MECHANISM OF PUROMYCIN INHIBITION OF HEMOGLOBIN SYNTHESIS*

Allan Morris+, Susana Favelukes,
Ralph Arlinghaus+ and Richard Schweet

Department of Biochemistry, University of
Kentucky Medical Center, Lexington, Kentucky

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The inhibition of protein synthesis by the antibiotic, Puromycin, has been reported by Yarmolinsky et al (1959). This inhibition has now been studied in various systems (Nathans et al, 1961; Schweet et al, 1961; Allen et al, 1962). A direct effect of Puromycin on the ribosome resulting in the release of soluble protein was reported by Morris et al (1961), and confirmed using liver ribosomes, by Hultin (1961).

C^{14}-leucine-labeled ribosomes were incubated in the presence of Puromycin. After incubation, 35 to 45% of the original counts were found in the trichloroacetic acid (TCA)-precipitable material in the supernatant fraction (Table I). Incorporation of C^{14}-leucine into protein under these conditions was inhibited by 95% or more (Allen et al, 1962). The total amount of TCA-precipitable material released into the supernatant with Puromycin alone was always less than that released in the complete system minus Puromycin (Table I).

The amount of TCA-precipitable material released into the supernatant by Puromycin was greatest in the absence of added energy or supernatant enzyme fraction. Also, release of TCA-precipitable material by Puromycin occurred at 4°, although much more slowly than at 37°. Examination of the ribosomes after incubation with Puromycin revealed no alteration of the sedimentation velocity pattern. The total nitrogen in the supernatants of ribosomes incubated with

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+Postdoctoral fellows of the National Heart Institute, U.S. Public Health Service.

TABLE I

EFFECT OF PUROMYCIN ON C^{14}-LEUCINE LABELED RIBOSOMES

Incubation Mixture	Supernatant		Ribosomes
	Acid Soluble	Acid Insoluble	Acid Insoluble
	c.p.m.	c.p.m.	c.p.m.
1. Complete, no Puromycin	0	1252	632
2. Complete, plus Puromycin	463	685	736
3. Plus Puromycin, no energy, no enzyme	63	790	1031
4. Plus Puromycin, plus enzyme, no energy	473	573	838

Ribosomes were labeled by a 10 minute incubation in the complete cell-free system with C^{14}-leucine (sp. act., 3 μC/μmole). The labeled ribosomes were diluted with C^{12}-leucine and re-isolated by centrifugation. The complete system contained about 7 mg. of these ribosomes plus the usual soluble enzyme fraction and other assay constituents with C^{12}-leucine (Allen, et al, 1962). After incubation as described above for 20 minutes at 37°, the ribosomes were isolated by sedimentation and the soluble and ribosome protein were washed and counted separately. The Puromycin concentration was 7.1×10^{-4}M. In 'no energy', the ATP, GTP, creatine phosphate and creatine kinase were omitted from the complete system. 'Acid Soluble' is the difference between total protein radioactivity recovered compared to that found in the absence of Puromycin (line 1).

or without Puromycin was the same. These results are evidence for the release of C^{14}-labeled material by Puromycin without measurable ribosome breakdown, and in the absence of added supernatant enzyme.

In the absence of added enzyme, the radioactivity in the supernatant following treatment with Puromycin was largely protein, based on its precipitability with TCA after treatment with 1N NaOH. Solubility and other properties indicated that most of this material was not hemoglobin, in contrast to supernatant protein formed in the absence of Puromycin (Table I), which was largely hemoglobin (Bishop et al, 1960). However, after trypsin digestion of Puromycin-released supernatant material and fingerprinting (Katz et al, 1959), the radioactive peptides had the mobilities of hemoglobin peptides. The valine present as N-terminal residues in hemoglobin (Bishop et al, 1960) is 8.1%. When ribosomes labeled with C^{14}-valine in the intact cell were isolated and treated with Puromycin, the supernatant contained 10 to 12% C^{14}-valine in N-terminal residues. These data provide strong evidence that Puromycin releases incomplete globin chains from ribosomes, and that

these are synthesized from the N-terminal end, as indicated previously (Bishop et al, 1960; Dintzis, 1961).

