15. M. Szymoniski, H. Overeijunder, A. E. de Vries, *Radiat. Eff.* **36**, 189 (1978).
16. R. W. Carlson *et al.*, *Science* **182**, 53 (1973).
17. Y. L. Yung and M. B. McElroy, *Icarus* **30**, 97 (1977).
18. A. L. Broadfoot *et al.*, *Science* **204**, 979 (1979).
19. F. Neubauer, *Geophys. Res. Lett.* **5**, 905 (1978).
20. A portion of the work at the University of Virginia was supported by NSF grant AST-79-12690. A portion of the work at the University of Kansas was supported by subcontract from the Johns Hopkins University Applied Physics Laboratory, at which the LECP program is supported by NASA under task 1 of contract N00024-78-C5384 between Johns Hopkins University and the Department of Navy. We thank S. M. Krimigis of the Applied Physics Laboratory for profitable discussions.

16 September 1980; revised 19 January 1981

Interferon Action: RNA Cleavage Pattern of a (2′-5′)Oligoadenylate–Dependent Endonuclease

Abstract. *One of the mediators of interferon action is a latent endoribonuclease (ribonuclease L) that is activated by (2′-5′)oligoadenylates. Among the homopolymers of the four common ribonucleotides, activated ribonuclease L degrades at an appreciable rate only polyuridylic acid. In two natural RNA's tested the most frequent ribonuclease L cleavages occur after UA, UG, and UU (A, adenine; U, uracil; and G, guanine) and much less frequent cleavages after CA and AC (C, cytosine).*

Interferons are components of the antiviral and antitumor defenses of vertebrates. The treatment of cells with interferons is revealed in the cell extracts in several ways (*1*). Thus in an extract from interferon-treated cells (such as Ehrlich ascites tumor or HeLa cells) added messenger RNA is cleaved much faster than in a corresponding extract from control cells. The faster cleavage requires adenosine triphosphate (ATP) and is manifested only if the extract is supplemented with double-stranded RNA (*2*). The enzyme system catalyzing the cleavage consists of two components (*3*). The first component is (2′-5′)oligoadenylate synthetase, an enzyme induced by interferon, which if activated by double-stranded RNA converts ATP to (2′-5′)oligoadenylates, such as (2′-5′)pppApApA (*4*). This enzyme was purified to homogeneity and characterized (*5, 6*). The second component is ribonuclease L, a latent endoribonuclease, which can be activated by (2′-5′)oligoadenylates (*3, 7–9*). The activation is reversible. It has been shown that on removal of (2′-5′)oligoadenylates the enzyme returns to the latent state and on readdition of (2′-5′)oligoadenylates it becomes reactivated (*10*). Here we present data on the unusual RNA cleavage pattern of the enzyme.

Partial purification of ribonuclease L by differential precipitation with ammonium sulfate and chromatography on DEAE-cellulose have been reported (*10*). Further purification by chromatography on phosphocellulose and poly(A)-agarose (*11*) increased the specific activity of the enzyme preparation about 14-fold. We have tested the cleavage preference of the ribonuclease L preparation first with poly(U) (polyuridylate), poly(A) (polyadenylate), poly(C) (polycytidylate), and poly(G) (polyguanylate). Of

