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**CHROMOSOME STUDIES  
ON HETEROSEXUAL TWINS IN CATTLE\*<sup>1</sup>**

**I SEX-CHROMOSOME CHIMERISM (XX/XY)**

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Recent progress in techniques for chromosome study in mammals including man has facilitated the analysis of chromosomes to a great extent: this has brought about a tremendous increase of knowledge in mammalian cytogenetics. Current literature indicates that patients with abnormal sexual development have been associated in most cases with sex-chromosome abnormalities, well-known examples having been reported in patients with Klinefelter's or Turner's syndrome. Nowadays, chromosome analysis has become increasing essential for diagnosis of sexual abnormalities in man.

In domestic animals, on the other hand, information is very poor on the chromosomes in the related field<sup>11,12,15</sup>). One of the well-known cases involving sexual anomaly in farm animals is the intersex condition in heterosexual twins of cattle, generally referred to as freemartin. Chromosomal investigations of the bovine freemartin have recently been carried out by MAKINO et al.<sup>11</sup>), OHNO et al. and FECHHEIMER et al. The former authors<sup>11</sup>) have failed to detect any abnormality in the chromosomes of freemartins, reporting a female chromosome complement, while the latter authors<sup>6,13</sup>) have confirmed the occurrence of sex-chromosome chimerism in both freemartins and male co-twins. The chromosome chimerism occurring in heterosexual bovine twins may be a significant factor for the solution of the freemartin mechanism. The number of animals used in the reported cases is insufficient for conclusive statements on the cause of freemartin. This situation prompted the present authors to take up a re-investigation with sufficient affected animals, use being made of current cytogenetic techniques.

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\*<sup>1</sup> A part of this work was communicated at the 8th Meeting of the Hokkaido Branch of the Japanese Society of Fertility and Sterility held on the 23rd January 1965 in Sapporo, and the 59th Meeting of the Japanese Society of Veterinary Science on the 7th April 1965 in Tokyo.

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TABLE 1 *Materials and results of chromosome counts in controls*

NO.	ANIMAL NUMBER	BREED	SEX	AGE	SOURCE	NO. OF CELLS COUNTED	CHROMOSOME NUMBER	SEX CHROMOSOME
C 1	F 10C	Hereford	} Homosexual } twin male	yr. mo. 0.5	Blood	56	} 60	} XY
C 2	F 10D	"		0.5	"	55		
C 3	H-100	Holstein	} Single born } male	0.7	Testis	50		
C 4	H-102	"		1.0	"	53		
C 5	P. C.	"	} Homosexual } twin male	3.6	Blood	56		
C 6	H-105	"		} Single born } male	5.0	Testis		
C 7	F 10A	Hereford	6.7		Blood	55		
C 8	F 70	Holstein	} Single born } female	Unknown	} Blood	57	} 60	} XX
C 9	9F 2	"		2.7		51		
C 10	F 10E	"		4.2		51		
C 11	G-298	Guernsey		4.5		52		
C 12	H-631	Holstein		5.2		52		
C 13	F 10B	Hereford		5.6		58		
C 14	9F 1	Holstein		5.9		54		
C 15	F 14M	"	8.1	56				

In the following, the present authors have attempted to carry out a chromosomal study on both homosexual and heterosexual twins in cattle including freemartins, with the application of current tissue culture methods. This paper describes the results of chromosome analyses of normal males and females in single birth and in homosexual twins as controls, and of heterosexual twins with or without freemartinism.

## MATERIALS

The materials used in the present study consisted of 15 cattle as controls—single birth and homosexual twins, and 16 heterosexual twins.

1) The control group consisted of 3 homosexual male twins, 4 single born males and 8 single born females (1 heifer & 7 cows). An account of materials dealt with in this study is given in table 1 together with chromosomal data.

2) Heterosexual twins consisted of 9 freemartins, 6 male co-twins with freemartin, and one non-freemartin female with one calving. In 5 co-twin pairs, both twins were examined. One pair of fetal twins was estimated as of about five months' gestation by the crown-rump length (male: 37 cm, freemartin: 40 cm). An account of this group is presented in tables 2 and 3.

