

EMBRYOTOXIC EFFECTS OF TISSUE ANTISERA
ON THE EARLY CHICK EMBRYO *IN VITRO*

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ON THE EARLY CHICK EMBRYO *IN VITRO*

By

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SCOPE AND CONTENTS:

Chick embryos at stages primitive streak to three somites were explanted on the vitelline membrane and cultured by the method of New (1955) or by Gallera's modification of the method (Nicolet and Gallera, 1963). Antisera produced in rabbits against adult chicken brain extract, adult chicken kidney extract, and embryo brain extract were placed on the uppermost side of the embryo preparation. The embryos were recovered after 24 to 36 hours further incubation. Defects of the central nervous system, the posterior trunk, and the extra-embryonic membranes occurred in embryos exposed to adult brain antiserum. Embryos exposed to adult kidney antiserum developed exactly the same kinds of defects. Embryo brain antiserum produced similar abnormalities which included defects of the central nervous

system and of the extra-embryonic membranes, and in addition defective somites. However, embryos exposed to gamma globulin solutions containing antibodies against neural-specific antigens and not against common tissue antigens were normal. In control experiments, embryos exposed to saline solution, to normal rabbit serum, and to normal rabbit serum gamma globulins developed normally.

Histological examination of representative antisera treated embryos revealed that there were extensive areas of disorganization and necrosis of neural tissue. In embryos with short trunks, the caudal proliferation centre was necrotic. Embryos exposed to gamma globulins of absorbed adult brain antiserum were histologically normal.

The antisera used in these experiments were characterized by double diffusion in agar gel. It was demonstrated by this method that the antisera contained antibodies against common tissue antigens as well as against tissue-specific antigens. It was also shown that certain antigens common to all adult organs were present in the embryo and in the extra-embryonic membranes during the time that the embryos were exposed to the antisera.

Embryos which had been exposed to various of the tissue antisera for 8.5, 21.5 or 32 hours were sectioned. The sections were stained with FITC-labelled goat anti-rabbit gamma globulins in order to localize distribution of the antibodies. Fluorescence was located on the ectoderm of the embryos and of their extra-embryonic membranes,

in the lumen of the neural tube and in the cavity of the otic vesicles. This demonstrated that, under the conditions of the experiment, the antibodies were available to the embryo.

Antisera which affected the embryo and the extra-embryonic membranes were shown to contain antibodies against antigens actually present in the embryos and in the extra-embryonic membranes during the time of exposure. The only antiserum which had no adverse effects on the embryos was the one which contained only antibodies against antigens not demonstrated to be present in the embryo during the time of exposure.

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TABLE OF CONTENTS

	<u>Page</u>
Acknowledgements.	v
List of Figures.	vii
List of Tables.	viii
 Introduction.	 1
Materials and Methods.	5
Results.	11
Discussion.	22
Bibliography.	38

LIST OF FIGURES

Figure	Title	Following Page
1	Drawing of Ouchterlony plate	8
2	Diagrams showing the two explant methods	9
3-11	Photographs of Ouchterlony double diffusion plates	12
12, 13	Photographs of embryos exposed to saline solution	15
14	Photograph of embryo exposed to 9dABS	15
15	Photograph of embryo exposed to NRS	15
16, 17	Photographs of sections through embryo in Fig. 15	15
18-24	Photographs of embryos exposed to ABS	16
25	Photograph of section through embryo in Fig. 24	16
26-30	Photographs of embryos exposed to AKS	18
31	Photograph of section through embryo in Fig. 30	18
32, 33	Photographs of embryos exposed to AKS	18
34, 35	Photographs of sections through embryo in Fig. 33	18
36, 37	Photographs of extra-embryonic membranes of embryos exposed to AKS	19
38	Photograph of section through membrane in Fig. 37	19
39	Photograph of embryo exposed to NRSYg	19
40, 41	Photographs of embryos exposed to ABSYg	19
42	Photograph of section through embryo in Fig. 15	20
43	Photograph of section through embryo in Fig. 33	20
44-49	Photographs of sections of antisera treated embryos stained with FITC-labelled goat anti-rabbit gamma globulins	21

LIST OF TABLES

Table	Title	Following Page
I	Treatment of embryos explanted by New's method	10
II	Treatment of embryos explanted by Gallera's method	10
III	Treatment and recovery schedule for embryos used in the immunofluorescence study	11
IV	A summary of the incidence of abnormal embryos and abnormal membranes with each treatment	14
V	The incidence of the major types of abnormalities present in embryos exposed to adult antiserum	17

INTRODUCTION

The application of immunochemical methods in the study of chemical embryology began about thirty years ago. Saline soluble substances of various adult tissues have proved to be immunogenic in rabbits. Antisera against these substances have been used in precipitin reactions (double diffusion in agar, Ouchterlony, 1953; immunoelectrophoresis, Grabar and Williams, 1955) to detect the presence of adult substances in embryonic tissues. In the chick embryo as early as primitive streak and neurula stages the presence of an antigen common to the brain, heart, liver, and muscle of chicks at hatching was first demonstrated by Schechtman (1948). Ebert (1950) confirmed this by showing that substances which are immunologically identical to those in adult heart, in adult spleen, and in adult brain were present in the early chick blastoderm. These antigens are probably substances common to all tissues since absorption of the test antisera on heterologous tissue removed the antibodies which cross-reacted with the early embryo. It has also been demonstrated that tissue-specific antigens appear in embryonic organs during the course of development (liver, Croisille, 1960; D'Amelio *et al.*, 1963; Okada and Yamamura, 1964; kidney, Okada and Sato, 1963; Okada, 1965; McCallion *et al.*, 1970; lens, Langman, 1959; Maisel and Langman, 1961; heart, Ebert, 1953; brain, Burke *et al.*, 1944; Schalekamp, 1963; McCallion and Langman, 1964; McCallion, 1970). These investigators have shown that the adult antigens appear sequentially during the development of the embryo and that each steadily increases in concentration.

Adult chicken brain contains a number of antigens some of which are tissue-specific. Using rabbit anti-chicken brain serum, Schalekamp (1963) detected 3 neural-specific antigens which appeared sequentially in the embryo on days 2, 7 and 14 of incubation. McCallion and Langman (1964) also detected 3 neural-specific antigens which appeared on days 5, 9 and 12 of incubation. More recently McCallion (1970) has described at least 6 neural-specific antigens, of which 2 are present by the fifth day of incubation, the third by the tenth day, and the fourth and fifth by the fifteenth day and the sixth somewhat later.

Antigens common to all tissues also appeared progressively in the neural tissues of the embryo, as many as 3 antigens being present by 24 hours, 5 by 48 hours and 7 by 96 hours of incubation (McCallion and Langman, 1964). It is obvious, therefore, that common tissue antigens are the first antigens to appear in the developing embryo. Transient embryonic antigens, both neural-specific and common tissue, have also been detected in the embryo brain (McCallion and Trott, 1964, 1965).

The appearance of new antigens is thought to reflect the progress of differentiation of the embryo and in some cases the appearance of a new antigen is, in fact, accompanied by a functional or structural change in the embryo (D'Amelio *et al.*, 1963; Okada and Sato, 1963; Okada, 1965; Maisel and Langman, 1961; Friedman and Wenger, 1965; 1970). The antigens may be either structural or functional end-products of differentiation, or important substances to the process of differentiation. However, the role of antigens, whether common tissue or tissue-specific, in embryonic development is still obscure. One method of determining

the significance of antigens in normal embryonic development has been to expose developing embryos to antisera against adult tissue. In the past 20 years several investigators have attempted to use antisera as tissue disorganizers and growth inhibitors (Ebert, 1950; Nettleship, 1953; Mun, 1958). Cytolysis and degeneration have been described in amphibian embryos exposed to tissue antisera (Clayton, 1953; Nace and Inoue, 1957). Ebert (1950) obtained cell clumping and degeneration in predifferentiated chick blastoderms cultured on a medium containing antisera to heart, spleen or brain. Using progressively diluted antisera, he obtained largely mesodermal deficiencies with antiheart and antispleen sera and largely neural defects with antibrain serum at moderate dilutions but only growth retardation at greater dilutions. He also found that antiheart serum affected heart development while antispleen serum did not. Johnson and Leone (1955) have reported that heart development *in ovo* and *in vitro* was inhibited by anti-actomyosin but they found that somites and general growth were also affected. Heart deformities were obtained by Licata *et al.*, (1962) when older embryos were injected with antiheart serum but the heart defects were always accompanied by many other abnormalities. Adult heart antigens (cardiac myosin) begin to appear very early in development (Ebert, 1953; Johnson and Leone, 1955) but the presence of a particular antigen has not been correlated with the effect of the antiserum on the target organ.

Langman, (Langman, 1959, 1960; Langman *et al.*, 1962) obtained only abnormalities of the eye and brain with antilens serum and anti-alpha-crystallin serum. These antisera had a cytolytic effect on ectodermal tissues. Embryos younger than 17 somites were affected

while embryos older than 17 somites were not. Antisera to beta- and gamma-crystallins had no effect on embryos of any age. The presence of lens antigens in tissues of the eye other than lens has been demonstrated by Langman and Prescott (1959) and there is some evidence that the crystallins are also present in embryonic brain tissue at least to the time of lens induction (Clarke and Fowler, 1960). Of the 7 lens antigens, the first lens antigen detectable by double diffusion in agar appeared at 50 to 60 hours of incubation (18-24 somites) (Langman, 1959) and was identified as alpha-crystallin (Maisel and Langman, 1961). Two other lens antigens which have been identified as beta-crystallin and gamma-crystallin appear at 72 hours and 10 days respectively (Maisel and Langman, 1961). The effects in the embryo of specific antisera to lens antigens appear to be related to the stage of development at which the antigens appear.

Specific neural defects have been described in chick embryos exposed *in ovo* at stage 8-9 (Hamburger and Hamilton, 1951) to antibodies against specific brain components (McCallion, 1970). Ebert (1950) obtained largely neural defects in embryos cultured at stage 4-8- (Hamburger and Hamilton, 1951) in the presence of diluted antibrain serum. The present work was undertaken in order to re-examine, in the light of current knowledge of embryonic antigens, the effects of antibodies to specific neural antigens and of antibodies to common tissue antigens on the early chick embryo.

MATERIALS AND METHODS

Preparation of antigens

Fresh chicken heads and kidneys were obtained from a local poultry processing plant and transported to the laboratory on dry ice. Brains were dissected from the heads, freed from meninges, and homogenized in a Sorval omnimixer with an equal volume of 0.9 per cent NaCl solution. After extraction for one hour, the homogenate was centrifuged at 2600 g for 30 minutes to remove debris. Kidneys were freed of surrounding tissues, homogenized and extracted by the same procedures as for brain. These procedures were all carried out at 4°C. The supernatants were recovered and stored in glass vials in a domestic freezer at -25°C until required for use in immunization of rabbits.

White Leghorn eggs obtained from a commercial hatchery were incubated at $37.5 \pm 1^\circ\text{C}$ for nine days. The embryos were removed from the eggs, freed of their membranes, and decapitated. The brains were freed of surrounding tissue, washed in 0.9 per cent NaCl solution and homogenized in a tissue grinder with a small amount of 0.9 per cent NaCl solution. The homogenate was allowed to extract at 4°C for one hour and then stored in vials in a domestic freezer at -25°C.

Preparation of antisera

Antisera to adult chicken brain, adult chicken kidney and nine day chick embryo brain were obtained in New Zealand white rabbits. Frequent injections of adult chicken brain extract either alone or suspended in Freund's complete adjuvant were made either intra-muscularly or sub-

cutaneously at several sites. Samples of blood were collected from the marginal vein of the ear, allowed to clot, and centrifuged at 2600 g for 20 minutes. The resulting serum was processed further for experimental use. Antisera to adult chicken kidney and to nine day chick embryo brain were also obtained in rabbits by similar immunization procedures. Normal rabbit serum for use in control experiments was obtained from non-immunized rabbits.

The sera were processed in two different ways before use in experiments. Normal rabbit serum and antisera to adult brain, embryonic brain and adult kidney were concentrated by pressure dialysis in an Amicon filter unit containing a UM 10 membrane (Molecules above molecular weight 10,000 were retained). Immediately before use the sera were heated at 56°C for 30 minutes in order to inactivate complement and other small toxic molecules (Witebsky and Neter, 1935).

