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Digynic triploidy in rabbit blastocysts after delayed insemination

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SUMMARY

The origin of triploid (3N) 5½-day blastocysts in rabbits is inferred from the segregation of sex chromosomes and of an autosomal *M*-marker whose properties are described. 39 triploids and no tetraploids were scored among 1454 chromosomally scored blastocysts. A delay of 8 h between an ovulatory injection and subsequent insemination raised the estimated normal incidence of 0·59 % triploid blastocysts to 3·13 %. The increase is ascribed primarily to digyny (17 blastocysts), and to diandry probably mediated by dispermy (1 blastocyst). The triploid components of the two 2N/3N mosaics and the one 3N/6N were digynic. Neither superovulation nor insemination of excessive numbers of spermatozoa could be shown to give rise to triploid embryos. The diandric triploid was *XYY*, the first of this constitution apparently reported in the rabbit. There was some evidence that *XXY* triploid blastocysts up to 5½-day gestation are more viable than *XXX*. In the 2N/3N mosaics each component had been entered by one spermatozoon, and the diploid component could not have been merely a contamination by diploid maternal somatic cells. In 2N/4N, 2N/4N/8N and 3N/6N mosaics, each polyploid component showed an exact doubling of the marker chromosome constitution of a component of lower ploidy; their origin is ascribed to doubling or redoubling of chromosome number in isolated embryonic cells. With earlier data included, 49·08 (± S.E. 1·96) % of 652 diploid blastocysts were *XY*. 460 non-experimental weaned rabbits were all diploid.

1. INTRODUCTION

The normal or 'background' incidence of triploid (3N) blastocysts in the rabbit is low. In non-experimental rabbits not subjected to superovulation, there were 4 3N and 665 non-3N, or 0·60 % 3N (0, 18 (Hofsaess & Meacham, 1971); 0, 105 (Martin & Shaver, 1972); 1, 71 (Widmeyer & Shaver, 1972); 0, 196 (Martin-DeLeon, 1972); 0, 75 (Shaver, 1970); 3, 69 (Fujimoto, Pahlavan & Dukelow, 1974); 0, 131 (Shaver & Carr, 1967, 1969)). The same category of rabbits, but after superovulation, yielded 2 3N and 344 non-3N, or 0·58 % 3N (0, 36 (Fujimoto *et al.* 1974); 2, 265 (Fechheimer & Beatty, 1974 'fresh semen'); 0, 43 (present work)). Since the superovulation had no significant effect on the incidence of triploidy the data may be combined to give 6 3N and 1009 non-3N (0·59 % 3N) as an estimate of the normal background incidence of triploidy in non-experimental rabbits.

When does are inseminated several hours after an ovulatory injection, the incidence of 3N blastocysts is raised well above this normal background level (Shaver & Carr, 1967, 1969; and the present work). The possible modes of origin of triploidy are commonly specified as digyny (two maternal sets of chromosomes) arising from doubling of the maternal chromosome complement at either the 1st or 2nd meiotic division, or diandry (two paternal sets of chromosomes) mediated either by dispermy (fertilization by two spermatozoa) or diplospermy (fertilization by a diploid spermatozoon). Delayed insemination cannot affect the origin of diploid spermatozoa in the testis, nor doubling of the female chromosome complement at the 1st meiotic division in the ovary. Only two possible causes remain for the increased incidence of triploidy after delayed insemination: *digyny* mediated by doubling of chromosome number at the 2nd meiotic division of the female, and *diandry* mediated by dispermy. The present work was designed to assess their relative importance.

Cytologically marked chromosomes in parents and progeny were necessary for tracing the origin of haploid sets of chromosomes. The sex chromosomes provided one pair of markers. An autosomal pair (*M* and *m*) was discovered during the work and shown to constitute a reliable 'neutral' marker segregating in a simple Mendelian fashion.

2. MATERIALS AND METHODS

The related AS and HS strains of *Oryctolagus cuniculus* maintained at the Department were used, and also an *X*-stock consisting of various crosses and individuals of other strains.

