THE LETHAL EFFECT OF ENDOTOXINS ON THE CHICK EMBRYO*

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The physiological alterations produced in animals by the endotoxins of Gram-negative bacteria include such diverse reactions as fever, leucopenia, hyperglycemia, and a series of vasomotor reactions terminating in a state similar to irreversible traumatic shock (1). The pathological effects of endotoxins suggest that the end result of their action is damage to the blood vessels, as exemplified by the hemorrhagic and necrotizing lesions of the local and generalized Shwartzman phenomena, but the mode of action of endotoxin remains unexplained. Studies of the latter reactions have indicated that they do not represent direct, destructive effects in blood vessels, but seem rather to involve a series of secondary events in which the animals' own mechanisms play a determining role in the production of tissue damage. For example, the hemorrhagic skin lesions of the local Shwartzman reaction develop as the result of occlusion of venules and capillaries by platelet-leucocyte thrombi, followed by dilatation of these vessels and rupture of their walls (2). The characteristic lesions of the generalized Shwartzman reaction are fibrinoid necrosis of the coronary arteries and bilateral renal cortical necrosis; these seem to be the result of the deposition of precipitated fibrinogen within the walls and lumen of blood vessels, leading to ischemic necrosis of the tissues which they supply (3–5).

Although the numerous manifestations of endotoxin have been widely investigated, little is known about the pharmacological properties which cause these responses. It has been suggested that the systemic vasomotor effects in rabbits are similar to those of adrenalin (6), and that endotoxins may be toxic because of a direct action on autonomic centers or conduction pathways (7). Recent studies indicate that endotoxins have the property of greatly enhanc-

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ing the reactivity of small vessels to adrenalin, with the result that adrenalin itself becomes a potent necrotizing agent (8). On the other hand, the suggestion has been made that the properties of endotoxin are in many ways similar to those of tuberculin in tuberculous animals, and the systemic response may represent a manifestation of bacterial allergy (9).

The complexity of the reactions to endotoxins in experimental animals, and the implication of numerous secondary effects in the events leading to tissue damage, have made it difficult to identify the events which represent primary, direct actions of these materials. Indeed, no satisfactory *in vitro* technique has yet been devised by which it can be shown that endotoxins have a direct toxic effect on any known cell or system of cells.

In an attempt to develop a simpler experimental model in which the effects of endotoxin might be more closely observed and manipulated, the response of developing chick embryos to endotoxin has been studied. It has been found that embryos are extremely vulnerable to the lethal effect of small doses of endotoxin, and, as was previously shown in rabbits (10), death may be prevented by cortisone. The present report is concerned with the influence of the age of embryos, the route of inoculation, the temperature of incubation, and the administration of 17-hydroxycorticosteroids on the response to endotoxin.

Materials and Methods

Eggs.—Large brown eggs obtained from a single flock of New Hampshire hens were used in all experiments reported herein. In other experiments White Leghorn eggs were found to be equally susceptible. The eggs were incubated in a Jamesway self-turning incubator regulated at 38.0 ±0.5°C. unless otherwise indicated. Fertility of the eggs varied between 70 and 85 per cent.

On the day of an experiment, the eggs were candled, an 0.5 × 0.5 cm. window drilled over the chorioallantoic membrane (CAM) and the membrane dropped by suction over the air sac. The substance to be tested was inoculated upon this membrane, the window sealed with cellophane tape and the eggs returned to a non-turning tray in the incubator. The eggs were broken in a Petri dish 18 to 24 hours later, deaths determined, and the incidence of hemorrhages noted in living embryos. Fifty per cent lethal endpoints were determined by the method of Reed and Muench (11).

Routine bacteriological studies of the injected materials, and the chick embryos (CE) at the time of sacrifice, included smears and cultures on blood agar plates and in thioglycollate broth. Bacterial contamination was extremely rare; experiments in which it occurred were discarded.

For intravenous injections, a vein in the chorioallantoic membrane was identified by candling, a small oblong window cut over it, the shell membrane cleared by swabbing with sterile mineral oil, and the injection made using a 27 gauge needle. Eggs observed to bleed significantly from the injected vein were discarded.

Allantoic sac, yolk sac, and amniotic sac inoculations were made by standard techniques described elsewhere (12).

