# The Biological Activity of Tenuazonic Acid*

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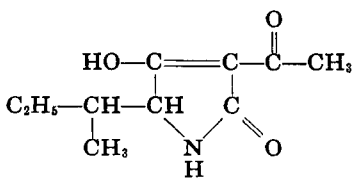
The possible nature of the biological activity of tenuazonic acid, a compound isolated from the culture filtrate of *Alternaria*, was investigated *in vivo* with intact rats and *in vitro* with Ehrlich ascites tumor and rat liver cells. The results obtained demonstrated that the new antibiotic inhibited the incorporation *in vivo* and *in vitro* of amino acids into proteins. Studies with cell-free systems have indicated that the inhibition by tenuazonic acid of protein synthesis was due essentially to the suppression of release of newly formed microsomal proteins into the supernatant fraction.

Kaczka *et al.* (1963) isolated from the filtrate of *Alternaria* a material that exhibited growth-inhibitory action against human tumors growing in the embryonated egg. The compound was found to be identical with tenuazonic acid that had previously been isolated by Rosett *et al.* (1957) from the culture filtrate of *Alternaria tenius* Auct and shown by Stickings (1959) to have the structure indicted in formula I.



Formula I

The effects of tenuazonic acid on the incorporation of amino acids into microsomal proteins and subsequent release of newly formed proteins into the supernatant fluid are described in this communication.

## Experimental Procedure

*Materials.*—The following chemicals were used: glycine-2C$^{14}$, formate C$^{14}$, L-leucine-UC$^{14}$, L-phenylalanine-UC$^{14}$, L-lysine-UC$^{14}$ (Nuclear Chicago Corporation), L-valine-UC$^{14}$ (New England Nuclear Corporation); ATP disodium salt (Sigma Chemical Company); GTP sodium salt (Pabst Laboratories); 3-phosphoglycerate barium salt (C. F. Boehringer and Sons); phosphoenolpyruvate trisodium salt, and phosphoenolpyruvate kinase (California Corporation for Biochemical Research).

*Experiments in vivo.*—Two male Sprague-Dawley rats weighing 100–110 g were fasted for about 18 hours. Specified amounts of tenuazonate dissolved in 0.5 ml 0.9% NaCl and 0.5 ml labeled amino acids were injected intraperitoneally into a rat. The control animal received the same amount of labeled amino acid in 0.9% NaCl. After 60 minutes, the animals were killed and liver, spleen, thymus, and intestinal mucosa were removed, homogenized in cold 0.9% NaCl, and precipitated in 5% TCA.[1] Proteins were purified as described below.

*Ehrlich Ascites Tumor Cells.*—Tumor-bearing Swiss albino mice were used 7 days after transplantation. The cells were collected and washed essentially free of erythrocytes as described previously for method A (Shigeura and Gordon, 1962), and incubation experiments were performed with cells suspended in modified Robinson's medium (Henderson and LePage, 1959) and containing 6–9 $\times 10^7$ cells per ml.

*Preparation of Ascites Tumor Cell Fractions.*—For this purpose, Ehrlich ascites cells were collected and washed in ice-cold medium containing 0.14 M NaCl, 0.02 M glucose, and 0.04 M Tris buffer (*p*H 8.5) according to the method of Hecht *et al.* (1958). The washed cells were then suspended in about 10 volumes of distilled $H_2O$ and disrupted in a glass homogenizer equipped with a Teflon pestle. Appropriate amounts of KCl, $MgCl_2$, Tris buffer (*p*H 8.5) and sucrose were added to give a homogenate in medium A (Keller and Zamecnik, 1956). After centrifugation at 15,000 $\times$ *g* for 10 minutes, a portion of the supernatant fluid containing microsomes and cell sap fractions were used for certain experiments. The microsome pellet obtained upon centrifugation of the remaining 15,000 $\times$ *g* supernatant fluid at 105,000 $\times$ *g* for 90 minutes was rinsed and suspended in medium A. The *p*H 5 precipitate was prepared from the 105,000 $\times$ *g* supernatant fluid as described by Keller and Zamecnik (1956) and suspended in medium A. Precipitates of *p*H 5, labeled with radioactive amino acids, were prepared as described by Hoagland *et al.* (1958). Ribonucleoprotein particles and deoxycholate-soluble fractions were prepared according to the method of Kirsch *et al.* (1962).