(i) *Air-dried preparations of cultured blood cells from weaned rabbits*

The technique was developed from the technique for human blood used by the M.R.C. Clinical and Population Cytogenetics Unit (Edinburgh) in 1972. A dramatic improvement resulted when the original incubation temperature of 37·5 °C was raised to that of rabbits (39 °C). The culture medium was: 800 ml Ham's F.10 without glutamine added (Flow Labs); 4·6 ml 200 millimolar glutamine; 100 ml bovine serum; 12·5 ml phytohaemagglutinin (Wellcome HA 15); 0·4 ml benzyl penicillin (sodium) BP (Glaxo Labs); 0·5 ml streptomycin sulphate BP (Glaxo Labs). Blood from an incised ear vein was collected in 1–2 ml heparinized tubes. 0·4 ml was injected into a sterile bottle containing 4·5 ml culture medium. The culture was incubated at 39 °C for 2 days (0·125 ml of 0·165 mg/ml colchicine being added for the last 3 h), centrifuged at 2000 rpm for 5 min, and the supernatant discarded. 5–10 ml of 0·075 M-KCl pre-heated to 39 °C was added. After exactly 6 min, the culture was recentrifuged and the supernatant discarded. The tube was shaken gently and fixative (3:1 methyl alcohol:glacial acetic acid) was added slowly (drop by drop for the first 10–15 drops) until 5–10 ml had been added. The fixative was replaced 3 times between further centrifugations. Cells were resuspended in fresh fixative and single drops placed immediately on clean slides were air-dried. The spreading of chromosomes was improved by cooling the slide

or warming it on the back of the hand in accordance with the air humidity: this required judgment.

(ii) *Air-dried preparations of blastocyst cells*

The procedures of Fechheimer & Beatty (1974) were followed with modifications included in the following summary. After a 4-day superovulation regime with FSH (Sigma), does were ovulated with 25 i.u. of LH (Pregnyl, Organon) at the beginning of the 4th day. Does were mated 0, 7, 8 or 9 h later, these figures being recorded as 'hours of delayed insemination'. In a few recalcitrant does mating was substituted by artificial insemination without further LH administration. Six days after insemination (about $5\frac{1}{2}$ days after fertilization) each dam was injected intravenously with 20 mg of colchicine in 2 ml deionized water. $1\frac{1}{4}$ h later, blastocysts were flushed from the excised uteri with Hanks's Balanced Salt Solution (BSS). Trypsinization, hypotonic treatment and fixation followed before air-drying. In one series with undelayed insemination each doe was artificially inseminated with the pooled ejaculates of 10 males.

(iii) *Air-dried preparations of testis cells*

A technique close to that of Evans, Breckon & Ford (1964) was employed, but with colchicine injection $1\frac{1}{4}$ h before sacrifice (as described above for blastocysts), and using slices of fresh testis *c.* 2 mm thick.

(iv) *C-banding and staining*

A C-banding technique was developed for showing the *M*-marker, though it was not optimal for banding in general and other aspects of chromosome structure: slides were placed in 0·2 M-HCl for 1 h, rinsed in deionized water, placed in 4% barium hydroxide octahydrate solution at a final temperature of 40 °C for 1 min after removing barium carbonate scum, rinsed twice in deionized water, placed in SSC solution at double strength (8·675 g NaCl and 4·41 g tri-sodium citrate with distilled water to 500 ml) at 60 °C for 1 h and rinsed in deionized water. Some earlier slides of blastocysts and blood cells were not C-banded. Slides were stained for $1\frac{1}{2}$ h in a mixture of 5 ml Gurr's R66 Giemsa and 300 ml Gurr's buffer solution (pH 6·8), rinsed in distilled water, air-dried and mounted in DePeX.