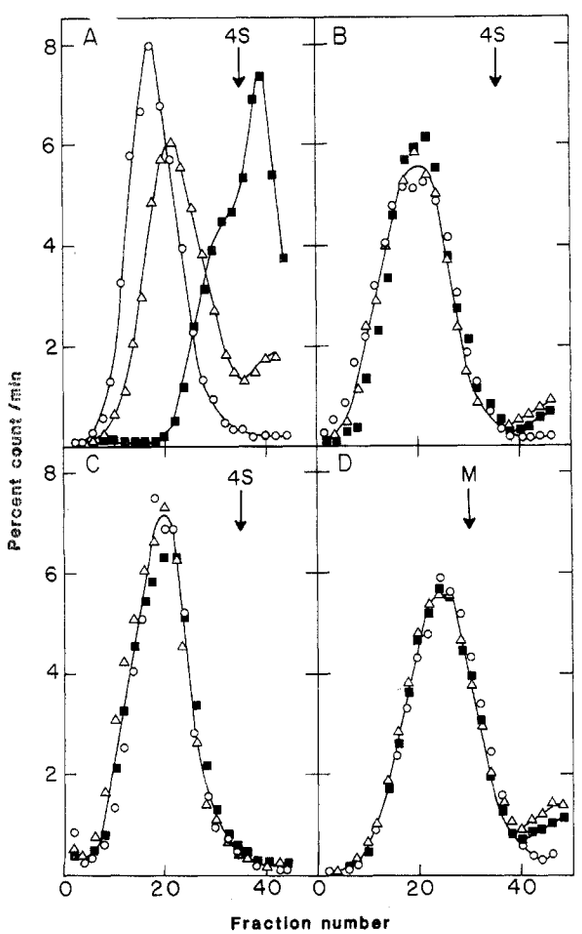

Fig. 1. RNA cleavage patterns of ribonuclease L: Experiments with homopolyribonucleotides. Ribonuclease L from Ehrlich ascites tumor cells was further purified from the DEAE-cellulose fraction of Slattery *et al.* (*10*) by chromatography on phosphocellulose and poly(A) agarose (*11*). The (2′-5′)pppApApA was prepared according to Samanta *et al.* (*6*). [5-^{3}H]Poly(U) (39 μCi per micromole of P), [8-^{3}H]poly(A) (50.9 μCi per micromole of P), and [5-^{3}H]poly(C) (41 μCi per micromole of P) were obtained from Miles Laboratories; [8-^{14}C]poly(G) (3.2 μCi per micromole of guanine) and [8-^{14}C]poly(A) (3 μCi per micromole of adenine) were obtained from P-L Biochemicals. Ribonuclease L was assayed as follows: the homopolyribonucleotide (50 μg/ml) was incubated in buffer A, consisting of 25 m*M* tris-Cl, *p*H 8.2, 75 m*M* KCl, 5 m*M* magnesium acetate, and 10 m*M* β-mercaptoethanol without or with ribonuclease L (12 μg/ml) and without or with (2′-5′)pppApApA (1 μ*M* in adenosine monophosphate equivalents) in a final volume of 30 μl at 30°C. After 10 minutes the reaction was terminated by the addition of 200 μl of buffer B [10 m*M* tris-Cl, *p*H 7.5, 100 m*M* NaCl, 1 m*M* EDTA, 0.1 percent (weight to volume) sodium dodecyl sulfate], and a 200-μl portion of the reaction mixture was applied on top of a 11.5-ml linear sucrose gradient [15 to 40 percent (weight to volume) in buffer B] and centrifuged at 177,000*g* at 15°C for 21 hours. In the analyses of experiments with [^{3}H]poly(U), [^{3}H]poly(C), and [^{14}C]poly(G), *Escherichia coli* transfer RNA (0.25 μg/ml) was used as an internal marker (sedimentation coefficient, 4*S*); in those with [^{3}H]poly(A), the internal marker was [^{14}C]poly(A) (sedimentation coefficient, 9*S*). Fractions (300 μl) were collected and counted in a scintillation counter. (A) Experiments with poly(U); (B) experiments with poly(C); (C) experiments with poly(G); (D) experiments with poly(A). ○, Incubation without ribonuclease L and (2′-5′)pppApApA; △, incubation with ribonuclease L but without (2′-5′)pppApApA; ■, incubation with both ribonuclease L and (2′-5′)pppApApA. The locations in the gradients of tRNA (4*S*) and of 9*S*-[^{14}C]poly(A) (*M*) are indicated by arrows. *Percent count/min* is the ratio of the net counts per minute in the fraction to the sum of the net counts per minutes in all fractions (the net counts per minute is the number of counts per minute in the fraction minus the background).

0036-8075/81/0529-1030$00.50/0

these homopolymers only poly(U) was appreciably degraded under our conditions (12 μg of enzyme preparation per milliliter of reaction mixture at 30°C for 10 minutes), as revealed by sucrose gradient centrifugation (Fig. 1). Although some poly(U) cleavage occurred without added (2′-5′)pppApApA, the addition of this activator resulted in a strong enhancement of the cleavage. Further studies are needed to establish whether the slow and apparently (2′-5′)pppApApA–independent poly(U) cleavage is catalyzed by either native ribonuclease L or by a form of the enzyme that has lost its absolute dependence on (2′-5′)pppApApA in consequence of partial denaturation or by an unknown contaminating nuclease degrading poly(U) but not any of the other three homopolyribonucleotides.

