United States Patent
Mintz

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[45] Sept. 12, 1972

[54] METHOD OF BIOASSAY OF FERTILITY AND CONTRACEPTIVE DRUGS

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[73] Assignee: The Institute for Cancer Research, Philadelphia, Pa.

[22] Filed: March 15, 1971

[21] Appl. No.: 124,307

[52] U.S. Cl. .....128/2 R, 195/62, 195/103.5 R
[51] Int. Cl. A61b 10/00
[58] Field of Search .....128/2 R; 195/103.5 R

[56] References Cited
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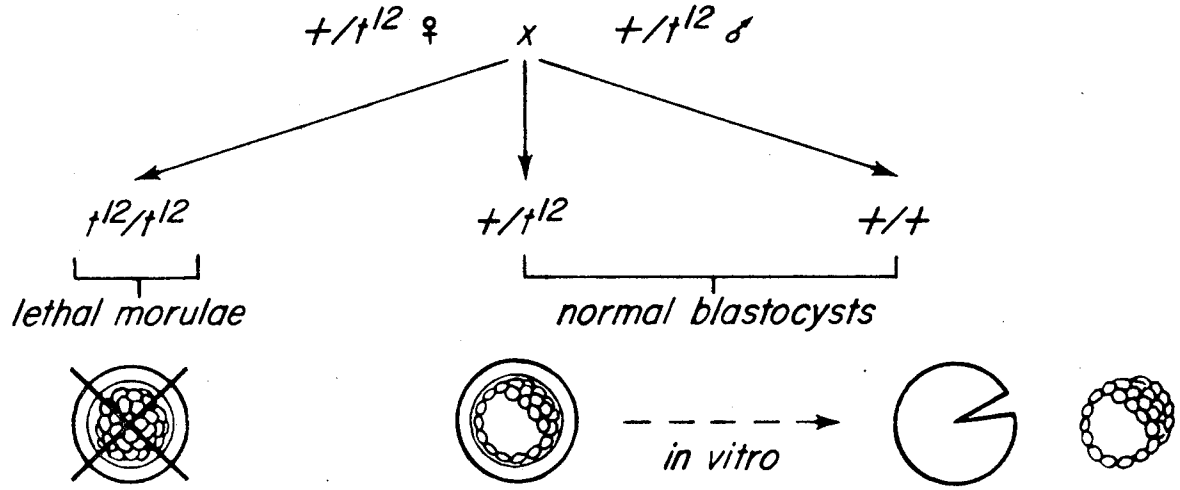
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Primary Examiner—Kyle L. Howell
Attorney—Jackson, Jackson & Chovanes

[57] ABSTRACT

A method of bioassay including the mating of female and male mice, introducing a test drug into the females within a time which begins before mating and ends within a few days after mating, sacrificing the female at various times within a few days of mating, flushing out the contents of the oviducts and of the uterine horns separately and examining the flushed out contents.

3 Claims, 16 Drawing Figures



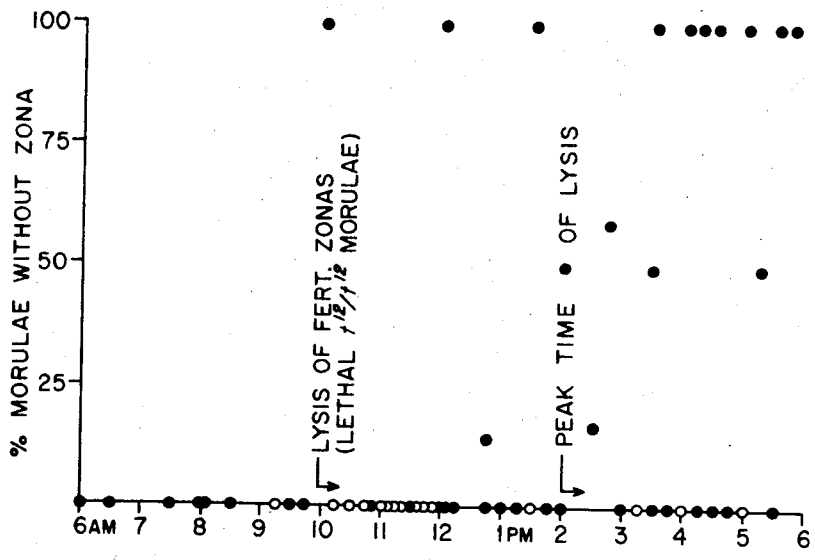


Fig. 3.

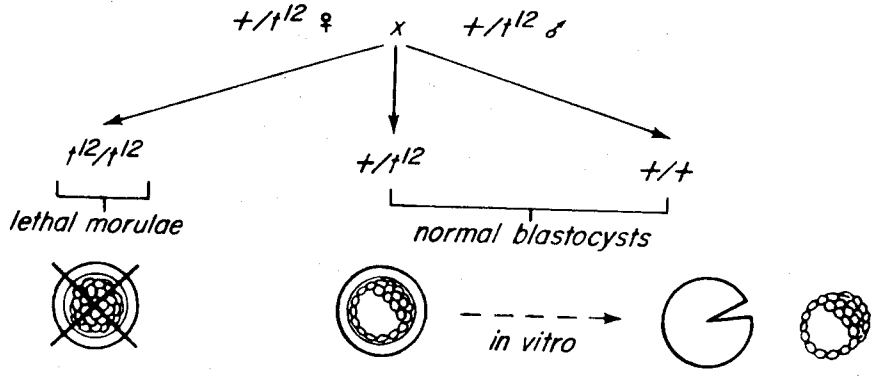


Fig. 1.

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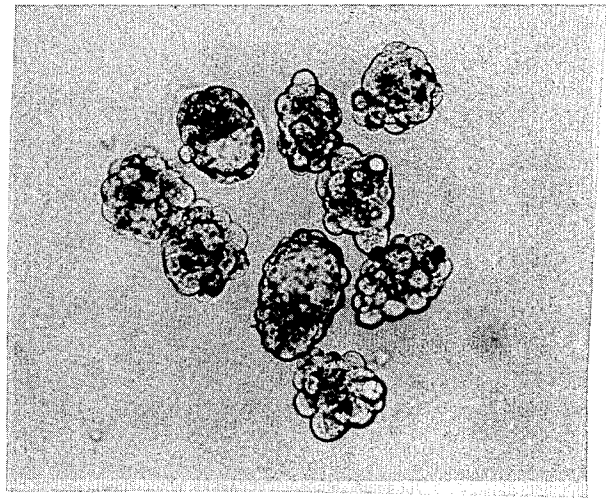


FIG. 2

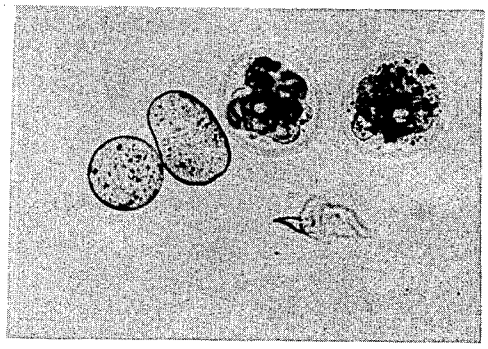


FIG. 4

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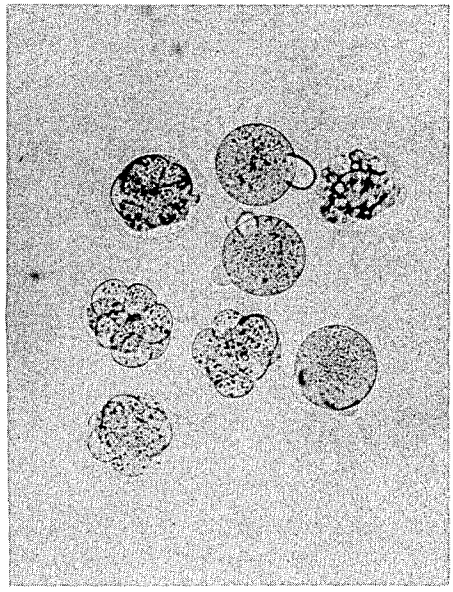


