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SELECTED CELLULAR AND SUBCELLULAR INTERACTIONS OF TOXIC AND DETOXIFIED ENDOTOXIN WITH EUCARYOTIC CELLS

By

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ABSTRACT

SELECTED CELLULAR AND SUBCELLULAR INTERACTIONS OF TOXIC AND EXPERIMENTALLY MODIFIED ENDOTOXIN WITH EUCARYOTIC CELLS

By

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Organ distribution and cell association of toxic and experimentally modified endotoxin *in vivo* and *in vitro* was compared. More than 50% of intravenously (i.v.) injected toxic endotoxin or poly L-α-ornithine mixed endotoxin (each 200 μg/mouse) became associated with the liver and spleen. The pattern of distribution did not change between 1 and 5 h. Alkaline detoxification of endotoxin, which changed the physical and chemical properties of endotoxin, also changed the organ distribution in that significantly less detoxified endotoxin (180 μg/mouse) was recovered from liver and spleen. Once fixed, the cellular and subcellular distribution of detoxified endotoxin did not significantly change between 1 and 5 h.

Association of endotoxin with hepatoma tissue culture cells (HTC) cells differed in several ways from *in vivo* organ distribution. For example, HTC-cell association and nuclear transfer of toxic and poly L-α-ornithine mixed endotoxin was a gradual and time dependent process. Poly L-α-ornithine increased *in vitro* cell association of

endotoxin by almost 10-fold. HTC-cell association of alkaline-treated detoxified endotoxin was 3- to 8-fold higher than toxic endotoxin and increased with time. Cumulatively, these observations indicate that while tissue culture cells could conceivably provide a more controllable experimental system in which to study the fate and pathogenic mechanisms of endotoxin at the cellular and subcellular level, HTC-cells, under the conditions employed herein did not yield binding data which compared favorably to in vivo results.

The present study also examined the in vitro interaction of toxic and experimentally detoxified endotoxin with chromatin, DNA and steroid hormone-receptor complexes. Toxic endotoxin was not able to bind to DNA alone, but relatively higher amounts could slightly inhibit the binding of glucocorticoid hormone-receptor complexes binding to DNA (14.6% inhibition/300 µg endotoxin). Toxic endotoxin was capable of interacting with HTC-cell chromatin (1 µg endotoxin/20 µg DNA) almost four times more efficiently than alkaline-treated detoxified endotoxin (0.24 µg endotoxin/ 20 µg DNA). Interaction of glucocorticoid-receptor steroid complexes with chromatin was inhibited in a dose dependent manner by both toxic and detoxified endotoxin, but again relatively high concentrations of endotoxin were needed. Under certain conditions endotoxin can block hormone-receptor complex binding to the DNA and chromatin. The biological

significance of this observation is complicated by the need of high concentrations of toxic endotoxin and by the fact that biologically detoxified endotoxin works even more efficiently than toxic endotoxin.

DEDICATION

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INTRODUCTION

The chemical and biological properties of bacterial endotoxin have been under investigation for more than a century. Thousands of papers dealing with chemical structure, purification and biological effects of endotoxin have been published during the past two decades. Unfortunately, the time and effort devoted to endotoxin has not produced the same level of understanding of its biological action as exists for many protein toxins. Endotoxin manifests a surprising multiplicity of biological effects in animals, but it is still not clear which of these are important in lethality.

Endotoxin has a wide range of biological activities that affects all systems of the host. Some of the major effects of endotoxin are shock, lethality, cardiovascular changes, pyrogenicity, immunogenicity, antitumor activity, Shwartzman reaction, mobilization of interferon, metabolic changes, cytotoxicity, interaction with complement, release and sensitization to histamine, abortion, changes in blood clotting, development of tolerance, effect on the reticulo-endothelial system (RES), protection against irradiation, leukopenia and leukocytosis, effects on properdin or natural antibody levels and adjuvant effects (7,61).

Despite the wide range of the biological changes which have been associated with bacterial endotoxin it elicits few cytological or morphological changes. The varied biological effects of endotoxin make it clear why so much attention, time and effort has been devoted to study of this bacterial poison.

One major school of thought on the significant factors in the pathophysiology of endotoxemia lies in its ability to induce host metabolic alterations, particularly in liver parenchymal cells (12-14,16,52-55). These changes range from depletion of carbohydrate reserves to the inhibition of gluconeogenesis and hormonal induction of certain liver enzymes (43,72,73). Whether endotoxin exerts its effects on parenchymal cells directly (90) or through soluble mediators (57) has not been definitively established. A previous report from this laboratory (90) indicates that endotoxin becomes associated with liver parenchymal cells in vivo and tissue culture cells in vitro. If the interaction of endotoxin with tissue culture cells mimics its interaction in vivo, then tissue culture cells would provide a more controlled environment for studying endotoxin-cell interactions. In the present study, cell and tissue association and distribution of toxic and modified endotoxin in whole animals and in hepatoma tissue culture cells is compared. The second goal of this study is to gain insight into the nature of the inhibition of hepatic enzyme induction and the mechanism of the protective activity of cortisone against endotoxin lethality.

The results of these experiments show that caution should be exercised in using tissue culture cells as a model to study endotoxin cell interaction and the fate of endotoxin in the cell. This study also indicates that ^{51}Cr-labeled endotoxin can interact with chromatin but not with DNA in the form of DNA-cellulose. Endotoxin in able to inhibit the binding of activated-hormone-receptor-complex (AHRC) to chromatin and DNA in a dose dependent manner, but the amount of endotoxin which is needed for significant inhibition of binding of AHRC to DNA and chromatin is far higher than is required to elicit the biological consequences of endotoxin.

Chemical Structure of Endotoxin

It is well established that endotoxin is an integral part of the outer membrane of gram negative bacteria (29). Endotoxin is a lipopolysaccharide (LPS) having a molecular weight ranging from 1 to 20 million daltons depending upon the method of purification. It forms aggregates easily which explains its very high and variable molecular weight (61). Chemically endotoxin consists of three parts: the lipid moiety, the R-core and the O-polysaccharide. The basic structure of the lipid moiety, known as lipid A, is a diglucoseamine unit that contains fatty acids attached to amino and hydroxyl groups (19). A fatty acid which seems to be unique to lipopolysaccharide (LPS) and which is usually found in the highest concentration is β-hydroxymyristic acid (46). The glucose-amine units are bound together by 1-6 glycosidic linkages and 1,4-phosphodiester bridges (1,32,64). The R-core region links lipid A to the O-polysaccharide moiety. The existence of a group of mutant bacteria termed R or rough forms which lack the O-polysaccharide has facilitated the investigation of the R-core structure. Chemical analysis of the R-core has shown five sugars, phosphate, and O-phosphorylethanolamine (37). The O-polysaccharide structure of bacterial endotoxin determines the O-antigenic specificity of the bacterium. Biochemical analyses of this portion of LPS molecule have demonstrated a repeating sequence of sugars (38).

Modification of Biophysical and Endotoxic Properties of Endotoxin

Chemical and biological modifications of endotoxin have been used in the search for an explanation of endotoxin toxicity. Goodman and Sultzer (33) studied the effects of mild alkaline hydrolysis on bacterial endotoxin. They obtained partial alkaline hydrolysis of LPS by dissolving 50 mg of LPS in 3 ml of 0.25 N NaOH and heating the solution at 56°C for 60 min as originally described by Neter et al. (60). Their results indicated that while the treatment reduced the lethality of endotoxin for mice by about 100 fold, there were no significant changes in the fatty acids of lipid A. Particle size was significantly reduced, and the material was more homogeneous and soluble than untreated LPS. Mild alkaline hydrolysis was found to enhance lps mitogenicity for murine B lymphocytes.

Skidmore et al. (78) prepared detoxified endotoxin by incubating LPS at a concentration of 1 mg per ml in 1.0 N NaOH at 56°C for 60 min. The solution was then neutralized with HCl and dialyzed against phosphate buffered saline. This type of treatment greatly reduced *in vitro* mitogenic activity and toxicity of LPS without affecting the chemical structure of the antigenic polysaccharide (65). Skidmore et al. (78) established that 1 N NaOH treated LPS was non-mitogenic for spleen cells and also showed a positive correlation between the *in vitro* activity of LPS as a lymphocyte mitogen and its *in vivo* activity as adjuvant and

as an immunogen. Tauber et al. (84) reported that hydroxylaminolysis is an effective method for detoxifying endotoxin and for removing almost all of its fatty acid ester groups.

While endotoxin-blood interactions have been intensively studied, the mechanism of LPS detoxification in the vascular compartment remains a subject of controversy. The mechanisms proposed to account for LPS detoxification involve either the cells of the reticuloendothelial system or humoral factors. Skarnes (77) believes that circulating plasma represents a principal site of detoxification and that plasma esterases of the nonspecific, carboxylic types are of major concern in defense against circulating endotoxins. His results indicate that within the first few hours following intravenous injection of endotoxin a decrease in ionized calcium, a three-fold increase in heat-stable esterase levels and a striking increase in the endotoxin detoxifying capacity of plasma all occur in the circulating plasma of the recipient.

Ulevitch and Johnston have recently reported (85) that normal rabbit, human, or mouse serum (or plasma) reduces the buoyant density of endotoxin from Escherichia coli O111:B4 ($d = 1.44\ g/cm^3$) and Salmonella minnesota R595 ($d = 1.38\ g/cm^3$) to a value $<1.2\ g/cm^3$. This density shift was associated with the inhibition of pyrogenic activity, the ability to produce an immediate neutropenia, and the anticomplement activity of endotoxin. Preliminary results from their laboratory indicated that the density shift did not occur as a result of the degradation of the glycolipid backbone of the

LPS. These data suggest that the interactions of LPS with plasma (or serum) components leading to reduction in buoyant density without degradation of LPS may account for a major pathway of LPS detoxification. In another report, Ulevich et al. (86) indicate that this reduction in buoyant density of endotoxin requires a plasma (or serum) lipid. Delipidation of plasma (or serum) prevented the conversion of the parent entotoxin to a low density form. Addition of purified high density lipoproteins (HDL) to delipidated plasma (or serum) restored both the ability to reduce the buoyant density of endotoxin and the capacity to abrogate the activities of endotoxin.

Freudenberg et al. (30) used the method of crossed immunoelectrophoresis to investigate early changes in plasma proteins of rats treated with LPS. Their results indicated that intravenous injection of either a smooth (S) or rough (R)-form of endotoxin leads to alterations in the HDL of plasma. The changes were dose dependent and identified as being due to the formation of a complex of LPS with HDL. They followed the fate of the complex in the plasma of injected rats, and showed that the R-form LPS complex disappeared after several hours, whereas the S-form LPS complex was still partly present after 2 days. Their results indicate that the time endotoxin circulates in the blood may depend on several factors, one of which may concern the type (S or R) endotoxin. Freudenberg et al. suggested that HDL may represent a transport protein for LPS in plasma to organs of clearance or to other cellular targets.

Radiolabeling of Endotoxin

To study the mode of endotoxin action, it is necessary to study the fate of endotoxin in the host and in the cell. The ability to radiolabel endotoxin has facilitated this type of study in recent years. Radioactive iodine (71), ^{32}P (40) and ^{14}C (48) have all been used to label endotoxin. Braude et al. (17) devised a method of firmly attaching ^{51}chromium in the form of sodium chromate or chromic chloride to the endotoxin of Escherichia coli. The toxicity of ^{51}Cr-labeled endotoxin was not altered as a result of the labeling procedure. Both the sodium chromate and the chromium chloride were able to bind to endotoxin firmly, however the acidic condition in the case of chromium chloride resulted in the loss of toxicity of endotoxin. His observation also indicated that serial dilutions of labeled endotoxin resulted in a proportional decrease in both radioactivity and toxicity. Braude et al. concluded that the close quantitative correlation between toxicity and radioactivity justifies the use of ^{51}Cr-labeled endotoxin. In a subsequent study Braude et al. (18) compared the organ distribution of the free ^{51}Cr to the ^{51}Cr bound endotoxin. Their results indicated that there is a significant difference in the distribution of free ^{51}Cr and ^{51}Cr-labeled endotoxin. These data support the stable nature of the ^{51}Cr-labeled endotoxin and reveal that the ^{51}Cr does not break away from endotoxin upon injection.

Chedid et al. (21) undertook a series of experiments to further clarify the character of ^{51}Cr-labeled endotoxin. Their results indicated that centrifugation of a freshly

prepared ^{51}Cr-labeled endotoxin mixture yielded a heavy, highly labeled pellet containing the toxic, labeled moiety. The supernatant contained a weakly labeled product which was nontoxic. Further studies showed that 9% of an injected dose (50 micrograms) of ^{51}Cr-labeled endotoxin was still recoverable from the plasma 6 hours after injection. Recovered endotoxin proved to be lethal for adrenalectomized mice, which had been previously shown to be susceptible to as little as 0.05 micrograms of endotoxin. Furthermore, autoradiographic studies demonstrated that the recoverable, toxic material was still labeled. Chedid's et al. work clearly demonstrate that ^{51}Cr does not dissociate from ^{51}Cr-labeled endotoxin after injection of toxic material.

Effect of Polycationic Polymers on the Entry of Macromolecules Into Mammalian Cells

Uptake of a macromolecule is the first necessary step toward a biological effect in a host cell. Ryser et al. (69,70) used albumin-I^{131} and monolayers of sarcoma S-180II cells as a model to study penetration of foreign proteins into mammalian cells. Their results indicated that transport proceeds at very low rates, requires little energy, and is markedly enhanced by polycationic polymers and certain polynucleotide aggregates. This enhancement phenomenon increases with the molecular size of the polymers. The stimulation of albumin uptake by 3 µg of poly-D-lysine or poly-L-ornithine per milliliter is approximately ten fold. Ryser et al. (70) suggested that polycationic polymers initiate the entry of macromolecules into the cells by

making multiple attachments with the negatively charged cell surface. While the sites of cell surface interactions of polynucleotide aggregates might not be the same as for polycationic polymers, it is likely that multiple contacts occur between polynucleotides and the cell membrane to trigger the entry of macromolecules. This enhancement phenomenon should be useful to investigators concerned with the intracellular functions of foreign macromolecules.

Interaction of Endotoxin with Cells

The problem of how such a large and complex molecule interacts with cells or membranes has not been resolved. Anderson et al. (3) and Dumont (26) have suggested that lipid A inserts into the lipid bilayer of cell membranes. Endotoxin has been shown to rapidly adhere to the surface of red blood cells (RBC). Coated RBC can be used to detect antibodies against endotoxin by indirect hemagglutination. In 1970, Springer et al. (83) described an inhibitory substance from the ghosts of human RBC which prevented attachment of LPS to RBC. The inhibitory material had no apparent effect on the binding of Salmonella Vi (virulence) antigens or binding of group and common antigens from the gram positive bacteria investigated. Springer et al. demonstrated the interaction between the chemical groups of LPS that attach to RBC and the inhibitory material. This inhibitory fraction was named LPS receptor. The receptor can displace the endotoxin after it has attached to RBC leading the authors to suggest that its blocking is physical and not enzymatic.

