this serum fraction. Confirmatory evidence on this point was obtained when it was shown that this more rapidly migrating antigen was unable to be sedimented by relatively high centrifugal forces, while the main endotoxin complex and its contaminating components were sedimentable. The formation of lighter components resulting from the incubation of endotoxin with the human plasma altering factor was observed earlier by Stauch and Johnson(2).

This work confirms, and extends with a purified fraction of human serum, the studies of Cluff(12), who recorded similar changes in the agar gel diffusion pattern of Shigella endotoxin when incubated in normal rabbit or human serum. In addition, the results presented herein make a tentative identification of the product released by serum-endotoxin interaction as the O polysaccharide hapten. A similar identification was made independently by using human plasma by Skarnes(13). In addition, Landy, Trapani, and Shear(14) have reported that resin treated plasma destroyed the capacity of endotoxin to stimulate antibody in rabbits, but the ability to precipitate anti-endotoxin antibody was retained. The observed precipitation reaction was characteristic of the haptenic polysaccharide rather than the unaltered endotoxin.

Summary. A substantial loss in the ability of endotoxin to be precipitated by homologous antibody occurs following incubation with Fraction IV_1 derived from normal human serum by cold ethanol fractionation. Evidence is presented that this loss in serologic reactivity is not associated with release of the major determinant sugar, *per se.* Rather, immunodiffusion analysis revealed the dispersion of a major antigen, with formation of a more rapidly migrating component. The latter was shown to form a line of identity with the O polysaccharide hapten.

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Hypoglycemia in Chick Embryos Infected with *S. typhosa via* the Allantoic Cavity.* (27883)

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Since the work of Goodpasture and Anderson which showed that the embryonated chicken egg is susceptible to infection with *Salmonella typhosa,* investigators have utilized this model to study various aspects of

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pathogenesis and sero-protection in experimental typhoid(1-9). It was observed that: embryos of 10-12 days' prior incubation were more susceptible than 14-15-day embryos; the route of inoculation was not crucial since a fatal outcome followed introduction of organisms *via* chorio-allantoic membrane, allantoic cavity, amniotic cavity or yolk sac; at death, positive cultures from heart's blood provided evidence of invasion of the embryo proper.

The immediate cause of death has not been established. It has been suggested that this may be due to the action of unidentified toxic substances elaborated by *S. typhosa*(1,5). Although it has been demonstrated that the 10-day-old chick embryo may be killed by endotoxin preparations from several Gram-negative species(9,10), Vil'ner(7) noted that these animals were less susceptible than adult mice to Boivin type endotoxin or heat-killed *S. typhosa*. Moreover while strains possessing both Vi and O antigens have been considered by some workers to be significantly more virulent in the embryonated egg, it has been reported(3,4,7,9) that lethal infections may be initiated by relatively small numbers of bacilli from Vi-deficient strains or the serologically rough variant Vi I (Bhatnagar) which is essentially devoid of the specific O antigen.

In the normal chick embryo the level of liver glycogen falls around the 10th day of incubation and does not rise again until the 13th day; during this interval the blood glucose level remains constant(11,12). Such embryos die within 24 hours following injection of 4-8 units of insulin, presumably because they cannot compensate for the induced hypoglycemia until the 14th day of incubation(13,14); further evidence for their physiologic immaturity is shown by the hypoglycemic response to ephedrine in embryos 9-12 days old(15).

In the light of this information we considered it important to determine whether altered carbohydrate metabolism might lead to the death of embryos infected at the age of 10 days. Strains Ty 2 and Vi I were selected to permit some evaluation of the endotoxic role of O antigen in the system; the allantoic route of inoculation was selected since at the outset the organisms would be growing largely in an extra-embryonic cavity thus permitting repeated assays of the microbial population of the fluid.

Materials and methods. Inocula. Lyophilized *S. typhosa* strains were reconstituted as needed and grown in heart infusion broth (Difco) for 6 hours at 37°C. The number of viables was determined by colony counts from serial dilutions, spread on plates of heart infusion agar.

For control purposes 0.3 ml of sterile skim milk was added to 5 ml of broth, the mixture being diluted 1:1000 in buffered saline and injected in eggs as were the bacterial inocula. *Embryos.* Fertile White Leghorn eggs were incubated in a forced-draft incubator at approximately 39°C prior to inoculation. They were inoculated (0.1 ml) *via* the allantoic cavity(16) and incubated at 37°C; death of the embryo was determined by candling; samples of allantoic fluid were routinely cultured to rule out contamination.

