Toxic Effect on the Chick Embryo of Homologous Tissue Suspensions Following Intravenous Inoculation. (17941)

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In attempting to adapt a neurotropic virus to chick embryos by intravenous passage the initial injection of a 10% infected mouse brain suspension produced no abnormal reaction. However, when 10% infected chick embryo brain suspension was similarly inoculated the embryos died within a few hours. Upon examination, multiple hemorrhages were noted in the skin and viscera. Brain suspensions of normal chick embryos were found to produce the same lesions. Similar phenomena in chick embryos have been briefly mentioned by Eichhorn(1) and Beveridge and Burnet(2). It has long been known that the intravenous inoculation of homologous and heterologous tissue extracts may be followed by sudden death or less severe reactions (3). In certain instances these effects appeared to be dependent upon the intravascular clotting of the blood. An example of such phenomena is afforded by the observations of Thomas(4,5). In mice injected with suspensions of normal mouse brain, an immediate reaction occurred consisting of ataxia, convulsions and coma. Administration of heparin prevented the reaction. Thomas attributed the phenomenon to the clotting action of thromboplastin. Green and Stoner(6) also recently analyzed the factors underlying the immediate lethal response of rats to intravenous inoculation of homologous tissue extracts. They concluded that it depends upon the combined action of thromboplastin and adenosine triphosphate and/or related compounds.

As far as we are aware, differences in the capacity of the extracts of tissue from various classes of animals to induce such reactions *in vivo* have not hitherto been clearly defined, although *in vitro* differences in thromboplastins have frequently been described. Accordingly, the phenomenon we have encountered has been further investigated.

Preparation of tissue suspensions. Suspensions used for injection were prepared by grinding the tissues in a mortar with a small amount of alundum. Sufficient isotonic buffer solution (pH 7.2) was added to make a 10% suspension based on the wet weight of the tissue. This material was centrifuged at 1500 r.p.m. for 15 minutes in an International Centrifuge fitted with a No. 2 head. The opalescent supernatant fluid was employed for injection. As a rule the suspensions were prepared on the day that they were used.

Technic of inoculation. Chick embryos were inoculated intravenously by the method of Eichhorn(1). Mice were injected into the tail vein, and hens into the wing vein.

Test for thromboplastin activity. Blood, with precautions to minimize trauma, was obtained in a syringe containing enough of a solution of 1% sodium oxalate in isotonic saline to give a final concentration of 0.1%. The plasma was separated by centrifugation. To 5 drops of plasma were added 3 drops of 0.5% calcium chloride and 2 drops of either tissue suspension or commercial thromboplastin prepared from beef tissue. The tubes were

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1. Eichhorn, E. A., *Science,* 1940, v92, 245.

2. Beveridge, W. I. B., and Burnet, F. M., The Cultivation of Viruses and Rickettsiae in the Chick Embryo, Med. Res. Council, Spec. Rep. Series No. 256, His Majesty's Stationery Office, London, 1946, p. 23.

3. Wooldridge, L. C., *Arch f. Physiol.,* 1886, p. 397.

4. Thomas, L., *Bull. Johns Hopkins Hosp.,* 1947, v81, 1.

5. Thomas, L., *Bull. Johns Hopkins Hosp.,* 1947, v81, 26.

6. Green, H. N., and Stoner, H. B., *Brit. J. Exp. Path.,* 1947, v28, 189.

TABLE I.
Effect of Intravenous Injection of Chick Embryo Brain Suspension Into 14-day-old Chick Embryos.

	Dilution of tissue suspension				
	1:10*	1:20	1:40	1:80	1:160
Mortality	4/4†	4/4	3/5	0/5	1/5
Hemorrhages‡	+	+	+	—	0

* Dilution based on wet weight of tissues.
† Numerator represents number of embryos dying, denominator the number injected.
‡ Plus indicates that multiple hemorrhages were observed upon inspection of the intact embryo after death.

agitated frequently and clotting determined by tilting the tube.

