Monier, R., Stephenson, M. L., and Zamecnik, P. C. (1960), *Biochim. Biophys. Acta 43*, 1.
Nirenberg, M. W., and Matthaei, J. H. (1961), *Proc. Nat. Acad. Sci. U. S. 47*, 1558.
Nirenberg, M. W., Matthaei, J. H., and Jones, O. W. (1962), *Proc. Nat. Acad. Sci. U. S. 48*, 104.
Risebrough, R. W., Tissiéres, A., and Watson, J. D. (1962), *Proc. Nat. Acad. Sci. U. S. 48*, 430.
So, A. G., and Davie, E. W. (1963), *Biochemistry 2*, 132.
Spyrides, G., and Lipmann, F. (1962), *Proc. Nat. Acad. Sci. U. S. 48*, 1977.
von Ehrenstein, G., and Lipmann, F. (1961), *Proc. Nat. Acad. Sci. U. S. 47*, 941.
Warner, J. R., Knopf, P. M., and Rich, A. (1963), *Proc. Nat. Acad. Sci. U. S. 49*, 122.
Warner, J. R., Rich, A., and Hall, C. E. (1962), *Science 138*, 1399.
Webster, G. C. (1957), *J. Biol. Chem. 229*, 535.
Weinstein, I. B., and Schechter, A. N. (1962), *Proc. Nat. Acad. Sci. U. S. 48*, 1686.
Young, R. J., Kihara, H. K., and Halvorson, H. O. (1961), *Proc. Nat. Acad. Sci. U. S. 47*, 1415.

Inhibition of Hemoglobin Synthesis by Puromycin*

ALLAN MORRIS,† RALPH ARLINGHAUS,† SUSANNA FAVELUKES, AND RICHARD SCHWEET‡

From the Department of Biochemistry, University of Kentucky College of Medicine, Lexington

Received April 11, 1963

The inhibition of hemoglobin synthesis by puromycin was proportional to inhibitor concentration. However, inhibition at low levels of puromycin increased with time. Preincubation studies showed that a small amount of protein synthesis in the presence of puromycin resulted in almost complete inhibition at 2×10^{-5} M concentration of inhibitor. The inhibitor caused the release of labeled protein from the ribosomes, and this release was related to the degree of inhibition. The released material contained incomplete globin chains, as shown by solubility, chromatographic behavior, and *N*-terminal analysis. About 25% of the released material contained chains which were newly started in the cell-free system. Release of incomplete chains took place at 4° in the absence of added enzyme, although this was a slow reaction. The mechanism of puromycin action suggested was that the inhibitor substitutes for the next incoming amino acid at the growing point of the peptide chain (the carboxyl end). This displaces the chain. Further synthesis can occur, but yields only small peptides which are displaced, and are acid soluble.

The inhibition of protein synthesis by puromycin, first reported by Yarmolinsky and de la Haba (1959), has been confirmed in various systems (Nathans and Lipmann, 1961; Morris and Schweet, 1961; Hultin, 1961). More complex effects of puromycin have been reported by Nemeth and de la Haba (1962), Mueller *et al.* (1961), and Rabinovitz and Fisher (1962). A direct effect of puromycin on ribosomes resulting in the release of soluble protein was found by Morris and Schweet (1961), Hultin (1961), Morris *et al.* (1962), Allen and Zamecnik (1962), and Lamborg (1962). Detailed studies of the mechanism of puromycin action on reticulocyte ribosomes are reported here.

EXPERIMENTAL

Materials.—DL-1-C^{14}-leucine was purchased from the California Corporation for Biochemical Research and had a specific activity of 10.3 $\mu c/\mu$mole. Uniformly labeled L-valine, L-arginine, and L-lysine were obtained from the Nuclear-Chicago Corporation and had specific activities of 6.5, 2.5, and 8.3 $\mu c/\mu$mole, respectively. Puromycin hydrochloride was kindly donated by Dr. E. Stokstad of Lederle Laboratories. Rabbit reticulocytes, enzyme fractions, and other components of the cell-free system have been described (Allen and Schweet, 1962). The data have been calculated for C^{14}-amino acid at a specific activity of 7 $\mu c/\mu$mole, which gave 2.3×10^6 cpm per μmole in the thin-window Geiger counter used.

Preparation of Labeled Ribosomes.—Incubations of intact cells were carried out by a modification of the procedure of Borsook *et al.* (1957). For each 2 ml of washed reticulocytes was added 0.33 ml of rabbit plasma, 1.84 ml. of twice-concentrated NKM[1] salt solution, 0.067 ml. of 1 M Tris buffer, *p*H 7.5, 1.0 ml of amino acid mixture less valine, 0.067 ml. of 0.01 M ferrous ammonium sulfate, 0.15 ml of 1×10^{-3} M C^{14}-L-valine, and 1.5 ml of water. The mixture was incubated 15 minutes at 37° and the cells were then centrifuged, resuspended in cold NKM solution, and recentrifuged. Labeled ribosomes were then isolated in the usual manner, except that unlabeled high-speed supernatant (approximately 4 mg protein/mg ribosomal protein) was added to the ribosome suspension after the first sedimentation of the particles in order to dilute any adsorbed or occluded *labeled* hemoglobin.