The shell over the air space was removed and the blood vessels of the allantois made visible by application of sterile mineral oil to the exposed shell membrane. A ½", 26-gauge needle, affixed to a tuberculin syringe mounted on a ring-stand, was passed into the lumen of a vessel and blood withdrawn. *Chemical determinations. Glucose.* The volume (0.025-0.050 ml) of blood to be tested was measured with a pipette. Natelson's method(17) was employed with either a Beckman DU spectrophotometer set at 540 μ or a Bausch and Lomb "Spectronic 20" instrument. *Glycogen.* Samples of yolk sac were removed and treated as recommended for embryonic liver(11), before assaying the glycogen content(18).

Experimental. To ascertain the rate at which the 2 strains grew in the allantoic cavity, 2 groups of 3 embryos each were inoculated with Ty 2 and Vi I respectively; at 4-hour intervals, over a period of 16 hours, samples of allantoic fluid were removed and the viable count was determined by colony counts from suitable dilutions. At the various time intervals similar values were obtained for the individual eggs of each group

TABLE I. Accumulative Deaths Resulting from Allantoic Infection of 10-Day-Old Chick Embryos with *S. typhosa.*

Infecting strain	Group	Inoculum (viables)	Specific deaths* within the following interval (hr)						
			12	24	36	48	72	96	120
Ty 2	A	1500	0/9†	3/9	6/9	8/9	8/9	8/9	9/9
	B	150	0/9	0/9	6/9	7/9	7/9	7/9	8/9
	C	15	1/10	2/10	6/10	7/10	7/10	7/10	8/10
	D	2	0/9	1/9	2/9	4/9	5/9	7/9	8/9
Vi I	E	11,500	0/9	2/9	2/9	4/9	6/9	6/9	6/9
	F	1150	0/10	5/10	7/10	7/10	7/10	7/10	7/10
	G	115	0/9	4/9	4/9	4/9	4/9	4/9	4/9
	H	12	0/9	2/9	3/9	3/9	3/9	3/9	3/9

* Contaminated embryos are not included in tabulation.
† Total No. of embryos dead/No. inoculated.

i.e., no differences were seen between the growth rates for the 2 strains. With an initial concentration of less than 1000/ml in each instance, the numbers reached approximately 10^6/ml in 8 hours and 10^9/ml by 16 hours, at which time the slope of the growth curve indicated that the stationary phase was being approached.

To determine the relative lethality of infection under the experimental conditions, eighty 10-day-old eggs were distributed randomly into 8 groups with 10 embryos in each. Four groups were inoculated with varying doses of Ty 2; the remaining 4 groups received varying doses of Vi I. The embryos were examined thereafter at intervals of 12 hours. With both strains the bulk of deaths occurred between 24 and 48 hours post-inoculation (Table I); for comparable inocula, deaths from Vi I infection usually occurred within the same period as for Ty 2.

In the light of results with O-deficient strain Vi I, it was thought improbable that the observed deaths were due largely to absorption of endotoxin derived from the bacterial population developing in the allantoic cavity; nevertheless an effort was made to rule out this possibility. *S. typhosa* in heavily infected allantoic fluids were separated by centrifugation, resuspended in smaller volumes of saline to concentrations of 4.4×10^{10} (Ty 2) and 9×10^{10} (Vi I) per ml, then killed by heating at 70°C. With these suspensions, groups of 11-day embryos were injected *via* allantoic route with 0.1 ml, then incubated and observed for several days longer. No deaths occurred.

Eggs with embryos of varying age were injected with *S. typhosa* and were later bled to determine the effect of infection on the blood glucose level. The results of 5 such experiments are summarized in Table II, showing that: a) in uninfected embryos of 11-17 days' incubation the values ranged from 105 to 136 mg %; b) in eggs inoculated at 10-11 days of incubation with Ty 2 or Vi I, the levels did not fall below normal during the first 12 hours of infection but after 16-20 hours the values were significantly lower; c) in eggs incubated for 16 days prior to inoculation, the blood glucose was maintained within the normal range during the next 28 hours.

The lower blood glucose levels did not seem related to a high bacterial content within the embryo carcass. In Experiment 1, at the time the embryos were bled the brains were removed aseptically, emulsified and cultured in poured plates of medium; only 7 of the 17 tested were positive and the numbers of colonies were not great. Again in Experiment 3, at the time the blood was taken for assaying glucose levels, samples of 0.01-0.05 ml were cultured; only 1 of 14 samples was positive.

In another trial, 10-day-old embryos were inoculated with approximately 100 (Vi I) or 170 (Ty 2) viables. From 23 to 29½ hours thereafter, the infected and control eggs were sampled and glycogen content of the yolk sacs was determined. The results summarized in Table III were obtained for the 7 eggs in each group; they show no significant differences among the groups.

Discussion. Strains of *S. typhosa* repre-

TABLE II. Comparison of Blood Glucose Levels in Normal and *S. typhosa*-Infected Embryos of Varying Age.