The receptor was shown to be sensitive to pH changes on either side of pH 7 and to be heat labile. In later publications (81,82) Springer et al. isolated a lipoglycoprotein (receptor) from smooth and rough strains of all gram negative bacteria tested. This "receptor" was able to block endotoxin's attachment to red cells. Receptor activity was destroyed by proteases and was found to be rich in N-acetylneuraminic acid, galactose, and hexosamines. They estimated that the LPS receptor molecule weighed about 256,000 daltons. Another report from Springer and Adye (80) indicated that they had been able to extract the LPS receptor with n-butanol water from isolated platelets, granulocytes, and mononuclear leukocytes of humans. The extracts contained 40 to 52% glycerophosphatides, 15 to 22% sphingomyelin, 17% cholesterol, less than 2 to 5% triglycerides and 7 to 13% inactive peptides. According to Springer and Adye (80) lipid A inhibited the binding of LPS to RBC. Studies of this type may elucidate how endotoxin gets into cells.

Alkaline-treated LPS had a greater affinity for RBC than toxic endotoxin (22). Hemolysis results when excessive quantities of alkaline-treated LPS are added to RBC. Benedetto et al. (6) used artificially produced phospholipid bilayer and monolayer membrane models. Their results indicated that alkaline treated LPS was at least ten times more efficient than the native product in penetration. Benedetto et al. concluded that the interaction of LPS with phospholipid monolayers was by a combination of penetration and adsorption to the undersurface.

Fate of Endotoxin In vivo and In vitro

The results of numerous investigations on the fate of endotoxin in the host indicates that endotoxin rapidly accumulates in the reticuloendothelial-cell-rich organs after iv injection. The spleen and especially the liver appear to be primarily involved (44). Other sites of considerable endotoxin accumulation are the endothelium of blood vessels and the lung alveoli. The reaction of endotoxin with white blood cells is very rapid. The results of Rubenstein et al. (68) indicate that within 10 min after injection of radiolabeled endotoxin, polymorphonuclear leukocytes are labeled with endotoxin. Herring et al. (39) found that platelets absorb endotoxin. Most endotoxin became fixed to tissues or organs by 30-60 min and little change was seen in this distribution even after several days (41,90). However, the Musson et al. report (59) indicated a significant change in the organ distribution of endotoxin with time. Inconsistancy in observations may reflect differences in animal species, in the type of endotoxin or perhaps, most importantly, the amount of particulate material in endotoxin preparations. In this regard, it is worth noting the nonparticulate nature of endotoxin used by Musson et al. (59).

In order to gain insight into the potential target tissues of endotoxin the distribution of LPS in LPS-responsive (C_3H/st) and LPS-unresponsive (C_3H/HeJ) mice was studied and compared (59). The results of this study demonstrated that C_3H/st spleens accumulated significantly more LPS than C_3H/HeJ

spleens after intravenous injection of either an immunogenic or a toxic dose of LPS. After a toxic dose of LPS, there is more LPS in C_3H/st lymph nodes, adrenals, lungs, kidneys, and heart than in the corresponding C_3H/HeJ tissue. Musson et al. (59) believe that the differential accumulation of LPS in the tissues of these two strains may be the reason for the decreased responses of C_3H/HeJ mice to LPS.

The liver contains a number of cell types. While the macrophages (Kupffer cells) are thought to be the main reservoir of in vivo clearance (41,47) there is evidence that liver hepatocytes are also important for the clearance of endotoxin (87,90). With autoradiography, Willerson et al. (87) demonstrated the presence of ^{14}C-labeled endotoxin in liver hepatocytes as well as Kupffer cells.

Zlydaszyk and Moon (90) found that over 80% of intravenously injected ^{51}Cr-labeled endotoxin was trapped in livers of normal mice 1 hr after the injection. Liver fractionation studies revealed that nearly 45% of the labeled toxin was associated with cell nuclei within the liver, 20% with the mitochondrial-lysosomal fraction and approximately 30% with the cell sap. To distinguish the distribution of labeled endotoxin between parenchymal and Kupffer cells, a population of mixed liver cells was exposed to 1% pronase for 1 hr at 37°C to lyse the parenchymal cells. After this treatment all the counts that were not sedimentable were assumed to be parenchymal cell associated. It was found that over 75% of the counts were parenchymal cell associated and the Kupffer

cells contained only 25% of the labeled endotoxin. In an *in vitro* study they tested the endotoxin uptake of six non-reticuloendothelial system tissue culture cell lines. All cell lines were able to internalize endotoxin after 3 hr incubation at 37°C. Once internalized, the labeled endotoxin was predominantly associated with the nuclear fraction. Zlydaszyk and Moon (90) believe that both *in vivo* and *in vitro* nonreticuloendothelial system cells, as well as reticuloendothelial cells, can sequester endotoxin.

Pathogenesis of Endotoxemia

Several investigators have tried to link the pathogenesis of endotoxin to its effects on mitochondria. Pioneer work of Fonnesu and Severi (27,28) has shown that mitochondria isolated from livers of endotoxin-poisoned rats had a depressed rate of oxidative phosphorylation compared with those from normal rats. Harris et al. (36) isolated mitochondria from beef heart and added endotoxin directly to mitochondria. They observed a loss of respiratory control, inhibition of respiration of NADH-linked substrates, inhibition of coupling of phosphorylation to terminal electron transport, and a change in morphology of the inner membrane of mitochondria. De Palma's et al. (24) results indicated that endotoxin altered the structure of hepatic mitochondria and depressed their energy production.

One of the characteristics of endotoxin shock is lowered blood flow to vital organs such as brain and kidney. Mela et al. (50) investigated the effect of tissue ischemia on the

mitochondria isolated from guinea pig brain and kidney after 18 to 24 hr of Escherichia coli endotoxemia. They found that at 18 hr brain mitochondrial Ca^{++} transport was inhibited by 35%, while kidney mitochronria transport function was still normal. At 24 hr, both brain and kidney mitochondria exhibited severe inhibition of Ca^{++} transport and ATP synthesis. Mela et al. (50) believe that because of the brain's sensitivity to tissue isochemia, it becomes an important metabolic target organ in septic and endotoxic shock.

Several metabolic changes after endotoxin poisoning have been detected in liver parenchymal cells (12-14,16,52-55). These metabolic changes may be significant contributing factors to the pathophysiology of endotoxemia.

Bacterial endotoxin has been known for many years to lower the carbohydrate reserves in the mammalian host (51). Abnormal carbohydrate metabolism, characterized by an initial hyperglycemia, followed by a prompt decrease in blood sugar to hypoglycemic levels and a concomitant decrease in liver glycogen (16), contributes to the pathogenesis of endotoxemia.

Protective Role of Adrenocortical Hormones Against Endotoxin Poisoning

The importance of adrenocortical (ACTH) hormones in an animal's response to endotoxin has been established by removal of the adrenal gland, which causes a thousand fold decrease in the LD_{50} of endotoxin. ACTH is used therapeutically in

the treatment of gram negative septicemic shock. Pioneer work of Duffy and Morgan (25) established the protective effects of ACTH and cortisone against the febril response of endotoxin in rabbits. They found that the dosage and the pretreatment of animals with ACTH or cortisone are two critical factors with respect to the ability of the hormone to alter the toxic effects of endotoxin. Geller et al. (31) supported Duffy's et al. results by demonstrating that pretreatment of mice with 5 mg cortisone acetate 1 hr prior to a lethal dose of endotoxin consistantly protected 60 to 90% of the mice. Berry (7) found that the injection of endotoxin and 5 mg cortisone acetate simultaneously protected 78% of mice from the lethal effect of endotoxin. If cortisone acetate was give 4 hr after endotoxin injection only 16% of the animals survived. These results support the importance of time of administration and dosage of cortisone in altering the toxic effect of endotoxin.

Several investigators have attempted to elucidate the protective mechanism of corticosteroids in lethal endotoxemia. Ribble et al. (63) investigated the distribution of ^{51}Cr-labeled endotoxin in mice which were pretreated with 5 mg of cortisone acetate 8 hr prior to the injection of endotoxin. Results from this study indicated that there was no significant difference in the tissue distribution of endotoxin in cortisone pretreated animals compared to untreated controls. Cremer and Watson (23), used fluorescent antibody techniques to detect the alteration in the tissue distribution of intravenously

injected endotoxin in cortisone acetate-pretreated animals. Their results indicated that the distribution of endotoxin in the RES of the rabbits studied was unchanged following cortisone acetate pretreatment. There was no significant difference in the rate of phagocytosis by steroid treated animals compared to untreated controls; however, degradation and elimination seemed impaired. Agarwal (2) found that endotoxin inhibits the binding of tritiated cortisone to nuclei in liver and spleen of Swiss albino mice. He suggested that cortisone and endotoxin may compete for the same receptor or proreceptor entities. Cumulatively, these data do not support the idea that the antagonistic nature of glucocorticoids in endotoxin poisoning is related to the alteration in the tissue distribution of endotoxin. The activity of the steroid hormones in the maintenance of near-normal carbohydrate levels in endotoxin-poisoned mice (see above) suggests a possible action at the metabolic level.

The Mediation of Endotoxemic Effects

There are uncertainties as to whether the parenchymal cells are the direct targets of the endotoxin or whether the toxic effects of endotoxin on parenchymal cells are mediated by factors within the circulatory system.

It has been known for several years that endotoxin antagonizes the glucocorticoid induction of hepatic tryptophan oxygenase (TO) and phosphoenolpyruvate carboxykinase (PEPCK) (7). Endotoxin has a similar effect on glycogen synthase (49) but does not have any effect on tyrosin aminotransferase (TAT). By developing and using specific antiserum

to TO and PEPCK Rippe and Berry (66,67) and Berry and Rippe (11) established that endotoxin inhibits the synthesis of these enzymes rather than inactivating them in the liver. Shtasel and Berry (74) determined the effect of cortisone and endotoxin, singly and in combination on the ribonucleic acid (RNA) synthesis in livers of mice by measuring the incorporation either of inorganic ^{32}P or of ^{14}C-orotic acid into the RNA. The effect of these agents on protein synthesis was evaluated with the use of ^{14}C-leucine. The results of this study indicated that bacterial endotoxin increased the synthesis of RNA and protein in the liver. Cortisone increased the uptake of isotope into liver RNA but depressed the incorporation of leucine into hepatic protein. Shtasel and Berry (74) assumed that since there was a net increase in hepatic protein, and mRNA synthesis, the block in the hepatic enzyme induction in response to endotoxin must be selective. They have suggested that the block in the hepatic enzyme induction is mediated. Berry (8) considered interferon as a possible mediator of endotoxin action but their results indicated that mice injected with either living or ultraviolet-irradiated Sindbis virus were unable to induce TO, PEPCK and TAT in response to hydrocortisone injection. Berry et al. (15) and Moore and Berry (56) found that synthetic double stranded RNA poly I:C, an inducer of interferon, increased the sensitivity of mice to LPS and mimicked the antagonism of endotoxin on glucocorticoid induction of PEPCK and TO.

Recent reports have suggested the existance of a plasma mediator called glucocorticoid-antagonizing factor (GAF) (10). Serum from endotoxin-poisoned mice was able to inhibit the hormonal induction of PEPCK in endotoxin-tolerant mice, whereas endotoxin itself did not, suggesting that endotoxin-tolerant animals fail to produce GAF in response to LPS but if GAF is provided exogenously they are able to respond normally. Moore et al. (57) used antimacrophage serum to demonstrate the role of macrophages in the production of GAF. Moore et al (57) injected mice with mouse antimacrophage serum and observed a full induction of TO and PEPCK in response to concurrent administration of hydrocortisone and endotoxin. Additional data (57) also indicate that the supernatant fluid from an overnight culture of macrophages incubated in the presence of endotoxin, also inhibited the hormonal induction of PEPCK in endotoxin-tolerant mice. Because of these results, Moore et al. have suggested that macrophages are the source of GAF. Since endotoxin fails to block the glucocorticoid induction of TO and PEPCK in nu/nu (nude) mice, Moore et al (58) used these animals to demonstrate an apparent need for T cells in the release of GAF. When serum or peritoneal cells from zymosan-pretreated animals was combined with endotoxin *in vitro* and introduced into nude mice, PEPCK induction by glucocorticoids was blocked. From these observations they concluded that either T-cells are needed for GAF formation or that macrophages of athymic mice are deficient in their ability to produce GAF. Goodrum et al. (34) found that

indomethacin, a nonsteroidal, anti-inflammatory agent suppresses the appearance of GAF in serum as well as its production by peritoneal exudate cells. Indomethacin did not interfere with the animal's response to GAF. It is not clear how indomethacin prevents the production of GAF. Berry et al. (9) tried to analyze the mode of action of GAF by examining its effect on PEPCK induction in mice and hepatoma cell cultures. They tentatively suggest that GAF acts at the level of transcription. GAF is able to inhibit the induction of only PEPCK, not TO in both conventional and athymic mice (79). Whether GAF and interferon are identical is not clear.

Mechanism of Action of Steroid Hormones

Steroid hormones play a central role in development and physiological regulation in animals ranging from arthropods to primates. A large body of circumstantial evidence suggests that all steroid hormones act via basically the same mechanism. Because of their lipophilic nature, steroids readily pass into the cytoplasm of the target cells where they bind to specific receptors (88). This binding process results in an "activated" state of the steroid receptor which now has an increased affinity for chromosomal binding sites in the nucleus. The consequence of the presence of steroid-receptor in the nucleus is determined by the particular biological response characteristic of the target tissue. It has been widely assumed that because of their lipophilic properties, the steroids enter the cells by diffusion, and the cell membrane provides little or no barrier to them. Target

tissue contains receptors that avidly bind the hormones. This observation has led to the assumption that the greater accumulation of hormone in target tissues results from their ability to retain steroids rather than a difference in the uptake of the hormone (35). When steroids enter the cells they bind both to receptors as well as many nonspecific proteins. The binding of steroid to receptor is specific and characterized by a high affinity. It is believed that a conformational change in the receptor occurs when the steroid is bound. By using sucrose density gradients a good body of evidence indicates a gross change in the size of the receptor after estrogen binding and exposure to temperatures of 20°C or above. The estrogen-receptor complex may take a variety of forms (4S, 5S, 6S, or 8S) in the cytoplasm, and it is not clear in which form the receptor exists prior to estrogen binding or entry into the nucleus (35). Translocation of steroid-receptor complexes to nuclei occurs via nuclear pores and does not seem to be an energy-dependent process because various metabolic inhibitors have no effect on the process (35).

