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Germ-Cell Line and Sexual Differentiation in Birds [and Discussion]

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Germ-cell line and sexual differentiation in birds

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[Plates 7 to 9]

Contents

The present paper deals with recent investigations on the germ line and the sexual organogenesis in birds. The analysis has been limited to the following problems: origin of the germ line, physiology of the germinal epithelia, determinism of the migration of gonocytes, and differentiation of the germ cells during sexual organogenesis.

It has been clearly established that in birds the germ line is precociously determined and that the anterior germinal crescent is a secondary formation. The hypothesis according to which the primary localization of the germ cells in birds (and more generally in Amniotes) would be posterior, is discussed. It appears to be the most plausible.

The germinal epithelia are secretory and excretory organs. The excretory function is of the merocrine type. Kinetic studies of the protein turnover in the germinal epithelia demonstrate the existence of at least two categories of cytoplasmic proteins: structural proteins, and exportable proteins; the latter are excreted via protrusions of the epithelial cells.

The hypothesis according to which the excretory function is associated with the attractive power exerted by the sexual primordia upon the migrating primordial germ cells is examined. The mechanism controlling the entry into the genital ridge of the gonocytes circulating in the embryonic blood stream is of a chemotactic nature.

Finally, during early sexual organogenesis, as well as during later stages of sexual differentiation, the germ cells undergo important ultrastructural, histochemical and physiological (migratory properties) changes.

Introduction

It has been clearly established that, in vertebrates, the germ cells are directly derived from a cell cluster which segregates very early from the purely somatic elements. The germ-cell line is the first expression of sexuality in the embryo. It can sometimes be recognized as soon as the first blastomeres have formed, thus well before the determination and localization of the presumptive sexual areas. This is essentially the case in some amphibians (anurans).

In general the primordial germ cells (p.g.c.) appear at the posterior part of the germ, in the region of the most vegetative blastomeres, and outside the sexual areas which, as they derive from the mesoderm, are of completely different embryological origin. This situation applies to some amphibians (anurans), chelonians, some lacertilians and to mammals. It implies that, to reach the sterile gonadal primordia, the p.g.c. have to move by an interstitial type of migration.

In birds the p.g.c. have been shown to appear at the neurula stage, rather late during embryonic development, and in the anterior part of the germ, in close correlation with the extra-embryonic endoblast and the mesoblast of the blood islands. Furthermore, their long-distance migration is known to be of the vascular type. This situation has long been considered to be characteristic of birds and to constitute an exception to the general schema that applied to most vertebrates. From recent experiments it appears, however, that similar situations exist in many reptiles (Rhyncocephala and lacertilians) and particularly in the viper, where the behaviour of the germ-cell lineage is typically of the avian type (Hubert 1966).

During the last 15 years new techniques such as the explantation of blastoderms on culture media *in vitro*, circulatory parabiosis, association of tissues *in vitro*, and use of radioactive precursors have been made available; their use has led to a better understanding of the different events occurring during sexual organogenesis in the chick embryo.

The present paper essentially deals with some recent contributions to the following three problems: primordial sexual events in the avian germ including the origin of the germ-cell line, the determinism governing early sexual organogenesis in birds and the entry of primary germ cells into the gonadal primordia, and the differentiation of the germ cells during embryonic sexual organogenesis.

Primordial sexual events in the avian germ

These primordial sexual events are on the one hand the segregation of the germ-cell line, and, on the other hand, the differentiation of the germinal epithelia.

The germ-cell line

The primary germ cells of the chick can be directly recognized for the first time in the anterior germinal crescent. At that stage (intermediate primitive streak stage) the p.g.c. have already acquired a certain number of cytological and histochemical characteristics permitting their identification (Reynaud 1967). At earlier stages, there is no such specific marker, and it seems almost impossible as yet to recognize a germ cell with certainty. The problems of the origin and primary localization of the p.g.c. in the chick could therefore not be investigated satisfactorily by histological and histochemical techniques, but had to be approached by indirect methods.

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Origin and primary localization of the primary germ cells

It has long been suspected that the p.g.c. originate in the deep layers of the germ, i.e. in the endophyll. This particular point has been demonstrated recently by means of *in vitro* cultures, of non-incubated blastoderms (Dubois 1967, 1968*a*, 1969*b*).

The primary localization of the p.g.c. cannot be in the anterior germinal crescent. First, the anterior localization is exceptional. Secondly, this localization is observed at a relatively late stage during early embryonic development. Furthermore, it is not rigidly defined, since many embryos are not totally sterilized after excision or irradiation of the germinal crescent (Dubois 1962). In certain blastoderms the germ cells are more or less disseminated as far as the posterior region (Matsumoto 1932; Fargeix 1966; Bruel-Beaudenon & Hubert 1968). The hypothesis according to which the caudo-cephalic movements of the endophyll during the pregastrula and gastrula stages result in the laying down of the germinal crescent (Vakaet 1962)

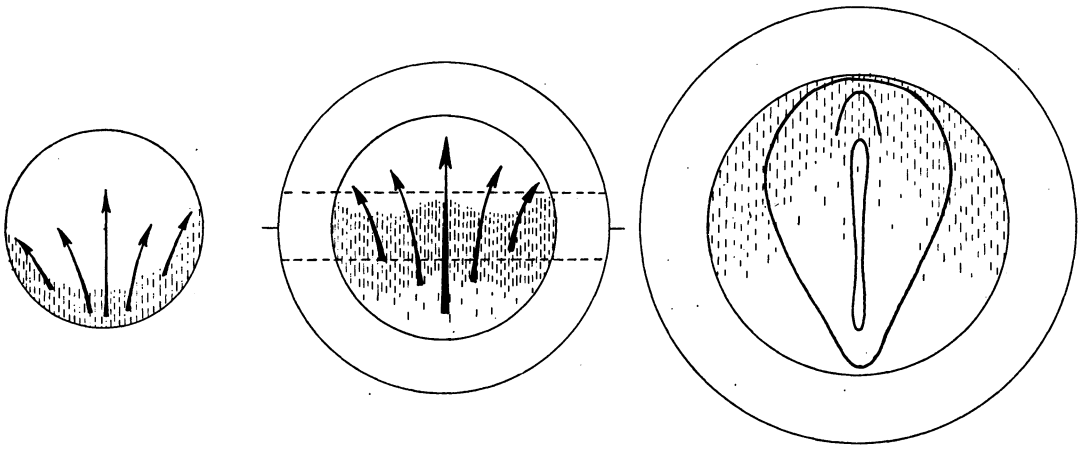
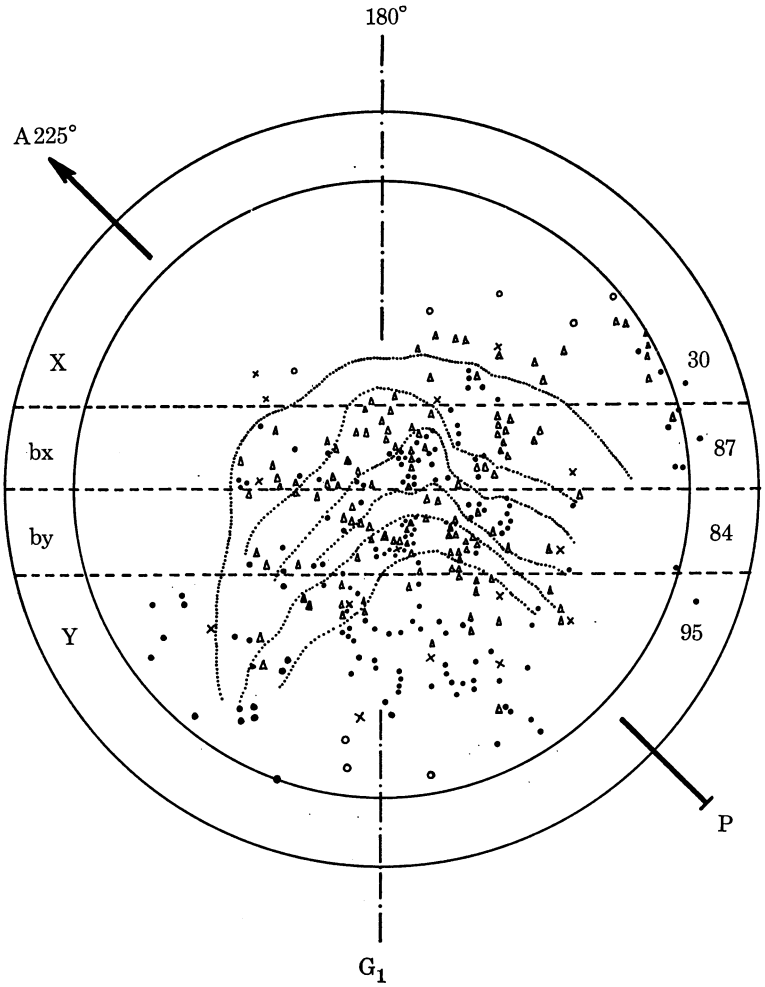


FIGURE 1. Evolution of the germinal area at the very early stages of ontogenesis in the chick embryo. −10 h, hypothetical stage of the posterior localization; 0 h, intermediate localization (interpretative schema, see text); +20 h, anterior localization (interpretative schema).

has been tested experimentally. These investigations have been carried out simultaneously in different laboratories by means either of the fissuration technique of the duck blastoderm, as described by Wolff & Lutz in 1947 (Fargeix 1967; Rogulska 1968), or the *in vitro* culture technique according to Wolff & Haffen (1952) and Wolff (1961) (Dubois 1967, 1968*b*). The results can be schematically summarized as follows: (i) when the non-incubated chick or duck blastoderm is transected according to its diameter, the anterior and posterior halves are always fertile (fissuration *in ovo*, or half-blastoderms cultivated *in vitro*); and (ii) when a diametrical narrow transverse band is dissected out, one of the fragments is sterile in an appreciable number of cases. A statistical analysis of the numerical data demonstrates that this is the anterior fragment. To explain these results, Dubois has proposed a theoretical interpretative schema according to which, in the non-incubated blastula, the p.g.c. are in a position intermediate between the posterior localization (common to most vertebrates) and the anterior localization that had been considered to be specific for birds (Swift 1914; Simon 1960) and of the viper (figure 1, 0h).

In order to provide more concrete evidence for the validity of that schema, the following

experiments have been carried out (Dubois 1969*b*). First of all, the relative fertility of the previously mentioned diametrical transverse band has been verified numerically. The number of gonocytes found in fragments X, bx, by and Y (see figure 2, G_1 and G_2) isolated from non-incubated eggs and cultivated for 48 h, are summarized in the upper part of table 1. Only those embryos in which the orientations, calculated from the relative amounts of gonocytes in fragments X and Y, were near 180° have been considered (Dubois 1967). One can see that the diametrical band perpendicular to the embryonic axis is always more fertile than the rest of the germ. The degree of fertility is sometimes very high: 76% for LG 56. In general, the real posterior half of the germ is richer in p.g.c. than the anterior half.



For legend see facing page.

Secondly, an attempt has been made to clarify the localization of the germ cells in blastoderms in which the movements of the endophyll are mechanically blocked. Two blastulae, oriented according to von Baer's law (1828), were explanted onto a tight lattice of cotton resting on a culture medium. After 36 h of culture, the distribution of the gonocytes was mapped with the aid of an ocular micrometer. The results are presented in figure 2 (G_1 and G_2) and in the lower part of table 1.

Since the embryos did not undergo any organized morphogenesis, it was not possible to

recognize the embryonic axis; histological sections have therefore been prepared parallel to the orientation axis (180°). The distribution maps of the *in vitro* differentiated gonocytes show at once that the p.g.c. are not confined to a crescentic array. Nor do they seem to be randomly distributed, but their localization appears rather to be in close correlation with the extension of the endophyll (especially in G_1), the highest concentration of p.g.c. being observed in the central zone of the germ. These observations demonstrate that blocking of the endophyll was efficient and that after 36 h culture the localization of the gonocytes corresponds nearly exactly to that observed in non-incubated blastulae.

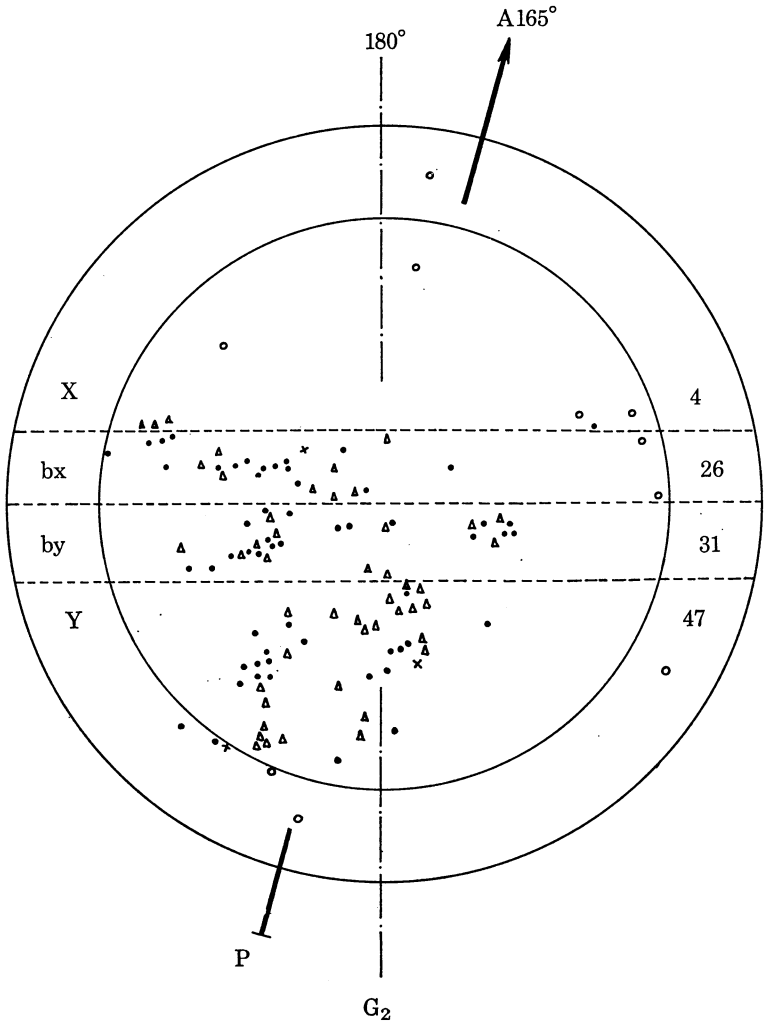


FIGURE 2, G_1 and G_2. Localization of the germ cells in two blastulae cultured for 36 h, with mechanical blockage of the endophyll. 180°, orientation axis (von Baer's law); P–A, probable embryonic axis; ●, gonocytes in the endophyll; △, gonocytes in the lacunar system; +, gonocytes in the ectomesoblast; ○, cells which could not be identified as p.g.c. with enough certainty; – – – –, outline of the endophyll. For the explanations and interpretation see text.

If each of the reconstituted germs G_1 and G_2 is cut into fragments X, bx, by and Y, the p.g.c. which have differentiated *in situ* in X and Y can be counted. Their relative proportions permit a calculation of the approximate orientations of the embryonic axis. Thus, G_1 would be orientated at 225° and G_2 at 165° (table 1; figure 2 G_1 and G_2). These orientations are further justified by the following observations: (i) the topographical reconstitution of the endophyll

shows an outline which corresponds perfectly to the well-known patterns of cell movements affecting that deep embryonic layer during the pregastrular period; (ii) the embryonic axis is the geometrically symmetrical axis of the fertile zone (G_1); (iii) the sectioning of the germs perpendicularly to the embryonic axis (in the case of coincidence between the symmetry axis and the orientation axis) permits one to calculate that the orientations of G_1 and G_2 would have been respectively estimated at 195 and 175° (instead of 180°). It appears therefore that, for each germ, the schema of an intermediate fertile zone perpendicular to the embryonic axis and of clear posterior predominance is the most plausible.