In an alternative procedure antiserum to adult brain was absorbed on adult kidney extract and adult chicken serum to remove the antibodies to common tissue antigens. Absorption was accomplished by mixing anti-brain serum, kidney extract and chicken serum in a ratio 10:2:1 and incubating the mixture at 37°C for 30 minutes. Following absorption the mixture was centrifuged at 2600 g for 30 minutes and the supernatant collected. Normal rabbit serum for use in control experiments was processed in the same manner. Gamma globulins were prepared from the antiserum to adult brain and from similarly treated rabbit serum by precipitation with ammonium sulphate. The sera were diluted with cold 0.9 per cent NaCl solution in the proportion 1:2. Cold saturated ammonium sulphate was added dropwise with stirring to an equal volume

of diluted serum to make the final mixture half-saturated with ammonium sulphate. The mixture was allowed to stand one hour at 6°C. The precipitate was recovered in a Büchner funnel, washed repeatedly with cold half-saturated ammonium sulphate solution and then redissolved in a minimum amount of 0.9 per cent NaCl. This solution was dialyzed against running tap water until all the ammonium sulphate was removed. The pH of the gamma globulin solution was adjusted to 7.8 with 1N NaOH. The protein concentration of the solution was determined by a modified biuret test and was increased by pressure dialysis to about 5g/100 ml. The gamma globulin solution was sterilized before use by millipore filtration.

The serum preparations were stored in a domestic freezer at -25°C. Some were thawed several times during a series of experiments. In such cases the quality of the antisera was tested at intervals by double diffusion in agar gel.

Agar Diffusion Technique

Each antiserum was tested against its corresponding tissue extract and other tissue extracts by the double diffusion method for antigen-antibody reactions in agar gels described by Ouchterlony (1953) (Fig. 1). Agar plates were prepared as described by McCallion and Langman (1964), with 0.2 per cent filtered agar (Ionagar, No. 2, pH 7.2, Oxoid) to which 0.01 per cent merthiolate (1:1,000) had been added. The plates containing the test materials were incubated at 39°C for 2 to 5 days, dialyzed against 0.9 per cent NaCl solution and photographed.

Treatment of embryos

Fresh fertile White Leghorn eggs were obtained from a commercial

FIGURE 1

A drawing of an Ouchterlony plate showing the positioning of the wells. The centre well contains antibrain serum. The peripheral wells contain adult kidney extract, adult brain extract, and adult chicken serum.

ABS - Antibrain serum

K - Kidney extract

B - Brain extract

S - Chicken serum

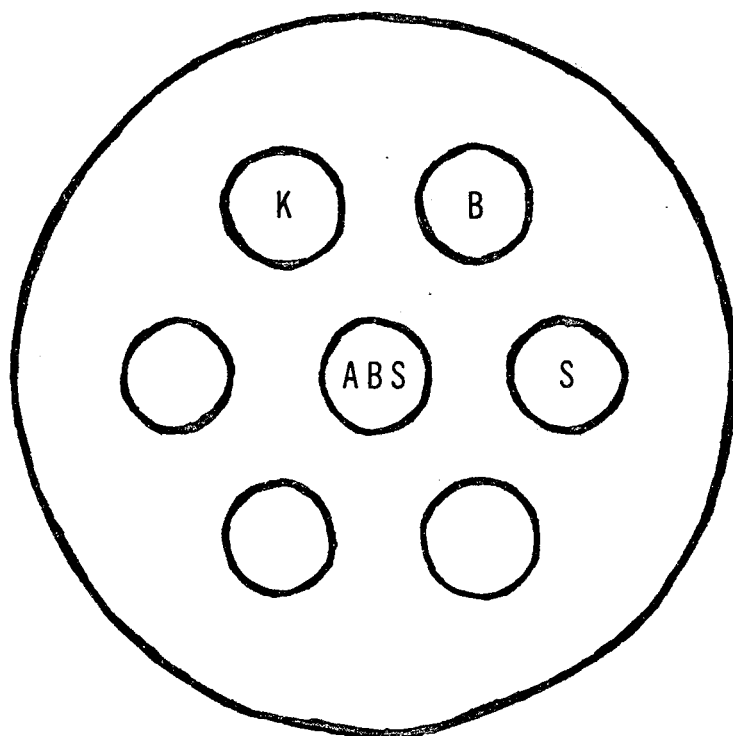


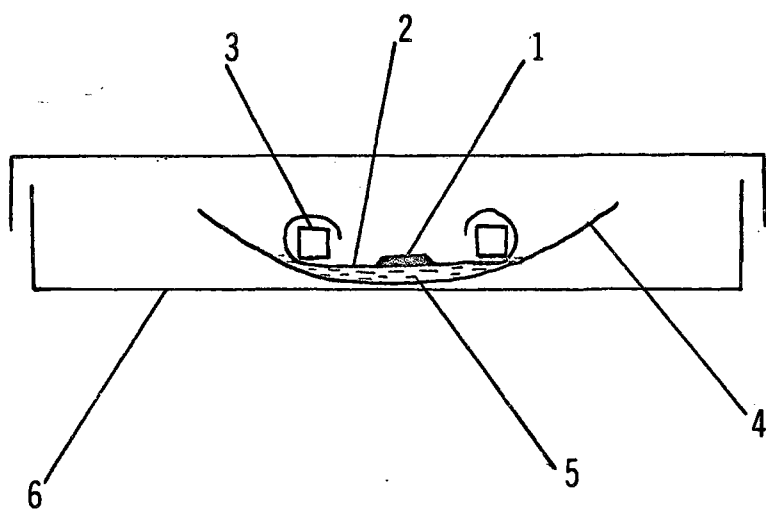
FIG. 1

hatchery and stored in the dark at 15°C. The eggs were incubated at 37.5±1°C in a humidified, forced draft incubator for about 25 hours to obtain embryos of selected stages (St. 4-9) (Hamburger and Hamilton, 1951). The embryos were explanted under sterile conditions by either the original method of New (1955) or by Gallera's modification of this method (Nicolet and Gallera, 1963). Howard's saline (Howard, 1953) was used in place of Pannett-Compton saline. The albumen was removed from the egg by means of a pipette, and the yolk, with the embryo uppermost was floated in a dish of saline. Scissors were used to cut the vitelline membrane around the perimeter and the membrane with its attached blastoderm was removed to a watch glass. The vitelline membrane with the blastoderm side up was anchored with a glass ring, OD 25 mm, ID 20 mm and 5 mm thick. The watch glass was removed to a plastic petri dish where excess saline was removed, the membrane secured around the glass ring and thin albumen injected under the ring (Fig. 2A). A graduated syringe was used to place the antiserum or a control solution on the ventral surface of the embryo. The petri dish was closed and the culture was incubated immediately in a Hotpack humidity controlled cabinet (5% CO₂ in air) at 37.5±0.5°C for 24 to 35 hours. When explants were incubated for more than 24 hours they were examined briefly at about 20 hours.

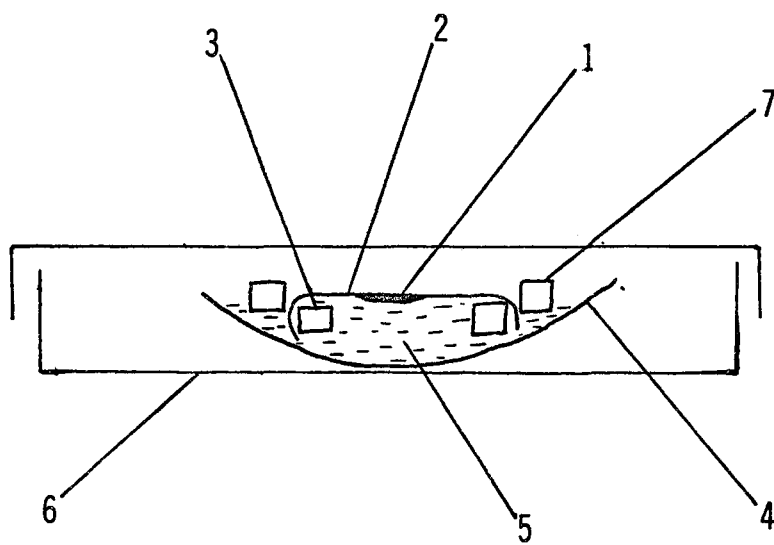
In Gallera's modification of the method, the vitelline membrane with the blastoderm side down is placed over one ring and a second, larger ring is fitted over the smaller ring to anchor the membrane. (Fig. 2B). The use of the second ring is not always necessary. In this preparation thin albumen was injected under the ring(s) and antiserum

FIGURE 2

- A. A diagram of an embryo explanted by the method of New (1955).
 - B. A diagram of an embryo explanted by the method of Gallera (Nicolet and Gallera, 1963).
-
- 1. Blastoderm
 - 2. Vitelline membrane
 - 3. Glass ring
 - 4. Watch glass
 - 5. Thin egg albumen
 - 6. Plastic petri dish
 - 7. Second glass ring (opt.)



A



B

FIG. 2

or a control solution was placed on the dorsal surface of the embryo. Each embryo preparation was numbered and the stage and treatment recorded. The Hamburger and Hamilton (1951) method of staging chick embryos was used throughout. Cultures in which blastoderm edges detached from the vitelline membrane or which had obviously suffered mechanical damage were rejected prior to treatment.

A summary of the details of the treatment of embryos is shown in Tables I and II. The data in Table I refer to embryos explanted by New's method in which the test solution was placed on the ventral side. The data in Table II refer to embryos explanted by Gallera's method in which the test solution was applied to the dorsal side of the embryo. Treatment of embryos with a given antiserum required several culture sessions; each culture session included approximately equal numbers of control and antiserum treated embryos. The control solutions used were Howard's saline or normal rabbit serum.

The embryos were recovered after 24-36 hours further incubation, separated from the vitelline membrane, washed with Howard's saline and fixed in Bouin's solution. They were stained with borax-carmin, mounted on microscope slides and photographed. Selected embryos were removed from the slides, embedded in paraffin, serially sectioned at 5 microns, destained of borax-carmin and stained with haematoxylin and eosin.

Immunofluorescent localization of antibodies

In order to demonstrate the presence of rabbit gamma globulins in embryos exposed to antisera the method of Sainte-Marie (1962) was

TABLE I

Treatment of embryos explanted by New's method in which the test solution was placed on the ventral side.

Test Solution	Dose Volume ml.	Protein con- centration g/100 ml	Number of cultures
Howard's saline	0.1	----	12
NRS	0.1	20*	18
9dABS	0.1	20*	30

NRS - Normal rabbit serum

9dABS - Antiserum to nine day chick embryo brain

* - Approximate values based on original concentration of protein in serum and volume reduction during pressure dialysis.

TABLE II

Treatment of embryos explanted by Gallera's method in which the test solution was placed on the dorsal side.

Test Solution	Dose Volume ml.	Protein con- centration g/100 ml.	Number of cultures
Howard's saline	0.05	----	139
NRS	0.02*	13.2	18
AKS	0.02*	9.2	21
ABS	0.02*	10.8	32
NRS γ g	0.02*	5.4	20
ABS γ g	0.02*	4.5	24

NRS - Normal rabbit serum

AKS - Antiserum to adult kidney

ABS - Antiserum to adult brain

NRS γ g - Gamma globulin fraction of treated normal rabbit serum

ABS γ g - Gamma globulin fraction absorbed adult brain antiserum

* - Approximate volume of one drop delivered by syringe

used. Embryos of stages 6-9 were cultured by Gallera's method and were exposed to serum preparations or to saline placed on the dorsal side as previously described. The embryos were subsequently recovered (Table III), washed in cold 0.05 M phosphate-buffered saline, pH 7.0, fixed overnight in cold 95 per cent alcohol, immediately dehydrated and embedded in paraffin (M.P. 56°C). All fixation and dehydration processes were performed at 4°C. The anterior portions of the embryos were sectioned at 5 microns, stained with FITC-labelled goat anti-rabbit gamma globulins (Cappel Laboratories, Downington, Pa.) and mounted in Harleco Fluorescence Mountant (Hartman-Leddon, Philadelphia, Pa.). The sections were examined with blue light fluorescence and photographed.

TABLE III

Treatment and recovery schedule for embryos used in the immunofluorescence study.

Test Solution	Dose volume ml.	Protein concentration g/100 ml.	Time exposed hrs.	Number of cultures
Howard's saline	0.05	----	8.5	1
Howard's saline	0.05	----	32.5	6
ABS	0.02*	10.8	23.0	3
AKS	0.02*	9.2	23.0	3
ABS _{Yg}	0.02*	4.5	8.5	2
ABS _{Yg}	0.02*	4.5	21.0	2
ABS _{Yg}	0.02*	4.5	32.5	4

ABS - Antiserum to adult brain

AKS - Antiserum to adult kidney

ABS_{Yg} - Gamma globulins of absorbed adult brain antiserum

* - Approximate volume of one drop delivered by syringe

RESULTS

Characterization of the antisera

When tested by double diffusion in agar gels adult chicken brain antiserum formed at least 8 precipitin lines with adult chicken brain extract, at least 6 precipitin lines with adult chicken kidney extract and 4 precipitin lines with adult chicken serum (Fig. 3). When the gamma globulin fraction of adult chicken brain antiserum previously absorbed on adult chicken kidney extract and adult chicken serum was tested against adult chicken brain extract 4 precipitin lines were formed (Fig. 4). There was no cross reaction between the gamma globulins and adult chicken kidney extract. This indicates that the gamma globulin solution did not contain antibodies to common tissue antigens but did contain antibodies to neural-specific antigens.