TABLE 2 *Materials and results of chromosome counts in blood of heterosexual twins (Holstein)*

NO.	ANIMAL NUMBER	AGE	MALE			FREEMARTIN		
			No. of cells counted	Sex-chromosome		No. of cells counted	Sex-chromosome	
				XX	XY		XX	XY
1* <sup>1</sup>	F 6	days Fetus	4	2	2	24	22	2
2	F 14	4	—			56	12	44
3* <sup>1</sup>	F 8	16	65	33	32	74	45	29
			55* <sup>2</sup>	34	21	57* <sup>2</sup>	32	25
4	F 3	25	—			239	7	232
			—			50* <sup>2</sup>	2	48
5* <sup>1</sup>	F 10	25	58	12	46	56	13	43
6	F 2	53	60	24	36	—		
7* <sup>1</sup>	F 1	169	146	133	13	111	98	13
8* <sup>3</sup>	F 9	yr. mo. 7.2	—			75	75	0

Notes: \*<sup>1</sup> Both of the twins were examined.  
\*<sup>2</sup> Bone marrow cells  
\*<sup>3</sup> Non-freemartin  
— Not chromosomally examined

TABLE 3 *Materials and results of chromosome counts in cultured tissues of heterosexual twins (Holstein)*

NO.	ANIMAL NUMBER	AGE	SOURCE	TISSUE ORIGIN	MALE			FREEMARTIN				
					No. of cells counted	Sex-chromosome		No. of cells counted	Sex-chromosome			
					XX	XY	Chimera		XX	XY	Chimera	
		days										
1*	F 6	Fetus	Skin	Ectoderm	108	0	108	—	116	116	0	—
			Lung	Endoderm	71	0	71	—	24	22	2	+
			Liver	"	64	0	64	—	—			
			Kidney	Mesoderm	42	2	40	+	40	40	0	—
			Gonad* <sup>2</sup>	"	139	0	139	—	138	136	2	+
9	1023	1	Lung	Endoderm	—				37	37	0	—
10*	F 4	2	Kidney	Mesoderm	151	0	151	—	215	215	0	—
11	F 5	7	"	"	—				93	93	0	—
5	F 10	25	Gonad* <sup>2</sup>	"	164	0	164	—	—			
			Liver	Endoderm	—				4	4	0	—
7	F 1	169	Kidney	Mesoderm	—				107	101	6	+
			Gonad* <sup>2</sup>	"	—				195	192	3	+

Notes: \*<sup>1</sup> Both of the twins were examined.  
\*<sup>2</sup> Interstitial or connective tissue cells  
— Not chromosomally examined

## METHODS

1) For leukocyte cultures, approximately 10 ml of blood was obtained from the jugular vein by means of a syringe and inoculated into a heparinized tube. Cultures were set up by a mixture of the heparinized blood and the culture medium in proportion of 1 : 3 with addition of phytohemagglutinin-M, and cultivated in TD-15 flasks. After incubation of 3 or 4 days, mitotic cells were arrested by colchicine treatment ( $50 \times 10^{-8}$  M) for about 1.5 hours. Following the water pretreatment, chromosomal slides were made according to the routine air-drying method and stained with Giemsa. The above procedure provided excellent results for the chromosome study of cattle.

2) Short-term cultures were set up for bone marrow: marrow specimens were suspended in a solution consisting of 9 parts of culture medium and 1 part of inactivated bovine serum to which about 0.1 part of colchicine ( $50 \times 10^{-8}$  M) was added. Then the suspension was incubated for 30 to 40 minutes at 37°C, and collected by means of centrifugal sedimentation at 1,000 rpm for 5 minutes. After the water pretreatment, the cell suspension was air-dried and stained with Giemsa.