*Preparation of Rat Liver Cell Fractions.*—Livers obtained from male Sprague-Dawley rats weighing about 160 g were homogenized in a loosely fitting Teflon homogenizer with 2.5 volumes of medium A. The 15,000 $\times$ *g* and 105,000 $\times$ *g* supernatant fluids, microsomes, *p*H 5 precipitates, and RNP particles were prepared in the same manner as described above for ascites tumor cells.

*Incubation.*—Incubation experiments with cell suspensions or cell-free preparations were performed in 10-ml Erlenmeyer flasks placed in a Dubnoff metabolic incubator. The vessels were shaken constantly in air at 37° for a specified time and reactions were terminated by the addition of trichloroacetic acid or perchloric acid to a final concentration of 5%.

In experiments where microsomes were separated before acid precipitation, the reaction mixtures at the end of the incubation period were mixed with cold medium A and centrifuged at 105,000 $\times$ *g* for 90 minutes. The microsome pellets thus obtained were rinsed with medium A and either suspended in 5% TCA or, when necessary, used to prepare ribonucleoprotein particles and deoxycholate-soluble fraction. The various fractions were then precipitated in 5% TCA.

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[1] Abbreviations used in this paper: TCA, trichloroacetic acid; PEP, phosphoenolpyruvate; PGA, 3-phosphoglyceric acid.

TABLE I

EFFECT OF TENUAZONIC ACID ON THE INCORPORATION *in vivo* OF AMINO ACIDS INTO PROTEINS

| Organ | Experiment 1[a] cpm/mg Protein Control | Experiment 1[a] cpm/mg Protein Test | Experiment 1[a] Per Cent Inhibition | Experiment 2[b] cpm/mg Protein Control | Experiment 2[b] cpm/mg Protein Test | Experiment 2[b] Per Cent Inhibition |
|---|---|---|---|---|---|---|
| Liver | 120 | 9 | 93 | 183 | 61 | 67 |
| Spleen | 91 | 10 | 89 | 438 | 212 | 52 |
| Thymus | 39 | 4 | 90 | 317 | 110 | 64 |
| Intestinal mucosa | 173 | 13 | 92 | 577 | 203 | 65 |

[a] The animals in experiment 1 received 0.5 ml glycine-UC$^{14}$ (10 $\mu$c/0.2 $\mu$mole). The test animal received 50 mg tenuazonate (500 mg/kg body weight). [b] The animals in experiment 2 received 0.5 ml L-lysine-UC$^{14}$ (12.5 $\mu$c/0.05 $\mu$mole). The test animal received 11 mg tenuazonate (100 mg/kg body weight).

TABLE II

EFFECT OF TENUAZONIC ACID ON THE INCORPORATION OF LABELED PRECURSORS INTO CELLULAR PROTEINS OF INTACT EHRLICH ASCITES TUMOR CELLS

Ehrlich ascites cells were suspended in modified Robinson's medium (Henderson and LePage, 1959) containing 0.1% glucose, 0.02 M $KHCO_3$, indicated amounts of tenuazonic acid, and radioactive precursors in a total volume of 2.5 ml. The mixtures were incubated at 37° for 45 minutes.

| Radioactive Precursor | Total Activity Added ($\mu$c) | Concentration of Tenuazonic Acid | cpm/mg Protein Control | cpm/mg Protein Test | Per Cent Inhibition |
|---|---|---|---|---|---|
| Glycine-2C$^{14}$ | 10.00 | $4 \times 10^{-3}$ M | 1805 | 357 | 80 |
| Formate-C$^{14}$ | 10.00 | 4 | 1975 | 375 | 81 |
| L-Leucine-UC$^{14}$ | 0.50 | 4 | 2310 | 592 | 75 |
| L-Leucine-UC$^{14}$ | 0.25 | 2 | 307 | 110 | 64 |
| L-Phenylalanine-UC$^{14}$ | 0.50 | 4 | 2130 | 520 | 75 |
| L-Lysine-UC$^{14}$ | 0.50 | 4 | 1475 | 330 | 78 |
| L-Valine-UC$^{14}$ | 0.25 | 2 | 85 | 43 | 49 |

For experiments in which the effect of tenuazonic acid on the release of prelabeled proteins from the microsomes was studied, the 15,000 $\times$ *g* supernatant fluid was incubated at 37° for 10 minutes with labeled amino acid and 3-phosphoglycerate. The labeled microsomes, separated by centrifugation at 105,000 $\times$ *g* for 1 hour, were rinsed with cold medium A, suspended by gentle homogenization in unlabeled 105,000 $\times$ *g* supernatant fluid containing 3-phosphoglycerate, and incubated at 37° with or without tenuazonic acid. Aliquots were removed at specified intervals and microsomes and supernatant fluids were separated by centrifugation, purified, and examined for radioactivity as described below.

