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Molecular Aspects of Endotoxic Reactions

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"Febrim laudamus medici instrumentum felicissimum . . ." (H. Boerhaave, Leyden, 1731).

INTRODUCTION

The most common procedure in searching for active sites in biological macromolecules is partial hydrolysis. In a few cases, acidic, alkaline, or enzymatic breakdown of the macromolecule results in the removal of the inert sites of the complex, reducing the size of the remainder to the active core structure. Similar treatments frequently serve as steps of purification by removing noncovalently bound contaminants.

Isolation of the active materials in a homogeneous state often decreases biological activity by separating either the solubilizing factor or a carrier constituent, or by removing a cofactor the presence of which is essential for full biological potency. Several examples are also known wherein dissociation of the macromolecular complex with mild methods, such as the use of surfactants, depolymerizes the structure without introducing hydrolytic cleavage of covalent bonds. These depolymerized structures were shown to have altered biological effectiveness, indicating that these activities require a certain organization of subunits.

Blocking some parts of the molecule has been successfully used in enzymology. The existence of functional groups and their specific steric arrangement in the structure is the molecular explanation of their activity. The same statement can be applied to a very large number of other biologically active substances. Interference with this steric arrangement by alteration of the functional groups achieved by their blockage or removal, or by substitution, leads to changes in activity. Similarly, modification between the distances of the important functional groups by distortion of the structure also results in changes in biological properties.

Most of these approaches have been applied in the investigation of gram-negative endotoxins. The present review of the achievements will not include a discussion in detail of the chemical aspects of endotoxin research, because several extensive surveys were recently published on this subject. The most impressive array of different

biological effects elicited by endotoxins will be merely outlined, because a full discussion of this aspect alone would require a separate monograph. The relationship of structure to biological effects in endotoxins is the subject of this review. Although no final answer has been found in this research, it seems timely to review this field and to evaluate the achievements critically.

CHEMICAL PROPERTIES AND CONSTITUENTS

Endotoxins (frequent synonyms: "lipopolysaccharides," "pyrogens," "Boivin antigens") are constituents of the walls of gram-negative bacteria, forming the outer layer of the cell body. They were detected in cell-free filtrates of autolyzed gram-negative cultures more than 100 years ago, indicating that some cells release these substances spontaneously into the medium (33, 245). Some cells release endotoxin readily under the effect of mild treatments or due to specific nutritional environments (375). In the majority of gram-negative families, the endotoxin-containing outer layers are so closely associated with the other constituents of the cell wall that their separation requires strong chemical treatment.

Endotoxic substances are not extracted in the form of dissolved monomers. They form aggregates easily and also complex with a number of other natural products. This indicates difficulties in obtaining the endotoxin in a purified, homogeneous state, free from other constituents of the cell walls. It also explains the very high molecular weight of endotoxic materials, measured either by sedimentation in analytical ultracentrifuge or by light-scattering photometry. The values obtained vary from 1 to 20 million, depending mainly upon the method of isolation used and the steps of purification and further treatments involved.

The two major constituents of endotoxins were discovered by Boivin, Mesrobeanu, and Mesrobeanu (41, 42), who described these materials as glycolipids. Mild acidic hydrolysis precipitated a lipid and left a degraded polysaccharide in the supernatant fluid. Almost all authors claimed the absence of proteins in their preparations, but more careful analysis usually revealed the presence of a low percentage of bound peptides which form the third characteristic component of such preparations. Phosphorus was also found in all endotoxins hitherto described, and several authors also reported other inorganic constituents, such as calcium, magnesium, or sodium.

The polysaccharide consists in most cases of a large number of different carbohydrates, the most common being glucose, galactose, and mannose. In addition, pentoses, hexosamines, heptoses, octonic acid derivatives, and different deoxy sugars are frequently present in similar endotoxin preparations. The carboxylic acids of the lipid moiety are the usual even-numbered, saturated and unsaturated fatty acids. Odd-numbered acids were observed in only a few cases, but hydroxy-acids are probably the most characteristic constituents of all endotoxins.

No unusual amino acids have been found thus far. The most commonly occurring amino acids in the few preparations which have been analyzed are aspartic acid, glutamic acid, cysteine, valine, leucines, alanine, serine, arginine, and lysine, and a few other amino acids found in much smaller amounts.

Detailed reviews of the chemistry of lipopolysaccharides have been published by Davies (74), Lüderitz, Staub, and Westphal, (194), and by Lüderitz, Jann, and Wheat (192). Therefore, no further discussion of this aspect needs to be included in this chapter.

BIOLOGICAL PROPERTIES

Characteristic Endotoxic Reactions

Inflammation is the summation of actions taken by the defense system of the host after infection. Besides the indications of the activated natural resistance, symptoms of damage initiated by the invading microorganisms are also characteristic of inflammation. Enhanced phagocytosis, fibrin formation, and activation of some metabolic enzymes are units of the mobilized defense. Enhanced capillary permeability facilitates the exit of phagocytic cells and plasma constituents from the vessels and makes it possible for them to reach the site of invasion. Additional symptoms are numerous; their listing would be superfluous. It is of interest that the most characteristic inflammatory reactions can be elicited by injecting crude or purified endotoxic lipopolysaccharide preparations obtained from gram-negative bacterial cell walls. There is little doubt that some pathological effects of gram-negative bacterial infections are caused by the endotoxin content of the bacteria, but it is equally important to note that stimulation of the host resistance can be initiated by isolated endotoxins.

In addition to those endotoxic reactions which are identical with some signs of inflammation, there are many others not obviously related to it. These are the profound effects of endotoxins on the production of antibodies against many different antigens, the "flushing out" of interferon, or clearing of experimental lipemia, and several others. There are very few biological

TABLE 1. *Review of characteristic endotoxic reactions*

Endotoxic reaction	Type of study	References
Pyrogenicity	Description or review	8, 21, 80
	Pathogenesis	25, 109, 315
	Measurement	73, 167, 359, 371
	Therapeutic application	24, 91, 138, 357
Release of endogenous pyrogen	Description or review	10, 11, 373
	Comparison with endotoxin	9
	Isolation from leukocytes	22, 113, 155, 158
	Role in tolerance	247
	Role in pathogenesis of fever	8
Immunogenicity	Description or review	42, 66, 181, 235
	Role of endotoxicity	183, 320, 321, 323
Adjuvant effect and inhibition of antibody production	Description or review	151, 153
	Other effects on antibody production	50, 96, 97, 112, 203
	Inhibition of antibody formation	44, 226, 367, 368
Effect on "properdin" or natural antibody levels	Description	180, 272, 358
	Relation to resistance	81
	Bactericidal antibodies	211
	Role in endotoxicity	183
Leukopenia and leukocytosis	Description	75–77, 159
	Determination	13, 101, 377
	Leukotaxis	52, 76, 288
	Other effects on white blood cells	130, 175, 218, 363, 372
	Effects on platelets	78, 79
	Effects on macrophages	95, 127
	Cytotoxic effects	369
	Effect on leukopoiesis	314
Protection against irradiation	Description	5, 204, 311
	Relation to phagocytosis	246
	Possible mechanism	310, 312–314
Effect on RES	Description	19, 35, 36
	Cytologica investigation	123–126
	Determination in different species	7, 57, 188
	Relation of RES to nonspecific resistance	38
Development of tolerance	Description	18, 216, 217
	Mechanism	16, 17, 359
	Reviews	23, 111, 247
Enhancement of nonspecific resistance	Description or review	1, 48, 81, 144, 268–270, 298
	Comparison with other natural products	221
	Estimation	154
Mobilization of interferon	Description	136, 324
	Characterization of induced interferon	137, 252
	Mechanism	287
Changes in blood clotting	Description	86, 142, 164
	Cellular mechanism	78, 79
	Role in shock	116, 117

TABLE 1.—*Continued*

Endotoxic reaction	Type of study	References
Metabolic changes	Carbohydrate mechanism	55, 115
	Lipaemia clearing	189, 289, 290
	Effect on enzyme levels	4, 29, 31, 32, 82–84, 129, 199, 248
	Effect on serum iron content	12, 156, 157
Endocrinological changes	Description and morphology	370
	Relation to endotoxin susceptibility	58
	Effect and role of cortisone	114, 162, 205, 341, 342
	Endotoxin shock therapy by cortisone	361
Release of and sensitization to histamine	Description and review	110, 133, 325
	Histamine sensitization	249, 352
Vascular effects	Reviews	104, 322
	Mechanism	134, 135, 148, 302, 381
	Role of epinephrine	382
	Histological changes	325, 338, 340, 349, 350
Sanarelli-Shwartzman phenomenon	Description or review	284, 299, 300
	Local Shwartzman assay	301
	Mechanism	108, 186, 338, 339, 341, 342
	Clinical observations	307
	Relationship to hypersensitivity	320, 323
	Estimation	185, 253
Cytotoxicity	Description	28, 49, 123, 124, 126, 207, 208, 369
Abortion	Description	262
	Mode of action	58, 94, 202, 347, 348
Tumor-necrotizing effect	Description	63–65, 147, 293
	Mode of action	62, 69, 119, 294, 296
	Review of clinical applications	220, 379
	Estimation	295
Interaction with complement	Description	37, 177, 219
	Role in endotoxicity	103, 176, 206, 227
Shock and lethality	Description and mechanism	51, 134, 191, 285, 343, 355
	In pregnancy	94
	Role of blood coagulation	116, 117
	Therapy	308, 361
	Species sensitivity	27, 133, 309

systems which would not be affected by endotoxins.

