mechanical change can be modulated by varying either the mechanical threshold or the duration of the spike. It is of interest to consider such alterations in terms of the role of the action potential in excitation-contraction coupling, the role of releasing an agent into the myoplasm that activates contraction of the actomyosin sliding-filaments; this agent—the link that couples excitation to contraction—may be the calcium ion (*11*). The work of Bianchi and Shanes (see *12*) indicates that such a release of Ca^{2+} is increased in nitrate-potentiated twitches. But our results suggest that the action potential causes an increased release of Ca^{2+} whenever the mechanical threshold is lowered or the spike is prolonged. This inference is supported by consideration that the rate of contraction of the sliding-filament mechanism of contraction is inherently dependent on the rate of hydrolysis of adenosine triphosphate (*13*), and that this in turn is, within limits, proportional to the concentration of the free Ca^{2+} (*14*). It is thus possible that the differences we observed in dP/dt indicate corresponding differences in the level of the Ca^{2+} very early after its release in excitation-contraction coupling. It is conceivable that the higher this initial level, the longer the Ca^{2+} may continue at sufficiently high concentration to activate contraction and thus prolong the active state.

ALEXANDER SANDOW
HANNA PREISER

Institute for Muscle Disease, New York 21, New York

References and Notes

1. A. Sandow, S. R. Taylor, A. Isaacson, J. J. Seguin, *Science* **143**, 577 (1964).
2. A. L. Hodgkin and P. Horowicz, *J. Physiol.* **153**, 404 (1960); J. Etzensperger, *Compt. Rend. Soc. Biol.* **157**, 1776 (1963); G. Falk, in *Biophysics of Physiological and Pharmacological Actions*, A. M. Shanes, Ed. (AAAS, Washington, D.C., 1961), p. 259.
3. J. Etzensperger, *Compt. Rend. Soc. Biol.* **151**, 587 (1957).
4. A. Sandow, *J. Cellular Comp. Physiol.* **24**, 221 (1944).
5. Both Zn^{2+} at 0.2 to 0.5 m*M* and eserine at 0.5 m*M* potentiate by prolonging the action potential (without lowering the mechanical threshold) and thereby cause a characteristic relatively late increase in dP/dt. They also produce an earlier decrease in dP/dt, which is evidently due to a reduction in rate of rise of the spike caused by these agents. In experiments at 0°C, R. Close [*J. Gen. Physiol.* **46**, 1 (1962)] reports that NO_3^- retards tension development in the twitch of the frog sartorius. In experiments at 0.5°C, we find that the effects on dP/dt of NO_3^- (generally) and of caffeine (always) are essentially the same as they are at room temperature. These various results will be discussed elsewhere in conjunction with a longer account of the effects of potentiators at both 0°C and room temperature.
6. A. Sandow and T. Seaman, *Life Sci.* **3**, 91 (1964).
7. A. V. Hill, *Proc. Roy. Soc. Ser. B* **138**, 329 (1951).
8. For review, see A. Sandow, *Arch. Phys. Med. Rehabil.* **45**, 62 (1964).
9. A. V. Hill and L. Macpherson, *Proc. Roy. Soc. Ser. B* **143**, 81 (1954).
10. A. Fleckenstein, in *The Cellular Functions of Membrane Transport*, J. F. Hoffman, Ed. (Prentice-Hall, Englewood Cliffs, N.J., 1963), p. 71.
11. L. V. Heilbrunn and F. J. Wiercinzki, *J. Cellular Comp. Physiol.* **29**, 15 (1947); A. Sandow, *Yale J. Biol. Med.* **25**, 176 (1952); G. Frank, *Nature* **182**, 1800 (1958); C. P. Bianchi and A. M. Shanes, *J. Gen. Physiol.* **42**, 803 (1959); R. J. Podolsky and C. E. Hubert, *Federation Proc.* **20**, 301b (1961); A. Weber, R. Herz, I. Reiss, *J. Gen. Physiol.* **46**, 679 (1963).
12. C. P. Bianchi and A. M. Shanes, *J. Gen. Physiol.* **42**, 803 (1959).
13. H. E. Huxley, in *The Cell*, J. Brachet and A. E. Mirsky, Eds. (Academic Press, New York, 1961), vol. 4, p. 365.
14. A. Weber and S. Winicur, *J. Biol. Chem.* **236**, 3198 (1961).
15. Supported by grants from PHS (NB 04262-03) and the Muscular Dystrophy Associations of America.

