiting the premature release of incomplete peptides; on the basis of their solubility, these would be measured as free amino acids. This observation that puromycin could diminish substrate-induced elevation of tryptophane pyrrolase activity was confirmed by Greengard and Acs (40) in studies on rats.

Inhibition of the enzyme tyramine methylpherase formation during the germination of barley has been obtained with puromycin by Mann *et al.* (58). With a concentration of 5×10^{-5} M, which did not inhibit germination, 50% inhibition of enzyme formation occurred. When the concentration of the antibiotic was raised to 10^{-3} M, partial inhibition of germination occurred and enzyme formation was further reduced (to 16%).

Click and Hackett (19) found that low concentrations of puromycin (5×10^{-4} M) blocked the development of rapid respiration in potato tuber slices and inhibited the incorporation of C^{14}-leucine. The degree of inhibition of both of these processes was similar. The incorporation of C^{14}-uracil into RNA was less sensitive to the antibiotic, a result which suggests that inhibition of synthesis of an enzyme was required for RNA synthesis.

In a preliminary paper, Sankaranarayanan *et al.* (82) reported that when mice bearing the Ehrlich ascites tumor were treated with puromycin (100 mg/kg/day $\times$ 7), increases of protein-bound sulfhydryl groups (−SH) and a reduction in the disulfide linkages (−S−S−) were observed. Combination treatment of puromycin and 5-fluorouracil (which decreased basic protein formation) resulted in only a slight increase of −SH groups and no difference in the −S−S− linkages.

3. Cell division. The effect of puromycin upon cell division was investigated in fertilized eggs of the sea urchin *Paracentrotus lividus* by Hultin (49). A mitotic blockage was induced, and this was assumed to be an effect of impaired protein metabolism. The antibiotic was added to unfertilized eggs 10 minutes before fertilization. At a concentration of 10^{-4} M puromycin, 50% of the eggs stopped developing after the first division and development of all eggs was arrested after

the second cleavage. At lower concentrations of puromycin a larger portion of the eggs divided, and at 10^{-5} M 40% of the eggs reached the 4-cell stage. Inhibition of the incorporation of C^{14}-valine into protein was at the 50% level at 10^{-5} M and at the 60% level at 10^{-4} M. A cell-free incorporation system was considerably more sensitive to the antibiotic than were the intact eggs. The effect of puromycin upon morphogenesis in amphibian eggs was investigated in *Pleurodeles* and *Rana temporaria* by Brachet (15). Inhibition of neurulation occurred at concentrations of 2 to 6 $\times$ 10^{-5} M when the membranes surrounding the embryos were removed in the late gastrula stage. A lack of effect at earlier stages of development may have been caused by poor permeability to the antibiotic.

In studies of the relationship between RNA synthesis and loop structure in the giant lampbrush chromosomes of amphibian oocytes, Izawa *et al.* (52) found the DNA-containing loops were not affected by puromycin. Although the uptake of radioactive amino acids into the chromosomal protein was markedly inhibited at a concentration of 10^{-4} M, puromycin did not destroy the loop structure. The results suggest that the morphology of the active chromosomal site is directly dependent upon its capacity to synthesize RNA rather than DNA. Brewen (16) has used puromycin to study the rejoining system of chromosomal aberrations induced by X-rays in the corneal epithelium of the Chinese hamster. Production of both ATP and protein must take place if the chromatin break is to be closed. Puromycin at a concentration of 2 $\times$ 10^{-4} M abolished the protection derived from a low dose of X-ray. The equivalence of the breakage rate at three doses of X-ray when the antibiotic was present probably resulted from equal inhibition of protein synthesis.

