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STUDIES ON THE MECHANISMS OF THE CARDIOVASCULAR EFFECTS OF BACTERIAL ENDOTOXIN

by

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Dedicated with thanks to my friends, Steven and Janice, who helped; but especially with love to my wife, Lynn.

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ABSTRACT

Septic shock can not be effectively treated because pathophysiologic mechanisms are unknown. Clinical shock has two characteristic phases: early cardiovascular instability and late metabolic inadequacy. Both phases can be reproduced in animal models with endotoxins extracted from bacterial cell walls.

Endotoxin, a complex macromolecule, comprises an antigenic polysaccharide (O-antigen), a core polysaccharide (R-antigen) and lipid (lipid-A). Toxicity depends on core polysaccharide combined with a minimum amount of lipid.

Cardiovascular instability occurs in all species during endotoxemia, but endotoxin does not directly injure the heart or vessels ; interaction with cellular elements of the blood is required, and vasoactive substances histamine, bradykinin or serotonin are released. Vascular reactivity and vasoactive mediators are species-specific, making interspecies comparisons difficult. In human and subhuman primates, early endotoxemia is characterized by vasodilation. Bradykinin, a potent vasodilator, may be the vasoactive mediator and primate leucocytes contain kallikrein-like activity not found in other species.

A series of questions was tested:

1) "Is the lipid portion of endotoxin responsible for bradykinin generation *in vivo* in the primate?" Endotoxin

lipopolysaccharide (LPS) or fractions containing 6.3% (PS_1) or 0.5% (PS_2) of original lipid were infused into monkeys. Systemic and regional hemodynamics, plasma bradykinin levels, effects on granulocytes and metabolism were monitored. Early effects (2 min.) were similar in animals receiving LPS (10 mg/kg or 2.5 mg/kg) and PS_1 (2.5 mg/kg). All had hypotension (40-44 mmHg decrease in mean BP), decreased peripheral resistance (-7% to -22%) and elevated bradykinin concentrations (14 ± 6 ng/ml) two hours after infusion. Animals given PS_2 had increased blood pressure, increased PVR and no kinin generation. LPS and PS_1 caused prolonged granulocytopenia (2 hours). PS_2 caused only transient granulocytopenia (< 1 hour). All animals receiving at least 6.3% lipid (LPS and PS_1) had vasodilation except in spleen and skin. Animals receiving no lipid (PS_2) had vasoconstriction in all vascular beds.

Late effects (6 hours) were more clearly lipid-dose related. 10 mg/kg LPS caused hypotension, severely increased regional vascular resistance and acidosis. 2.5 mg/kg LPS caused similar, but less marked changes. Animals receiving PS_1 recovered to baseline. Animals given PS_2 had not changed. Plasma bradykinin concentrations returned to baseline.

Early cardiovascular insufficiency and bradykinin generation are closely related and require a minimum amount of endotoxin lipid. Interaction of LPS with leucocytes may be

of importance. Later toxicity also is dependent on the dose of lipid. The early cardiovascular events and late toxicity may be independent effects of endotoxin.

2) "Is bradykinin a sufficient mediator for the cardiovascular events of endotoxemia in the primate?" The bradykinin blood pressure dose-effect relationship is linear. Infusion of bradykinin (15-18 μg/kg/min) produces plasma arterial concentrations similar to those measured during experimental endotoxemia (14.5 ± 3 ng/ml); but does not reproduce the systemic or regional hemodynamic characteristics. Bradykinin infusion causes initial decrease of vascular resistance (-14 ± 10 U) at 3 min. but this returns to baseline after 10 min. Hypotension persists (-26 ± 14 mmHg) due to diminished cardiac output.

Systemic and regional hemodynamics during simultaneous sympathetic ganglionic blockade and kinin infusion reproduce the effects of endotoxemia. Bradykinin alone does not cause the cardiovascular insufficiency of endotoxemia, but the doses of kinin may have been insufficient to reproduce the tissue levels of peptide. Alternatively, endotoxin could inhibit autonomic reflex mechanisms.

3) "Does endotoxin inhibit reflex cardiovascular control mechanisms?" The baroreceptor-pressor reflex was tested in cats and monkeys. In six cats transient inhibition of responsiveness occurred after injection of endotoxin. But, when baroreceptor blood pressure was controlled inhibition

did not occur. Reflex responsiveness is related to mean systemic blood pressure ($Y = -17.4 + 0.39\ X$) $r = 0.92$. Arterial pH also has influences on pressor responsiveness ($Y = 1.45 + 72.5x$, pH_7 as ordinate) $r = 0.39$. These results were similar in cat and primate.

These experiments show:

1) Cardiovascular effects and bradykinin generation (in the primate) both depend upon the lipid in endotoxin, but;

2) Bradykinin can not alone cause the cardiovascular effects of endotoxin in the primate.

3) Endotoxin does not, per se, alter baroreceptor-pressor reflex mechanisms.

I. INTRODUCTION

In 1907, T. C. Janeway wrote concerning the treatment of shock due to infection, "There must be no symptomatic treatment without adequate physiological concepts" (Janeway, 1907). During the subsequent 65 years, primarily in the last decade, the pathophysiology and pathogenesis of septic shock have been studied extensively to define rational bases for therapy. Septic shock has been differentiated from shock of other causes; endotoxin, derived from gram-negative bacterial cell walls, has been shown to be a causative substance; the lipopolysaccharide has been extracted, partially purified and studied chemically and physically; and assay methods have been derived. The direct and indirect effects of endotoxin have been studied in vivo and in vitro, and have been shown to be extremely complex.

The therapy of septic shock also has been evaluated, primarily by empiric methods, with but few improvements having been made. As in 1907, saline infusions remain the mainstay of treatment. Antibiotics have been a significant addition, when appropriately used, but the cardiovascular effects of endotoxemia have proven very difficult to treat. Vasoactive drugs actually may be detrimental (Smith and Corbascio, 1970; Perey, 1971). Corticosteroids lack theoretical or empirical justification for prescription (Reichgott and Melmon, 1972a). Other therapeutic agents also have not proven significantly effective when evaluated clinically.

The urging of Dr. Janeway to develop therapy based upon understanding of pathophysiology is reasonable. Septic shock is the second most common cause of death on university medical services (Weinstein and Klainer, 1966) and has 60-80% mortality (Altemeier, Todd and Inge, 1967). But septic shock presently cannot be treated effectively because it is understood inadequately. The studies presented in this dissertation were undertaken in an attempt to improve that understanding. Clarification of the mechanisms by which endotoxin may cause cardiovascular instability is necessary for the development of rational, effective therapy of the highly significant clinical problem of septic shock.

A. STUDY QUESTIONS

A series of experiments were undertaken to test three related hypotheses. The specific hypotheses and the general aspects of the analysis of each are as follows:

1. Does endotoxin interact with cellular elements of the blood to cause release or generation of vasoactive substances? Are lipid components of endotoxin required for such interactions with cells?

This question was tested in the primate <u>in vivo</u>. Generation of bradykinin was measured following injection of endotoxin preparations of known, varying, lipid content. The effects of these same endotoxin preparations on systemic and regional hemodynamics, leucocyte counts and blood plasma biochemical factors were also evaluated. Positive correlation

was demonstrated between the presence of some lipid, in the endotoxin fraction, and bradykinin generation. The late cardiovascular and metabolic effects of endotoxin were shown to have a definite dose-response relationship to the total amount of lipid infused. But the early cardiovascular events were not clearly lipid-dose related. This dichotomy of lipid dose-effect between early and late phases was unexpected.

2. Can the cardiovascular effects of early endotoxemia be caused by excessive amounts of vasoactive substances in the blood? The primary vasoactive agent in the primate is thought to be bradykinin. Therefore, to test this question bradykinin was infused into primates in doses sufficient to produce arterial plasma concentrations equivalent to those measured during experimental endotoxemia. Systemic and regional hemodynamic effects of the peptide were measured and compared with the patterns of effects produced by endotoxin. The results suggest that bradykinin cannot alone account for the cardiovascular events of endotoxemia in the primate. Bradykinin infusion was also performed during sustained blockade of autonomic ganglionic transmission. Under these conditions the pattern of hemodynamic response was much more like that caused by endotoxin. The third hypothesis was developed in part upon this observation.

3. Can endotoxin inhibit normal autonomic reflex mechanisms, thereby limiting the effectiveness of homeostatic defenses and allowing vasoactive substances to act unopposed?

This question was tested in a model using hypotension at the carotid baroreceptor as the stimulus and the vascular resistance changes in a perfused hind limb as the response. The resistance responses to baroreceptor hypotension in animals infused with endotoxin was compared to the responses in saline treated controls. Both feline and primate models were studied. Reflex inhibition could not be demonstrated.

The testing of these three study questions has provided further understanding of the pathophysiologic mechanisms of cardiovascular effects in septic shock. The presence, and probable participation of bradykinin as an important mediator of endotoxemia in the primate, has been reconfirmed. The importance of lipid portions of endotoxin to the cellular effects of the lipopolysaccharide has been demonstrated. Endotoxin has been shown not to directly inhibit baroreceptor reflex functioning.

More important, perhaps, are the questions which have developed from this work but have not been tested. Can the endotoxin-leucocyte interaction be used as a model for the effects of endotoxin on all cells? Can release or generation of vasoactive substances be used as markers for cellular toxic effects? Are early phase cardiovascular events of endotoxemia important in the outcome of septic shock? And, if autonomic reflexes are intact, why is cardiovascular compensation so poor?

These results and questions are discussed in the final

section of this dissertation. I will first present a review of the historical and clinical aspects of septic shock. The evidence establishing the relationship of endotoxin to sepsis, the role of vasoactive substances in endotoxemia, and characteristics of bradykinin and endotoxin will be discussed. The original research examining the study questions will then be presented. Finally, conclusions, questions and speculations will be discussed.

B. LITERATURE REVIEW

1. Historical

a. Mechanisms of septic shock: Shock has been recognized by each generation of physicians as a preterminal event in severe infection, and many attempts have been made to define its presence and pathogenesis. Laennec, in 1831, described "weakness of the heart sounds in severe febrile conditions." German physicians proposed in 1872 that pyrogenic substances produced fever and vasomotor collapse by action on vasomotor centers (Dubczanski and Naunyn, 1872). Other German (Romberg et al,1899) and French (Charrin and Gley, 1890) workers then examined blood pressure responses in rabbits with severe pneumococcal sepsis. Electrical stimulation of the nasal mucosa, a pressor stimulus, was not effective in elevating the blood pressure in severely infected animals. However, abdominal compression, which increased venous return to the heart, did elevate the blood pressure in these same animals. These investigators concluded that during sepsis cardiac function was normal but vasomotor mechanisms were ineffective. This was

subsequently interpreted to mean that the animals were "being bled to death in their own veins" (Janeway, 1907).

Physicians in England disagreed with that concept. They thought shock was the result of vasoconstriction, from their observations on dogs, cats and rabbits with severe pneumococcal sepsis (Malcolm, 1909; Mapother Report , 1879). Arterial blood pressure responses to electrical stimulation of the sciatic nerve or the aortic depressor nerves in infected animals could not be differentiated from responses in normal animals (Porter, Newburgh and Newburgh, 1914). A comparison, during shock, of the vascular responses of intact and denervated rabbit ears showed that the denervated ear became vasodilated while the innervated ear vasoconstricted (Sielig and Joseph, 1916). These studies were interpreted as indicating that the vasomotor center and nervous system were not impaired during terminal pneumonia. This controversy has not yet been resolved.

World War I shifted research attention from medical to surgical causes of shock, but clinicians continued to be confronted by the complications of sepsis. Atchley, in 1930 (Atchley, 1930), commented on the frequency of shock accompanying severe infection, but noted the lack of contemporary literature. He described several cases, including that of an elderly man who developed shock following urethral dilatation and was successfully treated with only intravenous saline infusion. Atchley speculated that the shock had resulted from

bacterial invasion of the blood during the dilatation procedure. Unfortunately, blood cultures were not obtained to confirm this suspicion.

Warfield, discussing circulatory failure in 1936, said, "When bacteria invade the body and set up disease, certain histamine-like substances are formed which circulate in the blood." During sepsis he also noted "toxic products of bacterial action...causing increased capillary permeability," and he described stagnation of blood resulting in "inadequate circulatory volume" (Warfield, 1936).

In 1941, Ebert and Stead (Ebert and Stead, 1941) reported eight cases of severe bacterial septicemia. They stated that the clinical and hemodynamic characteristics of the terminal stages of septic shock were similar to shock of other causes: traumatic, hemorrhagic, or "nitrite collapse". These few papers offer the first modern considerations of the pathophysiology of septic shock.

During World War II, attention was again shifted from infectious to surgical causes of shock. However, the extensive use of blood transfusion and other volume-maintaining and expanding substances during that conflict provided the observations which have led to the intensive examination of shock due to sepsis.

b. Pyrogenic Substances: The study of pyrogens has progressed in parallel with the considerations of the mechanisms

of septic shock, but only in the last decade have pyrogenic substances, derived from bacteria, been recognized a significant cause of shock. German physicians first described pyrogenic substances. Panum, in 1855, had described fever-producing substances in decaying meat and in 1865 Bilroth noted fever in dogs given injections of distilled water. The name "pyrogen" was given to an extract of putrifying meat prepared by Brendon-Sanderson in 1876 (Bennett and Beeson, 1950).

In 1911 it was found that febrile reactions to injected substances could be eliminated by preparation of the injectates in freshly distilled, bacteria-free water. Pyrogenicity persisted in preparations made up in bacterially contaminated water; even after they had been autoclaved and rendered sterile (Bennett and Beeson, 1950). In studies reported in the 1920's, Siebert characterized the pyrogenic substance, a water-soluble, heat-stable, filterable factor produced by gram-negative bacteria (Siebert, 1925; Bourn and Siebert, 1925).

c. Role of Pyrogens in Septic Shock: There is no indication that the association between pyrogen reactions and septic shock was well appreciated prior to 1950. In the early part of the twentieth century, pyrogens had been adopted as therapy for a variety of conditions (Hibler and McBride, 1917; Gow, 1919). The clinical literature of septicemia was primarily devoted to case descriptions (Brill and Libman, 1899; Eastman and Keene, 1904; Felty and Keefer, 1924; Atchley, 1930). Only brief reference was made to the possible role of pyrogenic substances

in the development of shock (Freedberg and Altschule, 1945; Altschule, Freedberg and McManus, 1945). However, vasodilatation was recognized as a major cardiovascular effect of pyrogen, and hypotension resulting from the associated decrease in cardiac output was well recognized (Freedberg and Altschule, 1945; Bradley et al, 1945). On this basis, Pyromen^{R} *, a soluble bacterial pyrogen, was recommended in the therapy of malignant hypertension (Page and Taylor, 1949). Positive results were claimed in twenty patients, but the therapy was never pursued.

In 1951, a report linked fatal septic shock in humans with the transfusion of blood contaminated with pyrogen-producing bacteria (Borden and Hall, 1951). A characteristic shock syndrome was demonstrated in animals following injection of the bacteria cultured from the contaminated blood. A review of twenty-nine cases of gram-negative bacteremia, published later that year , pointed out the high incidence of shock (52%) and significant mortality (35%) of bacteremic patients (Waisbren, 1951). Another report, appearing at that time, confirmed the lethal potential and difficulty of therapy of gram-negative bacterial infection (Braude et al, 1953).

Septic shock subsequently has been recognized as a major clinical problem and has become the second most common cause of death on hospital medical services (Weinstein and Klainer,

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* Produced by Travenol Co. (PDR 6th ed. 1952)

1966). A period of intense research into pathophysiology and therapy has accompanied this recognition.

2. Clinical Characteristics of Septic Shock

Shock is a non-specific term used to describe a clinical syndrome which can develop in the course of several, varied disease processes. It is defined as "a state of reduced tissue perfusion leading to generalized cellular hypoxia and vital organ damage" (Thal et al, 1971). This definition has been derived from the study of patients and animal models with hypovolemia, or extensive trauma. Because of apparent clinical and biochemical similarities between shock of different etiologies, the definition has been broadened to include 'medical' shock. This definition may be adequate to describe cardiogenic shock, in which condition cardiac function is inadequate to maintain effective systemic blood flow; but the shock developing during infection does not fit easily into this definition.

a. General Appearances: The cardiovascular instability of septicemia can be divided into two major phases (Gilbert et al, 1955; Weil, Shubin and Biddle, 1964; MacLean et al, 1967; Siegel, Greenspan and Del Guercio, 1967; Bell and Thal, 1970; MacLean, McLean, and Duff, 1970; Neely et al 1971; Siegel, Goldwyn and Friedman, 1971). These phases can be distinguished by their clinical signs and by differences in the results of clinical laboratory studies. The early phase is characterized (Table 1) by increased cardiac output and

Table 1

CHARACTERISTICS OF THE EARLY (WARM) PHASE OF SEPSIS

Clinical Signs:

Headache, malaise

Nausea, vomiting

Flushed, warm, dry skin

Fever (often preceded by chill)

Tachycardia

Moderate hypotension (blood pressure may be normal

Normal or increased cardiac output

Hyperventilation

Fluctuating sensorium

Normal or low urine output

Laboratory Signs:

Normal PO_2 (slightly decreased Arterio-Venous O_2 saturation difference)

Decreased PCO_2 (respiratory alkalosis)

Normal electrolytes

Normal to slightly elevated blood lactate

vasodilatation (Hermreck and Thal, 1969; Bell and Thal, 1970). Tissue perfusion, as measured by determination of oxygen extraction, pH, or plasma lactic acid concentrations, is apparently adequate. This has been called the "hyperdynamic" period (Siegel, Greenspan and Del Guercio, 1967). It may go unrecognized if it is not specifically sought (Lambert, 1969).

It septicemia persists the late, cold, hypodynamic phase will usually develop. This period more closely fits the standard definition of "shock" and is characterized (Table 2) by decreased cardiac output and hypotension and laboratory evidence of inadequate tissue perfusion and progressive metabolic dysfunction (Udhoji and Weil, 1955; Wilson et al 1967; Siegel, Greenspan and Del Guercio, 1967; MacLean, McLean and Duff, 1970).

The early, warm phase (Table 1) is recognized most often in patients without severe underlying disease, especially those initially normovolemic (Wilson, Sarver and LeBlanc, 1971). This phase probably occurs in all patients developing septicemia. However, patient-determined factors such as the underlying cardiovascular status, total blood volume, and the functional status of the autonomic nervous system may influence the duration of this early phase and lead to a rapid transition to the later cold phase (MacLean et al, 1967; Bryant et al, 1971; Kwaan and Weil, 1969). Shock due to gram-positive bacterial infection usually exhibits only the early phase characteristics (MacLean et al, 1967; Blair et al, 1971). The signs present

Table 2

CHARACTERISTICS OF THE LATE (COLD) PHASE OF SEPSIS

Clinical Signs:

- Mottled, cyanotic, cool, damp skin
- Fever (hypothermia may occur in elderly patients)
- Tachycardia
- Severe hypotension
- Decreased cardiac output
- Oliguria, or anuria
- Coma

Laboratory Signs:

- Low PO_2 (progressive increase of Arterio-Venous O_2 saturation difference)
- Elevated K , BUN, uric acid
- Marked elevation of blood lactate

during this early phase are identical with the observed effects of pyrogens on the circulatory system (Freedberg and Altschule, 1945).

The late, cold phase of sepsis (Table 2) is difficult to distinguish from terminal shock of any other cause. It may occur with any infecting organism and is a common terminal event in patients with any severe debilitating disease or those with ineffective defenses (e.g., from prior cancer chemotherapy, immunosuppression or corticosteroid therapy) (Altemeier, Todd and Inge, 1967; Bryant et al, 1971). The mechanism of transition from early to late phase is unknown. Transition may in part be due to prolonged exposure to endotoxin which can cause progressive deterioration of cellular respiratory function (Harris, Harris and Green, 1968; Plant and Goldman, 1970). In addition, factors such as activation of intrinsic clotting mechanisms (Margaretten and McKay, 1969; Corrigan, Ray and May, 1968) and disruption of endothelial integrity (McGrath and Stewart, 1969) may result in local perfusion deficits leading to "stagnant anoxia" (Dietzman et al, 1967), progressive acidosis (MacLean, McLean and Duff, 1970; Bell and Thal, 1970) and the death of cells.

The warm phase of sepsis generally responds to therapy which maintains effective cardiac output and removes the source of bacterial toxin (Altemeier, Todd and Inge, 1967; Baue, 1968; Lambert, 1969). Infusion of saline solutions or volume expanders such as Dextran or plasma, or whole blood can be used to

maintain cardiac output. The addition of vasoactive drugs to the therapeutic regimen has not greatly increased survival rates. In fact, some vasoactive agents, particularly vasoconstrictors, may be detrimental in the management of this phase (Perey, 1971). The source of bacterial toxin must be eliminated by surgical drainage of local sites of infection and/or the use of intensive antibiotic therapy. It is essential to select an antibiotic to which the causative organism is sensitive (Petersdorf and Beaty, 1967; Christy, 1971).

The late phase is much less responsive to therapy. Many different modes of treatment have been attempted, but none has achieved significant success. Some, such as the use of isoproterenol, have had apparent experimental justification (Kardos, 1966; Talley et al, 1969). Others, such as corticosteroids, have had little experimental or clinical rationale (Reichgott and Melmon, 1972) but have been used none the less. Despite these various therapeutic attempts, the mortality of patients developing the late phase of septicemia has been little altered since the studies of the pre-antibiotic era.

Early studies indicated that the risk of death was approximately 35% in patients with septicemia (Waisbren, 1951). Most of the deaths occurred among patients who developed septic shock (Spink, 1960). More recent analyses have confirmed the extremely high risk of mortality, approaching 80%, in patients developing the late phase of septicemia (Baue, 1969; Christy, 1971). Many factors increase the probability that the late

phase and death will occur. Some of those are outlined in Table 3. Recognition of the early phase of septicemia has led to earlier institution of therapy, and some improvement in overall mortality statistics (Altemeier, Todd and Inge, 1967).

As indicated in Table 4, a variety of diseases are associated with uniformly high mortality when complicated by infection. In many of these the transition to late, cold shock may occur rapidly, making early institution of therapy difficult (Bryant, et al, 1971). Improvements in the general medical treatment of the underling conditions has increased the risk that patients will die of the complications of infection.

Antibiotic therapy has also contributed to the continued risk of late sepsis. Prior to the antibiotic era, gram-negative organisms were responsible for a much smaller number of severe infections than at present (Rogers, 1959; McCabe and Jackson, 1962). Most instances of sepsis were due to gram-positive infection (Ebert and Stead, 1941). Since gram-positive organisms can now be treated with relative ease, gram-negative bacteria have become the primary agents of severe septicemia (Yow, 1955). These organism are both more difficult to treat, and more likely to cause late shock in spite of treatment, than are the gram positive group. Gram negative organisms are opportunistic pathogens causing infection in previously ill or debilitated patients; as noted, this will predispose to the more severe complications and higher mortality of late sepsis. The relative frequency of infection by gram-negative organisms

Table 3

FACTORS INCREASING RISK OF MORTALITY IN SEVERE INFECTION

Age and Sex:

- **Neonates - first year of life**
- **Women - child bearing years (septicabortion)**
- **Men - over age 50**

Chronic or Debilitating Illnesses: See Table 4

Hospital-acquired infection

Preceding Therapy:

- **Corticosteroids**
- **Immunosuppressive drugs**
- **Antibiotics, especially 'Broad Sprectrum'**

Infecting Organisms:

- **Gram-negative - Gram-positive**
- **Non-bacterial infections - fungal, protozoal**

Table 4

THE RELATIONSHIP BETWEEN PREEXISTING DISEASE AND MORTALITY DURING INTERCURRENT INFECTION

	No. of Patients	% Mortality
Immuno-suppressive or cancer chemotherapy	49	85%
Renal failure	41	80%
Neoplasia	76	68%
Pulmonary disease	32	55%
Trauma (Burns)	40	67%
Arteriosclerotic Cardio-Vascular Disease	49	60%
Hypertension	26	62%
Diabetes Mellitus	55	63%
Cirrhosis	29	45%
Peritonitis	29	51%
Pyelophritis	73	42%

References:
(Altemeier, Todd and Inge, 1967; Bell and Thal, 1970; Neely et al, 1971)

is indicated in Table 5.

The mortality rates for infection with any of these gram-negative organisms are approximately the same. Pseudomonas aeruginosa may be associated with slightly increased mortality because it is pathogenic in severely traumatized or burned patients. E. Coli is the common pathogen in acute infection in otherwise healthy people (acute pyelonephitis, septic abortion) and is therefore associated with slightly lower mortality. The patterns of cardiovascular instability, and progression to late phase shock, caused by different organisms are also essentially identical. This suggests that a common pathogenetic mechanism is involved.

These clinical and historical observations raise several questions. What is the causative substance in septic shock? What are the mechanisms by which this material can cause shock? Are different mechanisms involved in the pathophysiology of the early and late phases of septicemia? What role does the early phase play in the development of the late phase? Is late shock as irreversible as it appears to be? Would prevention of the cardiovascular phenomena of the early phase influence the development of the later phase?

b. Causative Substances: Severe infection with any organism can lead to shock as a terminal episode. As noted, gram-positive bacteria were the primary agents in the pre

Table 5

ORGANISMS ISOLATED IN 2176 CASES OF GRAM-NEGATIVE INFECTION

	No. of Patients	% of Patients
E. Coli	787	36%
Pseudomonas	326	15%
Klebsiella-Aerobacter	441	20%
Proteus	180	8%
Paracolon	43	2%
Salmonella	12	0.5%
Serratia	1	-
Bacteroides	65	3%
Unidentified	136	6%
Mixed	105	9%
Total	2176	99.5%

References:
(Weil, Shubin and Biddle, 1964; Wilson et al, 1967; Bryant et al, 1971; Christy, 1971; Neely et al, 1971)

antibiotic era. At present, gram-negative bacteria are most commonly associated with severe septicemia leading to shock (Table 5) (Rogers, 1959). The study of causative substances has been directed primarily toward materials derived from this latter group of organisms. Are bacterial products sufficient to cause septic shock, or is the whole, live organism required (Waisbren, 1964)? Pyrogens produce effects very similar to bacterial infection, and direct comparison of whole organisms with extracted substances has confirmed that endotoxin, extracted from bacterial cells, and live bacteria can cause identical effects (Guenter, Fiorica and Hinshaw, 1969; Thomas et al, 1969; Hinshaw, 1972).

Injection of live E. coli bacteria into unanesthetized primates reproduced the early hypotension, vasodilation and well-maintained cardiac output observed in animals given E. coli endotoxin (Neis et al, 1969; Buckberg, Kohn and Darling, 1971). Studies in pentobarbital-anesthetized primates also demonstrated that live organisms and endotoxin produce identical effects (Guenter, Fiorica and Hinshaw, 1969). The cardiovascular responses observed in the latter study differed from those of the former two, but this difference probably was due to the influences of anesthesia. In another report, identical effects of live organisms and cell extracts were confirmed in the dog (Hinshaw et al, 1968). Species differences in response between dog and primate are well recognized (Gilbert, 1960; Kuida et al, 1961), but within each species, prepared endotoxin and live bacteria produce the same effects.

These results indicate that endotoxin extracted from bacterial cell walls can be used to reproduce the events of septicemia, and that laboratory models employing endotoxin have validity in the study of septic shock.

3. Endotoxin

Endotoxin is a complex mixture of glycolipid, protein and nucleic acid extracted from gram-negative bacterial cell walls by a variety of methods (Milner, Rudbach and Ribi, 1971). The toxic effects of endotoxins are similar, no matter what the bacterial sources, but different endotoxins are chemically quite variable (Nowotny, 1969; Work, 1970). Preparation of endotoxin requires separation of bacterial cell wall components from other portions of the cell. The chemical heterogeneity of endotoxins from different bacterial species has required that a variety of extraction procedures be devised (Luderitz et al, 1971). Extraction may add to chemical heterogeneity by altering the chemical characteristics of the end products (Nowotny, 1971). Comparisons of results obtained with different endotoxin preparations, therefore, must be attempted with some caution.

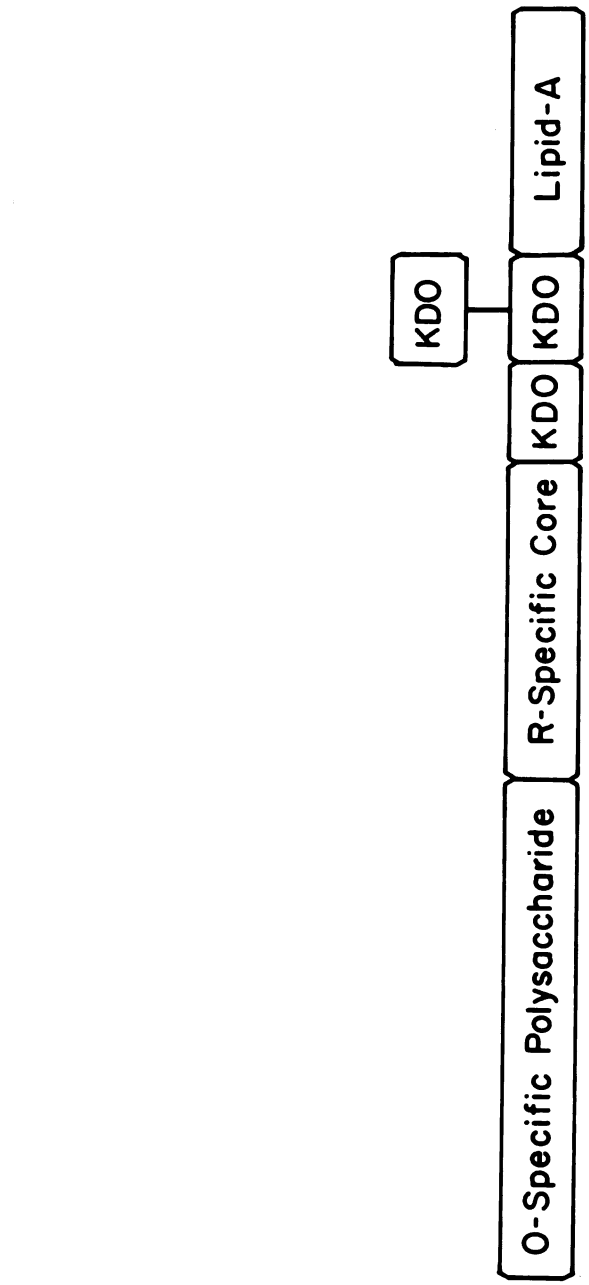
a. Physical and Chemical characteristics of endotoxin: Following initial separation, the crude cell wall extract is subjected to several steps of further preparation and purification. Protein and nucleic acid residues can be removed without loss of toxic potential (Nowotny, 1965; Nowotny, 1969). The remaining glycolipid, called lipopolysaccharide (LPS) con-

tains the active components of endotoxin.

LPS has three primary portions (Fig. 1) (Work, 1970): a polysaccharide portion, composed of repeating units of oligosaccharides, is the O-antigenic material of the bacterium and is the strain-specific antigenic determinant. Argentinian workers first identified this antigen with bacterial pyrogens (Mandolfo and Houme, 1947). This antigenic polysaccharide, however, is not necessary for endotoxin effects. Mutant strains of bacteria, called R or rough-colony forms, are spontaneously lacking in this moiely. Endotoxins prepared from these R strains retain full toxic potency but lack O-antigen (Luderitz, Staub and Westphal, 1966; Luderitz et al, 1971). Recent clinical reports indicate that antibodies to O polysaccharide are not associated with protection in spontaneous septicemia (McCabe, Kreger and Johns, 1972). Some workers have suggested that the complexity of the O polysaccharide portions of LPS may contribute to toxicity (Makela, 1972). However, this has not been well established.

A second portion of the LPS macromolecule is "Lipid A." Originally this designation was assigned to the highly insoluble mixture of fatty acids and phospholipids removed from endotoxin by acid hydrolysis (Nowotny, 1965). At present, the lipid components of the intact LPS complex are referred to generally as "Lipid A" (Luderitz et al, 1971) Studies of the toxicity of LPS have indicated that a minimum amount of this lipid material is required for toxic effects (Galanos et al,

Fig. 1



Schematic representation of lipopolysaccharide molecule

1971). The required moiety appears to be β-hydroxy myristic acid, which is bound tightly to the polysaccharide core of endotoxin (Luderitz, 1972).

The third portion of the LPS complex is the core polysaccharide. This portion is antigenic and has been called "R antigen" (Work, 1970; Luderitz et al, 1971). It represents a backbone to which O-antigens and "Lipid A" moieties are attached. Whereas the O-antigen is strain specific, "R antigen" is not, but varies only between species. The variability between species is due to characteristic units of polysaccharides, unique sugars, and the ratios of various types of bonding. One consistent component of this core polysaccharide is an 8-carbon keto-sugar (2keto-3deoxyoctonate (KDO)) found as a trisaccharide (Fig. 1), which may provide an important reactive site or be a major contributor to endotoxin effect. Alternatively, this moiety may contribute to the solubility of otherwise insoluble, but toxic, "Lipid A" (Galanos, 1971; Luderitz, 1972). Antibodies to R-antigen are associated with increased survival in septicemia, confirming the toxic role of this portion of LPS (Work, 1970; McCabe, Kreger and Johns, 1972).

Study of LPS by physical techniques has revealed that the native preparation consists of two sedimentable portions. The majority, which retains full toxicity, sediments at 60S. A minor fraction, with no toxicity, sediments at 2S (Ribi, Milner and Perrine, 1959). The latter material has O-antigenic capability. Electron microscopy of the 60S fraction reveals

a heterogeneous mixture thought to represent cell wall fragments. Further study indicates that these fragments retain a bilamellar structure and confirms that they are derived from bacterial cell walls (Shands, 1971).

Alkaline hydrolysis of the 60S fraction results in formation of smaller fragments. These sediment at approximately 20S, and again are a heterogeneous mixture, both by electron-microscopy and immunologic technics; this mixture retains full toxic potential. Further hydrolysis can reduce fragment size. Toxicity begins to decrease and is lost completely when fragment size or weight are reduced sufficiently. There seems to be a critical, minimum size for endotoxin activity (Nowotny, 1965; Shands, 1971). But the specifics of minimal size needed for toxicity, and the relationship of different toxic aspects of endotoxin to different sized aggregates have not been completely evaluated.

4. Pathophysiology of Endotoxin-caused Cardiovascular Instability

This dissertation is limited to considering the mechanisms of the early cardiovascular and hemodynamic abnormalities of endotoxemia. These preceed, and may underlie later shock, but this is unknown. The significance of the early phase to the development of the later phase will be considered in the conclusions.

