ANTIGENS IN EGGS AND DEVELOPMENTAL STAGES OF THE SEA URCHIN

I. Immunological and Physicochemical Properties

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ABSTRACT

A number of antigens in unfertilized eggs and embryos of the sea urchin *Paracentrotus lividus* were characterized with respect to both immunological and physicochemical properties. Experiments involved single diffusion in agar (Oudin technique) combined with mutual dilution, serial dilution, and heating of antigenic extracts, as well as immunoelectrophoresis with normal and heated extracts and agar electrophoresis followed by staining of the antigenic spots with protein specific dyes. The gradual transition in migration rates of bands of precipitates in Oudin tubes following mutual dilution of either extracts or antisera allowed the identification of 6 immunologically identical antigens in eggs and embryonic stages. Similarities with respect to diffusion coefficients, sensitivity to heat, electrophoretic mobility, and reaction to protein specific dyes indicated that the antigens in extracts of eggs and various developmental stages also had certain physicochemical properties in common. Such knowledge is of importance for an understanding of antigenic changes occurring during ontogenesis.

INTRODUCTION

Knowledge concerning the synthesis or breakdown of macromolecules during different phases of embryonic development is important for an understanding of cellular differentiation. Antibodies against intracellular macromolecules constitute valuable tools for obtaining such knowledge. They not only possess a high power of biochemical discrimination but are also the specific reagents for macromolecular fine structures which depend upon genetic qualities inherent in the cells producing these structures. Thus, variations in antigens reflect variations in genetic activities, occurring, for example, during ontogenesis.

Developmental changes in the relative concentrations of 2 soluble protein antigens of the sea urchin *Paracentrotus lividus* have been described briefly (13). With this as a basis, the present series of investigations was undertaken in order to characterize certain of the antigens present both in unfertilized eggs and in later stages of development; to determine their distribution within the eggs; and to study changes in their relative concentrations during normal and abnormal development.

Such a study requires that the identities of the various antigens extracted from different developmental stages be established both immunologically and chemically. Immunological identification alone would not suffice, since, at least hypothetically, a particular antigenic determinant present

in various embryonic stages might belong to physically or chemically different macromolecules. Therefore, in this first paper, both immunological and chemical properties of several antigens extracted from unfertilized eggs and from developmental stages will be described.

MATERIAL AND METHODS

This investigation was carried out on unfertilized eggs and developmental stages of the sea urchin *Paracentrotus lividus*, collected at the Stazione Zoologica in Naples, Italy. Ovaries from mature females were placed in bowls of filtered sea water, whereupon release of eggs was spontaneous. Eggs from 10 to 15 females were filtered, pooled, and washed with large volumes of filtered sea water. Eggs in one aliquot were freed from their jelly layers by gentle treatment with sea water acidified with HCl to pH 5.2 (18). They were then washed twice with sea water, concentrated by settling, and stored at −15°C. until future extraction. Other aliquots were diluted and the eggs in each fertilized by the addition of a few drops of dry sperm. Details of this procedure and the subsequent rearing of the developmental stages will be given in a future paper of this series. Most of the embryos were harvested 48 hours after fertilization, when they had developed into fully mature plutei. For the present study, only a few earlier developmental stages were harvested (one 5 hours old cleavage stages, two 8 hours blastulae and two 24 hours gastrulae). The embryos were concentrated by centrifugation, washed, and stored at −15°C.

Extraction: Both eggs and early embryos were suspended in 5 to 10 volumes of ice cold 0.15 M NaCl solution containing phosphate buffer of pH 7.4. However, the 48 hour plutei were suspended in only 2 to 3 volumes of this medium. The suspensions were homogenized in a motor driven Potter-Elvehjem homogenizer packed in ice. They were dialyzed for 24 hours in the cold against the same medium, centrifuged at 15,000 *g* for 1 hour, and the clear supernatants used for testing. Non-dialyzable nitrogen was determined electrometrically by means of a micro-Kjeldahl procedure (4).

Preparation of Antisera: Samples of the dialyzed homogenates of eggs and plutei were lyophilized, taken up in saline, centrifuged, and the supernatants used for preparation of antisera. Male albino rabbits, weighing about 3 kg. each, were given a single intravenous injection. Then, after a rest period of 3 weeks, they were given a series of 12 subcutaneous injections (2 ml. every other day). When the titers of the antisera, as determined in preliminary precipitin tests, were sufficiently high, the rabbits were exsanguinated 1 week after the last injection. The blood was allowed to clot at room temperature and to stand in the refrigerator over night. Then the antisera from 5 rabbits which had been injected with material from the same origin were pooled, centrifuged, Seitz filtered, and stored at −15°C. Accordingly, each type of antiserum used in this series of investigations was a pool from 5 rabbits similarly treated.

Immunological Procedure: Oudin's single diffusion agar tests (9, 10) were performed essentially as described elsewhere (13, 14). Glass tubes, 2.5 mm. inside diameter and 70 mm. in length, were coated with a 1 per cent salt-free agar solution to cause the antiserum-agar mixtures to adhere to the walls of the tubes. Such mixtures, containing various concentrations of antiserum in 0.25 per cent agar (in physiological saline containing phosphate buffer of pH 7.4) were prepared at 48°C. and pipetted into the lower 30 mm. of the tubes. Although the concentration (*v/v*) of antiserum was varied in different experiments, the total concentration of serum was kept constant by the addition of appropriate amounts of normal rabbit serum. In all experiments 8 parts of serum (normal + antiserum) were mixed with 2 parts of agar. Then 0.2 to 0.3 ml. of the antigenic extracts were pipetted over the antiserum-agar columns. The tubes were sealed with paraffin oil and stored in a constant temperature incubator at 20°C. Tubes were always prepared in duplicate.