Endotoxin.—Meningococcal endotoxin in the form of "agar washings" was prepared by a method previously described (13). The preparations employed were known to be potent

pyrogens, produced the local and generalized Shwartzman reactions, and were lethal when injected into mature rabbits. Other endotoxin preparations used included the following: *Serratia marcescens* endotoxin, lot P-25 provided by Dr. Murray Shear of the National Cancer Institute, Bethesda, Maryland; a purified preparation of *Shigella paradysenteriae* endotoxin provided by Dr. Walther Goebel of the Rockefeller Institute for Medical Research, New York; *Escherichia coli* 0127-B8 endotoxin prepared by Webster's modification of the method of Boivin and Mesrobeanu (27) at Difco Laboratories, Detroit; a crude dialyzed trichloroacetie acid extract of *E. coli*; and a Boivin-type extract of *E. coli* purified by alcohol fractionation obtained from Dr. A. I. Braude, Southwestern Medical School, Dallas.

Serial tenfold dilutions of the endotoxin preparation to be inoculated were made in sterile pyrogen-free saline, and inoculated in a 0.05 ml. volume using a tuberculin syringe and a 27 gauge needle. Non-sterile purified endotoxin preparations were heated for 10 minutes in a boiling water bath, and cultured to assure sterility prior to use.

Steroids.—Cortisone (free alcohol), hydrocortisone, and 9-alpha fluorohydrocortisone were obtained through the courtesy of Dr. Elmer Alpert, Merck and Co., Rahway, New Jersey. A preparation of hydrocortisone in 50 per cent ethyl alcohol (cortef) and preparations of crystalline 1-dehydrocortisone, and desoxycorticosterone were provided by the Upjohn Company, Kalamazoo. Because of the low solubility of some of the crystalline steroids in aqueous solution, the crystalline steroids were dissolved in absolute ethanol and diluted appropriately in ethanol-saline mixtures to contain, in the final dilution, the desired amount of steroid in 10 per cent ethanol-saline. Ten per cent ethanol-saline had no demonstrable effect in the normal CE, and did not influence the lethal outcome of endotoxin-inoculated CE. In all experiments steroids were inoculated on the CAM or injected intravenously in a 0.05 ml. volume.

EXPERIMENTAL

The Lethal Effect of Meningococcal Endotoxin.—Groups of 10 day old chick embryos were inoculated on the chorioallantoic membrane (CAM) with undiluted meningococcal endotoxin in a 0.05 ml. volume, and the eggs incubated at 38.0°C. At various times after inoculation, groups of eggs were opened and placed in Petri dishes, in which direct observations of the embryo and membranes were made using a stereomicroscope and a direct cool light source.

Following a latent period of 1 to 2 hours, minute hemorrhages became visible in the skin of the embryo, particularly along the dorsal surface and about the head and eyes. These hemorrhages increased in size during the next hour and often became confluent, particularly over the head and eyes. Hemorrhages in the region of the dorsal vascular plexi were prominent. Blood flow in the small arterioles and venules of the extraembryonic membranes was observed through large shell windows in eggs maintained at 38°C. Concurrently with the appearance of hemorrhages in the embryo, 1 or 2 hours after inoculation, flow was observed to slow gradually, with agglomerates of "sludged" blood in many of the small vessels. Complete cessation of blood flow in the extraembryonic membranes often occurred at about 4 hours after injection.

To obtain histopathological data, embryos were inoculated with a lethal dose of endotoxin, and sacrificed after varying periods of time. The embryos were fixed in formalin and sections were stained with hematoxylin and eosin.

Congestion of vessels and numerous perivascular hemorrhages were present in almost all embryos, particularly in the mesenchymal connective tissues. No changes, other than hemorrhages, were found in any of the organs. The result of a titration of meningococcal endotoxin, using tenfold falling dilutions of endotoxin, in 10-day embryos, is shown in Table I. In this experiment the eggs were opened 18 hours after inoculation. An LD_{50} value (0.05 ml.) of $10^{-3.12}$ was obtained with this batch of endotoxin. Similar titrations have been performed on approximately fifty different lots of meningococcal endotoxin. Although each sample was found to be lethal, some variation of the LD_{50} in different lots, and also in titrations of the same lot on different days, was

