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PREFACE

In 1968 we published the collected papers of the first Hungarian conference on endotoxins, as volume 12 of the Annales Immunologiae Hungaricae, that was held in 1967 in the „Frédéric Joliot-Curie" National Research Institute for Radiobiology and Radiohygiene in Budapest-Budafok. In 1968 we arranged an international conference on the biochemistry of lipopolysaccharides, which was organized by Professor K. Rauss and held in Pécs, Hungary. The collected papers in this volume represent the third conference. These conferences, as well as the lectures on endotoxins delivered at the usual meetings of the Immunological Section of the Hungarian Society of Microbiologists, illustrate the increasing interest in endotoxin research in Hungary.

I would like to express our thanks to the Medical Section of the Hungarian Academy of Sciences, to the Hungarian Society of Microbiologists, especially to Professor G. Ivánovics, the President of our Society, to Professor K. Rauss, and to Professor V. Várterész, Director of our Institute, for sponsoring this conference. I would also like to thank the scientific staff and the technical co-workers of our Institute for their helpful assistance in the organization of the conference.

It is especially pleasant to thank Professor L. J. Berry for his active participation in the conference and for this interest in the production of this volume. I am intebted to Dr. Vida Beaven and Dr. George N. Eaves of the National Institutes of Health for their valuable assistance in editing this volume. My special thanks are due Mrs. Edith Stefániay for the care and devotion with which she assisted whith the preparation of the original manuscripts and to Mrs. Helen Marie Smith, National Institutes of Health, for the typing of the final volume.

Budapest – Budafok, December 1971.

Lóránd Bertók

EFFECT OF ENDOTOXIN ON METABOLIC REGULATION IN MICE[1]

By

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The work I wish to present summarizes some of our efforts to analyze the metabolic effects of bacterial endotoxin in mice. This is a formidable undertaking because the many metabolic pathways in the mammalian organism make it difficult to know where to begin the study.

It would have been impossible to initiate the experimentation on a rational basis were it not that in 1954 *Geller et al.* [11] found that cortisone protected mice against the lethal effect of bacterial endotoxin. Subsequently it was recognized that, in addition to the lethal effect, most biological effects of endotoxin are suppressed or eliminated by concurrent treatment with the hormone. Therefore, it must be assumed that endotoxin and cortisone have antagonistic actions in the mammalian host, including actions that are metabolic. It thus becomes important to learn how cortisone is able to protect animals against nearly all types of stress. Professor *Hans Selye*, a former Hungarian now in Montreal, has contributed significantly to our understanding of the function of the adrenal cortex in animals responding to stress.

Since about 1960, a number of investigators have focused their studies on the regulatory effect of adrenal cortical hormones on certain hepatic enzymes. There are several liver enzymes that are known to be rapidly induced by cortisone or hydrocortisone. Among these enzymes is tryptophan oxygenase, which converts tryptophan, in combination with molecular oxygen, into formylkynurenine. A noninducible enzyme present in excess in liver converts formylkynurenine into kynurenine, which, through a series of reactions, gives rise to nicotinamide, a compound necessary for the synthesis of NAD and NADP, the important coenzymes in energy metabolism.

A second enzyme induced by cortisone is tyrosine aminotransferase, which converts tyrosine into a product that is utilized, finally, in the genera-

[1] These investigations were supported by grants from the National Institute of Allergy and Infectious Diseases (National Institutes of Health) and the National Science Foundation.

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tion of energy and which also functions in gluconeogenesis. Other inducible enzymes are phosphoenolpyruvate carboxykinase and pyruvate carboxylase, both of which occupy pivotal roles in gluconeogenesis, a process of especial importance in stressed animals. All of the aforementioned enzymes will double or triple within three of four hours in rats, rabbits, men—and apparently in most mammals—given cortisone or hydrocortisone. The guinea pig is an exception; it fails to respond to an adrenocortical hormone, for some unexplained reason. It has been established that the inducible enzymes increase in activity as a result of an augmentation in protein (enzyme) synthesis.

With this background as a guide, I will describe some of the results of our research. Most of the investigations that I will describe here have been concerned with the enzyme tryptophan oxygenase. A dose of intraperitoneally-injected endotoxin that results in 46 percent survival kills only three percent of mice that were also given cortisone subcutaneously. If the injection of cortisone is given four hours after the endotoxin, no increase in survival results [3]. These results are shown in *Table I*, which illustrates that the effect of endotoxin becomes irreversible within four hours under these conditions. With larger doses of endotoxin or with smaller amounts of cortisone, irreversibility occurs earlier. Thus, metabolic changes within the mice must have progressed to a point where the hormone can no longer exert its influence *Fig. 1* shows the behavior of tryptophan oxygenase in livers of mice given either cortisone or endotoxin alone and the two concurrently [5]. The enzyme assays, which utilize a liver homogenate to which tryptophan and hematin are added, are incubated in an oxygen atmosphere in a water bath shaker maintained at 37 °C. The reaction is stopped by the addition of metaphosphate and, after centrifugation, the amount of kynurenine that accumulates is measured spectrophotometrically at 360 nm. Activity is expressed as micromoles of kynurenine formed per gram dry weight of liver per hour.

The induction of the enzyme by 5.0 mg cortisone is illustrated by the upper curve in *Fig. 1*, while the lowering of the activity of the enzyme by the LD_{50} of endotoxin is seen in the bottom curve. Even more clearly shown is the ability of endotoxin to greatly suppress hormonal induction when the two substances are given concurrently, as illustrated by the middle curve. Nevertheless, it is evident that activity of the enzyme is maintained at a higher level, under these conditions, than with endotoxin alone.

Tryptophan oxygenase is induced not only by cortisone but also by its substrate, tryptophan.

Table I

Reversal by cortisone of endotoxin lethality in mice

Treatment	Number of living mice/total injected	Percent survival
Endotoxin* alone	18/39	46
Endotoxin +5 mg cortisone acetate	39/40	98
Endotoxin +5 mg cortisone acetate injected 4 hours later	16/33	48

* 1 LD_{50} endotoxin used throughout these experiments

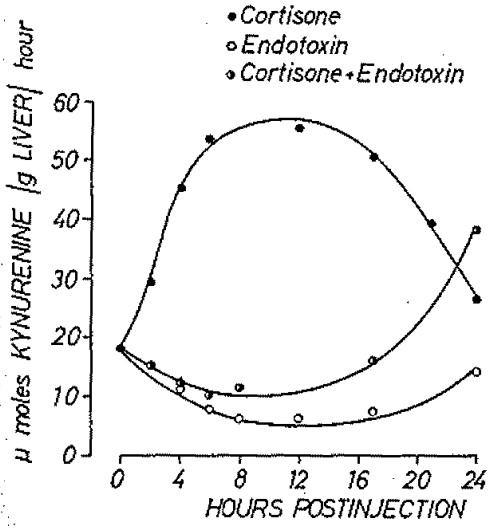

Fig. 1. Tryptophan oxygenase activity in livers of mice injected at time zero with 5 mg of cortisone acetate subcutaneously (closed circles), or the LD_{50} of endotoxin intraperitoneally (open circles), or both substances (half open-half closed circles

Table II

Liver tryptophan oxygenase activity after injection of mice with tryptophan and endotoxin

Treatment	Tryptophan oxygenase activity (μmoles kynurenine per g liver per hour)
Control	21.4
1 hour after 20 mg tryptophan	58.4
1 hour after 20 mg tryptophan + 1 LD_{50} endotoxin	58.1
4 hours after 1 LD_{50} endotoxin and 1 hour after 20 mg tryptophan	33.4

Table II. shows that one hour after the intraperitoneal injection of 20 mg of tryptophan the activity of the enzyme is increased nearly three-fold. It is well established that induction by substrate is transitory and does not involve *de novo* synthesis of enzyme. The enzyme increases in activity when tryptophan is injected because breakdown is prevented and because an inactive form of the enzyme is activated. Activity returns to normal, for example, within three to four hours after injection of the amino acid.

The inability of endotoxin to prevent substrate induction of tryptophan oxygenase is also shown by the data presented in *Table II.* These experiments were done by a former student, *Dr. Robert J. Moon* [15]. It is of interest that a delayed injection of tryptophan, given four hours after endotoxin, results in less than half as much increase as that seen after concurrent injection of the two substances. One might conclude as a possible interpretation that endotoxin after four hours has lowered the rate of enzyme synthesis.

Mice injected with tryptophan four hours after the LD_{50} of endotoxin begin to die convulsively within less than an hour [15]. We

Table III

Augmentation of endotoxin lethality by delayed injection of tryptophan

Treatment	Number of living mice/total injected	Percent survival
Endotoxin* alone	41/70	59
Endotoxin +15 mg tryptophan	35/60	58
Endotoxin +15 mg tryptophan injected 4 hours later	14/70	20

* 1 LD_{50} endotoxin used throughout these experiments

Table IV

Prevention by pretreatment with cyproheptadine of the augmented lethality that results from delayed injection of tryptophan

Treatment	Number of living mice/total injected	Percent survival
Endotoxin* alone	26/40	65
Endotoxin +1 mg serotonin injected 4 hours later	4/20	20
Endotoxin +15 mg tryptophan injected 4 hours later	0/20	0
5 mg cyproheptadine +endotoxin injected 4 hours later +15 mg tryptophan injected 8 hours later	11/20	55

* 1 LD_{50} endotoxin used throughout these experiments

first discovered this during an attempt to determine whether the maintenance of tryptophan oxygenase activity at a normal, or above level bymeans of repeated injections of substrate would augment survival. The reverse was observed. All mice died rapidly. Typical results are presented in *Table III*.

Since tryptophan can be converted into 5-hydroxytryptamine (serotonin) as well as be oxidized by tryptophan oxygenase, we decided to investigate whether this occurred in mice injected with the amino acid four hours after endotoxin. The results are shown in *Table IV* [15]. Concurrent injections of endotoxin and tryptophan result in the same mortality as that seen with endotoxin alone. Delaying the injection of tryptophan significantly augments the number of deaths. Notice that 1 mg of serotonin given four hours after endotoxin increases the mortality of the mice. The same amount of serotonin given alone was sublethal and when it was given at the same time as endotoxin no increased lethality ensued. In this set of experiments, a delayed injection of tryptophan killed all mice, while pretreatment with an antiserotonin drug, cyproheptadine, prevented the augmented mortality. This is seen in the last line of *Table IV*. The several interpretations of these results include the possibility that endotoxin lowers the *in vivo* activity of tryptophan oxygenase so that more of the injected tryptophan is converted to serotonin.

Concern with whether the *in vitro* measurement of tryptophan oxygenase reflects events that occurred *in*

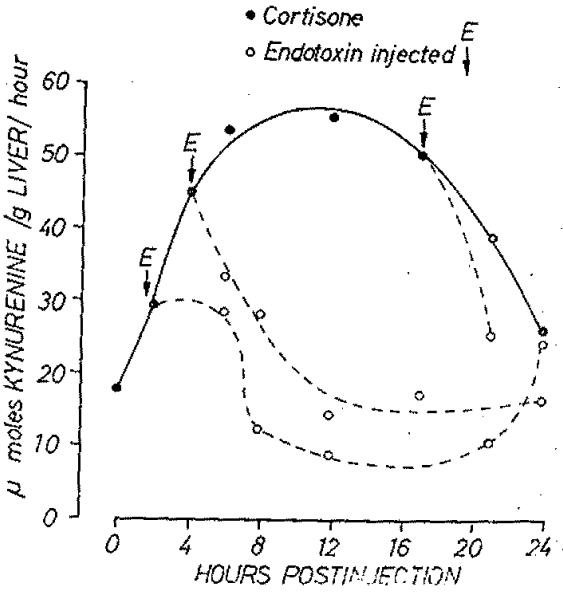

Fig. 2. Tryptophan oxygenase activity in livers of mice injected at time zero with 5 mg of cortisone acetate subcutaneously (closed circles) and at 2, 6, or 18 hours with the LD_{50} of endotoxin intraperitoneally (open circles)

vivo led us to compare the effect of endotoxin on the inductive process with that of actinomycin D, a well known inhibitor of protein synthesis. The data illustrated in *Fig. 2* show the result with endotoxin [5]. The same results were obtained with the an-tibiotic. When either substance was injected two, six, or 18 hours after 5 mg of cortisone, the increase in enzyme activity was stopped or rapidly diminished. The differences between the control value and the value observed four hours after endotoxin are the same in the first two situations, and it is also the same four hours after the injection at 18 hours. It looks as if a fixed amount of inhibition (total cessation of synthesis?) results.

In an attempt to obtain direct experimental evidence for an effect of endotoxin on hepatic protein synthesis, *Dr. Thelma Shtasel*, another former student, initiated a series of experiments [18] based on reports in the literature that hydrocortisone increases RNA synthesis, as measured by a greater incorporation of isotopically labeled orotic acid into the RNA isolated from livers of rats [10]. Our similar determinations utilized mice, and endotoxin was included in the studies. Typical results are summarized in *Table V*. Under the specific conditions shown, endotoxin increased RNA synthesis almost twice as much as cortisone. The two substances together gave results similar to those seen with cortisone alone. This is another example of the antagonistic action of these substances.

Hepatic protein synthesis, as measured by the incorporation of (^{14}C)leucine into acid insoluble polypeptides, was decreased by cortisone and increased by endotoxin, while the two combined were without measurable effect. These results are shown by the data presented in *Table VI*.

These observations merit special comment. Endotoxin is known to induce a proliferation of reticuloendothelial cells in liver and other organs, while cortisone results in atrophy. In an organ as complex as the liver, substances such as those under study could have different effects on different tissues and, indeed, on different proteins. It would be erroneous to conclude, therefore, that endotoxin does not inhibit the synthesis of tryptophan oxygenase, and possibly other enzymes, because it results in a net

Table V

Increased incorporation of (^{14}C)orotic acid into liver RNA of endotoxin-poisoned mice

Treatment	Specific radioactivity*	Percent change
Control	462	
5 mg cortisone	667	+45
1 LD_{50} endotoxin	861	+86
Cortisone + endotoxin	637	+38

* Each value based on assay 2 hours after treatment and 45 minutes after injection of (^{14}C)orotic acid.

Table VI

Increased incorporation of (^{14}C)leucine into liver protein of endotoxin-poisoned mice

Treatment	Specific radioactivity*	Percent change
Control	252	
5 mg cortisone	210	−16
1 LD_{50} endotoxin	330	+31
Cortisone + endotoxin	265	+ 5

* Each value based on assay 2 hours after treatment and 45 minutes after injection of (^{14}C)leucine

increase in the total protein moiety of the liver. Until it becomes possible to determine the effect of endotoxin on leucine incorporation into tryptophan oxygenase alone, uncertainty will remain.