When the antiserum to nine day chick embryo brain was tested against the homologous tissue extract at least 8 precipitin lines were formed. One precipitin line was formed with adult chicken kidney extract (Fig. 6).

Adult chicken kidney antiserum formed 9 or 10 precipitin lines with adult chicken kidney extract, 3 or 4 precipitin lines with adult chicken brain extract and 5 precipitin lines with adult chicken serum (Fig. 5).

Of the 7 precipitin lines that adult chicken brain antiserum formed with adult chicken brain extract, 3 were fused with precipitin lines that adult chicken kidney antiserum formed with that same extract

FIGURE 3

A photograph of an Ouchterlony double diffusion plate. Adult brain antiserum is in the centre well. Clockwise in the upper peripheral wells are adult chicken serum, adult chicken kidney extract and adult chicken brain extract.

FIGURE 4

A photograph of an Ouchterlony double diffusion plate. A gamma globulin solution of absorbed adult brain antiserum is in the top centre well; adult chicken serum in the right well; adult chicken kidney extract in the left well and adult chicken brain extract in the bottom centre well.

FIGURE 5

A photograph of an Ouchterlony double diffusion plate. Adult kidney antiserum is in the centre well. Clockwise in the upper peripheral wells are adult chicken serum, adult chicken brain extract and adult chicken kidney extract.

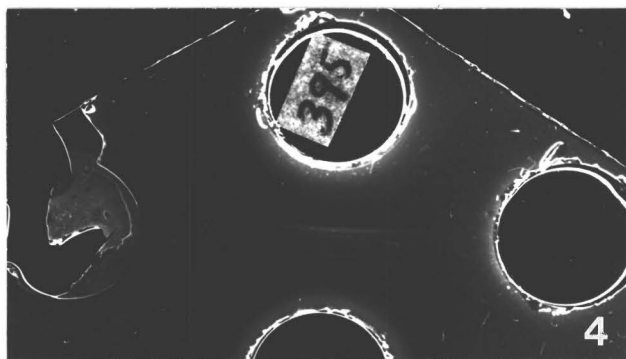
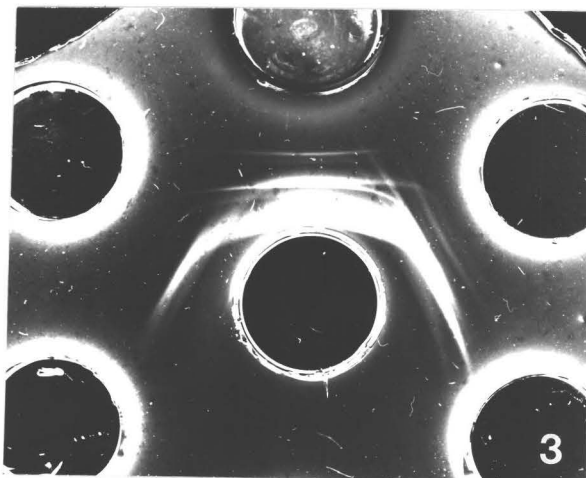


FIGURE 6

A photograph of an Ouchterlony double diffusion plate with 9-day embryo brain antiserum in the top centre well, 9-day embryo brain extract in the bottom centre well and adult chicken kidney extract in the left well.

FIGURE 7

A photograph of an Ouchterlony double diffusion plate with adult chicken kidney extract in the bottom centre well, adult chicken kidney antiserum in the top left well and adult chicken brain antiserum in the top right well.

FIGURE 8

A photograph of an Ouchterlony double diffusion plate with adult chicken brain extract in the bottom centre well, adult chicken kidney antiserum in the top left well and adult chicken brain antiserum in the top right well.

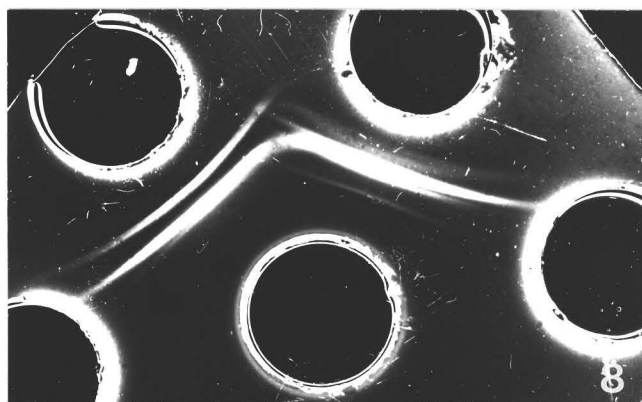
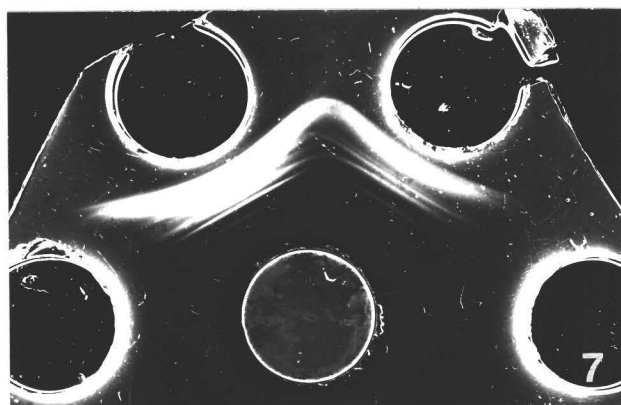
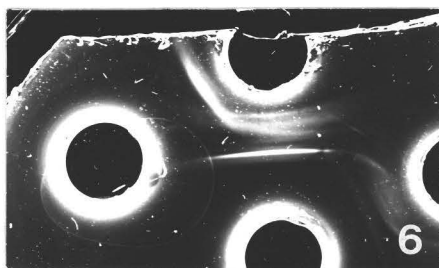


FIGURE 9

A photograph of an Ouchterlony double diffusion plate with adult kidney antiserum in the top left well, adult brain antiserum in the top right well, extract of extra-embryonic membranes from eggs incubated for 48 hours in the centre well and extract of extra-embryonic membranes from eggs incubated for 34 hours in the centre left and bottom right wells.

FIGURE 10

A photograph of an Ouchterlony double diffusion plate with adult kidney antiserum in the top left well, adult brain antiserum in the top right well and extracts of embryos of stages 14 to 16 from eggs incubated for 48 hours in the bottom centre well.

FIGURE 11

A photograph of an Ouchterlony double diffusion plate with adult chicken kidney antiserum in the top left well, adult chicken brain antiserum in the top right well and extract of stage 14 to 16 embryos raised in culture from definitive primitive streak and open neural plate stages in the bottom centre well.



(Fig. 8). Of the 9 precipitin lines that adult chicken kidney antiserum formed with adult chicken kidney extract, 6 were fused with precipitin lines that adult chicken brain antiserum formed with the same tissue extract (Fig. 7). These observations indicate that both antisera contain antibodies to the same antigens.

Adult chicken kidney antiserum and adult chicken brain antiserum both formed several precipitin lines with extracts of the extra-embryonic membranes from eggs incubated for 34 hours and from eggs incubated for 48 hours (Fig. 9). Of the precipitin lines that formed between adult chicken kidney antiserum and extract of 48-hour extra-embryonic membranes, 3 were fused with the precipitin lines that formed between adult chicken brain antiserum and the extract. Similarly, extract of 34-hour extra-embryonic membrane formed 2 to 3 precipitin lines with these antisera which fused with those formed between 48-hour extra-embryonic membrane and the antisera. Both antisera, therefore, contain antibodies to antigens in the extra-embryonic membranes of 34- and 48-hours chick embryo. Adult chicken kidney antiserum and adult chicken brain antiserum both cross-reacted with extracts of embryos from eggs incubated for 34 hours. These antisera cross-reacted with extracts of stage 14 to 16 embryos from eggs incubated for 48 hours (Fig. 10). They also cross-reacted with extracts of stage 14 to 16 embryos raised in culture from definitive primitive streak and open neural plate stages (Fig. 11).

Effects of antisera on embryos

The whole mounts of all embryos exposed to antisera, normal rabbit serum, and saline solution were examined under a dissection

microscope for morphological abnormalities. The stage of development achieved by experimental embryos was compared with that achieved by control embryos explanted at the same stage of development during the same culture session. Embryos which were younger than control embryos by more than one stage (Hamburger and Hamilton, 1951) were classed as abnormal and the abnormality was termed growth retardation. A small number of embryos explanted by Gallera's method, whether they were saline controls or treated with normal rabbit serum or with antisera, developed a hole in the posterior section of the embryo adjacent to the neural tube (Fig. 12). Disruption of the neural tube and somites occurred at this point. This defect was probably the result of mechanical damage during the preparation of the culture and, therefore, these embryos were classed as normal. The incidence of abnormal embryos and of abnormal extra-embryonic membranes with each type of treatment is summarized in Table IV.

Embryos treated with Howard's saline had a very low frequency of abnormalities (less than 6.5 %) whether they were explanted by New's method or by Gallera's method. A typical control embryo which was explanted at stage 8⁻ and which reached stage 15 after 32 hours in culture is shown in Fig. 13. The incidence of abnormal embryos in the group in which normal rabbit serum was applied to the ventral side was significantly greater at the 5% level than that of saline controls (The test for difference in proportions was used). The incidence of abnormal embryos in the group in which the dorsal surface was exposed to normal rabbit serum was not significantly greater (at the 5% level) than that in the

TABLE IV

A summary of the incidence of abnormal embryos and abnormal membranes with each treatment.

Test solution	Side exposed	Protein concentration g/100 ml.	Dose ⁺ ml.	Abnormal Embryos	Abnormal Membranes	Total cultures
Saline	ventral	----	0.1	0	0	12
NRS	ventral	20*	0.1	7	1	18
9dABS	ventral	20*	0.1	28	25	30
Saline	dorsal	----	0.05	9	5	139
NRS	dorsal	13.2	0.02	2	1	18
AKS	dorsal	9.2	0.02	21	8	21
ABS	dorsal	10.8	0.02	23	10	32
NRS _γ	dorsal	5.4	0.02	3	0	20
ABS _γ	dorsal	4.5	0.02	2	0	24

NRS - Normal rabbit serum

9dABS - Antiserum to nine day chick embryo brain

AKS - Adult kidney antiserum

ABS - Adult brain antiserum

NRS_γ - Gamma Globulins of treated normal rabbit serum

ABS_γ - Gamma globulins of absorbed antiserum to adult chicken brain

* - Approximate concentration

+ - Approximate volume

saline control group. Furthermore the protein concentration and the volume of normal rabbit serum applied was different in the two cases. A typical normal rabbit serum treated embryo explanted at stage 8⁻ and raised in culture 32 hours is shown in Fig. 15.

The incidence of abnormal embryos in the group treated on the ventral side with embryo brain antiserum was significantly greater (at the 5 % level) than either that in the group treated on the ventral side with normal rabbit serum or that in the group treated with saline. The abnormalities in the embryos treated with embryo brain antiserum consisted of open neural tubes (16), failure of posterior somite genesis (7) (Fig. 14), and complete absence of the embryo (4). In most instances when the embryo was abnormal it was accompanied by abnormal extra-embryonic membranes. The abnormalities of the extra-embryonic membranes were of two types, detachment of the edge of the blastoderm from the vitelline membrane (6) and failure of normal expansion of the blastoderm (19). In the former case, the extent of distortion of the blastoderm depended on the extent of the detachment and was extremely variable. Complete detachment resulted in small rounded blastoderms in which the embryo was no longer present. When blastoderms failed to expand normally, the area vitellina appeared to be thicker than normal. In control experiments the blastoderm expanded to meet the glass ring and to fill completely the area inside the ring.

In preliminary experiments in which embryo brain antiserum was mixed with albumen and injected under the preparation there were no more abnormalities of the embryos or of the membranes than in embryos similarly

FIGURE 12

A photograph of an embryo explanted at stage 6 and cultured in the presence of saline solution for 31.5 hours. Note the disruption of neural tube and somites (arrow) which is probably the result of mechanical damage.

FIGURE 13

A photograph of an embryo explanted at stage 8⁻ and cultured in the presence of saline solution for 32 hours.