One major unanswered question is the chemical nature of the chromatin binding site for the receptor steroid complex. Several lines of evidence suggest that glucocorticoid-receptor complexes bind directly to DNA. Baxter et al. (5) found that receptor-steroid binding to isolated hepatoma tissue culture (HTC)-cell nuclei was greatly diminished by pretreatment of nuclei with DNase, but if complexes were bound to nuclei prior

to DNase treatment, they remained bound during the digestion process. Their results also indicated that receptor-steroid complexes bind to purified HTC-cell DNA with characteristics similar to the binding of receptor-steroid complexes to isolated nuclei. Tomkins and his group (89), by a new genetic approach, provided evidence that the glucocorticoid-receptor binding site on chromatin is DNA. They developed a glucocorticoid-resistant mutant cell from mouse lymphoma tissue culture cells. Normally, these cells were killed by the addition of glucocorticoids (75). Further studies (89) indicated that the mutant cells fell into three classes: one class of mutants had 8-26% of the receptors in the nucleus, a second class of mutants had 85-93% of the receptors in the nucleus and a third class had no receptors, whereas the wild type cells had 50% of the receptors in the nucleus. Those mutants with fewer nuclear receptors were called nt^- (nuclear transfer deficient) and those with more nuclear receptors than the wild type were called nt^i (increased nuclear transfer). Yamamoto et al. (89) examined the binding of the receptors from these mutants to DNA on DNA-cellulose columns. They found that nt^- mutant glucocorticoid-receptors had little affinity for the DNA, but the nt^i showed approximately the same affinity as the wild type. The authors concluded that the critical interaction of the receptors in the nucleus occurs with the DNA. Another report from Tomkins's (76) laboratory indicated that chromosomal proteins reduce the number of acceptor sites while increasing the affinity of DNA for glucocorticoid-receptors. Other reports indicated

chromosomal proteins (62), ribonucleoprotein particles (45) and the nuclear membrane (42) as potential nuclear acceptor sites for steroid-receptors. Even though several reports (75,76,89) strongly suggest that DNA is the primary nuclear binding site for steroid-receptors, firm conclusion in this area may have to await more definitive biochemical assays.

The demonstration that nuclear translocation of receptors in vivo is a nonsaturable process, was in conflict with reports of saturable binding to nuclei (35,88). Chamness et al. (20) resolved this problem by demonstrating that apparent saturation of nuclear sites in vitro was due to competition of excess nonreceptor components for receptor binding sites. In their experiments they added increasing concentrations of receptor to nuclei in the presence of constant protein concentration. By eliminating the differential effect of the competing nonreceptor components in this way, they observed that a constant number of the receptors bound to nuclei regardless of the receptor:nucleus ratio. These results clearly demonstrated that nuclear binding of receptors was not saturable, thus conforming more closely with in vivo observations. The observation of Chamness et al. (20) led the way for the discovery of an inhibitory material which is present in cytosol and which inhibits the binding of steroid-receptor complexes to nuclei, chromatin and DNA. The experiments of Simons et al. (76) showed that HTC-cell cytosol contains a material which inhibits the binding of homologous receptor-steroid complex to HTC-cell nuclei or chromatin and DNA. The inhibitors appear to be macromolecular and can be

removed without destroying the receptor-steroid complex. Simons et al. (76) suggested that the most likely mechanism of inhibitor action involved association of inhibitor with the receptor to diminish the affinity of the complex for acceptor sites.

Baily et al. (4) have described another type of steroid-receptor inhibitor which is a low molecular weight, thermostable (resistant at 100°C for 30 min) molecule. Their primary results have indicated that the inhibitor acts on the activation process of steroid-receptor complexes.

Rationale for the Present Study

In summary, the mechanism by which steroid hormones cause specific gene transcription and exert their biological activity is not well advanced, but the basic features of steroid action have been outlined and are similar for all steroids. The initial steps in hormone action involve the rapid entry of the steroid into the cells followed by binding to cytoplasmic receptor proteins. The receptor-steroid complexes eventually become concentrated in the cell nucleus and this association apparently results in an increase in specific mRNA. The product of translation of mRNA leads to the biological reactions (76). Whatever the precise mechanism of steroid action may be, it is important to note that in endotoxin shock early glucocorticoid therapy has been shown to be protective against endotoxin lethality (7,25,31). The *in vitro* model of the steroid-receptor system is a much more controllable model than an *in vivo* system. In theory the *in vitro* system provides an excellent model from which to gain insight into the pathogenic

mechanisms of bacterial endotoxin and the protective nature of glucocorticoids in endotoxin shock. Studies that critically analyze the effect of endotoxin on glucocorticoid binding might also be of value in elucidating the mechanism of endotoxin action at the molecular level.

Despite extensive effort in the past two decades, the mechanism(s) by which bacterial endotoxin exerts its pathophysiological changes has not been definitively explained. Several metabolic changes in liver parenchymal cells after endotoxin poisoning (12-14,16,52-55) have been considered significant contributors in the overall pathophysiology of endotoxin shock. Whether these changes are mediated through soluble products released from macrophages or other cells after phagocytosis of endotoxin (8,9) or whether liver parenchymal cells are directly affected by endotoxin is not clear (90). It is known that substantial amounts of endotoxin are trapped in the liver *in vivo* and become associated with parenchymal cells (87,90). If endotoxin is directly affecting liver parenchymal cells, the *in vivo* and *in vitro* interaction of endotoxin with parenchymal cells should be similar. Beginning from the hypothesis that interaction of endotoxin with non-RES hepatic cells is basically similar *in vivo* and *in vitro*, experiments were designed to gain insight into the nature of this interaction. Experimentally, toxic and a variety of modified endotoxin were used to test the validity of this hypothesis.

In early preliminary experiments attempts were made to make primary cell culture preparations of parenchymal cells directly from the liver to use in *in vitro* studies on endo-

toxin uptake. Cell viability was very poor and *in vitro* uptake studies yielded unreliable data. Since no permanent non-transformed cell lines were available, the decision was made to do the bulk of our studies on the well characterized hepatoma tissue culture (HTC) cell line. This parenchymal-derived cell line seemed particularly well suited for our studies for a number of reasons. First, HTC-cells are a parenchymal-derived hepatoma cell line and in our study we were particularly interested in the direct interaction of endotoxin with non-phagocytic hepatic cells. Several reports (87,90) have indicated that substantial amounts of endotoxin trapped in the liver *in vivo* become associated with parenchymal cells. Parenchymal cells exhibit most of the metabolic changes associated with endotoxin shock thus making hepatic derived cell lines an excellent candidate in which to evaluate and compare the basic interactions of endotoxin with cells *in vitro*. Second, this cell line is the most extensively characterized hepatoma cell line with respect to interaction with steroid hormones *in vitro*. The cells are steroid-responsive (5) and hence provide an excellent cell line to explain endotoxin's ability to inhibit the induction of selected hepatic enzymes by glucocorticoids (8).

The cell line does not behave exactly like hapatic cells *in vivo* in all respects. For example, two glucocorticoid inducible enzymes namely TO and PEPCK are not found in these cells. Despite these selected drawbacks, the fact that these are a suspension culture cell line and demonstrate selected responses consistent with those *in vivo* made them by far the best choice of cells to use at the inception of our study.

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ABSTRACT

In vitro and In vivo Association and Subcellular Distribution of Toxic and Experimentally Modified Endotoxin

Cell association and organ distribution of toxic and experimentally modified endotoxin in whole animals and hepatoma tissue culture (HTC) cells was compared. Mouse organ distribution of toxic and poly L-α-ornithine mixed endotoxin indicates that most of the endotoxin is associated with the reticuloendothelial (RES) rich organs, and organ distribution of neither one changes over a 5 hour period. Significantly less detoxified endotoxin is recovered from RES rich organs of mice. HTC-cell association and nuclear transfer of toxic endotoxin is a gradual and time-dependent process. Poly L-α-ornithine at a concentration of 4 μg/ml significantly increases HTC-cell association of endotoxin. Nuclear transfer of endotoxin mixed with poly L-α-ornithine, like toxic endotoxin, is a gradual and time-dependent process. HTC-cell association of alkaline-treated detoxified endotoxin is significantly higher than the association of toxic endotoxin and increases with time. In contrast to toxic and poly L-α-ornithine mixed endotoxin, nuclear association of alkaline-treated detoxified endotoxin does not significantly change during 5 hour incubation. These observations indicate that while tissue culture cells could provide a more controllable

experimental system in which to study the fate and pathogenic mechanism of endotoxin at the cellular and subcellular level, HTC-cells under the conditions employed here do not yield binding data which compare favorably to *in vivo* results. Caution must be exercised when extrapolating *in vitro* data to the actual *in vivo* action of endotoxin.

A previous report from this laboratory (21) indicates that non-RES cells such as mouse liver cells and tissue culture cells as well as cells of the RES system, can sequester endotoxin. If conditions could be established whereby binding and internalization of endotoxin by tissue culture cells would mimic *in vivo* realities tissue culture cells could provide a much more controllable model for detailed study of the biological action of endotoxin. This report compares toxic and a variety of experimentally modified endotoxin preparations with respect to their *in vivo* distribution among organs and tissues of mice. A more detailed comparison of binding to hepatic tissues *in vivo* and HTC-cells *in vitro* is also presented.

The various endotoxin preparations include alkaline detoxified and plasma treated endotoxin. Alkaline hydrolysis cleaves ester linked fatty acids from lipid A and significantly reduces lethality and pyrogenicity *in vivo*. Alkaline detoxification also enhances binding to red blood cells and reduces endotoxin particle size but does not affect its antigenic specificity (5). Normal rabbit, human or mouse serum (or plasma) also reduces toxicity and buoyant density of endotoxin (18,19). The density shift is associated with the inhibition of pyrogenic activity, the ability to produce an immediate neutropenia and anti-complementary activity of endotoxin.

A third manipulation of endotoxin was exposure to polycationic polymers. Polycationic polymers and certain

polynucleotide aggregates enhance the entry of macromolecules into cultured mammalian cells (16,17). Ryser has suggested that polycationic polymers initiate the entry of macromolecules into cells by making multiple attachment with the negatively charged cell surface (16).

Chemicals

Lyophilized Salmonella typhimurium lipopolysaccharide (LPS) extracted by the Westphal procedure was obtained from Difco Laboratories (Detroit, MI). It was stored at 4°C and dissolved in isotonic, pyrogen free sterile saline.

Chromium-51 ($Na_2{}^{51}CrO_4$) was purchased from New England Nuclear Corp. (Boston, MA) in 2 mCi lots, with a specific activity of approximately 300-500 Ci/g. Limulus amebocyte lysate was obtained from Microbiological Associates (Walkersville, MD) and stored in a lyophilized form at -20°C. Pyrogen-free saline was purchased from Cutter Laboratories (Berkeley, CA). Poly-L-α-ornithine was obtained from Sigma (St. Louis, MO).

Alkaline Hydrolysis of Endotoxin

Mild alkaline-treated endotoxin was prepared by dissolving 50 mg of LPS in 3 ml of 0.25 N NaOH. The solution was heated at 56°C for 60 minutes (7). The modified endotoxin was precipitated with cold anhydrous ethanol, washed three times with ethanol, dialyzed against deionized distilled water and lyophilized.

In vivo Plasma-treated Endotoxin

In vivo plasma-treated ^{51}Cr-labeled endotoxin was prepared by injecting 15 mice intravenously (i.v.) with 400 μg ^{51}Cr-labeled endotoxin per mouse. Mice were bled into heparinized tubes 1 minute later and the plasma prepared.

Plasma was incubated with excess HTC-cells at 37°C for 20 minutes to adsorb nonspecific agglutinins. After agglutinin adsorption, the whole plasma was used to study HTC-cell association and cell distribution of plasma-treated endotoxin.

For some experiments, ^{51}Cr-labeled endotoxin was recovered from plasma by centrifuging at 100,000xg for 10-12 hours. The pellets (CA 50% of the label usually recovered) were resuspended in 1.8 ml MEM and 250 µl (about 70 µg LPS) was added to 1.25 x 107 HTC-cells.

Preparation of Chromium Labeled Endotoxin

To 100 mg of LPS, 1.5 mCi of chromium-51 was added. The mixture was dissolved in 10 ml of normal physiological saline and incubated at 37°C for 48 hours with constant stirring. This step was followed by dialysis at 4°C against deionized water for 3 to 5 days. The labeled endotoxin was centrifuged at 8000xg for 1 hour and the supernatant was recentrifuged at 100,000xg for 8 hour. The latter pellet was suspended in 4.0 ml of deionized distilled water and kept at -70°C. This preparation contained high molecular weight, highly toxic lipopolysaccharide fraction (4), with a specific activity of CA 7 µCi/ml for toxic endotoxin and 3.5 µCi/mg for 0.25 N NaOH treated endotoxin. For individual experiments, the extent of ^{51}Cr decay was calculated and incorporated into the final calculations (20).

Distribution of ^{51}Cr-labeled Endotoxin in Experimental Animals

Mice were injected intravenously with 0.20 mg of ^{51}Cr-labeled endotoxin, ^{51}Cr-labeled endotoxin mixed with 48 µg

poly-L-α-ornithine, or 0.18 mg ^{51}Cr-labeled 0.25 N NaOH treated endotoxin. After 1, 3 and 5 hours, mice were killed by cervical dislocation and the amount of radioactivity determined in liver, gall bladder, spleen, kidneys, intestine, heart, lung, brain, carcass, skin and feet. Subcellular distribution of ^{51}Cr-labeled endotoxin within the liver was determined by homogenizing the organ in two ml of TKM-0.25 M sucrose (0.05 M Tris HCl, pH 7.5 at 20°C; 0.025 M KCl; 0.005 M $MgCl_2$; and 0.25 M sucrose) in a glass homogenizing tube with a Teflon pestle. Two ml of the homogenate was centrifuged at 800 x g for 15 minutes. Counts per min in the pellet (crude nuclear fraction) after one wash with TKM-0.25 M sucrose and supernatant (non-nuclear fraction) were determined. Pure nuclei were prepared by mixing 1.0 ml of liver homogenate with 2.0 ml of 2.3 M sucrose in TKM. The mixture was then underlaid with 1.0 ml 2.3 M sucrose in TKM and centrifuged for 40 minutes at 120,000 x g at 0°C (2). After centrifugation the supernatant was poured off and the wall of the tubes were wiped with a spatula and Kimwipe. The pellet (pure nuclei) was counted in a gamma counter. About 50% of the nuclei were recovered in the pellet and 50% remained in the supernatant as measured by the distribution of DNA (3). Between 85 and 90% of the injected counts were routinely recovered from the whole animal.

Cell Association and Intracellular Distribution of ^{51}Cr-labeled Endotoxin by Hepatoma Tissue Culture Cells (HTC)

HTC-cells were grown in MEM supplemented with 5% heat inactivated fetal calf serum. The cells were harvested and

suspended in 25 ml MEM at a concentration of $5x10^5$ cells per ml. Approximately $1.2-2.5x10^5$ cpm of either ^{51}Cr-labeled toxic endotoxin (23 µl of 20 mg/ml), toxic endotoxin mixed with poly-L-α-ornithine (4 µg/ml) or alkaline treated endotoxin (50 µl of 6.3 mg/ml) was added to the cells. The mixture was incubated at 37°C with constant stirring in a suspension culture flask. At 1, 3 and 5 hour time intervals, 5 ml samples were removed, washed twice with Tris buffer [0.04 M Tris (hydroxymethyl) aminomethane-hydrochloride, 0.1 M KCl, 0.001 M $MgCl_2$; pH 7.2]. The pellet was transferred to a fresh tube to avoid increased counts stuck to the glass and counted in a Packard gamma counter. To determine the extent of nuclear association of LPS, the counted cells were treated with 0.5% Nonidet P-40 (NP-40) for 10 minutes on ice and centrifuged at 800xg for 10 minutes. Counts per minute in the supernatant (non-nuclear fraction) and the pellet (crude nuclear fraction) were determined.

