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THE KARYOTYPE OF THE DOG (CANIS FAMILIARIS) OBTAINED FROM CULTURES OF PERIPHERAL BLOOD LYMPHOCYTES

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THE KARYOTYPE OF THE DOG (CANIS FAMILIARIS) OBTAINED FROM CULTURES OF PERIPHERAL BLOOD LYMPHOCYTES

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(M. S. Thesis)

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THE KARYOTYPE OF THE DOG (*Canis familiaris*) OBTAINED FROM CULTURES OF PERIPHERAL BLOOD LYMPHOCYTES.

INTRODUCTION

The first observations on canine chromosomes appear to be those of Rath in 1894 (20). Using sectioned germinal tissue of a puppy of unspecified sex, as well as leukocyte precursors, he determined the chromosome number to be more than 32, and perhaps as high as 64. In 1918, Malone (12) worked with testicular tissue of eleven male mongrels and one purebred animal; he reported 21 chromosomes. Using various tissues from a female fetus, he reported 22 chromosomes. From these data, he believed the dog to have an XO/XX sex chromosome complement. Allocyclic behavior of the X-chromosome was noted, and Malone thought the X was associated with the nucleolus in some way. In 1925, Painter (18) also used testicular cells; he described "... at least 50 chromosomes" in spermatogonial cells (p.396). However, he placed the upper limit at close to 52. The sex elements were not described, but from context it is safe to assume his belief in an XX female, XY male, sex determining mechanism for the dog. He also believed the sex elements to be associated with the nucleolus.

With the advent of more satisfactory fixation techniques for mammalian chromosomes in 1927 (14), enumeration of dog chromosomes became more precise. Minouchi (15) used sectioned germinal tissue of dogs of both sexes, ranging in age from a fetus to fully mature animals. He found 78 chromosomes in both sexes, and described the X-chromosome at metaphase as "... atelomitic V-shaped..." and "... lying always at the periphery of the plate..." (p.257). The autosomes are described as having terminal centromeres. While he found two X-chromosomes in the female, and one in the male, he was unable to identify

the Y-chromosome. From observations of meiotic prophase, Minouchi believed the Y-chromosome might be a small rod-shaped element. Ahmed (1), in 1941, used Minouchi's techniques and confirmed his results. He could find no morphological differences among chromosomes of three breeds, although he found a difference in chiasma distribution. Ahmed, as had some earlier investigators, also noted allocyclic behavior of the sex chromosomes with respect to movements at division and intensity of staining. He indicated that portions of the sex chromosomes exhibited retarded prophase condensation, and that terminal knobs were often visible at meiotic prophase. Like Minouchi, Ahmed identified the Y-chromosome only by its association with the X during meiotic prophase. In the same decade, Makino (10,11) used sectioned material in a broad survey of Eutherian orders. He confirmed 78 as the chromosome number in both sexes of dogs, and identified both the X- and Y-chromosomes by their behavior at meiotic prophase. He noted the frequent association of the X-chromosome with the nucleolus (11), and indicated the distinctive V-shape of the X-chromosome.

Due to the relatively small size of somatic mammalian cells and chromosomes, early workers relied heavily on observation of the larger, more easily managed cells of germinal tissue. With perfection of techniques of tissue and cell culture early in the 1950's, somatic mammalian chromosomes could be visualized more satisfactorily. With special pretreatments, these chromosomes have been visualized with a clarity not obtained with older methods. Hsu and Pomerat (8), in 1953, used a cycle of cell culture - metaphase arrest - hypotonic swelling - fixation to study canine chromosomes in metaphase cells from an embryonic lung culture. They confirmed the earlier findings of 78 chromosomes in both sexes, and described the autosomes as being all acrocentric. They described the X as a large submetacentric element, but did not describe the Y. Awa, et al (3), used the same technique in 1959, and confirmed the results of Hsu and Pomerat. They believed some of the autosomes

might be subterminal, and they thought the Y-chromosome was the "...smallest of rod-shaped bodies." (p.262). Takayama and Makino, in 1961 (26), used a tissue culture method to investigate the chromosomes of canine venereal tumors, and they confirmed the ideogram for the normal dog.

Additional confirmation of the previously described normal dog karyotype is found in the recent work of Reiter, et al (21), and of Moore and Lambert (16). Both authors confirmed the general features of the dog karyotype by observations made on cultured solid tissues from dogs of various ages, sexes, and breeds. Both authors agreed that the Y-chromosome was probably a very small meta- or submetacentric element. Moore and Lambert implied that some autosomes might possess short arms and possibly secondary constrictions as well.