Endotoxin could cause early cardiovascular changes via three different mechanisms: (1) LPS could directly effect the functioning of cardiac or vascular tissue. (2) Endotoxin

could interact with endogenous substrates in blood or tissue to release vasoactive substances which could then serve as mediators of cardiovascular changes. (3) Endotoxin could cause dysfunction of autonomic nervous system mechanisms which maintain normal homeostasis.

a. Direct Effects of Endotoxin on the Myocardium: Endotoxin could directly influence myocardial contractility or the energy systems which support contractility. Gilbert, in 1960 (Gilbert, 1960), noted that there was little evidence for such a mechanism. However, subsequent reports did state that the primary cardiovascular effect of endotoxemia was direct myocardial depression (Maxwell et al, 1959; Moses and MacIntyre, 1963; Solis and Downing, 1966). The conclusions of these workers were questionable since their investigations of cardiac function did not exclude other effects of endotoxin which may have influenced the results. For example, Solis and Downing (1966) attributed death in cats injected with endotoxin to cardiac failure. However, they disregarded the acute changes in pulmonary vascular resistance which are probably the primary response to endotoxin in this species. Kuida and coworkers (1961) have reported that cats exposed to endotoxin exhibited respiratory insufficiency, and that the pulmonary artery pressure rose 12 ± 8.6mmHg (fifteen animals) representing an increment of 73 ± 15% over resting values. Several of their animals died with pulmonary edema. Gilbert (1960) had described elevated venous resistances in dogs and cats. More recently these observations have been reconfirmed (Greenway and Murthy,

1971) and a possible mechanism suggested (Kux et al, 1972).

More recent studies have demonstrated no direct myocardial depression by endotoxin. Thomas et al (1969) exposed dogs to both endotoxin and live E. coli. An identical shock syndrome developed and myocardial contractility was not depressed. Starzecki and Spink (1968) were able to demonstrate decreased myocardial contractility in dogs two hours after exposure to endotoxin, but Gomez and Hamilton (1964) have demonstrated similar late deterioration in animals made hypotensive by hemorrhage, suggesting that myocardial depression is not a specific response to endotoxin.

These results are supported by in vitro studies utilizing cat papillary muscles (Kutner and Cohen, 1966; Hinshaw et al, 1971 b), dog heart-lung preparations (Priano, Wilson and Traber, 1970; Hinshaw et al, 1971 b; Hinshaw et al, 1971 c) and guinea-pig atrial muscle strips (Bhagat et al, 1970). Endotoxin did not cause depression of contractility in any of these preparations. The most recently reported studies, by Chiba and associates, have examined endotoxin effects on the SA-node (Chiba, Shigetoshi and Nakajima, 1971) and AV-node (Chiba and Nakajima, 1972) in vagotomized dogs. At doses of endotoxin sufficient to cause shock there were no effects on the specialized tissue of the canine heart.

The results indicate that endotoxin does not have direct depressant effects on automaticity, conductivity or contractility in myocardial tissue. Myocardial failure does, however,

accompany both clinical and experimental septic shock, probably as the result ofmetabolic dysfunction and/or release of toxic substances (Gomez and Hamilton, 1964; Goodyer, 1967) in prolonged shock. Lefer and his co-workers have studied 'myocardial depressant factor' (MDF) (Lefer, 1970; Lefer and Rovetto, 1970; Lefer and Martin, 1970). This is reported to be a polypeptide of molecular weight 800-1000 which is released by the action of proteolytic enzymes from pancreatic or splanchnic lysosomes. The myocardial depressant peptide has been found in several species, and during ischemic, hemorrhagic and endotoxin shock. The substance is reported also to depress reticuloendothelial cell function (Lefer and Rovetto, 1970). Hypoxemia, but not acidosis, will lead to release of MDF. Its importance to clinical septic shock is not known.

b. Effects of Endotoxin on Cardiovascular Reflexes : Inhibition of normal autonomic nervous system fructions will result in cardiovascular instability. The abnormalities of cardiovascular function occurring during endotoxemia could be the result of a toxic effect of the lipopolysaccharide on the autonomic reflex mechanisms. This possibility has been little studied. Excessive vasoconstriction is proposed as a cause of inadequate tissue perfusion and 'stagnant anoxia' of late shock (Block, Pierce and Lillehei, 1966). Adrenegic blocking agents have been proposed for the correction of this abnormality, but have not had significant success.

Early phase responses to endotoxin also may be due to

autonomic dysfunction. The bradycardia occurring following endotoxin administration to dogs is inappropriate for the low level of blood pressure that is induced. Stimulation of vagal parasympathetic outflow by endotoxin was proposed as the cause of this reaction (Blattberg and Levy, 1969). More recently, bradycardia has been shown to be resistant to atropine (Chiba and Nakajima, 1972). Therefore vagal stimulation is less likely. The mechanism remains undefined.

The rates of carotid nerve discharge also are abnormal in the dog exposed to endotoxin (Trank and Visscher, 1962). The firing rates in this species were found to be inappropriately high for every level of blood pressure tested, when compared to controls. The significance of this finding, which should result in inappropriate vasodilatation, is unknown. The vasoconstrictive response to hypotension is apparently unimpaired in this species.

In primates, the cardiac and vascular responses to endotoxemia also are not appropriate to the level of blood pressure. Vasodilation persists despite profound hypotension (Nies et al, 1968). Bradycardia may also occur. Plasma catecholamine levels do not rise in early endotoxemia in the primate exposed to slow infusion endotoxin or infusion of live organisms (Hinshaw et al, 1967). These observations suggest that there may be an abnormality of the pressor reflex, caused by the endotoxin. The baroreceptor-pressor reflex arc has not been studied systematically in any species during endotoxemia.

c. Effects of Endotoxin on Capillary Endothelium:

Endotoxin is known to affect vascular permeability. Increased vascular permeability is manifested by marked hemoconcentration and decreased plasma volume in the dog exposed to endotoxin (Lillehei and MacLean, 1959). Increased permeability of the vasculature of limbs and mesentery of the dog has been confirmed by gravimetric technics using organs perfused with heparinized whole blood (Motsay et al, 1972); and by injection of radio-tagged macromolecules (Chien et al, 1964). Increased vascular permeability is manifested in the cat by development of severe pulmonary edema (Kuida et al, 1961; Solis and Downing, 1966; Greenway and Murthy, 1971).

Microscopic study of sheets of cells obtained from the epithelial surfaces of arteries of the rabbit injected with endotoxin demonstrated that "bacterial endotoxin causes detectable changes in almost every cell in the vascular lining . . ." (McGrath and Stewart, 1969). These observations have been confirmed by a report of extensive changes, nuclear disintegration, irregular staining and "spindling" of smooth muscle cells of veins of animals exposed to endotoxin (Fine, Palmerio and Rutenberg, 1963). Majno and co-workers (Cotran and Majno, 1964; Magno, Gilmore and Levinthal, 1967; Majno, Shea and Levinthal, 1969) have shown, using light and electron microscopy, that "histamine-like" vasoactive substances produce injury to and contraction of the epithelium of the venule. The epithelial cell contraction exposes the basement membrane

and can result in increased permeability.

The effects of endotoxin on the permeability of vascular tissue are very similar to the effects of "histamine-like" mediators, as demonstrated by transucation of injected carbon. Endotoxin interacts with both plasma and cellular elements of the blood to release potent vasoactive substances such as histamine (Hinshaw, 1971), serotonin (Greenway and Murthy, 1971) and bradykinin (Nies et al, 1968). (See below Pg.33) Since all studies of endotoxin effect on vascular endothelium or permeability have been performed in living animals or in limbs or tissues perfused with whole blood, these vasoactive substances may have contributed to the observed results.

d. Effects of Endotoxin on vasomotor activity: Endotoxin also can influence the activity of vascular smooth muscle. The observed effects depend upon the species being tested and the specific vascular bed being examined. Vasoconstriction occurs in some vascular beds and vasodilation in others (Gilbert, 1960; Wyler et al, 1969). Experiments have been performed using lungs (Hinshaw et al, 1957; Kux et al, 1972), intestinal mucosa (Penner and Bernheim, 1942), rabbit ear vessels and mesenteric vessels (Delaunay et al, 1948), isolated veins (Spink and Vick, 1961; Vick, 1964), intact dog limbs (Hinshaw et al, 1962) and perfused rat livers (Nolan and O'Connell, 1965; Filkins, 1969) to determine whether endotoxin can cause contraction of vascular smooth muscle. All of these studies have shown that endotoxin, in the presence of heat-

labile factors found in plasma, and cellular elements of the blood, can stimulate vascular smooth muscle. But if the endogenous components are not present, the vasoactivity does not occur. In a single study in which vasoconstriction did occur in the apparent absence of blood components (Nolan and O'Connell, 1965), blood may not have been completely removed from the perfusion system (Filkins, 1969).

e. Effects of Endotoxin on the Pharmacologic activity of vasoactive agents: Endotoxin can change vascular responses to circulating amines. French workers in the 1940's described increased microvascular reactivity in the mesenteric vessels of animals treated with endotoxin (Delaunay et al, 1948). These observations led others to examine the association of endotoxin with the dermal Schwartzman reaction (Thomas, 1956; Brunson, 1964). The dermal reaction could be reproduced by intravenous injection of endotoxin followed by intradermal injection of epinephrine within four hours. Similar lesions could be produced by intradermal injection of norepinephrine, but not by ephedrine, pitressin, histamine or serotonin, and the lesions could be prevented by pretreatment of the animals with dibenzylene or chlorpromazine (Thomas, 1956).

Subsequent studies demonstrated that mesenteric vasculature in rabbit and cat exhibited increased responsiveness to catecholamines following exposure to endotoxin (Zweifach, Nagler and Thomas, 1956; Zweifach, 1964). Studies of responses of rabbit ear vessels, perfused with Tyrode's solution, did

not confirm the enhancement of vascular response to epinephrine after exposure to endotoxin. The differences between these results were explained by experiments in which rabbit aortic strips were exposed to endotoxin dissolved in Krebs-Ringer solution. Responses to epinephrine were unchanged until whole blood or leucocytes were added to the bath. Responsiveness then increased significantly (Gouryis, Hollenberg and Nickerson, 1961).

The dermal Schwartzman phenomenon requires the presence of leucocytes or their granules and is closely related to activation of coagulation processes (Thomas, Zweifach and Benacerraf, 1957). In addition, recent studies have shown that when activation of coagulation and infusion of catecholamines are simultaneous injury and fibrin deposition occurs within arterioles (McKay, Linder and Cruse, 1971). Endotoxin can activate the coagulation mechanisms directly and via interaction with cellular elements of the blood (See below). The change in response to catecholamines caused by endotoxin is probably mediated via these mechanisms.

Another recent study (Weiner and Zweifach, 1966) indicates that endotoxin may be able to enhance vascular responsiveness to other vasoactive substances in the absence of blood components. Strips of rabbit aorta were exposed to endotoxin, 40 ug/ml in tissue bath. Basal tone was unchanged and there was no effect on the contractile response to epinephrine and norepinephrine. However, there was an 18% increase in contractile

response to serotonin, 5μg/ml, and 12% increase in response to 400μg/ml of the amine. The responses returned to normal when endotoxin was washed out of the system.

The evidence presented indicates that endotoxin does not directly influence cardiovascular functions. Myocardial depression requires metabolic changes or the release of endogenous substances; vasomotor responses also require the presence of endogenous vasoactive agents; and the interaction of endotoxin with vasoactive amines is enhanced through involvement of other plasma systems such as coagulation. The presence of cellular elements of the blood may also be required for endotoxin induced effects. Other effects of endotoxin, such as inhibition of corticosteroid output from perfused adrenals, also occurs only when whole blood was the perfusate (Rosenfeld, 1955).

5.Endotoxin induced release and generation of vasoactive substances

Clinical septic shock has often been attributed to circulatory vasoactive substances. The concept of the "histamine-like" substances mediating septic shock was initially proposed by Warfield in 1936 (Warfield, 1936). Similarities between endotoxic and anaphylactic shock have been noted (Weil and Spink, 1957) and histamine and serotonin may be mediators of sepsis in different species. Endotoxin also can activate several plasma enzyme systems, (see below) and the interaction of endotoxin with leucocytes and platelets adds another possi-

ble source of vasoactive agents and enzymes. Several mediators have been studied during endotoxemia.

a.) Catecholamines: Plasma levels of catecholamines may rise, after injection of endotoxin, in some species. In dogs, the rise begins just after injection, and generally continues, with the highest levels occuring in moribund animals (Rosenberg et al, 1959, 1961). In an occasional experiment the rise may be transient: the reason for this is unknown. In the primate exposed either to whole bacterial organisms or slow infusion of endotoxin, plasma catecholamines rise, but only after several hours (Hinshaw et al, 1962). The reason for this delay is unknown. Rapid injection of highly concentrated endotoxin, in a small volume, has been followed by brisk rise of plasma catecholamine concentrations in anesthetized primates (Cavanaugh et al, 1970). The response of catecholamine release following endotoxin is in part dependent upon species selection and experimental design. The role of these amines as mediators of clinical septicemia is not known.

b.) Histamine: Histamine was studied as the first potential mediator of endotoxin induced cardiovascular changes. It is found in high concentrations in the blood of dogs following injection of endotoxin. The methods originally used for its determination were nonspecific (Hinshaw et al, 1960; Hinshaw, Jordan and Vick, 1961a) but recently release of histamine in this species has been reconfirmed (Vick, Mehlman and Heiffer, 1971). The amine is present only briefly in the

earliest minutes following injection of endotoxin. Since high concentrations of plasma histamine occur in the dog when plasma catecholamines are also elevated, Schayer's suggestion (1960) of a balance between histamine and catecholamines influencing cardiovascular function may be correct.

The species-specific response of the dog to endotoxin injection is a brisk, but transient increase in hepatic and portal venous resistance, resulting in intense engorgement of the liver and splanchnic vasculature. Histamine injection or infusion can closely reproduce this phenomenon (Jordan, Holmes and Hinshaw, 1966), but this does not prove that the amine is a mediator sufficient to cause all of the cardiovascular events of endotoxemia.

Histamine is also thought to be present in high concentrations in the plasma of some primates exposed to endotoxin (Hinshaw, Jordan and Vick, 1961b). This observation has not been confirmed in a recent evaluation.* Experimental design may have influenced these results since blood samples were obtained, in the latter study, at a time when histamine may already have been cleared from the circulation.

c.) Serotonin: In the blood, serotonin is stored primarily in platelets (Engelman, 1971). It is also found in mast cells of some species, but not in the mast cells of humans. The amine is capable of exerting effects on pulmonary vascular resistance in some species (Miller and Melmon, 1970). The role of serotonin as a mediator of endotoxemia is unclear. Studies in

* Miller, R. D., Personal Communication

which blood levels of serotonin were measured during endotoxemia noted a sharp drop in its concentration in blood immediately following endotoxin injection. In one such study, the fall in whole blood serotonin was accompanied by a rise in the plasma concentration, suggesting, that some of the amine was released from platelets (Rosenberg et al, 1959; Rosenberg et al, 1961).

Endotoxemia is associated with transient thrombocytopenia (Gilbert, 1960; Kuida et al, 1971; Greenberg and Murthy, 1971; Kux et al, 1972). Serotonin could be released from platelets during or as a result of, interaction with endotoxin. As noted, serotonin may produce pulmonary vasoconstriction; and increased pulmonary vascular resistance has been reported to occur, in most species, following endotoxin injection (Gilbert, 1960; Kuida et al, 1961; Halmagyi, Starzecki and Horner, 1963; Tikoff, Kuida and Chiga, 1966; Brockman, Thomas and Vasco, 1967). Greenberg and Murthy (1971) reported that both aggregation of platelets and acute pulmonary vascular resistance changes, occurring following injection of endotoxin, could be blocked by pretreatment of the animals with aspirin. They suggested that the drug blocked the release of serotonin, which may occur during aggregation (Movat et al, 1965), and thereby prevented the pulmonary vascular reaction. They did not document their speculation by measurement of serotonin. The role of serotonin, as a mediator of the pulmonary vascular effects of early endotoxemia, requires further study since others (Kux et al, 1972) have presented evidence that proteo-

lytic enzymes, released from leucocytes, may be more important in the pulmonary vascular response.

d.) Bradykinin: Another potential mediator of the cardiovascular effects of endotoxin is bradykinin (Nies et al, 1968; Reichgott and Melmon, 1972) The interaction of bacterial endotoxin with several plasma and cellular systems may result in kinin generation (see below). Bradykinin is a multipotential substance which can exert many cardiovascular effects consistent with those occurring during endotoxemia in the primate. The experiments to be described explore both the role of bradykinin in the development of the cardiovascular events of endotoxemia and the relationships between endotoxin and kinin generation. The following will briefly outline the physiology and pharmacology of bradykinin which make it a potential mediator of endotoxemia.

Bradykinin is one of the most potent endogenous substances known (Erdos, 1966; Melmon and Cline, 1967; Collier, 1968). Its effects are diverse. At 10^{-9} molar concentrations in artificial media or plasma, it can affect blood vessels, and smooth muscle reacts to 10^{-10} molar bradykinin. Potentially 2-14 g of bradykinin are available in each ml of human plasma (Margolis and Bishop, 1963), and sufficient kinin could easily be generated at a local site to produce a significant vascular response. The doses of bradykinin needed to produce some effects in a variety of tissues are outlined in Table 6.

The amino acid sequence of bradykinin is:

Table 6

ACTIONS OF PURE BRADYKININ

Tissue	Preparation	Species	Response	Dose
Smooth muscle	Ileum	Guinea pig	Contraction	1 ng/ml
	Uterus	Rat	Contraction	0.1 ng/ml
	Duodenum	Rat	Relaxation	1 ng/ml
Blood vessels	Blood pressure	Cat	Decrease	1 µg IV
	Skin vessels	Cat	Dilation	0.1 µg IV
	Skin capillaries	Guinea pig	Increased permeability	0.1-1 ng/ml
	Ear veins	Rabbit	Constriction	0.08-20 ng
	Pulmonary arterioles	Lamb fetus	Dilation	2 ng IV
	Umbilical artery	Human fetus	Constriction	1.5 ng/ml
Pain	Blister base	Human	Pain	0.1-1 µg/ml
	Intraperi-toneal	Human	Pain	1 µg/ml

(Adapted from Erdos, 1966)

H-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg-OH

This amino acid sequence is critical to the biological effects of the peptide (Stewart, 1968; Brady et al, 1970). Even simple changes alter the pharmacologic characteristics of the molecule (Erdos, 1966; Stewart, 1968; Suzuki et al, 1969)

Bradykinin exerts its pharmacologic effects in several areas:

1 The cardiovascular system
2 The inflammatory process
3 Smooth muscle contraction (vascular, bronchial, intestinal, uterine)
4 Interaction with other vasoactive substances

Other kinin effects of possible biological importance have been imcompletely investigated.

1) Pharmacologic effects of Bradykinin - Cardiovascular: The effects and duration of action of bradykinin on the cardiovascular system are dose-dependent. The species and age of the animal and the particular vascular bed under observation also influence the measured effects.

Bradykinin does not appear to have any direct influence on the myocardium (Harrison et al, 1968; Bassenge et al, 1970). When the peptide is administered in vivo the heart rate, stroke volume and cardiac output increase significantly, but ventricular contractile force does not change (Table 7). The effects on cardiac function are secondary to vasodilation and associated hypotension, and are mediated by baro-receptor reflexes. They

CARDIOVASCULAR EFFECTS OF BRADYKININ INFUSION IN DOGS (Mean ± SEM)

	Control	Absolute Change	% Change
Heart rate (beats/min)	150.9 ± 6.9	4.1 ± 1.1*	2.6 ± 0.7*
Ventricular force (grams)	88.7 ± 12.6	11.6 ± 8.2	11.1 ± 7.7
Aortic pressure (mm Hg)	132.1 ± 3.6	56.6 ± 2.5*	43.0 ± 1.7*
Aortic flow (liters/min)	2.86 ± 0.3	1.4 ± 0.27*	48.8 ± 6.9*
Stroke volume (ml/beat)	18.9 ± 3.1	8.9 ± 1.8*	45.4 ± 7.6*
Systemic resistance (units)	46.2 ± 3.6	-28.1 ± 1.9*	61.2 ± 2.0*
Femoral artery flow (ml/min)	122.6 ± 20	30.7 ± 16.8	22.1 ± 10.9
Hind limb resistance (units)	1.2 ± 0.12	-0.59 ± 0.1*	-49.6 ± 6.1*

Table 7

*P 0.01

Maximal changes in cardiovascular function occurring in dogs receiving bradykinin injections 1 ug/kg into right atrium.

Reference:
(Harrison et al, 1968)

are dependent on the activity of the autonomic nervous system (Pearson and Lang, 1967; Lang and Pearson, 1968) and on release of catecholamines from the adrenal medulla (Feldberg and Lewis, 1964; Staszewska-Barczak and Vane, 1967; Lewis, 1970) and adrenergic nerve endings (Lewis, 1970).

The direct effects of bradykinin or kinins in general, on the cardiovascular system are limited to the blood vessels. Extensive arteriolar dilatation is well documented in almost all peripheral vascular beds and in almost every species studied. Of particular importance, bradykinin dilates cerebral (Carpi and Pinto-Corrado, 1961), coronary (Antonio and Rocha e Silva, 1962; Bassenge et al, 1970), splanchnic (Chour et al, 1963), renal (Gill et al, 1965; Willis et al, 1969), and muscular and cutaneous arterioles (Mason and Melmon, 1966), to varying extent (Harrison et al, 1968).

The effects of bradykinin on veins are less well defined than effects on arterioles. In the human forearm the peptide is a venodilator (Mason and Melmon, 1966), whereas in the rabbit ear it causes venoconstriction (Bobbin and Guth, 1968). The basis for the divergent effects on different venous beds is not known.

Bradykinin-induced vascular changes in the adult pulmonary bed also are poorly understood. The peptide may dilate pulmonary arterial vessels causing pulmonary arterial hypotension (DePasquale et al, 1969; Joshi et al, 1969). But pulmonary venous resistance increases due to active constriction (Hyman,

1968), or the passive effect of an increased pulmonary blood volume (DePasquale et al, 1969).

The vascular effects of bradykinin links it to production of inflammatory edema. The peptide increases capillary permeability (Lewis, 1965; Erdos, 1966; Kellermeyer and Graham, 1968; Northover and Northover, 1969) by producing capillary endothelial separation as shown by electron microscopic studies (Cotran and Majno, 1964), by increasing capillary hydrostatic pressure (Haddy, 1970), or by indirect effects which include release of catecholamines or other vasoactive substances such as histamine and serotonin (Lewis, 1965; Melmon and Cline, 1967; Kellermeyer and Graham, 1968).

ii) Pharmacologic effects of Bradykinin - Inflammation: Bradykinin probably is involved in the inflammatory process (Lewis, 1964, 1965; Greaves and Shuster, 1967; Eisen et al, 1968). In adequate concentration, the peptide can produce all of the cardinal signs of inflammation: pain, redness and warmth (vasodilation) and swelling (increased capillary permeability). In addition, it stimulates migration of granulocytes into an inflamed area (Lewis, 1965; Becker, 1969). Granulocytes contain kallikrein or kallikrein activators, as well as kininases (Greenbaum and Yamafuji, 1966; Melmon and Cline, 1968; Greenbaum, Chang and Freer, 1970) (See below for further discussion). Bradykinin is found in high concentration in the lymph draining from areas of spontaneous or experimental injury to tissues (Rocha e Silva, 1963;

Lewis, 1964, 1965). It is also found in abnormal quantities in synovial fluids from several forms of arthritis, including traumatic, septic, psoriatic, rheumatoid and gout (Melmon et al, 1967; Nies and Melmon, 1968; Jasani, Kabri and Lewis, 1969).

iii) Pharmacologic effects of Bradykinin - Smooth muscle: Organs containing smooth muscle (e.g., the gut, uterus and bronchi) contract when exposed to bradykinin. The physiologic or pathologic significance of the stimulation, by the peptide, or smooth muscle contraction is unknown. In man, bradykinin has minimal oxytocic action; it is not an important bronchoconstrictor; and although it may be produced to excess in some clinical conditions that are associated with intestinal hypermotility (e.g. carcinoid syndrome), it is apparently not required for normal peristaltic processes.

iv) Pharmacologic effects of Bradykinin - Interaction with other Vasoactive Substances: The catecholamines may release active kallikrein from salivary glands (Hilton and Lewis, 1956) and carcinoid tumor tissue (Oates and Melmon, 1966). Bradykinin may in turn release catecholamines directly from the adrenal medulla (Feldberg and Lewis, 1964; Staszewska-Barzac and Vane, 1967) or indirectly as a consequence of baro-receptor-mediated sympathetic reflexes (Lang and Pearson, 1968) and direct effects on sympathetic ganglia and nerve endings (Lewis 1970). Kinin can release histamine, and the amine can stimulate kinin generation (Lewis, 1965; Melmon and Cline, 1967).

Bradykinin and serotonin may exert synergistic effects on capillary beds (Weiner and Altura, 1967) or venules (Zweifach, 1964) and on pain production (Sicuteri et al, 1965); and may even be stored in the same cellular sites (Zeitlin, 1970). The potentiation of kinin effect by fibrinopeptide-B (Gladner, 1966) provides a link with clotting mechanisms (See below).

v.) Bradykinin - Interaction with Endotoxin: Endotoxemia is associated with generation of bradykinin in the human and subhuman primate (Nies et al, 1968; Kimball, Melmon and Wolff, 1972). This further suggests a relationship between the peptide and the cardiovascular events of endotoxemia. Several mechanisms of endotoxin-induced bradykinin generation have been described.

Incubation of endotoxin with whole blood results in bradykinin generation. This will occur in the blood of all species tested in vitro, but has been shown to be of consequence, in vivo, only in primates (Erdos and Miwa, 1968; Urbanitz, Sailer and Habermann, 1970). Several mechanisms are known to exist whereby endotoxin can initiate kinin generation, but not all are operative in all species. These mechanisms are:

1 Activation of Hageman factor (Factor XII)

2 Activation of Plasmin

3 Immune complex formation and activation of complement

4 Interaction with leucocytes

Direct kinin formation from kininogen by the action of kallikrein-like bacterial enzymes has been described (Erdos, 1966).

Endotoxin itself does not directly convert kininogen to kinin but the lipopolysaccharide component of endotoxin, in the presence of plasma can initiate kinin generation.

Endotoxin can activate Hageman factor (Rodriguez-Erdman, 1964; Muller-Berghaus, 1969; Beller, 1969) In vitro studies have confirmed that Hageman factor disappears from blood incubated with endotoxin (Rodriguez-Erdman, 1964). Also, endotoxin can cause the generalized Schwartzman phenomenon (Thomas, 1954; Margaretten and McKay, 1969), a condition characterized by intravascular coagulation and shock. The Schwartzman phenomenon also can be caused by other agents which act through activation of Hageman Factor (e.g. ellagic acid, sodium stearate, etc.) (Muller-Berghaus, 1969; McKay, Muller-Berghaus and Cruse, 1969). This enzyme, when activated, can initiate a sequence of proteolytic enzyme steps resulting in kinin generation (Ratnoff and Coloby, 1955; Margolis, 1958; Margolis, 1961; Margolis, 1963; Webster, 1968).

But Factor XII, when activated, only slowly releases kinin from purified kininogen (Kaplan and Austin, 1970; Kaplan and Austin, 1971). In vitro studies have also demonstrated bradykinin generation in Hageman factor deficient plasma (Nies and Melmon, 1971). Therefore, activation of factor XII probably represents only one of several mechanisms by which endotoxin promotes kinin production in plasma.

Endotoxin can activate plasmin. Several bacterial substances will act as proactivators of plasmin (Tillet and

Garner, 1933; Lack, 1948; Astrup, 1956). Release of tissue factors by at least one endotoxin (S. Abortus Equi) can also activate plasmin (Beller, 1969). Other endotoxins probably act in the same way. Large amounts of plasmin are required before bradykinin is slowly released from purified kininogen (Lewis, 1960; Webster and Pierce, 1960; Eisen, 1963). The most significant mechanism by which endotoxin initiates kinin generation in the plasma is probably a close interrelationship between activated plasmin and Hageman factor (Vogt, 1964; Burrowes, 1971; Kaplan and Austin, 1970, 1971). Plasmin proteolytically releases several peptide fragments from factor XII. Some of these fragments retain a specific active site that rapidly converts prekallekrein to kallekrein with subsequent generation of large amounts of bradykinin (Kaplan and Austin, 1970, 1971).

Complement and Immune Mechanisms A macroglobulin antibody (directed toward the polysaccharide component of endotoxin) is present in the serum of most animals (Landy and Weidanz, 1964). Both complement and this antibody are required for bradykinin generation in plasma incubated with endotoxin (Nies and Melmon, 1971). 42% of available kininogen was depleted upon incubation of complement-containing plasma with endotoxin. Kininogen depletion did not occur in complement-free plasma. Newborn human plasma, lacking in 19S anti-endotoxin antibody, did not exhibit kinin generation when incubated with endotoxin (Nies and Melmon, 1971). Addition of 19S antibody to new-born plasma allowed kininogen depletion to occur when incubated with endo-

toxin. Hageman factor was not required for kinin production in this sytem.

Complement may have other roles in endotoxin-mediated kinin production. The direct interaction of endotoxin with complement causes release of a substance chemotactic for polymorphonuclear leukocytes (Snyderman, Gewurz, and Mergenhagen, 1968). In addition, bacterial endotoxins and other substances that activate complement may initiate blood coagulation via a complement-mediated pathway (Zimmerman and Muller-Eberhard, 1971). Such pathways also may involve kinin production. Finally, complement seems to be required for some endotoxin-leukocyte interactions (Mergenhagen et al, 1969)

Leukocytes influence both generation and destruction of kinins. They contain kininogenase, or kallikrein activator activity, and kininase enzymes. These activities are found primarily in neutrophils and eosinophils ((Cline and Melmon, 1966; Melmon and Cline, 1967;1968). Kininogenase activity or kininogenase activating factors are in both the cell sap and granular fractions of the polymorphonuclear cells. The cytoplasmic enzymes act rapidly, generate kinin from both whole plasma and partially purified kininogen, and are most active at pH 7.4 - 7.6. The granular fraction of leucocytes contains kinin-forming enzyme with optimum activity at pH 6 of less. This enzyme activity probably is due to non-specific lysosomal cathepsins described by Greenbaum and Kim (1967).

Hageman factor may be required for kinin generation by the

leukocyte cytoplasmic factors. Both Hageman-deficient plasma, and kininogen prepared from Hageman-deficient plasma, are unable to support kinin generation by whole granulocytes or the cytoplasmic fractions of sonicated or homogenized cells.

Kininase activity is found primarily in the granule fraction of the cell, but there is some activity present in the cytosol. The optimum activities of cytoplasmic and granule fractions are found at pH 7.0. As the pH is lowered, cytoplasmic activity rapidly diminishes; granular kininases are most active below pH 5.5 (Zachariae, Malmquist and Oates, 1966).

Greenbaum and his co-workers have studied extensively kininogenase and kininase activities in rabbit peritoneal leukocytes and macrophages (Greenbaum and Yamafuji, 1966; Greenbaum and Kim, 1967; Greenbaum *et al*, 1969; Greenbaum, Carrara and Freer, 1971). Cells which were harvested after instillation of glycogen or mineral oil into the peritoneal cavities of rabbits (Cohn and Hirsch, 1960) contained kallikrein-like enzymes, primarily in the lysosomes. They were active at acid pH. These enzymes released kinin-like peptides from human and bovine kininogen preparations but acted much more slowly than the enzymes in human leukocytes. One of the released peptides - PMN-kinin - has chemical and pharmacologic characteristics clearly distinguishing it from bradykinin (Greenbaum and Kim, 1966; Greenbaum *et al*, 1969; Greenbaum, Carrara and Freer, 1971).

Other workers have demonstrated the presence of non-lyso-

somal proteases in human granulocytes. Janoff and Zeligs (1968) demonstrated one such enzyme, released by phagocytosis and active at neutral pH. which was capable of causing injury to epithelial cells, disruption of capillary basement membrane and hemorrhagic edema. Movat et al (1964) described an enzyme, released during phagocytosis of antigen-antibody complexes by leukocytes, which injured capillaries and caused edema. This enzyme activity also was released, but poorly, during incubation of leukocytes in the absence of particles for phagocytosis. In addition, human and dog leukocytes have been shown to release plasminogen-activating enzymes when placed on plasminogen-rich fibrin plates (Goldstein et al, 1971). This is noteworthy since the interaction of plasmin with Hageman factor (see above) represents the most important mechanism leading to kinin generation in plasma. Other studies (Matsuoka, Sakurugawa and Shimaoka, 1969) indicate that enzymes found in granulocytes, and capable of activating fibrinolytic mechanisms, are inactivated at temperatures above 56° and are inhibited by soybean trypsin inhibitor, the same inhibitor which blocks plasma kallikrein. The cytoplasmic protease discovered by Janoff and Zeligs (1968) was similarly heat-sensitive and inhibited by soybean trypsin inhibitor. All of these studies may actually have been dealing with a single enzyme or a small group of neutral proteases, each capable of kinin generation. The results indicate that the leukocytes of humans and other species may directly or indirectly contribute to kinin formation. The acid-active enzymes found in the granular fraction of leukocytes, are a clearly defined second group of kinino-

genases. They are identifiable with the acid proteases (Greenbaum et al, 1969), and are presumably non-specific. The acid proteases of rabbit cells act so slowly as kininogenases that their contribution to kinin generation is unlikely to be of pathophysiologic importance in the kinin generation associated with endotoxemia. In addition, these authors present evidence that the kinin-like polypeptide released by these acid proteases is not bradykinin.

Endotoxin has profound effects on polymorphonuclear leukocytes. In vivo, one of the earliest manifestations of endotoxemia is granulocytopenia (Gow, 1919; Bennett and Beeson, 1950; Cluff, 1971). This is caused by adherence of the cells to vascular (capillary) epithelial surfaces (Mulholland and Cluff, 1964). Granulocytopenia persists for 1-3 hours and is followed both by return of the sequestered leukocytes to the circulating pool and by an outpouring of newly matured cells from the bone marrow to the periphery (Quesenberry et al, 1972).

Endotoxin is rapidly cleared from the circulation by initial binding to leukocytes and platelets (Cooper, 1971). It is subsequently taken up and degraded by macrophages in the liver and spleen (Filkins, 1971). In vitro experiments (Gimber and Rafter, 1969) have confirmed that endotoxin rapidly binds to rabbit polymorphoneutrophils. The binding is a passive phenomenon, occurs in physiologic salt solution, and is dependent on divalent cations but is independent of metabolic function or availability of glucose to the cell.

The *in vivo* interaction of endotoxin with leukocytes is probably more extensive than simple binding of the lipopolysaccharide to the cell surface. Addition of endotoxin to whole blood and incubation of the mixture in a Warburg flask resulted in increased O_2 consumption in human or canine cells. The increased respiratory activity of the cells was proportional to the dose of endotoxin, occurred about ten minutes after endotoxin was added, and could be inhibited by NaF or iodoacetate (Strauss and Stetson, 1960). Rough or excessive handling of white cells eliminated this respiratory response to endotoxin. Cells exposed to endotoxin have also increased glucose utilization and lactic acid production, and decreased glycogen synthesis (Cohn and Morse, 1960). The capability for phagocytosis increased in cells incubated with endotoxin for 20-30 minutes. The metabolic and phagocytic effects occurred in physiologic saline solution and did not require serum. But, the cells, stimulated to more effectively phagocytize and kill microorganisms in the absence of serum, could then do so only when serum opsonins were present (Cohn and Morse, 1960).