FIGURE 14

A photograph of an embryo explanted at stage 6 and cultured in the presence of antiserum to embryo brain for 21 hours. Note the absence of posterior somites.

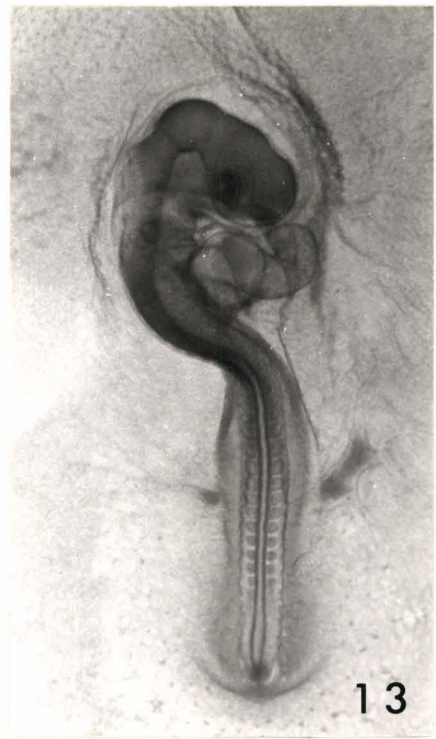


FIGURE 15

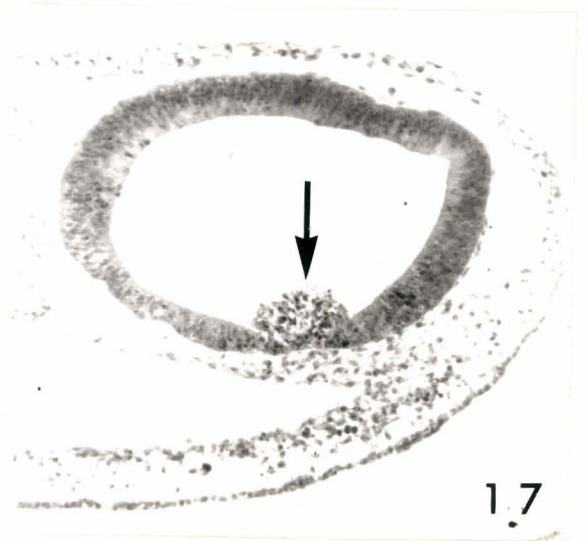
A photograph of an embryo explanted at stage 8⁻ and cultured in the presence of 0.02 ml normal rabbit serum at 13.2 g/100 ml. for 32 hours.

FIGURE 16

A photograph of a section through the prosencephalon of the embryo shown in Fig. 15. Note the disorganization in the wall of the neural tube (arrow). X740.

FIGURE 17

A photograph of a section through the mesencephalon of the embryo shown in Fig. 15. Note the small area of necrosis (arrow) in the wall of the neural tube. X740.



treated with normal rabbit serum. When the antiserum-albumen mixture was tested by double diffusion in agar gel against the homologous tissue extract, precipitin lines were still formed.

In the group of embryos treated on the dorsal side with antiserum to adult brain the incidence of abnormal embryos and abnormal membranes was greater than in the group similarly treated with normal rabbit serum, or in the saline control group (significant at the 5 % level). Of the 23 abnormal embryos, 17 had some defect of the central nervous system, and 11 had some defect of the posterior trunk (Table V). Frequently more than one abnormality occurred in the same embryo. Defects of the central nervous system consisted of open neural tubes (11), enlarged brain ventricles (4), abnormal cranial flexure and abnormal torsion (5), and retardation of brain development (12). Open neural tube involved only the prosencephalon and mesencephalon (6) (Fig. 18), or it occurred at more posterior levels (3) (Fig. 19) or extended the whole length of the neural tube (2). All of the embryos with enlarged brain ventricles lacked the normal cranial flexure (Fig. 21). The flexion either did not occur at all or was much less than normal. This defect was usually accompanied by failure of the anterior portion of the embryo to turn on its side. Growth retardation was a general abnormality of antisera treated embryos. In most cases retardation involved only the brain, that is, the stage of development achieved by the brain lagged behind the caudal development of the axis and somites and behind the stage of development achieved by the brains of control embryos explanted at the same stage during the same culture session. In a few cases the retardation was associated with failure of the neural tube to close and

FIGURE 18

A photograph of an embryo explanted at stage 8⁻ and cultured in the presence of adult brain antiserum for 28 hours. Note the opening (arrow) in the neural tube in the prosencephalon-mesencephalon.

FIGURE 19

A photograph of an embryo explanted at stage 8⁻ and cultured in the presence of adult brain antiserum for 32 hours. Note the opening (arrow) in the neural tube.

FIGURE 20

A photograph of an embryo explanted at stage 5 and cultured in the presence of adult brain antiserum for 28 hours. Note the abnormal caudal tip.

FIGURE 21

A photograph of an embryo explanted at stage 8⁻ and cultured in the presence of adult brain antiserum for 32 hours. Note the enlarged brain ventricles, the abnormal cranial flexure, the slightly short trunk and the abnormal caudal tip.

FIGURE 22

A photograph of an embryo explanted at stage 5 and cultured in the presence of adult brain antiserum for 32 hours. Note the small size of the prosencephalon (arrow), the short trunk and the abnormal caudal tip.

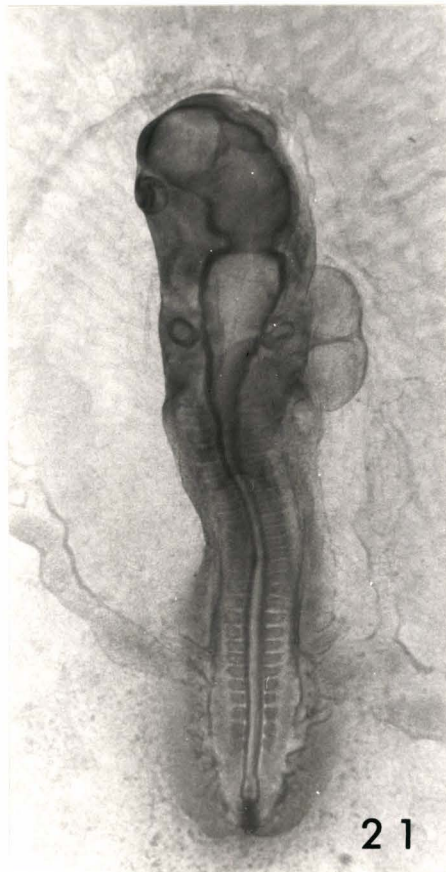
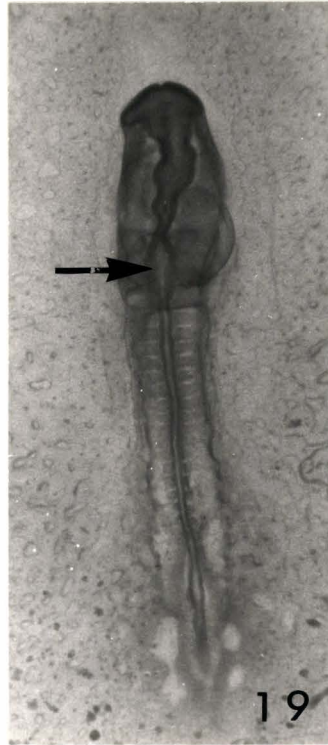
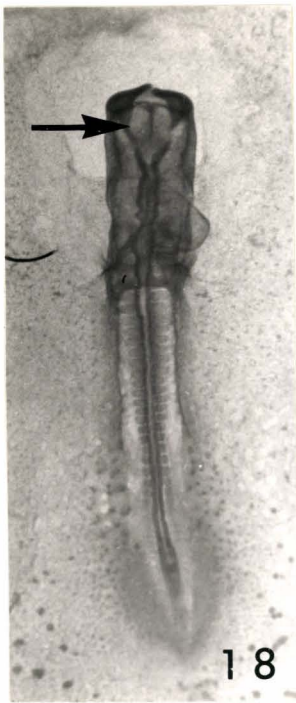


FIGURE 23

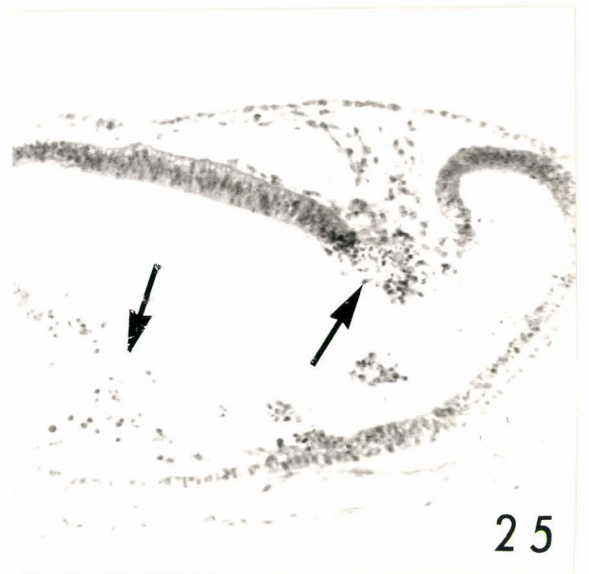
A photograph of an embryo explanted at stage 7 and cultured in the presence of adult brain antiserum for 31 hours. Note that the embryo appears to be normal in spite of the detachment of the edge of the extra-embryonic membrane from the vitelline membrane.

FIGURE 24

A photograph of an embryo explanted at stage 7 and cultured in the presence of adult brain antiserum for 33 hours. Note the normal appearance of the embryo.

FIGURE 25

A photograph of a section through the prosencephalon of the embryo shown in Fig. 24. Note the extensive necrosis (arrows) of the neural epithelium. X740.



more often with abnormal cranial flexure.

Defects of the posterior trunk consisted of short trunks (9) and some degree of abnormality of the caudal tip of the embryo (6). The posterior expansion of the trunk was obviously inhibited in some cases (Fig. 22) but less so in others (Fig. 21). Defects of the caudal tip of the trunk showed considerable variation (cf. Figs. 20 & 22).

Embryos treated with adult brain antiserum also developed a small number of rare abnormalities. There were 2 cases of absence of one lens and one case of absence of an otic vesicle. In all cases the opposite lens or otic vesicle was well developed. There were 3 cases in which the prosencephalon consisted of only a small mass with no recognizable structure (Fig. 22) in 2 of which the neural tube remained open anteriorly.

Abnormalities of the extra-embryonic membranes of the embryos in the group treated with adult brain antiserum consisted of detachment of the blastoderm edge from the vitelline membrane. This was a consistent phenomenon in two of the sessions but it did not occur in the remaining three sessions which made up the group. Antiserum from a single immunized rabbit was used in both cases but was obtained from the rabbit on different days. Of the 10 embryos with extra-embryonic membranes detached from the vitelline membranes, 5 were abnormal, 3 were disintegrated and the remaining 2 embryos were normal (Fig. 23). When the extra-embryonic membranes were severely distorted or when the embryos had disintegrated, these abnormal embryos were omitted from the data.

All of the embryos treated with antiserum to adult chicken kidney were abnormal. This represents a significant increase (at the 5 % level)

TABLE V

The incidence of the major types of abnormalities present in embryos exposed to adult antiserum

Treatment	No. Embryos	Abnormal Embryos	Abnormal Membranes	CNS D E F E C T S				POSTERIOR TRUNK DEFECTS	
				Open Neural Tube	Enlarged Brain Ventricles	Abnormal Flexion	Brain Retar- dation	Short Trunk	Abnormal Caudal Tip
ABS	32	23	10	11	4	5	12	9	6
AKS	21	21	8	11	7	5	10	8	10
NRS	18	2	1	0	0	0	0	0	0

ABS - Adult brain antiserum

AKS - Adult kidney antiserum

NRS - Normal rabbit serum

CNS - Central nervous system

over the normal rabbit serum treated and saline control groups. The types of abnormalities occurring in embryos treated with adult kidney antiserum were similar to those occurring in embryos treated with antiserum to adult brain, but the severity of the defect was greater. Of the 21 abnormal embryos, 20 had defects of the central nervous system and 13 had defects of the posterior trunk (Table V). Usually more than one defect occurred in the same embryo. Defects of the central nervous system included open neural tubes (11), enlarged brain ventricles (7), abnormal cranial flexure (5) and retardation of brain development (10). An opening in the neural tube occurred most often in the prosencephalon and mesencephalon (7) (Fig. 26) and rarely involved the whole neural tube (1). In 3 embryos a small to large area of the neural tube at the somite level remained open (Fig. 27). All but 2 of the 7 embryos with enlarged brain ventricles had an abnormal cranial flexure (Figs. 28, 29, 30). Abnormal torsion usually accompanied this defect. Retarded brain development was associated with partially open neural tubes (4) or with enlarged brain ventricles (4) but also existed alone without other abnormalities.