*Purification of RNA and Protein, and Determination of Radioactivity.*—Soluble RNA was extracted from the *p*H 5 precipitate with hot 10% NaCl according to the method of Hoagland *et al.* (1958) and TCA-insoluble proteins were purified as described previously (Shigeura and Gordon, 1960). Radioactive materials were plated on 5-cm$^2$ stainless steel planchets and dried, and radioactivity was counted in a windowless gas-flow counter.

## RESULTS

*Effect of Tenuazonic Acid on the Uptake in vivo of Amino Acids into Proteins.*—When tenuazonic acid and a radioactive amino acid were administered simultaneously to rats, the per cent inhibition of incorporation after 60 minutes of either glycine or L-lysine into proteins of various tissues was approximately equal (Table I).

*Effect of Tenuazonic Acid on the Incorporation of Labeled Precursors into Proteins in Whole Ascites Cells.*—As shown in Table II, the incorporation of a variety of labeled compounds into total proteins of intact Ehrlich ascites cells was also markedly suppressed by the antibiotic. In the presence of $4 \times 10^{-3}$ M tenuazonic acid, the per cent inhibition of incorporation of the compounds tested was approximately the same. These results suggested that the antibiotic acted as an inhibitor of a reaction in the protein synthetic process common to all the precursors rather than as an antagonist of a single amino acid.

*Inhibition of Amino Acid Incorporation into Microsomal Proteins in Cell-free Systems.*—Studies on the kinetics of radioactive amino acid incorporation into microsomal proteins in cell-free system of Ehrlich ascites cells showed that maximal incorporation occurred at 10 minutes. The specific activities of microsomal protein after 3, 5, 10, 20, and 30 minutes' incubation were 110, 165, 205, 170, and 150 cpm, respectively. Similar measurements with cell-free system of rat liver have also indicated that maximal incorporation was reached after approximately 10 minutes of incubation (Zamecnik and Keller, 1954).

The effects of tenuazonic acid on the maximal incorporation of labeled amino acids into microsomal proteins of both types of cells were investigated. When the 15,000 $\times$ *g* supernatant fluids containing both microsomes and cell sap were incubated with ATP-generating system and varying concentrations of the antibiotic for 10 minutes, it was observed that the uptake of amino acids into microsomal proteins of both tissues was inhibited (Table III). In the presence of $0.78 \times 10^{-3}$ M tenuazonic acid, the incorporation of both leucine and valine was inhibited by more than 58%. Negligible incorporation was obtained in the absence of a source of energy.

*Lack of Effect of Tenuazonic Acid on the Incorporation of Amino Acids into Soluble RNA.*—Since tenuazonic acid was found to inhibit the over-all proteosynthetic process in ascites and rat liver cells, a study was undertaken to determine the specific locus of action of the antibiotic. It is now well established that enyzmes present in the high speed supernatant fluid and sedimentable at *p*H 5 catalyze the first and second steps of

TABLE III

INHIBITION BY TENUAZONIC ACID OF AMINO ACID INCORPORATION INTO MICROSOMAL PROTEINS IN CELL-FREE SYSTEMS

| No. | Tissue | Labeled Amino Acid | Concentration of Tenuazonic Acid | cpm/mg Protein | Per Cent Inhibition |
|---|---|---|---|---|---|
| 1[a] | Ehrlich ascites | L-valine-UC$^{14}$ | 0 × 10$^{-3}$ M | 166 | — |
| | | | 0.78 | 57 | 66 |
| | | | 1.95 | 44 | 74 |
| | | | 3.90 | 40 | 76 |
| | | | 0 | 5[d] | — |
| 2[b] | Ehrlich ascites | L-leucine-UC$^{14}$ | 0 × 10$^{-3}$ M | 384 | — |
| | | | 0.78 | 163 | 58 |
| | | | 1.95 | 141 | 63 |
| | | | 3.90 | 123 | 68 |
| | | | 0 | 15[d] | — |
| 3[c] | Rat liver | L-leucine-UC$^{14}$ | 0 × 10$^{-3}$ M | 108 | — |
| | | | 0.78 | 29 | 73 |
| | | | 1.55 | 26 | 76 |
| | | | 3.11 | 24 | 78 |
| | | | 4.70 | 17 | 84 |
| | | | 0 | 3[d] | — |