For the labeling of ribosomes in the cell-free system, the ribosome pellet after the first centrifugation (Allen and Schweet, 1962) was suspended in 0.25 M sucrose to a final concentration of approximately 14 mg of ribosomes per ml (6–7 mg of ribosomal protein) and incubated 10 minutes at 37° in the usual complete system with C^{14}-leucine or other C^{14}-amino acid and other components of the cell-free system (Allen and Schweet, 1962). The reaction was terminated by the addition of 6–10 volumes of cold medium B (0.25 M sucrose, 0.0175 M $KHCO_3$, 0.002 M $MgCl_2$) containing a 50-fold excess of C^{12}-leucine. The labeled ribosomes were then reisolated by centrifugation.

Studies of Released Components.—The release of labeled material from labeled ribosomes either with or

* This study was supported by a grant (H-5293) from the U. S. Public Health Service and a grant (G-21026) from the National Science Foundation.

† Postdoctoral fellow of the National Institutes of Health, U. S. Public Health Service.

‡ Career Research Awardee of the U. S. Public Health Service.

[1] Abbreviations used in this work: TCA, trichloroacetic acid; chloramine T, sodium *para*-toluenesulfonchloramine; DIFP, diisopropylfluorophosphate; NKM, 0.13 M NaCl, 0.005 M KCl, 0.0075 M $MgCl_2$.

without puromycin was studied as follows. After incubation, the contents of a single incubation tube (usually 1.4 ml) were cooled and transferred quantitatively to 4-ml centrifuge tubes (Spinco centrifuge rotor No. 40) with the aid of 2 ml of 0.25 M sucrose. The ribosomes were removed by centrifugation at 40,000 rpm for 60 minutes. The supernatant solution containing the released material was removed and the ribosome pellet was rinsed with small amounts of sucrose solution. Casein solution (15 mg/ml) was added to the supernatant plus washings to a total protein content of 15 mg, and the mixtures were precipitated with trichloroacetic acid (TCA) at a final concentration of 5%. The ribosome pellet was made into a paste with a glass rod and then suspended by the addition of 1 ml of 0.1 M Tris-HCl buffer, *p*H 7.5. The suspension was then decanted into a tube containing casein in an amount calculated to give approximately 15 mg of total protein. Quantitative transfer of the ribosomes was ensured by washes with 0.5 ml of 1 N NaOH and two small volumes of 5% TCA. The ribosome and supernatant solutions were then washed and plated as described for the usual incubation mixture. Certain types of incubations resulted in the conversion of TCA-precipitable radioactivity originally present in labeled ribosomes into TCA-soluble radioactivity. The acid-soluble radioactivity was calculated by the difference in TCA-precipitable radioactivity before and after incubation. In all cases, the TCA-precipitable material was taken through the complete washing procedure before determination of radioactivity. The radioactivity of the acid-soluble materials could be determined *directly* only after dialysis of labeled ribosomes to remove adsorbed C^{14}-leucine. Ribosomes were suspended in a medium containing 0.25 M sucrose, 0.0175 M $KHCO_3$, 0.001 M $MgCl_2$, and 2×10^{-4} M C^{12}-leucine and dialyzed for 12 hours against the same medium without the leucine.

N-Terminal valine analysis followed the procedure of Bishop *et al.* (1960). Protein (10 mg) was precipitated with 5% TCA, washed twice with 1% TCA, dissolved in 0.1 N NaOH, and immediately adjusted to *p*H 9.5 as described.

Quantitative decarboxylation with chloramine-T was used to determine the percentage of C^{14}-leucine present as free amino acid in the acid-soluble fraction, which also contained labeled peptides (see below). The following procedure was used. Acid insoluble material was precipitated with 5% TCA and centri fuged. The supernatant was decanted, and the in soluble material was washed twice with small amounts of 5% TCA. The combined supernatants and washes were filtered through Whatman No. 4 paper and TCA was removed by extraction with ether. Residual ether was then removed by bubbling nitrogen through the solution, and aliquots were used for the decarboxylation analysis. The reaction was carried out in a vessel which allowed 1 ml of 6% (w/v) solution of chlor amine-T to be placed in one part of the container, the analytical sample plus 3 mg of C^{12}-glycine plus 0.4 ml of citrate buffer (0.1 M, *p*H 2.5) in another portion of the vessel, and 0.3 ml of 1 M hyamine base in a small cup in the center of the reaction chamber. After a partial vacuum was applied to the system, the vessels were closed to the outside atmosphere and the chloramine and C^{14}-samples were mixed and incubated for 30 minutes at 30°. The vessels were then opened and the hyamine C^{14}-carbonates were dissolved in 20-ml counting vials containing 15 ml of counting fluid (Bishop *et al.*, 1961). The radioactivity was then determined in a Packard Liquid Scintillation Spectrometer.

Fingerprint studies were conducted according to the method of Katz *et al.* (1959). Chromatography was done in butanol-pyridine-water (4:3:3) for 25 hours.

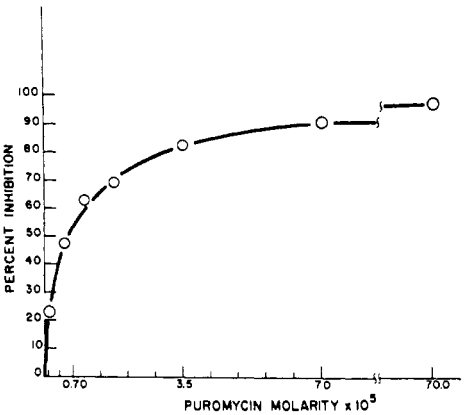

FIG. 1.—Inhibition of amino acid incorporation by puromycin. Puromycin was added to the complete system containing 6 mg of ribosomes and 1-C-^{14}-leucine and incubated 40 minutes at 37°. All values are compared to a complete system with no puromycin.