Bennett and Beeson (22), Burrows (54), Zahl and Hutner (380), Todd (344), Hoff (138), and Raskova and Vanacek (254) have surveyed the biological effects of endotoxins. The most valuable reference source for the biological activities of endotoxins at the present time is the book *Bacterial Endotoxins*, edited by M. Landy and W. Braun, which is the compilation of the lectures and discussions of the International Endotoxin Conference held at Rutgers University in 1963.

In the present review, the most characteristic endotoxic reactions will be merely listed in Table 1 and, therefore, only a few of the relevant publications can be quoted.

Sensitization and Desensitization Against Endotoxic Effects

The effect of environmental temperature was studied by Berry (30). It was observed that the

mice showed remarkable sensitivity to *Serratia marcescens* endotoxin at the extreme temperatures of +5 or 37 C, while showing relative resistance at +25 C. When the animals were acclimatized to the extreme temperatures, the LD_{50} of the endotoxin preparation increased and approached normal values. This seems to indicate that endotoxin sensitizes the animals to heat or cold. Rubenstein and Worcester reported similar results (274).

The lethal effect of endotoxin in mice could be enhanced by different chemicals and also by different biological and immunological preparations. Triiodothyronine was found effective by Melby and Spink (205). Selye, Tuchweber, and Bertók (292) found that sublethal intravenous (IV) injection of lead acetate increases the sensitivity of rats to endotoxins of different origin about 100,000 times above normal. The same treatment in mice proved to be less effective. Suter and associates reported the enhanced sensitivity of mice to endotoxin by several thousandfold after vaccination with BCG (327–330). A review covering the hyperreactivity to endotoxin injection was published by Suter (326). The sensitivity of pertussis-inoculated mice to endotoxin was reported by Kind (174). Abernathy, Bradley, and Spink (2) reported the effect of brucellosis on the sensitivity of mice. Barlow (14) described hyperreactivity in mice infected with choriomeningitis virus. The effect of BCG infection in the resistance of mice to endotoxin and bacterial infection was studied by Howard et al. (143). Rutenberg and Michael (281) described the reduced endotoxin-detoxifying capacity of the reticuloendothelial system after treatment with pertussis.

Reduced lethality to endotoxin in mice was reported by Freedman and Sultzer (98) after zymosan application. Benacerraf, Thorbecke, and Jacoby (20) studied the effect of zymosan on endotoxin sensitivity of mice. Several authors investigated the effect of antibiotics on endotoxin toxicity. Rifkind and Palmer (264) described the neutralization of endotoxin toxicity in chick embryos by three cationic polypeptide antibiotics. Rifkind (263) reported that the mouse lethality of endotoxins could be reduced by polymyxin B. The action of sulfanilamide compounds on mouse lethality was investigated by Hutner and Zahl (146), and protective action was observed. Spink and Su (316) found a protective action of unsaturated fatty acids in similar systems. Condie, Staab, and Good (67) observed that endotoxin enhances susceptibility to snake venom. Tolerance to bacterial endotoxins induced increasing resistance to snake venom. Further studies on the biological relationship of endotoxins and other toxic proteins were published by Staab, Good, and Condie (317, 318).

Relationships Among Endotoxic Reactions

If all endotoxic reactions are elicited by one single structural part or property of the endotoxin complex molecule, all these reactions should run parallel in different endotoxic preparations. In other words, an endotoxin which demonstrates low reactivity in the Shwartzman assay should be similarly less active in serological reactivity, pyrogenicity, or chick embryo lethality determinations. The fact that serological reactivity and immunogenicity or toxicity are not related has been demonstrated by the earlier experiments of Boivin, Mesrobeanu, and Mesrobeanu (41), as well as by several other authors who isolated alkali-degraded or acid-degraded polysaccharides which still precipitated with antisera but elicited none of the characteristic endotoxic reactions. The experiments of Thomas and Good (341, 342) in dissociating lethality from Shwartzman reaction by the use of cortisone gave the first indication that these endotoxic reactions do not show an all-out parallelism. Cortisone pretreatment prevents lethality in mice or in chick embryos but does not have any effect on the local Shwartzman reaction. In some experiments, the generalized Shwartzman reaction could be enhanced through prior application of cortisone. This observation does not necessarily indicate that the two biological reactions are elicited by two chemically different structural entities of the endotoxin macromolecule. It is also possible that cortisone may affect the development of one reaction in the host but does not interfere with the other.

Chemical detoxification of endotoxin preparations indicated that the different biological effects elicited may be selectively eliminated while others maintain their original activity. The most striking difference could be observed between the toxic and the protective effects of partially or completely detoxified endotoxin preparations. Toxic properties could be diminished or completely abolished, whereas the stimulation of the host defense demonstrable in the nonspecific resistance reaction or the adjuvant effect of the preparations was preserved (Noll and Braude, 228; Nowotny, 230 and 232; Johnson and Nowotny, 154). The mechanisms of chemical detoxification will be discussed in a later chapter.

Milner and Finkelstein (213) compared pyrogenicity for rabbits and lethality for chick embryos, applying the samples intravenously. Analyzing 182 different preparations containing endotoxin, they found that the two tests could be

employed interchangeably. Cundy and Nowotny (73) followed the alkaline detoxification of endotoxin preparations by using five different toxicity measurements. It was found that during mild alkaline inactivation, chick embryo lethality is rapidly diminished and completely destroyed in 30 min. During the same period, pyrogenicity is enhanced and is followed by a gradual decrease of activity. The activity is still demonstrable after 24 hr of treatment. Mouse lethality showed a somewhat parallel course, with the difference of complete inactivation in 24 hr. Shwartzman reactivity showed a steady increase up to 6 hr of NaOH treatment. During the same period, the chick embryo lethality was completely abolished, pyrogenicity was reduced to approximately 20% of the original value, and mouse lethality was almost completely abolished. These results gave further support to the lack of relationships among certain endotoxic reactions.

Fate of Injected Endotoxins

It is obvious that for the studies of the mode of endotoxic action, the first step is to investigate the fate of endotoxin in the host. Different methods were used to label the endotoxins. Shear's tumor-necrotizing preparations were marked by radioactive iodine (291). The preparation and use of ^{32}P-labeled endotoxins was first described by Homma et al. (139). Similar preparations were used later by Rowley, Howard, and Jenkin (271), by Howard, Rowley and Wardlaw (145), and by Ravin et al. (255). Braude and associates (46) used ^{51}Cr-labeled endotoxin and followed the accumulation of ^{51}Cr in different organs. According to Braude (45), the complex between the hexavalent chromium and the negatively charged endotoxin is firm enough to assume that detection of the labels in the organs indicates the presence of undissociated ^{51}Cr-endotoxin complex. Similar labeling methods were used by Skarnes and Chedid (305), and they reported that liberation of ^{51}Cr from its complex indicated inactivation of toxicity. Naturally occurring or chemically treated nontoxic derivatives of endotoxin do not have the capacity to complex with ^{51}Cr to the same degree as toxic preparations.

