BACTERIAL PYROGENS

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The history of science abounds with instances in which a crucial experiment, a chance observation, or a painstaking and thoughtful review has led to recognition of a general, unifying principle. The sources of our present knowledge of bacterial endotoxins furnish a striking example of this. Gradual appreciation of their ubiquity in nature and of the multiplicity of physiological alterations that they can elicit has led to intensive and continuing study of these substances. It is now known that these bacterial products are a common denominator that explains seemingly unrelated observations and experiments carried out by microbiologists, physiologists, pathologists, and clinicians over a period of almost a century.

It is the purpose of this review to describe concisely present concepts of the nature and structure of bacterial endotoxins, the reactions they produce, the mechanisms of these reactions, and to emphasize problems in this field that remain to be investigated. Because there have been several detailed reviews and bibliographies of this subject in recent years (30, 38, 58, 113, 129, 235, 244, 251, 265), historical aspects will be mentioned only briefly. The references have been selected carefully with no effort to document each point exhaustively. Whenever possible, reference has been made to previously published bibliographical listings where the interested reader will find several hundred specific citations dealing with the many aspects of research on endotoxins. Some repetition of presentation has been necessary to enable clarity of discussion and interpretation.

I. HISTORY

It was recognized in the latter part of the nineteenth and early in the twentieth century that injection of a variety of substances, including distilled water, saline solution, tissue extracts, milk, serum, salvarsan, colloidal metals, bacterial vaccines, and others, particularly by the intravenous route, would elicit fever and other "toxic" reactions in man or experimental animals. The first experi-

mental study of these troublesome "injection fevers" or, as they came to be called, "protein fevers" was probably that of Billroth (42) who produced hyperthermia in dogs by injection of ordinary distilled water in 1865. A few years later, Burdon-Sanderson (57) coined the term "pyrogen" to designate fever-producing substances he had extracted from putrefying meat, and there followed a period of extensive study of pyrogenic materials by many observers.

These investigations can be said generally to have followed three lines; although such categorization is convenient for descriptive purposes, it is readily apparent as one reads the older papers on pyrogens and fever that there was little exchange of information in the field and that many important studies were entirely unnoticed for several years.

The first approach was a clinical one, a result of recognition of the therapeutic efficacy of artificially induced fever; it was characterized by a search for pyrogenic substances. Bacterial vaccines were soon found to be potent and relatively consistent preparations for pyretotherapy. While there were those who continued for several years to advocate therapeutic injection of milk, proteoses, or other substances, it was finally apparent that most of these materials (particularly milk) owed their pyrogenic property to their content of contaminating bacteria. The details of the widespread enthusiasm for so called "protein shock" therapy are available elsewhere (30). Peterson's monograph (264) and Hektoen's review (158) are good samples of the claims made for this form of treatment. The apparent amelioration of symptoms in many diseases led to a few studies of the physiologic alterations accompanying pyretotherapy. None of the hematologic, hemodynamic, or metabolic changes described, however, explained the curative action of fever. The details of the reactions produced by pyrogens are better understood at the present time but it cannot yet be said that the unquestionable benefit of hyperpyrexia (in disorders of the eye, in arthritis, or in neurosyphilis) has been clarified.

A second line of investigation probably began with the work of Roussy (286) in 1889 when he attempted to separate from bacteria a purified fever-producing substance. The history of subsequent attempts by other workers to concentrate and fractionate the fever-producing substances of bacteria has been reviewed in detail in the excellent monograph of Berger *et al.* (38). Suffice it here to mention the important (and apparently, long-forgotten) demonstration by Centanni in 1894 (65) that the substance in bacterial cultures that produced fever was not a protein and that it was heat-stable. In 1916, Jona (177) prepared fractions of typhoid cultures and colon bacillus cultures that produced fever in rabbits in a dosage of a few micrograms; these were heat-stable and contained little or no protein. The curious neglect or unawareness of the investigations of others that has characterized the study of these bacterial substances through the years is nowhere more strikingly illustrated than in the first U.S.P. collaborative study of pyrogens (381) in 1943 in which, nearly 50 years after Centanni's experiments and 30 years after Jona, *nitrogen content* was adopted as an index of the pyrogenic activity of bacterial filtrates! The present status of our knowledge of the chemistry of endotoxins and the influence of methods of preparation upon the structure

and potency of the final product is summarized in a later section of this paper. It is of interest that it was not until 1944 that Co Tui (80) pointed out that the methods used to fractionate bacterial "pyrogens" through the years were similar to those employed by immunochemists to purify the "somatic antigens" of bacteria; it is now known that the pyrogen, the somatic antigen, and the endotoxin of an organism are closely related, if not identical, although toxicity and antigenicity are not necessarily dependent upon the same chemical groups.

The third, and eventually the most informative and important, series of investigations of injection fevers was aimed at finding and eliminating their causes to make possible the parenteral administration of therapeutic agents without unpleasant or dangerous side-reactions. Both Müller (233) and Wechselmann (377) called attention to the possible role of bacterial contaminants in distilled water used for preparing solutions of salvarsan for injection but were puzzled by the failure of elimination of micro-organisms by filtration or autoclaving to destroy the pyrogenic property. Samelson (289) and Bendix and Bergmann (22) also emphasized that "salt fevers" were not caused by salt but by the use of contaminated water in preparing solutions. The important investigations of Hort and Penfold, however, marked the beginning of the first real understanding of injection fevers (167a, b, 168). These investigators devised a standardized method of testing for pyrogens in rabbits and assayed bacteria for ability to produce fever. They were able to classify micro-organisms into pyrogenic types, predominantly the Gram-negative bacilli, and non-pyrogenic types, the Gram-positive cocci, and showed that dead bacteria were fully as capable of producing fever as living cultures. They showed that the toxicity of distilled water was usually correlated with its bacterial content and concluded that a filterable, heat-stable substance, probably of bacterial origin, was the cause of all injection fevers. Finally, they pointed out that many vaccines in use for fever therapy contained non-pyrogenic bacteria and owed their efficacy to the fact that the suspending medium was contaminated.

The studies of Hort and Penfold which were completed in 1912 were accorded almost no attention until the classic investigations of Seibert in 1923–1925 (300–303) eliminated all doubt and established conclusively that all injection fevers were a result of contamination by filterable, heat-stable pyrogens and that these pyrogens were produced by Gram-negative bacilli. The avoidance of febrile reactions, as outlined by Rademaker (273), who confirmed Seibert's findings, depends upon the avoidance of bacterial contamination at any stage in the preparation of pharmaceuticals and, because sterility is no guarantee of the absence of pyrogen, the injectability of fluids must be determined by assay in animals for ability to produce fever. Up to the present time, this is the method still employed for the detection of pyrogenic contaminants.

Gram-negative bacilli or their products produce many reactions other than fever, including leukopenia followed by leukocytosis, hemorrhagic necrosis in normal or neoplastic tissues, vasomotor disturbances, metabolic disturbances, and death (30). Without going into details, it can now be said that all of these and many curious "non-specific" alterations in resistance are now known to be

attributable to the filterable, heat-stable substance that is responsible for injection fevers, the endotoxin of Gram-negative bacteria.

II. BACTERIOLOGY AND TERMINOLOGY

Surveys have shown that endotoxins are characteristic of Gram-negative bacteria, particularly of coliform and related bacilli (79, 271, 404). An occasional Gram-positive organism (128) or fungus (151) has been reported to contain pyrogenic material, and Stetson (330) has recently shown that lysates of hemolytic streptococci possess endotoxic properties. It is not surprising that other organisms might contain chemical substances similar to endotoxin and these exceptions do not alter the fact that Gram-negative bacteria are by far the predominant source of endotoxins.

The endotoxins produced by Gram-negative bacilli differ in their antigenic properties but all that have been isolated are similar in structure, being complex polysaccharides or "lipopolysaccharides"; all produce the same physiologic changes and pathologic lesions in man or experimental animals; none of these alterations is detectably modified by antiserum (in contrast to so-called exotoxins), and the amount or potency of endotoxin possessed by a given species bears no relationship to its pathogenicity, virulence, or invasiveness.

There is considerable variation in the potency of purified endotoxins that are isolated from different species of bacteria or different strains of the same species, but in many instances this is as much the result of the methods used in extraction and concentration as it is of any intrinsic property of the bacteria themselves.

Studies such as that of Mondolfo and Hounie (223) indicate that conditions of growth of a bacterial culture and such properties as "smoothness" or "roughness" may influence endotoxin production. However, the excellent studies of Westphal (387, 388) have shown conclusively that "rough" strains are often more potent sources of toxin than the "smooth", and because quantitation of toxicity is difficult and the method of chemical fractionation so important, there seems little to be gained by drawing any other conclusions from the available data.

Many different names have been applied to the toxic fractions of Gram-negative bacilli, most of them reflecting the particular interests of the investigator. At the present time, however, it seems to the authors that "endotoxin" is preferable to such terms as antigen, somatic antigen, toxic antigen, Boivin antigen, Shear polysaccharide, tumor necrotizing substance, Shwartzman toxin, pyrogen, lipopolysaccharide, adrenal-hemorrhagic fraction, toxic gluco-lipids, Westphal toxin, leukopenia-producing fraction, pyretogenic factor, and the many others that have been used in the past. *Endotoxin* will be used in this review to designate the bacterial substance responsible for all of the reactions implied in the foregoing list.

III. PHYSICAL AND CHEMICAL PROPERTIES

The methods which have been used for fractionation and separation of purified endotoxin have been reviewed by Burrows (58), Van Heyningen (369) and West-

phal and Lüderitz (388). Most of these procedures require vigorous chemical treatment of Gram-negative bacteria or their filtrates, including the use of phenol, trichloracetic acid, pyridine or other organic solvents. Fractionation of the components of endotoxin has necessitated hydrolysis with hot acid or alkali. These measures have complicated the isolation of the toxic and antigenic constituents because of degradation, denaturation and detoxification. The use of enzymes for fractionation of endotoxin has not met with much success, although a number of microörganisms have been reported to produce substances capable of digesting the toxin complex.

It seems fairly certain that the pyrogenic action of Gram-negative bacteria is attributable to a phospho-lipid conjugated to polysaccharide and protein (387, 388). The lipo-carbohydrate-protein complex, or endotoxin, is antigenic as well as toxic. Westphal (387) has demonstrated that the lipid is probably toxic only when conjugated to polysaccharide or protein. It has been shown by several investigators (92, 180, 231, 232, 257, 343) that chemical separation of the polysaccharide results in loss of its antigenicity, but the carbohydrate may retain serological activity. Although the polysaccharide is the principal antigenic determinant (hapten) of the complex, the protein also possesses immunological properties.

The endotoxin polysaccharide is responsible for the serological specificity of different strains of Gram-negative bacilli, and its chemical characterization has shown distinct similarities between polysaccharides of various endotoxins. All seem to contain hexosamine and glucose, and many include also a pentose, usually rhamnose (38, 58, 128, 319). The specific physical-chemical properties responsible for determining antigenic specificity, however, have not been defined. Morgan and co-workers (231, 232, 257) and Westphal and Lüderitz (388) have demonstrated the ability of unconjugated polysaccharide to combine with protein derived from heterologous Gram-negative bacteria, conferring the serological specificity of the homologous antigen upon the prepared protein-polysaccharide conjugate. Illustrative of the close association but probable non-identity of the polysaccharide hapten to the toxic moiety of endotoxin is the observation by Morgan (225) showing that the precipitation of endotoxin with antiserum is not accompanied by detoxification.

Comparison of the immunological activity of a lipo-carbohydrate toxin prepared from *Salmonella typhosa* with its separated polysaccharide hapten has shown that not all of the serological activity of the endotoxin can be accounted for by the hapten (195, 376). Correspondingly, the lipo-carbohydrate is immunologically heterogenous when analyzed by precipitation-in-gel (72). In the rabbit, 0.001 μg of the purified Salmonella endotoxin is antigenic, and this amount also possesses pyrogenic activity.

Immunochemical and electrophoretic analysis of a purified endotoxin from *Shigella flexneri* (69) reveals that the lipopolysaccharide is conjugated to three protein substances with different electrophoretic and immunological properties. Two of the proteins with which the lipopolysaccharide is bound are susceptible to proteolytic action and their digestion with trypsin allows the freed lipo-

carbohydrate to combine with the trypsin-resistant protein. Analysis of the purified toxin by precipitation-in-gel shows three immunologically active constituents. Treatment with trypsin obliterates the evidence for this immunological heterogeneity. The three protein-lipo-carbohydrate complexes in the initially purified preparation are pyrogenic, indicating that the nature of the protein is probably not important in determining the biological action of endotoxin.