RESULTS

Inhibition of Incorporation.—Inhibition of amino acid incorporation by puromycin was first studied in the complete cell-free system containing soluble enzymes and unlabeled ribosomes (Allen and Schweet, 1962). Inhibition was proportional to puromycin concentration (Fig. 1), and 50% inhibition was obtained at 7×10^{-6} M inhibitor. Very large amounts of inhibitor were needed to obtain more than 85% inhibition. However, it was noted that incorporation, even with small amounts of puromycin, stopped after a short period of incubation. To study the development of inhibition, the time course of incorporation with various levels of puromycin was studied (Fig. 2). This was done at 25° in order to obtain a more accurate picture of early time periods, although similar results were obtained at 37°. For all concentrations of inhibitor tested, incorporation essentially ceased after a short time. The time required to obtain this cessation of incorporation decreased with increasing concentrations of puromycin (see arrows, Fig. 2), so that at high concentrations of puromycin complete inhibition occurred at zero time. These results suggested the possibility that some enzymatic reaction occurred

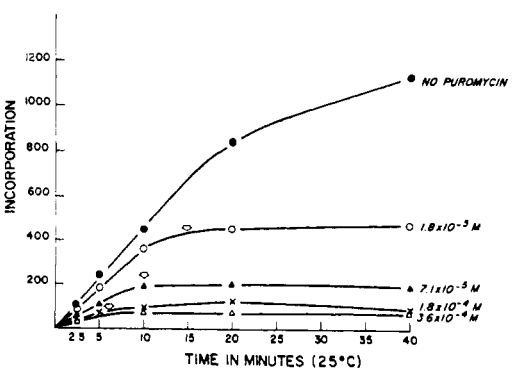

FIG. 2.—Time course of inhibition of amino acid inporation with various levels of puromycin. Data are given as cpm/mg ribosomes. Puromycin was added to the complete system containing 1-C-^{14}-leucine at a specific activity of 7 μc/μmole and incubated at 25° for the indicated time. The arrows show the point where the incorporation rate becomes very low.

TABLE I

PUROMYCIN INHIBITION OF INITIAL RATE OF AMINO ACID INCORPORATION

The components given below were preincubated for 10 minutes at 37° without C^{14}-leucine. After the preincubation, the missing components to make the usual complete system were added and the reaction was terminated after 5 minutes at 37°. In these assays the level of GTP was increased to 1 μmole. The data are given in cpm per mg ribosomes using C^{14}-leucine at a specific activity of 7 μc/μmole. Puromycin was added at 1.8×10^{-5} M final concentration. The enzyme fraction used was the AS_{70} described previously (Allen and Schweet, 1962).

Components of Preincubation	Incorporation (specific activity)	Inhibition (%)
Not preincubated	450	
Same, plus puromycin	290	35
Complete system	390	92
Same, plus puromycin	30	
Complete minus energy[a]	455	
Same, plus puromycin	265	42
Complete minus soluble enzymes	450	
Same, plus puromycin	260	42
Complete, no energy plus GTP[a]	480	
Same, plus puromycin	180	63

[a] No energy indicates omission of ATP, GTP, phosphocreatine, and creatine kinase.

which was required for puromycin inhibition. Preincubations with various components of the system, followed by a 5-minute assay in the complete system, were used to study the factors needed for development of inhibition (Table I). Conditions which normally permitted incorporation (of C^{12}-amino acids) led to development of inhibition during preincubation in the presence of puromycin. However, an exception is the increase in inhibition obtained by preincubation with GTP, since little incorporation would take place in the absence of puromycin under these conditions. It should be noted that any type of preincubation in the *absence* of puromycin did not lead to increased inhibition of the initial rate (5-minute assay). Thus these results indicate that an enzymatic, energy-requiring reaction in the presence of puromycin is needed for rapid establishment of puromycin inhibition. In other studies, similar to those of Allen and Zamecnik (1962), a small increase in inhibition was obtained by preincubation in the cold without added enzyme, but with puromycin. This inhibition was proportional to the amount of release (see below). It is of interest that, after preincubation, almost complete inhibition was obtained with 2×10^{-5} M puromycin. This corresponds to 10 molecules of inhibitor present per ribosome and emphasizes the specificity of the inhibition.

Reversal of Puromycin Inhibition.—Since it was previously shown that puromycin did not act by inhibiting any of the soluble components of the system (Allen and Schweet, 1962), it is the ribosome which is the site of puromycin inhibition, as noted previously (Morris *et al.*, 1962; Allen and Zamecnik, 1962). One explanation for the development of inhibition might be that puromycin was bound to ribosomes in some way. To test this possibility, ribosomes were incubated with the complete system containing 1.8×10^{-5} M puromycin and C^{12}-leucine. Under these conditions (Table II), incorporation after 10 minutes essentially ceased since the 10–15 minute increment of incorporation in the presence of puromycin was negligible compared to that

TABLE II

REVERSAL OF PUROMYCIN INHIBITION

I. Ribosomes isolated after the first centrifugation were incubated for 10 minutes at 37° in the complete system with *C^{12}-leucine* with and without 1.8×10^{-5} M puromycin. The mixtures were chilled and diluted with 8.5 volumes of cold medium B, and the ribosomes were reisolated by centrifugation. To be sure of the inhibition during this incubation, duplicate assays were incubated with *C^{14}-leucine* plus and minus puromycin for 10 and 15 minutes, then washed and counted in the usual way (lines 1–4).