Limulus Lysate Test

All glassware was sterilized and made pyrogen-free by dry heat (180°C for 4 hours). Disposable sterile pyrogen-free pipets were used for all experiments. The limulus lysate test was performed on toxic endotoxin, endotoxin mixed with poly L-α-ornithine (20 µg LPS/4 µg ornithine) and 0.25 N NaOH treated endotoxin. Toxic endotoxin and pyrogen-free saline were used as positive and negative controls.

Ten-fold serial dilutions of the respective samples were prepared in pyrogen free saline. An aliquot (0.1 ml) of the sample was mixed with an equal volume of limulus lysate and

incubated at 37°C. Changes in the opacity and clotting were judged visually on a scale of 1 to 4.

Isopycnic Density Gradient

Isopycnic density gradient ultracentrifugation was performed with CsCl solutions of an average density (d) of 1.40 to 1.50 g/cm^3. Samples were centrifuged to equilibrium at 40,000 rpm for 60 hours at 20°C in the Spinco model L3-50 ultracentrifuge (Beckman Instruments Co.) with the SW 50.1 rotor. Fractions of 0.1 ml were collected and the densities calculated from the index of refraction measured with a refractometer (Bausch and Lomb).

Lethality Studies in Mice

Charles River CD-1 mice (18-22 g) sensitized with lead acetate were used to determine LD_{50} of the various endotoxin preparations. The median lethal dosage was determined by injecting mice with 5 mg of lead acetate in 0.2 ml saline intraperitoneally (IP). Animals receiving lead acetate alone served as controls. For each concentration of endotoxin, at least six animals were used. Survivors were scored after 72 hours and the LD_{50} was calculated by the method of Reed and Meunch (15).

Comparison of Selected Biological and Physical Properties of Toxic and Experimentally Modified Endotoxin

Both similarities and differences were noted in the various endotoxin preparations used in this study. There was no significant difference in the limulus lysate reactivity of endotoxin and endotoxin mixed with poly L-α-ornithine (20 μg LPS/4 μg ornithine) (Table 1). Alkaline-treated endotoxin was about 1,000-3,000-fold less toxic as indicated by both limulus lysate assay and LD_{50} analysis. The LD_{50} of endotoxin mixed with ornithine was two-fold lower than untreated endotoxin.

The buoyant density of endotoxin and endotoxin mixed with ornithine was the same (1.51 g/cm^3). There was a decrease in the buoyant density of plasma-treated endotoxin (1.44 g/cm^3) and an increase in the density of 0.25 N NaOH treated endotoxin (1.59 g/cm^3).

Distribution of Toxic and Experimentally Modified Endotoxin Among Organs of Normal Mice.

Table 2 shows the organ distribution of labeled toxic endotoxin at 1, 3 and 5 hours after iv injection. At all time points about 50% of the ^{51}Cr-labeled endotoxin was found in the liver. There was no significant difference in the organ distribution of endotoxin throughout the ensuing 5 hour study. About 7,000-8,000 cpm (12%) of the liver-associated counts were recovered from crude nuclear fraction. Only a much

TABLE 1

Comparison of selected biological and physical properties of toxic and modified endotoxin

Experimental	LD_{50} in lead acetate treated mice (μg)	Limulus lysate (μg/ml)	Buoyant density (g/cm^3)
Toxic LPS*	0.31	1×10^{-3}	1.51
Ornithine mixed LPS	0.15	1×10^{-3}	1.51
0.25 N NaOH treated LPS	1000	1	1.59
In vivo plasma treated LPS	NP**	NP	1.44

*Lipopolysaccharide

**Not performed

smaller fraction of labeled endotoxin could be recovered from pure hepatic nuclei which suggests that most of the labeled endotoxin was actually bound either to the surface of nuclei or other contaminating cellular debries. There was no gradual increase in the nuclear transfer of endotoxin in vivo between 1 and 5 hours as seen in hepatoma cells.

In vivo whole organ distribution of endotoxin mixed with ornithine, was not significantly different from endotoxin alone through the entire 5 hour study (Table 3). Liver nuclear association of endotoxin mixed with ornithine fluctuated in a somewhat random manner. The significant increase in the pure nuclei association of endotoxin mixed with ornithine at 3 hours cannot be explained.

The organ distribution of 0.25 N NaOH-treated endotoxin differed significantly from toxic endotoxin. After 1 hour significant differences could be observed in the liver and spleen, and the carcass, skin and feet. Only 20-26% of 0.25 N NaOH-treated endotoxin was associated with the liver. Significantly less alkaline-treated endotoxin was also recovered from the spleen and significantly more was associated with the carcass, skin and feet than for toxic endotoxin. There was no significant redistribution of 0.25 N NaOH-treated endotoxin during the 5 hour study. No gradual nuclear transfer of alkaline treated endotoxin was observed.

Association and Subcellular Distribution of Toxic and Mouse Plasma-treated Endotoxin by HTC-cells.

Table 5 compares association and subcellular distribution of toxic and mouse plasma-treated endotoxin within HTC-cells.

TABLE 2

Organ and tissue distribution of toxic ^{51}Cr-labeled endotoxin (200 µg) in mice

	Liver Nuclei cpm[a]		Counts Per Minute[a]									
	Pure	Crude	Liver	Spleen	Kidney	Intestine	Heart	Lung	Brain	Carcass	Skin & Feet	Gall Bladder
1 hr	336 ± 104	7189 ± 441	60640 ± 4213	9839 ± 1431	1867 ± 305	5375 ± 1624	952 ± 315	2951 ± 404	180 ± 72	27976 ± 5918	5193 ± 1455	23 ± 15
3 hr	252 ± 25	8142 ± 674	65231 ± 4511	10208 ± 861	2823 ± 250	10372 ± 1123	710 ± 85	1866 ± 108	150 ± 29	28365 ± 2595	6712 ± 516	97 ± 15
5 hr	366 ± 27	7462 ± 775	65643 ± 4787	7553 ± 358	2147 ± 235	8990 ± 1012	481 ± 60	2049 ± 305	123 ± 16	26583 ± 2248	6849 ± 945	95 ± 31

a = Mean of cpm ± standard error of the mean obtained from six experiments

Table 3

Organ and tissue distribution of ^{51}Cr-labeled toxic endotoxin (200 µg) mixed with poly L-α-ornithine (48 µg) in mice

	Liver Nuclei cpm[a]		Counts Per Minute[a]									
	Pure	Crude	Liver	Spleen	Kidney	Intestine	Heart	Lung	Brain	Carcass	Skin & Feet	Gall Bladder
1 hr	284[b] ± 17	5685 ± 321	52745[c] ± 2005	8588 ± 915	3120 ± 298	8795 ± 1504	1201 ± 140	3566 ± 443	641 ± 286	40076[d] ± 4216	10930 ± 3114	18 ± 6
3 hr	553 ± 60	13237 ± 994	60392 ± 3675	8349 ± 806	2174 ± 190	7987 ± 886	574 ± 95	2252 ± 416	149 ± 29	29410 ± 2773	8255 ± 1572	32 ± 12
5 hr	322 ± 37	13419 ± 1076	61585 ± 3617	8598 ± 823	2115 ± 186	9446 ± 743	491 ± 75	2155 ± 327	135 ± 17	28878 ± 2183	6465 ± 573	44 ± 15

a = Mean of cpm ± standard error of the mean obtained from six experiments

b = Not significantly different from 5 h

c = Not significantly different from 3 and 5 h

d = Not significantly different from 3 and 5 h

Table 4

Organ and tissue distribution of ^{51}Cr-labeled 0.25 N NaOH treated endotoxin (180 μg) in mice

	Liver Nuclei cpm		Counts Per Minute									
	Pure	Crude	Liver	Spleen	Kidney	Intestine	Heart	Lung	Brain	Carcass	Skin & Feet	Gall Bladder
1 hr[a]	187 ± 35	1424 ± 240	9603 ± 790	464 ± 44	1128 ± 110	4020 ± 310	664 ± 119	688 ± 68	169 ± 40	22376 ± 1657	7391 ± 581	56 ± 10
3 hr[b]	216 ± 33	1623 ± 161	11550 ± 961	525 ± 104	1038 ± 62	3913 ± 524	579 ± 160	537 ± 28	92 ± 18	22192 ± 1770	6712 ± 618	0
5 hr[c]	139 ± 22	1140 ± 50	10878 ± 674	506 ± 51	978 ± 110	4561 ± 399	432 ± 82	503 ± 102	63 ± 16	18503 ± 587	5044 ± 141	14 ± 7

a = Mean of cpm ± standard error of the mean obtained from six experiments

b,c = Mean of cpm ± standard error of the mean obtained from four experiments

Experimentally, 1.25 X 10^7 HTC-cells were mixed with about 2.5 x 10^5 cpm (ca 460 µg) of labeled toxic endotoxin. After 1 hour incubation at 37°C, only 63 cpm of the toxic endotoxin became associated with 2.5 x 10^6 HTC-cells. By 3 and 5 hours, association per 2.5 x 10^6 cells was 122 and 189 cpm respectively. After 1 hour very few of the cell-associated counts were recovered from the crude nuclear fraction. Nuclear transfer of toxic endotoxin gradually increased after 1 hour so that by 3 and 5 hours 42 and 65 cpm were associated with the crude nuclear fraction respectively. The presence of toxic endotoxin in both whole cells and nuclear fractions was confirmed by the limulus lysate test (data not shown).

Table 5 also shows binding of uncentrifuged in vivo exposed endotoxin to HTC-cells. From 1.1 x 10^5 cpm (ca 142.5 µg/1.25 x 10^7 cells) of endotoxin added to cells, 82 cpm became associated with 2.5 x 10^6 cells after 1 hour, gradually increasing to 255 cpm after 5 hours. Nuclear association of plasma-treated endotoxin changed only slightly through the ensuing 5 hours. When in vivo plasma-treated endotoxin was recovered by centrifugation and then incubated with HTC-cells, of the 5 x 10^4 cpm (ca 70 µg/1.25 x 10^7 cells) of endotoxin added to cells, 167 cpm became associated with 2.5 x 10^6 cells after 1 hour, gradually increasing to 277 cpm after 5 hours. Relative to the starting counts this preparation more than doubled in binding compared to uncentrifuged in vivo exposed endotoxin. After 1 and 3 hour incubation there was no significant change in the nuclear association of plasma-treated centrifuged endotoxin. After 5 hours of incubation,

Table 5

HTC-cell association and cell distribution of toxic endotoxin (92 µg/5 ml cell), *in vivo* mouse plasma-treated (28.5 µg LPS/5 ml cell) and *in vivo* mouse plasma-treated centrifuged (14 µg LPS/5 ml cell) endotoxin after 1, 3 and 5 h incubation at 37°C

	Toxic Endotoxin[a]			*In vivo* plasma treated endotoxin[a]			*In vivo* plasma treated centrifuged endotoxin[a]		
Experimental	1	3	5	1	3	5	1	3	5
Counts to start with	43620 ± 3268	51704 ± 1900	51482 ± 1941	21271 ± 2782	21841 ± 2873	21773 ± 2887	10237 ± 314	10205 ± 315	10405 ± 355
Whole cell	63 ± 9	122 ± 12	189 ± 22	82 ± 18	170 ± 27	255 ± 35	167 ± 10	229 ± 8	277 ± 9
Nuclei	10 ± 1	42 ± 8	65 ± 13	38 ± 7	58 ± 10	51 ± 10	47 ± 5	51 ± 4	105 ± 11
Non-nuclear fraction	35 ± 3	59 ± 5	95 ± 8	61 ± 6	108 ± 20	173 ± 37	118 ± 11	206 ± 8	295 ± 22

a = Mean of cpm ± standard error of the mean obtained from 14 experiments

105 cpm of the cell associated counts were recovered from crude nuclear fraction.

The decrease in the amount of plasma-treated endotoxin relative to toxic endotoxin used in these experiments reflects the difficulty in recovering substantial amounts of endotoxin from the blood of mice. Endotoxin is cleared very rapidly from the circulation. The amounts used per experimental flask reflects the total counts recoverable from pooling plasma from at least 4 mice.

HTC-cell Association and Cell Distribution of Endotoxin Mixed With Poly L-α-ornithine

Table 6 shows HTC-cell association and subcellular distribution of endotoxin mixed with poly L-α-ornithine. Experimentally, 1.25×10^7 HTC-cells were mixed with about 2.5×10^5 cpm (ca 460 µg) of labeled endotoxin mixed with poly L-α-ornithine. Mixing endotoxin with ornithine significantly enhanced endotoxin binding to HTC-cells. After 1 hour incubation at 37°C, 1399 cpm of endotoxin were cell associated with 2.5×10^6 cells and this increased after 3 and 5 hours incubation to 1618 and 1850 cpm respectively. After 1 hour 451 cpm of the cell associated counts were recovered from crude nuclear fraction and after 3 and 5 hours 932 and 1106 cpm of the cell associated counts were bound to crude nuclear fraction. At the concentration used (4 µg/ml), ornithine (which did not markedly change the physical structure of endotoxin, Table 1) increased the cell association and subcellular distribution of endotoxin at least 10 times compared to toxic endotoxin.

Table 6

HTC-cell association and cell distribution of endotoxin mixed with poly L-α-ornithine (92 µg/5ml cell) after 1, 3 and 5 h incubation at 37°C[a]

Experimental	Incubation Time in Hour		
	1	3	5
Counts to start with	40300 ± 537	42135 ± 418	42893 ± 617
Whole cell	1399 ± 49	1618 ± 104	1850 ± 129
Nuclei	451 ± 38	932 ± 67	1106 ± 95
Non-nuclear fraction	949 ± 37	723 ± 21	917 ± 49

a = Mean of cpm ± standard error of the mean obtained from at least six experiments

HTC-cell Association and Cell Distribution of 0.25 N NaOH Treated Endotoxin

Alkaline-detoxified endotoxin showed a third type of binding pattern (Table 7). After 1 hour incubation of 1.25 x 10^7 HTC-cells with about 1.25 x 10^5 cpm (CA 315 µg) of labeled 0.25 N NaOH treated endotoxin, 507 cpm became associated with 2.5 x 10^6 cells. Binding did not significantly increase after 1 hour, since only 600 and 594 cpm were observed after 3 and 5 hours respectively. After 1, 3 and 5 hours incubation 172, 205 and 175 cpm of the cell associated counts were recovered from crude nuclear fraction respectively. The extent of crude nuclear fraction transfer of alkaline-treated endotoxin was significantly lower than toxic endotoxin at 5 hours and endotoxin mixed with ornithine at 3 and 5 hours.