Jesaitis and Goebel (175) have studied the relationship of the lipo-carbohydrate of *Shigella sonnei* phase II to inactivation of bacteriophage. The lipid component seems to act as a co-factor in the lysis of T4 phage. The polysaccharide component determines the affinity of T4 phage for the lipopolysaccharide. This relationship between endotoxin and activity of bacteriophage deserves investigation to determine what effect bacteriophage has upon the toxic action of the lipopolysaccharide.

It is difficult to prepare solutions for parenteral use free of contaminating endotoxin, as mentioned above. The methods which have been developed for eliminating pyrogenic contaminants are considered in detail by Berger *et al.* (38). These pyrogens resist autoclaving and boiling, and are destroyed only by long drastic heating. They are stable on storage, particularly in the dried state. Heating with alkali completely inactivates pyrogenic solutions. Oxidation with hydrogen peroxide results in a decrease of pyrogenic action. Acetylation and esterification of endotoxin reduces its pyrogenicity. The most practical means of removing pyrogen from solutions is by the use of adsorptive agents such as activated carbon or asbestos.

Although Gram-negative bacteria are generally considered to be the principal source of endotoxin, recent studies by Stetson (330) have shown that lysates of Group A hemolytic streptococci possess similar toxic actions, resulting in the elicitation of fever, the Shwartzman reaction and production of non-specific pyrogen tolerance. Identification of the toxin in streptococcal lysates has not been reported. It is of interest, however, that an adaptive enzyme prepared from soil bacteria and directed against the group-specific carbohydrate of a Group A streptococcus is capable of reducing the serological activity of a purified endotoxin derived from *S. flexneri* (75).

IV. BIOLOGICAL EFFECTS

The toxic activities of endotoxins are remarkably similar irrespective of the species of micro-organism from which they are derived. Their biological effects include production of fever, changes in leukocytes and platelets, production of hemorrhagic lesions in most viscera and skin, hypotension, tumor necrosis, adrenal and renal necrosis, and metabolic changes involving electrolytes and glucose (30). In addition endotoxins may cause death when inoculated in large dosage.

1. Distribution. A few studies have been performed attempting to delineate the distribution of endotoxin following inoculation. Two principal difficulties in performance and interpretation of such investigations are: 1) the necessity for injecting doses which are lethal; 2) the use of labels probably incorporated

in the polysaccharide or protein component of the endotoxin complex, which may not be directly concerned in toxicity.

When radioactive endotoxin is inoculated intravenously into normal rabbits or mice the substance is at first partitioned between leukocytes (buffy coat) and plasma but then becomes localized in the liver, spleen and lung (9, 52a, 52b, 88). No toxin can be found by isotope assay in the brain and there is no concentration in the kidney. Pretreatment of rabbits with thorium dioxide (Thorotrast) inhibits the uptake of toxin by the reticuloendothelial cells, and this is associated with an increase in the lethality of endotoxin. X-irradiation and cortisone do not interfere with the collection of labelled material in liver, spleen and lung (88). Endotoxin is not localized in the kidney, although pretreatment of rabbits with toxin or cortisone results in bilateral renal cortical necrosis.

Braude *et al.* (51) compared the distribution of radioactively labelled endotoxin in normal and pyrogen-tolerant rabbits. In normal animals there is clearance of toxin from the blood within two hours and large amounts appear in the liver. Ninety-five per cent of the toxin disappears from the blood in fifteen minutes in tolerant animals, and accumulates chiefly in lung and liver. Pretreatment with X-irradiation results in persistence of blood levels of toxin at about 50% for a prolonged period of time. In rabbits having specific serum antibody, but in which pyrogen tolerance has subsided, the radioactive toxin disappears from the blood rapidly, although taken up avidly by leukocytes, and accumulates in the lung and liver. After two hours the level in the lung subsides and toxin reappears in the blood.

2. Hemodynamic effects. A sudden increase in peripheral blood flow followed by generalized arteriolar constriction has been described (96, 97, 99) after intravenous or intraperitoneal inoculation of endotoxin. Waves of vaso-dilatation and vaso-constriction follow the initial blood vessel reaction, and may last for several hours. Lethal doses of endotoxin produce increasing vaso-dilatation, accompanied by the development of shock. Although sympathectomy does not prevent the arteriolar changes (48), treatment of the animal with dibenzylamine prevents arteriolar dilatation but does not prevent death (46). The adrenergic characteristics of these effects of endotoxin are particularly interesting in light of recent experiments (350) showing that endotoxin sensitizes blood vessels to the action of epinephrine and norepinephrine. Furthermore, intradermal injection of epinephrine into rabbits, followed or preceded by an intravenous inoculation of endotoxin, results in hemorrhagic necrosis at the site of epinephrine injection. This action may represent a fundamental mechanism in the hemodynamic and other effects of endotoxin.

Renal blood flow is increased for several hours after injection of endotoxin in man and experimental animals. The changes in renal circulation are attributable to dilatation of efferent glomerular arterioles (316), and are not related to the development of fever, as suppression of fever with antipyretics does not influence this effect of endotoxin. Renal hyperemia is prevented by dihydroergocornine (342).

Weil and his co-workers (380) have demonstrated an increase in *portal vein*

pressure after inoculation of endotoxin, occurring within five minutes and lasting about one hour. The rise in portal vein pressure is associated with a fall in cardiac output and hypotension. Decreased venous return to the heart is probably responsible for the fall in cardiac output, although vena cava pressures remain the same as normal, and blood is pooled in the portal circulation. Peripheral vascular resistance is normal and hypotension is most likely accounted for by the decrease in cardiac output (105, 209, 380). Hamrick and Myers (149) found an increase in *hepatic blood flow* in man given sub-febrile doses of endotoxin, associated with a decrease in bromsulphalein excretion and an increase in oxygen consumption by the liver. In these experiments in man there was no change in cardiac output.

The *central nervous system* may be implicated in some of the hemodynamic effects of endotoxin (262). Cross-circulation of the brain of normal dogs with blood from donor animals given an injection of endotoxin has shown that sympathicomimetic and other effects can be induced in the recipient dog.

Heyman *et al.* (162) studied the effect of endotoxin on the *cerebral circulation* of man and found no change in cerebral blood flow or oxygen consumption four hours after injection in patients with asymptomatic neurosyphilis, although in paresis the endotoxin caused an increase in cerebral blood flow and oxygen consumption, but not above normal levels. The cerebrovascular resistance decreased in both groups of patients. The findings in patients with asymptomatic neurosyphilis are believed to be equivalent to those expected in normal persons.

3. Hematological effects. The most extensively studied hematological effect of endotoxin is the pronounced influence on circulating leukocytes. Within a few minutes after intravenous injection there is a striking *leukopenia* followed after about two hours by increasing *leukocytosis*. Granulocytopenia is primarily responsible for the leukopenia. The decrease in number of circulating granulocytes is largely attributable to sequestration of these white cells within the pulmonary blood vessels and spleen. The rebound leukocytosis may be associated with release of white cells from the lung, and an increase in bone marrow activity.

Intravenous inoculation of endotoxin into normal rabbits produces inhibition of the *migratory activity of leukocytes* from the buffy coat of centrifuged blood (41). This effect is demonstrable only in the packed white cell layer, becomes evident within five minutes and lasts about six to twelve hours. Changes in the surface activity of leukocytes increasing their adhesiveness are probably responsible for this effect (39, 122, 123). Interference with the motility of leukocytes is not detectable in pyrogen-tolerant rabbits given an injection of endotoxin (68). An effect of endotoxin on migration of leukocytes in tissue culture is not regularly observed; however, Martin and Chaudhuri (214) have shown that the toxin may have a partial influence on the motility of white cells *in vitro*.

Increased aerobic glycolysis of granulocytes in contact with endotoxin has been described, and Kerby (180) has shown liberation of lysozyme by leukocytes in contact with the toxin.

Transient *thrombocytopenia* occurs following intravenous injection of endotoxin (30, 348), and recently it has been demonstrated that *fibrinogenopenia*

develops when an intravenous inoculation of endotoxin or synthetic acidic polymer is given to rabbits which have been given an initial intravenous injection of toxin a few hours previously (360). A single injection of endotoxin or polymer does not produce a fall in blood fibrinogen levels. Administration of heparin before the second injection of toxin or polymer prevents the fibrinogenopenia. Within four hours after a single injection of endotoxin a substance appears in rabbit plasma that is precipitated in the cold by heparin. However, this precipitable protein substance fails to develop in the plasma of animals treated with heparin.

4. Tissue-injurious effects. Endotoxin injected into a variety of experimental animals can produce mucosal and submucosal hemorrhages in the gastrointestinal tract (260, 261, 315), and necrosis of hepatic cells (including Kupffer cells), myocardium, adrenal cortex, bone marrow, kidneys and lymphoid tissue of the spleen and ileum (56, 226). Inoculation of endotoxin into joints of rabbits produces inflammation, degeneration of cartilage, synovial proliferation and articular fixation (230). Stuart (335–337) has demonstrated that oral and intravenous administration of *Pseudomonas* endotoxin (Pyromen) produces dispersion and then disappearance of the cytoplasmic granulation of mast cells, leading to pyknosis and cellular deterioration. These effects are most pronounced in mast cells of the vascular system. Intravenous inoculation of endotoxin into rabbits with sublethal burns of the skin inhibits fibroblastic growth (337). Glial scar formation in the transected spinal cord of cats is inhibited by giving toxin intravenously, facilitating regeneration of axons by relieving obliterative fibrosis (395). Repeated injection of sublethal doses of endotoxin into several animal species produces few pathologic alterations, including cloudy swelling of the kidneys and increased production of macrophages and lymphocytes (394). Increased production of myelocytes and granulocytes in the spleen and hypertrophy of the adrenals, occasionally with vascuolization of the zona reticularis, are also observed.

5. Tumor-necrotizing effects. Hemorrhage and necrosis has been produced in a variety of tumors, chiefly sarcomas, by the intravenous injection of endotoxin (101, 102, 141, 235, 236, 309, 405). Algire *et al.* (2) studied the effect of endotoxin upon transplanted tumors in mice by observations through window implants and observed slowing of circulation, thrombosis, necrosis and hemorrhage in the area of neoplastic growth. These changes did not occur in normal tissues. Mechanical circulatory obstruction duplicates the effect of endotoxin on tumors (401). There is a relationship between the degree and duration of hypotension after injection of the toxin and the extent of tumor damage; however, it is not certain that the tumor-necrotizing action of endotoxin is entirely attributable to circulatory stasis (1). Induction of tolerance to pyrogen in animals receiving tumor transplants nullifies the ability of endotoxin to produce hemorrhage and necrosis.

There is no evidence that endotoxin has a direct effect upon tumor cells in tissue culture (304), and the resemblance of tumor necrosis to the Shwartzman phenomenon suggests that both may have a similar pathogenesis. The decidual

part of the placenta in rabbits undergoes hemorrhagic necrosis after injection of endotoxin and this rapidly growing tissue may be damaged in the same way as rapidly growing tumors (402).

6. Shwartzman reaction. The local skin phenomenon of hemorrhagic necrosis classically elicited by a preparatory intradermal injection of endotoxin followed within 18 to 24 hours by an intravenous injection of toxin has been extensively studied, and important observations made within the past few years have helped to define the probable pathogenetic mechanisms. Similarly, a good deal has been learned about the generalized Shwartzman reaction, classically elicited by two intravenous inoculations of endotoxin separated by 18 to 24 hours, and characterized most specifically by bilateral renal cortical necrosis.

Endotoxins from many Gram-negative bacteria have been demonstrated to be capable of producing the Shwartzman reaction (35, 128, 308). Besides endotoxins it has been shown that some non-bacterial substances such as glycogen and starch (355), native Dextran (27), antigen-antibody reactions (329) and lysates of Group A hemolytic streptococcus (330) will elicit the reaction. The non-bacterial material, although capable of provoking a local skin reaction, will not elicit cortical necrosis or cause deposition of fibrinoid material in the renal glomeruli—characteristic features of the generalized reaction.