II. Each set of preincubated ribosomes (inhibited and uninhibited) were then assayed in the complete system for 40 minutes with C^{14}-leucine. The data are given as in Table I.

Incubation	Conditions of Assay	Specific Activity	Inhibition (%)
I	1. 10 minutes, plus puromycin	530	
	2. 15 minutes, plus puromycin	580	92.0
	3. 10 minutes, minus puromycin	770	
	4. 15 minutes, minus puromycin	1280	
II	Preincubated plus puromycin	630	31.5
	Preincubated minus puromycin	920	

in the absence of puromycin. Inhibited ribosomes were then washed by dilution and recentrifugation. These ribosomes, when assayed, had approximately 70% of the activity of a control set of ribosomes carried through the same procedure without puromycin. Thus the inhibition was reversible. In other studies, dialysis of ribosomes or passage through Sephadex G-50 did not result in better reversal of puromycin inhibition.

Effect of Puromycin on Labeled Ribosomes.—When puromycin was added to ribosomes which had been labeled with C^{14}-leucine in the cell-free system, a considerable portion of the radioactivity was released into the supernatant (Table III) under conditions where amino acid incorporation was almost completely inhibited (Morris *et al.*, 1962; Allen and Zamecnik, 1962). It should be noted that the material released into the supernatant in the *absence* of puromycin contained 75% of the original radioactivity in the ribosomes and was all TCA-insoluble. This material was largely hemoglobin (Bishop *et al.*, 1960), and the completion and release of hemoglobin chains required soluble enzymes and energy (Morris and Schweet, 1961; also see Lamborg, 1962). In contrast, the release by puromycin did not require added enzymes and energy, and proceeded at 4° (Table III). However, release at 4° was much slower than at 37° and required 40 minutes for completion at 7×10^{-4} M puromycin. Under these conditions but at 37°, release was completed in less than 5 minutes, but at lower puromycin concentrations longer periods were needed for complete release at both 4° and 37°. In addition to TCA-insoluble material, in the presence of puromycin TCA-soluble radioactive material was released. Formation of TCA-soluble material occurred only when soluble enzymes were present. The nature of this material is discussed below.

To study whether release of labeled materials involved ribosome degradation, samples of ribosomes incubated in the complete system plus and minus puromycin were studied in the analytical ultracentrifuge. No difference in the schlieren patterns was found (Fig. 3). As a further check, two ribosome samples were incubated for 30 minutes at 4° in a solution containing only buffer, salts, and glutathione (at the same concentrations as for the complete system) plus and minus puromycin (1×10^{-3} M). The ribosomes were re-

TABLE III

RELEASE OF LABELED COMPONENTS FROM RIBOSOMES BY PUROMYCIN

C^{14}-labeled ribosomes (see Methods) containing 3600 cpm were incubated as described. The ribosomes and supernatant were then separated and counted separately. The "acid-soluble" radioactivity was that remaining in the supernatant after TCA precipitation of the "acid-insoluble." Puromycin concentration was 7×10^{-4} M. Incubation time, 40 minutes at 37° unless noted otherwise.

Incubation Conditions	Supernatant		Ribosomes
	Acid Soluble (cpm)	Acid Insoluble (cpm)	Acid Insoluble (cpm)
1. Complete system, no puromycin	0	2820	942
2. Complete system, plus puromycin	708	1464	1596
3. Complete system for 20 minutes, then puromycin, 20 minutes	0	2700	1194
4. No energy,[a] no enzyme plus puromycin	96	1648	1992
5. Same, at 4°	0	1512	2052
6. No energy,[a] plus puromycin	840	1215	1704

[a] No energy refers to omission of GTP, ATP, creatine phosphate, and creatine kinase. Controls incubated under these conditions *without* puromycin released approximately 270 cpm, which was all acid insoluble. Zero time controls gave similar results, and this has been subtracted from the data. The enzyme fraction used was the AS_{70} previously described (Allen and Schweet, 1962).

moved by centrifugation (16.8 mg was recovered in each case). The protein content of the supernatant solutions was 546 and 525 μg, respectively, as determined by the Lowry method (Lowry *et al.*, 1951). Thus, no ribosome degradation was found, although the supernatant from the puromycin-treated sample contained 35% of the radioactivity originally present in the ribosomes, and the other sample contained 4%.

This fact indicates that the TCA-insoluble material released by puromycin had a high specific radioactivity, as would be expected for nascent hemoglobin or intermediates. However, the released material was not

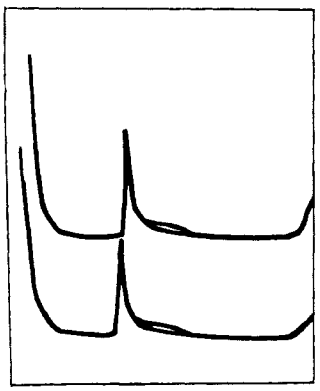

FIG. 3.—Sedimentation patterns of ribosomes in the presence and absence of puromycin. C^{14}-labeled ribosomes were incubated for 40 minutes at 37° in the complete system in the presence (regular cell-bottom) or absence (prismatic cell-top) of 1.4×10^{-4}M puromycin. The reaction mixtures were chilled and an aliquot from each placed in optical cells of a Model E analytical ultracentrifuge. Analyses were carried out at 42,040 rpm at 5° for 16 minutes. The $s_{20,w}$ of the major peak was 76.

