The plates were dried at 105°C for 15 minutes.

Samples were streaked in 10-μl quantities so that a narrow band about 30 mm wide formed at 25 mm from one edge of the plate.

Sodium formate buffer, *p*H 3.4, ionic strength 0.1, was used to separate the four mononucleotides. The glass plate was dipped into the buffer except for the region to which the nucleotide band had been applied. Two paper wicks were placed at the ends of the wet plate and held in place by a second glass placed over the first. The buffer was applied to the wicks and flowed by capillary action over the region of the plate containing the nucleotide band. The two plates and wicks were placed in the electrophoresis chamber with the wicks extending into the buffer which was covered with Varsol to cool the plates during electrophoresis.

The four compounds were resolved in 75 minutes when a potential difference of 450 volts was applied across the plate (Fig. 1). With a formate buffer at *p*H 3.1, guanylic acid and uridylic acid were separated from each other and the other nucleotides in 50 minutes. These times are comparable to those obtained with high-voltage (3000 volts) paper electrophoresis, which requires an expensive power supply. Complete resolution of these compounds with a potential difference of 450 volts takes 10 to 20 hours with ordinary paper electrophoresis (*7*, *8*). Another advantage of the thin-layer method is that the thickness of the layer can be continuously varied to suit the amount of the sample.

After drying the layers, the bands were located with a Mineralight UV lamp and removed from the plate with a razor blade. The thin roll of cellulose obtained was placed in a small conical centrifuge tube to which 2.0 ml of 0.05*M* *tris*-HCl buffer, *p*H 7.2 was added. The centrifuge tube was placed in a bath at 50°C and shaken vigorously several times. After 10 minutes the cellulose was removed by centrifugation and the supernatant was filtered through Schleicher and Schuell No. 589 Blue Ribbon filter paper. The bands were then identified by their ultraviolet spectrum.

FRANK M. DEFILIPPES
Laboratory of Biology of Viruses, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20014

References and Notes

1. K. Randerath, *Biochem. Biophys. Res. Commun.* **6**, 452 (1962); ———, *Angew. Chem. Intern. Ed. Engl.* **1**, 435 (1962).
2. R. G. Coffey and R. W. Newburgh, *J. Chromatog.* **11**, 376 (1963).
3. T. A. Dyer, *ibid.* **11**, 414 (1963).
4. G. Weimann and K. Randerath, *Experientia* **19**, 49 (1963).
5. K. Randerath, *Thin-Layer Chromatography* (Verlag Chemie, Weinheim, 1963), pp. 195–199.
6. From Machery, Nagel and Co., Düren, Germany.
7. Purchased from DeSaga, Brinkmann Corp.
8. J. M. Davidson and R. M. S. Smellie, *Biochem. J.* **52**, 594 (1952); R. Markham and J. D. Smith, *ibid.* **52**, 552 (1952).

10 April 1964

Development of Mouse Eggs in Diffusion Chambers

Abstract. *Mouse eggs in intraperitoneal diffusion chambers continued normal development up to the stage of implantation, and shedding of the zona pellucida occurred. At the time corresponding to implantation within the uterus, eggs attached to the Millipore surface, where both embryonic and trophoblastic cell populations developed. There was migration of cells, especially of trophoblastic giant cells, away from the point of egg attachment. Egg development was not influenced by the endocrine state of the host.*

Studies in vivo on the development of early mouse eggs in extrauterine locations have met with only limited success (*1*, *2*). In vitro, mouse eggs have never been cultured beyond the blastocyst stage. Since the advantages of techniques used in vivo and in vitro can be combined in the diffusion chamber (*3*), the idea that the chamber might allow even further development of mouse eggs was tested.