Bands of antigen-antibody precipitate formed and migrated down the antiserum-agar columns. The distances from the agar-liquid interface to the leading edges of the bands were measured on 5 successive days with the apparatus described in (14). The migration rates of the bands, termed *k* values, were determined graphically as the slopes of the straight lines obtained by plotting the distances (in mm.) from the interface to the leading edges of the bands against the square root of time (in hours) (3). The method has a high degree of accuracy (14).

Immunoelectrophoresis: Both immunoelectrophoretic and electrophoretic studies on agar were carried out according to the techniques of Grabar and Williams (6) with supporting glass plates of 6 × 10 cm. After addition of antiserum, the immunoelectrophoretic plates were incubated for 48 hours at 37°C. and stored for an additional 14 days in the refrigerator. They were then washed, dried, and stained according to standard procedures (5). Photographs were taken either before the plates had been dried or after they had been stained. Additional experimental details are given in the text below.

RESULTS

Double diffusion agar experiments according to Ouchterlony (8) reveal that both eggs and develop-

mental stages of the sea urchin *Paracentrotus lividus* contain a great number of saline soluble antigens in common (12). Thus, common precipitates form between either anti-egg or anti-pluteus serum, on the one hand, and the two corresponding antigenic extracts on the other. This suggests that most of the easily extractable egg or pluteus antigens are immunologically identical. Similar results were obtained when extracts of unfertilized eggs were compared with extracts of other early developmental stages.

When an extract of unfertilized eggs or embryos was pipetted over a mixture of antiserum-agar in Oudin tubes, the number of precipitation bands was smaller than that in Ouchterlony plates, due to the lower resolution of the Oudin technique. Appearance of only a small number of bands in Oudin tubes is, however, advantageous in that it leads to easier and more certain identification of the various bands present in different tubes. In order to study a relatively large number of bands, Oudin tubes with different antigen/antibody ratios were prepared. When the concentration of antiserum was high relative to the antigen concentration of the extracts being tested, only a few bands of precipitate traveled down the column at a conveniently measurable speed. The migration of the other precipitates was suppressed with the result that they remained in more or less stationary positions at the agar/liquid interface. On the other hand, when the concentration of antiserum was low, these stationary precipitates migrated at a measurable rate. Under such conditions those precipitates which had migrated at the higher antiserum concentration migrated even more rapidly and were soon out of the measuring area. Thus, they did not interfere with the measurements of the more slowly migrating bands.

Mutual Dilution: When extracts of unfertilized eggs and of embryos were compared by testing against antiserum of a given concentration, the pattern in the various Oudin tubes was similar with regard to the number, relative positions, and densities of the bands. However, in order to establish more definitely immunological identity of the bands formed by the antigens in the various ex-

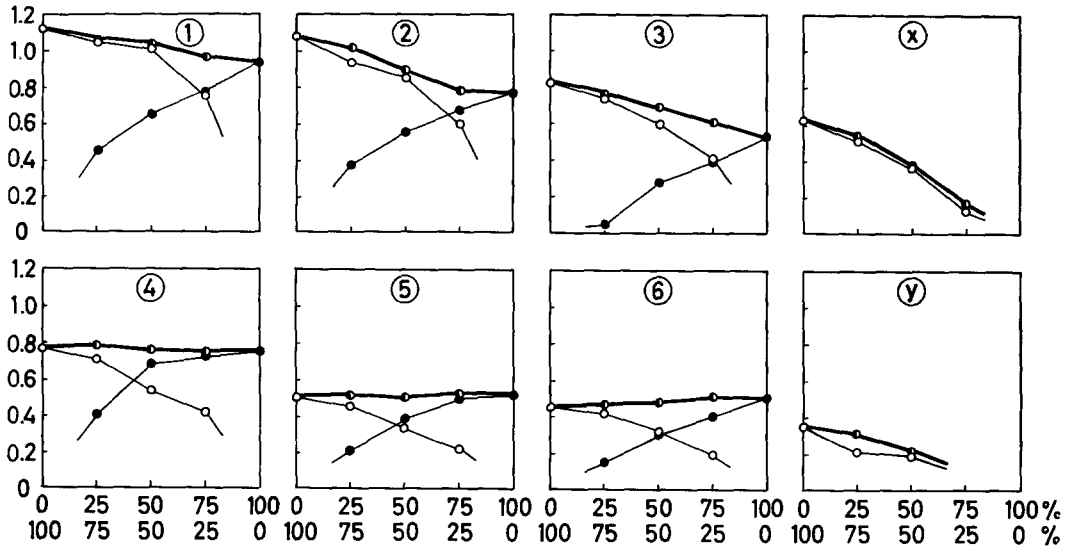


FIGURE 1

Migration rates (mm. × hours$^{-1/2}$) of leading edges of precipitates which formed in anti-egg serum–agar columns overlayered with extracts of unfertilized eggs and of 48 hour plutei, mutually diluted with each other (*half-filled circles*) or diluted with saline (*empty circles*: egg extracts, *filled circles*: pluteus extracts). *Ordinates*: migration rates. *Abscissae, upper scales*: concentration (per cent) of pluteus extract; *lower scales*: concentration of egg extract. The concentration of non-dialyzable nitrogen in the undiluted egg- and pluteus extracts (= 100 per cent) was 2.50 mg./ml. The diagrams in the upper row refer to measurements made at 70 per cent concentration of antiserum, those in the lower row to measurements at 30 per cent concentration of antiserum. *1–6, x, y*: different antigens.