Table 7

HTC-cell association and cell distribution of 0.25 N NaOH treated-endotoxin (63 μg/5 ml cell) after 1, 3 and 5 h incubation at 37°C[a]

Experimental	Incubation Time in Hour		
	1	3	5
Counts to start with	23770 ± 244	23420 ± 260	23567 ± 384
Whole cell	507 ± 13	600 ± 24	594 ± 27
Nuclei	172 ± 5	205 ± 16	175 ± 10
Non-nuclear fraction	384 ± 6	485 ± 6	531 ± 21

a = Mean of cpm ± standard error of the mean obtained from at least six experiments

DISCUSSION

The pattern of toxic endotoxin distribution among organs and tissues of normal mice has been reported previously (9,11, 21). To a large extent this distribution follows the distribution of the RES system and the clearance rates from the circulation are very rapid. Most toxic endotoxin becomes fixed to tissues or organs by 30-60 minutes (11) and there is little change in this distribution with time as seen by results up to 5 hours presented in this manuscript and longer (9,21) by others.

Alkaline detoxification changes not only the chemical and physical properties of endotoxin but also the rate of clearance and distribution of endotoxin in the host (6) (see Table 4). Once the detoxified endotoxin is fixed in tissues after 1 hour it does not significantly change location up to 5 hours. The bulk of the detoxified endotoxin is found in the liver, kidney, intestine and carcass. Since the mice were not perfused free of blood it is difficult to determine whether the relatively increased carcass counts reflect increased binding to the host muscle mass or just free detoxified endotoxin in the blood. It was not surprising that ornithine, which has dramatic effects on uptake of endotoxin in HTC-cells (see Table 6) did not alter distribution in vivo. This compound appears to have little effect on the physical or biological properties of endotoxin per se (see Table 1) but instead affects permeability of the cells in vitro. In all likelihood most of these effects in the complex

in vivo environment would be neutralized by normal physiological processes of metabolism and excretion.

The increasing interest in studying the biological actions of endotoxin in vitro (8,12,13,21) makes it incumbent upon the researcher to establish that in vitro actions approximate in vivo realities. Despite experimental modifications in the endotoxin molecule all of our whole animal studies reaffirm the notion that the liver is the major organ responsible for endotoxin clearance from the blood. This fact alone makes hepatic derived cell lines an excellent candidate in which to evaluate and compare the basic interactions of endotoxin with the cell in vitro and in vivo. As will be seen below, this comparison is not favorable.

The liver contains a number of cell types. While the macrophage (Kupffer cell) are thought to be the main reservoir of in vivo clearance (11) there is also evidence that about 75% of endotoxin trapped in the liver is associated with parenchymal cells (21). What this means is that if 50% of intravenously injected endotoxin is found in the liver, it might be assumed that about 35% is associated with parenchymal cells. Hence, the parenchymal cell binds endotoxin in vivo very rapidly. By contrast, the HTC-cell line used in this study binds little endotoxin (see Table 5) even after 5 hours. Reasoning that in vivo endotoxin is exposed to plasma before binding to parenchymal tissue we tried to enhance binding in culture by using endotoxin exposed to plasma in vivo but again, binding was discouragingly low (see Table 5).

Binding of alkaline-treated endotoxin to HTC-cells was 3 to 8 fold greater than toxic endotoxin. This is opposite to endotoxin binding in vivo. When the HTC-cell membrane was influenced by ornithine (16) we observed our greatest binding and subcellular uptake of endotoxin yet ornithine in vivo had little effect on endotoxin distribution. Further evidence of a lack of correlation between in vitro tissue culture models with in vivo realities is seen from the data on nuclear association. With toxic endotoxin nuclear association gradually increases with time, while in vivo no such pattern developes. One consistancy is that nuclear accumulation of detoxified endotoxin does not seem to increase with time in vitro or in vivo.

Cumulatively, we must conclude that conditions have not yet been identified by which endotoxin binds to HTC-cells to the extent it binds to liver in vivo. Before meaningful biological studies on in vitro toxicity can be performed, conditions which compare favorably to in vivo realities must be defined.

A preponderance of current literature suggests that endotoxin toxicities in vivo might be mediated by release of soluble materials into the circulation (14). Both our laboratory (Werner and Moon in preparation) and others (1) have been able to demonstrate, by addition of plasma from endotoxin poisoned mice, biological actions on tissue culture cells which seem to mimic in vivo changes. While in the final analysis, these mediators of endotoxin action might prove to, in fact, be the key to understanding the pathophysiology of endotoxin actions in vivo, study on direct toxicities of endotoxin on

non-RES cells remains a plausable alternative. A prerequisite to such studies is that *in vivo* and *in vitro* binding should be similar and, in our opinion we have not yet been able to achieve these experimental conditions *in vitro*.

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APPENDIX I

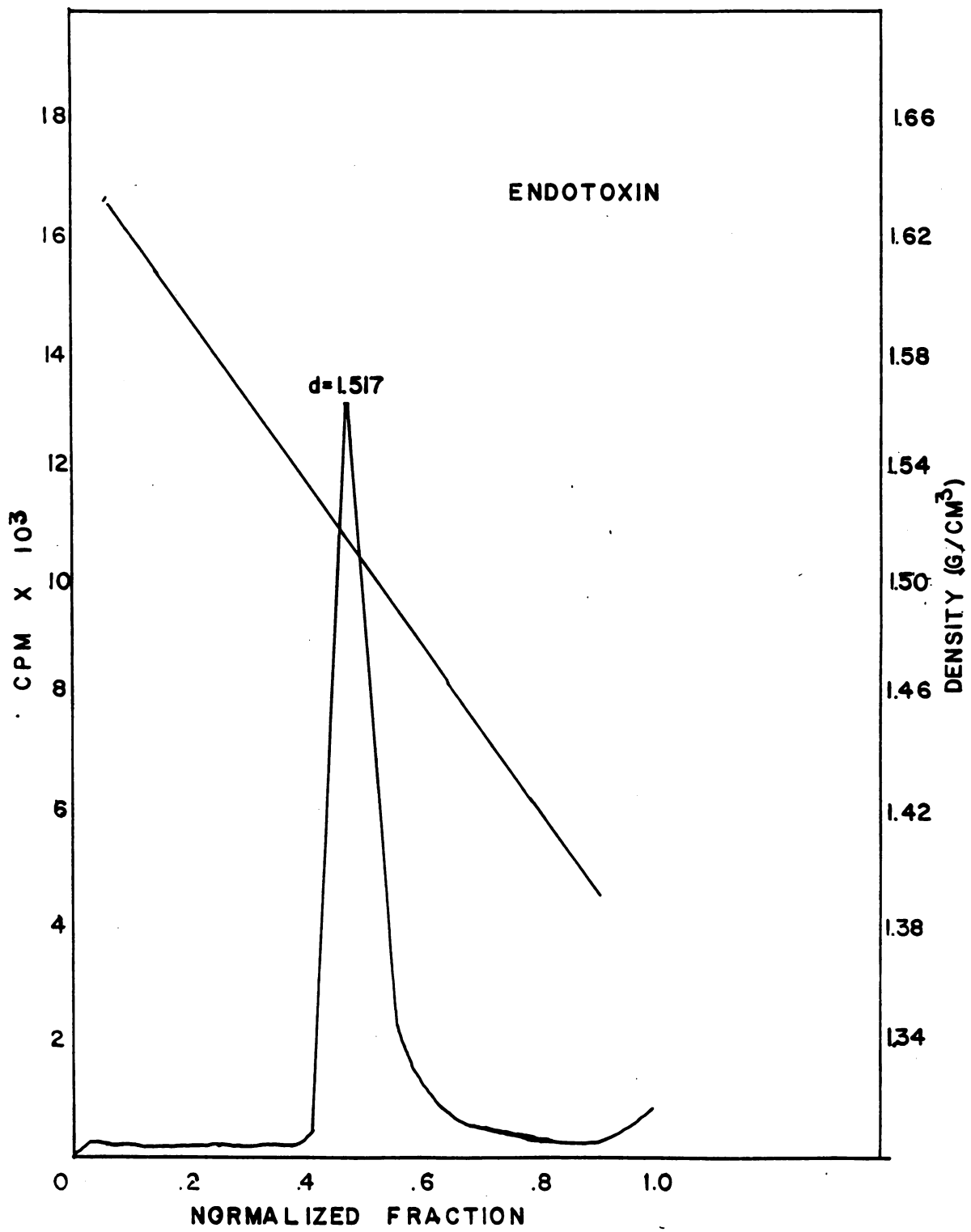

FIG. 1. CsCl ULTRACENTRIFUGATION OF UNTREATED ENDOTOXIN

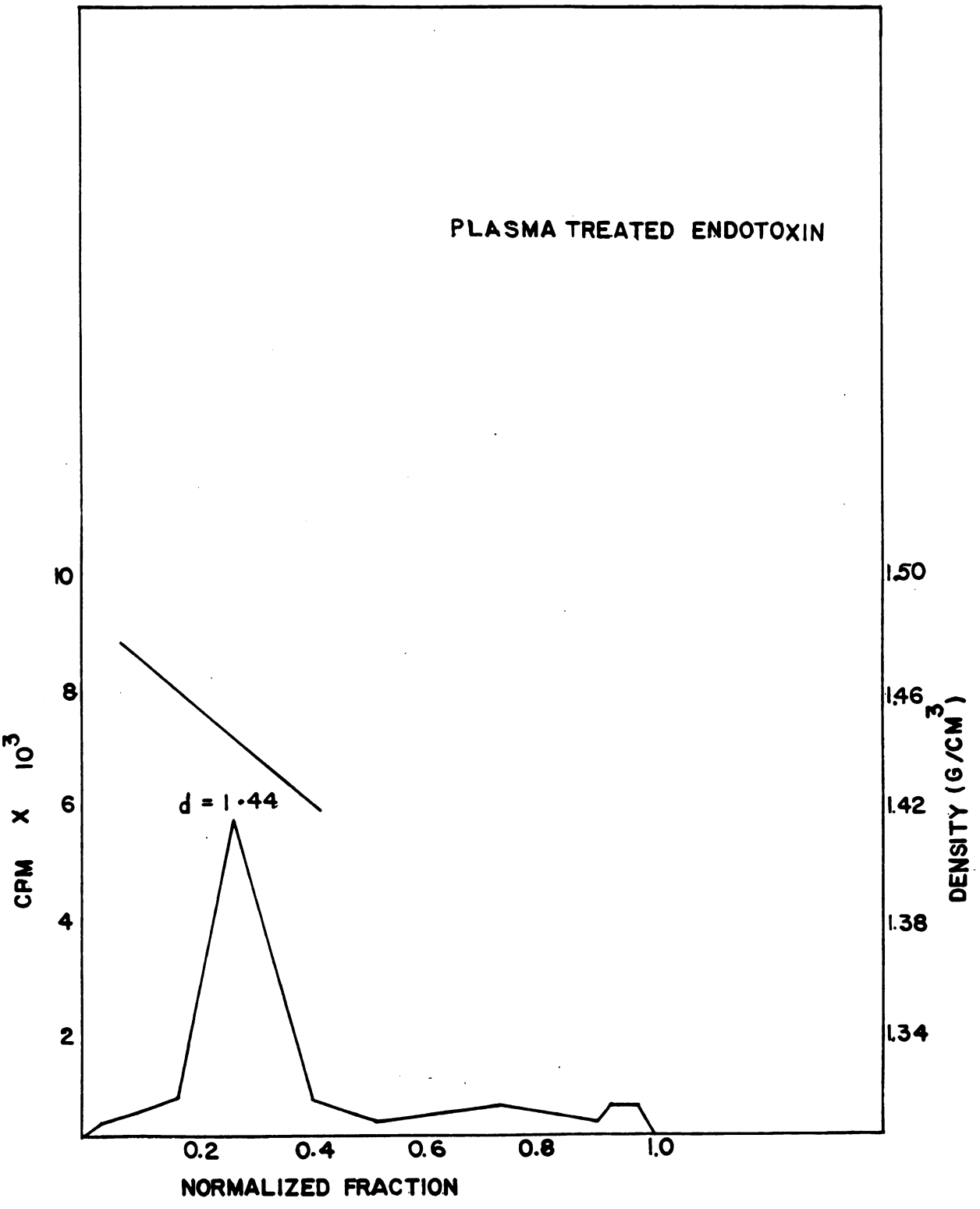

FIG 2. CsCl ULTRACENTRIFUGATION OF NORMAL MOUSE PLASMA TREATED ENDOTOXIN

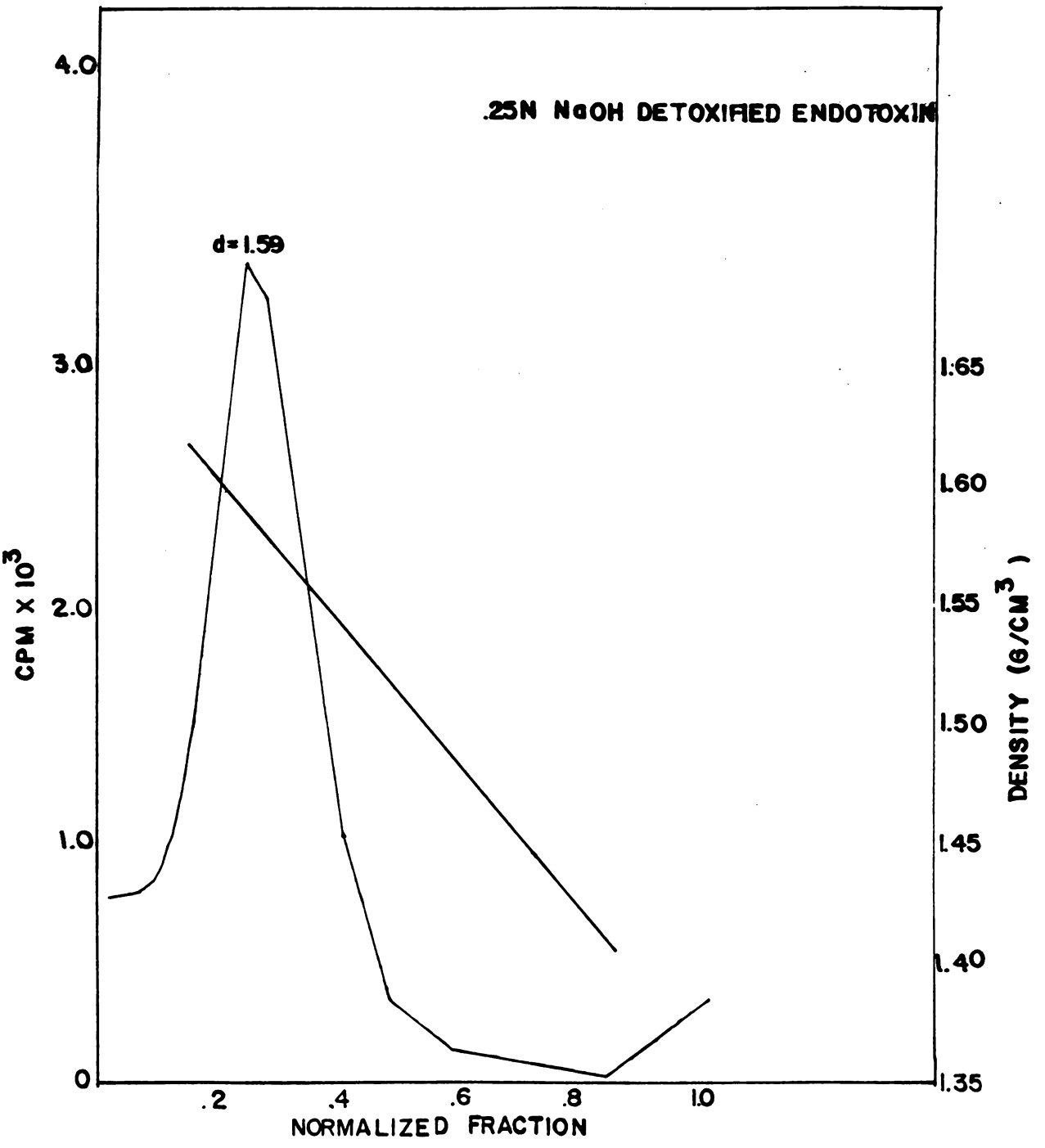

FIG 3. CsCl ULTRACENTRIFUGATION OF 0.25 N NaOH TREATED ENDOTOXIN

Association and Subcellular Distribution of ^{51}Cr-sodium Chromate, ^{51}Cr-sodium Chromate Mixed with Endotoxin, and ^{51}Cr-bound to Endotoxin

