Table 2. Infection-enhancing effect of an enzyme preparation. The enzyme was injected intraperitoneally 6 hours after infection with 0.2×10^{-4} ml of *Klebsiella* culture.

Enzyme injected (μg)	Percentage of deaths (cumulative) at indicated days after infection*			
	1	2	3	4
400	24	49	93	100
40	13	37	68	91
4	12	23	28	31
0	0	0	0	7

* The total numbers of animals (from 12 different experiments) exceeded 100 for each group.

arations were hemagglutinating but not hemolytic or antibacterial, and vice versa. Some preparations did not exhibit any in vitro activity in our tests, even though they were extremely active in depressing the body weight of mice and in aggravating infections. There seemed to be an association between the latter two activities.

Table 1 presents results with eight different enzyme preparations, selected to illustrate the wide range of differences in their activities in vitro.

The experiments dealing with the enhancement of *Klebsiella* infection in mice have been carried out under a wide range of conditions with each of the enzyme preparations available. Table 2 includes the results of 12 different tests with one single preparation over a period of 8 months.

Conclusions. All preparations of *Bacillus subttilis* autolyzates (so-called enzymes) tested were capable of causing a rapid loss of body weight when injected by the peritoneal route into specific pathogen-free mice; they also aggravated experimental infection with *Klebsiella pneumoniae* and staphylococcus. Some of the preparations, but not all, caused hemagglutination, hemolysis, and other cytotoxic effects. These various in vivo and in vitro activities differed qualitatively and quantitatively from preparation to preparation.

In all cases, the in vivo and in vitro activities persisted unaltered after the enzyme preparation had been heated at boiling temperature for 15 minutes, then clarified by centrifugation and filtration.

Although no attempt has been made to compare the immunological activities of the enzymatic preparations, the present findings may have a bearing on this problem. The profound differences observed among the various preparations probably have their origin in the characteristics of the bacterial strains of *Bacillus subtilis* from which they were prepared. It is not unlikely therefore that the various preparations also differ in immunological specificity and sensitizing ability—a fact which would greatly complicate the formulation of safety controls in their manufacture and use.

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Antiviral Activity of Polyribocytidylic Acid in Cells Primed with Polyriboinosinic Acid

Abstract. *Separate administration of polyribocytidylic acid [poly(rC)] and polyriboinosinic acid [poly(rI)] to cell cultures in vitro resulted in an antiviral activity identical to or greater than that resulting from addition of the poly(rI) • poly(rC) complex. Priming of cells with poly(rI), followed by treatment with poly(rC), gave a consistently greater antiviral activity than poly(rI) • poly(rC) itself. This priming effect was obtained in several cell cultures challenged with different viruses. In vivo, the antiviral activity of poly(rI) • poly(rC) was only partially restored if poly(rI) and poly(rC) were injected separately; prior injection of poly(rI) proved superior in restoring this antiviral activity as compared to prior injection of poly(rC).*

Homopolynucleotide complexes such as poly(rI) • poly(rC) (homopolymer pair of polyriboinosinic acid and polyribocytidylic acid) are highly active in inducing interferon and cellular resistance to virus infection, both in vitro and in vivo, as originally described by Field *et al.* (*1*). The antiviral activity of poly(rI) • poly(rC) and other polynucleotide complexes has been related to their stable, highly ordered, double helical structure and to their resistance against premature enzymatic degradation (*2–5*). In these and several other studies on the antiviral and antitumor activity, and toxic properties of poly(rI) • poly(rC), the two constituent homopolymers were always administered in the form of the double-stranded complex but never separately, one after the other. In a study on the toxicity of poly(rI) • poly(rC) in adrenalectomized rats, separate injection of the individual homopolymers in rapid succession proved as lethal as injection of the complex (*6*).

Separate administration of poly(rC) and poly(rI) to cell cultures in vitro resulted in an identical or greater antiviral activity than addition of the poly(rI) • poly(rC) complex itself. These findings may open new avenues in deciphering the mechanism by which (double-stranded) polynucleotide complexes trigger the production of interferon and other host cell responses.

