EMBRYOLOGY

ANALYSIS OF THE DEVELOPMENT OF THE EYE-LENS IN CHICKEN AND FROG EMBRYOS BY MEANS OF THE PRECIPITIN REACTION

BY

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Introduction.

The developmental physiology of the eye-lens appears to be a very suitable subject for a biochemical analysis of some aspects of the general problem of organ-differentiation, because this part of the eye has a relative simple structure and a remarkable chemical specificity.

This organ-specificity, probably due to its protein components, has been detected by UHLENHUTH (1903) by means of the precipitin reaction, and several authors since then have substantiated his result (e.g. KRAUS, c.s., 1908, KRUSIUS 1910, WOLLMAN, a.o. 1938 *a*, *b*.). The precipitin test enables us to differentiate simple aqueous extracts of the lens from similar preparations from any other organ of the body, even other parts of the eye itself. Moreover, especially when carried out on a micro scale, this test is an extremely sensitive reaction, so that a hundredth of a microgram of protein substance can be detected. Eventually it seems to become possible to combine this test with the biochemical technique of isolating and concentrating lens proteins, in order to find the slightest traces of these substances in very young embryos.

This possibility opens perspectives for an analysis of lens induction and regeneration but, although these may be very attractive objects to direct the course of investigations, biochemical work on lens proteins in our laboratory has only been started this year and results cannot be awaited within short time.

Meanwhile, applying the precipitin reaction on simple extracts, we carried out an investigation about the presence of adult lens antigens in embryonic lens vesicles before the beginning of morphological differentiation. The reason for this study has been the question, discussed among embryologists, whether the substances, constituting the adult organism, are present already in the youngest stages of ontogenetic development or whether these materials originate only in the course of the differentiation of the embryo.

The old controversy between preformationists and epigeneticists seems to be continued on this chemical level.

Recognizing the important role of the genes, the preformationist standpoint supposes that, at least qualitatively, all of the building substances of the adult organism will be present at the onset of embryonic development, either bound in the genes of the nucleus, in plasmagenes, or free in the cytoplasm. Perhaps the great mass of yolk may conceal the minute amounts of these specialised substances, so that only in the course of development will they become detectable because of their increasing quantity by assimilation of the food from yolk; nevertheless these materials will be present already at the beginning.

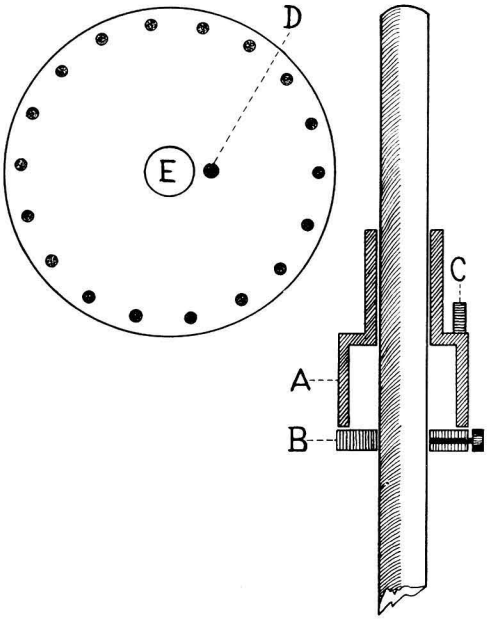

Fig. 1

The epigenetic view supposes that, may it be under the influence of the genes, most of the adult materials will become newly created qualitatively in different stages of development. Perhaps under the impression of the developing complicated form out of a simple egg, many embryologists adhere to the epigenetic viewpoint, which in a biochemical sense has been expressed by NEEDHAM (1942) in the following words: "Antigens come into being at successive points in time" (l.c., p. 349). Such opinion, however, based upon negative evidence, has to be changed as soon as positive experimental results are favoring an opposite view. A short survey of the literature concerning the antigenetic properties of the embryonic organism will demonstrate this.

In 1906, BRAUS arrived to the conclusion that embryos are devoid of any antigenetic action. This was a result of experiments in which he

tried to immunize rabbits by three intraperitoneal injections of a small quantity of an extract, made from Bombinator larvae.

In the same decade, however, other investigators were able to immunise rabbits by eggs of fishes and amphibia; they found a difference between embryos and adult tissues (DUNBAR 1910, UHLENHUTH and HAENDEL 1910). Also KRITCHEVSKI (1914) showed that amphibian larvae have antigenetic capacities, using larvae of Rana esculenta. For his conclusion that, corresponding with HAECKEL's biogenetic law, there is a difference between the chemical structure of embryos and that of adult frogs, the results of the complement fixation tests were not giving very strong evidence; in two out of seven tests the larval antigen reacted with the heterologous (adult) antiserum almost as strongly as with the homologous one and with heterologous antiserum a very great number of plus minus reactions was noted (in 33 out of 40 dilutions).