In a study of the relationship between endotoxin and granulocytes, Cline *et al* have also demonstrated the augmented metabolic processes that are closely associated with phagocytosis. Endotoxin stimulated incorporation of uridine ^{3}H into RNA and of ^{14}C-amino acids into protein, and production of lactic acid and $^{14}CO_2$ from glucose-1-^{14}C (Cline et al, 1968). Lysosomal enzymes were released into the suspending medium, and cellular content of α-glucosidase (a lysosomal enzyme)

correspondingly decreased. This system required complement for maximal activity, and none of these effects were seen when the cells and endotoxin were incubated in a serum-free system. The same study examined the uptake of ^{14}C-labelled endotoxin by leukocytes. Only in complement-containing systems was there significant uptake of label. Radioautography and electron microscopy confirmed that endotoxin was actually taken up into the granulocyte and note merely bound to the surface. Others (Wiener, Beck and Shjlo, 1965; Graham et al, 1967; Filkins, 1971) have confirmed the central role of phagocytosis in the endotoxin-leukocyte interaction when the system contains complement and opsonin. In the absence of these serum factors, endotoxin binds only to the cell surface and is not phagocytized, but it can still activate cellular metabolic systems (Cohn and Morse, 1960; Graham et al, 1967; Gimber and Rafter, 1969). This phenomenon has given rise to the term "sham phagocytosis" (Cohn and Morse, 1960).

Phagocytosis of endotoxin is closely associated with the release of lysosomal and cytoplasmic enzymes (Weissman and Thomas, 1962; Selvaraj and Sbarra, 1966; Cline et al, 1968; Nies et al, 1971; Weissman et al, 1971). Endotoxin also releases lysosomal enzymes from other tissues (Janoff et al, 1962; Janoff, 1964; Glenn and Lefer, 1970). The relationship of lysosomal acid proteases to kinin generation has already been discussed. A recent paper (Weissman et al, 1971) stated that phagocytosis of inert particles does not result in loss of cytoplasmic enzymes from cells. The direct or indirect effects

(e.g. complement activation) of endotoxin might be expected to damage the cell extensively, allowing release of cytoplasmic proteases that could contribute to kinin generation at neutral pH. A similar mechanism could also be operating in other tissues.

The lipopolysaccharide portion of endotoxin stimulates fibrinolytic activity of leukocytes (Goldstein et al, 1971) by releasing plasminogen activators and lysosomal enzymes. It is not known whether phagocytosis is required for these effects. In addition, endotoxin interacts with leukocytes to activate coagulation mechanisms. The lipopolysaccharide can stimulate formation of leukocytic thromboplastic substances which directly activate factors VII and X, bypassing Hageman factor (Niemetz and Farri, 1971). Endotoxin can also activate the intrinsic clotting system by releasing enzymes from leukocytes (Margaretten and McKay, 1969; Lerner, Goldsteen and Cummings, 1971).

In summary, the interactions of endotoxin with leukocytes provide multiple possible mechanisms of kinin generation, including phagocytosis with release of lysosomal and cytoplasmic enzymes, initiation of coagulation, via Hageman factor, thromboplastin generation and plasminogen activation.

vi.) Species Variability in Bradykinin Generation Induced by Endotoxin: Potentially there are multiple mechanisms in all species for endotoxin-induced bradykinin generation. Also, kininogens and kininogenases are universal. Nonetheless,

only very low levels of bradykinin have been found in the dog exposed to endotoxin (Shah et al, 1970; Carretero, Nasjletti and Fasciolo, 1970). Even in the rabbit, in which kinin generation by incubation of endotoxin with plasma is well documented (Erdos and Miwa, 1968; Urbanitz, Sailer and Habermann, 1970; Nies and Melmon, 1972), the peptide concentration is not elevated in vivo, and in these animals the cardiovascular abnormalities of endotoxemia are not consistent with elaboration of bradykinin or other vasodilators (Kuida et al, 1961).

Initial studies attempting to define the significance of the endotoxin-kinin interrelationship have been reported (Nies et al, 1971; Nies and Melmon, 1972). These studies compared the effects of crude endotoxin (LPS), the 165,000 g pellet (165P) obtained from a suspension of (LPS), and the polysaccharide (PS) obtained by mild alkaline hydrolysis of 165P. In in vitro studies, kininogen was depleted when monkey serum was incubated with LPS, 165P and PS (Table 9). Depletion was greatest with PS alone. Rabbit plasma also responded to incubation with PS by generation of kinin. Both the primate and rabbit models required complement and macroglobulin antibody.

Monkey and human leukocytes also interacted with endotoxin fractions (Nies et al, 1971). This cellular interaction resembled phagocytosis and relased lysosomal α-glucosidase into the suspending medium. It was demonstrable only with LPS and 165P, both of which have high fatty acid content. The polysaccharide alone did not affect the metabol-

ism of the cell or become phagocytized; presumably the leukocyte required at least some lipid or a relatively intact endotoxin molecule for the interaction.

6. Summary

The preceding review has presented currently available information related to the historical, clinical and biochemical aspects of septic shock. Evidence is presented that gram negative bacterial endotoxin is an important causative agent in this syndrome. The data linking endotoxin to the release of generation of vasoactive substances has suggested that these latter agents may play an important role as mediators of the cardiovascular events of endotoxemia. Species differences in response to endotoxin make the study of the primate necessary for the understanding of human septicemia. Primates have a unique cardiovascular response to endotoxin (vasodilation) and a unique mediator (bradykinin). Bradykinin generation, in the primate, apparently depends upon the interaction of a specific portion of the endotoxin molecule with leukocytes. However, the complexity of the cardiovascular response to endotoxin suggests that other mechanisms (independent of mediator release) may also be involved. The possibility also exists that endotoxin may cause autonomic dysfunction.

Several other questions also arise when considering the cardiovascular effects of endotoxemia. Are the pathogenetic mechanisms of early and late phases of endotoxemia different? Does early cardiovascular instability contribute to late

shock? Is therapy of the early phase unimportant to the ultimate outcome of shock? Finally, is there a truly irreversible state of shock? There is little information available to answer these questions.

Three questions, outlined on pages 2-5, have been derived from the observations presented in this review. These specific questions are tested in the original studies which follow. The results of these experiments also relate to the group of questions posed above and these relationships will be further discussed.

II Original Investigations:

Three specific questions have been used to test the proposed hypotheses. Those questions are:

A. Is the lipid portion of endotoxin responsible for bradykinin generation in vivo in the primate?

B. Is bradykinin a sufficient mediator for the cardiovascular events of early endotoxemia in the primate?

C. Does endotoxin inhibit reflex cardiovascular control mechanisms?

A. Is the lipid portion of endotoxin responsible for bradykinin generation in vivo in the primate?

1. Rationale:

These experiments have specifically tested the relationship between bradykinin generation in vivo, and the lipid content of an infused endotoxin preparation. They are an extension of work, discussed in the introduction, which has produced the following group of observations: Bradykinin is apparently a mediator of endotoxemia in the primate, but not other species (see pp. 47-49). Primate leukocytes, but not those of other species tested, have rapidly active kinin generating enzymes. Whole endotoxin, or lipid containing portions of endotoxin are required for the activation of leukocyte kinin generating systems (pp. 48-49); but antigenic, polysaccharide portions of endotoxin, activate only plasma kinin generating systems (p. 48). In the rabbit, which lacks

active leukocyte kinin-generating enzymes, bradykinin does not play an important role in septic shock. These observations suggest that the interaction of endotoxin with leukocytes is responsible for the release of significant amounts of bradykinin and that this interaction may require the lipid moiety of endotoxin.

In addition to directly testing the requirement of lipid for bradykinin generation, these experiments have provided further evidence suggesting the necessity of bradykinin for the development of the cardiovascular events of early endotoxemia. They may also reveal, however, that kinin generation in vivo is not closely related to the dose of endotoxin-lipid to which the animal is exposed, nor is the severity of late endotoxemia apparently closely related to the extent of kinin generation.

The results of these experiments must be interpreted with some caution. The supply of characterized endotoxin fractions available for study was extremely limited. Of necessity, observations could only be made in pairs of animals. Certain results are so definite, however, that their meaning is not obscured by a lack of numbers.

2. Methods:

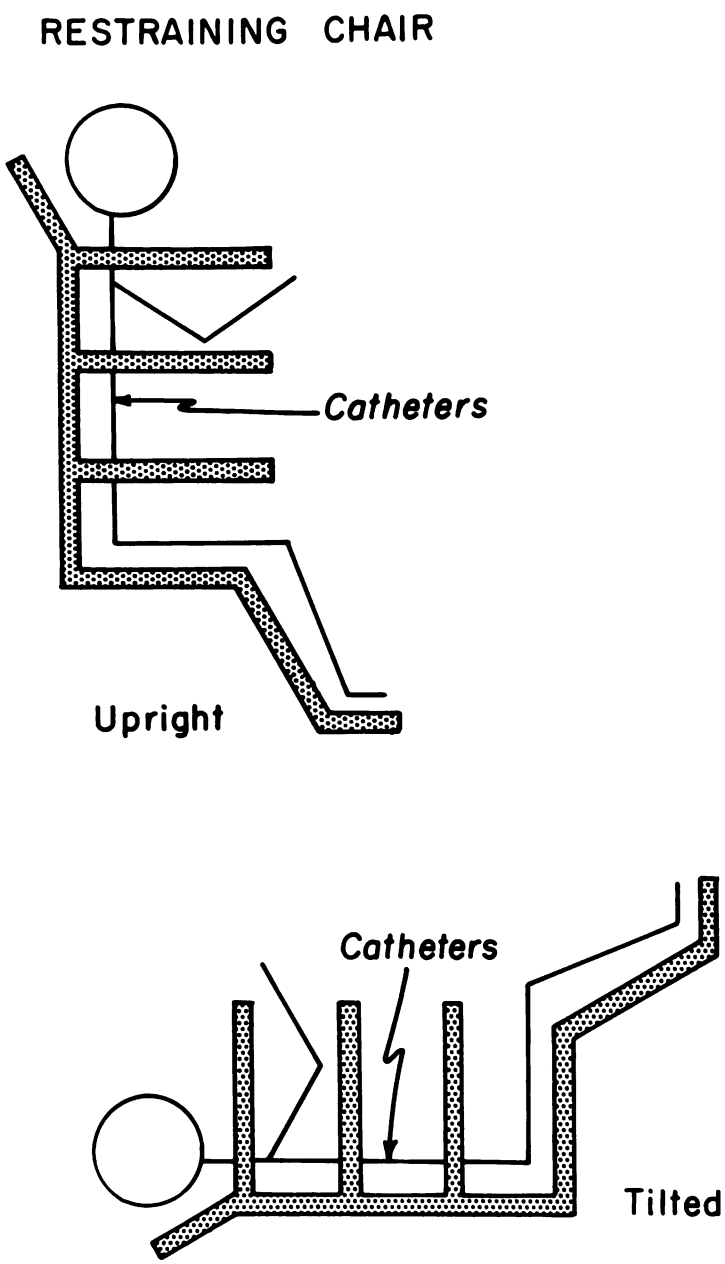
Studies were performed in nine rhesus monkeys (Macaca Mulatta), of either sex, weighing 4.3 to 7.4 kg. The animals were anesthetized for surgery by intravenous injection of pentobarbital sodium (Diabutal^{R} Diamond Laboratories)

25-30 mg/kg. Polyvinyl catheters were inserted into the inferior vena cava and abdominal aorta below the renal arteries via the left common iliac vessels. At a separate operation, using fluoroscopic observation, a third catheter was passed retrograde through the left common carotid artery into the left ventricle. The catheters were guided through prepared subcutaneous tracts and exteriorized at the level of the umbilicus.

After recovering from anesthesia, the animals were placed into restraining chairs, modified to allow tilting inside isolation booths (Foringer Co.), and the catheter lines were brought to the outside of the booths. The catheters were kept patent by continuous infusion of lightly heparinized (USP 5 units/ml) 0.9% NaCl at 1 ml/hr. Animals have been shown to remain stable for several months under these conditions (Forsyth and Baireuther, 1967). All experiments were performed a minimum of seven to ten days postoperatively to avoid the cardiovascular effects of recent surgical procedures and anesthesia. The animals were tilted to a semi-recumbent position at least one hour before the experiments were begun and blood pressure and heart rate were allowed to stabilize. The design of the restraining chair causes the animal's legs to remain flexed at both hips and knees, and to be slightly higher than the thorax and abdomen with the monkey tilted. (Fig. 2)

Measurements of arterial and central venous pressures

Fig. 2



Position of monkey in restraining chair

were obtained continuously using Statham 23Gb strain gauges (Statham Co.), adjusted to the midthoracic level. Mean blood pressure was derived electronically. A cardiotachometer coupler was used to derive the heart rate from the pressure pulse. All recordings were made on a Beckman type R recorder (Beckman Co.) using rectilinear pens, paper speed was 2.5 mm/sec. Blood samples were obtained from the arterial catheter at intervals. pH, PaO_2, and $PaCO_2$ were measured using Radiometer microelectrodes and corrected to 38 degrees Centigrade. Hematocrit was measured by the microtechnic and complete and differential leukocyte counts were made.

a. Cardiac output was determined by the indicator-dilution technic using indocyanine green dye. Injections of 1 ml of dye, 0.5 mg/ml, were made into the left ventricle. The ventricular catheter was filled with dye and then exactly 1 ml was injected using a spring-loaded constant volume syringe. Blood was withdrawn from the arterial catheter (length 75 cm, internal volume 1 ml) at constant rate (10 ml/min) under sterile conditions using a Harvard constant speed withdrawal pump. The dye curve was recorded using a Waters XP 302 densitometer and the blood was immediately returned to the animal. Dye curves were replotted onto semi-logarithmic paper. Measurements of the height of the dye curve above baseline were taken at $\frac{1}{2}$ second intervals. The disappearance curve of dye concentration was extrapolated to 0 according to the method of Hamilton and co-workers (Kinsman, Moore and Hamilton, 1929). The area under the curve was then determined by the traper-

zoidal rule (Franklin, 1953) and cardiac output calculated from the formula

$$C.O. = \frac{I \times 60}{A} C$$

I is the amount of dye injected (mg); A, area under the dye curve (mm·sec); C, dye calibration factor (mm $(mg/L)^{-1}$) (Kinsman, Moore and Hamilton, 1929; Dow, 1955). Dye calibration (C) was determined at the conclusion of each days experiments by preparation of standard concentrations of dye (3 mg/L and 6 mg/L) in the blood of the animal studied. This calibration value established the relationship between concentration of dye (mg/L) and pen deflection (mm) allowing calculation of C.O. in L/min.

b. Total peripheral resistance was calculated as mean arterial blood pressure minus venous blood pressure divided by the cardiac output

$$TPR = \frac{Pa - Pv}{C.O.}$$

and expressed in peripheral resistance units (mmHg $(L/min)^{-1}$) $(kg \cdot \text{body weight})^{-1}$ (Green, 1950).

c. The regional distribution of cardiac output was determined using the method described by Rudolph and Heymann (1967), based upon an original investigation using glass microspheres (Lindseth, E., 1960). The technic has become widely applicable since the introduction of commercially available plastic spheres (Ryan, 1969). Rudolph and Heymann adapted

the technic to allow repeated distribution determinations, in the same preparation, by using several injections of microspheres, each containing a different radioactive label (Rudolph and Heymann, 1967), and the method has been used to define normal hemodynamics (Forsyth et al,1968) and effects of endotoxin (Wyler et al, 1969) on the circulation of the rhesus monkey.

The microspheres consist of carbon, hydrogen and oxygen and a trace of a radionuclide. The radiotracer is incorporated into the plastic and there is no loss of tracer into the suspending medium even with prolonged storage (Ryan, 1969). The spheres used were 50 ± 10μ in diameter and separate batches were labeled with ^{125}Iodine, ^{141}Cerium, ^{51}Chromium, ^{85}Strontium or ^{95}Niobium. The microspheres were obtained in 20% dextran suspension and aggregation was inhibited by the addition of a small amount of Tween-80 (Polyoxyethylene sorbitan mono-oleate) (Ryan, 1969).

A different radionuclide was used for each determination of regional blood flow, allowing five such determinations. A well shaken suspension of microspheres was withdrawn into small glass injection vials and counted. 1-2 x 10^6 CPM were used for each measurement. The actual number of spheres injected varied with the age of the batch, the half-life of the nuclide and the actual number of counts, but approximated 5-10 x 10^3 spheres/injection. Immediately prior to injection of the microspheres, cardiac output was measured in duplicate

by dye-dilution. The vial containing the labeled microspheres was then connected to the left ventricular catheter. Blood was allowed to flow into the injection vial spontaneously; when full, the vial was briskly shaken to suspend the spheres in the blood. The suspended microspheres were then rapidly flushed into the left ventricle with 10 ml 0.9% NaCl. Occasionally cardiac extrasystoles would occur at the time of injection, but these were infrequent and of brief duration.

The use of this method for determination of regional blood flow is dependent upon the fact that the microspheres are actually distributed to the tissues in proportion to the distribution of cardiac output. The spheres must also remain in the arterioles and not transit arterio-venous shunts. Finally the presence of microspheres in the arterolar bed must not introduce an obstruction to effective perfusion, or change the peripheral vascular resistance.

Validity studies defining the reliability of the method have been reported and were not repeated in the present investigation. Rudolph and Heymann examined the possibility of shunting in the placenta and found none (Rudolph and Heymann, 1967). Studies conducted in the monkey revealed minimal amounts of radioactivity in portal blood, right ventricle, or lung (after injection into decending aorta) following sphere injection (Forsyth et al, 1968). Further experiments in unanesthetized monkeys have shown effective mixing of the microspheres in the left ventricle and no evidence of arter-

ial streaming. Studies with a mechanical model also have confirmed that the microspheres are distributed in proportion to flow (Rudolph and Heymann, 1967). Small increases of peripheral vascular resistance do occur with microsphere injection. These, however, occur with the first injection and subsequent injections are not associated with further increases in resistance. The resistance changes are thought to represent reflex responses rather than direct vascular effects of injected microspheres (Hoffbrand and Forsyth, 1969).

When the final set of measurements were completed the animals were killed by intravenous injection of pentobarbital. Extensive dissection was then undertaken and twenty organs and tissues were isolated (Table 8), weighed, minced and placed into individual glass vials. The samples were placed into the bottom 3 cm of the counting vials to limit geometric effects on reliability of counting. Radioactivity was measured with a Nuclear Chicago gamma-scintillation counter with an automatic sample changer. A well-scintillation detector, $1\frac{1}{8}$ inches in diameter and $1\frac{3}{4}$ inches deep, with a 3 inch thallium activated NaI crystal was used. 1100 volts were applied to the photo multiplier tube. The detector output was connected to a multichannel analyzer calibrated so that each channel spanned 10 KEV giving a total range of 1000 KEV. The system was calibrated with Cesium - 137. The gain was adjusted so the major energy peak of this nuclide (662 KEV) would fall in Channels #73-74. A Resolver integrator (Technical Measurement Corp 552A) allowed display or integra-

Table 8

ORGANS AND TISSUES COUNTED FOR RADIOACTIVITY

Brain (Separated into hemispheres, diencephalon, midbrain, medulla, cerebellum)

Heart (Separated into atria, ventricles, septum)

Kidneys (Right and left counted separately)

Skin *

Bone (Axial bone and skull, long bones counted separately)*

Stomach*

Small intestine (Duodenum, jejunum, ileum counted separately)

Large intestine (Cecum, colon counted separately)

Spleen

Pancreas

Liver (Hepatic artery)*

Lungs (Bronchial artery)*

Adrenals

Thyroid

Eyes

Lymph Nodes*

Body Fat*

Mesentery*

Pituitary

Miscellaneous (The remainder of unspecified organs)*

* Larger organs, and tissues, were weighed and measured aliquots taken for counting.

tion of individual channels of groups of channels. Data were printed on punched tape, and evaluated using an IBM 360 Computer. Programs were developed and written by Dr. R. P. Forsyth.

Gamma ray emitting radionuclides have specific and characteristic energy peaks. Each of the nuclides employed in these studies has a single major energy peak within the energy range examined. These are listed in Table 9 . Very little energy is found above the primary energy peak for each of these nuclides. These energy characteristics allow determination of the contribution of individual isotopes to the total radioactivity measured in an organ. To do this the 1000 KEV range of analysis was divided into five channel groupings. Each group of channels was selected to contain the major energy peak of a single isotope. The fractional distribution of counts within these five channel groups was determined individually for each nuclide (Table10) in preliminary experiments.

The predetermined distribution of energies was used to calculate the individual nuclide contributions following multiple injections in the following manner:

Only Niobium contributes to the counts measured in channels 72 - 100 (E), and 0.4362 is the fraction of Niobium counts in this channel, therefore the total Niobium - 95 count equals $\frac{E}{0.4362}$. Both Strontium - 85 and Niobium - 95 contribute to the counts measured in channels 46 - 72 (D).

Table 9

CHARACTERISTIC ENERGY PEAKS OF RADIONUCLIDES *

Radionuclide	Energy Peak
^{125} Iodine (^{125}I)	35 KEV
^{141} Cerium (^{141}Ce)	145 KEV
^{51} Chromium (^{51}Cr)	324 KEV
^{85} Strontium (^{85}Sr)	514 KEV
^{95} Niobium (^{95}Nb)	766 KEV

* From Chemical Rubber Co. Handbook of Chemistry and Physics 52nd Ed. 1971-72.

Table 10

FRACTIONAL DISTRIBUTION OF COUNTS PER MINUTE BY MULTICHANNEL PULSE-HEIGHT ANALYZER

Channels	^{125}I	^{141}Ce	^{51}Cr	^{85}Sr	^{95}Nb
2-12 (A)	0.9994*	0.1955	0.1110	0.1307	0.0918
13-29 (B)	0.0006	0.8024*	0.1161	0.2095	0.1857
30-45 (C)	0.00	0.0016	0.7711*	0.0805	0.1480
46-72 (D)	0.00	0.00	0.0013	0.5745*	0.1332
73-100 (E)	0.00	0.00	0.00	0.00	0.4362*

* Characteristic Energy Peak

The counts in this group of channels, due to Strontium - 85 alone, are D - 0.1332 (Nb) and total Strontium counts in the counted sample are:

$$\frac{D - 0.1332 \left(\frac{E}{0.4360}\right)}{0.5745}$$

Similar calculations can be made for each isotope taking into account the fraction of counts in each channel contributed by higher energy nuclides (Table 10). The equation for total ^{125}I CPM in the sample is:

$$^{125}I = \frac{(A-(0.1955Ce)-(0.1110Cr)-(0.1307Sr)-(0.0968Nb))}{0.9994}$$

The radioactivity due to each nuclide in the whole animal was determined by summation of the radioactivity of all the organs and tissues. The amount of radioactivity due to each nuclide within each organ, compared to whole body radioactivity due to that nuclide, defines the fractional distribution of cardiac output to that organ at the moment that nuclide was injected. When this fraction is multiplied by the cardiac output determined immediately prior to microsphere injection by dye dilution, the actual blood flow to each organ can be calculated. Regional vascular resistance can then be calculated by the formula:

$$R = \frac{Pa - Pv}{Flow}$$

with the assumption that the mean arterial-central venous pressure difference accurately reflects the pressure drop across individual organ vascular beds.

d. Bradykinin Extraction and Assay: (Webster and Pierce, 1961; Webster and Gilmore, 1965; Nies et al, 1968) Five ml of whole blood was drawn into plastic syringes and immediately mixed with two volumes of cold perchloric acid 0.5M (8.2%). The mixture was centrifuged for twenty minutes at 3,500 x G, zero degrees Centigrade, in a Sovall RC - 2B refrigerated centrifuge. The precipitate was discarded, and the supernatant was adjusted to pH 7.5 with KOH. A white precipitate formed and was removed by recentrifugation for ten minutes at 3,500 x G, zero degrees Centigrade. The resulting supernatant was diluted with five ml of deionized water and reacidified to pH 5.4 with HCl. Bradykinin was then separated by ion exchange chromatography using IRC - 50 resin (100 - 200 mesh) which had been prepared as follows: (Hirs, 1955)

After settling in water to remove the finest particles, 200 gms of the resin were dried on a Buchner funnel, washed with acetone, air dried, resuspended in water and washed five times with deionized water to remove all traces of acetone. The resin was then stirred overnight with concentrated NH_4OH (500ml). It was separated from excess alkali and washed in deionized water by resuspension and settling five times. The resin was air dried and then stirred in 3NHCl (1 liter) for four hours and again washed on a funnel with deionized water until the filtrate was no longer acidic. Finally, the resin was suspended in two volumes of 0.1N acetic acid. When prepared in this manner, the resin will absorb kinin peptides, but will exclude amines and acetyl-

choline (Webster and Pierce, 1961; Webster and Gilmore, 1965; Pierce, 1970).

1.2 ml of well mixed resin suspension was placed into small vitamin columns stoppered with glass wool. After the resin settled, the bradykinin containing solution was poured on to the column and allowed to drain through. The resin, with adsorbed kinin, was washed with 10 ml 0.1 NHAc and the peptide was then eluted with 6 ml of 8N acetic acid (Talamo, Haber and Austin, 1969; Pierce, 1970). The elute was freeze dried, and the residue kept frozen until assayed.

Assay was performed using a minor modification of the radioimmunoassay technic (Talamo, Haber and Austin, 1969). Radioimmunoassay technics depend upon competition between labeled and unlabeled hapten for specific antibody binding sites. As the concentration of unlabeled hapten is increased, the fractional binding of the labeled species will be proportionately decreased. A standard curve is established using a constant amount of labeled and increasing amounts of unlabeled compound in a constant final volume. Concentrations of hapten in unknown samples can be determined by extrapolation from this curve (Berson et al, 1964).

Conical plastic tubes (1 ml) (Fisher Scientific Co.) were rinsed with 1 ml of Veronal acetate buffer pH 7.4 containing lysozyme (1 mg/ml) (Worthington Biochem Co.). All measurements were made in duplicate. 100 microliters of the same buffer solution (pH 7.4) were placed into each of the

first two pairs of tubes. Standard concentrations of bradykinin were prepared in Veronal acetate buffer pH 7.4 and the unknown extracts were reconstituted in 1 ml of the same buffer. 100 microliters of standard or of unknown kinin solution were then placed into rinsed tubes. All procedures were conducted in duplicate in an ice-bath. At timed intervals, 20 microliters of ^{125}I-tyrosine-8-bradykinin (New England Nuclear Co), diluted in Veronal Acetate pH 7.4 to contain 15,000 DPM in 20 microliters, were added to each tube. This was followed immediately by the addition of 20 microliters of rabbit antibradykinin antibody to all but the first pair of tubes. To this pair was added 20 microliters of normal rabbit serum, to establish "nonspecific" binding. The tubes were mixed gently and allowed to incubate for two hours at four degrees Centigrade. Iced veronal acetate buffer was then added to each tube and antibody-bound peptide was separated from unbound peptide by filtration on Millipore^{R} filter discs (Millipore Corp. HAWP 025-00 0.45r pore).

There are several equally effective technics for separation of antibody-bound from unbound peptides (Palmeri, Yalow and Berson, 1971). Each of these methods requires centrifugation and handling which may result in decreased peptide recovery. Filtration using the Millipore^{R} filter eliminates most of the losses. The filter discs bind up to 200 ug protein/disc (Gilman, 1970). Although small amounts of unbound kinin may also adsorb to cellulose (Gilman, 1970), the nonspecific binding blank takes this factor into account.

The filters, with adsorbed peptide, were then dissolved in Liquifluor^{R} solution (New England Nuclear Co.) and radioactivity counted by liquid scintillation using the Packard Tricarb Model 3375 liquid scintillation counter. The kinin concentration was calculated in the following manner:

The observed average counts per minute (CPM) in the nonspecific binding tubes were subtracted from CPM of each subsequent pair of tubes. The corrected CPM found in each tube was then subtracted from the CPM in the 100% binding tube (no unlabeled bradykinin). The difference between 100% binding and binding in the sample tube is the effect of the competition of unlabeled bradykinin for binding sites. The value of this difference was then divided by the CPM 100% binding to give a value representing fractional inhibition of binding (FIB). FIB was then plotted against the log of bradykinin concentration, providing a standard line, and unknown values were determined by extrapolation from this line.

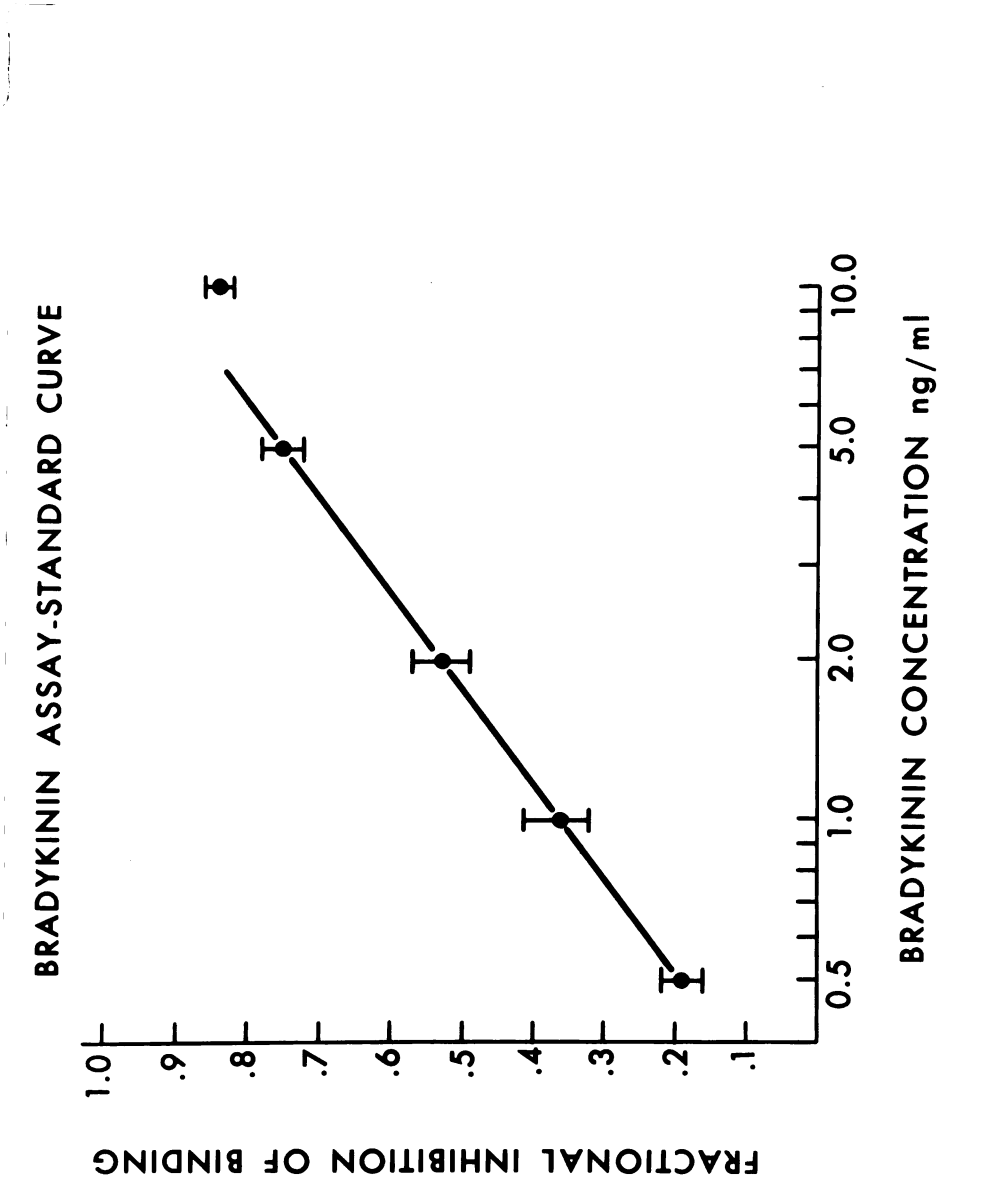
$$\text{CPM(observed)} - \text{CPM(nonspecific)} = \text{CPM(corrected)}$$

$$\frac{\text{100\% binding CPM(corrected)} - \text{sample CPM(corrected)}}{\text{100\% binding CPM (corrected)}} = \text{FIB (fractional inhibition of binding)}$$

Figure 3 illustrates the composite standard obtained by grouping 18 separate assays. The correlation coefficient (r) of the points establishing this line is 0.83 ($p < 0.001$).

Fractional recovery of bradykinin was measured in each

Fig. 3



Composite standard curve derived from 18 bradykinin assays (Mean ± SEM)

experiment. 200 millimicrograms of synthetic bradykinin were added to five ml of whole blood obtained at the same time the control period samples were obtained. The recovery sample was then processed through extraction and assay with the experimental samples. All unknowns were corrected for fractional recovery. Fractional recovery was 0.286 ± 0.019 (mean $\pm$ SEM) in the series of 18 experiments depicted in Fig. 3.

e. Endotoxin and Fractions: The endotoxin preparations used in these studies were produced and characterized by Dirk K. Greineder, M.D. in completion of his PhD in Pharmacology (Greineder, 1970). The preparations were used as provided.

Crude bacterial cell wall extract was produced by hot-phenol-water extraction. This extract was then treated with ribonuclease and incubated for four hours at 37 degrees Centigrade. After extensive dialysis the lyophilized product, "crude lypopolysaccharide" was centrifuged at 165,000 x G for 90 minutes. The pellet produced consisted of "pure" lypopolysaccharide (LPS) and portions of this material were used in the present experiments.

Subsequent subfractionation of LPS was performed by acid hydrolysis with 0.02N acetic acid at 100 degrees Centigrade. Centrifugation produced a precipitate, 'Lipid A', in 40-50% yield by weight. The supernatant liquid was then chromatographed on a charcoal column and eluted in steps. An initial water wash eluted a polysaccharide fraction (PS_1), containing

6.3% of the total fatty acids present in LPS, at yields of 20-25% by weight. A second elution, using 0.05 NH_2SO_4 in 50% EtOH, produced fraction PS_2. This polysaccharide was also obtained in approximately 20% yield and was found, on subsequent analysis, to contain less than 0.5% of the total fatty acid content of LPS. It is considered to be lipid free.