For examining the cleavage preference of ribonuclease L in a natural polyribonucleotide we used as substrate a bacteriophage T7–specific RNA segment (designated as R5) that was labeled at its 5′ end and whose nucleotide sequence has been established (*12, 13*) (Fig. 2). Gel electrophoresis (Fig. 2) of uncleaved (control) R5 (track A), of R5 partially cleaved by the guanine-specific ribonuclease T1 (used to verify the sequence, track B), of R5 partially cleaved by alkali (to generate the sequence ladder presented from top to bottom in the 3′ to 5′ direction, tracks C and H) (*14*), and of R5 incubated with ribonuclease L at 12 or 24 μg/ml without (tracks D and F) and with (2′-5′)pppApApA (tracks E and G) reveals that cleavage by ribonuclease L without (2′-5′)pppApApA is barely detectable, an indication that in the course of ribonuclease L purification most of the contaminating nucleases have been removed. In the presence of (2′-5′)pppApApA the major cleavages (whose sequence context could be determined) occur after UA, UA, UU, UA, UG, and UG (if read from bottom to top). Minor cleavages can be found after CA, UU, and UU, and very rare cleavages occur after AC, AC, CG, and UU. The effect, if any, on the rate of cleavage of the nucleotide at the 3′ side of the cleavage point is not obvious.

The sequence of the R5 RNA segment indicates no strongly hydrogen-bonded regions (*12, 13*). We also studied, however, the preferred ribonuclease L cleavage sites in an RNA segment with a well-defined secondary structure, that is, the 3′ terminal region of bacteriophage R17 RNA (*15*). The major RNA cleavage sites in this occurred after three UU sequences, a minor cleavage after UG, and a barely detectable one after GA. All of these cleavages occurred in single-stranded loop structures and none of them in self-complementary, hydrogen-bonded regions (*11*).