Injection of endotoxin into rabbit skin leads to inflammation, with polymorphonuclear leukocyte infiltration, increased local production of lactic acid and vascular dilatation (362). The permeability of blood vessels is probably impaired, as indicated by the failure of intravenously injected Evans blue dye to diffuse into the site of endotoxin inoculation (361). Following the provocative intravenous injection of endotoxin or glycogen there is intravascular aggregation of leukocytes which is probably attributable to the increased adhesive properties and surface alterations of white cells, discussed above (333). Stetson (332) has illustrated the development of thrombosis in blood vessels at the prepared site after intravenous injection of endotoxin, and has attributed to this a fundamental role in production of the hemorrhagic necrosis. These thrombi are composed of leukocytes, platelets and fibrin, and their production parallels the occurrence of leukopenia and thrombocytopenia which develops following the intravenous injection of toxin.

The probable role of leukocyte thrombosis in the hemorrhagic necrosis of the local Shwartzman reaction is supported by the finding that leukopenia induced in rabbits with nitrogen mustards (HN2) inhibits its production (333). Furthermore, anticoagulation with heparin or ethylbiscoumacetate (Tromexan) prevents the Shwartzman phenomenon (74, 132, 274). Of additional interest is the fact (34, 74) that heparin and HN2 will not significantly suppress the pyrogenic or lethal action of endotoxin. Becker (14) has previously shown that X-irradiation and benzene also inhibit the Shwartzman reaction, probably by a similar mechanism of leukopenia.

The reasons for the peculiar susceptibility of the prepared skin site to production of hemorrhagic necrosis with thrombosis, following the provocative intravenous injection of endotoxin, are not entirely clear. The altered pH and

accumulation of lactic acid at the prepared site may be important predisposing factors; however, other factors probably are operative as well. Epinephrine inoculated with endotoxin into skin of rabbits results in production of hemorrhagic necrosis without thrombosis playing a particular role (350). Similarly, injection of epinephrine into the skin followed or preceded by an intravenous injection of endotoxin leads to hemorrhagic necrosis. These findings suggest that adrenergic mechanisms probably play a significant role in elicitation of the local Shwartzman reaction.

Renal cortical necrosis is the specific feature of the generalized Shwartzman reaction but leukocytic infiltration and leukocyte thrombi are not seen in the kidney. Deposition of a fibrinoid material in the glomerular capillaries, however, has been described (353, 360) which is probably attributable to precipitation of altered fibrinogen, as discussed before. Fibrinoid deposits do not occur and there is no fall in blood fibrinogen after a single injection of endotoxin. The exact mechanism by which the initial injection of endotoxin sensitizes to a second injection some hours later is quite obscure. It is of interest, however, that HN2 or heparin will prevent the generalized Shwartzman reaction (132, 333). The localization of the generalized reaction to the renal cortex is not associated with concentration of labeled endotoxin in the kidney (52a, 52b, 88, 317). Certainly, it would appear that the generalized phenomenon is dependent upon other mechanisms than those which seem important in production of the local Shwartzman reaction. This is further defined by the fact that nonbacterial substances such as glycogen and starch are active in provoking the local but not the generalized phenomenon (332).

A preparatory inoculation of endotoxin into the skin of rabbits treated with cortisone results in production of a local hemorrhagic lesion and can elicit renal cortical necrosis (356–358). Increased diffusion of toxin from the site of inoculation may be responsible for this effect of cortisone, the single injection of endotoxin serving as the preparative and provocative dose.

Thomas *et al.* (354) have described myocardial necrosis and coronary thrombosis by fibrinoid substance in rabbits with Group A hemolytic streptococcus infection when injected with endotoxin. Systemic and cutaneous streptococcus infection will prepare for elicitation of the Shwartzman reaction. Injection of endotoxin into animals with systemic streptococcus infection results in development of bilateral cortical necrosis of the kidneys, and hemorrhagic necrosis of lungs, liver, spleen and myocardium.

Adrenocorticotrophic hormone and cortisone have an inhibitory effect on the Shwartzman phenomenon under certain conditions, and this is discussed elsewhere (311). Substances which block the reticulo-endothelial system, such as Thorotrast, increase the susceptibility of rabbits to the phenomenon of local and generalized reactivity to endotoxin. The ability of antihistamines, salicylates and other substances to interfere with production of the Shwartzman reaction is irregular, and is discussed in greater detail in reference to the lethality of endotoxin.

7. *Effect upon resistance to infection.* Endotoxins are antigenic but production

of antibody towards these substances plays a minor role in development of resistance to infection by homologous microörganisms. Resistance or tolerance to endotoxin action is not dependent upon antibody and is discussed elsewhere (p. 452). These toxins, however, do exert a non-specific effect upon resistance to infection, and this has been extensively studied in recent years.

Polymorphonuclear leukocyte infiltration induced by injection of endotoxin into skin or other tissues is regularly observed. Gram-negative bacteria, living or dead, and their filtrates have a *chemotactic* influence, *in vitro* and *in vivo* (152, 153). This action of endotoxin is quite different from the effect on leukocyte migration described above (41, 214). The effect on leukocyte migration is probably attributable to an action on white cells but does not concern the ability of endotoxin to attract leukocytes by chemotaxis.

Interference with *diapedesis* of leukocytes from capillaries has been described by Delaunay and Lebrun (98), but this effect is probably attributable to the shock and hypotension resulting from the injection of endotoxin, comparable to that observed in other types of circulatory failure (221).

Endotoxin has an *antiphagocytic* action upon leukocytes, which may be the result of changes in the surface properties of white cells, as described above (400).

The alterations in leukocyte function induced by endotoxin might be expected to result in changes in resistance to infection. The transient increase in resistance to infection by heterologous bacteria injected intraperitoneally a short while after killed Gram-negative bacilli have been injected by the same route may be illustrative of this (114, 248). In addition, Condie *et al.* (77, 78) have found that an intravenous injection of endotoxin increases the susceptibility of rabbits to pneumococcal skin infection. What role the effect of the toxin upon leukocytes has in production of this increased susceptibility remains to be determined. Rabbits made tolerant to the pyrogenic action of endotoxin clear *Staphylococcus aureus* and *K. pneumoniae* from their splanchnic circulation as readily as do normal animals (181).

Intravenous injection into rabbits of endotoxin derived from *Serratia marcescens*, *S. typhosa* and meningococcus enhances *antibody production* to a variety of protein and polysaccharide antigens (77, 78, 176, 334). This effect could be responsible for increasing resistance to infection by other microörganisms. There is no immunological enhancement in rabbits made tolerant or refractory to the pyrogenic action of endotoxin (76).

Serum properdin, in combination with magnesium and complement, participates in the destruction of Gram-negative bacteria (267, 375). Landy and Pillemer (197) and Rowley (287) have shown an elevation of serum properdin levels in mice following administration of endotoxin, and there appears to be a close relationship between the level of serum properdin and non-specific resistance to infection (196). A similar elevation of properdin level has been observed in man following intravenous injection of endotoxin (293).

The action of injected endotoxin or killed Gram-negative bacteria in producing a transient non-specific resistance ("pro-immunity") to infection with homologous or heterologous microörganisms, within a few hours (50, 253) is probably

explained by the effect on serum properdin. The transient non-specific resistance to the adreno-hemorrhagic effect of endotoxin induced by a prior injection of a bacterial suspension, described by Olitzki and co-workers (252, 344), is also probably explained by elevation in serum properdin levels.

8. Lethal effects. That death may occur following injection of endotoxin is well known, although the mechanism of the lethal action of the toxin is not always well defined. Many factors influence the lethality of endotoxin and it is not possible to predict this event with certainty. For this reason, the lethal effect of these substances is not a reliable criterion for testing their biological action.

a. Factors increasing the susceptibility of an animal. Young rabbits, four to seven weeks old, are less susceptible than older animals to the lethal action of endotoxin (318). The susceptibility of young rabbits is $\frac{1}{50}$ that of fully grown animals. A similar difference in the response of young and mature rats has been described by Zahl *et al.* (408).

Endotoxins are lethal to a number of *animal species*, including man (367), mice, rats, rabbits, dogs, horses, horse-shoe crab (*Limulus polyphemus*) (8) and the chick embryo (319). Man, rabbits, and dogs are probably the most susceptible. Goebel *et al.* (130) found that 20 μg of purified Shigella endotoxin given intravenously to rabbits was frequently lethal, whereas about 500 μg given intraperitoneally was required to kill mice. Dare and Mogey (91) studied the relative susceptibility of the rabbit and man to pyrogenic action of endotoxin and found that the sensitivity of rabbits can range from one-third to seven times that of human beings.

Hill *et al.* (164) have demonstrated differences in susceptibility of mice to endotoxin upon an *heritable basis*. Within the same animal species, therefore, there may be variations in the lethal effect of the toxins which are genetically determined.

Injection of colloidal substances such as Thorotrast, saccharated iron or organic iodide increases the susceptibility of rabbits to endotoxin, resulting in more rapid and frequent death, an increase in febrile responsiveness and enhanced susceptibility to the Shwartzman reaction (16, 18, 133, 318). Injection of trypan blue or India ink has a similar effect. The increased lethal action of endotoxin in animals treated with colloidal or particulate substances is probably related to a reduction of the function of reticulo-endothelial cells as discussed elsewhere in this review. Beeson (16, 18) observed that inoculation of Thorotrast into rabbits 18 hours before intravenous injection of endotoxin is associated with higher concentration of toxin in the blood for a longer period of time than in normal animals. Pretreatment of rabbits with Thorotrast inhibits the uptake of radioactive toxin by the RES (88), increasing mortality. Good and Thomas (133) have estimated that injection of Thorotrast into rabbits increases the lethal effect of meningococcus endotoxin about one-thousand times. Young as well as mature animals are susceptible to the increase in lethal effect of toxin induced by injection of colloidal material (318).

Adrenalectomy increases the susceptibility of animals to the lethal action of endotoxin (107, 155). Treatment with adreno-cortical extract restores to ad-

renalectomized animals the normal reactivity to this effect (155), whereas desoxycorticosterone acetate does not (106).

Fine (115) and his co-workers have demonstrated an increase in the susceptibility of rabbits to the lethal effect of endotoxin during *hemorrhagic shock*. It has been postulated that Gram-negative bacterial toxins enter the circulation after production of experimental shock and this is at least partly responsible for the irreversibility of the hypotension. They have recently described an endotoxin-like effect of plasma from shocked animals (297a). There is a relationship between the increased susceptibility to endotoxin of animals with shock and lowered levels of serum properdin.

Heyman and Beeson (161) detected an increased susceptibility to the pyrogenic action of endotoxin in patients with chronic *liver disease*. It is likely that these persons are also more susceptible to the lethal effect of the toxin.

Thomas *et al.* (354) have described an increase in the lethal action of meningococcus endotoxin in rabbits with *systemic infection* produced by Group A hemolytic streptococcus.

The lethality of endotoxin is principally demonstrated by intravenous or intraperitoneal inoculation. Injection by the subcutaneous, intramuscular or intracutaneous route infrequently leads to an animal's death, except in mice. Death is preceded by falling temperature, stertorous respiration, cardiac arrythmia, cyanosis, and occasionally paralysis and convulsions. Depending upon the dose of toxin the animal may die abruptly or survive a few hours.

b. Factors which protect against the lethal action of endotoxin. The effect of *adreno-cortical hormone* on lethality varies considerably in animals given endotoxin, depending upon the animal species, dose and route of inoculation. Spink and Anderson (327), and Geller *et al.* (125) found that cortisone protects mice from death if given one hour before, at the time of, or one hour after injection of toxin. Thomas and Smith (359) showed that cortisone protects mature rabbits from the early prostration and death due to large doses of endotoxin, but that pretreatment for three days with cortisone results in bilateral cortical necrosis of the kidneys. In addition, early death attributed to pretreatment with colloidal iron is prevented with cortisone. Reilly *et al.* (277) observed no protection against death in guinea pigs treated with adreno-cortical steroid. Furthermore, Ebert and co-workers (105) detected no beneficial effect of adrenal steroid in dogs. Levittin *et al.* (203) found that corticosterone and cortisol are equally effective in preventing the lethal action of endotoxin in rats. However, this is true only if the steroid is given intravenously at the time the toxin is injected. Recently, Gordon and Lipton (134) have shown that *serotonin* administered immediately before or immediately after an intraperitoneal injection of *E. coli* endotoxin into mice significantly protects animals against death. Combination of serotonin with Compound F has an enhancing effect in reducing mortality, whereas Compound F alone has no effect.

Development of *tolerance* to the pyrogenic action of endotoxin is accompanied by a decrease in the lethality of the toxin, which is discussed elsewhere (p. 452). Experiments showing development of resistance to the lethal action of endotoxin

by repeated inoculation of sublethal doses probably illustrate development of non-specific tolerance not due to antibody (84–87). The increased resistance detected after a single dose of toxin, however, may be attributable to elevations of serum properdin, discussed above (p. 438).