TABLE 1. NUMBERS OF DIFFERENTIATED P.G.C. IN FRAGMENTS OF BLASTULAE AFTER 48 h *IN VITRO* CULTURE

sample	fragments	total	relative percentages of gonocytes in X	relative percentages of gonocytes in Y	orientation of the embryo
	extrapolation for G_1 and G_2 (see text)				
L G 53	X bx 18 70 by Y 61 88	237	17	83	150° or 210°
	55%				
L G 54	0 6 109 98	213	0	100	180°
	55%				
L G 56	2 84 49 40	175	5	95	170° or 190°
	76%				
	extrapolation for G_1 and G_2				
(*a*) fragments perpendicular to the orientation axis					
G_1	X bx 30 87 by Y 84 95	296	24	76	135° or 225°
	57%				
G_2	4 26 31 47	108	8	92	165° or 195°
	53%				
(*b*) fragments perpendicular to the embryonic axis					
G_1	A bA 8 80 bP 110 P 98	296	(7.5)	(92.5)	180° (195°)
	64%				
G_2	1 24 32 51	108	(2)	(98)	180° (175°)
	52%				

Do these observations apply to birds in general? It really seems that they do, in spite of a slight divergence between the results of Fargeix and Rogulska on the one hand, and our own observations on the other hand; a divergence which most probably rests upon no more than the differences in the techniques used. Fargeix and Rogulska report a geometrical and numerical dissymmetry of the germinal crescents in regulation embryos obtained by means of median transections parallel to the embryonic axis of the blastula (see figure 3, 1g to 5d). In our opinion, the numerical dissymmetry which has been observed supports the concept according to which in birds the majority—if not all—of the p.g.c. arises in the central and posterior regions of the non-incubated germ. It is conspicuous that the geometrical asymmetry results from the slowing down or arrest, along the fissuration line, of the postero-anterior movements of the

endophyll. The fact that a very marked, and sometimes considerable numerical asymmetry (figure 3: 3d, e, 5d) occurs together with a geometrical dissymmetry proves that the deep layers in which the caudo-cephalic progression has been slowed down or blocked contain an appreciable number of p.g.c. In other words, the results obtained with the fissuration technique permit us to think that there is a direct relation between the degree of numerical asymmetry in the germinal crescents of parallel regulation embryos and the degree of fertility in the central and posterior endophyll of the non-incubated germ. This hypothesis also has the advantage of helping us to understand why, during *normal* development, the anterior germinal crescent contains more

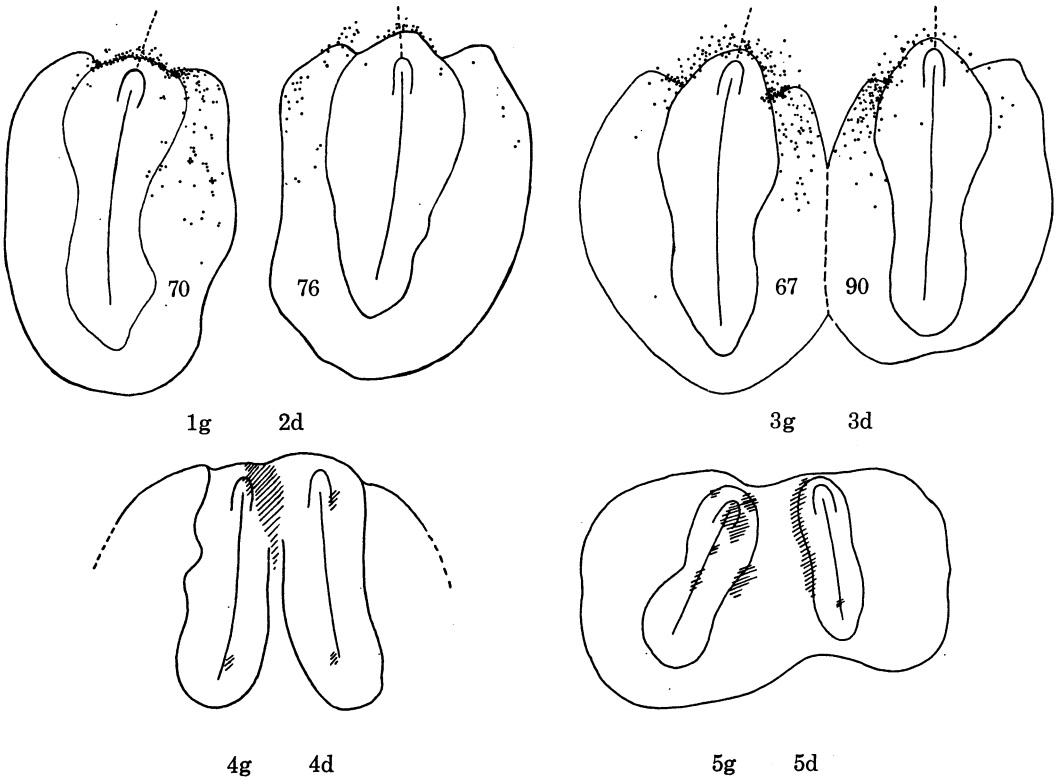


FIGURE 3. Localization and numerical distribution of the p.g.c. in regulation embryos obtained after median transection, parallel to the embryonic axis. 1g, 2d, 3g and 3d: embryos originating from the right (d) and left (g) lateral halves (according to Fargeix, thesis). Notice the coincidence between the geometrical asymmetry of the germinal crescents and the numerical asymmetry. The percentages indicate the approximate degree of the numerical asymmetry which is in favour of the side turned towards the fissuration line. 4g and 4d; 5g and 5d: same experiment as before (according to Rogulska). The hatched zones indicate the distribution areas of the p.g.c. The geometrical and numerical asymmetry is even more striking than in the examples described by Fargeix. Although these results are not numerical one can see that the degree of numerical asymmetry is sometimes near 100% (5d for example) (for explanation see text).

p.g.c. in the central part. Summing up, it does not seem that in the non-incubated embryo the p.g.c. are homogeneously distributed over the whole blastula; nor are they localized all around the germ wall (Vakaet's hypothesis). Instead they seem to be in a position which is intermediate, central, between the posterior localization of the chelonian type and the anterior localization of the avian type.

The fact that this temporary intermediate localization shows a clear posterior predominance has led us to carry the primary origin of the p.g.c. back by extrapolation to the posterior blastomeres of the morula, which have to be considered as the most vegetative. In this connexion

we would like to recall briefly that the localization of the germinal plasma at the vegetative pole of the anuran amphibian egg results from a reorganization and symmetrization of the fertilized egg under the influence of gravity. By analogy we may then suppose that the posterior localization of the p.g.c. in amniotes results from the effect of the gravitational forces and of the frictional forces that are developed during the rotation of the egg in the oviduct. These forces are most probably very subtle, but we know that they influence the germ since they are sufficient to print in an orientated axis of symmetry (Vintemberger & Clavert 1959; Wolff 1969).

The concept of a strict posterior localization of the p.g.c. in birds obviously remains hypothetical. It has, however, a certain number of advantages over the theories based on a diffuse or peripherical origin of the germ line. Indeed, in the case of a scattered distribution of the p.g.c. in the germ of Sauropsidea, one has to conceive of caudocephalic morphogenetic movements which bring them together in the anterior region. Such movements have in fact, been described in lacertilians and in birds. But, to explain the strict posterior localization observed in chelonians and some lacertilians one would also have to imagine movements in exactly the opposite direction. Such movements however have never been described. In contrast, the hypothesis of the posterior origin satisfactorily accounts for the extreme diversity which is observed in the localization of p.g.c. in sauropsidean germs, without having to advance an additional theory concerning the direction of the morphogenetic movements occurring during the very early stages of embryonic development. It also permits the connexion of birds with other groups in which the posterior origin of p.g.c. in the most vegetative blastomeres of the very early germ has been convincingly established, and contributes to the formulation of a unitarian theory of the germ cell line in vertebrates.

The germinal epithelia

The stimuli which determine that a given region of the coelomic epithelium differentiates into the genital primordia are unknown. In the chick embryo that region is approximately localized between the 22nd and 28th somites and can be recognized by the fact that it contains p.g.c.

Because she believed in a mechanical retention of the p.g.c. in that region of the blastoderm, Dantchakoff (1932) asserted that the gonocytes were the primary inducers of gonadal differentiation. Although most fascinating, this concept has been abandoned. Simon (1960), and most recently Reynaud (1969), have experimentally demonstrated that in completely sterilized embryos, the gonads of both sexes differentiate normally, in the absence of any germinal element.

In fact, the situation is exactly opposite: it is not because the gonocytes concentrate in a particular region of the embryo that it differentiates into a gonad, but because the germinal epithelia have already undergone profound changes and acquired several characteristics so as to become the presumptive sexual primordia that the gonocytes concentrate in the vicinity and invade them.

A study of the germinal epithelia is currently being carried out. The present paper is limited to some observations that have been made during a structural and autoradiographic study under the electron microscope. The germinal epithelia are essentially characterized by their syncytial aspect. The cellular limits are not very precisely marked and the basal membrane is poorly, or not at all, represented. This disaggregated aspect is most conspicuous on the proximal side of the epithelium. It could be demonstrated that these ultrastructural features are due to both the lytic and phagocytic activities of the gonocytes (Cuminge & Dubois 1969*a*), and the excretory properties of the epithelial cells.

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In the epithelial cells one can observe the formation of protrusions which are filled with cytoplasm and then burst, thus pouring their contents outside. Study of the movement of the radioactivity bound to leucine, 3 h after a short pulse followed by a chase period in the presence of cold leucine, demonstrates that the epithelial cells synthesize proteins which are excreted via the protrusions (figure 5*a*) (Cuminge & Dubois 1969*b*). Thus protrusions are not simple cellular extensions, but their formation appears to be related to a particular mechanism. The hypothesis according to which the protrusions are supplied with a pool of substances, elaborated by the epithelial cell and then excreted by a mechanism of the merocrine type, has been investigated experimentally.

From kinetic studies it appears that the turnover of the cytoplasmic proteins takes place at two different rates, whereas only one rate is observed for the nuclear proteins (figures 4 and 5) (Dubois & Cuminge 1969).

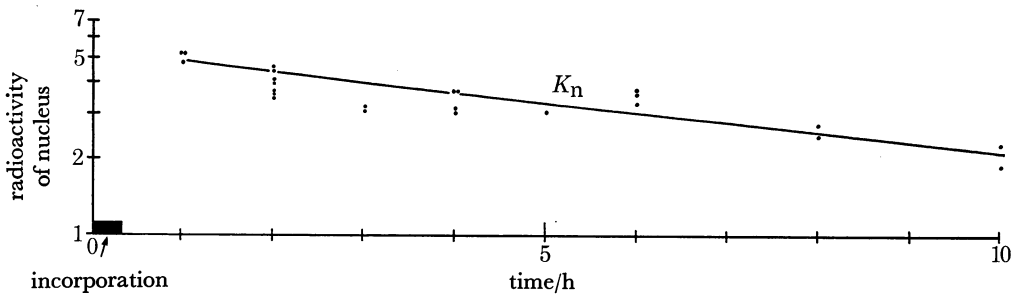

FIGURE 4. Turnover of the nuclear proteins in the germinal epithelium. If radioactivity is plotted logarithmically against time the experimental points yield approximately a straight line. One infers that at the selected time scale, the turnover of nuclear proteins is correctly described by a simple decreasing exponential, the turnover rate being K_n. One single kinetic process is involved.

Turnover of the nuclear proteins. Under the experimental conditions used, only one rate of turnover (K_n) has been observed, and only one protein pool has been found in the nucleus (figure 4).

Turnover of the cytoplasmic proteins. The analysis of the experimental data demonstrates that the turnover of the cytoplasmic proteins actually represents the sum of two exponential terms:

$$A_t = A_{0,s} \exp(-K_s t) + A_{0,e} \exp(-K_e t)$$

K_s and K_e representing the turnover rates of two different kinetic processes, A being the measured activity, $A_{0,s}$ and $A_{0,e}$ being the initial activities of the 2 distinct pools, calculated by extrapolation (figure 5*b*).

The biological interpretation of these data is that the straight line with the smallest slope can be considered as representing the turnover kinetics of the cytoplasmic sedentary or structural proteins. This pool is characterized by a low turnover rate K_s. The steeply sloping line (the value of K_e is about 30 times that of K_s) obtained by calculating the differences between the experimental and extrapolated values (figure 5*b*) most probably corresponds to a category of proteins which are rapidly synthesized by the cell and excreted by a merocrine-type process. This second cytoplasmic pool represents *exportable* proteins, synthesized in the cytoplasm, and excreted via the cellular protrusions. This interpretation is further supported by the close time correspondence between the migration of the radioactivity wave in the protrusions (figure 5*a*) and the rapid turnover kinetics.

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In conclusion, the germinal epithelia of the chick embryo have to be considered as secretory and excretory organs. The epithelial cells of the early gonadal primordia elaborate exportable cytoplasmic proteins which are rapidly excreted by a mechanism of the merocrine type. Can other substances than proteins be excreted by the germinal epithelia? The great complexity of the material constituting the protrusions on the one hand, and the probable role of the Golgi apparatus on the other hand, stress the necessity to augment this preliminary investigation by using more specific precursors than an essential amino acid.

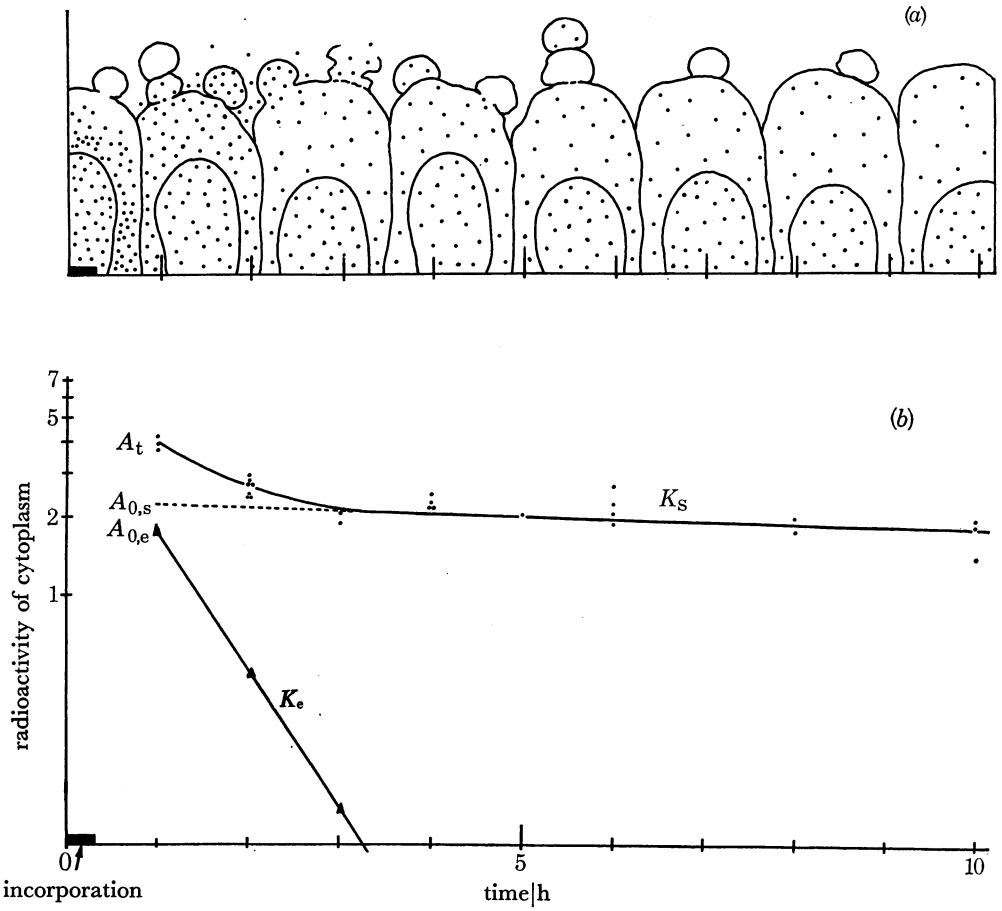

FIGURE 5. Turnover of the cytoplasmic proteins in the germinal epithelium. (*a*) Migration of the radioactivity in the cellular protrusions after a short pulse with [^{3}H]leucine. About 1 h after incorporation, radioactive proteins are expelled by a merocrine-type excretory mechanism (exportable proteins). The radioactivity associated with that category of proteins has almost disappeared after 4 h. (*b*) Turnover kinetics of the cytoplasmic proteins. If radioactivity is plotted logarithmically against time, the curve has a straight-line portion, which may be extrapolated to time 0, thus yielding the dashed line (– – – – –). By subtracting the extrapolated values from the experimental values, one obtains a second straight line (——▲——). Thus, the experimental curve may be considered to consist of two decreasing exponential components. The straight line with the lowest logarithmic decrement (K_s) represents the turnover kinetics of structural proteins. The kinetic process with a high turnover rate (K_e) corresponds to exportable proteins, which are rapidly excreted by the germinal epithelium. Notice the close time correspondence between the rapid turnover kinetics and the migration of the radioactivity wave in the cellular protrusions.