Both PS_1 and PS_2 were shown to have molecular weights above 100,000 by chromatography on Bio-Gel P100 equilibrated with 0.1M NaCl and 0.01M Tris buffer, pH 7.5. More than 90% of each fraction was excluded by the molecular-sieve. Small amounts of each of these three materials (LPS, PS_1 and PS_2) were available for these studies; they were prepared for intravenous administration by dilution in sterile 0.9% NaCl, at initial concentration 1 mg/ml, followed by a shaking for 24 hours at room temperature. The resultant suspensions were centrifuged at 1000 x G in a Sorvall RC - 2B refrigerated centrifuge with SS34 head. The supernatant fluids were saved and refrigerated until used. Aliquots of these supernatants were examined in a Beckman D.U. spectrophotometer using glass cells with a 10 mm light path, at wavelength of 500 mu. Absorbency was 0.050 with LPS, 0.036 with PS_1, and 0.015 with PS_2 at this wavelength. The relationships of OD were constant at other wave lengths as well.

LPS was administered at a dose of 10 mg/kg (LPS-10) as has been done in previous experiments in primates (Nies et al, 1968; Wyler et al, 1969). The 1 mg/ml suspension was infused slowly, intravenously, at a rate of 1 ml/min using a

Harvard infusion pump. The total infusion required approximately one hour in each of three animals studies. PS_1 and PS_2 were also prepared in initial concentration of 1 mg/ml. Each of these fractions was obtained in approximately 20% yield from the original LPS as noted. Each was administered at a dose of 2.5 mg/kg. This dose represents an approximation of the amount of each fraction to be found in the total infused dose of LPS. All infusions were diluted to 60 ml with saline to approximate the total volume infused into animals receiving the whole dose of LPS. Infusion rate was 1 ml/min. LPS was also administered at 2.5 mg/kg (LPS 2.5) in 60 ml final volume to control for the smaller amounts and lower concentrations of foreign material in the PS fraction infusions. LPS_{10} was administered to three animals, $LPS_{2.5}$ to two animals, and each PS fraction was administered to two animals.

f. Measurement Periods: On the day of experiment, each animal was tilted to the semi-supine position. They remained undisturbed for at least one hour to allow for adjustment to the experimental position. Blood pressure and heart rate were monitored during this time but the experimental measurements were not made until both had become stable. Baseline measurements of systemic blood pressure and heart rate, regional distribution of cardiac output were then obtained. Samples of arterial blood were withdrawn for assay of bradykinin, and for determination of hematocrit, leucocyte count, and arterial blood gases and pH.

Intravenous infusion of a preparation of whole or frac-

tionated endotoxin was then begun. Blood pressure and heart rate were continuously monitored. All other measurements were repeated at 15 minutes (during continuous infusion), at 45 minutes to 1 hour (at the end of infusion) and again at 2 hours and 6 hours. Following completion of the last measurements, the animals were killed and dissected as described.

3. Results:

a. Systemic Hemodynamic Effects of Endotoxin Fractions: Grouped baseline measurements for all nine animals (Table 11) did not differ from measurements in the other groups of animals similarly prepared and previously reported (Forsyth et al, 1968; Nies et al, 1968; Wyler et al, 1968). Systemic hemodynamic measurements obtained during the experiment are outlined by groups in Tables 12-13; only mean values are presented since the groups were small.

Figures 4 - 7 illustrate the effects of whole LPS or fractions on mean arterial pressure, peripheral vascular resistance (PVR), arterial plasma bradykinin, and total leukocyte counts. LPS_{10} (Fig. 4) produced a transient early increase in peripheral vascular resistance, followed by progressive vasodilation. By two hours after infusion, resistance had fallen to 7% below baseline although mean systemic arterial pressure had also fallen an average of 41 mmHg. LPS-2.5 (Fig. 5) produced progressive vasodilation; there was no initial vasoconstriction. At 2 hours, resistance had fallen 22%, mean systemic arterial pressure was 44 mmHg below baseline values.

Table 11

GROUPED BASELINE PERIOD MEASUREMENTS IN NINE UNANESTHETIZED RHESUS MONKEYS

Measurement	Mean	SEM
Systolic Blood pressure (mmHg)	136	7
Mean Blood pressure (mmHg)	102	5
Diastolic Blood pressure (mmHg)	73	4
Heart Rate (Beats/min)	178	9
Cardiac Output (L/min) (kg^{-1})	0.364	0.03
Total Peripheral resistance $mmHg(L/min)^{-1}(kg)^{-1}$	295	29
PO_2 (mmHg)	99	3
PCO_2 (mmHg)	39	4
pH (arterial)	7.45	0.01
White Blood Cells (mm^{-3})	13,583	1,650
Polymorphonuclear Cells (%)	58%	11%
Arterial Bradykinin (ng/ml)	0	0

EFFECTS OF WHOLE ENDOTOXIN ON SYSTEMIC CARDIOVASCULAR MEASUREMENTS AT BASELINE, 2 HOURS AND 6 HOURS AFTER INFUSION *

	(10mg/kg) N 3			(2.5mg/kg) N 2		
	Baseline	2 hr	6 hr	Baseline	2 hr	6 hr
Systolic Blood Pressure (mmHg)	134	92	109	110	90	98
Mean Blood Pressure (mmHg)	100	59	74	81	53	70
Diastolic Blood Pressure (mmHg)	70	34	55	59	33	46
Heart Rate (Beats/min)	172	232	232	197	223	211
Cardiac Output (ml/min)(kg^{-1})	336	219	117	388	327	207
Total peripheral resistance mmHg(ml/min)$^{-1}$ $\cdot$(kg^{-1})	311	278	650	217	164	342
PO_2 (mmHg)	97	94	94	108	102	96
PCO_2 (mmHg)	39	28	23	35	25	25
pH (arterial)	7.42	7.42	7.47	7.46	7.46	7.47
WBC/mm^3	15,050	5,850	24,550	11,750	5,470	23,500
Polymorphonuclear leucocytes (%)	45	32	65	50	32	70

* Values represent group mean.

Table 12

EFFECTS OF ENDOTOXIN FRACTIONS ON SYSTEMIC CARDIOVASCULAR MEASUREMENTS AT BASELINE, 2 HOURS AND 6 HOURS AFTER INFUSION *

	PS_1* (2.5mg/kg) N 2			PS_2 (2.5 mg/kg) N 2		
	Baseline	2 hr	6 hr	Baseline	2hr	6 hr
Systolic Blood Pressure (mmHg)	147	113	133	159	133	134
Mean Blood Pressure (mmHg)	117	74	104	111	103	104
Diastolic Blood Pressure (mmHg)	89	47	78	76	70	70
Heart Rate (Beats/min)	174	219	197	169	197	187
Cardiac Output (ml/min)(kg^{-1})	309	252	294	439	333	336
Total Peripheral Resistance mmHg$(ml/min)^{-1}$.(kg^{-1})	395	298	386	253	370	310
PO_2 (mmHg)	89	95	91	103	101	97
PCO_2 (mmHg)	43	33	41	37	29	31
pH (arterial)	7.48	7.47	7.49	7.45	7.49	7.52
WBC/mm^3	12,850	5,470	27,176	13,970	24,950	23,680
Polymorphonuclear leucocytes (%)	65	30	70	70	74	75

* Values represent group mean.
PS_1 fraction containing 6.3% lipid.
PS_2 fraction containing 0.5% lipid.

Table 13

Figs. 4-7

Effects of whole endotoxin (LPS) or characterized polysaccharide fractions (PS) on absolute mean blood pressure, relative peripheral resistance and plasma bradykinin levels.

4. LPS 10 mg/kg (pg. 68e)
5. LPS 2.5 mg/kg (pg. 68f)
6. PS_1 (6.3% lipid) 2.5 mg/kg (pg. 68g)
7. PS_2 (0.5% lipid) 2.5 mg/kg (pg. 68h)

o———o resistance, % of baseline

●———● mean BP, mmHg

□- - - -□ plasma bradykinin, ng/ml

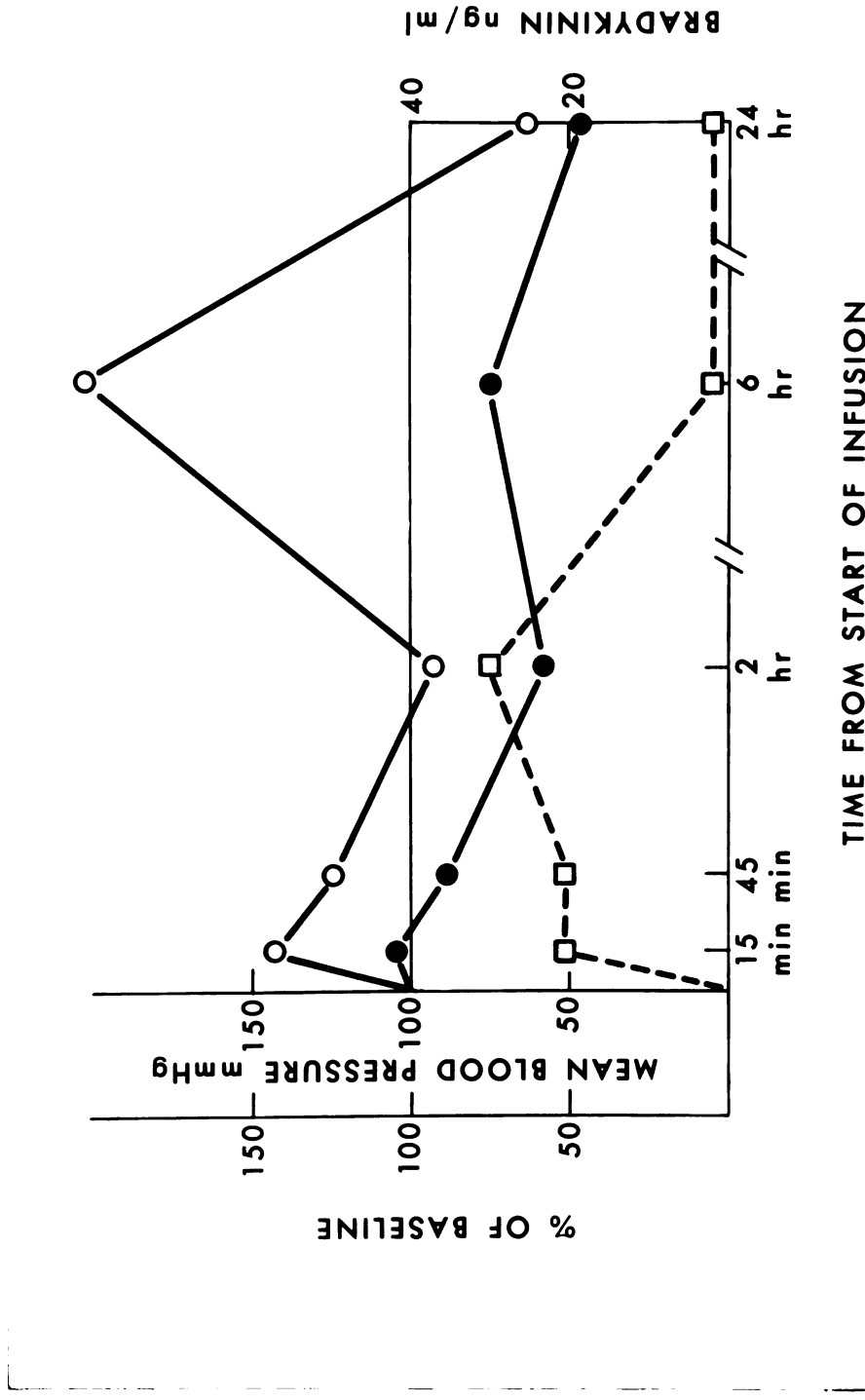

% OF BASELINE
150
100
50
MEAN BLOOD PRESSURE mmHg
150
100
50
BRADYKININ ng/ml
40
20
15 45
min min
2
hr
6
hr
24
hr
TIME FROM START OF INFUSION

Fig. 4

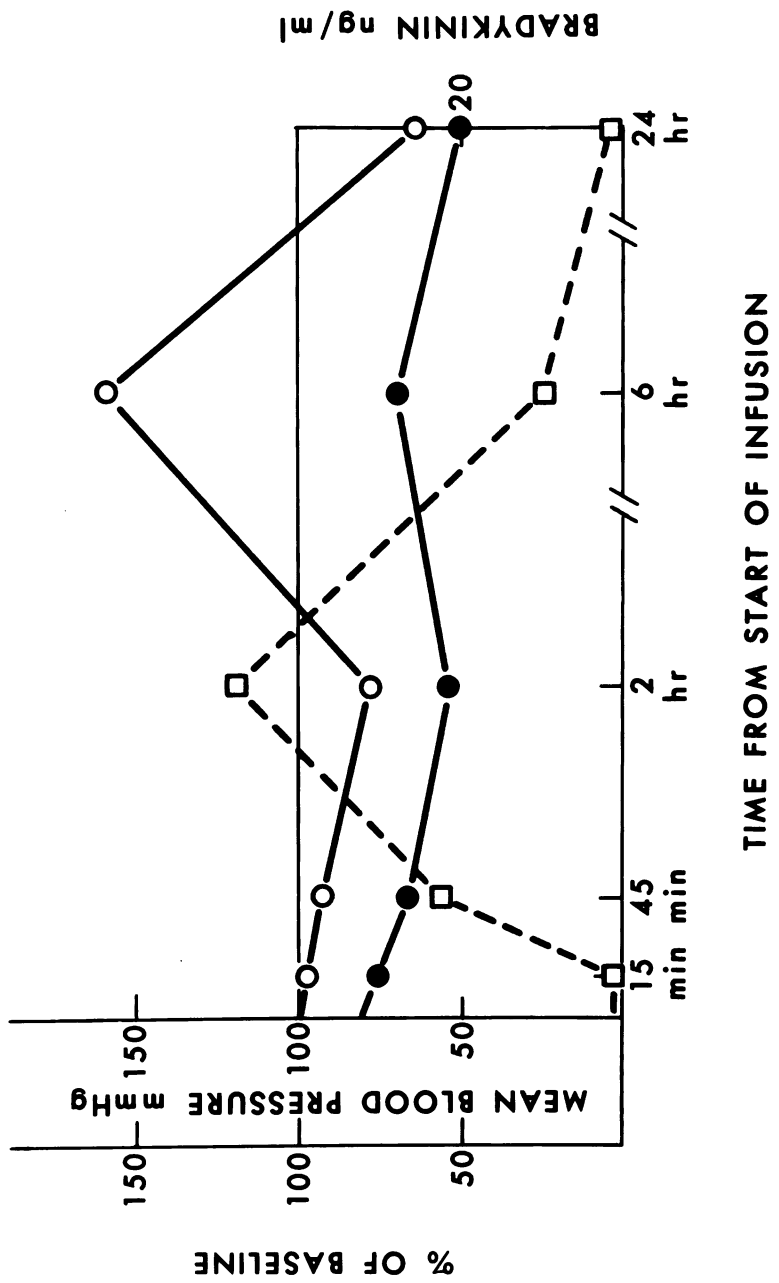

BRADYKININ ng/ml
20
24 hr
6 hr
2 hr
15 45
min min
TIME FROM START OF INFUSION
MEAN BLOOD PRESSURE mmHg
150
100
50
% OF BASELINE
150
100
50

Fig. 5

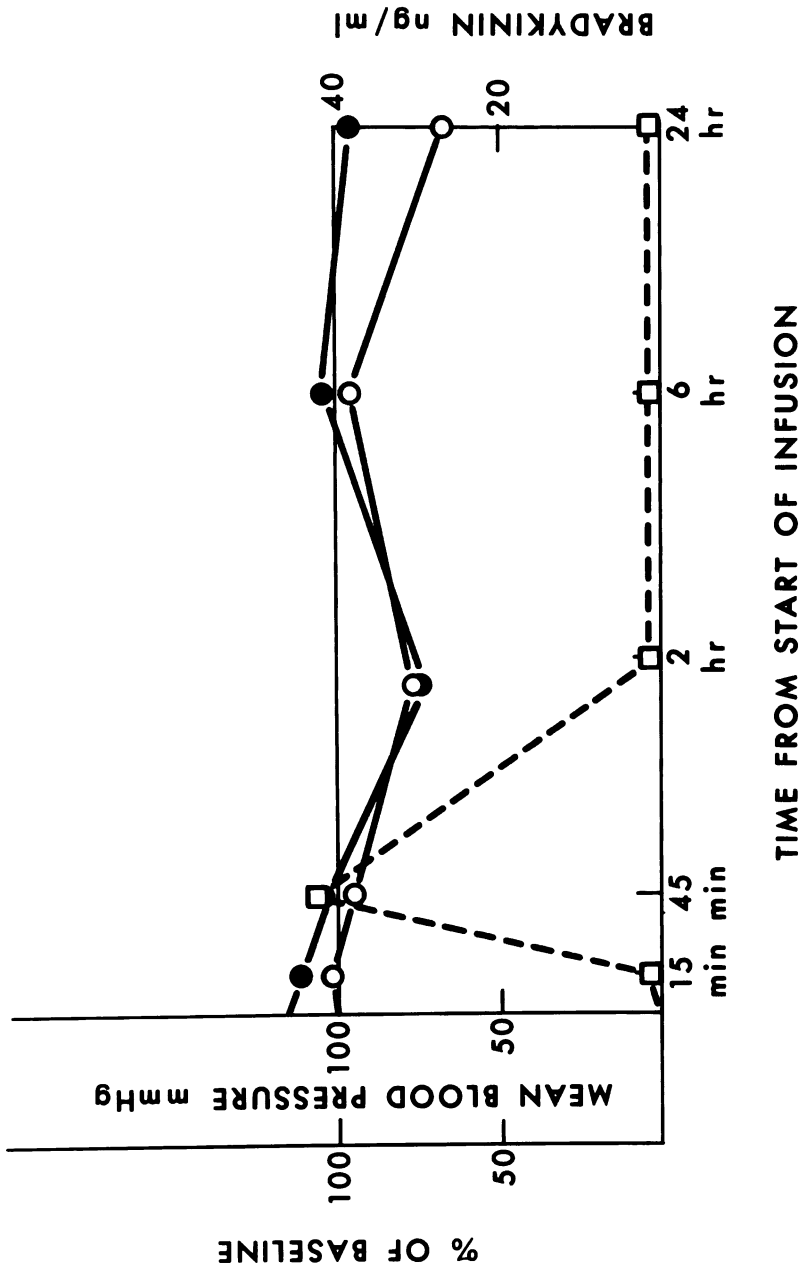

% OF BASELINE
100
50
MEAN BLOOD PRESSURE mmHg
100
50
BRADYKININ ng/ml
40
20
15 min
45 min
2 hr
6 hr
24 hr
TIME FROM START OF INFUSION

Fig. 6

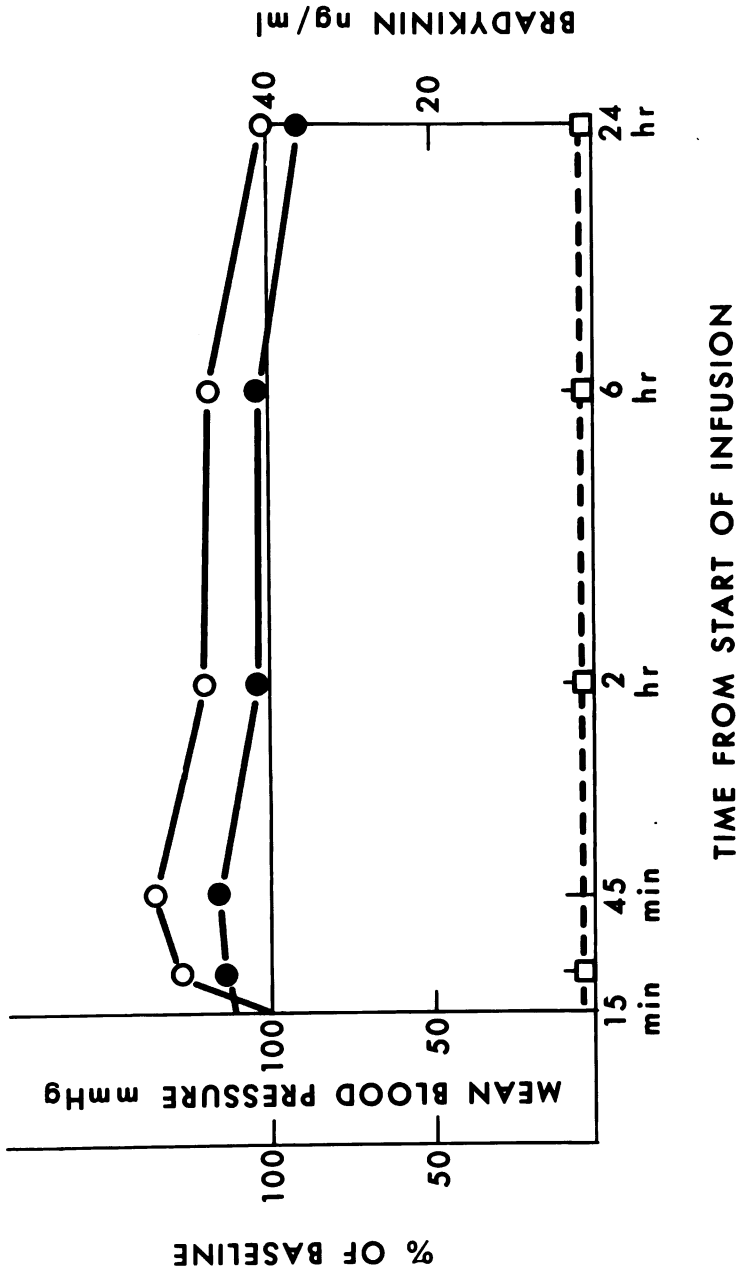

% OF BASELINE
100
50
MEAN BLOOD PRESSURE mmHg
100
50
BRADYKININ ng/ml
40
20
15 min
45 min
2 hr
6 hr
24 hr
TIME FROM START OF INFUSION

Fig. 7

Six hours after the beginning of the infusion, both LPS-10 and LPS-2.5 groups demonstrated marked vasoconstriction. Total peripheral resistance had increased 113% and 58% respectively. Arterial hypotension persisted in both of these groups.

Animals given PS_1 (6.3% lipid) (Fig. 6) developed progressive vasodilation with PVR decreased 22%; blood pressure had fallen 40 mmHg at 2 hours. In these animals, both peripheral resistance and mean pressures returned to baseline by 6 hours. Conversely, animals given PS_2 (0.5% lipid) (Fig. 7) had an early 35% rise of PVR. Blood pressure rose slightly and cardiac output decreased approximately 25%. By 2 hours these animals had reached a plateau; both pressure and resistance then remained constant for the remainder of the observation period. A similar pattern of resistance and pressure changes have been noted previously in animals tilted and subjected to microsphere infusions only (Nies et al, 1968; Hoffbrand and Forsyth, 1969); this response is considered to be primarily a nonspecific effect of the experimental design. PS_2 may actually have increased these nonspecific effects on cardiac output and peripheral resistance but the group size is too small to allow this conclusion.

b. Regional Hemodynamic Effects of Endotoxin Fractions: Regional hemodynamic changes 2 hours and 6 hours after infusion of LPS and fractions PS_1 and PS_2 are outlined in Tables 14 - 17 . The biphasic patterns of change caused by both

Table 14

RELATIVE FRACTION OF CARDIAC OUTPUT DELIVERED TO REGIONAL VASCULAR BEDS AFTER EXPOSURE TO WHOLE ENDOTOXIN

Organ	LPS-10@ Baseline*	N 3 2 hr#	6 hr#	LPS-2.5@ Baseline*	N 2 2 hr#	6 hr#
Heart	5.5	141	120	4.9	197	126
Brain	4.2	97	165	5.1	119	113
Kidney	14.8	90	40	15.5	113	89
Liver (total)	22.3	123	188	17.1	132	145
Hepatic artery	9.1	175	401	4.5	83	232
Portal vein	13.3	108	103	12.6	117	108
GI organs	7.1	143	155	7.8	160	139
Spleen	2.3	41	23	2.4	9	57
Pancreas	1.3	88	68	1.7	85	99
Skin	7.1	71	57	4.5	66	89
Skel. musc.	22.9	78	83	29.6	65	70
Bone	2.8	130	115	4.7	58	74
Adrenal	0.3	163	332	0.2	277	123

* Baseline values expressed as group mean percentage of actual cardiac output (ml/min/kg).
\# Expressed as % of baseline -- values represent group means.
@ LPS-10 (10mg/kg whole endotoxin), LPS-2.5 (2.5mg/kg whole endotoxin).

Table 15

RELATIVE FRACTION OF CARDIAC OUTPUT DELIVERED TO REGIONAL VASCULAR BEDS AFTER EXPOSURE TO CHARACTERIZED FRACTIONS OF ENDOTOXIN

Organ	PS_1@ N 2 Baseline*	2 hr#	6 hr#	PS_2@ N 2 Baseline*	2 hr#	6 hr#
Heart	5.7	187	132	5.6	131	119
Brain	5.8	88	100	6.0	102	112
Kidney	14.2	93	96	16.9	115	100
Liver (total)	19.4	149	130	19.2	100	103
Hepatic artery	5.8	218	212	5.7	113	115
Portal vein	13.5	114	92	13.3	99	101
GI organs	8.5	139	103	7.9	92	110
Spleen	1.9	50	35	2.0	98	131
Pancreas	1.8	92	91	2.3	99	79
Skin	5.0	98	86	5.4	123	93
Skel musc.	29.7	63	85	23.7	78	111
Bone	4.3	96	63	5.4	94	59
Adrenal	0.2	264	214	0.2	157	110

* Baseline values expressed as group mean percentage of actual cardiac output (ml/min/kg).
\# Expressed as % of baseline -- values represent group means.
@ PS_1 (6.3% lipid), PS_2 (0.5% lipid).

Table 16

REGIONAL VASCULAR RESISTANCE AFTER EXPOSURE TO WHOLE ENDOTOXIN

Organ	LPS-10[@] N 3 Baseline*	2 hr[#]	6 hr[#]	LPS-2.5[@] N 2 Baseline*	2 hr[#]	6 hr[#]
Heart	2	65	186	1	39	129
Brain	10	99	136	8	71	140
Kidney	1	108	623	1	71	181
Liver (total)	4	78	121	4	58	109
Hepatic artery	12	54	66	16	42	68
Portal vein	11	92	252	7	66	140
GI organs	11	68	174	9	48	114
Spleen	3	243	999	2	885	317
Pancreas	4	106	334	2	90	169
Skin	42	163	434	35	117	178
Skel. musc.	55	130	269	28	142	229
Bone	117	75	213	57	132	223
Adrenal	2	58	72	2	29	129

* Baseline units mmHg (ml/min)-1 per gram of wet tissue (means only).
\# Expressed as percent of baseline value.
@ LPS-10 (10 mg/kg whole endotoxin), LPS-2.5 (2.5 mg/kg whole endotoxin).

Table 17

REGIONAL VASCULAR RESISTANCE AFTER EXPOSURE TO CHARACTERIZED FRACTIONS OF ENDOTOXIN *

Organ	PS_1@ N 2 Baseline*	2 hr#	6 hr#	PS_2@ N 2 Baseline*	2 hr#	6 hr#
Heart	3	43	73	2	97	105
Brain	12	88	99	8	123	111
Kidney	1	79	111	1	110	125
Liver (total)	6	56	75	4	126	119
Hepatic artery	21	38	46	14	114	111
Portal vein	12	70	105	8	129	121
GI organs	13	59	94	11	140	111
Spleen	4	157	284	2	134	93
Pancreas	3	84	108	2	130	154
Skin	73	80	113	35	102	125
Skel. musc.	46	170	116	37	166	110
Bone	105	88	160	53	135	209
Adrenal	6	55	48	3	81	111

* Baseline units mmHg $(ml/min)^{-1}$ per gram of wet tissue (means only).
# Expressed as percent of baseline value.
@ PS_1 (6.3% lipid), PS_2 (0.5% lipid).

doses of whole lipopolysaccharide and by fraction PS_1 are again evident. Similarly, the degree of change appears to be related to the total dose of lipid. A well marked difference is evident between the patterns of regional blood flow resulting from the infusion of lipid-containing endotoxin preparations and the lipid free PS_2 fraction.

At 2 hours, in animals receiving 10 mg/kg whole LPS, an increased fraction of the cardiac output was delivered to hepatic and coronary arteries, gastrointestinal organs, and adrenal glands at the expense of spleen, skin and muscle (Table 14). Brain and kidney did not receive increased fractions of output and therefore suffered reduced flow, since total cardiac output had fallen. Flow was reduced in spleen, pancreas, skin and muscle. Resistance (Table 16) was decreased or only minimally elevated in most tissues with the exception of spleen and skin. Similar changes were present in the LPS-2.5 and the PS_1 fraction groups. In contrast, the animals receiving the PS_2 fraction showed minimal redistribution of cardiac ouput, primarily the diversion of flow from muscle; resistance was elevated in all organs except skin, heart and adrenal gland (Tables 15, 17).

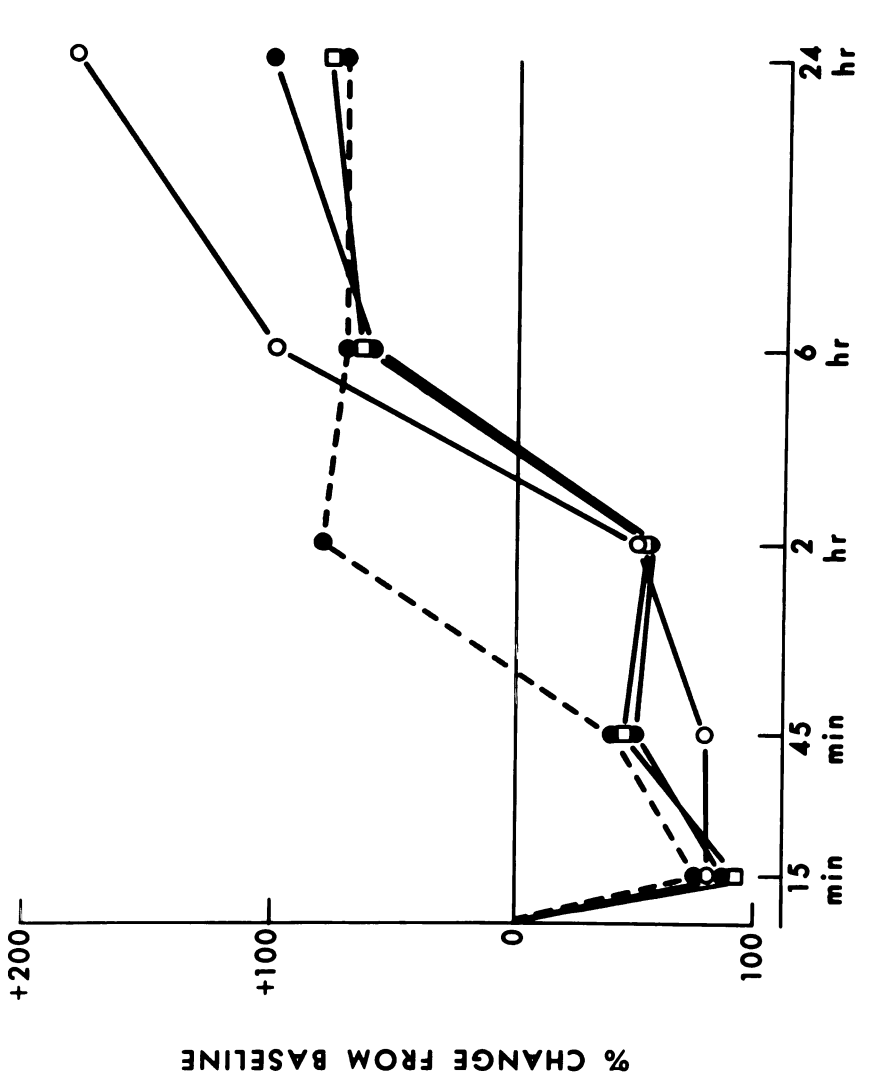
The late phase (6 hour) patterns were different (Tables 14 - 17). The animals exposed to both doses of whole LPS had increased vascular resistance in all of their organs except the hepatic artery. Blood flow was reduced except in the hepatic artery, and cardiac output was diverted to the heart, brain, hepatic artery, gastrointestinal organs and

adrenal, at the expense of kidney, spleen, skin and muscle. Animals receiving PS_1 had reverted to the baseline pattern except that vascular resistance elevation persisted in the vasculature of spleen and bone; blood flow was decreased to these same tissues. The animals which had received fraction PS_2 had further increases in vascular resistance in the pancreas and bone, but resistance had decreased in the spleen.

c. Metabolic Effects of Endotoxin Fractions: Animals in all four groups tended to hyperventilate as indicated by the decreases of $PaCO_2$ (Tables 12-13). Alkalosis developed only in those animals given PS_2. The constancy of pH in the animals receiving lipid containing preparations suggests the simultaneous development of metabolic acidosis. Transient leucopenia occurred in all animals (Fig. 8) but persisted , without recovery at 2 hours, only in animals given preparations containing more lipid than contained in fraction PS_2. The leucopenia was due primarily to disappearance of granulocytes (Tables 12-13).

d. Bradykinin Generation: Animals receiving LPS-10, LPS-2.5 and PS_1 developed high concentrations of bradykinin in arterial blood. Bradykinin generation did not occur in animals infused with fraction PS_2 (Tables 16-19, Fig. 9). The highest levels of bradykinin generally occurred at the same time as the nadir of peripheral resistance (Figs. 4-7). Bradykinin generation occurred only in those groups of animals exhibiting prolonged granulocytopenia (Table 19).