Another characteristic of Puromycin action is the production of TCA-soluble radioactive material in the presence of the supernatant enzyme fraction. Comparison of lines 1 and 3 with either 2 or 4 (Table I) show a decrease in the total TCA-insoluble radioactivity which appears as acid-soluble radioactive material. Acid-soluble material originates from the acid-insoluble fraction of the supernatant. This was shown by treating labeled ribosomes with Puromycin at 4° without added enzyme and then removing the ribosomes. When the labeled supernatant material was then re-incubated with the usual soluble enzyme preparation, the production of acid-soluble radioactive material was observed. No acid-soluble C^{14}-labeled material was produced with boiled enzyme under these conditions. The formation of acid-soluble labeled material in the presence of added enzyme was complete after 30 minutes of incubation at 37° and incubation with fresh enzyme did not produce additional C^{14}-labeled acid-soluble material. No acid-soluble, labeled material was found when labeled supernatants produced in the absence of Puromycin (Table I, line 1) were incubated under these conditions. The acid-soluble, labeled material has been studied by column chromatography and quantitative decarboxylation with chloramine T. The results of these studies suggest that a complex mixture of free amino acids and small peptides is present. The nature of the enzymatic reaction which produces the TCA-soluble material from the TCA-precipitable material released by Puromycin is not known, but the reaction is not inhibited by di-isopropylfluorophosphate and as noted above only part of the total TCA-precipitable material is made acid-soluble. These results suggest that in addition to incomplete globin chains, Puromycin releases other precursors of globin from the ribosome. The postulated precursors are TCA-precipitable, alkali-stable and can be converted to free amino acids and small peptides by an unknown enzymatic process.

Evidence that the postulated compounds are intermediates in hemoglobin synthesis was obtained in "chase" experiments. Labeled ribosomes were incubated with C^{12}-amino acids in the complete system and Puromycin added at various times

(Table II). As the labeled components of the ribosome were converted to completed hemoglobin chains and released into the supernatant, the TCA-soluble material produced when Puromycin was added, decreased. Similar results were obtained with ribosomes labeled in the intact cell (Table II). The decrease in the labeled TCA-soluble material only occurred when C^{12}-amino acids were being incorporated, as expected for an intermediate in the synthesis of globin chains.

TABLE II

RELEASE OF LABELED COMPONENTS FROM RIBOSOMES BY PUROMYCIN ADDED AT VARIOUS TIMES

Expt.	Incubation Time Before Puromycin min.	Supernatant Acid-soluble c.p.m.	Supernatant Acid-insoluble c.p.m.	Ribosomes Acid-insoluble c.p.m.
1	0	362	765	718
	10	241	980	602
	40	146	1113	568
	No Puromycin	0	1255	575
2	0	432	1280	1360
	10	245	1678	1160
	40	52	1978	1100
	No Puromycin	0	2250	1048

In Expt. 1, ribosomes were labeled in the cell-free system as described for Table I. In Expt. 2, ribosomes were isolated from intact cells incubated with C^{14}-leucine (Bishop et al, 1960). In both cases, ribosomes were incubated with C^{12}-amino acids in the complete, cell-free system for the indicated times. Puromycin (7×10^{-4}M) was added and incubation was continued for an additional 40 minutes.

The inhibition of protein synthesis by Puromycin is considered to result from the displacement of incomplete globin chains and the postulated earlier intermediates from the ribosome. In the presence of Puromycin, further formation of TCA-precipitable peptides would be blocked by a continuation of the same process. This mechanism implies reversibility of Puromycin inhibition, in contrast to irreversible mechanisms such as ribosome breakdown or removal of an essential component from the ribosome. Reversal of Puromycin inhibition by washing of inhibited ribosomes has been obtained (S. Favelukes, unpublished data).

REFERENCES

Allen, E. and Schweet, R., J. Biol. Chem., (March, 1962) in press.
Bishop, J., Leahy, J. and Schweet, R., Proc. Natl. Acad. Sci., 46, 1030 (1960).
Dintzis, H., Proc. Natl. Acad. Sci., 47, 247 (1961).
Hultin, T., Biochim. Biophys. Acta, 51, 219 (1961).
Katz, A., Dreyer, W., and Anfinsen, C., J. Biol. Chem., 235, 2897 (1959).
Morris, A. and Schweet, R., Biochim. Biophys. Acta, 47, 415 (1961).
Nathans, D. and Lipmann, F., Proc. Natl. Acad. Sci., 47, 497 (1961).
Schweet, R., Bishop, J., and Morris, A., Lab. Invest., 10, 992 (1961).
Yarmolinsky, M. and de la Haba, G., Proc. Natl. Acad. Sci., 45, 1232 (1959).