A variety of *chemical agents* have a partially suppressive effect upon the local Shwartzman reaction, including salicylates (310, 311, 320), antihistamines (368), thiouracil (403), and adreno-corticotropic hormone (321). In addition, crude penicillin (45), sulfonamides (63, 202, 403), oxytetracycline, chlortetracycline (272), streptomycin (125) and chloramphenicol (89, 272) partially suppress an animal's susceptibility to the lethal effect of endotoxin. The influence of antibiotics in preventing death may be partly attributable to a suppression of the bacteremia which frequently occurs after giving a large dose of toxin (125).

9. Fever. Historically, interest in endotoxins was first aroused by the fever-producing action of Gram-negative bacteria and their products. Through the years, and continuing up to the present time, the practical problem of avoiding contamination of solutions for parenteral injection and the devising of reliable methods for detecting endotoxins in pharmaceuticals have commanded a great deal of attention. The U.S.P. test for pyrogens employs bioassay in rabbits. Berger *et al.* (38) have reviewed this test in detail and have also attempted to evaluate various chemical or *in vivo* methods that have been suggested as possible substitutes. Such factors as sex, feeding, housing, and restraint of rabbits, methods of recording temperature, volume of injected material, frequency of use of animals, and attempts to make the test quantitative are evaluated critically by Berger and his colleagues. Their conclusion, that many of the present regulations are arbitrary and deserve further study, is in keeping with the data presented and the interested reader is referred to their monograph for details. A number of attempts have been made to adopt a pyrogen standard but, at present, there is no agreement on this subject. The discussion that followed the presentation of one such suggestion was characterized by striking evidence of a lack of awareness on the part of the pharmacologists with much of the work that has been carried out with endotoxins (93). The present authors have had no direct experience with large-scale testing of pharmaceuticals for pyrogenic contaminants and this aspect will not be further discussed in this paper.

a. Host range. Injection of crude vaccines or purified endotoxins will elicit fever in man, monkeys, horses, dogs, cats, and rabbits (379). Despite a few claims to the contrary, the temperature response to these substances in guinea pigs, rats, mice, hamsters, and chicks is so irregular and unpredictable that these species are unsatisfactory for investigations of fever. The rabbit has been used most frequently in studies on the mechanism of endotoxin fever and the cat has been employed in many investigations of the role of the central nervous system. Dogs are also very reliable test animals and, in general, react in the same fashion as rabbits. In the few studies devoted to the subject (79, 91) it has appeared that both the rabbit and the dog are considerably less sensitive to the pyrogenic action of endotoxin than is man.

b. Route of injection. Most studies of endotoxin fever have utilized the in-

travenous route of administration. Fever will also result from intramuscular, subcutaneous, or intraperitoneal inoculations but responses are less consistent and larger doses are required. It has been stated that slow intravenous infusion will result in higher fever from a given dose of endotoxin in man (59, 90) but in the rabbit, within wide limits, neither volume nor speed of injection appears to influence appreciably the magnitude of fever (15). An exception to this is the fact that minimal pyrogenic doses of endotoxin may elicit no response if infused over a period of several hours (37c).

The effect of *intrathecal* administration of endotoxin will be discussed separately in a later section (p. 446).

c. Central nervous system. There is little question about the fact that the final pathway in the production of fever by bacterial endotoxin is the nervous system. While other mechanisms such as endocrine hyperactivity (82) or shifts in water balance (7) have been suggested, these changes are far more likely to be secondary to hyperpyrexia.

The role of the hypothalamus in controlling body temperature is well recognized (179) and there is evidence that the activity of the thermoregulatory centers in this area is influenced by changes in the temperature of the arterial blood (210). However, numerous studies in cats and dogs with lesions placed in various portions of the brain (30) have been so inconsistent that it is difficult to make a case for the midbrain or any other localized area of the nervous system as being essential for the febrile response to endotoxin. In preliminary studies, Grant (139) found that injection of endotoxin directly into the midbrain of the rabbit had a negligible effect upon body temperature. His conclusion, that endotoxin has no direct action on the brain seems too general, however, and it is perhaps more proper to interpret his experiments as indicating that endotoxin does not act directly on the hypothalamus. The only central nervous system lesion that consistently abolishes the febrile response to intravenously administered endotoxin is high spinal transection.

The role of the autonomic nervous system in the febrile response is unquestionable. Complete sympathectomy interferes with the response of the cat to endotoxin (112). Curarized dogs respond normally to endotoxin but adrenergic blocking drugs abolish fever and, in rabbits, the febrile response to endotoxin is accounted for almost entirely by a sharp decrease in heat loss rather than increase in production (146, 382, 383). In man, the fever produced by endotoxin is the result of a combination of increased retention and increased production of heat (103).

Grant has emphasized an "emotional hypothermic" reaction in untrained rabbits (136) and, for some unknown reason, rabbits tied on their backs do not respond with fever to injection of endotoxin (12).

d. Effect of antipyretics and other drugs. Acetylsalicylic acid, amidopyrine, and similar compounds will suppress the febrile response to endotoxin in man and in animals (30). Other effects such as leukopenia, hypotension, or the Shwartzman reaction are almost wholly unaffected by antipyretics, the exception being incomplete protection against the local Shwartzman reaction by large

doses of salicylates (311). ACTH and cortisone are potent antipyretics but, by manipulation of dosage schedules and amounts of endotoxin used for testing, these compounds can be shown to suppress or aggravate the febrile response (31, 104). Similar results have been obtained with adrenal steroids in the Shwartzman and related reactions (31, 213, 311, 357).

Morphine is said to have an antipyretic action in man (212), and barbiturates (30), or ether (263b) will interfere with the ability of rabbits or dogs to respond with fever to endotoxin injection. This makes it necessary to use unanesthetized animals in studies of this type of fever.

Lastly, the action of calcium salts in abolishing the chill reaction deserves mention (19). It is of great interest that the cessation of chill or rigor after administration of intravenous calcium gluconate is followed by no diminution in the febrile response to endotoxin. This is, perhaps, more evidence for the predominant role of cutaneous vasoconstriction and decrease in heat loss in the pyrogenic reaction.

e. The febrile response. The intravenous injection of endotoxin in man or experimental animals is followed by a *lag-period* of 18 to 90 min (shorter in rabbits than in man) before the onset of abrupt rise in body temperature. This delay between introduction of the toxin directly into the circulating blood, and fever, referred to by Grant as pyrogen "latency" (140), is influenced by the dose of endotoxin but reaches an irreducible minimum of 15 to 18 min in the rabbit.

The onset of fever in man is often accompanied by shivering or rigor with cool, cyanotic skin, dilated pupils, piloerection, decrease in respiratory volume and transient, mild hypertension, evidences of sympathetic hyperactivity (110). Fever reaches a peak during the second or third hour and drops rapidly thereafter, although with large doses, a small secondary rise is sometimes seen. Defervescence is accompanied by cutaneous vasodilatation, diaphoresis, pupillary constriction, hypotension and, occasionally, involuntary urination or defecation and penile erection.

Shivering can accompany onset of fever in the dog and the cat but is less striking than in man. Normal rabbits do not shiver after injection of endotoxin. Grant (138) has shown that adrenalectomy or thyroidectomy in the rabbit will lead to shivering after injection of endotoxin, perhaps a result of loss of normal epinephrine inhibition of the shivering mechanism (147).

Endotoxin fever in normal rabbits or dogs follows a typical biphasic pattern after all save minimal doses. There is a rapid rise to a peak during the first two hours, followed by transient and incomplete defervescence and then a second rise with final return to the baseline after 6 to 9 hours. In the cat, there are sometimes three distinct peaks of fever, but this animal has not been studied very intensively. The constancy of the biphasic response to endotoxin was first pointed out by Grant and this pattern has turned out to be an important point of discussion in hypotheses about the mechanism of the pyrogenic action of these bacterial products.

f. Other characteristics of endotoxin fever. Before discussing present concepts of the pyrogenic action of bacterial endotoxins, it is necessary to emphasize two

other phenomena, both of which are discussed in more detail elsewhere in this review, *i.e.*, acquired resistance or *tolerance* to endotoxin fever and the *augmenting effect of normal plasma or serum* upon the pyrogenic action of endotoxins.

It is well-known to clinicians who have used endotoxins for fever therapy that repeated injections of these materials at frequent intervals render patients refractory to their pyrogenic action; it becomes necessary to increase dosage with successive injections in order to elicit comparable febrile responses. This refractoriness was investigated by Beeson (17, 18), and has been studied by many others. It is now known that resistance to the pyrogenic action of endotoxin is accompanied by resistance to its many other actions including hemodynamic changes, tissue necrosis, *etc.*, but the present discussion will concern only the febrile response, which is easily quantified and has been extensively studied (30).

Rabbits given daily injections of the same dose of an endotoxin preparation respond with progressively smaller fever until a "minimum response" is reached after 7 to 10 days. This resistance or "tolerance" is never complete; animals continue to show small, but definite fever no matter how long injections are continued. This tolerance is independent of antibody, it is non-specific in the sense that it applies to endotoxins from all bacterial species, it is transient and will subside within 3 weeks if daily injections are discontinued, and it can be abolished completely by injection of colloidal materials such as trypan blue, colloidal iron, or Thorotrast. The mechanisms believed to be responsible for this tolerance are discussed in a later section (p. 452).

Farr (109) and Grant (137) showed several years ago that mixing endotoxin with normal plasma, serum, or blood before injection resulted in striking augmentation of the febrile response of normal animals to the toxin. The details of various augmenting and inhibiting substances in serum are covered in another section and it is only necessary to mention here the fact that the augmentation of endotoxin fever by blood or serum is evidenced by an increase in the height of the temperature response and also, a decrease in the lag-period between injection and onset of fever. The blood or serum of "tolerant" animals will not augment endotoxin fever as strikingly as that of normal animals but tolerant animals, given endotoxin mixed with normal blood or plasma, react with "augmented" fevers even more readily than do normal animals. Indeed, for purposes of testing this action of serum, tolerant animals are preferable to normal ones.

g. The mechanism of the febrile response to endotoxin. While, as already discussed, the participation of the central nervous system in the febrile response to endotoxin seems well-established, there remains uncertainty about the train of events that precedes the triggering of the nervous system.

Prominent among the observed events that require explanation is the lag-period between injection of toxin into the blood stream and onset of fever. The constancy of this delay led Beeson (17) to suggest that the injected toxin itself does not act upon the brain and that some indirect mechanism is involved. The traditional explanation of fever accompanying diseases of various types is the release of fever-producing substances from injured tissues and cells (31).

Because endotoxins are capable of producing widespread cellular injury, it seemed reasonable to postulate that the lag-period is an interval during which the toxin-injured cells release a second substance, and that this product of tissue injury acts upon the thermoregulatory centers to cause fever.

Until recent years, there has been little or no accurate study of pyrogenic substances from normal or injured tissues. Detailed discussion of this subject falls outside the scope of this review; it is sufficient to state that there have been numerous studies of this problem in the past but the results of almost all of these cannot be evaluated because they were carried out without attention to the possibility of contamination of test materials by endotoxins (30, 224, 389) and are, therefore, likely to represent additional examples of "injection fevers". For example, *pyrexin*, a material prepared by Menkin from inflammatory exudates (219a) and thought by him to be a substance responsible for fevers accompanying inflammatory disorders, has been shown to resemble endotoxins in that it is heat-stable, induces leukopenia, and produces a biphasic fever in rabbits; repeated injections elicit the development of tolerance to known endotoxins. Endotoxin-tolerant animals are also refractory to pyrexin, and the injection of Thorotrast will restore reactivity to endotoxin and to pyrexin (32). Menkin has shown that daily injection of large doses of pyrexin does not elicit tolerance in rabbits and has interpreted this as indicating that pyrexin is different from endotoxin and that its action cannot be attributed to contamination (219b). However, failure of animals, given daily injections of large doses of endotoxin, to show appreciable decrease in febrile responses is easily demonstrable (263d), probably as a result of exceeding the capacity of the tolerance mechanisms. The effect of small doses given repeatedly is far more important in assessing similarity of a pyrogen to bacterial endotoxins. Menkins' publications on pyrexin failed to mention precautions against the possibility of endotoxin contamination until he reported the above-mentioned studies of tolerance using large doses of the exudate fraction. The present authors feel that the available data do not permit any final decision upon the question of whether pyrexin is, as claimed, an endogenous product of injured tissue. Its uncertain status, however, illustrates well the pitfalls and difficulties of interpretation that can arise in the study of tissue pyrogens.

