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THE ROLE OF CELLULAR ADHESIVENESS IN AMPHIBIAN GASTRULATION

By

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SUMMARY

Five different methods were used to examine cell contact behavior in normal and hybrid amphibian embryos in an attempt to elucidate the role of cell surface adhesiveness in gastrulation. The results, summarized for each type of embryo, are as follows. 1.) pipiens-pipiens- In this normal embryo, prior to gastrulation, all cells show an increasing tendency to attach to glass substrata in vitro with advancing developmental age. Dissociated presumptive mesodermal cells from early gastrulae show a high ability to attach to glass and to reaggregate. The rates of disaggregation of whole embryos show marked decreases with advancing age from the blastula to late gastrula period. Binary combinations of explanted embryonic germ layer fragments from early gastrulae undergo movements that are in part reminiscent of portions of gastrulation. At the level of fine structure, presumptive ectodermal cells form cell contacts which become increasingly intimate, as judged by closer apposition of plasma membranes and overlapping filopodia, from the blastula to the late gastrula period. Presumptive mesodermal cells in early gastrulae have numerous close cell contacts and some tight junctions. 2.) pipiens-palustris- This viable hybrid embryo, which occasionally shows slight abnormality in invagination, is quite similar to pipiens-pipiens in all respects. Slight alterations in cell contact behavior correlate positively with slight abnormalities in gastrulation. 3.) pipiens-sylvatica- This hybrid undergoes a moderately severe developmental arrest at the onset of gastrulation. During a short time, when controls are undergoing epiboly, a severe wrinkling appears in the roof of the blastocoel. No extensive invagination or archenteron formation occurs. Animal cells show an increase in frequency of attachment while vegetal cells show none. Rates of disaggregation decrease moderately with advancing age. Explanted fragments show movements similar to those seen in normal fragment combinations. Presumptive mesodermal cells have a low frequency of attachment, moderately reduced ability to reaggregate, moderately increased separation between plasma membranes, and no tight junctions. 4.) pipiens-catesbeiana- This embryo undergoes a severe developmental arrest at the onset of gastrulation. Little or no increase in frequency of attachment occurs in any cells. All stages disaggregate rapidly. Explanted fragments show no movements. Presumptive mesodermal cells have a low frequency of attachment, very little ability to reaggregate, moderately increased separation between plasma membranes, and no tight junctions. At the level of fine structure, presumptive ectodermal cells do not form increasingly intimate contacts. 5.) pipiens-clamitans- This hybrid undergoes the most severe arrest at the onset of gastrulation. No increase in frequency of attachment occurs in any cells. Disaggregation rates remain high at all stages. Explanted fragments show no movements. Presumptive mesodermal cells have a low frequency of attachment, no ability to reaggregate, grossly increased separation between plasma membranes, and no tight junctions. These results indicate that extent of increase in cell surface adhesiveness and extent of normal gastrulation correlate positively. This suggests that increased cell surface adhesiveness is necessary for the morphogenetic movements of gastrulation.

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INTRODUCTION

Amphibian gastrulation is a complex, integrated, morphogenetic process whereby the cavitated cluster of blastomeres known as the blastula develops into the triploblastic late gastrula via a coordinated migration of embryonic cellular masses. Blastopore initiation and subsequent invagination begin at a site below the equator of the late blastula and proceed inward, antero-dorsally and laterally, until the blastocoel is obliterated by the enlarging archenteron. Concurrently with this invagination of presumptive mesoderm and endoderm, the presumptive ectoderm undergoes an epibolic spreading, eventually to cover the entire surface of the embryo. The normal course of these cellular movements remained obscure until the application of the technique of vital staining, first used extensively by Goerttler ('25) and Vogt ('25). Later, Vogt ('29) used vital staining and dissection of living embryos to construct his well known fate maps for a number of urodeles and anurans. Although widely believed to be accurate, Vogt's classical description was shown by Pasteels ('40; '42; '49) to be incorrect in several important details. Vital staining of living embryos made it possible to understand the normal course of mass cellular movements in amphibian gastrulation.

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Little is known, however, concerning the mechanism(s) whereby gastrulation is initiated and gains its direction. Most attempts to explain morphogenetic cell movements suggest that alterations in cell contact behavior and cell surface properties lie at the basis of the changes that occur in locomotory behavior. Gustafson and Wolpert ('67) postulated that sea urchin gastrulation can be explained largely in terms of changes in cell contact behavior and pseudopodial activity. They proposed that the thickening of the vegetal portion of the late blastula and the subsequent release of primary mesenchyme cells could be accounted for by an initial increase in cellular adhesiveness (and consequently an increase in area of cell contact), followed by a decrease in cellular adhesiveness (leading to the release of the primary mesenchyme cells into the blastocoel). In addition, they presented evidence that the primary mesenchyme cells distributed themselves in a characteristic pattern on the inner surface of the blastocoel wall due to selectivity of pseudopodial adhesion.

Working with a different system, teleost epiboly, Trinkaus ('63) showed that alterations in cell contact properties occur in cells derived from the Fundulus blastoderm prior to the onset of gastrulation. Specifically, cells derived from early blastulae show little or no attachment to each other or flattening on a glass substratum during the first 6 hours in vitro; whereas, cells derived from early gastrulae show extensive attachment and flattening within 1-2 hours in vitro. Advanced blastula cells show increased flattening after 4-6 hours in vitro, when control embryos are beginning epiboly. Early blastula cells do not change during a similar time period. In a later study, Trinkaus and Lentz ('67) showed that there are alterations of cell contact behavior in the living Fundulus blastoderm. For example, in the

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blastula, the freely motile deep cells often form lobopodia and collide, without adhering to one another. In the gastrula stage, however, these same cells begin to adhere to one another and other cells. These observations in living material were supported by observations at the level of fine structure. Deep blastomere contacts, with a separation of 150-200 Å between the plasma membranes, become numerous at the time when the blastomeres begin to adhere to one another in vivo. These three lines of evidence indicate that Fundulus blastomeres show changes in cell contact behavior just prior to and at the onset of epiboly and suggest that increases in adhesiveness may be important for the initiation of morphogenetic movements.

Recently, Trelstad et al. ('66; '67) and Hay ('68), working on morphogenetic movements in chick blastoderms, speculated that there was a relationship between cell contact behavior and the direction of morphogenesis. They proposed that the lateral emigration of the primary mesenchymal sheet (mesoblast) from the primitive streak can be viewed as a spreading epithelium with the mesoblast spreading as a sheet due to the well known phenomenon of contact inhibition of ruffled membrane formation (Abercrombie, '61). This proposal, based entirely on fine-structural evidence, assumes that contact inhibition is mediated by way of tight or close junctions. The trailing edges of mesoblast cells contact their neighbors by close (25-100 Å gap) or focal tight (no gap) junctions, while the leading edges possess numerous filopodia. Mesoblast cells also make close and tight junctions with cells in the epiblast and hypoblast. Trinkaus ('69) points out that if contact inhibition is at work it is necessary to postulate that it is selective, operating only in mesoblast-mesoblast contacts, and not in the migration of

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mesoblast over epiblast and hypoblast. Such selectivity of contact inhibition, while possible, has never been demonstrated. The results of electron microscopy are suggestive in this instance, but cannot give positive evidence of contact inhibition. Direct observation of the contact behavior of living cells in vitro and in vivo is required for this.

Careful experiments have proved that many of the early theories to account for amphibian gastrulation were inadequate [see Holtfreter ('39) for review]. Holtfreter ('39) introduced a new concept, tissue affinities, to explain amphibian gastrulation. He felt that gastrulation was controlled by the inherent associative properties of prospective germ layers and their constituent cells. For example, when a fragment of presumptive endoderm is isolated from a blastula and cultured in a simple salt solution, this loose collection of cells soon forms a compact, spherical aggregate which subsequently flattens and spreads on a glass substratum. The appearance of this endogenous spreading capacity is temporally correlated with the dorsad spreading and fusion of the endodermal tissues in the late gastrula. "On glass, cells tend to spread in all directions, but in the embryo local configurations guide the cells." (Harrison, '28). By combining fragments of different germ layers, Holtfreter showed, in addition, that the final configuration of germ layers, attained through the expression of attractive and repulsive affinities, depended on the type of the cells which constituted the combined fragments. For example, when ectoderm and endoderm were combined, the two fragments initially rounded up into a compact mass, but subsequently underwent self-isolation. If small amounts of mesoderm were included in such a combination, however, the final result of the expression of repulsive affinities was modified in two different ways.

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If a small amount of mesoderm is included with approximately equal amounts of ectoderm and endoderm, self-isolation began, but then was prevented by mesoderm. In this case, the ectodermal and endodermal fragments remained discrete but did not self-isolate. Instead, they contributed equally to the formation of an epithelium surrounding an inner core of mesenchymal cells. If the ectodermal fragment was much larger than the endodermal fragment, the former engulfed the latter. Subsequently, the endoderm and mesoderm segregated into two distinct phases. The final configuration, an epithelium of ectodermal origin, an intermediate layer of mesenchymal elements, and an inner, tubular epithelium of endodermal origin, resembled a normal triploblastic embryo. In this case, the negative affinity of ectoderm and endoderm gained full expression. The relationship between cell contact behavior and tissue affinities in these systems was unclear at that time.

Soon after, Holtfreter ('43; '44) attempted to relate cell contact behavior, tissue affinities, and behavior of germ layers, both as isolated fragments and in the intact gastrula, by means of a surface tension model. Specifically, he proposed that isolated and recombined germ layer fragments could be treated as liquid phases that altered their relationships to one another in response to changes in surface tension (surface free energy). Later, Townes and Holtfreter ('55) showed that type-specific segregation of coaggregated amphibian cells resulted in the attainment of final configurations similar to those seen earlier by combining intact embryonic fragments. It appeared that cells, considered individually or as a group in a fragment, exhibited preferential associative behavior. Holtfreter thus made a remarkable contribution to our present understanding of amphibian gastrulation.

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Several authors have submitted other evidence to show that changes in cell contact behavior are important for the ordered progression of amphibian gastrulation. Steinberg ('64) proposed a model to explain histotypic segregation in coaggregates of chick tissues that extended to germ layer segregation in amphibian embryos. This differential adhesion hypothesis attempts to explain cell sorting out and germ layer segregation by the attainment of equilibrium cellular configurations which minimize surface free energy of adhesion. Recently the differential adhesion hypothesis has been stated in rigorous physical terms (Phillips, '69; Phillips and Steinberg, '69) and measurements of a parameter, specific interfacial free energy, have been made. Specific interfacial free energy is presumably closely related to cell adhesiveness. The differential adhesion hypothesis serves as a useful set of guiding principles for an investigation of the relationship between changes in cell contact behavior and amphibian gastrulation. Jones and Elsdale ('63), working with amphibian embryos, noted that blastula and early gastrula stages disaggregate readily with Ca^{2+}, Mg^{2+}-free saline but older embryos require the presence of chelating agents. Patricolo ('67) recently showed that there are differences in the reaggregative abilities of cell suspensions derived from whole embryos of Discoglossus pictus of different stages. Cells from dissociated blastulae have a low ability to reaggregate in vitro; whereas, cells from early or late gastrulae show a progressively increased ability to form compact aggregates. The question before us is are these changes in cell contact behavior related to changes in cellular adhesiveness and to the control of gastrulation and if so how.

Any discussion of cellular adhesiveness requires a careful definition of the term. Adhesiveness is the force acting between the constituent molecules of apposed surfaces that holds them together,

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once they make contact. No method is available at present for making direct measurements of this force. Therefore, one is forced to accept a compromise by using whatever methods are available for monitoring cell contact behavior which presumably reflects various cell surface properties, among them, adhesiveness. Adhesiveness has been defined operationally in a number of ways. For embryonic amphibian cells, only a limited number of methods are useful for measuring adhesiveness [see Curtis ('67) and Weiss ('67)]. These cells are large, extremely fragile, and undergo hourly changes in cell contact behavior, volume, and number from the early blastula to late gastrula stage. The method of Roth and Weston ('67; '68), which operationally defines the strength of intercellular adhesion as the probability that an adhesion, once formed, will be maintained against a constant shear, is unsuitable for the present material. For example, even though this method might be used to determine the relative adhesive stability of blastula-gastrula and gastrula-gastrula cell contacts by collecting labeled gastrula cells on unlabeled blastula and gastrula aggregates of the same diameter in the same cell suspension, blastula cells, unfortunately, are 2-4 times greater in diameter than gastrula cells and, therefore, would be subjected to different shear forces. In addition, Roth and Weston's experiments require periods of 3-6 hours. During this period, blastula cells would become gastrula-like in cell contact behavior. Experiments to measure the rate or extent of reaggregation, which probably reflects some cell surface property closely related to adhesiveness, performed on a Gyrotory Shaker (Moscona, '61), are also unsuitable for a number of reasons. First,

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this method requires a time course of 24 hours. Second, embryonic amphibian cells are difficult to reaggregate by this method (Steinberg, personal communication). Third, there is some uncertainty involved in the interpretation of the relationship between aggregate size and degree of adhesiveness (Ede and Agerbak, '68).

Certain techniques for monitoring cell contact behavior, however, are suitable for amphibian material. Among them are: 1.) frequency of cell attachment to glass in vitro, 2.) ability of stationary, dense populations of dissociated cells to undergo reaggregation in vitro, 3.) rate of disaggregation of whole, bisected embryos in Ca^{2+}, Mg^{2+}-free (CMF) solutions, 4.) behavior in vitro of recombined fragments of germ layers, designed to assess the morphogenetic potential of two germ layers, and 5.) cell contact relations at the level of fine structure. When monitoring cell contact behavior in an attempt to gain information about adhesiveness, it is advisable to employ several distinct methods which are independent of the phenomenon (gastrulation) to be explained. For example, if one monitors the ability to attach to glass (a foreign substratum) in vitro, one must contend with certain problems. Other modes of cell behavior, unrelated to surface adhesiveness, such as surface blebbing, could prevent attachment to glass, even though surface adhesiveness remained unchanged. Each method for examining cell contact behavior is subject to similar limitations. But when independent methods yield positively correlated results, one gains increasing confidence that the various methods of monitoring cell contact behavior are indeed direct reflections of cellular adhesiveness. Given confidence in the occurrence of changes in cellular adhesiveness, a specific hypothesis about its role in amphibian gastrulation can be tested.

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Increased cell surface adhesiveness appears to be necessary for the initiation of the cell movements of gastrulation. To test this hypothesis, interspecific hybridization was used to produce embryos which undergo developmental arrest at gastrulation (Moore, '55), and then these hybrid embryos were compared with normal embryos vis à vis their cell surface adhesiveness. Preliminary reports (Johnson, '69a; '69b; '69c) document the suitability of this strategy. The present work is an investigation of the changes in cell surface adhesiveness that begin in late normal blastulae prior to the onset of gastrulation and continue during normal gastrulation but which fail to occur in hybrid embryos arrested at the onset of gastrulation.

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MATERIALS AND METHODS

Xenopus laevis embryos, obtained by injection of human chorionic gonadotrophin (Sigma, CG-B) into adults [see Gurdon ('67) for details], were raised at 23-25° C. Rana pipiens, R. sylvatica, and R. catesbeiana were obtained from the Connecticut Valley Biological Supply Company (Valley Road, Southampton, Massachusetts). All R. palustris, all R. clamitans, some R. sylvatica, and some R. catesbeiana were collected near New Haven. Ovulation of females and fertilization was carried out according to Rugh ('62). For each clutch of hybrid embryos, there was a control clutch of normal embryos. On rare occasions, when control embryos failed to undergo normal gastrulation, both hybrid and normal embryos were discarded. All R. pipiens and R. clamitans embryos and all hybrid embryos, derived by fertilizing eggs of R. pipiens with sperm of another species, were raised at 23-25° C. All R. sylvatica embryos and all hybrids, derived by fertilizing the eggs of R. sylvatica with sperm of another species, were raised at 21° C. The upper temperature tolerance for R. sylvatica is 25° C. (Rugh, '62). Since at equivalent temperatures R. sylvatica develops more rapidly than R. pipiens, a rearing temperature of 21° C. was chosen, so that R. sylvatica embryos would develop at approximately the same rate as R. pipiens embryos raised at 23-25° C. To remove tenacious inner jelly coats, 10 hour embryos were freed from their outer protective jelly with forceps, incubated for 15-30 minutes in 0.7% mercaptoacetic acid (Matheson, Coleman, and Bell Co.) in 50% Wolf-Quimby balanced salt solution (WQBSS) (Wolf and Quimby, '62), with the pH adjusted to 8.6 with 5.0 N NaOH, and then rinsed ten times in 10% WQBSS. This treatment does not affect later development. Follow-

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ing removal of jelly, embryos were reared in 10% WQBSS. Whenever possible, experiments were run in parallel so that measurements could be made on hybrid and normal embryos derived from one clutch of eggs. For example, all reaggregation experiments on hybrid presumptive mesodermal cells were also performed with normal control cells.

For conventional histological examination, embryos and recombined germ layer fragments cultured in vitro, were fixed for 12-24 hours at 4° C. in Smith's fixative, rinsed 12-24 hours in running tap water, dehydrated with a graded se· ·s (10%, 20%, 30% ... 100%) of aqueous dioxane solutions, and embedded in 53-55° C. paraffin. Details for this excellent method for handling brittle, yolky, amphibian material can be found in Rugh ('62). The embedded material was sectioned at 15 μ and stained with the Feulgen reaction (5 minutes hydrolysis in 1 N HCl at 60° C. followed by 1-2 hours in Schiff's reagent at room temperature) followed by a light counterstain with 0.02% aqueous fast green-FCF for 30 seconds at room temperature.

To determine the frequency of cell attachment in vitro, as a function of embryonic age, embryos were dejellied, rinsed, and allowed to develop to an appropriate age. Then the embryos were placed in about 3 ml of Ca^{2+}, Mg^{2+}- free WQBSS (CMF) in a 35 X 10 mm Falcon dish (Falcon Plastics Company, #3001). Vitelline membranes were removed by means of sharpened Dumont #5 watchmaker's forceps and the embryos were cut into animal and vegetal halves. Later gastrula stages of normal embryos were cut into four equal fragments. The CMF was replaced with three washes of about 2 ml of 10^{-3} M EDTA in CMF and the embryos were left undisturbed to dissociate for 30 minutes. After dissociation, embryonic fragments were aspirated into a Pasteur pipette and gently

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expelled into a culture medium of WQBSS containing 0.5% bovine serum albumin, fraction V (Sigma) (Stearns and Kostellow, '58), penicillin G (200,000 IU/liter), and streptomycin sulfate (200 mg/liter). The culture medium was contained by a dam of Vaseline approximately 18 X 35 mm on a microscope slide. Dispersed cells were given one minute to settle out of suspension; most of the culture medium containing EDTA was removed, and replaced with an excess of fresh medium. After another one minute settling period, the excess medium was aspirated, and the culture was sealed with a 20 X 40 mm #1 coverslip. Following a 30 minute attachment period, 100-200 cells from either the animal or vegetal halves or from whole embryos in 5-10 replica preparations (one embryo/ preparation) were counted to determine the percentage of attachment and then averaged. Attached cells were flat, thin, polygonal, non-refractile, and often possessed ruffled membranes [see Johnson ('69a) for a detailed description and illustration]. For either animal halves, vegetal halves, or whole embryos of Xenopus laevis, counts were made on cells that were 6, 7, 8, 9 (beginning of gastrulation), 10, 11, 12, 13, 14, or 15 hours old. For example, to make a count of cell attachment at 6 hours, 5 hour embryos were dissociated for 30 minutes and allowed to attach for 30 minutes, so that the cells were 6 hours old at the time of the count. For either animal halves, vegetal halves, or whole embryos of R. pipiens, R. sylvatica, or hybrids using R. pipiens eggs, counts were made on cells that were 12, 14, 16, 18 (beginning of gastrulation or arrest), 20, 22, 24, 26, 28, or 30 hours old.

To determine the frequency of attachment of presumptive mesodermal cells, dejellied early gastrulae were placed in about 3 ml of CMF in a 35 X 10 mm Falcon dish. Vitelline membranes were removed by means of

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forceps and the roof of the blastocoel was cut away to expose the endodermal mass and invaginated presumptive mesodermal cells. Then the CMF was replaced with three washes of about 2 ml of 10^{-3} M EDTA in CMF and the embryos were left for 30 minutes. At the end of this time, the presumptive mesodermal cells were drawn into a micropipette (inner bore ~ 50 μ) with the aid of a microinjection apparatus on a Leitz micromanipulator. The the micropipette was removed from the dissociation medium and the dissociation vessel was replaced by a microscope slide with a Vaseline dam and culture medium as described before. Next, the tip of the micropipette was lowered into the culture medium and a stream of 500-1000 dissociated cells was expelled. The bulk of the culture medium and EDTA was removed and an excess of fresh medium was added to rinse and disperse the cells. After a one minute settling period, the excess culture medium was removed and the culture was sealed with a coverslip. Thirty minutes after dispersal, counts were made of the number of attached and unattached cells. Preparations for studying reaggregation were set up in a similar manner, except that the cells were not dispersed during rinsing. Photographs were taken of these preparations immediately after they were set up (0 hours), and after 1, 2, and 3 hours in vitro.

To measure the kinetics of disaggregation, dejellied, rinsed embryos of 15 hours (mid-blastula), 21 hours (early gastrula or early arrest), and 27 hours (yolk plug gastrula or late arrest) were placed in 10.0 ml of CMF in a 60 X 15 mm Falcon dish (Falcon Plastics Company, #1007). Vitelline membranes were removed by means of forceps and the embryos were cut into approximately equal halves. This cut was made along the future axis of bilateral symmetry in gastrulae. Then the bisected embryos were rotated at 84 RPM on a Gyrotory Shaker (New Brunswick Scientific Company) for 5, 10, 15, 20, 25, 30, 40, 50, 60, or 120 minutes. Almost

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complete disaggregation of all stages occurred after 120 minutes. One preliminary experiment showed that a normal 27 hour embryo, after 120 minutes of agitation, dissociated into a suspension consisting of 89% single cells, 9% double cell clusters, and 2% clusters of three or more cells. At each time point, the Falcon dishes were removed from the shaker, undissociated embryonic material was removed with a Pasteur pipette, and 1.0 ml of glacial acetic acid: formalin (1:1) fixative was added to prevent further cell division or dissociation. Then the suspension was mixed in the dish and allowed to stand until settling had occurred. There was a non-random, radially symmetric distribution of cells and cell clusters in the dish; smaller cells were at the periphery and larger cells and cell clusters were at the center. Thus a series of photographs, taken across any diameter of the dish, gave a reasonably accurate representation of the number of cells in the entire dish. In a preliminary experiment, forty negatives, ten taken at approximately equally spaced intervals across each of four different diametric transects, were taken of one dish containing one 27 hour normal embryo dissociated for 120 minutes. The number of cells in the dish was calculated from the first ten negatives and from all forty negatives, since the area of the dish and the area in the negatives was known. The number of cells, based on ten negatives, agreed within 2% with the number calculated from forty negatives. Therefore, a determination of the number of cells in the dish was made from 10 negatives taken at approximately equally spaced intervals across the diameter of each dish. In counting cells in projected negatives, singles and doubles received equal weight, since it was impossible to determine which doubles were the result of incomplete cytokinesis occurring during the dissociation period and which consisted

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of two cells that remained attached during the entire course of the dissociation. The average number of single cells and double cell clusters in ten negatives was then used to calculate the number of "cells" released from the embryo. Each kinetic experiment was repeated three times and the results were pooled for statistical analysis. Since the blastula, early gastrula, and late gastrula stages contain different numbers of cells, the number of cells released at each time point was expressed as a percentage of the total number of cells released after 120 minutes of agitation. All curves were extrapolated to 0% cells released at 0 minutes of disaggregation. This extrapolation is valid for normal embryos, but in several of the more severely arrested embryos free cells, floating in the blastocoel, were released during bisection. The kinetic data were fitted, by computerized curvilinear regression analysis, to curves of the form:

$$Y = \beta_o + \beta_1 X + \beta_2 X^2 + \beta_3 X^3 + \beta_4 X^4.$$

This regression analysis is similar to linear regression analysis, except that "least-squares" analysis is done for a complex polynomial describing a curve rather than a simple monomial describing a line. The computer output supplied, among other things, regression coefficients (βs), expected values for each point (these were used for curve plotting in the Results section), and plots of expected values. The β_1s were compared to one another using a modified t-test to see if they were similar or different. The β_1 is the regression coefficient for the linear portion of the fourth degree polynomial. Since the greatest differences between the curves occur in their linear portions, it was decided to compare

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curves based on the relative sizes of the first order regression coefficients.

For experiments involving in vitro culture of recombined germ layer fragments, Steinberg's solution (Hamburger, '60), modified by substitution of equimolar quantities of $CaCl_2$ for $Ca(NO_3)_2 \cdot 4H_2O$ (Steinberg, personal communication) and by addition of 100 mg/liter of phenol red, was used instead of WQBSS. The former has a Tris-HCl buffer system that gives a pH of 7.4 without CO_2 gassing; whereas, the latter has a bicarbonate buffer system that requires gassing to achieve and maintain the desired pH. Once embryos were dejellied and rinsed, aseptic technique was used in all further operations. Dejellied, rinsed, late blastulae were sterilized by washing 10 times in 60 X 15 mm Falcon dishes (Falcon Plastics Company, #1007) containing 10% Steinberg's solution with penicillin G (200,000 IU/liter), streptomycin (1 gm/liter), and sulfadiazine (saturated). This was followed by incubation for an additional 2-4 hours until embryos had reached the early gastrula stage. All solutions were sterilized by vacuum filtration through 0.45 μ Millipore filters (Millipore Filter Corporation, HAWP). Following sterilization, early gastrulae were transfered to 100 X 200 mm Falcon dishes (Falcon Plastics Company, #3003) which contained 30 ml of sterile Steinberg's solution. Vitelline membranes were removed by means of forceps and two fragments were isolated and recombined. Most of the presumptive ectoderm was isolated and placed with the pigmented side down in the Falcon dish. Subsequently, a fragment of presumptive endoderm, consisting of 100-200 cells, was isolated from the center of the floor of the blastocoel and placed upon the ectodermal fragment. In all, thirteen different binary combinations were formed. One was

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normal ectoderm with normal endoderm. The other twelve were of three types from four different hybrid embryos, namely hybrid ectoderm with normal endoderm, normal ectoderm with hybrid endoderm, and hybrid ectoderm with hybrid endoderm. Each different combination was repeated 15-40 times. Whenever hybrid germ layer fragments were combined with normal germ layer fragments, five control combinations were made by confronting ectoderm and endoderm from the same normal clutch of eggs. This control procedure was instituted to ensure that alterations in spreading potential of ectoderm on endoderm in hybrid fragment-containing combinations were due only to changes in the hybrid fragments. Combinations were cultured for 30 hours at room temperature, scored, and fixed or discarded. Individual combinations were scored as follows:

5 = ectodermal spreading complete, endoderm completely engulfed

4 = ectodermal spreading 70-90% complete

3 = ectodermal spreading 40-70% complete

2 = ectodermal spreading marginally detectable

1 = no ectodermal spreading, no healing of fragments, in some cases, even disintegration of fragments.

Individual scores were added to give a total raw score for each different combination. Each total raw score was then normalized to correct for different numbers of combinations, so that results would be comparable with control results. For normalization, the total raw score for each combination was multiplied by a ratio: number of control combinations/ number of combinations of that type.

Embryonic material was prepared for electron microscopy in the

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following manner. Glass distilled water was used to make all solutions. Commercially available 50% glutaraldehyde (Fisher) was shaken for 30 minutes with an excess of activated charcoal and $BaCO_3$ on a Gyrotory Shaker, filtered through Whatman #1 filter paper in a Buchner funnel, and dilute 1:50 with glass distilled water. The optical density of this solution was measured at 280 nm and 235 nm using glass distilled water as a blank. Then the OD_{280}/OD_{235} rato was calculated. This procedure was repeated 3-5 times until the OD ratio was between 1.5 and 2.0. The 50% solution was then diluted to 10% with glass distilled water and stored in a brown bottle at 4° C. until use. Whole, dejellied, rinsed embryos, with vitelline membranes intact, were fixed in 2.5% glutaraldehyde in 0.10 M sodium cacodylate, with the pH adjusted to 7.4 with 1N HCl, for two hours at room temperature. Following glutaraldehyde fixation, the embryos were rinsed three times for one hour each in 0.10 M cacodylate buffer and stored in this solution at 4° C. from 12 hours to three months for convenience. Next, the groups of cells of interest were dissected from the embryo, rinsed in 0.20 M collidine, with the pH adjusted to 7.4 with 10 N HCl, and chilled to 0° C. in an ice bath. The fragments were post-fixed for 3.5 hours in 1% osmium tetroxide in 0.20 M collidine buffer, pH 7.4, at 0° C. Following the osmication, fragments were rinsed in dilute HCl at pH 3-5 and stained for 1.5-4.0 hours in 1% uranyl acetate in 0.20 M collidine, with the pH adjusted to 6.1 with 10 N HCl before the addition of uranyl acetate crystals. This procedure for staining <u>en bloc</u> is slightly modified from Trelstad et al. ('67). Addition of HCl to achieve the desired pH must be done with care, for if, in using HCl to lower pH, the pH should go below 6.1, one cannot resort to the use of NaOH to raise

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pH as the resulting uranyl acetate solution will be turbid and must be discarded. Uranyl acetate solutions were prepared immediately before use. Following uranyl acetate staining, the fragments were rinsed quickly in glass distilled water (essential step to prevent precipitation of uranyl acetate by ethanol used in dehydration), dehydrated rapidly in ethanol, and embedded in Epon. Following curing for 24-48 hours at 60° C., silver and grey sections were cut using glass knives and a Porter-Blum MT-2 ultramicrotome. Sections were collected on 200 or 300 mesh copper grids, stained with Reynold's lead citrate for 10 minutes, dried, carbon coated, and examined at 60 or 80 kV in either a Phillips 200 or a Phillips 300 electron microscope.

Gap measurements in presumptive mesodermal cells were made on grey sections from blocks derived from several embryos of each gamete combination. For each block (embryo), sections were scanned at intermediate power to find a contact area which had a good combination of closely apposed, normally sectioned, well fixed, and resolvable plasma membranes. One or several photographs were taken of such a contact area (depending on its size) at 41,000 X. Then the negatives were projected at 100,000 X on 8 X 10 inch photographic paper so that contact areas were roughly parallel to the long axis of the paper. In the case of exceptionally long contact areas, negatives were projected so that the contact area extended along a diagonal of the paper. In either case, the right hand margin of the print was marked, Measurements of gaps were then made from right to left along the contact area, beginning at the first point where a measurement could be made, and thereafter at 500 Å intervals to the end of the contact area or the end of the photograph. Frequently, measurements could not be made because membranes were sectioned obliquely.