A concern using ^{51}Cr-LPS is that Cr might become dissociated from the LPS complex. To address this possibility directly, we incubated free chromium with HTC-cells in the presence and absence of endotoxin and compared uptake and subcellular distribution data with that obtained for ^{51}Cr-LPS complex. The results of this experiment (Table 1) clearly show significant differences in the cellular association and subcellular distribution of the bound vs free isotope. For example, when $Na_2{}^{51}CrO_4$ alone or $Na_2{}^{51}CrO_4$ mixed with endotoxin was incubated for 1 hr at 37°C, 1192 and 998 cpm of the counts respectively were associated with the cells compared to 63 cpm when ^{51}Cr-labeled endotoxin was used. Cell association of unbound $Na_2{}^{51}CrO_4$ increased with time but nuclear transfer did not. Cell association and nuclear transfer of ^{51}Cr-labeled endotoxin increased with time. With unbound $Na_2{}^{51}CrO_4$ the nuclear-cytoplasmic ratios are opposite those obtained with ^{51}Cr-labeled endotoxin. The significant differences between the cell association and cell distribution of ^{51}Cr-labeled endotoxin and $Na_2{}^{51}CrO_4$, rules out the possibility that unbound $Na_2{}^{51}CrO_4$ instead of ^{51}Cr-labeled endotoxin was taken up by the cells and nuclei.

Table 1

HTC-cell association and cell distribution of $Na_2{}^{51}CrO_4$, $Na_2{}^{51}CrO_4$ mixed with endotoxin and $Na_2{}^{51}CrO_4$-labeled endotoxin at 37°C.

	$Na_2{}^{51}CrO_4$			$Na_2{}^{51}CrO_4$ mixed with LPS			$Na_2{}^{51}CrO_4$-labeled LPS		
Experimental	1	3	5	1	3	5	1	3	5
Counts to start with	35488 ± 745	34866 ± 672	35239 ± 663	32768 ± 1290	31815 ± 1096	32361 ± 1201	43620 ± 3268	51704 ± 1900	51482 ± 1941
Whole cell	1192 ± 197	1725 ± 215	2425 ± 321	998 ± 100	1703 ± 158	2326 ± 175	63 ± 9	122 ± 12	189 ± 22
Nuclei	645 ± 176	447 ± 92	342 ± 52	457 ± 62	404 ± 42	421 ± 53	10 ± 1	42 ± 8	65 ± 13
Non-nuclear fraction	640 ± 96	1396 ± 181	2258 ± 299	597 ± 78	1321 ± 136	2031 ± 206	35 ± 3	59 ± 5	95 ± 8

a=Mean of cpm ± standard error of the mean obtained from at least 6 experiments. Cell association and cell distribution of $Na_2{}^{51}CrO_4$ and $Na_2{}^{51}CrO_4$ mixed with endotoxin was significantly different from $Na_2{}^{51}CrO_4$-labeled endotoxin at all time points that were studied.

Limulus Lysate Test

Presence of toxic endotoxin in HTC-cells and nuclei was further confirmed by the limulus lysate assay. This test can detect as little as 0.125 nanogram/ml endotoxin (Microbiological Associates Lot. No. L09578).

HTC-cells were grown, harvested and resuspended in 10 ml of MEM at a concentration of $5x10^5$ cells per ml. ^{51}Cr-labeled endotoxin (58 µg) was added to the cells and incubated for 3-4 hr. After incubation the cells were washed four times with pyrogen-free saline and resuspended in 1 ml of deionized pyrogen-free distilled water. The cells were broken, as judged by microscopic examination, by passing through a 25 gauge needle. A ten-fold serial dilution series of the broken cells was prepared in pyrogen-free saline. A sample (0.1 ml) of broken cells was mixed with an equal volume of limulus lysate and incubated at 37°C. Changes in the opacity and clotting were recorded on a scale of 1 to 4 by eye.

For limulus lysate tests on cell nuclei, the nuclei were prepared by treating HTC-cells with 0.5% Nonidet P-40 for 10 min on ice. Nuclei were harvested by centrifugation at 800xg for 10 min, washed once with pyrogen-free saline and resuspended in 1 ml pyrogen-free distilled water. Nonidet P-40 at the concentration of 0.5% did not clot the limulus lysate. The nuclei were broken, as judged by microscopic examination, with a glass Dounce homogenizer. Ten-fold serial dilutions of nuclei were prepared and the limulus lysate test was performed as described above.

TABLE 2

Limulus lysate test on HTC whole cell and nuclear lysate incubated with endotoxin for 3 hr

Experimental	µg of LPS or dilution of cells or nuclei				
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
Toxic endotoxin	4+	4+	4+	2+	-
Whole cell lysate	4+	3+	1+	-	-
Nuclei lysate	4+	-	-	-	-

Normal HTC-cells and nuclei were processed to serve as negative controls. Positive controls were ten-fold dilutions of normal endotoxin ranging from 1 μg to $1x10^{-6}$ μg per ml.

Table 2 shows the results of the limulus lysate test on whole cells and nuclear lysates. Cell lysate from cell incubated with endotoxin was able to initiate the clotting reaction at 10^{-3} dilution. A strong clotting reaction was visible at 10^{-1} dilution of the nuclear lysate. Neither normal HTC-cell lysate nor nuclear lysate was alone able to clot the limulus lysate.

Cell Association and Cell Distribution of ^{51}Cr-labeled Endotoxin by Hepatoma Tissue Culture Cells at 4°C

HTC-cells were grown in MEM supplemented with 5% heat inactivated fetal calf serum. The cells were harvested and suspended in 25 ml MEM at the presence of toxic endotoxin with constant stirring at 4°C. At 1, 3 and 5 hr intervals, 5 ml aliquots were removed and cell association and cell distribution of endotoxin was detected as stated before (first paper). The accumulation rate of ^{51}Cr-labeled endotoxin at 4°C was significantly lower than 37°C. After 1 h only 23 cpm of the initial counts became cell associated (Table 3). At 3 hr and up to 24 hr cell association of endotoxin at 4°C was significantly lower than cell association of endotoxin at 37°C. After 1 and 3 hr incubation negligable numbers of the cell-associated counts were recovered from crude nuclear fraction. Nuclear transfer of endotoxin increased slightly so that by 24 hr of incubation there were

Table 3

HTC-cell association and cell-distribution of ^{51}Cr-labeled toxic endotoxin after 1, 3, 5 and 24 h incubation at 4°C.

	Counts per minute[ab]			
Experimental	1	3	5	24
Counts to start with	51593 ± 904	51459 ± 426	51249 ± 672	47962 ± 843
Whole cell	23 ± 5	58 ± 6	69 ± 7	91 ± 6
Crude nuclear fraction	13 ± 2	18 ± 2	28 ± 7	40 ± 4
Non-nuclear fraction	21 ± 2	38 ± 8	36 ± 3	34 ± 2

a=Mean of cpm ± standard error of the mean obtained from at least 6 experiments.

b=Background was between 50-60 cpm.

an average of 40 cpm above background associated with the crude nuclear fraction. It is not clear whether lower cell association of labeled endotoxin at 4°C was due to lower binding of endotoxin to the cells or because of a decrease in the cell internalization of labeled endotoxin. This observation is consistent with Ryser's report (26, first paper) that monolayers of Sarcoma S180II adsorb less albumin-I^{131} at 2°C than at 37°C.

Organ and Tissue Distribution of 0.25 N NaOH treated ^{51}Cr-Labeled Endotoxin in Mice

Mice were injected intravenously with 0.18 mg ^{51}Cr-labeled 0.25 N NaOH treated endotoxin. After 1, 3 and 5 hr, mice were killed by cervical dislocation, and blood was collected by perfusion of 20 ml saline through the apex of the heart. The results of this experiment indicated that, in contrast to toxic endotoxin, only a small fraction of 0.25 N NaOH treated endotoxin was associated with the liver and spleen. Most of the counts were in the blood and carcass. With time less radiolabeled 0.25 N NaOH treated endotoxin was found in the blood and more recovered from liver and intestine. Association of detoxified endotoxin with liver and spleen was significantly lower than for toxic endotoxin. This observation clearly demonstrates that detoxified endotoxin behaves differently from toxic endotoxin *in vivo*.

Table 4

Organ and tissue distribution of (180 μg) ^{51}Cr-labeled 0.25 N NaOH treated endotoxin in mice.

	Liver Nuclei cpm		Counts per minute										
	Pure	Crude	Liver	Spleen	Kidney	Intestine	Heart	Lung	Brain	Carcass	Skin & Feet	Gall Bladder	Blood
1 hr[a]	221	1516	9592	501	761	2540	111	508	41	15799	5638	18	18093
	117	1333	9803	453	949	2517	213	291	1	12849	8202	10	20883
	224	1506	10175	667	866	2964	123	663	5	23816	6269	16	17694
3 hr[b]	132	1794	11958	478	1014	3743	149	260	31	20633	7544	15	8055
	361	1548	10316	344	814	4233	197	506	29	17978	5471	69	13458
5 hr[c]	194	1605	12164	301	928	4133	164	314	0	13243	5408	7	6445
	224	1043	10642	440	791	4037	117	1045	0	17878	5005	0	7986

a=cpm of three separate experiments.

b,c=cpm of two separate experiments.

ABSTRACT

In vitro Interactions of Endotoxin, Chromatin, DNA, and Steroid Hormone-receptors

The ability of toxic and alkaline-detoxified endotoxin to bind directly to chromatin and DNA and to inhibit glucocorticoid-receptor binding to these materials has been characterized. Toxic endotoxin does not bind to DNA in the form of DNA cellulose, but was capable of binding to HTC-cell chromatin (1 μg/20 μg DNA) four times more efficiently than alkaline-treated detoxified endotoxin. Interaction of glucocorticoid-receptor complexes with chromatin and DNA was inhibited in a dose-dependent manner by toxic endotoxin. Alkaline-treated detoxified endotoxin was even more effective. It appears most likely that endotoxin interacts directly with activated hormone-receptor complex, blocking its ability to bind to the DNA and chromatin. The biological significance of these observations is clouded by the need for high concentrations of endotoxin and the observation that biologically detoxified endotoxin in some instances works even better than toxic endotoxin.

INTRODUCTION

Glucocorticoid hormones stimulate a wide variety of biological responses in animal tissues including protection against the lethal effects of bacterial endotoxin (7,8). A large body of circumstantial evidence suggests that all steroid hormones act via basically the same mechanism. The hormones readily pass into the cytoplasm of cells (3,23) and bind to specific receptors (4,33). After a possible "activation" step (11,18,19,31,32) receptor-steroid complexes become concentrated in the nucleus (11,18,31,32). The extent to which the receptor-steroid complexes bind to chromatin (5,25,31,37) or DNA (2,33,37,41) has not been definitively established. Whatever the precise mechanism(s), receptor-steroid complexes are thought to alter the transcription process resulting in increased levels of specific messenger RNAs (31,37). The products of translation of these messenger RNAs leads to the observable biological effects (25,31,37), one of which is the induction of selected hepatic enzymes (15,16,20,21,24,27,29, 30,34,38,39,40). Bacterial endotoxin inhibits the induction of some, but not all of these adaptive enzymes (8,9). One possible molecular mechanism to explain endotoxin's ability to inhibit enzyme induction is that the lipopolysaccharide (LPS) directly interferes with glucocorticoid-receptor binding to cells or subcellular targets. Experiments described herein were designed to test the hypothesis that endotoxin can block steroid binding to hepatic cell chromatin and/or DNA. This hypothesis seems plausible since a previous report

from this laboratory (42) has shown that endotoxin associates directly with hepatic parenchymal cells and their nuclei *in vivo*. To evaluate the biological significance of the observed *in vitro* interactions, most experiments described herein compare particular variables using both toxic and alkaline detoxified endotoxins.

METHODS AND MATERIALS

All procedures were performed at 0-4°C unless otherwise specified.

Animals

Female Spartan HA-ICR mice were used for enzyme studies. Female Charles river CD-1 mice were used for organ distribution of endotoxin. Food and water were available ad libitum.

Chemicals

Minimum essential medium (MEM) and fetal calf serum (FCS) were obtained from GIBCO (Grand Island, NY).

[1,2,^{3}H] Dexamethasone (20-40 Ci/mmol) was purchased from New England Nuclear (Boston, MA) or Amersham/Searle (Arlington Heights, IL). Micrococcal nuclease, calf thymus deoxyribonucleic acid and hydrocortisone-21-sodium succinate was purchased from Sigma Chemical Company (St. Louis, MO).

Phosphoenolpyruvate Carboxykinase Assay

Phosphoenolpyruvate carboxykinase (PEPCK) activity was assayed by the method of Lane et al., (22). PEPCK was induced by injecting 5 mg of hydrocortisone-21-sodium succinate suspended in 0.5 ml of sterile saline subcutaneously into the interscapular region. Toxic or alkaline-treated detoxified endotoxin (50 µg/mouse), dissolved in saline, was injected intraperitoneally (i.p.) immediately after hydrocortisone. PEPCK activity was determined in liver homogenates 4 hr later.

Preparation of Radiolabeled Endotoxin and Pure Nuclei

^{51}Cr-labeled endotoxin was prepared according to the method of Chedid et al., (14). Blobel and Potter's (10)

method was used to prepare nuclei free of debrie by sucrose gradient centrifugation.