TABLE IV

PROPERTIES OF SUPERNATANT PROTEIN RELEASED BY PUROMYCIN

Labeled ribosomes (6 mg, containing 4200 cpm of C^{14}-leucine) were incubated for 30 minutes at 4° with 3.6×10^{-4} M puromycin. The incubation medium contained the salts and buffer of the complete system (0.036 M Tris buffer, *p*H 7.5; 0.0036 M $MgCl_2$; 0.036 M KCl; and 0.015 M GSH). The ribosomes were removed by centrifugation, 20 mg of unlabeled supernatant protein was added as carrier, and the combined supernatant fraction was used as described below.

Treatment	TCA-Insoluble Radioactivity (cpm)
None	1580
Precipitate at *p*H 5.2	570
Precipitate at 45% sat. ammonium sulfate	415
Precipitate at 95% sat. ammonium sulfate	340
Ammonium sulfate supernatant	50

hemoglobin, since a large percentage was precipitated at *p*H 5 (Table IV) and at 45% saturation with ammonium sulfate. Little or no hemoglobin is precipitated under these conditions. In addition, the puromycin-released material was strongly adsorbed to Sephadex G-25 and IRC-50 columns, while hemoglobin was eluted. Dialysis of the material against distilled water overnight at 4° resulted in precipitation, in contrast to hemoglobin. These data suggest that the puromycin-released materials are hemoglobin intermediates.

Evidence which supports this conclusion was obtained from studies of the peptide composition and the *N*-terminal valine content of labeled material released by puromycin. The pattern of radioactive peptides obtained after tryptic digestion of puromycin-released material was similar to that of globin, although not all the peptides from globin were labeled (Fig. 4). The percentage of C^{14}-valine in the *N*-terminal position of hemoglobin synthesized in the intact cell is about 8% (Bishop *et al.*, 1960). This corresponds to that expected for uniformly labeled hemoglobin. When ribosomes labeled in the *intact cell* were incubated in the *cell-free* system with C^{12}-amino acids, the hemoglobin isolated contained 12.5% *N*-terminal C^{14}-valine (Bishop

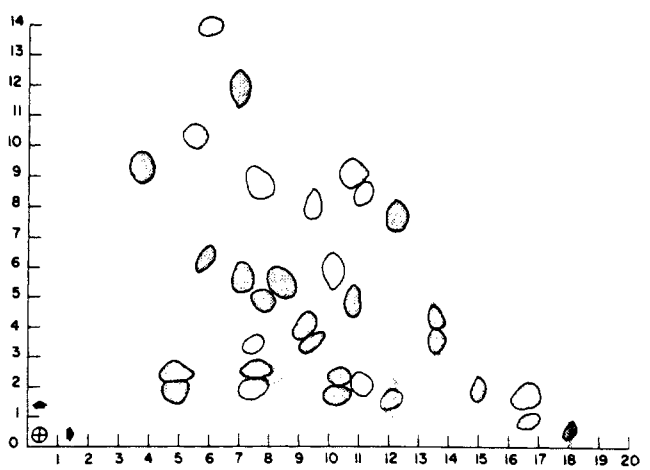

FIG. 4.—Radioautography of "fingerprint" of tryptic digest of puromycin-released material. The cross-hatching shows radioactive spots taken from X-ray film, superimposed on "fingerprint" of hemoglobin sprayed with ninhydrin (circles). See Methods for details.

TABLE V

N-TERMINAL C^{14}-VALINE CONTENT OF VARIOUS PROTEIN FRACTIONS

In each case, incubations with C^{14}-valine were performed as described. The ribosomes were separated and the C^{14}-valine in *N*-terminal position was determined on the supernatant (1 and 3). The labeled ribosomes were incubated with puromycin (see Table IV for conditions), the ribosomes were removed, and the "released" material was analyzed for *N*-terminal C^{14}-valine (2 and 4). The values are given as % of the total C^{14}-valine in the fraction.

	Material Analyzed	Conditions of Labeling	% *N*-Terminal Valine
1.	Hemoglobin	Whole cell	7.8
2.	Puromycin-released material	Whole cell	12.0
3.	Incubation supernatant	Cell-free	0.25
4.	Puromycin-released material	Cell-free	2.9

et al., 1960). When ribosomes labeled in the same way were treated with puromycin, a similar *N*-terminal C^{14}-valine value was found in the "released" material (Table V). This is evidence for the identity of the puromycin-released material and that "chased" into hemoglobin in the earlier experiments. As noted earlier, the soluble hemoglobin labeled in the cell-free system in a 10-minute incubation contained little *N*-terminal C^{14}-valine (Table V, line 3). However, the material released by puromycin from ribosomes labeled under these conditions contained 2.9% *N*-terminal C^{14}-valine (Table V, line 4). If this "released" material had the same average chain length as the whole-cell labeled ribosomes, this indicates that about 25% of the growing chains in *ribosomes* labeled in the cell-free system are *new* chains started from their *N*-terminus in the cell-free system.