All of the experiments described to this point were done with single doses of endotoxin (the LD_{50}) and of cortisone acetate (5 mg). In a mouse, 5 mg of cortisone is a massive amount, it is approximately the same weight as the two adrenal cortices in a 20—25 g animal. To be sure, the low solubility of this form of the hormone almost assures that the effective amount is much less than that injected. An examination of the site of injection after an interval of 24 hours reveals the presence of a significant amount of residual hormone. The 5 mg dose was chosen because of an earlier observation that mice are protected against endotoxin lethality by that amount but not at all by 1 mg [8]. We decided, therefore, to conduct a dose-response study in order to ascertain the least amount of cortisone acetate that would cause a statistically significant induction of tryptophan oxygenase and the least amount of endotoxin that, in turn, would inhibit the induction. *Table VII* shows that 50 μg of cortisone acetate caused induction of the enzyme (with no hormone the activity is about 20 units), while 1.0 μg of endotoxin (LD_{50} is about 400 μg) significantly reduced the activity of the enzyme when given with either 50 or 100 μg of hormone but not with 5.0 mg.

Table VII

Relation of concentration of cortisone to induction of tryptophan oxygenase in the presence of endotoxin

Amounts of cortisone	Tryptophan oxygenase activity* (μmoles kynurenine per liver per hour)	
	Control	1.0 μg endotoxin
50 μg	30.2	17.7
100 μg	48.8	30.7
5 mg	49.0	43.5

* Each value based on assay 6 hours after injection of cortisone and endotoxin

We explored the dose-response relationships further by comparing intraperitoneal and intravenous injections of endotoxin using the least amount of hormone that could be detected: 25 μg. The results are given in *Table VIII*. Here it becomes apparent that as little as 0.01 μg of endotoxin, 1/40.000 of the LD_{50}, when given intravenously, significantly lowered the hormonal induction of tryptophan oxygenase. By the intraperitoneal route of injection, ten times as much endotoxin was required for comparable results. As reported in the same

Table VIII

Relation of concentration of endotoxin and route of injection to induction of tryptophan oxygenase in the presence of cortisone*

Amounts of endotoxin	Tryptophan oxygenase activity† (μmoles kynurenine per g liver per hour)	
	intraperitoneal	intravenous
0	22.8	24.7
0.01 μg	§	15.3
0.05 μg	24.9	9.4
0.10 μg	12.6	§

* 25 mg cortisone injected subcutaneously.
† Each value based on assay 6 hours after injection of cortisone and endotoxin.
§ Not done, since 0.05 μg endotoxin intraperitoneally did not inhibit induction and because 0.05 μg intravenously did inhibit induction of the enzyme

publication from which these data were derived [6], this biochemical effect of endotoxin appears to be linked directly to the mouse-toxicity of the specific preparation under study.

In this presentation, as indicated previously, the focus has been on one inducible enzyme, tryptophan oxygenase. Tyrosine aminotransferase is induced by endotoxin in intact mice but not in adrenalectomized animals. The activity of this enzyme has not been detectably influenced by the bacterial poison [5]. The activity of phosphoenolpyruvate carboxykinase does not seem to be lowered by endotoxin alone, but its induction by cortisone is inhibited [6]. This is believed to be related to the rapid loss in carbohydrate reserves after an injection of endotoxin [7]. Pyruvate kinase, an enzyme that is inhibited by cortisone, increases in activity after an injection of endotoxin. This last enzyme has been studied in my laboratory by two former students (unpublished observations of *Harriet Coblentz* and *Eugene Morris*), and similar results have been obtained by *Dr. Irvin S. Snyder*, University of Iowa (personal communication). These changes would help explain the loss in carbohydrate seen in endotoxicosis.

Our ultimate objective in conducting these studies is to explain how endotoxin exerts its effect on the inducible enzymes. Once this can be accomplished, a real advance in understanding a basic problem in host-parasite interactions at the molecular level will have been made.

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Discussion

Dr. Bertók: In the published results of your studies of the metabolic effects of ethionine, actinomycin D, and other compounds on the development of tolerance and on the induction of enzymes, you reported that ethionine had no effect on the development of endotoxin tolerance. We found, however, that chronic ethionine treatment (0.36 percent ethionine in a half methionine-deficient diet) decreased or abolished tolerance induced by a single intravenous injection of 100 μg endotoxin in rats (Annales Immunologiae Hungaricae *12:* 133, 1968). How do you explain this discrepancy?

Dr. Berry: As Dr. Bertók mentioned, in a number of our experiments different inhibitors of protein synthesis were found to sensitize mice to endotoxin. Ethionine, an analog of methionine, was employed in these experiments, as were 8-azaguanine, 2-thiouracil, actinomycin D, and chloramphenicol [3]. Chloramphenicol is not a very good inhibitor of protein synthesis in mammalian cells, and it was the only compound in this group that failed to sensitize mice to endotoxin to prevent protection with cortisone. These results offer good evidence that synthesis of protein—synthesis of enzymes—is essential for the normal response of an animal to endotoxin. Actinomycin D prevented the development of tolerance, but ethionine, under the conditions of our experiments, did not. I cannot explain why, nor do I know why our results differ. Ethionine is known to be more than just an analog of methionine, but it is not as good as actinomycin D in preventing protein synthesis [4].

Dr. Bertók: I would like to emphasize that in your experiments the ethionine was given in a single dose. In our experiments, however, it was given in a half methionine-deficient, semi-synthetic diet. This resulted in

a chronic state. Induction of endotoxin tolerance was not attempted until the animals had been on the diet 14 days. At that time the protein metabolism of the rats was really disturbed. The initial body weight had decreased about 20—25 per cent. A single large dose of ethionine, as you know, can provoke an acute toxicosis, but no characteristic antimetabolite-induced methionine deficiency. Perhaps this is the explanation for the discrepancy between your experiments and ours.

Dr. Berry: Yes, I agree. Our procedure for making mice tolerant was to give a small dose of endotoxin daily from Monday through Saturday. An injection of the LD_{50} of endotoxin on the following Monday killed no mice. The same amount of endotoxin failed to lower the activity of tryptophan oxygenase. Neither ethionine nor actinomycin D at dose levels that lower tryptophan oxygenase in normal mice had an equivalent inhibitory effect in tolerant mice [4]. This aspect of tolerance has not been explained, but it is an intriguing observation.

Dr. Kontrohr: What has been done with tryptophan oxygenase in tissue culture systems?

Dr. Berry: Hepatoma cells grown in a tissue culture system have been found to have very low tryptophan oxygenase activity, and the enzyme is noninducible with hydrocortisone [16]. Dr. Joyce Greene and I have done some interesting experiments, however, with liver slices. In slices from normal mice the initial activity is essentially normal. It decreases with time and the addition of neither cortisone nor endotoxin alters the course of the decline. If slices are prepared from livers of mice one hour after an injection of cortisone and put into tissue culture, the enzyme will at least double in activity over a period of four to six hours. If endotoxin is added to the medium, induction of the enzyme is significantly lowered. The enzyme in a perfused whole liver behaves essentially as it does in the intact animal [12].

Dr. Fachet: What is the activity of this enzyme in endotoxin tolerant animals?

Dr. Berry: It is about normal or slightly higher than normal.

Dr. Fachet: Do you think there is any role for this enzyme in the tolerant state?

Dr. Berry: That is a difficult question to answer. The development of tolerance can be prevented by inhibiting protein synthesis, which we have done acutely with actinomycin D [4] and which *Dr. Bertók* has observed in chronic poisoning with ethionine. The effect could be attributed to an inhibition of antibody synthesis or formation of an endotoxin-detoxyfying enzyme. It might also be due to a stabilization of tryptophan oxygenase or to some other inducible enzyme[s]. The whole question is unresolved.

Dr. Fachet: If most of the endotoxin is in the blood, then why is the enzyme inhibited? The effect must be mediated. It could result from changes induced in lysosomal membranes or in receptor sites that combine with the endotoxin to release active particles.

Dr. Berry: Yes, there are a number of possibilities. *Dr. Moon* and I (unpublished observations) prepared lysosomal enzymes in quantity and injected them into normal mice. We were attempting to see if it were

possible to imitate the effects of endotoxin by this procedure. We found nothing. Perhaps the pH at the site of action was too high; possibly the enzymes never reached the sites of effectiveness. A positive result would have been informative, but a negative finding is, of course, meaningless.

Dr. Ralovich: What is your opinion about the role of endotoxin in infectious diseases, especially enteric diseases? Why is endotoxin not toxic after oral administration?

Dr. Berry: The role of endotoxin in an infection caused by Gram-negative organisms has not been resolved. We have done a number of experiments in attempts to determine whether changes induced by endotoxin appear in infected mice. The answer is yes for mice infected with *Salmonella typhimurium* [2], but the relationships are complicated. In our experiments, induction of tryptophan oxygenase in infected mice, after an injection of cortisone, is lower than normal and suggests that endotoxin is present. Other measurements based on the ingestion of food and the intake of water, for example, point to the same conclusion. Whatever we have done indicates that infected animals have endotoxemia. They are poisoned. I should say, however, that the results of experiments by *Dr. Sheldon Greisman* and his associates indicate that endotoxin plays no role in infections caused by Gram-negative organisms [13]. In reference to orally administered endotoxin, one of my former students, *Dr. J. J. Previte*, has shown that a large enough dose of endotoxin (several milligrams) given orally kills a mouse. Although between 10—100 times as much orally-administered endotoxin is required for this [17], such quantities are probably present in some enteric infections. Living bacteria in the intestinal tract get into the blood stream and many are killed, probably by mesenteric phagocytes. *Dr. Kocsár* and *Dr. Bertók* have shown that in bile deficient rats radioactive endotoxin passes into the blood stream. They have been able to kill rats in this way [14]. There seems to be no doubt, therefore, that endotoxin gets through the intestinal wall. The question, then, is 'how much endotoxin gets through?' It is also important to know whether it is the living or the dead bacteria that yield endotoxin. The extent of absorption of endotoxin and the function of bile in the process are important points yet to be resolved.

Dr. Kováts: Have you done any experiments with species other than mice?

Dr. Berry: We have done some experiments with rats and rabbits, which behave the same as mice [1]. Tryptophan oxygenase is lowered by endotoxin and its induction by cortisone is inhibited in poisoned animals. The enzyme in guinea pigs is not induced by cortisone and the hormone does not protect guinea pigs against endotoxin. *Chedid* had found this earlier [9].

Dr. Rédey: Can you influence the local effects of endotoxin, such as local haemorrhagia?

Dr. Berry: We have done no experiments of that type.

Dr. Csizér: Have you done any experiments with endotoxin from rough mutants or with chemically-detoxified endotoxin?

Dr. Berry: Yes, one of your former countrymen, *Dr. A. Nowotny*, has provided me with one of his detoxified preparations. When we administer

it in proportion to its LD_{50}, we get the same results as those obtained with the same proportional dose of a toxic preparation [6].

Dr. Merétey: Is enzyme induction in germ-free animals the same as in conventional animals? Is there any correlation between monoamineoxydase and the other liver enzymes?

Dr. Berry: We have done no experiments with germ-free mice, or with monoamineoxydases. There are some reports in the literature concerned with the response of germ-free mice to endotoxin, but they are contradictory and not easily interpreted.

IMMUNOLOGICAL INVESTIGATIONS OF THE STRUCTURE AND ANTIGENS OF SHIGELLA SONNEI AND SEROLOGICALLY RELATED BACTERIA

By
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For more than twenty years it has been known that a serologically distinct intermediate form appears during the course of the mutation from smooth (S)-form to rough (R)-form in *Shigella sonnei*. The investigators who described the phenomenon called this intermediate form "phase II" in order to distinguish it from the parent S-form (phase I) and from the classical R-mutant. Actually, the description of this intermediate form included the first reference to the fact that the S-R mutation in enterobacteria takes place step by step, that it is a process of dissociation, and that serologically distinct mutants can be isolated on the basis of the rate of dissociation.

Although our investigations have followed several directions during recent years, only a brief account of the main lines of our studies is presented here. In the first part of our survey, the serological and chemical analyses of the lipopolysaccharide antigens of the S-form (phase I), phase II, and R-form of *Sh. sonnei* were accomplished. It was shown that the serologically distinct antigens of phase I and phase II are quite similar in chemical composition, qualitatively and quantitatively. The antigen of the R-mutant, however, was shown to possess a qualitative distinction in its lack of galactose. Consequently, we initiated an analysis of the structure of these lipopolysaccharides. Previous experiments had revealed that this lipopolysaccharide, in contrast to that from other enterobacteria, is extremely labile to acid hydrolysis. Hence, oligosaccharides could not be obtained by the usual techniques. Rather, acetolysis was found to be the most suitable method. Because of the high content of lipids in the lipopolysaccharides, however, we considered it preferable to start the procedure with the *Freeman*'s degraded polysaccharide hydrolyzed by acetic acid. The degraded polysaccharide was found to be a heterogeneous material: three fractions were obtained by the use of Biogel P-2 and by Sephadex G-10 columns, and the distribution of sugar components in the particular fractions was investigated subsequently. The results revealed that *Freeman*'s degrada-

Table I

Distribution of the sugar constituents of the Shigella sonnei phase I lipopolysaccharides

Sugar	Percent sugar constituent in			
	"Lipid A"	Fraction 1	Fraction 2	Fraction 3
Galactose	16	52	13	20
Glucose	22	74	3	0.5
Glucosamine	86	13	0.5	2
Heptose	30	70	1	0.5
KDO*	—	70	5	26

* KDO = 2-keto-3-deoxy-D-mannooctonic acid

tion did not completely free the polysaccharide components of the molecule from the so-called "lipid A", and that a significant amount of low molecular weight fragments was formed even by weak acidic treatment *(Table I)*. An analysis of the structures of the oligosaccharides is being conducted at the present time.

With the aid of phages, we have attempted to isolate additional R mutants that represent the different phases of the biosynthesis of the lipopolysaccharides. The T phages of *Escherichia coli* were used for the isolation of four previously unknown mutants that differ from each other chemically as well as serologically, as in the R mutants of *Salmonella*. Unfortunately, these mutants were not stable. Consequently, we are attempting to isolate more stable mutants by other methods.

In another aspect of these studies, we used the results of serological and chemical tests to make a comparison of the antigens of the A and R mutants of Bader's *Pseudomonas shigelloides* and Ferguson's *Aeromonas shigelloides* C-27, which are identical serologically with the phase I and phase II antigens of *Sh. sonnei*. While the antigenic structure of the phase I antigen of *Sh. sonnei* is completely identical serologically to that of the S strain of *Ps. shigelloides* C-27, the antigen of the R mutants is identical serologically with the phase II antigen of *Sh. sonnei*. On the basis of chemical analyses, we found that the fractions of lipopolysaccharides from *Ps. shigelloides* and *A. shigelloides* C-27 corresponded qualitatively to the lipopolysaccharides of phase I and II of *Sh. sonnei*, respectively, except that the lipopolysaccharide of *Ps. shigelloides* and that of the strain C-27 contain galacturonic acid, as shown in *Table II*. The latter was quite surprising, since only a few lipopolysaccharides are known that contain uronic acid. The uronic acid was identified by chromatographic and microchemical methods. The lipopolysaccharides and the degraded polysaccharides derived from them were analyzed by chromatography on ion exchange gel. All of the serologically active peaks contained D-galacturonic acid. We concluded, therefore, that the galacturonic acid is a component of the lipopolysaccharide, but that it does not have a function in the formation of serologic specificity. The percentage of other sugars in the lipopolysaccharides of the serologically related bacteria differed in comparison to the lipopolysaccharide of *Sh. sonnei*, but the ratio of sugar components within these related antigens was quite similar to the ratios found in the lipopolysaccharides of *Sh. sonnei (Table II)*.