Abnormalities of the trunk consisted of very short trunks (8) (Figs. 29, 30, 32 & 33) and some abnormality of the caudal tip of the embryo (10) which showed considerable variation (Fig. 29, 33).

Abnormalities of the extra-embryonic membranes in the group treated with antiserum to adult kidney were significantly (at the 5 % level) more numerous than those in the groups treated with normal rabbit serum or saline but were not significantly different from those in the group treated with adult brain antiserum. The membrane abnormality

FIGURE 26

A photograph of an embryo explanted at stage 6 and cultured in the presence of adult kidney antiserum for 32 hours. Note the open neural tube (arrow) in the prosencephalon-mesencephalon.

FIGURE 27

A photograph of an embryo explanted at stage 8- and cultured in the presence of adult kidney antiserum for 32 hours. Note the large area of the posterior neural tube which is open (between arrows).

FIGURE 28

A photograph of an embryo explanted at stage 8- and cultured in the presence of adult kidney antiserum for 32 hours. Note the enlarged brain ventricles and the abnormal cranial flexure.

FIGURE 29

A photograph of an embryo explanted at stage 7 and cultured in the presence of adult kidney antiserum for 32 hours. Note the enlarged brain ventricles, abnormal cranial flexure, and abnormal caudal tip.

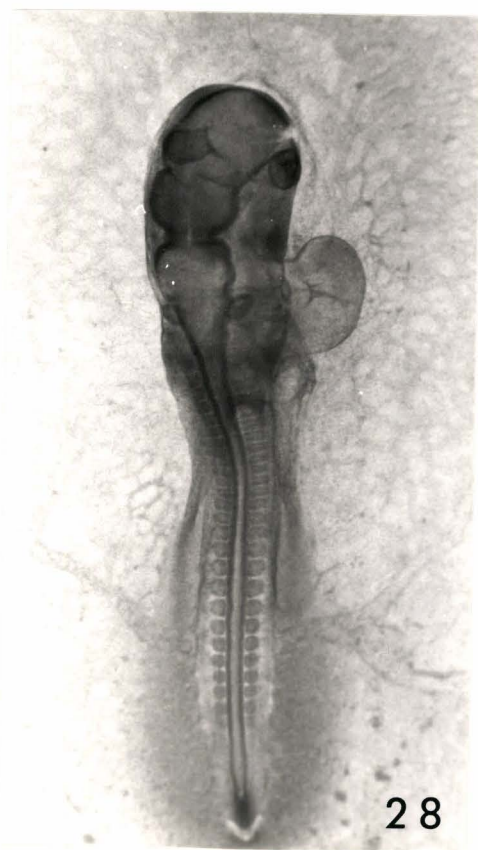
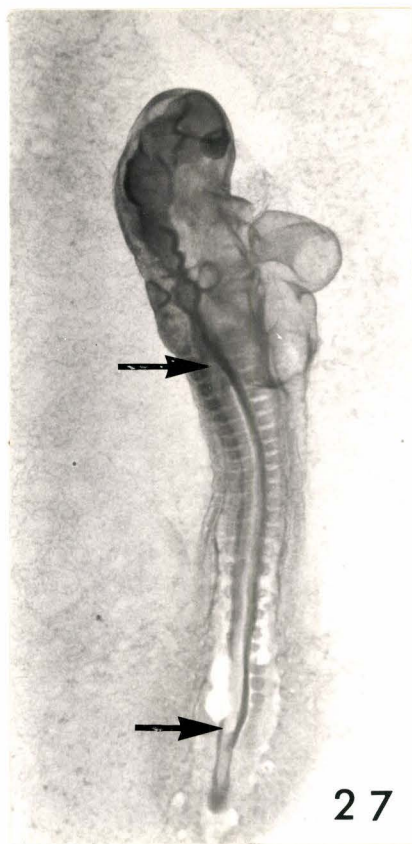
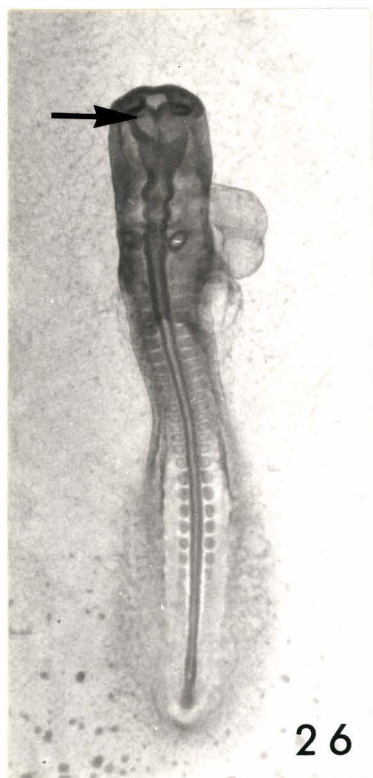


FIGURE 30

A photograph of an embryo explanted at stage 8⁻ and cultured in the presence of adult kidney antiserum for 31 hours. Note the enlarged brain ventricles, abnormal cranial flexure, short trunk and the abnormal caudal tip.

FIGURE 31

A photograph of a section through the mesencephalon of the embryo shown in Fig. 30. Note the size of the lumen and thickness of the walls of the neural tube. Also note the active cell division in the ependyma cells (arrow). X740.

FIGURE 32

A photograph of an embryo explanted at stage 7 and cultured in the presence of adult kidney antiserum for 32 hours. Note the extremely short trunk and the abnormal caudal tip.

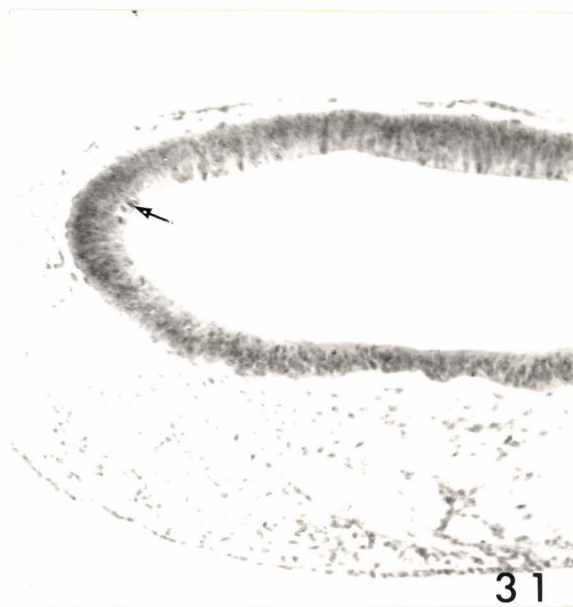
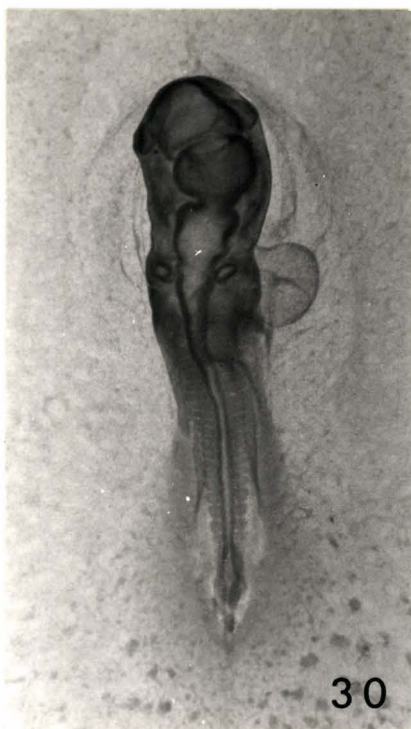


FIGURE 33

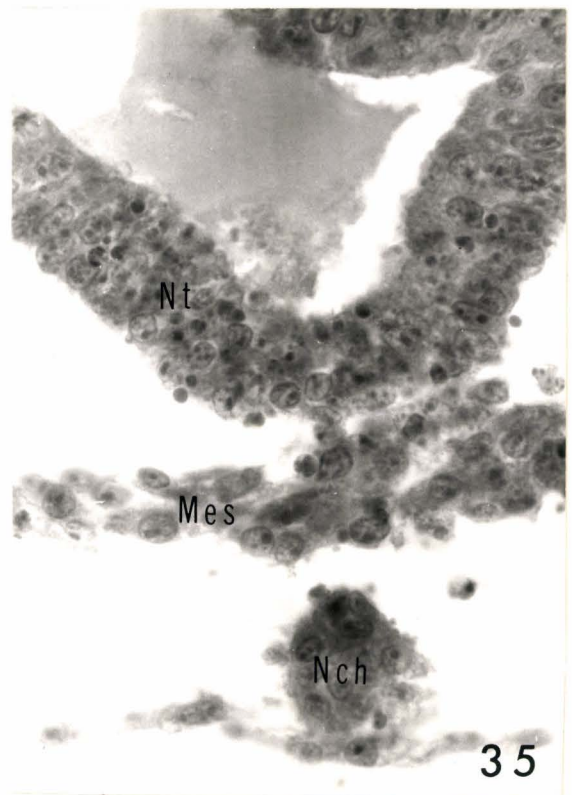
A photograph of an embryo explanted at stage 7 and cultured in the presence of adult kidney antiserum for 32 hours. Note the short trunk and the abnormal caudal tip.

FIGURE 34

A photograph of a section through the prosencephalon of the embryo shown in Fig. 33. Note the extensive disorganization and necrosis of the neural epithelium and also the overgrowth of neural tissue. X740.

FIGURE 35

A photograph of a section through the posterior neural tube of the embryo shown in Fig. 33. Note the necrosis of the ventral part of the neural tube. (Nt, Neural tube; Mes, mesoderm; Nch, notochord), X1800.



consisted of a failure of normal expansion of the blastoderm and of a thickening of the area vitellina. This defect is similar to that of the extra-embryonic membranes in the group treated with embryo brain anti-serum. Some of the membranes were more severely damaged (Fig. 37) than others (Fig. 36).

The incidence of abnormal embryos in the group exposed to gamma globulins of treated normal rabbit serum was very small and not significantly different (at the 5 % level) from that in the saline control group. Of the embryos treated with gamma globulins of absorbed adult brain anti-serum, few were abnormal. This was not significantly different (at the 5 % level) from the incidence of abnormal embryos in the group treated with gamma globulins of normal rabbit serum or in the saline control group. A typical morphologically normal embryo from the group treated with normal rabbit serum gamma globulins is shown in Fig. 39. Figs. 40 & 41 show typical normal embryos from the group treated with gamma globulins of absorbed adult brain antiserum.

Embryos were between stages 4 and 8- when they were explanted. A careful record was kept of the stage of development of each explanted embryo and of the treatment applied to each embryo. There was no apparent correlation between the stage of development at the time of treatment and the type of abnormality.

The few abnormalities which did occur in the saline control group, the normal rabbit serum control groups and the gamma globulin treated groups never included short trunks, enlarged brain ventricles, abnormal cranial flexure, brain retardation, or failure of normal blastoderm expansion but did include open neural tubes and detachment of the

FIGURE 36

A photograph of part of the extra-embryonic membrane of an embryo explanted at stage 7 and cultured in the presence of adult kidney antiserum for 31 hours. Note the abnormal margin of the membrane.

FIGURE 37

A photograph of part of the extra-embryonic membrane of an embryo explanted at stage 8 and cultured in the presence of adult kidney antiserum for 31 hours. Note the severe damage to the membrane.

FIGURE 38

A photograph of a portion of a section through the extra-embryonic membrane of the embryo shown in Fig. 37. Note the abnormal condition of the ectoderm. (Ect, ectoderm), X740.