According to the numerous investigators in this field, endotoxin seems to accumulate rapidly after iv injection in the reticulum cell-rich organs. The spleen and especially the liver appear to be primarily involved (85, 190). Other organs where accumulation could be observed were the endothelium of blood vessels (273), the lung alveoli (15), and the spleen (47, 72). The appearance of endotoxin in the liver is rapid; the accumulation in the Kupffer cells can be seen within a few minutes. The reaction of labeled endotoxin with white blood cells seems to be even more rapid. Immediately after the injection of isotopically labeled endotoxin, heavy radioactivity could be seen in the buffy coat of blood samples (57). Herring et al. (130) found that platelets absorb endotoxin. Erythrocytes do not fix in vivo-labeled endotoxin. The effect of endotoxin on the macrophage migration as well as its cytotoxic effects were studied (127). Detailed studies of bacterial endotoxins on rabbit platelets were carried out (78, 79). Cytotoxic effects of bacterial lipopolysaccharides on mouse peritoneal leukocytes were reported by Wiener, Beck, and Shilo (369). Rubenstein, Fine and Coons (273) found polymorphonuclear leukocytes to be tagged with endotoxin 10 min after injection. Detection of endotoxin in the brain after iv administration was unsuccessful. The absorption, distribution, and elimination of endotoxins was thoroughly reviewed by Braude (45), whose research team made the greatest contribution to our knowledge in this field.

The reticuloendothelial system (RES) uptake of toxic and chemically detoxified endotoxin was investigated by Golub, Gröschel, and Nowotny (106, 107), using the method of Cremer and Watson (72). Fluorescein-labeled antibodies were used to detect the two preparations in BRVR mouse organs, and it was found that toxic endotoxin will start accumulating in the spleen and liver, as reported earlier by several investigators. In sharp contrast, detoxified endotoxin was not taken up in measurable amounts by these organs but remained in the circulation for a relatively long time. Since detoxified endotoxin retains the first peak of the pyrogenicity curve, this gave a biological assay for following the fate of detoxified endotoxin as well as of toxic parent material in the same animal. It was found that, whereas toxic endotoxin is eliminated from the circulation relatively rapidly by the RES, the detoxified material remains in the circulation. In mice which were either actively or passively immunized to endotoxin, the uptake of detoxified endotoxin by the RES occurred at the same rate as the uptake of toxic material. It was found that passively transferred immunoglobulin G is capable of facilitating the RES uptake of endotoxoids.

Whether these organs or cells are the direct or indirect targets of endotoxic action is still not known. Virchow (356) was the first to suppose, in 1854, that a paralysis of the central nervous system must be responsible for the fever-inducing effect of certain agents. Since then, investigators have claimed that endotoxin acts on the central nervous system (92, 93, 163, 251,

297, 303). While it is possible that the central nervous system is involved in some of the endotoxic reactions, it is probably not the primary target. Certain evidence shows that the primary targets of endotoxin may be the platelets or the leukocytes and that damage caused by endotoxin releases certain materials (such as endogenous pyrogen, blood clotting factors, and others) which elicit a chain reaction, possibly acting on the central nervous system as well as on the vascular system.

RELATION OF STRUCTURAL PARTS TO BIOLOGICAL FUNCTIONS

The problem may be summarized as follows. Are polysaccharides or lipids or protein residues responsible for the biological activities? Are the biological activities all due to the existence of one structural moiety, or are the diverse biological properties elicited by different parts or properties of the structure? There are significant achievements as well as sharp disputes resulting from the research related to these questions.

Role of Polysaccharides

It has been established by the thorough and elegant work of several scientific groups that the polysaccharide moiety of endotoxin is the carrier of O-antigenic specificity. Major contributions have been made through cooperative efforts among the teams of Lüderitz and Westphal in Germany, Kauffmann in Denmark, and Anne Marie Staub in France. Publications of these results are numerous, the latest and most detailed review having been published by Lüderitz, Staub, and Westphal (194).

Role of the Polypeptides

It has been known since the work of Panum (245) that the pyrogenic materials are relatively heat-resistant, a finding which has been confirmed by practically every later publication. This rules out exotoxin-like proteins as carriers of toxicity but does not entirely eliminate the possibility that small peptides, resistant to 100 C or to usual sterilization procedures, may be involved either in toxic manifestations or in other biological effects.

Homma and associates showed in a number of publications that protein residues in *Pseudomonas aeruginosa* endotoxins obtained from the autolysis of cell culture or from mechanically disintegrated and washed cell walls were identical both chemically and biologically. Pyocinic activities were attributed to the proteins. This activity could be demonstrated only after their separation from lipopolysaccharide. These protein moieties did not show resemblance to the mucopeptide layer of the same cells. A review of the work of Homma and Suzuki has been published (141).

Jenkin and Rowley (150) isolated a toxic protein from the gram-negative *Vibrio cholerae*. This protein accounted for a major portion of the toxicity of the whole cell. It was suggested, based on chemical and immunological data, that this toxic protein is identical with the protein moiety of the endotoxic trichloroacetic acid-extracted antigen obtained from the same strain by Boivin and Mesrobeanu (39). Dissociation of the Boivin type antigen isolated from *V. cholera* was achieved by Jenkin and Rowley by using urea and by precipitation with ammonium sulfate.

L. Mesrobeanu, I. Mesrobeanu, and N. Mitrica (209) reported the isolation of heat-labile neurotoxic endotoxins from the autolysate of gram-negative bacteria. These preparations have a high nitrogen content and represent the peptide fraction of the Boivin-type antigen. Biological, immunological, and some chemical properties of these neurotoxic proteins were reviewed by the same authors (210). Other protein toxins were described as present in gram-negative bacteria, such as "L toxin" in *Salmonella enteritidis* (165) and several others.

The readiness of bacterial endotoxins to form complexes with other biological macromolecules is one of the most characteristic features of these preparations. In relation to the above-mentioned publications, one may not overlook the possibility that the toxic proteins observed are not covalently bound moieties of the endotoxic lipopolysaccharide, but merely adsorbed to it or extracted together with the endotoxins, thus occurring as a contaminant in these preparations.

Lipid Moiety

The supposition regarding the governing role of lipids of endotoxin in the various biological activities can be traced back to the work of Boivin, Mesrobeanu, and Mesrobeanu (41), who obtained a phosphorus-containing lipid precipitate during mild acidic hydrolysis. This preparation was called "fraction A." A degraded polysaccharide, "fraction B," was found in the supernatant fraction of the acidic hydrolysate. Whereas the latter fraction was nontoxic and nonimmunogenic but reacted with O-antiserum, the lipid "fraction A" showed residual toxicity in rabbits, without being antigenic or serologically reactive.

Several other authors, listed earlier (231), re-

ported the isolation and analysis of similar lipids from endotoxins by using acidic hydrolysis. The biological activity of this lipid precipitate was also investigated, and the findings corresponded to the observations of Boivin, Mesrobeanu, and Mesrobeanu.

Binkley, Goebel, and Perlman (34) used acidic and alkaline hydrolysis to obtain large breakdown products of endotoxin. By investigating the chemical nature and toxicity of the preparations obtained, they concluded that a toxic factor T must exist, which is neither protein nor polysaccharide (335).

Intensive investigation of the lipids precipitated by acidic hydrolysis was initiated by the detailed work of Westphal and Lüderitz (365). This precipitate, called "lipid A," was isolated and its constituents were analyzed. The biological properties of "lipid A" were investigated by Westphal and associates as well as by a number of different laboratories. The results demonstrated a 5 to 10% residual activity in the "lipid A" (144, 160, 212, 222).

Westphal, Lüderitz, and co-workers assumed that the toxic T factor is "lipid A" (364–366). The lipid is kept in solution in the lipopolysaccharide by the lyophilic polysaccharide. The removal of polysaccharide by acidic hydrolysis reduces the solubility, thus resulting in the precipitation of the lipids. It was assumed that reduced solubility of the isolated "lipid A" is responsible for the reduced biological activity. Analysis of the biological properties revealed that a slight enhancement of pyrogenicity can be achieved if the material is brought into a stable colloidal form.

Ribi and associates disagreed with this assumption. They obtained a highly toxic endotoxin from *S. enteritidis* by using a mild extraction procedure (259, 261). This preparation has been claimed to be very low in fatty acid content, a claim which was used by these authors to prove a lack of relationship between lipid content and endotoxic activity. A completely fatty acid-free endotoxin with full biological activity could not be obtained, however. In another series of experiments, Ribi and associates isolated lipids from endotoxins by using partial acidic hydrolysis (257, 258, 260). With the chloroform-soluble fraction of this precipitated "lipid A," biological activities were measured. The results showed that not 5 to 10%, but 0.1% or less, of the original endotoxic activity can be demonstrated in this preparation. The discrepancy is probably due to the different preparations investigated. Whereas Westphal and associates measured and reported the biological activity of the entire "lipid A" mixture, Ribi et al. used only the chloroform-soluble fraction which is especially rich in free fatty acids and lacks the more polar constituents of the "lipid A" precipitate (231).