3) For cultures of viscera and skin, fresh specimens were placed in the culture medium for 5 to 10 minutes, and then minced with scissors. To the specimens thus prepared was added culture medium in the proportion of 1 : 10 and the mixture was centrifuged for about 5 minutes. The resultant supernatant was utilized for cultivation. Chromosome slides were prepared as mentioned above.

## RESULTS

### 1 Results of chromosome counts in controls (single born or homosexual twin males and single born females)

In the control group, chromosome analysis was made of 375 cultured cells at metaphase from blood or testis cultures of 7 males, and of 431 cells from blood cultures of 8 females. As shown in table 1, the chromosome number in a cultured cell was 60 without exception, regardless of sex. The identification of individual chromosomes was based on size, shape and position of centromere, and 29 pairs of autosomes and one pair of sex-chromosomes were identified (fig. 1). The autosomes in 29 pairs were acrocentric and of various sizes. The X and Y chromosomes were easily identified by their submetacentric character, and could be distinguished from each other by size—the X chromosome being approximately equal to the largest autosomal pair and the Y chromosome to the smallest. Thus, the composition and morphological characteristics of chromosomes in the cultured cells were quite in accordance with those in either normal male or female cattle. Microphotographs (figs. 1, 3 & 7) clearly show karyotypes in cultured cells.

### 2 Results of chromosome counts in blood and bone marrow specimens of heterosexual twins

In table 2 are presented chromosomal findings involving the ratio of XX-cells to XY-cells obtained from blood cultures and bone marrow cells of heterosexual twins. Both freemartins and their co-twins (Nos. 1~7) showed 2A-XX/2A-XY chimerism. The degree of chimera ratio

varied considerably from individual to individual. For example, in some males (Nos. 3 & 7) the number of cells with 2A-XY was rather smaller than that of cells with 2A-XX, although these animals were complete males in phenotype. Likewise, in some freemartins (Nos. 2 & 5) the number of cells with 2A-XY was greater than those with 2A-XX, although the freemartin was observed to be a modified female.

On the other hand, observations of a fertile female (No. 8) from among the non-freemartin heterosexual twins revealed a normal female complement with an XX-mechanism in cultured leukocytes without exception.

### 3 Results of chromosome counts in cultured tissues of heterosexual twins

Table 3 represents chromosomal findings involving the ratio of XX-cells to XY-cells obtained from various tissues and organs of heterosexual twins under study. Five organs (2 kidneys, 2 gonads and one lung) derived from 3 animals (a freemartin and its male co-twin, and one freemartin) showed sex-chromosome chimerism, the degree of which was lower than in blood or bone marrow specimens. For example, a freemartin fetus (No. 1) supplied data showing that about 1.4 per cent of the cells from gonadal cultures, and approximately 8 per cent of the cells from lung cultures were of the XY constitution, leaving the remaining cells with the XX complex in each tissue. The cells from skin and kidney cultures had exclusively the 2A-XX constitution. Kidney cultures from the male of No. 1 provided metaphase cells of the XX/XY chimera constitution without exception. In No. 7 (freemartin), about 6 per cent of the metaphase cells from kidney cultures and 1.5 per cent of the cells from gonadal cultures showed the 2A-XY constitution, while the remaining cells in the tissues from kidney and gonads were of 2A-XX constitution. It should be mentioned that mitotic cells in the gonadal tissue may be from the interstitial or connective tissue. Morphologically and histologically the seminiferous tubules showed an apparent likeness to those of the immature testis of a normal bull. Figures 12 and 13 show chromosomes at metaphase in a cultured gonadal tissue from a freemartin.

### DISCUSSION

Recently, SASAKI & MAKINO reported that the diploid number of domestic cattle (*Bos taurus*) is 60, and that the X chromosome is one of the larger chromosomes, characterized by a submedian centromere, while the Y approximates in size to the smallest autosomes, being submetacentric in nature and positively heteropycnotic at metaphase. The present findings in controls agreed with those of SASAKI & MAKINO.