An antigen that is independent of the lipopolysaccharides was discovered in the phase II and R mutants of *Sh. sonnei*. Since it was demonstrated

Table II

Approximate ratios of the sugar constituents of Shigella sonnei and serologically related bacteria

	Galactose	Glucose	Glucosamine	Heptose	KDO*	Galacturonic acid
Shigella sonnei, phase I	1	1	1	2	2	—
Shigella sonnei, phase II	0.5	1	1	2	2	—
Shigella sonnei, R-form	—	1	1	2	3	—
Pseudomonas shigelloides, S-form	1	1	1	2	2	1
Pseudomonas shigelloides, R-form	0.5	1	1	2	2	0.5
Aeromonas shigelloides C-27, S-form	1	1	1	3	3	1
Aeromonas shigelloides C-27, R-form	0.5	1	1	2	2	0.5

* KDO = 2-keto-3-deoxy-D-mannooctonic acid

by the bacterial agglutination of serum absorbed by lipopolysaccharide, this antigen is referred to as "bacterial agglutinogen". This agglutinogen is characteristically bound firmly to the cell wall, and it is not extracted by the use of routine methods for preparing antigens. It is not inactivated by lysozyme or by protein-splitting enzymes, such as trypsin, pepsin, papain, pronase, and subtilisin. The agglutinogen is fairly stable in an acidic medium, at about pH 2; however, it is inactivated by treatment with weak alkali, at pH 8.5, for one hour at 37 °C. The ultimate purification of this agglutinogen would depend, of course, upon our ability to measure the material quantitatively. In this case, the usual haemagglutination test would not be suitable, because it has not been possible to solubilize the antigen. Consequently, the antigen cannot be used to sensitize red blood cells for an haemagglutination test.

INCREASED SERUM RIBONUCLEASE ACTIVITY DURING ENDOTOXIN SHOCK IN RATS

By

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The results of earlier studies from this laboratory [4] demonstrated that whole-body irradiation, as a shock-eliciting factor, provoked a significant increase in the serum ribonuclease activity of rabbits. Since it is possible that bacterial endotoxins released from intestinal bacteria may have an important function in this response to irradiation, we have attempted to determine whether endotoxin could produce a change in the serum ribonuclease activity of rats.

A total of 120 female rats of Wistar origin, weighing 100 to 150 g, were used in these investigations. The experimental materials used included *Escherichia coli* 0 89 lipopolysaccharide, Westphal-type endotoxin prepared by the warm phenol-water method [7], ribonucleic acid prepared according to the phenolic method of *Kirby* [3], and lead acetate supplied by the Fischer Scientific Company (Fair Lawn, New Jersey, USA).

The activity of acidic serum ribonuclease was measured by the method of *Kalnitsky*, *Hummel*, and *Dierkes* [2]. Blood samples were taken from the retroorbital venous plexus of rats according to the technique of *Riley* [5].

Endotoxin shock was provoked by the intravenous injection of 10 mg of endotoxin into each rat. The serum ribonuclease activity was measured immediately before and three hours after the injection of endotoxin. This time interval was selected because the animals were in severe endotoxin shock three hours after the injection of endotoxin. Since it is well known that the values for serum ribonuclease activity are rather variable, we determined the enzyme activity of 75 normal rats at each experimental interval. (Double controls were used in all subsequent experiments.) Immediately before the injection of endotoxin the average value of the serum ribonuclease activity, expressed as UV absorbance at 260 nm, was 14.23 $\pm$4.10. The results of these experiments are presented in *Fig. 3*, where it can be seen that the serum ribonuclease activity increased significantly after the administration of endotoxin ($P < 0.01$).

We also attempted to determine whether induced tolerance to endotoxin would prevent the increase of serum ribonuclease activity while protecting the animal from endotoxin shock. Tolerance to endotoxin was induced by the intravenous injection of 100 μg of endotoxin. The amount of endotoxin used to induce tolerance caused no change in the level of serum ribonuclease activity ($P < 0.50$), as shown in *Fig. 4*. Moreover, there was no increase in the level of serum ribonuclease activity after the injection of the challenging dose (10 mg) of endotoxin.

Since previous investigations in this laboratory have resulted in the demonstration that the administration of normal, well tolerated amounts of lead acetate to rats causes a hypersensitivity to endotoxin [1, 6], we measured serum ribonuclease activity in animals given lead acetate with a small amount of endotoxin. First of all, we investigated the effect of lead acetate alone on serum ribonuclease activity. As shown in *Fig. 5*, a single intravenous injection of 5 mg of lead acetate caused a nonsigni-

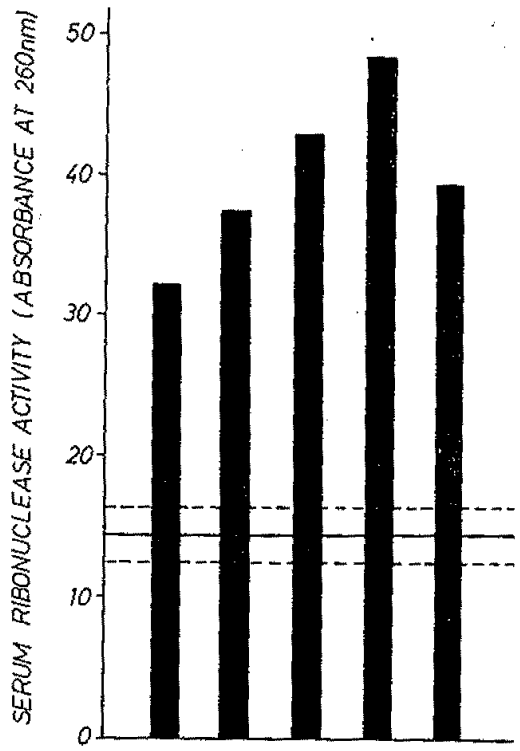

Fig. 3. Increased serum ribonuclease activity of rats in endotoxin shock. The continuous line represents the mean value of the controls (n = 75); the dotted lines represent the standard deviation of the mean. Each column represents an individual value of ribonuclease activity an the serum from a single rat 3 hours after in intravenous injection of 10 mg of endotoxin

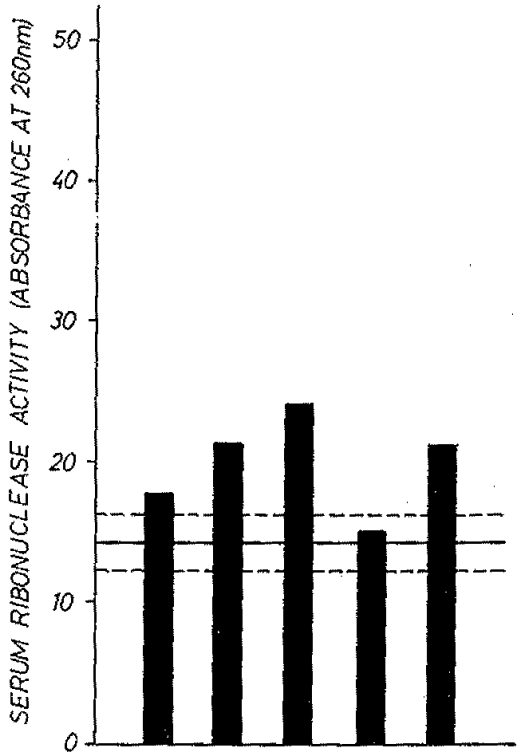

Fig. 4. Normal serum ribonuclease activity of endotoxin-tolerant rats after injection of amount of endotoxin used to induce tolerance. The continuous line represents the mean value of the controls (n = 75); the dotted lines represent the standard deviation of the mean. Each column represents an individual value of ribonuclease activity in the serum from a single rat 3 hours after an intravenous injection of 100 μg of endotoxin

ficant ($0.05 < P < 0.10$) decrease in serum ribonuclease activity. When the lead acetate was given with 3 μg of endotoxin, however, the serum ribonuclease activity was increased to a value almost as high as that observed in endotoxin shock, as shown in *Fig. 6*.

In conclusion, we suggest that the observed changes in the levels of serum ribonuclease activity after the administration of endotoxin under various experimental conditions could represent a secondary consequence of a primary damaging effect of endotoxin. Specifically, the serum ribonuclease activity measured in these experiments may originate from lysosomes damaged by endotoxin. There was no increase, however, in ribonuclease activity in the serum of endotoxin-tolerant animals that were challenged with an amount of endotoxin that normally elicits shock. The results suggest the possibility that there may be an increase in ribonuclease activity during shock caused by various factors and, perhaps, during anaphylactic shock.

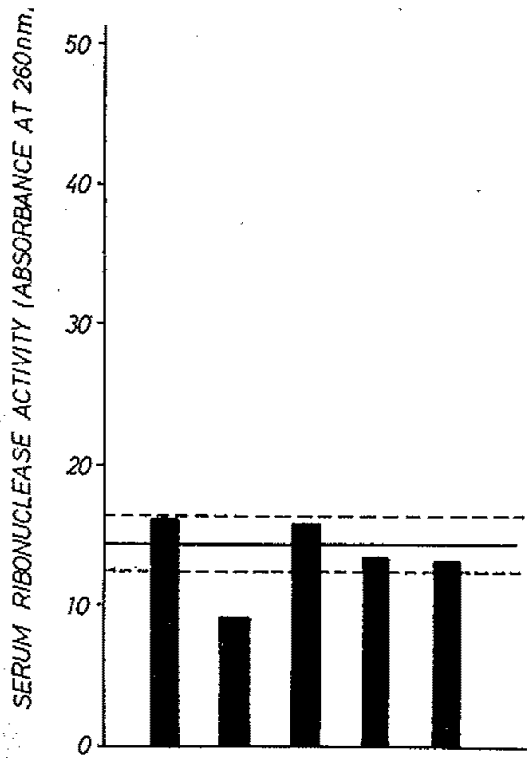

Fig. 5. Normal serum ribonuclease activity of rats after an intravenous injection of lead acetate. The continuous line represents the mean value of the controls (n = 75); the dotted lines represent the standard deviation of the mean. Each column represents an individual value of ribonuclease activity in the serum from a single rat 3 hours after an intravenous injection of 5 mg of lead acetate

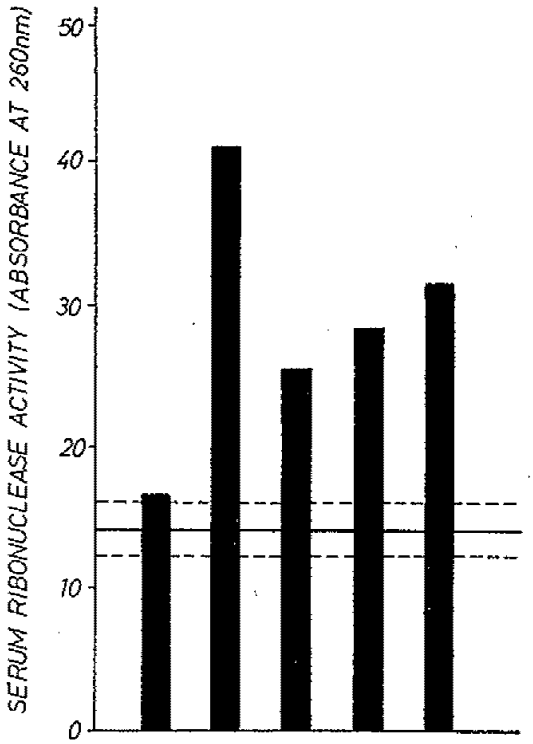

Fig. 6. Increased serum ribonuclease activity of rats after injection of lead acetate and a minute amount of endotoxin. The continuous line represents the mean value of the controls (n = 75); the dotted lines represent the standard deviation of the mean. Each column represents an individual value of ribonuclease activity in the serum from a single rat 3 hours after an intravenous injection of 5 mg of lead acetate and 3 μg of endotoxin

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Discussion

Dr. Berry: Since endotoxin shock can produce the same effect as the irradiation, what is your opinion about the origin of the enzyme activity in endotoxin shock? Why do you suppose the enzyme activity does not change in endotoxin-tolerant animals?

Dr. Kutas: We suppose that the increased enzyme activity originates from damaged lysosomes in endotoxin shock. Endotoxin tolerance may prevent damage to the lysosomal membranes. Tolerance may actually stabilize the membranes.

PLASMINOGEN-PLASMIN SYSTEM IN COMPLEMENT ACTIVATION INDUCED BY ENDOTOXIN

By

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The activity of bacterial endotoxin that affects the most important functions of an organism is only partly due to a direct toxic effect of endotoxin; most of the activity is the consequence of the indirect action of mediators. Although several chemical mediators have been described, such as catecholamines, serotonin, and histamine, their effect is only similar to that of endotoxin.

Previous reports [4, 7] and our experiments [3] indicate that the endotoxin-induced activation of the complement system is one of the important processes responsible for the resulting endotoxin effect. This consideration led us to investigate more intensively the mechanism of the complement reaction induced by endotoxin. In previous investigations [3], we attempted to determine the function of natural antibodies in the course of the reaction. The experiments reported here represent our attempts to clarify any relationships between the endotoxin-induced activation of the complement system and that of the plasminogen system. *Serratia marcescens* endotoxin was prepared by the method of *Boivin* and *Mesrobeanu* [1]. The activity of the plasminogen-plasmin system was measured by euglobulin lysis time according to the method of

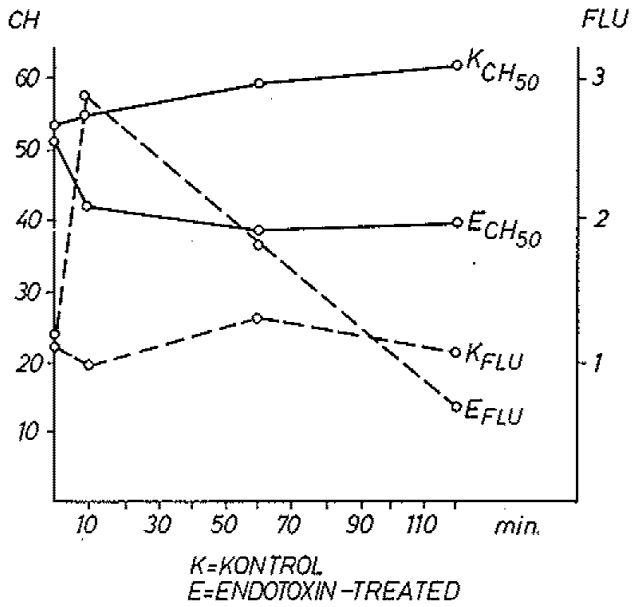

Fig. 7. Effect of endotoxin on the complement level (CH_{50}) of dog serum and on the fibrinolytic activity (FLU) of dog plasma *in vivo*. (Mean values of eight experiments)

Table I

Increased fibrinolytic activity and decreased complement titer 10 minutes after the administration of endotoxin

Experiment	Increased fibrinolytic activity* (x)	Decreased complement titer* (y)
1	465	60
2	259	20
3	54	40
4	45	23
5	560	42
6	253	59
7	42	28
8	6	4

* Percentage of control values

Correllation coefficient (r)

$$(r) = \frac{Qxy}{\sqrt{Qx \cdot Qy}} = \frac{22{,}865}{28{,}800} = 0.7939$$

$(p < 0.02)$

von Kaulla [9]. Lysis time was converted to a fibrinolytic unit by the method of *Sherry et al.* [8]. Complement was titrated according to the method already described [2], except that the optimum time for the lysis of canine complement was 60 minutes.