FIG. 5

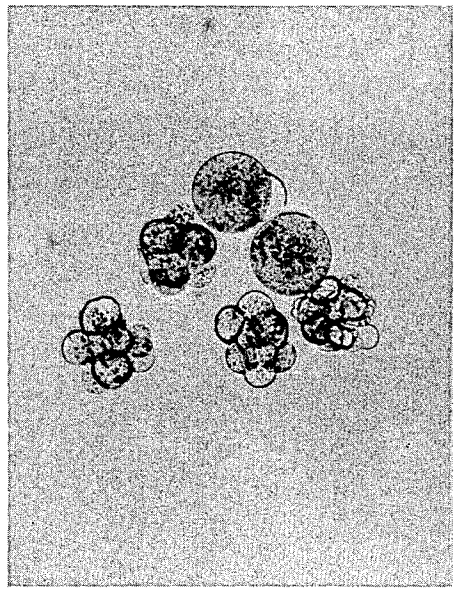


FIG. 6

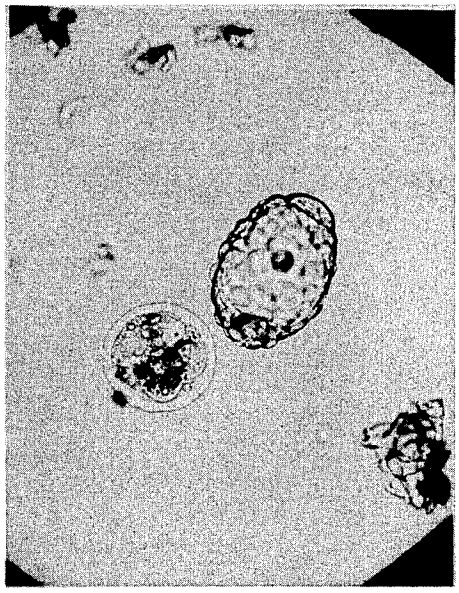


FIG. 7 LEFT

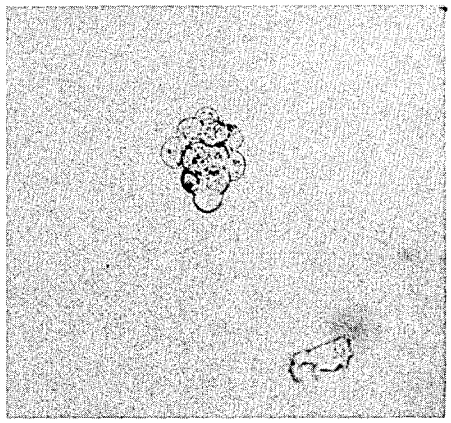


FIG. 7 RIGHT

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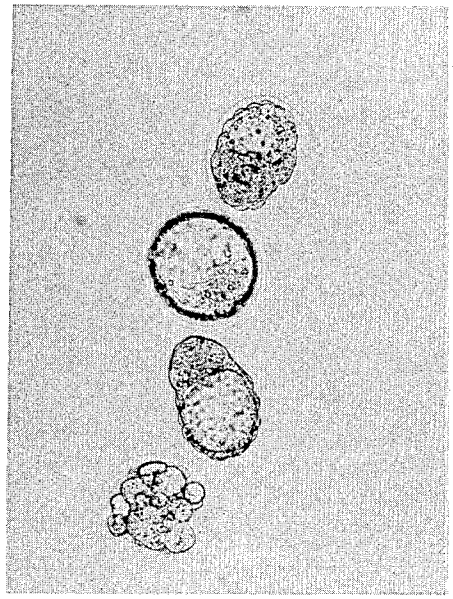


FIG. 8

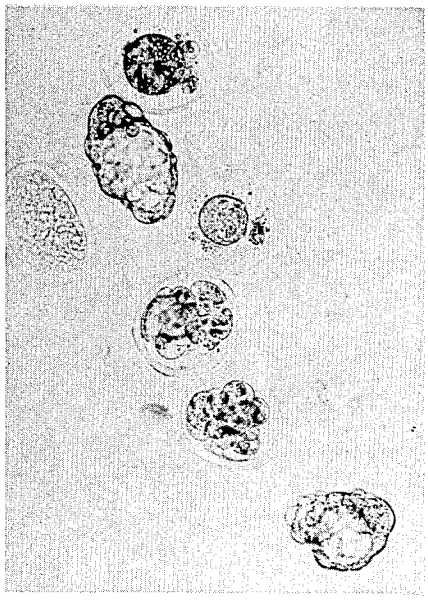


FIG. 11

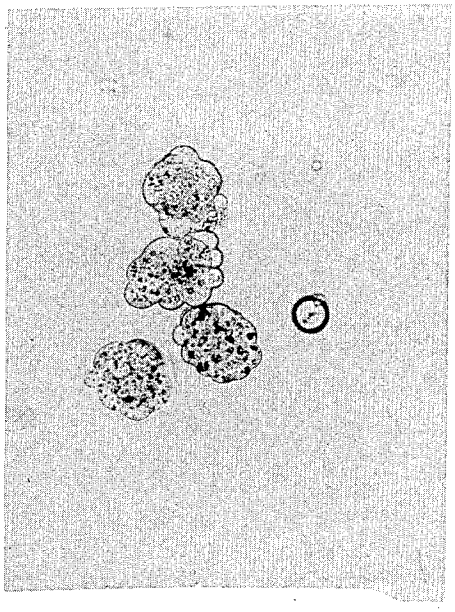


FIG. 12

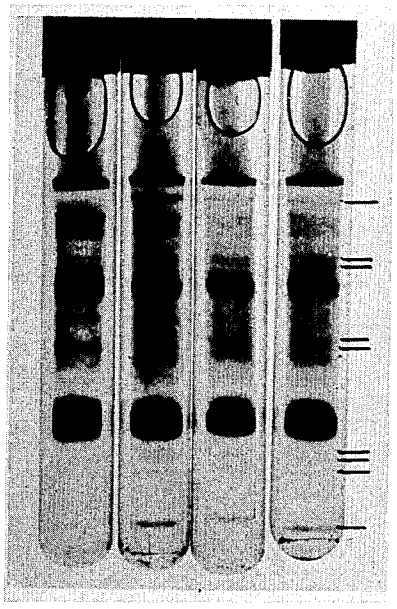


FIG. 13

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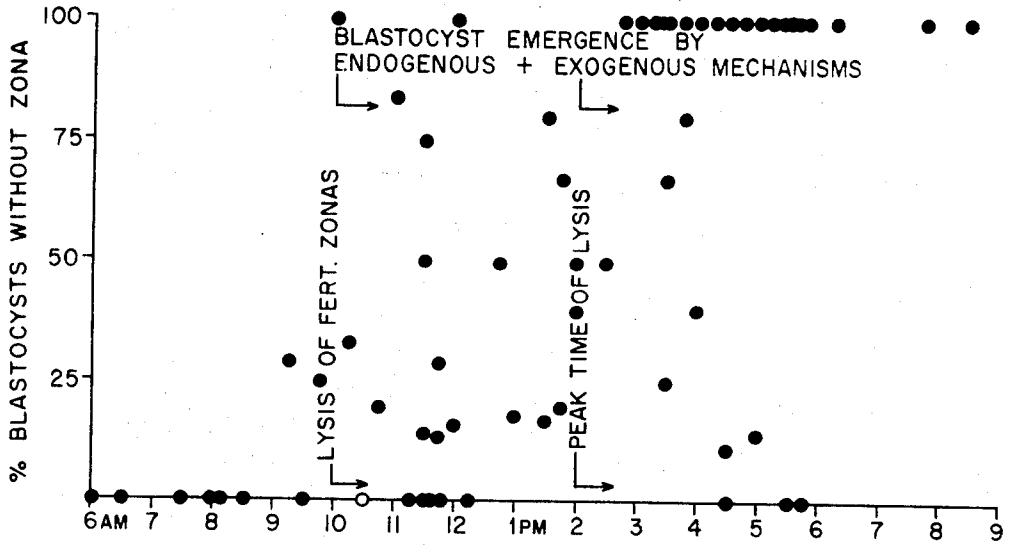


Fig. 9.

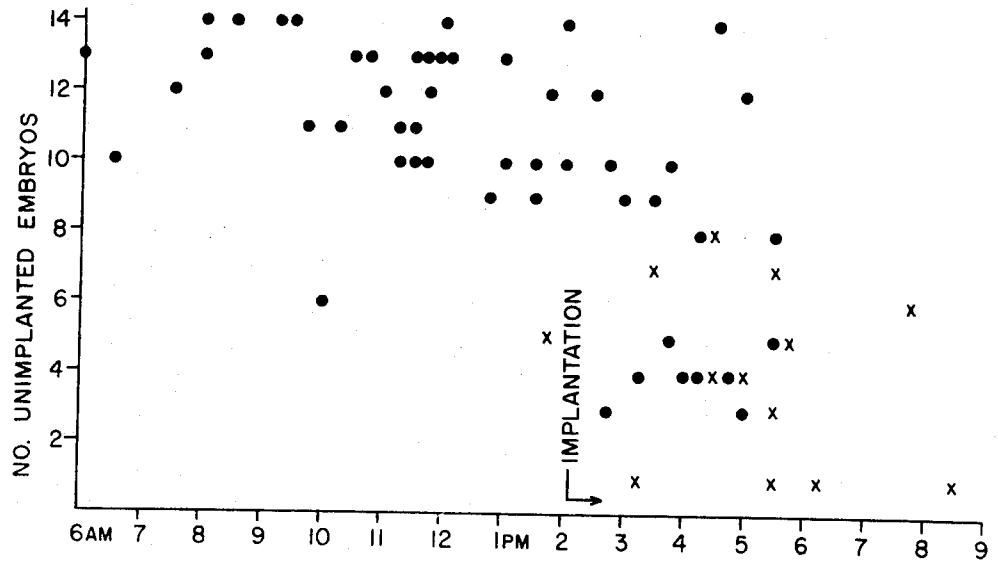


Fig. 10.