LILLIE<sup>10</sup> reported that in about 87 per cent of heterosexual bovine twins, the females were generally sterile, and only the remaining female twins were fertile. According to RICHTER freemartins seldom occur in cattle—approximately 2 per cent of births. The vascular anastomosis between twins provides evidence essential for defining the freemartin. Chorionic fusion and vascular anastomosis in two-sexed twins induce the hormonal influence from the immatured male twin upon the gonad of the female co-twin, and as a result the female calf develops into a

freemartin, but the bull calf is normal at birth<sup>9,10</sup>). OWEN expressed a view on the basis of his immunological studies that the existence of a vascular anastomosis results in an interchange of cells which are ancestral to the hemopoietic cells of postnatal life. Further, ANDERSON et al. and BILLINGHAM et al. reported that the dizygotic twin pairs were tolerant to grafts of each other's skin. If chorionic fusion results in blood chimeras, the migration of primordial germ cells may possibly occur between dizygotic twins.

There are several reports about the XX/XY chimerism of bone marrow, blood and gonad of heterozygotic sexed twins: BENIRSCHKE et al. in the marmoset monkey; BENIRSCHKE & BROWNHILL<sup>3,4</sup>) in the marmoset monkey; OHNO et al. in cattle; UCHIDA et al. in human beings. In this study, the authors observed the chromosome constitutions of various organs in bovine heterosexual twins. The results are as shown in tables 2 and 3. Organs of endodermal and mesodermal origin generally consisted of 2A-XX cells and 2A-XY cells in both freemartins and co-twins, but in those of ectodermal origin no XX/XY chimerism was detected (tab. 3). Further, 2A-XX/2A-XY cell chimerism was usually detected in both freemartins and their co-twins, with various ratios of cell chimerism in tissues. One heterosexual female twin without freemartinism showed no such cell chimerism. The observed facts may be consistent with the assumption that cell chimerism occurring in the freemartin and its co-twin suggests an interchange of cells between them, and that the interchange of cells occurs as a result of vascular anastomosis. According to the immunological observations on dizygotic bovine twin embryos by OWEN, and HOSODA et al., most pairs of bovine twins have an identical blood type. Further, the 2A-XX/2A-XY chimerism detected in organs of endodermal and mesodermal origin alone seems to indicate that vascular anastomosis occurs generally in certain embryonic stages after the completion of the blastoderm differentiation. LILLIE<sup>9,10</sup>) reported that the bovine freemartin was produced by the effect of hormones on sex-differentiation and sexual development. The existence of XX/XY chimerism in the freemartin does not always exclude the hormone theory of LILLIE<sup>9,10</sup>) to explain the bovine freemartin. The question arises whether the exceptional chimera ratios in the present study (Nos. 4 & 7) might affect to some extent the differentiation of their gonads, but no anatomical or histological examinations useful for the elucidation of the above view were carried out. It should be mentioned, however, that cases have been noted of bovine freemartins in which no cell mosaicism occurs (MAKINO et al.<sup>11</sup>). However, FECHHEIMER et al., in confirming recent reports that freemartins and male co-twins are mosaics containing both male and female cells, have now postulated that the freemartin condition may be caused directly by the sex-chromosome chimerism. It is very likely that the sex-chromosome chimerism is a significant factor for the

cause of the freemartin condition.

#### SUMMARY

The chromosome constitution of 16 cases of bovine heterosexual twins consisting of 9 freemartins, 6 males co-twinning with the freemartin and one non-freemartin female, and also 15 control animals consisting of 3 homosexual twin males, 4 single born males and 8 single born females were examined in blood cultures as well as in tissue cultures from various organs.

The results are summarized as follows:

- 1) The diploid number of chromosomes in controls was 60. The 29 autosomal pairs were acrocentric in nature, while X and Y chromosomes were submetacentric.
- 2) In both freemartins and their co-twins, tissue cells of mesodermal and endodermal origin generally showed 2A-XX/2A-XY chimerism. A normal cow from the non-freemartin heterosexual twins was found to possess a normal female complement of chromosomes. Cell chimerism occurring in both the freemartins and co-twins is ascribed to a cell interchange as a result of vascular anastomosis.

