Temporal Protection of Chick Embryo against Endotoxin by Isoproterenol, Polymyxin B Sulfate, and Hydrocortisone* (33754)

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The lethal effect of endotoxin in the 10–11 day chick embryo could be prevented by the administration of hydrocortisone (1), isoproterenol (2), or polymyxin B sulfate (3) when endotoxin and the protectorant were given simultaneously. Since the properties of these agents differ, a variance in protection could be anticipated with a change in the time between the administration of endotoxin and protectorant. To preclude a nonspecific effect of *in vitro* mixing of endotoxin and a protectorant, separate administration was desirable. This is a further report on lethality studies in the chick embryo when the three aforementioned agents were administered at varying time intervals before and after the administration of LD_{50-100} dose of endotoxin.

Methods. White leghorn fertile eggs (Ghostley Pearl, Anoka, Minnesota) were used throughout. Ten-day eggs were candled and a large vein was exposed by removal of the overlying shell. After a 60-min interval which enhanced the exposed vessel's resistance to the trauma of injection, a drop of sterile mineral oil was placed on the membrane for better visualization of the vessel when transilluminated. The same vessel or a similarly prepared one was used for the second intravenous injection. A total of 1.5 minims was injected each time. Eggs with traumatic bleeding were discarded. The eggs were placed in a humidified incubator set at 99.5°F between the first and second injection and were returned to the incubator after the second injection for 18–24 hr, at which time the viability of embryos was determined by candling. All nonviable embryos and those with questionable viability were examined grossly. Only those embryos with clear evidence of intra-embryo hemorrhage were recorded as experimental deaths.

Materials. Endotoxin: a single lot of *Escherichia coli* endotoxin prepared in our laboratory as previously described (5) was used in all experiments. Protectorants: sterile injectable isoproterenol hydrochloride (Isuprel),[1] polymyxin B sulfate,[2] and hydrocortisone sodium succinate (Solu-Cortef)[3] were used throughout. Each 10 μg of Isuprel contained 6 μg of lactic acid, 90 μg of sodium lactate, 350 μg of sodium chloride and 50 μg of sodium bisulfite. Each 25 μg of hydrocortisone sodium succinate contained 0.2 μg of anhydrous sodium biphosphate, 0.62 μg of methyl paraben, 0.02 μg of propyl paraben and 2.2 μg of exsiccated sodium phosphate. These additives with the respective drugs were not independently controlled. Incubator: a single stage humidity and temperature controlled incubator was used to house the developing and treated embryos.[4] Syringes: disposable sterile, nontoxic, nonpyrogenic, monojet 501-M 1 cc syringes graduated in minims with a 27-gauge needle were used for all injections.[5]

Results. A total of 25 experiments with appropriate saline controls were carried out. Each experiment compared endotoxin with one or more protectorants used independently and with saline control at one or more time intervals. The results in the Tables represent the sum total of embryos in each experiment given the same protectorant at the same time

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[1] Winthrop Laboratories, New York, New York.
[2] Pfizer Laboratories, New York, New York.
[3] The Upjohn Company, Kalamazoo, Michigan.
[4] Jamesway Single-Stage Incubator, James Manufacturing Company, Inc., Fort Atkinson, Wisconsin (model 252 B).
[5] Roehr Products Company, Inc., Deland, Florida.

TABLE I. Effect of Isoproterenol HCl (10 μg) at Varying Time Intervals.

Time (hr)	No. of expts.	Saline		Isoproterenol HCl	
		Alive	Dead (%)	Alive	Dead (%)
		A. After endotoxin, 0.05 μg			
0	5	3	24 (89)	22	7 (24)
0.5	5	15	37 (71)	44	5 (10)
1	10	27	55 (67)	56	30 (35)
1.5	1	3	6 (67)	4	7 (64)
2	4	9	25 (74)	9	21 (70)
3	4	10	22 (69)	11	21 (64)
		B. Before endotoxin			
0	2	0	14 (100)	11	8 (42)
1	3	2	30 (91)	20	7 (26)
2	2	1	19 (95)	1	24 (96)

relative to endotoxin. Eighteen experiments compared a single protectorant with saline control. In 4 experiments 2 protectorants were evaluated independently, and in 3 all 3 protectorants were studied independently with saline controls. All but one experiment included more than one time interval between the administration of protectorant and endotoxin. The amount of endotoxin in each injection was 0.05 μg. This quantity, with four exceptions, gave an LD_{50-100} in each experiment, and an ID_{50-100} in the summary of all experiments included in the tabulated data.