Fig. 8



Effect of LPS or PS fractions on leucocyte count

LPS - 10 o——o LPS - 2.5 ●——● PS_1 □——□ PS_2 ●- - -●

Table 18

ARTERIAL PLASMA BRADYKININ CONCENTRATIONS IN PRIMATES EXPOSED TO CHARACTERIZED ENDOTOXIN FRACTIONS

Time of observation	Lipid		
	Present (LPS, PS_1)*	Absent (PS_2)	
0-2 hr (grouped)	16 ± 3.6ng/ml (21)	0 (2)	P<0.001
15 min	12 ± 6ng/ml (7)	0 (2)	P<0.05
1 hr	18 ± 6ng/ml (7)	0 (2)	P<0.01
2 hr	14 ± 6ng/ml (7)	0 (2)	P<0.02

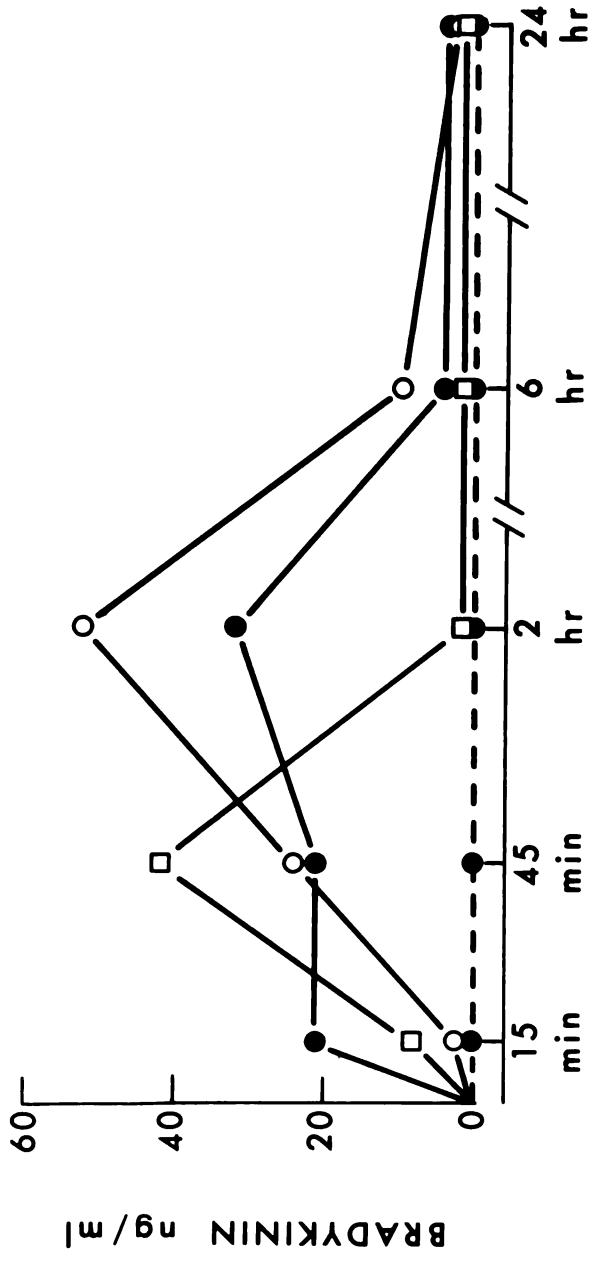
* Animals receiving lipid containing Endotoxin fractions grouped together

RELATIONSHIPS BETWEEN TOTAL DOSE OF LIPID AND RESPONSES OF PERIPHERAL VASCULAR RESISTANCE (PVR), ARTERIAL PLASMA BRADYKININ CONCENTRATIONS AND CHANGE IN TOTAL LEUCOCYTE COUNT

Fraction infused	Lipid dose	2 HR			6 HR	
		PVR	Kinin	WBC	PVR	WBC
LPS - 10	5mg/kg	-7%	32ng/ml	-61%	113%	63%
LPS - 2.5	1.25mg/kg	-22%	52ng/ml	-53%	58%	100%
PS_1	0.075mg/kg	-22%	42ng/ml	-57%	0%	111%
PS_2	0.006mg/kg	-35%	0	79%	15%	69%

Table 19

Fig. 9



Effect of LPS or PS fractions on arterial plasma bradykinin concentration

LPS 10 o——o LPS 2.5 ●——● PS_1 □——□ PS_2●- - -●

4. Discussion:

The results of these experiments demonstrate that there is an association between bradykinin generation in vivo in the primate, and the lipid content of infused fractions of endotoxin. This supports the hypothesis that generation of significant amounts of bradykinin is the result of the interaction of lipid containing endotoxin with leucocytes capable of initiating kinin generation. The present experiments do not demonstrate a dose relationship between kinin generation and the total lipid content of the infused endotoxin. The PS_1 fraction (6.3% original lipid) contained enough of the lipid material to be able to maximally activate kinin generation mechanisms, whereas the PS_2 fraction did not. Fractions of endotoxin containing intermediate amounts of lipid would be required to test for dose-response, but are not available.

The possibility that molecular size was a determinant of kinin generation cannot be excluded. During the preparation of the fractions used, they were screened for molecular weight by chromatography on a molecular sieve gel (Greineder, 1970). Both fractions PS_1 and PS_2 were excluded by the gel, indicating molecular weights greater than 100,000. However, the fractions were not further characterized, and it is possible that the PS_1 fraction was significantly larger than the PS_2.

An alternative consideration is more consistent with recent proposals that the toxicity of an endotoxin molecule

is a function of a small central component containing only a minimal amount of lipid material (Galanos et al, 1971; Luderitz et al, 1971; Westphal, 1972). Fraction PS_1 consists of molecules each retaining the small amount of lipid necessary to fully activate the kinin generating system. Conversely, there are insufficient lipid containing molecules to cause measurable activation of kinin generating mechanisms by fraction PS_2.

Similar considerations can be used in attempting to understand the leucopenic responses which occurred during these experiments. Leucopenia occurred in all experimental groups (Fig. 8); in the animals receiving lipid free PS_2, it was of short duration and was unassociated with any measurable kinin generation. However, in the three groups given material with larger amounts of lipid, leucopenia was prolonged for at least two hours, and was closely correlated with marked kinin generation, which also peaked at 2 hours.

Transient leucopenia occurs in all species exposed to endotoxin and may be nonspecific. White cells are known to ingest both endotoxin (Cline et al, 1968; Nies et al, 1971) and immune complexes (Cochrane, Weigele and Dixon, 1969). The polysaccharide portions of endotoxin are antigenic and can interact with plasma immune mechanisms (Work, 1970; Nies and Melmon, 1971; Nies et al, 1971). Therefore, it is probable that as the endotoxin fractions were infused, phagocytoses occurred. Immune complexes can cause some of

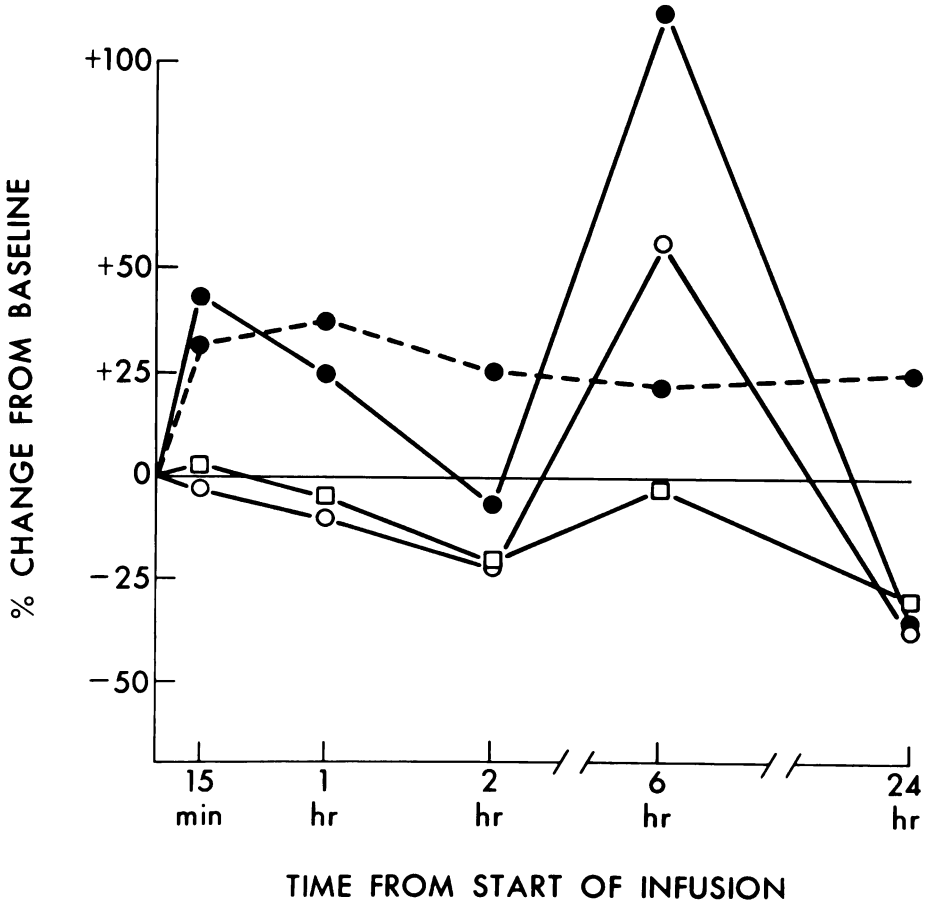
the effects on leucocytes that endotoxin is known to cause (Movat et al, 1964). However, in the present experiment, prolonged granulocytopenia occurred only in animals receiving lipid-containing endotoxin fractions. An activation period has been demonstrated in the endotoxin-granulocyte interaction resulting in pyrogen production (Nordlund, Root and Wolff, 1970). This interaction represents a function of endotoxin cellular toxicity. Prolonged granulocytopenia resulting from lipid containing endotoxin probably represents another, similar function, of cellular toxicity. Kinin generation probably does also.

Persistent granulocytopenia, release of active materials into the circulation, and development of metabolic acidosis, are clearly related to exposure to lipid containing endotoxin fractions. PS_2, although essentially lipid free, is not completely devoid of effects on the cardiovascular responses of the whole animal or on leucocytes, but, the very different pattern of cardiovascular events, the transient leucopenia, lack of kinin generation and metabolic acidosis only serve to underscore the importance of a mimimum lipid content of the molecule to these toxic effects. Extensive analysis of these fractions (Greineder, 1970) has shown that the only chemical difference between PS_1 and PS_2 is in the content of lipid material. It appears that the endotoxin molecule, retaining a certain minimum fatty acid content, can interact with leucocytes to cause both cellular dysfunction and release of bradykinin.

The changes in blood pressure occurring during early endotoxemia also can be correlated with bradykinin levels generated. The correlation coefficient $r = -0.338$, $P < 0.05$ ($N = 35$). The equation for the regression line is $y = 92.9 - 0.561X$. It is not possible to similarly correlate vascular resistance changes because of the acute rise in peripheral resistance occurring in the animals infused with LPS-10. This rise in resistance may have been caused by the particulate nature of the suspension of endotoxin infused at this high dose, indicated by nonspecific light absorption (Pg.66). Similar acute increases in resistance have been reported to occur in primates by Cavanaugh et al (1970). But these workers injected highly concentrated endotoxin as a bolus and caused increased vascular resistance in their primate preparations. These acute resistance changes were probably nonspecific reactions to the injection of particulates.

The patterns of peripheral resistance developing after endotoxin fraction infusion are illustrated in Figure 10. Animals generating bradykinin all had diminished resistance at 2 hours: (Table 19). These early phase resistance changes were independent of total dose of endotoxin, or of lipid. The lack of endotoxin-dose relationship, and the association of vasodilatation with generation of bradykinin, suggest that this early phase cardiovascular response is mediated via the release of the vasoactive peptide. It confirms the observations of Nies et al (1968). The patterns

Fig. 10



Effects of LPS or PS fractions on peripheral vascular resistance

LPS 10 — LPS 2.5 — PS_1 — PS_2

of regional distribution of cardiac output are also similar to those previously described in early endotoxemia in the primate (Wyler et al, 1969).

The data indicate that there is a relationship between the dose of endotoxin and the degree of change in regional and systemic hemodynamics during the later (6 hour) phases of endotoxemia. The severity of rise of vascular resistance is related to the total amount of lipid infused (Table 19). Conversely, there is no apparent relationship of these later phase changes to the early phase vascular effects of endotoxemia. Endotoxin exerts direct cellular toxicity. This direct toxicity would appear to be of greater importance in the development of late septic shock than are the cardiovascular events of early endotoxemia.

B. Is Bradykinin a sufficient mediator for the cardiovascular events of early endotoxemia in the primate?

1. Rationale:

Bradykinin is closely associated with endotoxemia in the primate. Endotoxin can activate several kinin generating mechanisms in both plasma and leucocytes, as discussed in the introductory review. Bradykinin is present in the plasma of humans and subhuman primates following exposure to endotoxin (Nies et al, 1968; Pettinger and Young, 1969) (Table 18). Bradykinin, a potent vasodilator, could cause a decrease of peripheral vascular resistance when generated during endotoxemia and could, thereby, reproduce the characteristic cardiovascular effects occurring during early sepsis. And, as indicated by the results of the prior experiments, as well as those of Nies et al (1968) and Kimball, Melmon and Wolff (1972), the time course of vasodilatation and kinin generation, during endotoxemia, are closely related. Bradykinin has been proposed as the mediator, in the primate, of the effects of endotoxemia (Nies et al, 1968). To test this possibility, bradykinin was infused into unanesthetized primates and the systemic and regional hemodynamic effects of the peptide were measured and compared with the known effects of endotoxin. Kinin infusions were also made during sustained autonomic ganglionic blockade. The role of autonomic reflex mechanisms in the systemic vascular response to bradykinin were evaluated.

2. Methods:

Rhesus monkeys (Macaca Mulatta), of either sex, weighing 4.4 to 7.8 kg, were used. The animals were prepared and maintained as described in the prior study. Measurements of systemic blood pressure, heart rate, cardiac output, regional distribution of cardiac output, and arterial blood gas tensions, pH, bradykinin concentrations, hematocrit and leucocyte counts were all performed by the methods previously described (see pages of methods from first study).

Bradykinin triacetate trihydrate (Cal Biochem Co.) MW 1294.41 was diluted to 1 mg/ml in 0.9% NaCl. 2 ml aliquots were frozen and new aliquots thawed for each day's experiment. Prior to infusion, the bradykinin was further diluted in 0.9% NaCl. Infusions were made intravenously using a Harvard constant speed infusion pump (Harvard Co. Model #930). The bradykinin was diluted, stored and infused using sterile plastic labware and syringes to avoid losses by adsorbtion to glass surfaces.

3. Results:

a. Dose Response Studies:

i) Bradykinin infusion: Freshly thawed bradykinin was diluted and then infused intravenously in doses ranging between four and 37 μg/kg/min in six animals, while continuous records of blood pressure and heart rate were obtained. Infusions lasted three minutes, with ten minutes between each infusion. A total of 64 such infusions were performed, and each animal received at least four. The order of doses

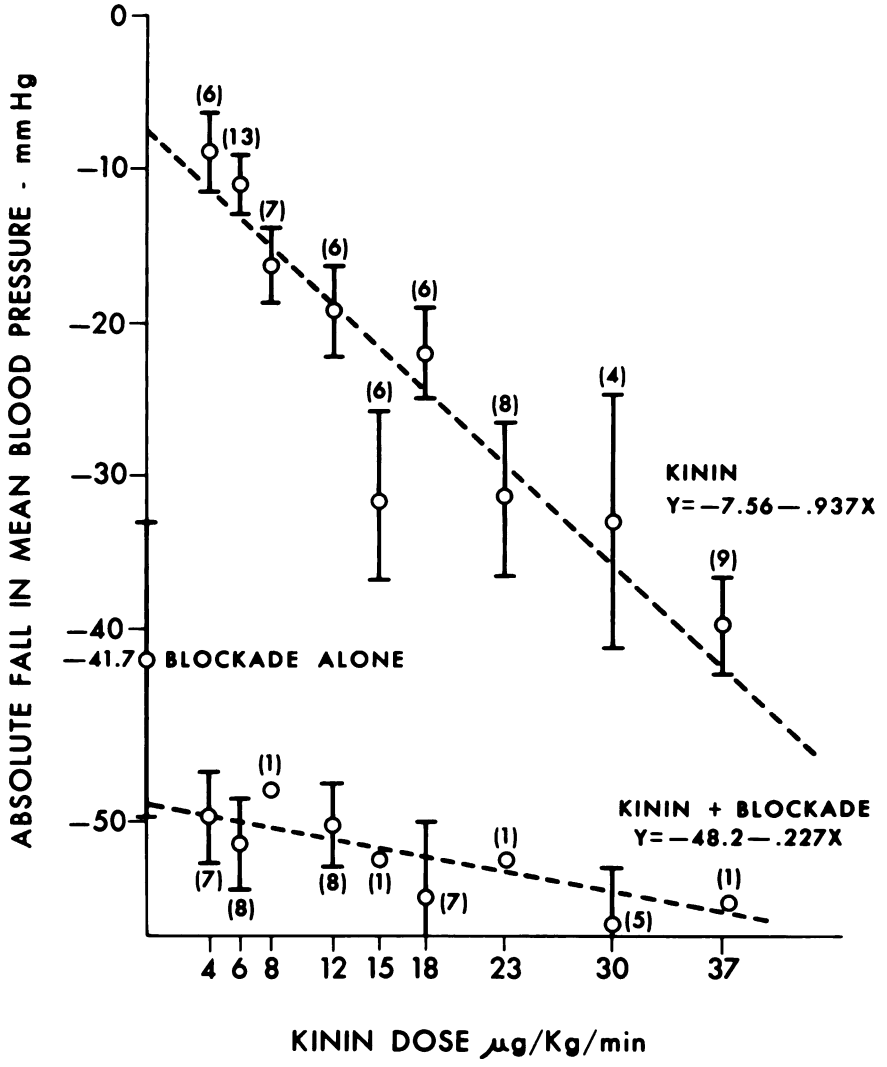
was randomized. Animals received a maximum of three mg of bradykinin in any single day.

In Figure 11 is illustrated the dose-response relationship between bradykinin and mean arterial blood pressure. Blood pressure fell sharply approximately 30 seconds after the start of each bradykinin infusion. Pressure then stabilized and the graphed values were obtained during this stable period. In the range of doses examined, a significant Pearson Product-moment correlation (Colquhoun, 1971) was found ($r=-0.711$, $p<0.001$) between dose of kinin and fall of pressure. The formula for the regression line was $Y=-7.56-0.937X$.

Each monkey received multiple infusions of bradykinin during the determination of the dose-response relationship. As the total amount of bradykinin infused into each animal increased, significant changes occurred in all hemodynamic measurements. The earliest changes were noted in heart rate and total peripheral vascular resistance, both of which rose rapidly. Cardiac output fell simultaneously with these changes, the hematocrit increased more slowly. The baseline value of blood pressure remained stable until more than two mg of kinin had been infused, when it too began to fall. These cumulative effects of multiple doses of bradykinin are illustrated in Figure 12. Metabolic measurements were not made during these studies. None of the animals died.

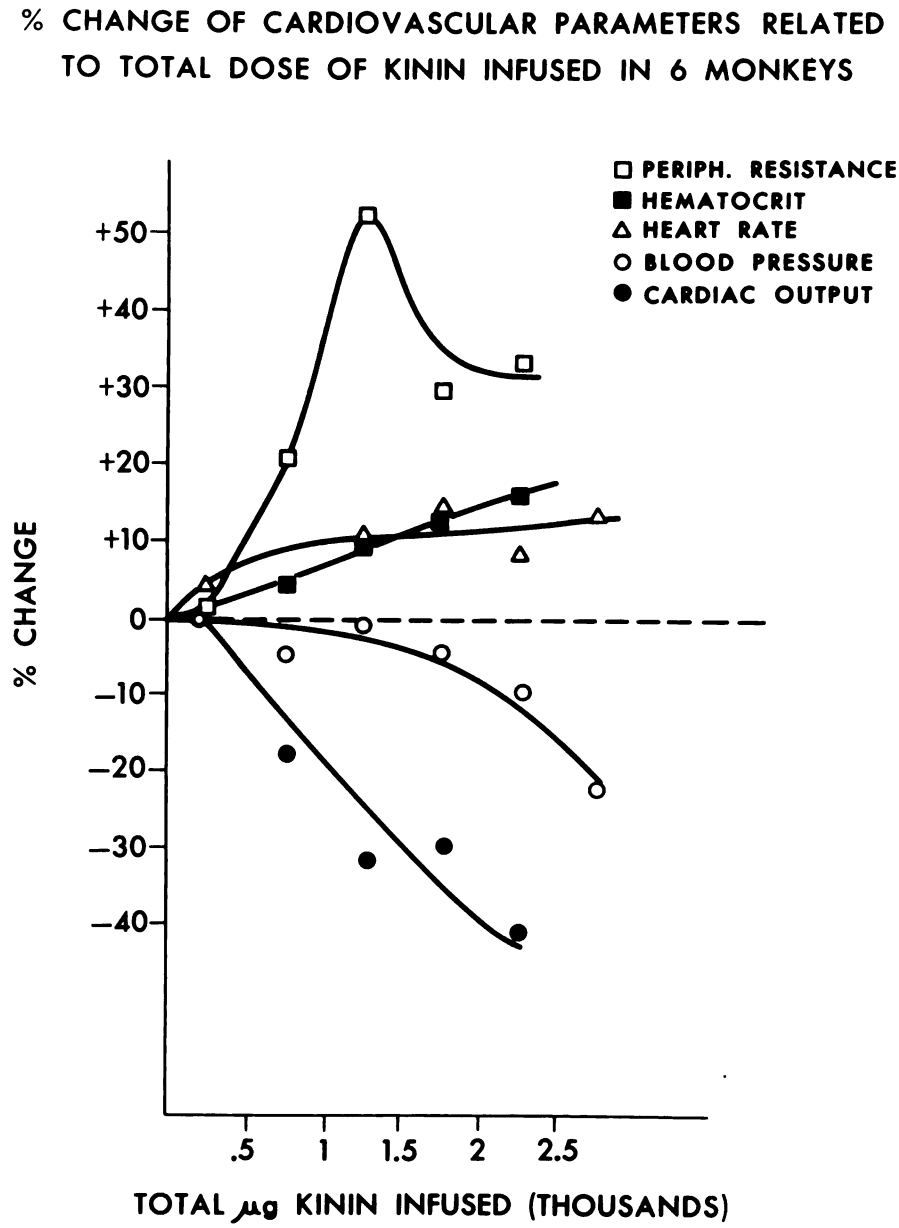
Because of the significant changes in the cardiovascular status of the animals, as the total dose of bradykinin accumu-

Fig. 11



Effects of bradykinin on mean arterial blood pressure. Upper line represents bradykinin alone. Lower line represents bradykinin after ganglionic blockade.

Fig. 12



Effects of cumulative doses of bradykinin on cardiovascular parameters

lated, the effects of identical single doses of kinin, given before and after the midpoint of the total dose (1200μg), were compared. There were no significant differences in effect (Table 20). Therefore, all dose-responses were grouped and analyzed without regard to the time of their administration.

ii) Bradykinin infusion during sustained autonomic blockade: In five tilted animals, the bradykinin dose-response relationship also was determined during sustained autonomic ganglionic blockade. Autonomic ganglionic blockade was established by intravenous administration of trimethaphan camsylate, one mg/ml, at a rate adjusted so that the mean arterial pressure would fall approximately 40 mmHg without associated increase in heart rate. The infusion was maintained at this constant rate and bradykinin was administered intravenously through the other arm of a 'Y' tube. A total of 39 bradykinin infusions were performed at doses of four to 30μg/kg/min. Each animal received at least five randomly ordered doses of kinin.

Continuous recording of blood pressure and heart rate were obtained during these dose response infusions. All other measurements also were made in the manner previously described. Plasma levels of bradykinin were not measured during ganglionic blockade.

As illustrated in Figure 11, ganglionic blockade caused the mean blood pressure to fall 42 $\pm$ 8.6 mmHg ($P<0.001$).

Table 20

INFLUENCE OF MULTIPLE DOSES OF BRADYKININ ON THE FALL OF MEAN ARTERIAL BLOOD PRESSURE CAUSED BY SINGLE DOSES OF BRADYKININ

Cumulative dose	Individual Dose Infused $\mu g/kg\ min^{-1}$ 4	6	8	15	18	23	37	
1200 ug	11.0 ±2.9	11.8 ±2.2	13.9 ±4.3	15.8 ±2.7	17.5 ±4.8	40.5 ±7.4	42.7 ±5.7	Fall of mean arterial blood pressure (mm Hg) (Mean ± SEM)
1200 ug	5.0 ±2.6	9.5 ±2.0	15.6 ±4.5	23.7 ±1.9	25.5 ±2.1	29.7 ±4.9	37.5 ±4.7	
t *	1.53	0.74	0.28	1.48	1.59	1.20	0.72	
n **	6	15	11	9	6	11	13	

* Unpaired t test

** Observations were made in six different animals. n represents the total number of infusions performed at each dose, and was used to determine the pooled variance.

Infusion of bradykinin during sustained ganglionic blockade produced a further fall of $\bar{P}a$. The fall of blood pressure was again directly related to the kinin dose but the slope is much less steep than in animals not subjected to autonomic blockade.

$$Y = -48.2 - 0.227x$$

$$r = -0.294 \ (P<0.05)$$

b. Measurement of Arterial Plasma Bradykinin Concentrations during Intravenous Bradykinin Infusion: Arterial plasma concentrations of bradykinin were determined during intravenous infusion of bradykinin at two different dose levels. This was done to establish that plasma levels achieved during infusion at these doses were comparable to levels measured during spontaneous kinin generation in endotoxemia, and that plasma kinin levels remained constant during a 10 minute observation period. Kinin concentrations were determined before infusion, and again after three and 10 minutes of kinin infusion. The infusion was stopped and repeat determinations of bradykinin blood levels were made at 30 minutes and 60 minutes. Plasma kinin levels were measured six times in four monkeys at infusion rates of 15-18 μg/kg/min, and twice in two monkeys at 9-10 μg/kg/min. The results of these infusions are listed in Table 21 .

c. Systemic Hemodynamic Effects of Bradykinin Infusion: Bradykinin was infused intravenously for ten minutes at doses of 15-18 μg/kg/min, 11 times in seven monkeys, to

Table 21

ARTERIAL PLASMA BRADYKININ CONCENTRATIONS (ng/ml ± SEM)

DURING INTRAVENOUS BRADYKININ INFUSION

Time	Infused Dose 15-18 μg/kg min^{-1}*	9 μg/kg min^{-1}
0 min	0.5 ± 1.0	0, 2 #
3 min	14.0 ± 3.5	5, 6
10 min (End Infusion)	14.5 ± 3.0	8, 6
30 min	1.0 ± 1.5	1, 1
60 min	0.5 ± 1.0	

* 5 observations in 3 monkeys

\# 2 observations in 2 monkeys

characterize, in this species, the systemic hemodynamic effects caused by the peptide. The four animals receiving more than one such infusion were rested for at least three days between experimental studies, although all hemodynamic measurements had returned to baseline within six hours of the completion of the first infusion. In each animal, a single value for blood pressure or heart rate represents the average of at least five observations under stable conditions. Blood pressures and heart rate were measured continuously, and cardiac output determined three and ten minutes after the start of the bradykinin infusion.

Table 22 illustrates the effects of bradykinin infusion at a dose of 15-18 μg/kg/min on blood pressure, heart rate, cardiac output and total peripheral resistance after three and ten minutes of infusion. Both pressure and resistance were significantly decreased by three minutes; cardiac output had fallen significantly and resistance had returned to baseline. Blood pressures remained significantly decreased.

d. Effects of Bradykinin Infusion on Systemic and Regional Hemodynamics before and after Ganglionic Blockade: The hemodynamic effects of kinin infusion were studied in 10 different monkeys before and after the establishment of autonomic ganglionic blockade. Following an initial baseline period (I), kinin was infused at 15-18 μg/kg/min for ten minutes and measurements made (II). The animals were then rested for six or more hours and repeat baseline (III) measurements obtained.

Table 22

SYSTEMIC HEMODYNAMIC CHANGES AFTER THREE AND TEN MINUTES OF INTRAVENOUS INFUSION OF BRADYKININ (15-18 $\mu g/kg\ min^{-1}$)

Measurement	Base line	Bradykinin Infusion 3 Min	Bradykinin Infusion 10 Min
Mean arterial pressure (mm Hg)	102 ± 6	76 ± 14*	74 ± 13*
Heart rate (beats/min)	176 ± 26	199 ± 22*	186 ± 29*
Cardiac output (L/min)	1.99 ± 0.73	2.03 ± 0.73	1.46 ± 0.43*
Total peripheral resistance (mm Hg/L min^{-1})	54 ± 15	40 ± 10*	53 ± 10

Values are means ± SD of 11 observations in seven monkeys.

* P 0.01, paired 't' test, infusion period compared to base line.

Ganglionic blockade was then established with trimethaphan and measurements were repeated (IV). Finally, kinin was infused as before, with blockade maintained by constant trimethaphan infusion, and a final set of measurements was obtained (V).

The systemic hemodynamic measurements obtained during these experiments are presented in Table 23. Effects of ten minute infusions of bradykinin, at 15-18 μg/kg/min, on cardiovascular variables in this group of animals are the same as those previously obtained in other animals (Table 18). Repeat baseline values, obtained after the initial infusion (III) did not differ significantly from initial values (I). Blood pressure, cardiac output and total peripheral resistance all fell after autonomic blockade (IV). Bradykinin infusion during maintained ganglionic blockade resulted in further fall in all these measurements (V).

The hematocrit increased slightly during kinin infusion and fell significantly during combined infusion. Arterial blood gases remained stable throughout the study (pH 7.45 ±0.02, PaO_2 106 ±4.3, $PaCO_2$ 35.1 ± 1.4).

The effects of bradykinin alone (II), autonomic blockade alone (IV), and the combined effects (V) on distribution of cardiac output, blood flow and resistance in those organs and systems showing significant changes are presented in Table 24-26 and Figures 13-14. These changes in flow and resistance were compared to changes previously determined in seven normal

Table 23

SYSTEMIC EFFECTS OF BRADYKININ INFUSION (15-18 μg/kg min^{-1}) FOR TEN MINUTES IN TEN MONKEYS BEFORE AND AFTER GANGLIONIC BLOCKADE

	First base line	Bradykinin infusion	Second base line	Ganglionic blockade	Bradykinin ganglionic blockade
Systolic arterial pressure (mm Hg)	128 ± 13	102 ± 12*	131 ± 13	90 ± 15*	76 ± 12*
Mean arterial pressure (mm Hg)#	97 ± 14	75 ± 11*	99 ± 13	66 ± 15*	54 ± 9*
Diastolic arterial pressure (mm Hg)	68 ± 13	51 ± 11*	71 ± 11	44 ± 15*	35 ± 9*
Heart rate (beats/min)	184 ± 22	196 ± 25	185 ± 32	176 ± 22	175 ± 20
Cardiac output (L/min)	2.44 ± 0.7	1.75 ± 0.9*	2.20 ± 0.8	1.62 ± 0.5	1.46 ± 0.5
Total peripheral resistance (mm Hg/L min^{-1})	42 ± 10	49 ± 15	49 ± 13	43 ± 14**	41 ± 10**
Hematocrit	31 ± 1	32 ± 2	32 ± 3	28 ± 3	28 ± 3

Values are means ± SD.
* $P < 0.01$, t-test ; # Electronically averaged; ** $P < 0.05$ compared to changes in seven control monkeys (Forsyth et al, 1968)

Table 24

EFFECT OF BRADYKININ INFUSION ON FRACTION OF CARDIAC OUTPUT, BLOOD FLOW AND VASCULAR RESISTANCE IN ORGANS AND TISSUES SHOWING SIGNIFICANT CHANGES

	Cardiac output (L/min)	Blood flow (ml/min 100 g tissue^{-1})	Resistance (mmHg/L min^{-1} 100 g tissue^{-1})
Heart	148	106	86
Brain	116	79*	102
Kidney	138*	96	86*
Skin	60*	44*	208*
Skel. musc.	64*	46*	197*
Stomach	92	65*	134
Spleen	74*	33*	415*
Pancreas	72	52*	179*
Liver (hepatic artery)	180*	125*	71*
Bone	75*	55*	177
Lung (bronchial artery)	287*	189*	45*

Values are given as percent of baseline values in ten monkeys.
* P 0.05 (Mann-Whitney U-test) compared to changes in seven control monkeys.

Table 25

EFFECT OF GANGLIONIC BLOCKADE ON FRACTION OF CARDIAC OUTPUT, BLOOD FLOW, AND VASCULAR RESISTANCE IN ORGANS AND TISSUES SHOWING SIGNIFICANT CHANGES

	Cardiac output (L/min)	Blood flow (ml/min 100 g tissue $^{-1}$)	Resistance$_{-1}$ (mmHg/L min^{-1} 100 g tissue^{-1})
Heart	80*	60*	155
Brain	114	83*	77
Kidney	137*	102	64*
Skin	69*	52*	127
Skel. musc.	82*	62*	108
Stomach	62*	46*	143
Spleen	153	110	68*
Pancreas	80	61*	113
Liver (hepatic artery)	126	95	80
Bone	97	71*	96
Lung (bronchial artery)	442*	332*	28*

Values are given as percent of baseline values in ten monkeys.
* P 0.05 (Mann-Whitney U-test) compared to changes in seven control monkeys.