29 September 1964

Interferon-Like Viral Inhibitor in Rabbits after Intravenous Administration of Endotoxin

Abstract. *Intravenous injection into rabbits of endotoxin or killed cells of* Escherichia coli *induced, in 1 hour, a viral inhibitor detectable in serum. The inhibitor disappeared from the serum in 7 to 24 hours, and was only active after incubation with rabbit cell cultures. Like interferon, it did not preferentially inactivate virus directly, was ineffective in chick cells, was inactivated by trypsin, and was not sedimentable. Unlike interferon, the inhibitor was heat labile. Nucleic acid or nucleotides apparently play no role in its induction.*

While studying circulating interferon, which appears after an intravenous inoculation of certain viruses in rabbits, a rough correlation between the capacity of the virus to induce inteferon and its pyrogenicity was noted (*1*). This observation stimulated a reexamination of the hypothesis that bacterial endotoxins (pyrogens) may induce interferon or interferon-like viral inhibitors. Gledhill found in mice and a serum factor which "spared" viral infections appeared after inoculation with endotoxin (*2*). Whether this factor acts within the cell, directly on the virus, or by way of other host defense mechanisms is not known. Youngner and Stinebring showed that an intracellular bacterium, such as *Brucella abortus*, induced interferon in chickens, but endotoxin was not an effective inducer (*3*). However, they later reported that 2 hours after inoculation with 40 mg of endotoxin or other bacteria, an inhibitor appeared in the blood of chickens (*4*).

The lethal (LD_{50}) and pyrogenic doses of the endotoxin (*5*) used in these experiments were 468 and 0.00045 μg, respectively, in rabbits weighing 2.6 to 3.0 kg. The endotoxin was suspended in phosphate buffered saline (*p*H 7.2) and inoculated in ear veins of albino male rabbits (1 to 2 kg). Serums were titrated for viral inhibitory activity as follows. Serial twofold dilutions (0.3 ml) were overlaid on duplicate tube cultures of rabbit kidney cells and incubated at 37°C for 18 hours. The fluids were then decanted, and each tube was inoculated with 10^4 to 10^5 plaque-forming units (PFU) of vesicular stomatitis virus (VSV, Indiana) suspended in 1.0 ml of medium consisting of lactalbumin hydrolyzate (0.5 percent) in Hanks balanced salt solution and 6 percent calf serum. Observations of cytopathic effect were made at 24 and 48 hours. Inhibitory effect was expressed as the last dilution of rabbit serum which inhibited cytopathic effect in 50 percent of the cultures as calculated by the Reed-Muench method. Virus was titrated by plaque formation in chick embryo cell cultures (*6*).

While normal serums showed no inhibitory effect when tested as described, an inhibitory factor developed in all rabbits 1 to 7 hours after inoculation of 2 μg of endotoxin or more (Table 1). Inhibition appeared to be greatest about 2 hours after inoculation and disappeared in 24 hours. In separate experiments those serum dilutions which inhibited the cytopathic effect of VSV also inhibited virus replication.

Whereas 0.5 μg did not consistently elicit the inhibitory factor, doses equal to or greater than 2.0 μg did (Fig. 1). There was little increase in the inhibitory titer of serum obtained from rabbits receiving exponentially increasing doses of endotoxin.

Properties of this inhibitor were studied with a pool of rabbit serum (titer of 1 : 32) obtained 2 hours after inoculation of 100 μg of endotoxin. The inhibitory effect was largely eliminated by heating at 56°C for 30 min-

utes and by digestion with trypsin (6.6 mg/ml) for 2 hours at 37°C. The titer was reduced to 1:16 if the serum was kept at *p*H 2.5 for 5 hours. It was not affected by dialysis in phosphate-buffered saline, and all inhibitory activity remained in the supernatant after centrifugation at 104,500*g* for 2 hours.

To determine approximately which serum protein was responsible for the inhibitory effect, eight fractions were obtained from a serum sample by adding increasing concentrations of ammonium sulfate (from 30 to 100 percent saturation) and testing for inhibitory effect. Most of the activity was in the fractions precipitated by 50 and 60 percent saturation of ammonium sulfate; this is also the case with interferon (*7*).

To test whether the activity of the inhibitor was due to direct viral inactivation or to an interferon-like material, three portions (0.2 ml) of the stock preparation of VSV were incubated at 37°C for 1 hour with 0.2 ml of (i) inhibitory serum, (ii) normal rabbit serum, or (iii) medium. After incubation, the mixtures were titrated for VSV. The titers for these three categories (in log PFU per 0.1 ml) were: (i) 5.65, (ii) 5.10, and (iii) 5.17. The inhibitor did not inactivate VSV when compared with normal serum or medium. Similar results were obtained with eastern equine encephalomyelitis (EEE) virus but not with Newcastle disease virus (NDV), which is inactivated directly by fresh rabbit serum (*8*). Inhibitory serum had the same inactivating effect, but not to a greater extent than fresh normal serum.