Ten μg of Isuprel was used in each injection, which had proved protective in previous studies of simultaneous administration (2). The amount of Solu-Cortef in the initial two experiments was 500 μg and in the remainder 25 μg, the two amounts proving equally effective. The results of both doses of Solu-Cortef are included in the data. The amount of polymyxin B sulfate was 25 μg and was determined in part by reported studies (3) and confirmation in our laboratory. This quantity proved consistently effective when given simultaneously with endotoxin. The time intervals between the injection of protectorants and endotoxin varied from zero (immediately) to 4 hr, and before endotoxin from zero (immediately) to 24 hr.

Isuprel showed definite protection when given as much as 1 hr after endotoxin, but no protection at 2 or 3 hr after endotoxin (Table I). It was protective 1 hr prior to endotoxin, but ineffective at 2 hr before.

Polymyxin B sulfate given 0.5 hr after endotoxin was protective whereas at 1 and 2 hr there was no protection (Table II). When it was given before endotoxin it was protective at 6 hr, but not at 24 hr.

Solu-Cortef in contrast to both Isuprel and polymyxin B sulfate showed protection for a longer period of time when given after or before endotoxin (Table III). Protection was clearly present when given 2 hr after endotoxin. At 4 hr after endotoxin it was also protective but less marked. It was distinctly protective 24 hr before endotoxin. A greater pre-endotoxin treatment interval was not evaluated, because the susceptibility of the 12-day embryo is inherently less than the 10- or 11-day embryo. A longer postendotoxin period was not evaluated because endotoxin deaths occurred between 4 and 18 hr after injection. Therefore, some of the embryos in the 4-hr postendotoxin experiments were dead when saline or Solu-Cortef was to be given.

Discussion. The 10–11-day-old chick embryo is a useful model for the study of endotoxin. The mechanism of endotoxin death in the embryo has not been resolved. The original hypothesis (1) of asynchronous development of the adrenal medulla and cortex, which permitted release of catecholamine from the medulla, while precluding release of sufficient protective cortical glucocorticoid

TABLE II. Effect of Polymyxin B Sulfate (25 μg) at Varying Time Intervals.

Time (hr)	No. of expts.	Saline		Polymyxin B	
		Alive	Dead (%)	Alive	Dead (%)
		A. After endotoxin, 0.05 μg			
0	3	1	29 (97)	31	3 (9)
0.5	2	1	18 (95)	11	7 (26)
1	3	5	30 (86)	10	33 (77)
2	2	1	18 (95)	3	24 (89)
		B. Before endotoxin			
0	7	15	69 (82)	59	6 (9)
1	3	3	34 (92)	30	5 (14)
2	4	3	37 (93)	33	7 (19)
3	1	0	5 (100)	7	0 (0)
4	2	4	8 (67)	17	0 (0)
6	1	6	9 (60)	16	4 (20)
24	2	15	21 (58)	11	28 (72)

has been questioned by Finkelstein (6). He suggested that an insulin-like effect might be more important and demonstrated a hypoglycemic effect of endotoxin in the 10–11-day embryo. How glucocorticoid (Solu-Cortef), a cationic cyclic polypeptide (polymyxin B sulfate) and a *N*-alkyl substituted sympathomimetic amine (Isuprel) protect against the lethal effect of endotoxin in the embryo is not entirely clear.