Formation of Acid-Soluble, Labeled Material.—As noted previously (Morris *et al.*, 1962), when labeled ribosomes were incubated in the complete system a significant fraction of the radioactive material released into the supernatant was not precipitable by TCA (acid-soluble). The formation of acid-soluble material did not require nucleoside triphosphates or the energy-generating system, but did require the presence of the AS_{70} enzyme fraction used for incorporation (Table III). The acid-soluble material was formed only from the material released from *ribosomes* by puromycin, since when labeled ribosomes were incubated for 20 minutes (Table III, line 3) in the complete system with C^{12}-amino acids *before* puromycin addition, acid-soluble, labeled material was not formed. In this case, most of the radioactivity was in hemoglobin prior to puromycin addition.

The study of this material was simplified by the finding that the TCA-insoluble material released by puromycin in the *absence* of added enzyme could be separated from the ribosomes and then used to study formation of acid-soluble material. The formation of acid-soluble components from this supernatant material required the addition of enzyme and was largely completed after 20 minutes of incubation (Table VI). Usually, about 25–30% of the total material was made acid-soluble and further incubation or addition of fresh enzyme did not increase this amount. The reaction was not inhibited by diisopropylfluorophosphate. Treatment of supernatant material with ribonuclease or carboxypeptidase instead of the AS_{70} enzyme fraction did not produce acid-soluble material. When globin or soluble protein labeled in a cell-free incubation (without puromycin) were incubated in the same way, either with or without puromycin, no acid-soluble material was formed. The material which becomes acid soluble after incubation is *not* like amino acyl-RNA, since this would be hydrolyzed by the washing procedure used to determine the radioactivity in the original acid-insoluble material. When C^{14}-phenylalanine, C^{14}-leucine, C^{14}-valine, C^{14}-histidine, C^{14}-threonine, or a C^{14}-algal protein hydrolysate was used to label the ribosomes, essentially the same percentage of material was made acid-soluble after incubation.

TABLE VI

INCUBATION OF SUPERNATANTS FROM PUROMYCIN-TREATED RIBOSOMES

Ribosomes were labeled in the complete, cell-free system for 10 minutes with 1-C^{14}-leucine and reisolated by centrifugation. The ribosomes were dialyzed as described. These ribosomes were incubated with puromycin at 4° for 1 hour (see Table IV for conditions). The ribosomes were removed by centrifugation and the supernatant material was incubated with 10–12 mg of crude AS_{70} fraction in a final volume of 1.4 ml at 37°. The data are given as % of the original radioactivity.

Puromycin Supernatant Incubated with	Incubation Time (min)	Acid-Soluble Material (%)
No enzymes	40	6
Enzymes	5	17
	10	19
	40	27
Above plus fresh enzymes	80	26
Boiled soluble enzymes	40	6
Enzymes preincubated with DIFP	40	25

An extensive series of experiments was done in an attempt to characterize the acid-soluble materials. These included fractionation on Dowex-50 columns and on paper, and studies of the free amino acid content using chloramine-T decarboxylation (see Methods). When AS_{70} enzymes were used a mixture of free amino acids and peptidelike material was found in the acid-soluble material. The evidence for the presence of peptides was based on separation from free amino acid on Dowex-50 columns and rates of hydrolysis in acid and base. However, since the amount of free amino acid increased with incubation time, it appeared likely that the free amino acid was formed from the peptide material, possibly by proteolytic action (Table VII). This was confirmed when a purified enzyme fraction

TABLE VII

DECARBOXYLATION OF ACID-SOLUBLE MATERIAL FORMED BY VARIOUS ENZYME FRACTIONS

Ribosomes labeled with 1-C^{14}-leucine were incubated with puromycin and the supernatant was prepared (see Table VI). This supernatant contained 14,930 cpm per ml, all TCA insoluble. One ml of supernatant was incubated with AS_{70} enzyme (10–12 mg) or S_2-2 enzyme (2.5 mg; see Bishop and Schweet, 1961). The acid-soluble material formed was isolated and decarboxylated with chloramine-T (see Methods).

Incubation Conditions	Acid-Soluble Materials (cpm)	CO_2 Formed (cpm)	Free Amino Acid (%)
AS_{70} enzymes, 5 minutes	1488	507	34.2
AS_{70} enzymes, 40 minutes	3125	1970	64.2
S_2-2 enzyme, 40 minutes	4800	576	12.0

was used to form the acid-soluble material. In this case, very little free amino acid was present, as shown by the failure to decarboxylate with chloramine-T. In other studies, it was found that when ribosomes were labeled for periods of time from 1 to 30 minutes, the percentage of acid-soluble material formed by incubation with puromycin and enzymes decreased with increased time of labeling. This is consistent with the acid-soluble materials being small peptides. As the peptide chains increase in length during the incubation with C^{14}-amino acid, the percentage of small labeled peptides would be expected to decrease. This interpretation implies that puromycin releases small peptides in some bound form, since they are acid-insoluble when enzymes are omitted. The AS_{70} enzyme is postulated to release the peptide fraction from its binding, resulting in the formation of acid-soluble material.

Discussion

These results confirm and extend earlier reports (Allen and Zamecnik, 1962; Morris and Schweet, 1961; Morris *et al.*, 1962) that puromycin acts directly on the ribosome and does not compete with *free* amino acyl-RNA (Allen and Schweet, 1962). The result of puromycin action is the release of incomplete globin chains from the ribosome. This point seems well established, based on "fingerprinting," solubility, chromatographic behavior, and *N*-terminal valine analysis of this material. Similar results were reported by Allen and Zamecnik (1962) using ribosomes labeled in the intact cell, while ribosomes labeled in the cell-free system were used in most of these studies. In addition, evidence presented here indicates that this release of incomplete chains from the ribosome does not involve ribosome breakdown. This is also shown by the reversal of puromycin inhibition by washing. An important aspect of hemoglobin synthesis in the cell-free system was the presence of some *N*-terminal C^{14}-valine in the incomplete chains in the ribosome. This was shown by releasing these with puromycin. This supports earlier evidence (Bishop *et al.*, 1960) that new chains can be started in the cell-free system, although only a small percentage of chains started in the cell-free system are completed.

