(*11*). Other experiments showed that labeled antibody can also be used to detect vaccinia antigens within the cytoplasm of infected cells if the cells are fixed with acetone before they are incubated with [^{125}I]IgG against vaccinia (*11*).

The labeled antibody technique can also be used to quantitate the amount of antibody to viral antigens in a particular serum. This can be done either by labeling the serum immunoglobulin with ^{125}I and measuring radioactivity bound to infected cells (Fig. 2A) or by quantitating the amount of unlabeled serum required to block the attachment of a standard amount of labeled IgG (Fig. 2B). The advantage of the latter approach is that a single preparation of labeled IgG can be used to measure the concentration of specific antibody in an unknown serum. An even simpler approach might be the use of isotopically labeled antibody to immunoglobulins (anti-immunoglobulin) (*12*). We showed that the binding of labeled anti-immunoglobulin to vaccinia or HSV infected cells was directly related to the concentration of virus-specific antibody that had attached to the cells (*13*). By this procedure a single reagent (for example, labeled goat antibody to human immunoglobulins) might be used to measure the binding of virus-specific antibody to cells infected with different viruses.

The binding of labeled antibody to infected cells appears to have certain advantages over several of the widely used diagnostic procedures for detecting viral antigens and antibody to virus. For example, the binding of labeled antibody would not be affected by factors that inhibit complement fixation (anticomplimentary factors). Moreover, the radiolabeled antibody technique could be used to detect virus-specific antibody that does not fix complement. In comparison to immunofluorescence, the radiolabeled antibody technique is objective, quantitative, and unaffected by autofluorescence. Moreover, samples can be counted rapidly in an automated gamma counter. The attachment of isotopically labeled antibody to virus-infected cells also has potential advantages over immunoprecipitation procedures in which labeled viral antigens are used. These antigens are difficult to prepare and purify, but antibody to virus is plentiful and easy to label. In addition, labeled immunoglobulins that do not bind to infected cells can be rapidly separated from cell-bound antiviral antibody by simply washing the monolayer. By adapting the isotopically labeled antibody technique to microplates and by using fixed cells which are stable when stored, it should be possible to develop simple, rapid, and sensitive techniques for screening and quantitating large numbers of serums for antibody against different viruses.

KOZABURO HAYASHI
JOEL ROSENTHAL
ABNER LOUIS NOTKINS
Virology Section, Laboratory of Microbiology and Immunology, National Institute of Dental Research, Bethesda, Maryland 20014

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10. From the specific activity of the [^{125}I]IgG and from the number of counts bound to the monolayers (10^6 cells), it is possible to estimate the number of antibody molecules attached to each cell. In initial experiments, more than $7.5 \times 10^{5.0}$ molecules of IgG attached to cells infected for 12 hours. From this type of information an estimate can be made of the number of virus-induced antigenic sites on the surface of each cell.
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Delayed Fertilization and Chromosome Anomalies in the Hamster Embryo

Abstract. *Polyploidy and aneuploidy in hamster embryos are produced as manifestations of pregnancy wastage in surviving young when the interval between the estimated time of ovulation and copulation and hence fertilization is progressively increased. Significant delays resulted in triploidy in runted embryos, and the sporadic occurrence of mosaic aneuploidies.*

We report here the experimental production of polyploidy and aneuploidy in hamster embryos by lengthening progressively the intervals between copulation and the estimated time of ovulation, hence fertilization. At 9 hours after ovulation, sexual activity diminished and if mating did occur, it did not result in fertilization. Even as short a delay as 3 hours after the estimated time of ovulation resulted in a measurable increase of pregnancy wastage and, more importantly, in demonstrable polyploid and aneuploid chromosome patterns of developing embryos and fetuses.

Others have reviewed background work on the impact of delayed fertilization on the subsequent development of eggs in amphibians and mammals (*1–3*). Important contributions include the demonstrations of triploid embryos stemming from aging ova from dispermy (diandry) in rats and from suppression of second polar body sequences (digyny) in mice and rabbits. Not so well documented or confirmed are the occasional observations of monosomy, trisomy, and other aneuploidies (*1–3*) that develop under similar conditions and are suggestive of a cause and effect relation.

Large elements of uncertainty exist about the origin of these less frequently observed anomalies because of the number of critical variables involved, including species variation, circadian rhythms, ovulation and aging of ova, mating behavior, fertilization times, differences in preimplantation development, and chromosomal patterns. Such variables make confirmation or even comparison of rare observations difficult and inconclusive.