tracts, mutual dilution tests (17) were performed. This procedure is best described by referring to a specific experiment (Fig. 1). An extract of unfertilized eggs and one of plutei were either mixed with each other in 3 different proportions, or each was diluted with corresponding volumes of saline. The original extracts, mixtures, and saline dilutions were each reacted with antiserum-agar in Oudin tubes. As can be seen from the upper row of Fig. 1, at 70 per cent antiserum concentration the pure egg extract produced 4 distinct bands which migrated at a measurable rate. With undiluted pluteus extract, 3 migrating bands appeared. Mixtures of the 2 extracts produced 3 bands with migration rates intermediate in value between those produced by the 2 undiluted extracts. Therefore, when migration rates were plotted against dilutions, smooth curves could be drawn between corresponding points. On the other hand, when either egg or pluteus extract was diluted with saline, the migration rates of the 3 bands decreased as the antigen concentration decreased. The results indicated that a minimum number of 3 antigens (designated *1* to *3*) were present in both egg and pluteus extracts. The migration rate of the fourth, slow moving band (designated *x*) produced by the egg extract not only decreased with progressive dilution with saline, but also decreased equally much upon dilution with pluteus extract. This indicates that the particular egg antigen responsible for formation of this band was not present in the pluteus extract, or was present in suboptimal concentration.

The lower row of Fig. 1 shows the results when aliquots of the same extract were tested with antiserum at 30 per cent concentration. The bands corresponding to those in the upper row of Fig. 1 migrated much faster and are not shown in the graphs. The migration rates of the bands which are recorded here were too low to be measured at the 70 per cent antiserum concentration. These bands were found to be immunologically identical in both extracts and are designated as antigens *4* to *6*. As at 70 per cent antiserum concentration, an additional band which was not formed by the pluteus extract was formed by the egg extract (designated *y*).

When extracts of eggs were diluted with extracts of blastulae or gastrulae, similar results were obtained. Moreover, smooth curves were obtained when the migration rates of bands produced by egg extracts were plotted against mutually diluted

TABLE I

Migration Rates of Six Precipitates Formed by Antigens 1 to 6 in Extracts of Unfertilized Eggs and 48 Hour Plutei*

Antigen		Relative concentration of antigen (%)‡					Relative concentration of antiserum in agar
		20	40	60	80	100	
1	eggs	0.571	0.785	0.907	0.982	1.057	70%
	plutei	0.353	0.540	0.679	0.765	0.823	
2	eggs	0.493	0.708	0.851	0.936	1.012	
	plutei	0.098	0.319	0.448	0.535	0.586	
3	eggs	0.365	0.563	0.680	0.766	0.823	
	plutei	0.015	0.231	0.340	0.422	0.487	
4	eggs	0.383	0.551	0.660	0.712	0.779	30%
	plutei	0.407	0.576	0.702	0.750	0.813	
5	eggs	0.292	0.417	0.485	0.551	0.570	
	plutei	0.299	0.426	0.496	0.536	0.574	
6	eggs	0.218	0.370	0.439	0.496	0.557	
	plutei	0.195	0.342	0.435	0.476	0.534	

* The migration rates are the slopes of the straight lines obtained by plotting the distances (in mm.) from the agar/liquid interface to the leading edge of each precipitate against the square root of time (in hours) of migration. The slopes were determined graphically from 5 daily measurements made on duplicate tubes.
‡ The concentration of non-dialyzable nitrogen in both extracts at "100 per cent" was 1.50 mg./ml. The dilution series were made from these stock solutions with 0.15 M saline containing phosphate buffer, pH 7.4.

TABLE II

Slopes for Plots of Migration Rates vs. Log (Antigen), their Errors and t-Values, Calculated from the Data of Table I

Antigen	Slopes	s_b‡	$t_{(eggs-plutei)}$§	$t_{(antigens)}$‖
1 eggs	0.6907	0.0101	0.327	
plutei	0.6846	0.0156		$t_{1,2} = 9.156$
2 eggs	0.7450	0.0096	1.849	$t_{1,3} = 6.002$
plutei	0.7086	0.0172		$t_{2,3} = 16.384$
3 eggs	0.6603	0.0054	1.630	
plutei	0.6722	0.0115		
4 eggs	0.5622	0.0139	1.527	
plutei	0.5834	0.0208		$t_{4,5} = 34.553$
5 eggs	0.4089	0.0159	1.544	$t_{4,6} = 18.537$
plutei	0.3927	0.0104		$t_{5,6} = 16.790$
6 eggs	0.4727	0.0156	0.645	
plutei	0.4803	0.0144		

* The slopes (b) were calculated by least squares, $b = \frac{S(x - \bar{x})(y - \bar{y})}{S(x - \bar{x})^2}$ where $x = log\ (antigen)$ and $y =$ migration rates according to Table I.