Effect of the injection of homologous tissue suspensions on the chick embryo and adult chicken. In 11- to 14-day-old chick embryos death with multiple hemorrhages regularly followed the intravenous injection of various quantities of brain suspension prepared from 13-day-old normal chick embryos. The data of one experiment are given in Table I. Several other experiments with brain suspensions prepared from 11- to 14-day-old embryos gave essentially the same results as well as preparations from the brains of newly hatched chicks and adult chickens. Suspensions of the following tissues induced the same response: Whole embryo (5 days); whole embryo without brain (13 days); chorioallantoic membrane; yolk sac. In accordance with the findings of Eichhorn(1), normal allantoic fluid from 12-day embryos caused death upon intravenous inoculation into 13-day embryos. Whether or not this effect depended upon the same factor as was demonstrated in the materials just enumerated is rendered doubtful by the absence of definite hemorrhage.

The following materials proved innocuous: egg yolk (diluted 1:2); defibrinated chick embryo blood (diluted 1:10); amniotic fluid. It is evident, therefore, that the active principle is present in many tissues, although it is not universally distributed.

In distinction to the findings in embryos 11- to 14-day-old, no hemorrhages were seen following the inoculation of comparable tissue suspensions into 18-day embryos, although death regularly ensued. Instead large clots were observed in the heart. Intravascular clots, in the absence of hemorrhage were also found in a hen that died two minutes after the intravenous inoculation of about 3 cc of a 20% chick embryo brain suspension.

Histologic examination of embryos of different ages after inoculation of 20% chick brain suspension revealed changes compatible with the findings in the gross. Interstitial hemorrhages and vascular congestion without evident deposition of fibrin were observed in embryos ranging from 9 to 15 days in age. In 18-day embryos and in the hen interstitial hemorrhages were not seen. Marked vascular congestion, however, with fibrin deposits in the large vessels and heart were observed.‡

Effect of injection of mouse tissue extract in the chick embryo and adult chicken. Twenty per cent suspensions of normal mouse brain, capable of reproducing the phenomenon described by Thomas(4) in mice were without effect when introduced intravenously into chick embryos. In addition suspensions of mouse liver, lung and spleen proved inactive. Comparable suspensions of mouse embryonic brain and of whole mouse embryo produced no toxic effect in chick embryos, nor were any symptoms noted after intravenous inoculation of about 3 cc of 20% mouse brain suspension into an adult chicken.

Effect of chick embryonic tissues in the mouse. Conversely, no effect was produced in mice by the intravenous injection of suspensions of various chick embryonic tissues (whole embryos, brain, chorioallantoic membrane, yolk sac).

Effect of various agents upon the toxicity of tissue suspensions. Heat. The activity of chick embryonic brain suspensions was not reduced by heating at 55° for 30 minutes, but was completely destroyed by heating at 65° for the same period. *Heparin.* It was hoped that the use of heparin would afford a practical means of neutralizing the activity of chick embryonic material and thus permit the injection of homologous tissue in chick embryos by the intravenous route. It was found

‡ We are very grateful to Dr. Orville Bailey for the preparation of the material for histologic examination and for his aid in interpreting the lesions observed.

TABLE II.
Effect of Heparin on Toxicity of Chick Embryo Brain Suspension.

	Dilution of heparin*						
	1:20	1:40	1:80	1:160	1:200	1:320	No heparin
Deaths†	0/3	1/3	2/3	3/3	3/3	2/3	3/3
Hemorrhages‡	—	+	++	+++	+++	+++	+++

* The stock heparin preparation used was Liquaemin (Roche-Organon) solution containing 10 mg/cc of sodium heparin. One volume of this solution in the dilutions indicated was mixed with one volume of 10% chick brain suspension. 0.05 cc of the mixture was inoculated intravenously into 13-day chick embryos.

† The numerator refers to the number of embryos that died, the denominator to the number injected.

‡ The number of hemorrhages visible upon inspection of the intact embryo after death was roughly estimated and graded from 0 = none to ++++ = many.

TABLE III.
Capacity of Tissue Suspensions from Various Species to Clot Human and Chicken Plasma.