The homopolynucleotides poly(rI) and poly(rC) (*7*) were dissolved in phosphate-buffered saline (PBS) and stored at −20°C. Concentrations of the polymers were determined spectrophotometrically (*2*). The homopolymer pair poly(rI) • poly(rC) was prepared by annealing the individual homopolymers (*2*). Before use, poly(rI), poly(rC), and poly(rI) • poly(rC) were diluted in Eagle's minimum essential medium (MEM) to the appropriate concentrations. As described previously (*4*), poly(rI) • poly(rC) was heated at 37°C in MEM before exposure to the cells.

Cellular resistance to bovine vesicular stomatitis virus (VSV) (Indiana strain) was determined in seven different cell cultures [five continuous cell lines: HSF (human skin fibroblasts), RK 13 (rabbit kidney) cells, mouse L 929 cells, HeLa cells, HK (human kidney) cells; and in two primary cell cultures: MEF (mouse embryo fibroblasts), PRK (primary rabbit kidney) cells] by inhibition of virus plaque formation or viral cytopathogenicity. Cellular resistance to vaccinia virus was determined in RK 13 cells by inhibition of virus plaque formation. Twenty-four hours after exposure of the cell cultures to the polymers (the second polymer, if the individual homopolymers were administered in succession), virus was added, and virus plaque formation (or cytopathogenicity) was recorded 2 to 3 days later. Interferon production was measured in vivo (young NMRI mice, 12 to 14 g) after intravenous or intraperitoneal injection of the polymers; serum interferon titers were determined in a plaque inhibition

assay on L 929 cells with VSV as challenge virus.

In a first set of experiments, HSF and RK 13 cell cultures were exposed to either poly(rC) (10 μg/ml) or poly(rI) (10 μg/ml) for various periods of time (Table 1), washed three times and further incubated with different concentrations (10^{-5} to 10 μg/ml) of either poly(rI) or poly(rC), respectively. Sequential administration of the homopolymers in the order poly(rI), poly(rC) induced a higher degree of resistance to virus infection than administration of the poly(rI) • poly(rC) complex. Whether the cells were first treated with poly(rI) for only 10 minutes or for more than 1 day, addition of poly(rC) resulted in a consistently greater antiviral activity than incubation of the cells with the complex itself. Separate administration of the individual homopolymers in the order poly(rC), poly(rI) afforded the same protection as the complex, but only if poly(rI) was added shortly (within 10 minutes) after poly(rC). Poly(rC) alone, poly(rI) alone, poly(rC) followed by poly(rC), and poly(rI) followed by poly(rI) were inactive at 10 μg/ml, the highest concentration tested. Table 1 shows that a very short contact of either homopolymer with the cells, such as 1 minute for poly(rC), is sufficient to prime the cells successfully for the antiviral effect of the complementary homopolymer. These results indicate that both poly(rI) and poly(rC), when given separately, interact very rapidly with the cell, as seen before with the alternating copolymer poly(rA-rU) (*5*).

Similar results were obtained in several other cell cultures (L 929, HeLa, HK, MEF, and PRK) with either VSV or vaccinia as challenge virus (Table 2). The cells were exposed to either poly(rC) (10 μg/ml) for 30 minutes or poly(rI) (10 μg/ml) for 1 hour, washed three times, and further incubated with different concentrations (10^{-5} to 10 μg/ml) of poly(rI) and poly(rC), respectively. In all cell cultures tested, sequential administration of the homopolymers in the order poly(rI), poly(rC) reduced virus infection more effectively than administration of the complex poly(rI) • poly(rC).

When poly(rI) and poly(rC) were injected successively into mice (either intravenously or intraperitoneally), significantly lower interferon levels were produced than with injection of the poly(rI) • poly(rC) complex (Table 3). Relatively high interferon titers were observed when intravenous injection of poly(rI) was followed 1 minute later by intravenous injection of poly(rC) or vice versa. In all conditions, interferon titers were higher when poly(rI) was injected first than when poly(rC) was injected first (Table 3).

The surprisingly high antiviral activity obtained with successive administration of poly(rI) and poly(rC) to cell cultures in vitro can be explained by assuming (i) that the homopolymers form a double-stranded complex at the outer cell membrane or within the cell, or (ii) that the two strands do not reunite but act independently on the cell. The first alternative may stand or fall on the question of whether the

Table 1. Antiviral activity of poly(rC) in cells primed with poly(rI) for different times, and of poly(rI) in cells primed with poly(rC) for different times. Antiviral activity was measured by cellular resistance to vesicular stomatitis virus infection in human skin fibroblasts (HSF) and rabbit kidney (RK 13) cells.