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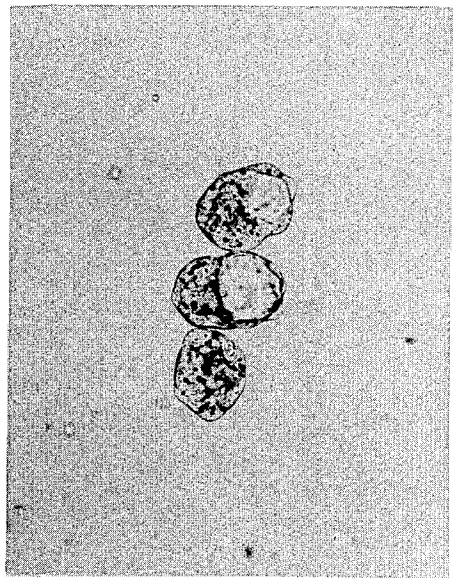


FIG. 14

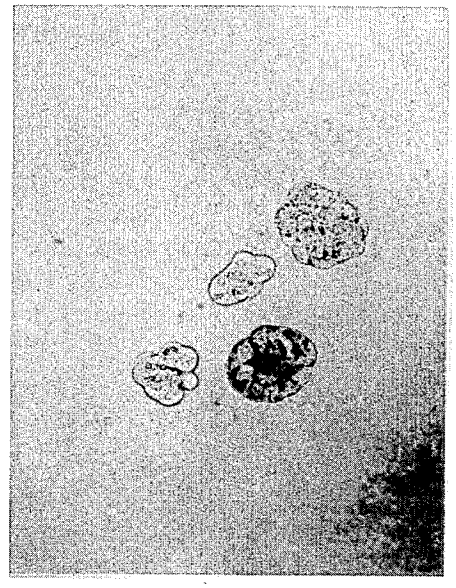


FIG. 15

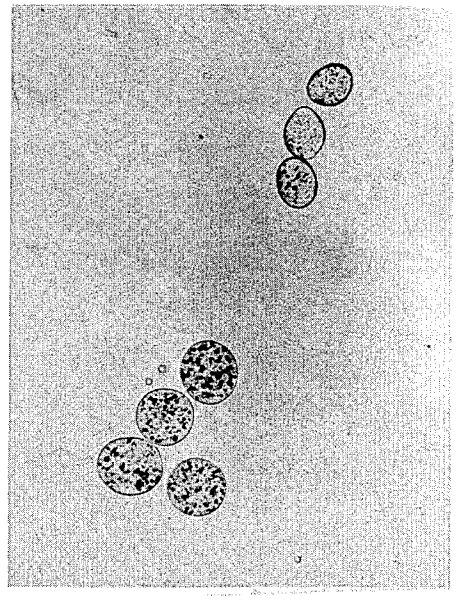


FIG. 16

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METHOD OF BIOASSAY OF FERTILITY AND CONTRACEPTIVE DRUGS

The present invention relates to a method of bioassay or evaluation of fertility and contraceptive drugs in a test animal.

Working with the mouse, it has been found that mechanisms critical for implantation and survival of mouse embryos can be detected in matings of female and male test animals in which both parents are heterozygous $+/t^{12}$, and will produce some homozygous lethal embryos of genotype t^{12}/t^{12}. These lethals arrest and die in the morula stage and are obtained in the same uteri as normal +/+ and $+/t^{12}$ blastocysts, from matings between heterozygotes. The zona pellucida was completely lysed on the lethal embryos in utero on day 3 of gestation (counting the vaginal plug date as day zero), whereas their zonas remained entirely intact in vitro. The dead embryos therefore demonstrated the existence of a uterine zonalytic factor, possibly an enzyme or enzymic complex. Further experiments in which the dead morulae were employed as an in vivo bioassay showed a sharp peak of the lysin on day 3. This coincided with a sudden attachment of the accompanying hatched blastocysts to the uterine wall, even though some of the blastocysts had hatched earlier, partly by pulsating and breaking out of their own zona pellucida. The evidence suggests that the uterine lysin acts as an implantation initiating factor, hereinafter called IIF, possibly by exposing, changing, or combining with glycoprotein or other molecules on the surface of blastocyst cells (but not of earlier stages), making the surfaces more adhesive to the uterine wall. The IIF is present in all strains, as shown by zona lysis of unfertilized eggs on day 3. The more rapid lysis of unfertilized than of fertilized zonas reveals a change toward increased resistance of the zona to lysis after fertilization.

Deficiency of IIF is the immediate cause of delayed implantation in postpartum and prepuberal pregnancies. IIF production is estrogen dependent and can therefore be influenced or controlled by hormonal manipulation. It is produced in the uterus and is not found in the oviduct. Fractionation of mouse uterine fluid proteins by electrophoresis on polyacrylamide gel columns reveals a number of bands (possibly as many as (10) present in the uterine fluid of various stages but not in serum. Whether any of the bands corresponds to the IIF will be determined from other biochemical studies in progress.

The peak IIF time is strain-specific. In some all-normal-genotype strains (for example, BALB/c), embryos are still often in the morula stage when the IIF is produced. These denuded morulae may become dissociated into two (or more) groups of cells, forming viable twins. Alternatively, a pair of whole morulae may adhere, forming one aggregate capable of becoming a single mouse. The latter corresponds to the experimental allophenic mice with genetic mosaicism, produced by aggregating embryos at 37° C. after zona removal with pronase. XX⟷XY sex chromosomal mosaicism may result. Such animals are occasionally intersexes, but usually males or females with sterility infrequent. If still younger (cleavage stage) embryos are denuded by IIF, blastomeres tend to dissociate and die.

IIF constitutes a normal system with an important biological function at implantation. It presents new possibilities for experimental control of fertility, via promotion or prevention of implantation. Some of its effects may, however, cause viable anomalies of development.

THE PRESENT INVENTION

A purpose of the invention is to evaluate the effect on IIF of drugs which promote or prevent fertility in humans and other vertebrates by introducing such drugs either into the uterus or via any indirect route (such as orally, subcutaneously, intramuscularly, intraperitoneally, or intravenously) in female test animals before or after mating of heterozygous $+/t^{12}$ female and male test animals, and examining the contents of the uterus of the females at various stages within a few days of mating.

A further purpose is to examine for lysis of the zona pellucida of lethal morula embryos of the type t^{12}/t^{12}.

A further purpose is to compare the number of normal blastocysts attached to the uterine wall in treated as compared with untreated control animals.

A further purpose is to determine whether precocious transport of fertilized ova from the oviducts into the uteri has occurred at a stage before it would normally occur, or whether such transport has been abnormally delayed.

A further purpose is to examine for lysis of the zona pellucida of living morulae in strains that normally have some embryos still in this stage on day 3, because morulae are incapable of hatching out of their own zonas in the absence of an external lysin. An example of a strain that can be used for this purpose is the BALB/*c* strain, including the BALB/*c*AnNi*cr* subline bred at The Institute for Cancer Research in Fox Chase, Philadelphia. Mice of this strain are available generally to researchers.

A further purpose in the case of pseudopregnancy, following matings of females of any strain with sterile males, is to determine whether lysis has occurred in the zona pellucida from unfertilized eggs; this is more sensitive than the lysis from fertilized eggs.

Further purposes appear in the specification and the claims.

DESCRIPTION OF DRAWINGS

Each of the photographs herein is at a magnification of 300 diameters.

FIG. 1 shows the experimental cross used to produce co-existing lethal morulae and normal blastocysts. In vitro, only the blastocysts hatch by stretching and breaking the zona.

FIG. 2 is a photograph showing lysis of zonas of dead morulae by the uterine lysin. It will be noted that there are thinned zonas still visible on a morula in the upper right and a blastocyst in the upper left. Eggs were flushed from the uteri on day 3 after a $+/t^{12} \times +/t^{12}$ mating.

FIG. 3 is a plot showing the percent of dead morulae that are zona-free in each female (each circle) in day 3 uteri after a $+/t^{12} \times +/t^{12}$ mating as a bioassay of the uterine lysin in vivo. Open circles designate zonas that are sticky.

FIG. 4 is a photograph of uterine eggs from a $+/t^{12} \times +/t^{12}$ mating early on day 3. Two zona-free unfertilized eggs reveal a low level of uterine lysin, still inadequate to lyse the dead morula zonas.

FIG. 5 is a photograph showing that fibrinolysin in vitro lyses all unfertilized egg zonas first. The blebs are formed from the denuded eggs.

FIG. 6 is a photograph showing that fibrinolysin in vitro lyses the fertilized egg zonas only after the step seen in FIG. 5.

FIG. 7 is a photograph showing the eggs on day 4 after ligature of the uterotubal junction on day 2 subsequent to a $+/t^{12} \times +/t^{12}$ mating. The dead morula zona bioassay shows lysin absent in the oviduct (to the left) and present in the uterus (to the right). Blastocysts hatch mechanically in the oviduct.

FIG. 8 is a photograph showing that blastocyst self-hatching (note split zona at the top) has started in this female ($+/t^{12} \times +/t^{12}$, early day 3) before uterine lysin is adequate to digest unfertilized zonas (fragmented egg at bottom).

FIG. 9 shows the percent of blastocysts that are zona-free in each of the same females (each circle) examined in FIG. 3. The information on lysis start and peak for fertilized zonas is superimposed from FIG. 3. Blastocysts are denuded faster than dead morulae, showing that they use endogenous in addition to exogenous hatching mechanisms.

FIG. 10 is a plot which shows that implantation starts rapidly (abrupt drop in unimplanted embryos) at the uterine lytic peak (2 P.M.) in each of the same females shown in FIGS. 3 and 9. The crosses are females with unimplanted embryos in only one uterine horn.

FIG. 11 is a photograph showing the deficiency of uterine IIF in delayed implantation. This is a prepuberal female on day 4 of a $+/t^{12} \times +/t^{12}$ mating, after pretreatment with pregnant mare serum (PMS) and human chorionic gonadotropin (HCG) to induce ovulation. The zonas are intact on unfertilized eggs and dead morulae, but the blastocysts have split their zonas and hatched.