Tissue suspension	Source of plasma		
	Adult chicken	Chick embryo	Man
Chick embryo brain	+	+	0 or ±
Adult chicken brain	+	+	0 or ±
Mouse brain	0 or ±	0 or ±	+
Beef lung*	0 or ±	0 or ±	+

* Desiccated beef lung (Upjohn).

that in sufficient concentration heparin, mixed with an appropriate quantity of homologous brain suspension, prevented multiple hemorrhage in the chick embryo. (Table II). However, following the injection of heparin, either alone or with tissue suspensions, massive hemorrhage often followed at the site of inoculation resulting in rapid death. For example 1 mg of heparin was inoculated into each of 14 embryos (12 days). Hemorrhage at the point of inoculation and death occurred in 8. Death occurred in only one case after the intravenous inoculation of 14 embryos with isotonic buffer solution. *Homologous and heterologous sera.* Thomas(5) reported that the toxic effect for mice or rabbits of mouse tissue suspensions could be neutralized by incubation with normal rabbit serum. The reaction took place only when calcium was present in the system. Using his technic, various quantities of chick embryo brain suspension were incubated with normal rabbit serum, normal mouse serum and with the serum of a normal adult chicken. When these mixtures were injected into chick embryos and the results compared with those obtained with the same tissue suspensions incubated in the presence of isotonic salt solution, no evidence of an inhibitory effect of the sera was demonstrated.

Possible relationship between the toxic factor and thromboplastin. Specificity of thromboplastin. The pathologic changes induced by the homologous tissue suspensions suggested that the active principle was thromboplastin. Accordingly the capacities of tissue suspensions derived from the chicken, mouse and ox to clot human and chicken plasma *in vitro* were investigated. The results are summarized in Table III. Human plasma was employed in place of mouse plasma, since we were unsuccessful in obtaining specimens of the latter that did not either clot spontaneously or upon the addition of calcium alone. These findings indicate a specific difference between avian and mammalian thromboplastins. Moreover the identity of the toxic factor with thromboplastin is suggested by their parallel specificity.

This evidence for a difference in the specificity of thromboplastins supplements that provided by the early work of Delezenne(7), Loeb(8,9), Hewlett(10) and Nolf(11), all of whom demonstrated *in vitro* differences in

TABLE IV.
Clotting of Plasma from Chick Embryos of Varying Ages by 10% Suspension of Chick Embryonic Brain.

	Age in days of embryos yielding plasma			
	10	13 and 15	17	19
Characteristic of clot	slight ppt.	heavy ppt.	stringy clot	solid clot

thromboplastins derived from various species or classes of animals. These old observations, however, appear to have frequently been disregarded by modern writers on blood coagulation.

Removal of the toxic factor during the clotting of plasma by tissue extracts. Additional evidence for the close association of the toxic factor with thromboplastin was obtained in the following manner. A 10% chick embryonic brain suspension, shown to be toxic for chick embryos, was mixed with an equal volume of adult chicken plasma and the clotted plasma centrifuged. It was found that the supernatant fluid was not toxic for embryos and no toxic property could be extracted from the clot after grinding in isotonic buffer at pH 7.2.

Possibility that the toxic factor is distinct from thromboplastin. There is, therefore, much evidence to suggest the identification of the toxic factor with thromboplastin. The fact, however, that the blood of the chick embryo does not acquire the capacity to form a typical clot during the earlier stages of development, makes it impossible to assert that all the effects observed *in vitro* are the result of thromboplastic activity. That the blood of the embryo fails to clot or to clot typically before the latter phases of embryonic development was observed long ago(12,13,14). In experiments of our own, as indicated in Table IV, it was found that when homologous embryonic brain suspension and calcium chloride were added to oxalated plasma from 10-day chick embryos no true coagulum developed; however a fine, very scanty precipitate slowly formed. The plasma from 13- and 15-day embryos yielded a more voluminous precipitate. In that from 17-day embryos a definite but loose and stringy coagulum was generated. Only from 19-day embryos was plasma obtained which produced a firm clot comparable to that characteristic of adult chicken plasma.

If these phenomena observed *in vitro* occur also *in vivo* when chick embryos of varying ages are inoculated with homologous tissue suspensions, it is evident that the formation of large firm intravascular thrombi cannot account for the presence of multiple hemorrhages in the younger embryos, or their death. It is possible that finely dispersed particles of precipitate comparable to those formed *in vitro* in the plasma of young embryos may develop *in vivo* and cause capillary thrombosis, with subsequent formation of multiple small hemorrhages. But in the light of available evidence, it cannot be concluded that the characteristic changes in young embryos and their death are dependent on the intravascular deposition of fibrin.