Usually one, but up to eight eggs were placed in 0.1-ml Lucite diffusion chambers of drum shape, bounded by cell-impermeable Millipore filters (pore size, 0.45 μ). The sealed chambers were placed in the peritoneal cavity of male mice, and pregnant and nonpregnant female mice. Peritoneal fluid soon displaced the original chamber medium, which consisted of equal volumes of calf serum and 0.9 percent NaCl solution. After 1 to 27 days, studies were made of the eggs and the cellular derivatives of eggs attached to the filters (see Figs. 1 and 2), the contents of the chamber fluid, and the contents of the fibrin clot (Fig. 3) formed after 1 day in the chamber cavity. The clot formed after 1 day in control chambers was also studied, and was uniformly acellular.

At least one egg continued development in nine out of 19 chambers into which eggs were placed at the 2-cell stage (1½ days after fertilization) and in all 30 chambers that received eggs at the 8- to 16-cell and early blastocyst stages (2½ days and 3½ days). The older stages also develop better in vitro (*4*). However, among eggs that did develop in the chambers, there was no systematic difference in development according to whether the eggs had been transferred at an early or late stage. The interpretation is that the 2-cell eggs which did develop became normal blastocysts in the chamber cavity, and thus exhibited the same subsequent behavior as those eggs introduced directly at the blastocyst stage.

When the developing egg reached the late blastocyst stage, it attached to the filter surface. This attachment is considered analogous to egg implantation within the uterus, because of both the chronology and the morphology of the attachment. Implantation in the mouse occurs on the 5th day of pregnancy (*5*), and in chambers containing either originally 2-cell eggs or later stages, attached eggs were on the Millipore surface on the 5th day after fertilization, but never before this time. Unlike the uterus, the Millipore filter provides an impenetrable barrier to vertical invasion by eggs, and thus implantation must be transcribed into two dimensions on the filter surface. As in the uterus, the peripheral cells of the attached egg differentiated into trophoblastic giant cells which migrated away from the

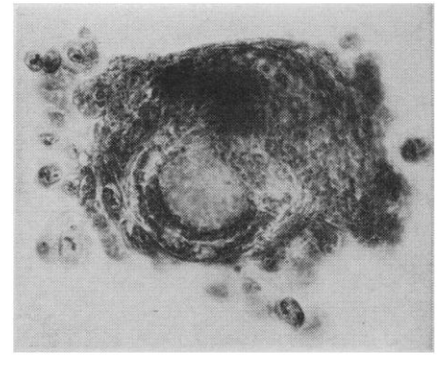

Fig. 1. Stained blastocyst attached to filter surface after 4 days in chamber. The zona pellucida no longer surrounds the egg. The inner cell mass has escaped and spread to the top and to the right. Trophoblastic giant cells surround the other side of the egg, where the inner cell mass has differentiated (gastrulated) into primitive ectoderm and endoderm. (× 150)

point of attachment. The inner cell mass, destined in vivo to become the embryo proper, sometimes differentiated into primary germ layers before becoming a population of migratory, embryonic cells (Fig. 1).

In normal development of the mouse, the zona pellucida disappears before implantation (*6*). Similarly, in the diffusion chamber, the zona was not present around the eggs attached to the filter surface. Thus, whatever the mechanism of zona pellucida loss may be, it appears to be an intrinsic manifestation of egg development, rather than a consequence of the uterine environment acting on the zona from the outside.

Of the 39 successful chambers, 30 were in males, 6 in nonpregnant females, and 3 in pregnant females. The early development, attachment, and continued development of the eggs was not correlated with the endocrine state of the hosts. Similarly, hormones have no apparent effects on the development of mouse eggs in other extrauterine locations (*1*). Hormones do have a critical effect on intrauterine egg development, especially during and after implantation (*7*).

The physical surface of the Millipore filter may have been an important factor in the attachment and continued development of the eggs, since mouse eggs injected freely into the peritoneal cavity show a very low rate of attachment (*2*).

Trophoblastic giant cells made their first appearance in the diffusion chambers during the early stages of egg attachment, surrounding the inner cell mass (Fig. 1). Frequently, instead of migrating across the filter surface, some of the giant cells would remain surrounding the inner cell mass at the attachment site, causing the eventual destruction of the inner cells by cytolysis and phagocytosis. In such cases, no derivatives of the inner cell mass developed.