(v) *Chromosome scoring*

The total chromosome number per metaphase was counted. Chromosome groups were as defined by Issa, Atherton & Blank (1968). In diploids (2N = 44) an *XY* constitution was recognized by the small *Y* chromosome and a count of 11 in the $9-13+X$ group (Plate 1*a*, *b*), *XX* by absence of a *Y* and a count of 12 in the group (Plate 1*c*, *d*). An *M*-chromosome (identified as probably chromosome 20, though it may possibly have been chromosome 21) was recognizable even under low power magnification by the presence of three dark spots; one the centromere region, the other two the distal ends of the chromatids of the long arm. *m*-chromosomes were devoid of terminal spots, or occasionally had small lightly stained ones. *M* and *m*

are merely symbols for a chromosomal polymorphism and no dominance or recessiveness is implied. *MM*, *Mm* and *mm* constitutions were scored by the respective presence of 2, 1 or 0 *M*-chromosomes among the 4 chromosomes of the 20–21 group (Plate 1 *h*, *i*, *j*). About 5–20 metaphases per animal were scored in blood cultures, and *c.* 5 cells per blastocyst. Triploid blastocysts were scored on the same principles from up to 10 metaphases per blastocyst, taking into account part-information from difficult metaphases, but with at least one unequivocally scored metaphase available: examples are shown in Plate 1 (*e*), (*f*), (*g*).

3. RESULTS

(i) *Fertility of does*

Among superovulated does with 8 h delayed insemination, up to 1st July 1976, there was 1 doe with no corpora lutea, 3 with corpora lutea but no recovered eggs or blastocysts, 31 with unfertilized eggs only, 89 with blastocysts. After that date, corresponding numbers were 0, 0, 24, 5. In the period after 1st July one of 2 does sacrificed on the last day of the superovulation regime, without mating or LH injection or any known sexual stimulation, was found to contain corpora lutea and unfertilized eggs and had therefore ovulated.

(ii) *Segregation of sex chromosomes among diploid blastocysts*

The numbers of *XY* and *XX* recorded among superovulated blastocysts after undelayed insemination were respectively 20, 15; after 8 h delay, 89, 94. The sum of these statistically homogeneous figures is 109, 109.

(iii) *Segregation of the* M-*marker among diploids*

The numbers of weaned offspring recorded as *MM*, *Mm* and *mm* were respectively 0, 1 and 39 in AS strain; 0, 0 and 58 in the related HS strain; and 51, 141 and 133 in X-stock.

Including reciprocal crosses, there are nine possible ways in which *MM*, *Mm* and *mm* parents can be crossed. Segregation data were first classified in detail by the nine crosses, by sex of offspring, and by the stage at which progeny were scored (blastocysts or weaned young). Preliminary χ^2 tests showed no significant association between *M* segregation and sex of offspring, nor a difference between stages or between matings reciprocal for parental sex. The data were therefore sum-

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Plate 1

Sex chromosomes among metaphase chromosomes of the 9–13 + *X* group, and *M*-markers among those of the 20–21 group. *Y* and *M* are individually identifiable. The seriation of homologous chromosomes, their setting one below the other, and the individual identity of *X* and *m* chromosomes among other members of their groups are all necessarily tentative. The variant of C-banding technique adopted was for the specific purpose of differentiating *M* and *m* chromosomes and does not reveal C-bands in general. (*a*), (*c*), (*h*), (*j*) from diploid blastocysts, (*b*), (*d*), (*i*) from diploid blood cultures of weaned offspring. (*e*), (*g*) from one metaphase of a digynic triploid *XXY Mmm* blastocyst. (*f*) from the diandric triploid *XYY* blastocyst. Reproduced at magnification × 2500.