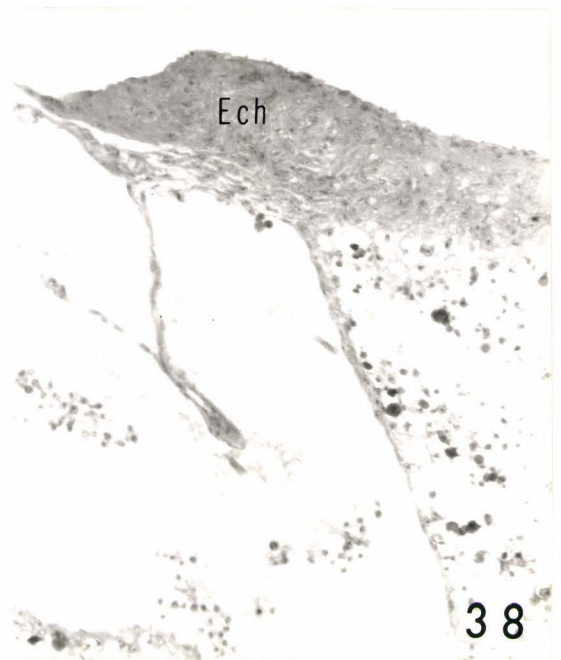


FIGURE 39

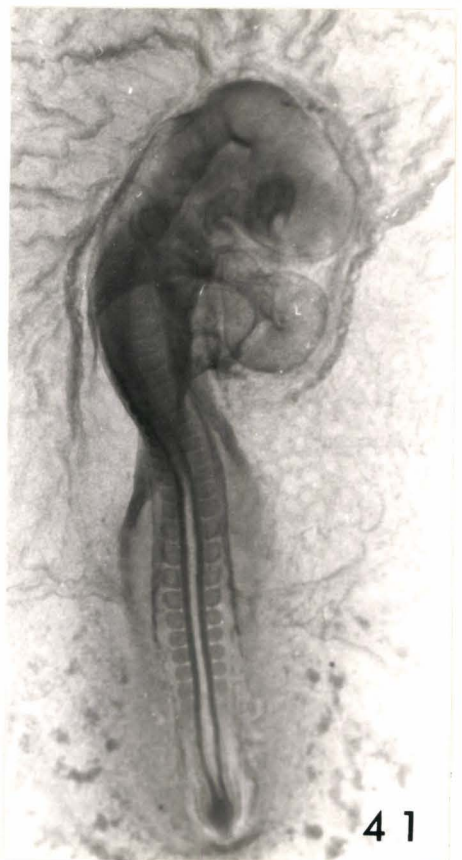
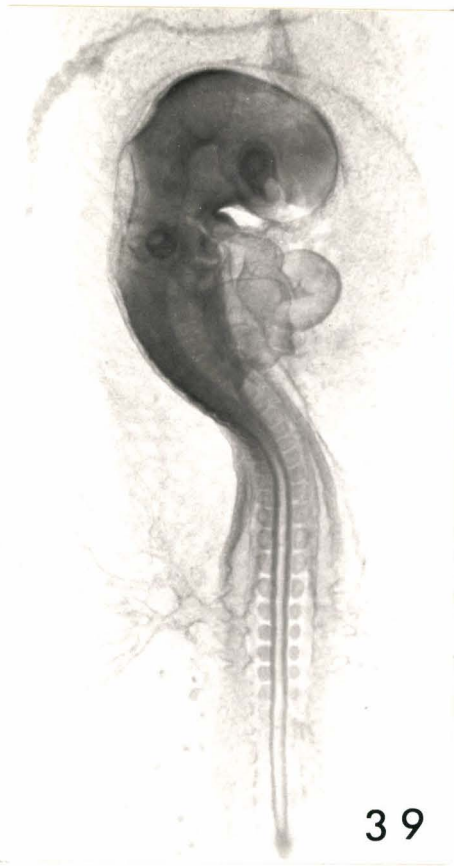
A photograph of an embryo explanted at stage 5 and cultured in the presence of gamma globulins of treated normal rabbit serum for 33 hours. Note that this embryo is normal.

FIGURE 40

A photograph of an embryo explanted at stage 6 and cultured in the presence of gamma globulins of absorbed adult brain antiserum for 35 hours. Note that this embryo is normal.

FIGURE 41

A photograph of an embryo explanted at stage 6 and cultured in the presence of gamma globulins of absorbed adult brain antiserum for 35 hours. Note that this embryo is normal.



blastoderm edge from the vitelline membrane.

The abnormalities in embryos exposed either to adult kidney antiserum or to adult brain antiserum were the same. Typical abnormal embryos from both groups were examined histologically. Examination of a few embryos with short trunks revealed that the caudal proliferation centre had undergone progressive cytolysis and necrosis. There was no evidence of continued mitotic activity (Fig. 43). Furthermore, even in antisera treated embryos which had no apparent morphological abnormality of the caudal tip the growth centre was similarly affected. In embryos with morphological abnormalities of the caudal tip there was, in addition to the destruction of the caudal proliferation centre, necrosis of the posterior end of the neural tube and of the notochord (Fig. 35). By comparison, the caudal growth centres of embryos exposed to normal rabbit serum were very healthy and the cells were actively dividing (Fig. 42).

In embryos with open neural tubes there were extensive areas of disorganization and necrosis of neural epithelium at the site of the lesion (Fig. 34). Smaller areas of disorganization and necrosis also occurred elsewhere in the neural tube. There is good evidence of mitosis and some overgrowth of neural tissue in or near the defect. Even in an apparently morphologically normal embryo which had been exposed to adult brain antiserum there were extensive areas of necrosis of the neural epithelium of the brain (Fig. 25). Disorganization and necrosis rarely occurred in other tissues.

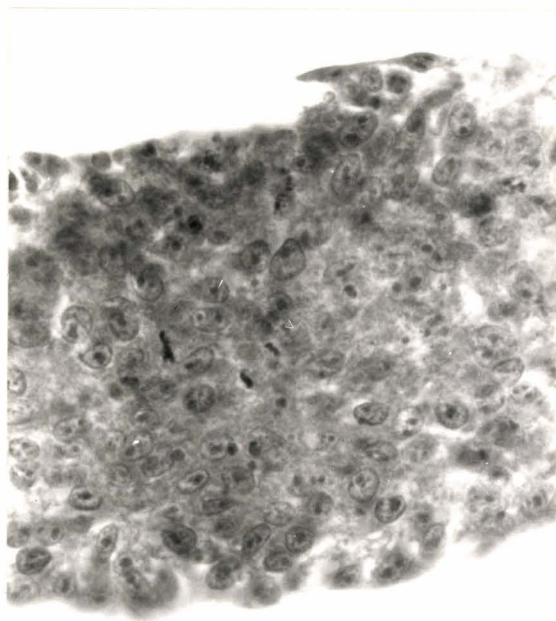
In embryos with enlarged brain ventricles the lumen of the neural tube was very large and the walls were normal with many dividing

FIGURE 42

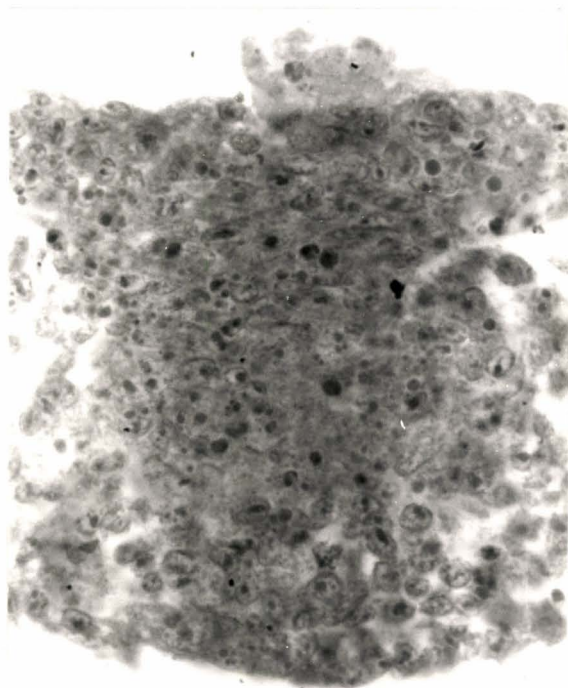
A photograph of a section through the caudal proliferation centre of the embryo shown in Fig. 15. Note the actively dividing cells. X1800.

FIGURE 43

A photograph of a section through the caudal proliferation centre of the embryo shown in Fig. 33. Note the absence of mitotic activity and the necrosis of the tissue. X1800.



4 2



4 3

cells in the ependyma (Fig. 31). The walls of the neural tube did not appear to be appreciably thicker than normal.

The tissues of embryos treated with gamma globulins of either normal rabbit serum or of adult brain antiserum, or with whole normal rabbit serum were essentially normal. In one case there was some tissue disorganization in the wall of the underside of the prosencephalon (Fig. 16) and a small area of necrosis in the mesencephalon (Fig. 17).

Only one representative of an embryo with damaged extra-embryonic membranes was sectioned and examined histologically. At the inner edge of the area vitellina the ectoderm was very thick with irregular layers of cells having abnormal cellular relationships (Fig. 38). The inner margin of the ectodermal layer was indistinct and merged with a thick mass of scattered cells containing yolk material. This condition of ectoderm is usually described as ectodermal dysplasia. In addition, there were smaller plaques and streaks of ectodermal dysplasia elsewhere in the surface of the membrane.

Fluorescent localization of rabbit gamma globulins

Fluorescent material was located on the ectoderm of the embryos, especially on the head ectoderm, and of the adjacent extra-embryonic membranes (Fig. 44), in the lumen of the neural tube (Fig. 45), and in the cavities of the otic vesicles (Fig. 47). Most of the fluorescent material was distributed over the ventral floor of the lumen of the neural tube (Fig. 45) and occasionally over the dorsal and lateral walls of the neural tube (Fig. 46). Limited evidence (Fig. 48) suggests that this material was attached to the surface of the neural epithelium.

FIGURE 44

A photograph of a section through the brain of an embryo explanted at stage 9 and cultured in the presence of gamma globulins of absorbed adult brain antiserum for 32.5 hours. The sections were stained with FITC-labelled goat anti-rabbit gamma globulins. Note the fluorescence on the ectoderm of the head and adjacent extra-embryonic membrane.

FIGURE 45

A photograph of a section through the prosencephalon of an embryo explanted at stage 9 and cultured in the presence of gamma globulins of absorbed adult brain antiserum for 8.5 hours. The sections were stained with FITC-labelled goat anti-rabbit gamma globulins. Note the fluorescent material distributed over the floor of the neural tube.

FIGURE 46

A photograph of a section through the neural tube of an embryo explanted at stage 8⁻ and cultured in the presence of adult brain antiserum for 23.0 hours. The sections were stained with FITC-labelled goat anti-rabbit gamma globulins. Note that the fluorescent material is distributed over the dorsal and lateral walls of the neural tube.

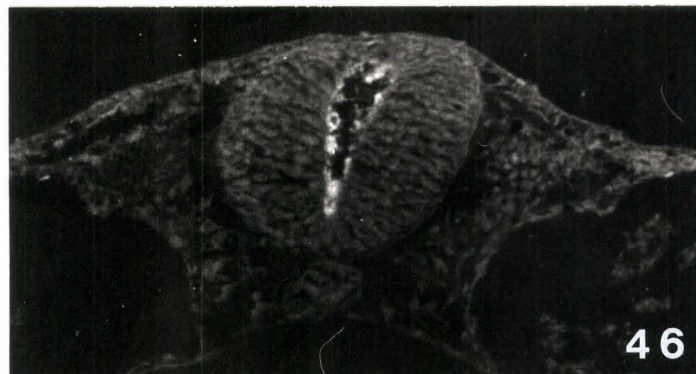
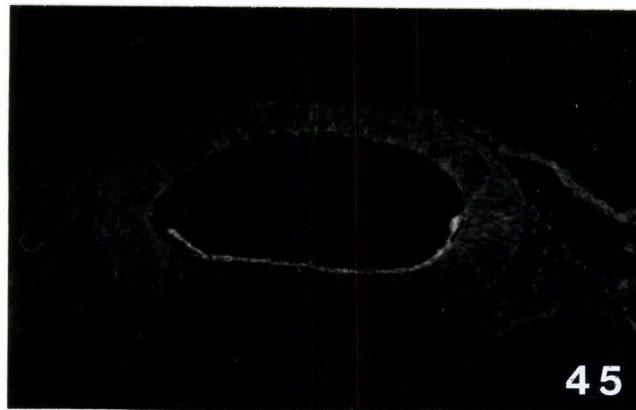
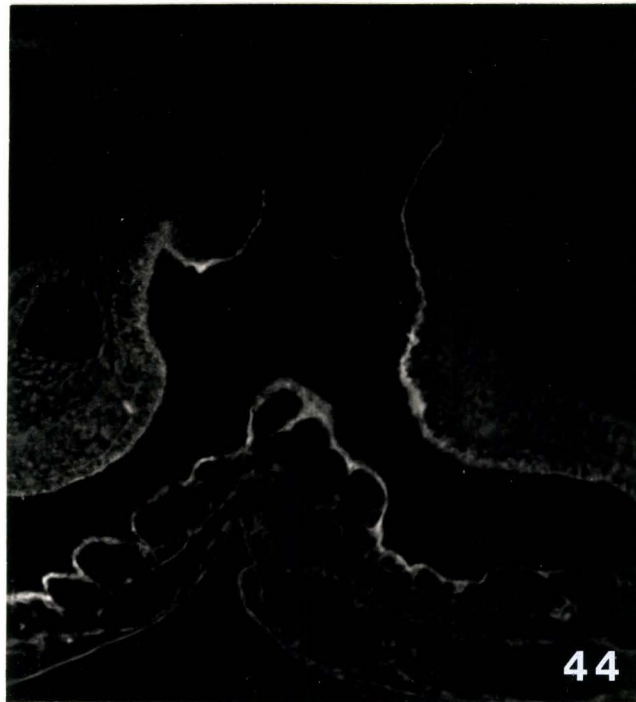