[a] Reaction mixture in a total volume of 2.32 ml contained 2 ml of 15,000 × *g* supernatant fluid (20 mg protein), 23 μmoles of PGA, 3.2 μmoles of ATP, and 0.45 μc (0.08 μmole) L-valine-UC$^{14}$, incubated at 37° for 10 minutes. [b] Reaction mixture in a total volume of 2.32 ml contained 2 ml of 15,000 × *g* supernatant fluid (20 mg protein), 23 μmoles of PGA, 3.2 μmoles of ATP, and 0.45 μc (0.08 μmole) L-leucine-UC$^{14}$, incubated at 37° for 10 minutes. [c] Reaction mixture in a total volume of 2.92 ml contained 2.5 ml of 15,000 × *g* supernatant fluid (64 mg protein), 30 μmoles of PGA, 4 μmoles of ATP, and 0.45 μc (0.08 μmole) L-leucine-UC$^{14}$, incubated at 37° for 30 minutes. In all the experiments, microsomes were separated after incubation by centrifugation at 105,000 × *g* for 1 hour. [d] Energy source omitted.

TABLE IV

LACK OF EFFECT OF TENUAZONIC ACID ON THE INCORPORATION OF AMINO ACID INTO SOLUBLE RNA

| No. | Tissue | Labeled Amino Acid | Concentration of Tenuazonic Acid | cpm/mg RNA |
|---|---|---|---|---|
| 1[a] | Ehrlich ascites | L-valine-UC$^{14}$ | 0 × 10$^{-3}$ M | 390 |
| | | | 0.72 | 373 |
| | | | 1.43 | 401 |
| | | | 2.87 | 414 |
| | | | 0 | 30[d] |
| 2[b] | Ehrlich ascites | L-leucine-UC$^{14}$ | 0 × 10$^{-3}$ M | 199 |
| | | | 0.08 | 196 |
| | | | 0.19 | 196 |
| | | | 0.38 | 200 |
| | | | 1.52 | 209 |
| | | | 0 | 0[d] |
| 3[c] | Rat liver | L-leucine-UC$^{14}$ | 0 × 10$^{-3}$ M | 1105 |
| | | | 0.65 | 1180 |
| | | | 1.46 | 1250 |
| | | | 1.75 | 1215 |
| | | | 3.50 | 1340 |
| | | | 0 | 0[d] |

[a] Reaction mixture in a final volume of 2.54 ml contained 2.20 ml *p*H 5 precipitate (5.0 mg protein), 24 μmoles of ATP, 0.5 μc (0.086 μmole) of L-valine-UC$^{14}$, and indicated amounts of tenuazonic acid, incubated at 37° for 15 minutes. [b] Reaction mixture in a final volume of 2.40 ml contained 2.00 ml *p*H 5 fraction (5.8 mg protein), 20 μmoles of ATP, 0.5 μc (0.09 μmole) of L-leucine-UC$^{14}$, and indicated amounts of tenuazonic acid, incubated at 37° for 15 minutes. [c] Reaction mixture in a final volume of 2.81 ml contained 2.50 ml of *p*H 5 fraction (5.0 mg protein), 24 μmoles of ATP, 0.5 μc (0.09 μmole) L-leucine-UC$^{14}$, and indicated amounts of tenuazonic acid, incubated at 37° for 15 minutes. [d] Energy source omitted.

the reaction sequence leading to protein synthesis; namely, the activation of amino acids with ATP and the subsequent transfer of aminoacyladenylate to soluble RNA (Hoagland, 1960).