‡ The errors of the slopes were calculated as

$$s_b = \frac{s}{\sqrt{(x - \bar{x})^2}} \quad where \quad s = \sqrt{\frac{S(y - \bar{y})^2 - \frac{[S(x - \bar{x})(y - \bar{y})]^2}{S(x - \bar{x})^2}}{n - 2}}; \qquad n = 5$$

§ t-values for each of the 6 antigens for comparison of the slopes obtained with egg or pluteus extracts. $t = \frac{b_e - b_p}{s_{b_e - b_p}}$ where b_e and b_p are the slopes of the curves which are being compared (e = eggs, p = plutei).

$$s_{b_e - b_p} = \sqrt{s^2_{e,p}\left[\frac{1}{S(x_1 - \bar{x}_1)^2} + \frac{1}{S(x_2 - \bar{x}_2)^2}\right]} = \sqrt{\frac{2\, s^2_{e\,p}}{S(x - \bar{x})^2}}$$

since $log\ (antigen)$, x_1 and x_2, are identical in both solutions. $s^2_{e,p} = \frac{s^2_e - s^2_p}{2}$ where s_e and s_p correspond to s in footnote‡. Degrees of freedom, $d.f. = n_1 + n_2 - 4 = 6$.

‖ t-values for comparison of average slopes of the different antigens, *e.g.* $t_{1,2} = \frac{\bar{b}_1 - \bar{b}_2}{s_{\bar{b}_1 - \bar{b}_2}}$ where $\bar{b}_1$ and $\bar{b}_2$ are the averages, from the slopes obtained with egg and pluteus extracts, for antigens 1 and 2, respectively

$$s_{\bar{b}_1\ \bar{b}_2} = \sqrt{s^2_{1,2}\left[\frac{1}{S(x_1 - \bar{x}_1)^2 + S(x_2 - \bar{x}_2)^2} + \frac{1}{S(x_3 - \bar{x}_3)^2 + S(x_4 - \bar{x}_4)^2}\right]} = \sqrt{\frac{s^2_{1,2}}{S(x - \bar{x})^2}}$$

since log (antigen), $x_1 = x_2 = x_3 = x_4$. $s^2_{1,2} = \frac{s^2_{e1} + s^2_{p1} + s^2_{e2} + s^2_{p2}}{4}$ where s_{e1}, s_{p1}... correspond to s in footnote‡. $d.f. = 12$. The indices 1 to 6 of the t-values stand for the corresponding antigens.

anti-egg and anti-pluteus sera or mutually diluted anti-egg and anti-gastrula sera. It was noted that with some lots of anti-pluteus serum one or two more bands formed in reactions with pluteus extracts than with egg extracts. It is likely that these were caused by "pluteus antigens" which were not detectable in the eggs (12).

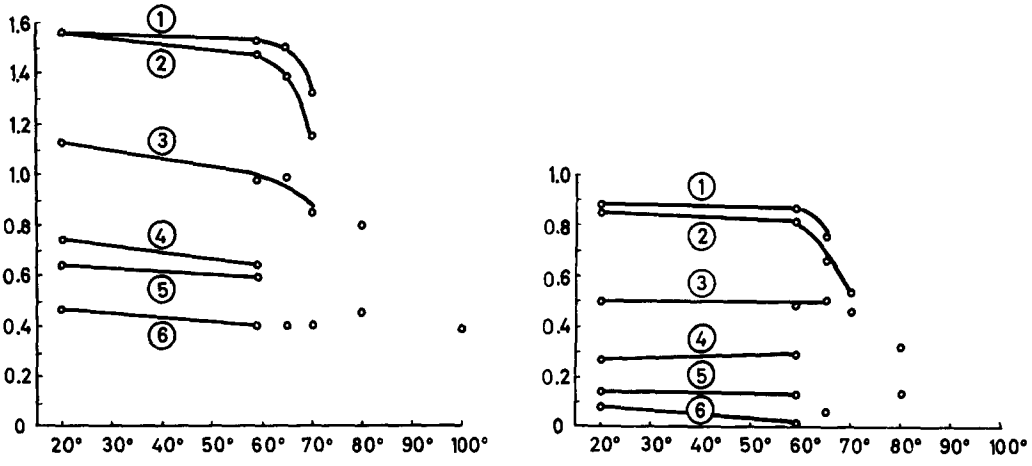
With different lots of extracts or of antisera, slight variations did sometimes appear in the total number of bands formed. Further discussion in this paper will be restricted to the common antigens designated *1* to *6* in Fig. 1.

Serial Dilution: Other variables being constant, the migration rate of a band of precipitate in an

Oudin tube increases with increasing concentration of antigen (3, 10, 16). The concentration of antigen being sufficiently low and that of antibody sufficiently high, the migration rate k increases linearly with *log* (*antigen*). Moreover, under these conditions, the slope of the lines in a k vs. *log* (*antigen*) plot is a measure of the diffusion coefficient of the antigen forming the precipitate in question (2, 7). Hence, a comparison of the slopes of bands formed by immunologically identical antigens from extracts of various developmental stages should give information about some of the physicochemical properties of the antigen molecules. A comparison of this kind also forms the basis for quantitative determinations of concentrations of antigens in extracts of different developmental stages, when the results are calculated from a standard curve (*k/log* (*antigen*)) obtained with extracts of unfertilized eggs (13).