In 1923, KRITCHEVSKI studied the same problem in chick embryos and from his results inferred the following statement: "The facts presented in this article suggest the conclusion that the biochemical properties of animal cells are subject to transformations during the ontogenetic development" (l.c. p. 194). The author presented the following facts: he had been able to demonstrate the existence of FORSSMAN antigen in embryos of four days and older by the results of immunisation experiments in rabbits, whereas he failed to obtain the same result, using two days old embryos and unincubated eggs. Having indicated that this antigen is contained in the cell nucleus (1916), KRITCHEVSKI also stated that the chemical structure of the nucleus changes during ontogenesis, an opinion that probably will be refuted by most embryologists.

IWAE (1915), using the same method as KRITCHEVSKI, however had demonstrated the presence of the FORSSMAN antigen in yolk of chicken eggs and IDZUMI (1924), GUGGENHEIM (1929), WITEBSKY and SZEPSENWOL (1934) confirmed this. The antigen has also been detected in one day old chick embryos by the last authors, who found its concentration in young embryos to be about the same as in adult tissues.

IDZUMI (1924), injecting the whole content of chicken eggs in the peritoneal cavity of rabbits for immunisation, found an increase in quantity of both FORSSMAN and serum antigen during the incubation period and a decrease in that of the egg-white, determined after the titer of the antisera he could obtain. Although it may be very comprehensible that these quantitative changes are occurring during development, the results do not indicate any alteration in quality. The method of determining the amount of an antigen by the effect of immunisation is not very quantitative, because great differences exist in reaction of the injected rabbits. Therefore, exceptions in the rather regular quantitative changes, noted by IDZUMI, might be expected; so the serum antigenicity in chickens, four days after hatching, was found to be six times weaker than in unincubated eggs.

The results of ABE (1931) led this author to believe that the species specificity of organ lipoids is stronger in human adults than in fetuses and new-borns, but this belief is based upon rather subtile and only quantitative differences in the results of complement fixation tests.

PERLMAN and GUSTAFSON (1948) demonstrated the presence of certain antigens in plutei of Paracentrotus lividus and found them missing in young embryos, from which result the authors concluded that these antigens "are not present at all, or perhaps in only undetectable quantities" in the younger stages. Certainly this careful statement is likely to express the true situation; it corresponds with the opinion, expressed by COOPER (1948): "the failure so far to demonstrate the presence of some complex molecules in early embryos does not necessarily indicate that they are all absent" (l.c., p. 430).

The article of AVRECH and HERONIMUS (1937) has not been available. Results of recent biochemical investigations about the blood proteins are indicating that some differences do exist between embryonic proteins and those of adults. It has been found that the amino-acid composition of the fetal hemoglobin in mammals differs from that of the adult type, but still the possibility remains that both types of the globin molecules may be present in both life periods but that one type is predominating quantitatively.

The different composition of the blood serum too may be caused by differences in quantity of the constituent proteins. Even the special embryonic protein fetuin, discovered by PEDERSEN in fetuses of the cow perhaps may be present in minor quantities in adults. However, it is questionable if such functionally inactive proteins will continue their existence in the cytoplasm, but perhaps they remain preserved in the genome.

Besides these differences, some similarity between the embryonic and adult organism has been noted, e.g. by RÖSSLE (1905) and WILKOEWITZ a.o. (1928), who could not find any seizable difference in antigenicity, but like many authors in their time, both used a rather obsolete technique, causing denaturation of the antigens, so that their results cannot be useful in the present discussion.

Furthermore, evidence has been given that in the yolk several substances, found in the blood of the mothers may be present, e.g. serum proteins and antigens. (SCHECHTMAN 1947, 1948 and COOPER 1946, 1948). The article of COOPER (1948) contains an extensive review of literature on this subject to which may be referred.

Finally, the presence of many non-antigenetic enzymes in the unfer tilized egg may be taken as an evidence of similarity.

The problem about the presence of more specialised materials, building up the organs, is more interesting however, and has been attracting our attention in connection with the development of the eye-lens. There is one report already, given by BURKE, and collaborators (1944), de-

scribing the presence of organ specific lens substances in chick and frog embryos. In embryos of the chicken, however, younger than 146 hours, the adult antigens could not be found by means of the complement fixation test, and using the precipitin reaction only embryos of 250 hours of age and older reacted positively.

Although they used these reactions "... to determine the age at which adult lens antigen appears in sufficient amount to be detected by these methods" (l.c., p. 229), the authors, in accordance with the epigenetic theory of changing chemical structure during morphological development, arrive at the conclusion that: "Adult organ specificity does not arise in the chick and frog until the organs are well differentiated morphologically" (l.c., p. 232). This conclusion is very interesting because it may be related to the problem of the organ-forming substances.