The levels of puromycin which result in release of labeled incomplete chains are similar to those which yield inhibition (also see Allen and Zamecnik, 1962) and, as shown in Table I, conditions which favor release also yield increased inhibition. However, the question of why the release of incomplete chains should result in inhibition of further synthesis has not been answered. The reversal of puromycin inhibition by washing shown here, plus the evidence that some puromycin is bound to the released chains (Allen and Zamecnik, 1962), indicates that binding of puromycin to ribosomes is not the cause of the inhibition.

An attractive hypothesis which has been described briefly (Schweet and Bishop, 1962) is that puromycin acts at the point of growth of the peptide chain, that is, the carboxyl terminus, and releases the chain from the ribosome by substituting for the next incoming amino acid. This could be accomplished by the incorporation of puromycin into the chain itself, as indicated by Allen and Zamecnik (1962), or by the attachment of puromycin at an enzyme site to which the carboxyl terminus of the growing chain attaches prior to addition of the next amino acid. It should be noted in this connection that puromycin does not have any effect on *free* amino acyl-RNA, or on amino acyl-RNA bound to ribosomes (Arlinghaus *et al.*, 1963). Therefore, in the presence of puromycin formation of peptide bonds continues, but the small peptides, or possibly amino acid–puromycin compounds, formed are continually released. Since these small compounds are not TCA-precipitable they would not be measured as protein synthesis in the usual assay. The existence of such compounds as a result of puromycin inhibition has been noted (Nathans *et al.*, 1962; Arlinghaus *et al.*, 1963).

However, at the start of an incubation with regular ribosomes, any amount of peptide-bond formation, however small, will show up as TCA-precipitable protein because of the presence of unlabeled, long chains to which a few C^{14}-amino acids can add before the chain is released by puromycin. Once these long chains are released by the inhibitor, incorporation continues, but only TCA-soluble peptides are produced. This would be measured, as indicated above, as complete inhibition of amino acid incorporation, and would account for the time curves of Figure 1 and the effect of preincubation on inhibition. This hypothesis can be tested in the case of poly U–directed polyphenylalanine synthesis, where ribosomes with partially completed chains, or without chains, can be studied. Such studies are in progress. One might expect on this basis that an enzyme would be required for puromycin action. So far no enzymatic stimulation of release in the presence of puromycin has been found (Allen and Zamecnik, 1962; Morris *et al.*, 1962), but recent studies show that enzymes in small amounts are bound to ribosomes washed by the techniques used here (Arlinghaus *et al.*, 1963).

The enzymatic reaction involved in the formation of *TCA-soluble* material from the incomplete chains released by puromycin is not understood. This reaction seems quite specific, and the data seem to exclude the action of the common proteolytic enzymes. The data are consistent with the suggestion that these are small peptides originally present in the ribosome and released by puromycin bound to TCA-precipitable material until after incubation with the enzyme fraction. It is possible, however, that the small peptides are artifacts produced by proteolytic action of an unknown kind. The release of small peptides from ribosomes has been reported by Lamborg (1962).

Acknowledgments

The authors wish to thank Mrs. Frances Chrostowski and Mrs. Janice Miller for excellent technical assistance.

References

Allen, D., and Zamecnik, P. (1962), *Biochim. Biophys. Acta 55*, 865.

Allen, E., and Schweet, R. (1962), *J. Biol. Chem. 237*, 760.

Arlinghaus, R., Favelukes, G., and Schweet, R. (1963), *Biochem. Biophys. Res. Commun. 11*, 92.

Bishop, J., Favelukes, G., Schweet, R., and Russell, E. (1961), *Nature 191*, 1365.

Bishop, J., Leahy, J., and Schweet, R. (1960), *Proc. Nat. Acad. Sci. U.S. 46*, 1030.

Bishop, J., and Schweet R. (1961), *Biochim. Biophys. Acta 54*, 617.

Borsook, H., Fischer, E., and Keighley, G. (1957), *J. Biol. Chem. 229*, 1059.

Hultin, T. (1961), *Biochim. Biophys. Acta 51*, 219.

Katz, A., Dreyer, W., and Anfinsen, C. (1959), *J. Biol. Chem. 234*, 2987.

Lamborg, M. (1962), *Biochim. Biophys. Acta 55*, 719.

Lowry, O., Rosebrough, N., Farr, A., and Randall, R. (1951), *J. Biol. Chem. 193*, 265.

Morris, A., Favelukes, S., Arlinghaus, R., and Schweet, R. (1962), *Biochem. Biophys. Res. Commun. 7*, 326.