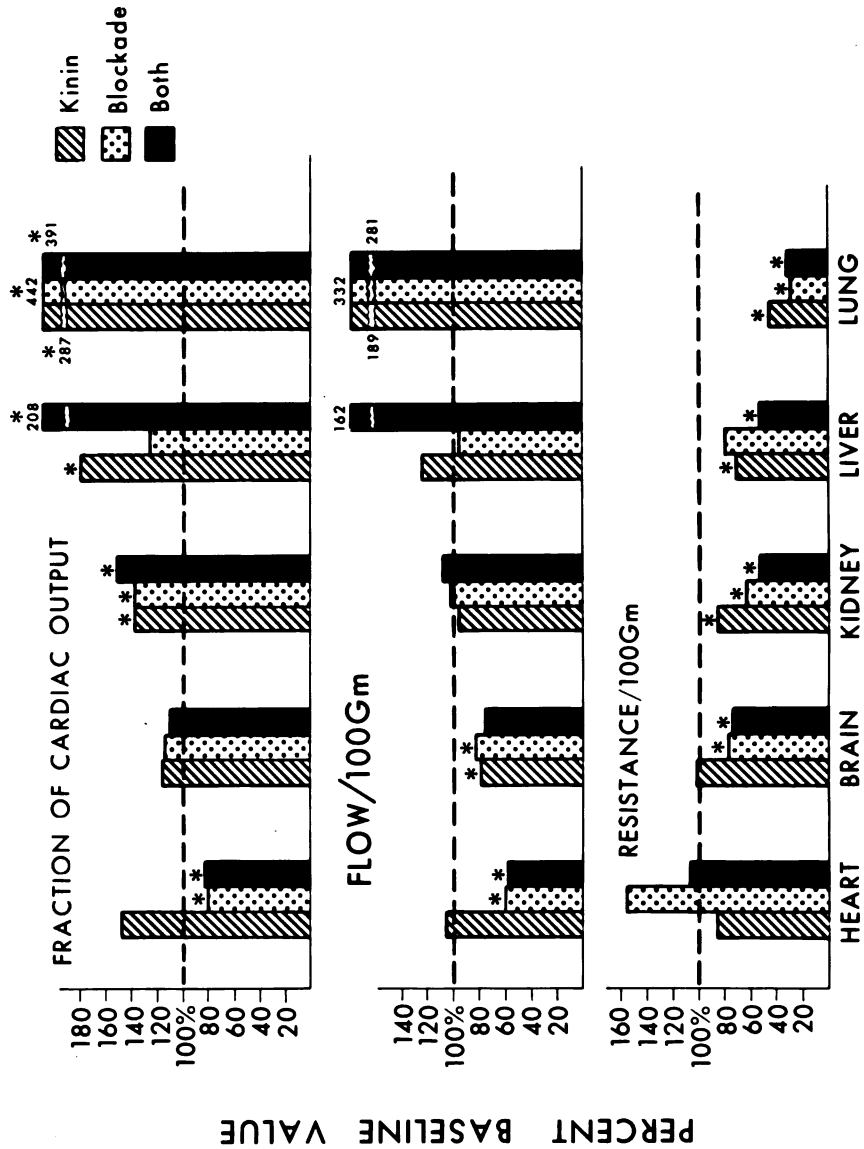
Table 26

EFFECT OF COMBINED INFUSION ON FRACTION OF CARDIAC OUTPUT, BLOOD FLOW, AND VASCULAR RESISTANCE IN ORGANS AND TISSUES SHOWING SIGNIFICANT CHANGES

	Cardiac output (L/min)	Blood flow (ml/min 100 g tissue^{-1})	Resistance (mmHg/L min^{-1} 100 g tissue^{-1})
Heart	83*	58*	107
Brain	110	76	79*
Kidney	152*	108	54*
Skin	60*	42*	146
Skel. musc.	111	32	129
Stomach	72*	53*	125
Spleen	92	58*	128
Pancreas	80	58*	128
Liver (hepatic artery)	202*	162	53*
Bone	82*	60*	107
Lung (bronchial artery)	391*	281*	31*

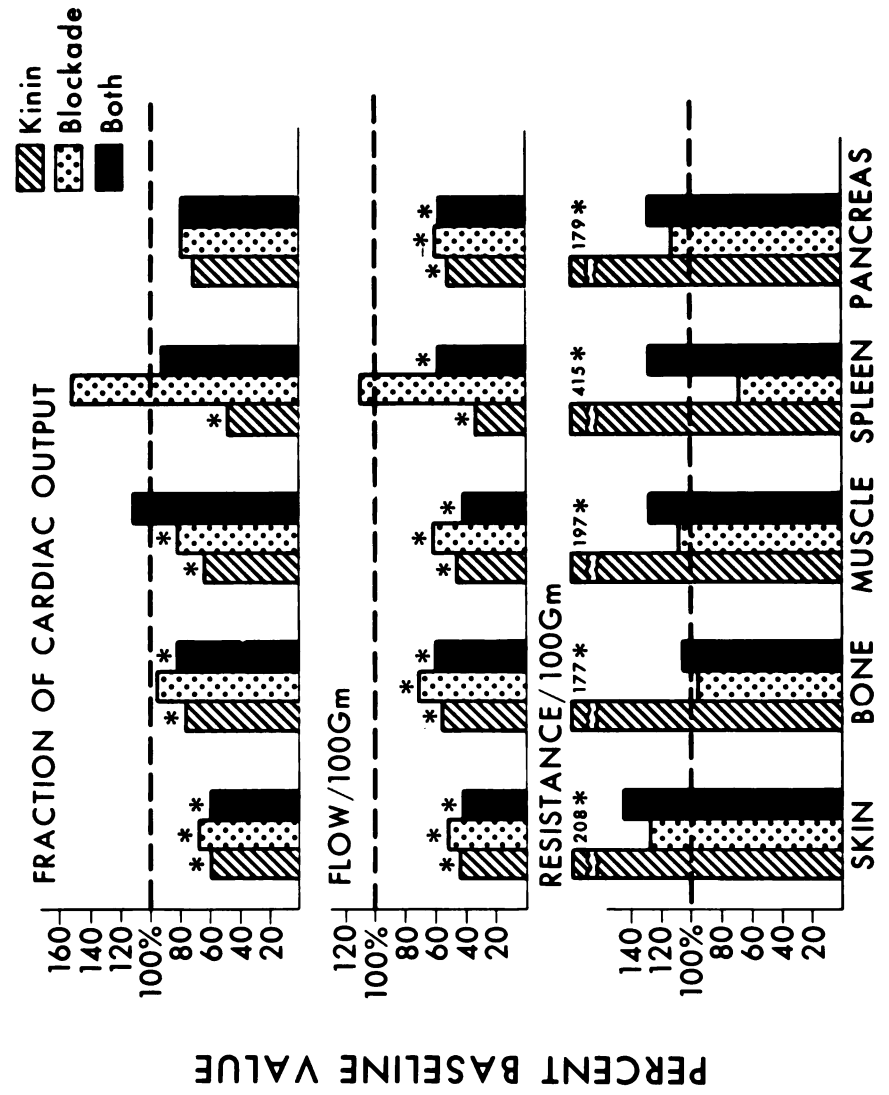
Values are given as percent of baseline values in ten monkeys.
* $P < 0.05$ (Mann-Whitney U-test) compared to changes in seven control monkeys.

Fig. 13



Effects of bradykinin alone and during ganglionic blockade on regional vascular hemodynamics

Fig. 14



Effects of bradykinin alone and during ganglionic blockade on regional vascular hemodynamics

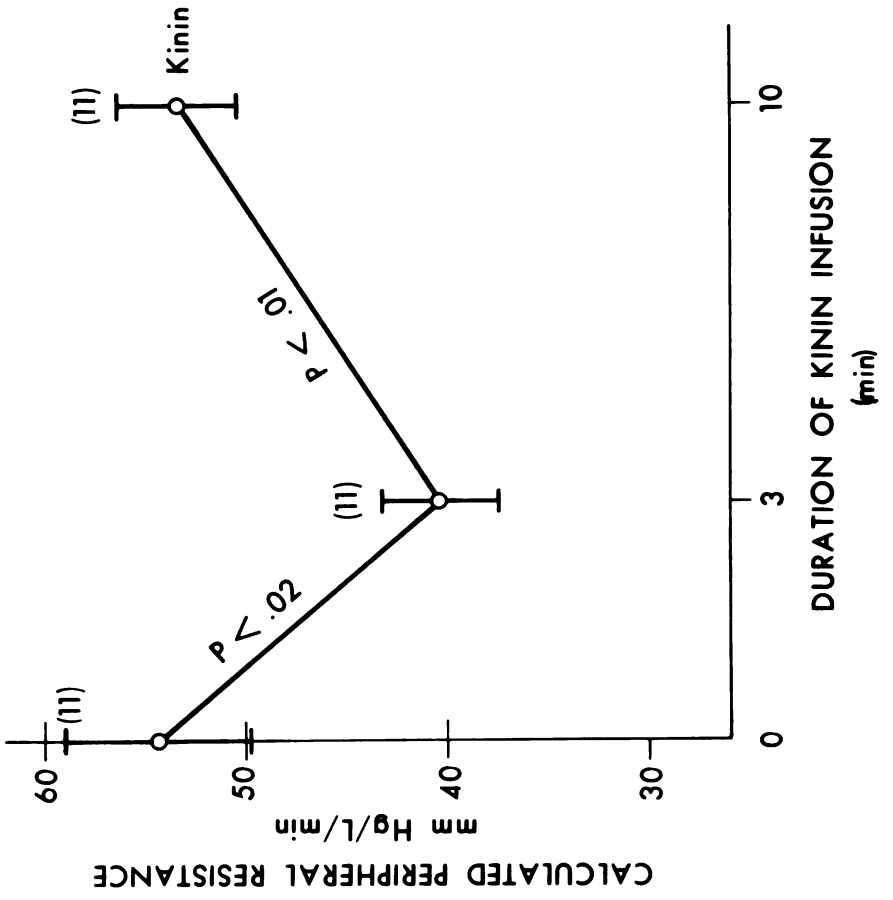
animals, tilted and subjected only to microsphere injections (control experiment). Comparisons were made by determination of rank-order significance using the Mann-Whitney U test (Colquhoun, 1971).

4. Discussion

Bradykinin could function in the primate as the primary mediator of the cardiovascular events of early endotoxemia. That potential has been established and the evidence discussed in the introduction. The present experiments were intended to test the possibility that bradykinin is the sufficient mediator for the cardiovascular events caused by endotoxin. Were this true, the infusion of bradykinin would reproduce the specific cardiovascular effects of endotoxin. The results obtained in these experiments suggest, but do not prove, that kinin is not a sufficient mediator of these events. These results also suggest that endotoxin may inhibit normal autonomic cardiovascular reflexes.

As in other species, bradykinin is a potent hypotensive agent in the primate. Figure 12 demonstrates the linear relationship between the dose of bradykinin and the fall in mean arterial blood pressure. But, bradykinin does not cause persistent hypotension via vasodilation. The initial response to the peptide, as indicated in Table 18, is vasodilation. However, when the infusion is maintained for ten minutes, peripheral arterial resistance returns to normal, or may actually exceed baseline values (Fig. 15; Tables 18-19).

Fig. 15



Effect of infusion of bradykinin (15-18 mg/kg/min) on peripheral vascular resistance (11 observations)

Hypotension persists at the expense of decreased cardiac output.

Prior observations, in a variety of species, have shown that there is a biphasic cardiovascular response to the peptide (Pearson and Lang, 1967; Lang and Pearson, 1968). Initial, direct vasodilation is rapidly replaced by vasoconstriction as a result of activation of autonomic cardiovascular reflexes. The peptide has also been shown to cause direct release of catecholamines from the adrenal medulla (Feldberg and Lewis, 1964; Staszewska-Barczak and Vane, 1967; Lewis, 1970) and adrenergic nerve endings (Lewis, 1970).

The role of autonomic cardiovascular reflexes in the biphasic resistance response of the animals studied is confirmed by the results obtained during simultaneous infusions of trimethaphan and bradykinin. Under these conditions resistance did not recover during sustained kinin infusion. The recovery of resistance was therefore due to a reflex event, which was inhibited by the blocking agent.

Cardiac output fell significantly during sustained kinin infusion. There are several mechanisms by which this could have occurred: 1)The peptide could be a myocardial depressant. But, tests of this possibility in other species, as discussed in the introduction (pg. 34), have shown that bradykinin is not a direct myocardial depressant. 2)The peptide could cause venous return to be diminished. No direct measurements were made of venous capacitance in these prepara-

tions, but prior measurements in humans have indicated that bradykinin may be a venodilator (Mason and Melmon, 1966). The resultant loss of venous return to the heart could easily account for the observed decrease in cardiac output. 3)Finally, the peptide could have caused hemoconcentration or edema formation, resulting in a decreased blood volume. Known effects of bradykinin on capillary and venular epithelia would support this possibility. However, in these acute experiments the hematocrit did not rise. The mechanism by which cardiac output falls during kinin infusion cannot be specifically determined in these studies. However, the fact that cardiac output during peptide infusion is reduced differentiates the effects of bradykinin from those of early endotoxemia.

The characteristics of early phases of endotoxemia are vasodilation with normal or increased cardiac output. Thus, the systemic hemodynamic responses to bradykinin, both during acute infusion and with accumulation of high doses, cannot be claimed to reproduce the unique cardiovascular events of endotoxemia. Furthermore, the animals receiving high cumulative doses of bradykinin did not die, despite the development of a "shock-like" hemodynamic state. This supports the suggestion, arising from the first study, that the late toxicity of endotoxin is not simply an extension of the cardiovascular instability of early phases of endotoxemia.

The lack of congruence between the effects of bradykinin and those of endotoxin is further illustrated by an analysis

of patterns of regional blood flow and resistance occurring during infusion of peptide. Forsyth, Hoffbrand and Melmon (1970; Forsyth, 1972) have demonstrated the patterns of regional blood flow and organ resistances occurring with sympathetic cardiovascular reflex activation. The pattern of flow and resistance occurring with kinin infusion is quite similar (Figs. 13-14). Differences between the effects of the peptide and other mechanisms of sympathetic reflex activation may be attributable to the fact that during kinin infusion the reflex response is constantly opposed by the presence of an active, intravascular vasodilating substance. The observed response in an individual vascular bed therefore represents the balance between the opposing direct hormonal vasodilatation and reflex neurogenic vasoconstriction.

By the same analysis of patterns it is possible to evaluate the effects on flow and resistance occurring during simultaneous kinin infusion and ganglionic blockade. This analysis further confirms the role of sympathetic reflex mechanisms in modifying response to infused bradykinin. And there is a suggestion that superimposition of autonomic blockade on the presence of bradykinin may more closely reproduce the regional hemodynamic patterns occurring during endotoxemia (Wyler et al, 1968). The questions arising from this observation are tested and discussed later in this dissertation.

The studies reported imply that bradykinin is not a sufficient mediator for the cardiovascular events occurring

during endotoxemia. However, the evidence does not allow for the rejection of the peptide as one important mediator of these events. Several objections can be raised. First, the dose-response curves show that the hypotensive response to kinin infusion is linear throughout the range of doses tested. This indicates that the maximum response was not closely approached, since even at the highest doses tested there was no tendency for the curve to flatten out. The lack of maximum stress is also suggested by another observation. During tremethaphan infusion the response to infused bradykinin was still linear but the slope of the dose-response curve was much less steep. A paradox is apparent when the dose-effects of bradykinin occurring before and after autonomic blockade are compared (Fig. 11). Low doses of peptide caused equal depression of mean arterial pressure under both conditions; but, high doses of peptide caused more severe hypotension in animals with intact reflex mechanisms than in animals with ganglionic blockade. This is the opposite of what would be expected and suggests that there is a limit to vasodilatation that was more closely approached in blocked animals (baseline blood pressures were 40 mmHg lower after blockade). It also suggests that extremely high doses of bradykinin may be able to overcome autonomic reflex vasoconstriction. These doses were not approached during the dose-response infusions, and were certainly not approached by the 15-18 μg/kg/min dose used in the regional blood flow studies.

Second, infusion of peptide was performed at doses

which would reproduce the arterial blood levels of bradykinin reported during endotoxemia by Nies et al (1968). However, the gradient of bradykinin between the central and peripheral vasculature was not reproduced. In fact, the gradient during experimental infusion was reversed relative to that which would be expected during exposure to endotoxin. In the former case the concentration of kinin would be lowest in the peripheral vasculature, whereas in the latter situation bradykinin concentrations would be the same throughout the intravascular space or perhaps highest in the peripheral vessels. Thus, the dose of kinin infused, while reproducing arterial blood concentrations may have been much too low to effectively reproduce the concentrations of bradykinin occurring in the capillaries during endotoxemia.

Finally, when blood concentrations of bradykinin are elevated the observed peripheral vascular resistance is due to a balance between neurogenic vasoconstriction and hormonal vasodilation. It is possible, therefore, that during endotoxemia, concentrations of bradykinin in the tissue vascular beds are high enough to completely overcome reflex vasoconstriction. Since the infused doses of peptide were insufficient to test this possibility, the question of the sufficiency of bradykinin remains incompletely answered.

C. Does Endotoxin inhibit reflex cardiovascular control mechanisms?

1. Rationale:

Bacterial substances can directly influence neuronal function. Exotoxins, produced by some gram-positive bacteria, inhibit or distort neuromuscular or central synaptic transmission. By analogy, endotoxin might be capable of inhibiting or otherwise altering autonomic nervous system functions.

Hinshaw and his coworkers (1966, 1967) have suggested that inhibition of autonomic function, by endotoxin, was a possible mechanism of the action of the bacterial product. They did not pursue the question further. Other investigators have reported effects of endotoxin on parasympathetic function (Blattberg and Levy, 1969). This study, done in the dog, attributed bradycardia during endotoxemia to vagal stimulation since it was blocked by atropine. A more recent report (Chiba and Nakajima, 1971) did not confirm this, however. Trank and Visscher (1962) also demonstrated an abnormality of baroreceptor functioning in the dog exposed to endotoxin. The baroreceptor was found to respond with an abnormally high firing rate, at any level of systemic blood pressure, following exposure to endotoxin. This was interpreted as a basis for "tolerance" of hypotension. These studies have not been repeated nor confirmed.

The clinical observation of persistent vasodilation despite hypotension, during spontaneous septicemia, has been discussed. Vasodilation during experimental endotoxemia

has been described in humans (Kimball, Melmon and Wolff, 1972); and in the monkey (Nies et al, 1968). These observations have been reconfirmed in studies reported in this dissertation.

The results of the preceding experiments demonstrate that infusion of bradykinin, at doses sufficient to reproduce the arterial plasma kinin concentrations found during experimental endotoxemia, did not reproduce systemic or regional hemodynamic effects of endotoxemia. But, simultaneous infusion of bradykinin, at those same doses, and blockade of peripheral autonomic responsiveness by addition of a ganglionic blocking agent, more closely reproduced the peripheral hemodynamic patterns of endotoxemia. Therefore, the possibility was considered that endotoxin could alter cardiovascular reflexes.

The effects of endotoxin on the baroreceptor-pressor reflex were tested directly to evaluate this possibility. Sudden lowering of carotid-artery pressure was used as the stimulus to the baroreceptor. The change of perfusion pressure at constant flow, in the innervated hind limb, was used as an index of the peripheral vascular resistance response. Cats and primates were tested before and after exposure to endotoxin. The responses of the reflex arc were compared in pre- and post-endotoxin exposure periods, and in treated and untreated animals. Those changes in responsiveness which did occur could be shown to be due to secondary effects of endo-

toxemia. They are not due to direct influences of LPS on the reflex arc.

2. Methods:

Adult cats of either sex, weighting 2.2 to 5.2 kg were anesthetized by intravenous injection of thiopental sodium 20 mg/kg, followed by ∝-chloralose 10 mg/ml (in ethanol: water 2:3) 50 mg/kg. Supplemental ∝-chloralose anesthesia was used, as necessary, to maintain in surgical anesthesia. After tracheostomy, the left femoral vessels were exposed by sharp and blunt dissection. Poly-ethylene cannulae (Intramedic PE 100 I.D. 0.034" x O.D. 0.060") were inserted retrograde into the aorta and into the inferior vena cava. The cannulae were filled with heparinized saline solution (5 USP units/ml of 0.9% NaCl). The venous cannula was used only for injection. The arterial cannula was attached, via a plastic three way stopcock (Ipco Hospital Supply) to a Statham 23 Gb arterial strain gauge for measurement of systemic arterial blood pressure.

The right femoral vessels were exposed by dissection and the artery was isolated in the upper thigh from the inguinal ligament to below the branch to the gracilis muscle. This small branch was ligated and cut between ties. The cat's femoral artery has no other branches in the upper thigh. Using this free length of artery (approximately 2-2.5 cm), a retrograde cannula was inserted into the aorta to the level of the umbilicus. An anterograde cannula was

inserted into the femoral artery far enough for secure ligation using a 1.5 cm length of poly-ethylene tubing attached to a #17 plastic luer stub adapter (Intramedic A-1030/15). Both cannulae were PE 190 tubing (Intramedic ID 0.047" xOD 0.067".). The outflow of arterial blood from the aortic cannula was conducted through plastic connectors and tubing (Tygon OD 7/16" x ID 5/16") which served as the pumping chamber of a Harvard Peristaltic Pump Model 1202. The output of this pumping chamber was then directed through two plastic "T" connectors in series. An air filled length of tubing, occluded by a screw clamp, was attached to the side arm of the first "T". This sidearm functioned as an elastic capacitance to reduce the pulsations of the pump outflow, and as a bubble trap. The side arm of the second "T" was connected, via a saline filled catheter, to a Statham 23 Gb arterial strain gauge which recorded the limb perfusion pressure (Pf.P.). Blood flowing from the pump was then directed into the femoral artery through the short, anterograde arterial cannula. The animals were heparinized with sodium heparin, 1000 U/kg initially and supplemented with 1000 U/hr.

The volume of blood in the extracorporeal pump circuit was 11.5 ml. The pressure drop across the luer stub and short anterograde catheter was negligible with saline, but reached 10-15 mmHg (depending upon flow) during blood perfusion. Limb perfusion flow rates were constant within each experiment; therefore, the small additional resistence to flow offered by the cannula was also constant. Changes in

perfusion pressure recorded at the sidearm reflected changes in the vascular resistence of the perfused arterial bed of the limb. This bed was comprised of vessels in skin, bone and muscle.

The perfusion pump was tested for constancy of flow. Determinations were made at several pump speeds with no resistence using a stop watch and graduated cylinder. Flow at each tested pump speed (within 3% tolerance) was constant. When outflow pressure was increased from 0 to 250 mmHg, in 50 mmHg steps, by increasing resistence with a screw clamp, flow also remained constant ($\pm$ 3%) at each of four pump speeds tested. The pump was occlusive to back flow at pressures above 300 mmHg, and the perfusion circuit exhibited no leaks at these pressures.

Fresh cannulae, luer stubs and stopcocks were used for each days experiments. The pumping chamber, plastic connectors and "T" devices were cleaned immediately after use. They were rinsed three times in freshly distilled water. Then they were immersed in 8 M urea and flushed several times with the urea solution. After three more distilled water rinses the tubing and connectors were boiled for thirty minutes in fresh distilled water and allowed to air dry. Prior to the start of each experiment the perfusion system was completely reconnected and pumping was started to check for leaks. Three changes of sterile 0.9% NaCl were used and the system remained filled with saline until connected to the hind limb.

Baroreceptor stimulation was initially imposed by bi-

lateral occlusion of the common carotid arteries with arterial clips. The carotids were carefully exposed as low as possible in the neck and isolated using loops of saline-wetted umbilical tape. Occlusion was then obtained with clips applied on the tapes. The mean pressure in the carotid sinus following bilateral carotid occlusion was measured with a poly-ethylene catheter (PE 10 ID 0.011" x OD 0.024") inserted via the left lingual artery. Mean carotid artery blood pressure fell to 21 ± 3.5 mmHg.

Continuous records of systemic blood pressure and hind limb perfusion pressure were obtained using a Beckman-type R dynograph with curvilinear pens. Paper speed was 2.5 mm/sec. Heart rate was derived from the arterial pressure tracing using a cardiotachometer coupler (Beckman-type 9857B) and mean blood pressure was obtained by electronic averaging.

Endotoxin (Difco Co. E. Coli 0127:B8 lot #567458) was prepared by shaking in 0.9% NaCl for eighteen hours at an initial concentration of 1 mg/ml. This suspension was centrifuged at 1000 x G for twenty minutes in a Sorvall RC-2B centrifuge and the supernatant was refrigerated. Fresh material was prepared weekly, or more frequently if needed.

Preliminary experiments confirmed previous reports (Gilbert, 1960; Kuida et al, 1961; Kadowitz and Yard, 1970; Greenway and Murthy, 1971) that rapid injection of endotoxin into the cat caused sudden death. Pulmonary edema, cyanosis, hypoxemia and cardiac arrythmias were noted. Endotoxin was

therefore injected slowly (10 minutes) intravenously. The dose was 2.5 mg/kg (based on initial endotoxin concentration); the final volume of injection was 10 ml.

Following endotoxin injection, systemic blood pressure fell and there was an appropriate rise of limb perfusion pressure. But the responses to acute carotid occlusion appeared to be transiently inhibited (see below). To resolve this incompatibility it was felt to be necessary to repeat these experiments while protecting the baroreceptors from spontaneous blood pressure changes. To do this, the carotid sinuses were isolated, perfused at constant mean pressure and intermittently subjected to controlled pressure changes of 30 or 50 mmHg. The perfusion system represents an adaptation of the classic Mossiejeff preparation (1927) and was designed to maintain an adequate flow of well oxygenated blood to the chemoreceptors during baroreceptor hypotension.

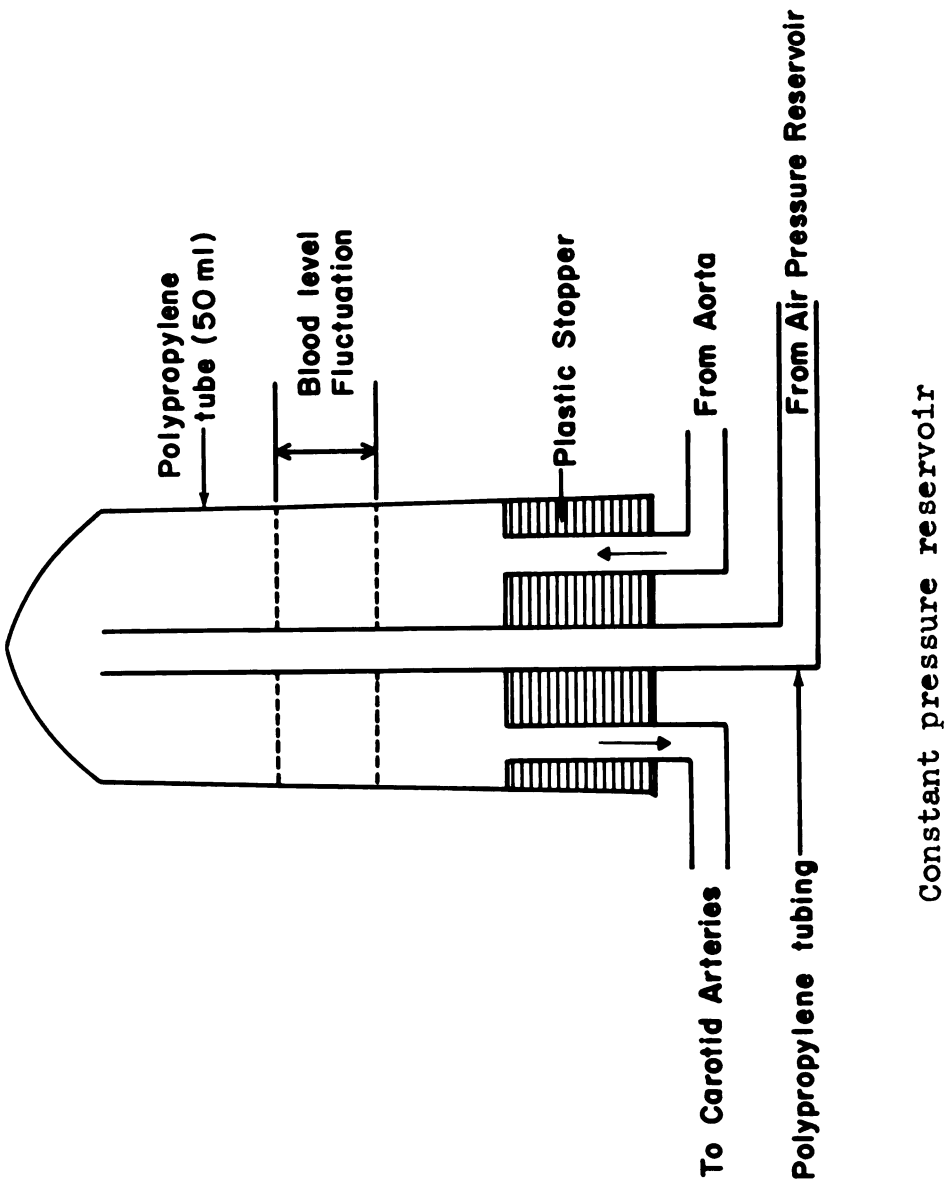
Adult cats were anesthetized using thropental-∝chloralose. As already described, the left femoral vessels were cannulated and the right femoral artery perfused. A midline incision was made in the neck from the sternal notch to the thyroid cartilage and the trachea was cannulated as low as possible. The rostral trachea and subjacent esophagus were then dissected free of the anterior spinal musculature and reflected rostrally. The carotid vasculature and associated nerves, and the vagus nerves were carefully preserved.

The region of the carotid bifurcation was located and

the occipital and ascending pharyngeal arteries identified. The following vessels were ligated bilaterally: dorsal muscular artery, superior thyroid artery, external carotid arteries. The internal carotid is a degenerated vessel in the cat and is usually imperforate (Davis and Story, 1943); therefore it was not ligated. In two animals the right lingual (a branch of the ascending pharyngeal) was cannulated with PE 10 tubing as before. The left lingual artery was tied. In the remainder of the animals the lingual arteries both were tied. No attempt was made to ligate the occipital or ascending pharyngeal vessels. These vessels were left undisturbed to minimize the possibility of damage to the carotid sinus nerve. They also allowed for continuous flow of blood to maintain oxygenation. The common carotid arteries were cannulated in an anterograde direction with PE 190 tubing (ID 0.047" x ID 0.067"). A retrograde cannula was inserted via the left common carotid into the aortic arch; the right common carotid was ligated low in the neck. The output of the aortic arch cannula was pumped through plastic tubing into a pressure control device (Fig. 16).

The pressure reservoir consisted of a 50 ml conical plastic tube stoppered with a plastic, three-holed stopper. Polypropylene tubes were inserted into the holes in the stopper. Two of these tubes were cut flush with the inner surface of the stopper; the third extended deeply into the conical tube. Blood was pumped into the pressure chamber through one of the short tubes. The pressure in the chamber

Fig. 16


Polypropylene tube (50 ml)
Blood level Fluctuation
Plastic Stopper
From Aorta
From Air Pressure Reservoir
To Carotid Arteries
Polypropylene tubing
Constant pressure reservoir

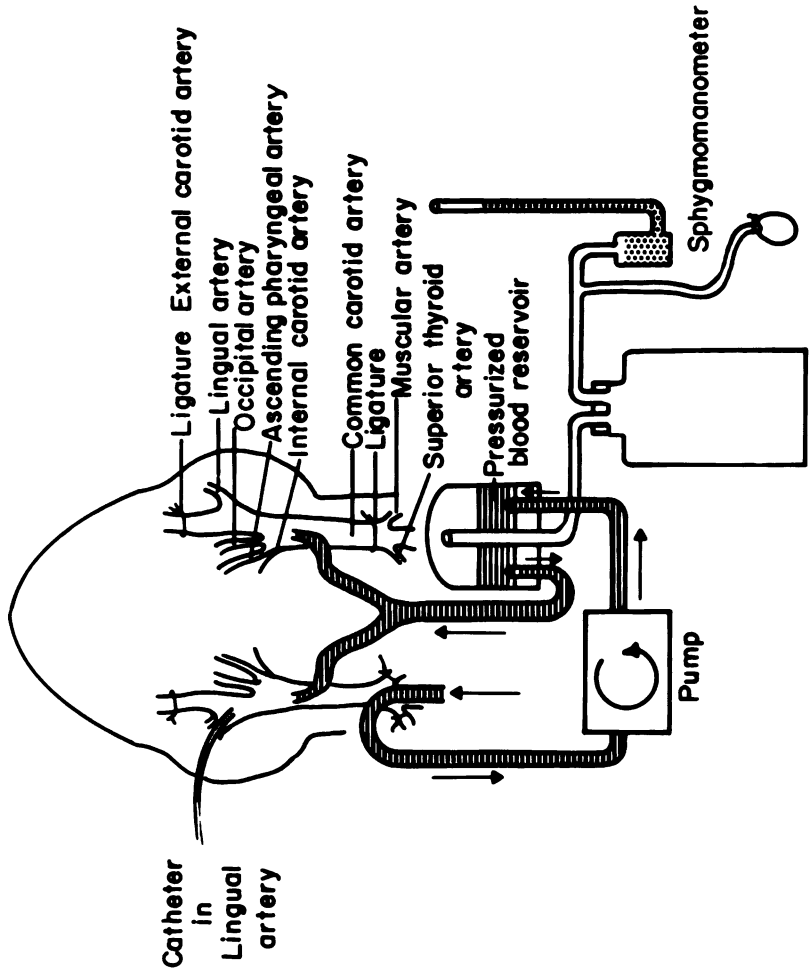
was controlled with a sphygmomanometer-reservoir bottle system attached to the long center polypropylene tube. Blood flowed out of the pressure chamber through the other tube cut flush with the stopper. A magnetic stirring bar was used to provide gentle agitation to prevent erythrocyte sedimentation.

The non pulsatile blood flow was then directed into both carotid sinus areas through a "Y" tube attached to the antero-grade cannulae. The volume of blood in the reservoir system fluctuated as the imposed pressure was varied. The rate of the pump was varied to keep the pressure chamber at a relatively constant volume. The entire perfusion system is schematically illustrated in Figure 17.

All components of this perfusion system which came into contact with the blood were made of plastic. New arterial cannulae were made for each days experiments . The pumping tubing and blood reservoir were cleaned as described before, and pumping was started with three changes of sterile saline before blood perfusion was begun. The possibility that pumping would cause trauma to red cells was considered (Bernstein et al, 1965). Blood samples were checked for gross hemolysis and none was noted. The hematocrit was noted to fall slowly in experimental and control animals. This may have represented pump-induced cell injury, but was not further evaluated.

Once the complete perfusion systems for limb and neck had been established, a series of responses were tested to be sure that the reflex arc was functional. Unresponsive

Fig. 17



Modified Mossiejeff Preparation

animals, those exhbiting no change in limb perfusion pressure with changes of carotid perfusion pressure, were not used. Several animals were examined after both the vagi and cardio-accellerator nerves had been cut. These animals were extremely unstable. They deteriorated rapidly and exhibited striking changes of systemic blood pressure as the volume in the constant pressure reservoir fluctuated. Because of this instability, results obtained with these animals were not included in the analysis.

Ten animals were prepared with the vagi intact. Two were completely unresponsive in the baseline period. Three of the eight responsive animals were used as controls, receiving only saline injection. The other five were given intravenous endotoxin, 2.5 mg/kg, as previously described. Continuous records were made of systemic blood pressure and limb perfusion. Heart rate was recorded using a cardiotachometer coupler. Blood samples were obtained at intervals for measurement of pH, PaO_2, $PaCO_2$, using Radiometer microelectrodes. Total leucocyte counts and hematocrit were also measured.

Baroreceptor stimuli were imposed in the following manner: The pressure in the carotid sinus perfusion system was initially set at a level sufficient to maintain the hind limb perfusion pressure at the same mean arterial pressure (measured prior to any intervention in the neck). At three to five minute intervals the pressure in the carotid sinus was changed suddenly. Reservoir pressure was lowered either

30 mmHg or 50 mmHg from the baseline pressure. Multiple measurements were made at each pressure step, before and after endotoxin injection, in each cat. Observations were grouped in the following intervals for analysis: baseline (before endotoxin), 0-15 min, 15-45 min, 45-90 min after endotoxin. All observations at each pressure step were then used to compute the data points presented in the results.

b. Studies in primates:

Species differences in response to injected endotoxin have been well documented (Gilbert, 1960; Kuida et al, 1961). Studies of reflex responsiveness were repeated in a small group of primates to exclude possible species-related effects. Four monkeys were prepared for study. One animal was completely unresponsive and was not included in analysis. The remaining three animals weighed 4-5 kg. They were initially tranquillized by injection of Sernylan^{R} (phencyclidine hydrochloride) 10 mg IM. This was followed by slow intravenous injection of α-chloralose in alcohol:water 50 mg/kg. Effective anesthesia was achieved with good relaxation and well maintained respiration. Tracheostomy was performed and the left femoral vessels were cannulated for injection, blood sampling and continuous arterial blood pressure monitoring. The right femoral artery was prepared by retrograde and anterograde cannulation, as described in the cat, and pump perfusion was started. The animals were heparinized with 1000 U/kg initially and an additional 1000 U/hr.

Endotoxin was prepared from the same lot previously used in the same concentrations. The suspension was infused slowly into two animals using a Harvard pump set to deliver 1 ml/min. The dose of endotoxin was 10 mg/kg and the infusion lasted 40-50 min. The control animal was given an infusion of 0.9% NaCl, total volume 45 ml, rate 1 ml/min, in place of the endotoxin infusion. Blood samples for PaO_2, $PaCO_2$, pH, total and differential white blood cell counts and hematocrit were obtained and cardiac output was measured by dye-dilution at baseline, at the end of infusion, one hour after completion of the infusion and again immediately prior to termination of the experiment (approximately two hours after completion of endotoxin infusion).