The following observations further suggest that this inhibitor acts intracellularly in an interferon-like manner and is distinct from the heat-labile serum inactivators of virus. (i) Inhibitory action of the serum could not be demonstrated without prior incubation with rabbit kidney cell cultures. Routinely, the inhibitory titer of the pooled serum was obtained after 18 hours of incubation with rabbit kidney cultures at 37°C. Six hours of such incubation was equally effective, but the inhibitory titer diminished to 1 : 2 with only 1 hour of this prior incubation. This suggests that time for proper cell-inhibitor interaction is required for the manifestation of the inhibitory action of the serum. (ii) In titrating the inhibitory action of the pooled serum the titer remained at 1 : 32 if, prior to challenge with VSV, the serum dilutions were first decanted and then washed off the cell monolayers with three portions (5 ml each) of medium. (iii) When the inhibitor was tested against VSV or EEE in chick fibroblast cell cultures, it was ineffective. This shows that the inhibitor exhibits species specificity, which is one of the hallmarks of interferon (*9*).

Table 1. Summary of serum inhibitory titers after intravenous injection in 21 rabbits of 2 to 2000 μg of endotoxin. Titers are expressed in reciprocal of dilutions. All serums were tested in twofold dilutions. None was tested at a dilution of less than 1 : 2 because undiluted serums occasionally cause detachment of cells.

No. of samples	Time obtained (hr)	Range of titers	Mean
16	*	<2	<2
2	½	<2	<2
2	1	8–32	20
2	1½	8–64	36
21	2	8–80	44
17	4	4–64	23
12	7	<2–64	9
6	24	<2	<2

* Prior to administration of endotoxin.

The action of the inhibitor was not restricted to VSV; it was equally effective against EEE virus. To test whether the induction of the serum inhibitor was limited to purified endotoxin, *Escherichia coli* (0113) cells, which had been dried and killed in a vacuum oven at 65°C, were tested. Serums were collected at intervals after inoculating two rabbits with 2.0 and 0.2 mg of dried bacteria intravenously. Both serums obtained after 2 hours had inhibitory titers of 1 : 64. After inoculation of 0.02 mg bacteria, no inhibitory effect could be found in the serums after 2, 7, or 24 hours. Hence bacterial cells were also effective in inducing this viral inhibitor. To test whether the mechanism of induction of the inhibitor is related to the nucleic acid or nucleotide content of endotoxin or bacteria in view of the purported role of foreign nucleic acids in the induction of interferon (*10*), a preparation of endotoxin, free of nucleotides, was inoculated in rabbits. After inoculation of 20 μg of this material, the titer of inhibitory activity in the serum was higher than 1 : 64 in 2 and 4 hours, but in 7 hours the titer decreased to 1 : 8. Thus nucleic acids or nucleotides are probably not the inductive substance. To test whether endotoxin which may persist in the circulation of injected rabbits was responsible for the inhibitory effect, cell cultures of rabbit kidney were incubated with 10 μg of endotoxin for 18 hours at 37°C. These treated cultures showed no inhibition of cytopathic effect or virus replication when challenged with VSV. Prior incubation of endotoxin (5 μg/ml) with fresh normal rabbit serum at 37°C for 1 or 2 hours did not alter the results; this suggests that the inhibitor is not a simple reaction product of endotoxin and serum.