A total of eight mongrel dogs weighing from eight to 11 kilograms was used in the experiments. In the control experiments, the dogs were injected intravenously with 1.8 ml of pyrogen-free isotonic saline per kilogram body weight and, after a week, they were injected intravenously with *Serratia marcescens* endotoxin of the same volume. Blood was withdrawn before and 10, 60, and 120 minutes after each injection; 3 ml of citrated blood and 4 ml of blood without an anticoagulant were collected at each interval. The citrated blood was used immediately for the determination of the lysis time of euglobulin. The blood without anticoagulant was stored at −18 °C until the following day, when complement was titrated.

Fig. 7. shows the changes in complement titer and fibrinolytic activity in the controls and in the animals given endotoxin. Within 10 minutes after the administration of endotoxin the complement titer decreased significantly and the low value remained unchanged during the period of investigation. Within 10 minutes after the administration of endotoxin the fibrinolytic activity increased almost twofold and then decreased until at 120 minutes the value was lower than that of the control. (Values for each sample of blood withdrawn 10 minutes after the administration of endotoxin are shown in *Table I.*) Our previous investigations [3] have shown that activation of the complement system is the result of an endotoxin-induced decrease in complement titer; the results of the present experiment indicate that within 10 minutes after the administration of endotoxin both the complement system and the plasminogen-plasmin system become activated.

In the course of our previous experiments [3], several disorders associated with blood clotting were detected in samples of blood from endotoxin-treated dogs. These changes always paralleled the decrease in complement titer and could be correlated with the activation of the plasminogen-plasmin system. On the basis of our experimental results and the reports of others [5, 6], we assumed that the plasminogen-plasmin system was at least partly responsible for the endotoxin-induced activation of the complement system. The results of our present experiments have supported this assumption and demonstrated a relation between the increase of fibrinolytic activity and the activation of the complement system. Of course, the involvement of other mechanisms cannot be excluded.

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DIMINISHED TURNOVER OF PLASMA IRON IN PIGS AFTER INJECTION OF ENDOTOXIN

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The "coli toxicosis of piglets" (coli diarrhea or oedema disease) often occurs simultaneously with iron deficient anaemia or develops as a consequence of decreasing resistance. The coincidence of these two disease states and the recognition that bacterial endotoxins can induce an experimental hypoferremia in laboratory animals [1, 2] prompted our attemps to deter-

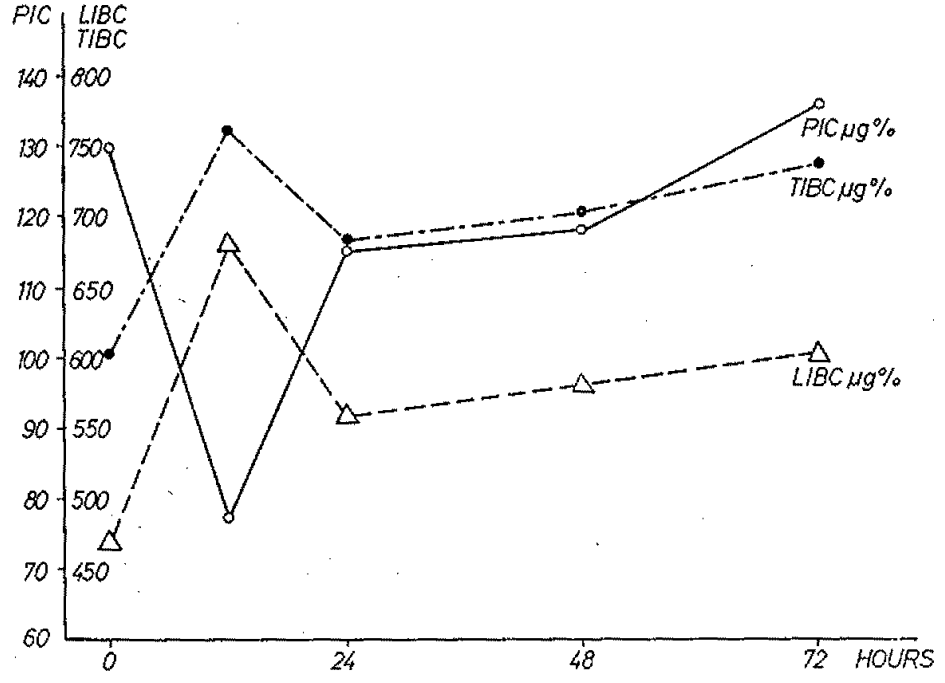

Fig. 8. Effect of endotoxin on plasma iron capacity (PIC), latent iron-binding capacity (LIBC), and total iron-binding capacity (TIBC) of pigs 6 weeks of age injected intreperitoneally with a single dose of 0.3 mg of endotoxin per kg body weight. PIC, LIBC, and TIBC were measured at the time of the injection of endotoxin and at 12, 24, 48, and 72 hours afterwards

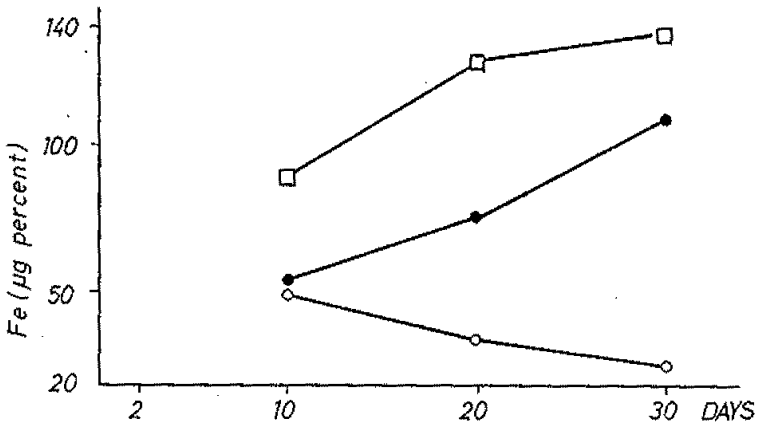

Fig. 9. Concentration of plasma iron in pigs given iron-dextran complex. In the negative or normal control (open circles), the pig was given neither additional iron nor endotoxin. In one of the positive controls (closed circles), the pig was given a single dose of 150 mg of iron-dextran complex intramuscularly on the third day after birth, but no endotoxin. In the second positive control (open squares), the pig was given a dose of 150 mg of iron-dextran complex intramuscularly on the third and tenth days after birth

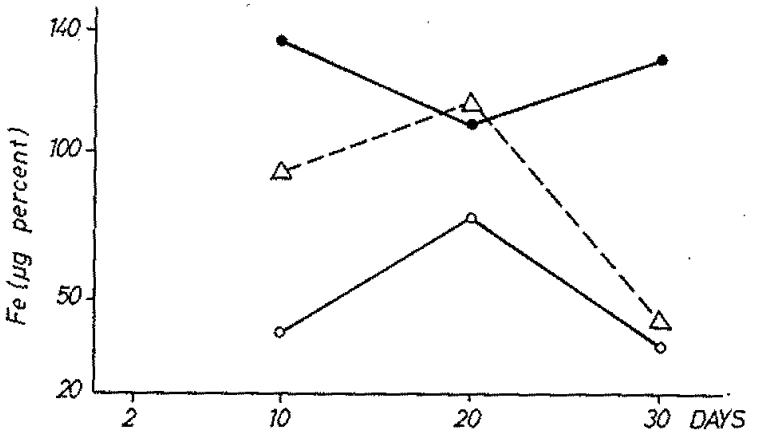

Fig. 10. Concentration of plasma iron in pigs given continuous doses of endotoxin. All experimental pigs were injected subcutaneously with 0.1, 0.2, and 1.0 mg of endotoxin per kilogram body weight daily during the first, second, and third weeks after birth, respectively. Open circles and open triangles = the level of plasma iron in each of two pigs given a single dose of 150 mg of iron-dextran complex intramuscularly on the third day after birth; closed circles = the level of plasma iron in a pig given a dose of 150 mg of iron-dextran complex intramuscularly on the third and tenth days after birth

mine how and by what mechanism *Escherichia coli* endotoxin affects the concentration of plasma iron and the uptake of iron by plasma in pigs. *Escherichia coli* 0 89 lipopolysaccharide — Westphal-type endotoxin [3] —was used in all experiments.

Each suckling pig was given 150 mg of iron-dextran intramuscularly either once, on the third day after birth, or twice, on the third and tenth days after birth. The control animals were not given additional iron.

In the first experiment, our aim was to assess the amount of iron in the plasma and the amount of iron taken up by the plasma after the injection of endotoxin. Pigs (6 weeks of age) were injected intraperitoneally with a single dose of 0.3 mg of endotoxin per kg body weight. Plasma iron and iron uptake were measured at the time of the injection of endotoxin and every 12 hours afterwards. The average values are presented in *Fig. 8*, which shows that by 12 hours after the injection of endotoxin the level of plasma iron had decreased by approximately 50 percent, whereas the latent and total iron-binding capacity of the plasma in-

creased. The changes in plasma iron capacity, latent iron-binding capacity, and total iron-binding capacity that resulted from a single dose of endotoxin lasted only 24 hours.

In the second experiment, we determined the fluctuations in the concentration of iron in the plasma of piglets injected subcutaneously with endotoxin: 0.1, 0.2, and 1.0 mg of endotoxin per kilogram body weight daily during the first, second, and third weeks, respectively. One animal, the negative control, was given neither additional iron nor endotoxin; one animal, a positive control, was given a single dose of iron-dextran but no endotoxin; and one animal, a second positive control, was given two doses of iron-dextran but no endotoxin. Fluctuations in the level of plasma iron are shown in *Fig. 9*. During the three weeks, the level of plasma iron decreased continuously in the negative control animal, increased moderately in the positive control given a single dose of iron-dextran, and increased distinctly in the positive control given two doses of iron dextran. As shown in *Fig. 10*, the first week after the administration of endotoxin, the level of plasma iron in one of the experimental animals given a single dose of iron-dextran complex was very low; by the twentieth day after birth there was a slight rise; and after injection of a massive dose of endotoxin (1 mg/kg body weight), there was an abrupt decrease. In the second experimental animal given a single dose of iron-dextran complex, the level of plasma iron remained close to normal until it decreased after the administration of the massive dose of endotoxin. In the experimental animal given two doses of iron-dextran complex, there was a slight increase in the level of plasma iron after the massive doses of endotoxin. These results, which were confirmed by repeated experiments, indicate that two large (150 mg) doses of iron-dextran complex negated the decrease in plasma iron mediated by massive doses of endotoxin.

In the third experiment, our aim was to examine the uptake and release of plasma iron in pigs given endotoxin 6 weeks after birth. ([^{59}Fe]dextran complex was injected intravenously; all other experimental conditions were the same as

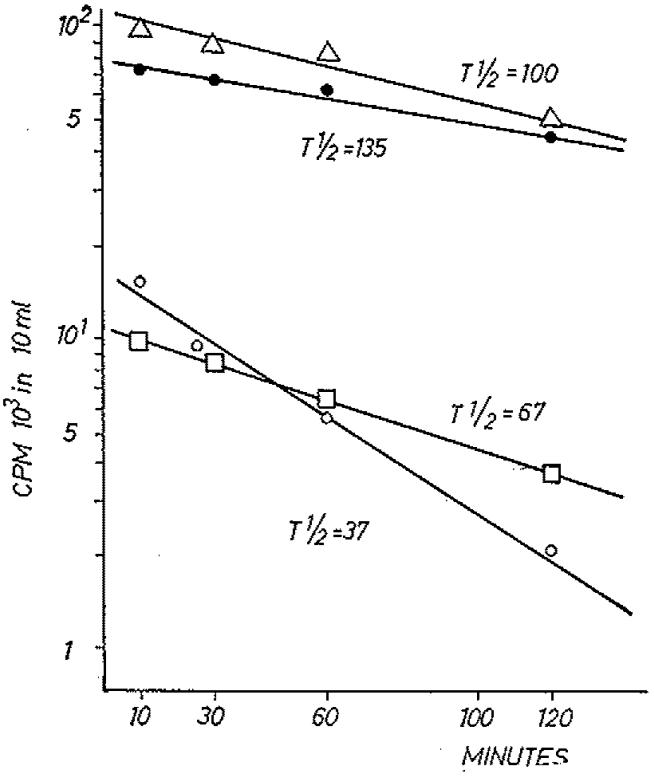

Fig. 11. Release of ^{59}Fe from the plasma of pigs injected intraperitoneally with 0.3 mg endotoxin per kilogram body weight. Open triangles and closed circles = animals injected with endotoxin; open circles and open squares = controls; and T 1/2 = half-life time of ^{59}Fe in minutes

for the first experiment.) As shown in *Fig. 11*, iron was released fairly rapidly ($T^1/_2$=37 and 67 minutes) from the plasma of pigs not given endotoxin. In contrast, iron was released more slowly ($T^1/_2$=100 and 135 minutes) from the plasma of pigs previously treated with endotoxin.

Previous studies have shown that the rate of plasma iron turnover was nearly identical in pigs either given endotoxin or not given endotoxin, but that the incorporation of iron (^{59}Fe) into red blood cells was, nevertheless, dissimilar. In pigs given endotoxin, the incorporation of ^{59}Fe increased to 76—87 percent after the effect of endotoxin on plasma iron concentration was no longer apparent, in contrast to an incorporation of only 50.3—60.7 percent in the controls.

In the fourth and last experiment, we observed the release of ^{59}Fe-labeled haemoglobin-iron in pigs given continuous doses of endotoxin, as in the second experiment. The results showed that the level of released ^{59}Fe was higher in the plasma of the control animals than in the pigs given endotoxin.

In relation to the controversial explanation of the mechanism by which the endotoxin-induced hyposideremia develops, the results of our experiments on pigs have shown that *E. coli* endotoxin effects not only an increase in haemolysis but also an inhibition of the reutilization of the iron released from the erythrocytes into the plasma. Therefore, in summary, we have demonstrated that endotoxin diminishes the rate of the release of haemoglobin iron into the plasma and that, correspondingly, the rate of incorporation of iron into the erythrocytes is increased after the apparent expiration of the effects of endotoxin. Thus, we suggest that the hyposideremia which occurs after the injection of *E. coli* endotoxin results from a decrease in the reutilization of haemoglobin iron and that this endotoxin-mediated decrease may be sufficient for the development of the so-called infectious anaemia.