TABLE I

The Lethal Effect of Meningococcal Endotoxin on the 10-Day Chick Embryo

Material inoculated*	No. inoculated	No. of deaths	Deaths
			per cent
Meningococcal endotoxin Lot D105			
Undiluted	18	18	100
1–10	14	14	100
1–100	17	16	94
1–1000	16	6	37.5
1–10000	14	0	0
Saline	20	0	0
Agar washings, undiluted	20	0	0
Old tuberculin, undiluted	10	0	0
Streptolysin 0, undiluted	10	0	0

* The material indicated, diluted in saline, was inoculated on the CAM of 10-day chick embryos in a 0.05 ml. volume; deaths were determined after 18 hours incubation at 38.5°C.

noted in the early phase of this study. However, when the crucial importance of embryonic age and the temperature of incubation were recognized and these variables controlled as described below, the results of consecutive titrations of any particular batch of this endotoxin were substantially the same.

Comparison of the Lethal Effect of Various Endotoxin Preparations.—Following the observation of a lethal action of meningococcal filtrates on the chick embryo, it was necessary to learn whether the lethal activity represented an effect of the endotoxin of the meningococcus, or some other product contained in the crude agar washings. Several types of evidence were obtained which indicate that endotoxin was the cause of death.

Control inoculations of agar washings harvested from sterile media caused no deaths, indicating that no constituent of the media was responsible for the lethal effect. Also, inoculation of such diverse substances as tuberculin, strep-

tolysin O, foreign serum, and acid-hydrolyzed meningococcal filtrates under the same conditions failed to cause embryonic hemorrhage or death.

Heat stability, a characteristic property of endotoxins (14), was demonstrated for the lethal property of meningococcal culture filtrates, in the following experiment.

Three aliquots from a lot of endotoxin were treated as follows: one was heated 30 minutes at 56°, a second for 30 minutes in a boiling water bath, and a third aliquot was untreated. Tenfold dilutions of these samples were inoculated in 10-day embryos.

As is shown in Table II, the heated preparations possessed the same toxicity as the unheated control.

Presumptive evidence that the lethal property under study is attributable

TABLE II

Lethal Effect of Meningococcal Endotoxin on the Chick Embryo; Heat Stability of the Lethal Factor

Treatment of endotoxin	Deaths			
	Endotoxin dilutions*			
	10^{0}	10^{-1}	10^{-2}	10^{-3}
None	8/8‡	6/8	2/8	0/8
Heated 56°C. fo 30 min	7/8	5/8	2/8	
In boiling water bath for 30 min	8/8	7/9	3/8	

* 0.05 ml. of indicated endotoxin dilution inoculated on CAM of 10-day chick embryos. Deaths determined after 18 hours.

‡ Numerator, No. dead; denominator, No. inoculated.

to endotoxin was provided by tests with endotoxins derived from other microorganisms, including several highly purified lipopolysaccharide preparations. The results, summarized in Table III, showed that each of the endotoxins caused hemorrhage of the embryo and death within a few hours. Considerable variation in activity was encountered, perhaps depending on the source or the degree of purity of the preparations.

Preparation A, a purified lipopolysaccharide endotoxin prepared from *E. coli* by trichloracetic acid extraction, consistently yielded LD_{50} values between 0.4 and 1.2 μg., representing the most potent lethal activity, based upon dry weight, of all preparations tested. Preparation B, a purified endotoxin from *Shigella paradysenteriae*, prepared by the pyridine extraction method of Goebel *et al.* (15), was lethal in doses of less than 5 μg. Preparation C, the "tumor-necrotizing" polysaccharide endotoxin of *S. marcescens* prepared by Shear, and preparation D, derived from *E. coli* by the method of Braude (16), showed considerably less lethal activity.