According to a theory which has been accepted by many embryologists, morphological differentiation is preceded by a chemo-differentiation (HUXLEY, 1924), by which the fate of organ-forming regions is determined. There is a common opinion that the determination of the organ-development is due to the presence of certain organ-forming or perhaps organ-determining substances. It has, however, not yet been possible to demonstrate irrefutably the existence of such an organ-determining or organ-forming substance. Histochemical evidence for the action of certain enzymes in this respect could not become substantiated by the results of microchemical investigations, and the enzymes which have been indicated by the histochemical pictures can be found in several organs.

Now there seems to exist the possibility that the organ specific substances may be responsible for the determination of organ-differentiation and especially in the development of the eye-lens it might be feasible to try and analyse this problem of the relation between organ-specific, organ-forming and organ-determining substances.

Two questions may be asked:

First, are organ specific substances present before morphological differentiation or do they arise only after or during this differentiation? And second:

If the organ specific substances might be detected before the stage of morphological differentiation, may they be indicated as organ-forming (organ-building) substances or do they determine the fate of the presumptive lens-region in still younger stages where no indication of a beginning lens-formation can be found?

The former question has already been answered by BURKE c.s., who only found the specific adult lens antigen present after morphological differentiation, but, using a very careful technique and isolating a greater number of young lens vesicles by means of a micro surgical method we made an attempt to improve the sensitivity of the reaction. Indeed this has been successful, so that by demonstrating the presence

of adult antigens in the embryonic lens before its morphological differentiation, our answer to the first question has become contrary to that, given by BURKE c.s.

Methods.

1. *Immunisation.*

The adult antigen was prepared from fresh lenses, carefully dissected out and weighed. Then the material was thoroughly ground in a mortar and a suspension prepared, adding nine volumes of saline ($p_H = 7$). After centrifuging at 4000 r.p.m., the supernatant opalescent solution was used for immunisation. Strong rabbits, about three kilograms of body weight, were immunised by seven to eight injections of 2 to 3 ml. of the 10 % antigen solution in the marginal vein of the ear, once in two or three days. Beginning with the fourth injection the rabbits received a subcutaneous injection of the same dose one hour before the intravenous injection in order to prevent shock. (In the last year this injection was given on the night before). One week after the last injection the antiserum was tested and if it had a sufficient high titer (1 : 20000 to 1 : 50000), the animal was bled to death from the carotid artery or the blood was obtained by puncture of the heart. After clotting, the serum was removed, sterilized by filtration through a Seitz filter, distributed over a series of ampullae and stored in the refrigerator.

2. *Serological test.*

In 1948 we used the complement fixation test, as it is a most sensitive reaction. The results, however, were unsatisfactory for several reasons. First, specificity was low, probably caused by the presence of non-specific lipoids in the eye-lens; KRAUSE (1935) estimated the lipoid content to be 1 % of dry weight. We tried to improve specificity by absorbing the antisera with other antigens or by eliminating the lipoids with ether in the cold, but these methods reduced the strength of the sera too much. Furthermore, especially in extracts from whole parts of frog larvae, a sensitizing action was met with, so that more embryonic material had to be used for control titrations of complement, and finally these extracts sometimes caused a spontaneous hemolysis.

Therefore, several experiments were made for comparing the specificity of the complement fixation test with that of the precipitin reaction. The results of these experiments demonstrated a very great difference between the two reactions, the precipitin test being almost completely specific. This phenomenon perhaps may be explained by the possibility that the precipitin test in our experiments reacted with the proteins more than with lipoids or lipoid complexes (cf. WITEBSKY, 1928). In our definitive experiments we thus made use of the precipitin reaction.

Some details of the micro-technique we have been using may be described here:

a. BOYD's micro-method.

A series of antigen dilutions was prepared in micro test-tubes (25 × 2.5 mm). Each tube received 20 microliters of antigen solution and then an equal amount of antiserum was layered carefully beneath the antigen. After a 20 minutes period of incubation at 37° C. the presence of a ring at the interface between the two layers could be noted in case of a positive reaction. The result was expressed by recording the last positive dilution. This maximum dilution could be taken as an index for the strength of the antiserum as well as for estimating the amount of antigen present in a solution of unknown concentration.

It must be remarked that in mixtures of several antigens it is rather difficult to find the exact concentration of one antigen because one protein may change the reactivity of another, so that, if the concentration of the separate components is not known, any quantitative determination will be impossible. This could be demonstrated by the reaction between antisera prepared to a complete extract of the lens and different lens proteins. The α-cristallin fraction reacts more intensively than the other components and after mixing this fraction with the β-cristallin the reactivity of the α-fraction is decreased. This phenomenon must be taken into account if a comparison has to be made between antigens from different species or different stages of embryonic development, because the relative concentrations of the fractions may be different in different objects.

For filling the micro test-tubes with antigen and serum and for the preparation of dilutions, microburettes are used, calibrated in microliters, in which the fluid can be moved by air-pressure changes. For this purpose the microburette, by means of a narrow and thick-walled rubber tube, is connected with a small reservoir, made from a piece of rubber tubing (internal diameter about 10 mm). This reservoir and a great part of the narrow tube are filled with water, so that between the water and the fluid in the burette only a small air volume remains. By changing the volume of the reservoir with a screw-clamp the burette can be filled or emptied (fig. 2).