Because of its many advantages (*4, 5*) we selected the golden hamster for our experiments. The aim was to examine, under as optimal circumstances as possible, the mutagenic impact of delayed fertilization on the chromosome complement of the preimplantation embryo and the implanted fetus.

The 76 females of the test group were exposed, when in estrus, to healthy males for 15 minutes at intervals of 0, 3, 6, and 9 hours after the estimated time of ovulation (2 p.m., daylight time). Estrous rhythms and ovulation pattern had been reversed by substituting darkness for daylight from 6 a.m. to 6 p.m. and electric lighting for darkness from 6 p.m. to 6 a.m. (*5, 6*).

The 47 females comprising the control group were mated when in estrus, 5 hours before the estimated time of ovulation, on the assumption that 4 to 6 hours would elapse before motile sperm reached, capacitated, and fertilized a batch of ovulated eggs (*6*). Pregnant animals were killed 3 days later and the embryos were harvested; fetuses were examined at the organogenetic stage of development 9 days after copulation.

We analyzed the chromosome constitution of progeny at 3 days and also at 9 days, after copulation. The preparation of embryos and fetuses for direct examination of the chromosomes has been described (*4, 5*). In the 3-day-old embryos, all available metaphase plates were examined and chromosomes counted if they were satisfactorily spread. At least ten metaphase plates from 9-day-old fetuses were counted in order to establish both the chromosome number and sex of the fetus.

Increasing the interval between ovulation and copulation resulted in increased pregnancy wastage (Table 1). Delays in copulation of 3 to 6 hours resulted in decreased yields from completed matings and correspondingly increased mortality rates of uncleaved ova and absorbed fetuses. In addition, sexual behavior was altered. After 3-hour delays, females began to show less interest in the males; after a 9-hour delay, the typical lordotic reactions and sticky estrous discharge had generally disappeared. Also, after a 9-hour delay, we witnessed only three matings from which there was no reproductive yield.

As judged by the presence of sperm tails in uncleaved ova, penetration but lack of fertilization must be considered to be as much of a factor in causing reduced reproductive yields as is embryonic death. Unfertilized ova accounted for 84 percent of uncleaved eggs in the control group and for 71, 72, and 75 percent of the uncleaved ova in the three test groups. The high prevalence of chromosomal aberrations in metaphase plates of surviving embryos (Table 2) was a finding of greater biologic significance. Such changes were already evident at 3 days after copulation when, of a total of 98 developing embryos, 15 (15.3 percent) from 29 mothers showed anomalous chromosome constitutions (Table 2, Fig. 1). Of 134 control embryos, only one (0.7 percent) showed an abnormal chromosomal pattern—tetraploidy in one metaphase plate from an eight-cell morula.

Although only limited numbers of metaphase plates could be obtained from preimplantation morulas, karyotyping and sex determination were possible when made from well-spread metaphase formations. The sex ratios of control and test animals were not significantly different, either in the morulas (1.05 and 1.50 in test animals and 1.29 among controls) or in fetuses

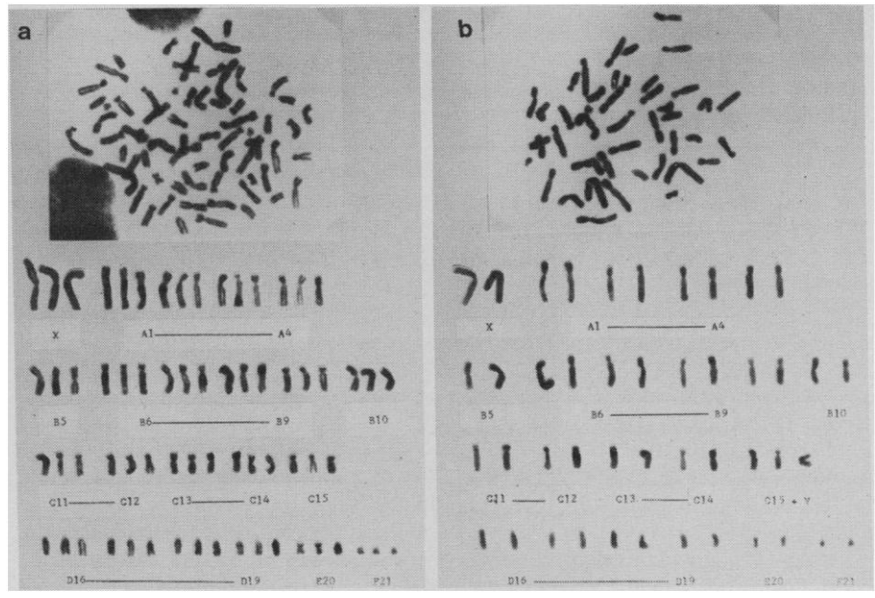

Fig. 1. (a) Triploid karyotype with XXX sex chromosome complex (3-hour delay after the estimated time of ovulation) (× 900); (b) 45 XXY karyotype (3-hour delay after the estimated time of ovulation) (× 900).