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Discussion

Dr. Bertók: Although *Dr. Horváth* was interested in the effects of endotoxin on the metabolism of iron, *Dr. Bereznay* and I found, in a preliminary experiment conducted a few years ago, that iron-deficient animals were more sensitive to endotoxin than were normal animals. I think that the enhanced effect of endotoxin on iron-deficient animals may be related primarily to haemodynamics.

ENDOTOXIN-MEDIATED DECREASE IN CONTENT OF RNA, DNA, AND PROTEIN OF SPLEEN AND LYMPH NODES IN NEONATALLY THYMECTOMIZED RATS IMMUNIZED WITH SHEEP ERYTHROCYTES

By

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Miller and *Osoba* [11] reported that inbred mice thymectomized on the first day of life developed a wasting-syndrome which was characterized by a retardation of growth, a decreased number of small lymphocytes in the blood an lymphatic organs, and and increased number of reticular elements. In our earlier experiments, however, neonatal thymectomy led to a wasting-syndrome in only 15 or 20 per cent of Wistar rats [6], which agrees with the earlier report of *Jankovic*, *Waksman*, and *Arnason* [7]. Almost every animal that died of the wasting-syndrome showed evidence of bacterial infections [1]. Moreover, neither the wasting-syndrome nor the hyperactivity of the reticulo-endothelial system occurred in neonatally thymectomized animals maintained in germ-free conditions [3, 10, 15]. Consequently, we have concluded that neonatally thymectomized animals maintained in a conventional environment fail to develop an essential immunological defense against the effects of toxins and infectious agents.

It has been demonstrated that bacterial endotoxins increase nonspecific resistance, as well as humoral immunological reactions, in rodents [8]. As part of our goal to pursue this phenomenon in detail, we have determined the content of RNA, DNA and protein of spleen and lymph nodes in neonatally thymectomized rats before and after injection of endotoxin.

Newborn rats of the CFE (random) strain under light ether anaesthesia were thymectomized or sham-operated 24 hours after birth [6]. Endotoxin was extracted from *Serratia marcescens* according to the method of *Boivin* and *Mesrobeanu* [4]. The final preparation contained the active endotoxin of 4×10^9 bacteria in a volume of 2 ml. Animals were given simultaneous injections of endotoxin (0.1 ml/100 g body weight) intraperitoneally and of 10 percent thrice-washed sheep erythrocytes (0.5 ml/100 g body weight) intravenously. Blood samples were taken at different times after injection

of endotoxin and immunization with erythrocytes. On the fifteenth day after immunization, the rats were given a second injection of erythrocytes. On the eighteenth day, the animals were decapitated and the spleens and the six lymph nodes from the inguinal and iliac regions were removed immediately and weighed. Part of the spleen and two or three of the lymph nodes were processed for histological examination, the detailed results of which will be reported elsewhere, and the remaining tissue was utilized for the determination of RNA, DNA and protein content [9, 14], the results of which are presented in *Tables I* and *II*.

Table I shows that endotoxin treatment in sham-operated rats resulted in significant increases ($p < 0.01$) in the relative weight of the spleen and in the total content of RNA, DNA and protein. In the neonatally thymectomized rats which had not been given endotoxin, all values were lower than those of the sham-operated controls, and the differences were greater after endotoxin treatment.

The results summarized in *Table II* show that in the sham-operated rats the content of RNA, DNA and protein of the lymph nodes increased after injection of endotoxin but did not increase ($p < 0.01$) in the neonatally thymectomized rats, when compared to the sham-operated controls before the injection of endotoxin.

In summary, we have shown that the content of RNA, DNA and protein of the spleen and lymph nodes is lower in neonatally thymectomized rats than in sham-operated controls. The content of RNA, DNA and protein of the spleen and lymph nodes increased significantly after simultaneous injections of endotoxin and antigen (sheep erythrocytes) in sham-operated rats but did not increase in neonatally thymectomized animals.

Table I

Content of RNA, DNA and protein and weight of spleen of neonatally thymectomized rats injected with sheep erythrocytes or with endotoxin and sheep erythrocytes

Experimental (number of animals)	Body Weight g	Spleen Weight mg/100 g body weight	RNA Content total μg (μg/100 mg)	DNA Content total μg (μg/100 mg)	Protein Content total mg (μg/100 mg)
Sham-operated: erythrocytes (6)	223 ± 8	296 ± 26	398 ± 65 (58.7 ± 2.7)	572 ± 98 (83.8 ± 5.4)	87.2 ± 10.3 (13.09 ± 0.20)
Endotoxin and erythrocytes (5)	222 ± 6	354 ± 5	448 ± 31 (57.6 ± 2.3)	750 ± 37 (95.4 ± 3.1)	100.2 ± 15 (12.79 ± 0.08)
Thymectomized: erythrocytes (6)	218 ± 6	247 ± 18	287 ± 16 (53.5 ± 1.1)	355 ± 36 (66.0 ± 5.9)	72.2 ± 3.6 (12.58 ± 0.23)
Endotoxin and erythrocytes (5)	210 ± 7	210 ± 7	347 ± 24 (56.4 ± 1.1)	476 ± 37 (77.0 ± 3.7)	80.2 ± 4.7 (13.03 ± 0.31)

All values are expressed as the mean ± the standard error.

Table II

Content of RNA, DNA, and protein and weight of lymph nodes of neonatally thymectomized rats injected with sheep erythrocytes or with endotoxin and sheep erythrocytes

Experimental (number of animals)	Body Weight g	Lymph Node Weight mg/100 g body weight	RNA Content total µg (µg/100 mg)	DNA Content total µg (µg/100 mg)	Protein Content total mg (mg/100 mg)
Sham-operated: erythrocytes (6)	223 ±8	59 ±8	52 ±3 (76.7 ±1.3)	105 +7 (91.3 ±3.6)	11.3 ±0.9 (9.59 ±0.36)
Endotoxin and erythrocytes (5)	222 ±6	65 ±5	78 ±6 (55.2 ±0.7)	131 ±18 (92.0 ±7.8)	14.7 ±1.0 (10.47 ±0.18)
Thymectomized: erythrocytes (6)	218 ±6	40 ±4	46 ±6 (52.8 ±3.1)	67 +8 (77.0 ±1.7)	8.9 ±0.7 (10.22 ±0.24)
Endotoxin and erythrocytes (5)	210 ±7	42 ±4	43 ±4 (48.4 ±2.0)	70 ±7 (79.4 ±3.7)	8.6 ±0.5 (9.98 ±0.28)

All values are expressed as the mean ± the standard error.

The observations of *Rieke* [12, 13] that certain essential metabolic responses were impaired in the lymphatic organs of neonatally thymectomized animals may be related to our studies of the relatively lower specific [2] and nonspecific [5] resistance of neonatally thymectomized animals injected with endotoxin. Thus, we suggest that the metabolism of RNA, DNA and protein is impaired in neonatally thymectomized rats because the animals do not develop appropriate metabolic responses in their lymphatic organs when endotoxin is injected simultaneously with the erythrocyte antigen.

ACKNOWLEDGEMENTS. We are indebted to *Dr. E. Stark* for his help and guidance throughout our experiments, to *M. R. Hajtman* for the statistical analyses, and to *I. Szabó* and *I. Moldován* for their valuable technical assistance.

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STIMULATION OF IN VITRO RELEASE OF FREE FATTY ACIDS FROM ADIPOSE TISSUE BY ADENOHYPOPHYSEAL HOMOGENATES FROM ENDOTOXIN-TREATED RABBITS

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The observation that a single injection of endotoxin is followed by an increase in the level of the free fatty acids of serum was first described by *Hirsch et al.* [2] and later confirmed by *Tuzson* and *Kertai* [5], who also showed that the lipid-mobilizing effect of endotoxin was inhibited by drugs that block alpha receptors, by transection of the cervical spinal cord, and by ablation of the hypophysis. We have demonstrated that an injection of endotoxin results in an increased level of the fatty acids produced by interscapular adipose tissue and by the epididymal fat pad. This lipolytic effect of endotoxin was not affected by the denervation of the adipose tissue, but it was inhibited by the removal of the hypophysis. Since the endotoxin had no direct lipolytic effect on the adipose tissue, as demonstrated by *in vitro* experiments, we assumed that the lipolytic effect of endotoxin was indirect and mediated by the central nervous system and the hypophysis.

In our attempts to elucidate the relation of the hypophysis to the lipolytic effect of endotoxin, we injected *Serratia marcescens* endotoxin of the Boivin-type [1] intravenously intomale rabbits weighing 2.5—3 kg and examined the lipolytic activity of the hypophysis in an *in vitro* system that utilized the adipose tissue of a normal (control) rabbit. The amount of fatty acid released by the adipose tissue in-

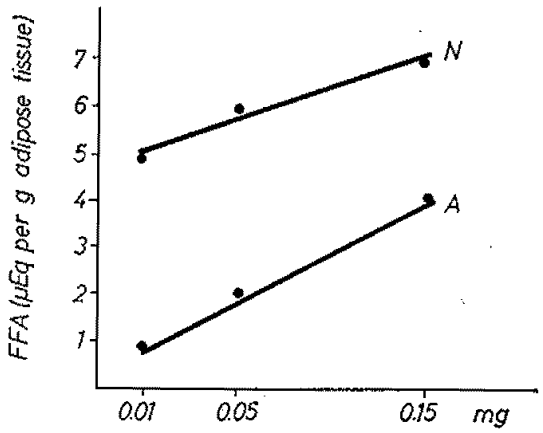

Fig. 12. Relation of concentration (mg) of homogenates of the adenohypophysis (A) and the neurohypophysis (N) to the *in vitro* release of free fatty acids (FFA) from adipose tissue of a rabbit. The amount of FFA released in 3 hours is expressed as μEq per gram of adipose tissue

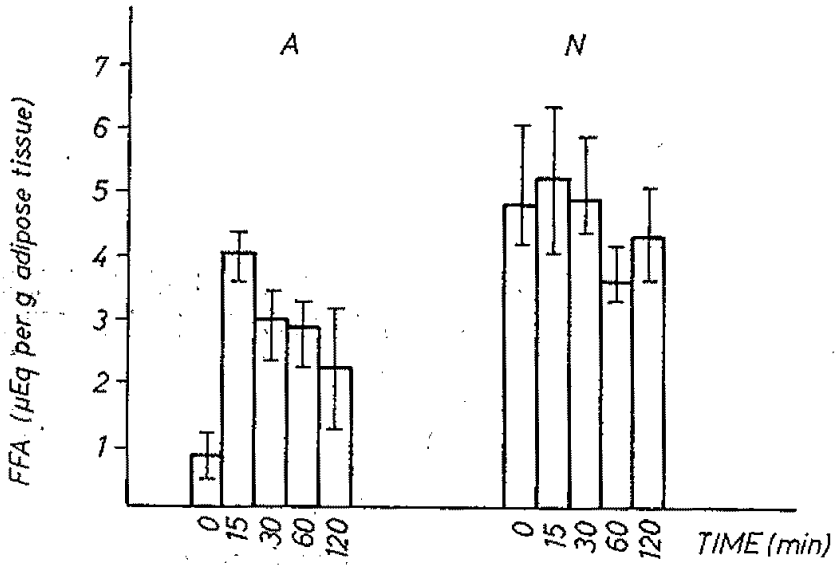

Fig. 13. Augmentation of lipolytic activity of adipose tissue *in vitro* by the adenohypophyseal homogenates (A), but not by the neurohypophyseal homogenates (N), obtained from rabbits at various times after the injection of endotoxin. Lipolytic activity, or the amount of free fatty acids (FFA) released in 3 hours, is expressed as μEq per gram of adipose tissue. The vertical (I-shaped) lines represent the standard error of the mean

cubated for 3 hours at 37 °C was measured according to the method of *Mosinger* [3]. The ACTH activity of the hypophysis was measured according to the method of *Sayers et al.* [4] and compared with international working-standards.

In the initial experiments, we found that addition of the homogenate of the whole hypophysis to the reaction mixture resulted in a considerable increase in the release of fatty acids from the adipose tissue. In order to determine the specific anatomical component responsible for the *in vitro* lipolytic effect, we homogenized the adenohypophysis and neurohypophysis and added different concentrations of each to separate reaction mixtures.

As shown in *Fig. 12*, the release of fatty acids from the adipose tissue was augmented by an increase in the concentration of hypophyseal homogenate added to the reaction mixture. The lipolytic action of the extract of the neurohypophysis was greater than that of the adenohypophysis.

In the subsequent experiments, we removed the hypophysis of rabbits at different times after the injection of 1 LD_{50} endotoxin per kg body weight and measured the lipolytic activity of the neurohypophysis and adenohypophysis as before. As shown in *Fig. 13*, lipolytic activity was markedly increased by the adenohypophyseal homogenate as compared to the control (0 time), but the activity was not changed significantly by the neurohypophyseal homogenate. In each case, lipolytic activity was maximal at 15 minutes after the injection of endotoxin.

The stimulation of lipolytic activity by the adenohypophysis of rabbits injected with endotoxin may be related to an increased amount of peptide I,

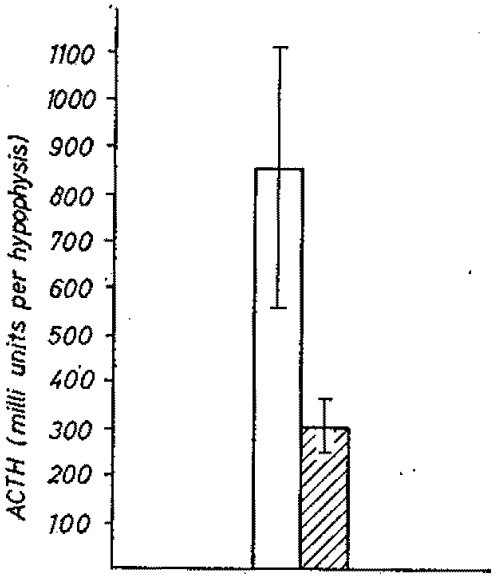

Fig. 14. ACTH content, in milli units, of the hypophysis of hormal rabbits (open bar) and of rabbits injected with endotoxin 15 minutes before sacrifice (bar with cross hatching). The vertical (I-shaped) lines represent the standard error of the mean

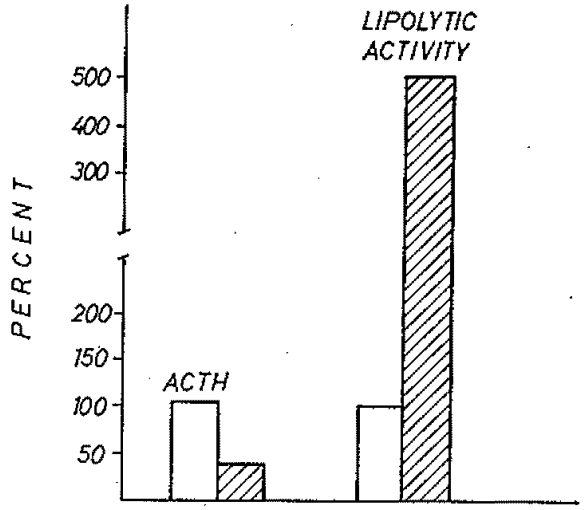

Fig. 15. Percent change in ACTH content of, and stimulation of lipolytic activity by hypophyseal homogenates of normal rabbits (open bars) and of rabbits injected with endotoxin 15 minutes before sacrifice (bars with cross hatching)

of ACTH, or, possibly, of an unknown substance. As shown in *Fig. 14* and *15* participation of ACTH could be excluded. At 15 minutes after injection of endotoxin, when the stimulation of lipolytic activity by the adenohypophysis is maximal, there was no ACTH activity at all. We are currently investigating the possible participation of peptide I and attempting to find the unknown compound or compounds which could be responsible for the stimulation of lipolytic activity.