Extracts of eggs and plutei were tested in 5 different concentrations against aliquots of a given antiserum. The migration rates of bands *1* to *3* were again measured at an antiserum concentration of 70 per cent and those of bands *4* to *6* at a concentration of 30 per cent. The total concentration of serum was the same (80 per cent) in all experiments (see Material and Methods). Moreover, in an attempt to equalize any influence from additional nonspecific factors (2, 11, 15, 16) the extracts were prepared so as to contain the same concentration of non-dialyzable nitrogen. Since the solubility of the proteins in the 48 hour plutei is apparently less than that of egg proteins, approximately twice the number of plutei as eggs were extracted.

The migration rates of the bands formed by the serially diluted extracts are listed in Table I. The values of the slopes in the k vs. *log* (*antigen*) plots and their errors, all calculated by the method of least squares, are shown in Table II. On the average, the standard deviation, s_b, of the slopes, amounted to 2.4 per cent. As can be seen from Table II, the bands formed by the corresponding antigens in egg and pluteus extracts had similar slopes. The t test showed that differences between the slopes of such bands were not statistically significant (p for antigen *1*: 0.7 to 0.8; for antigens *2* to *5*: 0.1 to 0.2; for antigen *6*: 0.5 to 0.6). This suggests that the physicochemical properties, with respect to diffusion coefficients, of the corresponding antigens in the 2 extracts were similar. On the other hand, when the average slopes, calculated for each of the 6 pairs of egg-pluteus antigens were compared, the differences between the slopes were highly significant ($p \ll 0.001$ in all cases indicated in Table II). It may, therefore, be assumed that



FIGURES 2 and 3

Migration rates (mm. × hours$^{-1/2}$, along the *ordinates*) of leading edges of precipitates which formed in agar columns containing anti-egg serum (concentration 30 per cent v/v) tested with an extract of unfertilized eggs (Fig. 2) or plutei (Fig. 3) previously heated for 30 minutes to various temperatures (°C.) as indicated along the *abscissae*. *1–6*: different antigens. No curves have been drawn through points, the identities of which could not be established with certainty. For further explanations see text.

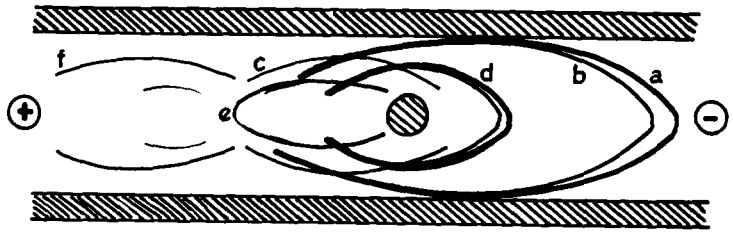


FIGURE 4

Diagram of an immunoelectrophoretic plate. An extract of unfertilized eggs was added at the *shaded circle*. After completion of the electrophoresis (phosphate buffer, pH 7.2, μ = 0.05, 0.7 mA/cm. cross-section, agar layer 2 mm. high, 16 hours) an anti-egg serum was added to the *shaded lateral channels*. *a to f*: the 6 predominant precipitates.

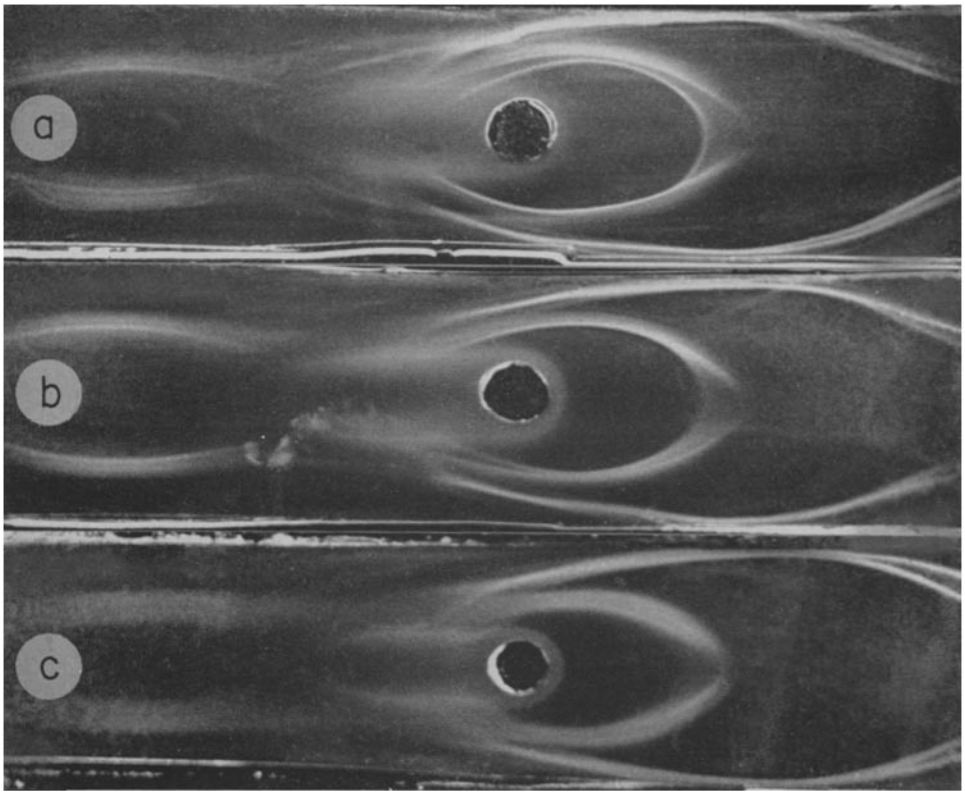


FIGURE 5

Darkfield photographs of washed, undried immunoelectrophoretic plates. Experiments were made with extracts of 3 developmental stages: (*a*) unfertilized eggs, (*b*) 24 hour gastrulae, (*c*) 48 hour plutei. After electrophoresis (same conditions as given in legend to Fig. 4) an anti-egg serum was added to the lateral channels. In the experiments depicted in this figure, the doubling of the precipitates is due to repeated addition of antiserum.