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These areas were passed over. When breaks in one or both plasma membranes were encountered, measurements could not be made. If breaks were less than 1000 Å, they were passed over. If breaks were greater than or equal to 1000 Å, a new measurement was made at the extreme right end of the break and thereafter at 500 Å intervals. In some prints of contact areas, regions were encountered where the plasma membranes of adjacent cells were continuously divergent and cells were no longer in contact. Measurements were terminated when the gap distance became equal to or greater than 300 Å. Gap measurements were then assigned to one of the following classes, TJ (tight junction, where outer leaflets of apposed plasma membranes apparently fuse)[1], 20-50 Å, "X" (a contact point where both plasma membranes were not clearly resolved but the combined width of both membranes and gap did not exceed 200 Å), >50 Å but ≤ 100 Å, >100 Å but ≤ 150 Å, and so on in 50 Å intervals to 1000 Å, and greater than 1000 Å. For each gap class, a percentage of the total number of measurements was calculated. Cumulative percentiles were also calculated to give a number which indicated the percentage of the total number of measurements which were equal to or less than a certain gap size. For example, in normal R. pipiens embryos 4/269 = 1.5% of the total number of gaps were TJ, while 30/269 = 11.2% of the total number of gaps were 20-50 Å, and 33/269 = 12.3% of the total number of gaps were "X". Thus the cumulative percentile at 50 Å gap

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1. Strictly speaking, one cannot consider a tight junction "tight" unless lead citrate is not used as a stain, since lead citrate is thought to fill a 20 Å gap to make a gap junction look like a tight junction (Hay, personal communication).

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width is 1.5% + 11.2% + 12.3% = 25.0%, i.e., of all measurements, 25.0% were less than or equal to 50 Å.

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RESULTS

In all results described below, special terminology will be used to describe each gamete combination. The species designation of the egg source will be given first, followed by the species designation of the sperm source, e. g. the normal R. pipiens and R. sylvatica embryos will be called pipiens-pipiens and sylvatica-sylvatica. The egg of R. pipiens fertilized by the sperm of R. sylvatica will be called pipiens-sylvatica.

The morphological characteristics of the gamete combinations used throughout this study may be summarized as follows. 1.) pipiens-pipiens- This is the normal embryo which undergoes normal gastrulation. 2.) pipiens-palustris- This is a viable hybrid combination. In any clutch, some embryos develop normally, some undergo morphologically normal but temporally retarded gastrulation, and others undergo abnormal gastrulation, characterized by incomplete endodermal invagination (Moore, '41). 3.) pipiens-sylvatica- This is a hybrid that undergoes arrest at gastrulation. These embryos appear normal until the beginning of gastrulation. Blastopore initiation invariably occurs and occasionally proceeds until there is a distinct invagination with a crescent-shaped blastopore. During the time that control embryos are undergoing epiboly, there is a severe wrinkling and thickening of the roof of the blastocoel, while that in controls is thinning and spreading. Following arrest, the embryo survives in an apparently healthy state for up to five days. The presumptive ectodermal cells differentiate cilia and the arrested embryos often can be seen moving in the perivitelline fluid. Histologically,

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the presumptive ectodermal cells appear to adhere to one another to form a cell sheet varying in thickness from 2-3 cell layers, at areas of folding in wrinkles, to 6-8 cell layers, where no wrinkling occurs. Previously, Moore ('46) and Ting ('51) have described this hybrid. 4.) pipiens-catesbeiana- This is also a hybrid embryo arrested at the beginning of gastrulation. All of the cells comprising this grossly abnormal embryo appear to be less adherent to one another. Frequently, single cells are seen floating in the blastocoel. The embryo dies, apparently due to extensive endodermal cytolysis, one to two days after arrest. Blastopore initiation is often slight and transient and invagination is rarely seen. This description is a confirmation of the previous account of Briggs et al. ('51). 5.) pipiens-clamitans- This arrested hybrid is quite similar to pipiens-catesbeiana in gross morphology, but frequently shows endodermal cytolysis and death in less than one day after arrest. Ting ('51) also described this hybrid. Figures 1 and 2 show representative cross sections of pipiens-pipiens, pipiens-sylvatica, pipiens-catesbeiana, and pipiens-clamitans embryos.

For all gamete combinations, cleavage rates are maternally determined, resulting in identical rates of pre-gastrular development (Moore, '41). Ting ('51) and Barth and Barth ('66) have reported that pipiens-sylvatica hybrids occasionally complete gastrulation. This was not observed in over 20 different clutches in the present study, but considerable variability was noted in extent of gastrulation seen in this hybrid. For example, some clutches of pipiens-sylvatica embryos showed a small amount of invagination while others showed none. This result was quite common. In several clutches of pipiens-catesbeiana embryos, unusually extensive invagination was seen. The same was true for one clutch of

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pipiens-clamitans embryos, which ordinarily show only the faintest trace of blastopore initiation and no invagination.

1. Attachment of cells derived from animal and vegetal portions of embryos, and from whole embryos.

Normal cells have a pattern of attachment to glass in vitro that reveals changes in cell contact behavior as a function of embryonic age. Figure 3 shows the nature of this relationship for Xenopus laevis embryos. For these data, and the data shown in Figures 4-9, the standard deviation for each point is 5-30%. Thus it is not possible to make judgments concerning small differences in the shapes of the curves or in degree of attachment of animal and vegetal cells. Figures 4 and 5 show the nature of this relationship for pipiens-pipiens and sylvatica-sylvatica embryos. In all three normal embryos, the frequency of attachment of cells, derived from both the animal and vegetal portions of the embryo, is at a low level during the blastula stage and increases steadily until it reaches a maximum during the early gastrula stage. It then drops off rapidly. It should be noted that samples in the descending portion of the curve, those cells which are not attached are most frequently involved in active ameboid blebbing. This frantic activity, known as limicola movement (Holtfreter, '43), may preclude settling and flattening on the glass. It is possible, of course, that blebbing is occurring because the cells are less adhesive, but in the light of evidence to be presented in the Discussion (pp.57-58), it appears likely that the descending portion of the curve is due to increased ameboid activity rather than decreased adhesiveness of the cells. In Figures 6-9, the results of similar experiments are presented for the viable hybrid,

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pipiens-palustris, and for the arrested hybrids, pipiens-sylvatica, sylvatica-pipiens, pipiens-catesbeiana, and pipiens-clamitans. The pipiens-palustris hybrid shows normal attachment levels for cells in the animal region and a slight decrease in the peak of attachment for cells in the vegetal region. This result may well be related to abnormalities in invagination commonly observed. The sylvatica-pipiens hybrid shows nearly normal increases in both the animal and vegetal portions. This hybrid would be useful for testing the hypothesis that the descending portion of the curves of normal embryos is due to an increasing frequency of limicola movement. No decreases in frequency of cell attachment occur in sylvatica-pipiens. This may be due to a lack of appearance of ameboid motility. The pipiens-sylvatica hybrid shows a nearly normal pattern in its animal region, but a grossly abnormal, reduced level of attachment in the vegetal region. These results are of interest since both hybrids are arrested at the onset of gastrulation and yet show extensive but abortive morphogenesis (see Discussion, pp. 58-60)..The pipiens-catesbeiana and pipiens-clamitans hybrids show strikingly abnormal attachment patterns, with little or no increase in frequency of cell attachment for either animal or vegetal regions throughout the course of the experiment, but rather, a constant, low level of attachment in the 10-30% range, characteristic of the blastula.

2. Attachment of presumptive mesodermal cells from early gastrulae.

The results on the attachment of presumptive mesodermal cells, reported recently (Johnson, '69a), have been extended and confirmed; namely, cells isolated from early gastrulae of normal embryos show a high frequency of attachment, cells isolated from a viable hybrid with

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some abnormalities in invagination show an intermediate frequency of attachment, and cells isolated from hybrid embryos arrested at gastrulation show a low frequency of attachment. The extended results are summarized in Table 1. It should be noted that the sylvatica-pipiens hybrid shows a relatively high level of attachment, comparable to that seen for pipiens-palustris, the viable hybrid that undergoes gastrulation, yet does not undergo normal gastrulation. This apparently contradictory result will be discussed later (pp. 58-60).

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TABLE 1

ATTACHMENT LEVELS FOR PRESUMPTIVE MESODERMAL CELLS FROM NORMAL AND HYBRID EARLY GASTRULAE (OR ARRESTED GASTRULAE)

GAMETE COMBINATION	NUMBER OF DIFFERENT GAMETE COMBINATIONS	NUMBER OF EMBRYOS	TOTAL ATTACHED	TOTAL COUNTED	% ATTACHED ± S.D.	% CONTROL[1] VALUE	STATISTICAL TREATMENT[2] $t = n_1$	$p < n_2$
pipiens-pipiens	10	38	3922	5341	73±12	100	0.000	1.000
sylvatica-sylvatica	5	18	1305	1800	73± 8	100	0.000	1.000
clamitans-clamitans	1	11	839	1100	76± 9	100	0.000	1.000
pipiens-palustris	10	37	2592	4788	53±16	73	6.580	0.001
pipiens-sylvatica	10	34	1092	4478	26±13	36	15.11	0.001
pipiens-catesbeiana	10	50	1920	8267	22±10	30	21.34	0.001
pipiens-clamitans	10	35	928	4031	23±16	32	14.88	0.001
sylvatica-pipiens[3]	10	27	1576	2900	55±11	76	5.900	0.001
sylvatica-catesbeiana	6	17	431	1800	24±12	33	14.45	0.001
sylvatica-clamitans	3	9	171	900	18± 9	25	16.67	0.001

FOOTNOTES FOR TABLE 1

1. The control for any attachment value is the percentage attachment of the normal embryo formed from that egg, e.g. the control for pipiens-sylvatica is pipiens-pipiens and the control for sylvatica-pipiens is sylvatica-sylvatica.
2. For a statistical treatment, a t-test was performed, comparing the control value with the tabulated value.
3. The sylvatica-pipiens hybrid is a severe arrest hybrid combination that nonetheless appears to have at least as high an attachment level in its presumptive mesodermal cells as does pipiens-palustris, where apparently normal gastrulation occurs. This result will be considered further in the Discussion (pp. 58-59).

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3. Reaggregation of dissociated presumptive mesodermal cells.

The methods employed in a preliminary report of these observations (Johnson, '69a) have been altered slightly. Instead of taking photographs at 0 and 3 hours in vitro, photographs were taken at 0, 1, 2, and 3 hours in vitro. This modification was used to gain a more detailed impression of the kinetics of reaggregation. This technique allows a more refined judgment of the degree and rate of reaggregation, as shown in Figure 10. It is evident that detectable reaggregation has occurred in pipiens-pipiens (A) and pipiens-palustris (B) presumptive mesodermal cells after one hour in culture. This reaggregation continues until it is quite striking at three hours, and continues thereafter until multicellular chains are formed. In the pipiens-sylvatica (C) sample, reaggregation is not detectable until two hours, and at three hours is maximal and clearly less than that found in the pipiens-pipiens and pipiens-palustris cells. In the pipiens-catesbeiana (D) and pipiens-clamitans (E) cells, little or no reaggregation is evident after three hours and does not increase with longer time in culture. Observations of the reaggregation of other cultures, derived from different embryos of the same or different clutches, when taken with the preceding observations, reveal the following relationship for amount of reaggregation: pipiens-pipiens = pipiens-palustris > pipiens-sylvatica > pipiens-catesbeiana = pipiens-clamitans. There is a fairly good agreement between the results obtained in the frequency of attachment studies and the reaggregation studies. It should be noted that no differences were detected between the reaggregative abilities of pipiens-pipiens and

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pipiens-palustris samples comparable to the clear differences observed in the frequency of attachment. Also, pipiens-sylvatica appeared to reaggregate more extensively than pipiens-catesbeiana and pipiens-clamitans, an occurrence that would not be predicted from frequency of attachment data. It is possible that the reaggregation method is not sensitive enough to detect small differences in cell contact behavior, or that attachment to glass and reaggregation are fundamentally different processes. Currently, the former interpretation is favored (see Discussion, p. 61).

4. The rate of disaggregation of whole embryos (a measurement of overall embryonic coherence).

These experiments were designed to measure the coherence of whole embryos by measuring the rate of disaggregation, which is defined as the rate of release of single cells and double cell clusters from whole, bisected embryos. The results show that gastrulating embryos become increasingly coherent as development proceeds up to and through the gastrula stage. Non-gastrulating hybrid embryos, on the contrary, show either smaller increases or no increases at all (possibly even a decrease) in overall coherence. When one compares the rates of disaggregation of pipiens-pipiens embryos at 15, 21, and 27 hours of development, the increasing coherence of the cell masses that constitute the embryo is obvious, as shown in Figure 11 and 14. The blastula has a high rate of disaggregation; the early gastrula has an intermediate rate of disaggregation; the late gastrula has a low rate of disaggre-

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gation. In Figures 11, 12, and 13 the points of each curve are the estimated values for each point, i.e. the Ys at each X in the fourth degree polynomial (p. 15) of the regression analysis. Figure 14 illustrates these differences convincingly. The pipiens-palustris viable hybrid has rates of disaggregation similar to pipiens-pipiens. Figure 12 shows the curves of rates of disaggregation for 15, 21, and 27 hour pipiens-sylvatica, a hybrid embryo arrested at gastrulation. The rate of disaggregation decreases with increasing age in this embryo too, but clearly decreases less than in pipiens-pipiens. This result is not surprising, since the animal portion of the embryos, containing about 70% of the total cell number, undergoes an increase in adhesiveness between 15 and 21 hours which presumably continues between 21 and 27 hours. Figure 15 shows that although the rate of disaggregation shows a downward trend during development, the embryo, considered as a whole, does not show increasing overall coherence with increasing age. These results indicate that although adhesions occur between small groups of cells with increasing frequency during development, these adhesions are not sufficient to hold the embryo together in a coherent mass, nor are they sufficient to allow gastrulation. In Figure 13, the rate of disaggregation for 15, 21, and 27 hour pipiens-catesbeiana embryos is shown. There is clearly no decrease in the rate of disaggregation and there may even be an increase in the rate of disaggregation with advancing age. This, of course, is the reverse of the situation found in pipiens-pipiens, pipiens-palustris, and pipiens-sylvatica. Figure 16 illustrates the lack of increasing overall

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coherence in pipiens-catesbeiana. Similar results were obtained with pipiens-clamitans embryos. For both pipiens-catesbeiana and pipiens-clamitans, a blastula-like rate of disaggregation is observed at all three developmental ages tested.

Statistically, the picture is somewhat more complex. First, it should be remembered that a large value for β_1 from the fourth degree polynomial indicates a high rate of disaggregation. Ideally, the following relationships should exist between the β_1s of the fourth degree polynomials:

pipiens-pipiens 15 hours = pipiens-palustris 15 hours
pipiens-sylvatica 15 hours = pipiens-catesbeiana 15 hours =
pipiens-clamitans 15 hours

pipiens-pipiens 21 hours $\leq$ pipiens-palustris 21 hours $<$
pipiens-sylvatica 21 hours $<$ pipiens-catesbeiana 21 hours $\leq$
pipiens-clamitans 21 hours

pipiens-pipiens 27 hours $\leq$ pipiens-palustris 27 hours $<$
pipiens-sylvatica 27 hours $<$ pipiens-catesbeiana 27 hours $\leq$
pipiens-clamitans 27 hours

The actual results are suggestive of this pattern, but by no means follow these predictions exactly. The computed values for the $4^{\circ}\beta_1$s are shown in Table 2.

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TABLE 2

COMPUTED VALUES OF FIRST ORDER REGRESSION COEFFICIENTS

FOR THE FOURTH DEGREE POLYNOMIALS

GAMETE COMBINATION	AGE IN HOURS AT ROOM TEMPERATURE	COMPUTED VALUE OF $4°\beta_1$	EXPECTED VALUE for $4°\beta_1$
pipiens-pipiens	15 hours	7.7397	expected high and equal
pipiens-palustris	15 hours	6.9986	
pipiens-sylvatica	15 hours	8.3272	
pipiens-catesbeiana	15 hours	7.4922	
pipiens-clamitans	15 hours	8.3605	
pipiens-pipiens	21 hours	5.3116	expected low and equal
pipiens-palustris	21 hours	6.4200	
pipiens-sylvatica	21 hours	4.6768	expected intermediate
pipiens-catesbeiana	21 hours	3.9271	expected high and equal
pipiens-clamitans	21 hours	8.3605	

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TABLE 2 (CONT.)

COMPUTED VALUES OF FIRST ORDER REGRESSION COEFFICIENTS

FOR THE FOURTH DEGREE POLYNOMIALS

GAMETE COMBINATION	AGE IN HOURS AT ROOM TEMPERATURE	COMPUTED VALUE OF $4°\beta_1$	EXPECTED VALUE OF $4°\beta_1$
pipiens-pipiens	27 hours	1.8436	} ——— expected low and equal
pipiens-palustris	27 hours	0.6914	
pipiens-sylvatica	27 hours	3.2005	} ——— expected intermediate
pipiens-catesbeiana	27 hours	8.3656	} ——— expected high and equal
pipiens-clamitans	27 hours	7.6132	

The regression coefficients were compared by a modified t-test. The results of this analysis follow. Each logical statement is made with a statistical confidence of 99.9%. The $4^{\circ}\beta_1$ for each combination is listed below the appropriate combination.

OBSERVED RESULTS

pipiens-pipiens 15 hours > pipiens-palustris 15 hours <

(7.7397) (6.9986)

pipiens-sylvatica 15 hours > pipiens-catesbeiana 15 hours <

(8.3272) (7.4922)

pipiens-clamitans 15 hours

(8.3605)

pipiens-pipiens 21 hours < pipiens-palustris 21 hours >

(5.3116) (6.4200)

pipiens-sylvatica 21 hours > pipiens-catesbeiana 21 hours <

(4.6768) (3.9271)

pipiens-clamitans 21 hours

(8.8291)

pipiens-pipiens 27 hours > pipiens-palustris 27 hours <

(1.8436) (0.6914)

pipiens-sylvatica 27 hours < pipiens-catesbeiana 27 hours >

(3.2005) (8.3656)

pipiens-clamitans 27 hours

(7.6132)

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Although all the results are not matched to the expectations, most are. The $4°\beta_1$s at 15 hours for all combinations are high and not greatly different. In addition, the $4°\beta_1$s for pipiens-pipiens and pipiens-palustris at 27 hours are low, that for pipiens-sylvatica is intermediate, and those for pipiens-catesbeiana and pipiens-clamitans are high. The results for 21 hour embryos are not well matched. Only pipiens-clamitans shows the predicted high value. These discrepancies will be considered at length in the Discussion (pp. 64-65).

5. Recombination of germ layer fragments derived from normal and hybrid embryos.

These experiments were performed in an attempt to understand more fully the relationship between morphogenetic potential of a germ layer fragment and cell contact behavior of the cells in the fragment. Since these are complex experiments, a brief reiteration of the methods used is necessary. Fragments of ectoderm and endoderm were isolated from early gastrulae or early-arrested gastrulae (18-20 hours). Thirteen different combinations of ectodermal and endodermal fragments were made from normal and hybrid embryos. One combination was ectoderm and endoderm from pipiens-pipiens. The other twelve combinations were of three types for four different hybrid embryos (pipiens-palustris, pipiens-sylvatica, pipiens-catesbeiana, and pipiens-clamitans), namely, hybrid ectoderm and normal endoderm, normal ectoderm and hybrid endoderm, and hybrid ectoderm with hybrid endoderm. A number of combinations were formed in a large Falcon dish

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and cultured for 30 hours. At the end of this time, each combination was scored for degree of spreading of ectoderm on endoderm. Every time hybrid-normal recombinants were made, control normal-normal recombinants were also made from the same clutch of normal eggs and cultured under identical conditions. Scores for individual combinations were added to give a total raw score for each combination. Each total raw score was then normalized by multiplying it by a ratio: total number of normal-normal recombinants/ total number of normal-hybrid or hybrid-hybrid recombinants of each type. This normalization was used to correct for differences in sample size.

Although certain results are hard to explain, the behavior of most germ layer fragments is consistent with one interpretation. For a fragment of presumptive ectoderm to engulf a fragment of presumptive endoderm, the cells of each must be adherent to one another. In addition, the endodermal cells must be capable of serving as a substratum for ectodermal spreading, i.e. ectodermal and endodermal cells must be firmly adherent to one another. No assumptions are made about the relative strengths of adhesions. For example, all cells in fragments derived from pipiens-pipiens and pipiens-palustris embryos appear to be adhesive to one another, and extensive spreading and engulfment occurs in all combinations made between germ layer fragments from these embryos. All cells in fragments derived from pipiens-catesbeiana and pipiens-clamitans embryos are less adhesive to one another. There is also a great reduction in the amount of spreading observed in any combination of fragments where one fragment is derived from either of these two hybrid embryos. The situation involving combi-

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nations of fragments from pipiens-sylvatica hybrids is more difficult to interpret. One would predict that pipiens-sylvatica ectoderm would engulf pipiens-pipiens endoderm, since both are known to show cell contact behavior which indicates high adhesiveness in all cells in each fragment. Pipiens-sylvatica ectoderm does engulf pipiens-pipiens endoderm. On the other hand, it has been shown that there are gross reductions in the adhesiveness of endodermal cells in pipiens-sylvatica hybrids. This fragment, however, contrary to expectations, serves as an excellent substratum for spreading of either pipiens-pipiens or pipiens-sylvatica ectoderm. This paradoxical situation will be discussed further (pp. 67-68). The results of these experiments are summarized in Table 3. In Figure 17, the results of Table 3 are presented graphically using the symbolic designation for each ectoderm-endoderm combination of Table 3. Figure 18 illustrates the clear differences in the spreading ability of pipiens-pipiens and pipiens-catesbeiana ectoderm on pipiens-pipiens and pipiens-catesbeiana endoderm. Higher magnification of the ectoderm of pipiens-pipiens and pipiens-catesbeiana in the combinations in Figure 18A and 18C show that the pipiens-pipiens ectoderm forms a tightly coherent and exceedingly thin spreading epithelium; whereas, the pipiens-catesbeiana ectoderm forms a non-coherent, thickened mass of non-spreading cells that could not be described as an epithelium.

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TABLE 3

BINARY COMBINATIONS OF EXPLANTED GERM LAYER FRAGMENTS

EXPLANT COMBINATION					
ECTODERMAL SOURCE	ENDODERMAL SOURCE	SYMBOL	NUMBER OF COMBINATIONS	TOTAL RAW SCORE[1]	NORMALIZED SCORE[2]
pipiens-pipiens	pipiens-pipiens	A	40	166	166
pipiens-pipiens	pipiens-palustris	B	24	93	155
pipiens-palustris	pipiens-pipiens	C	24	91	152
pipiens-palustris	pipiens-palustris	D	30	117	156
pipiens-pipiens	pipiens-sylvatica	E	15	59	157
pipiens-sylvatica	pipiens-pipiens	F	15	60	160
pipiens-sylvatica	pipiens-sylvatica	G	15	58	155
pipiens-pipiens	pipiens-catesbeiana	H	30	75	100
pipiens-catesbeiana	pipiens-pipiens	I	30	47	63
pipiens-catesbeiana	pipiens-catesbeiana	J	30	47	63
pipiens-pipiens	pipiens-clamitans	K	15	41	109
pipiens-clamitans	pipiens-pipiens	L	15	15	40
pipiens-clamitans	pipiens-clamitans	M	15	15	40

FOOTNOTES FOR TABLE 3

1. The total raw score is the total of the individual scores for each binary combination.
2. The normalized score is the total raw score for each combination multiplied by the ratio: number of control combinations/ number of combinations of that particular type. For example, combination B has a total raw score of 93 from 24 different combinations. This total raw score was normalized by multiplying it by 40/24 to be comparable with the A score.

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6. Fine-Structural Investigations-

The results already described lead one to believe that there is a marked alteration in the adhesiveness of certain cells in normal embryos that does not occur in arrested hybrid embryos. This raises the possibility that there are differences in the ways cells contact each other that may be revealed in the electron microscope. Two approaches were employed. First, comparisons were made of the types of contacts formed between presumptive mesodermal cells of normal and hybrid embryos at the early gastrula stage. Second, an attempt was made to detect changes in number or in types of contacts between presumptive ectodermal cells in gastrulating embryos from the blastula to the late gastrula period and in one hybrid, arrested at the onset of gastrulation, at similar developmental ages.

Presumptive mesodermal cells from all gamete combinations studied have certain similarities. Much of the similarity, of course, is due to their common maternal component. Thus the numerous yolk platelets and vesicles, filled with moderately electron dense material and scattered throughout the cytoplasm, are similar. Likewise, the mitochondria are indistinguishable, having an ovoid shape and sparse cristae. No endoplasmic reticulum or Golgi apparatus was detected at the early gastrula stage. Microtubules are not seen, but there are many glycogen granules.

The most striking difference between presumptive mesodermal cells from different gamete combinations occurs in the amount of

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contact area between cells and in the distances that separate the plasma membranes of apposed cells in areas of contact. To determine the relative frequencies of distances that separate plasma membranes for all gamete combinations, an unbiased method to determine measurement locations along contact areas was devised (see Material and Methods). Measurements were made and assigned to different size classes. The number of embryos used, the total number of measurements, the numbers in each gap class, percentage of total in each gap class, and cumulative percentile for all gap classes, is presented for each gamete combination in Table 4.

The contact relations of presumptive mesodermal cells from pipiens-pipiens and pipiens-palustris consist characteristically of small, triangular, intercellular spaces where three cells join (Figure 19) and long regions, 1-8 μ in extent and probably longer, where cell membranes run parallel to one another and are closely apposed. Junctions with gaps of 50-100 Å between the apposed plasma membranes are quite common in these regions; gaps of 20-50 Å are nearly as common; tight junctions also occur but are rare. Between regions of close apposition of plasma membranes, larger gaps, where the cells are not in contact, are found. Typical contacts between presumptive mesodermal cells of pipiens-pipiens and pipiens-palustris are shown in Figures 21 and 22. Note especially the extensive region (>1.5 μ) of close apposition (maximum gap = 130 Å) of plasma membranes of pipiens-pipiens cells illustrated in Figure 21B. It should be noted that tight junctions are seen with less frequency in pipiens-palustris than in pipiens-pipiens, and in general, there is a slight shift toward larger gaps.

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These differences may not be significant, but they correlate with slight alterations in cell contact behavior detected by other means. Presumptive mesodermal cells from two different sylvatica-sylvatica embryos of one clutch were also examined and they appear, superficially at least, to resemble similar cells in pipiens-pipiens and pipiens-palustris.

Presumptive mesodermal cells from pipiens-sylvatica and pipiens-catesbeiana show an increase in amount of intercellular space and a decrease in the area of cell contact, but also have fairly extensive regions of cell contact where the membranes run parallel to each other. In these embryos, however, the contacts are different from gastrulating embryos in two ways. First, larger gaps are more common. There is a marked reduction in the frequency of 20-50 Å and "X" gaps (a contact point where both plasma membranes were not clearly resolved but the combined width of both membranes and gap did not exceed 200 Å) and more frequent regions where the plasma membranes are separated by gaps of greater than 200 Å. Second, no tight junctions were observed. Typical cell contacts for presumptive mesodermal cells of pipiens-sylvatica and pipiens-catesbeiana are shown in Figures 23 and 24 respectively.

When one compares presumptive mesodermal cells from the arrested hybrid pipiens-clamitans with the same cells in gastrulating embryos, striking differences are immediately apparent (Figures 20 and 25 should be compared with Figures 19 and 21). Large intercellular spaces are frequently seen. There is a reduction in the area of cell contact. The surfaces of apposed cells have a highly irregular contour,

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so that plasma membranes no longer run parallel for great distances. Rather, they occasionally approach one another with gaps of 100-200 Å, but rarely less, and then are separated by much larger spaces of 250-1000 Å and more. Again, no tight junctions were seen. A typical contact area between four pipiens-clamitans presumptive mesodermal cells is shown in Figure 25. Note especially the large gaps between cells.

Three generalizations can be drawn from the information provided above and the data in Table 4. 1.) It is clear that the plasma membranes of presumptive mesodermal cells in non-gastrulating hybrids make contact over smaller areas and do not approach one another as closely in contact areas as cells of gastrulating embryos. This point is clearly illustrated in Figures 19 and 20 and in Figure 26, which is a histogram of the data of Table 4. 2.) It is equally clear that as hybrid incompatibility becomes more severe, as judged by degree of severity of arrest of gastrulation, the distances separating plasma membranes becomes greater. This fact is illustrated in Figure 27, which shows the cumulative percentiles as a function of gap size for different gamete combinations. Objective criteria for severity of incompatibility will be considered fully in the Discussion (p. 71). 3.) There appears to be a severe reduction in number, and possibly a complete absence, of tight junctions, as well as a severe reduction in the number of 20-50 Å and "X" gaps in non-gastrulating hybrids. This finding suggests that these specialized cell contacts play a role in gastrulation.

The results of an ultrastructural examination of cell contacts in presumptive ectodermal cells from pipiens-pipiens and pipiens-pal-

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ustris are well correlated with other results on changes in cell contact behavior during development. Both of these gastrulating embryos yield similar results. At 15 hours of development (blastula), inner blastomeres in the roof of the blastocoel do not appear to be firmly adherent to one another. There is a relatively large amount of intercellular space and relatively small area of cell contact. Intercellular gaps are commonly 300-800 Å and are rarely less that 100-200 Å. The cell surfaces are quite smooth, but occasionally are thrown into small undulations where the gaps are 100-200 Å. A typical region of contact between three inner blastomeres in the roof of the blastocoel of the pipiens-palustris blastula is shown in Figure 28. At 21 hours (early gastrula) and 27 hours (late gastrula), the inner ectodermal blastomeres appear to be firmly adherent to one another (Figures 29-32). There is less intercellular space and more area of cell contact, where 100-200 Å gaps are common. Some regions of the plasma membranes are separated by gaps of 50 Å and tight or close junctions can be observed, as shown in Figures 29 and 30. No gross differences in numbers or types of junctions were observed between 21 hour and 27 hour ectodermal blastomeres. There is, however, one conspicuous difference in surface morphology. There is an increase in number of filopodial processes seen extending between blastomeres from the early gastrula and late gastrula. In 21 hour material, occasional filopodia project from the otherwise smooth cell surfaces a distance of 0.1-0.2 μ. The tips of these projections commonly contact adjacent cells and form either a tight junction or a gap of about 50 Å. By 27 hours, these processes are more numerous, longer (up to 1.3 μ), and

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frequently contain short segments of microtubules (Figure 31). These processes sometimes overlap, especially along the inner surface of the spreading ectodermal sheet, (Figure 33), i.e. the surface adjacent to the nearly obliterated blastocoel, and may well be designed to withstand the tangential stresses felt by cells as they participate in epiboly. It is quite clear that the presumptive ectodermal cells of both normal and viable hybrid embryos form an increased number of close appositions during gastrulation. In addition, there is an increase in the number of filopodia.

The fine structure of cell contacts in presumptive ectodermal cells in one non-gastrulating hybrid, pipiens-catesbeiana, has also been examined. At 15 hours of development (blastula), inner blastomeres in the roof of the blastocoel have contact relations similar to those seen in pipiens-pipiens and pipiens-palustris (Figure 34). There are relatively small areas of cell contact. In areas of cell contact, gaps are usually 100-200 Å but rarely less. The cell surfaces are quite smooth. At 21 hours (early gastrula arrest) there is a reduction in area of cell contact. Again, in contact areas, plasma membranes are separated by gaps of 100-200 Å but rarely less. Cell surfaces remain smooth (Figure 35). At 27 hours (late gastrula arrest) there is a further "degeneration" in area of cell contact, such that great cell-free spaces, especially among inner blastomeres, are common (Figures 36 and 37). The blastomeres which compose the outer surface layer of the roof of the blastocoel have long regions of contact along their lateral surfaces (Figure 38) where gaps are 100-200 Å but rarely less. The long filopodia containing microtubules, seen in normal 27 hour

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ectoderm (Figures 31 and 33) are lacking at 27 hours in pipiens-catesbeiana ectoderm. Microtubules are abundant in hybrid cells at this stage (Figure 38).