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## EXPLANATION OF PLATES

### PLATE I

Fig. 1 Serial alignments of 60 supposed homologous pairs of chromosomes in cattle, showing submetacentric X and Y (right), and 29 acrocentric autosomal pairs

Metaphase chromosomes from leukocyte cultures of a normal male (No. C 5, bottom left) and a normal female (No. C 8, bottom right)

Arrows show X and Y, respectively.

× 1,000

Fig. 2 Normal female (No. C 11)

Slide from a leukocyte culture of 72 hours cultivation

Arrows show metaphase cells.

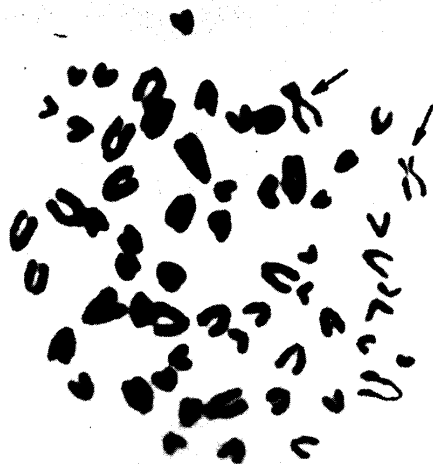
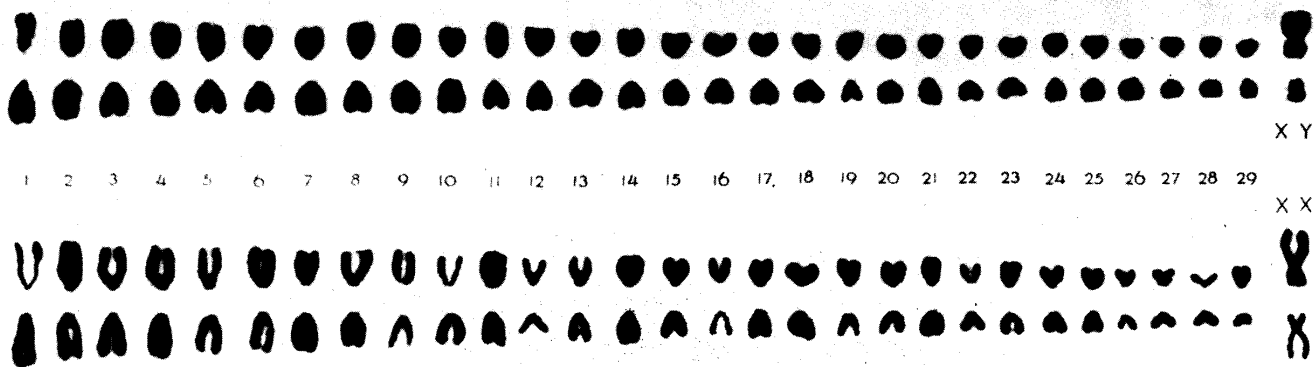
Giemsa stain

