bioautograph technic of developing paper chromatograms of antibacterial agents can also be applied to antiviral materials. It remains to be seen, however, whether antiviral substances discovered by this technic will eventually be useful in human and veterinary medicine. It is encouraging, in this regard, that at least one antiviral agent—isatin-thiosemicarbazone—which is highly active in vaccinia virus infected mice(7), also was extremely active in this tissue culture system. It can only be hoped that the success observed in discovering clinically useful antibacterial agents can be paralleled in virology.

Summary. A procedure has been developed whereby agar diffusion technics used in bacteriology can be applied to antiviral agents. Chick embryo cell monolayers were grown in Pyrex baking dishes, infected with virus and overlaid with agar. Filter paper discs impregnated with the test materials were deposited on the hardened agar surface. After proper incubation the virus produced plaques in the cell sheet, except in the area around a disc containing an antiviral agent. Since size of the plaque-free zone was proportioned to concentration of the antiviral agent present in the disc, bioassays were simply performed in exactly the same manner as done with antibacterial antibiotics.

1. De Somer, P., Prinzie, A., *Virology,* 1957, v4, 387.
2. Porterfield, J. S., *Lancet,* 1959, vII, 326.
3. Dulbecco, R., *Proc. Nat. Acad. Sci., Wash.,* 1952, v38, 747.
4. Melnick, J. L., *Diagnostic Procedures for Virus and Rickettsial Diseases,* Am. Public Health Assn., 2nd Ed., 1956, 142.
5. Klein, S. W., Goodall, S. H., *Science,* 1959, v130, 629.
6. Gavin, J. J., *Appl. Microbiol.,* 1957, v5, 25.
7. Bauer, D. J., *Brit. J. Exp. Path.,* 1955, v36, 105.

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Activation of Histamine-Releasing Factor in Normal Rat Plasma by *E. coli* Endotoxin. (25618)

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The mechanisms whereby Gram-negative bacterial endotoxins (somatic antigens) produce the various pathophysiologic alterations in the mammalian host(1) are poorly defined. The basic problem of whether endotoxins act directly at the cellular level, or through an intermediate step whereby composition of blood is altered, or both, is unresolved. The toxic activity of bacterial endotoxins, which are protein-lipid-polysaccharide complexes, appears to reside in the lipid-polysaccharide moiety(2). In a previous study(3), polysaccharides prepared from somatic antigens of *Salmonella typhi* and *Salmonella paratyphi* were included among a list of numerous high molecular weight polysaccharides capable of activating, *in vitro,* a factor in normal rat plasma with potent histamine-liberating activity. It seemed possible, therefore, that certain of the pathologic effects induced by bacterial endotoxin might be attributable to activation of such plasma histamine-liberating factors. The previous study, however, utilized large quantities of the bacterial polysaccharides (20 mg/ml rat plasma) and failed to specify whether these a) were combined with or dissociated from their lipid or protein components, b) exhibited the characteristic activities of endotoxin *in vivo,* or c) released some portion of histamine by direct tissue action. The present study was designed to determine whether smaller quantities of physiologically active, purified lipopolysaccharides from Gram-negative bacteria could activate plasma histamine-releasing factors and to exclude the possibility that such histamine release might be attributable to direct endotoxin-tissue interaction.

Materials and methods. The lower ileum was resected from normal 250 g guinea pigs

TABLE I. Lethality of Single Intravenous *E. coli* Endotoxin Injections for Immature Albino Rabits.*

E. coli endotoxin† μg/kg	96 hr mortality (deaths/total)
200	0/30
500	1/20
1000	1/10
2000	0/5
4000	4/8
5000	2/6
7000	3/6
10,000	7/8

* Rabbit wt = 1.1 (range 0.9-1.5) kg.
† Supplied by Difco Laboratories as Lipopolysaccharide *E. coli* 0127B8.