There are two conspicuous differences in the nuclei of presumptive ectodermal cells of gastrulating and non-gastrulating embryos. The most striking difference occurs in the distribution of electron density in the nucleoplasm. At all stages examined, nuclei in gastrulating embryos always have a homogeneous distribution of moderate electron density. In contrast, at all stages examined, interphase nuclei in non-gastrulating embryos always have a heterogeneous distribution of electron density with numerous areas of high density distributed in a haphazard fashion throughout the otherwise moderately dense nucleoplasm. These dense areas are quite similar to metaphase chromosomes (Figures 34 and 37). Nucleoli are first seen in the nuclei of gastrulating embryos at the early gastrula and are more common and larger by the late gastrula stage. In contrast, nucleoli are never seen in the nuclei of non-gastrulating embryos. These observations are based on material taken from one or two embryos and thus may not be truly representative (see Discussion, p. 72).

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TABLE 4

GAP DIMENSIONS IN CONTACT AREAS BETWEEN PRESUMPTIVE MESODERMAL CELLS IN EARLY GASTRULAE OR EARLY ARRESTED HYBRIDS (21 HOURS)

PIPIENS-PIPIENS

GAP IN Å	NUMBER	PERCENT TOTAL	CUMULATIVE PERCENTILE
TJ	4	1.5	1.5
"X"	33	12.3	13.8
20-50	30	11.2	25.0
>50 but≤100	56	20.8	45.8
>100 but≤150	36	13.4	59.2
>150 but≤200	46	17.1	76.3
>200 but≤250	26	9.7	86.0
>250 but≤300	8	3.0	89.0
>300 but≤350	9	3.3	92.3
>350 but≤400	7	2.6	94.9
>400 but≤450	3	1.1	96.0
>450 but≤500	5	1.9	97.9
>500 but≤550	3	1.1	99.0
>550 but≤600	3	1.1	100.1
>600 but≤650	-	-	-
>650 but≤700	-	-	-
>700 but≤750	-	-	-
>750 but≤800	-	-	-
>800 but≤850	-	-	-
>850 but≤900	-	-	-

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TABLE 4 (CONT.)

PIPIENS-PIPIENS (CONT.)

GAP IN Å	NUMBER	PERCENT TOTAL	CUMULATIVE PERCENTILE
>900 but≤950	-	-	-
>950 but≤1000	-	-	-
>1000	-	-	-
TOTAL NUMBER OF MEASUREMENTS	269		
TOTAL NUMBER OF EMBRYOS	5		

FOOTNOTE FOR TABLE 4

1. The cumulative percentile is a number that represents the percentage of the total number of gaps that are less than or equal to a certain gap size, e.g. the cumulative percentile for pipiens-pipiens at 50 Å is 1.5% + 12.3% + 11.2% = 25.0%.

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TABLE 4 (CONT.)

PIPIENS_PALUSTRIS

GAP IN Å	NUMBER	PERCENT TOTAL	CUMULATIVE PERCENTILE
TJ	1	0.3	0.3
"X"	20	5.2	5.5
20-50	37	9.7	15.2
>50 but≤100	142	37.2	52.4
>100 but≤150	70	18.3	70.7
>150 but≤200	52	13.6	84.3
>200 but≤250	26	6.8	91.1
>250 but≤300	20	5.2	96.3
>300 but≤350	6	1.6	97.9
>350 but≤400	4	1.0	98.9
>400 but≤450	4	1.0	99.9
>450 but≤500	-	-	-
>500 but≤550	-	-	-
>550 but≤600	-	-	-
>600 but≤650	-	-	-
>650 but≤700	-	-	-
>700 but≤750	-	-	-
>750 but≤800	-	-	-
>800 but≤850	-	-	-
>850 but≤900	-	-	-
>900 but≤950	-	-	-
>950 but≤1000	-	-	-
>1000	-	-	-

TOTAL NUMBER OF MEASUREMENTS = 382 TOTAL NUMBER OF EMBRYOS = 8

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TABLE 4 (CONT.)

PIPIENS-SYLVATICA

GAP IN Å	NUMBER	PERCENT TOTAL	CUMULATIVE PERCENTILE
TJ	-	-	-
"X"	-	-	-
20-50	5	2.1	2.1
>50 but≤100	32	13.7	15.8
> 100 but≤150	42	18.0	33.8
> 150 but≤200	54	23.2	57.0
> 200 but≤250	18	7.7	64.7
> 250 but≤300	30	12.9	77.6
> 300 but≤350	17	7.3	84.9
> 350 but≤400	16	6.9	91.8
> 400 but≤450	2	0.9	92.7
> 450 but≤500	5	2.1	94.8
> 500 but≤550	-	-	-
> 550 but≤600	1	0.4	95.2
> 600 but≤650	-	-	-
> 650 but≤700	2	0.9	96.1
> 700 but≤750	1	0.4	96.5
> 750 but≤800	2	0.9	97.4
> 800 but≤850	1	0.4	97.8
> 850 but≤900	3	1.3	99.1
> 900 but≤950	1	0.4	99.5
> 950 but≤1000	1	0.4	99.9
>1000	-	-	-

TOTAL NUMBER OF MEASUREMENTS = 233 TOTAL NUMBER OF EMBRYOS = 7

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TABLE 4 (CONT.)

PIPIENS-CATESBEIANA

GAP IN Å	NUMBER	PERCENT TOTAL	CUMULATIVE PERCENTILE
TJ	-	-	-
"X"	3	0.6	0.6
20-50	15	3.2	3.8
>50 but≤100	138	29.8	33.6
>100 but≤150	87	18.7	52.3
>150 but≤200	89	19.2	71.5
>200 but≤250	45	9.7	81.2
>250 but≤300	29	6.3	87.5
>300 but≤350	12	2.6	90.1
>350 but≤400	13	2.8	92.9
>400 but≤450	12	2.6	95.4
>450 but≤500	10	2.2	97.6
>500 but≤550	3	0.6	98.2
>550 but≤600	5	1.1	99.3
>600 but≤650	-	-	-
>650 but≤700	-	-	-
>700 but≤750	-	-	-
>750 but≤800	-	-	-
>800 but≤850	-	-	-
>850 but≤900	1	0.2	99.5
>900 but≤950	-	-	-
>950 but≤1000	1	0.2	99.7
>1000	-	-	-

TOTAL NUMBER OF MEASUREMENTS = 463 TOTAL NUMBER OF EMBRYOS = 6

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TABLE 4 (CONT.)

PIPIENS-CLAMITANS

GAP IN Å	NUMBER	PERCENT TOTAL	CUMULATIVE PERCENTILE
TJ	-	-	-
"X"	4	1.0	1.0
20-50	12	3.1	4.1
>50 but≤100	63	16.4	20.5
>100 but≤150	39	10.2	30.7
>150 but≤200	44	11.5	42.2
>200 but≤250	33	8.6	50.8
>250 but≤300	40	10.4	61.2
>300 but≤350	22	5.7	66.9
>350 but≤400	33	8.6	75.5
>400 but≤450	22	5.7	81.2
>450 but≤500	12	3.1	84.3
>500 but≤550	8	2.1	86.4
>550 but≤600	9	2.4	88.8
>600 but≤650	8	2.1	90.9
>650 but≤700	6	1.6	92.5
>700 but≤750	3	0.8	93.3
>750 but≤800	2	0.5	93.8
>800 but≤850	3	0.8	94.6
>850 but≤900	1	0.3	94.9
>900 but≤950	2	0.5	95.4
>950 but≤1000	1	0.3	95.7
>1000	16	4.2	99.9

TOTAL NUMBER OF MEASUREMENTS = 383 TOTAL NUMBER OF EMBRYOS = 8

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DISCUSSION

Five different experimental methods were employed in this study to investigate cell contact behavior of different groups of cells from different normal embryos and gastrulating or non-gastrulating hybrid embryos. For the most part, the presence or absence of a large alteration in cell contact behavior correlates with the presence or absence of gastrulation. Since the results of these different methods are similar, it seems probable that the same surface property was being measured. There is good reason to believe that these altered contact relations are due to alterations in cellular adhesiveness.

Adhesiveness is the force acting between the constituent molecules of apposed surfaces that holds them together, once they make contact. For cells adhering to other cells or to inert substrata, this force is notoriously difficult to measure and to this date no measurements have revealed a mechanism of cell adhesion (Curtis, '60; Weiss, '60; Moscona, '61; Curtis, '62; Steinberg, '64; Curtis, '67; Weiss, '67; Phillips and Steinberg, '69; Trinkaus, '69). Most "measurements" of adhesiveness are performed in systems which use an operational definition of adhesiveness which may or may not be accurate. In the present study, different methods were employed to examine cell contact behavior. The problem before us is whether these methods, individually or collectively, give any reliable information on cell surface adhesiveness.

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1. Attachment to Glass

The first method for examining changes in cell contact behavior was a measurement of the ability of cells to adhere to and flatten upon a glass substratum, with the formation of ruffled membranes. In a normal embryo and in a gastrulating hybrid embryo, all cells show an increasing ability to attach to glass, from the mid-blastula to early gastrula period. In non-gastrulating hybrid embryos, on the other hand, cells show little or no increases in frequency of attachment. Is flattening on a plane glass substratum and the formation of a ruffled membrane dependent on adhesiveness of the cell surface? To answer this question, it must be shown that flattened cells are indeed attached; and, in addition, it must be shown that attached cells are more adhesive than unattached cells.

Four lines of evidence indicate that flattened cells with ruffled membranes are attached to the glass substratum. 1.) All flattened cells with ruffled membranes from an early normal gastrula cannot be dislodged by inversion of a culture or a gentle stream of medium. Rounded cells can be dislodged readily by either treatment (Johnson, unpublished observations). Similar observations have been made by Taylor ('61) for conjunctiva cells. 2.) Taylor ('61) also observed that as conjunctiva cells attach to glass, they become progressively flattened, and that both rate of cell attachment and rate of cell flattening were equally depressed by serum in the medium. 3.) Ambrose ('61) used surface contact microscopy of moving fibroblasts to show that cell surfaces

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in the region of ruffled membranes make close contacts with the glass. More recently, A. Harris (unpublished observations) and Trinkaus, Betchaku, and Krulikowski (unpublished observations) have shown that if a migrating fibroblast has a large ruffled membrane at the leading edge and a smaller one at the trailing edge, the trailing edge of the cell can be seen to detach repeatedly and snap forward, as if there is no attachment other than at the ruffled membrane. 4.) Curtis ('64) used interference reflection microscopy to show that the surfaces of embryonic chick heart fibroblasts approach a glass substratum as close as 100 Å in regions of ruffled membranes and at a distance of 300-600 Å in other portions of the cell away from the ruffled membrane. From these lines of evidence it seems apparent that a flattened cell with a ruffled membrane is attached to a glass substratum.

Attachment to glass may not be related to attachment to another cell, however, and thus may not be a reliable measurement of cell-cell adhesiveness. There are four observations, however, that suggest that attachment to glass and attachment to cells are closely related processes. 1.) In this study, it has been shown that rate of disaggregation decreases during the same period that frequency of cell attachment increases. 2.) Changes in cell contact at the level of fine structure that occur during amphibian gastrulation are consistent with the notion of stronger cell-cell adhesion. 3.) In the present work, dissociated cells that attach to glass with high frequency also reaggregate; whereas, cells that attach to glass with a low frequency do not reaggregate. This point is illustrated as well in a study by Weiss ('68) on the cell contact behavior of two malignant tissue culture lines, a

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mouse mastocytoma and an osteogenic sarcoma. When mastocytoma cells are grown in spinner culture, they remain in single cell suspension, whereas, sarcoma cells form small clusters under the same conditions. In stationary cultures, in the presence of serum, mastocytoma cells fail to attach to glass, whereas, sarcoma cells attach readily. 4.) Trinkaus (unpublished observations) has shown that a Fundulus blastoderm, dissected from an early gastrula, adheres readily to the periblast (its normal substratum) but not to the yolk cytoplasmic layer. The periblast also adheres rapidly to glass, but the yolk cytoplasmic layer does not. 5.) Curtis ('64) showed that fibroblasts form gaps of 100-200 Å with a glass substratum in regions of the ruffled membrane, and, of course, 100-200 Å gaps are commonly seen in electron micrographs between cells that are thought to be adherent. This observation suggests that there is a universal mechanism of cell adhesion. On the basis of these several lines of evidence, it seems safe to conclude that the ability to attach to a glass substratum is one reliable method for determining cellular adhesiveness.

On the other hand, if the ability of dissociated cells to attach to glass were an adequate criterion in itself for assessing cell adhesiveness, one would be forced to conclude that a yolk plug embryo possesses less cell-cell adhesiveness than an early gastrula (see p. 24). This conclusion is probably erroneous for a number of reasons. 1.) Close observation in situ of the inner surface of the roof of the blastocoel in normal early gastrulae reveals some polygonal cells making contact with their neighbors and some rounded cells which are not contacting other cells over such an extensive area. Spaces can be seen

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between some cells. In the normal late gastrula, in contrast, the same cells form a uniform, closely packed, hexagonal array of cells which all appear to be firmly adherent to their neighbors, forming a coherent sheet. 2.) The rate of disaggregation of normal embryos decreases between the early and late gastrula period. 3.) Presumptive ectodermal cells of the late gastrula have large, over-lapping filopodia interconnecting adjacent cells that are lacking in the early gastrula. 4.) Patricolo ('67) showed that cells derived from late gastrulae of Discoglossus pictus reaggregate more readily than cells derived from early gastrulae. These observations suggest that late gastrula cells are actually more adhesive than early gastrula cells. How then can we explain the lack of adhesion to glass of dissociated cells from late gastrulae? It could be due to the surface activity of these cells. When dissociated they become rounded up and undergo frantic blebbing, known as limicola movement (Holtfreter, '43). This activity might preclude settling on the substratum, with subsequent formation of ruffled membranes and fan-like pseudopodia. It is also possible, of course, that limicola movements occur because attachment cannot occur, due to a decrease in cell-substratum adhesiveness. The possibility also remains that cells of the late gastrula have less "general" adhesiveness (and thus will not attach to glass) but more ability to form morphogenetically significant, cell-cell adhesions, hence cling closely together in situ.

Frequency of attachment studies reveal severe alterations in cell adhesiveness in all cells composing a hybrid that is severely arrested at the onset of gastrulation. Two hybrids, however, show

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quasi-normal patterns of adhesiveness in all or some cells of the embryo. The sylvatica-pipiens embryo, for example, shows a normal pattern of increase in adhesiveness from the blastula to early gastrula period but does not show the characteristic drop in frequency of attachment characteristic of all normal embryos tested. The embryo, described in a preliminary way by Moore ('41), has a number of unique morphogenetic characteristics. Blastopore initiation appears normal and continues until the blastopore has become a normal full circle. Bottle cells, however, do not appear to be formed. Little invagination and no archenteron formation occurs, but there is a pronounced dorsad shift of presumptive mesodermal and endodermal cells, so that the blastocoel is partially reduced in volume. At about 26 hours of development, superficial blastomeres loose their normal cortical pigmentation, followed by a rapid degeneration and death of the embryo. For this hybrid, therefore, development becomes abnormal at the onset of gastrulation, but, nevertheless, considerable morphogenesis does occur. It is significant that all cells from this arrested hybrid show an attachment level that is comparable to or even greater than that seen in the viable hybrid pipiens-palustris. More extensive investigations of this hybrid are planned. It may be that this hybrid possesses the "general" adhesiveness necessary for attachment to glass, but not the specific pattern of cell surface adhesiveness that directs normal gastrulation.

The recirocal hybrid, pipiens-sylvatica, shows a quasi-normal pattern of adhesiveness in cells of the animal portion and an abnormal pattern in the vegetal portion. This embryo has been described

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in detail by Moore ('46) and Ting ('51). Again, a number of unique and interesting morphogenetic characteristics are seen. Blastopore initiation invariably occurs with some invagination. Occasionally, embryos are seen with well-formed, horse shoe-shaped blastopores. Barth and Barth ('66) have reported that some embryos in a limited number of clutches complete gastrulation. This result was not encountered in over twenty different clutches in the present study. Other abortive morphogenesis occurs as well. During the time that control embryos are undergoing epiboly, pipiens-sylvatica embryos exhibit wrinkling in the roof of the blastocoel. It appears that the animal portions of these embryos have a tendency to spread which results in wrinkle formation, since no space is available for their spreading (invagination does not occur). This interpretation is similar to one presented by Devillers ('52) for a different experimental system. He analysed the coordination of epiboly in Salmo irideus by performing reciprocal transplantation of blastoderms between young and advanced embryos. The young blastoderm transplanted to an advanced periblast undergoes epiboly at the same time as the young control. The advanced blastoderm transplanted to the young periblast does not undergo epiboly. Rather, it forms a long protrusion from the surface of the egg. This is presumably due to the lack of coordination between the tendency toward epiboly in the advanced blastoderm and the inability of the young periblast to support epiboly.

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2. Reaggregation

If a universal mechanism of cell adhesion exists, regardless of substratum, one would predict, based on frequency of attachment studies reported here, that amount of reaggregation would have the following relationship: pipiens-pipiens > pipiens-palustris > pipiens-sylvatica = pipiens-catesbeiana = pipiens-clamitans. Actually, reaggregation shows the following relationship: pipiens-pipiens = pipiens-palustris > pipiens-sylvatica > pipiens-catesbeiana = pipiens-clamitans. It seems, therefore, that the tendency to adhere to glass and cells, although approximately the same, shows some discrepancies. These discrepancies may be due to a number of factors. There is some variability from clutch to clutch in the amount of reaggregation seen in cells from arrested hybrids, such that one pipiens-sylvatica preparation may reaggregate more extensively than another. There is a good deal of insensitivity in this method for measuring cell contact behavior; small differences may not be particularly significant. For example, it is very difficult to make cultures that have equal cell densities or are derived from precisely the same group of cells. Given these limitations, only gross differences in extent of reaggregation can be detected consistently. Finally, it may well be that slightly different levels of adhesiveness are being measured with the two different methods- adhesion to glass versus adhesion to cells.

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3. Disaggregation

The measurements of rates of disaggregation indicate that gastrulating embryos become more coherent throughout the course of blastula → late gastrula development while non-gastrulating embryos do not. In pipiens-pipiens and pipiens-palustris, there are large, progressive decreases in rates of disaggregation between the blastula and late gastrula stage (Figure 11). These changes are also reflected in the increases in overall coherence of the whole embryos (Figure 14). Pipiens-sylvatica embryos, in contrast, show only moderate progressive decreases in rates of disaggregation (Figure 12); moreover, the embryo, considered as a whole, does not show increasing overall coherence with increasing age (Figure 15). Pipiens-catesbeiana and pipiens-clamitans have high, possibly equal or increasing, rates of disaggregation at all stages tested (Figure 13); therefore, they could hardly be expected to show any change in overall coherence (Figure 16). Gastrulation involves the coordinated movement of masses of cells (Holtfreter, '39; Schechtman, '42; Holtfreter, '43, '44; Trinkaus, '65, '69). In the present study, the most striking difference between gastrulating and non-gastrulating embryos is the ability of gastrulating embryos to form progressively more coherent masses of cells, rather than simply the ability to form increasing numbers of adhesions between small numbers of cells. It seems likely then, that gastrulation requires the ability to form large groups of adherent cells. Clearly, the ability to form coherent masses of cells is only a necessary condition for

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gastrulation. It is difficult to imagine a mechanism whereby such collective adhesive behavior could by itself be summated into a morphogenetic event which invariably follows one route.

The rates of disaggregation of gastrulating and non-gastrulating embryos are well correlated with attachment and reaggregation studies. Pipiens-pipiens and pipiens-palustris, both gastrulating embryos, show large, progressive decreases in rate of disaggregation during development and moderate to high increases in frequency of attachment for all cells during the blastula to early gastrula period. Also, presumptive mesodermal cells show moderate to high frequencies of attachment and a high ability to reaggregate into multicellular chains. For pipiens-sylvatica, a non-gastrulating hybrid, rates of disaggregation show moderate, progressive decreases. This result is predictable from attachment studies, which show fairly normal increases in adhesiveness from the blastula to the early gastrula period in cells derived from the animal portion (approximately 70% of the total number of cells in the embryo). The remaining vegetal cells, however, do not show large increases in frequency of attachment. Presumptive mesodermal cells, which constitute a sizable fraction of the vegetal population, show both low frequencies of attachment and a severely reduced ability to reaggregate. For pipiens-catesbeiana and pipiens-clamitans, two more non-gastrulating hybrids, rates of disaggregation show no decreases. This result correlates with the lack of increase in frequency of attachment during the blastula to late gastrula period. Also, for presumptive mesodermal cells, there is likewise a low frequency of attachment and a low ability to reaggregate. In sum, the

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the correlations between rates of disaggregation, reaggregation, and attachment are quite good, considering the insensitivity inherent in the reaggregation and disaggregation methods. It appears, therefore, that severely arrested hybrids have some alteration in cell surface adhesiveness which causes striking alterations in cell contact behavior.

The interpretation of the different rates of disaggregation of normal and hybrid embryos is somewhat oversimplified. These generalizations apply well when one compares rates of disaggregation for blastulae and late gastrulae or late gastrula arrest hybrids, but there are certain discrepancies in the data for early gastrulae. For example, one would expect that the $4°\beta_1$ for 21 hour pipiens-catesbeiana would be greater than the $4°\beta_1$ for 21 hour pipiens-pipiens, since, the former shows lesser frequencies of attachment and a lesser ability to reaggregate. Other similar problems exist with the 21 hour $4°\beta_1$s. There are a number of experimental difficulties which may account for these discrepancies, at least in part. 1.) The kinetics of disaggregation is a complex function that is only approximated by a fourth order polynomial. 2.) Embryos used to determine the rate of disaggregation at 21 hours for each different gamete combination were necessarily from different egg clutches. There are inherent differences in rates of development as well as severity of arrest from one clutch to another (Ting, '51; Barth and Barth, '66), in spite of all efforts to control rearing conditions of temperature, oxygenation, and crowding. 3.) The counting method for these experiments only permitted detection of large differences in rates of disaggregation. Since nearly 100,000 cells were counted during the course of these particular experiments, a refinement of the counting technique by taking

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larger samples were not judged to be worth the small degree of increased accuracy it would bring.

It is important to note that earlier disaggregations of hybrid embryos provide similar, but less detailed information. In the course of work designed to test the competence and differentiation of cells derived from hybrid embryos, Barth and Barth ('66) noted that explants of presumptive epidermis and marginal zone cells from pipiens-sylvatica and pipiens-clamitans hybrids dissociate more readily than the same explants from pipiens-pipiens. This difference in rate of dissociation applies to dissociation in approximately 3 X 10^{-4} M EDTA, a condition which is quite comparable to dissociation in CMF. These earlier observations are consistent with the present results; indeed, they served as one of the stimuli for the present investigation.

4. Morphogenesis in vitro: Binary Combinations of Explanted Embryonic Fragments

In the light of the above studies of cell adhesiveness, the determination of the morphogenetic potential of explanted fragments by binary combination takes on added significance. Thus, for example, all combinations of ectoderm and endoderm from gastrulating embryos are equally effective in mimicing the spreading of ectoderm on endoderm that occurs in situ. It seems probable that they do this because they are composed of cells which possess the necessary level of adhesiveness to cause or allow spreading. In contrast, no combination of either pipiens-catesbeiana or pipiens-clamitans ectoderm or

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endoderm is effective, even in combination with pipiens-pipiens fragments, probably because at least one fragment in each combination is composed of cells with low adhesiveness. These results suggest that a certain minimal level of cell-cell adhesiveness is necessary in both fragments of a binary combination to ensure or allow spreading. This interpretation makes no assumptions about absolute magnitudes of adhesive strength. In these binary explant combinations, it would only be necessary to visualize the spreading ectoderm as an epithelial sheet that filled available space by a contact inhibition mechanism but which required an appropriate endodermal substratum for ruffled membrane traction. If the ectoderm were composed of cells of low adhesiveness, they could not form functional ruffled membranes; similarly, if the endoderm were composed of cells of low adhesiveness, it could not serve as a suitable substratum for ruffled membrane attachment. Currently, no direct evidence is available to support this speculation.

The results of these explant experiments could also be interpreted in terms of the differential adhesion hypothesis (Steinberg, '64). For example, it could be asserted that pipiens-pipiens ectoderm engulfs pipiens-pipiens endoderm because endoderm-endoderm work of adhesion is of greatest magnitude, ectoderm-ectoderm work of adhesion is of lowest magnitude, and the value of ectoderm-endoderm work of adhesion ranges between the average of ectoderm-ectoderm and endoderm-endoderm at a maximum and ectoderm-ectoderm at a minimum (Case 2 of Steinberg). It could also be argued that pipiens-pipiens ectoderm fails to engulf pipiens-catesbeiana endoderm because a marked alteration in the surface of endodermal cells in this instance results in a

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reduction of the value of ectoderm-endoderm work of adhesion to a level with a maximum at the strength of ectoderm-ectoderm adhesions (Case 3).

Two of the four combinations of pipiens-pipiens and pipiens-sylvatica fragments, however, present problems for both interpretations. Each interpretation can explain why pipiens-pipiens endoderm can be engulfed by either pipiens-pipiens ectoderm or pipiens-sylvatica ectoderm (both fragments are composed of "adhesive" cells). Unfortunately, pipiens-pipiens ectoderm can engulf pipiens-sylvatica endoderm (which has diminished "adhesiveness" by two different criteria) with scarcely less facility. Similarly, pipiens-sylvatica ectoderm can engulf pipiens-sylvatica endoderm with malicious disregard for explanations for such behavior. It should be pointed out that the diminished adhesiveness of endodermal cells should not support ectodermal spreading but does. This evidence also argues directly against the validity of the differential adhesion hypothesis as an explanation of this case. It would, of course, predict that pipiens-sylvatica endoderm, with its diminished adhesiveness, would not segregate internally to the ectodermal fragments.

The apparent paradox could be explained by assuming that these cells are only somewhat diminished in their adhesiveness, such that they cannot support invagination of bottle cells and presumptive mesodermal cells, attachment to glass, and cannot withstand the shear forces of disaggregation experiments, but nevertheless can support spreading of ectodermal tissues. It might also be that the particular group of endodermal cells used in these experiments

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were adherent to one another; however, since they constitute a small fraction of the total vegetal portion of the embryo, their adhesiveness was overshadowed by the low adhesiveness of the remainder of the vegetal population. This is possible but not probable. The differential adhesion hypothesis could explain the behavior of these paradoxical combinations by assuming that ectoderm-endoderm works of adhesion are at opposite ends of an allowable range, i.e. between the average value of endoderm-endoderm and ectoderm-ectoderm at a maximum and the value of ectoderm-ectoderm at a minimum. At this level of conjecture, however, both explanations become so speculative that they can be stretched to fit almost any observation. Neither explanation seems adequate at present.

This dilemma might be solved by assuming that a longer time course for the experiments is necessary for a realization of differences in spreading potential. For example, when explant combinations were scored at 30 hours, many pipiens-pipiens ectoderm-endoderm binary combinations had not completed engulfment of endoderm by ectoderm. It might be that the paradoxical combinations had undergone maximal spreading by 30 hours. The work of Gregg and Klein ('55), however, gives similar results to those reported here, and their explants were cultured for up to 48 hours, at which time maximal spreading of normal ectoderm on normal endoderm had occurred. Thus, the aberrant behavior of pipiens-sylvatica endoderm in supporting ectodermal spreading remains unexplained.

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5. Fine-Structural Investigations

Four different experimental methods were used to establish a picture of cell contact behavior for different cell groups in different ages of normal and hybrid embryos. In an effort to determine whether these differences are reflected in the contact relations of the cells at the level of fine structure, an electron microscopic study of cell contacts was undertaken. The differences in contact behavior of different kinds of presumptive mesodermal cells, as shown by the ability to attach to glass or reaggregate, are indeed reflected in the data on frequency of different gaps sizes. In presumptive ectodermal cells from gastrulating embryos, alterations in cell contact behavior were demonstrated which suggest that cells became more firmly adherent to one another as development proceeded from the blastula to late gastrula stage. In a similar period, gross fine-structural changes in area of cell contact, in distances that separate plasma membranes, and in the occurrence of large, overlapping filopodia, were also seen. In presumptive ectodermal cells from one non-gastrulating hybrid, pipiens-catesbeiana, no alteration in cell contact behavior and no fine-structural changes of the sort observed in gastrulating embryos were observed. Instead, there appeared to be a progressive degeneration in area of cell contact with advancing age. It appears quite safe to conclude that adjacent, adherent cells have closer apposition of plasma membranes over greater areas that adjacent, non-adherent cells.

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These generalizations about fine-structural observations apply well when one compares presumptive mesodermal cells from pipiens-pipiens and pipiens-clamitans, or when one compares presumptive ectodermal cells from a blastula with ectodermal cells from a late gastrula. The differences are large and invariably seen. On the other hand, there is some danger in electron microscopy that selection of areas to be photographed may influence the outcome of the result. Decisions on what contact areas to photograph were based on criteria that are presumably independent of gap dimension; namely, fixation quality, stain quality, angle of section of membrane, and length of contact area available for measurements. In addition, regions of closest apposition were chosen in all cases. This method for selecting areas is not free of potential bias, but only a random selection method would be objective. Given the technical limitations of electron microscopy, a random method would be prohibitively cumbersome. In selected areas of contact, an objective method was used to determine the loci for measurement of gap dimensions. This method ensures that there was no selection of loci for measurement.

The presence of tight or close junctions between cells in gastrulating embryos, and their lack or reduction in number in non-gastrulating hybrids is of considerable interest. Tight and close junctions are not seen between presumptive ectodermal blastomeres in blastula stages, appear by the early gastrula and persist through the late gastrula stage in normal embryos, and do not appear in arrested hybrids. These observations suggest that these particular cell contacts are particularly important in gastrulation in binding cells together.

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It is possible that tight junctions and close junctions are necessary for the coordination of gastrulation by way of ionic communication (Sheridan, '68). Unfortunately, electrophysiological data are as yet unavailable in these embryos.

6. Genetic Control of Amphibian Gastrulation

Throughout the course of this study, a firm impression was gained that there is a spectrum of severity of arrest in gastrulation. This spectrum, observed in amount of gastrulation occurring, time of death of hybrids after fertilization, frequency of cell attachment, amount of reaggregation, and rate of disaggregation, was also observed in relative frequency of gap classes (Figure 27). This spectrum is well correlated with phylogenetic relationships among Ranids (Moore, '49; Uzzel, personal communication). The existence of such correlations constitutes evidence that amphibian gastrulation is under genetic control.

The results of this study indicate that the arrest of hybrid embryos at gastrulation is due in part to alterations of the cell surface, presumably of cell adhesiveness. Thus, the nucleus, and presumably nuclear genes, must, in some way, control cell surface properties. If nuclear genes participate in the differentiation of cell surfaces, a good deal of the control of gastrulation and other aspects of morphogenesis may be due to differences in rates of synthesis of new surface constituents as well as to differences in temporal and spatial patterns of synthesis.