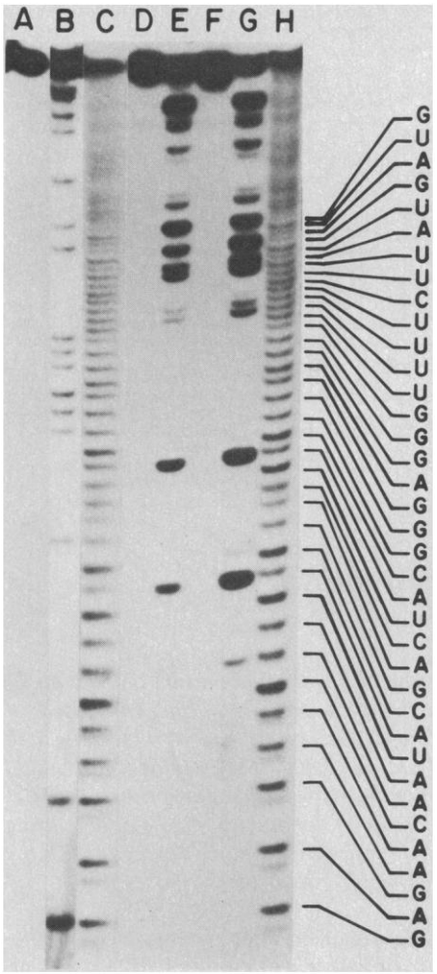

Fig. 2. RNA cleavage patterns of ribonuclease L: Experiments with a T7-specific RNA segment. A T7 DNA segment designated as Hpa II 140b was transcribed by means of T7 RNA polymerase with the use of γ-^{32}P–labeled guanosine triphosphate (specific activity, 1400 Ci/mmole; New England Nuclear) and three unlabeled nucleoside triphosphates (*12, 13*). An 85-nucleotide-long transcript obtained (R5) was purified by gel electrophoresis (*20*). The sequence of R5 had been determined (*12, 13*). The γ-^{32}P–labeled R5 RNA (4 μg, 30,000 count/min) was incubated without or with ribonuclease L (in the amounts indicated) and without or with (2′-5′)pppApApA (1 μ*M* in adenosine monophosphate equivalents) in buffer C in a final volume of 30 μl at 30°C for 30 minutes. Samples were prepared for electrophoresis on 20 percent polyacrylamide gel (*14*). Partial digestions of samples of R5 RNA (2 μg, 15,000 count/min) with ribonuclease T1 and alkali were also performed as described (*14*). The RNA samples were analyzed by electrophoresis at 1800 volts for 3 hours and radioautography (*14*). (Track A) Control (undigested) RNA; (track B) RNA treated with ribonuclease T1; (tracks C and H) treated with alkali; (track D) treated with ribonuclease L (12 μg/ml); (track E) treated with ribonuclease L (12 μg/ml) and (2′-5′)pppApApA; (track F) treated with ribonuclease L (24 μg/ml); and (track G) treated with ribonuclease L (24 μg/ml) and (2′-5′)pppApApA. The sequences are indicated from the top to bottom in the 3′ to 5′ direction.

Further experiments with poly(U) indicate that in the course of degradation the activated ribonuclease L generates products with phosphate at their 3′ termini and hydroxyl residues at their 5′ termini (*11*).

Our results establish that ribonuclease L, which is known to be distinctive among the nucleases in its dependence on an unusual oligonucleotide [(2′-5′)pppApApA] for activity (*3, 6–7*), is different from other nucleases (*16*) in its cleavage preference. The activated ribonuclease L can efficiently cleave single-stranded RNA after at least three dinucleotides (UA, UG, and UU) out of the 16 possible. This makes it most unlikely that, as one of the mediators of the antiviral action of interferons, the enzyme should cleave only viral RNA [see also Baglioni *et al.* (*7*), Clemens and Williams (*8*), and (*17*)]. It is curious that (i) the most preferred cleavage sites of activated ribonuclease L include a uridylate residue and (ii) the activators of ribonuclease L [(2′-5′)oligoadenylates] consist of nucleotides complementary to uridylates. Whether or not direct U to A pairing occurs during this enzyme's action remains to be seen (*18*).

Note added in proof: After this manuscript was submitted, I. M. Kerr and his colleagues reported that (2′-5′)oligoadenylate–dependent ribonucleases in rabbit reticulocyte lysates and in crude extracts from Krebs ascites cells and Daudi cells cleave several viral RNA's predominantly at the 3′ side of UA and UU sequences yielding 3′-phosphate terminated products (*19*).

GEORGIA FLOYD-SMITH
ELIZABETH SLATTERY*
PETER LENGYEL†

Department of Molecular Biophysics and Biochemistry, Yale University, New Haven, Connecticut 06511

References and Notes

1. W. E. Stewart II, *The Interferon System* (Springer-Verlag, New York, 1979).
2. G. E. Brown, B. Lebleu, M. Kawakita, S. Shaila, G. C. Sen, P. Lengyel. *Biochem. Biophys. Res. Commun.* **69**, 114 (1976); G. C. Sen, B. Lebleu, G. E. Brown, M. Kawakita, E. Slattery, P. Lengyel, *Nature (London)* **264**, 370 (1976); S. Shaila, B. Lebleu, G. E. Brown, G. C. Sen, P. Lengyel, *J. Gen. Virol.* **37**, 535 (1977).
3. L. Ratner, R. C. Wiegand, P. J. Farrell, G. C. Sen, B. Cabrer, P. Lengyel, *Biochem. Biophys. Res. Commun.* **81**, 947 (1978).
4. W. K. Roberts, A. G. Hovanessian, R. E. Brown, M. J. Clemens, I. M. Kerr, *Nature (London)* **264**, 477 (1976); A. G. Hovanessian, R. E. Brown, I. M. Kerr, *ibid.* **268**, 537 (1977).