In most chambers in which only one egg was placed, trophoblastic giant cells were present in large numbers on both filter walls of the chamber. Probably, some such cells were released from the filter on which the egg attached, to "metastasize" to the opposite side of the chamber. Migration may have been through both liquid and solid phases (peritoneal transudate and fibrin clot). In both phases, many giant cells were usually found.

The trophoblastic giant cells ranged greatly in size and shape (Fig. 2). This variation increased as a function of time, reaching a peak about 10 days after egg attachment. The giant cells were often large enough to be seen with the unaided eye (diameters up to 400 μ). Despite their wide variation in morphological appearance, trophoblastic giant cells could always be easily distinguished from derivatives of the inner cell mass. Most giant cells separated from the filters by the 15th day after fertilization, and floated in various stages of autolysis in the chamber fluid. Survival in the fibrin clot was longer.

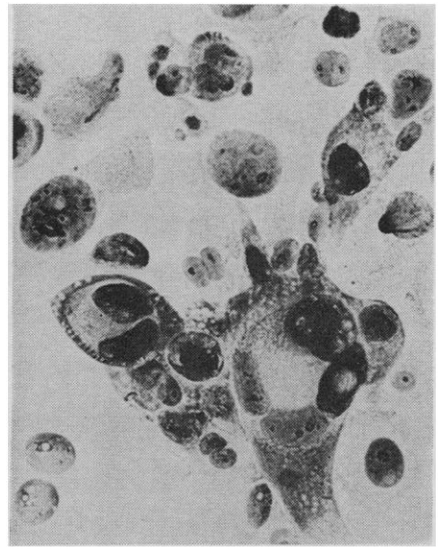

Fig. 2. Trophoblastic giant cells of a mouse blastocyst, after 12 days of cultivation in a Millipore diffusion chamber. The cells are attached to the filter surface, and have been fixed and stained. The multiple nuclei probably result from nuclear budding. The largest cells can be seen with unaided eye (diameters up to 400 μ). (× 150)

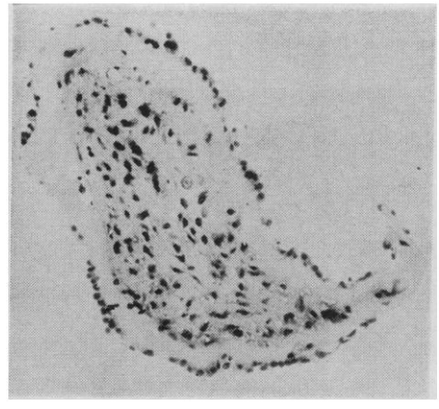

Fig. 3. Blastocyst after 27 days of cultivation. Stained section showing structure of teratoma growing in chamber cavity. Center composed of mesenchymatous spindle cells. Acellular rim covered by epithelioid round cells. (× 150)

As in vivo, trophoblastic giant cells had no mitotic figures, and nuclei were observed in different stages of budding. Multinucleate cells were also present (Fig. 2), and probably resulted from nuclear divisions that were not followed by cytoplasmic division, but it is also conceivable that some giant cells phagocytized or fused with each other.

In those cases where the inner cell mass was not completely surrounded by giant cells at the attachment site, the embryonic cells spread across the filter surface (upper right of Fig. 1). The time of attachment coincided with the initiation of the spread, suggesting that early embryonic cells need not "condition" themselves to a new environment, as do adult cells in tissue culture (*8*). Apparently, all the cells of the inner mass that were not trapped by giant cells took part in this migration. Thus, at least in this situation, the culture does not result from the selection of a few cells among many, as predicted by the "selection hypothesis" of culture initiation (*9*).