FIGURE 47

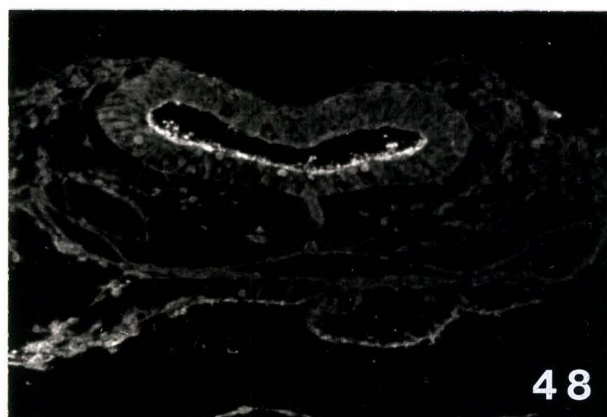
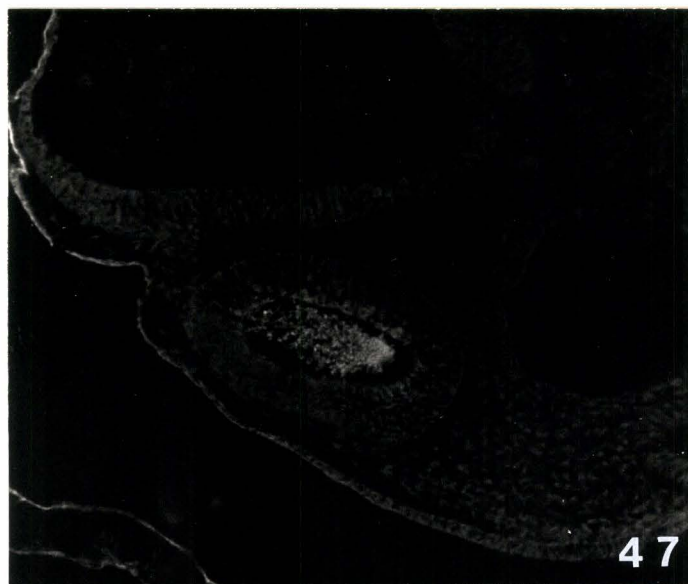
A photograph of a section through the head of an embryo explanted at stage 9 and cultured in the presence of gamma globulins of absorbed adult brain antiserum for 32.5 hours. The sections were stained with FITC-labelled goat anti-rabbit gamma globulins. Note the fluorescence in the lumen of the otic vesicle and on the head ectoderm.

FIGURE 48

A photograph of a section through the head of an embryo explanted at stage 8 and cultured in the presence of gamma globulins of absorbed adult brain antiserum for 21.0 hours. The sections were stained with FITC-labelled goat anti-rabbit gamma globulins. Note that the fluorescent material appears to be attached to the neural epithelium.

FIGURE 49

A photograph of a section through the brain of an embryo explanted at stage 6 and cultured in the presence of adult kidney antiserum for 23.0 hours. The sections were stained with FITC-labelled goat anti-rabbit gamma globulins. Note the presence of fluorescent material on the ectoderm and neural epithelium at the opening in the neural tube.



No difference in the distribution of fluorescent material was found in embryos exposed to gamma globulins of absorbed adult brain antiserum and in those exposed to adult brain antiserum or adult kidney antiserum. Embryos recovered 32.5 hours after treatment with gamma globulins of absorbed adult brain antiserum had no fluorescent material in the lumen of the brain although fluorescence was evident in the lumen of the remainder of the neural tube and in the cavities of the otic vesicles. Embryos recovered 8.5 or 21 hours after treatment had fluorescent material in the lumen of the brain as previously described, except in one case in which the embryo was at stage 9 when the antiserum was applied.

In one of the embryos treated with adult kidney antiserum with an open neural tube most of the fluorescent material appeared to be located at the edge of this opening (Fig. 49).

Saline control embryos which were sectioned and stained with the fluorescent stain, showed no detectable fluorescence.

DISCUSSION

Antisera to various adult tissue extracts have been shown to have adverse effects on the development of the chick embryo (Ebert, 1950; Johnson and Leone, 1955; Langman, 1959, 1960; Langman *et al.*, 1962; McCallion, 1970). In the present study, a comparison of the effects of antisera containing antibodies to common tissue antigens and antisera to tissue-specific antigens was made with reference to the development of embryonic antigens.

In control experiments, embryos exposed only to saline solution had a low incidence of abnormalities, less than 6.5 per cent. The incidence of abnormal and dead embryos in a control population of eggs is somewhat higher at 8 to 10 per cent (McCallion, 1971). The selection of healthy embryos for culture reduces the incidence of abnormalities in the final population. However, normal growth and differentiation will not be achieved by all cultured embryos due to handling and to the artificial environment of the culture. Previous investigators (Ebert, 1950; Johnson and Leone, 1955) employed a culture method in which the embryos were trimmed of most of the extra-embryonic membranes and placed on an agar-albumen clot (Spratt, 1947) in which antisera were incorporated. Although the method of New (1955) has not been used previously in the evaluation of antisera, it has been used successfully in developmental studies (Bellairs and New, 1962; Bellairs, 1963; Niu and Mulharker, 1970; Waheed and McCallion, 1970) and in drug teratology studies (Barron and McKenzie, 1962; O'Dell and McKenzie, 1963; Billett *et al.*,

1965). The technique imposes certain limitations on the age range of embryos studied, the length of the study period, and the volume of material that may be applied to the embryos. Embryos may be explanted as early as stage 4, definite primitive streak, and may be cultured for as long as 48 hours. However, growth rate decreases markedly after 36 hours and survival is poorer (New, 1955). For this reason, embryos were recovered after 24 to 36 hours in culture. The amount of liquid applied to the uppermost side of the embryo was critical, whether the embryos were explanted by the method of New (1955) or by the Gallera modification of this method (Nicolet and Gallera, 1963). An excess of liquid may have interfered with oxygen exchange. It is possible that culturing the embryos in an atmosphere with added CO₂ reduced the range of volumes that could be used without interfering with respiration. It was found that cultures were more successful in the humidified CO₂ incubator than in a moist chamber in a 37°C dry incubator even though CO₂ is thought to have adverse effects on embryos in culture (Klein *et al.*, 1964). In spite of its limitations, this culture method allowed the precise placement of serum preparations on embryos of known developmental stages. This precision is not possible in *in ovo* studies. Furthermore, the blastoderm expands normally over the vitelline membrane until it meets the glass ring, and therefore, the effects of antisera on the extra-embryonic membranes could also be studied. This is not possible with other culture methods.

Embryos exposed only to saline solution had a very low incidence of abnormalities of the extra-embryonic membranes. When detachment of the extra-embryonic membrane from the vitelline membrane occurred, it

probably resulted from distortion of the vitelline membrane during the preparation of the culture.

The question arises whether the factors in the antisera which cause the defects of embryos and their extra-embryonic membranes are antibodies. The toxicity of rabbit serum is well known and is due to the presence of complement and small toxic molecules both of which can be destroyed or inactivated by heating at 56°C for 30 minutes (Witebsky and Neter, 1935; Mun, 1958). Adequately heated normal rabbit serum appeared to have no adverse effects on cultured embryos when the dorsal side was exposed but did significantly increase the incidence of abnormal embryos when the ventral side was exposed. This was probably not an effect of embryotoxic factors in the serum but an effect of the volume and viscosity of the material applied. Whereas 0.1 ml of saline apparently allowed sufficient oxygen exchange, the same volume of concentrated protein possibly could have interfered with oxygen diffusion. The volume of serum applied to the dorsal side was much smaller (0.02 ml) than that applied to the ventral side. It was found that embryos in which the dorsal side was exposed to 2 to 3 times this volume of unconcentrated normal rabbit serum developed poorly, disintegrated and died. In order to increase the dose, the serum was concentrated to about 2 times the normal protein concentration for serum. The low volume (0.02 ml) of concentrated normal rabbit serum had no apparent adverse effects on embryos but slight defects at the tissue level were observed. Since embryos cultured by this method have a limited life, and since growth cessation is probably gradual, the slight tissue disorganization and necrosis in an embryo treated with normal rabbit serum may be regarded

as the beginning of this deterioration. It is reasonable to suggest that oxygen deprivation was a key limiting factor since the affected areas were neural and were located on the side of the head closest to the medium and furthest from the gaseous environment. Therefore, factors in normal rabbit serum were not responsible for the gross defects of treated embryos and probably the presence of a small volume of concentrated protein contributed very little to tissue defects. Since normal rabbit serum and the antisera differed only in that antibodies against chicken tissue antigens were present in the latter, the antibodies must have been the cause of the abnormalities.

Treatment of embryos with adult brain antiserum resulted in certain abnormalities of the central nervous system and of the trunk. The same abnormalities were also found in embryos treated with adult kidney antiserum; therefore, these defects could not have resulted from the effects of antibodies to specific neural tissue components. Both adult brain antiserum and adult kidney antiserum contain several antibodies against common tissue antigens as well as antibodies against tissue-specific antigens. Since these antisera contain a number of antibodies against the same common tissue antigens, it is reasonable to expect that the antisera would have the same effects on embryos. Furthermore, absorbed adult brain antiserum, which contained antibodies to specific neural antigens and not to common tissue antigens, had no adverse effect on embryos. This confirms the conclusion that the antibodies against common tissue antigens were responsible for the defects.

It may now be asked whether the effects of antibodies have any relationship to the presence of antigens in the chick embryo. The

presence of adult antigens in the chick embryo as early as the first day of incubation has been demonstrated in several studies (Schechtman, 1948; Ebert, 1950; McCallion and Langman, 1964). The antigens which appear first are common to all tissues. The adult brain antiserum and the adult kidney antiserum used in the present study each contained antibodies against antigens which are present in embryos and their extra-embryonic membranes from eggs incubated for 34 to 48 hours. It is possible that the mechanism by which these antibodies caused abnormal development of embryos was a direct interaction with the common tissue antigens.

The earliest time at which a neural-specific antigen can be detected by immunochemical methods is towards the end of the second day of incubation (Schalekamp, 1963). There are, however, some discrepancies in the reported times of appearance of adult antigens in chick embryos. Schalekamp (1963) and McCallion (McCallion and Langman, 1964) each independently found 3 neural-specific antigens but the times of appearance of these antigens in embryos did not coincide. Croisille (1969) has suggested that they were, in fact, describing 6 different antigens. The differences in their observations could be accounted for by differences in the quality of the antisera used for the detection of antigens. This lack of uniformity of antisera developed in different rabbits to a given tissue extract, under different courses of immunization, has been the subject of some concern (Dumonde, 1966; Clayton, 1970). More recently McCallion (1970) described 6 neural-specific antigens but these appear to coincide with only 5 of the antigens described previously by Schalekamp (1963) and McCallion

and Langman (1964). It seems possible, therefore, that again, due to differences in the quality of antisera used by these authors for the detection of antigens, there may be as many as 7 different neural-specific antigens. It is more likely, however, that one of the antigens detected by McCallion and Langman (1964) is identical to one of those detected by Schalekamp (1963). In any case, neither of these authors detected the first neural-specific antigen earlier than the end of the second day of incubation, that is prior to stage 14. It is reasonable to expect that antibodies against specific neural antigens which are not present in the embryo during the time of exposure would have no adverse effects on the development of the embryo. Thus, it seems that the failure of the neural-specific antibodies to affect the early embryo is due to the absence of the corresponding antigens at the time of treatment. McCallion (1971), using an absorbed antiserum containing at least 3 antibodies against neural-specific antigens, obtained defects restricted to nervous tissue. The embryos were treated *in ovo* at stage 8-9 and were recovered well after the stage at which the first neural-specific antigen has been demonstrated to be present. The fact that open neural tubes resulted implies that the embryo was affected prior to closure of the neural tube which begins at stage 9. Croisille (1969) has suggested that the first neural-specific antigen may appear as early as 15 hours before it is detectable by double diffusion in agar gel. He based this suggestion on the fact that the first neural-specific antigen was detected by this method about 30 hours after neural induction and that, in studies of lens and kidney, immunofluorescent techniques made possible the detection of tissue-specific

antigens at about 15 hours after induction. The first neural-specific antigen may not appear as early as 15 hours prior to stage 14 but it is reasonable to expect that the antigen does arise somewhat before the time at which it is detectable by double diffusion in agar gel. The beginning of neural tube closure does take place about 15 hours after induction and 15 hours before the presence of the antigen has been demonstrated. This may account for the effects that McCallion (1971) obtained, since exposure to the antiserum was begun at the estimated time of initiation of antigen synthesis. This explanation does not account for the absence of response of the cultured embryos to specific antisera containing antibodies against specific neural antigens and not against common tissue antigens. Although the antiserum used here contained antibodies to 4 of the 6 neural-specific antigens, the antibody against the first-appearing antigen may not have been present. It is also possible that the antibody could have been present but at a concentration too low to cause damage. The antiserum gamma globulin solution used by McCallion (1971) had a protein concentration of 20 mg/ml and the volume applied to each embryo was 0.15 ml. The solution used in the present work had a protein concentration of 45 mg/ml and the volume applied to each embryo was about 0.02 ml. Therefore, the amount of protein applied to the embryo by McCallion (1971) was about 3 times the amount applied to the embryo in the present study. Total amount of protein, however, may or may not be more important than the concentration of the protein. It may also be that the initiation of the synthesis of new proteins is delayed or inadequate in cultured embryos. Since the embryos differentiated normal structures under control conditions in

culture, it may be assumed that antigens develop normally, even if more slowly. The relationship between induction, initiation of new synthesis, and cellular differentiation requires further study.