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Determination of early sexual organogenesis in birds and the entry of primordial germ cells into the gonadal primordia

The p.g.c. appear in the extra embryonic endoblast of the anterior germinal crescent. On the other hand, the somatic gonadal primordia differentiate in a precise and limited region of the posterior embryonic mesoblast. Sexual organogenesis can therefore only occur if the p.g.c. undertake a migration which leads them to the gonadal primordia. The mechanisms whereby the migration is determined in birds have been made evident and clarified by experiments *in vitro* (Wolff & Haffen 1952). In the chick the p.g.c. migrate through the vascular route and concentrate in the genital ridges (Simon 1960). This observation raises several questions and more particularly the following: how do p.g.c. enter the vascular network, and how do they leave it again?

Mechanism of the penetration into the vascular network

Recent experiments (Dubois 1968*a*, 1969*a*) have shown that the p.g.c., located in the endoblast of the germinal crescent, penetrate into the vascular network by virtue of its proximity (figure 6, plate 7). They can also leave the endophyll spontaneously, and randomly invade a variety of tissues such as the skin (figures 7 and 8, plate 7), the liver, the lung (figure 9, plate 7), the mesonephros (figure 10, plate 7) or any other embryonic tissue (figure 11, plate 7). It follows that, if the vascular network exerts an attraction upon the p.g.c. of the endophyll, that attraction is not specific.

In contrast, if these different tissues are associated *in vitro* with early undifferentiated gonads ($5\frac{1}{2}$ to 6 days incubation), the primary gonocytes do not leave their gonadal environment and remain inside the gonad (figures 12 and 13, plate 7). The primary gonocytes of the early organized gonad do not invade the heterogeneous tissues which are invaded by the p.g.c. of the endophyll.

Mechanism of the retention of the germ cells in the vicinity of the genital primordia

The embryonic circulation plays an essential role in the movement of p.g.c. to the gonadal primordia (Simon 1960). However, it has been demonstrated that although the configuration of the vascular network, i.e. the dimension of blood vessels and blood flow, can influence the positioning of the p.g.c. it does not determine this positioning (Dubois 1968*b*).

The p.g.c., carried by the blood stream, seem to be subjected to two forces: the blood flow, and the attraction exerted by the gonadal primordia. When conditions dependant upon the dimension of blood vessels and blood flow are favourable, the attractive stimulus becomes predominant and orientates the migration of the p.g.c., which leave the embryonic vascular network (figure 14, plate 8).

Attraction of the germ cells by the early gonadal region cultured in vitro

The following associations have been realized *in vitro*:

sterile gonadal region + fertile germinal crescent;
sterile gonadal region + fertile undifferentiated gonad;
sterile gonadal region + embryonic testis;
sterile gonadal region + embryonic ovary.

In all these experiments, except the last one, the germ cells leave the fertile graft and invade the sterile germinal epithelia. The autonomous migration of the gonocytes is particularly well

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illustrated in figure 15 (plate 8) where a [^{3}H]thymidine labelled gonad is associated with a sterile germinal epithelium; the radioactive gonocytes leave the graft and penetrate into the non-radioactive germinal epithelium of the host. The numerical data of the different experimental series are summarized in table 2.

The *in vitro* culture technique eliminates all influence of the vascular system and realizes artificially the conditions for an interstitial migration. It permits a demonstration that in birds the p.g.c. migrate by active amoeboid movements towards the sexual primordia as soon as they come into the vicinity of the latter. The hypothesis according to which the configuration of the vascular system is the only force operating in determining the retention of p.g.c. in the genital primordia has definitely to be abandoned.

TABLE 2. NUMERICAL RESULTS (MEAN VALUES) OF THE ASSOCIATIONS *IN VITRO*

	controls (degree of sterility)		experimentals (degree of entry of the p.g.c. into the host)				
				number of gonocytes in			
fertile grafts	number of embryos	number of gonocytes	number of embryos	gonadal region	coelome	g.e.	total
p.g.c. (germinal crescent)	22	1.2	19	10	0	19.6	29.6
gonocytes I (gonads of 5 to 6 days incubation) series 1	12	0	12	18	0	26	44
series 2	8	0	8	0	24	86	110
spermatogonia							
7.5 days	2	1	2	—	14	30	44
8 days	3	0	3	—	14.5	30.5	45 } 46
12 days	2	0	2	—	9	40	49
ovogonia							
7.5 days	4	0	4	—	7	4	11
8 days	4	0	4	—	1	0	1 } 5
12 days	3	0	3	—	0	3	3

Note Gonocytes I: series 1, the early gonad is placed against the gonadal region of the host; series 2, the graft is introduced into the coelomic cavity of the host, against the germinal epithelium (g.e.).

After they have settled down in the embryonic gonad, the primary gonocytes maintain their migratory ability for several days. The migratory power persists in the male germ line (plate 8), while it diminishes and tends to disappear in female germ cells from the eighth day of incubation onwards (figure 17, plate 8).

On the other hand, one can see that in the case of primary gonocytes, the mode of association influences the total number of primary gonocytes that have migrated (110 against 44, see series 1 and 2 in table 2). The mean number of gonocytes which have colonized the germinal epithelium is much higher if the association is made on the side of the coelome (86 gonocytes against 26). This observation shows that a close association between the attractive germinal epithelium and the graft favours the attraction and suggests that the attractive power has to be attributed only to the germinal epithelium.

The fact that an early germinal epithelium promotes the exit of primary gonocytes out of an early gonad of five days incubation proves *a contrario* that the attractive power decreases, or even disappears, before the end of the fifth day of incubation. Recently, Reynaud (1969), using completely different techniques reached the same conclusions.

Finally it has to be stressed that the attraction is exerted *at a distance* and that it results in an

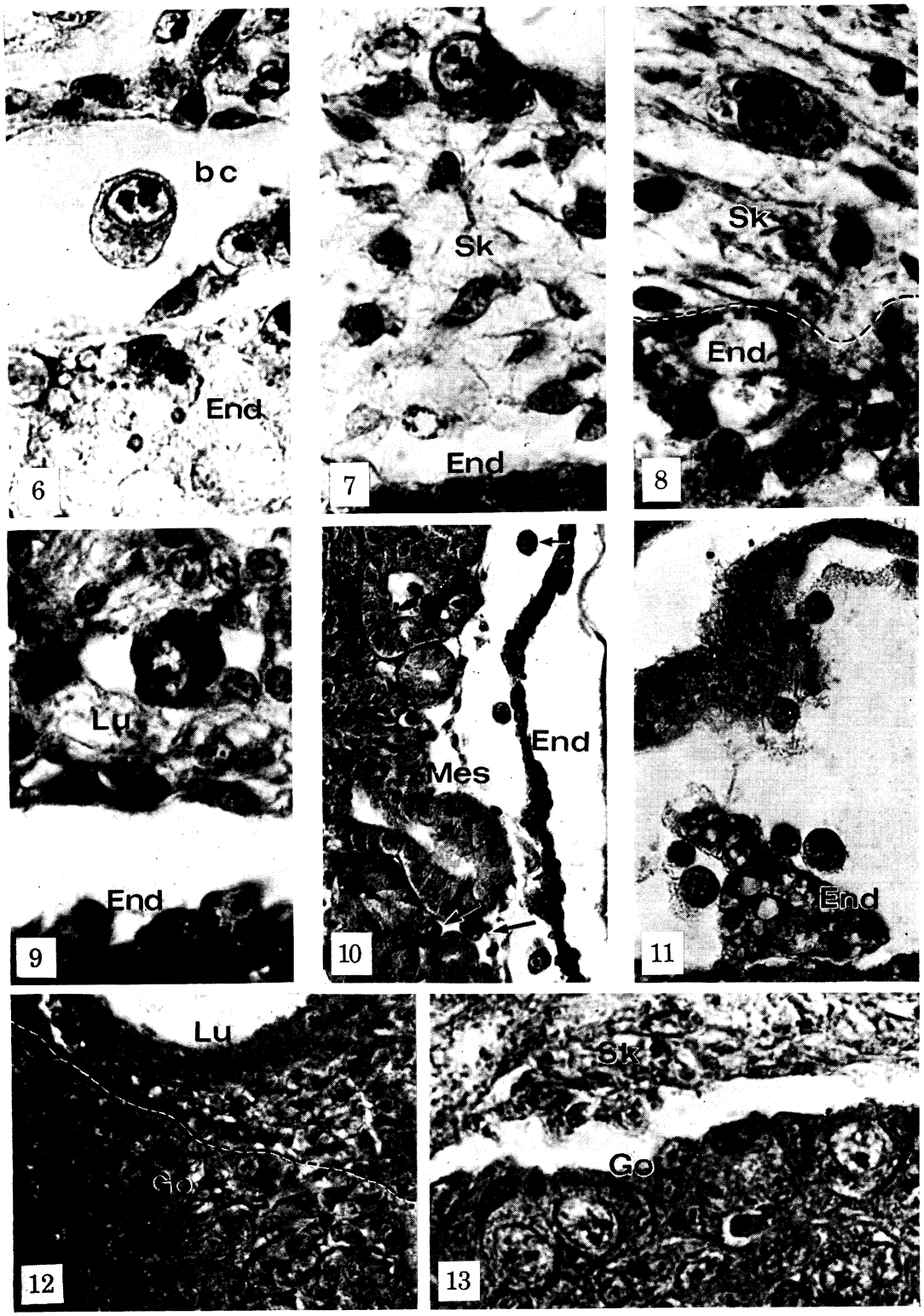


FIGURE 6. P.g.c. located in the anterior germinal crescent. The germ cell has left the endophyll (End) and floats in a newly developing blood vessel (bc) (normal development) (magn. × 800).

FIGURES 7 to 13. Heterogenous associations *in vitro*.

FIGURES 7, 8. P.g.c. invading the subdermal and dermal layers of the skin (Sk) from a 9-day-old embryo. Notice the lipid complexes (figure 7) and the vitelline globules (figure 8) in the cytoplasm of the gonocytes (magn. × 800).

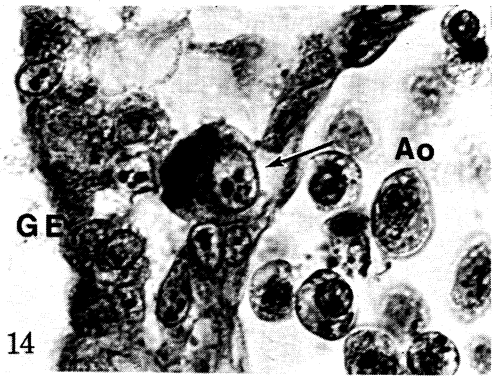
FIGURE 9. P.g.c., rich in sudanophilic cytoplasmic inclusions, and which has invaded the pulmonary mesenchyme (Lu) (magn. × 800).

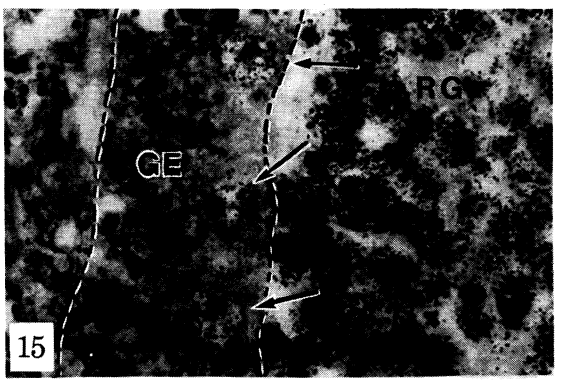
FIGURE 10. P.g.c. leaving the endophyll (End) and invading the mesonephros (Mes) (magn. × 200).

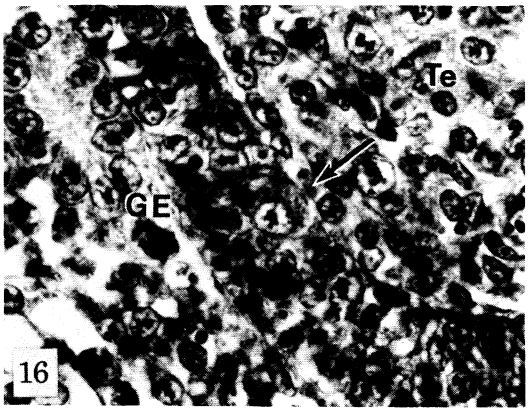
FIGURE 11. P.g.c. leaving the vitelline endoblast (End) and migrating towards the embryonic layers (fragments of blastulae cultured for 38 h) (magn. × 290).

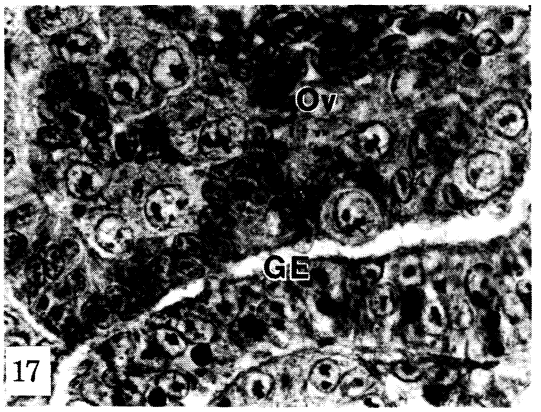
FIGURES 12, 13. The lung (Lu) (figure 12) and the skin (Sk) (figure 13) from 9-day-old embryos, are not invaded by the primary gonocytes of the morphologically undifferentiated gonad (Go). In contrast, the primary gonocytes leave the gonad and penetrate into the early germinal epithelia (see figure 15; plate 8). (figure 12: magn. × 320; figure 13: magn. × 800).

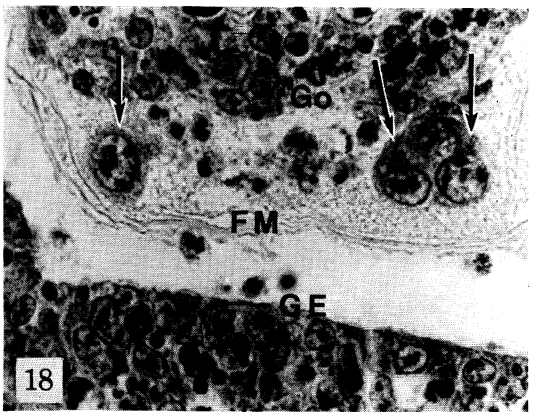
(*Facing p.* 84)











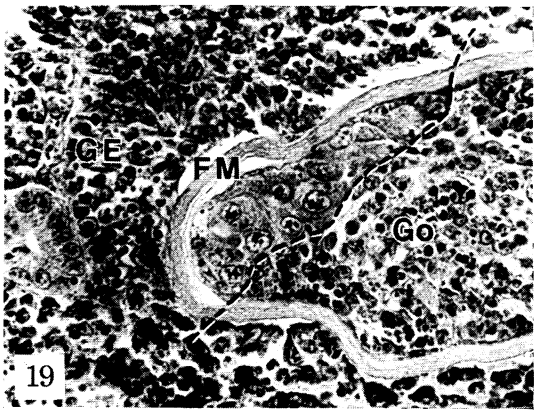


FIGURE 14. P.g.c. crossing the endothelium of the aorta by diapedesis in the vicinity of the gonadal region; the near germinal epithelium (GE) will be reached by amoeboid movements (magn. × 650.)