When the *p*H 5 precipitates of Ehrlich ascites or rat liver cells were incubated with labeled amino acids, a source of energy, and varying concentrations of tenuazonic acid, it was observed that the antibiotic at concentrations that were previously found to inhibit amino acid incorporation into microsomes did not suppress the formation of aminoacyl-soluble RNA (Table IV). On the contrary, addition of tenuazonic acid resulted in slight but noticeable increases in the specific activities of aminoacyl-soluble RNA. It may also be concluded from these results that the generation of high energy phosphate bonds from exogenously added phosphoglycerate and subsequent activation of amino acids with ATP to form aminoacyl-AMP were also not affected by the antibiotic. The results, therefore, implicated the site of action of tenuazonic acid to be at a stage subsequent to the formation of aminoacyl-soluble RNA.

TABLE V
EFFECT OF TENUAZONIC ACID ON AMINO ACID TRANSFER FROM $C^{14}$-LEUCINE-LABELED pH 5 PRECIPITATE TO MICROSOMES

| No. | Tissue | Labeled Amino Acid | Concentration of Tenuazonic Acid | Total Activity (cpm) | Per Cent Inhibition |
|---|---|---|---|---|---|
| 1[a] | Ehrlich ascites | L-leucine-$UC^{14}$ | $0 \times 10^{-3}$ M | 113 | — |
| | | | 0.90 | 91 | 20 |
| | | | 1.80 | 89 | 21 |
| | | | 3.60 | 76 | 33 |
| | | | 5.40 | 74 | 35 |
| | | | 0[c] | 3 | — |
| 2[b] | Rat liver | L-leucine-$UC^{14}$ | $0 \times 10^{-3}$ M | 594 | — |
| | | | 0.73 | 403 | 32 |
| | | | 1.49 | 370 | 38 |
| | | | 2.98 | 370 | 38 |
| | | | 4.30 | 328 | 45 |
| | | | 0[c] | 15 | — |

[a] Reaction mixture in a final volume of 1.26 ml contained 0.40 ml pH 5 precipitate labeled with L-leucine-$UC^{14}$ (1.8 mg protein), 0.40 ml of microsomes (3.36 mg protein), 10.5 μmoles of PEP, 1.6 μmoles of ATP, 0.6 μmoles of GTP, 35 μg of PEP kinase, and indicated amounts of tenuazonate, incubated at 37° for 10 minutes. [b] Reaction mixture in a final volume of 1.26 ml contained 0.30 ml pH 5 precipitate labeled with L-leucine-$UC^{14}$ (1.2 mg protein), 0.30 ml of microsomes (6 mg protein), 10.5 μmoles of PEP, 1.6 μmoles of ATP, 0.6 μmole of GTP, 35 μg of PEP kinase, and indicated amounts of tenuazonate, incubated at 37° for 15 minutes. [c] Energy source omitted.

TABLE VI
EFFECT OF TENUAZONIC ACID ON AMINO ACID INCORPORATION IN SYSTEMS CONTAINING $C^{14}$-LEUCINE-LABELED pH 5 PRECIPITATE AND RIBOSOMES

| No. | Tissue | Labeled Amino Acid | Concentration of Tenuazonic Acid | Total Activity (cpm) | Per Cent Inhibition |
|---|---|---|---|---|---|
| 1[a] | Ehrlich ascites | L-leucine-$UC^{14}$ | $0 \times 10^{-3}$ M | 824 | — |
| | | | 0.63 | 693 | 16 |
| | | | 1.26 | 678 | 17 |
| | | | 1.89 | 690 | 16 |
| | | | 2.50 | 682 | 17 |
| | | | 3.70 | 662 | 20 |
| 2[b] | Rat liver | L-leucine-$UC^{14}$ | $0 \times 10^{-3}$ M | 378 | — |
| | | | 0.63 | 312 | 18 |
| | | | 1.26 | 317 | 16 |
| | | | 1.89 | 305 | 19 |
| | | | 3.16 | 257 | 32 |

[a] Reaction mixture in a final volume of 1.46 ml contained 0.50 ml of pH 5 precipitate labeled with L-leucine-$UC^{14}$ (3.5 mg protein), 0.50 ml of ribosomes (1.1 mg protein), 10.5 μmoles of PEP, 1.6 μmoles of ATP, 0.6 μmole of GTP, 35 μg of PEP kinase, and indicated amounts of tenuazonate, incubated at 37° for 15 minutes. [b] Reaction mixture in a final volume of 1.44 ml contained 0.5 ml of pH 5 precipitate labeled with L-leucine-$UC^{14}$ (2.3 mg protein), 0.5 ml of ribosomes (0.9 mg protein), 10.5 μmoles of PEP, 1.6 μmoles of ATP, 0.6 μmole of GTP, 35 μg of PEP kinase, and indicated amounts of tenuazonate, incubated at 37° for 15 minutes.