A proper understanding of the heterogeneity as well as the origin of the components in the "lipid A" preparation should facilitate the explanation of the residual biological activities in these preparations. Westphal and Lüderitz consider "lipid A" as one molecular species built into the complete endotoxin structure. Attempts to fractionate "lipid A" soon revealed a great degree of heterogeneity; at least 16 different components could be detected. The precipitate obtained by acidic hydrolysis consists of free fatty acids, "phosphomucolipids" (the occurrence of which was reported for the first time in this precipitate), and amino acid-rich as well as 10 to 15% carbohydrate-containing phosphomucolipid fractions (53, 229, 231, 239). This is understandable if one considers that "lipid A" was obtained by partial acidic hydrolysis, which yields completely liberated building stones, barely altered acid-resistant cores, incompletely degraded lipopolysaccharides, and all intermediates. All split products which are insoluble in hot acid will be precipitated by this treatment. This means that the "lipid A" precipitate does not consist of one chemically well-defined molecular species, but is a mixture of diverse chemical entities.

Whether all these constituents of the "lipid A" precipitate are part of one or more lipid moieties in the lipopolysaccharide fraction is not known. It is difficult, although not entirely impossible, to visualize them as breakdown products of only one structural part, as Westphal and Lüderitz assumed. It seems more likely that the constituents of the "lipid A" precipitate derive from different subunits of the very complex endotoxin structure. Whether these subunits are chemically identical and occur as repeating units, or the different lipid-rich zones represent related but not identical structures, is the subject of current research in the author's laboratories.

The biological activity of some of the isolated fractions was investigated by Johnson and Nowotny (*unpublished*). It was found that purified fractions showed a very low biological potency in Shwartzman reactivity or in mouse LD_{50}. The crude mixture, "lipid A," showed significantly higher activity. Chemical analysis revealed that the crude mixture may contain incompletely degraded residual endotoxin. The components of the "lipid A" precipitate are not as toxic as they should be if they are the toxic constituents of endotoxin. This has been established by several authors. However, the possibility that these fragments were parts of a toxic

moiety in the intact endotoxin structure is not excluded. The present method used for their isolation is acidic hydrolysis, which not only degrades the polysaccharide part but also partially destroys or at least alters the structure of these constituents.

The term "lipid A" should be used only as a common name for all parts which become insoluble after acidic hydrolysis. We recommend the use of the term "lipid moiety" to designate the long-chain carboxylic acid-containing areas or zones in the endotoxin structure. These two may be similar but are obviously not identical.

Polysaccharide-free Endotoxic Glycolipids

Laboratory procedures which could isolate the lipid moieties in their intact native form are not available, but nature provides bacterial strains which lack the usual polysaccharide part of their endotoxins. Lüderitz et al. (193) reviewed their investigation on the immunochemistry or biochemistry of the S → R conversion and described a number of *Salmonella minnesota* strains which differ in the size of the O-antigenic polysaccharide part in the endotoxin structure. One of these, *S. minnesota* R595, did not contain pentoses, hexoses, or heptoses in the phenol-water-extracted preparation. The constituents present were hexosamines, 2-keto-3-deoxy-octonate, fatty acids, and phosphorus. Recent investigations on this preparation have shown that "lipid A" obtained from this preparation contains a structural unit of phosphorylated glucosaminyl-β-1,6-glucosamine. In the glycolipid, 2-keto-3-deoxy octonic acid-trisaccharide is linked to this unit, which carries the long-chain fatty acids (105).

Lüderitz et al. (193) as well as Tripodi and Nowotny (346) showed that the different R mutants, which lack some of the polysaccharide side chains, are still potent endotoxins. Kasai and Nowotny (161) isolated and purified a glycolipid from rough *S. minnesota* R595 strain and studied its chemical and biological properties. The glycolipids showed qualitative and quantitative similarities in chemical constituents to some fractions obtained from "lipid A" mixtures. On the other hand, in contrast to the biological activity of the "lipid A" preparations, it has been found that the glycolipid showed full endotoxic potencies in chick embryo lethality, Shwartzman reactivity, and pyrogenicity assays (*see also* 237). Interestingly, these polysaccharide-free glycolipids were also active in the consumption of complement as were smooth lipopolysaccharides (206). This glycolipid preparation showed a strong enhancement of nonspecific resistance in mice. The mouse lethality of the preparation was lower than that of the corresponding smooth lipopolysaccharide. Similar results were obtained by Kim and Watson (172). These results indicate that the polysaccharide part is probably not essential for the toxicity of endotoxins, and it is attractive to consider these results as support for the significant role of the lipid moieties in endotoxic reactions.

SEARCH FOR THE TOXIC PRINCIPLE

Besides the "T factor" of Goebel and associates and the assumed identification of the "lipid A" precipitate with the "T factor" by Westphal and associates, there are several other theories which claim to explain the biological activities of endotoxins. These theories have been substantiated by a few observations and experiments.

Role of Hypersensitivity

The most significant of these experiments is unquestionably the study by Stetson, who revealed similarities between the Arthus-type hypersensitivity reaction and the local Shwartzman phenomenon (320, 321). Histological studies of the skin area and of the surrounding tissues after intradermal endotoxin injection revealed changes resembling mild allergic reactions. Lee and Stetson (187) found that enhancement of the level of the so-called "natural antibodies" in rabbits leads to increased skin sensitivity. Farr (87) and Farr et al. (88) found that hypersensitivity to protein antigens results in a biphasic fever curve similar to that elicited by endotoxins (*see also* 149). Kováts (178) described a local endotoxin hypersensitivity and related this phenomenon to the Shwartzman reaction. Netzer and Vogt (227) reported anaphylatoxin formation induced by endotoxin. Malkiel and Hargis (195) described the observation of anaphylactic reactions in mice which could be induced by *Bordetella pertussis* endotoxin. Landy and associates (184) as well as Abernathy and Landy (3) reported decreased sensitivity in germ-free guinea pigs. In summary, it was stated by Stetson (322, 323) that all major endotoxic effects, such as fever, shock, and the local Shwartzman, as well as the generalized Sanarelli-Shwartzman phenomenon, may be reproduced experimentally by interaction of nontoxic antigens with the corresponding antibodies. It was assumed that normal animals are hypersensitized against gram-negative bacteria, and the existence of this hypersensitized state in all experimental laboratory animals as well as in man is responsible for endotoxic reactions. Recent publications of

Bonilla-Soto (43), Kováts and Végh (179), and of Pitkin (250) show further relationships between hypersensitivity reactions and endotoxic action.

Others found a number of differences between hypersensitivity reactions and endotoxic effects (123, 124). The results of Kim and Watson (171) are in disagreement with Stetson's postulate. These authors found that immunologically virgin piglets, obtained by Caesarian section and kept under strictly germ- and antigen-free conditions, were just as sensitive to endotoxin as fully grown animals. Watson and Kim (360) supposed that hypersensitivity may play a role in febrile response and that this is expressed in the second peak of the fever curve, but that a primary toxicity of the endotoxic preparation also exists which is responsible for the toxic manifestations in the immunologically virgin piglets.

Chemically detoxified endotoxins, obtained by potassium methylate treatment, resulted in enhanced reactivity with O-antiserum. The immunogenicity of this preparation if injected 8 to 10 times during a month resulted in an antibody titer, measured by passive hemagglutination, identical to toxic endotoxins (154, 230). More recent results demonstrated that, although no difference could be found in the quantity of the immunoglobulins produced, there are qualitative differences in the immune response (235).

Relation of Particle Size to Endotoxicity

Numerous publications describe the inactivation of biological macromolecules by simple dissociation using detergents or other dissociating agents. Most of these reactions are reversible. Rivkine used anionic surface-active agents in studying gram-positive bacterial antigens (265). Young, Harrington, and Kielley dissociated and reassociated myosin (378). Hersh used sodium lauryl sulfate for the reversible dissociation of alcohol dehydrogenase (131). Stellwagen and Schachman dissociated and reconstituted aldolase (319). Roberson et al. (266) demonstrated that a certain degree of polymerization is necessary for optimal activity in toxicity of staphylococcal cell wall preparations. Similar observations were reported by these same authors in measuring the potency of endotoxin preparations (267).

The same explanation for toxicity was advanced by Ribi and associates who, in a series of papers, elaborated the claim that a certain particle size of endotoxins is required for the toxic manifestations. Degradation by mild acidic hydrolysis was used for these studies and it was found that, below a certain particle size, the preparation is no longer toxic (118, 214, 257, 260). They hypothesized, therefore, that a causal relationship exists between the reduction of size and loss of toxicity.