FIGURES 15 to 19. Associations with the early gonadal region in *in vitro* culture.

FIGURE 15. Association of an early radioactive (TdR–^{3}H) gonad with a non-radioactive gonadal region. Three labelled gonocytes have left the radioactive graft (RG) and have penetrated into the non-radioactive germinal epithelium (GE) of the host (magn. × 650). The germinal epithelia attract the primary gonocytes whereas the lung and the skin do not (see figures 12 and 13; plate 7).

FIGURE 16. Association with a fragment of testis (Te) of 8 days incubation. A spermatogonia has settled down in the germinal epithelium (GE) of the previously sterilized host (magn. × 600).

FIGURE 17. Association with a fragment of the left ovary (Ov) of 8 days incubation. Several ovogonia are in a favourable position to be attracted by the germinal epithelium (GE). However none has invaded the gonadal primordium (magn. × 600). The penetration of an ovogonia into the germinal epithelium of the host is uncommon (see lower part of table 2).

FIGURE 18. Use of the egg vitelline membrane as a permeable barrier: the primary gonocytes leave the graft on the side which is turned towards the germinal epithelium (GE) of the host, but their progression is stopped by the vitelline membrane (FM) (magn. × 650).

FIGURE 19. Same experiment as in figure 18; the attraction exerted by the germinal epithelium (GE) reassembles the gonocytes against the membrane (FM) and leads to an almost perfect separation between the germinal and somatic elements of the gonad (magn. × 350).

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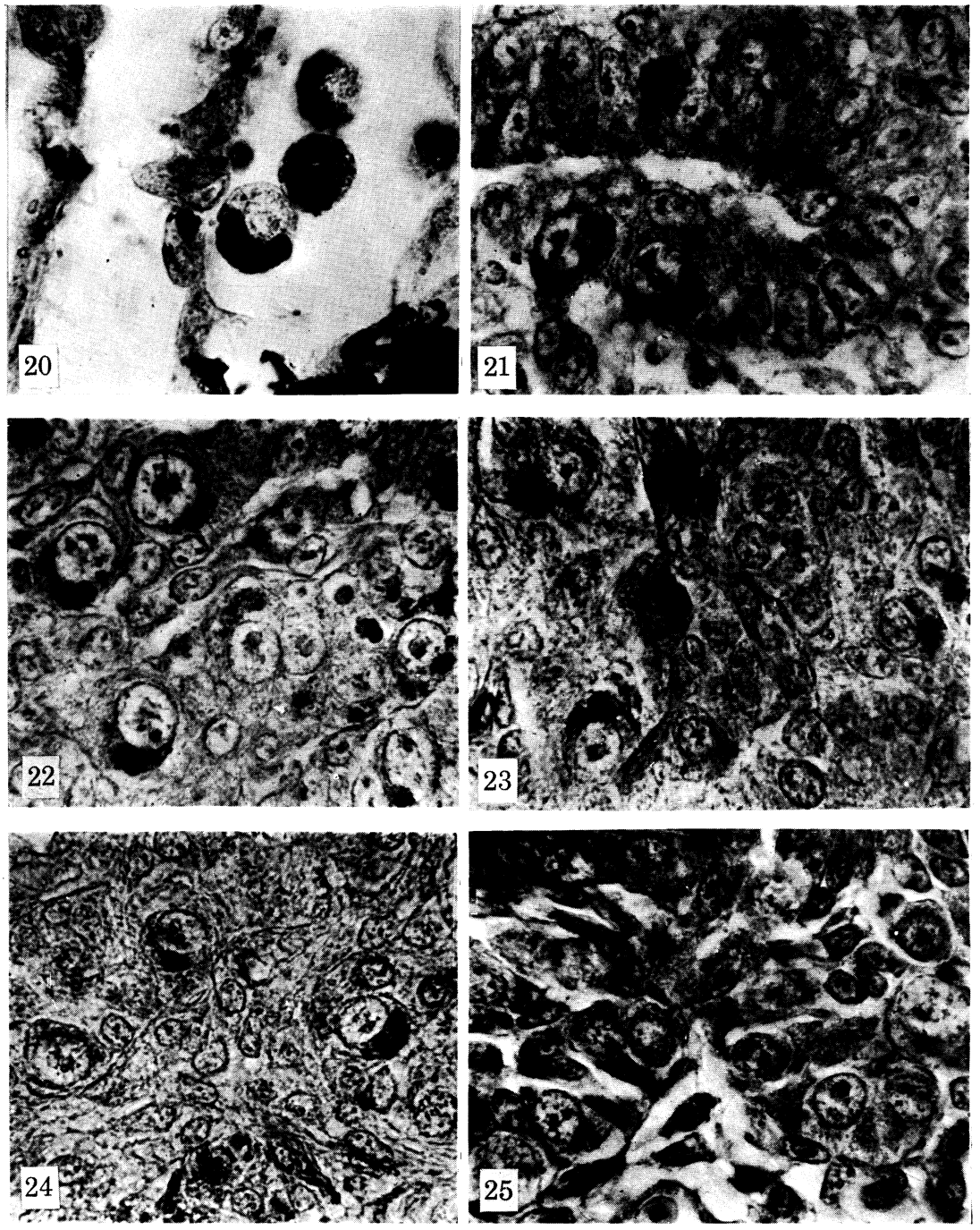

FIGURES 20 to 25. Histochemical differentiation of the germ cells (evolution of the lipid complexes).

FIGURE 20. Stage of the germinal crescent—important concentration of lipid complexes in the cytoplasm of three p.g.c. (magn. × 960).

FIGURE 21. 3½-day-old embryo. Sudanophilic cytoplasmic inclusions in the p.g.c. which have settled in the germinal epithelia. The reaction is weaker than figure 20 (magn. × 960).

FIGURE 22. Morphologically undifferentiated gonad (6 days incubation): the sudanophilic lipid droplets mark the primary gonocytes. The amount of lipid complexes is usually less important than at earlier stages (magn. × 960).

FIGURES 23, 24. Testes of 8 days (figure 23) and 14 days (figure 24) incubation. An appreciable amount of lipid complexes can still be seen in the cytoplasm of the spermatogonia (magn. × 960).

FIGURE 25. Left ovary of 8 days incubation: none of the cortical ovogonia is Sudan Black positive (magn. × 960).

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autonomous and oriented migration of the primary gonocytes out of the non-dissociated graft. Furthermore, the experiments in which a radioactive tracer has been used demonstrate that only the primary gonocytes respond to the attractive stimulus.

Thus, the hypothesis of a selective specific chemotaxis seems to be the most satisfactory interpretation of these observations.

Chemotactic aspect of the attraction of the germ cells to the germinal epithelium and their penetration into it

If a permeable barrier, such as the egg vitelline membrane (Wolff 1961) or a film of agar, is placed between the host and the graft, the gonadal attraction is exerted across the barrier. The gonocytes leave the graft (figure 18, plate 8) or assemble at the pole of the graft which is turned towards the germinal epithelium (figure 19, plate 8).

Thus, the factor excreted by the germinal epithelia and responsible for the attraction is soluble and diffusible. It acts selectively upon the germ cells; a direct contact between the germ cells and the germinal epithelia is not necessary. The orientated amoeboidism of the p.g.c. towards the gonadal primordia is thus a phenomenon of chemotactic order. The hypothesis of a positive chemotaxis implies two complementary facts: (i) the germinal epithelia elaborate and excrete soluble and diffusible substances; (ii) the germ cells have a particular affinity for the products elaborated, which are, as a consequence, attractive.

The latter point has been verified by an ultrastructural study of the p.g.c., at the moment when they colonize the genital primordia (Cuminge & Dubois 1969*a*). During their penetration into the germinal epithelia, the primordial gonocytes absorb intensively the soluble substances by pinocytosis, essentially in the region of the pseudopodia, and ingest the cellular debris of the dissociated epithelium by phagocytosis.

Thus, the chemotactic activity of the p.g.c. explains their migration and their regrouping in the vicinity of the gonadal primordia; their pinocytotic and phagocytic activity accounts for their penetration into the active epithelia. The behaviour of the p.g.c. at the time of their arrival in the sexual primordia perfectly agrees with the hypothesis according to which their migration has been guided by positive chemotaxis.

Differentiation of the germ cells and sexual organogenesis: general conclusions

Several attempts have been made to find a morphological or cytochemical characteristic that would specifically apply to the p.g.c. These attempts have been unsuccessful.

However, at later stages, the differentiation of the germinal cell line is marked by changes at the ultrastructural and histochemical levels and by modifications of certain physiological properties such as their amoeboidism. These changes seem to be related to the progress of sexual organogenesis and to the differentiation of both sexes.

Ultrastructural and histochemical differentiation

It is possible to identify with certainty the p.g.c. of birds at the stage of appearance of the first somites. They are usually described as large rounded cells (15μm diameter), with a clear and swollen nucleus which is bigger than in somatic cells (about 9μm diameter), and frequently contains two nucleoli. The importance of the Golgi apparatus, the presence of a granular chondrioma and of vitelline globules has also been mentioned by different investigators. Furthermore, p.g.c. contain an important stock of glycogen which, by means of the PAS

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reaction, permits their identification in the germinal crescent as early as the intermediate streak stage (Reynaud 1967). Finally it has been recently demonstrated that the p.g.c. contain an important amount of cytoplasmic lipid complexes. After treatment with Sudan Black they are selectively marked and can thus be easily traced before and after their settlement in the gonadal primordia (figures 20 and 21, plate 9) (Dubois & Cuminge 1968). An ultrastructural study of the p.g.c. in the germinal crescent, and of the primary gonocytes at the time they install themselves in the gonadal primordia, has completely confirmed these findings. Moreover, some complementary information has been obtained: the nucleoli are reticulated; the Golgi apparatus is highly developed (dictyosomes) and shows a vacuolar system containing a secretion product: accordingly, it is most probably functional. In the cytoplasm, the mitochondria are small, rounded, with few cristae; the ribosomes are distributed at random and the ergastoplasm is poorly represented. At the time when they colonize the gonadal primordia, the p.g.c. show a very intense amoeboid, phagocytic and pinocytotic activity. Their cytoplasm takes a vesicular aspect which is due to the accumulation of pinocytotic vesicles, especially in the pseudopodial extensions. This situation remains practically unchanged in the primary gonocytes of the morphologically undifferentiated gonad. However, ultrastructural and histochemical studies permit the conclusions that there is an appreciable decrease in the amount of glycogen and lipids (figure 22, plate 9). In contrast to the observations made on the viviparous lizard (Hubert 1968) and although an appreciable number of germ cells has been examined, it has not been possible to detect in the chick embryo a structure in keeping with the migratory activity of the primordial gonocytes (fibrils or microtubules).

Differences between the structure of germ cells of the male and female

From the eighth day of incubation onwards it is possible to distinguish the spermatogonia from the ovogonia by several features.

In the spermatogonia, the nucleus is clear and limited by a sinuous membrane. The mitochondria are slightly elongated, but remain clear and contain very few cristae. The pinocytotic activity remains important and the cytoplasm maintains its vesicular aspect. The cytoplasmic lipid droplets are still visible at the eighth day (figure 23, plate 9) and fourteenth day (figure 24, plate 9) of incubation.

In the cortical ovogonia, the mitochondria are dense and elongated; they contain a great number of cristae and are grouped at one pole of the germ cell, where they participate in the building of Balbiani's vitellin body. From the eighth day of incubation onward, the cytoplasmic lipid droplets disappear in the cortical ovogonia (figure 25, plate 9). In contrast, the ovogonia which are located in the medullar zone maintain the characteristics of the primary gonocytes and of the spermatogonia, e.g. rounded, clear and small mitochondria with very few cristae; one also observes the persistence of lipid droplets. With the techniques used, glycogen was no longer detectable in the germ cells of both sexes after the eighth day of incubation.

Physiological differentiation of the migratory power of the germ cells

During sexual organogenesis the embryonic germ cells undergo very marked morphological and histochemical changes. They also undergo physiological changes concerning essentially the migratory power of these cells.

At early stages of embryonic development the p.g.c. have very extended migratory properties: they can leave the endophyll and invade the vascular network. *In vitro* experiments demonstrate

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that they can equally well penetrate into various other tissues such as skin, liver, mesonephros and lung. If during normal development they colonize the blood islands of the vascular network, which is just beginning to form it seems only by virtue of their proximity (figures 6 and 11, plate 7). However, the colonization of the genital ridges cannot be explained by reasons of proximity, nor can it be explained on a purely mechanical basis. What then is its determinism?

The study of the migratory properties of the primary gonocytes of the undifferentiated gonad (and not of the p.g.c.) permits an approach to the study of this problem. Indeed, the primary gonocytes have a latent migratory power. They can still manifest a chemotactic amoeboid activity if a young germinal epithelium is placed in the vicinity (figures 15, 18, 19, plate 8). However, at that stage, other tissues no longer exert any attraction upon the primary gonocytes which then remain inside the gonad (figures 12 and 13, plate 7). Thus, as soon as they have settled in the sexual primordia, a difference appears in the migratory properties of the germ cells. This physiological difference justifies a different denomination: from this point of view a primary gonocyte is not equivalent to a p.g.c.

The determinism of the behaviour of the germ cells during early sexual organogenesis may be explained by the adjustment of two complementary mechanisms: the specificity of the attraction and the selectivity of the response to that attraction. At the very early stages of development the primordial germ cells would be particularly sensitive, and various stimuli would be able to trigger their amoeboid activity (non-specific and selective chemotaxis). At slightly later stages only the germinal epithelium is capable of promoting a response in the primary gonocytes (specific and selective chemotaxis). However, it is important to underline that this difference in the migration mechanisms, which could be made conspicuous by the remarkable migratory power of the primary gonocytes, necessarily exists in the p.g.c., because they settle down in the germinal epithelia. To explain that situation, two hypotheses may be advanced: the p.g.c. progressively lose their primitive responsiveness and remain sensitive only to the attraction exerted by the germinal epithelia, or that the attractive power of the germinal epithelia is stronger than that of other tissues. We may also consider that, so as to insure maximum efficiency, the two phenomena occur together and that the two explanations are complementary.

Whatever the explanation, the results which have been described in the present paper clearly indicate that in future experiments concerned with the nature of the attractive stimulus and its mode of action, the primary gonocytes and not the p.g.c. will have to be used. Indeed, whereas p.g.c. are excited by several tissues (including the gonadal primordia), the primary gonocytes respond specifically to the stimulus only of the germinal epithelia.

Migratory power of spermatogonia and ovogonia

Whereas the spermatogonia maintain the property of responding to the attractive stimulus of the germinal epithelia, the ovogonia lose their responsiveness. This physiological difference between the male and female germ lines seems to be contemporary with the first morphological and histochemical signs of sexual differentiation. It also appears at the time when the first ultrastructural differences between ovogonia and spermatogonia, and particularly the disappearance of the lipid droplets in the cortical ovogonia, can be seen. The time correspondence between the disappearance of the lipids and the loss of the migratory power in the female germ line suggests that the lipid metabolism may play an important role in the migratory activity

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of germ cells in birds. As yet there is no experimental evidence to substantiate this hypothesis. However, the use of radioactive precursors as well as the study of specific enzymatic activities should prove very helpful in approaching the problem successfully.

General conclusion

The anterior localization of the p.g.c. and their migration by the vascular route have long been considered as being characteristic of birds.

The experimental evidence discussed in the present paper leads to a complete revision of that concept. The p.g.c. of birds have probably the same embryological origin and the same primary posterior localization as those of other vertebrates. Their anterior localization is secondary and results from the effect of the pregastrular and gastrular caudocephalic morphogenetic movements, which convey them towards the anterior part of the embryo. These postero–anterior movements have been described in many Sauropsidian embryos. It appears more and more clearly that the p.g.c. undergo these movements passively and that these early rearrangements are responsible for the scattered distribution of the p.g.c. in many lacertilians and for their anterior localization in birds and in the viper. It is tempting to compare these phenomena with the pregastrular movements of the endodermal mass which bring the primordial gonocytes to the floor of the archenteron in the early anuran gastrula.