The tubes are placed in a circular rack (fig. 1) which fits on a stand (hole *E*) and rests upon a ring (*A*); this ring is freely movable along the rod and is resting itself on a second ring (*B*). The hole (*D*) in the bottom plate of the rack is fitted for a pin (*C*) on ring *A*. By taking ring *A* between thumb and fingers the rack may be turned and lifted and so each of the tubes can be brought around the tip of the microburette. For filling the burette, eventually one or two of the holes, designed for the test-tubes may be widened in order to contain a larger tube, supplying the saline or serum.

This design (fig. 2) has been found to be very practicable for more rapid work. The rack was placed in the incubator, after the incubating period the tubes taken out and the result read against a dark back ground.

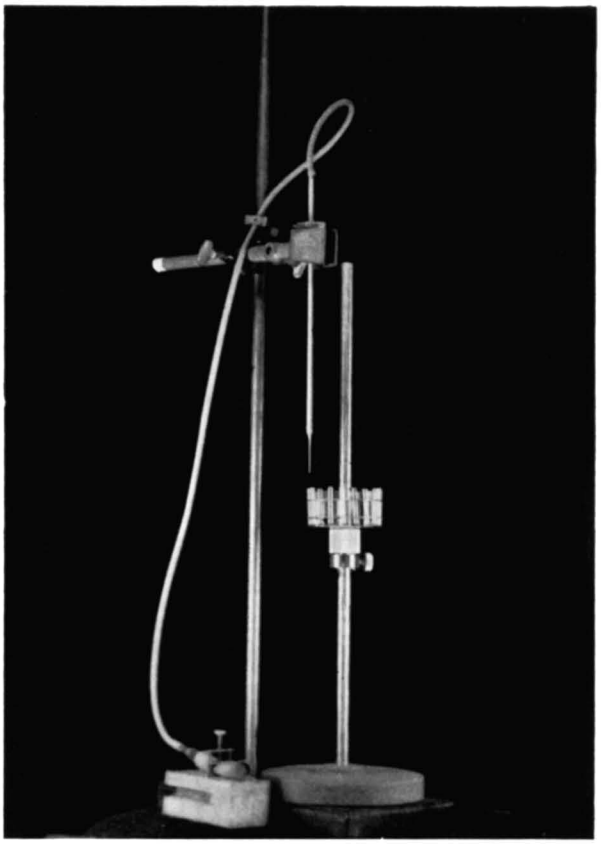

Fig. 2

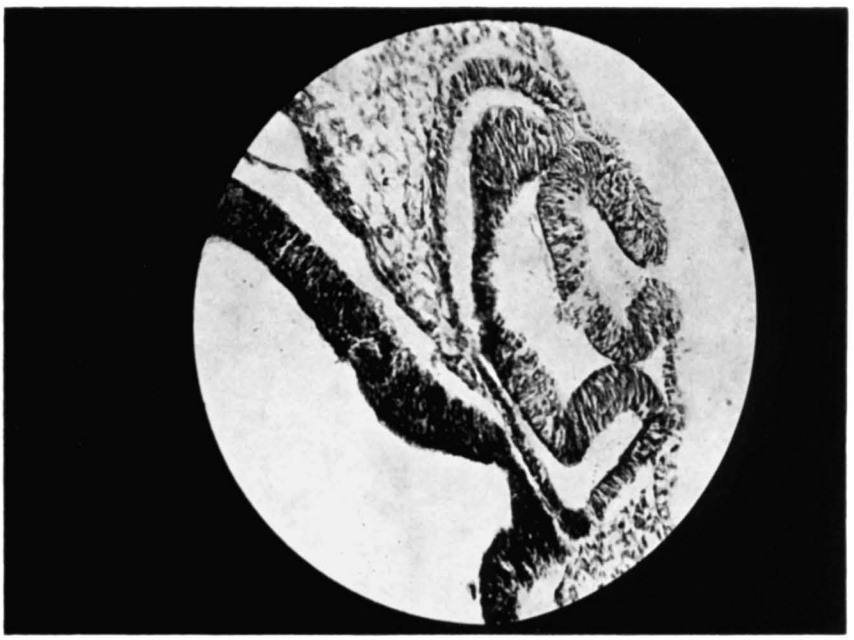

Fig. 3

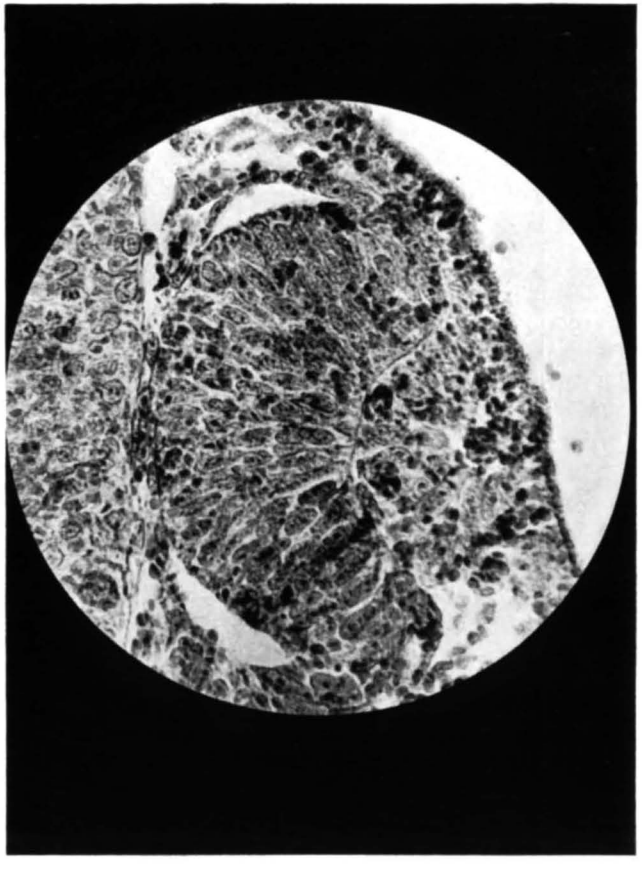

Fig. 4

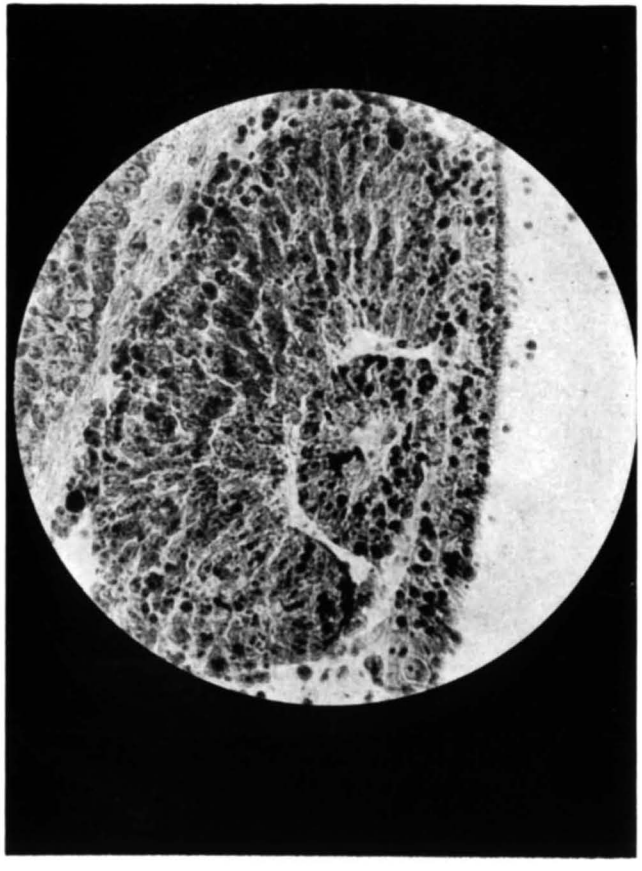

Fig. 5

b. Capillary method.

In experiments where only small amounts of antigen are present, it is useless to prepare a series of dilutions, and the initial concentration has to be taken as high as possible. Therefore, a method was developed, using narrow, thin-walled glass capillaries, so that for each test only one microliter was needed. From the extract this quantity was sucked up in the narrow capillary tube and then about the same volume of antiserum (or control fluid) followed. Both were blown out on a glass slide, mixed by stirring with the tube and the mixture again sucked up into the capillary. By repeating this a suitable mixing could be achieved and finally the mixture was brought in the middle of the tube, the ends of which were sealed by melting the glass in a micro flame. The tubes were put with their lower ends into plasticene and incubated during one hour, whereafter they were left standing over night at room temperature. Next day the result was read under a dissection microscope in dark-field illumination. Of course there were several control tubes, corresponding with the ordinary method.

The mixing of the reacting fluids is known to decrease the sensitivity as compared with the ring test. We found, by comparing both methods, the mean difference, expressed by the quotient of the last positive dilutions, to be about 5.

The increase in sensitivity by using the capillary method thus became about fourfold, using one microliter in stead of twenty in the ring test. (Of course, in both methods still smaller volumes may be used).

In some experiments only, a third method was used. The antigen extract was distributed over three watch-glasses and then frozen in dry ice and dried. For the reaction the antigen was dissolved directly in the antiserum or control fluid and with use of the capillary method sensitivity again was increased.

3. *Preparation of antigen from embryos.*

The young lens-vesicle was cut out from the embryo by means of the ordinary glass-needle technique. In this way as little yolk or other materials as possible have been taken together with the lens antigen itself.

For focussing the microscope during dissection we used a modification of the device, published by La Rue (1932).