Time of Death.—In experiments designed to determine the time of death in

TABLE III

The Lethal Effect of Various Purified Endotoxins

Preparation designation	Microorganism	Method of preparation	Amount of inoculum*	Deaths	LD_{50}‡
			μg.		μg.
A	*Escherichia coli* 0127-B8	Partially purified trichloracetic acid extract (Difco)	100	10/10	0.66
			50	10/10	
			10	9/10	
			1	7/10	
			0.1	1/10	
			0.01	1/10	
B	*Shigella paradysenteriae*	Purified "toxic antigen" (Goebel)	100	6/6	4.2
			50	4/6	
			10	5/6	
			1	2/6	
C	*Serratia marcescens*	Purified polysaccharide (Shear) Lot P-25	100	3/6	100
			50	1/6	
			10	0/6	
D	*Escherichia coli*	Purified trichloracetic acid extract (Braude) Lot 14	100	6/8	62
			50	4/8	
			25	1/8	
			5	0/8	
			1	0/8	

* The material indicated was inoculated on the CAM of 10-day chick embryos in a 0.05 ml. volume of saline; deaths were determined after 18 hours incubation at 38.5°C.

‡ LD_{50} calculated by method of Reed and Muench (11).

TABLE IV

Lethal Effect of Meningococcal Endotoxin in Chick Embryo; Time of Death

Time interval after endotoxin*	Deaths		
	Endotoxin dilution		
	Undiluted	10^{-1}	10^{-2}
hrs.			
24	6/6‡	5/6	2/6
12	4/4	6/6	1/3
6	3/4	2/6	0/2
4	4/4	2/4	1/4
2	1/4	0/4	1/7
1	0/4	0/4	0/4

* Groups of 10-day eggs inoculated on CAM with 0.05 ml. of the dilution of meningococcal endotoxin indicated; examined after the indicated time interval.

‡ Numerator, No. dead; denominator, No. inoculated.

embryos receiving various amounts of endotoxin on the CAM, groups of twenty-five to thirty eggs were inoculated with tenfold dilutions of meningococcal endotoxin. After intervals varying from 1 to 24 hours, four to six eggs from each group were opened and deaths determined. The results of an illustrative experiment are given in Table IV. At 2 hours, the embryos receiving the largest amount of endotoxin showed hemorrhages in all and death in one. At 4 hours, and at each of the subsequent harvestings, all such embryos were found dead. With the higher dilutions of endotoxin, the total mortality was lower, but death still occurred within 4 hours in some of the embryos. In experiments in which eggs were allowed to incubate for 48 or 72 hours, no additional deaths

TABLE V

Lethal Effect of Meningococcal Endotoxin on the Chick Embryo; Influence of Route of Inoculation

Route of inoculation	Deaths						
	Dilution of endotoxin*						
	Undiluted	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	Saline
Chorio-allantoic membrane	18/19‡	15/18	10/16	3/16	0/14	0/10	0/12
Intravenous		2/2	2/2	5/6	4/6	1/6	0/4
Yolk sac	3/10	1/10	2/10	0/10			0/10
Amniotic sac	2/7	1/6	0/6				0/6
Allantoic sac	0/5	0/6	0/6	0/6			0/6

* 0.05 ml. of the indicated dilution of meningococcal endotoxin inoculated into 10-day chick embryos by route shown. The embryos examined 24 hours later, deaths recorded.

‡ Numerator, No. dead at 24 hours; denominator, No. inoculated.

occurred in embryos receiving the higher dilutions of endotoxin, indicating that the issue was determined within 24 hours or less after inoculation.

Route of Inoculation.—

In an attempt to investigate the site of action of endotoxin, the lethal activity of meningococcal endotoxin was compared by various routes of inoculation. Groups of 10-day embryos were inoculated with tenfold dilutions of meningococcal endotoxin on the CAM, and into the yolk sac, the allantoic sac, the amniotic sac. Similar groups were injected intravenously with various dilutions of endotoxin.

The outcome of this experiment, shown in Table V, indicates that intravenous injection and CAM inoculation caused the highest incidence of deaths. Intravenous injection produced death in dilutions as high as 1–100,000, giving LD_{50} values slightly more than tenfold below those obtained in the groups inoculated on the CAM. Yolk sac and amniotic sac inoculation in this experiment gave low values for lethal activity. No deaths occured in any experiment when inoculation was made into the allantoic sac, even with doses as large

as 0.5 ml. of undiluted meningococcal endotoxin, and 100 μg. of *E. coli 0127-B8* endotoxin.