FIG. 12 shows a photograph of precocious IIF production on day 2, causing zona lysis after a small estradiol supplement to progesterone. The eggs are from an adult female in a $+/t^{12} \times +/t^{12}$ mating.

FIG. 13 is a photograph of the results of polyacrylamide gel electrophoresis at pH 8.3, tris-glycine buffer. It compares uterine fluid proteins (tubes 2–4 from the left) with serum proteins (tube 1 on the left). Bands specific for uterine fluid are marked at the right as they are seen in estrus (tube 2), and day 2 (tube 3) and day 3 (tube 4) of pregnancy, in the BALB/*c* strain.

FIG. 14 is a photograph showing zona lysis on a BALB/*c* viable (+/+) morula (and blastocysts) by IIF on day 3.

FIG. 15 is a photograph showing two miniature "twin" early morulae (+/+) along with two normal size morulae, after IIF action on day 3 in BALB/*c*.

FIG. 16 is a photograph showing zona lysis and blastomere dissociation by IIF, after 2-cell embryos with zonas were transferred to a day 3 host uterus. Blastomere cleavage continued (smaller size cells).

BACKGROUND OF THE INVENTION

The present research started as a result of a chance observation. In 1963, early mouse embryos were being explanted from the cross shown in FIG. 1, between heterozygous $+/t^{12}$ parents, in order to obtain embryos of the homozygous lethal t^{12}/t^{12} genotype. Individuals of the latter type are known to die in the morula stage. [Smith, L.J. 1956. A Morphological and Histochemical Investigation of a Pre-implantation Lethal (t^{12}) in the House Mouse. J. Exp. Zool., 132:51–83] The purpose of these explants was to combine blastomeres of the lethal genotype, prior to lethality, in a single aggregate together with blastomeres of normal (+/+) genotype so as to produce genetically mosaic or allophenic mice by the methods that had been worked out. [Mintz, B. 1962. Formation of Genotypically Mosaic Mouse Embryos. Amer. Zool., 2:432.; Mintz, B. 1962. Experimental Study of the Developing Mamalian Egg: Removal of the Zona Pellucida. Science, 138: 594– 595.; Mintz, B. Experimental Recombination of Cells in the Developing Mouse Egg: Normal and Lethal Mutant Genotypes. Amer. Zool., 2: 541–542.; Mintz, B. 1967. Gene Control of Mammalian Pigmentary Differentiation. I. Clonal Origin of Melanocytes. Proc. Nat. Acad. Sci. U.S., 58: 344–351.]

In the course of observing the explants, [Mintz, B. 1963. Growth In Vitro of t^{12}/t^{12} Lethal Mutant Mouse Eggs. Amer.Zool., 3:550–551.; Mintz, B. 1964. Formation of Genetically Mosaic Mouse Embryos and Early Development of "Lethal (t^{12}/t^{12})-Normal" Mosaics. J. Exp. Zool., 157: 273–292], it became evident that the two normal segregating genotypes ($+/t^{12}$ and +/+) developed to the late blastocyst stage and then hatched out of the enveloping zona pellucida in an essentially mechanical way, by repeatedly pulsating, splitting the zona during the stretched or expanded phase and then "crawling" out, leaving behind the empty, partially split zonal jacket. This form of hatching by normal mouse blastocysts had been seen in vitro by several earlier workers since at least 1935, [Lewis, W. H. and E.S. Wright 1935. On the Early Development of the Mouse Egg. Carneg. Inst. Contrib. to Embryol., 25: 115–143].

In the same explants, on the other hand, no morulae ever hatched out of their zonas. Thus, the arrested morulae (t^{12}/t^{12}) remained permanently encased in their zonas as would be expected. Despite autolysis of the embryo itself, the breakdown products failed to lyse the zona even when the cultures were kept for many months.

Referring to FIG. 1, none of these observations were surprising. It was surprising, however, that in an earlier paper describing these lethal embryos in utero in histological sections, Smith,[Smith, L.J. 1956. A Morphological and Histochemical Investigation of a Pre-implantation Lethal (t^{12}) in the House Mouse. J. Exp. Zool., 132: 51–83] reported that the lethals were at first present in their expected statistical frequency on day 3 of gestation, counting the plug date as day 0, but that they rapidly disappeared later that same day and could no longer be accounted for. No explanation of this phenomenon appeared. Thus, there was a paradox: the t^{12}/t^{12} dead morulae remained physically present in their zonas in vitro for an indefinite period but were quickly lost after their demise in vivo. Before observing the explants, this in vivo loss had seemed vaguely acceptable as a consequence in some way of the death and disintegration of the embryos. But after observing the long term persistence of dead morulae in culture, the present inventor was unwilling to accept lethality per se as an explanation of embryo disappearance in vivo. It seemed essential that there should be a more specific explanation of the reasons for this discrepancy.

As a purely hypothetical explanation that could be tested, the present inventor postulated that there might be some uterine product, possibly a proteolytic enzyme, normally present in vivo and absent in vitro that dissolved the zona pellucida in a way analogous to the experimental use of the enzyme pronase to dissolve the zona [Mintz, B. 1962. Experimental Study of the Developing Mammalian Egg: Removal of the Zona Pellucida. Science, 138: 594–595], so that the dead embryo's cells would then perhaps readily separate from each other, possibly accelerated by lysis of the intercellular matrix as well. Such a situation then would probably leave a mere scattering of small, isolated, dead cells that would be difficult to detect.

This hypothesis was tested by flushing out the embryos from the uterine horns of $+/t^{12}$ females following matings with $+/t^{12}$ males at progressively later times on day 3. Upon doing so, it became immediately evident that the hypothesis of a uterine lytic factor was correct. As FIG. 2 shows, the zonas of the lethal morulae were indeed being lysed in utero on day 3. This could not be due to the morulae themselves. The lytic mechanism, once present, is indifferent to either the stage or the viability of the embryo, and the blastocyst as well as morula zonas are visibly thinned, made stickier, and finally completely lysed at this time. However, it is the inviable morulae rather than the viable blastocysts that furnish unequivocal evidence of the lytic factor and of its extrinsic rather than embryonic origin. The reason that the loss of t^{12}/t^{12} morula zonae had escaped detection in the original histological study [Smith, L.J. 1956. A Morphological and Histochemical Investigation of a Pre-implantation Lethal (t^{12}) in the House Mouse. J. Exp. Zool., 132: 51–83.] now became clear. Acid fixation had been used, and the zona is known to dissolve at low pH [Hall, B.V. 1935. The Reactions of Rat and Mouse Eggs to Hydrogen Ions. Proc. Soc. Exp. Biol. Med., 32: 747–748.], below the pH range actually found in utero during life.

See FIG. 2. The mouse embryo therefore clearly has two distinct hatching mechanisms: an endogenous one, in which the blastocyst splits its own zona and emerges; and an exogenous one, due to the uterine lysin supplied by the embryo's normal environment. This conclusion was first stated in 1965 [Mintz, B. 1965. General Discussion. In "Ciba Foundation Symposium on Pre-implantation Stages of Pregnancy," Ed. by G. Wolstenholme and M. O'Connor, Churchill, London. pp. 167–168.].

It was considered likely that the embryo must be freed from its zona in order to make direct contact with the cells of the uterine wall and to implant. If, however, zona shedding could in fact be accomplished by the embryo in vivo as it is in vitro, by the self-hatching or endogenous mechanism, then the existence of the exogenous lytic mechanism would become in a sense superfluous for embryo hatching in vivo. Thus it appeared conceivable that the lytic mechanism might exist in utero primarily for biological purposes other than zona lysis. In order to clarify these and other questions, a series of experiments was undertaken based largely on the $+/t^{12} \times +/t^{12}$ cross described above as a simple and unambiguous in vivo bioassay for the presence and purpose of the lytic factor. If one were to examine only normal embryos, the dilemma persists that zona absence from a blastocyst can be accounted for by endogenous or exogenous mechanisms or both. If, however, genetically lethal morulae are present in the same uterus with the blastocysts, the morulae are built-in indicators of the presence of the uterine lytic factor under the same conditions as those surrounding the accompanying normal blastocysts.

In the preceding and ensuing investigations, examination of almost 10,000 embryos of this and many other strains was made with the results recorded below.

Timing of Uterine Lysin Effect on Embryo Zonas

FIG. 3 records the timing of the uterine lysin effect by plotting zona loss from only the lethal morulae on day 3 after spontaneous matings between $+/t^{12}$ individuals. Each circle represents all of the arrested lethal embryos from one female. Thus, prior to 10 A.M., no zonas whatever are lysed. At about 10 A.M., some females contain morulae with zonas that are very sticky, represented by the open circles, and there are also some females whose lethal morulae have lost their zonas entirely, in this case unquestionably as a result of the action of the lytic factor. Around 2 P.M., there is a great increase in the number of females with lysed morula zonas, and, later on that afternoon, clearly visible evidence of gradual disintegration of the denuded lethal embryos. These are found in small groups of cells and finally as single cells. Thus 10 A.M. is the beginning and 2 P.M. the peak of the lytic effect on zonas from post-fertilization eggs.

The genetic background in FIG. 3 is that of an F_1 hybrid between the balanced lethal T/t^{12} inbred strain and the BALB/c (+/+) strain; both strains are the ICR sublines at The Institute for Cancer Research in Philadelphia. The cross produces not only viable $+/t^{12}$ individuals but also T/+. In the experimental cross, between the $+/t^{12}$ heterozygotes, the embryos are of the F_2 generation, but the trait under examination, that of the uterine lysin, is in fact a maternal trait and therefore reflects the characteristics of the F_1 hybrid.