In an extensive survey of the tissues of normal rabbits for fever-producing substances, it was found that granulocytes, obtained from peripheral blood or from sterile exudates, contain a fever-producing substance that is destroyed by heating at 90°C., produces short, monophasic fever in normal rabbits, is fully active in animals tolerant to endotoxin and elicits no tolerance when injected daily (31, 32). Similar findings in dogs (263a, 263e) permit the conclusion that the granulocyte is a possible source of endogenous pyrogenic substance. Parenthetically, there is some evidence to indicate that the pyrogenic factor in leukocytes is active only in animals of homologous species (263c).

Beeson, using passive transfer of serum (17, 18), and Braude (52a, 52b), using isotopically labelled endotoxin, have shown that an injected dose of endotoxin is removed completely from the circulating blood within a few minutes,

predominantly by the liver and spleen and presumably by reticuloendothelial cells. However, Grant (140) and Atkins and Wood (5a) have shown that the serum of rabbits given endotoxin contains passively transferable pyrogen for as long as two hours after injection. Atkins and Wood have shown that the pyrogen in serum collected immediately after injection of endotoxin is the endotoxin itself. However, the ability of serum collected 30 to 120 min later to produce fever in test animals is the result of its content of a different pyrogen, and this substance has been called "endogenous serum pyrogen". It produces a monophasic fever, the lag-period between injection of serum and onset of fever is relatively short, it does not affect leukocytes as does endotoxin, it is active in tolerant animals, it produces no tolerance when injected daily and, by all testing methods that have been used, endogenous pyrogen differs from endotoxin and resembles the pyrogenic factor in leukocytes (263d). Grant has suggested that the late-appearing pyrogen in serum of febrile rabbits may simply be the equivalent of an *in vitro* plasma-endotoxin mixture resulting in augmentation of fever and shortening of the lag-period. It is more likely that the concept of Atkins and Wood of serum pyrogen as a new substance, probably released from polymorphonuclear leukocytes injured by the endotoxin, is correct. Some evidence to support this comes from the observation that granulocytopenia, induced by nitrogen mustard in dogs, results in the appearance of a lessened amount of serum pyrogen when endotoxin is administered (37b).

That the appearance of serum pyrogen is essential in the production of fever by endotoxin has not been established. Without going into detail, it may be said that, under certain appropriate conditions, the febrile response of animals given endotoxin can be shown to have no relationship to the presence of serum pyrogen (37b, 263d). For example, animals made granulocytopenic with nitrogen mustard do not show endogenous pyrogen in the serum, but their fevers are in every way similar to those elicited by endotoxin in normal animals (37b).

Recent studies of a possible *direct* action of endotoxin employing the technique of intrathecal administration of endotoxin in rabbits and in dogs (37b) have shown that these substances are very active by this route. Indeed, the amount of endotoxin required to elicit fever of a given magnitude by the intravenous route is 1000 to 4000 times the intrathecal dose.

Animals given daily intrathecal injections of endotoxin continue to react with high fevers and no "tolerance" develops. Animals that are tolerant to endotoxin as a result of repeated intravenous injections show no decrease in their reactivity to intrathecal endotoxin. The onset of fever after intrathecal injection of toxin is prompt with little or no lag-period and the fever is accompanied by no peripheral leukopenia. The serum of animals with fever elicited by injection of endotoxin into the subarachnoid space contains *no* endogenous pyrogen. Finally, within 15 to 30 min after *intravenous* injection of endotoxin into dogs, the cerebrospinal fluid is found to contain significant amounts of the originally injected endotoxin.

On the basis of these findings, the authors believe, at present, that the best explanation of the pyrogenic action of bacterial endotoxins would be a dual

mechanism involving a direct action of endotoxin and an indirect action of endogenous pyrogen. Such a course of events might account for the biphasic fever curve that characterizes endotoxin fever.

Finally, evidence is accumulating rapidly (5b, 398a) that fevers of other origins involve endogenous pyrogens similar to the serum pyrogen which appears in animals given endotoxin, and it seems that, in fevers produced by viruses and certain bacterial infections, endogenous pyrogen is the single important factor in the production of elevation of body temperature.

10. Other effects. In addition to those reactions already described, endotoxins have been shown to alter carbohydrate metabolism, gastric function, and adrenal function, as well as to bring about a host of minor metabolic and chemical abnormalities. The significance of many of these observations is incompletely understood at the present time and there is relatively little to be said about them other than to note their occurrence. Generally, the reactions to be described are not influenced by antipyretics and appear to be attributable to the action of the toxin rather than concomitant alterations in body temperature. "Tolerance" to many of them has been observed in animals given repeated injections of endotoxin although studies of this aspect of the reactions have been few.

Serum protein changes in patients given repeated injections of endotoxins for therapeutic purposes include decrease in albumin, increased gamma globulin, increased fibrinogen and concomitant acceleration of erythrocyte sedimentation rate (174, 187, 188). Alterations in liver function including impairment of bromsulphthalein excretion are common (163). Massive doses of salicylates appear to lessen serum protein changes (174). In rabbits bearing the Brown-Pearce carcinoma, blood proteose was unaffected but uric acid rose slightly after injection of endotoxin (385); blood uric acid increased in human subjects also (54). Blood lipids decrease sharply in rabbits or dogs after injection of endotoxin but return to normal within a few hours (246, 295). Cholesterol does not participate in these fluctuations to any great extent.

Injection of endotoxin is followed in man by a prompt decrease in serum iron and serum iron-binding capacity with a parallel rise in serum copper (53). Plasma magnesium (391) and blood calcium (121) are unchanged and blood inorganic phosphorus decreases (94) or increases (194) depending upon alterations in carbohydrate metabolism, discussed below. Additional reported effects of endotoxin include: increase in plasma fibrinolytic activity (372), increase in blood non-protein nitrogen and ascorbic acid (121), reduction in blood and increase in liver and muscle concentration of histamine (192). Despite extensive hemodynamic changes in the kidney, already mentioned, there is no significant alteration in urinary excretion of sodium, potassium, and water (199).

a. Carbohydrate metabolism. Injection of endotoxin alters carbohydrate metabolism in striking fashion. With moderate dosage there is hyperglycemia in man (100, 111), rabbits (47, 107, 194), rats (60, 112), and mice (60). The hyperglycemia is succeeded by hypoglycemia when lethal doses are given. In adrenalectomized animals hypoglycemia is the rule (107). The increase in blood glucose can be prevented by ergotamine (47, 107), but not by dibenamine (47) and is

partially reversible *in vivo* (107) and *in vitro* (191, 192) by insulin. Blood inorganic phosphate increases or decreases with blood glucose and there is accumulation of lactic acid in blood (194) and tissues (194, 362). *In vitro* there is inhibition of succinic dehydrogenase in muscle and liver, cytochrome oxidase is spared, and it is presumed that phosphorylation of glucose is impaired (193, 194). There is striking depletion of liver glycogen when endotoxin is given; glucose tolerance is impaired (107, 390); and insulin, glucose, and lactate are relatively ineffective in increasing liver glycogen storage (390). It is interesting that Delafield (95) described the development of "tolerance" to the hyperglycemic action of endotoxin in rabbits given repeated injections.

b. Gastric effects. It is well-known that febrile diseases are accompanied by hypochlorhydria (173). Attempts to separate a hormone from human urine that inhibits gastric secretion and motility (so-called urogastrone) were complicated by the finding that bacterial endotoxin produced similar effects on gastric function (142, 238). Necheles (239) particularly has pointed out the great danger of drawing false conclusions about the specific activity of substances on gastric function because of possible endotoxin contamination. He and his co-workers have shown that gastric motility in dogs, even under stimulation by neostigmine (Prostigmine), is decreased by amounts of endotoxin too small to produce a rise in rectal temperature (240). Grossman and Blickenstaff (145) showed that the action of endotoxin on gastric motility and secretion does not coincide with onset of fever, but tends to lag behind body temperature. As might be expected, antipyretics are without effect upon the action of endotoxin on the stomach (373). Endotoxins have been shown to inhibit the development of peptic ulceration in rats with pyloric ligations, in contrast to polysaccharides from pneumococci (217), and there is some evidence that they may temporarily alleviate peptic ulceration in human patients (292). There is a consistent decrease in gastric motility and volume of secretion but, usually, no decrease in concentration of hydrochloric acid.

Studies of the physiology of gastric secretion that have not utilized rigid precautions against contamination with endotoxin present the same problem as studies on experimental fever or leukopenia and leukocytosis in which this factor has not been controlled.

c. Adrenal function. It is difficult to be succinct in discussing the adrenals and endotoxins. There is no doubt, however, that adrenalectomized animals are more susceptible to the lethal action of these materials (106, 107, 112, 155, 204). Furthermore, there is good evidence that endotoxin will produce morphologic changes (393) and functional changes in the adrenal glands (4, 184, 278, 284) but there is nothing to support the idea that the physiologic effects of endotoxin are mediated directly by the pituitary-adrenal axis (184, 324), and claims equating the supposed beneficial action of endotoxin in various disease states with adrenal steroid therapy are not acceptable. The claim that the leukocyte changes produced by endotoxin are mediated by the adrenal (222) has not been born out in other studies (204, 324, 325). It may be pointed out that several of the effects of endotoxin are exactly the opposite of those that would be expected

from steroids—amelioration of peptic ulceration (217), aggravation of the Arthus reactions (334), failure to prevent serum arteritis (36), hypotension, *etc.*

The effects of administration of ACTH or cortisone to normal animals upon the actions of endotoxin have been shown to depend upon dosage schedules and inhibition or aggravation of fever (27, 104, 178), of the Shwartzman reaction (27, 213, 311, 357), of lethality, tumor necrosis (11, 13) and other alterations have been reported. It is, therefore, important to specify any effect of adrenal steroids upon the activity of endotoxin in terms of hormone, reaction, and dosage of toxin; it is not possible to generalize from specific observations. None of the studies that have been reported supports any concept of the role of the pituitary-adrenal axis as other than "permissive" in the activities of endotoxin.

V. MECHANISM OF ACTION

The multiple biological effects of endotoxin make it exceedingly difficult to define the mechanism by which it acts. Many organ systems are affected by the toxin, resulting in tissue injury with metabolic, vascular, hormonal, immunological, hematological and necrotizing consequences.

1. Role of white blood cells. The effect of endotoxin on circulating leukocytes, indicated by pronounced leukopenia followed by leukocytosis, may be responsible for some of the manifestations produced by endotoxin. Although there are irregular effects of endotoxin upon the migration of white cells *in vitro*, there is no question of the marked disturbance of leukocyte migration from the buffy coat of blood from rabbits injected intravenously (214). It is probable that this effect is attributable to changes in the surface activity (122, 123) and not to disintegration of the white cells (41). Whether or not the toxin injures the surface of leukocytes or becomes adsorbed to them to produce this effect has not been determined. The liberation of lysozyme by white blood cells in contact with endotoxin is of interest but does not clarify the mechanism of toxin action (180). The profound effect of endotoxin upon blood vessels, particularly suggesting a peripheral action (48, 350, 410), may indicate that the toxin acts directly upon vascular endothelium. This is further suggested by the failure of Evans blue dye to diffuse into a skin site inoculated with endotoxin (355). The formation of leukocyte thrombi in the local Shwartzman reaction may, therefore, be explained by a change in the vessel wall at the site of skin injection as well as a change in the surface properties of leukocytes, favoring the deposition of a clot, leading to infarction and hemorrhagic necrosis. The restriction of the thrombi to the veins and capillaries is of interest in this regard. Glycogen and other nonbacterial substances which can provoke a local Shwartzman reaction have the ability to cause clumping of leukocytes, probably attributable to changes in surface properties of these cells, and may, for this reason, be able to elicit the reaction, once the skin site (blood vessels) has been prepared with an injection of endotoxin.

It has been shown that substances affecting vascular reactivity (dibenzyline, chlorpromazine) (48, 350), causing leukopenia (HN2), or preventing coagulation are capable of inhibiting the local Shwartzman reaction, further illustrating the

importance of white blood cells, thrombus formation and the vascular system in the elicitation of this phenomenon.