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Little information is available on the nature of the genetic lesion of arrested hybrid embryos. From the mid-blastula to late gastrula arrest stages in pipiens-catesbeiana, nuclei appear to have abnormal condensation of chromatin and lack nucleoli. The condensed chromatin looks quite like metaphase chromosomes. These observations are based on small fragments of one or two embryos of each age (15, 21, and 27 hours) derived from one clutch of eggs and may well be the result of nuclei which were fixed in late prophase. On the other hand, it may well be that the chromatin in pipiens-catesbeiana nuclei is abnormally coiled in a nonfunctional state. Recent cytological studies on this hybrid at the blastula stage (Reynhout and Kimmel, '69a; '69b) show a diploid complement of 26 chromosomes and the presence of certain chromosome markers that indicate that the 26 chromosomes are derived equally from each parental gamete. No gross karyotypic alterations were detected at this early stage. There are numerous indications, however, that replication of chromosomes in a foreign cytoplasmic environment can result in the restriction of the capacity of these chromosomes to support gastrular and post-gastrular development (Moore, '60). This restriction is well correlated with gross chromosomal abnormalities, which are caused by replication of chromosomes in a foreign cytoplasmic environment (Hennen, '63). The pipiens-sylvatica hybrid has been examined biochemically (Gregg, '57) and shows depressed rates of oxygen consumption, anaerobic lactate production, and abnormalities in phosphate metabolism. It seems likely that little or no gastrulation or cell surface differentiation occurs in arrested hybrid embryos

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because little or no gene function occurs, due to alterations in chromosome structure.

Recent work demonstrates directly that nuclear gene products are needed for gastrulation. Humphrey ('66) described a recessive mutation O (for ova deficient) in Ambystoma mexicanum. Homozygous recessive females produce deficient ova which only rarely develop through the gastrula stage when fertilized by +/+, +/o, or o/o sperm. There is clearly some defect in the egg which inhibits embryonic development past the gastrula stage. Briggs and Cassens ('66) showed that cytoplasm from fertilized eggs of +/+ or +/o females would partially correct this defect in eggs from o/o females, so that they would now proceed through gastrulation and begin neurulation. They localized this corrective factor in the germinal vesicle. By injecting germinal vesicle contents from +/+ or +/o ova into embryos formed by fertilizing eggs from o/o females, they could get fairly normal development into the larval stages. Briggs and Justus ('67) have characterized this corrective activity as a high molecular weight gene product that requires associated proteins to become active. This is the best known example of nuclear gene activity being necessary for gastrulation. It would be of great interest to examine cell contact behavior in cells from early gastrulae derived from fertilizing eggs of +/+ and o/o females, and in corrected o/o embryos.

If cell surface properties are under the control of nuclear genes, it is reasonable to expect that new informational RNA for new surface proteins (or enzymatic proteins for the modification of cell surface composition) would be synthesized prior to and during

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the time of appearance of new surface properties. Several workers have injected actinomycin D into amphibian eggs (Brachet and Denis, '63; Wallace and Elsdale, '63; Brachet, et al., '64) and have found that cleavage is apparently normal but gastrulation is blocked. This result suggests that m-RNA synthesis is required for gastrulation. The histological similarity between a severely arrested hybrid in the present study (Figure 1C) and an actinomycin D treated pipiens-pipiens embryo [Figure 1 of Wallace and Elsdale ('63)] is striking but may only be coincidental. More recently, Bachvarova and Davidson ('66) and Davidson, et al. ('68) have shown that new species of heterogeneously sedimenting, DNA-like RNA are synthesized in Xenopus laevis in Stage 8 and 9 mid- to late blastulae (Nieuwkoop and Faber, '67). During that same time period in the present studies, marked changes in cell contact behavior have occured (Figure 3). New informational RNA is probably synthesized, therefore, during the time of appearance of new surface properties. Whether or not these new surface properties are due to the appearance of new surface proteins, is not known at present. If cell surface properties in the material studied in this investigation are under the control of nuclear genes in the way outlined above, informational RNA should not be synthesized in arrested hybrids and new surface proteins should not appear.

Other evidence indicates that cell contact behavior and presumably cell surface properties may be under the control of nuclear genes. Ede and Agerbak ('68) studied the cell contact behavior of wing bud mesenchyme cells from normal and mutant chick embryos. They used a pleotropic mutation known as talpid^3, which causes abnormalities

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in the condensation fields of limb bud mesenchymal cells leading to a condition of polydactyly. The mesencymal cells in the mutant form small, supernumerary, condensation fields, rather than the correct number of large condensation fields. The cell contact behavior of wing bud mesenchymal cells from normal and talpid^3 chicks was studied by rotation-mediated aggregation of suspensions of dissociated cells. It was found that normal aggregates were large, rather loose, and irregular in outline; while, talpid^3 aggregates were small, compact, and ovoid. Although such results are difficult to interpret, these authors made the reasonable assertion that talpid^3 cells were more adhesive to one another than were normal cells. Thus, for talpid^3 cells, initial aggregation would be rapid, resulting in a quick decline in collision frequency between aggregates and cells in suspension. On the other hand, normal cells would undergo gradual aggregation which would not lead to the formation of many small aggregates, but to the formation of a few large, loose aggregates. Although it is not certain that talpid^3 cells are more adhesive, the authors have convincingly demonstrated that talpid^3 aggregates differ from normal aggregates, and the differences correlate well with observations in vivo. Again, it can be seen that cell contact behavior is under nuclear control, presumably due to an effect on the cell surface.

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7. Are Cells in Arrested Hybrid Embryos Viable?

Many of the differences between the cell contact behavior of normal and hybrid embryos could be explained by assuming that massive cell death occurs, preventing changes in cell contact behavior, at the early gastrula stage in arrested hybrids. Four lines of evidence clearly refute this possibility. 1.) Barth and Barth ('66; '68) showed that fragments of the pipiens-sylvatica embryo would differentiate into a number of different tissue cell types when explanted. In the intact embryo, cilia invariably differentiate in the presumptive epidermis several days after arrest. 2.) The experiments to measure the rate of disaggregation show that in all hybrids, cell number continues to increse at about the same rate as in control embryos. Thus the pipiens-pipiens, pipiens-palustris, pipiens-sylvatica, pipiens-catesbeiana, and pipiens-clamitans embryos are composed of approximately equal numbers of cells at 15 (11,000-18,000 cells/embryo), 21 (35,000-45,000 cells/embryo), and 27 (44,000-52,000 cells/embryo) hours of development. 3.) If one dissociates the roof of the blastocoel of 30 hour (late gastrula) pipiens-pipiens embryos, all cells engage in limicola movements. If one dissociates the roof the the blastocoel from 30 hour (approximately 12 hours after arrest) pipiens-sylvatica or pipiens-catesbeiana embryos, 80-100% of the cells engage in limicola movements (Johnson, unpublished observations). Presumptive ectodermal cells from 27 hour pipiens-catesbeiana embryos have a fine structure which is consistent with viability. Mitochondria appear to be normal; 100-200 Å contacts are found; microtubules are evident and are commonly arrayed parallel to

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the plasma membrane; nuclear envelopes are invariably found surrounding all nuclei; and metaphase figures are common. In view of these several lines of evidence, cell death cannot account for the results presented in this thesis.

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8. Conclusions and a Speculation

There are a number of conclusions that should be drawn from the present study. First, it is clear that there are marked alterations in cell contact behavior in all cells prior to gastrulation and throughout the course of gastrulation in normal embryos and a hybrid embryo that is able to gastrulate. Second, it is equally clear, that these changes, to a greater or lesser extent, fail to occur in other hybrid embryos whose development is arrested just prior to or during the early phases of gastrulation. A number of different methods have been used to monitor changes in cell contact behavior in normal and hybrid embryos during their development. The results obtained by the application of one measurment of cell contact behavior, are well correlated with all other measurements of cell contact behavior. When considered together, these correlated results most probably indicate that there are increases in the degree of cell surface adhesiveness in gastrulating embryos that do not occur in non-gastrulating embryos. When adhesiveness increases, gastrulation occurs. Although the genetic basis is not understood, it seems probable that these alterations in cell contact behavior and cell surface adhesiveness are directed by nuclear genes. In addition, one might speculate that gastrulation, an exceedingly complex morphogenetic event, may thus be viewed as due in large measure to differentiation of the cell surface directed by nuclear genes. Thus the linear array of genetic information

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could be translated into the three dimensional event known as gastrulation by controlling temporal and spatial patterns of synthesis of enzymatic and structural surface proteins. In turn, controlled modification of cell surface biochemistry might lead to controlled modification of cell surface properties such as adhesiveness.

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BIBLIOGRAPHY

Abercrombie, M. 1961 The bases of the locomotory behavior of fibroblasts. Exp. Cell Res., Supp. 8:188-198

Ambrose, E. J. 1961 The movements of fibrocytes. Exp. Cell Res., Supp. 8:54-73.

Bachvarova, R., and E. H. Davidson 1966 Nuclear activation at the onset of amphibian gastrulation. J. Exp. Zool., 163:285-296.

Barth, L. J., and L. G. Barth 1966 Differentiation and competence of cells from hybrid embryos. Dev. Biol., 13:95-111.

_____ 1968 Further studies of differentiation of cells from hybrid gastrulae. Dev. Biol., 17:272-292.

Brachet, J., and H. Denis 1963 Effects of actinomycin D on morphogenesis. Nature, 198:205-206.

_____, and F. de Vitry 1964 The effects of actinomycin D and puromycin on morphogenesis in amphibian eggs and Acetabularia mediterranea. Dev. Biol., 9:398-434.

Briggs, R., and G. Cassens 1966 Accumulation in the oocyte nucleus of a gene product essential for embryonic development beyond gastrula-

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tion. Proc. Nat. Acad. Sci., 55:1103-1109.

_____, J. T. Justus 1967 Partial characterization of the component from normal eggs which corrects the maternal effect of gene O in the Mexican axolotl (Ambystoma mexicanum). J. Exp. Zool., 167:105-116.

_____, Green, E. U., and T. J. King 1951 An investigation of the capacity for cleavage and differentiation in Rana pipiens eggs lacking "functional" chromosomes. J. Exp. Zool., 116:455-500.

Curtis, A. S. G. 1960 Cell contacts: some physical considerations. Amer. Nat., 94:37-56.

_____ 1964 The mechanism of adhesion of cells to glass. A study by interference reflection microscopy. J. Cell Biol., 20:199-215.

_____ 1967 The Cell Surface: Its Molecular Role in Morphogenesis. Academic Press, New York.

Davidson, E. H., Crippa, M., and A. E. Mirsky 1968 Evidence for the appearance of novel gene products during amphibian blastulation. Proc. Nat. Acad. Sci., 60:152-159.

Devillers, Ch. 1952 Coordinations des forces épiboliques dans la gastrulation de Salmo. Bull. Soc. Zool. de France, 77:304-309.

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Ede, D. A., and G. S. Agerbak 1968 Cell adhesion and movement in relation to the developing limb pattern in normal and talpid^3-mutant chick embryos. J. Embryol. exp. Morph., 20:81-100.

Goerttler, K. 1925 Die Formbildung der Medullaranlage bei Urodelen. In rahmen der Verschiebungsvorgänge von Keimbezirken während der Gastrulation und als entwicklungsphysiologisches Problem. W. Roux' Arch. f. Ent. Org., 106:503-541.

Gregg, J. R. 1957 Morphogenesis and metabolism of gastrula-arrested embryos in the hybrid Rana pipiens ♀ X Rana sylvatica ♂. In: "The Beginnings of Embryonic Development" (Tyler, von Borstel, and Metz, eds.), pp. 231-261. AAAS, Washington.

_____, and D. Klein 1955 Morphogenetic movements of normal and gastrula-arrested hybrid amphibian tissues. Biol. Bull., 109:265-270.

Gurdon, J. B. 1967 African clawed frogs. In: "Methods in Developmental Biology" (Wilt and Wessells, eds.), pp. 75-84. Crowell, New York.

Gustafson, T., and L. Wolpert 1967 Cellular movement and contact in sea urchin morphogenesis. Biol. Rev., 42:442-498.

Hamburger, V. 1960 A Manual of Experimental Embryology. University of Chicago Press, Chicago.

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Harrison, R. G. 1928 On the status and significance of tissue culture. Arch. f. exp. Zellfors., 6:4-27.

Hay, E. D. 1968 Organization and fine structure of epithelium and mesenchyme in the developing chick embryo. In: "Epithelial-Mesenchymal Interactions: 18th Hahnemann Symposium" (Fleischmajer and Billingham, eds.), pp. 31-55. Williams and Wilkins, Baltimore.

Hennen, S. 1963 Chromosomal and embryological analyses of nuclear changes occuring in embryos derived from transfers of nuclei between Rana pipiens and Rana sylvatica. Dev. Biol., 6:133-183.

Holtfreter, J. 1939 Gewebeaffinität, ein Mittle der embryonalen Formbildung. Arch. f. exp. Zellfors., 23:169-209.

_____ 1943 A study of the mechanics of gastrulation, Part I. J. Exp. Zool., 94:261-318.

_____ 1944 A study of the mechanics of gastrulation, Part II. J. Exp. Zool., 95:171-212.

Humphrey, R. R. 1966 A recessive factor (O, for ova deficient) determining a complex of abnormalities in the Mexican axolotl (Ambystoma mexicanum). Dev. Biol., 13:57-76.

Johnson, K. E. 1969a Altered contact behavior of presumptive mesodermal cells from hybrid amphibian embryos arrested at gastrulation.

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J. Exp. Zool., 170:325-332.

_____ 1969b Adhesiveness of cells from normal amphibian embryos and hybrid embryos arrested at gastrulation. Amer. Zool., 9:601.

_____ 1969c Contact relations of cells from normal amphibian embryos and hybrid embryos arrested at gastrulation: fine-structural evidence. Amer. Zool., 9:1129.

Jones, K. W., and T. R. Elsdale 1963 The culture of small aggregates of amphibian embryonic cells in vitro. J. Embryol. exp. Morph., 11:135-154.

Moore, J. A. 1941 Developmental rate of hybrid frogs. J. Exp Zool., 86:405-422.

_____ 1946 Studies in the development of frog hybrids. I. Embryonic development in the cross Rana pipiens ♀ X Rana sylvatica ♂. J. Exp. Zool., 101:173-219.

_____ 1949 Patterns of evolution in the genus Rana. In: "Genetics, Paleontology, and Evolution" (Jepsen, Simpson, and Mayr, eds.), pp. 315-338. Princeton University Press, Princeton, New Jersey.

_____ 1955 Abnormal combinations of nuclear and cytoplasmic systems in frogs and toads. Adv. in Genetics, 7:139-182.

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_____ 1960 Serial back-transfer of nuclei in experiments involving two species of frogs. Dev. Biol., 2:535-550.

Moscona, A. A. 1961 Rotation-mediated histogenetic aggregation of dissociated cells. A quantifiable approach to cell interactions in vitro. Exp. Cell Res., 22:455-475.

Nieuwkoop, P. D., and J. Faber 1967 Normal Table of Xenopus laevis (Daudin). Second Edition. North-Holland, Amsterdam.

Pasteels, J. 1940 Un aperçu comparatif de la gastrulation chez les chordés. Biol. Rev., 15:59-106.

_____ 1942 New observations concerning the maps of the presumptive areas of the young amphibian gastrula (Amblystoma and Discoglossus). J. Exp. Zool., 89:255-281.

_____ 1949 Observations sur la localisation de la plaque préchordal et de l'entoblaste présomptifs au course de la gastrulation chez Xenopus laevis. Arch. de Biol., 60:235-250.

Patricolo, E. 1967 Differentiation of aggregated embryonic cells of amphibians (Discoglossus pictus). Acta Embryol. et Morph. Exp., 10:75-100.

Phillips, H. M. 1969 Equilibrium measurements of embryonic cell adhesiveness:physical formulation and testing of the differential

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adhesion hypothesis. Thesis, The Johns Hopkins University.

_____, and M. S. Steinberg 1969 Equilibrium measurements of embryonic chick cell adhesiveness, I. Shape equilibrium in centrifugal fields. Proc. Nat. Acad. Sci., 64:121-127.

Reynhout, J. K., and D. L. Kimmel, Jr. 1969a The chromosome complement of the Rana pipiens ♀ X Rana catesbeiana ♂ hybrid blastula. Amer. Zool., 9:599.

_____ 1969b Chromosome studies of the lethal hybrid Rana pipiens ♀ X Rana catesbeiana ♂. Dev. Biol., 20: 501-517.

Roth, S. A. 1968 Studies of intercellular adhesive stability. Dev. Biol., 18:602-631.

_____, and J. A. Weston 1967 The measurement of intercellular adhesion. Proc. Nat. Acad. Sci., 58:974-980.

Rugh, R. 1962 Experimental Embryology. Third Edition, Burgess, Minneapolis.

Schechtman, A. M. 1942 The mechanism of amphibian gastrulation. I. Gastrulation-promoting interactions between various regions of an anuran egg (Hyla regilla). Univ. Calif. Publ. Zool., 51:1-40.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Sheridan, J. D. 1968 Electrophysiological evidence for low-resistance intercellular junctions in the early chick embryo. J. Cell Biol., 37: 650-659.

Stearns, R. N., and A. B. Kostellow 1958 Enzyme induction in dissociated cells. In: "A Symposium on the Chemical Basis of Development" (McElroy and Glass, eds.), pp. 448-453. Johns Hopkins University Press, Baltimore.

Steinberg, M. S. 1964 The problem of adhesive selectivity in cellular interactions. In: "Cellular Membranes in Development: 22nd Symposium of the Society for the Study of Development and Growth" (Locke, ed.), pp. 321-365. Academic Press, New York.

Taylor, A. C. 1961 Attachment and spreading of cells in culture. Exp. Cell Res., Supp 8:154-173.

Ting, H-P. 1951 Diploid, androgenetic and gynogenetic haploid development in anuran hybridization. J. Exp. Zool., 116:21-57.

Townes, P. L., and J. Holtfreter 1955 Directed movements and selective adhesion of embryonic amphibian cells. J. Exp. Zool., 128:53-120.

Trelstad, R. L., Hay, E.D., and J-P. Revel 1967 Cell contact during early morphogenesis in the chick embryo. Dev. Biol., 16:78-106.

_____, Revel, J-P., and E. D. Hay 1966 Tight junctions between cells in the early chick embryo as visualized with the electron

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microscope. J. Cell Biol., 31:C6-C10.

Trinkaus, J. P. 1963 The cellular basis of Fundulus epiboly. Adhesivity of blastula and gastrula cells in culture. Dev. Biol., 7:513-532.

_____ 1965 Mechanisms of morphogenetic movements. In: "Organogenesis" (DeHaan and Ursprung, eds.), pp. 55-104. Holt, New York.

_____ 1969 Cells Into Organ, The Forces that Shape the Embryo. Prentice-Hall, Englewood Cliffs, New Jersey.

_____, and T. L. Lentz 1967 Surface specializations of Fundulus cells and their relation to cell movements during gastrulation. J. Cell Biol., 32:735-739.

Vogt, W. 1925 Gestaltungsanalyse am Amphibienkeim mit örtlicher Vitalfärbung. I. Teil. Methodik und Wirkungsweise der örtlicher Vitalfärbung mit Agar als Farbträger. W. Roux' Arch. f. Ent. Org., 106:542-610.

_____ 1929 Gestaltungsanalyse am Amphibienkeim mit örtlicher Vitalfärbung. II. Teil. Gastrulation und Mesodermbildung bei Urodelen und Anuren. W. Roux' Arch. f. Ent. Org., 120:384-706.

Wallace, H., and T. R. Elsdale 1963 Effects of actinomycin D on amphibian development. Acta Embryol. et Morph. Exp., 6:275-282.

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Weiss, L. 1960 The adhesion of cells. Int. Rev. Cytol., 9:187-225.

_____ 1967 The Cell Periphery, Metastasis, and other Contact Phenomena, Wiley, New York.

_____ 1968 Studies on cellular adhesion in tissue culture. IX. Electrophoretic mobility and contact phenomena. Exp. Cell Res., 51: 609-625.

Wolf, K., and M. C. Quimby 1964 Amphibian cell culture: permanent cell line from the bullfrog (Rana catesbeiana). Science, 144:1578-1580.

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FIGURE LEGENDS

Figure 1. Light micrographs of sagittal sections of normal and hybrid embryos. All embryos were derived by fertilizing R. pipiens eggs of one clutch will isologous and heterologous sperm and were 25 hours old at fixation. Scale mark equals 1 mm. A. pipiens-pipiens. B. pipiens-sylvatica. C. pipiens-catesbeiana. D. pipiens-clamitans. X 43.

Figure 2. Light micrographs of sagittal sections of normal and hybrid ectoderm. All embryos were derived by fertilizing R. pipiens eggs of one clutch with isologous and heterologous sperm and were 25 hours old at fixation. All scale marks equal 100 μ. A. pipiens-pipiens, X 220. Note that the ectoderm is smooth, uniformly thick, and composed of two layers of adherent, cuboidal cells. B. pipiens-sylvatica, X 80. Note the conspicuous wrinkle in the sheet of apparently adherent cells. Deeper wrinkles are commonly seen. The cell sheet is thinner at the base of the wrinkle than it is immediately lateral to the wrinkle. Deeper wrinkles have more pronounced thinning at their base. Individual cells are cuboidal, polyhedral, or pyramidal, but rapely sperical. C. pipiens-catesbeiana, X 110. Typical section, showing many spherical cells and intercellular spaces. D. pipiens-catesbeiana, X 110. Different portion of the same ectoderm as shown in 2C. Many spherical, unattached cells can be seen floating freely in the blastocoel.

Figure 3. Frequency of cell attachment as a function of embryonic age in Xenopus laevis. The G on the abscissa represents the approximate beginning of gastrulation. Open circles, animal portion; closed circles, vegetal portion; Xs, whole embryo. The same symbols are used in Figs. 3-9.

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Figure 4. Frequency of cell attachment as a function of embryonic age in pipiens-pipiens. The G on the abscissa represents the approximate beginning of gastrulation.

Figure 5. Frequency of cell attachment as a function of embryonic age in sylvatica-sylvatica. The G of the abscissa represents the approximate beginning of gastrulation.

Figure 6. Frequency of cell attachment as a function of embryonic age in pipiens-palustris. The G on the abscissa represents the approximate beginning of gastrulation.

Figure 7. Frequency of cell attachment as a function of embryonic age in pipiens-sylvatica (7A) and sylvatica-pipiens (7B). The GA on the abscissa represents the approximate beginning of gastrula arrest.

Figure 8. Frequency of cell attachment as a function of embryonic age in pipiens-catesbeiana. The GA on the abscissa represents the approximate beginning of gastrula arrest.

Figure 9. Frequency of cell attachment as a function of embryonic age in pipiens-clamitans. The GA on the abscissa represents the approximate beginning of gastrula arrest.

Figure 10. Light micrographs showing reaggregation in vitro of presumptive mesodermal cells from normal and hybrid embryos. Scale mark equals 200 μ. Each column represents one area of one culture photographed under

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phase contrast at 0, 1, 2, and 3 hours in vitro. Each row represents a different gamete combination. A. pipiens-pipiens, B. pipiens-palustris, C. pipiens-sylvatica, D. pipiens-catesbeiana, E. pipiens-clamitans.

Figure 11. Rate of disaggregation for pipiens-pipiens. Open circles, 15 hour blastula; closed circles, 21 hour early gastrula; Xs, 27 hour late gastrula. Each time point is the computed, estimated value.

Figure 12. Rate of disaggregation for pipiens-sylvatica. Open circles, 15 hour blastula; closed circles, 21 hour early gastrula arrest; Xs, 27 hour late gastrula arrest. Each time point is the computed, estimated value.

Figure 13. Rate of disaggregation for pipiens-catesbeiana. Open circles, 15 hour blastula; closed circles, 21 hour early gastrula arrest; Xs, 27 hour late gastrula arrest. Each time point is the computed, estimated value.

Figure 14. Photographs to illustrate decreasing rate of disaggregation of developing pipiens-pipiens embryos. 15 hour blastula after 0 (A) and 10 minutes (B) of agitation in CMF. 21 hour early gastrula after 0 (C) and 10 minutes (D) of agitation in CMF. 27 hour late gastrula after 0 (E) and 10 minutes (F) of agitation in CMF. In Figures 14,15, and 16, the left and right members of each pair are photographs of one embryo, before and after 10 minutes of agitation in CMF.

Figure 15. Photographs to illustrate lack of increase in overall coherence of developing pipiens-sylvatica embryos. 15 hour blastula

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after 0 (A) and 10 minutes (B) of agitation in CMF. 21 hour early gastrula arrest after 0 (C) and 10 minutes (D) of agitation in CMF. 27 hour late gastrula arrest after 0 (E) and 10 minutes (F) of agitation in CMF.

Figure 16. Photographs to illustrate the constant high rate of disaggregation of arrested pipiens-catesbeiana embryos. 15 hour blastula after 0 (A) and 10 minutes (B) of agitation in CMF. 21 hour early gastrula arrest after 0 (C) and 10 minutes (D) of agitation in CMF. 27 hour late gastrula arrest after 0 (E) and 10 minutes (F) of agitation in CMF.

Figure 17. Cumulative, normalized scores for binary explant combinations. The letter designations below each bar are described in Table 3.

Figure 18. Light micrographs of cross sections of binary explant combinations. All fragments were derived from two clutches of embryos, normal and hybrid, formed by fertilizing the eggs of one R. pipiens female. Combinations were fixed after 30 hours in vitro. Scale mark equals 500 μ. A. pipiens-pipiens ectoderm + pipiens-pipiens endoderm. The combination illustrated would receive a score of 5, since the ectoderm has completely engulfed the endoderm. Note the firm cohesiveness of the ectodermal cells, which are darkly pigmented. B. pipiens-pipiens ectoderm + pipiens-catesbeiana endoderm. The combination illustrated would receive a score of 3 since some spreading has occurred, but is not nearly complete. It should be noted the the pipiens-pipiens ectoderm is a coherent but non-spreading mass. C. pipiens-catesbeiana ectoderm + pipiens-pipiens endoderm. The combination illustrated

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would receive a score of 1 because of the lack of spreading and serious disintegration of the ectodermal fragment. D. pipiens-catesbeiana ectoderm + pipiens-catesbeiana endoderm. The combination illustrated would again receive a score of 1. All figures, X 42. Arrows in 18B-D point to the leading edge of the ectodermal fragment of the combination.

Figure 19. Electron micrograph of presumptive mesodermal cells of pipiens-pipiens. Scale mark equals 2 μ. Note the small, triangular intercellular space between three cells and the extensive regions of cell contact radiating away from the space. This triangular space and extensive area of contact was typical of normal presumptive mesodermal cells. X 11,800.

Figure 20. Electron micrograph of presumptive mesodermal cells of pipiens-clamitans. Scale mark equals 2 μ. Note the large intercellular spaces and small areas of cell contact. X 11,800.

Figure 21. Electron micrograph of presumptive mesodermal cells of pipiens-pipiens. Scale in 21A equals 2 μ. Figure 21 B and inset of 21 A, x 100,000. All insets in Figures 22-31 are X 100,000. Figure 21A is a low power (X 16,000) area of contact between three cells. The arrow indicates the area in the inset. A gap of 50-100 Å or less is shown in the inset. An area of specialized cell contact from a different embryo is shown in 21B, showing at arrows moving from left to right, tight junctions, "X" junctions, a gap of 50-100 Å, and a gap of 20-50 Å.

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Figure 22. Electron micrograph of presumptive mesodermal cells of pipiens-palustris. Scale mark equals 2 μ. The arrow indicates the area in the inset. An "X" junction is shown in the inset, where the total width of the gap and both plasma membranes is less than 200 Å but it is impossible to tell whether the junction is a tight junction or a gap junction, due to the unfavorable sectioning angle of the plasma membrane. X 16,000.

Figure 23. Electron micrograph of presumptive mesodermal cells of pipiens-sylvatica. Scale mark equals 2μ. The arrow indicates the area in the inset. A gap of 530 Å is shown in the inset at the arrow. X 12,000.

Figure 24. Electron micrograph of presumptive mesodermal cells of pipiens-catesbeiana. Scale mark equals 2 μ. The arrow indicates the area in the inset. A gap of 270 Å is shown at the arrow. X 18,000.

Figure 25. Electron micrograph of presumptive mesodermal cells of pipiens-clamitans. Scale mark equals 5 μ. Note the large gaps between adjacent cells. X 6,000.

Figure 26. Frequency distribution of gap classes from presumptive mesodermal cells of pipiens-palustris (open circles) and pipiens-sylvatica (closed circles). Note the higher frequency of larger gaps and the lack of tight and "X" junctions, as well as the reduced frequency of 20-50 Å gaps in pipiens-sylvatica.

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Figure 27. Graphic illustration of the difference in frequency of gap sizes in presumptive mesodermal cells of pipiens-pipiens (open circles), pipiens-sylvatica (closed circles), and pipiens-clamitans (Xs). This graph is an illustration of the data shown in column three for each gamete combination in Table 4. It shows, for example, that about 46% of all pipiens-pipiens gap measurements are less than or equal to 100 Å while only about 16% of all pipiens-clamitans gap measurements are less than or equal to 100 Å.

Figure 28. Electron micrograph of typical contact area in presumptive ectodermal cells in a 15 hour pipiens-palustris blastula. Scale mark equals 5 μ. The arrow indicates the area in the inset. A gap of 1100 Å is shown in the inset at the arrow. Note how the cell surfaces are relatively smooth and areas of contact reduced. Compare this Figure with Figures 29-31. X 6,000.

Figure 29. Electron micrograph of typical contact area in presumptive ectodermal cells in a 21 hour pipiens-pipiens early gastrula. Scale mark equals 10 μ. The arrow indicates the area in the inset. An "X" junction is shown in the inset, where the total width of the gap and both plasma membranes is 160 Å. This is probably a tight junction but may be a gap junction. Note that there are numerous undulations in the plasma membranes and more extensive areas of cell contact than in Figure 28. X 5,000.

Figure 30. Electron micrograph of typical contact area in presumptive

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ectodermal cells in a 21 hour pipiens-palustris early gastrula. Scale mark equals 5 μ. The arrow indicates the area in the inset. Another "X" junction is shown in the inset. Again there are surface undulations and broad areas of cell contact. X 10,000.

Figure 31. Electron micrograph of typical contact area in ectodermal cells in a 27 hour pipiens-palustris late gastrula. Scale mark equals 5 μ. The arrow indicates the area in the inset. A gap of 90 Å is shown in the inset at the arrow. The box encloses the area shown in Figure 32. Note that the filopodial processes are longer and have microtubules in them. X 9,000.

Figure 32. Electron micrograph of the tip of the process in Figure 31. Four arrows indicate small segments of microtubules. Two gaps were also measured at the other two arrows. X 121,000.

Figure 33. Electron micrograph of typical contact area in ectodermal cells in a 27 hour pipiens-pipiens late gastrula. Scale mark equals 2 μ. Long segments of microtubules are shown at the arrows. Complex junctions of this sort are commonly seen between the lower ectodermal blastomeres at their surface which lies immediately adjacent to the nearly obliterated blastocoel (B). X 22,000.

Figure 34. Electron micrograph of typical contact area in presumptive ectodermal cells in a 15 hour pipiens-catesbeiana blastula. Scale mark equals 10 μ. Note the clumping of the nuclear chromatin in the center of the micrograph and the similarity of this clumped chromatin to

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metaphase chromosomes, shown at the arrow at the bottom of the micrograph. X 4,000.