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ABSENCE OF EFFECT OF ENDOTOXIN ON THE ISOLATED ILEUM OF THE GUINEA-PIG

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There are few available data on the effect of endotoxins on smooth muscle. Although *Urbaschek et al.* [2, 3, 4] have reported that endotoxins derived from *Brucella abortus*, *Brucella melitensis*, and *Escherichia coli* 0 55 and 0 111 increased the sensitivity of the guinea-pig and rat uterus to oxytocin and histamine, they used a perfused recipient to test the possibility of a direct effect of endotoxin on rat uterus. *Wiedermann et al.* [5] described a direct effect of *E. coli* endotoxin on the contraction of the guinea-pig uterus in a perfused recipient, but *Urbaschek* [2] did not observe this effect in a stationary recipient nor did *Goodno* and *Kumar* [1] observe it in rat uterus or strips of human uterus.

Because the available data are inconsistent and because it is not known whether either a direct or an indirect effect of endotoxin on gut muscle is involved in the endotoxin enteritis, I have examined the effect of *E. coli* endotoxin on the isolated ileum of the guinea-pig. In my attempts to demonstrate whether endotoxin had a direct effect on smooth muscle or whether it exerted an indirect (histamine-sensitizing) effect, I utilized the standard techniques: a piece of the ileum was removed and suspended in isotonic Tyrode solution, which contained 1 mg/ml atropine, at 37 °C, and the contractions were recorded on smoked paper.

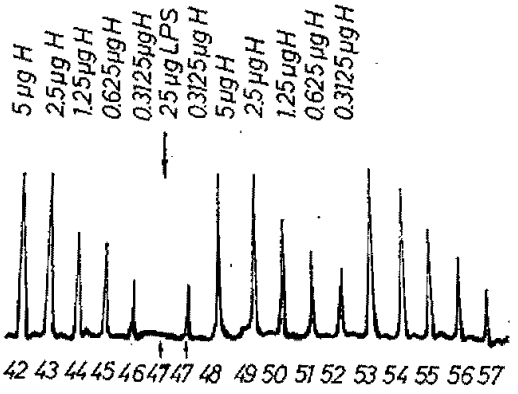

Fig. 16. Contraction of isolated guinea-pig ileum in response to decreasing amounts of histamine (H) in the absence and presence of 25 µg of endotoxin (LPS). The response of the ileum to decreasing amounts of histamine in the perfusion solution was recorded. When the amount of histamine (0.3125 µg) that stimulated contraction just above the threshold was reached, 25 µg of endotoxin was added to the bath; the incubation was continued for 4 minutes, and, without an intervening wash, decreasing amounts of histamine were again tested

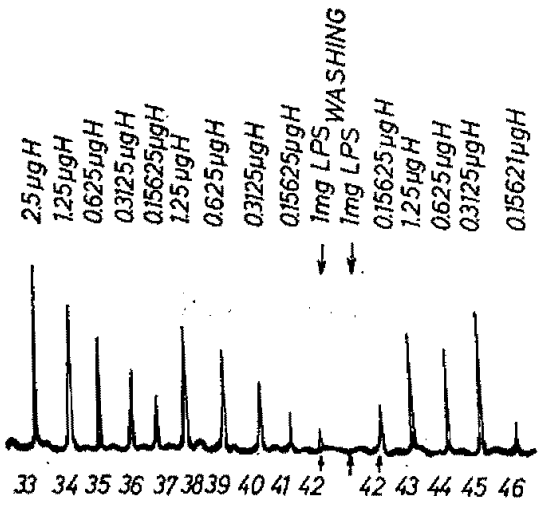

Fig. 17. Contraction of isolated ileum of the guinea-pig in response to decreasing amounts of histamine (H) in the absence and presence of 1 mg of endotoxin (LPS). The experimental conditions were the same as in *Fig. 16*, except that the ileum was washed before the second addition of endotoxin to the perfusion bath

In attempts to demonstrate a direct effect of endotoxin, I employed a perfused-organ bath and opened the bowel so that the mucosa could serve as receptor. The velocity of perfusion was 25 ml per minute. After the spontaneous contraction was recorded, endotoxin was added to the perfusion solution and perfusion was continued for 20–120 minutes. No effect of endotoxin on contraction could be observed in any of 12 experiments.

The results of my attempts to reveal a possible histamine-sensitizing effect of endotoxin are shown in *Fig. 16* and *17* No histamine-sensitizing effect of endotoxin was observed under the experimental conditions described. Thus, in my preliminary experiments, endotoxin had neither a direct effect nor a histamine-sensitizing effect on the isolated guinea-pig ileum.

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Discussion

Dr. Bertók: It is well known that endotoxin has no immediate effect on mast cells. Endotoxin affects mast cells through a delayed (perhaps indirect) reaction; there is no detectable change in the structure of these cells until after 24 hours. In anaphylactic shock, however, mast cells degranulate and disrupt immediately, with the release of heparin, histamine, serotonin, and other active substances. Although this may be but another difference between endotoxic and anaphylactic shock, it may be

relevant to the case of an apparent ineffectiveness of endotoxin on the isolated ileum. Also, in earlier, *in vivo* experiments, our research group, with *Dr. Benedeczky*, demonstrated a peculiar, rapid granulolysis of the cathecolamine-granules in the adrenal medulla of rats given a large dose of endotoxin, but our attempts to demonstrate this in tissue culture always yielded negative results. Perhaps, then, the so-called enterotoxin has a direct damaging effect.

Dr. Semjén: I do not know whether the so-called enterotoxin that was first described by *Smith* and his co-workers can cause a direct damaging effect on the gut, but it can produce a dilatation of ligated loops of the small intestine.

Dr. Bertók: In collaboration with *Dr. Kocsár*, we observed certain alterations, such as bleeding and necrosis, in the ligated segments of small intestine of rats if we washed out the intestinal contents before applying endotoxin. I am not sure, however, that those observations provided any evidence for the direct damaging-effect of endotoxin.

Dr. Kontrohr: An important, and perhaps critical, question here is what kind of endotoxin was used. In a lecture delivered in this Institute, Professor *Nowotny* stated that endotoxin activity depends upon the method of preparation.

Dr. Berry: Yes, this question is extremely important, primarily because we know that the toxicity of different preparations varies considerably.

FUNCTION OF BILE ACIDS IN THE INTESTINAL ABSORPTION OF ENDOTOXIN IN RATS

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Earlier investigations of the mechanism of absorption of endotoxin from the intestines have yielded contradictory results [1, 6, 7, 8, 10, 11]. *Rudbach et al.* [9], in their physico-chemical studies of bacterial endotoxin, demonstrated a reversible inactivation (detoxification and degradation) of endotoxin by sodium deoxycholate *in vitro*. In a preliminary experiment, we found that the intestinal contents, perhaps the surface active agents therein, have an important function in the inactivation and absorption of endotoxin under physiological conditions. Consequently, we have investigated the effect of the biological surface-active agents, such as bile acids, on the absorption of endotoxin in rats.

Tritiated *Westphal*-type endotoxin [13] from *E. coli* 0 89 was prepared in our laboratory according to the method of *Wilzbach* [14]. The specific activity of the tritiated endotoxin was 572 μCi/mg. Bile was collected with a sterile syringe from the gall bladders of starved rabbits immediately after sacrifice. For the production of a bile-deficient state, we canulated, with a polyethylene tube, the common bile-duct of rats anesthetized with Nembutal and fixed the tube to the abdominal wall with an opening on the surface of the skin below the *processus xiphoideus*. The animals were used in experiments one week after the operation.

First, we investigated the effect of bile and of one of the bile acids, sodium deoxycholate, on the absorption of endotoxin from the peritoneal cavity of rats. The bile and the sodium deoxycholate diminished the absorption of tritiated endotoxin 20–50 percent one hour after administration of the endotoxin. Then it was necessary for us to determine whether the radioactivity detected in the blood was associated with labeled endotoxin or with degradation products of the endotoxin preparation. We therefore induced hypersensitivity to endotoxin in rats by injecting 5 mg of lead acetate intravenously [4, 5, 12]. We tested the biological activity (endotoxin content) of the sera of the rats that had received ^{3}H-labeled

endotoxin intraperitoneally three hours before by injecting 1 ml of the serum intravenously into the sensitized animals. The normal rat serum had no effect on the five endotoxin-hypersensitive rats, but the sera of rats that had received ^{3}H-labeled endotoxin caused death in all five of the hypersensitized animals. Thus, the radioactivity measured in the blood of rats that had received ^{3}H-labeled endotoxin must have contained at least 3 or more μg of the endotoxin, since it was demonstrated earlier that 3 μg of endotoxin is necessary for the provocation of a fatal shock in the lead acetate-treated rats [4, 5].

It is known that in normal rats no measurable quantity of endotoxin absorption from the intestinal tract can be detected [1, 2, 3]; however, if we administered the endotoxin perorally to bile-deficient rats, a significant amount of endotoxin absorption could be demonstrated. For example, in one experiment, when 5 mg of the endotoxin was given perorally and 3 hours later 5 mg of lead acetate was injected intravenously, all of the animals died of typical endotoxic shock within 24 hours. The characteristic pathologic changes associated with endotoxin shock were apparent when the animals were subjected to autopsy.

We estimated the absorption of tritiated endotoxin by determining the radioactivity of blood samples at 90 minutes after treatment with endotoxin. In rats experimentally deprived of bile by the use of a bile fistula, 5 to 6 times higher activity could be detected 90 minutes after the peroral administration of ^{3}H-labeled endotoxin. For example, in a typical experiment, the radioactivity in counts per minute of a blood sample from a normal rat was 23.100, whereas the radioactivity in a blood sample from a rat with a bile fistula was 120.500.

Finally, we tried to prevent the intestinal absorption of endotoxin in bile-deprived rats by administering 40 mg of sodium deoxycholate perorally with the endotoxin and estimating the content of endotoxin in the serum by use of hypersensitive animals, as before. Of the six bile-deprived rats given endotoxin perorally and hypersensitized with lead acetate, five died. Of the six bile-deprived rats given endotoxin with 40 mg of sodium deoxycholate and hypersensitized with lead acetate, only two died. Thus, sodium deoxycholate may indeed protect bile-deprived rats from shock induced by perorally administered endotoxin. On the basis of our experiments, we therefore conclude that the bile acids have an important function in the defense of an animal against bacterial endotoxins.

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Discussion

Dr. Fachet: When, after the operation, is an animal suitable for use as an experimental subject?

Dr. Kocsár: From one week to a fortnight.

Dr. Ralovich: Although this experiment provides evidence of absorption of endotoxin, it does not explain the toxic effect of perorally administered endotoxin.

Dr. Bertók: Actually, we do not know what the mechanism is of the intestinal absorption of endotoxin in bile-deprived rats. We have found high radioactivity and biologically active endotoxin in the blood of the bile-deprived rats given ^{3}H-labeled endotoxin, and we have observed fatal enterotoxemia in these animals. Additional experimentation has been impeded because cannulation of the common bile duct and maintenance of the rats that have had this operation are very difficult. We realize, of course, that the demonstration of intestinal absorption of endotoxin in bile-deprived rats is just the first very small step toward the clarification of this complex problem.

Dr. Kontrohr: What is the first effect of sodium deoxycholate on the endotoxin? Since surface active agents could change the dispersity of the lipopolysaccharides, perhaps the higher dispersity indicates a higher toxi-

city because the smaller products of endotoxin could better penetrate the intestinal wall.

Dr. Kocsár: In *Rudbach*'s in *vitro* physico-chemical experiments, it was demonstrated that deoxycholate treatment reversibly detoxified the endotoxin molecule by splitting it into small fragments, but these fragments were not toxic. We were interested in the absorption of the intact macromolecule.

Dr. Semjén: We also conducted similar experiments in rats in which the common bile-duct was ligated. One day or one week after the operation we gave these rats 20–100 mg of endotoxin perorally. These animals were also sensitized by lead acetate, but were we unable to demonstrate any effect of endotoxin. We found, however, that the jaundice which resulted from ligature of the common bile-duct increased the sensitivity of rats to parenterally (intravenously) administered endotoxin.

A THEORY ON THE ACTION OF BACTERIAL ENDOTOXINS

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The complex effects of endotoxin in an animal are difficult to differentiate because most of the effects occur together [17] and are interdependent [30]; however, these effects can be classified. Moreover, the manifestations of different endotoxins are rather quantitatively influenced by differences in the host species and in the state of the native and immunological defense mechanism of the host. Previously, only the purely toxic activity of endotoxin was stressed. Available data indicate that this "primary toxic" effect of endotoxin is on the structures of the adrenergic nervous system—the first manifestation—and also on certain cells. Recently, a hypersensitivity mechanism was also proposed as an explanation for the mode of action of endotoxin. Specifically, some reactions of normal animals to endotoxins are very similar to those induced by other antigens in the specifically sensitized host. In the case of endotoxin, the hypersensitivity seems to be a natural type that is derived from the symbiotic association of the host with endotoxin-producing microorganisms and diseases of the host caused by endotoxin-producing microorganisms [4, 17, 30]. Thus, the effects of endotoxin can be related to both pharmacological and immunological factors in the normal animal: the susceptibility of the host to the primary toxicity and the hypersensitivity of the host in relation to the antigenic (haptenic) character of endotoxin, which was denoted by *Watson* and *Kim* [30] as secondary toxicity.

Our theory on the mechanism of action of endotoxins supposes a close interdependence of the many and complex effects of endotoxin, as illustrated in *Fig. 18*. References to supporting data and explanations concerning the diagram follow.