Complete carotid occlusion using arterial clips was used as the baroreceptor stimulus. This stimulus was applied for one minute every five minutes. Baseline period was 30 minutes. Blood samples were obtained and cardiac output was measured. Endotoxin or saline infusion was then begun, and carotid arterial occlusions repeated at five minute intervals until the termination of the experiment.

3. Results:

a. Cats - complete carotid occlusion: Six animals were studied, each animal served as its own control. After surgical preparation, the animals were allowed to stabilize for thirty minutes. Then carotid occlusion was applied for thirty seconds, every three to five minutes, for fifteen

minutes providing an average response for each cat. This was called baseline. The animals were then injected with endotoxin suspension as described and thirty second carotid occlusion was applied at three to five minute intervals for 90 minutes. Only observations falling within two minutes of a stated observation time were grouped at that time point.

The effects of endotoxin on the responses of systemic arterial pressure and limb perfusion pressure, before and during complete carotid artery occlusion, are presented in Tables 27 and 28 and Figures 18 and 19. These results illustrate an apparent decreased responsiveness to carotid occlusion of both systemic blood pressure and limb perfusion pressure in the first five minutes after endotoxin exposure. The spontaneous response of the animal to the falling systemic arterial pressure, following exposure to endotoxin, appears appropriate (Fig. 20) but the early response to acute baroreceptor stimulation (Figs. 18, 19) are significantly decreased ($P < 0.01$) compared to responses in the baseline period. In this series of animals, blood samples were obtained during the baseline period (0) at fifteen minutes and at one hour after endotoxin injection for leucocyte counts (Fig. 24) Baseline values (0 time) were 8330. ± 2148 cells/cmm. By fifteen minutes after endotoxin injection, leucocyte counts had fallen to 1660 ± 359 cells/cmm ($P < 0.02$) and the total leucocyte count remained reduced at 1760 ± 261 ($P < 0.02$) cells/cmm at one hour after injection. This leucopenia was due almost entirely to granulocytopenia.

Table 27

TIME COURSE OF EFFECT OF ENDOTOXIN ON SPONTANEOUS SYSTEMIC ARTERIAL BLOOD PRESSURE AND HIND LIMB PERFUSION PRESSURE IN THE CAT *

Blood pressure (mmHg)	Time			
	Control	5 min	15 min	20 min
Systolic	157 ± 4(20)	134 ± 7(7)	136 ± 5(4)	138 ± 9(7)
Diastolic	123 ± 4(20)	88 ± 15(7)	84 ± 15(4)	95 ± 11(7)
Limb	143 ± 9(20)	172 ± 20(7)	184 ± 33(4)	195 ± 19(7)

Blood pressure (mmHg)	30 min	45 min	60 min	90 min
Systolic	137 ± 9(7)	121 ± 3(7)	107 ± 9(6)	85 (2)#
Diastolic	95 ± 11(7)	78 ± 11(7)	61 ± 12(6)	40 (2)
Limb	195 ± 19(7)	179 ± 22(7)	193 ± 14(6)	141 (2)

* Mean ± SEM (n = total number of observations within two minutes of that time point)
# average only

Table 28

TIME COURSE OF EFFECT OF ENDOTOXIN ON RESPONSE OF PERFUSED HIND LIMB TO COMPLETE CAROTID OCCLUSION IN THE CAT

	Time			
mmHg*	Control	5 min	15 min	20 min
Actual maximum perfusion pressure	205 ± 10(20)	199 ± 18(7)	219 ± 25(4)	246 ± 10(5)
Pressure increase	59 ± 4(20)	27 ± 8(7)@	35 ± 14(4)	45 ± 20(5)

mmHg*	30 min	45 min	60 min	90 min
Actual maximum perfusion pressure	239 ± 16(5)	252 ± 11(7)	218 ± 19(5)	200 (2)
Pressure increase	50 ± 10(5)	11 ± 15(7)	46 ± 10(5)	55, 90

* Mean ± SEM (n total number of observations within two minutes of that time point.
@ less than baseline response $P < 0.01$

Fig. 18

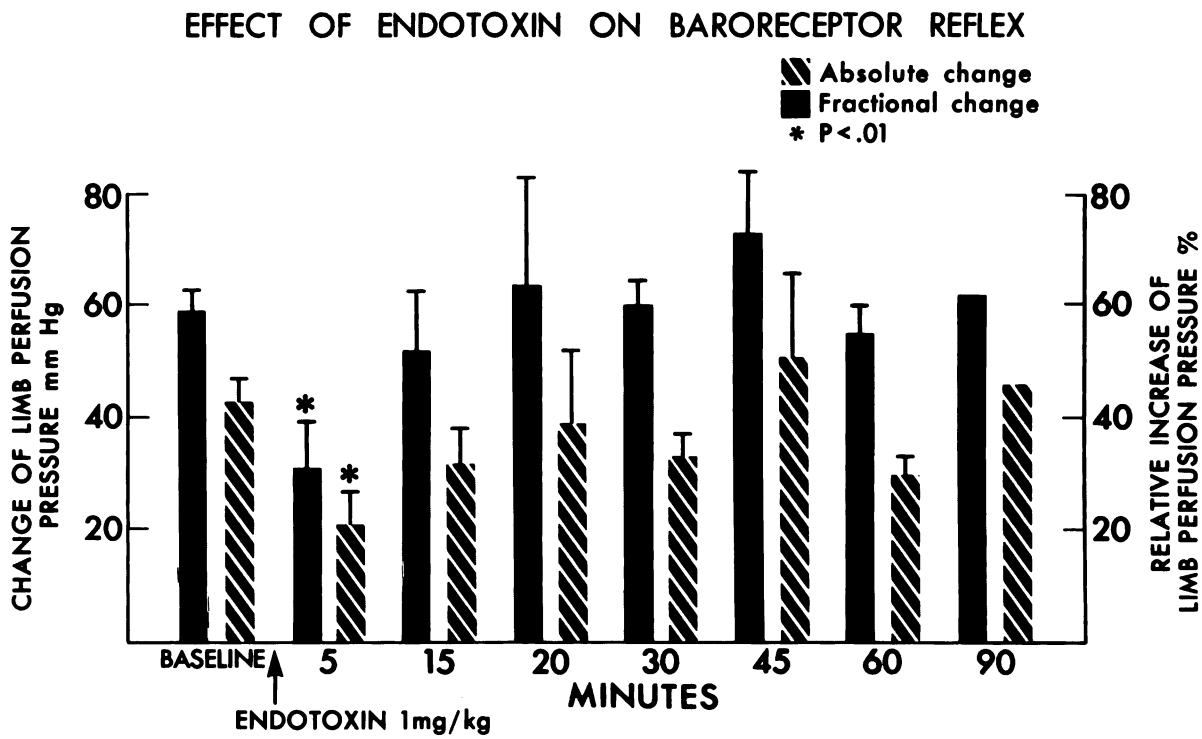

EFFECT OF ENDOTOXIN ON BARORECEPTOR REFLEX
Absolute change
Fractional change
* P<.01
CHANGE OF LIMB PERFUSION PRESSURE mm Hg
RELATIVE INCREASE OF LIMB PERFUSION PRESSURE %
80
60
40
20
BASELINE
5
15
20
30
45
60
90
MINUTES
ENDOTOXIN 1mg/kg

Fig. 19

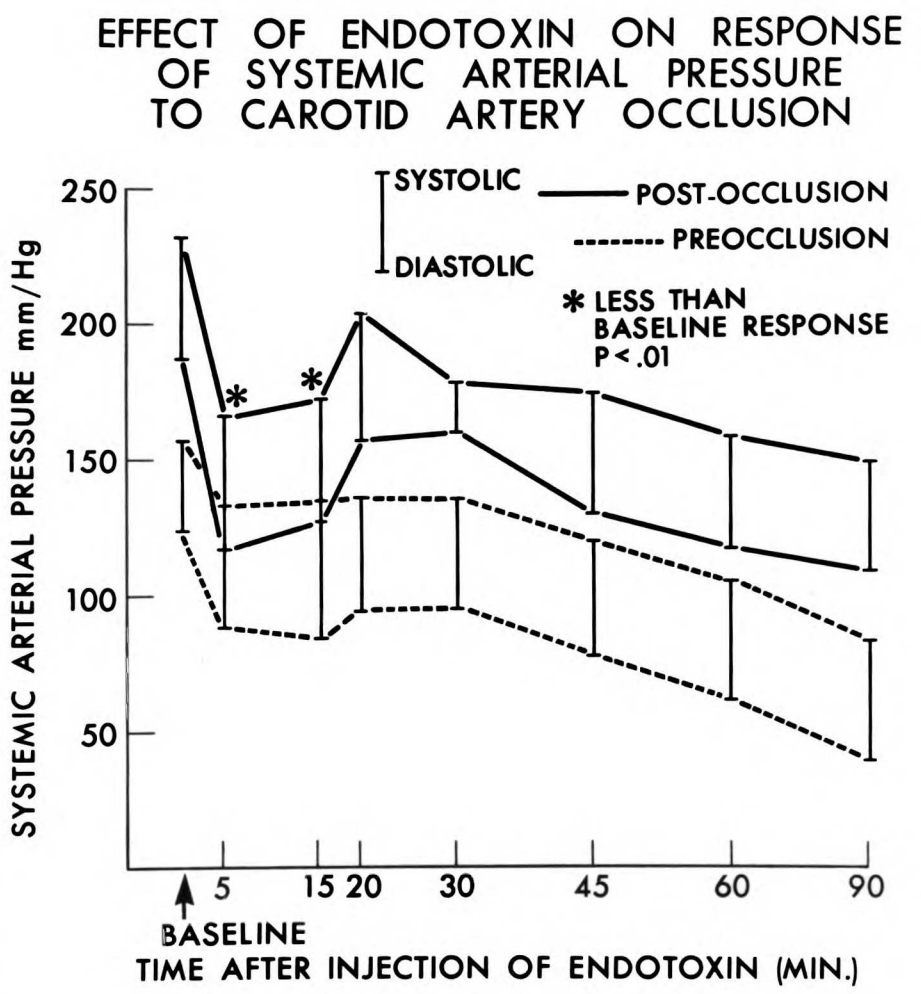
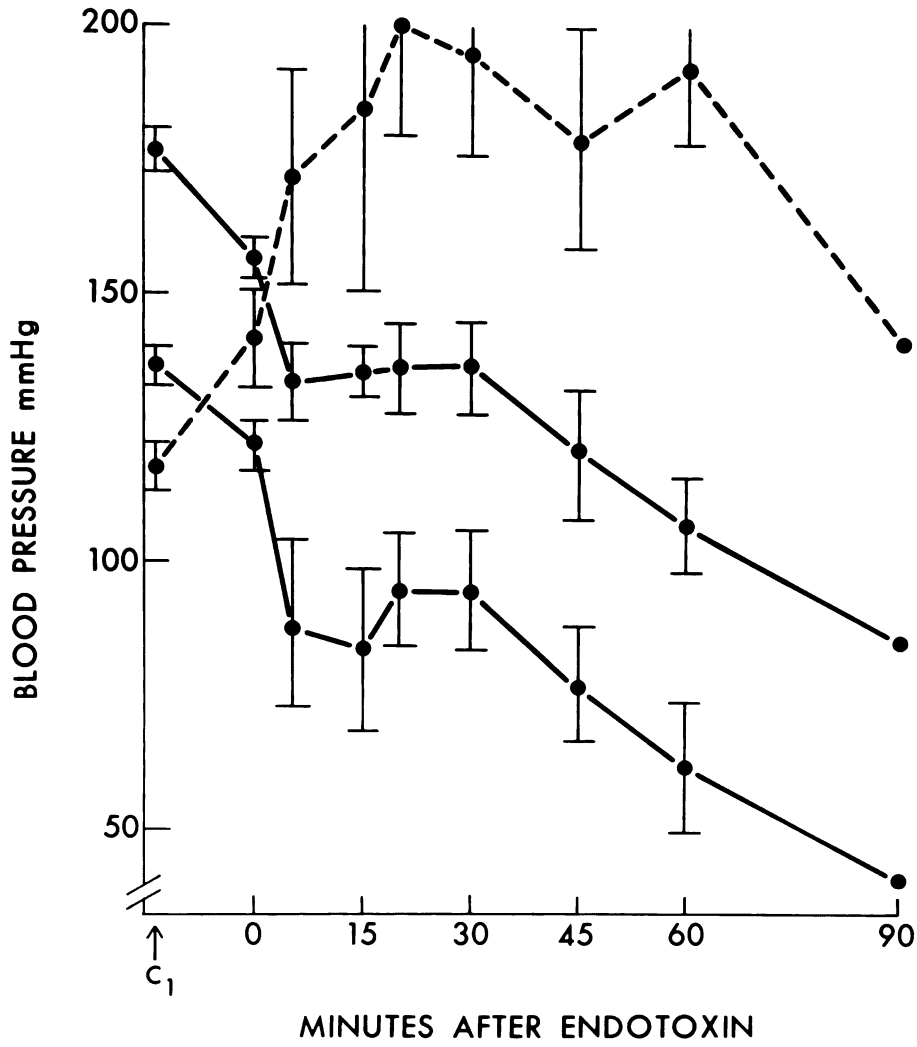

EFFECT OF ENDOTOXIN ON RESPONSE
OF SYSTEMIC ARTERIAL PRESSURE
TO CAROTID ARTERY OCCLUSION
SYSTOLIC
DIASTOLIC
POST-OCCLUSION
PREOCCLUSION
* LESS THAN
BASELINE RESPONSE
P < .01
SYSTEMIC ARTERIAL PRESSURE mm/Hg
250
200
150
100
50
5
15
20
30
45
60
90
BASELINE
TIME AFTER INJECTION OF ENDOTOXIN (MIN.)

Fig. 20

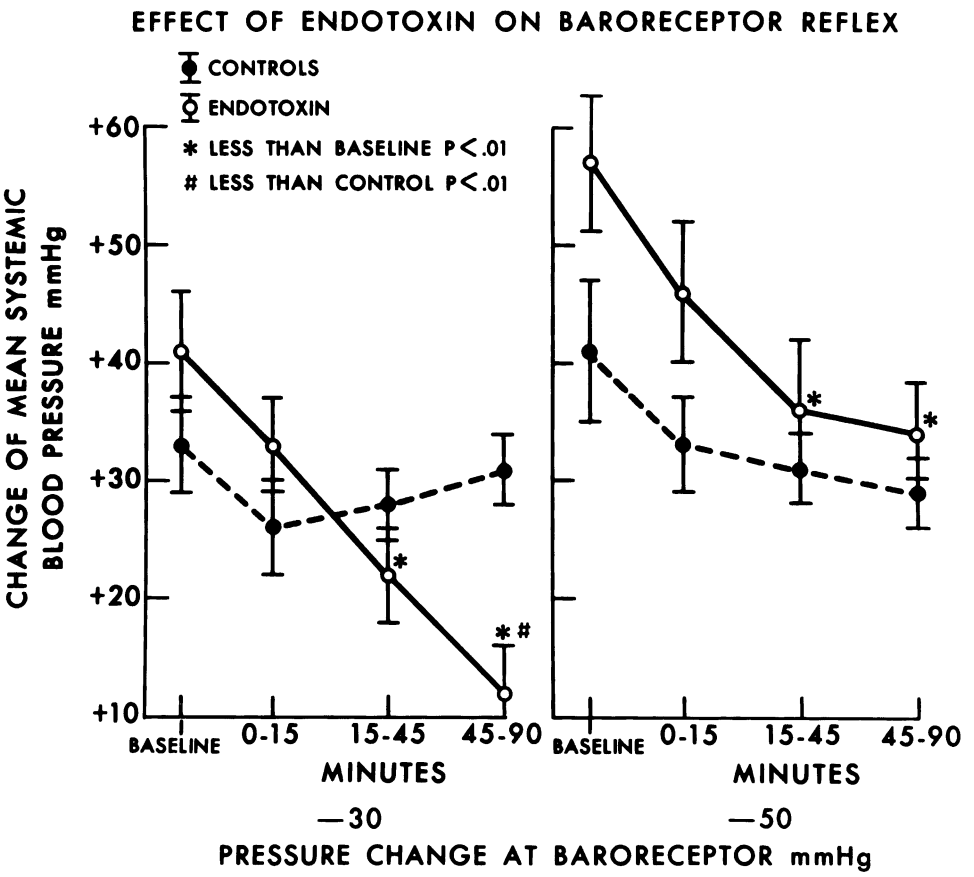


Effect of endotoxin on systemic and limb perfusion pressure (no carotid occlusion)

Systolic/Diastolic blood pressure ●——●

Limb perfusion pressure ●- - - -●

Fig. 21

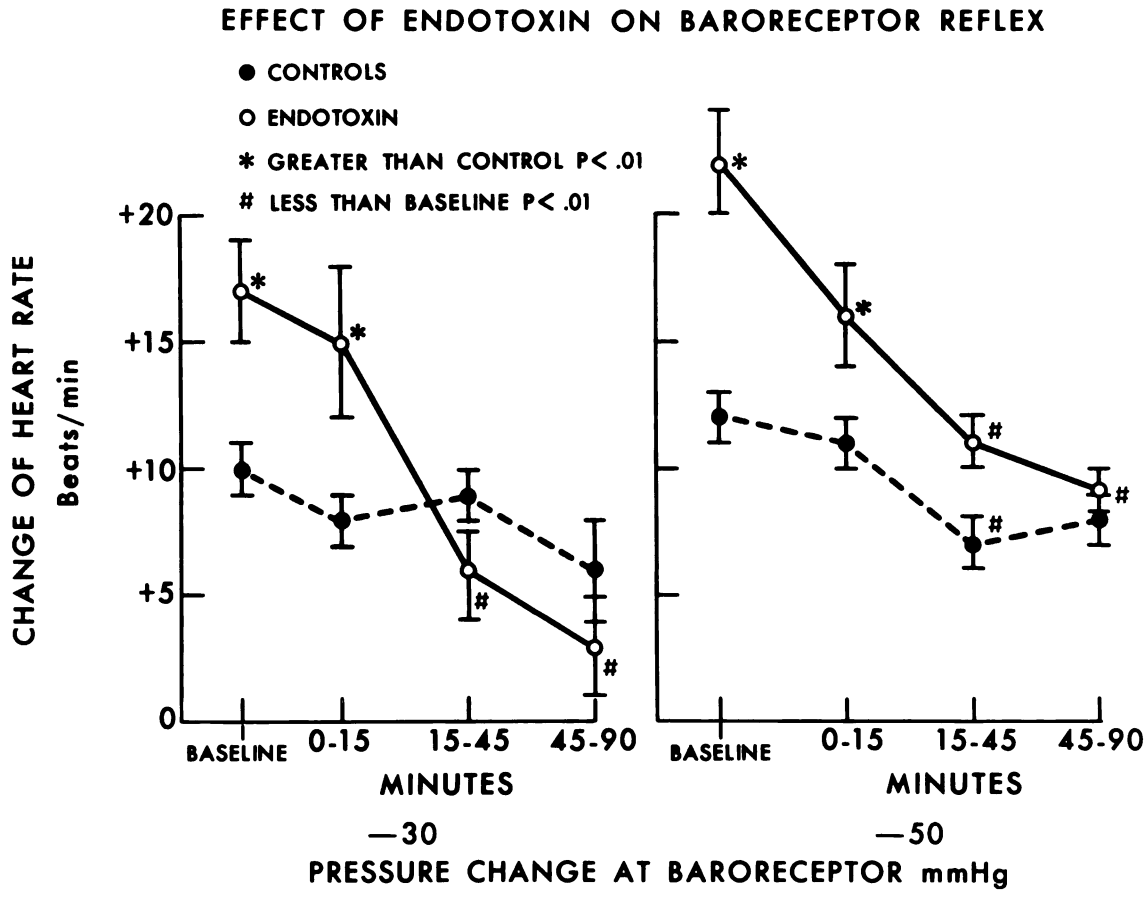

EFFECT OF ENDOTOXIN ON BARORECEPTOR REFLEX
CONTROLS
ENDOTOXIN
* LESS THAN BASELINE P<.01
# LESS THAN CONTROL P<.01
CHANGE OF MEAN SYSTEMIC BLOOD PRESSURE mmHg
+60
+50
+40
+30
+20
+10
BASELINE
0-15
15-45
45-90
MINUTES
—30
—50
PRESSURE CHANGE AT BARORECEPTOR mmHg

The results of these experiments suggest that in the earliest minutes following injection of endotoxin the responsiveness of the baroreceptor-pressor arc may be inhibited. However, the spontaneously occurring resistence change in the limb appears to be entirely appropriate. In order to resolve this seeming incompatibility of results it was necessary to repeat these experiments, while protecting the baroreceptor from the acute changes of arterial blood pressure.

b. Cats-controlled baroreceptor hypotension: Eight animals were studied. Three served as controls and were injected with saline. Five animals received an intravenous injection of endotoxin 2.5 mg/kg in ten ml saline.

Figure 21 illustrates effects of endotoxin on the response of mean systemic arterial blood pressure to pressure changes at the baroreceptor. Control animals (saline infused) remained responsive at baseline levels for the full experimental period. The slight decline in the response to 50 mm pressure change was insignificant. The endotoxin treated animals showed significant decreases in responsiveness, with time, as compared to baseline. The initial response of endotoxin treated animals, to the 50 mmHg pressure step, was greater than controls. Therefore, the later responses are not different than those of controls. However the response to 30 mmHg pressure change was initially the same in experimental and control animals and the late responses following endotoxin are significantly reduced when compared to both baseline and to control animals. There are

Fig. 22


EFFECT OF ENDOTOXIN ON BARORECEPTOR REFLEX
● CONTROLS
O ENDOTOXIN
* GREATER THAN CONTROL P< .01
# LESS THAN BASELINE P< .01
CHANGE OF HEART RATE
Beats/min
+20
+15
+10
+5
0
BASELINE
0-15
15-45
45-90
MINUTES
—30
—50
PRESSURE CHANGE AT BARORECEPTOR mmHg

no differences in the early (0-15 min) period either between endotoxin and control animals or between baseline and post endotoxin observation periods.

Parallel results were obtained in the measured responses of heart rate (Fig. 22). Endotoxin treated animals initially had greater increases of heart rate, with pressure changes, than did control animals. The responses in endotoxin treated animals declined significantly with a time related course. Control responses remained constant. No early inhibition of the heart rate response was noted following endotoxin.

The response of the reflex arc, as measured by the change in hind limb perfusion pressure (Fig. 23) remained constant throughout the period of observation in response to the 50 mmHg pressure step. Some decrease in the perfusion pressure response was noted with the 30 mmHg step. Again, the diminished responses occurred in the later observation periods and early responses were not different from controls or baseline.

The arterial blood pH fell rapidly following endotoxin administration after being stable throughout the baseline period (Fig. 25) The hematocrit remained essentially stable; initially 35 ± 3, it fell to 33 ± 2 after two hours on the pumping system and was not different in controls and endotoxin treated animals. PaO_2 and $PaCO_2$ also remained constant in both controls and endotoxin treated animals. PaO_2 was 90-100 mmHg and $PaCO_2$ ranged from 32-38 mmHg.

Fig. 23

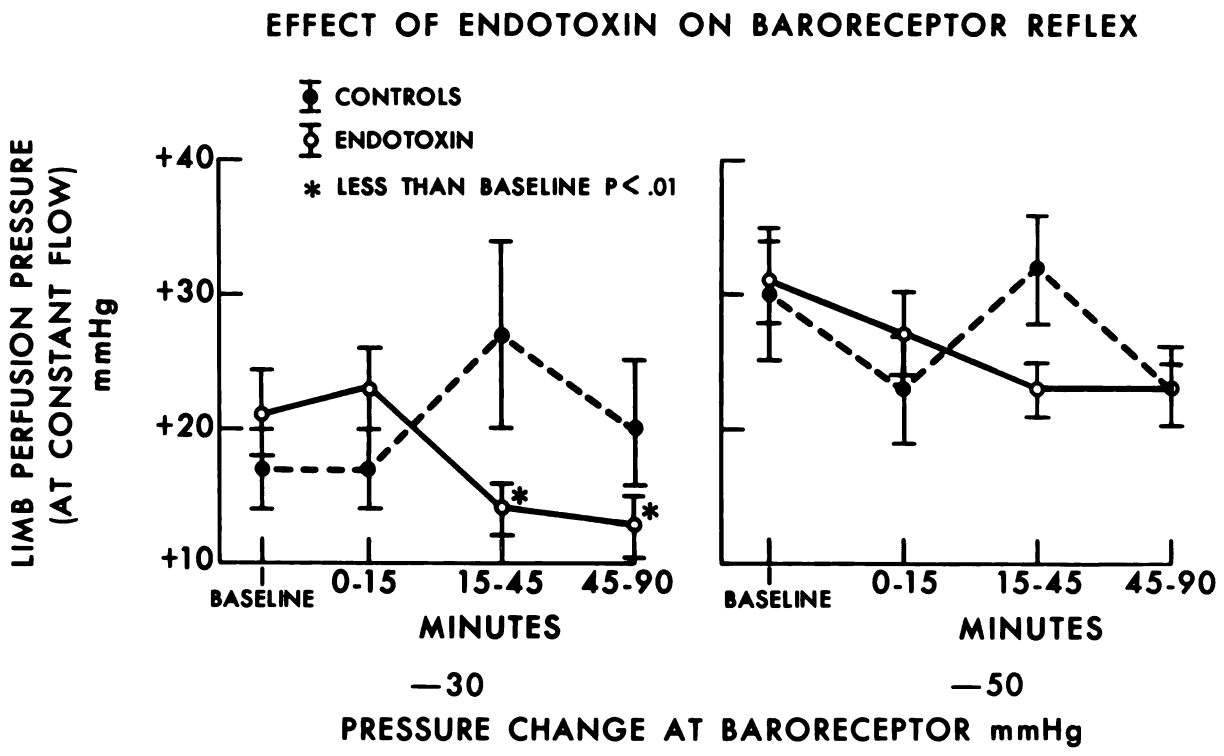

EFFECT OF ENDOTOXIN ON BARORECEPTOR REFLEX
CONTROLS
ENDOTOXIN
* LESS THAN BASELINE P < .01
LIMB PERFUSION PRESSURE (AT CONSTANT FLOW) mmHg
+40
+30
+20
+10
BASELINE
0-15
15-45
45-90
MINUTES
—30
—50
PRESSURE CHANGE AT BARORECEPTOR mmHg

Fig. 24

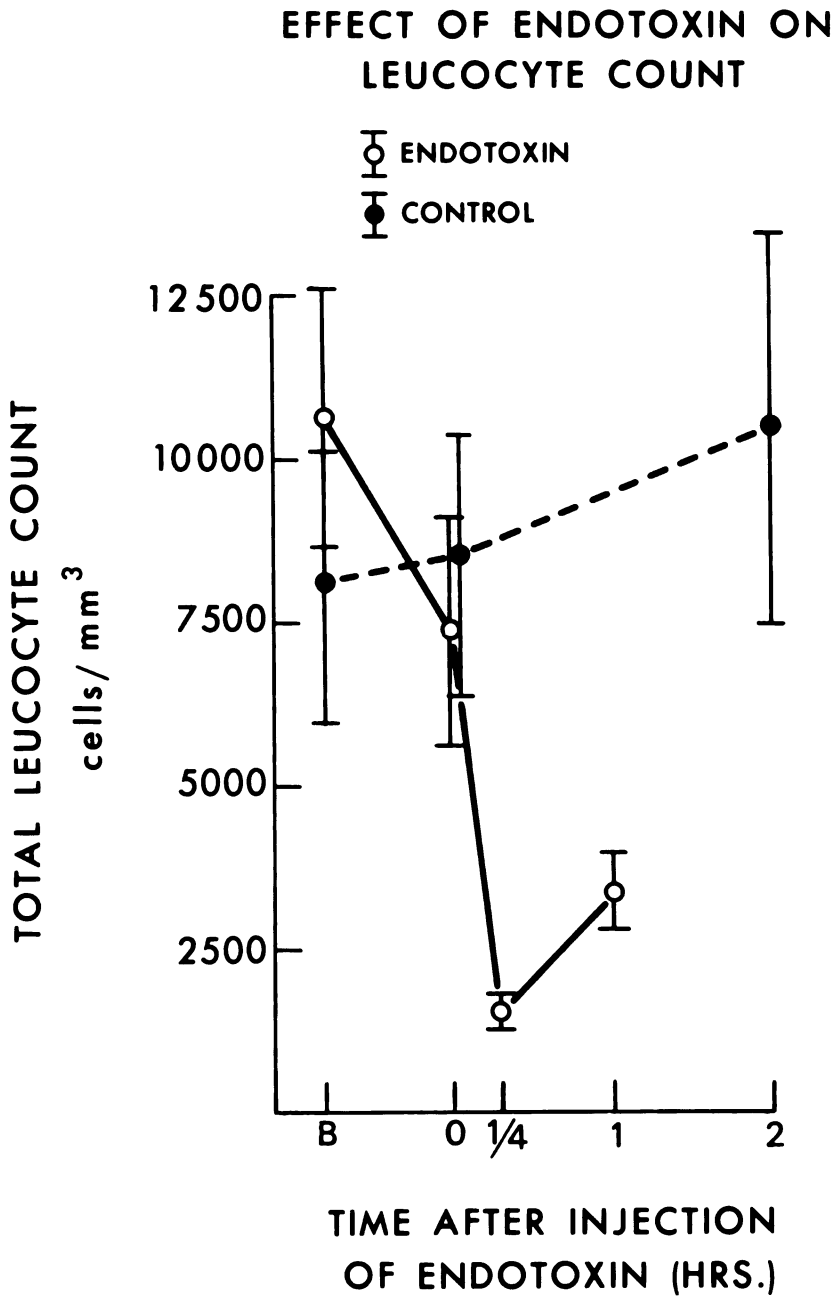
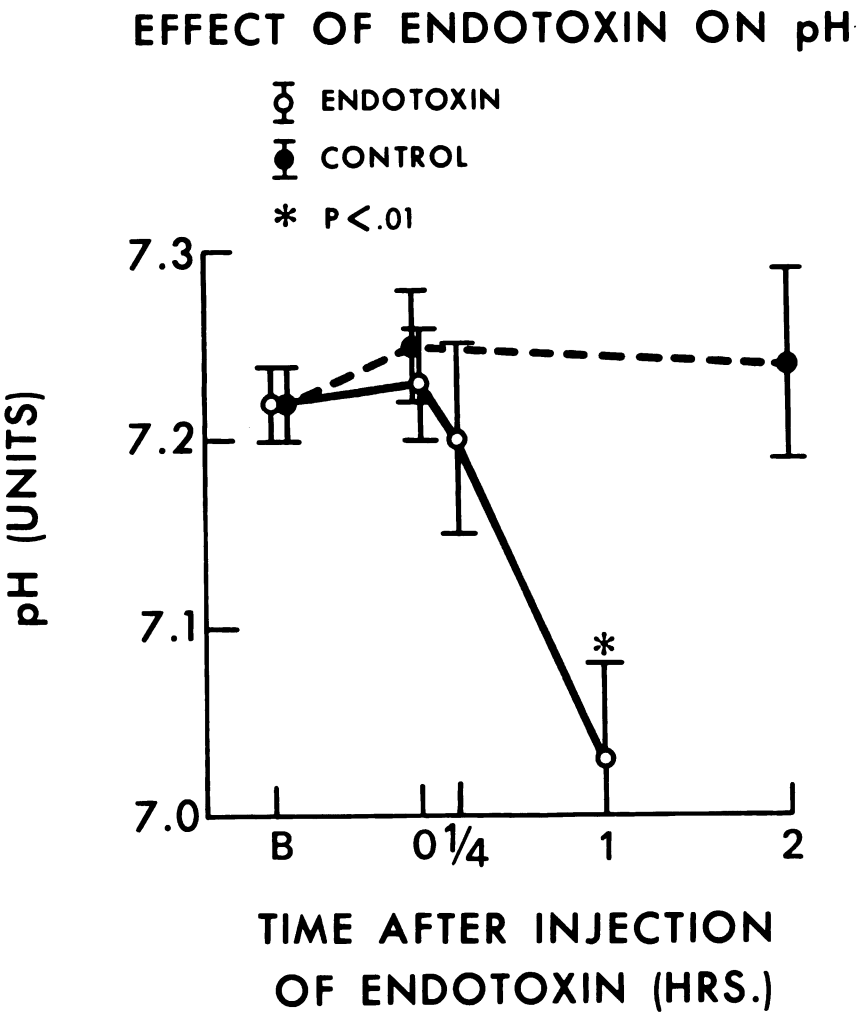

EFFECT OF ENDOTOXIN ON
LEUCOCYTE COUNT
ENDOTOXIN
CONTROL
12500
10000
7500
5000
2500
TOTAL LEUCOCYTE COUNT
cells/ mm3
B
0
1/4
1
2
TIME AFTER INJECTION
OF ENDOTOXIN (HRS.)

Fig. 25


EFFECT OF ENDOTOXIN ON pH
ENDOTOXIN
CONTROL
* P<.01
7.3
7.2
7.1
7.0
pH (UNITS)
B
0 1/4
1
2
*
TIME AFTER INJECTION
OF ENDOTOXIN (HRS.)

c. Cats-reflex exhaustion: Another possibility, that endotoxemia might lead to rapid exhaustion of the reflex vasoconstrictor response, was tested. I postulated that an appropriate initial response to baroreceptor hypotension might not be sustained. To test this possibility four animals were prepared for hind limb perfusion. The reflex stimulus used was complete carotid occlusion. Table 29 displays the results obtained in the two endotoxin treated and two control animals. Each occlusion of the carotid arteries was maintained for 60 seconds rather than 15-30 seconds as in previous experiments. The absolute values for limb perfusion pressure, and the change from baseline, at 10, 30 and 60 seconds after occlusion, are presented. As previously noted, there was progressive decrease in responsiveness with time following endotoxin infusion. However, the response to carotid occlusion was well maintained within each time period.

d. Primates-baroreceptor responses following endotoxin infusion: A small group of animals was studied to evaluate possible species differences between primate and feline. Four animals were prepared surgically. One animal proved unresponsive in the baseline period. Two animals received endotoxin infusions and the last served as a saline control. The control animals was stable throughout the observation period. There were 19 observations made in this animal (Table 30).