4. Viral replication. The syntheses of viral protein and RNA are coordinated in time; they have been elucidated in detail by Penman *et al.* (76). Levintow *et al.* (56) reported that the addition of puromycin (10^{-5} M) to a poliovirus-infected HeLa cell culture during the latent period (0 to 2 hours) prevented both the formation of the mature virus and the initiation of the synthesis of viral RNA. Its addition after RNA synthesis had begun (2.5 hours) permitted partial synthesis (10%) of RNA to occur after a lag period of one hour. These effects were reversed by removal of the drug. The data suggest that protein synthesis was necessary to permit the replication of RNA. Wecker and Richter (98) reported reversible inhibitions by puromycin (0.2 $\times$ 10^{-5} M) of the maturation of poliovirus in HeLa cells and equine encephalomyelitis virus in chicken embryo fibroblasts. With considerably higher concentrations of the antibiotic (1.6 $\times$ 10^{-5} M), the infectious RNA did not appear in the infected fibroblast cell provided the antibiotic was added sufficiently early. These data support the conclusion of Baltimore and Franklin (11) that synthesis of viral protein is required for the appearance of infectious viral RNA. In these studies, however, no appreciable lag in the production of infectious RNA followed the removal of puromycin. Additional work by Scharff *et al.* (83) with the interruption of poliovirus infection of HeLa cells by this antibiotic (5 $\times$ 10^{-5} M) is also in agreement with this concept.

Wheelock (100) studied the role of protein synthesis in the multiplication of Newcastle disease virus in HeLa cells using puromycin as an inhibitor. The anti-

biotic at a concentration of 10^{-4} M reversibly inhibited both protein synthesis and viral multiplication, and the interval between infection and the presence of the new virus antigen was prolonged from 2 to 12 hours. The rapid reversibility when the antibiotic was withdrawn indicates that some aspect of maturation may take place in the presence of puromycin, with the accumulation of virus precursors, and that early viral synthesis may be independent of protein synthesis.

Wecker (97) investigated further the inhibition by puromycin of western equine encephalitis and poliomyelitis viral replication. He found maturation more sensitive to the antibiotic than the synthesis of infectious RNA. With western equine encephalitis virus the inhibitory levels of puromycin ranged from 0.2 to 3.2×10^{-5} M. In chicken embryo fibroblasts viral replication was evident at a concentration of 0.4×10^{-5} M, whereas 3.2×10^{-5} M was necessary for suppression of infectious RNA synthesis. With the poliomyelitis virus 1.5×10^{-5} M puromycin completely inhibited maturation when added at the time of the infection of the HeLa cells. At this concentration the incorporation of C^{14}-lysine into the cellular protein was slightly inhibited (20%). When the antibiotic was removed, infectious RNA accumulated at the normal rate without an appreciable lag. These observations are in agreement with those of Wheelock (100) on Newcastle disease virus in HeLa cells.

In their studies with C^{14}-adenosine triphosphate and mouse fibroblast L-cells infected with mengovirus, Baltimore and Franklin (11) reported that puromycin (2×10^{-4} M) did not appear to act directly on the system synthesizing viral RNA. Kit *et al.* (54) found that puromycin (5×10^{-5} M) inhibited (by 62%) the incorporation of C^{14}-tryptophane into the cell protein of suspension cultures of L-M mouse fibroblasts. Although the antibiotic did not directly inhibit thymidine kinase activity, it did prevent induction of this activity after infection with vaccinia (after which viral RNA is synthesized actively).

Nathans *et al.* (68) noted that the RNA-dependent biosynthesis of the coat protein of coliphage f2 by extracts of *E. coli* also had the properties expected of a protein-synthesizing system, since puromycin at a concentration of 4×10^{-4} M inhibited the incorporation of C^{14}-leucine (99%).

5. Spore germination. The germination of saprophytic fungi is known to be accompanied by the synthesis of proteins and polynucleotides. The effect of puromycin upon spore germination in the corn rust fungus, *Puccinia sorghi*, was therefore investigated by Staples *et al.* (89). Exposure to puromycin (8×10^{-5} M) inhibited the incorporation of C^{14}-labeled leucine, glutamate, and glucose into the protein fraction of the uredospore. The treatment, however, also was associated with an accumulation of amino acids, nucleic acids, and protein within the uredospore itself. These findings were interpreted to indicate that puromycin could overcome partially a suspected inhibition of amino acid synthesis by the spores that exhibited growth failure. It is apparent that in plant tissue, as well as in bacterial and mammalian systems, puromycin inhibits some part of protein synthesis.