*Effect of Tenuazonic Acid on Amino Acid Transfer from $C^{14}$-Leucine-labeled pH 5 Precipitate to Microsomes or Ribosomes.*—It has been shown by several investigators that amino acids bound terminally to soluble RNA are assembled on the ribonucleoprotein particles or ribosomes and subsequently polymerized to form polypeptides (Hoagland, 1960). The effect of tenuazonate on this phase of protein synthesis was examined. When the pH 5 precipitates containing labeled aminoacyl-soluble RNA were incubated with microsome, a source of energy, and varying concentrations of the antibiotic, it was observed that the transfer of labeled amino acid to microsome was partially inhibited. As shown in Table V, the per cent inhibition was reduced to approximately one-half that obtained with whole cells and crude cell-free extracts. Addition of 1000-fold excess of nonradioactive L-leucine did not alter the results.

In view of the results obtained above, further studies on the effect of tenuazonate on this reaction were made by using purified ribonucleoprotein particles or ribosomes instead of microsomes. As shown in Table VI, the marked inhibitory effect of tenuazonate on amino acid incorporation into microsomes observed earlier with crude systems appeared to decrease upon further purification of the system.

*Effect of Tenuazonic Acid on the Release in vitro of Microsomal Proteins into Soluble Fraction of Ehrlich Ascites Cells.*—Peters (1957) described the release of newly synthesized albumin from particles to the soluble state in liver slices. Subsequent studies on the mechanism by which newly formed proteins on the ribosomes of *E. coli* (Lamberg, 1962), reticulocytes (Rabinovitz and Olson, 1958; Morris and Schweet, 1961; Lamfrom, 1961; Allen and Zamecnik, 1962), and pea seedlings (Webster, 1959) are released into the soluble fraction have demonstrated the requirement of energy and enzyme(s) present in the high speed supernatant fluid. Similar results have been obtained in studies with liver ribosomes (Simkin, 1958; Hultin, 1961).

The effect of varying concentrations of tenuazonic acid on the release *in vitro* of labeled proteins of Ehrlich ascites microsomes was examined by measuring the radioactivity present in the microsome pellets and supernatant fluids after incubation at 37°. As shown

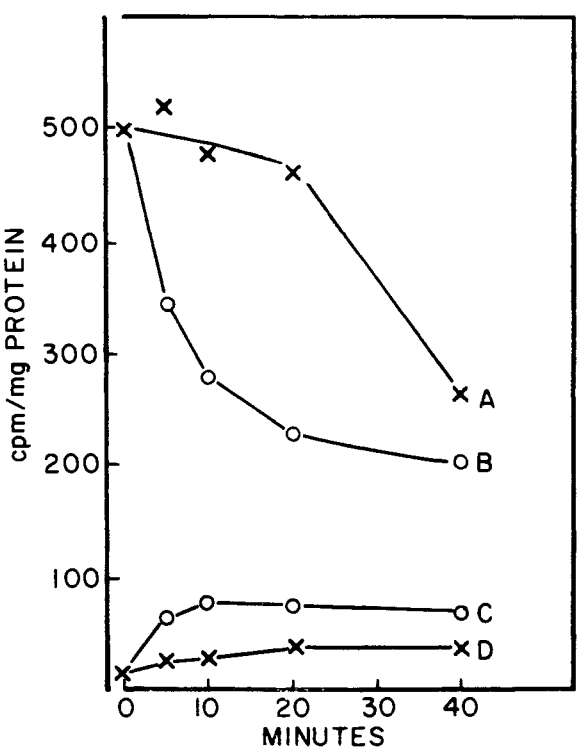
TABLE VII

EFFECT OF TENUAZONIC ACID ON THE RELEASE *in vitro* OF MICROSOMAL PROTEINS INTO THE SOLUBLE FRACTION OF EHRLICH ASCITES CELLS