The two animals given endotoxin developed profound, pro-

Table 29

EFFECT OF ENDOTOXIN ON THE ABILITY TO SUSTAIN THE PRESSOR RESPONSE TO PROLONGED COMPLETE CAROTID ARTERY OCCLUSION IN THE CAT@

Duration of Carotid Occlusion (sec)

Observation period		Actual Maximum Perfusion Pressure*				Change of Perfusion Pressure*		
		Baseline	10	30	60	10	30	60
Before infusion	C	170 ± 2	177 ± 1	182 ± 1	185 ± 1	8 ± 2	12 ± 2	16 ± 2
	E	213 ± 6	214 ± 4	228 ± 3	231 ± 3	3 ± 2	16 ± 4	19 ± 4
After infusion	C	171 ± 6	179 ± 5	184 ± 5	190 ± 5	8 ± 2	13 ± 3	18 ± 4
0-15 min	E	244 ± 7	251 ± 3	260 ± 6	265 ± 6	8 ± 4	16 ± 5	21 ± 6
15-45 min	C	187 ± 6	193 ± 6	199 ± 6	200 ± 7	6 ± 1	12 ± 1	13 ± 1
	E	237 ± 5	248 ± 4	252 ± 3	260 ± 3	11 ± 3	15 ± 3	22 ± 3
45 min	C	204 ± 4	210 ± 5	214 ± 6	219 ± 6	6 ± 1	10 ± 3	15 ± 3
	E	244 ± 4	247 ± 2	253 ± 2	256 ± 2	3 ± 3	8 ± 3	12 ± 4

* mmHg Mean ± SEM
@ observations in four animals (2 saline infused - C, 2 endotoxin infused - E)

Table 30

HEMODYNAMIC AND METABOLIC EFFECTS OF SURGICAL PREPARATION AND REPEATED CAROTID OCCLUSION ON A RHESUS MONKEY

(Mean ± SEM)

Mean Arterial Blood Pressure	117 ± 5 mmHg (19)*
Heart Rate	115 ± 4 Beats/min (19)
Limb Perfusion Pressure	153 ± 6 mm Hg (19)
Perfusion Pressure Response	43 ± 2 mm Hg (19)
Cardiac Output	Baseline -1620 ml/min
	45 min-1817 ml/min
	150 min-1400 ml/min
Total White Blood Cells	12, 237 ± 1340 (mm^{-3}) (4)
PO_2	94 ± 3 mm Hg (4)
PCO_2	22 ± 3 mm Hg (4)
pH	7.34 ± 0.02 units (4)

* ( ) number of observations made during the study

gressive hypotension during the two hour period following infusion of endotoxin. Hemodynamic responses are outline in Table 31 . There were ten baseline observations and two observations at each post endotoxin time point in these animals.

The time course of mean blood pressure changes is illustrated in Figure 26. In this figure the response of mean blood pressure to complete carotid occlusion is also illustrated. The time course pattern is very similar to that of the cat (Fig. 19). The early apparent inhibition of response of mean systemic arterial pressure is evident. This is followed by a period of normal responsiveness after which the systemic pressure falls, as does response to carotid occlusion.

In Table 27 are listed the measured responses of limb perfusion pressure to carotid artery occlusion. Despite progressive systemic hypotension the limb perfusion pressure remains essentially constant. Or, it may fall slightly at a time when systemic responsiveness is lowest. The perfusion pressure changes in response to carotid occlusion also fall during this time, but then recover in parallel with systemic responses.

The heart rate (Table 31) does not follow this pattern. As systemic blood pressure falls the heart rate increases reaching a significantly increased rate. This response to systemic hypotension, in the primate, remains intact. These dichotomous results are discussed below.

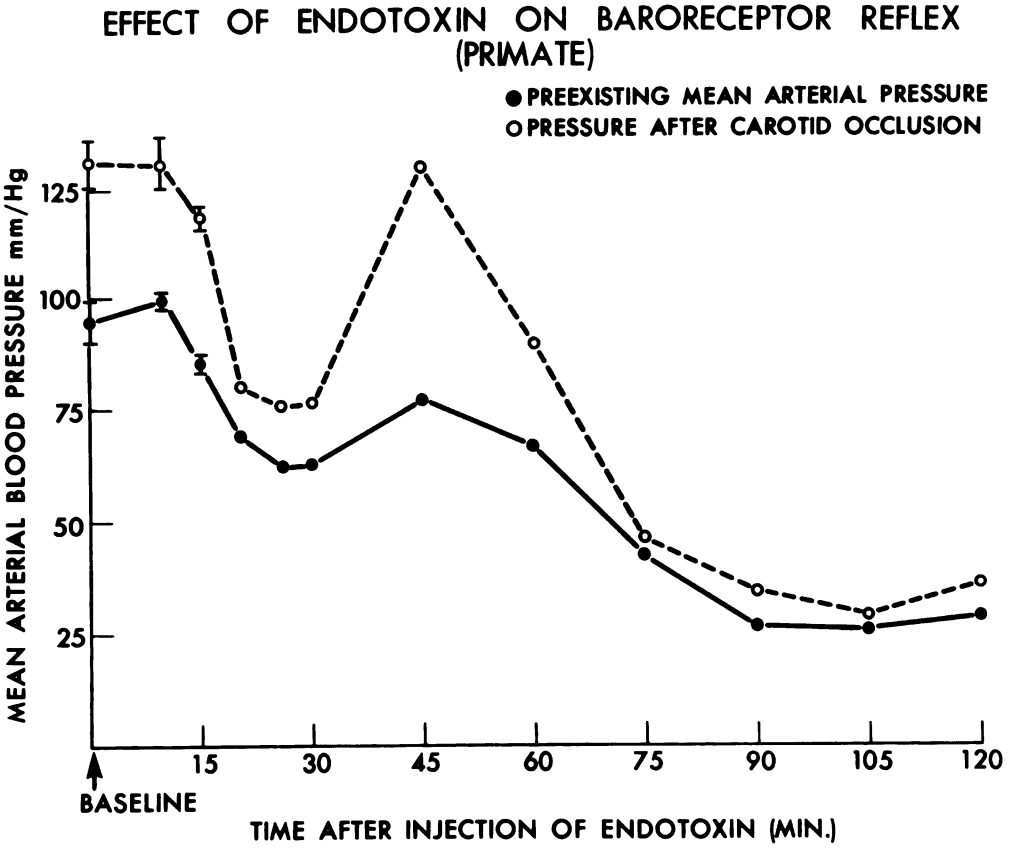
Table 31

EFFECTS OF ENDOTOXIN ON HEMODYNAMIC RESPONSES IN TWO ANESTHETIZED RHESUS MONKEYS

	Mean arterial blood pressure (mmHg)	Heart rate (Beats/min)	Hind limb perfusion pressure (mmHg)	Response to carotid occlusion (mmHg)	Cardiac output (cc/min)
Base	95 ± 5	173 ± 5	128 ± 4	25 ± 4	637, 890
10	100 ± 1	189 ± 29	135 ± 0	25 ± 5	
15	85 ± 1	193 ± 27	125 ± 1	29 ± 1	
20	70 ± 10	200 ± 19	115 ± 16	13 ± 10	
25	60 ± 10	210 ± 15	115 ± 9	10 ± 8	
30	65 ± 15	210 ± 18	120 ± 13	13 ± 7	
45	75 ± 25	215 ± 13	130 ± 0	22 ± 11	1270, ?
60	65 ± 30	215 ± 14	120 ± 7	20 ± 20	
75	40 ± 8	225 ± 5	125 ± 10	5 ± 5	
90	25 ± 8	220 ± 15	115 ± 19	12 ± 3	
120	25 ± 4	210 ± 20	100 ± 24	1 ± 1	540, ?

? Cardiac output measurement invalidated by recirculation of dye.

Fig. 26


EFFECT OF ENDOTOXIN ON BARORECEPTOR REFLEX
(PRIMATE)
● PREEXISTING MEAN ARTERIAL PRESSURE
○ PRESSURE AFTER CAROTID OCCLUSION
MEAN ARTERIAL BLOOD PRESSURE mm/Hg
125
100
75
50
25
15
30
45
60
75
90
105
120
BASELINE
TIME AFTER INJECTION OF ENDOTOXIN (MIN.)

4. Discussion:

The hypothesis that endotoxin inhibits autonomic cardiovascular reflexes, in a manner analogous to the somatic motor neuron inhibition caused by exotoxins, has been tested by direct measurements of the baroreceptor pressor reflex arc. The results of these experiments indicate that the reflex arc is intact. Apparent inhibition of responsiveness occurs, but can be shown to be indirect manifestations of endotoxin effects.

The baroreceptor pressor arc was selected for study for several reasons. The hypothesis to be tested presupposed some abnormality of this particular cardiovascular control mechanism. Using the baroreceptor itself as the site of reflex stimulus, and the resistance response as the end point of measurement, allowed testing of the entire autonomic reflex arc. Had an abnormality been uncovered, further testing would have been required to establish the particular level at which endotoxin was acting, but the lack of evidence of inhibition eliminated this requirement. Finally, it has been shown that carotid baroreceptor stimulation effects blood pressure primarily by changes in resistance, while cardiac output remains relatively constant.(Levy, Brend and Branden, 1955; Polosa and Rossi, 1961; Corcondilas, Donald and Shepherd, 1964; Olmstead, McCubben and Page, 1966; Dampney, Taylor and McLachlan, 1971).

The methods employed to examine the reflex arc were relatively simple. Perfusion of the hind limb at a constant rate of flow throughout each experiment allowed changes in

perfusion pressure to be used as a direct index of the vascular resistance in the perfused arterial bed. This fact is based upon the relationships between pressure, flow and resistance described by Poiseuille's law. That law states that flow (Q) is equal the pressure drop across a vascular system ($P_1 - P_2$) divided by the flow resistance (R). R is primarily determined by the radius of resistance vessels as discussed by Folkow and Neil in their recent text (1971).

Certain assumptions have been made in interpreting these data. The first assumption is that in the relationship ($P_1 - P_2$), the venous pressure of the perfused limb, P_2, is relatively constant under all conditions and is very small compared to P_1. P_1 is the perfusion pressure as measured at the inflow cannula. It is unlikely that P_2 is actually constant under the conditions of the experiment (Salzman, 1957). However, since venous pressure is measured in centimeters of water, and arterial pressure in hundreds of millimeters of mercury, and since each millimeter of mercury pressure represents 13.6 centimeters of water pressure, exclusion of P_2 is reasonable.

The second assumption is that the added resistance to flow imposed by the perfusion cannula can be disregarded. This assumption is valid since flow remained constant throughout each experiment. The measured perfusion pressure includes the contribution of the perfusion cannula, but changes in the perfusion pressure could result only from changes in the vascular bed.

The third assumption made is that the same vascular bed was perfused throughout the experiment. Vascular resistance is primarily determined by small muscular arterioles. If arteriovenous stunts opened during endotoxemia, and flow were diverted away from the arteriolar bed, the measured perfusion pressure would not reflect the increases in arteriolar tone and might suggest that the resistance response were decreased. Since the measured responses appeared to be normal, this possibility can be discounted. In all, the use of the controlled constant flow preparation is "the most practical expression for change in vascular tonus caused by vasomotor nerve activity. . ."(Green, 1950).

The initial input stimulus was equally simple and practical. Simultaneous, complete occlusion of both carotid arteries resulted in increased peripheral resistance. As noted, the changes in systemic pressure caused by this maneuver are primarily due to resistance increases rather than to changes in cardial output. Heymans has stated, "Carotid occlusion is not the ideal method to study the reflexes. . ."(Heymans and Niel, 1958). This statement was based upon two points. The first is the importance of pulsatile flow patterns to carotid baroreceptor function (Ead, Green and Neil, 1952). The second is the threshold concept of Koch which has been restudied and confirmed with pulsatile input (cf Heymans and Niel, 1958). These considerations are valid, but for the purposes of the present study can be excluded. Nonpulsatile stimuli produce linear baroreceptor pressor reflex

effects (Tedeschi et al, 1971). Finally, the intent of the studies reported was to examine the effects of endotoxemia on the reflex arc. The nonpulsatile stimulus, although perhaps less physiologic than a pulsatile stimulus, caused a reproduceable, controlled effect.

Initial studies suggested an apparent inhibition of responsiveness to acute carotid sinus hypotension during the first fifteen minutes following injection of endotoxin as illustrated in Figure 19. This was difficult to reconcile with the simultaneous observation, illustrated in Figure 20, that the vascular resistance, unstimulated by carotid clamping, was continuously rising during this same period. Several considerations were entertained to explain these results: Endotoxin could have been inhibiting acute responses to transient baroreceptor stimuli, while allowing slow, but progressive response to prolonged hypotension; Vascular contractility could have been compromised by some other mechanism; or the sharp fall in systemic pressure following endotoxin injection could have lowered systemic blood pressure toward the baroreceptor threshold and further decreases in carotid sinus pressure would produce relatively small effect. Finally, there could be a finite maximum to vasoconstriction, which was reached spontaneously, and which could not be exceeded even with further neurogenic stimulation caused by reduction of baroreceptor pressure.

The possibility of a finite maximum of vasoconstriction

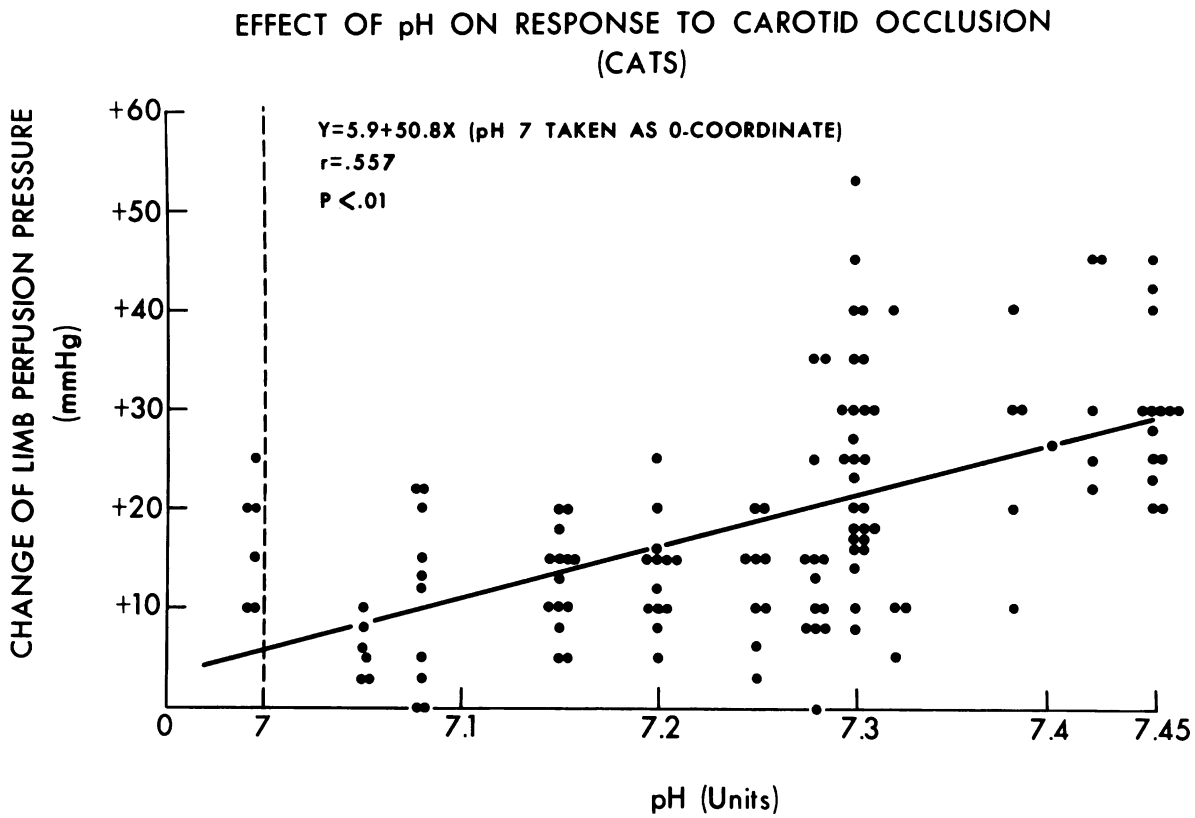
having been achieved is disproven by data obtained in later periods of endotoxemia. Observations at 30 and 45 minutes post endotoxin injection (Fig. 18) indicate that much higher maximal vascular resistances were achieved than had occurred at five of fifteen minutes. Similarly, no vasoactive substances which could have been acting as vasodilators were found. Bradykinin and histamine levels were measured in four cats injected with whole endotoxin during preliminary experiments. Plasma levels of these potential vasodilators were not elevated. Other substances may have been present, of course, but were not tested.

To determine if baroreceptor "responsiveness" were being influence by the spontaneous blood pressure change following endotoxin injection, the Mossiejeff preparation was employed. This preparation has been extensively described in the methods section. Recent use of a similar preparation in the cat produced values in close agreement with control observations in the present study (Takeguchi and Manning, 1971). With this preparation it became possible to isolate the carotid baroreceptors from the spontaneous change in pressure occurring after endotoxin injection. The baroreceptor, both before and after endotoxin injection, was exposed to a constant "baseline" and to constant pressure changes. Measurements of the end organ responses of resistance and heart rate, as well as systemic arterial pressure, were obtained. Endotoxin treated animals started from an apparently higher level of resting sympathetic tone and their

initial responsiveness was greater than nontreated animals with respect to change in heart rate. They retained this increased level of responsiveness during the first fifteen minutes of observation although the actual response in both injected and uninjected animals fell slightly. These results are taken to indicate that the apparent decrease in early responses noted with "unprotected" baroreceptors was due to the response characteristics of the receptor rather than to an inhibition of the reflex by endotoxin per se. In the Mossiejeff preparation the baroreceptor perfusate contained endotoxin and any generated vasoactive materials, so the tissue was protected only from acute mechanical changes. Since the baroreceptor can adapt slowly, perhaps the later recovery of responsiveness in animals with unprotected baroreceptors, despite persistent hypotension, was a function of such adaptation.

Early inhibition of reflex function did not occur in the controlled pressure preparations, but later periods of observation did demonstrate significant decreases in response. This decrease in response was associated with a sharp drop in systemic pH in the endotoxin treated animals as illustrated in Figure 25. Evaluation of the response to pressure change at the baroreceptor as a function of pH (Fig. 27) indicates that there is a high degree of correlation between these factors. The fall in pH can account for almost 30% of this decline of response. The regression relationship is $y = 5.9 + 50.8x$ (pH7 taken as ordinate) $r = 0.557$ ($P < 0.01$).

Fig. 27


EFFECT OF pH ON RESPONSE TO CAROTID OCCLUSION
(CATS)
Y=5.9+50.8X (pH 7 TAKEN AS 0-COORDINATE)
r=.557
P <.01
CHANGE OF LIMB PERFUSION PRESSURE (mmHg)
+60
+50
+40
+30
+20
+10
0
7
7.1
7.2
7.3
7.4
7.45
pH (Units)

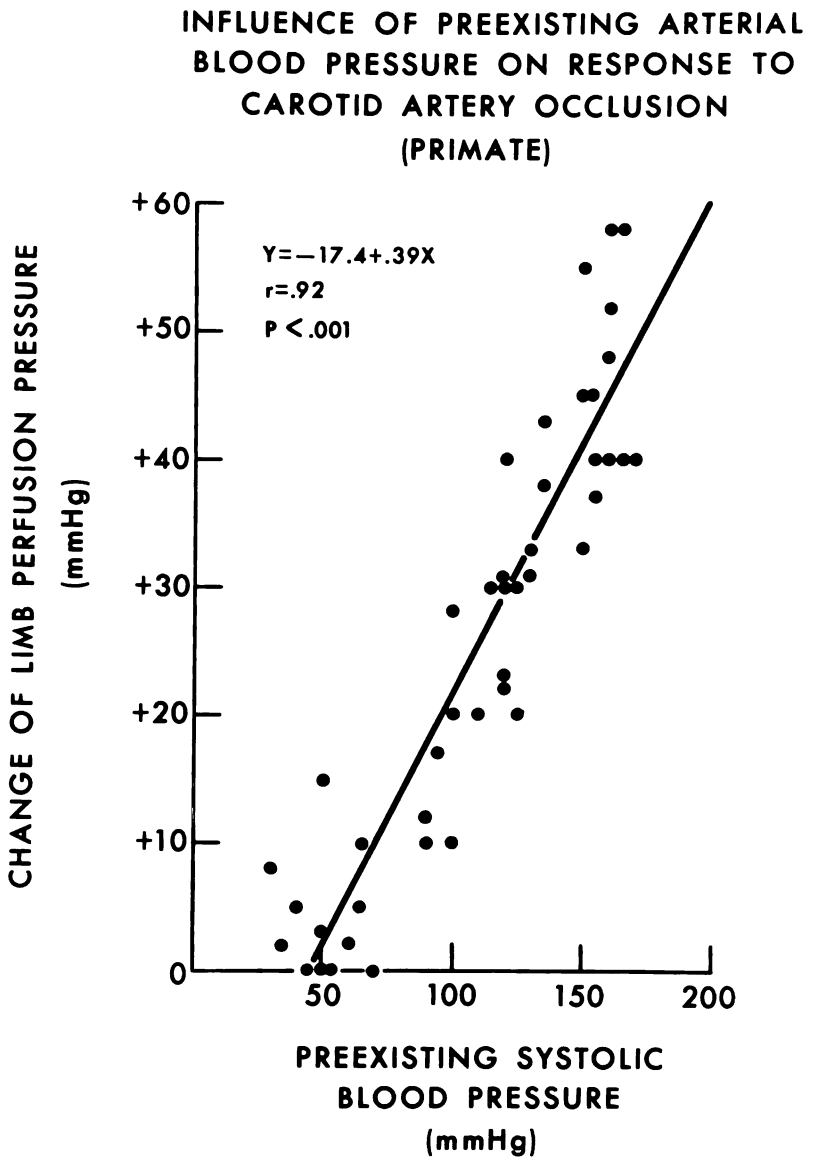
Calculation of the regression of limb response on pH in the primate gives the line $y = 1.45 + 72.5x$ where pH7 is taken for the ordinate; $r = 0.39$, $P < 0.01$. This relationship is similar to that defined for the cat. These relationships are consistent with previously documented effects of acidosis on the pressor reflex (Lewis and Mellander, 1962; Mellander and Lewis, 1963).

These results, obtained in cats, disprove the hypothesis that endotoxin inhibits the baroreceptor pressor reflex. However, species differences between cat and primate required that these observations be made in the monkey as well. In the primate, too, apparent changes in pressor reflex responsiveness following endotoxin injection are secondary effects of the lipopolysaccharide and do not indicate direct effects on the autonomic nervous system.

In both cat and primate, a period of seeming inhibition of baroreceptor pressor response occurred immediately after the injection of endotoxin. In both species, this period corresponded with a period of rapid decline of the systemic blood pressure; and in the cat responsiveness was "restored" by maintaining baseline pressures at the baroreceptor.

The Mossiejeff preparation was not used in the primate. However, it is possible to demonstrate that the responsivenexx of the pressor arc reflex is dependent upon prexisting baroreceptor pressure. This relationship is illustrated in Figure 28 and is defined by the line $Y = -17.4 + 0.39x$. The

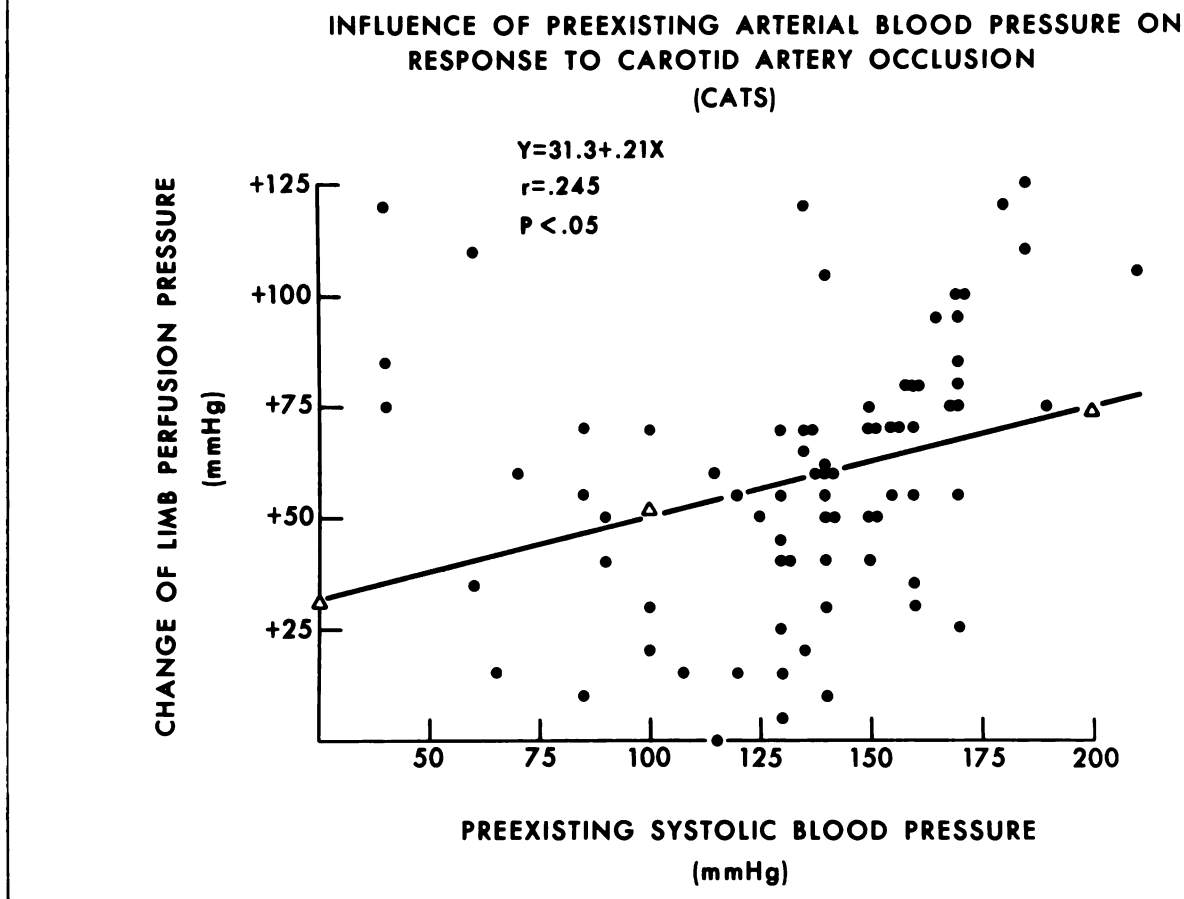
Fig. 28


INFLUENCE OF PREEXISTING ARTERIAL BLOOD PRESSURE ON RESPONSE TO CAROTID ARTERY OCCLUSION (PRIMATE)
Y=−17.4+.39X
r=.92
P < .001
+60
+50
+40
+30
+20
+10
0
50
100
150
200
CHANGE OF LIMB PERFUSION PRESSURE (mmHg)
PREEXISTING SYSTOLIC BLOOD PRESSURE (mmHg)

correlation $r = 0.92$ is highly significant ($P < 0.001$) and indicates that more than 80% of the apparent inhibition of reflex response results from the preexisting change in systemic blood pressure. A similar relationship is demonstrable in the cat (Fig. 29).

However, in the primate, the limb perfusion pressure did not rise as systemic pressure fell (Table 27). This is in contrast to the results in the cat. Since the vasodilator, bradykinin, is present in high concentration in the blood of the primate during this period following endotoxin infusion, this observation is not unexpected. In fact, the constancy of perfusion pressure offers further support to the consideration that a balance is struck between neurogenic vasoconstriction and hormonal vasodilatation during endotoxemia in the primate. The heart rate rises normally as indicated in Table 27. This further confirms that the autonomic reflex arc is intact in the primate.

Fig. 29


INFLUENCE OF PREEXISTING ARTERIAL BLOOD PRESSURE ON
RESPONSE TO CAROTID ARTERY OCCLUSION
(CATS)
Y=31.3+.21X
r=.245
P<.05
CHANGE OF LIMB PERFUSION PRESSURE
(mmHg)
+125
+100
+75
+50
+25
50
75
100
125
150
175
200
PREEXISTING SYSTOLIC BLOOD PRESSURE
(mmHg)

III CONCLUSIONS AND SPECULATIONS

The studies on mechanisms of the cardiovascular effects of gram-negative bacterial endotoxin reported in this dissertation have been only partially successful. They have provided limited answers to circumscribed questions; and for the most part those answers have been negative. In addition, many new questions have been raised.

1. Endotoxin-lipid was shown definitely to be important in the development of the characteristic cardiovascular effects of endotoxemia. As previously discussed (see pp. 72-76), the occurrence of vasodilation during early endotoxemia was related to the presence of a sufficient amount of endotoxin-lipid material in the injected preparation. A polysaccharide fraction of endotoxin (PS_2), almost lipid free and directly capable of producing effects on systemic hemodynamics, did not reproduce the cardiovascular effects of whole endotoxin. Whereas a second fraction (PS_1), similar in molecular weight and in the content of most constitutents, but containing 6.3% of the lipid present in whole LPS identically reproduced the cardiovascular effects of whole endotoxin. The similarities were manifested both in systemic and regional hemodynamics; and were apparent despite differences in the direct particulate effects of injected preparations.

Other events could also be closely linked with the presence of a sufficient amount of lipid-material in the injected endotoxin preparation. These were the generation of signifi-

cant amounts of bradykinin in the arterial blood, and prolonged granulocytopenia. These observations have also been discussed and support the hypothesis that lipid-containing endotoxin interacts with leucocytes to cause release of vasoactive substances *in vivo* as well as *in vitro*. In addition, the time course of endotoxin-leucocyte interaction and release of bradykinin closely correlates with the development of vasodilation. This reconfirms the apparent role of kinin in the development of the vasoactive events of early endotoxemia (see below).

Observations on the role of endotoxin-lipid in the development of the shock syndrome also were extended to the later phases of sepsis. The small group sizes studied preclude definite answers, but the observations raise important questions. The specific observation of note is the apparent dose-relationship between the development of late phase vasoconstriction and the total amount of endotoxin-lipid administered. This is clearly different than the lipid-dose-independent vasodilation and kinin generation of the early phase effects and suggests independent pathogenetic mechanisms for the early and late toxic manifestations of endotoxin. Both phases may be dependent upon the lipid portions of the molecule for their development, but the early cardiovascular effects of endotoxin do not appear to play an important role in late toxicity. Reports in the literature have previously suggested a disassociation of early and late phase mechanisms (Hildebrand *et al*, 1966; Rothschild and Castania, 1968; Greenway and Murthy, 1971). Clinical experience suggests that alteration of cardiovascular

effects of endotoxin does not significantly alter survival rate (Perey, 1971). The implications are, therefore, that direct cellular toxicity may be more important in clinical septicemia, and perhaps therapy should be designed to influence this aspect of endotoxin effect.

Although mediator release and early cardiovascular effects may not be important for late phase events, they may be useful as markers of cellular toxicity. Definition of the mechanisms by which endotoxin causes the release or generation of these substances by cells may provide useable in vitro models for pharmacologic testing.

2. The dose association of bradykinin generation with the cardiovascular events of early endotoxemia was directly explored in a second series of experiments. The hypothesis tested was; bradykinin is the vasoactive mediator of endotoxemia, therefore kinin infusion should reproduce the cardiovascular effects of endotoxin. However, the comparison of the cardiovascular effects of bradykinin with those known to be caused by endotoxin produced an apparently negative result. The pattern of cardiovascular response to kinin infusion, at doses sufficient to reproduce the arterial kinin concentrations occurring during endotoxemia, was similar to responses caused by activation of autonomic cardiovascular reflexes by a variety of mechanisms. Blockade of these reflexes more closely reproduced the endotoxin pattern (see below).

But, an important consideration, discussed on p. 89, was

not satisfied in the experiments conducted as described. During spontaneous endotoxemia bradykinin concentrations would presumably be highest in the microvasculature and lowest in arterial plasma. In the experimental situation the gradient would be reversed, and arterial plasma concentrations would be higher than microvascular concentrations. Under such circumstances, neurogenic mechanisms could be sufficiently effective so as to overcome the local peptide effects. In the spontaneous circumstance the concentrations of peptide could conceivably be so high as to completely obscure reflex vasoactive events. The possibility that the peptide can effectively overcome reflex events has been discussed (p. 88). Available evidence is insufficient to further evaluate this question. The fact that baroreceptor-mediated reflex events remain intact during endotoxemia (see below) suggests that the gradient hypothesis is true. Final confirmation will be obtained when a specific bradykinin blocking agent is developed. Specific blockade of kinin effect or generation will critically test the sufficiency and necessity of the peptide for the cardiovascular events of endotoxemia.

The possibility that other vasoactive agents play a role in these events remains open to speculation. There is no evidence available at present to support or refute such a contention. As noted, evaluation of such a possibility may prove useful in the development of <u>in vitro</u> models of cellular toxicity of endotoxin. Similarly, questions as to specific cardiovascular effects associated with endotoxemia, such as

alterations in cardiac output, or the precise mechanisms by which compensatory vasoconstriction is prevented, remain unanswerable with information currently available.

3. The possibility was examined that endotoxin can influence cardiovascular reflex mechanisms by inhibiting the baroreceptor-pressor reflex. Initial experiments suggested transient inhibition of reflex responsiveness during the initial systemic hypotension following endotoxin injection. Similar results were obtained in cats and primates.

In the former species, it was possible to obviate apparent reflex inhibition by maintenance of stable perfusion pressures at the carotid baroreceptors. The components of the entire reflex arc were exposed to endotoxin, even in animals with isolated baroreceptors. Therefore prevention of inhibition was the result of control of baroreceptor pressure and is consistent with theories of threshold responsiveness of these receptors.

The Mossiejeff preparation was not used in the primate. Correllation of the reflex response with preexisting blood pressure was significant, however. Although this does not prove that the Koch threshold mechanism was operational in the monkey it is highly suggestive. The similarity between the responses of cat and monkey also suggests that similar mechanisms were operational in both species.

The unique difference between the responses of these

animals was the lack of spontaneous vaso-constriction in the primate. As noted this is consistent with the fact that a vasodilator (bradykinin) is present in the monkey but not in the cat. This further supports the consideration of a balance between peptide induced vasodilation and neurogenic vasoconstriction, as previously discussed.

The results of the experiments reported in this dissertation do not define the mechanisms by which the cardiovascular effects of endotoxemia occur. They do, however, throw into sharper focus two questions of significance. Those questions are:

1. Can the role of bradykinin as a potential mediator of endotoxin effect be clarified? This is, in essence, the same question initially asked in these studies. The available results only further emphasize the need for an answer. Methodology and experimental design must be developed to provide new approaches before it will be solved, however. A solution to this question will also probably provide answers to ancillary points relating to the cardiovascular events of endotoxemia.

2. Do the early cardiovascular effects of endotoxemia play a significant role in the progression of shock? This is probably a more pressing question. If the toxicity of endotoxin directly depends upon effects on metabolism or cell function, and early hypotension is not a significant contributor to progression, then the activity of vasoactive mediators becomes moot. In addition, if direct cellular toxicity is of

primary importance, pharmacologic investigations should preferentially be directed at those mechanisms.

However, it may be possible to develop methods to explore these questions. Those methods would involve the control of the mechanisms by which vasoactive mediators are released in response to endotoxin. Such control would allow final definition of the role of these mediators; the significance of mediator-controlled events to the progression of shock; and would provide measurable markers for the cellular toxicity of endotoxin.

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