Interval between first and second polymer	Minimum inhibitory concentration* (μg/ml)			
	HSF		RK 13	
	Poly(rC) then poly(rI)	Poly(rI) then poly(rC)	Poly(rC) then poly(rI)	Poly(rI) then poly(rC)
1 minute	0.004		0.1	0.002
10 minutes	0.004		0.4	0.002
30 minutes	0.4	0.0001	4	0.002
1 hour	1	0.0001	10	0.002
2 hours		0.0001	>10	0.002
4 hours			>10	0.002
8 hours			>10	0.002
16 hours				0.002
28 hours				0.002
Poly(rI) • poly(rC) complex	0.004		0.1	

* Concentration polymer [either poly(rI) • poly(rC) or poly(rI), if poly(rI) is added second, or poly(rC), if poly(rC) is added second] required to reduce virus plaque formation by 50 percent.

Table 2. Antiviral activity of poly(rC) in cells primed with poly(rI), and of poly(rI) in cells primed with poly(rC), as measured by cellular resistance to vesicular stomatitis virus (VSV) or vaccinia virus in different cell cultures.

Cell culture	Virus challenge	Minimum inhibitory concentration* (μg/ml)		
		Poly(rI) • poly(rC) complex	Poly(rC) then poly(rI) in 30 minutes	Poly(rI) then poly(rC) in 1 hour
HSF	VSV	0.004	0.4	0.0001
RK 13	VSV	0.1	4	0.002
L 929	VSV	2	>10	0.2
HeLa	VSV	4	>10	0.4
HK	VSV	4	>10	0.4
MEF	VSV	0.01	1	0.001
PRK	VSV	0.001	0.01	0.00004
RK 13	Vaccinia	2	>10	0.01

* Concentration of polymer [either poly(rI) • poly(rC) or poly(rI), if poly(rI) is added second, or poly(rC), if poly(rC) is added second] required to reduce virus plaque formation (in RK 13, L 929, HeLa, HK, MEF, and PRK) or viral cytopathogenicity (in HSF) by 50 percent.

Table 3. Interferon production by successive administration of poly(rI) and poly (rC) to mice.

Interval between first and second polymer	Interferon (units per 4 ml of serum)*			
	Intravenous injection		Intraperitoneal injection	
	Poly(rC) then poly(rI)	Poly(rI) then poly(rC)	Poly(rC) then poly(rI)	Poly(rI) then poly(rC)
1 minute	225	400	≤ 10	92
10 minutes	≤ 10	80	≤ 10	69
1 hour	≤ 10	10	≤ 10	≤ 10
4 hours	≤ 10	≤ 10	≤ 10	≤ 10
Poly(rI) • poly(rC) complex	480		1480	

* Two hours after intravenous injection or 16 hours after intraperitoneal injection of either poly(rI) • poly(rC) or poly(rI), if poly(rI) is injected second, or poly(rC), if poly(rC) is injected second. Poly(rI) • poly(rC) was injected at 20 μg per mouse, and poly(rI) and poly(rC) at 10 μg per mouse.

second polymer is able to join the first polymer at the cellular level, even if it is added more than 1 day after the first polymer [for example, poly(rC) applied to RK 13 cell cultures 28 hours after poly(rI)] (Table 1). According to the second alternative, the polymer added first might exert an effect on the cell that is required for the antiviral activity of the second polymer.

The fact that separate administration of the individual homopolymers only partially restores the interferon inducing capacity of poly(rI) • poly(rC) in vivo, does not necessarily refute the hypothesis of a two-step action mechanism of the homopolymers. When injected separately in the whole animal, the two homopolymers are probably going to different cells and do not interact with the same cell, as they do in vitro. Hence, the partial antiviral effect obtained with poly(rI) and poly(rC) in vivo, when injected in rapid succession, may be ascribed to an association of the two homopolymers and to activity of the double-stranded complex.

The finding that the antiviral activity of the poly(rI) • poly(rC) complex in vitro can be equaled and even surpassed by successive administration of the component homopolymers may be an important step in the study of the mechanism of interferon production by nucleic acids of either synthetic or viral origin, and might help in identifying the ultimate trigger site for interferon production.