after head blow sacrifice and placed in Tyrode solution at room temperature. Four cm segments were removed, attached to a lever arm exerting 5 g tension, and suspended in a 25 ml aerated 37°C Tyrode bath stirred by a glass rod connected to a constant speed motor. Multiple segments of ileum from each of 8 guinea pigs were studied as follows: Baseline responses to a standard histamine solution (0.1 μg histamine base in 0.1 ml physiologic saline) were recorded kymographically by adding histamine immediately after interrupting the aerating system. Following each contraction, the ileum was washed 3 times with Tyrode solution and aeration restarted. When reproducible histamine responses were obtained, the effect of 0.5 ml of the following solutions was tested in the sequence indicated; a) physiologic saline containing 5, 50, or 250 μg *E. coli* endotoxin, b) normal heparinized fresh rat plasma (100 μg heparin/ml plasma) diluted 1:1 with physiologic saline, and c) aliquots of the same rat plasma diluted 1:1 with physiologic saline, containing a final content of 5, 50, or 250 μg *E. coli* endotoxin. All endotoxin and control solutions were incubated at 37°C for 30 minutes prior to guinea pig ileum assay. With each solution tested, the Tyrode bath aeration was temporarily discontinued to circumvent formation of noxious aerosols. After each assay, the ileum was washed 5 times with Tyrode solution and reactivity to exogenous histamine rechecked. Ability of an antihistaminic compound, pyribenzamine (Ciba) to alter the ileum responses was also determined. The *E. coli* endotoxin employed for the present study was supplied by Difco Laboratories as *E. coli* lipopolysaccharide 0127B8 and prepared by a modification of the Boivin trichloracetic acid extraction procedure which removes most of the protein components(4). The lethality of this endotoxin preparation following single intravenous injections into immature healthy albino rabbits is given in Table I. In addition, 1 to 10 μg of the endotoxin injected intravenously generally produced delayed hemorrhagic necrosis in areas of the rabbit abdominal wall simultaneously treated with 100 μg epinephrine intradermally; 100 μg of the endotoxin injected intravenously provoked the local Shwartzman reaction in most skin areas prepared 24 hours previously with 50 μg endotoxin.

Results. Ten segments of guinea pig ileum were tested with the 250 μg aliquots of *E. coli* endotoxin; Fig. 1 is representative of results. The *E. coli* endotoxin *per se,* incubated at 37°C for 30 minutes in physiologic saline, failed to evoke contractile response during ileum contact. Following endotoxin washout, the ileum remained fully responsive to exogenous histamine. Normal fresh rat plasma, incubated with physiologic saline as described above, elicited minimal or no ileum responses with the dosages employed and left the ileum fully responsive to exogenous histamine. In contrast, mixtures of the rat plasma incubated with 250 μg *E. coli* endotoxin in physiologic

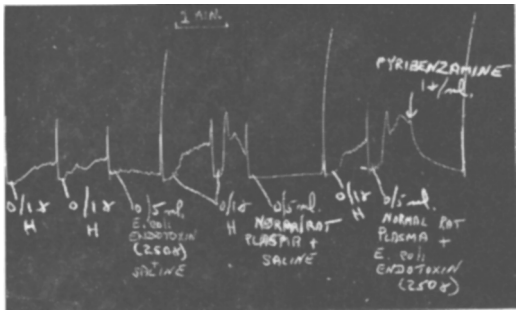

FIG. 1. Effect of a physiologically active lipopolysaccharide component of *E. coli* endotoxin on ability of fresh normal heparinized rat plasma to contract isolated guinea pig ileum segments at 37°C in a 25 ml Tyrode bath. (0/1γ H = 0.1 μg histamine base. The unique effect of 0.1 μg exogenous histamine in the 5th assay from the left was intentionally induced by introducing the histamine adjacent to the ileum for comparison with response to activated rat plasma. The pyribenzamine added, 1 μg/ml, is expressed as final concentration in the Tyrode bath.)

saline induced immediate ileum contraction which could be reversed rapidly by pyribenzamine in final concentrations of 1 μg/ml. Normal fresh rat plasma from 3 different pools was employed and each yielded comparable results.

The 50 μg aliquots of *E. coli* endotoxin were assayed on 12 fresh guinea pig ileum segments and the 5 μg aliquots on 8 other segments. A sequence of events entirely similar to that described above was obtained consistently with 3 different batches of fresh rat plasma. Although the assay system could not be employed for reliable quantitative data, when responsiveness of each ileum segment to the standard histamine solution was considered, it appeared that greater activation of the rat plasma "ileum contractor" factor could be accomplished with the 50 and 250 μg *E. coli* endotoxin than with the 5 μg aliquots.

In the above experiments, the *E. coli* endotoxin incubated in saline failed to induce contractions upon contact with guinea pig ileum segments during periods of 2 minutes or less. Although this was sufficient to indicate clearly the different response from that of endotoxin incubated in rat plasma, it seemed of interest also to determine whether endotoxin *per se*, if permitted longer tissue contact time or employed in large quantities, could directly induce ileum contraction. In addition, the previous experiments were performed without precautions to circumvent Tyrode solution or glassware contamination with traces of bacterial endotoxin. Conceivably, suspension of guinea pig ileum segments in Tyrode solution already containing endotoxin may render such segments refractory to endotoxin assay. Studies were therefore performed with pyrogen-free solutions and glassware rinsed with pyrogen-free saline and heated at 180°C for 2 hr. Each ileum segment was resected with strict aseptic technic and the ends firmly ligated to prevent fecal leakage. In 3 trials, 2000 μg aliquots of *E. coli* endotoxin were thus assayed, employing fresh guinea pig ileum segments from 2 pigs. No contractions or change in baseline tone resulted during contact periods of 20 minutes in each case. Similar results occurred in 3 other trials without pyrogen-free precautions. The baseline responsiveness of these ileum segments to standard exogenous histamine was ± 200% that shown in Fig. 1 and histamine responsiveness at completion of each assay remained essentially unaltered.