At the end of this early migration, the p.g.c. of birds swarm out of the endophyll and invade the anterior vascular network. This attractive mechanism, selective but not specific, reminds one again of a similar happening in anurans: in this group it has been experimentally demonstrated that the mesodermal dorsal organs exert an attraction upon the primordial gonocytes located in the endodermal mass (Gipouloux 1964).

After that period of amoeboid activity, the p.g.c. undergo again a passive migration, of great magnitude, by the vascular route. As they arrive in the vicinity of the gonadal primordia, they recover an autonomous activity which is directed by a selective and *specific* chemotactic mechanism. The transit through the vascular route appears as an adaptive process, consequent upon the remoteness of the gonocytes. This interpretation has a good chance of being correct, since it has been verified that the same process has been adopted not only by birds, but also by some lacertilians and by the viper, where the p.g.c. are far away from the genital sphere. Furthermore, when the two modes of localization occur in the same embryo, the two types of migration co-exist (Rhyncocephalia). Thus, the transfer by the vascular route is also a secondary character. *The fundamental biological mechanism which presides over the migration and over the settlement of the germ line in the sexual primordia is of a chemotactic nature*. This mechanism has long been suspected in embryos where the posterior localization of the p.g.c. and the interstitial type of migration are the rule; but it has never been unequivocally demonstrated. It is remarkable that it is in an embryo where the migration of the p.g.c. is of the vascular type, where such a demonstration could be made. Furthermore, it is the experimental evidence obtained in a group considered as an exception which permits the formulation of a unitarian theory of the germ cell line in vertebrates.

During the last years several investigations have also yielded some information on the different steps of the differentiation of germ cells. Care has to be taken not to confuse *determination of the germ line* and *differentiation of the germ cells*. These two concepts have long been intimately associated: thus, the organisms known for the precocity of the segregation of their germ line (*Ascaris* and frog for example) are precisely those in which the germ cells are precociously

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differentiated by a particular character (maintenance of a full set of chromosomes, germinal cytoplasm). The investigations made in birds show that a germ line can be determined long before the p.g.c. can be identified by appropriate conventional techniques. Indeed, indirect methods permit one to assert the existence of the germ cells in the blastula, and to delineate their precise localization in the germ well before they can be directly recognized. Thus, for a given organism, one cannot conclude that a germ-cell line has not yet segregated, or segregates tardily, if the only criterion at hand is an identification of the germ cells which depends upon the accuracy and the efficiency of the techniques used. Finally it appears from the present study that the germ cells do not remain passive and unchanged during ontogenesis. They undergo profound ultrastructural, histochemical and physiological changes, and have to be considered as very highly specialized cells.

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Discussion on paper by R. Dubois & Y. Croisille, p. 73

A. K. Tarkowski: (*University of Warsaw*): According to Drs Dubois & Croisille primordial germ cells are originally located in the posterior region of the blastoderm, and their position in the anterior part is secondary. How do the observations of Fargeix and Rogulska, who transected duck blastoderms and found germ cells both in the anterior and the posterior part, accord with this conclusion? Their observations would imply that primordial germ cells can form from any part of the unincubated blastoderm.

R. Dubois: The observations made by Fargeix and by Rogulska on the one hand, and those

presented in the present paper on the other hand, seem contradictory. A first point I would like to stress is that the experimental procedures are different. The excision of a diametrical band is essential and constitutes an important technical modification. I would also like briefly to recall that, using the same technique as Fargeix and Rogulska (but in *in vitro* culture), I have obtained exactly the same results in the chick: the anterior and posterior halves of the blastula develop and are nearly equally fertile. The excision of a diametrical band completely modifies the results. This observation has to be taken into account, and calls for an explanation. I have proposed an interpretation which obviously is largely open to speculation and to criticism. Particularly, the possibility that certain fragments remain sterile because of their low evolutive potentialities *in vitro*, remained an open question. It seems to me that the experiment in which the endophyll is mechanically blocked, and the integrity of the germ completely maintained, meets that objection.

Finally, I would like to make some comments about the methodologies. Fissuration experiments, *in vitro* cultures or grafts of fragments of blastoderms lead, in this kind of problem, to the interpretation of numerical data. These data are derived from an artificially decomposed system, each point of which has been developed separately. From the data, obtained from each fragment at the time, one tries to derive the state of the system at the time 0, i.e. the time at which the experiment was performed. In the case we are interested in here, the numerical analysis applies to a definite cellular population (the germ cell line) which is distributed in an orientated system (the unincubated blastula). Consequently, whatever the mode of distribution of the p.g.c. in the blastula may be, and whatever the real stage of the latter, the relative numbers of differentiated gonocytes in the isolated fragments or in regulation embryos cannot, and should by no means be compared if the following two conditions are not fully met:

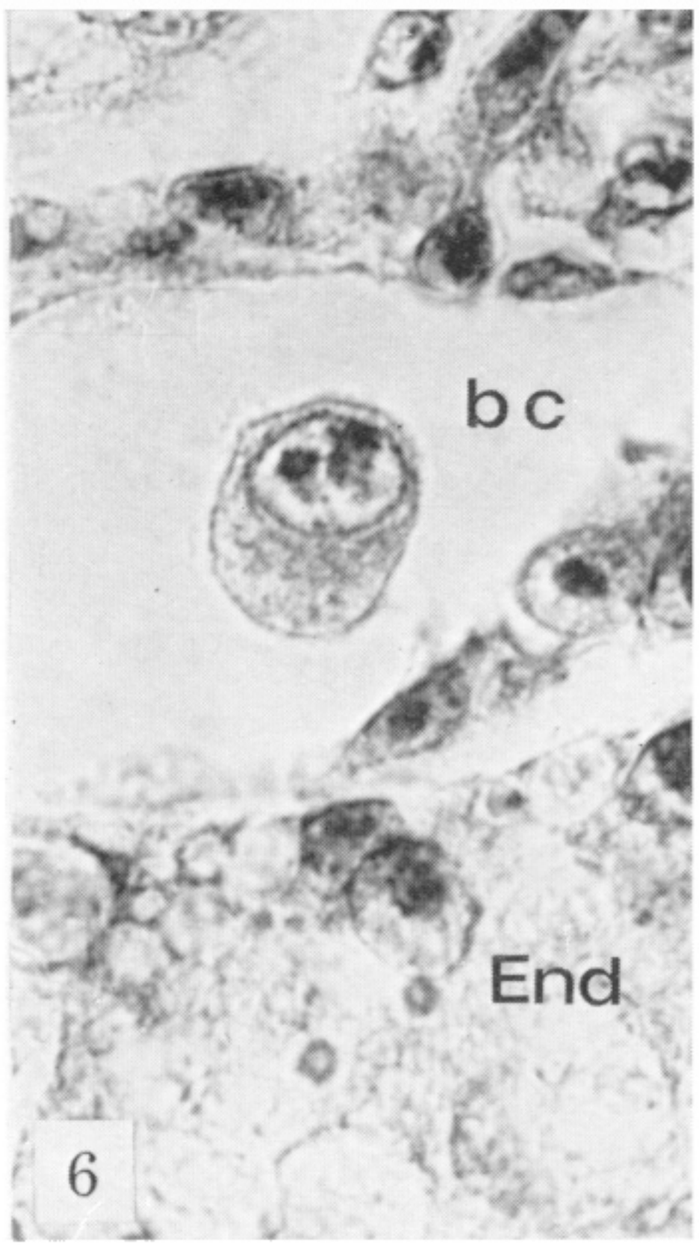
(i) The symmetry axis of the blastula has to be known (formally or statistically).

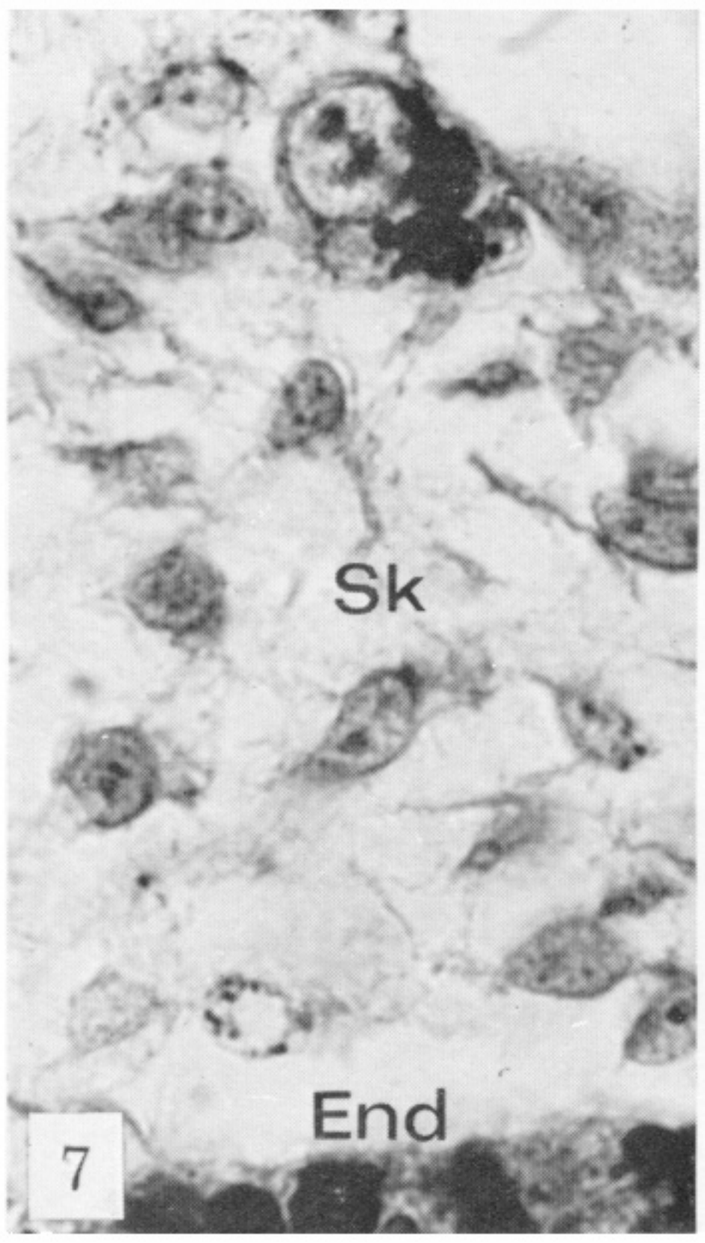
(ii) The numbers of the p.g.c. in the two incubated fragments have to be in the same ratio as the numbers of germ cells in these two fragments at the time the experiment is being done. The latter proposition requires the two fragments, or the two embryos, to develop during the same length of time, since the total number of cells in a cellular population at a given time is function of the multiplication rate, of the length of the incubation period, and of the initial number of cells. To estimate the last parameter, the first two must be kept constant by using strictly identical experimental conditions.

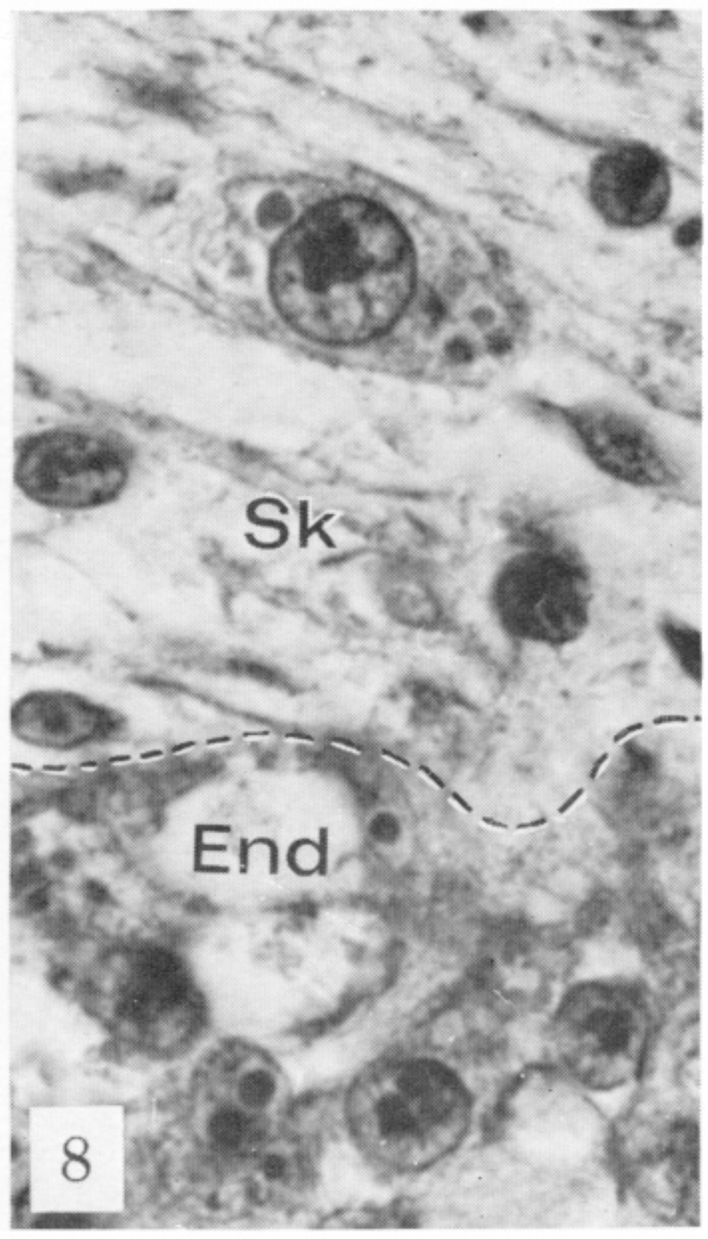
Concerning the *in vitro* cultures, it is important to notice that the two fragments come from the same blastula, and that they are separately cultivated, for the same length of time on the same medium. On the other hand, all the investigated blastulae are analysed and the embryonic axis is known from statistics using the conformity test to von Baer's law.

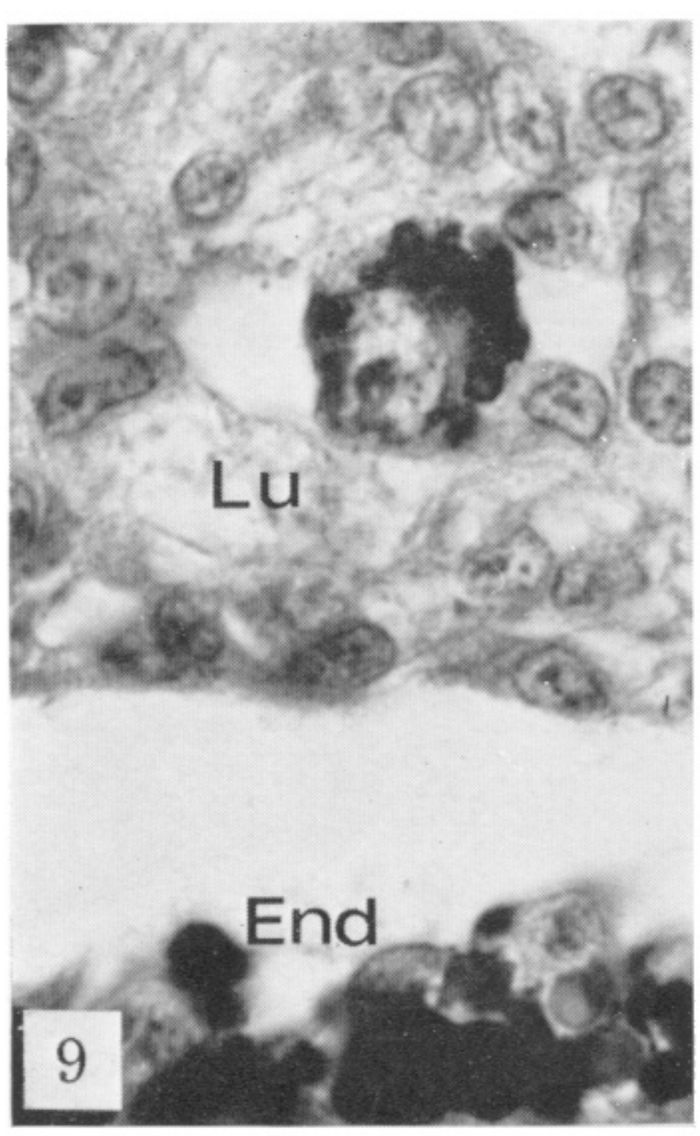
In the fissuration experiments, the comparison of the number of germ cells found in the anterior and posterior regulation embryos *of the same stage*, as well as the comparison of these numbers to the number of germ cells found in normal embryos of a corresponding stage, is not without certain drawbacks, since these different embryos reach identical degrees of development within different periods of time. Finally, an exact knowledge of the axis of symmetry of the transected blastula is illusory, since transection has an important effect on the orientation of regulation embryos.