Comment. Since we have already discussed certain of the theoretical aspects of the phenomena that have been investigated we shall here mention only their possible practical application. To a virologist desiring to employ the intravenous route in the serial passage of preparations of infected homologous tissues the rapid death of the chick embryo will present an obstacle that as

7. Delezenne, M. C., *Arch. de Physiol.*, 1897, v29, 333.

8. Loeb, Leo, *Virch. Arch. f. path. Anat. u Physiol. u.f. Klin. Med.*, 1903, v173, 35.

9. Loeb, Leo, *Virch. Arch. f. path. Anat. u Physiol. u.f. Klin. Med.*, 1904, v176, 10

10. Hewlett, A. W., *Arch. f. Exp. Path. u. Pharm.*, 1902-03, v49, 307.

11. Noll, P., *Arch. Internat. de Physiol.*, 1906-07, v4, 216.

12. Boll, F., *Arch. f. Anat.* (Reichert), 1870, p. 718.

13. Ascarelli, A., *Untersuch. z. Natur. d. Mensch, u.d. Thiere* (Moleschott), 1895, v15, 255.

14. Pickering, J. W., and Gladstone, R. J., *Proc. Roy. Soc. of London, Series B*, 1925, v98, 516.

far as we know now can be circumvented only by employing relatively high dilutions of such materials, or perhaps, in certain instances, by the simultaneous intravenous injection of heparin. In the preparation of tissue cultures where chicken plasma is used, mammalian tissues or tissue extracts cannot be relied upon to induce the formation of a firm coagulum. The results of experiments designed to determine the anaphylactogenic capacity of various tissues or organs might be misinterpreted if the investigator were unaware of the rapid death which may follow the intravenous injection of tissue suspensions from certain related classes of animals and careful post mortem examinations were not made.

Conclusion. The toxic effect *in vivo* of certain tissue derivatives exhibits biologic specificity. This specificity appears to parallel that of thromboplastin on the clotting of blood plasma *in vitro.*

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The Preparation and Biological Activity of Alpha Estradiol-Sensitized Collodion Particles.* (17942)

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In the course of studies on the antigenic properties of crystalline alpha estradiol† (in combination with protein substances) we included collodion particle agglutination(1) as one of the tests for the presence of serum antibody. This serological procedure has proved to be a sensitive and reliable method of antibody detection and titration in our laboratory(2), as well as in others(3,4,5). The usual method of sensitization of collodion particles consists of incubation of a mixture of particles and antigen in optimal proportions. Non-specific absorption of antigen occurs, and the sensitized particles may then be combined with antisera in proper dilutions. This method of sensitization was not successful when aqueous suspensions of alpha estradiol were incubated with the collodion particles. We were not able to overcome the insolubility of the steroid substance in the aqueous medium. However, sensitization of collodion particles has been accomplished by incorporation of the steroid in and on the particle at the time of preparation.

Preparation of sensitized particles. Collodion U. S. P. Merck was treated in the manner recommended by Cannon and Marshall (1). Either a 5 or 10% acetone solution of the dried collodion constituted the stock solution to be used as a source of particles. Arbitrary amounts of crystalline alpha estradiol were dissolved in 100 ml quantities of the stock solution. In most instances we added sufficient alpha estradiol to give 100 mg per 100 ml of the collodion solution. The particles were then prepared and washed in the usual manner. The alpha estradiol content of the final particle suspension was calculated by lyophilizing quantities of 1-5 ml, weighing of the dried material and comparison

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† We are indebted to the Schering Corporation for supplying the alpha estradiol used in these studies.

1. Cannon, P. R., and Marshall, C. E., *J. Immunol.,* 1940, v38, 365.

2. Weir, J. M., PROC. SOC. EXP. BIOL. AND MED., 1941, v46, 47.

3. Dulaney, Anna Dean, and House, Virginia, PROC. SOC. EXP. BIOL. AND MED., 1941, v48, 620.

4. Cavelti, P. A., and Cavelti, E. S., *Arch. Path.,* 1945, v39, 148.

5. Cavelti, P. A., PROC. SOC. EXP. BIOL. AND MED., 1945, v60, 379.