Effect of Age of the Embryo.—Early in the course of this study extreme variability in the susceptibility of batches of eggs to endotoxin was frequently encountered; in retrospect, it seemed that variation in the age of the embryos might be in part responsible for these irregularities. Experiments were therefore designed to study the influence of age on susceptibility. In a series of experiments, summarized in Table VI, batches of eggs of different ages were inoculated with serial dilutions of a single lot of meningococcal endotoxin, and

TABLE VI

Lethal Effect of Meningococcal Endotoxin on Chick Embryo; Influence of Age

Age of embryo*	Deaths					
	Endotoxin dilution					
	Undiluted		10^{-1}		10^{-2}	
	No.	Per cent	No.	Per cent	No.	Per cent
days						
6	1/10‡	10	0/10	0	0/10	0
8	25/58	43	17/55	31	10/50	20
10	55/64	86	35/63	56	18/61	30
12	20/44	45	13/40	33	6/41	15
14	5/21	24	4/19	21	1/22	5
16	1/10	10	0/10	0	0/10	0

* Eggs were placed in incubator simultaneously; groups of eggs were inoculated on the day indicated with 0.05 ml. of undiluted, 10^{-1}, or 10^{-2} dilutions of the same lot (D78) of endotoxin. Deaths determined after 18 hours incubation at 38.5°C.

‡ Numerator, No. dead; denominator, No. inoculated.

the number of deaths recorded 24 hours later. The results indicate a striking influence of age on susceptibility. The 10 day old embryos were highly susceptible, while the younger or older embryos showed much less reactivity to any of the doses of endotoxin employed. At 6 days, and at 16 days, the embryos appeared to be completely insusceptible.

In other experiments, maximum susceptibility at the age of 10 days was also demonstrated when endotoxin was administered by the yolk sac or intravenous routes. With intravenous endotoxin it was possible to cause death in a small proportion of 14-day embryos, but no deaths were produced by undiluted endotoxin in embryos of 16 days or older.

Effect of Endotoxin on Newly Hatched and Adult Chickens.—In view of the high degree of susceptibility of embryos to the lethal action of endotoxin at the age of 10 days, and the lack of susceptibility at 16 days and over, it was of interest to determine the effect of endotoxin in newly hatched chicks, and in

older fowl. No evidence of toxicity could be demonstrated in either group of birds. Doses of endotoxin 1,000 times larger than the 50 per cent lethal dose for 10-day embryos had no untoward effect when given intravenously to eight new-born chicks, or to eight mature hens.

Effect of the Temperature of Incubation.—It was found that the temperature at which the eggs were incubated after inoculation was of great importance in determining the outcome. The protective effect of lowered temperatures of incubation is illustrated by the experiment summarized in Table VII, in which groups of eggs were inoculated on the CAM with tenfold dilutions of meningococcal endotoxin, and incubated at 39.5, 36.0, and 28.0°C. After 18 hours

TABLE VII

Lethal Effect of Meningococcal Endotoxin in the Chick Embryo; Influence of Temperature of Incubation

Temperature of incubation	Deaths				
	Dilution of endotoxins*				
	Undiluted	10^{-1}	10^{-2}	10^{-3}	Saline
°C.					
39.5	9/9‡	9/9	7/9	0/9	0/9
36.0	10/10	7/9	1/9	0/9	
28.0	0/8	0/9	0/9	0/9	0/9

* 10-day chick embryos inoculated with 0.05 ml. of the indicated dilution of endotoxin on the CAM, immediately placed at the temperature indicated. After 24 hours incubation at that temperature, deaths were recorded.

‡ Numerator, No. dead; denominator, No. inoculated.

incubation at these temperatures, the number of deaths was determined. Maximum lethal effect for this lot of endotoxin was obtained in the group incubated at 39.5°C.; the group incubated at 36.0°C. was less susceptible, and no deaths occurred in the group incubated at 28.0°C. In repeated experiments conducted at various temperatures, it was found that maximal susceptibility occurred at incubation temperature of 38.0–39.5°C., and that incubation below 37°C. consistently resulted in a pronounced decrease in mortality. Complete protection was afforded by incubation at temperatures below 30°C.

In one experiment eggs were inoculated with lethal amounts of endotoxin then held at a temperature of 28°C., for a period of 24 hours. After examination of an aliquot group of the eggs had revealed that this inoculum had produced neither hemorrhagic lesions or death of the embryos, the remainder of the eggs were placed in the 38°C. incubator. The regular occurrence of death of the embryo after 12 hours of further incubation at this temperature indicated that the endotoxin retained its lethal effect in the cooled eggs.