Preparation of HTC-cell Cytosol and Chromatin

Hepatoma tissue culture (HTC) cells were grown at 37°C in suspension cultures in MEM supplemented with 5% heat inactivated fetal calf serum. Cells were harvested at 600xg for 5 min, washed at least twice with an ice cold buffer [0.04 M Tris (hydroxymethyl) aminomethane-hydrochloride, 0.1 M KCl, 0.001 M $MgCl_2$; pH 7.2], suspended in one volume homogenization buffer [0.02 M Tris (hydroxymethyl) methylglycine, 0.002 M $CaCl_2$, 0.001 M $MgCl_2$, 10^{-4} dithiothreitol, pH 7.4] and homogenized with a Dounce homogenizer. Homogenates were centrifuged at 800xg for 10 min and the supernatant solution (non-nuclear fraction) was separated from the pellet containing nuclei which was reserved and used as described below. The non-nuclear fraction was further centrifuged at 35,000xg for 15 min. Dexamethasone ($5x10^{-8}$ M) was added to the supernatant and the mixture centrifuged at 105,000xg for 90 min. The supernatant (cytosol) was then incubated for at least 2 hr to allow steroid binding, followed by activation of the steroid receptors by supplementing the cytosol with 0.15 M NaCl and incubating at 20-25°C for 30 min (18). Activated cytosol was finally passed through 20 ml of Sephadex G-25 equilibrated with homogenizing buffer containing 0.25 M sucrose to remove unbound steroid and salt. The macromolecular fraction containing activated hormone-receptor-complexes (AHRC) was used immediately. AHRC had about 5 mg/ml protein and was inactivated (IHRC) by incubation at 37°C for 1 hr.

Nuclear fraction was washed once in 10 vol of 0.01 M Tris, pH 8.0 at 0°C and allowed to swell for 2 hr in 3.5 vol of the same buffer. Nuclei were broken by sonication and centrifuged at 20,000xg for 15 min. The supernatant contained chromatin and had about 1 mg/ml DNA as measured by the diphenylamine technique (12).

Binding of ^{51}Cr-labeled Toxic and Alkaline-treated Detoxified Endotoxin to Chromatin

Alkaline-treated endotoxin was prepared by dissolving 50 mg of endotoxin in 3 ml of 0.25 N NaOH. The solution was heated at 56°C for 60 min (17). The modified endotoxin was precipitated with cold anhydrous ethanol, washed three times with ethanol, dialyzed against deionized distilled water and then lyophilized. Both toxic and detoxified endotoxin were labeled with $Na_2\ ^{51}CrO_4$ according to the method of Chedid et al., (14).

An aliquot (20 µl) of chromatin was incubated for 90 min with 10 µl of ^{51}Cr-labeled toxic or alkaline-treated detoxified endotoxin. Chromatin precipitation was achieved by adding 2 ml of 5 mM $MgCl_2$/0.01 M Tris buffer to the assay mixture. After at least 10 min, the mixture was centrifuged at 8,000xg for 10 min. As a control, 10 µl of ^{51}Cr-labeled toxic or alkaline-treated detoxified endotoxin was mixed with 20 µl homogenizing buffer and precipitated with $MgCl_2$ solution. The cpm of endotoxin precipitated by $MgCl_2$ in the control tubes was considered background and subtracted from the test.

Binding of Hormone-receptor to Chromatin in the Presence of Toxic and Detoxified endotoxin

Different concentrations of toxic or alkaline-treated detoxified endotoxin were mixed with 10 µl of chromatin. After making the volumes equal with homogenizing buffer, the endotoxin-chromatin mixtures or chromatin alone were incubated for 2.5 hr. To each tube 0.4 ml of the macromolecular fraction of activated cytosol was added. After 90 min incubation, 0.2 ml of the assay mixture was added to 1.8 ml of 5 mM $MgCl_2$/ 0.01 M Tris buffer. The tubes were mixed on a vortex mixer and after at least 10 min, centrifuged at 8,000xg for 10 min. The pellet was digested in 0.4 ml of 1 M NaCl/0.5 M NaOH overnight at room temperature and counted in 3a70B coctail (rpi). As a control, 0.2 ml of activated cytosol was added to 1.8 ml of $MgCl_2$ solution and processed as stated. Counts per minute of activated cytosol that were precipitated with $MgCl_2$ were subtracted from the test. The percent inhibition of binding of hormone-receptor to chromatin in the presence of endotoxin was calculated as follows: 100-[cpm of hormone-receptor bound to chromatin in the presence of endotoxin/cpm of hormone-receptor bound to chromatin]x100.

Binding of Endotoxin to DNA

DNA-cellulose (340 µg DNA/ml) was prepared according to the technique of Albert et al., (1). An aliquot of 0.1 ml or 0.3 ml DNA-cellulose or cellulose alone was centrifuged in 1.5 ml disposable conical tubes (Eppendorf) at 600xg for 5 min. The supernatant was removed by aspiration. To each

tube, 20 µl of labeled endotoxin was added and the total volume was made up to 0.6 ml with Tris/glycerol buffer (0.01 M Tris/10^{-4} M dithiothreitol/10% glycerol v/v). The tubes were capped, placed on a roller bottle apparatus at 10 rpm for 2.5 hr and turned 180° every 30 min during the assay to help keep the cellulose resuspended. The reaction was stopped by centrifugation at 600xg for 5 min. The supernatant was removed and the ^{51}Cr radioactivity was measured. The pellets were resuspended in Tris/glycerol buffer and counted.

Binding of Hormone-receptor to DNA in the Presence of Toxic Endotoxin

Aliquots of 0.1 ml of DNA-cellulose (34 µg) or cellulose alone were centrifuged in 1.5 ml disposable conical tubes at 600xg for 5 min. The supernatant was discarded and the pellet was suspended in 0.6 ml of Tris/glycerol buffer. Different amounts of toxic endotoxin (100 or 300 µg) or Tris/glycerol buffer were added to DNA-cellulose and left on a roller bottle (10 rpm) for 1.5 hr and turned 180° every 30 min to keep the DNA-cellulose in suspension. After incubation, some of the tubes which contained 100 µg of toxic endotoxin were centrifuged, the supernatant discarded, and the pellet resuspended in 0.6 ml of Tris/glycerol buffer. The binding of AHRC to DNA in the presence or absence of excess toxic endotoxin was determined by raising the total volume of all the tubes to 0.8 ml by adding 0.2 ml of AHRC.

The tubes were capped and left on a roller bottle for 2.5 hr. The reactions were stopped by centrifugation at 600xg for 5 min. The pellets were resuspended in 0.2 ml of Tris/glycerol buffer for at least 1 hr at room temperature before being counted. The percent of inhibition of binding of AHRC to DNA in the presence of toxic endotoxin was calculated as follows: 100-(cpm of AHRC bound to DNA cellulose which was incubated with endotoxin-cpm of AHRC bound to cellulose)/(cpm of AHRC bound to DNA cellulose-cpm of AHRC bound to cellulose)x100.

RESULTS

Cortisone Induction of PEPCK in Normal and Endotoxin-Poisoned Mice

The effect of toxic and detoxified endotoxin on the *in vivo* induction of PEPCK by cortisone is presented in Table 1. Cortisone administration significantly increased the level of PEPCK in the liver 4 hr after injection and toxic endotoxin inhibited this induction. When endotoxin was detoxified by treating with 0.25 N NaOH, the ability of endotoxin to inhibit the induction of PEPCK in whole animals was prevented.

Hepatic Association and Subcellular Distribution of Toxic and Detoxified Endotoxin

Table 2 shows the extent of binding of intravenously (i.v.) injected toxic and detoxified endotoxin to liver and hepatic nuclei. Experimentally, 200 µg (CA 120,000 cpm) of ^{51}Cr-labeled toxic endotoxin were injected into mice. After 5 hr about 50% (65643 cpm) were associated with the liver. Of this number 7462 cpm could be recovered from washed crude nuclei, and 366 cpm from pure nuclei. Five hours after injection of 180 µg (45,000 cpm) of detoxified endotoxin, hepatic association was significantly lower than with toxic endotoxin. About 50% of detoxified endotoxin remained in the circulation and carcass (data not shown) on a percentage basis. No significant difference in hepatic nuclear association of toxic and 0.25 N NaOH-treated endotoxin was observed.

Table 1. Hydrocortisone induction of PEPCK in the presence and absence of toxic and detoxified endotoxin.

Experimental	PEPCK Activity
Normal	19.7 ± 1.4[a]
Cortisone	34.2 ± 3.7[b]
Toxic endotoxin + cortisone	20.8 ± 2
Detoxified endotoxin + cortisone	34.9 ± 3

a=Mean ± standard error of the mean obtained from 6 separate experimental determinations.

b=Significantly different from toxic endotoxin + cortisone ($P < 0.05$).

Table 2. Hepatic association and subcellular distribution of toxic and detoxified endotoxin 5 hr after i.v. injection into mice.

Experimental	cpm of toxic endotoxin[a]	cpm of detoxified endotoxin[b]
Liver homogenate	65643 ± 4787[c]	10878 ± 674[d]
Crude nuclear fraction	7462 ± 775	1140 ± 50
Purified nuclei	366 ± 27	139 ± 22

a=200 μg (120,000 cpm)/mouse intravenously.

b=180 μg (45,000 cpm)/mouse intravenously.

c=Mean ± standard error of the mean obtained from 6 separate experimental determinations.

d=Mean ± standard error of the mean obtained from 4 separate experimental determinations.

Ability of Toxic and Detoxified Endotoxin to Bind to Chromatin

Table 3 shows the ability of ^{51}Cr-labeled toxic and detoxified endotoxin to bind to sonicated-chromatin. When 20 µl of chromatin (ca 20 µg DNA) was incubated at 0°C with 51.4 µg of toxic endotoxin, 1.9% of the counts were bound after 90 minutes. Detoxification of endotoxin lowered chromatin binding approximately four fold.

Effect of Graded Quantities of Toxic and Detoxified Endotoxin on Binding of AHRC to Chromatin

Graded quantities of unlabeled toxic endotoxin could inhibit binding of AHRC to a constant amount of sonicated-chromatin (10 µl). In all experiments sonicated-chromatin was used. No significant binding of AHRC to nuclease-extracted chromatin (28) was observed (data not shown).

Table 4 shows that, as the concentration of toxic unlabeled endotoxin increased from 5 to 500 µg, the percent inhibition of binding of AHRC to chromatin increased up to a maximum of slightly higher than 50% with 500 µg of endotoxin. There was about 345 cpm of AHRC bound per 10 µg of DNA in chromatin. When AHRC was inactivated at 37°C for 1 hr (IHRC) no binding of IHRC to chromatin was observed. By contrast, alkaline-treated endotoxin exerted more of an inhibitory effect on the binding of AHRC than the toxic endotoxin. In the presence of 500 µg of alkaline-treated endotoxin, there was essentially 100% inhibition in the binding of AHRC to chromatin (Table 4).

Table 3. Binding of ^{51}Cr-labeled toxic and detoxified endotoxin to chromatin.

Experimental	µg added	µg bound	% binding
Toxic endotoxin	51.4	1	1.9
Detoxified endotoxin	46	0.24	0.52

Table 4. Inhibition of AHRC binding to chromatin in the presence of toxic and detoxified endotoxin.

	µg of endotoxin							cpm of HRC bound to chromatin		
Type of endotoxin	0	5	30	100	200	300	500	AHRC[a]	IHRC[b]	Blank[c]
Toxic endotoxin	0[d]	4.1	7.3	10.8	18.3	33	51.2	798	403	453
Detoxified endotoxin	0	22.5	34.8	67	-	97.4	100	891	390	558

[a]AHRC = activated hormone-receptor complex.

[b]IHRC = inactivated hormone-receptor complex.

[c]Blank = $MgCl_2$ + AHRC.

[d]Percent inhibition of binding compared to AHRC binding to chromatin.

These results do not establish whether the inhibition of binding is a result of direct interaction of endotoxin with chromatin (cf Table 3) or whether free endotoxin remaining in solution interacts directly in AHRC discouraging binding.

Binding of Toxic Endotoxin to DNA and its Effect on the Binding of AHRC to DNA

Two different concentrations of DNA (34 µg and 100 µg) were used to investigate whether or not toxic endotoxin was able to bind directly to DNA conjugated to cellulose (DNA-cellulose). Data in Table 5 shows that toxic endotoxin does not bind directly to DNA. When toxic endotoxin (88 µg) was added to either 0.1 or 0.3 ml of cellulose alone 13,605 and 33,531 cpm of sedimentable label was observed respectively. When DNA was conjugated to the cellulose there was actually less binding than to cellulose alone, suggesting that endotoxin does not bind to DNA.

Table 6 shows the effect of two different concentrations of endotoxin on the binding of AHRC to 34 µg DNA in the form of DNA-cellulose. There was about 9.5% inhibition in the binding in the presence of 100 µg of endotoxin and 14.6% inhibition in the presence of 300 µg of endotoxin. When DNA-cellulose was centrifuged after incubation with 100 µg endotoxin and resuspended in Tris/glycerol buffer, there was no inhibition of AHRC binding (line 5).

Table 5. Binding of ^{51}Cr-labeled endotoxin to cellulose or DNA-cellulose.

Experimental	cpm bound
Cellulose (0.1 ml)	13605
Cellulose (0.3 ml)	33531
34 µg DNA-cellulose (0.1 ml)	10358
100 µg DNA-cellulose (0.3 ml)	26516

Table 6. Binding of AHRC to DNA-cellulose in the presence and absence of endotoxin.

Experimental	cpm of AHRC bound	% Inhibition
Cellulose	730	-
DNA-cellulose	3236	-
DNA-cellulose + 100 µg endotoxin	2999	9.5
DNA-cellulose + 300 µg endotoxin	2871	14.6
Endotoxin exposed DNA-cellulose	3300	-

DISCUSSION

Despite the extensive effort devoted to understanding the pathophysiology of bacterial endotoxin, so far there are no conclusive data as to whether endotoxin exerts its toxic effects directly on cells or through release of soluble mediator(s). In the present study we have explored an *in vitro* model which could conceivably yield insight into the nature of metabolic changes observed in endotoxemia and the protective mechanism of cortisone against endotoxin lethality. In this study we have used a line of glucocorticoid-responsive, rat hepatoma (HTC)-cells. This cell line is particularly well suited for such a study because at least five steroid-induced responses have been defined (36) in these cells.

Since glucocorticoids are believed to increase enzyme synthesis by elevating the production of specific messenger RNAs (26,31) it might be postulated that endotoxin suppresses the hormonal induction of hepatic enzymes through an effect on RNA synthesis. However, when a sublethal dose of toxic endotoxin is given to adrenalectomized mice total net RNA and protein synthesis in the liver increases (35). If endotoxin acts by suppressing RNA synthesis there must be a high degree of selectivity in the action of endotoxin. If hepatic parenchymal cells are among the direct targets of endotoxin action, endotoxin might be able to inhibit the hormonal induction of hepatic enzymes by competing with the

hormone for possible binding sites on or within the target cells. Our results tentatively suggest this possibility in that endotoxin can interfere with AHRC binding to chromatin in vitro.