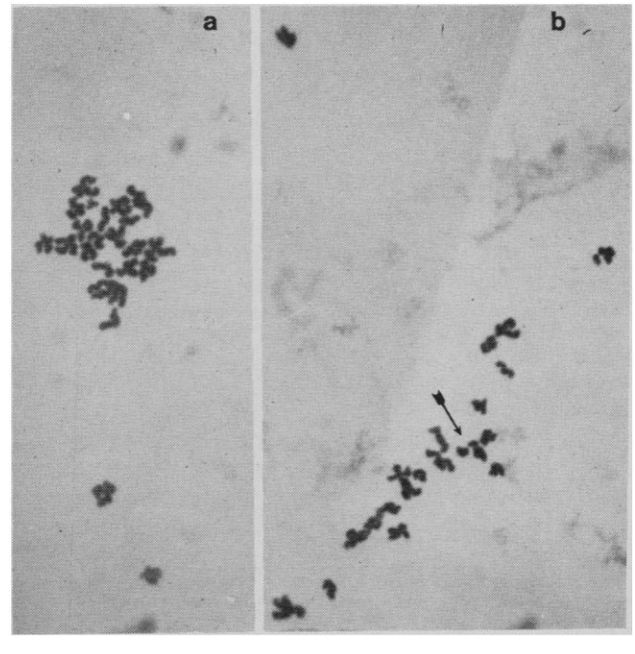

Fig. 2. (a) Normal appearing chromosome set of the metaphase II oocyte harvested shortly (about 30 minutes) after the estimated time of ovulation. (b) Disjoined chromatids (arrow) of a metaphase II oocyte 6 hours after the estimated time of ovulation (× 900).

Table 1. Effect of delayed fertilization on pregnancy wastage in hamsters examined at 3 and 9 days after copulation. The determinations of uncleaved ova, pregnant females, and absorbed fetuses are expressed as absolute numbers and percentages.

Specimen	Number of specimens at hours between ovulation and copulation				
	Control*	0	3	6	9
		3 days after copulation			
Successful matings	25	14	15	3	0
Ova recovered	259	144	178	36	
Uncleaved ova	13 (5%)	49† (34%)	111† (63%)	36 (100%)	
		9 days after copulation			
Successful matings	22	14	15	12	3
Pregnant females	20 (91%)	10 (71%)	7 (47%)	3 (25%)	0 (0%)
Implantation sites	262	136	96	39	
Absorbed fetuses	31 (12%)	32‡ (23%)	29‡ (30%)	3 (20%)	

* Mated 5 hours before estimated ovulation. † Significantly different from frequency in control group, $P < .001$. ‡ Significantly different from frequency in control group, $P < .01$.

examined at 9 days (Table 2). These results are in agreement with those of others (*3*).

Perhaps the most consistent chromosomal finding in both the preimplantation period and at 9 days after copulation (Table 2) was the presence of five triploid embryos (5.1 percent) among the 98 preimplantation specimens surveyed and four triploid runts (2.0 percent) among 202 9-day-old fetuses (Fig. 1A). None of 259 control specimens showed triploidy.

The overall figures are more impressive for triploidy than they are for the occasional case of aneuploidy. We did, however, find the rare occurrence of a single case of "pure" trisomy C [probably 45 XXY (Fig. 2); the Y chromosome is a member of group C-15] in a 9-day-old fetus (Fig. 1b), and we recovered another mosaic form of trisomy, a trisomic D/diploid mosaic in a 9-day-old fetus. We also encountered two monosomic D/diploid mosaic fetuses and one monosomic C-15/diploid mosaic fetus. In addition to these cases of aneuploidy in 9-day-old fetuses, five hypodiploid embryos were observed in the 3-day test group (Table 2). One of these was an eight-cell morula with seven mitoses of 35, 36, 36, 37, 40, 41, and 43 chromosomes. Metaphase plates from four other preimplantation embryos showed hypodiploid chromosome counts in the upper 30's. Such unpatterned findings are difficult to interpret; perhaps they signal the beginning dissolution and death of the developing morula.