5. J. B. Dougherty, H. Samanta, P. J. Farrell, P. Lengyel, *J. Biol. Chem.* **255**, 3813 (1980).
6. H. Samanta, J. B. Dougherty, P. Lengyel, *ibid.*, p. 9807.
7. C. Baglioni, M. A. Minks, P. A. Maroney, *Nature (London)* **273**, 684 (1978).
8. M. J. Clemens and B. R. G. Williams, *Cell* **13**, 565 (1978).
9. P. J. Farrell, G. C. Sen, M. F. Dubois, L. Ratner, E. Slattery, P. Lengyel, *Proc. Natl. Acad. Sci. U.S.A.* **75**, 5893 (1978); A. Schmidt, A. Zilberstein, L. Shulman, P. Federman, H. Berissi, M. Revel, *FEBS Lett.* **95**, 257 (1978); D. A. Eppstein and C. E. Samuel, *Virology* **89**, 240 (1978).
10. E. Slattery, N. Ghosh, H. Samanta, P. Lengyel, *Proc. Natl. Acad. Sci. U.S.A.* **76**, 4778 (1979).
11. G. Floyd-Smith, in preparation.
12. J. L. Oakley, R. E. Strothkamp, A. H. Sarris, J. E. Coleman, *Biochemistry* **18**, 328 (1979).
13. H. Osterman and J. E. Coleman, in preparation.
14. H. Donis-Keller, A. M. Maxam, W. Gilbert, *Nucleic Acids Res.* **4**, 2527 (1977).
15. S. Cory, J. M. Adams, P.-F. Spahr, *J. Mol. Biol.* **63**, 41 (1972).
16. D. Kowalski and M. Laskowski, in *Handbook of Biochemistry and Molecular Biology*, section B, vol. 2, *Nucleic Acids*, G. D. Fasman, Ed. (CRC Press, Cleveland, 1976), pp. 508–517.
17. L. Ratner *et al.*, *Eur. J. Biochem.* **79**, 565 (1977).
18. Some of these results were presented at the symposium on Frontiers in Molecular Biology, Nutley, N.J., September 1980. Studies on the cleavage specificity of ribonuclease L are also pursued in the laboratory of I. Kerr.
19. D. H. Wreschner, J. W. McCauley, J. J. Skehel, I. M. Kerr, *Nature (London)* **289**, 414 (1981).
20. C. L. Peebles, R. C. Ogden, G. Knapp, J. Abelson, *Cell* **18**, 27 (1979).
21. We thank H. Chao for help in preparing ribonuclease L and R17 RNA, Drs. H. Osterman and J. Coleman for the bacteriophage T7 Hpa II 140b segment and T7 RNA polymerase, D. Soll and S. Sharp for useful discussions, and T. Novicki for preparing cell extracts. This work was supported by NIH research grants AI-12320 and CA-16038 and a national research service award to G.F.S.
* Present address: Department of Biological Chemistry, Washington University School of Medicine, St. Louis, Mo. 63110.
† Address correspondence to P.L.
10 November 1980; revised 17 February 1981

Inflammatory Toxin from *Mycoplasma bovis*: Isolation and Characterization

Abstract. *An inflammatory toxin was extracted from* Mycoplasma bovis *with 75 percent aqueous ethanol. The toxin is a complex polysaccharide composed of glucose, glucosamine or galactosamine, and a heptose, is heat-stable, devoid of protein and lipid, and has a molecular weight of 73,000. The holotoxin in the cell membrane is a glycoprotein; however, it is the polysaccharide portion that is toxic. This inflammatory toxin increases vascular permeability and is capable of activating complement. Infusion of 0.9 milligram of toxin into the bovine udder resulted in the characteristic eosinophilic mastitis produced by* Mycoplasma bovis.

Most cases of bovine mycoplasmal mastitis are due to *Mycoplasma bovis* (*1*). In cattle with mastitis caused by this organism, the inflammation at the acute stage is characterized by exudation of mostly eosinophils in the alveoli. Later on an interstitial reaction with eosinophils and mononuclear cells, including plasma cells and lymphocytes, develops. The chronic stage is characterized by progressive fibroplasia around ductuli and alveoli, with hypertrophy of alveolar epithelium (*2*). Some animals may return to secretion of normal-appearing milk within a few weeks; others may not do so until the next lactation, if at all. In either situation the yield of milk is far below that prior to the disease (*3*).