L Leucine-UC$^{14}$-labeled microsomes from Ehrlich ascites cells prepared as described under Experimental Procedure were suspended in unlabeled 105,000 × *g* supernatant fluid. 1.20 ml of microsomal suspension was incubated with 12 μmoles of PGA, 1.6 μmoles of ATP, and indicated amount of tenuazonate. Tubes 1 to 8 contained unlabeled L-leucine 10,000-fold in excess of added L-leucine-UC$^{14}$. Energy was omitted from vessels 1 and 1a. After incubation at 37° for 10 minutes, the reaction mixtures were diluted with cold medium A and centrifuged at 105,000 × *g* for 1 hour. Total activities in the microsome pellets and supernatant fluids (hot TCA-insoluble proteins) were determined.

| Vessels | Energy | Concentration of Tenuazonic Acid | Microsome | | Supernatant Total cpm |
|---|---|---|---|---|---|
| | | | Total cpm | Per Cent of Original Activity[a] | |
| 1 | — | — | 107 | — | 10 |
| 2 | + | — | 61 | 57 | 56 |
| 3 | + | $0.33 \times 10^{-3}$ M | 90 | 84 | 27 |
| 4 | + | 0.66 | 92 | 86 | 25 |
| 5 | + | 1.31 | 98 | 92 | 23 |
| 6 | + | 1.96 | 97 | 91 | 24 |
| 7 | + | 2.62 | 101 | 95 | 23 |
| 8 | + | 3.93 | 105 | 98 | 19 |
| 1a | − | — | 130 | — | 11 |
| 2a | + | — | 75 | 58 | 68 |
| 3a | + | $0.05 \times 10^{-3}$ M | 89 | 69 | 60 |
| 4a | + | 0.16 | 99 | 76 | 50 |
| 5a | + | 0.32 | 107 | 82 | 52 |
| 6a | + | 0.64 | 103 | 79 | 44 |
| 7a | + | 1.28 | 114 | 88 | 38 |

[a] Values were obtained by dividing observed activity by 107 or 130 (activity of sedimented microsomes incubated in the absence of energy source) × 100. Labeled microsomes kept at 0° for 10 minutes without added energy and recentrifuged for 1 hour were found to retain about 95% of the original radioactivity.




IG. 1.—Microsome prelabeled with L-leucine-UC$^{14}$ and suspended in unlabeled 105,000 × *g* supernatant fluid as described under Experimental Procedure was incubated with 95 μmoles of PGA, 13 μmoles of ATP, and with or without 18 μmoles tenuazonic acid (total volume = 9.25 ml). Aliquots were removed at specified intervals and the specific activities of the TCA insoluble proteins of microsomes and supernatant fluids were measured. A. Microsome + tenuazonic acid; B. microsome (control); C. supernatant fluid (control); D. supernatant fluid + tenuazonic acid.

in Table VII, 43% of the radioactivity was released from the microsome in the presence of a source of energy. The release of radioactivity from the microsomes was inhibited in the presence of the antiobiotic. The radioactivity found in the supernatant fluid was found to decrease progressively with increasing concentration of tenuazonate. A large amount of nonradioactive L-leucine was added to minimize the influence of proteosynthetic reactions.

The kinetics of detachment of radioactive, hot TCA-insoluble proteins from the microsomes into the microsome-free supernatant fluid is shown in Figure 1. The specific activity of microsomal proteins of ascites cells decreased upon incubation in the presence of the supernatant fraction and ATP-generating system (line B). As expected, the release of radioactive protein from the microsome was accompanied by an increase in radioactivity of TCA-insoluble proteins present in the supernatant fraction (line C). Under identical conditions of incubation, except for the presence of 2 × $10^{-3}$ M tenuazonic acid, the specific activity of the microsomes remained essentially unchanged for about 20 minutes (line A). This apparent inhibitory activity of the antibiotic on the "releasing process" was also reflected in the significantly lower activities of the proteins in the supernatant fraction (line D) as compared to the corresponding fraction in which the antibiotic was omitted. The possible presence of radioactive substances in the supernatant fractions not precipitable with TCA was not examined.