The demonstration that polymorphonuclear leukocytes are pyrogenic when inoculated intravenously into normal rabbits, has led to the interpretation that endotoxin may produce fever by its effect on these cells. The role of white blood cells in the mechanism of endotoxin fever is discussed in detail elsewhere (p. 445).

Deposition of fibrinoid material in renal glomeruli during provocation of the generalized Shwartzman reaction, associated with a fall in levels of blood fibrinogen, has been discussed before (360). The mechanism by which an initial intravenous injection of toxin sensitizes the animal to a second injection resulting in fibrinoid deposits and depression of blood fibrinogen is unknown. Platelets are a common source of thromboplastic substance, however, and the thrombocytopenia induced by endotoxin given intravenously may lead to the intravascular precipitation of fibrinogen.

2. Interaction with plasma components. Endotoxin in the presence of normal blood, plasma and serum is altered in its physical-chemical and immunological properties, and its pyrogenic action is changed. Furthermore, the effects on endotoxin of serum from rabbits tolerant to bacterial pyrogen differ from those of normal serum. The implications of these influences of plasma components in the mechanism of action of endotoxin, however, have not been clarified.

a. Augmentation of the pyrogenic action of endotoxin. Mixing endotoxin with normal serum, plasma or blood prior to intravenous injection results in enhancement of the toxin's pyrogenic action, characterized by a shortened latent period before onset of fever and increased height of fever (73, 83, 109, 126, 137, 140). This effect has been demonstrated with normal rabbit and human serum (73, 156, 157). Augmentation of endotoxin fever by serum is demonstrable after the two have been mixed for a short period of time, and is not dependent upon incubation at 37°C., as mixing of endotoxin and serum at 4°C. results in a similar enhancement of the febrile response (109). Increase in the pyrogenic action of endotoxin by normal serum occurs in normal and pyrogen-tolerant or refractory rabbits, and is more readily demonstrated in the latter. Increasing the amount of rabbit serum in contact with endotoxin results in increasing enhancement of the toxin's pyrogenic action, suggesting a stoichiometric relationship (73). The augmenting property may be associated with the alpha globulin component of normal rabbit serum (72). Grant (140) described the appearance of a fast-acting pyrogen in the blood of normal rabbits given an intravenous injection of endotoxin and suggested that this "endogenous" pyrogen may be endotoxin which has reacted with plasma *in vivo*. The relationship of this "endogenous" pyrogen to the *in vitro* endotoxin-plasma mixture is discussed elsewhere, but will be summarized here.

Endogenous pyrogen is heat-labile, and produces fever in pyrogen-tolerant animals, whereas endotoxin is heat-stable and the febrile response of tolerant animals to it is less than that of normal rabbits. Endogenous pyrogen has been detected in the blood of rabbits infected with microörganisms which do not

possess a pyrogenic endotoxin. Furthermore, the pyrogenic action of endogenous pyrogen bears a striking resemblance to the action of leukocyte pyrogen. There are similarities between *endotoxin-in-serum* and endogenous pyrogen, however, which make it difficult to differentiate them completely from one another. Petersdorf and Bennett (263a) have shown that endotoxin-in-serum becomes heat-labile to essentially the same degree as endogenous pyrogen. It has also been found that repeated injection of endotoxin-in-serum into rabbits does not result in development of tolerance to the augmented febrile response, in the same way as it has been shown that repeated inoculation of endogenous pyrogen into rabbits does not result in tolerance (37b).

The observations by Braude (52a, 52b), however, illustrating a rapid clearance of radioactive endotoxin from blood of injected animals, casts significant doubt on endogenous pyrogen being an altered form of endotoxin.

It has been shown by precipitation-in-gel techniques that normal serum increases the rate of diffusion of endotoxin (Shigella) through agar and changes its reaction with antiserum (70). Neter *et al.* (241) have described an alteration of the erythrocyte adsorption and hemagglutination of endotoxin-modified erythrocytes by normal serum, which might be attributable to lecithin or cholesterol. These effects of serum on endotoxin are dependent upon contact for a short period of time at 37°C.

The possibility that the toxin might bind with a serum protein to result in augmentation of pyrogenic action has recently been investigated (72) and no change in the ultraviolet absorption spectrum, quantitative antibody precipitation or electrophoretic pattern of endotoxin was observed after mixing with normal serum or serum protein fractions for 30 to 60 min at 37°C. The possibility, therefore, that contact of normal serum with endotoxin for a short period of time results in a change in the physical properties of the toxin without change in its chemical characteristics seems probable. That lecithin or cholesterol may be responsible for this effect has not been thoroughly evaluated; the fact that the toxic moiety of endotoxin is probably a lipid strongly suggests this possibility.

b. Inhibition of the pyrogenic action of endotoxin. The effect of mixing normal serum (rabbit and human) with endotoxin for several hours at 37°C. has been studied (73, 156, 157), and it has been shown that there is inhibition rather than augmentation of the pyrogenic action of toxin. The property of normal serum responsible for this effect is labile to heat and storage, and can be removed by treating the serum with zymosan to deplete it of properdin. It seems fairly clear that the properdin system plays a major role in this inhibitory action of the normal serum upon pyrogen fever. Hegemann (156, 157) has defined this effect of properdin upon endotoxin as enzymatic.

As discussed elsewhere, serum from tolerant rabbits fails to enhance endotoxin fever in the same way as normal serum. This is not attributable to an increase of serum properdin in the blood of tolerant animals (73). The implications of this observation in the mechanism of pyrogen fever have been evaluated by replacement transfusion of tolerant animals with normal rabbit blood, and it has been shown that this does not restore the normal febrile reaction upon the

recipient refractory animals (73). This might suggest that a pyrogen inhibitor develops in serum of tolerant rabbits which suppresses the pyrogen-augmenting property of normal serum. Farr (109), indeed, has shown that serum of tolerant rabbits possesses the ability to inhibit the pyrogenic action of endotoxin. The relationship of these observations to an animal's tolerance to endotoxin is discussed elsewhere.

Recent studies by Stauch and Johnson (328) and Ho and Kass (165) indicate that normal serum can alter antigenicity and protect against the lethal action of crude endotoxin in the absence of properdin, suggesting that other factors may play a role in the animal's reaction to endotoxin.

3. Direct tissue injury. Liberation of lysozyme by white cells, impairment of leukocyte migration and changes in electrical surface properties of these cells by endotoxin *in vitro* are strong evidence for a direct injurious effect of the toxin on cells. However, the pronounced effect of endotoxin on leukocytes *in vivo* cannot be duplicated *in vitro*, and no injurious effects on tumor cells have been observed *in vitro* to explain the profound effect of toxin on transplanted tumors in mice. Although striking pharmacological and anatomical changes occur upon injection of endotoxin into animals and man, no satisfactory explanation has thus far been derived which suggests that these effects are entirely attributable to direct tissue injury.

4. The role of hypersensitivity. It is suggested occasionally that the physiological alterations produced by endotoxins might be the result of hypersensitivity or "natural antibodies" to Gram-negative bacteria. Reinjection of antigen into animals with induced hypersensitivity of the immediate (108) or delayed (331) type results in fever. There are also certain close resemblances between the Shwartzman and Arthus reactions, between the leukopenia of anaphylaxis and that produced by endotoxin (25), *etc.* However, it is well-known that things that look the same are not necessarily produced by the same agent and, at present, there is really no firm evidence for relating endotoxins and hypersensitivity in a causal fashion. This is a subject worthy of further investigation.

VI. TOLERANCE TO ENDOTOXINS

The phenomenon of acquired resistance to endotoxins has already been mentioned a number of times. It was probably first noted by clinicians who employed these substances for producing therapeutic hyperpyrexia (264, 370, 371). Human subjects given repeated injections of endotoxin develop remarkable resistance to its fever-producing action; in order to achieve febrile responses of comparable magnitude, it becomes necessary to increase dosage progressively, doubling or trebling it with successive injections (17, 227, 323). The same resistance occurs in animals and this has posed a special problem in the use of rabbits in testing pharmaceuticals for pyrogen content. This aspect of pyrogen-testing was investigated in great detail by Tennant and Ott (346) and the reader is referred to their paper for recommended procedures.

Repeated injections of endotoxins have been noted to elicit resistance not only to the fever-producing effect of these materials but also to their lethal (305,

406), leukopenic (16, 249, 251), leukocyte-inhibiting (41), adreno-hemorrhagic (252, 344), tumor-necrotizing (86, 87, 406, 408), hypotensive (255, 345), antibody-enhancing (77, 78), hyperglycemic effects (95) and to the Shwartzman phenomenon (17, 18, 33, 34).

There is every reason to believe that resistance to endotoxin which develops with repeated, frequent exposures involves the same basic mechanisms whether the criterion of an animal's ability to react is a change in leukocytes, elevation of body temperature, or the appearance of hemorrhage in normal or neoplastic tissues. Because many of the responses to endotoxin are "all-or-none" reactions, body temperature which is easily measured and quantified is a good indicator of the progressive development of resistance and is suitable for the study of its dynamics.

There is considerable confusion in the earlier literature about resistance or immunity to the action of endotoxins (30), and it was not until the definitive study of Beeson (17, 18) that many of the details of this striking phenomenon were appreciated. Of the several terms, including "immunity", "resistance", and "refractoriness", that have been suggested to designate the non-reactive state, the present authors prefer that used by Beeson, "tolerance".

After a description of tolerance to endotoxins, the present knowledge of mechanisms of its development will be discussed.

1. Development of tolerance. Animals given injections of the same dose of an endotoxin preparation show a diminution in response to the toxin beginning on the second day. As has already been mentioned, if the response being studied is tumor necrosis or the Shwartzman reaction, tolerance is apparent first as a decrease in the extent of the reaction and finally, its complete failure to occur. With fever or leukopenia, both of which are elicited by far smaller amounts of endotoxin (341), tolerance is never complete; animals continue to react to injection of endotoxin with small but definite "minimal responses". The level of an animal's tolerance is dependent upon the frequency of injection; rabbits given endotoxin three times weekly react with higher fevers than animals given the same dose each day (17). The dose of endotoxin is important. An animal that has been made tolerant by repeated injections of a given dose of endotoxin will react in an almost normal fashion to larger doses and will not even give a "minimal response" to smaller amounts. Furthermore, it is possible to administer amounts of endotoxin too large for the "tolerance" mechanisms in the sense that the animal may respond for many days with maximal degrees of fever or leukopenia (263c). Tolerance is also dependent upon continued exposure to endotoxin and, if injections are stopped, animals and man will completely lose resistance within a period of 2 to 3 weeks (17, 346).

When animals are given daily injections of endotoxin and febrile responses are measured, it is found that the pattern of diminution of the febrile responses is consistent (140). The typical "double-hump" of endotoxin fever on the first day usually is modified by the second or third day by disappearance of the second hump, and this is followed during the next few days by gradual diminution of the first hump and lengthening of the lag-period between injection and onset of

fever. By the seventh to tenth injection, a minimal level of response will have been established and, no matter how long daily injections are continued, this is not reduced further.

One of the most striking features of tolerance to bacterial endotoxins is its *non-specificity*; animals made tolerant by repeated injections of the endotoxin produced by one bacterial species are also resistant to the effects of the products of heterologous species. In demonstrating this phenomenon, the importance of dosage must be borne in mind and many apparent exceptions to this non-specific resistance are attributable to the difficulty encountered in selecting dosages of different endotoxin preparations that are comparable in biological activity.

In passing, it may be mentioned that the development of tolerance is not influenced by amidopyrine (17) and is only slightly delayed by certain dosages of adrenal steroids (33).

The resistance referred to here as tolerance becomes progressively greater for a period of several days when daily injections are administered. There is another form of refractoriness to endotoxins, especially apparent for their pyrogenic action (137, 263d, 407), which appears within a few hours after a first injection of endotoxin and subsides rapidly. This early, transient resistance may be related to the "promunity" of Orskov and Kauffman (253), the peculiar early protection against the lethal action of endotoxin described by Creech (86, 87), and the transient refractoriness to the Shwartzman reaction that follows a single intravenous injection of endotoxin (308). It is possible that many of these early, rapid alterations in resistance to the action of endotoxins will eventually be shown to bear a relationship to the properdin system, the activity of which is profoundly influenced by endotoxins (195, 196, 269).

2. The adrenals and tolerance. It has been suggested that tolerance to endotoxins may be the result of increased activity of the adrenal cortex. Actually, there is little to support this contention. The results of administration of adrenal steroids upon reactivity to endotoxins have been variable and in no way indicate a relationship between tolerance and adrenal function. Adrenal steroids and "tolerance" have had opposite effects in some situations (36, 334).