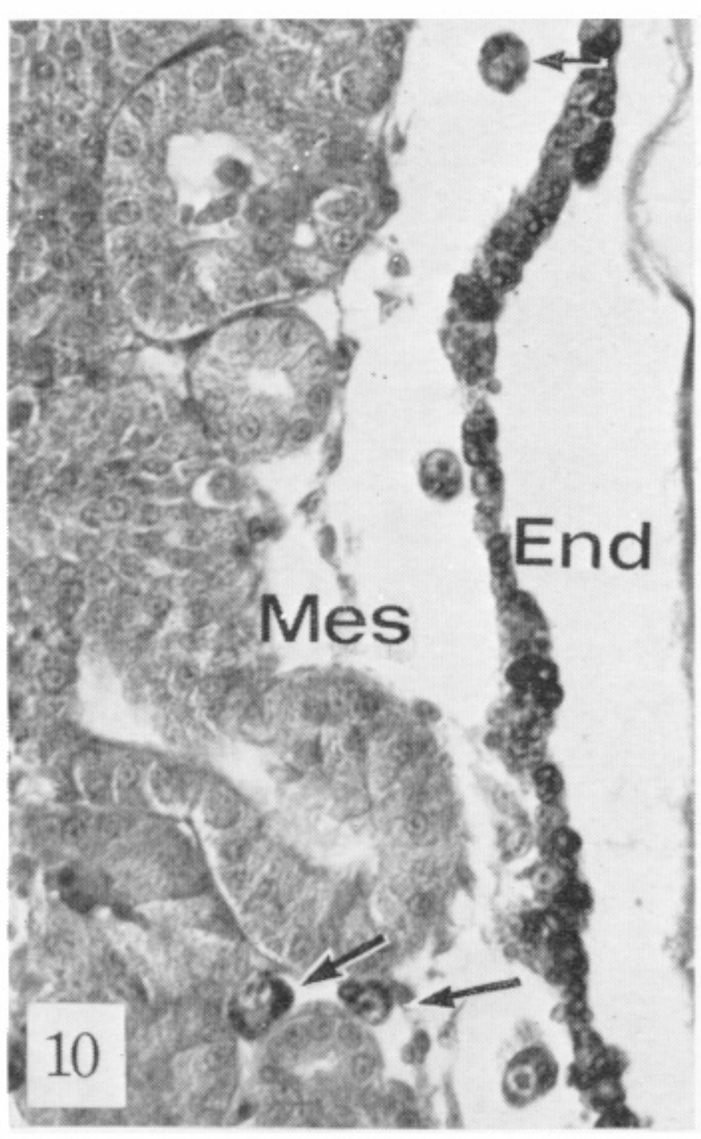
A. K. Tarkowski: The chemotactic attraction of the chick genital ridge is not class specific for it can attract mouse primordial germ cells, if those are experimentally placed in the coelom of the chick embryo (T. Rogulska, Ozdzenski & Komar, in preparation).











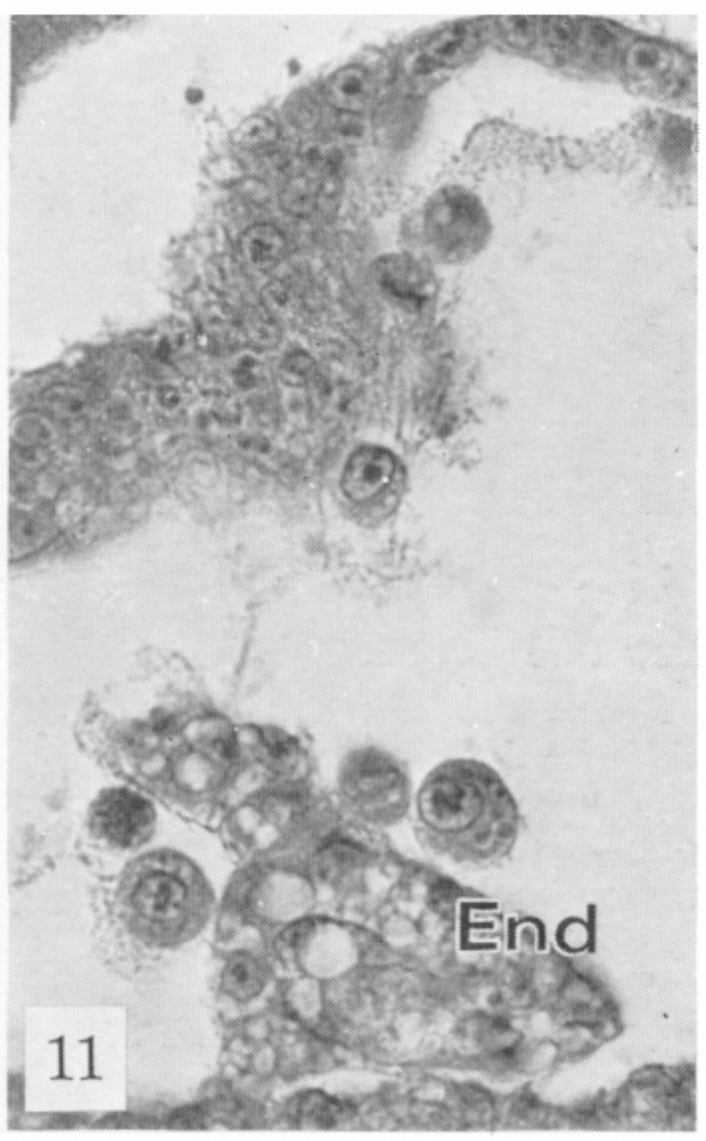


FIGURE 6. P.g.c. located in the anterior germinal crescent. The germ cell has left the endophyll (End) and floats in a newly developing blood vessel (bc) (normal development) (magn. × 800).

FIGURES 7 to 13. Heterogenous associations *in vitro*.

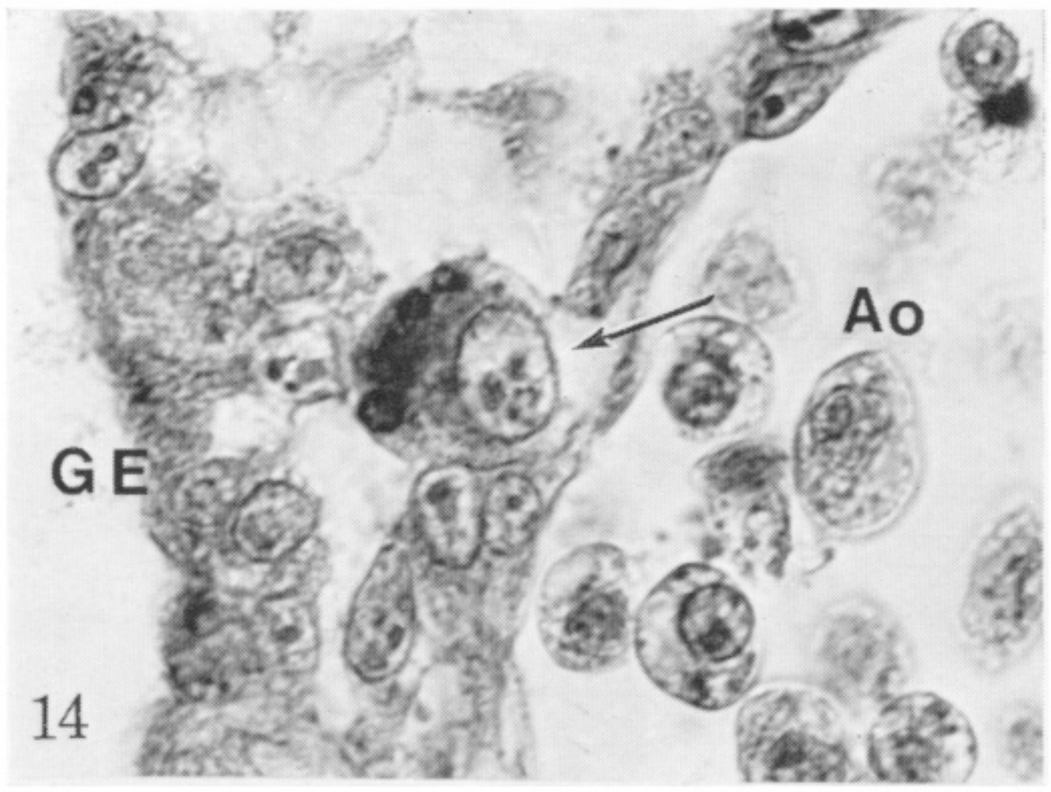
FIGURES 7, 8. P.g.c. invading the subdermal and dermal layers of the skin (Sk) from a 9-day-old embryo. Notice the lipid complexes (figure 7) and the vitelline globules (figure 8) in the cytoplasm of the gonocytes (magn. × 800).

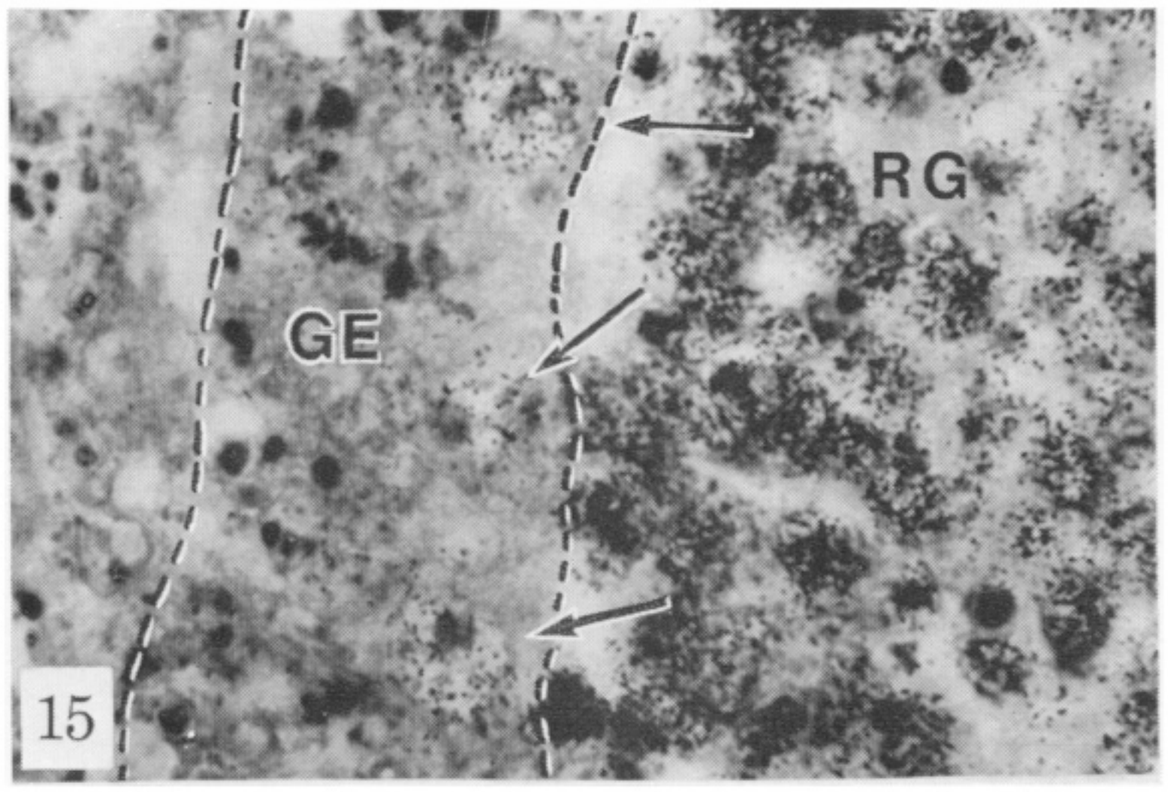
FIGURE 9. P.g.c., rich in sudanophilic cytoplasmic inclusions, and which has invaded the pulmonary mesenchyme (Lu) (magn. × 800).

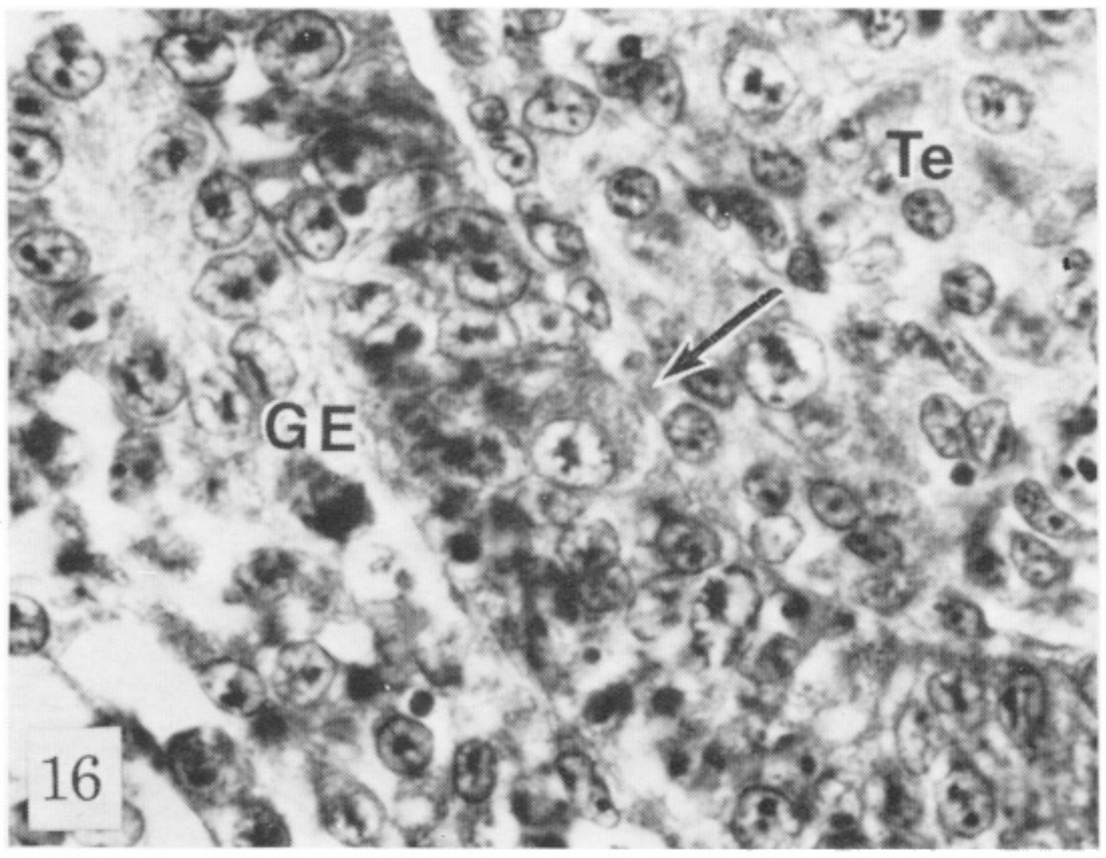
FIGURE 10. P.g.c. leaving the endophyll (End) and invading the mesonephros (Mes) (magn. × 200).