The embryonic cells in the chambers exhibited contact guidance along fibrin bands, and contact inhibition between fibroblastic monolayers. Total contact inhibition is much more readily formed in a two-dimensional system (*10*), which explains the high frequency of fibroblastic monolayers on the Millipore surface. In contrast to fibroblastic cells, epithelioid cells increase the area of intercellular contact after random association (*11*). Many aggregations of epithelioid cells in close contact were on the filters. In early cultures, some groups of embryonic cells were neither clearly fibroblastic nor epithelioid, stressing the gradual emergence of even such basic markers of differentiation as these.

As in tissue culture, cellular differentiation and cellular contact were correlated in the chambers. Isolated cells on the filter surface remained morphologically nonspecific, and often degenerated. Cellular contact on a local basis led to the formation of new cell types, as well as gland-like, circular clusters of cells. With aggregations of many cells on the filter surface, involving more contacts, vacuolated cells were extruded from the tissue margin in a fashion analogous to cornification in the skin. However, there was no organogenesis.

The highest level of differentiation was present in the fibrin clot in the chamber cavity, where three-dimensional interaction was possible (Fig. 3). This teratoma represented the end-stage of a process noted in other chambers, where embryonic cells often colonized various regions of the fibrin. In the presence of adequate cell numbers and contacts, the inherent tendency for embryonic cells to associate at a high level of integration was expressed here, even in an entirely abnormal fashion.

DAVID LYMAN BRYSON
Department of Graduate Studies, Rockefeller Institute, New York

References

1. D. W. Fawcett, G. B. Wislocki, C. W. Waldo, *Anat. Rec.* **108**, 71 (1947); D. R. S. Kirby, *Nature* **194**, 696 (1962).
2. A. McLaren and A. K. Tarkowski, *J. Reprod. Fertility* **6**, 385 (1963).
3. D. B. Amos, in *Transplantation of Tissues and Cells*, R. E. Billingham and W. K. Silvers, Eds. (Wistar Inst. Press, Philadelphia, 1961), p. 69.
4. J. Hammond, *Nature* **163**, 28 (1949).
5. G. D. Snell, *Biology of the Laboratory Mouse* (Blakiston, Philadelphia, 1941), pp. 1–54.
6. B. Mintz, *Science* **138**, 594 (1962).
7. M. Smithberg and M. N. Runner, *J. Exptl. Zool.* **143**, 21 (1960).
8. L. Levitow and H. Eagle, *Ann. Rev. Biochem.* **30**, 605 (1961).
9. G. Sato, L. Zaroff, S. Mills, *Proc. Natl. Acad. Sci. U.S.* **46**, 963 (1960).
10. M. Abercrombie and E. J. Ambrose, *Cancer Res.* **22**, 522 (1962).
11. P. Weiss, *Intern. Rev. Cytol.* **7**, 391 (1958).
12. I thank William U. Gardner of the Yale University School of Medicine for his constant encouragement and advice. This work represents part of a thesis, Yale University, 1963. It was supported by USPHS grants CA-5505 and C-343. I also thank Paul Weiss of the Rockefeller Institute for editorial suggestions.

18 March 1964

Lactate Dehydrogenase in Pigeon Testes: Genetic Control by Three Loci

Abstract. *Homogenates of pigeon testes show three types of lactate dehydrogenase isozyme patterns. Recombination of the polypeptide subunits of the dehydrogenase in mixtures of the different types, and the distribution of the three isozyme patterns in six different pigeon populations demonstrated that lactate dehydrogenase synthesis in pigeon testes is under the control of three distinct loci, A, B, and C.*

By the method of starch-gel electrophoresis five distinct types (isozymes) of lactate dehydrogenase (LDH) have been separated and identified in most mammalian and avian tissues (*1*). Information currently available (*2*) indicates that each isozyme is a tetramer formed by association of two polypeptides, A and B. Thus isozymes 1 to 5 have the following polypeptide compositions: A_0B_4, A_1B_3, A_2B_2, A_3B_1, A_4B_0. The synthesis of each of the polypeptides, A and B, appears to be under the control of two separate genetic loci (*3*), so that the isozyme composition of any tissue depends on the relative activity of the genes at these loci.