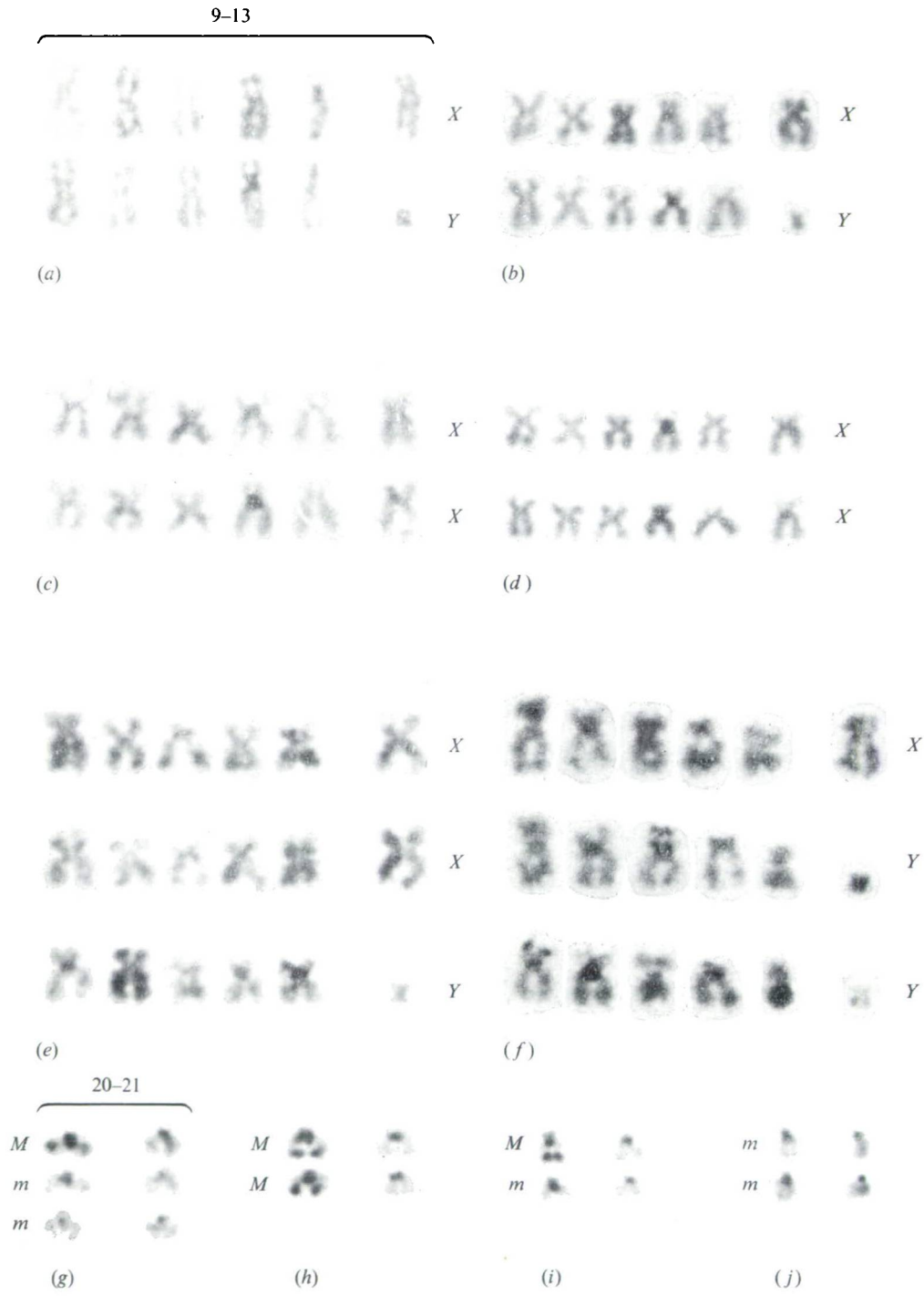

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Table 1. *Segregation of the* M*-marker among 824 diploid progeny, summed over reciprocal matings, sex of progeny and stages scored (566 blastocysts, 258 weaned young)*

Parental constitutions	Expected proportions in progeny			Observed numbers in progeny			Deviations from expected proportions	
	MM	*Mm*	*mm*	*MM*	*Mm*	*mm*	$\chi^2_{[D.F.]}$	*P*
$MM \times MM$	1	0	0	43	0	0	—	—
$MM \times Mm$	0·5	0·5	0	27	28	0	$0{\cdot}02_{[1]}$	0·9–0·8
$Mm \times Mm$	0·25	0·5	0·25	19	34	21	$0{\cdot}59_{[2]}$	0·8–0·7
$MM \times mm$	0	1	0	0	432	0	—	—
$mm \times Mm$	0	0·5	0·5	0	73	78	$0{\cdot}17_{[1]}$	0·7–0·5
$mm \times mm$	0	0	1	0	0	69	—	—
							Total χ^2 $0{\cdot}78_{[4]}$	0·95–0·90

marized as in Table 1, where all offspring are of the expected type when only one type of offspring is expected. When two or three types are expected, deviations from expected ratios are separately and collectively non-significant. It is concluded that the *M*-marker undergoes a simple Mendelian segregation.