To the focussing wheel a lever of variable length was attached which could be moved by means of a flexible cable (choke cable from an automobile). In stead of moving the pedal, to which this cable was connected, up and down with the foot, the displacement of the cable was obtained by moving the foot in a horizontal direction. The cable was attached at the fore-end of the pedal and the axis of motion placed near to the heel, so that by rotating the leg in the knee the microscope could be focussed. This modification of the original design, invented by our instrument maker de Vries, has become very useful, being less

tiresome because in every position the foot is resting on the floor

The material was assembled on the top of a small cylindrical glass pestle, cooled in ice. The extract was prepared by grinding the material in a small glass-mortar, made from a thick-walled glass capillary (pyrex) of 2 mm internal diameter, about 25 mm long, and widened at the upper end. After grinding and adding a few microliters of saline the "mortar" was centrifuged and the supernatant fluid used.

Results.

A. Control experiments.

1. *Comparative experiments with* BOYD*'s technique and capillary technique.*

The well-known fact that the precipitin reaction in a mixture is somewhat less sensitive than the ring test was also demonstrated by the results of our experiments (table 1). From each of the tubes, in which

TABLE 1

Relation (Q) between last dilution of antigen, reacting positive in Boyd's test (B) and in the capillary method (C)

	B	C	Q
Chicken lens (embryo) + frog lens antiserum	1600	400	4
Chicken lens (adult) + chicken lens antiserum	25600	3200	8
Frog lens + frog lens antiserum	51200	12800	4
Frog lens + frog lens antiserum	25600	12800	2
Frog lens + frog lens antiserum ($p_H = 9$) . .	6400	3200	2
			Mean $Q = 5$

an antigen dilution had been prepared for the ring test, one microliter had been taken out into a capillary tube, mixed with the same volume of antiserum and the results of both tests compared.

The difference in sensitivity has been expressed in the table by the quotient between the factors of the last positive dilutions. It may be concluded that the ring test is about five times more sensitive than the capillary test.

2. *Organ specificity of the antiserum.*

In table 2 the results have been given of precipitation reactions of lens-antiserum and extracts of other organs.

The number of experiments and of the negative and positive reactions as well as the last dilution index in case of positive tests has been presented. One of the antisera had to be rejected because of its weak specificity, but in all other sera this quality has been satisfactory, as may be seen from the facts in the table. Only the vitreous body has been reacting positively in three out of six experiments. Probably this can be explained by the possible presence of small parts of the lens, remaining adhered to the vitreous after this had been dissected out.

TABLE 2

Reactions between eye-lens antiserum and 5 % extracts (in saline) from other organs

Organ (part)	adult frog		adult chicken		chick embryo	
Results:	neg.	pos.	neg.	pos.	neg.	pos.
muscle (leg of embryo) . .	3	—	1	—	15	—
liver	1	—	1	—	3	—
skin	5	—	3	—	4	—
brain	3	—	3	—	4	—
retina	5	—	1	—	4	—
iris	4	—	1	—		
vitreous corpse	—	1 (160)	1	1 (80)	3	1 (40)
eye cup (complete)					12	—

(The number of experiments has been given. In case of a positive reaction the last positive dilution has been indicated in parentheses).

3. *Species specificity.*

By combining several portions of one antigen solution with different antisera and by combinations between one antiserum and different antigens an impression could be obtained about this quality. The results

TABLE 3

Species specificity

a. Reactions between one solution of antigen, combined with homologous and heterologous antisera.

Antigen from lens of:	Chicken lens antiserum	Frog lens antiserum
adult chicken	51200	25600
chick embryo (15 days)	25600	3200
chick embryo (9 days)	1600	800
adult frog	12800	51200

(indication of last positive dilution)

b. Reactions between one (adult frog lens) antiserum and lens antigens from different species.

antiserum no. 2		antiserum no. 7	
antigen	last dilution	antigen	last dilution
frog	32000	frog	16000
chicken	16000	chicken	8000

of the first method are presented in table 3*a* and those of the second in table 3*b*, the last positive dilution having been indicated. Certainly a small difference is present, caused by species specificity, although it is possible that in the second combination the exact concentration of the active antigen is varying with the species. Such a variation also may be present in the first experiments, if the antisera are gained by injections of quantitatively differently constituted antigens, but the effect may be less intense.

Obviously the lens antigen only is weakly species specific and strongly organ specific.

B. The presence of adult lens antigen in embryos.

1. *Chick embryos.*

By means of the BOYD technique the presence of adult lens antigen in embryos 74 hours of age and older clearly could be demonstrated. Probably this result was due to the careful dissection and assembling of the lens material.