The same group also isolated a biologically inert hapten from the protoplasm of *Escherichia coli* cells (257). According to their chemical data, this material had a composition identical to that of a fully active endotoxin, but its molecular weight was much lower. This material was first called "native hapten," which term was later modified to "native protoplasmic polysaccharide" (276). The first chemical analysis revealed no difference in the percentage of fatty acid or in other constituents present in these preparations. The hypothesis was that formation of active endotoxin from these inactive native haptens occurs through polymerization of the subunits into an active complex which now has the required size to elicit toxic reactions. This was offered as additional proof of the determining role of a certain particle size in endotoxic activities. The claim that the protoplasmic hapten is a precursor of endotoxin was later deemphasized by the same authors (276). Similarly, the application of improved chemical analytical procedures revealed that the major chemical difference between the protoplasmic hapten and toxic endotoxin is the lack of long-chain carboxylic acid in the former substance (6).

Oroszlan and Mora (243) initiated the use of the detergent SLS (sodium lauryl sulfate) for the dissociation of subunits of *S. marcescens* endotoxin. A strong reduction in the sedimentation rate was observed in an analytical ultracentrifuge, which was paralleled by inactivation of the tumor-necrotizing effect. If the detergent was removed by alcohol extraction, the activity was restored. Ribi and associates (256) and Rudbach et al. (275) reported the results of studies with applied sodium desoxycholate (NaD). Ribi, Rudbach, and associates observed that endotoxin treated with NaD shows degradation and simultaneous loss of pyrogenicity. Dilution or dialysis leads to recombination of endotoxin, achieving a molecular weight of 500,000, and the recovery of pyrogenicity. The presence of serum proteins inhibits the recombination of the subunits. The authors conclude that a certain micellar organization of inert subunits results in an active endotoxin macromolecule. Rudbach, Milner, and Ribi (279) reported that dissociated and inert subunits of different endotoxins can be combined into hybrid macromolecules. Such subunits will form a complex if the NaD is removed from the mixture, and the complex will carry both the serological specificity and the

characteristic biological activity. It is important to stress one of the findings of this team; i.e., the so-called "native protoplasmic polysaccharides" which lack long-chain fatty acids cannot be combined into hybrid endotoxin molecules. It seems to us that the constituents which take an active part in the reaggregation of the subunits may be identical with those responsible for the complex formation between endotoxin and its cellular targets.

The results of Oroszlan and Mora as well as of Ribi and associates appear to indicate that a certain organization of subunits into fringe micelles is the structural requirement of biological activity of endotoxin. One consideration may not be overlooked, and that is the possibility that the small subunits are inactive not because they are small or disorganized, but because they are complexed with the dissociating agents SLS or NaD or with proteins. These agents may mask or inhibit the reactivity of the subunits, preventing reaggregation as well as action on the targets. The problem could be answered only if subunits could be isolated without the presence of detergents or serum proteins. Such a preparation has not yet been obtained.

Recent results from our laboratory (*unpublished*) showed that mouse lethality and local Shwartzman skin reactivity of endotoxins in rabbits are enhanced or unchanged by the addition of NaD in a final concentration of 0.2%. This has been observed with *E. coli* 08, *Salmonella typhi* 0901, or *Serratia marcescens*, and with endotoxins extracted with phenol-water as well as with trichloroacetic acid-soluble endotoxins from these strains. No explanation for the different effects of NaD on pyrogenicity and on Shwartzman reactivity or mouse LD_{50} can be offered at the present time.

Toxic Constituents or Toxic Conformation?

In the search for a chemical explanation of toxicity, two working hypotheses can be advanced: (i) that the material contains subunits which are toxic even if cleaved from the macromolecule, or (ii) that the macromolecule does not contain such toxic groups but is built up of nontoxic functional groups in such an arrangement that the whole entity will have toxic effects on the subcellular targets of endotoxic action. Similar examples for the first as well as for the second possibility can be found among plant akaloids, enzymes, animal poisons, bacterial exotoxins, and in a number of other natural products.

That the first assumption would apply to bacterial endotoxins could not be substantiated by hitherto-described experimental results. The possibility that such toxic constituents exist, attached to the endotoxic molecule, still cannot be eliminated, but their isolation, free from the rest of the endotoxin, cannot be achieved by present procedures without the destruction of this constituent.

It seems most likely that toxic endotoxin is built of otherwise harmless constituents, such as naturally occurring carbohydrates, fatty acids, phosphoric acid, and amino acids. The incorporation of these constituents into a macromolecular structure may be such that they provide a "toxic conformation" (241, 346). Earlier as well as more recent results which will be discussed later indicate that the presence of long-chain carboxylic acids, ester- or amide-bound to a polysaccharide backbone, play a dominant role in the creation of the "toxic conformation" (230, 232, 233). It must be strongly emphasized that other functional groups may also be involved in endotoxicity.

DETOXIFICATION AS AN APPROACH

Several methods are known by which the toxic manifestations of endotoxic preparations can be eliminated. These range from biological observations through chemical and physicochemical changes in the endotoxin molecule. A review of the phenomena and a discussion of their mechanisms may aid in approaching a better understanding of the problem.

Biological and Biochemical Detoxification

Hegemann (120, 121) and Hegemann and Lessmann (122) described and studied the endotoxin pyrogenicity-inactivating effect of fresh human serum. Hegemann observed that incubation of endotoxin with serum or with plasma inactivates the pyrogenicity of the endotoxin within a few hours. Landy et al. (182) and Skarnes et al. (306) investigated this phenomenon in more detail. The enzymatic nature of the detoxifying effect was emphasized (166). Cluff and associates (59–61) observed that short incubation of endotoxin with serum has an enhancing effect on pyrogenicity. Kimball and Wolff (173) reinvestigated the experiments of Cluff and co-workers and were unable to observe enhanced pyrogenicity due to incubation with serum. Yoshioka and Johnson (376) as well as Rudbach and Johnson (277) fractionated serum and found that the Cohn fraction IV-1 contains the substance which decreases endotoxin pyrogenicity. They attempted, unsuccessfully, to isolate breakdown products after incubation of endotoxin with the Cohn fraction

IV-1. These authors found later that the detoxification of pyrogenicity by the serum proteins is due not to an enzymatic effect but to complexing proteins. Strong proteolytic enzymes, such as Pronase, removed the protein from the complex and restored the toxicity of the liberated endotoxin (278).

Skarnes and Chedid (305) reported the degradation and inactivation of endotoxin by serum. Skarnes' more recent results (304) showed that two serum enzymes are responsible for the detoxification. One of the enzymes can degrade the endotoxin into smaller particles without destroying its biological potency. Another enzyme also present in the serum detoxifies the already degraded endotoxin. Both enzymes were isolated from serum by column chromatographic procedures. The second enzyme showed esterase activities.

Other enzymes, although not isolated, were thought to be actively involved in the in vivo detoxification. One of these enzyme systems was studied by Corwin and Farrar (68, 89, 90). The detoxifying properties of the liver were studied by these authors who found that, if the liver has been damaged by carbon tetrachloride, it loses its capacity to destroy endotoxins. At the same time, the authors found that the lipoperoxidase activity of the liver is also diminished. These results indicate that lipoperoxidases may be involved in detoxification. Rutenburg and associates (281–283) studied the similar effect of the spleen in vivo. They observed that endotoxin mouse toxicity is diminished during perfusion through the dog spleen in vivo. The results indicate that the detoxifying process is rapid and the reaction rate has a different order of magnitude than was obtained with normal serum. Attempts to isolate these enzymes from tissue homogenates were unsuccessful. Oroszlan, Mora, and Shear (244) succeeded in neutralizing endotoxicity by incubating endotoxin with liver homogenates. This reaction, as well as the serum-neutralizing effect on endotoxin investigated by Oroszlan and associates (242), could be reversed by a strong polyanion, polyglucose sulfate, which dissociated the endotoxin from its complexing liver proteins or serum proteins.

Kim and Watson attempted to remove the peptide residues from their endotoxin by using papain (169). According to the already-mentioned hypothesis of Watson and Kim, these peptides may be responsible for the hypersensitivity-like reactions of endotoxin. Removal of the peptides by papain resulted in the loss of the second peak of the pyrogenicity curve. Rudbach, Ribi, and Milner (280) reported that inactivation of pyrogenicity with papain is due not to enzymatic action, but to complexing of papain with the endotoxin. The use of Pronase removed the complexing papain and restored the pyrogenicity.