Segregation among 150 metaphases of the second meiotic division of an adult *Mm* male was studied. 97·3 % of the *M*-marker chromosomes had one *M* and one *m* chromatid, thus indicating that the *M*-marker was primarily post-reductional. This is recorded only as indicative evidence, mainly because of an undue number of other metaphases that could not be scored at all.

(iv) *Yield of triploids*

None of 183 ♂ and 277 ♀ non-experimental weaned rabbits were triploid. All possessed the normal diploid number. These were selected rabbits and do not represent the normal sex ratio. Table 2 shows that 8 h delayed insemination gives a relatively high yield of triploid blastocysts (3·13 %). Insemination of excess spermatozoa (10 pooled ejaculates per insemination) yielded no triploids among 83 blastocysts.

(v) *Chromosomal scoring of triploid blastocysts*

Table 3 records 39 chromosomally scored blastocysts recovered after superovulation and 8 h insemination delay (35 after natural mating and 4 after artificial insemination). There were 12 *XXX*, 24 *XXY* and 1 *XYY*. Sex chromosomes could not be scored in 2. The *M*-marker segregations showed 17 to be digynic, 1 diandric and 6 uncertain. *M*-marker information was not available in 15.

(vi) *2N/3N mosaic blastocysts*

After superovulation and 8 h delayed insemination, a parental cross *MM* ♂ × *mm* ♀ gave a mosaic (*XX Mm*)/(*XXX Mmm*), and an *mm* ♂ × *MM* ♀ cross gave a mosaic (*XY Mm*)/(- - - *MMm*) in which sex chromosomes could not be scored in the 3N component. The 3N components of both mosaics are digynic.

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Table 2. *Yield of triploid blastocysts after superovulation, in relation to delayed insemination. Additional to the Table: 12 blastocysts, all diploid, with 8 h delay but no superovulation*

Delay in insemination (h)	Nos. of blastocysts scored: Triploid	Non-triploid	% triploid
0	0+0*	43+83*	0
7	0	67	0
8	39	1208	3·13
9	0	2	0
	39	1403	

* After insemination with 10 pooled ejaculates per doe.

Table 3. *Classification of 39 triploid blastocysts recovered after superovulation and an insemination delay of 8 h*

Sire	Dam	Triploid constitutions	Inferred origin: numbers digynic (DG), diandric (DA), or origin unknown (?)
MM	*mm*	5 *XXX Mmm*, 7 *XXY Mmm*, 2 *- - - Mmm*	14 DG
mm	*MM*	1 *XXX MMm*, 2 *XXY MMm*, 1 *XYY Mmm*	3 DG, 1 DA
MM	*Mm*	1 *XXX MMm*, 2 *XXY MMm*	3 ?
Mm	*mm*	1 *XXX Mmm*, 1 *XXY Mmm*	2 ?
Mm	*Mm*	1 *XXX Mmm*	1 ?
(*M*-marker not available)		3 *XXX*, 12 *XXY*	15 ?

(vii) *2N/4N, 2N/4N/8N and 3N/6N mosaic blastocysts*

There were 6 (*XX*)/(*XXXX*), 2 (*XX Mm*)/(*XXXX MMmm*), 1 (*XX mm*)/(*XXXX mmmm*), 8 (*XY*)/(*XXYY*), 4 (*XY Mm*)/(*XXYY MMmm*), 1 (*XY MM*)/(*XXYY MMMM*), 5 (*Mm*)/(*MMmm*), 1 (*XX*)/(*XXXX*)/(*XXXXXXXX*), 1 (*XY*)/(*XXYY*)/(*XXXXYYYY*). An (*XXY Mmm*)/(*XXXXYY MMmmmm*) mosaic with a digynic triploid component was recovered after an undelayed natural mating of *MM* ♂ × *mm* ♀.

(viii) *Other heteroploid blastocysts*

Additional heteroploids, not considered further in the present paper, included one 2N −1, five 2N + 1, one 2N + 2 and one each of the mosaics 2N/8N, 4N/8N and (3N −1)/(6N −2). In one diploid blastocyst the *M*-marker appeared to be represented by an isochromosome of the long arm.