Change in Zona Pellucida at Fertilization

From the present development, it would be expected that pseudopregnant females might also show the uterine lysin, inasmuch as they resemble pregnant females in many respects, during early stages. In this case detection would be possible by a bioassay similar in principle to the one discussed above, except that the eggs would be unfertilized. If the unfertilized eggs were lacking in zonas on day 3, it could be assumed, as in the case of the t^{12}/t^{12} lethals, that zona absence could be accounted for only by the influence of a uterine product. Zona-free eggs at this time would probably be relatively difficult to find because disintegration into fragments characterizes unfertilized eggs of advancing age, and the fragments would tend to become dispersed. This may in fact account for their non-detection in the past. The same kinds of F_1 hybrid $+/t^{12}$ females were mated with sterile vasectomized males in order to obtain only unfertilized eggs, and the flushings from the uteri were very carefully examined. Intact unfertilized eggs free of zonas were found, as well as considerably fragmented portions of eggs. [Mintz, B. 1965. General Discussion. In "Ciba Foundation Symposium on Pre-implantation Stages of Pregnancy," Ed. by G. Wolstenholme and M. O'Connor, Churchill, London. pp. 167–168.]

Thus the unfertilized egg bioassay demonstrated that the lytic factor is also present on day 3 of pseu-

dopregnancy. However, this experiment also revealed that the unfertilized egg zonas were much more rapidly lysed in utero than were the zonas of fertilized eggs, that is, the t^{12}/t^{12} morulae. In the unfertilized eggs, appreciable zona lysis started almost 5 hours earlier than in the case of fertilized zonas, at 5:30 A.M., and reached a peak correspondingly earlier, at about 9:30 A.M. The obvious explanation for the fact that the unfertilized egg's zona pellucida is much more readily lysed than is the zona from a fertilized egg is that some important change has occurred in the zona itself at the time of fertilization, causing it to become more resistant to enzyme digestion. The possibility of a zona change, termed a "zona reaction" was postulated some time ago by Braden et al. [Braden, A.W.H., C.R. Austin, and H.A. David 1954. The reaction of the Zona Pellucida to Sperm Penetration. Austral. J. Biol. Sci., 7:391–409.] with reference to some kind of alteration that, in effect, helped to prevent penetration by supernumerary sperms. The zona change observed in the present study seems especially relevant for this hypothesis inasmuch as increased resistance to a lytic agent, after fertilization with the first sperm, could account for decreased effectiveness of any sperm lytic enzymes of subsequent sperms in entering the zona.

In addition to the timing difference observed in comparing unfertilized and fertilized eggs, in the two kinds of matings on this same genetic background, occasional matings between $+/t^{12}$ heterozygotes also happened to yield some unfertilized eggs in the same females that contained fertilized but lethal t^{12}/t^{12} morulae and normal (+/+ and $+/t^{12}$) blastocysts. See FIG. 4. Such cases of side-by-side comparisons in a single uterus invariably showed dissolution of the unfertilized egg zonas prior to fertilized zona lysis (for which the lethal morulae furnished the diagnostic endpoint).

These in vivo indications of a zona change at fertilization were in fact merely a substantiation of in vitro observations that had been made in tests of zona digestibility with proteolytic enzymes including pronase. In the earliest in vitro tests with this enzyme at 0.5 percent concentration and 37° C. [Mintz, B. 1962. Experimental Study of the Developing Mammalian Egg: Removal of the Zona Pellucida. Science, 138: 594–595], zona lysis started very soon and was completed in all eggs at close to 3 minutes' elapsed time. It was subsequently decided to repeat this experiment, comparing zona lysis time for unfertilized and fertilized eggs in the same dish at 37° C., with progressively more dilute solutions of pronase. At concentrations between 0.05 percent and 0.5 percent, lysis of either kind of zona was still sufficiently rapid so that there was only a slight timing difference between them. However, at 0.05 percent or below, the unfertilized eggs zonas were invariably much more rapidly digested than were the fertilized ones. At first, only zonas from unfertilized eggs were digested; digestion of the fertilized zonas then began. The time gap increased as the enzyme concentration decreased. This is illustrated in Table 1 and FIGS. 5 and 6 for pronase and several other enzymes also found to lyse the zona without injuring normal eggs.

TABLE 1

Time (in Minutes) Required for Lysis of the Zona Pellucida from Unfertilized and Fertilized Mouse Eggs, by Enzymes Compatible with Egg Viability, Showing Increased Resistance to Lysis after Fertilizaton

	Concentration (percent)				
	0.5	0.05	0.025	0.005	0.0025
Pronase:					
Unfertilized	2.5	7	10	32	173
Fertilized	3	12	20	45	218
Subtilisin:					
Unfertilized	2.5	10	19	30	90
Fertilized	4.5	14	27	50	120
Fibrinolysin:	2	1	0.2	0.1	
Unfertilized	12	16	32	60	
Fertilized	13.5	27.5	49	115	
Aspergillin-O:	1	0.5			
Unfertilized	5	14			
Fertilized	8	22			
Keratinase:	Undiluted	50	10	1	
Unfertilized	4	7	12	100	
Fertilized	8	10	18	120	

NOTE.—All enzyme dilutions were in Hanks' balanced saline without bicarbonate, at pH. 7 For each solution, mixtures of unfertilized and fertilized eggs were tested simultaneously in repeated trials at 37° C.; mean zona lysis times (in minutes) are recorded here.

SPECIFIC UTERINE ORIGIN OF THE LYTIC FACTOR

It was decided to establish whether the in vivo lysin which is found in the uterus actually is produced in the oviducts or originates in the uterus. The answer was obtained from a simple ligature experiment, [Mintz, B., 1965. General Discussion. In "Ciba Foundation Symposium on Pre-implantation Stages of Pregnancy," Ed. by G. Wolstenholme and M. O'Connor, Churchill, London. pp. 167–168] in which the ligature was placed between the oviduct and the corresponding uterine horn on day 2 while the eggs were in transit between oviduct and uterus, in a mating of $+/t^{12}$ heterozygotes. As a result of the closure, some embryos are trapped in the oviduct above the ligature while others have already passed below it. Even if the examination of the embryos on the two sides of the barrier was deferred until day 4, there was still no lysis of the dead morula zonas in the oviduct above the ligature, whereas lysis had already occurred earlier, on day 3, below the ligature. In addition it was now evident that the viable blastocysts that had remained in the oviduct underwent self-hatching mechanically, [Mintz, B. 1965. General Discussion. In "Ciba Foundation Symposium on Pre-implantation Stages of Pregnancy," Ed. by G. Wolstenholme and M. O'Connor, Churchill, London. pp. 167–168.], as seen in figure 7, and their empty zonas remained present. The experiment therefore simultaneously demonstrates that the lytic factor is found in the uterus and not in the oviduct and also that blastocysts are capable of self-hatching in vivo, presumably by means of the same pulsating behavior that they exhibit in vitro. As will be shown presently, they are also capable of this form of hatching in the uterus as well as in the oviduct. Hatching by blastocysts retained in the oviducts by means of a ligature was also confirmed in an all-normal strain [Orsini, M.W., and A. McLaren 1967. Loss of the Zona Pellucida in Mice, and the Effect of Tubal Ligation and Ovariectomy. J. Reprod. Fertil., 13: 485–499].

SELF-HATCHING OF BLASTOCYSTS IN UTERO

If the uterine contents are examined relatively early on day 3, in matings of $+/t^{12}$ heterozygotes, some zona-free blastocysts are found, despite the fact that the accompanying lethal morulae are not yet deprived of their zonas. Such hatched blastocysts may even be found in small numbers before the occasionally present unfertilized eggs are zona-free as in FIG. 8. Here the egg at the bottom of the photograph is an unfertilized egg rather than a lethal morula as evidenced by fragmentation at a time prior to disintegration of lethal morulae. Among the three blastocysts shown, one is still encased in its zona, one has split its zona but has not yet emerged, and the third has left the empty zona behind. These data demonstrate occurrence of some blastocyst hatching independently of uterine lysin. Thus, blastocysts are perfectly capable of self-hatching in utero in normal pregnancies as well as in the oviduct under experimental conditions, or in vitro.

This self-hatching of blastocysts in utero is also observed in pregnancies in which implantation is delayed, either in postpartum lactational delay of adults or in prepuberal hormone-primed females. Others have similarly noted absence of zonas in mouse embryos undergoing postpartum delay. [McLaren, A. 1967. Delayed Loss of the Zona Pellucida from Blastocysts of Suckling Mice. J. Reprod. Fertil., 14:159–162.]

From another experiment, there is evidence that blastocysts in utero emerge from the zona by self-hatching if they are placed in a uterine environment that is physiologically less advanced than is characteristic for that embryo stage. Unhatched blastocysts were obtained from donor females early on day 3 and were surgically transferred to day 2 recipients that had previously been mated to sterile vasectomized males. Subsequent examination later that day or early the next day showed that the blastocysts actually emerged from their split (unlysed) zonas. At the same time, the intact zonas around the host's own unfertilized eggs were also evidence that uterine lysin was absent in the host, or else was present in insufficient amount even to lyse unfertilized zonas.