The embryo brain antiserum caused defects of the central nervous system, somites and membranes. Adult brain antiserum and adult kidney antiserum also caused defects of the central nervous system and the extra-embryonic membranes but not of the somites. The differences in the antibodies contained in the antisera may partially account for the different effect. Double diffusion tests showed that the embryo brain antiserum contained several antibodies against embryo brain (9 days of incubation) and at least one antibody against adult kidney. In the double diffusion technique, an excess of antigen results in the formation of soluble complexes (Clausen, 1969). It is likely that kidney proteins were in excess and that this antiserum contained more than one antibody against common tissue antigens. Unfortunately, a very small amount of this embryo brain antiserum was available and it was not adequately characterized. Other studies (McCallion and Trott, 1964, 1965) have shown that antisera to nine day embryo brain contain antibodies against some adult common tissue and neural-specific antigens and against transient embryonic antigens both common tissue and tissue-specific. The existence of these antigens in the embryo during the first 2 days of incubation has been demonstrated; therefore, the relationship between the effect of the antiserum and the antigens present in the embryo is only speculative. The side of the embryo which is exposed to the antisera may also partially account for the differences in the effects of the

antisera. The types of abnormalities obtained in some drug teratology studies differed with the side of the embryo exposed to the drug (McKenzie, 1969). When applied to the dorsal side, the same embryo brain antiserum had no detectable adverse effects on the embryo. The antiserum was mixed with the albumen under the embryo preparation and may have been too dilute to have an effect. Double diffusion tests showed that the antiserum was not inactivated by the albumen. Ebert (1950) did obtain destruction of blastoderms with a dilute antiserum in a medium containing egg albumen. It is impossible to compare the quality and antibody titre of the antisera in separate studies. It has also been suggested that large molecules do not diffuse readily through the vitelline membrane (O'Dell and McKenzie, 1963; Billett *et al.*, 1965; Klein *et al.*, 1964), but with the aid of fluorescent antibody techniques it was established, in the present study, that rabbit gamma globulins from antiserum placed directly on the vitelline membrane were able to pass through the vitelline membrane to the embryo. The possibility remains that antisera may not be available to the embryo when it is in the albumen under the dorsal side of the embryo.

An examination of the types of abnormalities caused by the antisera may shed some light on the mechanism of action of the antisera. Antisera in general have growth retarding effects on embryos (Ebert, 1950; Johnson and Leone, 1955). It appears that antisera affected the most rapidly growing parts of the embryos, the extra-embryonic membrane, the caudal proliferation centre, and the neural tube but had little effect on other tissues. All tissues probably contained the common tissue antigens against which these antibodies were directed, but the

inactivation of a functional antigen would have been more critical in a rapidly growing tissue.

Adult brain antiserum, adult kidney antiserum, and embryo brain antiserum all had some effect on the extra-embryonic membranes. Attachment of the blastoderm to the vitelline membrane is achieved by the amoeboid cells at the advancing margin. It is conceivable that an antigen-antibody reaction which inactivated molecules at the surface of these cells could cause the cells to lose their attachment. This could account for the effects of adult brain antiserum and embryo brain antiserum on the extra-embryonic membranes. Adult kidney antiserum and embryo brain antiserum caused a membrane abnormality which did not involve detachment from the vitelline membrane. The extra-embryonic membranes failed to expand normally. Expansion is a combined result of the multiplication of cells in the zone of junction of the blastoderm and of the amoeboid movements of the cells at the margin. An antigen-antibody reaction which interfered with the amoeboid movement without interfering with the attachment is conceivable. It is also possible that the antibodies affected growth at the zone of junction by interfering with functional common tissue antigens. The abnormality of the membrane ectoderm, revealed by histological examination, may be a direct or an indirect effect of the antibodies. A cell surface interaction of antibodies and antigens could have destroyed the organization of this cell layer. New (1959) reported that ectodermal cells of the membrane fail to arrange in flat sheets unless the moving edge creates tension; therefore, the abnormal ectoderm is more likely an indirect result of the effect of

antibodies on the outward moving edge of the membrane.

Of the two lots of adult brain antiserum, only one affected the extra-embryonic membranes. There was no apparent difference between the two antisera as shown by double diffusion in agar. However, the comparison of two different Ouchterlony plates is not very reliable (Clausen, 1969). Furthermore, antisera produced by a single rabbit to the same tissue extract contain different numbers and titres of antibodies at different times (Dumonde, 1966).

Adult kidney antiserum and adult brain antiserum both contain antibodies against the same antigens found in extra-embryonic membranes at 34 and 48 hours of incubation. Adult kidney antiserum contained one additional antibody against these membrane antigens which was not found in adult brain antiserum. This fact probably accounts for the differences in the effects of these two antisera on the membranes. Ebert (1950) reported cell clumping in the membranes with an antiserum concentration which destroyed the embryo. Since his explant technique required the removal of the blastoderm peripheral to the area pellucida, he would not have been able to observe an effect on the margin of the membrane. It has been demonstrated in pregnant mice and rats that the antibodies of adult kidney antiserum become fixed in the yolk sac endoderm of the embryo (Slotnik and Brent, 1966; Gebhardt *et al.*, 1970).

The short trunks resulting from the treatment of embryos with adult brain antiserum and adult kidney antiserum probably were caused by an effect of the antibodies on the caudal proliferation centre. This centre is responsible for the longitudinal growth of the embryo and is

located just anterior to Hensen's node (Seichert and Jelinek, 1968). According to these authors, maximal growth occurs at the transitional zone of the differentiated neural tube into the end-bud blastema. In all cases examined histologically, the growth centre was necrotic. Although the mechanism by which antibodies cause cell death is obscure, it may be assumed that they inactivate antigens which are somehow essential to the integrity of the cell. Short trunks have also been produced by exposing the early chick embryo to other teratogenic agents, for example 6-azauridine (Klika *et al.*, 1969), actinomycin D (Klein, 1964), amino acid analogues (Rothfels, 1954). Thus, it seems that any agent which interferes directly or indirectly with protein metabolism affects the caudal proliferation centre of the chick embryo. The similarities in effects suggest that antibodies have their effects on protein synthesis. Caudal abnormalities were also produced by the treatment of embryos with antibodies to specific neural antigens (McCallion, 1971). He failed to mention this effect although it is quite evident from his photographs. The indication is, however, that the defect is primarily of the posterior neural tube and only secondarily of the caudal growth centre.

Neural tube closure is very sensitive to many teratogenic agents including drugs such as insulin (Barron and McKenzie, 1962) and antisera (Licata *et al.*, 1962; McCallion, 1971). It is possible that the antibodies react with antigens at the cell surfaces of the neural epithelium preventing fusion of the appropriate cell layers. Fluorescent localization of rabbit gamma globulins from adult kidney serum in an embryo

which developed with an open neural tube, indicates that the antibodies are probably attached to or lying on the surfaces which would have been involved in fusion. The mechanism of neural tube closure is very complex and involves growth and morphogenetic movements as well as fusion of like layers. Since cell death, as evidence the large areas of necrosis of neural tissue, was also an effect of the antibodies, growth and morphogenesis probably were affected. Overgrowth of neural tissue occurred in some embryos with open neural tubes and was also described by McCallion (1971) in embryos with open neural tubes. This overgrowth is probably not a direct effect of the antibodies but a compensatory proliferation of the tissue as a reaction to excessive cell death.

In some cases, embryos exposed to antisera developed enlarged brain ventricles. This defect cannot be attributed to compensatory overgrowth since no damage to the neural epithelium was evident and since the neural epithelium appeared to be of normal thickness. Normal flexion results from the differential growth rates of different parts of the neural tube. For example, the roof of the mesencephalon grows more rapidly than the floor. Since flexion in treated embryos with enlarged brain ventricles was abnormal, the antibodies had an effect on differential growth of neural tissue although the cells were not killed and the ependyma cells were in active mitosis. The general observations in the present study indicate that the more rapidly growing parts of the embryo are more affected by the antibodies. It has been suggested previously that antibodies may have a sublethal growth inhibiting effect upon embryonic tissues (Langman *et al.*, 1962).

Treatment of embryos with embryo brain antiserum resulted in several cases of somite block, that is, the somites fail to segment from the mesoderm. A similar defect has been reported in embryos treated with antimetabolites (Rothfels, 1954). Any treatment which interferes with protein metabolism may cause somite block. The fact that the other antisera did not have this effect presents a problem since it was also suggested that the antibodies in these antisera interfere with protein metabolism. However, the other antisera were not placed on the ventral side but on the dorsal side of the embryo. The organ or tissue affected by an agent relies to some extent on the side of the embryo to which the agent is applied (McKenzie, 1969). The embryo brain antiserum probably contains antibodies which are not present in adult tissue antisera, and against embryonic antigens which may have a peculiar role somite development.

Frequently, antigen-antibody reactions have been proposed as the mechanism whereby antibodies affect the embryos but this reaction has never been demonstrated in tissues. It is quite possible that antigens are no longer functional when they are bound to antibodies. There is some evidence which suggests that an antigen-antibody reaction is possible in the embryo. The fluorescent antibody study indicated that when antisera were placed on the dorsal side of an embryo cultured by Gallera's method (dorsal side uppermost), the antisera passed through the vitelline membrane and came to lie in the neural tube and on the ectoderm of the embryo and of its extra-embryonic membrane. The neural tube closed and the antiserum remained inside the neural tube for up to 32

hours after application of the antiserum. Therefore, antisera applied by this method is available to the embryo for a considerable period of time. There is no evidence that antibodies were inside the cells or tissues of the embryo, but this may have been due to the limitations of the technique. The antibodies may not have been available to all the tissues of the embryo. Fluorescent antibody studies also gave some evidence that antibodies become attached to the cell surfaces of the neural epithelium.

If an antigen-antibody reaction is responsible for the adverse effects which tissue antisera have on embryonic development, then the antigen must be present in the embryo. Ebert (1950) obtained more or less specific results with adult spleen antiserum, adult heart antiserum, and adult brain antiserum. Since these antisera contained antibodies to common tissue antigens, as well as tissue-specific antigens, the specific results must be explained. The presence of common tissue antigens, a heart-specific antigen and possibly a neural-specific antigen are now recognized in embryos of the ages covered by his experiments. It is possible that dilution of the antiserum would leave only tissue-specific antibodies in effective concentrations, or that he was using antisera containing antibodies against quantitatively specific antigens. Only two investigators have shown that antisera containing antibodies against only tissue-specific antigens affect only those tissues which contain the antigens. Langman and co-workers (1962) have shown that embryo brain and eye tissues which contain alpha-crystallin are destroyed by alpha-crystallin antiserum and that tissues lacking this antigen are not affected. McCallion (1971) showed that specific neural

defects occurred in embryos treated with antibodies against only neural-specific antigens. The presence of at least one neural-specific antigen in embryos at the stages studied has been demonstrated (Schalekamp, 1963). In the present study, antisera which affected the embryo and the extra-embryonic membranes were shown to contain antibodies against antigens actually present in the embryos and in the extra-embryonic membranes during the time of exposure. The only antiserum that had no adverse effects on embryos was the one which contained only antibodies against antigens not demonstrated to be present in the embryo at that time.

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