Figure 35. Electron micrograph of typical contact area in presumptive ectodermal cells in a 21 hour pipiens-catesbeiana early gastrula arrest. Scale mark equals 10 μ. Note the clumping of the chromatin and reduction in area of cell contact when compared with Figure 34. X 4,000.

Figure 36. Electron micrograph of typical contact area in presumptive ectodermal cells in a 27 hour pipiens-catesbeiana late gastrula arrest. Scale mark equals 10 μ. The outer surface of the roof of the blastocoel is shown at the arrow. Note that the surface blastomeres appear to be firmly adherent along their lateral surfaces and the large intercellular spaces between the inner blastomeres. X 4,000.

Figure 37. Electron micrograph of typical contact area in presumptive ectodermal cells in a 27 hour pipiens-catesbeiana late gastrula arrest. Scale mark equals 10 μ. These cells are in the same embryo as illustrated in Figure 36, but located further away from the surface of the embryo. Note the clumping of the chromatin and the similarity of this clumped chromatin to metaphase chromosomes, shown at the arrow. X 2,800.

Figure 38. Electron micrograph of contact area between the lateral surfaces of two surface blastomeres in a 27 hour pipiens-catesbeiana late gastrula arrest. Scale mark equals 2 μ. Close examination of X 100,000 prints of this entire contact area reveal that gaps are usually

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100-200 Å and occasionally about 50 Å. Two long segments of microtubules are shown at the arrows. X 25,000.

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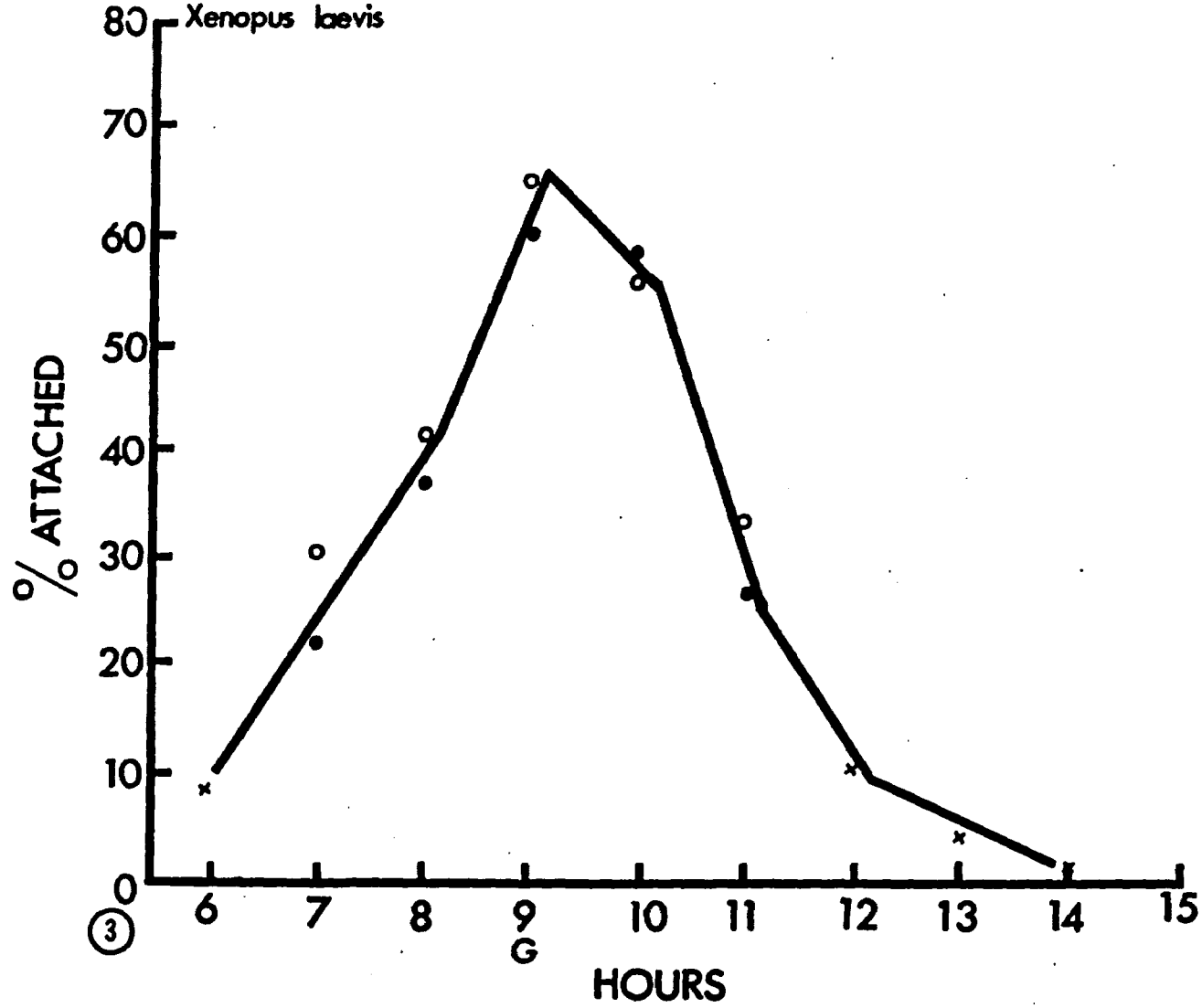

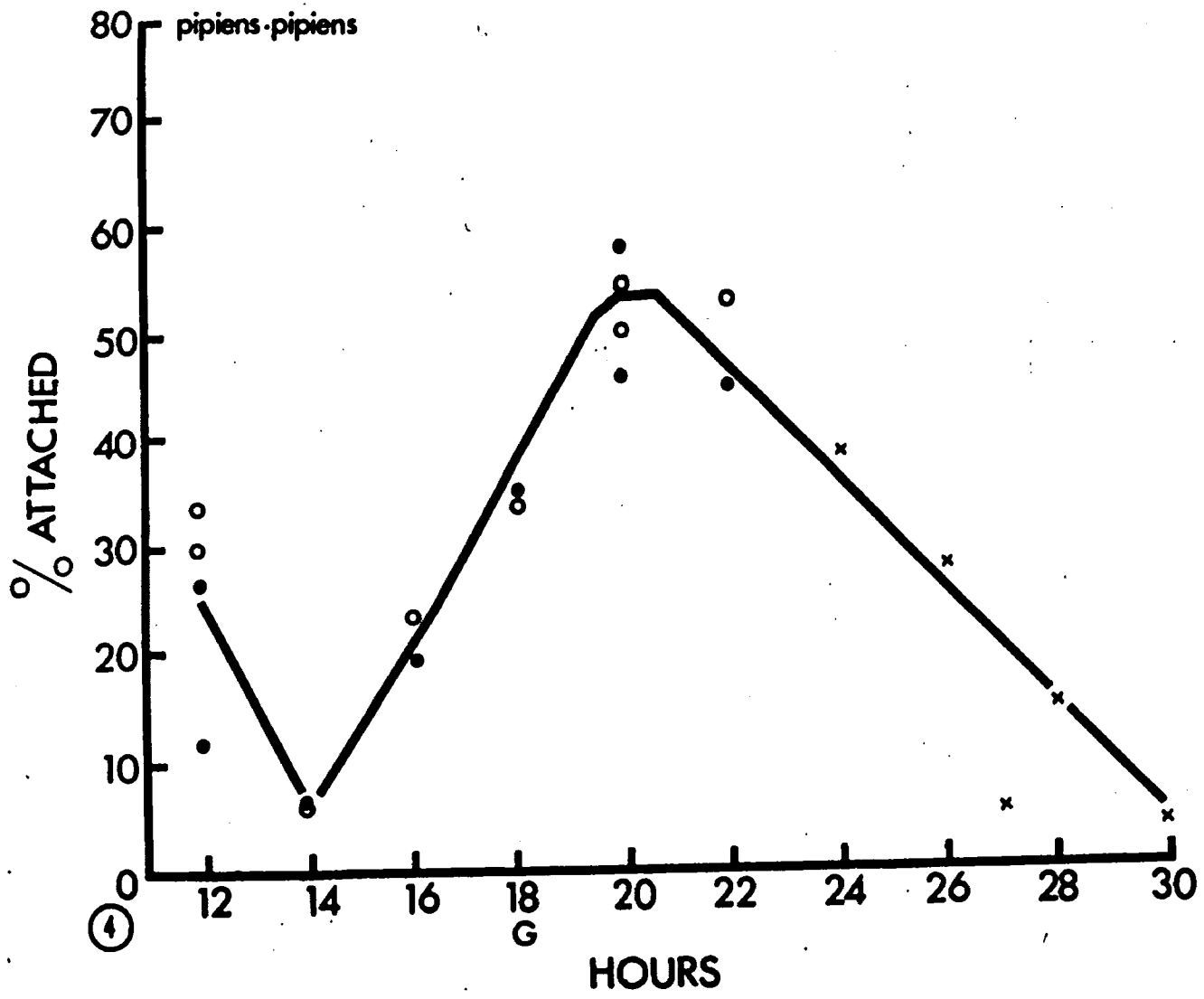

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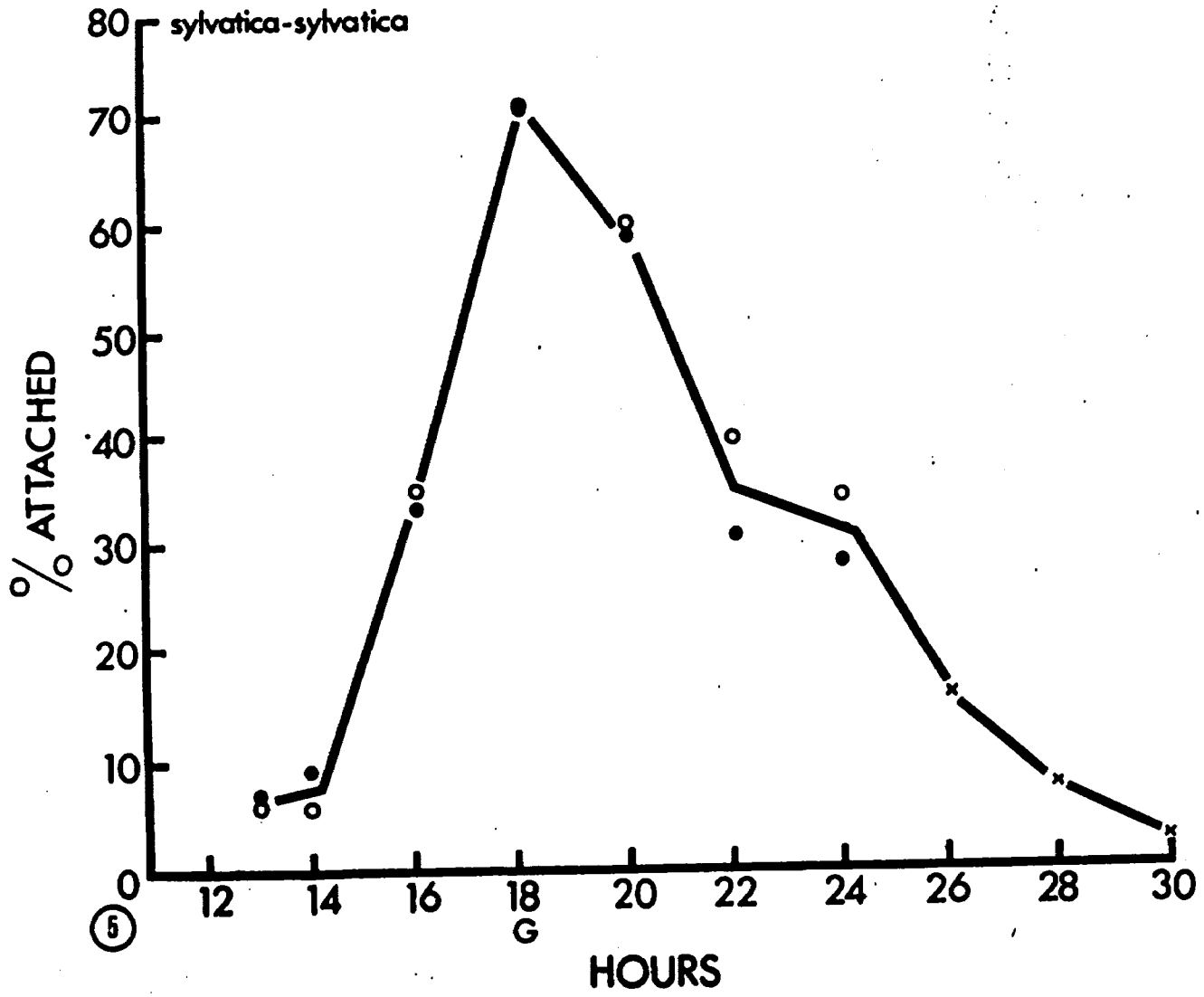

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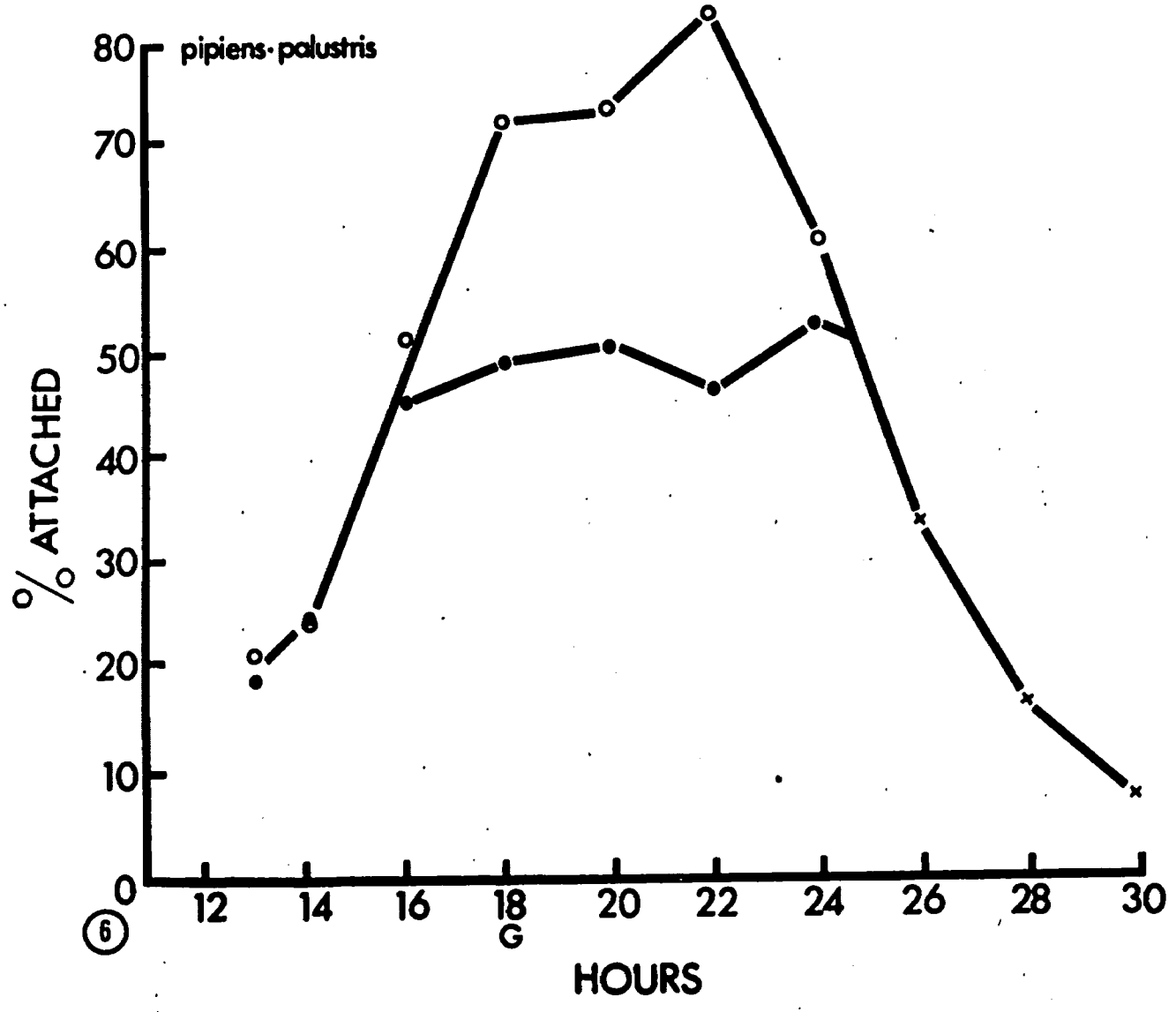

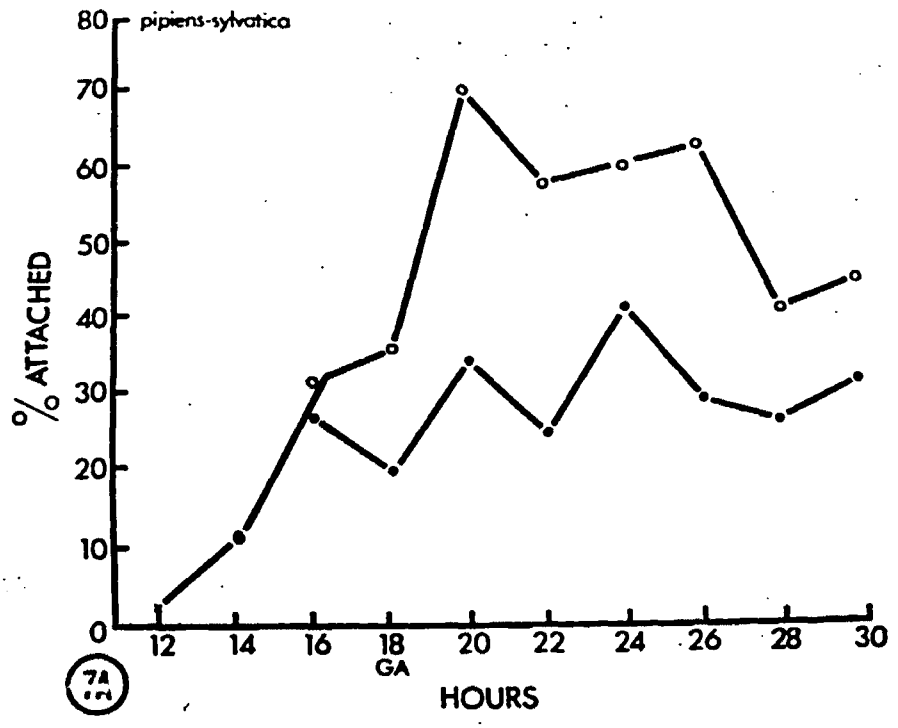

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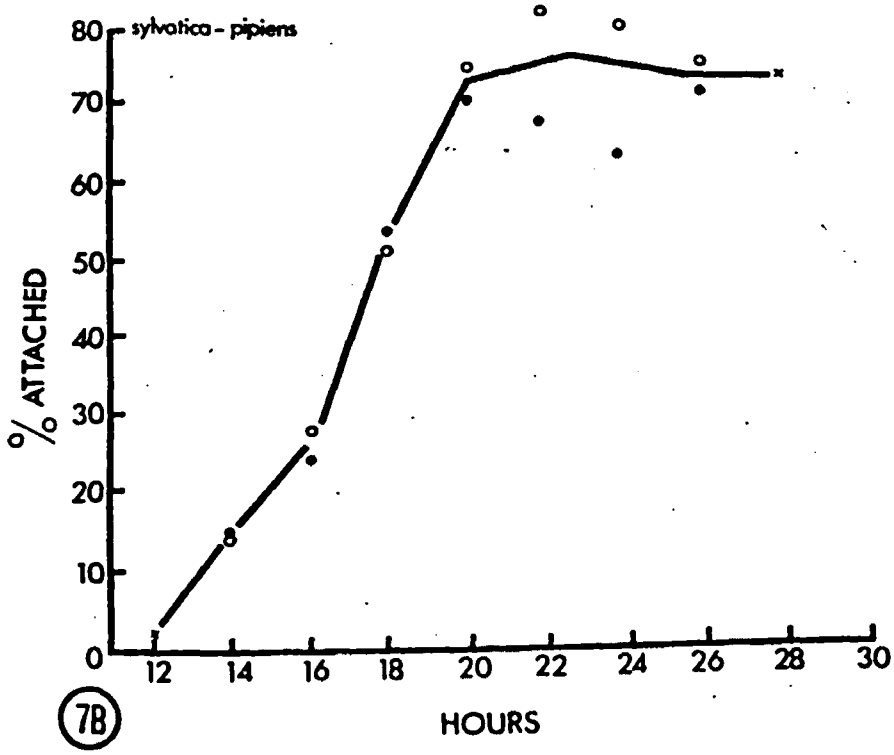

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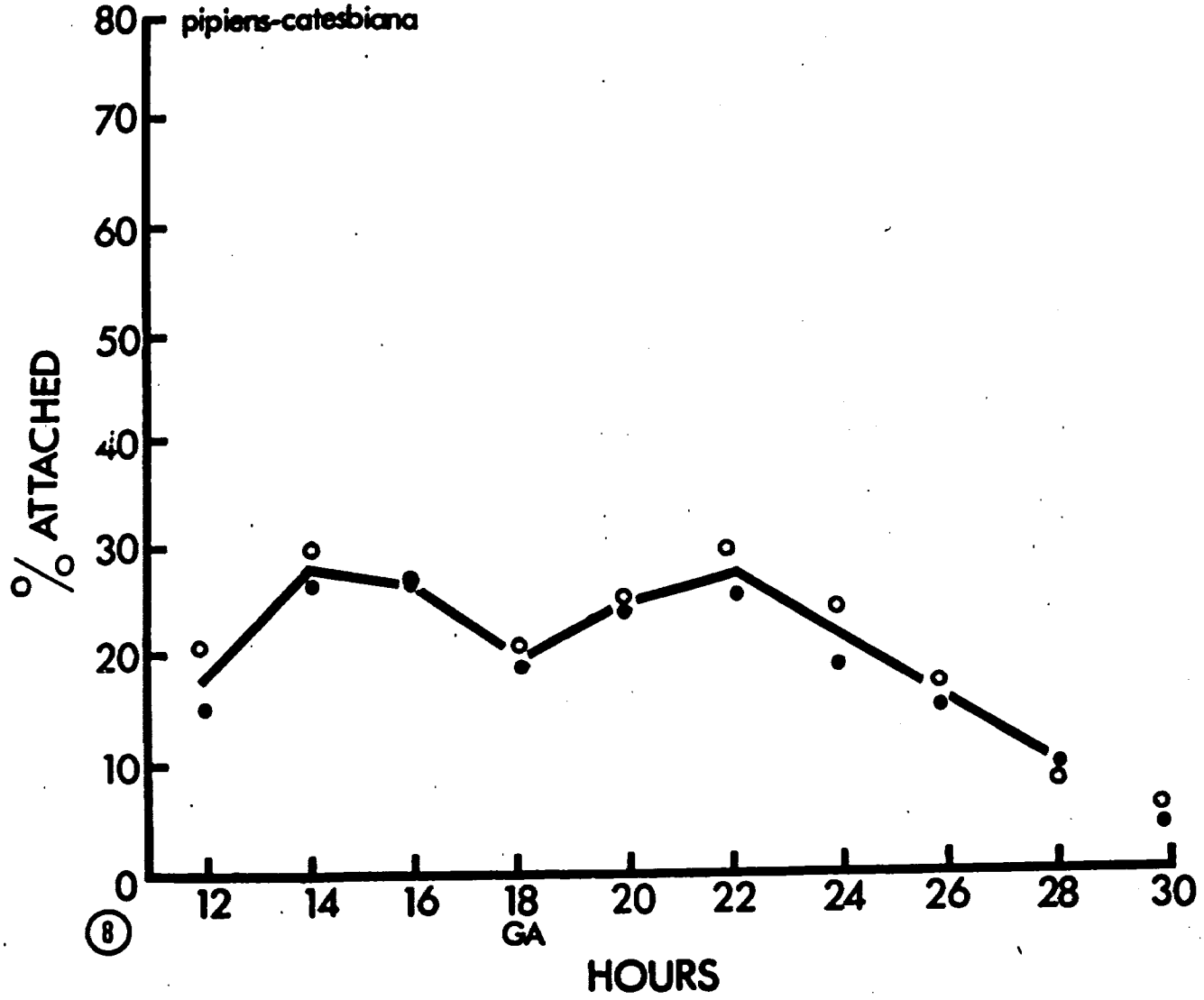

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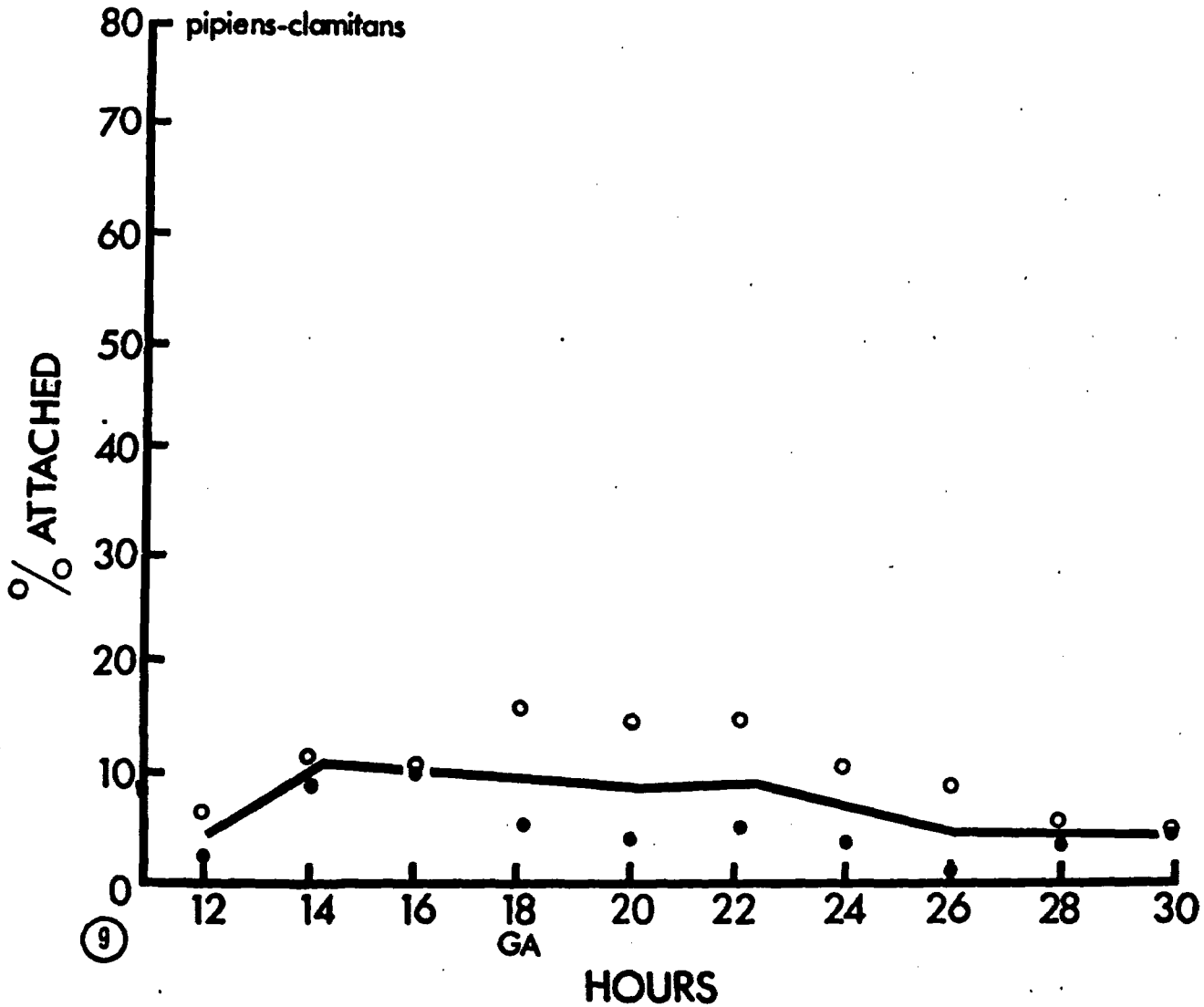