The extent to which blastocysts in this particular F_1 hybrid ordinarily avail themselves of this endogenous form of hatching in normal pregnancies, as opposed to hatching by zona lysis effected by the uterine factor, can now be readily inferred by comparing the timetable for zona loss from blastocysts with the timetable of the uterine lytic factor. The latter is measured by the lethal morula bioassay of zona loss. As already shown in FIG. 3, the bioassay for the uterine lytic factor indicates that lysis of fertilized egg zonas starts at 10 A.M. on day 3 and reaches a peak at 2 P.M. the same afternoon. In FIG. 9, the frequency of blastocysts without zonas is plotted from the same group of females. Again, each circle represents the embryos from a single female. The distribution clearly indicates that the loss of blastocyst zonas rises more rapidly than does the loss of morula zonas in the same females. Therefore, it can be concluded that in a normal pregnancy, blastocysts in this F_1 hybrid start to emerge by their own endogenous mechanisms first, and receive only partial assistance, that is, some softening up of their zonas, from the exogenous uterine factor, starting at approximately 10 A.M. Thus there is a very sudden peak of zona-free blastocysts evident at 2 P.M. when the lytic factor reaches its peak effect. After that time, all or almost all blastocysts in almost all these females are zona-free.

Function of Uterine Lysin as an Implantation Initiating Factor (IIF)

During the handling of these embryos, it began to become apparent that the population of free-living or unattached blastocysts was markedly declining co-incident with the peak lytic time of the uterine factor. Not only did unattached blastocysts seem much rarer than before, but more forceful flushing of uteri revealed that some of the ostensibly missing ones were in fact already superficially attached to the uterine wall and could only be dislodged with a strong stream of fluid. Therefore, the numbers of unimplanted embryos, including blastocysts as well as morulae, were tabulated. The results are indicated in FIG. 10.

In this figure, the important fact emerges that although there are fair numbers of hatched blastocysts before 2 P.M., as seen previously in FIG. 9, that is, before the uterine lysin peak, blastocyst attachment to the uterine wall is not initiated until the peak lysin time is reached. Before that time, the average number of embryos, 12 per mating for this F_1 hybrid mouse, is maintained. But after the uterine factor reaches its peak, there is an impressively abrupt drop in free-living embryos, even before the dead morulae have disintegrated. The crosses in FIG. 10 show females with unimplanted embryos in only one uterine horn, so that there are to some degree local uterine bilateral differences in lysin production or in uterine response to a systemic control. Thus there is a notable simultaneity of events: when the uterine factor reaches its peak biological effect on the zona, there is a virtually immediate rush of blastocyst attachment.

This concurrence of events would scarely seem to be an unrelated coincidence and, as will be shown shortly, a cause-and-effect relationship between them can be experimentally shown. On the total evidence to date, it therefore appears that the uterine factor is in some critical way actually responsible for initiating implantation and that (whatever other effects it may have), this is perhaps its chief biological role. For this reason it is designated as an implantation initiating factor or IIF.

Deficiency of IIF as the Immediate Cause of Delayed Implantation in Postpartum and Prepuberal Pregnancies

If the preceding interpretation of the role of this factor in implantation is correct, it should follow that lack or deficiency of the IIF should lead to failure of implantation. This failure may be temporary or merely delayed, if IIF insufficiency is corrected.

It is well known that there are at least two forms of delayed implantation in the mouse: postpartum or lactational delay, of variable length, when embryos from a postpartum mating are present while a female is nursing the previous litter; and delay in prepuberal mice, which may become a failure of implantation in the absence of hormone therapy. By the experimental use of heterozygous matings of $+/t^{12} \times +/t^{12}$, it has become clear that both the postpartum and the prepuberal forms of delayed implantation are indeed characterized by insufficiency of the implantation initiating factor, and that when the delay is spontaneously terminated and implantation is initiated, as in the postpartum case, this initiation of implantation is accompanied by increased production or effectiveness of the uterine lytic factor.

The first point, namely deficiency of IIF during delay, is shown in FIG. 11. These eggs were obtained from a prepuberal $+/t^{12}$ female after hormone-induced

ovulation with pregnant mare serum (PMS) and human chorionic gonadotropin (HCG), and mating with a $+/t^{12}$ male. The uterine complement of eggs is shown on day 4. This and some other females fortunately had not only normal blastocysts and lethal morulae, but also some unfertilized eggs, thereby providing a double bioassay of lytic factor, for levels adequate to lyse pre- or postfertilization zonas. As can be seen, both the unfertilized eggs and the disintegrating lethal morulae have remained in their zonas, indicating that even the low-level amount of the uterine factor needed to lyse the unfertilized zonas is not present. At the same time, the viable blastocysts have hatched. Thus, although it is a whole day later than the normal peak IIF time in adult pregnancies, in this pregnancy there is no effect of IIF apparent, as shown by the bioassays. The blastocysts have hatched out entirely by their own efforts as already seen earlier in certain adult cases, and their empty zonas can be recovered.

It has been found that spontaneous implantation of viable embryos does in fact occur in some prepuberal females after PMS and HCG treatment, and that such very young animals are then able to bear and care for litters. Experimentation with hormone-treated, mated prepuberal females at random has therefore turned up individuals in which implantation was successfully beginning. Unlike the prepuberal females described above with delayed implantation, these were all characterized by the presence of IIF, as seen by the disappearance of zonas from their t^{12}/t^{12} lethal morulae.

Comparable observations were made in postpartum delays of adult females from $+/t^{12}$ heterozygous matings. During delayed implantation, lethal morula zonas were intact, indicating that IIF was deficient, and blastocysts hatched mechanically. At termination of the delay, zonas were lysed on dead morulae when implantation of the blastocysts began.

Hormone Dependence of the IIF

The preceding observations suggested that IIF might be under hormonal control, possibly directly by ovarian hormones, chiefly estrogen, and indirectly by pituitary hormones. Attempts have been made to analyze hormonal control of IIF in a number of different ways. One approach is to terminate delayed implantation with specific hormone treatment. Another is to introduce hormone treatment at a time in pregnancy earlier than the usual time of the IIF peak of day 3. Ovariectomy can also be combined with such hormone replacements. In FIG. 12, precocious zona lysis is seen induced on day 2 of gestation in an adult $+/t^{12}$ female after mating with a $+/t^{12}$ male. This female had been treated with a small estrogen supplement to progesterone, the latter alone being ineffective. Although spontaneous zona lysis on morulae is not ordinarily seen on day 2, the results are clear, and the zonas are fast disappearing.

Both IIF and Embryo Maturation are Indispensable for Implantation

In experiments in which the lethal genotype was not involved, viable morulae were sometimes found to be denuded by the IIF. In some inbred strains, for example, the BALB/*c* strain, there is a somewhat different timing of the IIF, such that some perfectly normal and viable (+/+) morulae are precociously made zona-free. In these cases the morulae do not implant at that particular stage, but seem first to continue their development to blastocyst. Therefore, although the uterine factor is needed to initiate implantation, it appears that the factor must do so by acting on an embryo that has reached the appropriate blastocyst stage.

It is believed that IIF may either enzymatically change or open some molecules on the outer surface of blastocyst cells and expose active groups on these molecules, in effect making them "stickier" or more reactive, or else act as a linking molecule, leading to adhesion between the blastocyst surface and the surfaces of cells lining the uterus. Since many changes are undoubtedly occurring on cell surfaces between the morula and blastocyst stages, it is not surprising to find an embryo-stage requirement for successful IIF-mediated attachment to the uterine wall.

Zonalytic Enzymes

Preliminary tests of zona softening in vitro by heated, as compared with untreated, crude uterine extracts were made. The results, indicating loss of lytic action with heating, are consistent with the interpretation that the IIF is an enzyme or enzymic complex.

As a first approach to learning more about the properties of the uterine lysin, it is important to know whether there are other enzymes, in addition to pronase (obtained from Calbiochem), [Mintz, B. 1962. Experimental Study of the Developing Mammalian Egg: Removal of the Zona Pellucida. Science, 138:594–595] that might also be capable of lysing the zona pellucida without injuring the embryo. Enzymes like trypsin, that lyse the zona but are injurious to the embryo, are obviously not particularly relevant. In tests of a small number of available proteolytic enzymes, several others were found which, like pronase, are zona lysins and are compatible with embryo viability. They are: subtilisin (available from Sigma); fibrinolysin (from several sources, including General Biochemicals); aspergillin-O, also designated a "fibrinolytic agent" (from Connaught Medical Research Labs., made available through the courtesy of Dr. R.C. Parker), and keratinase (an impure preparation, kindly supplied by Dr. E. Nickerson). The data of Table 1 indicate the concentrations required for fairly rapid lysis of mouse zonas. They also substantiate the fact already stated for pronase that the zona becomes more resistant to enzymatic digestion at the time of fertilization.

A simple extrapolation concerning the concentration of the uterine lysin can be attempted from these data. Since there is a lag of approximately 5 hours in utero between complete digestion of unfertilized and of fertilized zonas, the uterine lysin may be present in rather low concentration equivalent to even greater dilutions than any tested for the enzymes in Table 1. However, precise quantitative comparisons cannot be drawn because the uterine product may be secreted in changing amounts and may differ in its stability at 37° C. from the stabilities of the other enzymes.

Most of these commercial enzymes are microbial products, but fibrinolysins are widespread enzymes also found in animal tissues. Of special interest is the fact that fibrinolysin is known to be present in uterine tissue and fluids and that inhibitors of this enzyme have also been found in rabbit uterine fluid [Beier, H. M. 1970. Hormonal Stimulation of Protease Inhibitor Activity in Endometrial Secretion During Early Pregnancy. Acta Endocr., 63: 141–149.]