Morris, A., and Schweet, R. (1961), *Biochim. Biophys. Acta 47*, 415.
Mueller, G., Gorski, J., and Aizawa, Y. (1961), *Proc. Nat. Acad. Sci. U.S. 47*, 164.
Nathans, D., von Ehrenstein, G., Monro, R., and Lipmann, F. (1962), *Fed. Proc. 21*, 127.
Nathans, D., and Lipmann, F. (1961), *Proc. Nat. Acad. Sci. U.S. 47*, 497.
Nemeth, A., and de la Haba, G., (1962), *J. Biol. Chem. 237*, 1190.
Rabinovitz, M., and Fisher, J. (1962), *J. Biol. Chem. 237*, 477.
Schweet, R., and Bishop, J. (1962), *in* Molecular Genetics, Taylor, J. H., ed., New York, Academic, p. 353.
Yarmolinsky, M., and de la Haba, G. (1959), *Proc. Nat. Acad. Sci. U.S. 45*, 1721.

The Mechanism of Hydrolysis of Amino Acyl RNA*

RICHARD WOLFENDEN

From The Rockefeller Institute, New York

Received April 9, 1963

The hydrolyses of leucyl RNA and leucine ethyl ester are proportional to hydroxide ion concentration below *p*H 8 and above *p*H 10, with a plateau between these values. The *p*H rate profiles for hydrolysis of the two compounds are strictly parallel between *p*H 6 and *p*H 11, leucyl-RNA reacting thirty times as rapidly as leucine ethyl ester. The rate behavior indicates a double mechanism of saponification, involving hydroxide attack on both the amino acid ester and its conjugate acid.

The chemical properties of amino acid esters have attracted increasing interest since the discovery that such esters are intermediates in protein synthesis (Hoagland *et al.*, 1957; Zachau *et al.*, 1958). The high free energy of hydrolysis of amino acid esters of soluble RNA at physiological *p*H (Berg *et al.*, 1961; Lipmann *et al.*, 1959; Allen *et al.*, 1960; Leahy *et al.*, 1960) has been shown to be a general property of amino acid esters and arises from destabilization, by the positively charged α-ammonium group, of the ester relative to the acid released by hydrolysis (Jencks *et al.*, 1960).

In experiments requiring the removal of amino acids from sRNA by treatment with alkali it was observed that in the region between *p*H 8 and 10, which is most commonly employed, the rate of hydrolysis increases with *p*H but not in proportion to the concentration of alkali.

A detailed study of the *p*H dependence of the rate of hydrolysis of leucyl-RNA and of leucine ethyl ester was undertaken in order to obtain information about the mechanism of amino acid ester hydrolysis.

Experimental Procedure

L-Leucine ethyl ester hydrochloride was prepared by the method of Greenstein and Winitz (1961) and gave mp 134° (uncorrected). Soluble RNA was prepared from *E. coli* strain B by phenol extraction and isopropanol fractionation, and was enzymatically esterified with L-leucine-1-C^{14} (20 mc/mmole; New England Nuclear Corp.) according to published procedures (Zubay, 1962). The product contained one mole of amino acid per 700 moles of nucleotide, based on a measured optical density of 2.00 for a 0.01% solution of sRNA (measured at 260 mμ, *p*H 7.0) and an average molecular weight of 240 per nucleotide.

Buffer solutions for hydrolysis contained potassium carbonate, tris(hydroxymethyl)aminomethane hydrochloride, potassium phosphate or potassium acetate buffers, 0.10 or 0.05 M, together with sufficient potassium chloride to make the ionic strength 0.30.

The hydrolysis mixture for leucyl-RNA contained 5 μg of RNA per ml of buffer solution. Aliquots of 0.25 ml removed at various time intervals were cooled and mixed, first with 0.12 ml of a solution containing 1% yeast sodium nucleate (Schwarz BioResearch, Inc.) and 4% potassium acetate, and second with 0.9 ml, 0.5 M HCl in ethanol. After removal of the RNA by centrifugation in the cold, aliquots of the clear supernatant were removed for radioactivity determination of the amino acid released.

In the case of leucine ethyl ester the reaction mixture contained 2.5 μmole of ester hydrochloride per ml of buffer solution. Aliquots of 1 ml were removed at timed intervals and the disappearance of hydroxylamine-reacting material was determined by the method applied to glycine ethyl ester by Jencks *et al.* (1960); the time allowed for reaction with hydroxylamine before addition of ferric chloride was extended to 20 minutes for maximum color development.

The *p*H determinations were carried out before and after reaction with a Radiometer Model 25 *p*H meter and showed that negligible change in *p*H occurred during the course of the reactions.

All rate measurements were made in duplicate and reactions were followed for at least two half-times with at least four measurements and usually at least ten. Typical observations on leucyl-RNA are recorded in Figure 1. Good first-order kinetics were obtained in all cases, and apparent first-order rate constants were calculated from the equation $k_{obs} = 0.693/t_{1/2}$. A small correction for buffer catalysis was made by extrapolation to zero buffer concentration using the values obtained in 0.10 and 0.05 M buffer, yielding corrected observed rate constants at zero buffer concentration and ionic strength 0.30. The required correction for buffer catalysis never exceeded 10% of the observed rate and was usually much less.

Results

In Figure 2 the observed rates of hydrolysis of leucyl-RNA and of leucine ethyl ester at 37° and ionic strength 0.30, extrapolated to zero buffer concentration, are plotted as a function of *p*H. This type of rate profile is consistent with a mechanism involving attack by hydroxide ion on the conjugate acid of the ester at neutral *p*H, superseded in importance at high *p*H by hydroxide attack on the free ester. At the plateau, the reaction between species of opposite charge ap-

* This work was done during the tenure of a graduate fellowship of the Rockefeller Institute, New York, and was supported in part by a grant from the National Science Foundation.