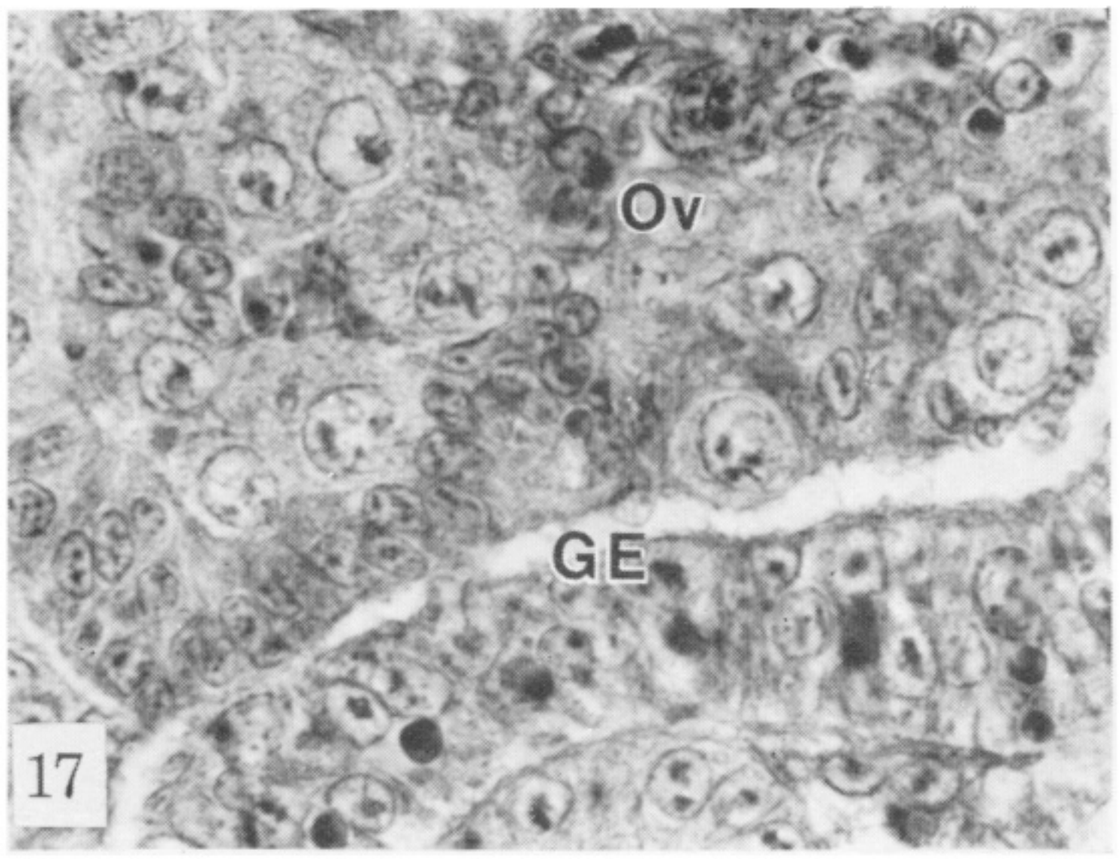
FIGURE 11. P.g.c. leaving the vitelline endoblast (End) and migrating towards the embryonic layers (fragments of blastulae cultured for 38 h) (magn. × 290).

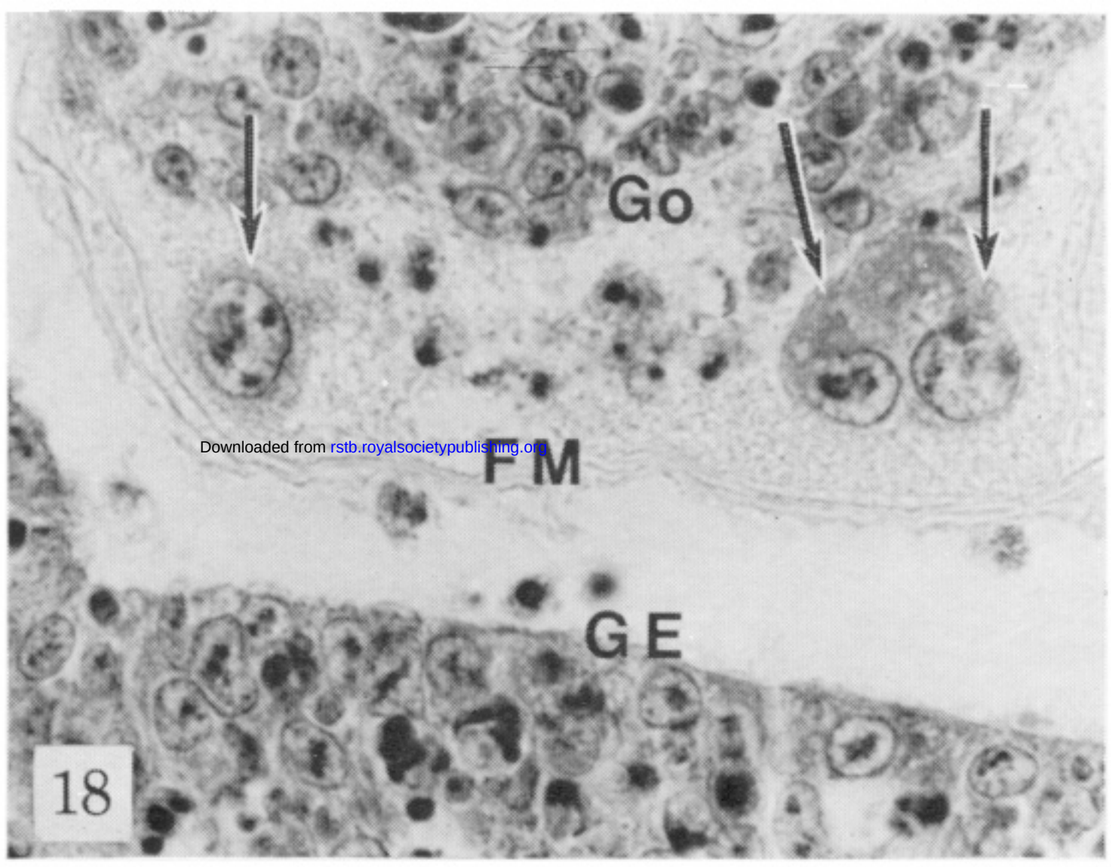
FIGURES 12, 13. The lung (Lu) (figure 12) and the skin (Sk) (figure 13) from 9-day-old embryos, are not invaded by the primary gonocytes of the morphologically undifferentiated gonad (Go). In contrast, the primary gonocytes leave the gonad and penetrate into the early germinal epithelia (see figure 15; plate 8). (figure 12: magn. × 320; figure 13: magn. × 800).











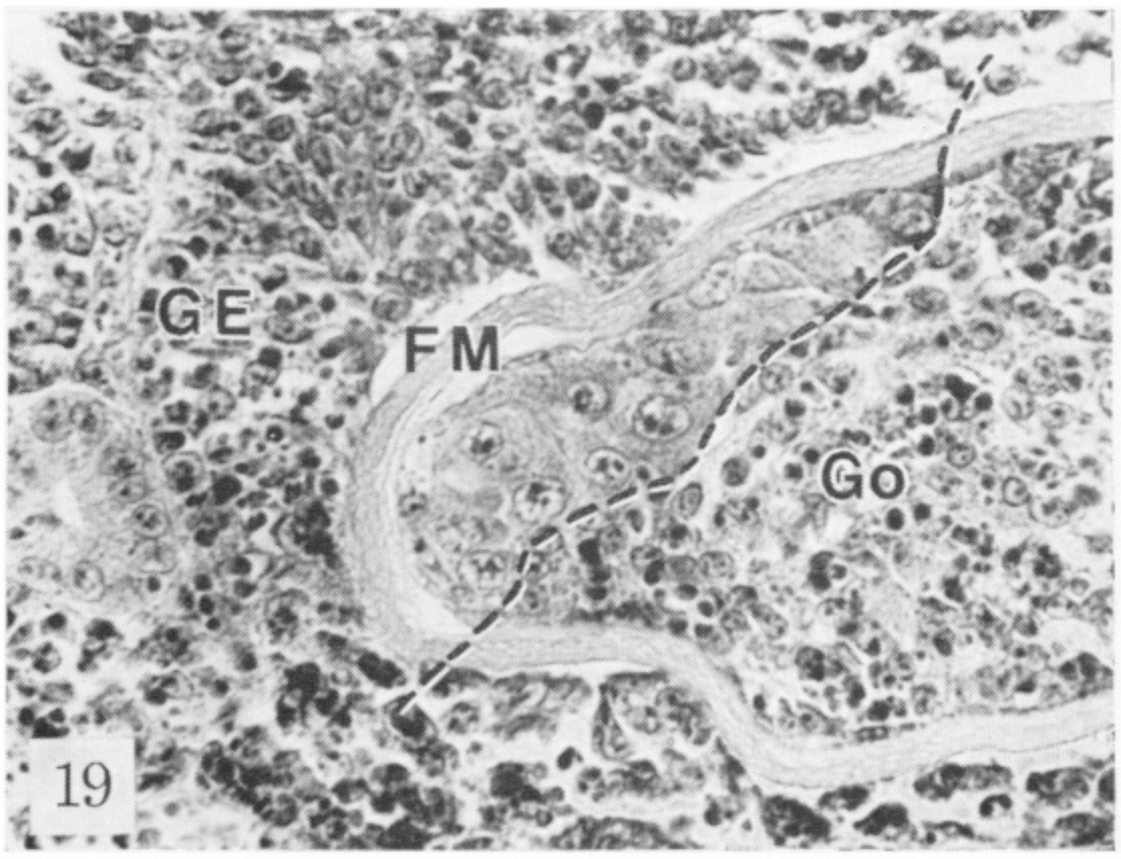


FIGURE 14. P.g.c. crossing the endothelium of the aorta by diapedesis in the vicinity of the gonadal region; the near germinal epithelium (GE) will be reached by amoeboid movements (magn. × 650.)

FIGURES 15 to 19. Associations with the early gonadal region in *in vitro* culture.

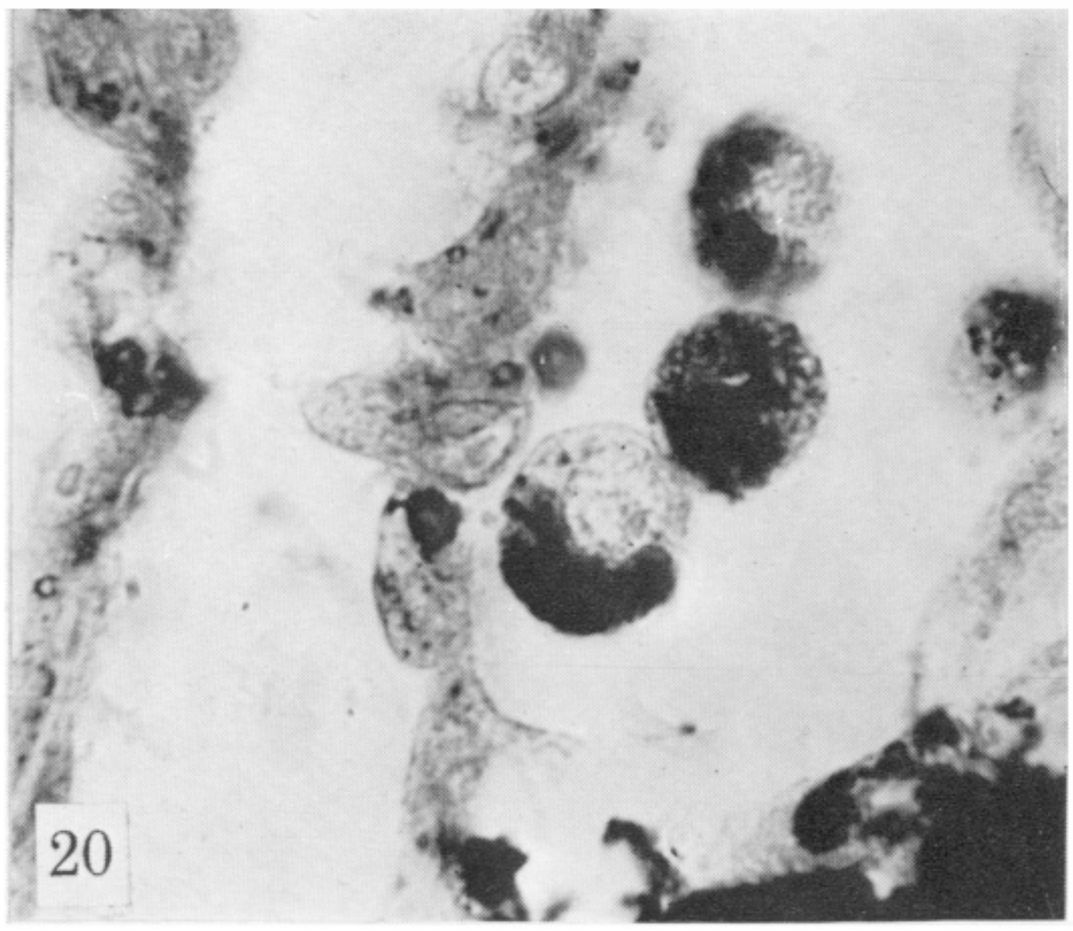
FIGURE 15. Association of an early radioactive (TdR–^{3}H) gonad with a non-radioactive gonadal region. Three labelled gonocytes have left the radioactive graft (RG) and have penetrated into the non-radioactive germinal epithelium (GE) of the host (magn. × 650). The germinal epithelia attract the primary gonocytes whereas the lung and the skin do not (see figures 12 and 13; plate 7).

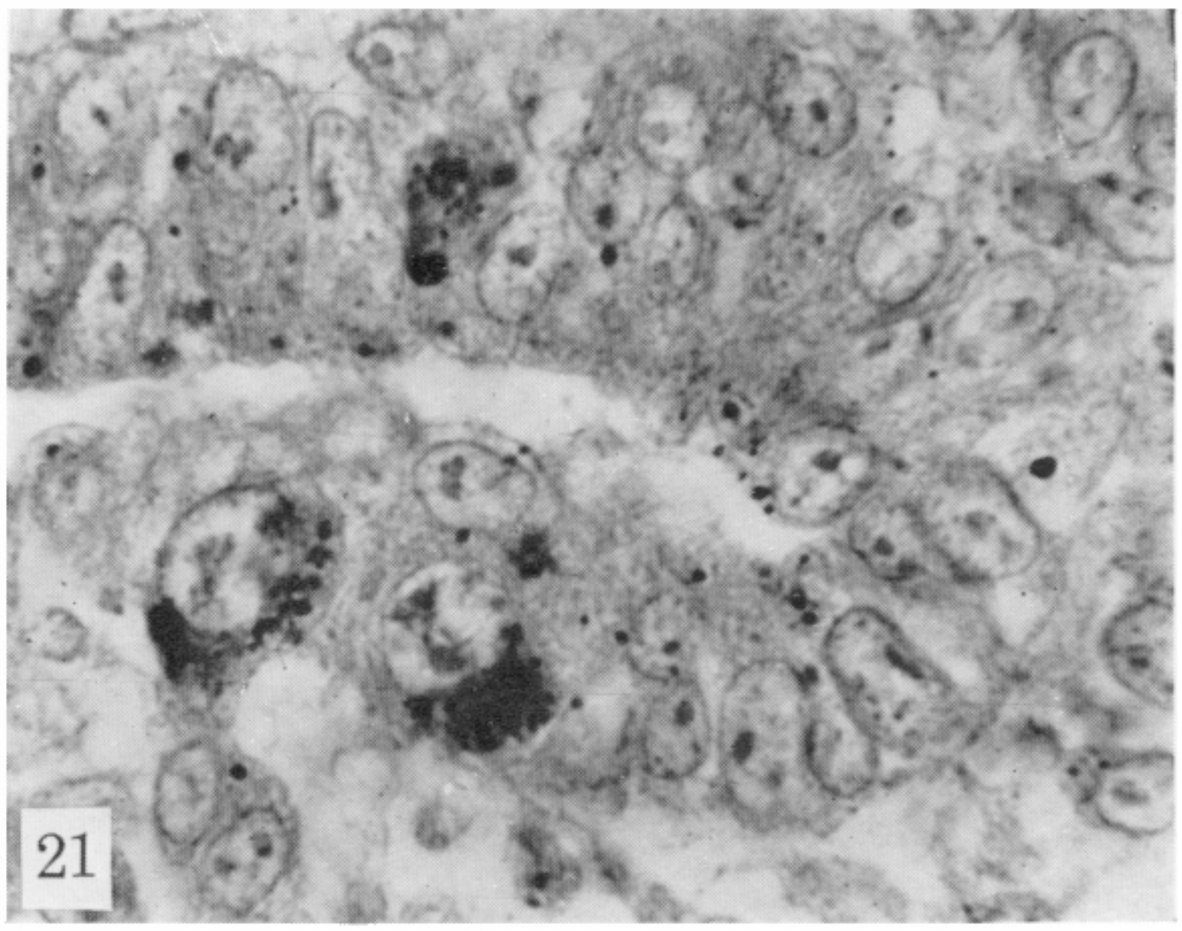
FIGURE 16. Association with a fragment of testis (Te) of 8 days incubation. A spermatogonia has settled down in the germinal epithelium (GE) of the previously sterilized host (magn. × 600).