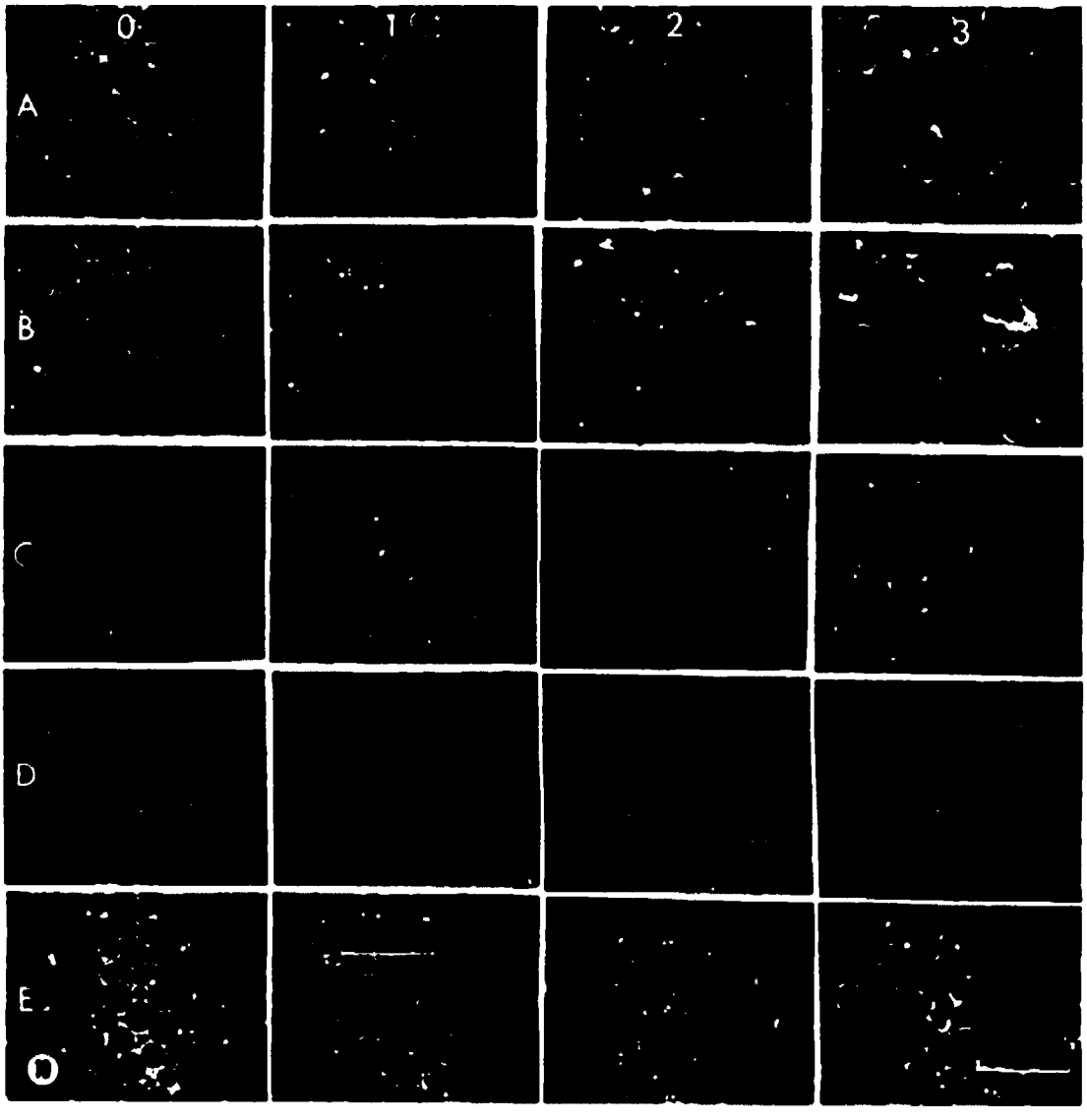

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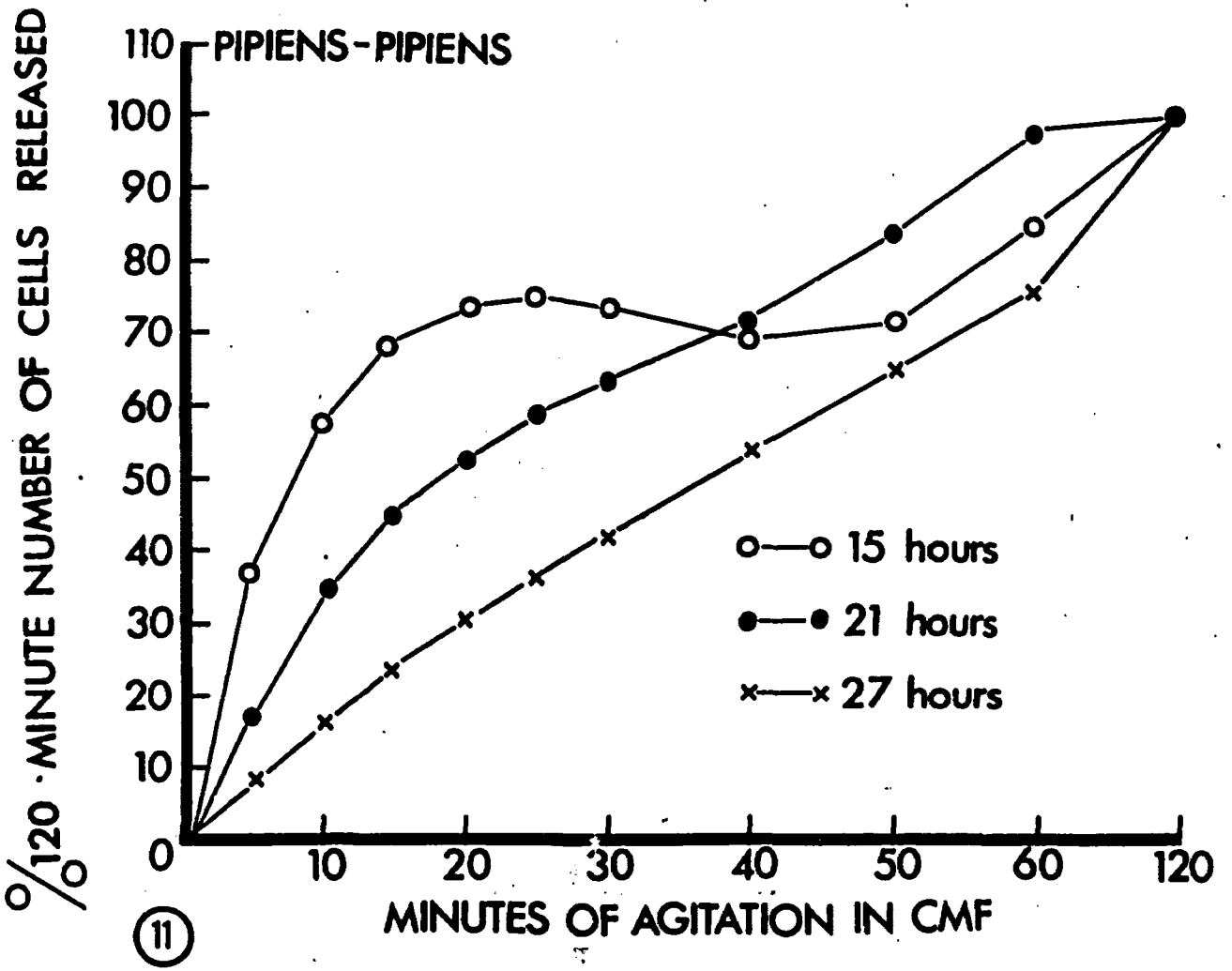

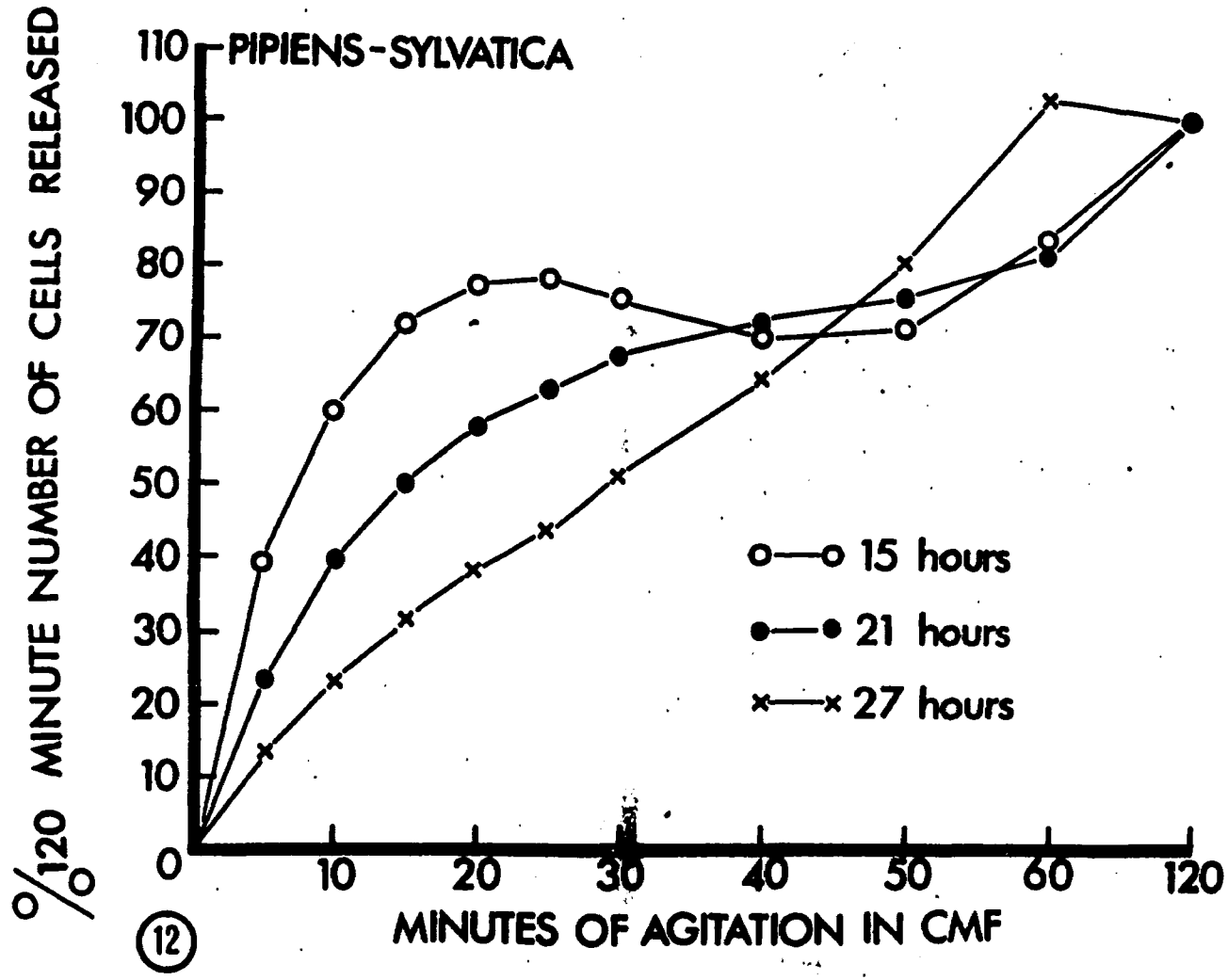

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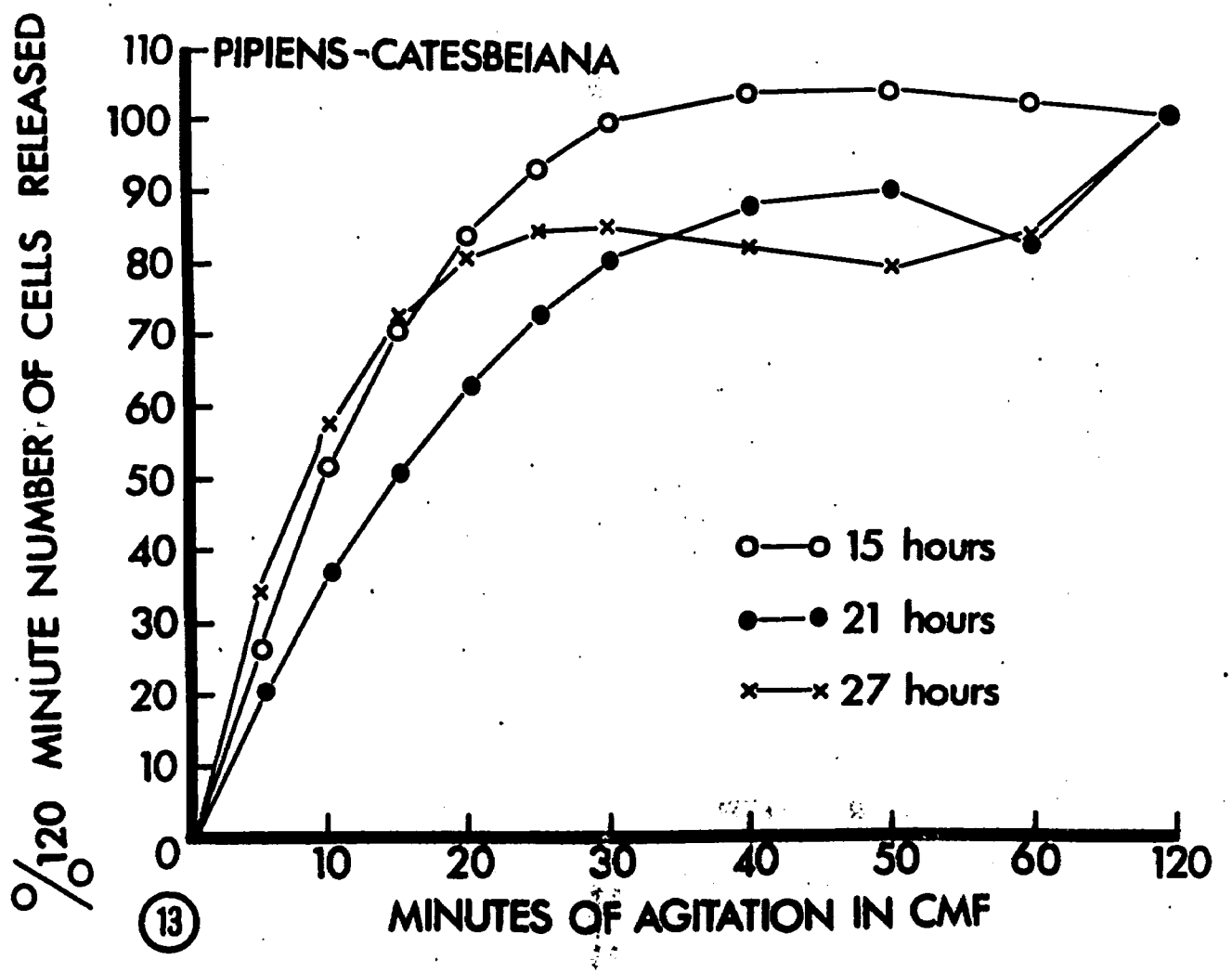

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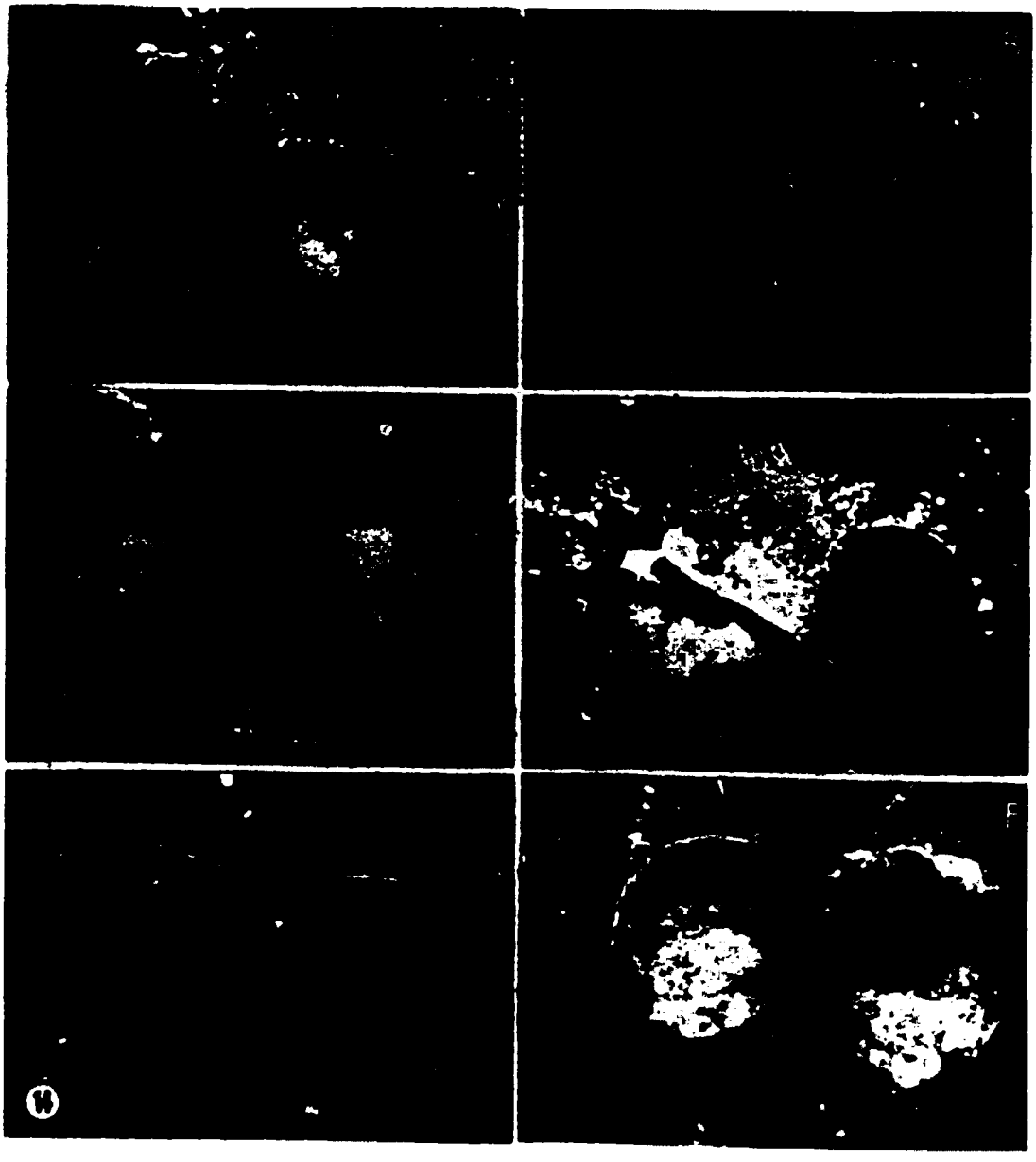

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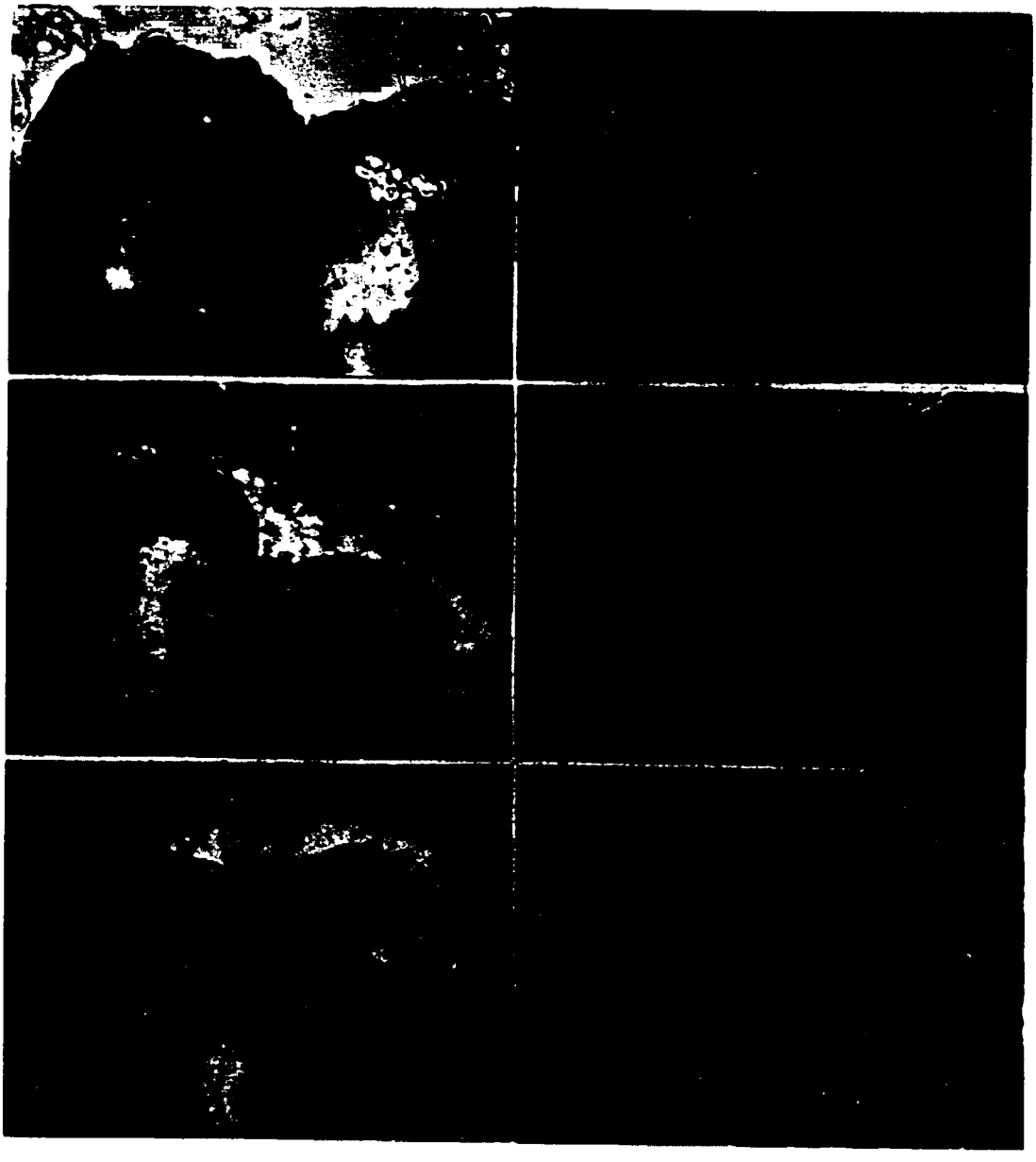

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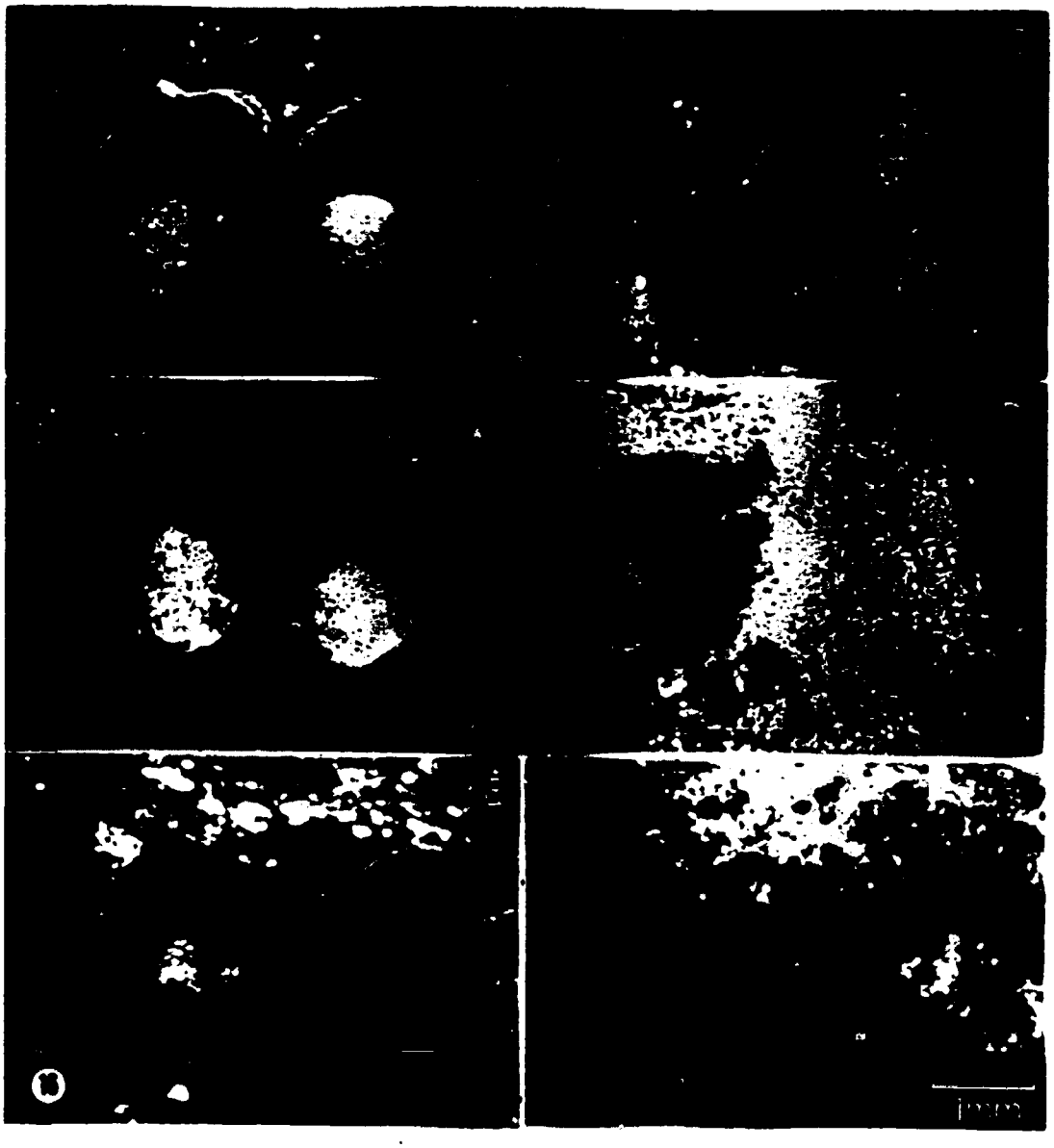

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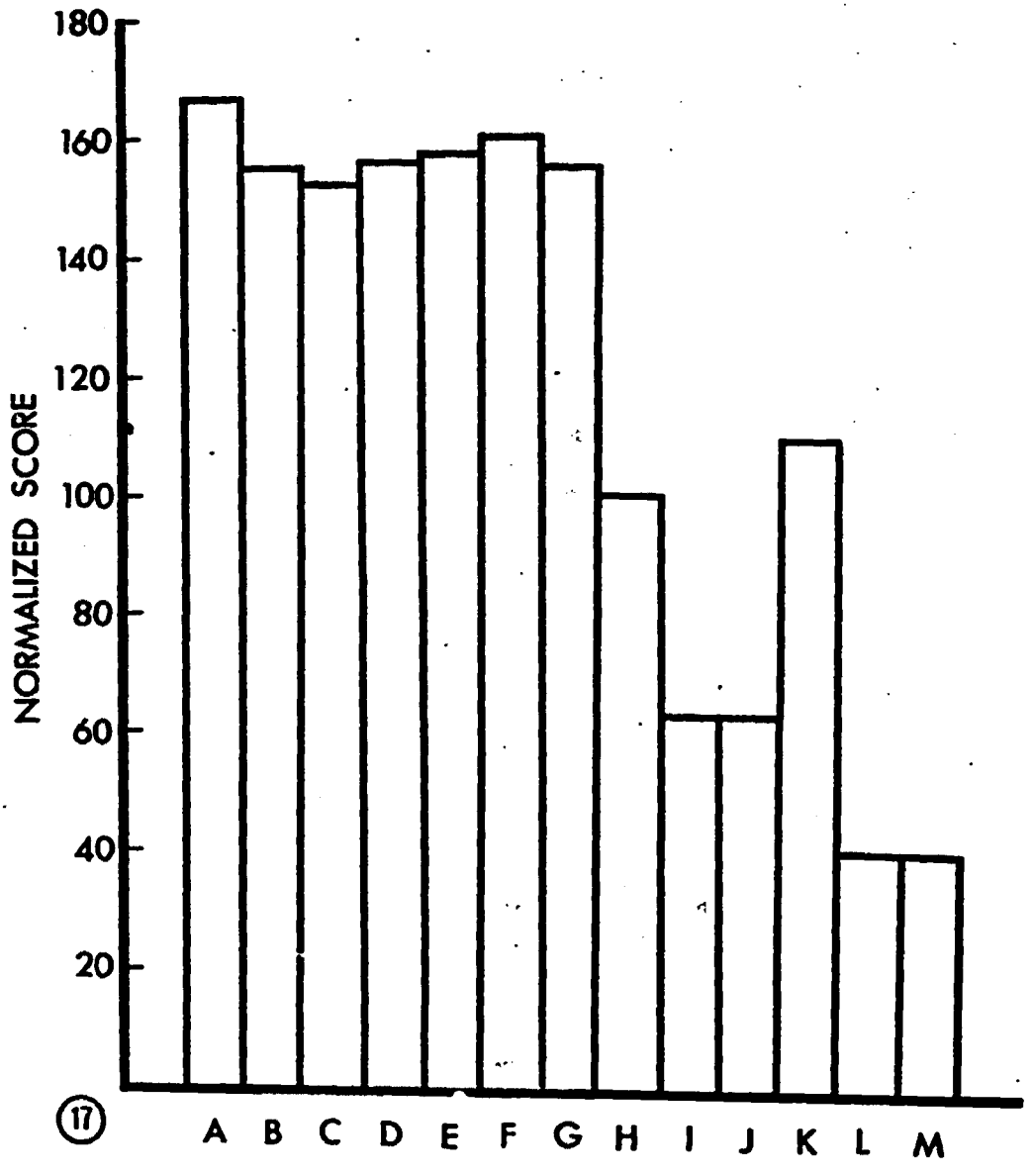

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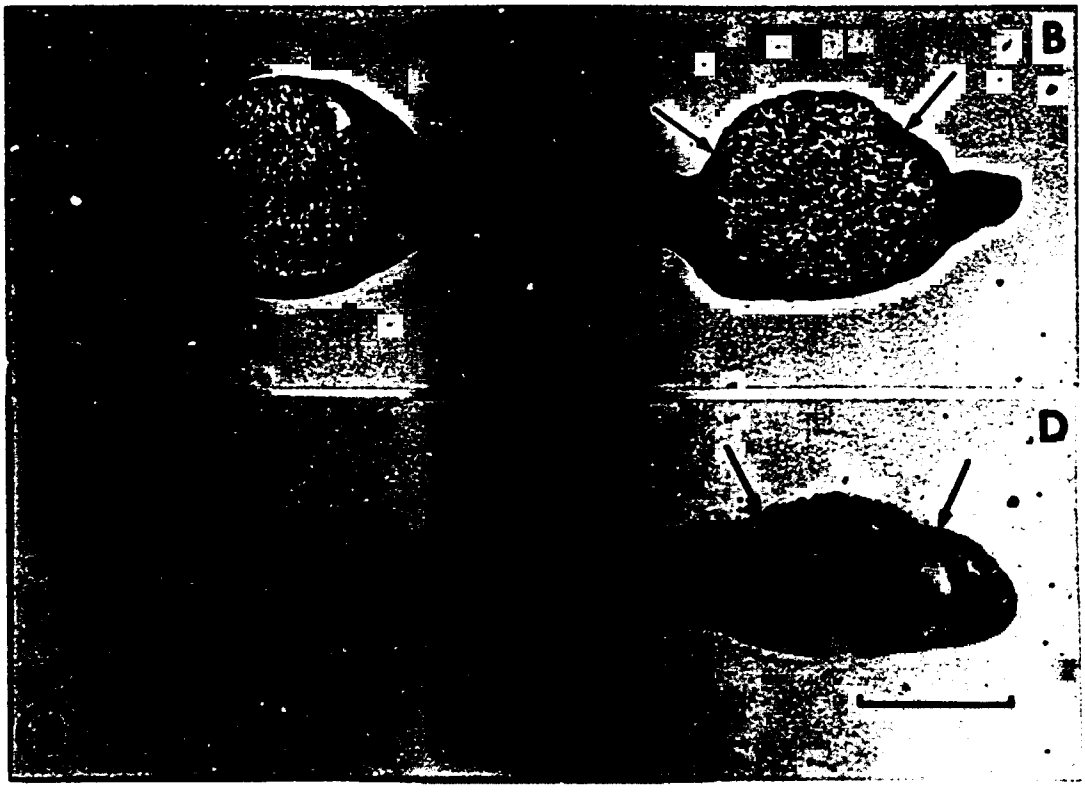