Puromycin has been reported to stimulate the release of newly formed proteins and peptides from prelabeled ribosomes obtained from various sources (Morris and Schweet, 1961; Nathans *et al.*, 1962; Lamberg, 1962; Allen and Zamecnik, 1962). It seemed of interest, therefore, to compare the effects of the two antibiotics on the protein releasing activity in ascites cells. As shown in Figure 2, in the presence of puromycin the

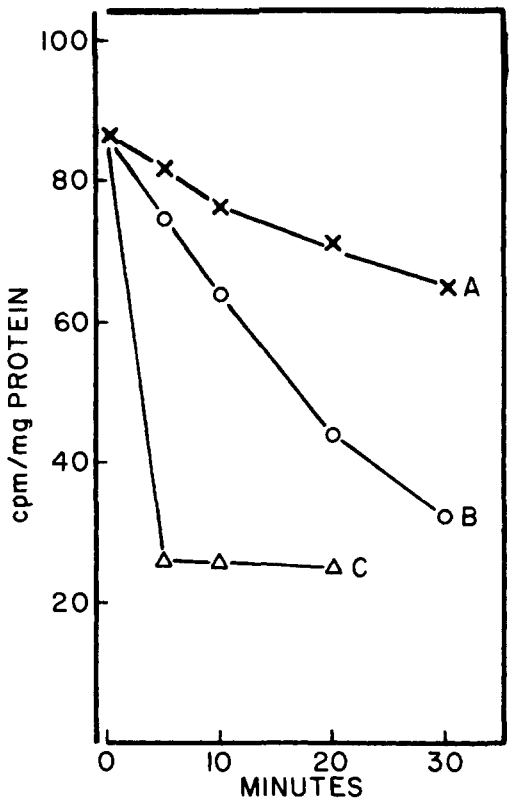



FIG. 2.—Microsome prelabeled with L-leucine-UC$^{14}$ and suspended in unlabeled 105,000 × *g* supernatant fluid (67 mg protein) was incubated with 75 μmoles of PGA, 8 μmoles of ATP, and 13 μmoles tenuazonic acid or 1.5 μmoles puromycin in a total volume of 7.6 ml. Antibiotic was omitted from control vessel. Aliquots were removed at specified intervals and microsomes were separated and tested for radioactivity. A. Plus tenuazonic acid; B. control; C. plus puromycin.

specific activity of microsomes dropped precipitously after a few minutes of incubation. Tenuazonic acid, as demonstrated earlier, inhibited the release of proteins.

## DISCUSSION

During the last few years, several antibiotics have been reported to inhibit the incorporation of amino acids into proteins. Chloramphenicol appears to be active primarily in bacterial systems (Lacks and Gros, 1959) although higher levels of the antibiotic have been found to inhibit protein synthesis in calf thymus nuclei (Hopkins, 1959). Streptomycin and dihydrostreptomycin were observed to suppress similar processes in *Mycobacterium fibrinogenesis* (Erdos and Ullman, 1959) and *Mycobacterium tuberculosis* (Stachiewicz and Quastel, 1959), respectively. Puromycin has been shown to be active in both bacterial (Nathans and Lipmann, 1961) and mammalian systems (Yarmolinsky and de la Haba, 1959).

The results reported in this communication have demonstrated that the new antibiotic, tenuazonic acid, inhibited the incorporation *in vivo* and *in vitro* of amino acids into proteins. Results with cell-free systems have further shown that the synthesis of adenylates was probably insensitive to tenuazonate and the transfer of amino acids from soluble RNA to ribosomes was only slightly inhibited. However, the fact that the inhibitory activity on the "transfer reaction" progressively decreased upon purification of the system suggested that the major site of action of tenuazonate was not directly associated with this reaction. The principle site of action of the antibiotic appears to be related to the release of newly formed proteins from the ribosomes into the cell sap or supernatant fluid. It is conceivable that ribosomes prevented from releasing newly synthesized proteins will be unable to accept amino acids from transfer RNA. Such a situation may explain the inhibition of amino acid incorporation into microsomal proteins observed with crude cell extracts, and the increase in specific activity of soluble RNA with increasing concentrations of tenuazonic acid.

It is of interest to note that puromycin has been shown to inhibit the transfer of amino acids from aminoacyl-soluble RNA to microsomes, and recent studies indicated that the drug becomes bonded to the ribosomes, resulting in the release of unfininished polypeptides (Allen and Zamecnik, 1962). Tenuazonic acid, as shown above, inhibited the release of labeled proteins from the microsomes. These results indicated that the modes of action of these two antibiotics are quite different. This unique property of tenuazonic acid may possibly be used to advantage in the study of synthesis and activation of a specific protein.

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