The finding of an increased prevalence of triploidy in the hamster embryo after delayed fertilization is compatible with the work of other investigators who used mice and rabbits (*2, 3*). It is consistent also with the observation of Chang and Fernandez-Cano (*7*) that 34 percent of eggs derived from hamsters who were prevented from mating for 4 hours after the estimated time of ovulation had 3 pronuclei; many of the eggs were surely destined to become triploid embryos. However, the investigators did not extend their study with cytogenetic methods. Delayed fertilization leading to either diandry or digyny will account reasonably for the origin of a triploid constitution. However, the latter mechanism appears more likely in the hamster, not only because of the observations of Chang and Fernandez-Cano (*7*), but also because of our own analyses of chromosome complexes, which revealed six XXX and three XXY constitutions but no XYY constitution in the nine cases we have encountered. In contrast, two cases of dispermy have been recognized among 196 uncleaved ova that were studied 3 days after copulation.

Although the origins of triploidy are clear, the origins of aneuploidy are far more speculative. However, the three cases of D mosaic aneuploidy and two cases of C-15 aneuploidy suggest to us that some chromosomes such as acrocentric or sex chromosomes in the hamster may be more susceptible to injury, replication, and deletion than are others. This inference can, we believe, be formulated into a hypothesis for scientific testing. Moreover, the findings here of seven cases of mosaic formations in test animals, and none among the controls, clearly relate their origin to aging sequences of oocytes.

In the hamster, as in mammals generally, the first meiotic division takes place in the ovary shortly before ovulation which, under the controlled lighting scheduled here, was expected to occur around 2 p.m., about 8 hours after the onset of estrus (*6*). The secondary oocyte would then remain in the metaphase state (Fig. 2a) until sperm entry which would occur about 2 to 4 hours after copulation. Within a few more hours the second maturation spindle would start to degenerate and by 9 hours after ovulation "aging" ova have generally lost their capacity to undergo fertilization at all. Recently, Rodman (*8*) has observed that aging secondary oocytes of the mouse may manifest a fragmentation of chromatids at their centromeric junctions, and he has suggested that disjoined chromatids may account for aneuploidies having their genesis at the second meiotic division.

We have observed similar manifestations in metaphase II formations of the hamster and we too postulate that the kind of chromosome aneuploidies we observed, monosomy and trisomy, in the hamster embryo may actually have their genesis in fragmented bonding of chromatid pairs at anaphase II, shortly before fertilization of the oocyte (Fig. 2b).

Whatever sequences may be involved

Table 2. Effect of delayed fertilization on the chromosomal patterns of hamster progeny at 3 and 9 days after copulation.

Chromosomal patterns	Number of specimens at hours between ovulation and copulation							
	3-day embryos				9-day fetuses			
	Control*	0	3	6	Control*	0	3	6
Specimens	135	51	47	0†	124	104	67	31
Normal diploid ($2n = 44$)	134	44	39		124 (100%)	99 (95%)	63 (94%)	31 (100%)
Triploid	0	3	2		0	1	3	0
Tetraploid	1	1	1					
Hypodiploid ($2n < 42$)	0	2	3					
Trisomy (45, XXY)					0	0	1	0
Mosaic	0	1‡	2‡		0	4§	0	0
Sex ratio (XY : XX)	1.29	1.05	1.50		0.98	1.13	0.60	1.82

* Mated 5 hours before estimated ovulation. † All of the 36 ova recovered in this group were uncleaved. ‡ These embryos were diploid/tetraploid. § The chromosomal patterns were: 45, D+/44 in one, 43, D−/44 in two and 43 XO/44, XY in one.

—whether they occur during meiosis, fertilization, or the preimplantation cleavage stages which follow—a study of them in the hamster should produce greater success than in other small animal models.

MASAHARU YAMAMOTO*
Epidemiology Study Center, Framingham, Massachusetts 01701
THEODORE H. INGALLS
Department of Community Medicine, Boston University School of Medicine, Boston, Massachusetts 02118, and *Epidemiology Study Center, Framingham, Massachusetts 01701*

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* Present address: Department of Hygiene and Preventive Medicine, Niigata University Medical School, Niigata City, Japan.