Endotoxin. In our view, the endotoxic part of the endotoxin molecule (the toxophore group) is a component of the larger 0-antigen complex that has one mutually common antigen-determinant. The effects of endotoxin are also caused by endotoxin obtained from smooth (S) strains and rough (R) antigen-containing mutants [9, 10, 14]. Chemically, the R-antigen differs in a lack of certain sugars [9, 20] in the endotoxin complex. Anti-

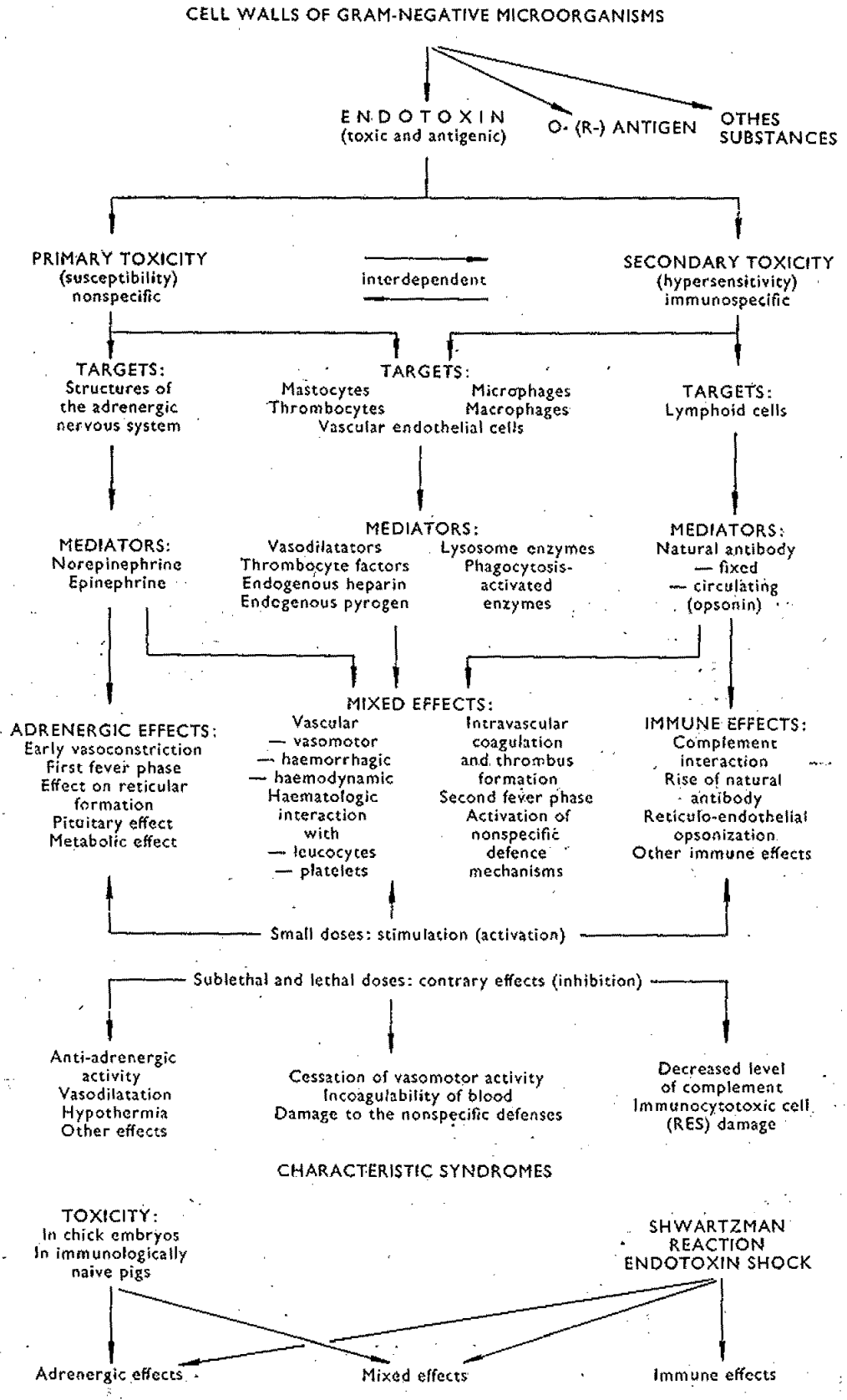

Fig. 18. Interdependence of the many and complex effects of endotoxin

serum induced by endotoxin from an R-mutant can also neutralize the toxicity of endotoxin from the wild strain [14]. According to our experiments, four different O-serotypes of *Escherichia coli* endotoxin [29] can be neutralized by a single type-specific antiserum. At the present time, the terms "lipid A–X" [30] and "glycolipid" [9] seem to be the most acceptable for denoting the portion of the endotoxin macromolecule that is both toxic and antigenic. Thus, the toxicity is very closely associated with the antigenicity or, rather, included in it. Use of less than the full O-antigen may modify the usual endotoxin reactions in normal animals; however, the protein is necessary for the primary sensitization [3].

Adrenergic effects. The targets of the primary toxic effect of endotoxin are the structures of the adrenergic nervous system. Experimental data indicate that epinephrine and norepinephrine are released from all of the adrenergic structures, the nerve-endings, the adrenal medulla, and the chromaffin cells [17]. Mastocytes, platelets, microphages, macrophages, and vascular endothelial cells, however, cannot be excluded as primary targets of endotoxin toxicity. *Thomas* [28] has postulated that the systemic effect of endotoxin is either to increase the reactivity of terminal vessels to epinephrine or to increase the amount of epinephrine released by the adrenal medulla or liberated in the peripheral tissues. Adrenergically-mediated effects of endotoxin have also been observed in isolated organs [6, 12, 17, 23, 27, 28].

Mixed effects. Cellular elements—mastocytes, platelets, microphages, macrophages, and vascular endothelial cells—may also be effected by the primary toxic activity of endotoxin, as well as by the secondary toxicity (hypersensitivity), which would lead to the release of vasoactive substances, coagulation factors, endogenous pyrogen, and enzymes, for example. In view of the relation of these targets and mediators to the adrenergic system and the lymphoid cells, mixed effects are predictable. Other types of cells are also considered by some investigators as targets of endotoxin [1, 2, 6, 17, 22, 23, 26, 27, 28, 30].

Immune effects. Apart from the adrenergic and the mixed effects of endotoxin, purely immunological effects can be distinguished. It is probable that some lymphoid cells other than those with fixed anti-endotoxin antibodies may also contain antibodies that would be released after injection of endotoxin. These antibodies are probably the so-called natural antibodies [19].

Previous experiments have demonstrated that complement has a function in several endotoxin-produced actions. The participation of antibody in the endotoxin-complement interaction is, of course, to be expected. Moreover, the natural antibodies (opsonin) that arise after the injection of small doses of endotoxin also influence the endotoxin effects. It is well documented that reticulo-endothelial opsonization (the host defence mechanism) is based on the interaction of opsonin, complement, and phagocytes stimulated by endotoxin [4, 7, 8, 11, 15, 19, 21, 29].

Sublethal and lethal doses of endotoxin. It is important to consider, in this theory on the interdependence of the complex effects of endotoxin, that massive doses of endotoxin cause effects, in general, that are contrary

to the effects caused by small or moderate doses, as outlined in *Fig. 18*. The specific phenomena associated with endotoxin resistance (tolerance) are not considered in this theory, which relates only to normal, not to endotoxin-pretreated, animals.

Characteristic syndromes. The toxicity of endotoxin in chick embryos [5, 25] may also be considered a purely primary toxic effect. A similar effect has also been produced by epinephrine and norepinephrine, but the mechanism by which this effect was obtained is not yer clear. In addition, toxicity of endotoxin in immunologically naïve pigs that lack detectable immunoglobulins is considered by *Kim* and *Watson* [13] to be a purely primary toxic effect. The participation of the adrenergic nervous system and the cellular targets was considered probable, but not proved.

Shwartzman reaction. Besides the obvious primary toxicity, an immunological mechanism seems to function also in the induction of the *Shwartzman* reaction. Skin hemorrhages that are *Shwartzman* reaction-like can be induced in non-reactive animals pretreated with vaccines of enterobacteria or of endotoxin [16, 24]. In endotoxin-resistant rabbits, an immunological specificity can be demonstrated by the use of heterologous endotoxin in the preparation of the *Shwartzman* reaction. It is suggested [17, 18] that the intravenous provocation of the *Shwartzman* reaction is nonspecific and occurs only at a cellular level.

Endotoxic shock. The endotoxic shock that results from lethal doses of endotoxin includes, apart from the most characteristic hemodynamic changes, all of the effects outlined in *Fig. 18*.

In summary, it is obvious that neither one substance nor one mechanism is responsible for the toxicity or lethality of endotoxin. The relationships between the complex effects of endotoxin are also fairly evident and support our theory that the manifestations of the effects of endotoxin are interdependent.

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Discussion

Dr. Berry: The investigations by *Watson* and *Kim* of the immunological aspects of the effects of endotoxin utilized colostrum-deprived pigs.These studies are extremely interesting and provide the best evidence, that I know, that the toxicity of endotoxin is independent of any response. Essentially, then, *Watson* and his co-workers have shown that endotoxin is toxic in itself.

Dr. Kováts: I agree that these studies of the toxicity of endotoxin is colostrum-deprived pigs raise a very interesting question, but I also recognize that it is very difficult to separate the immunological and toxicological effects of endotoxin in living animals.

SPECIFICITY OF BORDETELLA ENDOTOXINS IN BACTERICIDAL SYSTEMS

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Several immunogenic and pharmacologic effects of *Bordetella* are well known, but only some have been associated with chemically-defined components of the bacteria. The excellent adjuvant effect of *Bordetella pertussis* is attributed to the endotoxin content, as demonstrated by *Farthing* [2], who used purified endotoxin. Until now, however, *Bordetella* endotoxins have not been implicated in immune processes.

Purified endotoxin (LPS) was extracted from acetone-killed bacteria by a modification of the phenol-water method [3]. The yield of LPS from different strains varied from 1.5 to 5 percent. A dose of *B. pertussis* LPS of 1–10 μg/kg body weight caused a 1 °C, or higher, temperature elevation that lasted for 3–4 hours in rabbits; sometimes the dose was lethal. In immunodiffusion and immunoelectrophoretic tests, no precipitation was observed with either the antibacterial sera or with the anti-LPS sera. Binding antibodies were demonstrated by passive haemagglutination in nonimmune, hyperimmune, and anti-LPS sera. For the preparation of antisera to *B. pertussis* and to *B. parapertussis* LPS, rabbits were immunized by two or three injections of complete *Freund*'s adjuvant. A total dose of 35–40 mg of LPS was subsequently given intravenously at intervals of 4–7 days over a period of 4–5 weeks.

The bactericidal activity of immune sera produced by immunization with purified LPS suggested that in the case of *B. pertussis* and *B. parapertussis*, as in the case of other Gram-negative bacteria, the target of the action of the bactericidal antibody was the cell wall LPS [1]. It was thus implied that endotoxin is also on the surface of *Bordetella* cells and that the variation in amount in different strains would account for the variation in sensitivity observed among the strains examined. The bactericidal system has provided confirming evidence that the endotoxins of *B. pertussis* and *B. parapertussis* are of dissimilar specificity, but that the antigenic specificity of the LPS of different *B. pertussis* serotypes are identical.

The bactericidal specificity of anti-LPS sera is shown in *Table I.* The bactericidal activity of anti-LPS sera was eliminated only by absorption

Table I

Bactericidal specificity of Bordetella pertussis LPS antiserum and of Bordetella parapertussis LPS antiserum

Absorption of antiserum with	Strain used in bactericidal system (Origin of LPS antigen)	
	B. pertussis 18323 *(B. pertussis)*	*B. parapertussis* 7088 *(B. parapertussis)*
Salmonella typhi O 901 LPS	+	+
Salmonella typhi O 901 cells	+	+
B. pertussis LPS	—	+
B. pertussis cells	—	+
B. parapertussis LPS	+	—
B. parapertussis cells	+	—

Table II

Bactericidal specificity of "normal" antibodies in guinea-pig serum

Absorption of guinea-pig serum with	Strain used in bactericidal system		
	B. pertussis 18323	*B. parapertussis* 7088	*Salmonella typhi* O 901
Salmonella typhi LPS	+	+	—
Salmonella typhi cells	+	+	—
B. pertussis LPS	—	+	+
B. pertussis cells	—	+	+
B. parapertussis LPS	+	—	+
B. parapertussis cells	+	—	+

of the sera with homologous bacteria or with homologous LPS. The *B. pertussis* LPS antiserum was inactive against *B. parapertussis*, and vice versa.

The demonstration of the anti-LPS nature of "natural" bactericidal antibodies in fresh guinea-pig serum is summarized in *Table II*. The natural antibodies were absorbed by the homologous bacterial strain by the homologous endotoxin, without any decrease in bactericidal activity against the heterologous strain. The same results were obtained when we used non-immune rabbit sera in the bactericidal system.

These experiments confirm our suspicions that the natural antibodies against *Bordetella* that are active in a bactericidal system belong to the anti-LPS type. We postulate that under natural conditions the antigen enter the organism primarily by the oral route and that the immunogenic process is therefore initiated by the endotoxin, which is the component of the cell most resistant to enzymatic and chemical processes.

In addition to the bactericidal activity, we examined the agglutinin and protective-antibody content of the anti-LPS rabbit sera. In the case of *B. pertussis* LPS antiserum, the agglutinins were absorbed by cells of *B. pertussis* strains of different serotypes, but not by LPS. The protective antibodies were absorbed by homologous LPS only [4]. The experimental demonstration of the specificity and anti-LPS nature of the protective antibodies provoked by LPS suggests that in the control of bacterial infection with *B. pertussis* all antibodies capable of *in vivo* binding to the bacterial surface may be protective antibodies. At the same time, the data show that the *Bordetella* endotoxins have an important function not only as an adjuvant substance on the bacterial cell wall, but also as an immunogenic factor, as is the case with other Gram-negative bacteria.

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Discussion

Dr. Kováts: In my opinion, all antigens of microorganisms are responsible for the protective action of antisera, and this action is not necessarily directed toward the toxic part of the endotoxin molecule.

Dr. Pusztai: We know that in *Bordetella pertussis* there is a protective protein-antigen which is active in the mouse and in the human. In the evaluation of the efficacy of a vaccine, the determination of protective value is the most important step. But, on the other hand, we wanted to show that it is not only the protective antibody that can function in the immune processes of experimental animals and humans. In the case of *Bordetella*, for example, the normal bactericidal antibodies also have anti-endotoxin antibodies, as we have been able to demonstrate by using a bactericidal system.

ANTIBODY PRODUCTION IN RATS TREATED WITH ENDOTOXIN

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Neither the nature of endotoxin tolerance [1] nor the function of specific antibodies and nonspecific factors in endotoxin tolerance have been elucidated [9, 21]. Consequently, we have investigated both natural and specific antibody production in rats pretreated with endotoxin.

Female Wistar rats that weighed 150–200 g were used in all experiments. Endotoxin tolerance was induced by a single intravenous injection of 100 μg of endotoxin of either the *E. coli* O 26:B6 Boivin-type (Difco) or the *E. coli* O 18 Westphal-type [23] as prepared in our laboratory [2]. Plaque-forming spleen cells (PFC) were determined by the direct method of *Jerne et al.* [8] and the indirect method of *Dresser* and *Wortis* [4]. Anti-rat-IgG serum was used for the development of indirect plaques with low hemolyzing activity.

The antibody titer in rat sera was determined by micro-methods based on passive haemagglutination and haemolysis [12, 16]. Sensitivity to 2-mercapto-ethanol (2-ME) was studied according to the methods of *Hege* and *Cole* [7]. All ten animals survived that were given 100 μg of endotoxin intravenously 24 hours before an intravenous injection of 10 mg of endotoxin. All ten of the control animals not pretreated with endotoxin succumbed to intravenous injection of 10 mg of endotoxin.