4. DISCUSSION

(i) *Fertilization rate in superovulated does*

Up to 1st July 1976 the number of does with blastocysts was 89 and those with only unfertilized eggs 31. Thereafter there was a sudden and inexplicable change to numbers of 5 and 24 respectively and this led eventually to termination of the work. There had been no known change in technique. The doe found to have ovulated during the superovulation regime, without LH injection or mating or known sexual stimulation, may provide a clue. There had evidently been a premature ovulation, possibly due to changes in the quality of the FSH or to lack of knowledge about its optimal dosage. Premature ovulation would have two consequences relevant to the present work. At the time of a subsequent insemination such eggs might sometimes be too old for fertilization, thus accounting for a failure in fertility. If fertilized, then the true 'delay' between ovulation and fertilization would be greater than recorded.

(ii) *Chromosomal sex among diploid blastocysts*

Paternal X and Y chromosomes in mice are transmitted in virtually a 50:50 ratio at all stages studied between meiosis I of the male and the late blastocyst (cf. Beatty, Lim & Coulter, 1975). This period includes the time of fertilization and the primary sex ratio of the mouse must, therefore, be very close to 50:50. The present scorings of 109 XY and 109 XX rabbit blastocysts may be added to records of 211 XY and 223 XX (Fechheimer & Beatty, 1974) to yield a joint figure for $5\frac{1}{2}$-day rabbit blastocysts of 320 XY and 332 XX (49·08 ($\pm$ S.E. 1·96) % XY): this is consistent with the mouse data.

(iii) *Properties of the* M*-marker*

The M-marker is a terminal segment of heterochromatin on the long arm of what is probably chromosome 20, though it may possibly be chromosome 21. It is virtually strain-specific and segregates in a simple Mendelian fashion (Table 1) among diploid blastocysts and weaned young, without evidence of reciprocal mating effects or (in agreement with its autosomal location) of sex linkage. It is inferred with some confidence that scorings are objective and that there is no differential mortality of the MM, Mm and mm constitutions. It appears, therefore, to be a reliable 'neutral' marker.

There is evidence that the M-marker is primarily post-reductional when segregating in a heterozygous animal. A regular pre-reduction would not in any case be expected of a terminal locus on the long arm of an autosome. The indicative cytological evidence of $\sim$ 97 % post-reduction in male meiosis suggests a high figure in the female also. The following argument indicates post-reduction in female meiosis in the three analysable cases. Digyny was the major mode of origin in the 18 blastocysts where it could be distinguished unequivocally from diandry. The 3 MMm blastocysts from an MM ♂ × Mm ♀ cross must therefore have a high probability of being digynic; i.e. an M spermatozoon had fertilized an Mm oocyte.

Since doubling of the maternal complement in this material is likely to have taken place at the second meiotic division (cf. Introduction), it can be inferred that the *M*-marker segregated post-reductionally in all three. Pre-reduction would have given only *MM* or *mm* oocytes, and therefore only *MMM* or *Mmm* blastocysts.

(iv) *The mode of origin of triploid blastocysts after undelayed insemination and after 8 h delay*

In normal material not subjected to delayed insemination, superovulation did not cause triploidy (cf. Introduction). The 0·59 % incidence of triploidy was represented by 6 triploids that were all *XXY* (Widmeyer & Shaver, 1972; Fujimoto *et al.* 1974; Fechheimer & Beatty, 1974). Insemination of excessive numbers of spermatozoa might have been expected to increase the numbers of triploids by favouring dispermy, but there was no increase. The most that can be generalized from these figures for normal rabbits with undelayed insemination is that the incidence of triploids is low, *XXY* is a major class, and neither insemination of excessive numbers of spermatozoa nor superovulation are causes of triploidy. In mice, however, superovulation is a specific cause of triploidy (Takagi & Sasaki, 1976).