the diffusion coefficients of each of the 6 antigens were different from one another.

Effect of Temperature: In order to obtain additional information about the chemical properties of the various antigens, aliquots of egg and pluteus extracts were heated to different temperatures and for various periods. After the precipitates which formed at elevated temperatures had been removed by centrifugation, the extracts were tested against anti-egg serum in Oudin tubes in the usual way. Fig. 2 shows the results of a typical experiment with aliquots of an egg extract which had

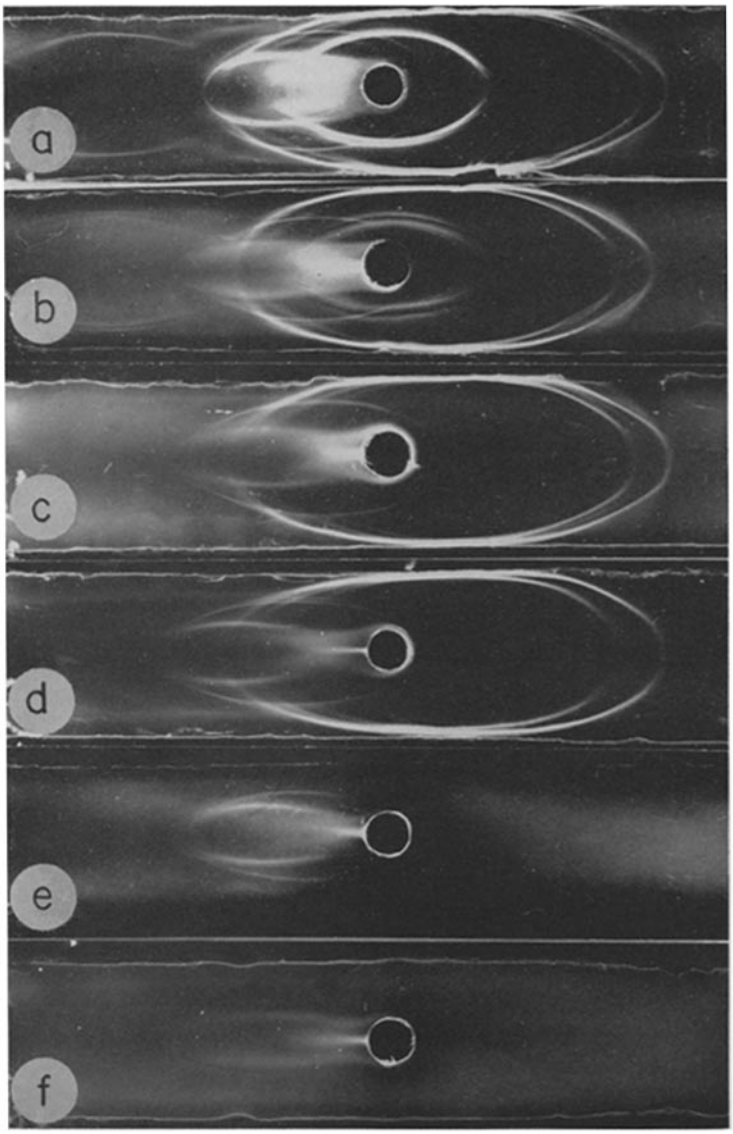


FIGURE 6

Photographic prints (obtained by direct enlargement through a photographic magnifier) of dried immunoelectrophoretic plates stained with azocarmine B. Experiments were made with heated (30 minutes) extracts of unfertilized eggs and anti-egg serum (aliquots of extracts and antiserum from experiment of Fig. 2). (*a*) room temperature, (*b*) 58°C., (*c*) 65°C., (*d*) 70°C., (*e*) 80°C., (*f*) 100°C. For further details see text and legend to Fig. 4.

been heated for 30 minutes. Similar results were obtained after a heating period of only 10 minutes. Although only one concentration (30 per cent) of antiserum was used in the experiment illustrated in Fig. 2, it was possible to record the migration rates of all 6 bands of precipitate by making frequent measurements of the 3 fast migrating bands during the first 24 hours of the experiment. (The accuracy of the measurements of these 3 bands was thereby reduced.) In Fig. 2 the migration rates of the various bands are plotted along the ordinate, while the various temperatures to which the extracts had been heated are given along the abscissa. Fig. 2 shows that after heating the extract to 58°C. all 6 bands were still present, though the amounts of the antigens had probably been slightly reduced. Between 58°–65°C. 2 bands disappeared. The remaining 4 bands were still present at 70°C., but at 80°C. only 2 bands were present, and at 100°C. only 1 band could be detected.