Table I

Direct and indirect counts of plaque-forming cells in spleens of rats after intravenous injection of 100 μg of Escherichia coli O 26 endotoxin

Days after injection of endotoxin	Plaque-forming cells x 10^3	
	Direct Method	Indirect Method
0 (untreated	0.003 ± 0.004 (7)	0.05 ± 0.02 (7)
2	0.16 ± 0.06 (6)	1.66 ± 0.64 (6)
3	0.99 ± 0.82 (6)	350.6 ± 13.0 (6)
4	4.66 ± 2.63 (6)	384.6 ± 9.22 (6)
5	2.38 ± 0.86 (7)	396.3 ± 61.0 (7)

Values represent the mean of counts from the number of animals shown in parentheses ± the standard error

As shown in *Table I*, by two days after an injection of 100 μg of endotoxin the number

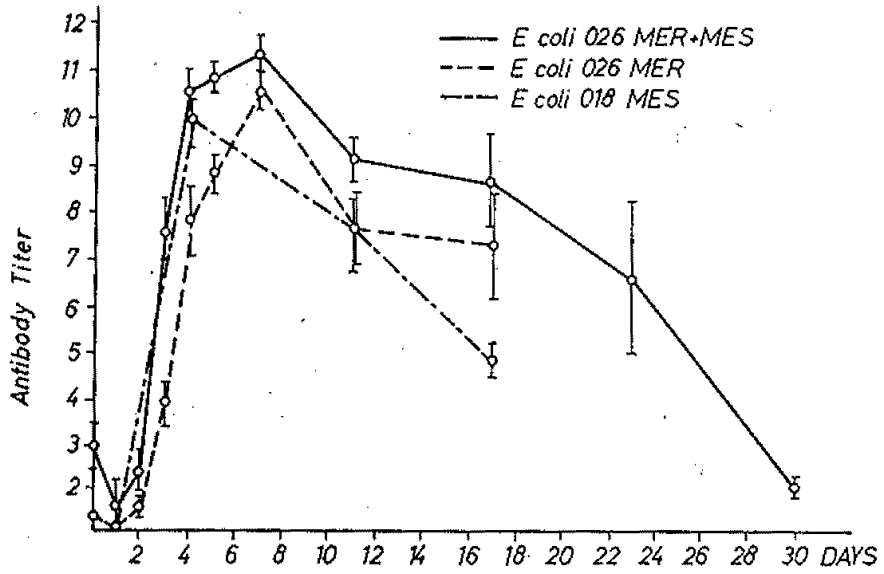

Fig. 19. Specific antibody titers in sera of rats given 100 μg of *E. coli* O 26 or *E. coli* O 18 endotoxin intravenously. MER = resistant to 2-mercaptoethanol; MES = sensitive to 2-mercaptoethanol

of plaque-forming cells in the spleen increased about 50 times by direct count and about 30 times by indirect count. After the peak on the fourth day, when there was a preponderance of plaque-forming cells detected by indirect count, the number of plaque-forming cells detected by both methods was declining.

As shown in *Fig. 19*, the rate of increase in antibody titers in sera of rats treated with endotoxin was slower than the rate of increase of plaque-forming cells. The decrease in natural *E. coli* O 26 haemagglutinin titer after endotoxin injection was succeeded by a significant increase in the titer of 2-ME-sensitive antibody. The titer of 2-ME-resistant antibody also increased by the fourth day and reached a maximum by the seventh day. Similar results were obtained with sera from animals given *E. coli* O 18 endotoxin.

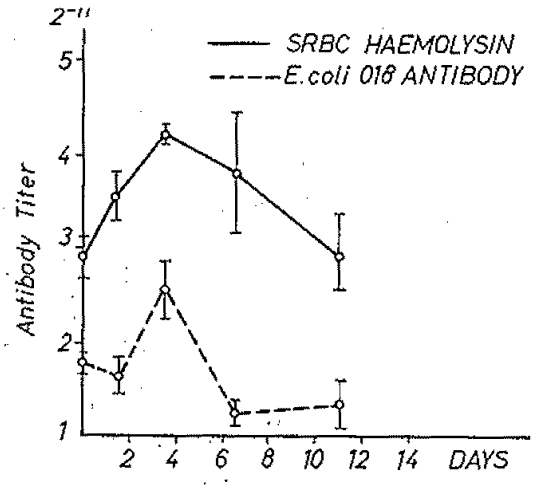

Fig. 20. Nonspecific increase in titers of natural sheep erythrocyte (SRBC) haemolysin and of *E. coli* O 18 antibody in sera of rats given *E. coli* O 26 endotoxin

The titers of natural sheep-erythrocyte haemolysin and of *E. coli* O 18 haemagglutinin in the sera of rats given *E. coli* O 26 endotoxin were also determined. As shown in *Fig. 20*, there was a transient increase in both natural antibody titers.

Two types of antibody-producing cells could be demonstrated by the plaque method. The plaques detected by direct count and the 2-ME-sensitive antibodies may represent the expected

production of IgM-type antibodies [11, 15]. The early appearance of 2-ME-resistant antibodies and of plaque-forming cells determined by indirect count is difficult to explain, although the production of IgG-type antibodies has been observed in other species too [3, 19, 24]. It should be taken into consideration that *E. coli* antibodies resistant to 2-ME could also be found in untreated rats [13]; however, the meaning of results obtained by use of the indirect plaque method is not definitely known [17, 18]. The plaque-forming cells detected by the indirect method are most probably producers of 7S antibody [22, 25]. The high level of 2-ME-resistant antibodies supports our assumption that it were plaque-forming cells of the IgG-type that were detected by the indirect method of counting. Moreover, the kinetics of the immune response seem to indicate a secondary-like reaction, a possibility that has also been discussed by others [10, 20]. Investigations of the action on endotoxin as a non-specific adjuvant [5, 6, 14] yielded results similar to those obtained from studies of the immune response.

In summary, our results suggest that there is a rapid proliferation of different types of antibody-producing cells after endotoxin treatment. It is possible that among these different types of antibody-producing cells there is a type that is responsible for the increased defense mechanism in endotoxin-tolerant rats.

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ENDOTOXIN-LIKE SUBSTANCES FROM FLAVOBACTERIA

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Large numbers of various species of *Flavobacteria* can frequently be demonstrated in foods that have induced enteric disturbances. Only a few cases of enteritis in humans have been associated with these microorganisms, which are usually referred to as saprophytes. The effects associated with such enteric disturbances were considered to be iatrogenic [3].

In our studies of the possible participation of *Flavobacteria* in the induction of enteric disturbances, 100 strains of the microorganisms were isolated from different foods and, on the basis of their biochemical properties (See *Tables I* and *II*), were identified as *Flavobacterium rhenanum*. Endotoxin-like substances were extracted from 9 of the 100 strains by the hot phenol-water method of *Westphal* [4]. The LD_{50} of the endotoxin-like substance was about 0.2 mg, as determined by the intraperitoneal inoculation of adult white mice. Investigations of the biological action of these substances have utilized rabbits, swine, and rats. For the determination of toxicity, the substances were given intraperitoneally, intravenously, or perorally. The *Sanarelli–Shwartzman* phenomenon was elicited by the usual method.

Table I

Biochemical properties of flavobacteria

Standard Test	*Flavobacteria* isolated from feedstuffs	*Flavobacterium rhenanum*
Indol	—	—
Methyl red	—	—
Voges-Proskauer	+	+
Citrate	±	+
Urea	—	—
H_2S	±	+
Gelatin liquefaction	+	ND
Hemolysis (type)	β	ND
Motility	±	+

ND = not determined

A provoking inoculation of the endotoxin-like substance of *Flavobacterium* given 24 hours after a sensitizing inoculation induced the *Sanarelli–Shwartzman* phenomenon in all cases. (See, for example, *Fig. 21*). The clinical symptoms and pathological lesions seen in animals injected intravenously or intraperitoneally with extracts of

Table II

Degradation of sugars and sugar-like alcohols by flavobacteria

Substrates	*Flavobacteria* isolated from feedstuffs*	*Flavobacterium rhenanum*
Adonitol	− − −	−
Arabinose	− − −	−
Dulcite	− − −	−
Glucose	+ + +	+
Glycerin	− − −	−
Inulin	− − −	−
Lactose	+ − −	−
Mannitol	+ + +	+
Raffinose	− − −	−
Saccharose	+ + +	+
Salicin	+ + +	+
Sorbitol	− + −	−
Trehalose	+ + +	+

* Degradation of substrates by 3 strains

Flavobacteria (see *Fig. 22*) were similar to those induced by *E. coli* endotoxin in mice [1, 2]. Peroral administration of the endotoxin-like substances to mice and swine produced a marked toxic effect in both species within 30 to 60 minutes. There was dullness, muscular tremor, and forced and rapid respiration in the mice, and scour in the swine. There were no fatalities.

It is therefore concluded that the large numbers of *Flavobacteria* found in foods may indeed produce enteric disturbances through the action of an endotoxin-like substance.

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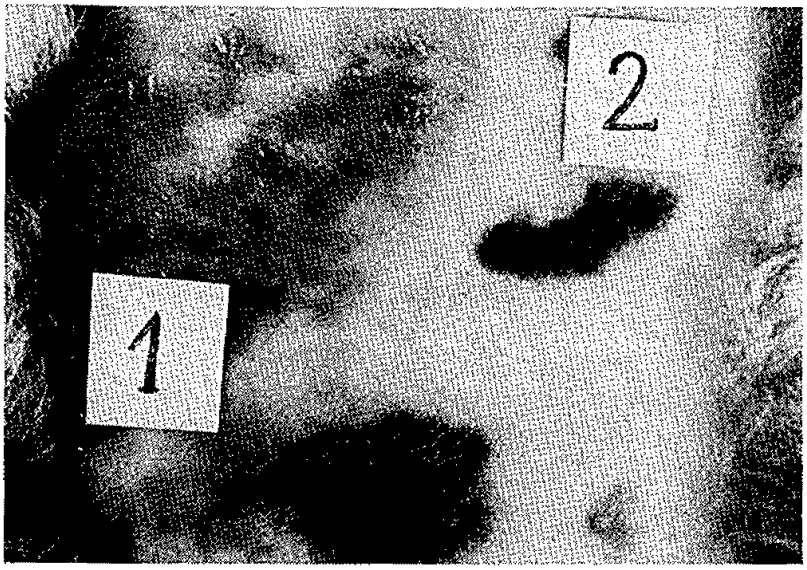

Fig 21. Sanarelli–Shwartzman reaction induced in the skin of a rabbit by endotoxin-like substances of *Flavobacteria*. 1 = site of sensitizing inoculation; 2 = site of the Sanarelli–Shwartzman reaction

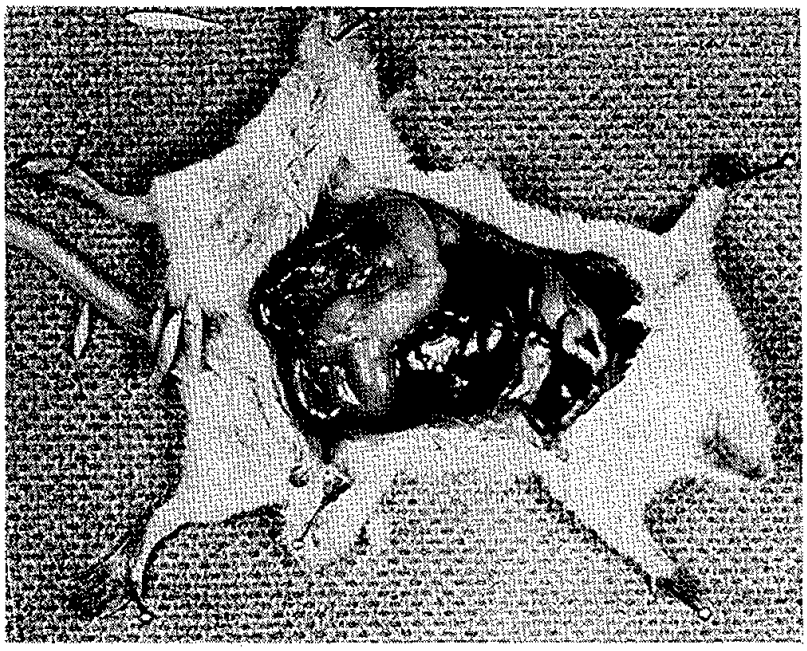

Fig. 22. Intestinal oedema, in a mouse, induced by endotoxin-like substance of *Flavobacteria*

Discussion

Dr. Bertók: I think that the term "endotoxin-like substances" is quite suitable for the extracts of *Flavobacteria* because a few of their characteristics remind us of the true endotoxins, while other of their properties do not. For example, endotoxins given perorally in experimental conditions cannot provoke anything. Perhaps *Dr. Etter's* extract is a mixture of true endotoxin and other toxic components of *Flavobacteria.* Further purification of these substances and determination of the biological effects of the various fractions in experimental animals will be necessary.

Dr. Etter: Yes, but although it is well known that endotoxin cannot be absorbed from the gut, there are data which show that extracts from *E. coli* could provoke something in the intestinal tract. Besides, I know from *Dr. Truszczynszky* (Pulawy, Poland) that *E. coli* extracts produced an enlargement of the ligated loops of the small intestine. Since our preparations were prepared by the phenol-water method of Westphal, it is possible that other toxic components are in the "endotoxin-like substances".

Dr. Berry: It is very difficult to characterize an endotoxin. In terms of toxicity, *Sanarelli–Shwartzman* properties do not provide sufficient evidence for the characterization of a substance as an endotoxin.

DOUBLE LABELING OF ENDOTOXIN WITH CHROMIUM AND TRITIUM: AN INTERIM REPORT

By

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Methods for labeling endotoxins with ^{32}P and ^{51}Cr [1, 2] have produced radioactive toxins with very low specific activities [3]. Consequently, labeled endotoxins have not been widely used in biolgocial experiments. Our aim was to obtain labeled endotoxins with a much higher specific activity and thus to expand the possibilities for the use of labeled endotoxin in biological research.

E. coli O 89 endotoxin of the Westphal-type [4] was prepared in our laboratory and labeled with chromium-51 according to our own method (to be reported) and with tritium according to the method of *Wilzbach* [5]. For tritium-labeled endotoxin, we obtained a specific activity of 500 to 600 μCi/mg without any change in biological properties. Neither the toxicity nor the tolerance-inducing capacities of the endotoxin were altered. For example, on the basis of our experiments on 120 rats, we concluded that the quantity of endotoxin absorbed from the peritoneal cavity paralleled the observed biological activity, which indicated that the labeling procedure did not change the biological activity.

The specific activity of the endotoxin labeled with chromium-51 according to our own method varied from 50 to 100 μCi/mg, in comparison to the 2 to 5 μCi/mg specific activity reported by *Braude* and co-workers [1, 2]. In addition, the biological characteristics of our ^{51}Cr-labeled endotoxin preparations were unaltered.

We have also succeeded in producing double-labeled (^{3}H and ^{51}Cr) endotoxin with a specific activity as high as that of the single-labeled compounds. Moreover, the original biological properties of the endotoxin were retained in the double-labeled compound.

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