A main conclusion from the work is that triploidy arising after 8 h delayed insemination is primarily digynic (17 digynic blastocysts, 1 diandric). The major role of digyny is reflected by the digynic triploid components of the two 2N/3N mosaics and the one 3N/6N mosaic. The six uncertain 3N with *M*-marker constitutions scored are not inconsistent with digyny. Both chromosome markers show the single *XYY Mmm* to be diandric. It appears to be the only *XYY* triploid rabbit blastocyst so far reported (Plate 1 *f*). Since few diploid spermatozoa reach the site of fertilization (Mortimer, 1977), it is probably dispermic in origin. The rarity of *XYY* is also a feature of human triploids (cf. Beatty, 1978). There have been arguments in human material that assumed delayed fertilization of 'overripe' oocytes leads to diandric triploidy mediated by dispermy (Schindler & Mikamo, 1970). The rabbit oocytes may well be 'overripe', but digyny rather than diandry usually results.

The experiments were not designed to estimate the optimum period of delay. However, in agreement with Shaver & Carr (1967, 1969), 8 h delay gave a relatively high yield of triploids (3·13 %), though not so high as reported by them (12·5 % after 6–9 h delay).

(v) *The relative viability of XXX and XXY triploids*

The relative viability of *XXX*, *XXY* and *XYY* triploids has to be taken into account when the origins of triploidy are being assessed quantitatively. In man, a maximum likelihood analysis indicated that *XXX* and *XXY* had viabilities of comparable magnitude (Beatty, 1978). In the rabbit, the *M*-marker segregations showed that nearly all triploidy after 8 h delayed insemination was digynic. The 12 *XXX* and 24 *XXY* were in a proportion deviating significantly from the digynic expectation of 50 : 50 at the time of fertilization (Shaver & Carr (1967, 1969) report 3 *XXX*, 7 *XXY* after 6–9 h delay). This suggests a higher relative viability

of XXY, but a detailed quantitative check on the lines of Beatty (1978) cannot be made until more data are available.

(vi) *Origin of 2N/3N mosaic blastocysts*

These are not uncommon in relation to the number of 3N. In the rabbit, one 2N/3N and eight 3N are recorded by Fechheimer & Beatty (1974), and the present work yields two 2N/3N and 39 3N. In man, Niebuhr (1974) lists ten 2N/3N and 233 3N. 2N/3N mosaics are of particular interest because they can survive as adults in cats (Centerwall & Benirschke, 1973) and cattle (Rieck, 1973) and to at least 13 years of age in man (Dewald *et al.* 1975).

Both of our 2N/3N mosaics have a digynic triploid component. In the ($XY\ Mm$) /(- - - MMm) mosaic blastocyst from an $mm\ ♂ \times MM\ ♀$ cross, the maternal contribution of (M)/(MM) could have had its origin in a binucleate oocyte (as in the chicken: Fechheimer & Jaap (1978)), or as a result of some but not all of the mechanisms reviewed by Niebuhr (1974). The paternal contribution of (m)/(m) indicates that each component of the oocyte has been fertilized by one spermatozoon. After exchanging the symbols M and m, analysis of our other 2N/3N mosaic yields the same conclusions. In both mosaics the chromosome constitutions exclude the possibilities that either component was parthenogenetic or androgenetic, or that the 2N component represented diploid somatic cells from the dam contaminating the cell suspension of an otherwise pure triploid.

(vii) *Origin of mosaics with a geometric progression of chromosome number* (*2N/4N, 2N/4N/8N, 3N/6N*)

In all 30 mosaic blastocysts where a polyploid component had twice the total chromosome count of another component and marker chromosomes were scored, there was also a precise doubling of all recorded sex chromosome and M-marker constitutions. The simplest explanation (also proposed for the chicken by Miller (1971) and Fechheimer & Jaap (1978)) is that the mosaics arose by doubling or redoubling of chromosome number in isolated blastomeres of the embryo. Fechheimer & Beatty (1974) suggested that simple doubling of chromosome number in a mosaic embryo could be attributed to 'an accumulation, in a moribund blastocyst, of cells that have undergone chromosomal but not cytoplasmic division': if this is so, morbidity would be a cause of mosaicism rather than vice versa, and the mosaicism would be of little interest. The complete absence of pure tetraploids among 1454 chromosomally scored blastocysts is to be noted; either they do not arise or do not survive to $5\frac{1}{2}$ days of gestation.

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