In conclusion, while the induction of

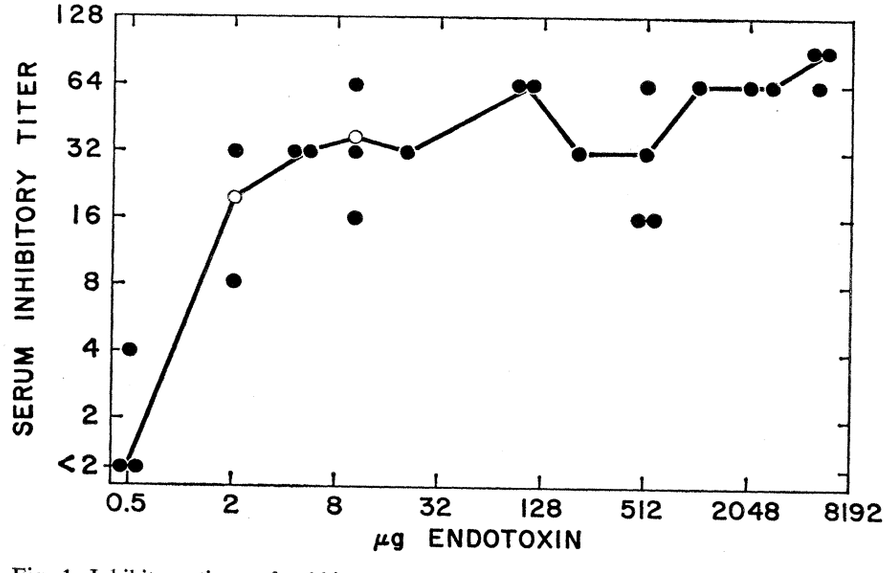

Fig. 1. Inhibitory titers of rabbit serum with varying doses of endotoxin. Two hours after intravenous injection serums were titrated against VSV for inhibitory effect in cell cultures of rabbit kidney tissue. Each solid dot represents a separate sample. The curve was drawn through the mean titers.

a viral inhibitor with killed *E. coli* and endotoxin has been demonstrated, its identity is not yet definite. Most of its properties suggest that it is an interferon, but this cannot be categorically stated since a precise definition of interferon is lacking (*9*). The characteristics of the inhibitor here described which differentiate it from interferon are its heat and acid lability. While heat-labile interferon from mouse cells has been described (*11*), circulating interferon of rabbits induced by viruses rather than by endotoxin was stable at 56°C for 1 hour (*1*). Possibly there may be more than one type of interferon-like inhibitors in one animal species. The phenomenon described here may explain the sparing effect of endotoxins on certain viral infections (*2, 12*). It suggests further proof of the relation between bacteria and viral inhibitors, and particularly between interferon, endotoxin, and fever.

Note added in proof: W. R. Stinebring and J. S. Youngner have obtained in mice an inhibitor in high titer which was more definitely characterized as interferon (*13*).

MONTO HO

Department of Epidemiology and Microbiology, Graduate School of Public Health, University of Pittsburgh, Pittsburgh, Pennsylvania

References and Notes

1. Y. Kono and M. Ho, *Virology*, in press, and unpublished data.
2. A. W. Gledhill, *Nature* **183**, 185 (1959); in *Bacterial Endotoxins*, M. Landy and W. Braun, Eds. (Rutgers Univ. Press, New Brunswick, N.J., 1964), pp. 410–20.
3. J. S. Youngner and W. R. Stinebring, *Science* **144**, 1022 (1964).
4. ———, Private communication.
5. A. I. Braude, in *Bacterial Endotoxins*, M. Landy and W. Braun, Eds. (Rutgers Univ. Press, New Brunswick, N.J., 1964), pp. 98–109; V. E. Gilbert and A. I. Braude, *J. Exptl. Med.* **116**, 477 (1962). *Escherichia coli* was cultured in defined medium and extracted by the Boivin method for endotoxin by Dr. Braude.
6. M. Ho, *Proc. Soc. Exptl. Biol. Med.* **107**, 639 (1961).
7. R. R. Wagner, *Bacteriol. Rev.* **24**, 151 (1960).
8. H. S. Ginsberg, *ibid.*, p. 141; ——— and F. L. Horsfall, *J. Exptl. Med.* **90**, 475 (1949).
9. D. A. J. Tyrrell, *Nature* **184**, 452 (1959); G. E. Gifford, *J. Gen. Microbiol.* **33**, 437 (1963); M. Ho, *Bacteriol. Rev.*, in press.
10. Z. Rotem, R. A. Cox, A. Isaacs, *Nature* **197**, 564 (1963); K. E. Jensen, A. L. Neal, R. E. Owens, J. Warren, *ibid.* **200**, 433 (1963).
11. T. C. Merigan, *Science* **145**, 811 (1964).
12. R. R. Wagner, R. M. Snyder, E. W. Hook, C. N. Luttrell, *J. Immunol.* **83**, 87 (1959); R. W. Finkelstein, *Proc. Soc. Exptl. Biol. Med.* **106**, 481 (1961).
13. W. R. Stinebring and J. S. Youngner, *Nature*, in press.
14. Supported by NIH grant AI-02953. I thank Dr. A. I. Braude for supplies of endotoxin and bacteria as well as for criticism. I also thank Mrs. S. P. Mitchell and Miss Mary Kay Breinig for technical assistance.