Protection against Lethal Effect of Endotoxin by 17-Hydroxycorticosteroids.—

The potent lethal effect of intravenously administered meningococcal endotoxin in mature rabbits has been shown to be completely prevented by the administration of cortisone or hydrocortisone prior to endotoxin injection

TABLE VIII

Lethal Effect of Meningococcal Endotoxin on the Chick Embryo; Protection by Various Amounts of Cortisone, Hydrocortisone, and 9-Alpha Fluorohydrocortisone

Group	Deaths				
	Endotoxin dilution*				
	Undiluted	10^{-1}	10^{-2}	10^{-3}	10^{-4}
Endotoxin plus cortisone					
20 μg	1/7‡	0/6			
10 "	2/6	0/5			
5 "	4/6	1/5			
1 "	5/6	1/5			
Endotoxin alone (controls)	6/6	6/6	6/6	4/6	0/6
Endotoxin plus hydrocortisone					
1.0 μg.	3/7	1/6	0/6		
0.1 "	6/8	5/7	1/7		
0.01 "	8/8	4/7	5/6	2/6	0/7
0.001 "	8/8	6/6	6/6	2/7	0/8
Endotoxin alone (controls)	7/7	7/7	5/7	3/7	1/7
Endotoxin plus 9-alpha fluorohydrocortisone					
2.5 μg.	0/7	0/7			
0.25 "	2/7	3/7	1/7		
0.05 "	5/7	2/7	3/7	3/6	
0.01 "	6/7	6/7	6/8	1/7	
Endotoxin alone (controls)	7/7	7/7	5/7	3/7	0/7

* 0.05 ml. of the indicated dilution of meningococcal endotoxin in saline on the CAM of 10-day CE. Various amounts of cortisone, hydrocortisone, or 9-alpha fluorohydrocortisone in 10 per cent ethanol-saline inoculated in 0.05 ml. volume on the CAM immediately before endotoxin. Controls receiving the largest amount of steroid indicated alone, were all living and are not included. Deaths were determined after 24 hours incubation at 38.5°C.

‡ Numerator, No. dead; denominator, No. inoculated.

(10), or by intravenous injection of soluble hydrocortisone up to 2 hours following endotoxin (17). It was of interest, therefore, to examine the effect of steroids on the lethal action of endotoxin in the chick embryo.

In preliminary experiments, 0.02 ml. of aqueous suspensions of cortisone acetate or hydrocortisone containing 25 mg. per ml. were inoculated on the

CAM simultaneously with endotoxin, and were found to protect against a dose of 800 LD_{50} of meningococcal endotoxin. Since it was difficult to control the amounts of steroid inoculated or estimate the activity on a weight basis, soluble preparations of cortisone, as well as hydrocortisone, 9-alpha fluorohydrocortisone, 1-dehydrocortisone, desoxycorticosterone, and cholesterol were prepared in 10 per cent ethanol-saline diluent. By themselves, doses of

TABLE IX

Effect of Various Steroids on the Lethal Effect of E. coli 0127-B8 Endotoxin in the Chick Embryo

Steroid inoculated,* μg.	Deaths		
	E. coli 0127-B8 endotoxin, μg.		
	50	10	1
None	8/8‡	8/8	3/8
Hydrocortisone, 1.0	3/10	0/10	0/10
0.1	—	2/10	0/10
Desoxycorticosterone, 5.0	10/10	8/10	2/8
1.0	—	10/10	4/8
1-dehydrocortisone, 5.0	—	8/8	—
1.0	—	8/8	—
0.1	—	6/8	—
Cholesterol, 5.0	—	8/8	4/8
10% ethanol-saline	—	8/8	4/8

* 0.05 ml. containing the indicated amount of steroid in 10 per cent ethanol-saline was inoculated on the CAM of 10-day CE simultaneously with 0.05 ml. containing the indicated amount of *E. coli 0127-B8* endotoxin. After 18 hours incubation at 38.5°C., deaths were determined.