In Fig. 2 curves were only drawn between points representing bands which could be identified beyond doubt by means of their relative positions and densities. Five points were not included in the curves, because it was not clear which bands they represented. The curves show that the 2 most labile antigens are among antigens *4* to *6*. It appears that antigens *1*, *2*, and *3* are among those still present at 70°C. Fig. 2 does not reveal which of the antigens are stable at 80°–100°C. The results in Fig. 2 were confirmed by similar experiments carried out at 50 and 70 per cent concentrations of antiserum. Each antiserum concentration was tested with 2 dilutions of the heated aliquots of the egg extract.

Fig. 3 shows the results of a corresponding experiment made with heated aliquots of an extract of 48 hour plutei tested with the anti-egg serum of Fig. 2 at 30 per cent concentration. The curves are very similar to those obtained with egg extracts. Somewhat fewer points are present in Fig. 3 than in Fig. 2, probably due to the lower concentrations of antigens in the pluteus extract than in the egg extract.

Immunoelectrophoretic Experiments: Immunoelectrophoresis provides a good means for further comparing chemical properties of antigens. Fig. 4 was drawn at the conclusion of an experiment in which an extract of unfertilized eggs was tested with an anti-egg serum. The predominant precipitates are labeled *a* to *f*. These precipitates were obtained consistently with all extracts or antisera tested. In addition, a variable number of weaker precipitates always appeared. Only precipitates *a* to *f* will be considered here.

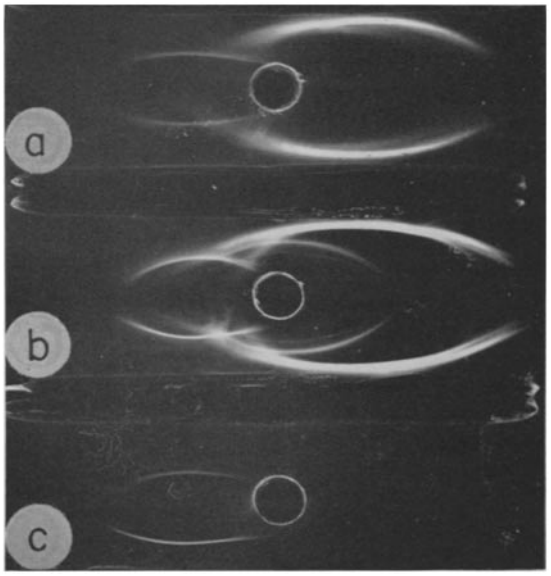


FIGURE 7

Photographic print of dried immunoelectrophoretic plate stained with Azocarmine B. Experiments were made with heated (30 minutes) extracts of unfertilized eggs and an anti-egg serum (both were from different lots than those used in the experiments of previous figures). (*a*) 70°C., (*b*) room temperature, (*c*) 100°C. For further details see text and legend to Fig. 4. The print was obtained as described for Fig. 6. Precipitates *c* and *f* which were visible on the original plate do not appear on this print.

Fig. 5 shows the photographic prints from a series of experiments in which extracts of various developmental stages after electrophoresis in agar had been tested against an anti-egg serum. As can be seen, the antigens responsible for the precipitates *a* to *f* are electrophoretically similar in the different extracts. Quantitative differences were present, but these will not be discussed in the present paper.

Fig. 6 shows a series of immunoelectrophoresis made with aliquots of the heated egg extract and the anti-egg serum used in the experiment of Fig. 2. Fig. 6 illustrates that antigen *e* is thermostable, present even after heating to 80° or 100°C. It probably contains a relatively immobile, heat labile component, disappearing already at 58°C. However, in the experiments of Figs. 6 *b* to *e*, the distance between the antiserum basin and the ver-

tex of the precipitate of antigen *e* is approximately the same. This indicates that the concentration of this antigen does not decrease severely between 58° and 80°C. Heating to temperatures above 80°C. led to the formation of a more diffuse precipitate, probably due to beginning denaturation of antigenic determinants (Fig. 6 *f*). This was accompanied by a slight change in electrophoretic mobility. However, in other experiments, antigen *e* formed a sharp and well defined precipitate even after 30 minutes of heating to 100°C. (Fig. 7). The thermostability of antigen *e* plus its relatively slow diffusion towards the antiserum basin suggest that it is identical to one of the antigens labeled *4* to *6* in the Oudin tests of Fig. 2. For similar reasons it appears likely that antigen *c* which disappears between 80° and 100°C. corresponds to antigen *3* of Fig. 2. Antigens *a* and *b* are completely stable up to 70°C., but they disappeared after heating to 80°C. (although their presence in reduced concentrations could occasionally be detected in such extracts). This and their position relative to the antiserum basin indicate that they probably correspond to the rapidly diffusing antigens *1* and *2* of Fig. 2. Of the remaining principal components, *d* and *f* disappeared between 58° and 65°C. This suggests that they are identical to two of the antigens of group *4* to *6* in the Oudin diffusion test.

Antigens in pluteus extract heated to various temperatures produced immunoelectrophoretic patterns similar to those produced by heated egg extract. Antigens *a*, *b*, *c*, *d*, and *f* in the pluteus extract showed the same sensitivity to heating as did the corresponding antigens in the egg extract. Possibly, antigen *e* was less stable, having usually disappeared from the aliquot of pluteus extract heated to 100°C.