Discussion. The findings suggest that the lipopolysaccharide *E. coli* endotoxin preparation employed, exhibiting lethal, epinephrine-sensitizing, and Shwartzman provoking activity in the rabbit, was capable of activating a potent histamine (or histamine-like) liberating factor in fresh heparinized rat plasma upon 37°C incubation *in vitro*. The possibility that the pristine endotoxin exerted a direct tissue effect in the test system was excluded, although the alternative that rat plasma converted the endotoxin to a direct histamine-releasing material was not ruled out. The latter, however, appears improbable in view of previous studies on the nature of plasma histamine-liberating factor activation by polysaccharides(3). It is of interest that whereas 5 μg *E. coli* endotoxin incubated in rat plasma induced immediate and definite histamine liberation, 2000 μg of the same endotoxin in physiologic saline failed to induce detectable histamine release despite 20 minutes of tissue contact. This inability of *E. coli* endotoxin to release histamine directly did not appear to result from use of an assay system already contaminated with endotoxin since precautions to eliminate such contamination failed to alter the results. Nor could the failure to detect direct histamine release by *E. coli* endotoxin be attributed to a toxic depression of the histamine sensitivity of the assay system.

Interpretation of the present observations in relation to physiologic activity of endotoxin is speculative and must be approached with caution. First, it is possible that some contaminant, rather than *E. coli* endotoxin *per se*, was responsible for the rat plasma histamine-liberating factor activation; this seems unlikely, however, since polysaccharides from somatic antigens of *Salm. typhi* and *Salm. paratyphi* also activate such factors(3). Secondly, species specificity for activation of plasma histamine-releasing agents is unknown, although if endotoxin reacts as do other polysaccharides, fresh rat plasma would constitute the major suitable substrate(3). Finally,

whether plasma activation can occur *in vivo,* and the plasma endotoxin concentrations required, remains unanswered. Since endotoxins can be cleared rapidly from the circulation(5), it is speculative whether endotoxin would remain in contact with plasma in concentrations and for periods sufficient to initiate histamine-releasing activation *in vivo;* presumably dosage, route of administration, and status of the reticulo-endothelial system would affect this factor(6). Nevertheless, if the rat behaves similarly to the mouse, *E. coli* endotoxin plasma levels after single intravenous dosages above LD_{50} may remain comparable to the present *in vitro* test levels for at least 3 hours(6). Moreover, considerably lower plasma concentrations of endotoxin might be effective *in vivo* since the *in vitro* treated rat plasma exhibited histamine-releasing activity after 100-fold dilution in the Tyrode bath.

Despite the most liberal assumptions, the ability of *E. coli* endotoxin to activate plasma histamine-releasing factors *in vitro* fails to account for many of the *in vivo* physiologic activities. Thus numerous other polysaccharides lacking classical endotoxic activity possess the same or greater *in vitro* plasma activating potential(3). Indeed, recent studies indicate that the lipid moiety of bacterial endotoxin is the toxic factor, and that the polysaccharide (which may constitute the plasma histamine-releasor activating factor) is important merely as a carrier to permit dispersion of the toxic lipid(7). The significance of the present observations therefore centers on the possibility that in some animal species, the plasma histamine-releasing mechanism might, by *contributing* to or *modifying* other concomitant reactions *in vivo,* participate in certain pathophysiologic alterations which characterize the endotoxic response. Some support is already available from evidence of Kuida *et al.*(8) that *E. coli* endotoxin action, at least on the vascular bed of the dog and cat lung, is not direct but mediated through liberation of some agent or agents in blood and that such intermediary substances have generalized smooth muscle constrictor activity. It was noted, however, that *exogenous* histamine in this system did not reproduce the entire endotoxin action. Additional evidence for a histamine-releasing agent is suggested by observations of Gilbert(9) that the initial acute circulatory responses of the cat to *E. coli* endotoxin are compatible with histamine and serotonin release; this parallels studies by Weil and Spink (10) in which histamine-like substances were detected in inferior vena caval blood of dogs within one to 2 minutes after *E. coli* injection. Finally, the striking clinical similarity between anaphylactic and endotoxin induced shock(10) is consistent with endotoxin activation of plasma histamine-liberating agents since such agents are believed capable of reproducing certain physiologic alterations of specific anaphylaxis(11,12). Future studies will be of interest to determine whether *in vitro* and *in vivo* histamine-liberating activity of *E. coli* endotoxin can be induced by the toxic lipid component described by Westphal (7) in the absence of the polysaccharide moiety.