Exp	Inoculum Strain	Inoculum Viables	Age of embryos inj (days)	Embryos per group	Interval before bleeding (hr)	Glucose levels (mg %) Mean	P†
1	Ty 2	1600	10	15	19	66 ± 10*	.001
	Control	None		17	23	136 ± 5	
2	Vi I	600	10	6	19	37 ± 13	.005
		None		6	19	105 ± 13	
3	Ty 2	1900	10	7	20	34 ± 7	.001
	Vi I	900		7	20	29 ± 8	.001
	Control	None		7	20	106 ± 10	
4	Ty 2	22	11	5	0	127 ± 5	>.05
				5	12	127 ± 5	>.05
				5	16	97 ± 10	.01
	Vi I	1700	11	5	1	128 ± 5	>.05
				5	12	108 ± 8	>.05
				4	16	40 ± 18	.001
	Control	None	11	5	0	125 ± 3	
				5	15	127 ± 2	
5	Ty 2	700	16	8	28	134 ± 6	>.05
	Control	None		8	28	134 ± 8	

* S.E. = Standard error of mean.

† Probability was calculated by means of the t test in which the mean of test sample was compared with that of control.

senting extremes of high and low invasive capability for the mouse were found to grow in the allantoic cavity of the embryonated egg at the same rate, yielding within 16 hours approximately 10^9 viables per ml (*cf* 7-9). Infected embryos began dying between 12 and 24 hours following inoculation. From our data it is difficult to conclude that the strain Ty 2 possessed greater virulence for the embryo than did Vi I; if such a difference does exist it is not of the order of magnitude as that seen in the mouse (*cf.* 3,4,7).

Our data support the hypothesis that in embryos exposed to *S. typhosa,* deaths might be related at least in part to hypoglycemia resulting from interaction of living bacteria and host. Thus in 10-11-day eggs infected with either strain, after 16 hours the blood glucose fell markedly below the normal level and in some instances was virtually undetectable. It may be pointed out here that in the experiments of Dalton and Hanzal (13) after injection of 4 units of insulin into the allantoic cavity embryos regularly died within 24 hours, the blood glucose level having fallen from an initial average value of 112 mg % to approximately 65 mg %. According to Zwilling(14), among insulin-treated embryos the glycogen content of the yolk sac membrane remained equal to or even greater than that of the controls, a finding similar to that presented above following *S. typhosa* infection. The definitely greater resistance to lethal outcome of infection exhibited by embryos older than 14 days, an observation of previous workers(2,5,7) as well as ourselves, is also correlated with the ability of the older animals to maintain a normal blood glucose level (see Table II, Exp. 5).

TABLE III. Glycogen Concentration (as % Glucose per mg Dry Tissue) in Yolk Sacs of Embryos Infected with *S. typhosa.*

	Ty 2	Vi I	Control
Mean	1.18 ± .10*	.98 ± .12	1.14 ± .14
P†	>.05	>.05	

* S.E. = Standard error of mean.

† Probability was calculated by means of the t test in which the mean of test sample was compared with that of control.

It has been shown that glycolysis of certain normal or neoplastic cells derived from various mammalian tissues is stimulated by endotoxins of Gram-negative bacteria in a manner similar to that of insulin(19-21); in a given species, the susceptibility of liver

cells may be particularly high(22,23). It is questionable whether the deaths we observed were due solely to systemic absorption of endotoxin or its damage to local tissues, since (a) large numbers of dead bacilli of either strain failed to kill when introduced into the allantoic cavity and (b) previous workers have shown that endotoxin preparations when introduced by this route are far less harmful than when dropped on the chorio-allantoic surface or given intravenously(9,10).

The relatively early hypoglycemia induced by *S. typhosa* appeared to be independent of systemic invasion, since the infection seemed to be largely extra-embryonic at the time blood glucose values were low. Whether the hypoglycemia is directly the result of microbial proliferation and metabolism, or whether it is an indirect response to injury on the part of the host remains to be determined. In any case, there is little doubt that lack of adequate quantities of glucose available as an energy source would compromise the normal defense mechanisms of the tissues and ultimately prove fatal.

Summary. *S. typhosa* strains Ty 2 and Vi I grew at the same rate when inoculated into the allantoic cavity of 10-day chick embryos, reaching concentrations of 10^9 viables per ml within 16 hours. Although Ty 2 in small inocula produced a fatal infection in more embryos, comparable numbers of Vi I also killed a significant proportion of the embryos within similar intervals. Both strains produced marked hypoglycemia in 10-day embryos after 16 hours although the yolk-sac glycogen levels were not reduced. Embryos 16 days old were more resistant to fatal infection; in these, normal blood glucose levels were maintained. Hypoglycemia did not appear to be due to extensive systemic invasion of the embryo by *S. typhosa*.

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