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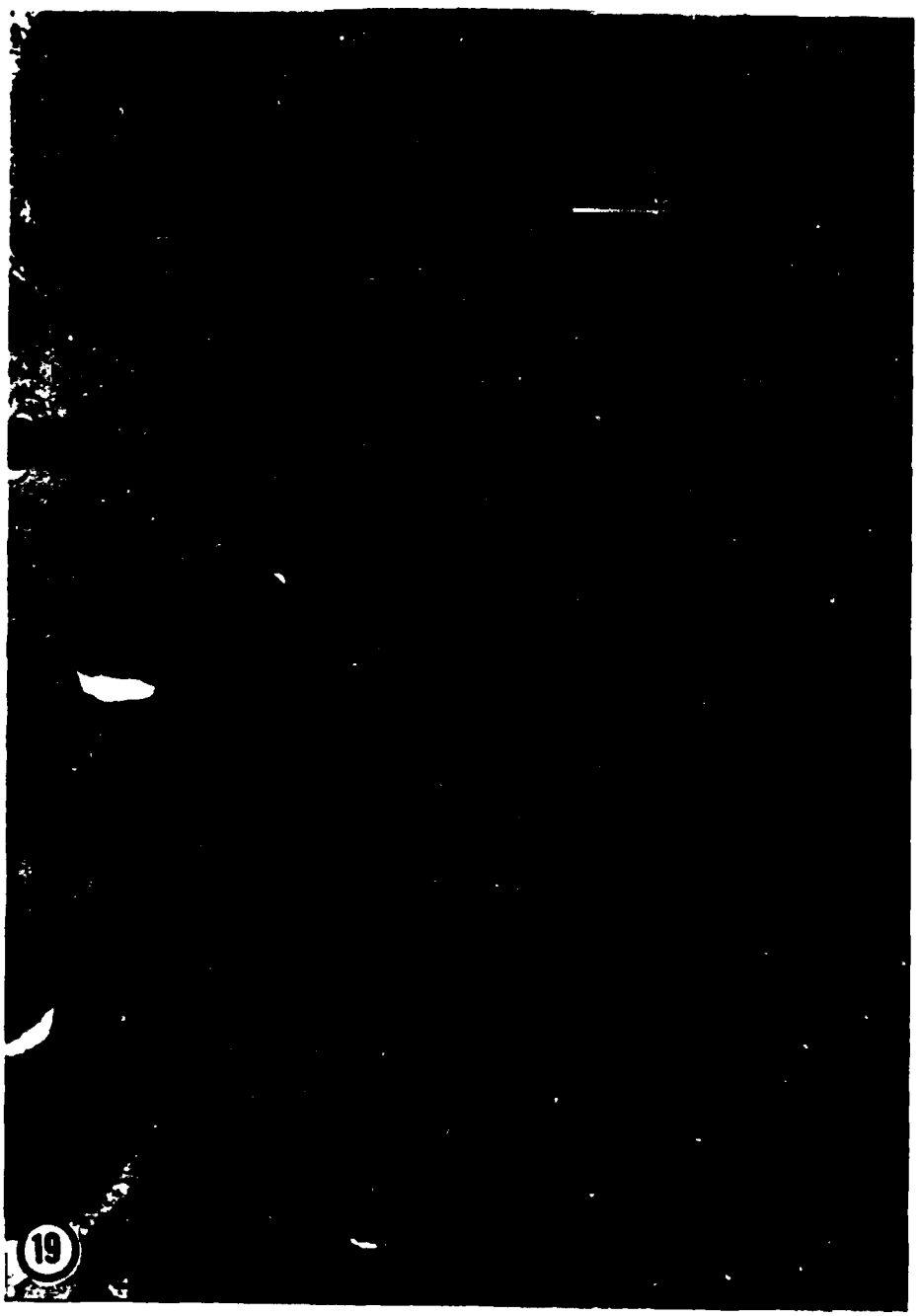

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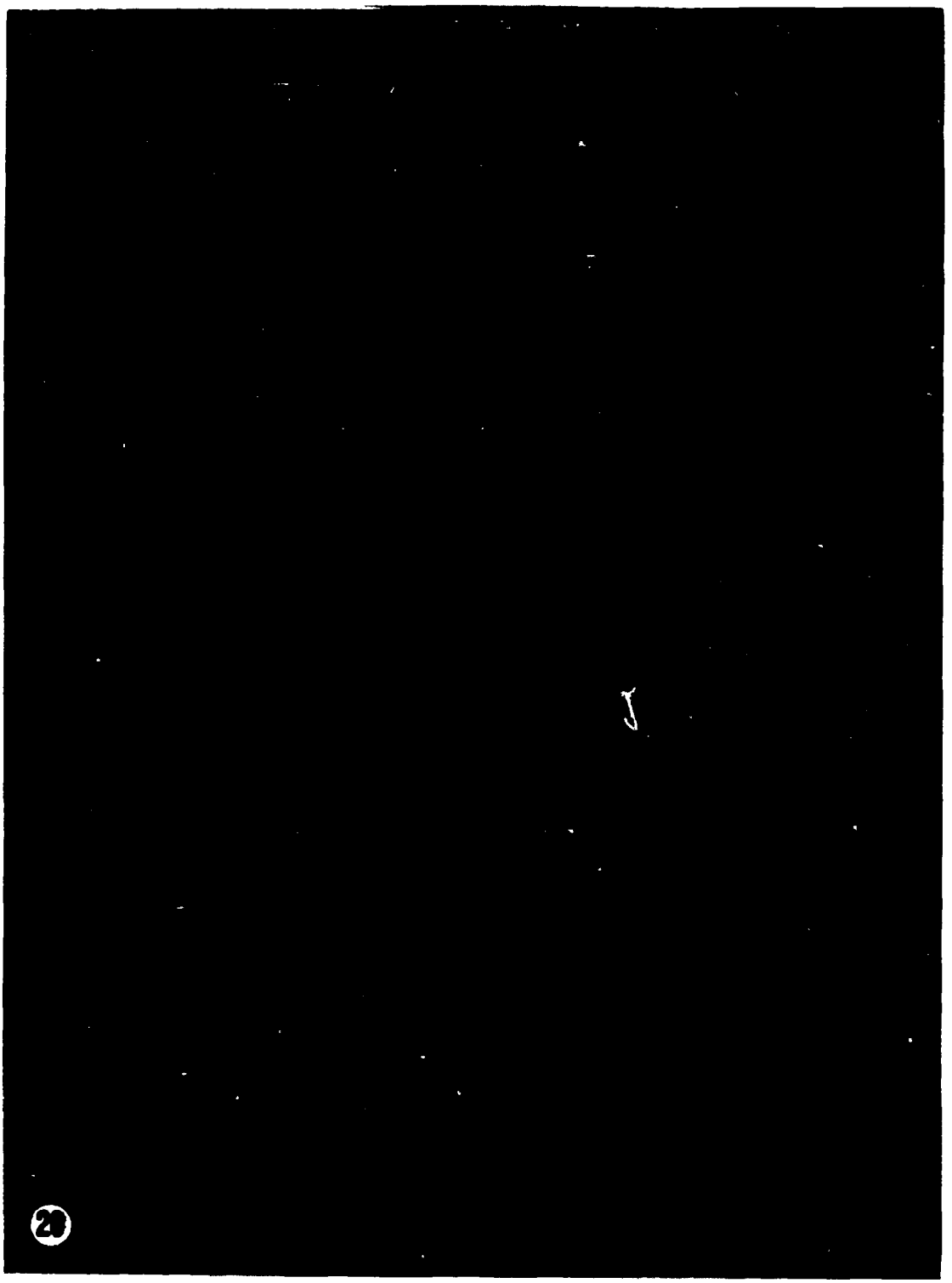

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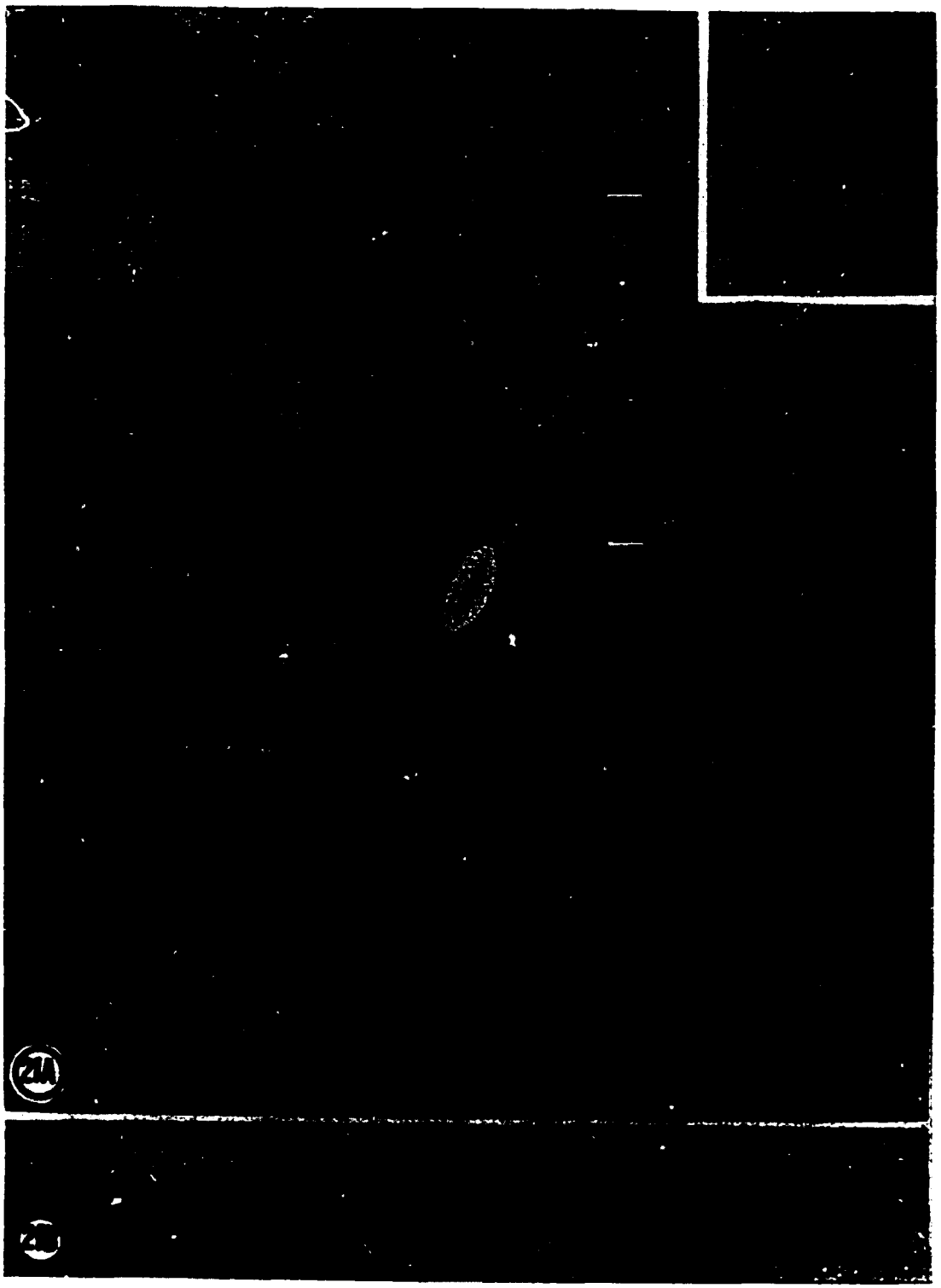

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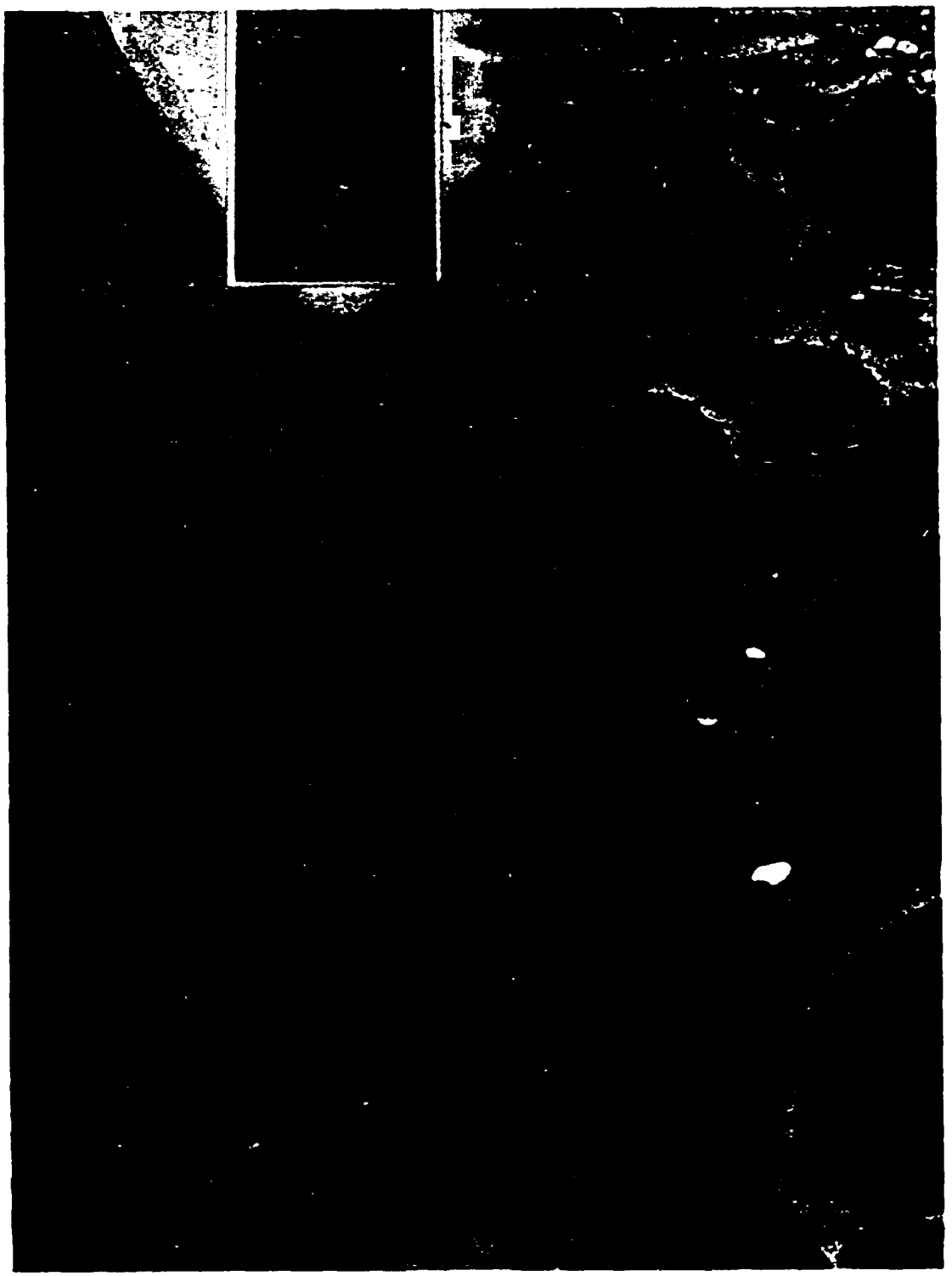

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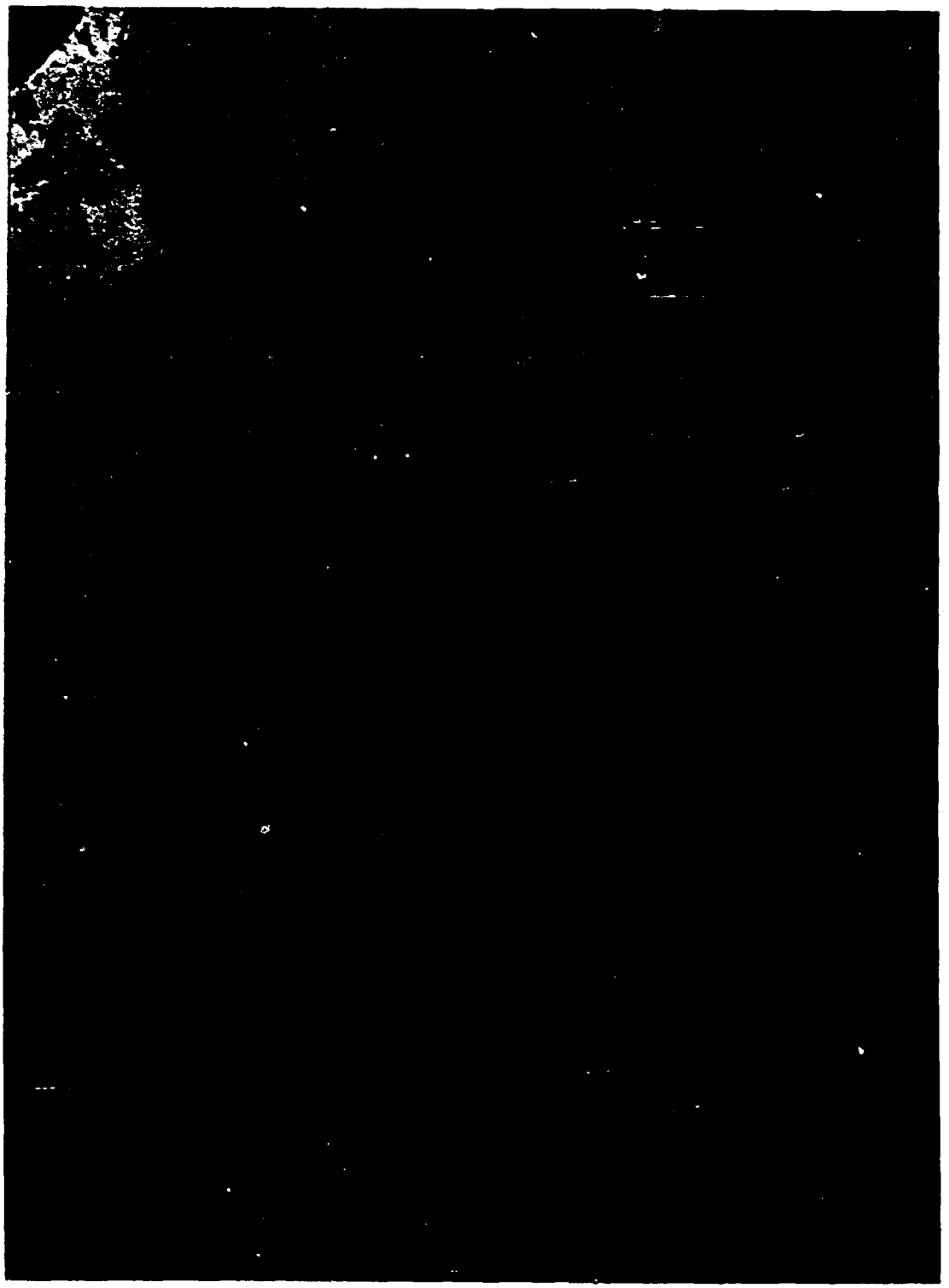

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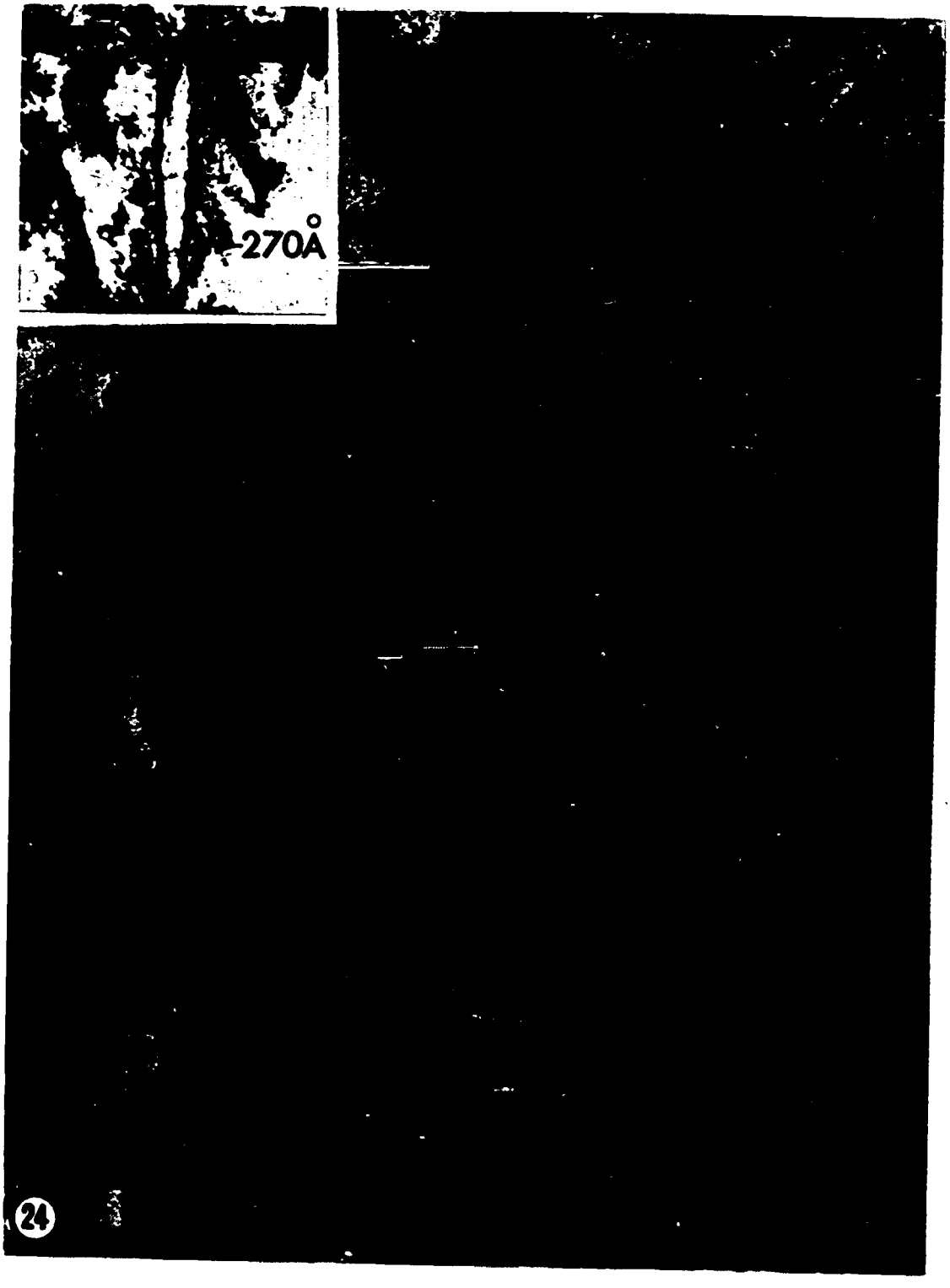

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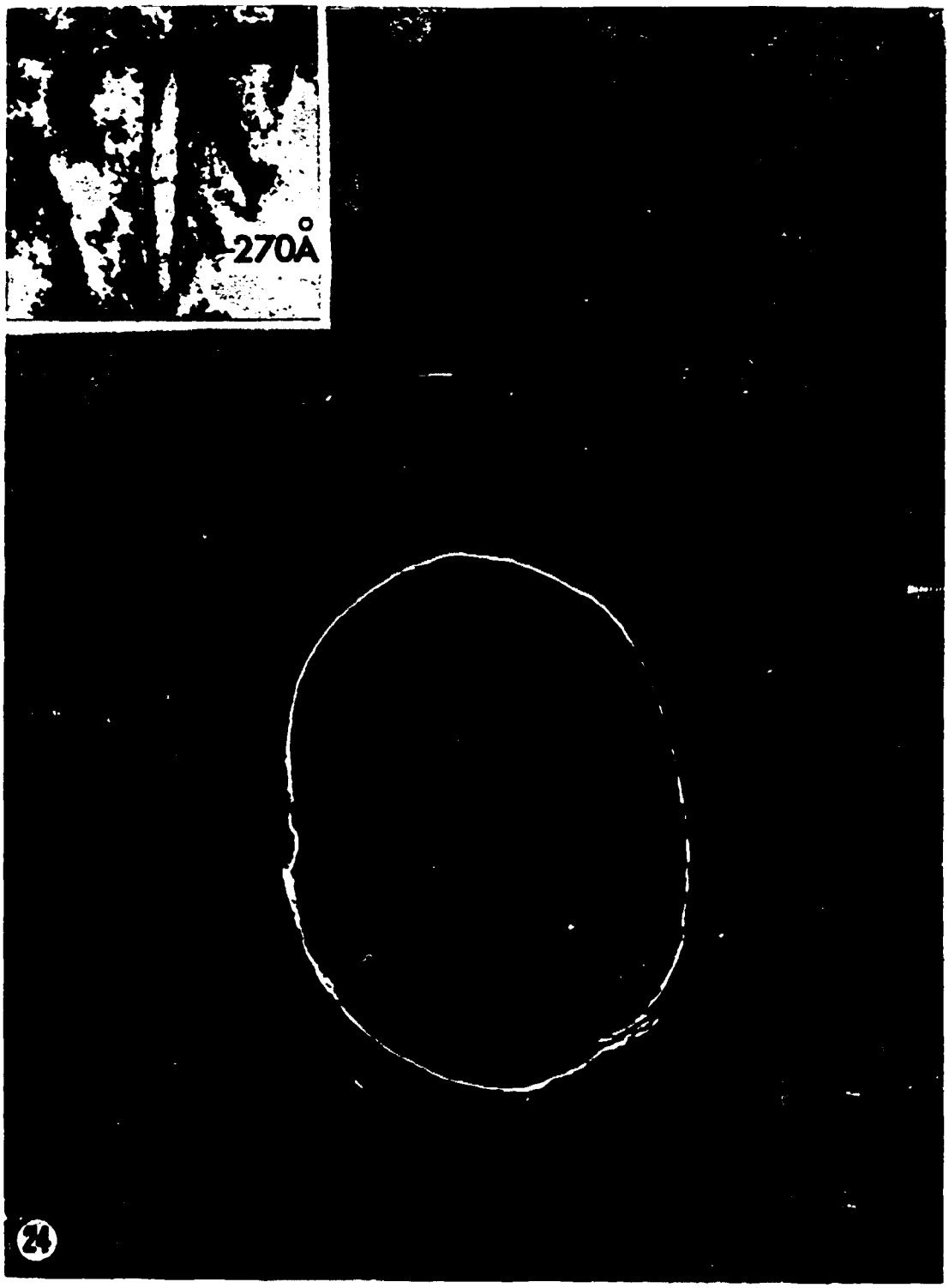

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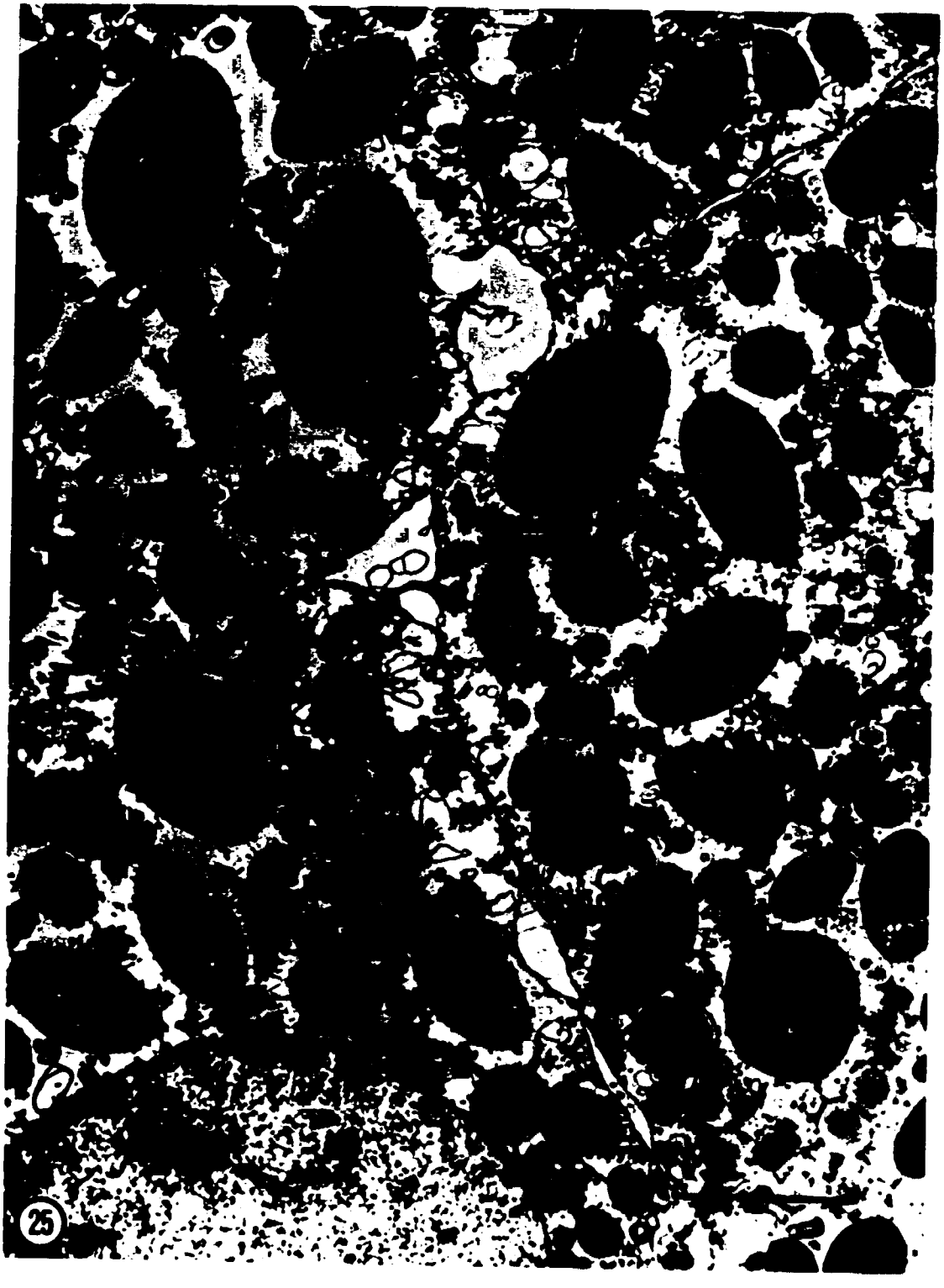

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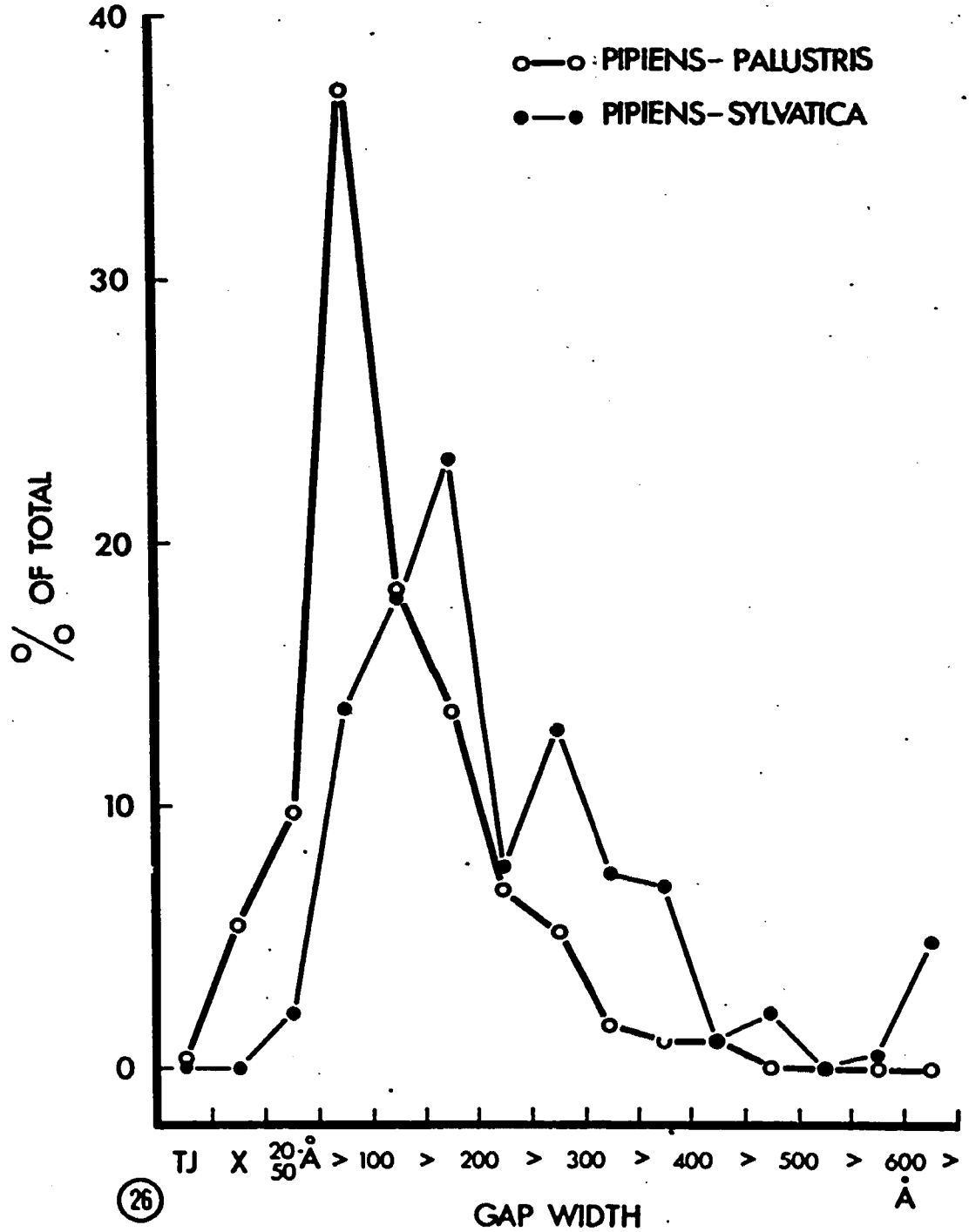