‡ Numerator, No. dead; denominator, No. inoculated.

these steroids up to 20 μg. had no apparent effect in the gross on the normal embryo within 18 hours. Representative experiments in which various amounts of cortisone (free alcohol), hydrocortisone, and 9-alpha fluorohydrocortisone were inoculated in a 0.05 ml. volume on the CAM simultaneously with tenfold dilutions of meningococcal endotoxin are shown in Table VIII. Table IX gives the result of an experiment in which hydrocortisone was found to afford protection against *E. coli* endotoxin, and in which the lack of protection by desoxycorticosterone, 1-dehydrocortisone, cholesterol, and the ethanol-saline diluent is demonstrated. To summarize the data, cortisone, hydrocortisone, and 9-alpha fluorohydrocortisone were protective in very small quantities

against the lethal effect of endotoxin. One μg. of hydrocortisone, for example, gave protection against approximately 1,000 LD_{50} of meningococcal endotoxin, and 0.1 μg. protected against 50–75 LD_{50}. Nine-alpha fluorohydrocortisone gave a comparable degree of protection.

DISCUSSION

Endotoxin preparations from a variety of Gram-negative microorganisms have been shown to be lethal for the chick embryo under defined experimental conditions. The lethal effect was maximally demonstrable in 10 day old embryos, while younger or older embryos were much less susceptible. Intravenous injection and CAM inoculation resulted in maximal activity, while other routes were relatively ineffective. Incubation temperatures of 38–39.5°C. were found to be optimal for the effect of endotoxin, and embryos incubated at 28°C. were fully protected against death. 17-hydroxycorticosteroids, including cortisone, hydrocortisone, and 9-alpha fluorohydrocortisone, in relatively small amounts, provided protection of embryos against the lethal action of large doses of endotoxin. Cholesterol, desoxycorticosterone, and 1-dehydrocortisone failed to give significant protection.

Evidence which indicates that the lethal effect of the preparations employed was attributable to their endotoxin content includes the following: (*a*) the effectiveness of preparations derived from a variety of Gram-negative microorganisms by a variety of methods, and the lack of effect of miscellaneous control substances; (*b*) the apparent vascular effect as indicated by the occurrence of widespread hemorrhage; (*c*) protective activity of 17-hydroxycorticosteroids analogous to that demonstrated against endotoxin in mammalian species (10, 18); and (*d*) heat stability, at 100°C. for 30 minutes, of the lethal factor in meningococcal and other preparations tested. It is of interest to compare the susceptibility of the chick embryo with that of other species which show a lethal response to endotoxin. Since 10-day old embryos, which weigh 4 to 5 gm., are susceptible to about 1 μg. of *E. coli* endotoxin, it may be calculated that the 50 per cent lethal dose is between 200 and 300 μg. per kg. This figure corresponds roughly to the general range of susceptibility of the adult rabbit to severely intoxicating or lethal doses of this preparation (10). On the other hand, the embryo appears to be much more susceptible than the mature mouse; in this species the 50 per cent lethal dose of this endotoxin is approximately 10 mg./kilo. The relatively high degree of sensitivity of the embryo to endotoxin indicated by these studies may offer a partial explanation for the observation that embryonated eggs up to 13 days of age infected by certain Gram-negative microorganisms, particularly the meningococcus (19), usually die with multiple petechiae and subcutaneous hemorrhages within less than 24 hours.

The protective action of 17-hydroxycorticosteroids is in agreement with

numerous studies in mammalian species. The mechanism of this protection remains unknown. It should be noted that the amounts of steroid needed for protection of the embryos are distinctly less than those which prevent the lethal effect in mammalian species. For example, it may be calculated roughly that a 10 day embryo is protected against 50–75 LD_{50} of endotoxin by hydrocortisone, in a range of 30 to 100 μg. per kg., as contrasted with the dose of 2.5 mg. per kg. required to protect the adult rabbit against a lethal amount of endotoxin (17). Protection of embryos by cortisone, hydrocortisone, and 9-alpha fluorohydrocortisone, and not by desoxycorticosterone is consistent with the findings in mammalian species. On the other hand, the failure of 1-dehydrocortisone to afford protection is surprising and unaccountable, in view of its close structural similarity to cortisone, and its potent cortisone-like effects in mammals.