Agar electrophoresis, without addition of antiserum, followed by staining of the antigen spots with protein stains such as Azocarmin B or Amidoblack after the plates had been fixed and dried (5) indicated that most of the antigens just described were proteins. The possible exception is the thermostable fraction of antigen *e* which, after having been heated to 80° or 100°C., gave only a very faint color with these stains.

DISCUSSION

The purpose of these experiments was to characterize both immunologically and chemically various macromolecules present in extracts of unfertilized eggs and of embryos in various developmental stages. The main emphasis has been given to a description of qualitative similarities, rather than differences, of antigens present in eggs and various developmental stages. Such similarities are predominant when total homogenates of eggs or embryos are used in preparation of antisera and when water or dilute salt solutions are used for extraction (12). However, qualitative immunological differences between eggs and various embryonic stages may exist to a greater degree than previously indicated. Such differences could probably be visualized by working with more elaborate extraction procedures and, above all, with antisera against entities smaller than total homogenates; *e.g.*, against intracellular fractions or special parts of embryos. No conclusions can be drawn from the present experiments concerning changes in relative concentrations of antigens occurring during embryogenesis. However, quantitative relationships will be described in a later paper.

In the present paper a number of antigens common to various embryonic stages have been chosen for a detailed study using the single diffusion technique of Oudin as well as immunoelectrophoretic techniques. As already pointed out, 6 bands of precipitate were obtained quite consistently in both types of experiments, when various antisera were reacted with extracts of different embryonic stages. These bands could conveniently be studied because of their characteristic densities and their favourable positions which made accurate measurements possible. It is likely that some of these bands, in each type of test, were heterogenous, consisting of several more or less related antigen-antibody complexes of similar densities and close leading edges or of similar electrophoretic mobilities.

Mutual dilution of antigenic extracts showed that the 6 antigens of the Oudin's experiments were apparently immunologically identical throughout embryonic development; that is, the antigenic determinants in different extracts were similar. The question then arises as to whether or not these determinants are part of chemically similar macromolecules. In the present study this problem has been approached in a number of different ways.

Determination of the slopes of the straight lines obtained by plotting diffusion rate *k* vs. *log* (*antigen*) constitutes an important approach to this problem, since the slopes reflect the diffusion coeffi-

cients of the antigens (2, 7). No difficulties were encountered in finding the ranges of antigen and antibody concentrations within which the curves for all 6 antigens were straight in a good approximation. The slopes obtained from the data given in Table I were typical for the particular lots of extracts and of the antiserum used in that experiment. When the results obtained with different lots of extracts and with different antisera were compared with one another, the variability was greater (2). However, using appropriate concentrations of antigen and antibody mostly gave satisfactory results. The influence of non-specific factors which act on migration rates of precipitation bands, *e.g.* viscosity of the antisera, non-reacting substances in the reagents, etc. (2, 11, 15, 16), was of minor importance in the present connection. As already pointed out, the experiments were run in such a way that these factors seemingly affected the migration of corresponding antigens in different extracts in a similar manner.

The true relationship between the slope of the k vs. *log (antigen)* plot and the real diffusion coefficient of an antigen is still open to discussion (1, 2). Moreover, because of a square root relationship, the slope of the k vs. *log (antigen)* plot cannot be considered as sensitive indicator of diffusion coefficients (7). Nevertheless, the results obtained in this way are sufficient to demonstrate (1) that the 6 antigens tested are significantly different from each other with regard to their diffusion in antiserum-agar and (2) that each is physicochemically similar to its corresponding antigen in extracts of other stages.

The sensitivity of antigens to heat, studied in conjunction with agar diffusion, provides an additional tool for the characterization of antigens. From the Oudin experiments it appears likely that the heat sensitivity of each of the 6 antigens is similar in extracts of different stages. However, as apparent from Figs. 2 and 3, it is difficult to make definite identifications of all bands which are produced by heated extracts.

Immunoelectrophoretic experiments indicate that the electrophoretic mobility of each of the most important antigens found in these tests is similar in extracts from various stages. Further information on the properties of the antigens was obtained by a combination of heating and immunoelectrophoretic experiments and also by conventional electrophoresis on agar. The results show that most of the antigens are proteins and that some of them are composed of molecules which are remarkably homogeneous with respect to heat sensitivity. Thus, an entire antigen (*c, f*) may disappear within a very narrow range of temperature. The molecular populations in other precipitates are more heterogeneous, that is, the molecules may disappear from a precipitate over a relatively wide range of temperature, without any apparent change in electrophoretic or immunological properties. This was noted with respect to antigens *a* and *b* when heated above 70°C., to antigen *d* between 58° and 65°C., and, at times, to the thermostable fraction of antigen *e*. It has not yet been established whether this is due to a difference in heat sensitivity of both immunologically and electrophoretically similar molecules within a precipitate, or whether it is due to an actual immunological or electrophoretic heterogeneity of an apparently homogeneous precipitate.

By a comparison of the results of the Oudin and the immunoelectrophoretic experiments, both carried out with heated extracts, it has been possible to correlate to a certain extent the migration rates of antigens in antiserum-agar columns and their electrophoretic mobilities. Such correlation is based on the assumption that the 6 predominant precipitates in the Oudin tubes correspond to the 6 predominant ones in the immunoelectrophoretic plates. Experiments to prove this finally are so far lacking.

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