3. Specific immunity. One of the characteristics of endotoxins is the failure of specific antiserum to neutralize their toxic action (58). Tolerance to endotoxins cannot be transferred passively by serum, and tolerance bears no relationship to circulating antibody levels (18, 68, 227). The precipitate formed by endotoxin and specific antibody retains toxicity (227). Finally, Good has shown that children with agammaglobulinemia develop tolerance to endotoxins without the appearance of serum antibody (131). In summary, specific immunity in the classic sense seems to play no part in resistance to endotoxins.

4. Plasma factors other than antibody. Farr (109a) has reported the presence of at least one and possibly two inhibitors of endotoxin in the serum of tolerant animals. However, the degree of potency of serum inhibitors is insufficient to account for more than a very small part of the resistance of tolerant animals and the exact role of these substances remains to be determined. As has already been mentioned, there is suggestive evidence that alterations in serum properdin

levels may account for transient resistance to endotoxin in some situations. However, it has been shown that the serum of rabbits with fully established tolerance to endotoxins contains only normal amounts of properdin (73) and it is obvious that tolerance depends upon another mechanism. The suggestion by Grant (137) that tolerance to endotoxins is a result of disappearance from the plasma of the "enhancing factor" present in normal plasma is interesting. However, the essential role of this augmenting factor in the genesis of endotoxin fever remains to be established and, in recent experiments (73), the present authors have been unable to establish that absence of this factor plays an important part in tolerance to endotoxins, although there is no question about the decreased ability of tolerant serum to augment the reaction to endotoxins.

5. The role of the "reticulo-endothelial" system (RES). There is abundant evidence to suggest that tolerance to bacterial endotoxins involves an alteration in the function of the fixed phagocytes of the liver and spleen—the RES. Using a method of passive transfer, Beeson (18) showed that tolerant animals clear injected endotoxin from the circulating blood more rapidly than do normal animals. This has been confirmed by others using the same method (5a, 140) and by Braude (51) using isotopically labelled endotoxin. Furthermore, the intravenous injection of colloidal materials including trypan blue, Thorotrast, and iron (18, 26, 33, 351) completely abolishes tolerance to the action of endotoxins. These materials, of course, are known to be removed by the cells of the RES and are among the agents known to produce so-called RES blockade. As Thomas (351) has emphasized, caution must be used in discussing reticulo-endothelial "function" and in interpreting the action of such a material as Thorotrast. Nevertheless, it is apparent that tolerance is accompanied by an increase in clearance of endotoxin from the blood, that colloidal materials interfere with this clearance and abolish tolerance, and that the increase in clearance coincides with a decrease in toxic action of the endotoxin. The concept that tolerance is an increase in the ability of the body to sequester toxin, mechanically or functionally, in certain cells of the RES has great appeal and is supported by the available evidence. That this description is an oversimplification is altogether probable. The end-result then, of injection of endotoxin into the tolerant subject is that a smaller dose actually reaches the "target" organs. Therefore, to overcome the "leak" of endotoxin, a larger dose is needed to produce a given effect.

Whatever future investigations of endotoxin tolerance may reveal about its underlying mechanisms, its occurrence is of considerable importance in the study of endotoxins. The progressive development of resistance which disappears when exposure ceases in any biological system should always call to mind the possibility that unsuspected endotoxin contaminants are at work. As will be mentioned in a later section, an assessment of the role of endotoxins in the pathogenesis of various disease states depends to a large extent upon the ability of these substances to elicit tolerance. It is difficult to believe that a biological phenomenon of such effectiveness is an artefact of laboratory investigations. Other examples of tolerance that are well-known and strikingly similar in their characteristics are the acquired resistance to opiates that disappears rapidly

when these drugs are withdrawn and the acquired resistance to the cardiovascular effects of nitrites that is so important in the industrial use of nitroglycerine, dynamite, and similar compounds. The relationship between these forms of "tolerance" is a fertile field for investigation.

VII. THE ROLE OF ENDOTOXINS IN HUMAN DISEASE

As has been mentioned, the endotoxins of Gram-negative bacteria bear no discernible relationship to pathogenicity, virulence or invasiveness. The endotoxins of the saprophytic bacilli of the human alimentary tract are fully as toxic as those of the most virulent typhoid bacilli.

The obvious similarity of the malaise, chill, fever, leukocyte changes, and other reactions produced by the injection of endotoxins to the clinical features of many acute infectious diseases makes it difficult not to believe that these substances are involved in the pathogenesis of these manifestations. Perhaps it is because the role of endotoxins in disease has been assumed so readily that there is relatively little firm evidence to indicate their importance in bacterial infections. For example, the statement has often been made that the leukopenia that characterizes typhoid fever results from the leukopenic action of the endotoxin of the causative organism (229, 281). This seems to be an oversimplification when one considers that the injection of typhoid vaccine produces initial leukopenia followed by leukocytosis in patients with active typhoid fever just as it does in normal subjects (81). Furthermore, in many other infections produced by Gram-negative bacilli the blood leukocyte count may be normal or tremendously elevated.

It is not at all certain that even the fever accompanying most bacterial infections is directly attributable to the pyrogenic action of endotoxin. Sebastiani (299) isolated crude endotoxin from *S. marcescens* in 1912 and showed that he could roughly duplicate various types of fever curves seen in disease by employing selected schedules of injection of this material. He concluded that bacteria elaborate "pyrotoxin" and that the sustained, remittent, or intermittent fevers that characterize various infections result from varying periodicity of release of pyrotoxin by different infecting organisms. In recent years, most investigations of the role of endotoxin in the fever of infection have been based on the assumption that fever over a period of several days that is produced by endotoxin should elicit tolerance to the pyrogenic action of these substances. Heyman and Beeson (161) found no evidence of tolerance in a group of patients convalescent from acute infections, including two with typhoid fever. An exception was malaria which resulted in striking refractoriness to endotoxin fever. This was suggested by these authors to have resulted from the proliferation of the reticulo-endothelial cells that accompanies malarial infection. Neva and Morgan, however, tested several patients during convalescence from typhoid or paratyphoid infection and found them to be relatively resistant to the pyrogenic action of endotoxins from *Salmonella typhosa* and *Shigella dysenteriae* (228, 243). These same workers found no evidence of tolerance in patients recovering from other Gram-negative bacterial infections including gonorrheal arthritis,

bacillary dysentery, and tularemia. Bennett (23, 24) could demonstrate no tolerance to homologous or heterologous endotoxins in rabbits convalescent from *Escherichia coli* or pneumococcal infections, although it was shown that infected animals could be rendered tolerant by daily injection of endotoxin during the illness. Spink (327) has actually found *increased* reactivity to endotoxins in patients recovered from brucellosis, and similar findings have been reported in cattle (206). On the basis of these few data, it seems clear that, while endotoxin may play a part in the fever of some infections, it is not the sole cause of elevation of body temperature, even in infections by endotoxin-producing bacteria.

The fever accompanying Gram-negative bacterial infections is likely to be higher than that with others (28), and shock is somewhat more frequent in bacteremias produced by Gram-negative bacteria (37). There is good experimental evidence linking the purpuric rash of meningococcemia and the vascular collapse of the Waterhouse-Friderichsen type to endotoxin and the Shwartzman reaction (43). Again, however, this type of collapse can accompany infection by Gram-positive cocci not known to possess any endotoxin. One peculiar fact that is difficult to reconcile with the idea that the manifestations of typhoid and brucellosis are caused by endotoxin is the rarity of herpes labialis as a complication of these diseases (37). "Fever blisters" are exceedingly common in patients given typhoid vaccine or other endotoxins for therapeutic purposes (189, 326). These bits of evidence suggest that endotoxin may indeed be important in the clinical picture of human disease but specific information is sorely needed.

1. Accidents with endotoxin contaminants. There is no question that accidental infusion of a large number of Gram-negative bacteria will produce an acute, occasionally fatal reaction in man that closely resembles endotoxin poisoning. A number of fatal accidents have been traced to contamination of blood for transfusion by cold-growing organisms. Geller and Jawetz (124) have reviewed this problem and the interested reader is referred to their excellent paper. Fatal pyrogenic reactions have followed infusion of other fluids also, a striking example being glucose administered to patients undergoing insulin shock therapy (220). In these situations, there can be no doubt about the primary role of endotoxin in the production of untoward reactions in human subjects.

2. Shwartzman reaction. The work of Black-Schaffer (43) on the relationship between the purpuric manifestations of meningococcal infections and the Shwartzman reaction has been mentioned. Among other diseases that have been suggested to have their etiology in some reaction resembling the Shwartzman phenomenon are acute hemorrhagic pancreatitis, focal reactions in tuberculosis, peptic ulcer, rheumatic fever, bilateral cortical necrosis of the kidneys, and certain drug eruptions. None of these has been established as definitely related to endotoxins. There are a number of isolated descriptions of human reactions to typhoid vaccine or other substances that closely resemble the Shwartzman reaction (150, 208, 367). Much remains to be learned about the significance of this type of hemorrhagic necrosis in the manifestations of human disease.

3. Endotoxins and shock. Fine and his associates have drawn attention to the similarity of irreversible shock produced by hemorrhage and intoxication by bacterial products, showing among other things that animals subjected to hemorrhage are tremendously susceptible to the lethal action of endotoxins (115, 120, 296, 297). The source of endotoxin in fatally shocked animals is obscure although it has been assumed that enteric organisms might be responsible. The recent demonstration by Landy and Shear (198) of endotoxin-like substances in mammalian tissues which duplicate the reactions produced by bacterial products, including the effect upon the properdin system (268), may clarify some of the events in hemorrhagic shock and other forms of vascular collapse and explain their similarity to endotoxin-induced reactions.

4. Inhalation of endotoxin. There is a group of disorders which are produced by inhalation of endotoxin, especially in dusty occupations. These have been discussed in detail elsewhere (30) and include "cotton fever", produced by inhalation of low-grade cotton fibers contaminated by *Aerobacter cloacae* (237, 279, 365), also known as byssinosis or Monday fever, and "Farmer's lung" (338). These and a miscellany of other brief febrile diseases in individuals exposed to organic dusts are characterized by cough, chill, fever, leukocytosis, and malaise for a few hours, rapidly developing tolerance with continued exposure, and loss of tolerance and recurrence of illness with interruption of exposure. They can be duplicated in all respects by inhalation of endotoxin-producing organisms or their products such as has been reported by Paine (256).

Finally, it may be said that "metal-fume fever" or "brass-founders ague" possesses all of the characteristics mentioned for cotton fever (201, 339), and it is now believed that metal fumes in some way allow absorption from the respiratory tract of bacterial products already present (283).

A summary of what is known about the role of the endotoxins of Gram-negative bacteria in the pathogenesis of human disease contains surprisingly little information. The investigation of these substances and their action in patients with infection caused by endotoxin-producing organisms is an important field and one which should eventually yield information about the mechanisms of the infectious process and diseases of non-infectious etiology.

VIII. ENDOTOXINS AS THERAPEUTIC AGENTS

With the possible exception of the use of vaccines for specific immunization, by far the greatest medical interest in Gram-negative bacteria and their endotoxins has centered around their use in so-called non-specific therapy. The wave of enthusiasm for this type of treatment in a wide variety of diseases in the early part of this century (30, 81, 166, 264, 270, 271) has subsided, but fever therapy continues intermittently to have its advocates up to the present time (113, 139, 366). Literally hundreds of articles describing good, bad, or indifferent results in disorders of every kind have been published, and reports continue to appear (38, 129) each year, particularly from European countries where this form of treatment is presently used on a much larger scale than it is in this country. It is appropriate to preface a discussion of specific diseases treated by

endotoxins and the possible mechanisms of the therapeutic benefits claimed for these substances by stating that the vast majority of the reports of their use have been based on completely uncontrolled clinical impression and are, therefore, to be viewed with considerable skepticism. There are, however, a number of situations in which the benefit of treatment with endotoxin cannot be doubted.

1. Untoward reactions to endotoxin therapy. It is not surprising that the deliberate injection of these substances has produced a number of unpleasant and dangerous effects. The headache, malaise, nausea, and muscle pain that characterize fever therapy can be relieved with analgesics and, like most of the other reactions, can be attributed to the endotoxin content of the preparations employed. Despite many proprietary claims, there is no real evidence that any preparation to date can produce a therapeutic effect without the danger of undesirable and unpleasant reaction to the endotoxin. Most "non-toxic" preparations are simply weaker substances.