Electrophoresis of testicular homogenates from several mature animals revealed one or more unusual LDH isozymes ("band X"), suggesting that a third genetic locus contributed to the synthesis of LDH isozymes in mature testes (*4*). Further evidence for this additional genetic locus has been obtained by studying the LDH isozymes in homogenates of pigeon testes.

We used racing homer pigeons, White Carneau and Silver King pigeons, and wild park pigeons (*5*). All of the birds were delivered to the laboratory, killed by decapitation, and dissected to determine sex and maturity. Most of the racing homer pigeons were males, whereas the wild population comprised approximately equal numbers of males and females. Testes and several other tissues from the males were frozen immediately after dissection, and enzyme studies were carried out within 2 weeks. The electrophoresis patterns of fresh and frozen tissues were the same. Methods for preparation and electrophoresis of tissue homogenates, localization of LDH isozymes in starch gel, dissociation and recombination of LDH polypeptide subunits, and determination of total LDH activity were similar to those previously described (*4*).

As shown in Fig. 1, homogenates of pigeon testes exhibited three different patterns of isozymes when lactate was used as substrate. Type I showed seven isozymes, including two new ones (designated "X"), type II showed a total of eight, and type III showed four. The bimodal distribution of activity among the isozyme bands in the type II pattern suggested the presence of a second isozymic group whose first two members were superimposed on LDH-4 and LDH-5.

This interpretation was supported by studies with α-hydroxybutyrate as substrate (*4*, *6*). Two of the bands in type I, five in type II, and one in type III (all designated "X" in Fig. 1) exhibited higher catalytic activity with this substrate than did the usual five isozymes. When α-hydroxybutyrate was substituted for lactate in the reaction mixture, LDH-1, -2, and -3 in testes, as well as LDH-4 and -5 in tissues containing large amounts of these isozymes, were barely visible.

In order to investigate the polypeptide composition of the "band X" complex in pigeon testes, various combinations of types I, II, and III testicular homogenates were treated with 0.5*M* sodium chloride and 0.1*M* phosphate and frozen for 24 hours prior to electrophoresis. This method was first used by Markert (*7*) to dissociate the polypeptide subunits of the usual five isozymes. After dissociation and random

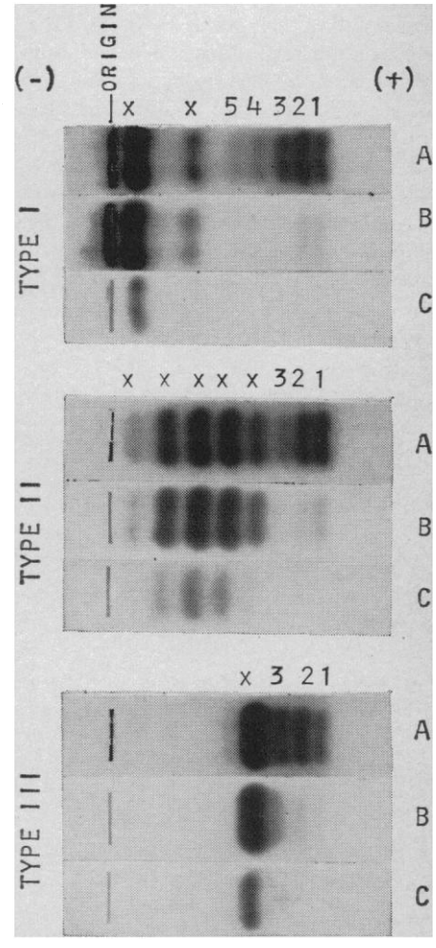

Fig. 1. Lactate dehydrogenase isozymes in mature testes from types I, II, and III pigeons. *A*, Enzymatic activity with 0.5*M* lactate; *B*, activity with 0.5*M* α-hydroxybutyrate; and *C*, activity with 0.5*M* α-hydroxyvalerate. The homogenates were electrophoresed simultaneously in the same starch gel, and the conditions for localizing LDH activity, except for substrate used, were identical.