4 November 1964

Cycloheximide: Aspects of Inhibition of Protein Synthesis in Mammalian Cells

Abstract. *Cycloheximide and acetoxycycloheximide specifically inhibit protein synthesis in L-cells growing in suspension culture. In extracts of rat liver, the drugs inhibit transfer of amino acid from soluble RNA to polypeptide. Unlike puromycin, these drugs do not accelerate release of nascent polypeptide chains. The drugs have no effect on protein synthesis in extracts of* Escherichia coli.

Cycloheximide (*1*), an antibiotic produced by *Streptomyces griseus*, inhibits the growth of many yeasts and fungi and inhibits protein synthesis in intact rats, but has little effect on growth of bacteria (*2*). Opinion is divided on the question of whether the primary effect of low doses of cycloheximide is on DNA synthesis or on protein synthesis (*3–5*). Our results reported here indicate that in L-cells the effect of this antibiotic is greater on protein synthesis.

Two laboratories have found that cycloheximide inhibits transfer of amino acids from aminoacyl sRNA (*6*) to polypeptide. Siegel and Sisler have recently reported that cycloheximide decreases transfer of amino acids to polypeptide in extracts of *Saccharomyces partorianus* by about two-thirds (*7*). Our results with extracts of rat liver (*8*) are given in detail below. The present report also describes experiments on the release of nascent polypeptide chains, on the reversibility of inhibition by cycloheximide and acetoxycycloheximide (*9*), and on the sensitivity of systems stimulated by natural and synthetic messenger RNA.

For measurements on intact cells, L-cells were grown in suspension culture, and various concentrations of drug and either C^{14}-leucine or C^{14}-uridine were added. After 1 hour of incubation the cells were collected, and the radioactivity in the fraction precipitated by cold TCA was determined. Cycloheximide and acetoxycycloheximide markedly inhibited incorporation of C^{14}-leucine, but, even at concentrations 100-fold greater than that required for maximum inhibition of protein synthesis, incorporation of C^{14}-uridine into the TCA precipitable fraction was not inhibited (Fig. 1). No inhibition of C^{14}-uridine incorporation was apparent until 90 to 120 minutes after addition of drug.

To measure the rate of DNA synthesis, portions of the cell suspension were removed at intervals during a 90-minute period of incubation in the presence of C^{14}-uridine, and 10*N* NaOH was added to produce a final concentration of 1*N*. The mixture was then kept at 37°C for 2 hours, by which time RNA had been degraded (*10*). The mixture was neutralized with concentrated HCl, an equal volume of 10 percent TCA was added, and the precipitate was collected and counted. At a concentration of cycloheximide (10 μg/ml) that inhibited protein synthesis by 95 percent, DNA synthesis was inhibited by only about 50 percent (three experiments). This result is similar to the findings reported for HeLa cells by Young *et al.* (*4*). On the other hand, Bennett *et al.* have reported that, in cell lines derived from human epidermoid carcinoma and from mouse adenocarcinoma, DNA and protein synthesis are nearly equally depressed by cycloheximide (*5*).

When cells were incubated in the presence of either drug for 3 hours and then washed free of drug, protein synthesis resumed promptly at nearly nor-

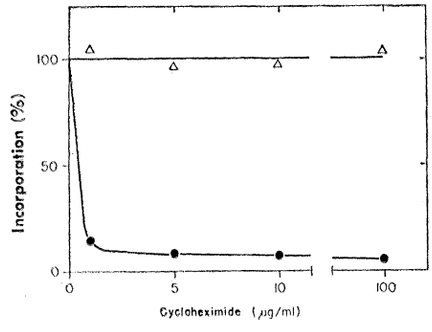

Fig. 1. Effect of cycloheximide on the incorporation of C^{14}-leucine and C^{14}-uridine into growing L-cells. A growing suspension (3×10^5 cells/ml) with a division time of about 24 hours was inoculated into Eagle's MEM medium (*16*) containing either C^{14}-leucine (0.1 μc/μmole, 0.3 μmole/ml) or C^{14}-uridine (1.5 μc/μmole, 0.24 μmole/ml). The indicated quantity of cycloheximide was added to 10-ml portions of each of the suspensions, and the cells were incubated at 37°C with shaking. At the end of 60 minutes, 1-ml samples (in duplicate) were filtered through Millipore filters (3-μ pore) under slight suction (2 cm H_2O). The cells were washed with 10 ml of Earle's salt solution, and then washed with 10 ml of 5 percent TCA containing 1 mg of nonradioactive leucine and uridine per milliliter. The filters were mounted on planchets and counted with a Nuclear-Chicago end-window counter. C^{14}-uridine, (△——△); C^{14}-leucine, (●——●).