It seems reasonable to assume that the underlying mechanisms involved in the action of endotoxin on the chick embryo must be similar or in some way related to the events which occur in mammalian species. However, as an experimental animal susceptible to endotoxin, the chick embryo differs from the mammals studied thus far in at least two important respects, both of which bear upon the various hypotheses which have been advanced to explain the mechanism of action of endotoxin. First, the chick embryo is generally considered to be an essentially "germ-free", immunologically pristine, experimental subject (20). Presumably, then, if bacterial allergy to Gram-negative microorganisms or their products, analogous to tuberculin hypersensitivity, were responsible for the endotoxin reaction, as recently proposed (9), it would appear that it is not acquired in this species by prior exposure to Gram-negative bacteria. The findings are more compatible with the interpretation that the response to endotoxin involves a pharmacologic action which is not dependent on allergy, although its other manifestations in animals may be similar.

Secondly, the chick embryo differs from other species in the influence of age on susceptibility to endotoxin. The embryo becomes maximally susceptible at about 10 days, then progressively less so at older ages, and newly hatched chickens and mature hens are completely refractory. In contrast to this age pattern of reactivity, rabbits become maximally susceptible at the time of full maturity, and immature rabbits are relatively resistant to the lethal effect of endotoxin (21). If the mechanism of action is the same in these two species, it would be necessary to postulate that an undefined state of affairs exists for a limited period during embryonic development of the chick which is comparable to conditions which are constantly present in fully matured rabbits. This set of circumstances is also difficult to reconcile with the interpretation of the reaction to endotoxin as representing bacterial allergy (9). It seems unlikely that bacterial allergy to the endotoxins of gram-negative micro-

organisms should appear between the 8th and 10th day in the chick embryo, and even more unlikely that it should disappear soon afterward. Nor can this sequence of events be explained satisfactorily on the basis of times of appearance of other known defense mechanisms in the embryo.

For example, it has been reported that the chick embryo, on about the 12th to 14th day of incubation, first acquires the capacity to develop an inflammatory response to bacterial infection (19), and to reject rat or rabbit heterografts (22). Although it is possible that these responses represent development of mechanisms of resistance which are also involved in the resistance to endotoxin appearing at this time during incubation, such an hypothesis would fail to account for the equal degree of resistance of the 6 and 7 day embryos.

It is possible that the peculiar age distribution of vulnerability to endotoxin in the chick embryo may be related to the timing of events in the development of the medullary and cortical portions of the adrenal gland.

There is evidence from other sources that the systemic response of rabbits to endotoxin may involve a release of adrenalin or a related compound (6), or a heightened reactivity to adrenalin (8). The possibility that a similar mechanism may be involved in the lethal response of the chick embryo to endotoxin is suggested by the findings of Brauer (23), who showed that chromaffin cells begin to differentiate at about the 7th day, and become complete at 10 days, and Lutz and Case (24), and Okuda (25), who demonstrated the presence of epinephrine in the adrenal glands of the embryonic chick between the 8th and 10th day. If the susceptibility to endotoxin involves the participation of adrenalin, the 10 day embryo is well equipped in this respect. But adrenal steroids, which have been shown here to protect embryos, probably are not available until the twelfth day, according to Dawson (26). The latter showed that morphological and histochemical differentiation of the developing cortex lags approximately two days behind the medulla.

Thus, it seems possible that the special vulnerability to endotoxin in the 10 day chick embryo may be related to the appearance of epinephrine-producing tissues at this time, and the rapid disappearance of susceptibility on the 12th day may be the result of maturation and adequate functioning of the adrenal cortical cells. Further studies of this aspect of the problem are currently in progress.

SUMMARY

Inoculation of the CAM of the 10-day chick embryo with endotoxin preparations derived from the meningococcus and other Gram-negative microorganisms has been shown to result in multiple hemorrhages and death of the embryo within a few hours. Evidence has been presented to indicate that this lethal effect is specific for the general class of endotoxins derived from Gram-negative bacteria. Susceptibility to endotoxin was maximal in 10-day old embryos,

and younger or older embryos showed little or no response. The optimal incubation temperature for the effect of endotoxin was 39.5°C., and embryos incubated at 28°C. were completely protected. The lethal effect was prevented by small amounts of cortisone, hydrocortisone, and 9-alpha fluorohydrocortisone, but not by cholesterol, desoxycorticosterone, or 1-dehydrocortisone.

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