The whole cell uptake of ^{3}H-dexamethasone is not altered in the presence of 350 μg endotoxin (results not presented). It seems unlikely that endotoxin interferes with the cell uptake of the hormone. The fact that endotoxin binds to isolated chromatin in vitro (Table 3) suggests that the association of endotoxin to chromatin may block the ability of hormone-receptors to bind to chromatin. While this tends to support the idea that endotoxin attaches to nuclear chromatin and inhibits AHRC binding, two lines of data suggest caution must be exercised in drawing this conclusion. First, endotoxin seems to bind to chromatin by interaction with proteins rather than DNA. This is suggested indirectly by the observation that endotoxin cannot bind to calf thymus DNA associated with cellulose (Table 5). Secondly, AHRC can bind to DNA (Table 6) and sonicated chromatin (Table 4) but does not bind to deoxyribonuclease-treated nuclei (2) or nuclease-extracted chromatin which might have lost between 25 to 50% of its DNA (28). If the AHRC is binding to DNA and the endotoxin is binding to protein a direct inhibition would not seem likely. Instead, some stereochemical alteration would have to be involved as an explanation. Even though our data do not offer conclusive evidence, what would appear most likely is that

the endotoxin interacts directly with the AHRC, blocking its ability to bind to DNA. This explanation is most consistant with our data since the presence of endotoxin in solution inhibits, in a dose-dependent manner, the binding of AHRC to both chromatin (Table 4) and DNA-cellulose (Table 6). It is possible that endotoxin acts like the inhibitory material which has been detected in the cytosol of HTC-cells and rat uterus (13,37). This inhibitor interacts with the receptor-steroid complex (37) and prevents its binding to the acceptor sites in the nuclei.

The biological significance of the ability of endotoxin to block AHRC binding is further complicated by the need for relatively high concentrations of toxic endotoxin and by the observation that biologically detoxified endotoxin inhibits binding even more efficiently than toxic endotoxin (Table 4). These observations and the fact that bacterial endotoxin cannot inhibit the induction of the hepatic enzyme phosphoenolpyruvate carboxykinase by cortisol <u>in vitro</u> (6) probably indicate that endogenous factors (8,9) or a host-modified form of endotoxin is more likely to be involved in the inhibition of PEPCK induction than is a direct toxicity of endotoxin on hepatic parenchymal cells.

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APPENDIX II

Sephadex G-25 Chromatography of Activated-hormone-receptor-complex (AHRC)

HTC-cell cytosol was incubated with dexamethasone for 2 hr to allow steroid binding to receptor. This was followed by activation of steroid-receptor by supplementing the cytosol with 0.15 M NaCl and incubating at 20-25°C for 30 min. Activated cytosol was passed through 20 ml of Sephadex G-25 equilibrated with homogenizing buffer containing 0.25 M sucrose to remove unbound steroid and salt. Fractions of 1.2 ml were collected. Counts per minute in 0.1 ml and absorbance at 280 nm of each fraction were measured (Fig. 1). The results of these observation indicated that hormone-receptor which is included in the macromolecular fraction of HTC-cell cytosol is coming off the column with the void volume. Dexamethasone and NaCl were held on the column and thus separated from the macromolecular-fraction. Fractions number 6 through 9 were used as crude AHRC.

Quantitation of Chromatin

For quantitation of chromatin, increasing amounts (0.05 to 0.8 ml) of crude AHRC (5.5 mg/ml protein) were mixed with steroid free HTC-cell cytosol (5.5 mg/ml protein) for a total volume of 0.8 ml. As a blank 0.1 ml of the mixture was added to 0.9 ml of 5 mM $MgCl_2$/0.01 M tris buffer. For binding a constant amount of chromatin (10 µg DNA) was added to 0.7 ml of crude AHRC (0.05-0.7 ml) and steroid free cytosol for 90 min at OC. After incubation and work up (Material and Methods for second paper), chromatin bound receptor-steroid

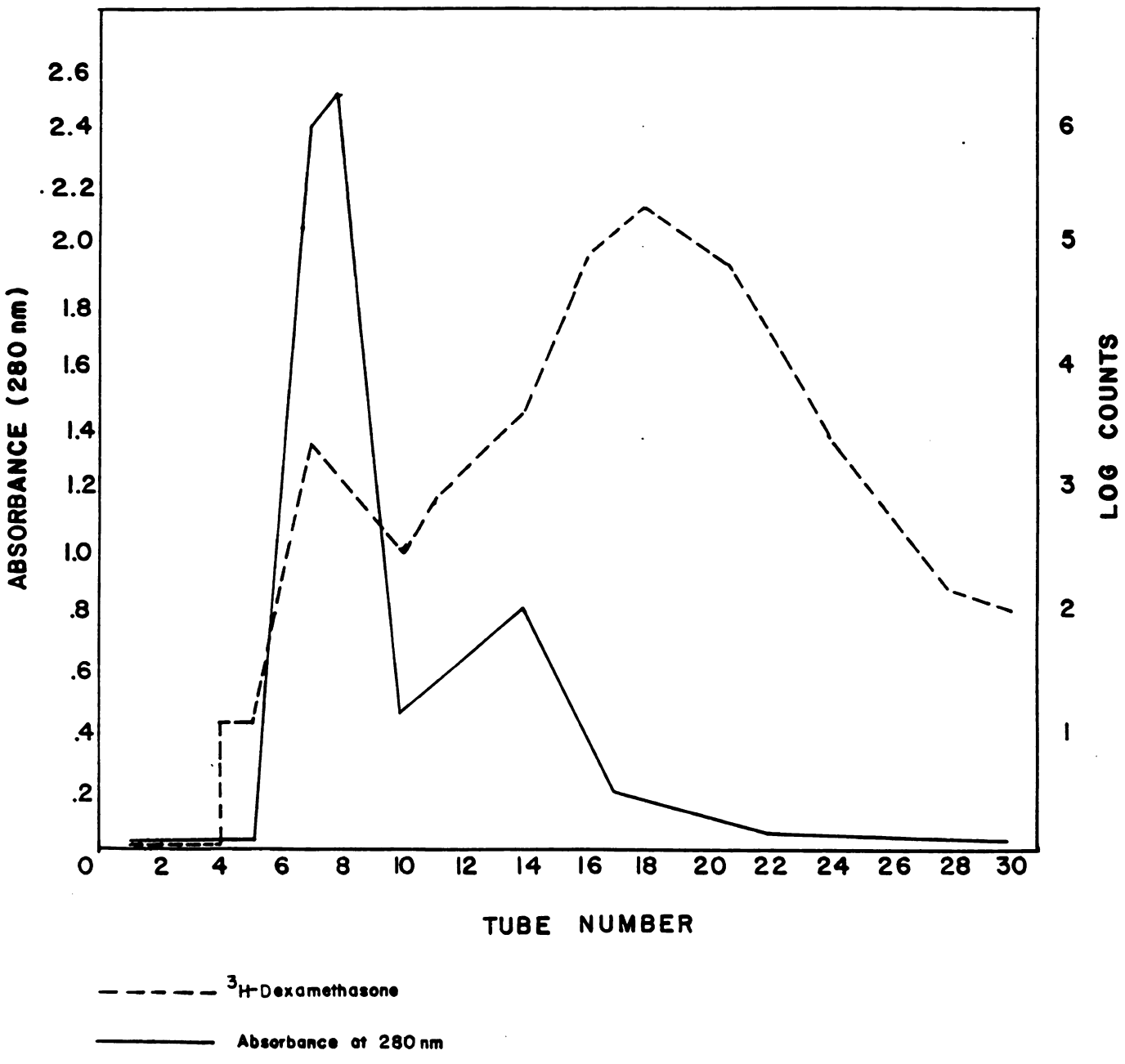

FIG. 1. SEPHADEX G-25 CHROMATOGRAPHY OF ACTIVATED HORMONE-RECEPTOR COMPLEX

complex was calculated as cpm of precipitated assay mixture minus blank . Fig. 2 shows that with constant protein when the concentration of ^{3}H-labeled AHRC reaches 3 mg per ml protein, chromatin (10 µg DNA) becomes saturated, and no more AHRC can bind to it.

Quantitation of Activated Hormone-receptor Complex

For quantitation of AHRC, 0.6 ml of crude AHRC (5.5 mg/ml protein) was mixed with 0.2 ml steroid free cytosol (5.5 mg/ml protein). As a blank, 0.1 ml of this mixture was mixed with 0.9 ml 5 mM $MgCl_2$/0.01 M tris buffer. Different amounts of (1 µl-40 µl) of chromatin (1 mg/ml DNA) was added to 0.7 ml of crude AHRC and steroid free cytosol for 90 min at 0°C. After incubation and work up (Materials and Methods), chromatin bound receptor-steroid complex was calculated as (CPM of precipitated assay mixture-blank). Fig. 3 shows that as the concentration of chromatin increases (1 µg to 40 µg DNA) more AHRC becomes associated with the chromatin.

In all of the hormone receptor binding experiments, 0.4 ml AHRC (5 mg/ml protein), either in the presence or absence of endotoxin, was incubated with 10 µl chromatin (10 µg DNA).

Binding of Activated Hormone-receptor Complex to Sonication- and Nuclease-extracted Chromatin

Since nuclease extracted chromatin is supposingly more close to the native form of chromatin, attempts were made to use the nuclease-extracted chromatin instead of sonication-

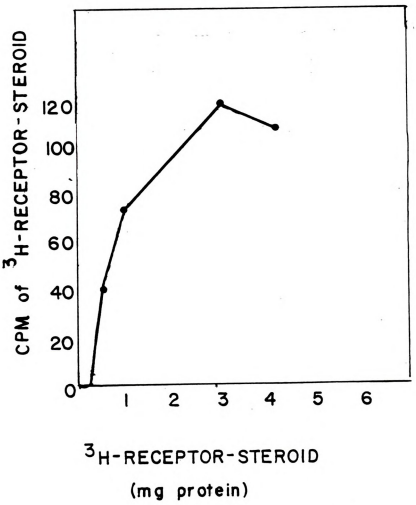

FIG. 2. BINDING OF INCREASING AMOUNTS OF ^{3}H-DEXAMETHASONE ACTIVATED-RECEPTOR TO CONSTANT AMOUNT OF CHROMATIN

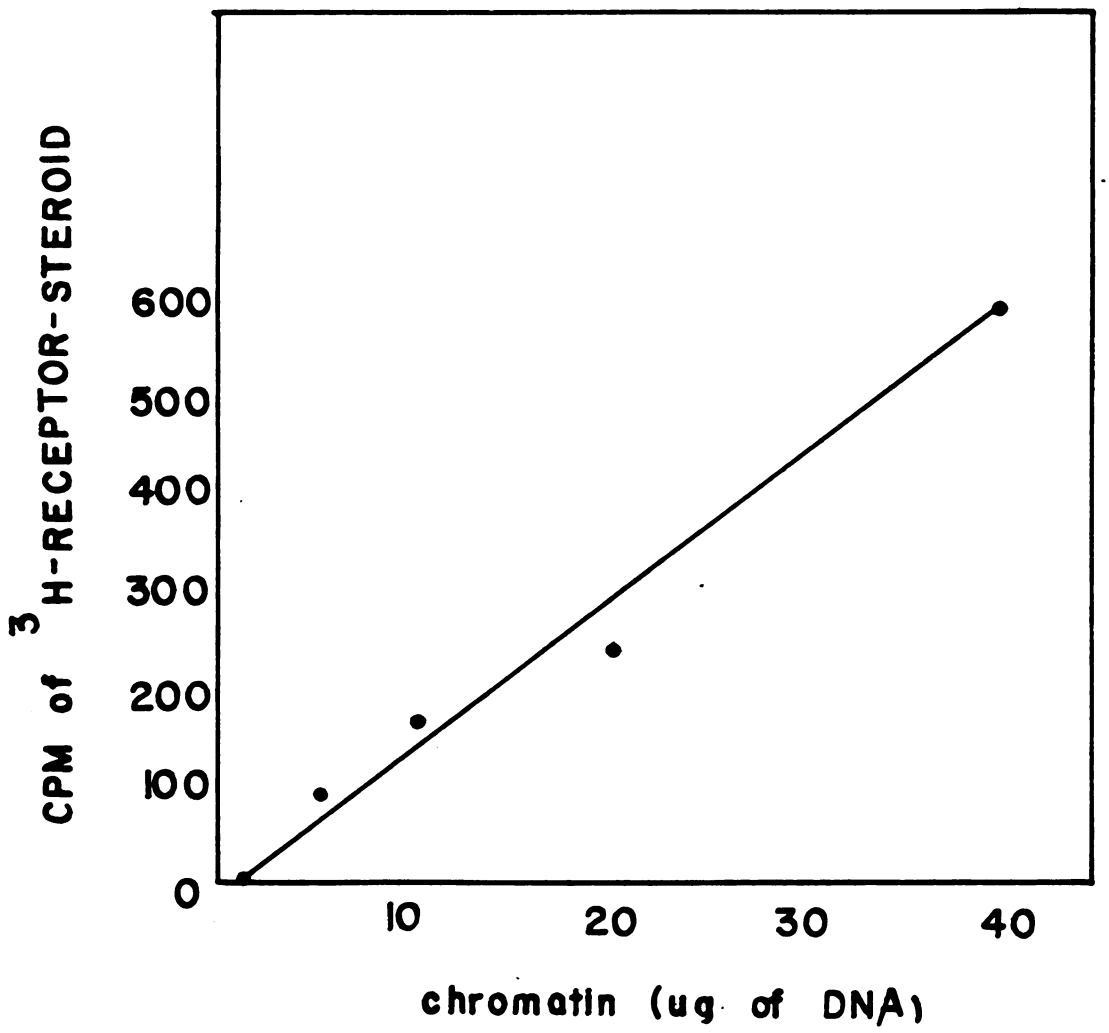

FIG. 3. BINDING OF CONSTANT AMOUNT OF ^{3}H-DEXAMETHASONE ACTIVATED-RECEPTOR TO INCREASING AMOUNTS OF CHROMATIN

prepared chromatin. Nuclease-extracted chromatin was prepared by suspending the nuclei at a concentration of $1x10^8$ per ml in 0.34 M sucrose buffer A [15 mM Tris (pH 7.4), 60 mM KCl, 1.5 mM NaCl, 0.15 mM spermine, 0.5 mM spermidine, 15 mM β-mercapto-ethanol, 67 mM phenylmethylsulfonyl fluoride]. The suspension was made 1 mM in $CaCl_2$ and digested with micrococcal nuclease (1 μg/ml) for 10 min at 37°C. The reaction was stopped by chilling on ice and addition of ethylenedinitrilotetraacetic acid (EDTA) to a final concentration of 2 mM. The nuclei were centrifuged for 5 min at 4,000xg, suspended in 1 ml of 0.2 mM EDTA, (pH 7) with a pasture pipette and recentrifuged for 2 min at 4,000xg. The opalescent supernatant was used as nuclease-extracted chromatin.

Table 1 shows that while there was about 345 cpm of AHRC bound per 10 μg of DNA in sonication-prepared chromatin, no significant binding of AHRC to nuclease-extracted chromatin was observed. Since nuclease-extracted chromatin might have lost between 25 to 50% of its DNA, the absence of significant binding of AHRC to nuclease-extracted chromatin tends to support the idea that the binding site of glucocorticoid-receptors in target cell nuclei is DNA.

Table 1

Binding of activated hormone-receptor complex to sonication-prepared and nuclease-extracted chromatin.

AHRC incubated with	CPM
Sonicated chromatin	793
$MgCl_2$ for sonicated chromatin	503
Nuclease-prepared chromatin	594
$MgCl_2$ for nuclease-prepared chromatin	541