× 140

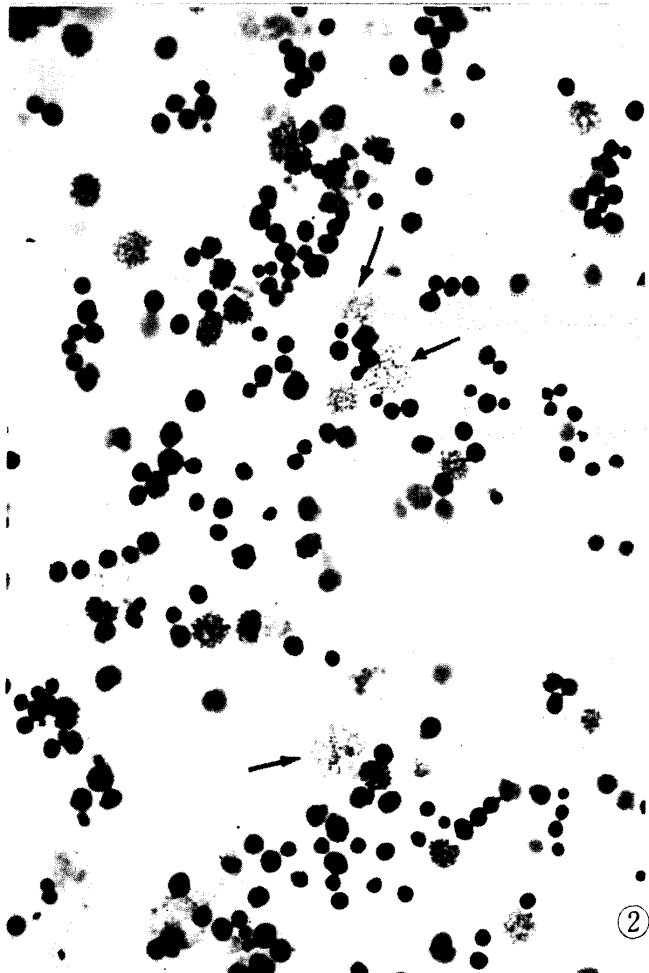
Fig. 3 The same

Arrows show sex-chromosomes (XX).

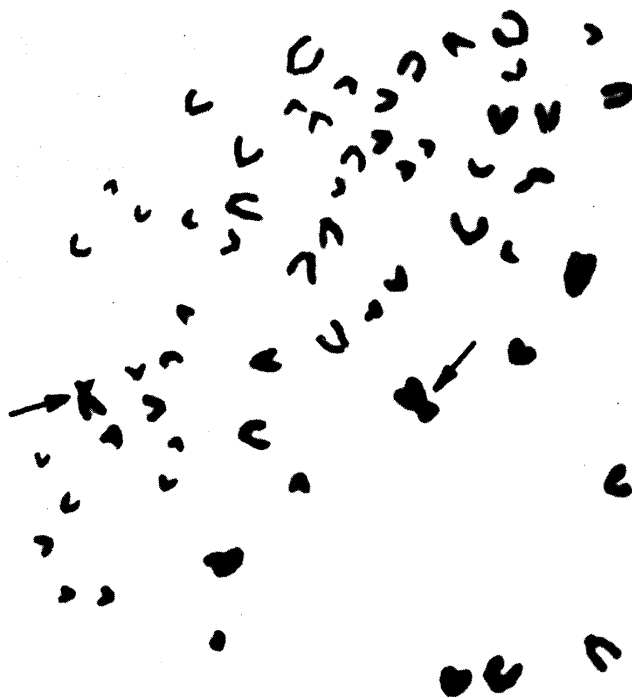
× 2,000



①



②



③

PLATE II

- Fig. 4 Testis culture from a normal male (No. C 6), 96 hours cultivation  
May-Grünwald-Giemsa stain  
× 150
- Fig. 5 The same  
High magnification of a part of figure 4  
Arrows indicate metaphase cells.  
May-Grünwald-Giemsa stain  
× 850
- Fig. 6 The same  
Air-drying preparation, stained with Giemsa  
Arrows show metaphase plates.  
× 115
- Fig. 7 The same  
Arrows show sex-chromosomes (XY).  
× 1,150