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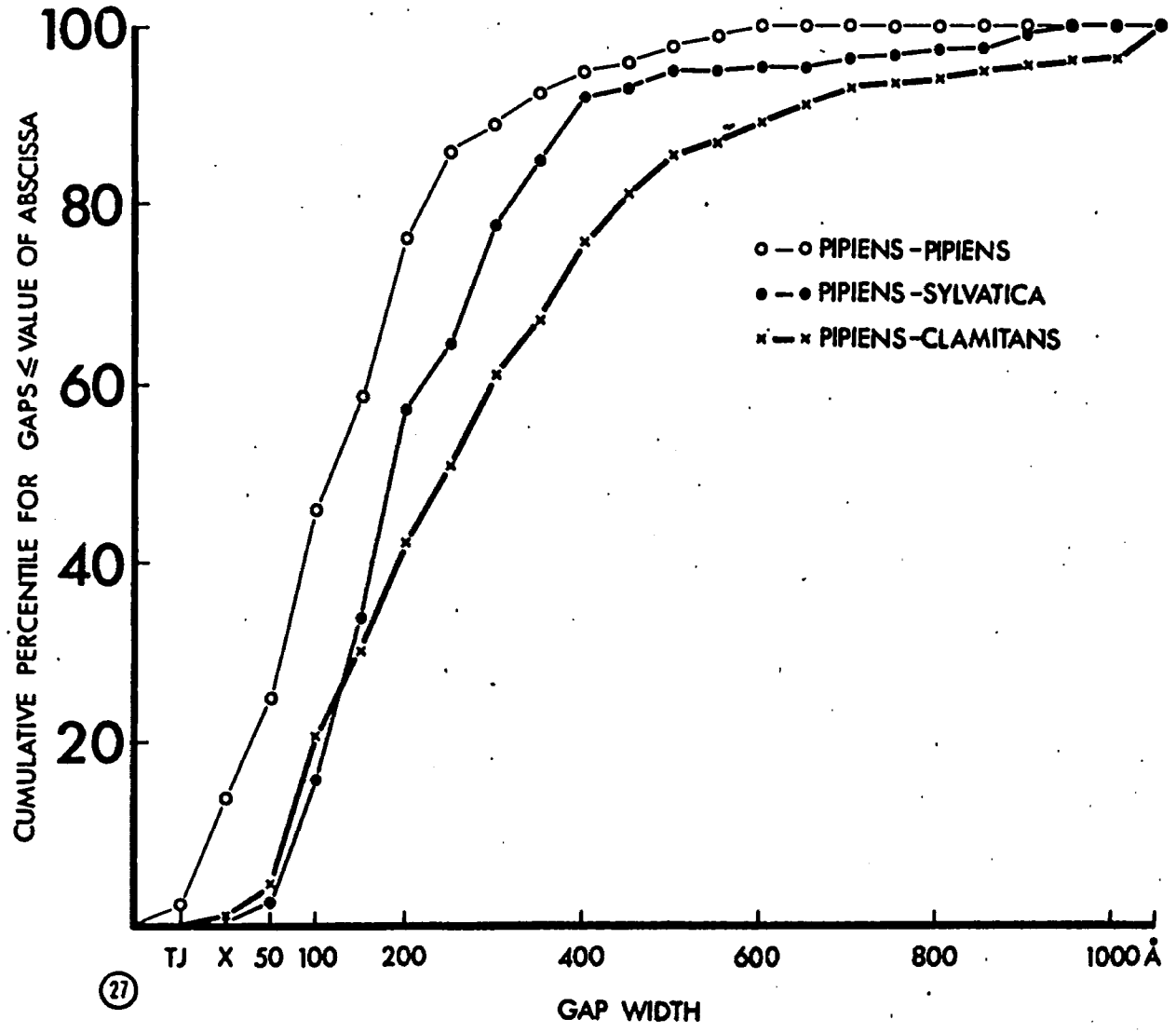

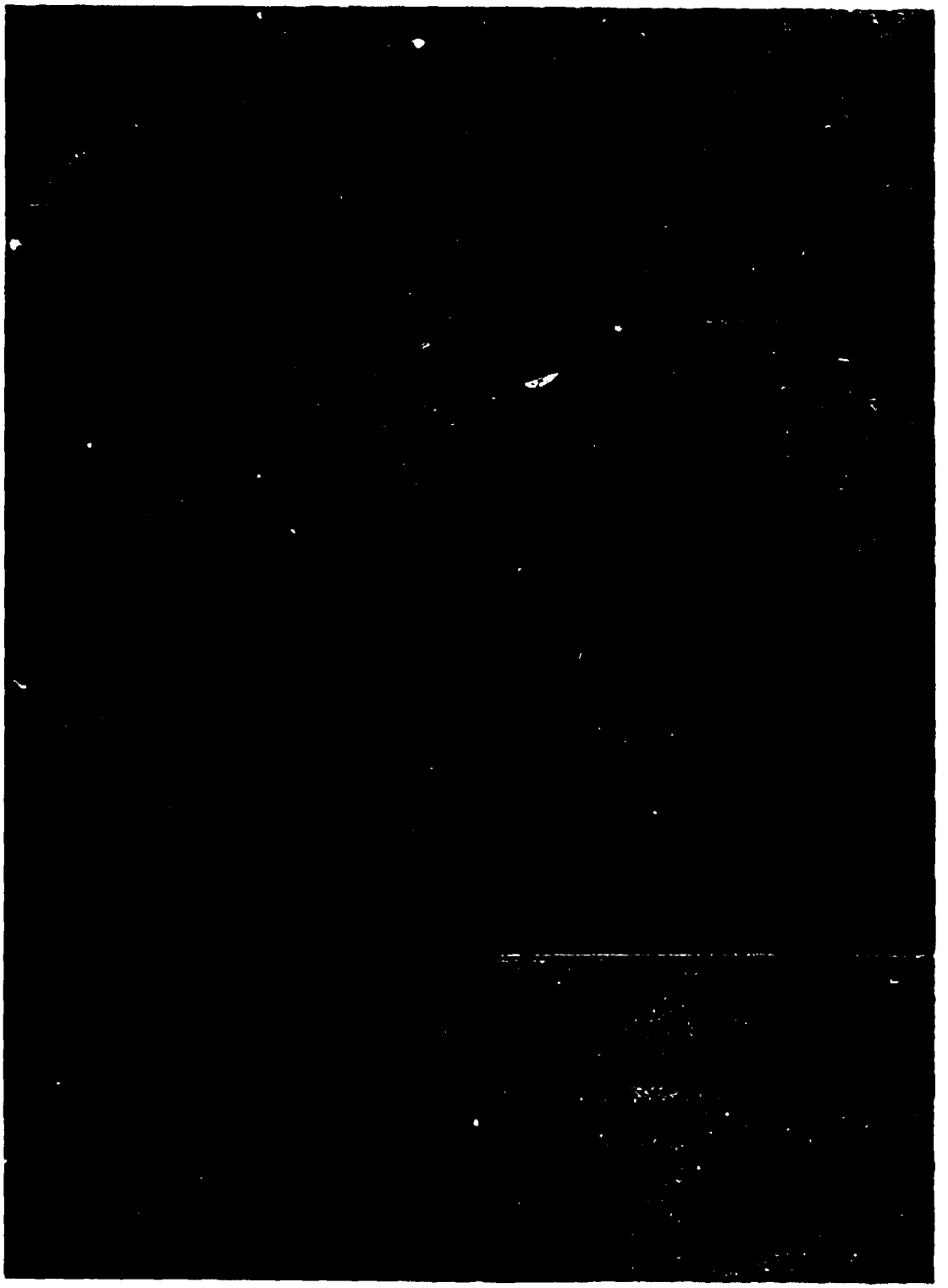

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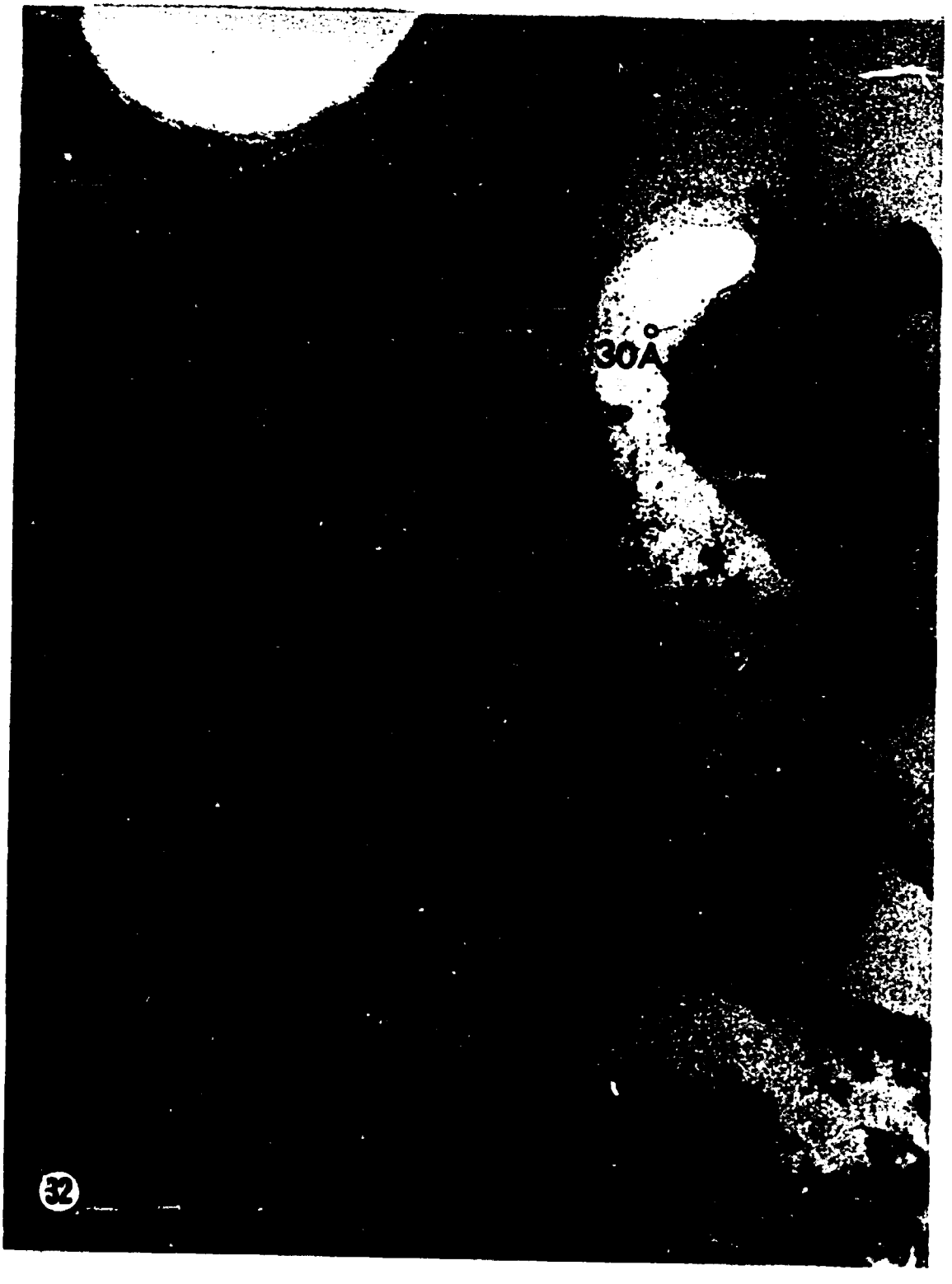

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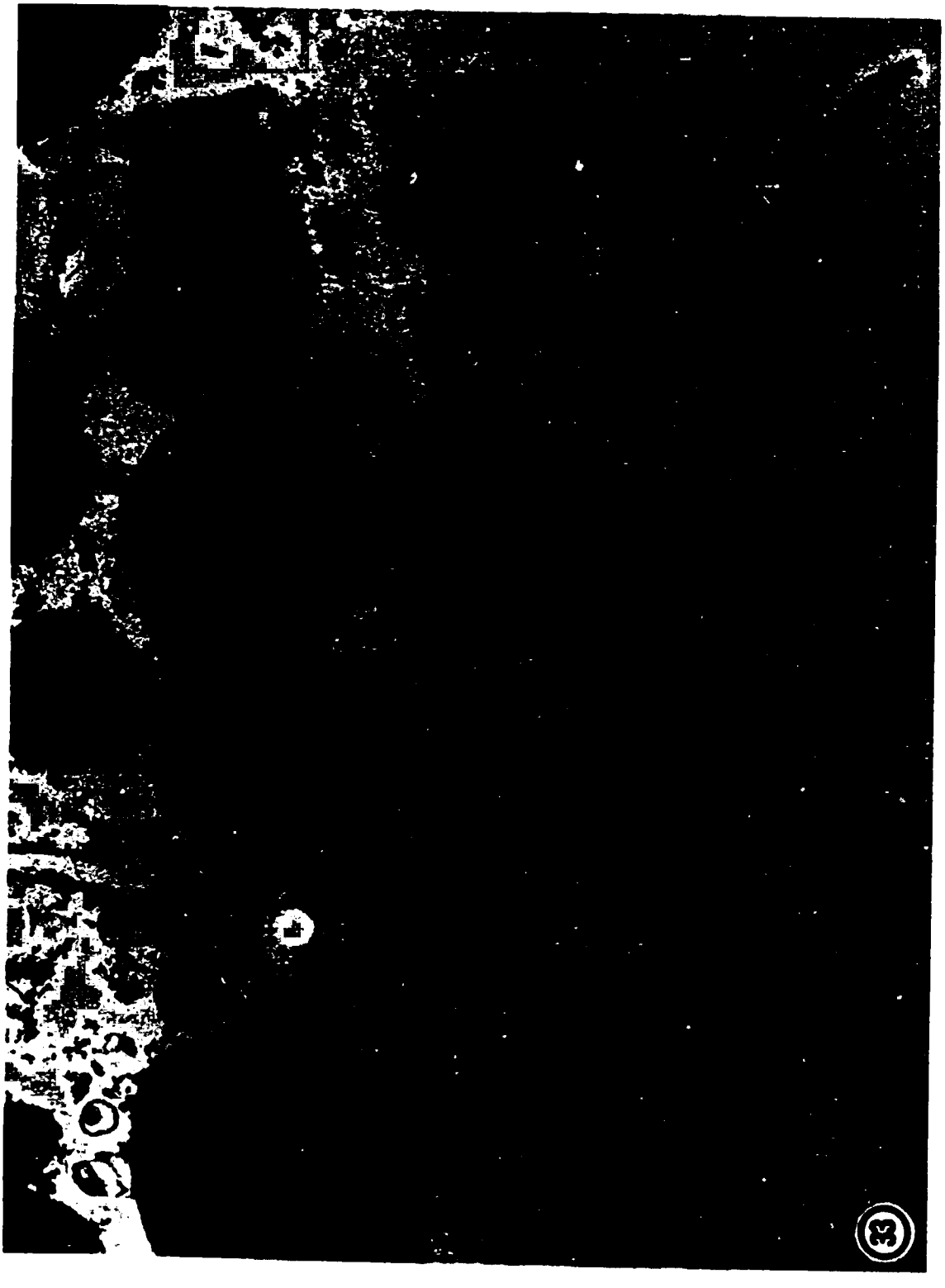

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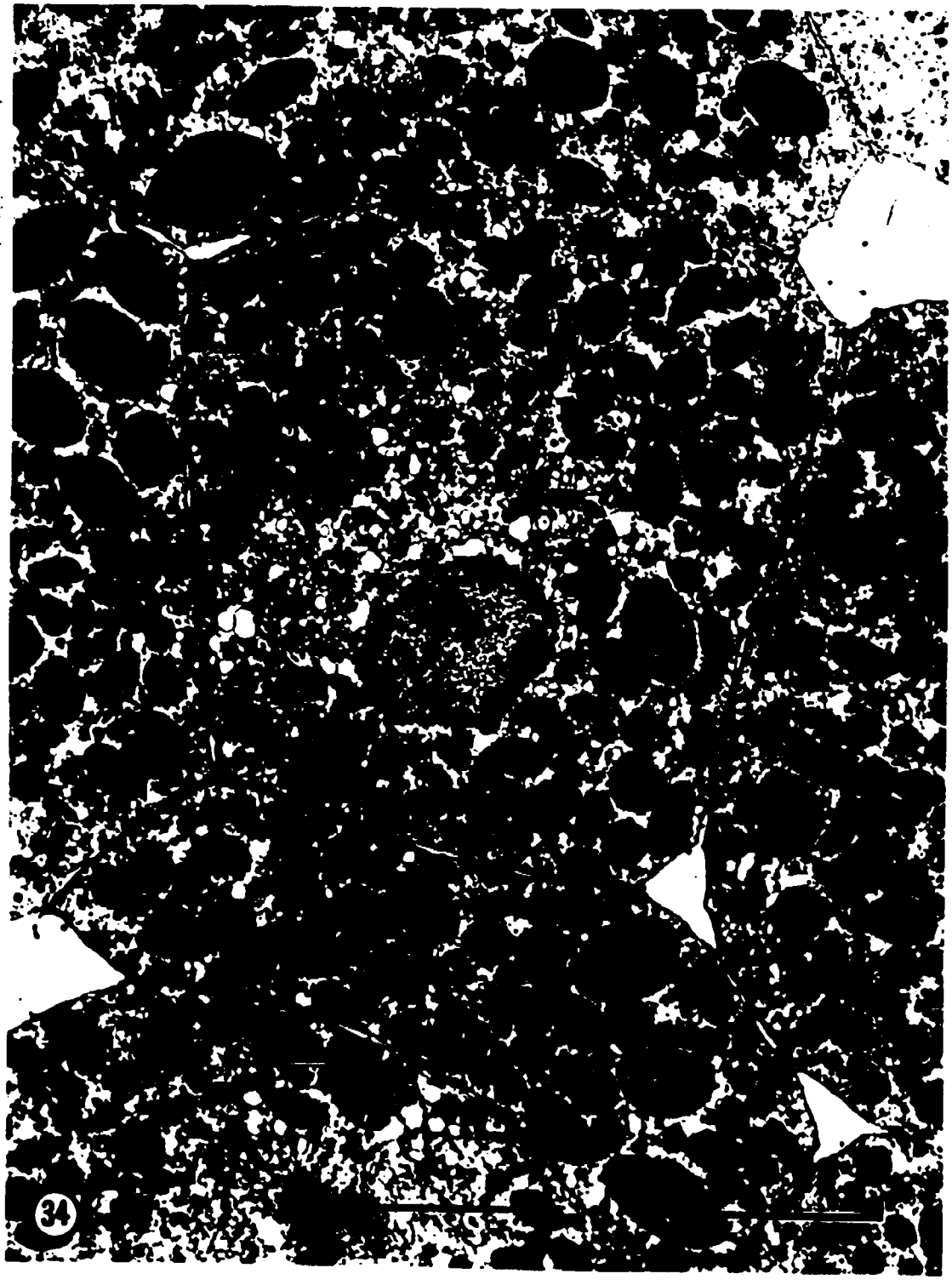

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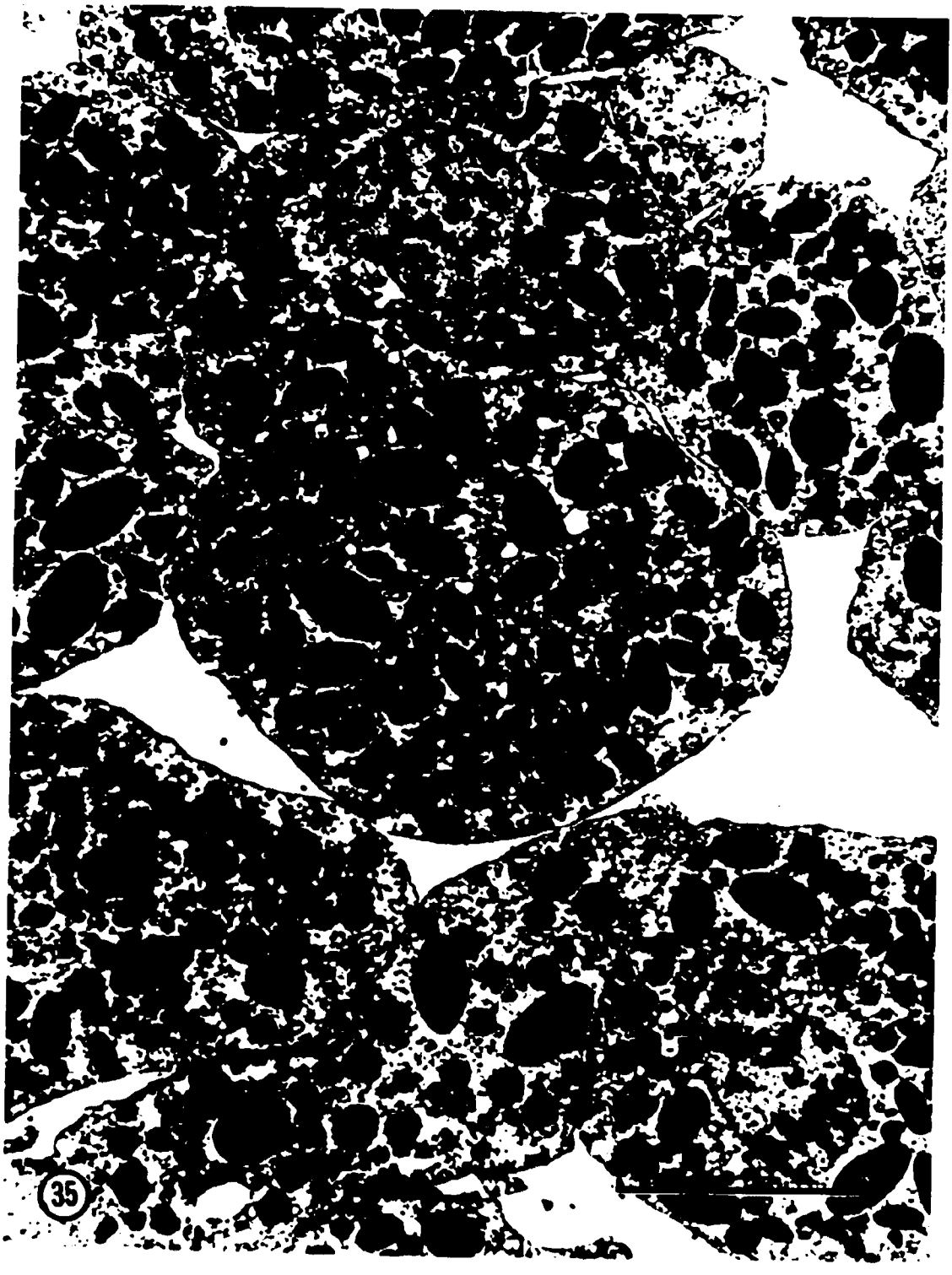

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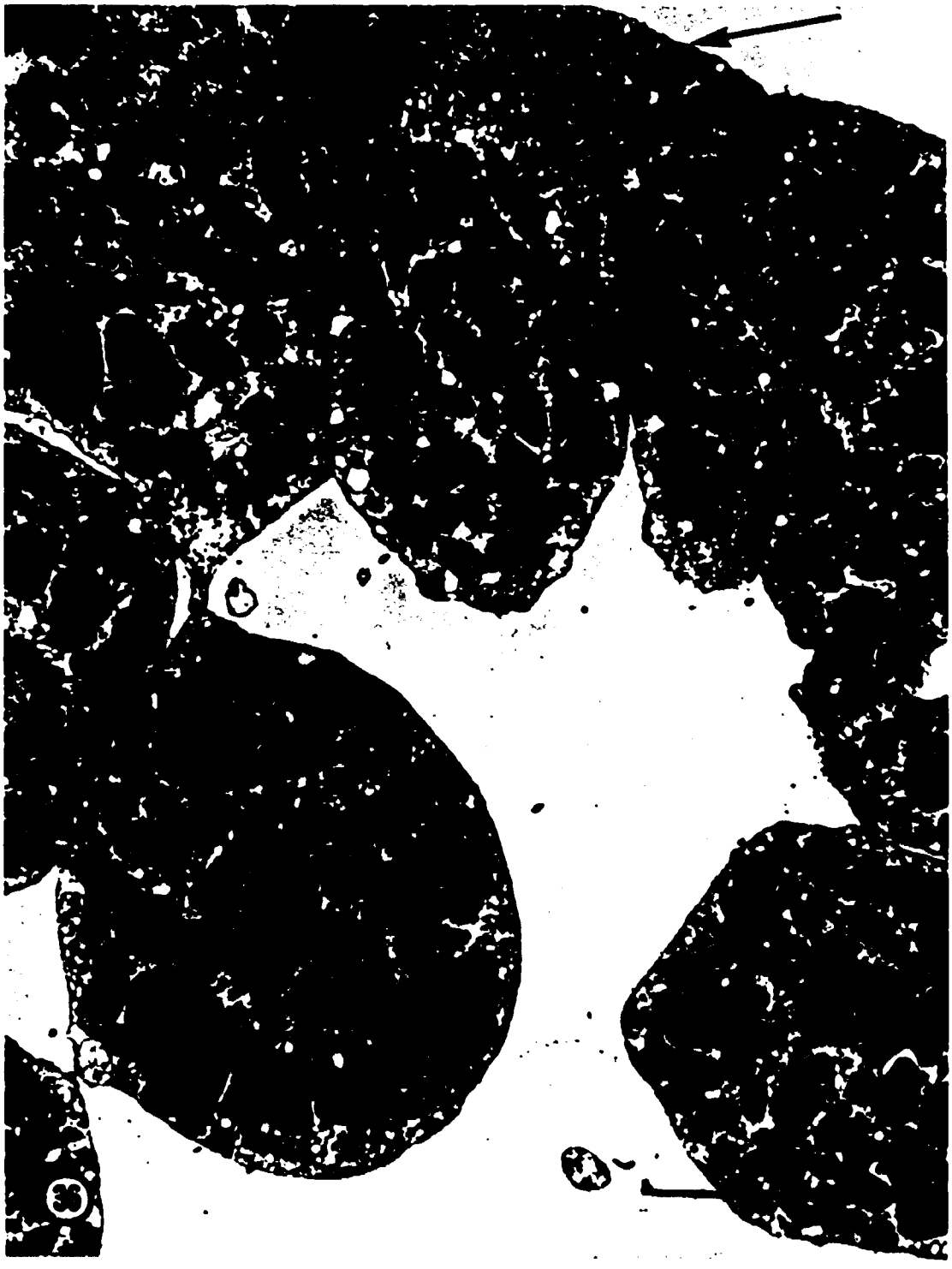

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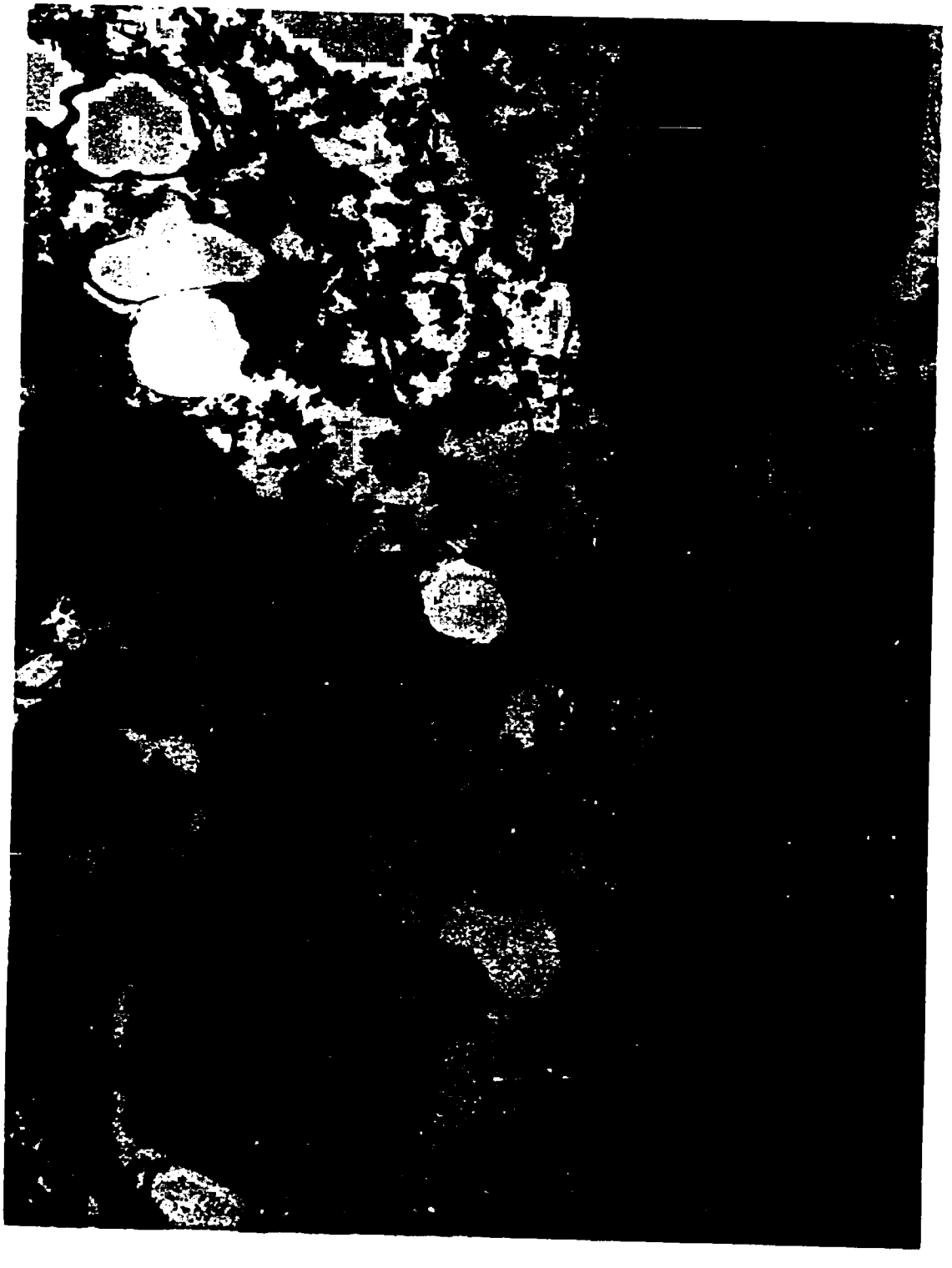

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