Detoxification by Complexing

Several examples in the preceding chapter clearly indicated that some detoxifications reported may involve complex formation of endotoxin with other nontoxic substances. Additional experimental data will be reviewed here, showing that the formation of complexes is one of the most characteristic features of endotoxins. It was shown by Sarvas, Lüderitz, and Westphal (286) that phenol extraction of a mixture of two different gram-negative bacterial cells will result in a hybrid endotoxin which will carry both serological characteristics. Rudbach, Milner, and Ribi (279) observed a similar phenomenon. Endotoxin forms aggregates but it also forms complexes with other materials. Takeda et al. (333) were the first to complex endotoxin with casein under alkaline conditions. This endotoxin-casein complex showed unchanged biological activity. Neter et al. (224) investigated the complex formation between basic proteins, such as histone and protamine, and endotoxins. Woodside and Fishel (374) reported that gelatin forms complexes with endotoxin and neutralizes its biological properties.

Complex formation may result in loss of toxicity if the nontoxic substance blocks the active site of the endotoxin and thus mechanically inhibits contact with the target. It is also feasible that complex formation results in distortion of the toxic structure. Neutralizing of endotoxicity by antibodies, which is discussed in the next chapter, is an example of these possibilities.

Immunochemical Detoxification

Passive immunization with antiendotoxin serum injected into experimental animals before or simultaneously with endotoxin did not achieve significant protection (40, 69–71, 215, 362). Kim and Watson (170) showed that endotoxin tolerance can be passively transferred by injection of isolated 19*S* immunoglobulins of a tolerant rabbit serum, but the same authors claimed that no relationship exists between this protection and the O-antibody titer of the sera. Tate and associates (336) described a subcutaneous injection of rabbit O-antiserum reducing the lethality of endotoxin in mice. Nowotny, Radvany, and Neale (238) and Radvany, Neale, and Nowotny (253) reported that O-antiserum can neutralize toxic reactions of endotoxic O-antigen preparations if it is incubated in vitro

with the endotoxin. If the endotoxin was mixed with the corresponding hyperimmune O-antiserum, an optimal 1:6 antigen/antibody ratio was established. This precipitate showed no signs of pyrogenicity, Shwartzman reactivity, or mouse lethality when injected ip (intraperitoneally) or iv. If less than optimal amounts of antibody were mixed with endotoxin and incubated, such as 3% of the optimal amount, abolishment of the second fever peak could be readily observed. This shows the presence of antibodies with high affinity for endotoxin, or it may indicate that those parts of the endotoxin molecule which elicit this hypersensitivity-like reaction are already blocked by the first few antibody molecules. If antiendotoxoid (endotoxoid = chemically detoxified endotoxin) serum was used for the neutralization of toxic endotoxins, no similar protection could be achieved. If rabbit hyperimmune serum was absorbed by endotoxoid (which does not contain the hypothetical toxic site or toxic conformation but still reacts with O-antibodies), the hyperimmune serum supernatant fluid retained its capacity to neutralize Shwartzman skin reactivity or pyrogenicity. Detoxification with antiserum may be due to simple coating of the endotoxin molecule with a sixfold amount of antibody proteins. On the other hand, the use of antiendotoxoid serum as well as adsorption of hyperimmune rabbit O-antiserum with endotoxoid indicates that probably two different structural parts of the lipopolysaccharide are responsible for O-antigenicity and for toxicity.

Although Kim and Watson were able to passively immunize animals with 19*S* immunoglobulins against pyrogenic and lethal effects of endotoxin, they could not neutralize the same effects by using hyperimmune rabbit serum (171). Berczi (26) reported that antiendotoxin serum prepared in rabbits neutralized the chick embryo lethality of endotoxin if incubated for 1 hr at 37 C. A 0.005-ml dose of the serum neutralized 100 LD_{100} for chick embryos. Normal rabbit serum had no neutralizing effect. These latter results support the observations of Radvany et al. (253).

Chemical Detoxification

In the past, numerous attempts have been made to destroy the pyrogenic materials present in certain pharmacological preparations and solutions. Campbell and Cherkin (56) destroyed pyrogens by hydrogen peroxide. Suzuki (331, 332) used other oxidizing agents and successfully eliminated pyrogenicity in different solutions. Treffers (345) detoxified whole bacteria by acetylating the entire cell. The preparation obtained was able to elicit antibody production but showed no toxic manifestations. Freedman, Sultzer, and Kleinberg (100) used basically the same procedure to inactivate endotoxins. These authors showed that, while acetylation of bacterial endotoxin leads to detoxification, the preparation retains its ability to stimulate nonspecific resistance. More detailed studies and comparisons of other different endotoxic properties were also published by Freedman and Sultzer (99). Noll and Braude (228) used lithium aluminum hydride to destroy pyrogenicity of a trichloroacetic acid-extracted endotoxin of *E. coli*. These authors showed by infrared spectroscopy that this procedure resulted in the elimination of some ester-bound fatty acids. The material, while nontoxic and nonpyrogenic, maintained full immunizing potency. Fukushi and co-workers (102) were unable to reproduce these results. Various other chemical procedures were used to obtain similar preparations (230). Transesterification with boron trifluoride, O-acyl cleavage with potassium methylate, and dissociation with an equimolar mixture of pyridine and formic acid were found to destroy mouse lethality in these preparations while maintaining serological reactivity. Further analysis of the most characteristic endotoxic reactions elicited by these chemically detoxified preparations was reported by Johnson and Nowotny (154). The chemical changes elicited by detoxification showed that these three detoxifying procedures have a common feature, the cleavage of ester-bound carboxylic acids (232). Martin and Marcus (197, 198) detoxified crude endotoxin from *Salmonella typhimurium* by acetylation and periodate oxidation, in addition to the above three procedures. Similar results were obtained, yielding a nontoxic but immunogenic preparation which they suggested for use as an enteric vaccine.

Alkaline Detoxification

Martin (196) observed that both alkaline and acidic hydrolysis destroy mouse LD_{50} of *S. typhimurium* endotoxin. Neter and co-workers (225) studied the effect of heat and chemicals on the erythrocyte-modifying, antigenic, toxic, and pyrogenic properties of lipopolysaccharides. Among the methods used was alkaline partial hydrolysis. It was observed that pyrogenicity is not completely lost during this treatment. The red cell-modifying capacity of endotoxin and its reactivity with homologous O-antiserum was maintained. Tauber, Russell, and Guest (337) used alkaline hydroxylamine to remove lipids from an endotoxic lipopolysaccharide. The

result was a highly soluble preparation which did not show endotoxic properties. Tripodi and Nowotny used alkaline hydrolysis by dilute NaOH and studied the kinetics of the loss of toxicity observed (346). Measurements with light-scattering photometry showed that the particle size of the endotoxin decreases rapidly after the addition of 0.1 N NaOH at 25 C (room temperature). This rapid fall in particle size levels off in approximately 2 to 3 hr, and thereafter no significant further decrease in particle size can be observed. After measuring the changes in mouse lethality during the same time, it was reported that mouse LD_{50} is not decreased in the first 3 hr, thus showing that there is no parallelism between a certain particle size and mouse lethality. The mouse lethality of this alkali-hydrolyzed endotoxin started to decrease after 3 hr and reached practically a nontoxic state in 6 to 8 hr of the above treatment. During this time, barely measurable further changes could be observed in the particle size measured by light-scattering photometry. On the other hand, measurement of the molecular dissymmetry by light-scattering photometry revealed that, whereas the dissymmetry of the particles seems to show no change in the first 3 hr, it starts to increase gradually between the 3rd and 8th hr, thus showing a time relationship between loss of toxicity and changes in molecular symmetry. It was postulated that unfolding or swelling of the molecule takes place and induces distortion of the original toxic structure. By analyzing the split products liberated during alkaline detoxification, fatty acids were found, the major percentage of these being palmitic acid.

Whether the distortion of the "toxic conformation" is due to the cleavage of weak forces or to the split of covalent linkages was also investigated (240). By investigating the kinetics of the alkaline inactivation, the activation energy of the reaction was measured. It was found that the magnitude of this reaction is 11 kcal, which is much higher than the energy requirements of simple dissociation.

McIntire and co-workers (201) studied endotoxic lipopolysaccharides from *E. coli* K cells and obtained similar results. The measurements included aggregation, lipid content, and molecular charge. These properties were related to toxicity, pyrogenicity, and serological reactivity. It was observed that disaggregation by sodium lauryl sulfate did not decrease pyrogenicity. Succinylation has little effect on the same parameter. A high degree of molecular assymmetry was indicated by the observed very low sedimentation values in an analytical centrifuge in relation to light-scattering figures.