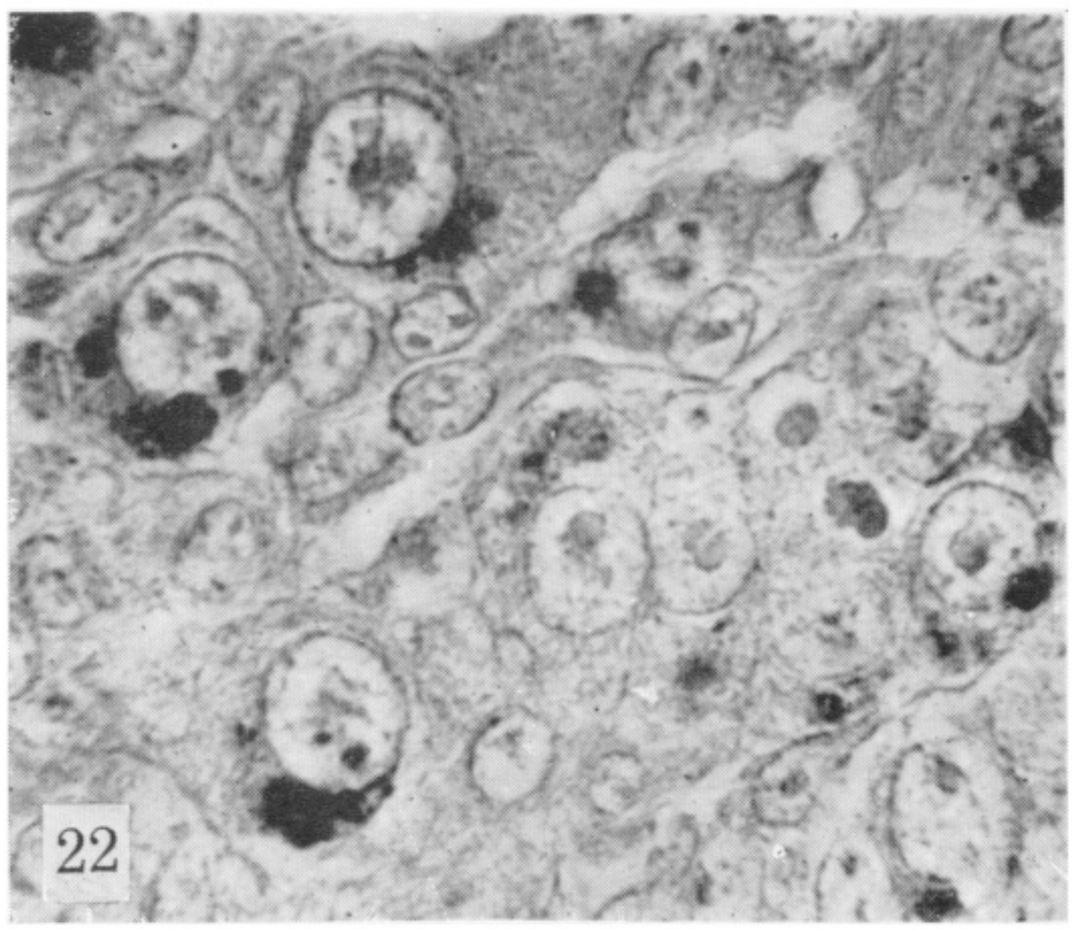
FIGURE 17. Association with a fragment of the left ovary (Ov) of 8 days incubation. Several ovogonia are in a favourable position to be attracted by the germinal epithelium (GE). However none has invaded the gonadal primordium (magn. × 600). The penetration of an ovogonia into the germinal epithelium of the host is uncommon (see lower part of table 2).

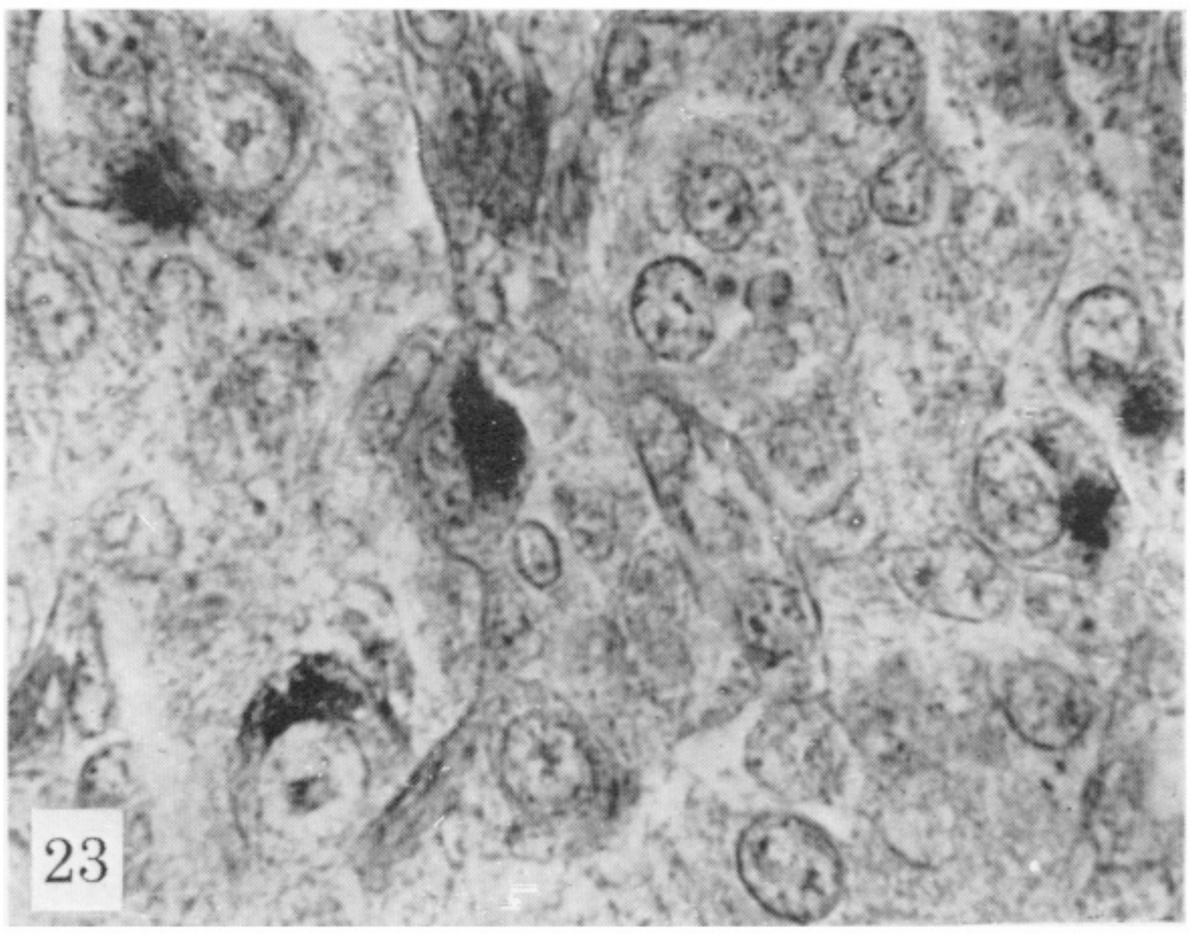
FIGURE 18. Use of the egg vitelline membrane as a permeable barrier: the primary gonocytes leave the graft on the side which is turned towards the germinal epithelium (GE) of the host, but their progression is stopped by the vitelline membrane (FM) (magn. × 650).

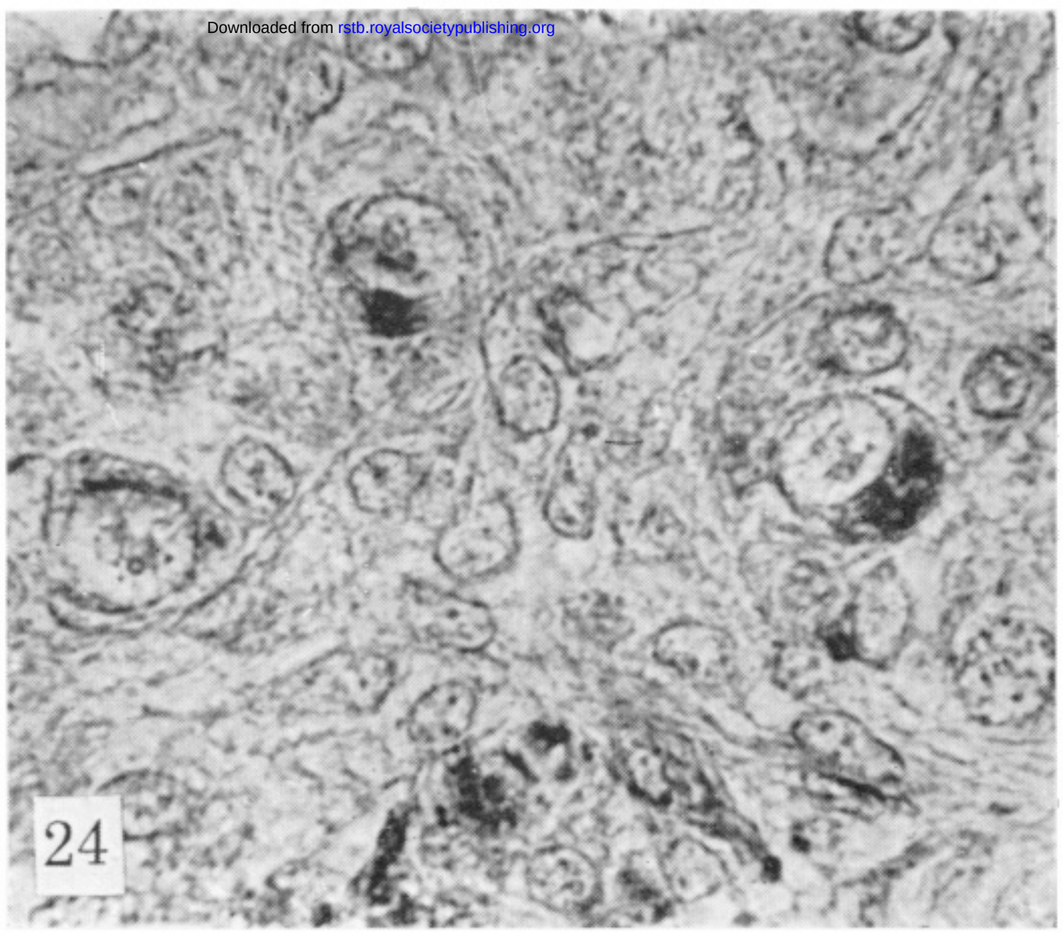
FIGURE 19. Same experiment as in figure 18; the attraction exerted by the germinal epithelium (GE) reassembles the gonocytes against the membrane (FM) and leads to an almost perfect separation between the germinal and somatic elements of the gonad (magn. × 350).

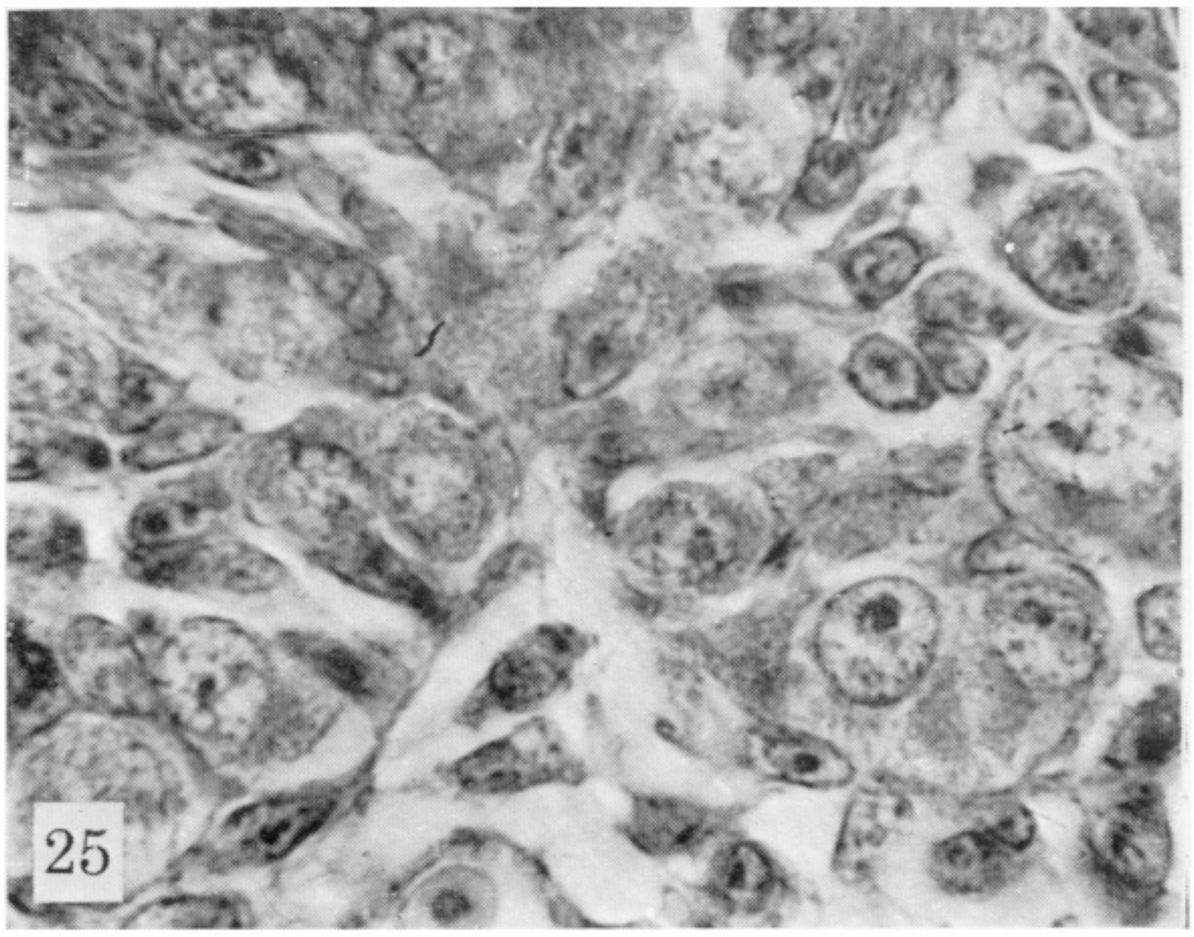












FIGURES 20 to 25. Histochemical differentiation of the germ cells (evolution of the lipid complexes).

FIGURE 20. Stage of the germinal crescent—important concentration of lipid complexes in the cytoplasm of three p.g.c. (magn. × 960).

FIGURE 21. $3\frac{1}{2}$-day-old embryo. Sudanophilic cytoplasmic inclusions in the p.g.c. which have settled in the germinal epithelia. The reaction is weaker than figure 20 (magn. × 960).

FIGURE 22. Morphologically undifferentiated gonad (6 days incubation): the sudanophilic lipid droplets mark the primary gonocytes. The amount of lipid complexes is usually less important than at earlier stages (magn. × 960).

FIGURES 23, 24. Testes of 8 days (figure 23) and 14 days (figure 24) incubation. An appreciable amount of lipid complexes can still be seen in the cytoplasm of the spermatogonia (magn. × 960).

FIGURE 25. Left ovary of 8 days incubation: none of the cortical ovogonia is Sudan Black positive (magn. × 960).