Fractionation of Proteins from Mouse Uterine Fluid

The task of identifying and isolating IIF has been undertaken. Collection, fractionation, and analysis of uterine proteins has been reported in several mammals. [Beier, H.M. 1968. Biochemish-entwicklungsphsiologische Untersuchungen am Proteinmilieu fur di Blastozystenentwicklung des Kaninchens (Oryctolagus cuniculus). Zool. Jahrb. Anat., 85: 72–190.; Daniel, J.C., Jr. and R.S. Krishnan 1969. Studies on the Relationship between Uterine Fluid Components and the Diapausing State of Blastocysts from Mammals Having Delayed Implantation. J. Exp. Zool. 172: 267–281.], but not previously in the mouse, which poses serious difficulties because of its small size.

FIG. 13 shows a series of small polyacrylamide gel columns with stained bands of proteins separated by electrophoresis of mouse uterine fluid at pH 8.3 compared with mouse serum. The uterine contents were obtained by flushing through with a small volume of 0.15M NaCl from the anterior end of each excised uterine horn after removal of the oviduct while carefully avoiding dislodging the cells from the cut ends. This yielded a protein profile also seen when the oviduct was left intact. Comparison of the uterine proteins (tubes 2–4) with those of serum (tube 1) under these conditions reveals a possible maximum of ten bands present in uterine fluid but absent in serum. These (starting at the moving front at the bottom of the tube) are: one fairly strong band at the moving front; three faint prealbumin bands; two bands with a different migratory distance between them, as compared with the corresponding pair in serum, so that one or two of the pair of uterine bands may differ from those in serum; another pair of uterine bands which also have a different migratory position from the corresponding ones in serum; and, finally, one or two bands near the origin at a position that shows nothing in serum. The postalbumin band seen in uterine fluid is also present in serum, but is only readily visible as distinct from the albumin band in the latter when the concentration of total serum protein is decreased. All these unique components found in utero and not found in serum are of potential interest. The presence of some shared components in both uterine and blood fluids but in different quantities, for example, albumin, is also noteworthy. Possibly some serum contamination from uterine blood vessels is partly involved.

It might be expected that uterine proteins either found only on day 3 or found in changing amounts might be the best candidates for further examination. A conspicuous component is present in uterine fluid at the various times of the estrous cycle of non-pregnant animals and also on progressive stages of early pregnancy, at least through the preimplantation period. (FIG. 13, tubes 2–3). However, it is present as changing amounts of the total protein, being most conspicuous at estrus and declining by day 2 of pregnancy. In addition, a faint band seems to accompany it on day 3 of pregnancy. Several hypotheses present themselves as to ways in which the proteins of this region might be implicated in zona lysis and implantation initiation.

One possibility is that the component that diminishes during gestation might be an inhibitor of the IIF, rather than the IIF itself, and thus be analogous to trypsin inhibitors or fibrinolysin inhibitors, [Beier, H. M. 1970. Hormonal Stimulation of Protease Inhibitor Activity in Endometrial Secretion During Early Pregnancy. Acta Endocr., 63: 141–149.] so that its decline on day 3 would permit the IIF to produce its effects.

Another hypothesis is that this same protein may in fact be the IIF (or a major component of it). Its peak at estrus, when eggs are not present in utero, might be associated with quite other biological activities at that time. Gradual entry of eggs into the uterus starts around midday on day 2 of gestation after the sample in FIG. 13 was taken and therefore after decline in the uterine product is well underway. Perhaps further decline is briefly and temporarily reversed by an estrogen surge [Shelesnyak, M.C. 1960. Nidation of the Fertilized Ovum. Endeavour, 74: 81–86.] for a limited period on day 3, permitting a minimal critical level of the IIF to be restored.

Still another view is that the IIF may be identified with the faint, possibly new band on day 3, appearing close to the one that has been declining in amount since estrus.

It is possible that the IIF is not unique to uterine fluid. Ectopic implantation in the organism may involve similar substances produced by other tissues. Or the IIF may also occur in serum with different activator or inhibitor arrangements.

Twinning or Death As Possible Results of Premature Embryo Exposure to IIF

It seems reasonably clear that IIF is a normal system and that it plays a key role in the economy of implantation. Moreover, since it is under hormonal control, it is a mechanism by which some significant measure of control of implantation itself can be accomplished. These important applications, that is, either triggering or preventing implantation experimentally, must of course be examined more carefully in order to learn whether manipulations of the system may have adverse effects.

Chang, [Chang, M.C. 1950. Development and Fate of Transferred Rabbit Ova or Blastocysts in Relation to the Ovulation Time of Recipients. J. Exp. Zool., 114: 197–226.] pioneered in the recognition of the importance of developmental synchrony between mammalian embryo and uterine stages. This general concept led to further experiments on whether any developmental anomalies might be produced in the embryo if the IIF were caused to be present in critical amounts unusually early in gestation or if embryos of unusually young (pre-blastocyst stages became exposed to the IIF.

In screening the various standard strains to verify whether the IIF was present in all, this has been found to be a universal mechanism in all strains examined (BALB/*c*, C57BL/6, C3H, C3Hf, AKR, DBA/2, A/He, ICR and others). This conclusion was reached in two ways for each mouse strain: by noting zona lysis in unfertilized eggs flushed out of pseudopregnant females at progressive times on day 3; and by noting time of zona loss in relation to inception of implantation in embryos from pregnant females at progressive times on day 3. These studies disclosed that although the factor was present and was temporally related to the beginning of implantation in all strains, the timing of peak effect of the factor was specific for each strain.

Quite fortuitously, one of the normal strains that was investigated—the BALB/*c* strain—proved to be charac-

terized by a relatively exceptional degree of spontaneous malsynchrony between embryo stage and IIF timing as noted above. In this strain, a substantial percent of the embryos are still in the morula stage with no visible cavity when the IIF effect starts to increase on day 3, as shown in FIGS. 14 and 15. In this case, the zona-free morulae were the indicators of the uterine lytic factor since morulae are incapable of shedding their own zonas, but, unlike the t^{12}/t^{12} genotype, the morulae are viable and no lethal genes are involved here. Among 232 total BALB/*c* embryos, there were 50 (22 percent) that were still in the morula stage when the zona pellucida was lysed by the uterine factor.

The same study also revealed at least one possible spontaneous consequence of this precocious embryo exposure to IIF: five of the zona-free morulae were half-size as seen in FIG. 15. In addition, there was one miniature, half-size blastocyst. Thus, it is apparent that embryo twinning is a possible result of exposure of morulae to IIF, and that such twins may be viable. The twinning can have come about after zona loss because cells are less tightly bound together in pre-blastocyst than in blastocyst stages.

Still younger stage embryos have their cells even more easily separable (as manipulation in vitro readily discloses), and cleavage-stage embryos would therefore be expected to dissociate still further into single blastomeres under IIF influence. Embryos in their zonas were therefore removed at 2-cell stages from day 1 pregnant females and were surgically transferred to uteri of day 3 females. After periods of up to 7 hours, the uterine contents of the recipients were flushed out and examined. As FIG. 16 strikingly shows, the donor embryos, which were identified by their cell sizes, had indeed become dissociated into single blastomeres. In some instances, cleavage had even continued during this time, as seen by reduction in cell size and increase in cell number. Such twins may not be viable.

These phenomena of twinning, cell dissociation, and embryo death can be brought about experimentally by treatments, for example with hormones, that would cause accelerated passage of eggs from oviduct to uterus, and also precocious production of IIF. Indeed, it seems possible that the embryocidal effects of some known antifertility agents (with still unknown mode of action) may be due to these or similar effects ascribable to the IIF.

On the other hand, day 3 blastocysts transferred to uteri of day 2 recipients hatch out of their own zonas mechanically, leaving the empty jackets otherwise intact. But, as the preceding facts indicate, the hatched blastocysts do not implant until day 3. This result, as compared with the dissociation of young embryos in a day 3 host, is also evidence for the general desirability of transferring any experimental morulae to a day 2 rather than a day 3 host.

Sex Chromosomal or Other Genetic Mosaicism as Possible Results of Premature Embryo Exposure to IIF

Another anomaly which is to be anticipated if pre-blastocyst-stage embryos of viable genotypes become precociously zona-free is the permanent adhesion between two such embryos. Denuded blastocysts adhere far less successfully than do earlier stages, [Mintz, B. 1964. Formation of Genetically Mosaic Mouse Embryos, and Early Development of "Lethal (t^{12}/t^{12})-Normal" Mosaics. J. Exp. Zool., 157:273–292.] presumably because tight junctions in the former limit their cell movements and keep the two embryo spheres from uniting into one. The formation of a single embryo by aggregation of two entire denuded embryos kept in contact at 37° C. has already been experimentally demonstrated in vitro [Mintz, B. 1962. Formation of Genotypically Mosaic Mouse Embryos. Amer. Zool., 2:432.; Mintz, B. 1964. Formation of Genetically Mosaic Mouse Embryos, and Early Development of "Lethal (t^{12}/t^{12})-Normal" Mosaics. J. Exp. Zool., 157:273–292.] It is known to lead to size regulation after transfer of the embryo in utero, and to result frequently in the birth of viable allophenic mice [Mintz, B. 1965. Experimental Genetic Mosaicism in the Mouse. In "Ciba Foundation Symposium on Preimplantation Stages of Pregnancy," Ed. by G. Wolstenholme and M. O'Connor, Churchill, London. pp. 194–207.] Thus, in spontaneous embryo adhesion *in vivo*, the IIF would substitute for any of the available *in vitro* zonalytic enzymes (pronase, fibrinolysin, etc.) and the normal body temperature of 37° C. takes the place of the artificially elevated temperature in explants of conjoined cleavage-stage embryos.