6. Vitamin production. In studies on the effect of purines on the biosynthesis

of riboflavin by the fungus *Eremothecium ashbyii*, puromycin was investigated as a possible inhibitor by Brown *et al.* (17). At concentrations of 1 to 2 $\times$ 10^{-4} M the antibiotic inhibited growth significantly but did not interfere specifically with riboflavin biosynthesis.

7. Antibiotic production. Puromycin has been used in investigations on the production of the antibiotic actinomycin by *Streptomyces antibioticus*. Katz and Weissbach (53) suggested that this process proceeds by a mechanism different from protein synthesis, although both processes compete for the available amino acids in the cell. Puromycin markedly inhibited the incorporation of C^{14}-valine into protein, but brought about a 2- to 3-fold stimulation of the rate of C^{14} incorporation into actinomycin. When the demand for amino acids for protein synthesis was high, as in early stages of growth, little if any antibiotic was synthesized; in contrast, when the requirement for protein synthesis was low (as at the end of the lag phase or when protein synthesis was inhibited by puromycin), antibiotic formation was markedly enhanced. It is postulated that synthesis of this antibiotic may represent a control mechanism that is initiated by a cell when certain types of normal metabolite, such as amino acids, accumulate during a phase in the development of an organism when growth is considerably reduced. The biosynthesis of the antibiotic polypeptide tyrocidine by the bacterium *Bacillus brevis* has also been separated from the biosynthesis of protein in studies with puromycin by Mach *et al.* (57). In this investigation the incorporation of S^{35}-amino acids into protein was sensitive to a low level of puromycin (2 $\times$ 10^{-5} M), while the incorporation of C^{14}-tyrosine into tyrocidine was not inhibited by even a higher level (4 $\times$ 10^{-4} M). Thus, a transfer reaction resembling that known for protein synthesis, and involving amino acyl-sRNA, was considered unlikely to be involved in tyrocidine formation. This suggested that the availability of free amino acids was not a rate-limiting factor in the biosynthesis of the polypeptide antibiotic.

8. Learning and memory. Nucleic acids or proteins may possibly be concerned with learning and memory, and Flexner *et al.* (32) have investigated the effects of puromycin on the central nervous system of mice. The various areas of the brain in control animals were remarkably alike in the rate at which labeled valine was incorporated into protein. The maximum amount of puromycin that could be tolerated in a single subcutaneous injection (0.42 mg per g) was administered to young adult, albino mice. Occasional animals did not survive this dose beyond 24 or 48 hours, but in the great majority of instances it was tolerated and the animals remained in good condition indefinitely. Although this treatment appeared to suppress the rate of protein synthesis (incorporation of C^{14}-valine) in various parts of the brain to 80% of the control value for a period of 6 hours, it was without effect on either learning or retention of simple or discrimination avoidance responses. Inhibition of the incorporation of C^{14}-valine was increased to 95% by combining one subcutaneous and six intracerebral injections (0.26 mg each) of puromycin. Animals treated in this way showed disorientation compatible with disturbances of either learning or memory. Reduced amounts of puromycin (0.09 mg) were then injected intracerebrally into each hemisphere

by Flexner *et al.* (31) so that disorientation of the animal at the time of testing was avoided. The results indicate that memory can be altered consistently, differences in the effective loci of recent and longer-term memory apparently established, and the time factor concerned in modification of the effective locus determined. Upon recovery, animals were capable of learning again. These behavioral effects cannot at this time be related to the suppression of protein synthesis.

VI. SUMMARY

Puromycin blocks protein synthesis *in vitro* and *in vivo* and is a useful agent in demonstrating the role of protein synthesis in acute physiological response. Its action appears to occur at the stage of formation of the polypeptide chain at the ribosomal site. The transfer of the amino acids from sRNA into ribosomal protein is inhibited in the presence of puromycin, and a subsequent release of incomplete, soluble protein from the ribosome then takes place without ribosomal breakdown. Maximum inhibition of protein synthesis is obtained with puromycin analogs that contain an aromatic amino acid attached to the 3′ amino group. Although the specificity of this antibiotic for the amino acid portion of the molecule is an established fact, the reasons for this specificity remain obscure, and current concepts include a direct reaction with the enzyme system and a metabolic analogue response. Because of this inhibition of protein synthesis, puromycin is a useful agent for metabolic studies involving either enzymatic or hormonal systems.

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