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7. Poly(rI) and poly(rC) were purchased from P-L Biochemicals, Milwaukee, Wisconsin, or from Miles Laboratories, Elkhart, Indiana. Most experiments were carried out with poly(rI) and poly(rC) obtained from P-L Biochemicals, but preparations of Miles Laboratories gave essentially identical results.
8. We thank Miss A. Van Lierde for technical assistance. E.D.C. is "Aangesteld navorser" of the Belgian Nationaal Fonds voor Wetenschappelijk Onderzoek.

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Habituation of Electrically Induced Readiness to Gnaw

Abstract. *Electrical stimulation of the hypothalamus in prairie dogs* (Cynomys ludovicianus) *produced a readiness to gnaw which decreased over time, exhibited spontaneous recovery, and could be dishabituated by foot shock. The response decrement was in part habituatory and could modify the interaction between a stimulation-induced readiness to gnaw and a physiologically induced hunger. Functional plasticity of stimulation-induced behavior might be accounted for, in part, by habituation.*

Electrical stimulation of the hypothalamus has been shown to produce a *readiness* for such behavior as eating, drinking, gnawing, nesting, attacking, and mating (*1*). The term "readiness" has been used because the stimulation-induced behavior is dependent upon access to appropriate goal objects (*2*). Valenstein, Cox, and Kakolewski (*3*) have described "a procedure for modifying behavior elicited by hypothalamic stimulation" in rats. This procedure first involved stimulating a rat in the presence of food, water, and gnawing material. The preferred goal object (for example, food) was then removed and the subject was repeatedly stimulated in the presence of the remaining goal objects.

After several nights of such "training" the subject was again stimulated in the presence of all three goal objects. When this procedure was used, significant preference changes were reported (for example, an animal that ate on a pretraining test drank or gnawed on the posttraining test). Valenstein *et al.* seemed to assume that the preference changes resulted primarily from environmental manipulations, that is, removal of the preferred goal object, during stimulation rather than the continued stimulation per se. But this assumption does not explain how a long series of stimulation periods with *all three* goal objects present changed the preference of several animals (*4*). Furthermore, these investigators did not include a control group stimulated in the absence of any goal object nor did they report any attempt to retrain an animals to its initial preference.

Questioning the assumption that environmental manipulations influence the preference changes, Wise (*5*) has advanced the alternative hypothesis that continued stimulation can reduce the threshold for an initially latent response to the extent that this response can compete with behavior shown to be prepotent on initial tests. A third possible explanation for the preference changes reported by Valenstein *et al.* is that prepotent behavior observed on the first test habituated (*6*) more rapidly than other initially latent tendencies. For example, if stimulation-induced hunger were prepotent on the initial test but habituated more rapidly than stimulation-induced thirst, we would expect the animal would eat when first stimulated but would, at some time, switch to drinking if stimulation continued long enough. The acceptability of such an explanation rests upon the assumption that a stimulation-induced readiness can habituate. Habituation of a variety of peripherally evoked behaviors has been demonstrated (*7*). Habituation of cortical and behavioral arousal elicited by intracranial stimulation has also been shown (*8*). We have attempted to demonstrate the habituation of a stimulation-induced readiness to gnaw and to determine how this habituation alters the interaction between gnawing and other motivational tendencies.

Bipolar electrodes made of Teflon-coated wire 0.20 mm in diameter, with insulation removed 0.4 mm from the tips, were implanted in the lateral hypothalamus (*9*) of 13 adult black-tailed prairie dogs (*Cynomys ludovicianus*). Six of these animals showing reliable gnawing on several daily stimulation tests were selected for study.

Each subject was placed in a 34 by 25 by 66 cm test chamber containing 16 loose white pine blocks (7.0 by 1.5 by 2.0 cm). (Foam rubber blocks of the same size were used in place of wood blocks for one animal with defective teeth.) Animals were adapted to the box for 10 minutes; none showed spontaneous gnawing. To determine each animal's gnawing threshold, continuous stimulation (*10*) was administered at 1.0 volt and then increased at a rate of 3 volt/min. When gnawing began, stimulation was turned off. The subject was then given a series of 30-second stimulation trials with 3 minutes between trials. On the first trial the voltage was set at 0.5 volt below threshold and increased in 0.2-volt steps on subsequent