TABLE 4

Adult lens antigen in chick embryos

Age of embryo (hours)	Number of expts.	Number of lenses	Last. dil. BOYD's method.
192	1		6400
168	1		3200
144	1		3200
120	1		1600
96	1		800
84	1		400
74	1		400
72	1	11	+
66	1	40	+
60	1	40	—
capillary method:			
60	3	40	+ ; + ; ++.
58	1	60	+
54	3	50; 30; 30.	± ; ± ; —.
51	1	50	—
48	1	50	—

In a 72-hour embryo the reaction still was positive when 11 lens vesicles were dissolved in 10 microliters of saline, and in the 66 hours stage a positive result could be got by preparing an extract from 40 lens vesicles in 20 microliters saline. In the 60-hour embryo this method failed, however, and henceforth the capillary method was used, extracting the material, assembled from 20 to 30 embryos with about 4 microliters of saline.

By this procedure positive results were obtained in embryos of 60 and 58 hours (four experiments) and weakly positive reactions in material from 54-hour embryos. In 51- and 48-hour embryos only negative results could be noted.

Taking into account the possible individual variations, we thus safely may draw the conclusion that the 60-hour embryo already possesses the adult antigen (or one of the lens antigens) and in a sufficient amount to be detectable by this method.

The change in quantity of the antigen is apparent from the fact that in younger stages the detection of it is becoming increasingly difficult. Also the results of our quantitative tests in older embryos demonstrate this phenomenon. The series of decreasing values of the last positive dilutions from 1/6400 in 8-day embryos to approximatively 1/400 in 3-day embryos makes this clear.

In nearly all experiments the results have been checked by comparing the lens material with extracts from other parts of the same embryos. In all these controls the reaction gave negative results, even in extracts, prepared from eye-cups. Other control experiments, using normal serum, muscle antiserum as well as saline have been negative.

Because the time, required for operating so great a number of embryos became rather long, extending to about 6 hours, it was possible that, if the embryos were stored in this period at room temperature, they were changing too much. We, therefore, carried out some experiments in which the total number of eggs was divided into portions of ten and these were incubated one after another with intervals of one hour. So, during the dissection period, at each hour a portion of ten embryos became available.

The individual variation in rate of development remained, but the few exceptional cases were excluded from the experiments. Ordinarily, about 30 embryos out of 50 incubated eggs could be used, because of mortality or of low fertilisation percentage.

The results of these experiments in chick embryos have been presented in table 4.

2. *Frog embryos.*

In these experiments, material from embryos of Rana esculenta has been investigated. In most cases the embryos were reared before the experiment at 12° C. for retarding the development. There was no opportunity to compare these results with the properties of embryos, reared at higher temperatures.

In the eldest stages the antigen material was assembled from about 50 to 100 embryos, in the younger stages some 200 embryos have been used for each experiment.

The results have been reported in table 5 in which also the control experiments have been presented, because of the small number of these experiments.

In stage 18—19 (according to SHUMWAY, 1940, 1942) one experiment was a failure, because of interference by a vast amount of yolk, resulting in a cloudiness in the extract that could not be removed by centrifuging at 4000 r.p.m. during a long period. In stage 19 two experiments gave negative results, whereas at the same stage a positive reaction could be obtained by drying the extract and dissolving the antigen directly in the sera, as has been described.

In stage 19—20 the result was positive, but no control experiments could be done.

From these facts it may be concluded that the appearance of the first positive reaction has been found at about stage 19 to 20.

TABLE 5

Adult lens antigen in frog embryos

Stage (SHUMWAY)	Antigen	Lens antiserum	Muscle antiserum	Normal serum
25	lens + eye-cup	+		
23	lens + eye-cup	+	—	
23	rest of embryo	—	—	
21—22	lens + eye-cup	+	—	
21—22	ventral epidermis . . .	—	± (adhering mesoderm?)	
19—20	lens + eye-cup	+		
19	lens	—		
19	lens	—		
	Antigen material dried and dissolved in the sera:			
19	lens	+	—	—
17—18	lensectoderm	—	—	—

3. *Morphology.*

According to the normal tables of development of the *chick embryo* (KEIBEL a.o. 1900) the formation of the lens-plate and its invagination takes place at the age of 42 to 50 hours. The separation of the lens vesicle from the epidermis can be found at the 60 to 63 hours' stage and thickening of its wall as a beginning of fiber differentiation takes place at about 70-hours stages. The microphotograph (fig. 3) shows a section through the lens vesicle of a 60-hour embryo, where it is still opening to the surface.

According to our experience, at the 60-hour stage this connection between ectoderm and lens vesicle is constantly present.

In embryos of *Rana esculenta*, stage 19, according to SHUMWAY, is characterized by the presence of a lens bud only, no vesicle having been formed yet (fig. 4). In stage 20 the formation of a lens vesicle has taken place, but the vesicle is still very primitive (fig. 5). Because of the presence of abundant yolk, the structure of the lens bud in fig. 4 seems to be more irregular than it is.

Consequently the conclusion may be drawn, that the adult lens antigen in both species could be detected in stages before the complete formation of the lens-vesicle in which no specific morphological differentiation could be found.

Discussion.