Marx and associates (200) studied the relationship between particle size measured by sedimentation in an ultracentrifuge and mouse lethality determined in adrenalectomized animals. It was observed that alkaline hydrolysis degrades endotoxin but decrease in particle size is not followed by detoxification. These findings are in agreement with earlier reports (346).

Chemical and Biological Changes Induced by Detoxification

The principal aim of detoxification was to introduce limited chemical changes in the structure, followed by determination of the changes in biological potency. If the chemical change is restricted to a certain group in the structure, the role of this group in one or several biological properties may be estimated.

There are two shortcomings to this approach. (i) The chemical changes induced in the structure are usually not restricted to a limited part or to one type of functional group in the structure. Acidic hydrolysis acts in a random manner, causing not only cleavage of acid-sensitive linkages but also transformations in the structure, many of them being irreversible. Alkaline hydrolysis is somewhat more selective but, in addition to fat saponification, distortions of the structure also occur. More specific chemical attacks were sought, but it is obvious that their action is not directed entirely against selected functional groups or linkages. It is especially difficult to trace all reactions in a structure as complex as endotoxin. (ii) The other difficulty is equally important. To follow the effect of chemical alterations on biological potencies, it is essential to apply at least semiquantitative biological measurements. Most of the assays either do not give a linear dose/response relationship, or do so in a narrow interval. Application of improper doses may give erratic information. These facts are well known to the pharmacologist and physiologist but are remarkably infrequently applied in other fields. Bearing these two pitfalls in mind, let us sum up and evaluate the results of the detoxification of endotoxins.

Regarding the changes in chemical structure, one cannot overlook the fact that fatty acids are involved in a large number of detoxifying procedures. Oxidation, acidic hydrolysis, acetylation, and especially deacylation alter the number of short- or long-chain carboxylic acids surrounding some regions of the endotoxin molecule with a nonpolar layer. Detoxification with $LiAlH_4$, alkaline hydroxylamine, boron trifluoride, or

potassium methylate cleaves ester linkages. The detoxifying effect of saponification with dilute alkali such as sodium hydroxide, potassium hydroxide, lithium hydroxide, or with concentrated ammonia fits into this picture. Nonionic detergents do not saponify, but they may act by dissociating nonpolar interactions between long aliphatic chains.

The results of structural changes during some chemical detoxifications have been investigated, but their chemical mechanisms are still far from completely clarified. These can be studied only on homogeneous endotoxin preparations.

Alterations in biological potency were also examined. In addition to those already discussed, a number of recent results will be summarized here. In these experiments, potassium methylate (endotoxoid-2)- or sodium hydroxide-detoxified preparations were used for the most part.

Investigating the lethality of the preparations, Chedid (*unpublished data*) used adrenalectomized mice. In these experiments it was shown that an approximately 100-fold decrease in lethality could be achieved by potassium methylate detoxification. The hemodynamic properties of the endotoxoids were investigated by Johnson and Anderson-Imbert (152). It was found that endotoxoid-2 has no effect on the blood pressure measured in the femoral artery of rabbits. Pretreatment of rabbits with detoxified endotoxin for 24 hr or up to 7 days before challenge with toxic endotoxin prevented the development of the typical hemodynamic changes elicited by endotoxins or by virulent gram-negative bacteria. The results of Urbaschek and Nowotny (350, 351) showed that endotoxoid-2 is able to elicit an endotoxin tolerance-like state. A single injection of 1 μg of endotoxoid-2 into guinea pigs 24 hr before they were challenged with a lethal dose of serologically unrelated toxic endotoxin prevented 50% mortality. Higher endotoxoid doses resulted in complete prevention of endotoxic shock. Burn-shock of guinea pigs was prevented by applying a 100 μg/100 g (body weight)-dose at 24 hr before the burn. Alterations were observed microscopically in the microcirculation of hamster cheek pouch shortly after administration of the endotoxins. This characteristic effect was eliminated by pretreatment of the animals with a single 100 μg/100 g (body weight)-dose of endotoxoid-2. Pyrogenicity tolerance in monkeys was achieved only after twice-repeated iv injections of endotoxoid-2, 24 hr apart. Tolerance of Shwartzman local skin reactivity in rabbits could not be induced even after five-times repeated endotoxiod-2 injections given iv (350).

The nonspecific resistance-enhancing effect of these preparations was investigated in several assays. The results in mice challenged with virulent *Salmonella typhosa* 0901 cells 24 hr after pretreatment with detoxified endotoxin showed that the detoxified material has activity comparable to that of toxic preparations. Wiener, Beck, and Shilo (*unpublished data*) found that detoxified endotoxins at higher levels showed protection in levanized rabbits comparable to that of toxic endotoxin. At lower dose ranges, the toxic material gave higher protection. The effect of detoxified preparations on the phagocytic index enhancement was similarly lower than that of the toxic materials.

Differences in the uptake of endotoxin and endotoxoid by the RES were investigated by Golub, Gröschel, and Nowotny (107), as discussed earlier. The results show that 2-mercaptoethanol-resistant immunoglobulins facilitate the entry of endotoxoid into the RES.

These results are in correlation with recent observations (235) on the immunogenicity of toxic and detoxified materials. By injecting toxic or nontoxic endotoxins several times in increasing doses, approximately the same antibody titer was achieved in 4 weeks. One significant difference was observed during the immunization. While toxic endotoxin produced 2-mercaptoethanol-sensitive and -resistant antibodies simultaneously, endotoxoid-2 produced mostly 2-mercaptoethanol-sensitive antibodies in the first 10 days. At this time, detectable amounts of resistant antibodies occurred in the peripheral blood, and from this time on, a rapid production of resistant antibodies was the response.

Gewurz et al. (103), who studied the consumption of complement by endotoxin without the addition of antibodies, observed that detoxified preparations lack this capacity. The degree of detoxification, measured by other biological parameters, showed parallelism with the diminished ability to fix complement. The gradual decrease of complement-fixing capacity of a sodium hydroxide-detoxified preparation showed an apparent parallelism with the pyrogenicity or mouse lethality measurements. These findings support the hypothesis that some endotoxic reactions may be mediated through complement.

The evaluation of these experiments with detoxified preparations is difficult at the present time. What seems to be an important achievement is the detection of a lack of relationships among certain biological properties, such as lethality and the development of endotoxin tolerance or adjuvant effect. Serological reactivity seems to be unrelated to toxicity. Endotoxoids seem to be able to stimulate the defenses of the host just as well as toxic endotoxins. A lack of relationship even among different toxic param-

eters was shown by using partial detoxification with NaOH.

On the other hand, the use of endotoxoids as well as partially detoxified preparations gave insight into relationships hitherto not recognized. Examples of this are the lethality and complement-fixing capacity, as well as pyrogenicity, RES uptake and toxicity, toxicity and immunoglobulin G production, and some others.

Summing up the studies of detoxification, the assumption is that not merely a certain particle size or shape, but the existence of functional groups in the endotoxin structure are essential for toxic manifestations. The distances between the essential functional groups and their arrangement seem to be important also to create a toxic conformation. If this is destroyed, by cleaving off some of the essential functional groups or by changing the distance between them through hydrolysis or through distortion or by masking these groups through complex formation, the result is detoxification.

THEORETICAL CONSIDERATIONS OF THE POSSIBLE ROLE OF FATTY ACIDS IN ENDOTOXICITY

The chemical structure of the lipid moiety of endotoxins is unique, and no other natural products of similar structure have been reported to date. The biological effects are also unique, especially with regard to the great variety of reactions elicited by endotoxins. The singular structural features of endotoxins are the fatty acid-carbohydrate linkages, which so far have not been found in any other natural products (229, 231, 239). Numerous data show that the presence of fatty acids and their derivatives is essential for the elicitation of endotoxic reactions (230, 232, 233, 346).

How these long-chain carboxylic acids can endow the lipopolysaccharide molecule with toxic properties has been discussed in the past (346). These theoretical considerations included facilitated passage of the endotoxin through the lipophilic membranes owing to its fatty acid content, and the possibility that the presence of long-chain acids in the lipopolysaccharide molecule will slow down enzymatic breakdown by enzymes present in normal hosts. Some other theoretical considerations were based on the possibility that the long aliphatic chains of the fatty acids form nonpolar binding forces. These may serve as intramolecular forces holding the endotoxin in a certain toxic conformation and may participate in aggregation, in polymer formation of endotoxin molecules.