Of these agents the action of polymyxin B sulfate appears most evident. It has been suggested that the polymyxin B sulfate may bind the endotoxin (7). Our results are consistent with this conclusion since polymyxin B sulfate can be detected in the serum for several hours in man and experimental animals (8). While definitive studies in the embryo concerning the fate of polymyxin B sulfate are lacking it would appear that some time between 6 and 24 hr after administration it is degradated, tissue bound, or "excreted" into the allantoic sac and is no longer able to bind endotoxin. For polymyxin to be protective 30 min after endotoxin, endotoxemia would have to be present for this peri-

TABLE III. Effect of Hydrocortisone Sodium Succinate (25 μg)[a] at Varying Time Intervals.

Time (hr)	No. of expts.	Saline		Solu-Cortef	
		Alive	Dead (%)	Alive	Dead (%)
		A. After endotoxin, 0.05 μg			
0	5	3	32 (91)	37	0 (0)
0.5	3	12	16 (52)	30	0 (0)
1	3	9	17 (65)	29	0 (0)
2	2	3	18 (86)	16	3 (16)
4	2	2	16 (89)	6	11 (65)
		B. Before endotoxin			
0	6	6	46 (87)	58	1 (<1)
1	2	2	24 (89)	22	0 (0)
2	3	2	24 (89)	29	1 (3)
3	2	2	14 (88)	14	0 (0)
4	2	4	8 (67)	16	0 (0)
6	1	7	7 (50)	14	0 (0)
24	2	13	23 (64)	35	0 (0)

[a] Solu-Cortef dose in the initial 2 experiments at 0, 0.5, and 1 hr was 500 μg.

od. While such evidence in the embryo is lacking, ^{51}Cr tagged endotoxin remains in the plasma of dogs for considerably longer periods of time (9). Presumably sufficient active endotoxin remains in the embryo circulation for at least 30 min, or at least remains accessible to binding, which blocks endotoxin effect. The data here suggest that if polymyxin acts directly on endotoxin in the embryo, it must take place within 30 min of the endotoxemia.

Isuprel has a short term effect, and possibly mediates its action through one of its β-adrenergic activities. Whether this is a chronotropic or inotropic effect on the heart, as demonstrated in tissue culture of heart muscle cells of the 10-day embryo (10); a metabolic effect counteracting the hypoglycemia; or an undefined effect is not known. We have demonstrated the failure of hypoglycemia to occur when a protective amount of Isuprel is given simultaneously with endotoxin, the significance of which requires further study. If the cardiovascular effects of Isuprel are as short-lived in the embryo, as has been demonstrated in other animals and in man, some unknown mechanism must be operative when protection is afforded when given an hour before endotoxin.

The protection of Solu-Cortef persisted for a longer period of time when given before or after endotoxin, although the steroid has been detected in the circulation for only a short time after administration in chickens (11). The present results support the hypothesis of maximum endotoxin susceptibility during the 10- to 11-day period as being related to insufficient endogenous glucocorticoid. There is no definitive evidence as to the nature of the protective action of glucocorticoid except its metabolic effect on glucose metabolism and lysosomal membrane preservation has been noted (12). Prevention of hypoglycemia could reverse the deleterious effect of endotoxin, and preservation of cell membrane function could prevent irreversible deterioration. It is noteworthy that in our experiments vessel damage with subsequent collapse due to an initial injection was prevented with Solu-Cortef and only rarely could such a vessel not be used for a second injection. This was not observed following the injection of polymyxin B sulfate or Isuprel.

The results demonstrate the variation in the temporal protection of 3 agents against an intravenous LD_{50-100} dose of endotoxin. The differences are probably related to specific biological properties of each of these substances.

Summary. The temporal relations of protection by a glucocorticoid (Solu-Cortef), a cationic cyclic polypeptide (polymyxin B sulfate), and an *N*-alkyl substituted sympathomimetic (Isuprel) against a lethal effect of *E. coli* endotoxin was shown. Solu-Cortef was protective at least 24 hr before and 4 hr after endotoxin; polymyxin G sulfate, 6 hr before and 0.5 hr after endotoxin; and Isuprel, 1 hr before and after endotoxin.

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