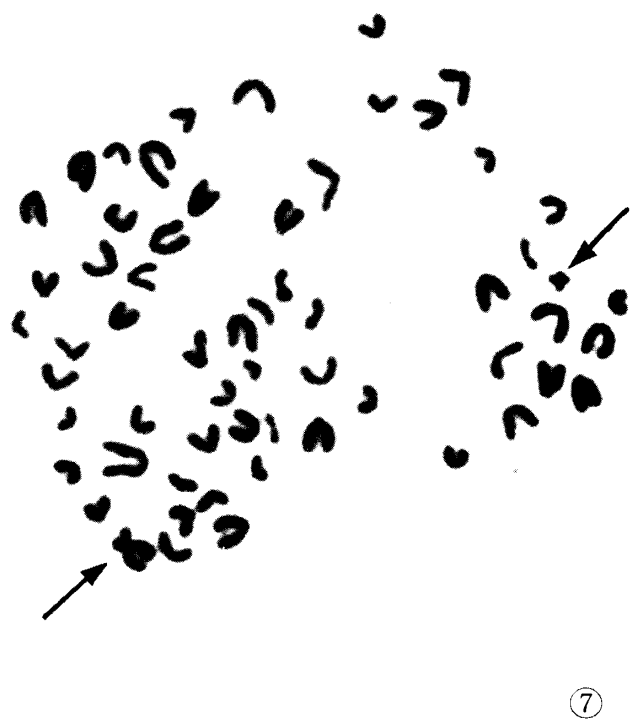
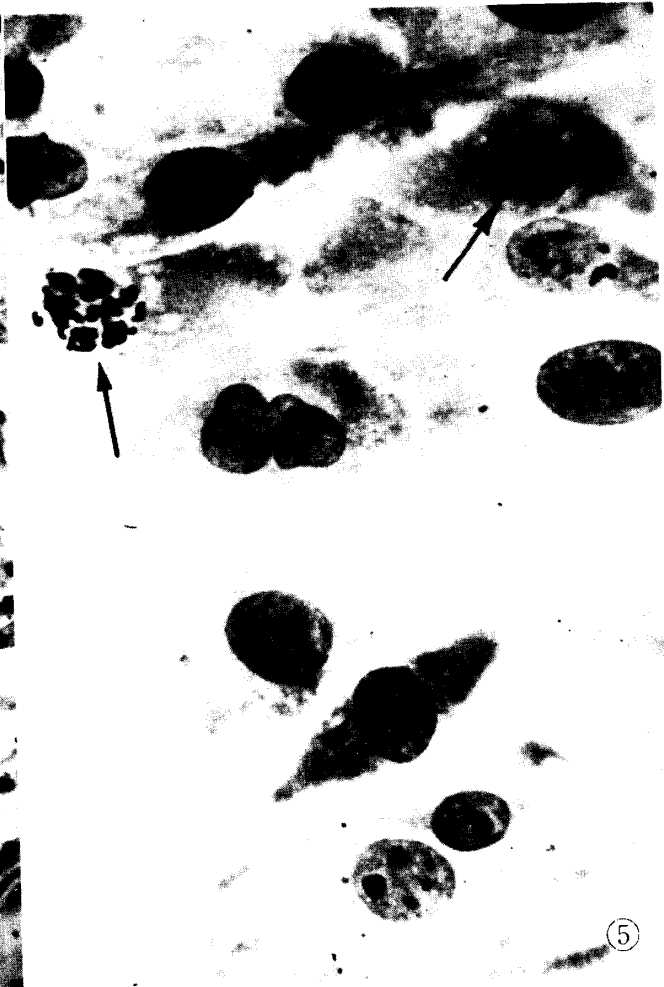
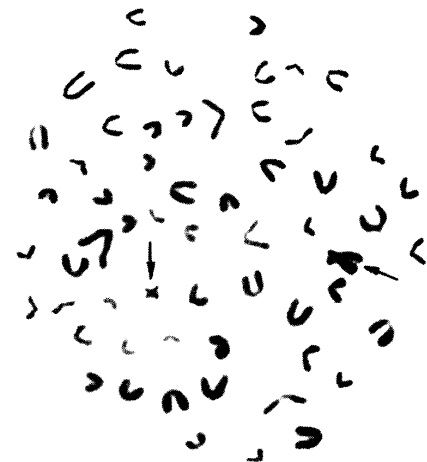
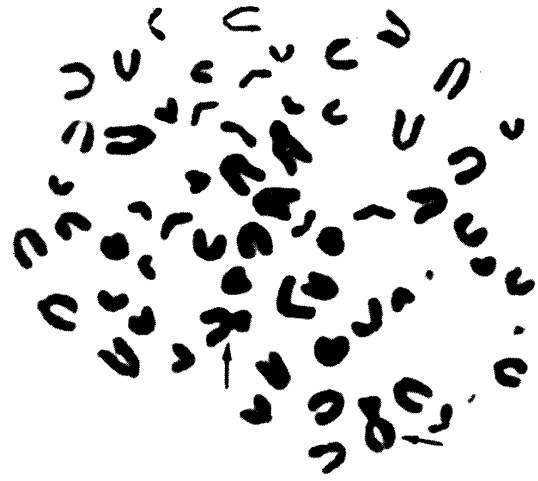
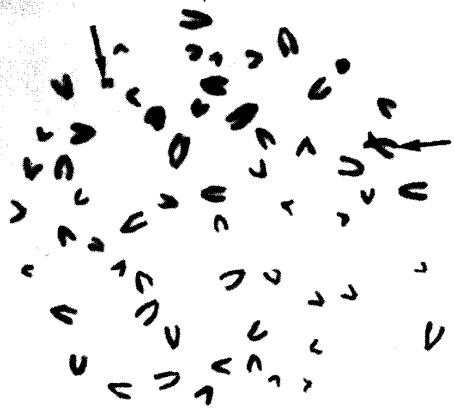