Some results seem to indicate that the role of fatty acids may be even more important in the complex formation between endotoxin and its subcellular targets. Adhesion of endotoxin to red blood cell membranes was the basis of passive hemagglutination developed by Neter and associates (223). Unusual firmness of the antigen-antibody complex was observed if endotoxin was precipitated with O-antibodies (253). Great affinity of endotoxin to certain ion-exchange polymers has been reported (140, 236). Removal of fatty acids from endotoxins by alkaline hydrolysis results in complete loss or great decrease in the firmness of such complexes in the above systems. Partial or complete removal of the fatty acids from endotoxin results in detoxification also.

How complex formation between endotoxin and its subcellular targets may result in harmful effects to the host may be visualized by inhibition of the normal function of the subcellular target or interference with its normal metabolism.

It seems important to emphasize that the presence of all the long-chain carboxylic acids found in an endotoxin are not necessary to elicit endotoxic manifestations. In fact, potassium methylate treatment carried out at 20 C indicated that almost 50% of the total fatty acids can be removed from a partially purified endotoxin without altering toxicity. If, however, potassium methylate treatment is continued at 56 C, an additional 24 to 26% of the total fatty acids will be cleaved, resulting in loss of toxicity. The identity and location of these apparently essential fatty acids are the subject of present investigations in our laboratories. The requirement for chromatographically homogeneous endotoxins in this type of study cannot be overemphasized.

SOME OF THE UNANSWERED PROBLEMS

Only a few of the most intriguing but little understood problems can be mentioned here. The first is the lack of a clear understanding of the target of endotoxic action. It is known that certain bacterial exotoxins find their receptors in the sialic acid of the brain gangliosides (354). The mechanism of many poisons is less clearly understood but quite well described in modern pharmacology. In the action of endotoxin, not even the cell types which may serve as the targets of endotoxin action are unequivocally identified. A proper knowledge of the endotoxic targets, cellular or subcellular, is essential for the clarification of the mechanism of endotoxic action, and it would also facilitate the identification of the active sites on the endotoxin structure.

The heterogeneity of endotoxic preparations (234, 236), demonstrable not only in polydispersity but in the chemical composition of the fractions, is unquestionably the most disturbing fea-

ture of this research. Results indicate that no one type of endotoxin molecule exists, but that several active and chemically different molecular complexes are present in some endotoxin preparations. Whether they act similarly or there is a difference in their mode of action is not known. The question of whether the isolated fractions have an active site identical in all molecular species of endotoxin or whether different active sites exist which carry out different roles in endotoxic action is similarly unanswered.

Recent observations from our laboratories showed that reduction in lethality (LD_{50} in chick embryos) occurs in endotoxin preparations during purification by column chromatography. Isolated components, showing a higher degree of homogeneity, were less active than the crude starting material. Recombination of the isolated fractions enhanced their lethality, although the original activity could not be restored. Whether this phenomenon is a simple loss of activity due to the procedure applied, or whether it indicates that synergistic effects of the several fractions are required for high toxicity, is under current investigation.

SUMMARY

No other natural product is known which would elicit such a great variety of reactions as do endotoxins when injected into the proper host. These characteristic endotoxic effects show a certain degree of interrelationship, but not all activities are present in all endotoxin or endotoxoid preparations to an equal degree. Selective elimination of certain activities became possible by using chemical alterations of the molecular structure.

The chemical structure of bacterial endotoxins is similarly unique. The chemical constituents of endotoxins are carbohydrates, short- and long-chain carboxylic acids, some amines, amino acids, and phosphorus. These are arranged in three major zones in the macromolecular structure, forming the polysaccharide, the lipid-rich, and the amino acid-rich moieties. The backbone of the structure is probably polysaccharide, which consists of a variety of different carbohydrates such as hexoses, heptoses, octonic acids, amino sugars, and their derivatives. Attached to this backbone are amino acids, probably through amino sugars, as well as carboxylic acids which are ester-bound to OH groups or amide-bound to NH_2 groups of carbohydrates, favoring glucosamine. The exact location of phosphoric acid residues is not known; the lipid-rich zones and the polysaccharide moiety both contain phosphoric acid linked to carbohydrates. The presence or absence of these different substituents on the polysaccharide chain endows certain parts of this backbone with lipidic or peptidic, highly polar or nonpolar, charged or neutral characteristics.

It is assumed that the entire lipopolysaccharide macromolecule is not involved in the elicitation of the diverse endotoxic reactions but that these are localized in certain active sites. These active sites are formed through specific steric arrangements of certain functional groups of the structure. The biological role of the above three major moieties has been investigated and it was found that the major role of the polysaccharide moiety lies in the determination of the serological specificity of the bacteria as well as of the lipopolysaccharide. The peptide-forming amino acids enhance the immunogenicity of the preparations and probably also serve as immunodeterminants. No convincing experimental evidence has shown that these parts play a role in the toxic manifestations of endotoxins, although their role in assumed "endotoxin-hypersensitivity" cannot be ruled out. Rough mutants, lacking the usual polysaccharide moiety, yielded a glycolipid which demonstrated full endotoxic potency. Similarly, almost complete removal of peptides by chemical means enhanced the endotoxicity. These results seem to indicate that the active sites may be within the lipid moiety.

The relationship between the lipid moiety and the so-called "lipid A" preparation is unquestionably close, although they are not identical. The so-called "lipid A" preparation is an extremely heterogeneous mixture which consists of different sizes of breakdown products, from free building stones to incompletely degraded residual endotoxins. It is obvious, therefore, that the whole mixture still shows residual endotoxic properties. In "lipid A," everything is present which became insoluble owing to acidic hydrolysis which destroyed the lyophilic polysaccharide carrier. Most of these originate from the lipid-rich moiety of the endotoxin but are obviously not identical with it. On the other hand, structural study of the different split products present in the "lipid A" mixture is one of the reasonable approaches to gain insight into the structure of the lipid moiety in the intact endotoxin.

The formation of complexes is one of the most characteristic physicochemical properties of endotoxin molecules. Endotoxin consists of associated subunits. According to our observation, these subunits are active even if they are dissociated from each other. The increase in some activities after short alkaline treatment seems to support this assumption. If the dissociation is carried out by or in the presence of substances which form complexes with the subunits, their biological activity may suffer. It is suggested herewith that

the sites involved in subunit aggregation are the same sites through which complexes with the targets of endotoxic action are formed, thus eliciting toxic manifestations.

The results covering the identification of the essential functional groups within the active sites are still incomplete. Some of the O-acyl-bound fatty acids may be incriminated as participants in endotoxic reactions. Experimental data indicate that the presence of fatty acids provides a structure which seems to be harmful to the cellular or subcellular targets. The role of fatty acids may be important in the formation of complexes between endotoxin and its targets, but the role of other functional groups such as phosphoric acid radicals or unusual carbohydrates may not be disregarded. These may also be involved in interactions with the targets.

It has been demonstrated by many authors that the essential functional groups are acid-labile. They can be split more selectively with other chemical procedures. Detoxification may be achieved either by cleaving some of the functional groups or by the distortion of the structure which changes the optimal steric arrangement of the functional groups, or both. Obviously, blockage of the essential functional groups through complex formation with antibodies, other proteins, detergents, or other chemicals will also lead to detoxification.

In the investigation of the mode of endotoxic action, the fate of injected endotoxin was followed and it was found that it accumulates in the reticulum cells. Whether this accumulation of endotoxin in the reticuloendothelial system means that these cells are the primary targets of endotoxin or represent only stations in usual clearing processes is uncertain. Our present knowledge of the primary, subcellular targets of endotoxic action is highly inadequate.

The recently discovered heterogeneity of all investigated endotoxic preparations makes the study of the mode of action even more difficult. It appears that the higher the heterogeneity, the greater the endotoxicity of the preparation. It is possible that the presence of several components is necessary for the elicitation of a full array of characteristic endotoxic reactions. At the present time, a picture of quite formidable complexity emerges from the published data with regard to the mode of endotoxic action. It is possible that several targets of endotoxic action may exist, some being hit directly, some indirectly. It seems reasonable to suppose that not only one molecular type of endotoxin exists, which makes it possible that several endotoxic components act simultaneously or in sequence on different targets, thus eliciting not one but a number of chain reactions in the organisms. The use of chemically homogeneous endotoxin preparations is indispensable not only for chemical structural studies but also for a better understanding of their mode of action.

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