It is now evident that the in vitro experiments leading to production of allophenic mice have their perfect counterpart in vivo. For those mouse strains, such as BALB/*c*, in which a known percent of embryos are still morulae when zona lysis by IIF occurs, there is a statistically predictable risk that two or more of these zona-free morulae will stick together in a group which will eventually form a single mouse. There is reason to think that a system similar to the IIF may exist in the human species and that adhesion of two co-existing embryos precociously deprived of their zonas is sometimes encountered here.

In cases where this occurs between embryos of different genotypes, in any species, various cellular markers could disclose the presence of genetic mosaicism in ways comparable to those already demonstrated in allophenic mice. [Mintz, B. 1967. Gene Control of Mammalian Pigmentary Differentiation. I. Clonal Origin of Melanocytes. Proc. Nat. Acad. Sci. U.S., 58:344–351.] In the human population, it is quite possible that some clinical cases of genetic mosaicism [Gartler, S.M., S.H. Waxman, and E. Giblett 1962. An XX/XY Human Hermaphrodite Resulting from Double Fertilization. Proc. Nat. Acad. Sci. U.S., 48:332–335; Zuelzer, W. W., K.M. Beattie, and L. E. Reisman 1964. Generalized Unbalanced Mosaicism Attributable to Dispermy and Probable Fertilization of a Polar Body. Am. J. Hum. Genet., 16:38–51.] may have had their origin by this route rather than by some others that have been suggested. The possibility of early embryo fusion in man has often been erroneously discounted, on the assumption that a mechanism to account for zona removal did not exist.

If embryos of the same genetic strain become joined, the resultant individual would show no cellular differences unless the two cell strains were of opposite chromosomal sex. Such XX⟷XY mosaics with both female and male cells have also been described in allophenic mice [Mintz, B. 1968. Hermaphroditism, Sex Chromosomal Mosaicism, and Germ Cell Selection in Allophenic Mice. J. Animal Sci., 27 (Suppl. I): 51–60.;

Mintz, B. 1969. Developmental Mechanisms Found in Allophenic Mice with Sex Chromosomal and Pigmentary Mosaicism. Birth Defects: Original Article Series, 5: 11–22.] Contrary to some stated preconceived views about the possible sex phenotypes of such animals, XX↔XY allophenic mice have been found to have virtually any gross sex phenotype. Sex chromosomal mosaics are in fact males, or females, or intersexes. Intersexuality is actually very rare (1 percent of all), and sterility among the males and females is also very rare. In the fertile individuals, the gametes are only from the cellular sex that corresponds to the gross sex phenotype. The spontaneous intersexes found by Hollander et al. [Hollander, W.F., J.W. Gowen, and J. Stadler 1956. A Study of 25 Gynandromorphic Mice of the Bagg Albino Strain. Anat. Rec., 124:223–224.] in his subline of BALB/*c* mice would appear, in the light of the observations on our own BALB/*c* subline, to have had a good probability of origin by embryo adhesion between denuded morulae. Presumably some human XX⟷XY mosaics [Gartler, S. M., S.H. Waxman, and E. Giblett 1962. An XX/XY Human Hermaphrodite Resulting from Double Fertilization. Proc. Nat. Acad. Sci. U.S., 48: 332–335.; Zuelzer, W.W., K.M. Beattie, and L.E. Reisman 1964. Generalized Unbalanced Mosaicism Attributable to Dispermy and Probable Fertilization of a Polar Body. Am. J. Hum. Genet., 16: 38–51.] may also have arisen in this way.

Bioassay of Fertility and Contraceptive Drugs

The invention can be used for bioassay of fertility or contraceptive drugs, and for the screening of drugs to see whether they possess any fertility-promoting or contraceptive activity. It can also be used to determine the mechanism by which fertility and contraceptive drugs act in the body of veterinary animals and humans.

There are several different procedures which may be used for bioassay according to the present invention.

a. t^{12}/t^{12}Lethals

In using this method of assay, test mice which are heterozygous $+/t^{12}$ females and males are mated, and the test drug is introduced into the females before or shortly after the time of mating. In terms of the mouse, shortly after should be within at least 2¼ days of the time of mating. The test drug, if it is to be used in a certain way, as for example orally in the human, can by preference be introduced in the same way in the body of the mouse. However, it is sufficient to introduce the test drug in any effective way, such as intraperitoneally, intravenously, subcutaneously, intramuscularly, orally, intravaginally, or directly in the uterus.

The female animals are sacrificed at various times within a few days of mating, and the contents of the oviducts and of the uterine horns are flushed out separately for a census and examination of the embryos. All embryos are examined to see whether they have been transported from the oviducts into the uterus precociously. Any embryos in pre-morula stages and the t^{12}/t^{12} homozygous lethal genotype embryos in the morula stage are examined to determine whether the zona pellucida has been lysed, and also whether the cells have been dissociated. The normal blastocysts (+/+ and $+/t^{12}$ genetypes) are counted and examined to determine whether attachment to the uterine wall has been initiated. Untreated controls of the same genetic matings are used.

b. BALB/*c* Inbred Strain

Using this method of bioassay, female and male test animals of the BALB/*c* inbred mouse strain are mated, and the test drug is introduced into the females by any effective route as described above before or shortly after mating. The females are sacrified at various times within a few days of mating, and the contents of the oviducts and of the uterine horns are flushed out separately for a census and examination of the embryos. All embryos are examined to see whether they have been transported into the uterus precociously. Those embryos which are still in cleavage or morula stages despite their genetic normalcy (+/+) genotypes) are examined to determine whether the zona pellucida has been lysed and also whether the cells have been dissociated. The normal blastocysts are counted and examined to see whether attachment to the uterine wall has been initiated. Untreated pregnant controls of the same strain are used.

c. Pseudopregnant Females

The method of bioassay in this aspect of the invention involves mating female mice of any strain or genotype with sterile vasectomized male mice of any strain or genotype, introducing the test drug into the females by any effective route as described above before or shortly after mating, sacrificing the female animals at various times within a few days of mating, and flushing out the contents of the oviducts and of the uterine horns separately for examination of the unfertilized eggs. The eggs are examined to see whether they have been transported into the uterus precociously and to see whether the zona pellucida has been lysed. Untreated pseudopregnant controls of the same strain are used.

d. General Observations Regarding Bioassay

The dosages which are administered of an unknown drug should cover a wide range, up to the highest practical dosage for administration until more data as to the effectiveness of the drug are obtained.

In place of the use of t^{12}/t^{12} mouse embryos in the bioassay involving lysis of zonas on dead embryos, another test genotype can be substituted if it is first discovered to be lethal at or prior to the early blastocyst stage. In place of the use of BALB/*c* embryos in the bioassay involving lysis of zonas on viable morulae, another strain can be substituted if it is first found to have some embryos still in early blastocyst stages or younger when the IIF lyses the zonas.

While specific illustrations have been given applied to the mouse, it will be understood that the invention can be applied to other test animals, with knowledge of what their normal characteristics are. Of course, controls should first be run unless the normal characteristics of the species and strain of animals are well known in advance.

In view of my invention and disclosure, variations and modifications to meet individual whim or particular need will doubtless become evident to others skilled in the art, to obtain all or part of the benefits of my invention without copying the process shown, and I therefore claim all such insofar as they fall within the reasonable spirit and scope of my claims.

Having thus described my invention, what I claim as new and desire to secure by Letters Patent is:

1. A method of bioassay which comprises mating female and male test mice which are heterozygous

$+/t^{12}$, introducing a test drug into the females within a time which begins before mating and ends within a few days after mating, sacrificing the female animals at various times within a few days of mating, flushing out the contents of the oviducts and of the uterine horns separately, examining all embryos to see whether they have been transported into the uterus precociously, examining any pre-morula stage embryos and t^{12}/t^{12} homozygous lethal morulae to determine whether the zona pellucida has been lysed and whether the cells have been dissociated, and counting and examining the normal blastocysts (+/+ and $+/t^{12}$ genotypes) to see whether attachment to the uterine wall has been initiated.

2. A method of bioassay which comprises mating female and male test mice of the BALB/*c* inbred strain, introducing a test drug into the females within a time which begins before mating and ends within a few days after mating, sacrificing the female mice at various times within a few days of mating, flushing out the contents of the oviducts and of the uterine horns separately, examining all embryos to see whether they have been transported into the uterus precociously, examining those embryos still in cleavage or in morula stages despite their genetic normalcy (+/+ genotypes) to determine whether the zona pellucida has been lysed and also whether the cells have been dissociated, and counting and examining the normal blastocysts to see whether attachment to the uterine wall has been initiated.

3. A method of bioassay which comprises mating females of any mouse strain or genotype with sterile vasectomized male mice of any strain or genotype, introducing a test drug into the females, within a time beginning before mating and ending within a few days after mating, sacrificing the female animals at various times within a few days of mating, flushing out the contents of the oviducts and of the uterine horns separately, and examining the eggs to see whether they have been transported into the uterus precociously and to see whether the zona pellucida has been lysed.

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