Of the mycoplasmas that are known pathogens, only a few appear to produce a pathogenic toxin. Relatively little is known about these toxins.

A neuroactive exotoxin is produced in cultures of *Mycoplasma neurolyticum*, which is the causative agent of "rolling disease" in mice (*4, 5*). The toxin is a protein with a molecular weight greater than 200,000. It is thermolabile, being destroyed by heating at 45°C for 15 minutes, and is destroyed by treatment with trypsin (*6*). *Mycoplasma gallisepticum* also has a neurotoxic property; however, the organism does not produce an exotoxin; all of its neurotoxicity is associated with the living organism (*7*). *Mycoplasma fermentans* possesses a toxic factor that is associated with both viable and lysed cells (*8*). When injected intraperitoneally into mice, a lethal toxicity syndrome is induced which resembles shock and is similar to that caused by endotoxins of Gram-negative organisms (*9*). Viable cells and membrane preparations of *Mycoplasma pneumoniae* induce necrosis of organ cultures of adult hamster trachea. The lesions produced include ciliostasis, vacuolization, loss of ciliated respiratory epithelial cells, disorganization, and a loss of polarity (*10*). *Mycoplasma pulmonis* and *Mycoplasma arthritidis* are toxic for mice and rats. Intravenous injection of either organism is usually lethal within 2 to 3 days (*11*). A galactan was isolated from *Mycoplasma mycoides*, the causative agent of contagious bovine pleuropneumonia, which is composed of 90 percent D-galactose, about 4 percent lipid, less than 2 percent nitrogen, and less than 0.1 percent phosphorus (*12*). Intravenous injection of this galactan into cattle causes dramatic stress: the rate of respiration increases, the animals cough and salivate, and then they collapse. The animals will, however, return to normal within a few days (*13*). Lysed *Mycoplasma bovis* cells are also toxic. Infusion of lysed *Mycoplasma bovis* into the udders of cows resulted in severe, acute mastitis characterized by an eosinophilic response (*14*).

In this report we describe the isolation and purification of an inflammatory toxin from *Mycoplasma bovis*, and discuss its biochemical and biological characteristics.

Mycoplasma bovis was cultured in Bacto PPLO broth (Difco) supplemented with 20 percent sterile swine serum at 37°C for 48 hours. The cells were centrifuged for 20 minutes at 12,500*g*, washed twice in phosphate-buffered saline (PBS), resuspended at 1000 times the original concentration, lysed by freezing and thawing, and washed twice in PBS. Lipids were extracted from this membrane preparation with a mixture of chloroform and methanol (2:1 by volume) and then with 75 percent aqueous ethanol (by volume) at 56°C with constant stirring for 1 hour. This membrane residue was hydrolyzed with the broad spectrum proteolytic enzyme, Pronase B (1.0 mg per 4.5 mg of membranes) for 18 hours at 37°C, after which it was again extracted with 75 percent aqueous ethanol. The toxin, which was extracted into this aqueous ethanol, was run on a Bio-Gel A-5M column, with PBS as the eluant. The molecular weight of the toxin was determined to be 73,000 by comparison to a curve for standard dextrans that were also run on this column.

Protein determination by the method of Lowry *et al.* (*15*) showed that the toxin contained no detectable protein. Sugar analysis by gas-liquid chromatography of trimethylsilyl derivatives (*16*) of the toxin revealed the toxin to be composed of glucose, glucosamine or galactosamine, and a heptose. Fatty acid analysis (*17*) of the toxin by gas-liquid chromatography failed to demonstrate the presence of any fatty acids, and a test for 2-keto-3-deoxyoctonate was also negative (*18*).

Since extraction of the toxin required prior digestion of the protein, it was possible that the toxin was covalently bound to a protein embedded in the membrane. We therefore solubilized *Mycoplasma bovis* membranes with sodium dodecyl sulfate and then ran them on 7.5 percent polyacrylamide gels. Scans of these gels, stained with Coomassie blue for proteins and periodic acid–Schiff for carbohydrate, revealed 32 protein bands and one carbohydrate band which corresponded to one of the protein peaks (Fig. 1). The presence of glycoproteins in

0036-8075/81/0529-1032$00.50/0