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Helical Filaments Produced by a Mycoplasma-Like Organism Associated with Corn Stunt Disease

Abstract. *Mycoplasma-like bodies with helical filaments were seen by phase contrast microscopy in juice expressed from tissues of plants infected with corn stunt agent. Each filament is bounded by a "unit membrane," and no cell wall, sheath, envelope, or second membrane has yet been discerned by electron microscopy. The association of these filaments with development of disease, their occurrence in phloem cells as seen by both freeze-etching and thin-section electron microscopy, the diagnosis of infection based on their presence in plants without symptoms, and their absence in noninfected corn are consistent with the hypothesis that these unusual filaments are formed by the mycoplasma-like organism presumed to be the corn stunt agent.*

The causal agents of the plant yellows diseases had been thought to be viruses for over 40 years prior to 1967, but now evidence indicates that these agents may be mycoplasmas (*1*, *2*). Among the morphological forms so far described for the presumed mycoplasma-like yellows agents, none are unique to these organisms. The mycoplasma-like bodies in yellows-diseased hosts are morphologically and ultrastructurally similar in every previously reported respect to members of the class Mollicutes (*3*, *4*). The present report, however, describes helical filaments produced by a mycoplasma-like organism in plants with corn stunt (CS) disease. Filaments with this morphology are previously undescribed for the organisms in plant yellows infections, are previously unreported for the described mycoplasmas, and to our knowledge are unknown among cell wall–deficient organisms of any type. A preliminary announcement of these findings has been made (*5*).

The mycoplasma-like helical filaments were first found in 1970 during the course of examining, by phase contrast microscopy under oil immersion (×1000), extracts from stems of corn (*Zea mays* L.) plants infected with the Rio Grande strain of corn stunt agent. In subsequent work, the filaments were found in juice expressed from leaves, tassels, or roots of more than 200 infected plants but have not been found in any of over 150 CS-free plants so far examined.

Under phase contrast, the helical filaments could readily be seen, although their very small width and Brownian motion made photography very difficult. By the constant motion of the filaments, however, and the consequent presentation of different views of the same filament to the eye, the helical morphology of the filaments was readily apparent. The filaments (Fig. 1A) typically were about 3 to 12 μm in length and roughly 250 nm in diameter. The amplitude of the spirals was usually about 0.4 μm. Spherical bodies, 400 to 600 nm in diameter, were often attached to the filaments.

In several tests, we examined juice expressed from various plant parts to determine the association of the helical filaments with CS disease development. Helical filaments first appeared in roots and later in the youngest leaves, where first symptoms of CS also appeared, and could be found in juice from some plants as early as 1 week before symptoms appeared. In some plants containing helical filaments, however, symptoms of CS failed to appear by 8 to 10 weeks after inoculation. By this time the plants had developed tassels and ears. The evidence that the helical filaments are diagnostic for CS infection suggests that such plants were subclinically infected. In plants with symptoms, numbers of helical filaments in leaves were directly correlated with age of infection and increasing severity of CS symptoms.

To gain information on the ultrastructure of the helical filaments, we turned to electron microscopy. Negative contrast [phosphotungstic acid (PTA) 2 percent, *p*H 7.0] of juice expressed from CS-infected plants characteristically revealed filamentous structures of even caliber and often attached

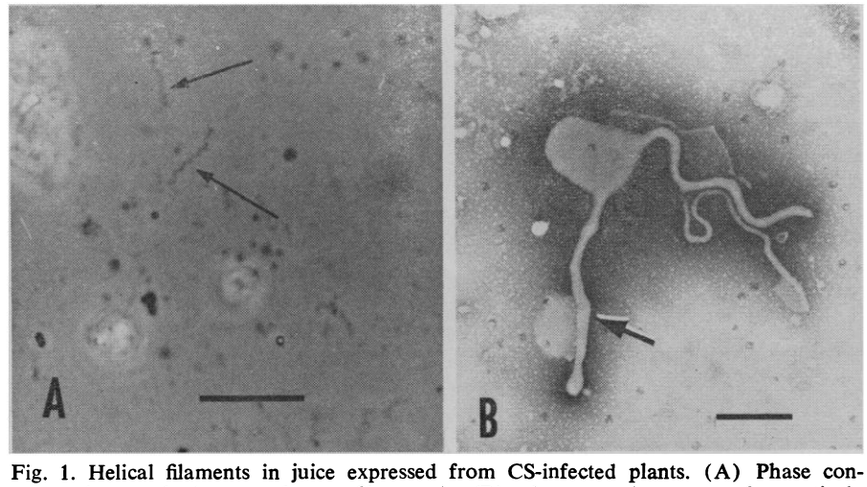

Fig. 1. Helical filaments in juice expressed from CS-infected plants. (A) Phase contrast micrograph (arrows denote filaments); (B) electron microgram of negatively contrasted mycoplasma-like body and attached filaments. Arrow denotes a filament lacking suggestion of helical shape. Bar equals 5 μm in (A) and 2 μm in (B).