Important reactions that have been reported include generalized Shwartzman phenomenon with renal cortical necrosis (208, 267), herpes labialis (189, 326). shock (20, 26, 166), cardiac enlargement (378), hyperpyrexia (117), renal dysfunction (117, 280), serum protein depletion (159), and fatal bronchial asthma (374). Undesirable reactions are unusual and proper control of dosage will largely avoid them.

2. Mechanism of the beneficial action of endotoxin. The mechanisms of the beneficial effects of fever or non-specific protein therapy are not known. Almost all of the changes that have been observed to result from administration of endotoxin have at one time or another been credited with therapeutic efficacy. There are those who believe that *elevation of body temperature* is important and the almost equal results obtained in neurosyphilis from endotoxin, malaria, or physical hyperthermia seem to substantiate this. Even rat-bite fever has helped (322) luetics. Speirer (326) pointed out that the gonococcus is killed outright by temperatures of 40°C. and suggested that this might account for the beneficial action in gonorrhea and its complications. Others (55) have said that fever itself is a minor matter and that the beneficial results in syphilis and other diseases were a result of *mobilization of body defense mechanisms* (207), *stimulation of reticuloendothelial macrophages* (55), effects on peripheral leukocytes (111, 158, 242), *stimulation* of antibody formation (55, 158, 205), or *activation of various enzymes* (205, 264, 271). The mild hepatic dysfunction produced by endotoxins (163) has been suggested as the cause for benefit in rheumatoid arthritis, similar to the amelioration of arthritis by hepatitis (3). In the treatment of hypertension, the *hypotensive action* of endotoxin would certainly seem to be important; indeed, it has been shown that suppression of fever does not interfere with benefit in hypertension. Similarly, the occasional use of endotoxins in patients with peptic ulcer has been suggested by the known *depression of gastric secretion and motility* that they produce.

In 1930, Krumbach suggested rather non-specifically that the beneficial action of endotoxins in tabes dorsalis might be a result of "increased circulation

of the blood". It is of considerable interest that it was shown nearly 20 years later that patients with symptomatic neurosyphilis show both an increase in cerebral blood flow and oxygen consumption when given endotoxin, in contrast to individuals with asymptomatic neurosyphilis who show no such changes (162, 259).

Because so many of the suggestions outlined above were made without any basis in experimental findings, and because it is not possible to link the alterations which undoubtedly occur with therapeutic benefit in any causal way, it is perhaps appropriate to mention three recent developments that may be of considerable significance in the question of non-specific therapy. Stuart (334) showed that endotoxin, under certain conditions, would enhance antibody formation and increase the severity of Arthus skin reactions in rabbits. Using another dosage schedule, others (36) were unable to modify the incidence of serum arteritis in rabbits by concomitant administration of endotoxin. It remained for Good (77) and Landy (176) to show conclusively that concomitant administration of endotoxin with another antigen definitely enhances specific antibody formation. It is, therefore, entirely conceivable that some of the beneficial results alleged to have occurred in infections when treatment with endotoxin was begun may have involved this type of reaction.

A second development has been the demonstration that polysaccharides influence profoundly the properdin level of the blood (269). Landy and Pillemer (196, 197) have shown conclusively that endotoxins elevate the serum properdin level and that this is accompanied by an increased resistance to infection. The role of these alterations in the properdin system in the therapeutic benefits of endotoxins in the past can only be speculated upon until further information is available.

Finally, the demonstration by Thomas of the increased sensitivity to epinephrine that follows administration of endotoxin is of interest not only because of its implications in terms of the mechanisms of tissue damage by these bacterial products (352, 410) but because it may offer an explanation for the occasional benefit derived from these substances in certain types of allergy.

Because the relationship of endotoxins and the adrenal cortex has been discussed thoroughly in previous sections of this paper, it will simply be stated here that there is nothing to support the contention that the action of these materials is mediated directly by the pituitary-adrenal axis. The hints that endotoxins may represent an inexpensive substitute for ACTH or adrenal steroids in the management of various diseases are not in accord with present information.

3. Application in specific diseases. a. Neurosyphilis. The beneficial effect of fever therapy in syphilis of the central nervous system has been clearly demonstrated (190, 200, 214, 247, 280, 314, 386), and in combination with arsenicals and in recent years, penicillin, it is a valuable form of treatment (211).

b. Psychoses. Results in various psychotic states have not been dramatic, although occasional benefit in patients with schizophrenia has appeared to follow injection of endotoxins (171, 347).

c. Other neurologic disorders. As a result of the studies of Windle and his asso-

ciates (67, 298, 392) on the effect of endotoxin upon neuronal regeneration in the central nervous system, there has been an increased interest in this form of treatment in neurologic diseases. Windle found that the administration of endotoxin to animals with various types of central nervous injuries facilitated histologic regeneration of neurones apparently by inhibiting the formation of glial scars at the site of trauma. The regenerating fibers were also shown to be capable of impulse conduction. Unfortunately, the functional results of treatment of animals with such injuries as transection of the spinal cord have not been dramatic. Clinical reports on the use of endotoxins in multiple sclerosis (66), various neuropathies (282) and poliomyelitis (62, 294) have been very poorly controlled. Until some objective assessment of therapeutic results can be substituted for such phrases as "seemed to improve" or "appeared to benefit", the evaluation of these studies will not be possible. In one of the most carefully conducted investigations of this type in which patients with multiple sclerosis, amyotrophic lateral sclerosis, arachnoiditis and residual disability from poliomyelitis were treated with endotoxin, there was found nothing to indicate that this form of therapy possesses any great usefulness (6).

d. Infections other than syphilis. As an adjunct to sulfonamide or antibiotic therapy in *gonorrhea* of the "intractable" or "resistant" type, endotoxin injection has been of value in the hands of many investigators (61, 148, 186, 326). One of the first infections in which benefit was thought to derive from endotoxin therapy was *typhoid fever* which Fraenkel (119) treated with typhoid vaccine and Rumpf (288) with a vaccine made from *Pseudomonas aeruginosa* with supposedly good results. One of the most recent studies of this type (21) reports "acceleration of recovery" in 48 of 50 cases of typhoid given intravenous typhoid vaccine. There were no control cases, and it seems probable that the availability of specific antimicrobial treatment in the form of chloramphenicol will eliminate this problem without solving it. Among other infections reported to benefit from fever therapy in recent years are *herpes zoster* (40), *sporotrichosis* (349), and *diphtheria* (397). *Herpes zoster*, of course, is notorious for the variability of its course and has "responded" to many remedies. In Wirth's (397) report on the use of endotoxin as an adjunct to penicillin and specific antiserum in diphtheria, despite what seemed to be speedier recovery and smaller mortality in treated patients, the incidence of neurologic complications in a group of controls was the same as that in patients given endotoxin. On the basis of this study, penicillin and antiserum seem to remain the best agents in diphtheria, and endotoxin is not to be recommended as an addition to routine treatment.

e. Gastrointestinal diseases. One study using pyrogenic urine extracts (292) in patients with peptic ulcer reported remission and total improvement in a large number of cases. There is little, however, to lead one to advocate endotoxins as a replacement for alkali, diet, and antispasmodics for this disorder. Occasional reports of benefit in chronic ulcerative colitis (170) are unimpressive, especially since "good" results in this disease have been reported after almost every form of medication tried for it, including horse-serum containing antibodies against the colon bacillus (396).

f. Vascular disease. The use of endotoxin has been advocated as beneficial

in occlusive arterial disease, especially thrombo-angiitis obliterans, by many reliable observers (44, 399). There now appears to be no question about the frequent effectiveness of prolonged administration of endotoxin in arresting the course of malignant hypertension (49, 254, 255, 345). This form of treatment is prolonged and expensive, however, and is not used extensively at present. A combination of endotoxin and hexamethonium has recently been suggested (364), but despite its effectiveness it requires much time, lengthy hospitalization, and careful supervision.

g. Neoplasms. The long history of the use of Coley's toxins for malignant tumors has been reviewed elsewhere (235) and will not be repeated here. Since the work of Shear and his colleagues (305, 307) in isolating the tumor-necrotizing endotoxin from *S. marcescens*, there have been many investigations of the effect of these materials upon tumors in experimental animals (306), and a few clinical trials in man (54, 166). There is no question about the ability of endotoxins to produce necrosis in some types of neoplasm but, in general, the amounts required elicit such severe reactions of fever and shock, and tolerance to the lytic action upon tumors develops so rapidly that this is not a practical form of therapy at present. It represents one of the more hopeful fields for investigation in tumor chemotherapy, however.

h. Arthritis. "Non-specific" protein therapy has long been used in the treatment of chronic rheumatoid arthritis (64) and other rheumatic disorders. Results have ranged from good (266, 366) or fair (234) to poor (118) in rheumatoid disease, and at present, it can only be said that occasional cases may possibly benefit from this form of treatment. Results in gonorrheal arthritis are good (118).

i. Diseases of the eye. In inflammatory diseases of the eye, especially uveitis, iritis, and keratitis of luetic or other origin, there is still widespread use of endotoxin therapy in this country (4). Even in ophthalmoplegic migraine, good results have been reported (258). The place of this form of therapy in modern ophthalmology has been reviewed periodically through the years and the interested reader is referred to these summaries (59, 90, 127, 169, 245, 291).

j. Diseases of the skin. As might be expected, endotoxins have been a part of the therapeutic armamentarium of dermatologists for many years and, recently, there has been renewed interest in the use of these materials. Because so few clinical reports are based on controlled study (172, 182, 183, 215, 312), it is difficult, if not impossible, to draw conclusions from reports of "improvement" in assorted cases of different dermatoses, and the question of the usefulness of endotoxins in diseases of the skin remains open until more careful studies have been made. It is of considerable interest that Rostenberg (285) found endotoxin to be without effect upon eczematous sensitization in guinea pigs.

k. Allergic disorders. In perhaps no other field has there been more uncontrolled "evaluation"of endotoxins given by injection or by mouth than in the management of allergic disorders (10, 185, 275, 276, 313, 384, 398, 409). Samter and Kofoed (290) conducted a controlled study, employing placebos, of the

effect of an endotoxin on the symptoms of allergy and concluded that there was no objective benefit from this form of treatment.

In asthma (116, 135, 212), there seems to be frequent benefit from the use of vaccines of various sorts. Swineford has reviewed the use of this form of treatment in an excellent paper (340) and concludes that this admittedly empirical procedure seems to benefit some patients and that it is worth undertaking in selected cases.

l. Other conditions. Hartmann (154) described an increased effectiveness of radiation in the management of *leukemia* when patients were given endotoxin. In contrast, others (160, 218, 363) have described protective action of endotoxins and pyrogenic preparations against the action of *ionizing radiation.*

Administration of endotoxin was said to increase survival and to speed healing following *extensive thermal burns* (143, 144) but carefully conducted studies by McCarthy and Blackburn failed completely to substantiate this (216).

IX. CONCLUDING REMARKS

The purpose of this review has been to summarize what is now known about the sources, chemical nature, and physiological action of bacterial endotoxins. Beginning with the "practical" problems of eliminating pyrogenic contaminants from fluids for parenteral infusion and the use of these substances in therapy, there has been an increasing interest in the role of these materials in the pathogenesis of infection. Endotoxins have been utilized in experimental studies of many diseases of non-infectious origin and have been invaluable in studies of fever and regulation of body temperature. The ubiquity of these bacterial products and their biological potency has led to misinterpretation of experimental results in many fields, and it is only in recent years that the tremendous importance of establishing that observed alterations in body temperature, leukocyte count, blood pressure, *etc.* are not the result of undetected endotoxin contamination has been recognized widely.

Recent advances in our understanding of the enhancement of antibody formation, stimulation of the properdin system, and sensitization to epinephrine by bacterial endotoxins are summarized, and it is concluded that these substances and the reactions elicited by them deserve further study. The discovery of lipopolysaccharides in mammalian and plant tissues that are capable of eliciting host responses that are identical with those produced by endotoxins may open the way to a better understanding of diseases not only of bacterial origin but of many other etiologies.

An evaluation of the efficacy of endotoxins in the therapy of human disease is handicapped by the uncontrolled studies that predominate in the published literature. For several disorders, however, there is no doubt about the beneficial action of endotoxins, and properly conducted clinical investigations of these materials as therapeutic agents are greatly needed.

The volume of information about endotoxins is so large that the present authors have attempted to supplement this paper by referring to detailed studies

in the appended bibliography. The references cited have been selected carefully and it is hoped that the interested reader will make use of them.

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