Summary. Employing isolated guinea pig ileum segments in aerated Tyrode solution, 5 to 250 μg aliquots of a physiologically active lipopolysaccharide component of *E. coli* endotoxin were found capable of activating a potent histamine-like releasing factor upon *in vitro* incubation with 0.5 ml diluted fresh heparinized normal rat plasma. The release of histamine by this rat plasma factor occurred within seconds after tissue contact. The *E. coli* endotoxin preparation *per se,* in the dose-time relations employed, did not liberate histamine directly from the ileum segments; indeed, 2000 μg *E. coli* endotoxin failed to release detectable histamine during 20 minute tissue contact periods. This lack of histamine-releasing activity could not be attributed to preexisting endotoxin contamination of the tissue bath nor to toxic depression of the histamine sensitivity of the assay system. The above findings, in conjunction with previous evidence, suggest that *E. coli* lipopolysaccharide behaves, at least in one manner, as do numerous other high molecular weight polysaccharides and justifies the hypothesis that activation of plasma histamine-releasing factors may account for certain of the pathophysiologic alterations which characterize the endotoxic response. Further studies

are required to define the importance of the polysaccharide moiety of the endotoxin molecule for the histamine-releasing activity.

1. Thomas, L., *Physiol. Rev.*, 1954, v16, 467.
2. Landy, M., Johnson, A. G., PROC. SOC. EXP. BIOL. AND MED., 1955, v90, 57.
3. Rothschild, A. M., Rocha e Silva, M., *Brit. J. Exp. Path.*, 1954, v35, 507.
4. Webster, M. E., Sagin, J. E., Landy, M., Johnson, A. G., *J. Immunol.*, 1955, v74, 455.
5. Carey, F. J., Braude, A. I., Zalesky, M., *J. Clin. Invest.*, 1958, v37, 441.
6. Noyes, H. E., McInturf, C. R., Blahuta, G. J., PROC. SOC. EXP. BIOL. AND MED., 1959, v100, 65.
7. Westphal, O., Presented at Nat. Inst. Health under sponsorship of Lab. Chem. Pharm., Nat. Cancer Inst., Nov. 20, 1959.
8. Kuida, H., Hinshaw, L. B., Gilbert, R. P., Visscher, M. B., *Am. J. Physiol.*, 1958, v192, 335.
9. Gilbert, R. P., PROC. SOC. EXP. BIOL. AND MED., 1959, v100, 346.
10. Weil, M. H., Spink, W. W., *J. Lab. and Clin. Med.*, 1957, v50, 501.
11. Novy, F. G., DeKruif, P. H., *J. Infect. Dis.*, 1917, v20, 776.
12. Rocha e Silva, M., *The Mechanism of Inflammation*, Pub. Acta, Inc., Montreal, 1953.

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Bactericidal Activity of Normal Serum Against Bacterial Cultures. II. Activity Against *Escherichia coli* Strains. (25619)

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The preceding paper of this series(1) on bactericidal action of normal serum against strains of *Salmonella typhosa* established that a strain's resistance to serum bactericidal action was associated with its O-inagglutinability or Vi content(2). The most resistant of the strains tested were a strain of maximum mouse virulence and of proven virulence for chimpanzees as well as another strain isolated during a severe human outbreak. From the point of view of the host, moreover, it was shown that natural bactericidal antibody of marked specificity played the major role in serum bactericidal action. The present report represents a study of bactericidal action of normal human serum against strains of *Escherichia coli*. Though usually non-pathogenic, this species was chosen for study because it may cause acute or chronic disease, and recognition of this disease is increasing. In addition, there is evidence that certain serological types of *E. coli* are responsible for acute enteritis in newborn infants and calves(3). For a given host, therefore, wide variations in virulence of different strains of the same species may exist. Distinguishing characteristics have been ascribed to potentially virulent strains of *E. coli*. These have included necrosis of the skin of rabbits and of cells in culture, hemolysis of horse erythrocytes, ability to elicit the Shwartzman phenomenon, greater mouse virulence, and O-inagglutinability(4,5). These properties have not led to well-defined distinctions between virulent and non-virulent strains. To these may now be added resistance of strains to bactericidal action of normal human serum. It would seem likely, therefore, that virulence results from an association of various properties, rather than from a single characteristic.

Materials and methods. E. coli strains. Most of the cultures and the information concerning their source were received through the generosity of Dr. W. H. Ewing, Enteric Bacteriology Unit, Communicable Disease Center, U.S.P.H.S., Chamblee, Ga., except for 0114 serotypes which were kindly provided by Dr. Joan Taylor, Central Public Health Lab., London. Most of the cultures were isolates from normal healthy children or from infants with diarrhea. *Sera.* Several pools of sera from normal humans were tested. Each pool was composed of equal volumes of sera of 4 individuals whose sera lacked detectable *E. coli* agglutinins against the test organisms. Tests with the different pools invariably gave