The experimental results have demonstrated that organ-specific antigen(s) is(are) present in developmental stages where no specific differentiation of the lens-vesicle is visible, and even practically no

difference can be found between the morphology of this primitive lens and the rudiments of nose and ear.

It therefore seems justifiable to draw the conclusion that chemical differentiation is preceding morphological differentiation. This answer to the question about the priority of these two modes of differentiation is contradicting the conclusion of BURKE c.s. (1944), but the conclusion of these authors has not been based upon positive results.

The early appearance of chemical differentiation is substantiating the theory of chemo-differentiation which, since HUXLEY, has been discussed in embryological literature. It seems very unlikely that these important constituents of the adult lens would not have any significance in the period of construction of this organ-part. At least it may be supposed that the adult antigens, being organ-specific substances, also may represent organ-forming materials, enabling the embryo to build up its eye-lens. Whether these antigens also may be representing organ-determining substances cannot yet be stated, because they have not yet been detected in the period before the first beginning of the morphogenesis of the lens.

It is clear that preformationists have strong trumps in hand, if they state that qualitatively all specialised materials of the adult organism will be present at the onset of ontogenesis, because a theory of epigenetic creation of such substances during embryonic development only can get its evidence from negative results of experiments and these always may be explained by deficiency of technique.

Another question is that of the presence of other than adult antigens in the lens. BURKE and collaborators are of the opinion that embryonic lens antigen, differing from that of the adult lens, may be present in young stages of development. These authors immunised rabbits by the 300-hour embryonic lens antigen and the immune sera reacted with younger stages than the adult antiserum. The explanation for this phenomenon seems to be at hand: there must be another antigen common to the 120-hour and 300-hour lens but not present in the adult lens. But now that we demonstrated the presence of adult antigen in still younger embryonic lenses the possibility remains that the different antisera, used by BURKE, have been reacting with different intensity; as no control experiments have been reported, solution of this problem is difficult. It also may be remarked that these results only have been obtained by the complement fixation test, and we were necessitated to leave this test for the precipitin reaction. The fact, that BURKE did not mention any difficulty when using this technique whereas we found a very weak specificity, is difficult to explain. Perhaps the mode of preparation of the antigen for immunization has been different. BURKE reports the use of formalin in some cases for preserving this antigen, while in our experiments only fresh preparations were used. Denaturation may be expected by the treatment with formalin, and perhaps the antigen

preparation of BURKE contained less lipoids than ours, because they centrifuged it during 20 to 30 minutes at high speed; no data about the centrifugal force have been given, however.

In the experiments with 160-hour lens antiserum the authors themselves remarked that these sera were rather unspecific. They have based their opinion on the general damage, inflicted to embryos, and no control experiments have been reported. It seems possible that the presence of yolk in the material, used for immunising the rabbits has resulted in the production of antiserum against yolk, more than against the very small quantity of lens antigen that has been present. The reactions with yolk also may have disturbed some of the precipitin or complement fixation tests. Whether the embryonic antigen, met in the 300-hour lens, also may be a yolk antigen cannot be excluded nor can it be proved.

The possibility remains that a special embryonic lens antigen may be present. Moreover, it is possible that the ratio between the concentration of the different protein fractions of the lens will be changing during ontogeny. Such changes have been reported to occur in the lens during post-natal life, where a shift is found in the relative concentration of α- and β-cristallin. Such a change in quantitative relation between the different protein components of the lens would correspond with the differences in protein composition of the blood that have been found.

The increasing indexes of the last positive dilutions during the development from the 3-day to the 8-day embryo suggest a gradual increase during this period of development. Whether periods of more rapid increase do occur cannot yet be stated. Perhaps the period of lens induction is characterised by a large increase in quantity of the lens antigen, caused by the stimulus.

Summary.

It has been possible to demonstrate the presence of adult lens antigen in young embryonic lenses by means of the precipitin technique. In the chick embryo the youngest stages where the adult antigen could be detected were about 60 hours old and in the embryo of Rana esculenta the adult antigen was present from stage 19 to 20 (SHUMWAY) on.

In the 60-hour chick embryo the young lens-vesicle still opens to the surface and no specific morphological differentiation can be observed. In the frog embryo the lens vesicle is absent in stage 19 and just has been formed in stage 20; in stage 19 only a lens bud is found.

Therefore it can be concluded that the chemical differentiation of the lens is preceding the morphological differentiation. This conclusion is contradictory to the results of BURKE and collaborators (1944), but their opinion, like that of many others, has been based upon negative experimental results.

Our results thus do not permit the conclusion that in still younger stages the characteristic adult substances may be lacking. Improving

the technique probably will result in positive reactions in these stages.

The suggestion has been made that the organ-specific lens substances really may be called organ-forming substances, whereas it still remains more doubtful whether they also can be ranged among the organ-determining substances.

The complement fixation test in our experiments has failed to give reliable and specific results. Some additional notes about the technique and the organ- and species-specificity of the antisera, used in these experiments, have been presented.

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