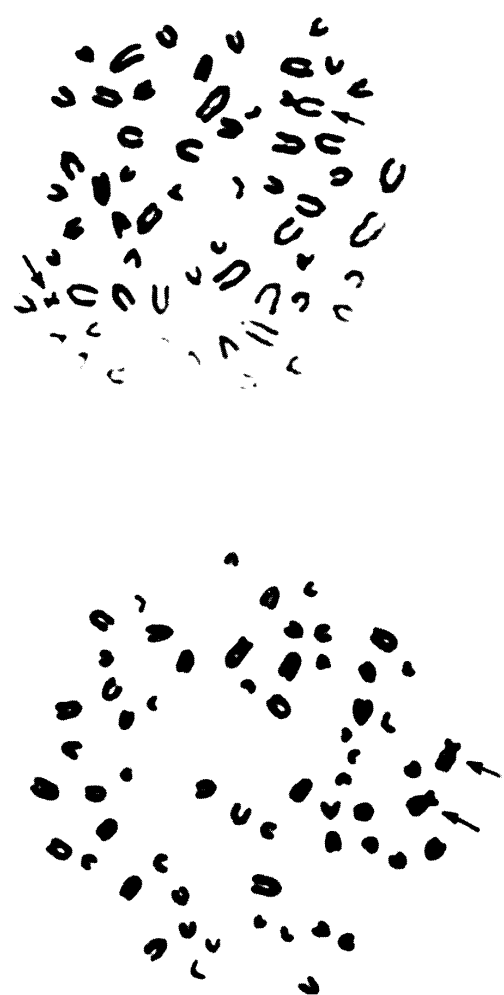
PLATE III

- Fig. 8 Metaphase figures from a blood culture of a male fetus (No. 1)  
Note sex-chromosome chimerism, showing XY (upper) and XX  
(bottom).  
× 1,000
- Fig. 9 Sex-chromosome chimerism from a blood culture of a freemartin fetus  
(No. 1), showing XX (upper) and XY (bottom)  
× 1,000
- Fig. 10 Male (No. 6)  
Chromosome preparation from leukocyte cells cultured for 72 hours  
Giemsa stain  
× 200
- Fig. 11 The same, showing sex-chromosome chimerism  
Arrows show XY (upper) and XX (bottom).  
× 850



⑧

⑨



⑩

⑪

PLATE IV

Fig. 12 Freemartin (No. 7)

Tissue culture of a gonad, showing mitosis at various stages

× 800

Fig. 13 The same, showing sex-chromosome chimerism in a gonadal culture

Arrows show XX (left) and XY (right).

× 1,000