All modern work firmly establishes the general characteristics of the canine karyotype, but leaves certain details in doubt. The morphology of the Y-chromosome, the reality of autosomal short arms, and the presence of secondary constrictions or satellites on some chromosomes, are all unsettled questions. There appears to be no record of a canine karyotype being obtained by the elegant peripheral blood culture method of Moorehead, et al (17). The abundant high quality metaphase figures obtained with this technique might permit a more detailed characterization of the canine karyotype than has been possible so far.

It has been shown that in cultures of peripheral blood cells from normal animals, the dividing cells arise almost entirely from lymphocytic elements (9,19). Cultures of lymphocytes obtained from peripheral blood might produce not only a detailed karyotype, but they might also be used for *in vitro* studies of the kinetics of donor and recipient cell populations following homologous lymphoid tissue transplantation. The latter studies would be made possible by utilizing the readily identifiable canine X

chromosomes as "markers" for donor cells of one sex and recipient cells of the opposite sex. Permanent or transient changes in the karyotypes of both donor and recipient cells could be induced by irradiation, or by immunological selection acting at the cell level, or both. These changes could be assessed *in vitro* by examining karyotypes obtained from cultured lymphocytes.

In order to establish a baseline for the studies indicated above, this paper will describe the normal canine karyotype obtained from cultures of peripheral blood lymphocytes. Methods for reliable canine lymphocyte culture, and for routine canine chromosome preparation, will be presented.

MATERIALS AND METHODS

Preliminary experiments using the methods of Moorehead, et al (17), yielded generally poor results. They served to demonstrate the advisability of finding improved methods for canine leukocyte culture and for preparation of canine cells for karyotype analysis. Several methods of leukocyte concentration, lymphocyte separation, cell culture, cell pretreatment, fixation, and final slide preparation were tried. Details are not presented here. The following method is a synthesis of these experiments, and it has been found highly suited to canine lymphocyte culture and to preparation of slides for chromosome analysis. It differs in certain important ways from methods now commonly used, primarily in the constitution of the cell culture medium and in the manner of producing air-dried chromosome spreads.

OBTAINING BLOOD SAMPLES

Mongrel dogs of various types, and purebred beagles, were used in this study. The animals were of both sexes, and ranged in age from two months to five years. Prior to drawing blood, most animals were fasted for 24 hours or more in order to avoid lipemic blood samples. All animals were awake at the time of blood sampling, or were lightly anesthetized with sodium thiopental ("Pentothal" Abbott) at a dose of 2-3 mg. per pound body weight. The cephalic, saphenous, or external jugular veins were the sites selected for venipuncture. Prior to blood sampling, these areas were closely shaved and then scrubbed with benzalkonium chloride 1:750 ("Zephiran" Winthrop). Entry into the vein was made with a sterile 18 g. disposable needle, and 10-20 cc. blood was withdrawn into a disposable plastic syringe containing 220 units of sterile preservative-free heparin (Riker) in 0.5 cc. sterile physiological saline solution. The blood and heparin solutions were mixed by repeated inversion of the syringe.

LYMPHOCYTE SEPARATION

In order to obtain a cell inoculum which contained primarily lymphocytes, for reasons to be discussed, a procedure was used which was essentially that of Hastings, et al (7). Heparinized blood was aseptically transferred to a sterile 30 cc. glass culture tube, which was tightly capped and placed at a slight angle in a 37 deg. C. water bath. The red cells were allowed to sediment by gravity for one hour or more, until several cc. of red-cell free supernate could be seen. The supernatant white-cell rich plasma (usually 5-25% of the original volume) was aseptically transferred to a sterile 125 cc. flat-bottomed culture flask. To this was added two volumes of NCTC 109 (Microbiological Assoc.; Difco) at 37 deg. C., and approximately ½ g. sterile iron powder (reduced electrolytic grade, Matheson, Coleman, and Bell, sterilized by exposure to UV radiation). The flask and contents was placed in a 37 deg. C. shaker water bath, tightly capped to prevent CO_2 loss, and agitated for 1½-2 hours with 100 ½-inch strokes per minute. In order to remove granulocytes which had phagocytized iron particles, the flask was removed from the water bath and placed on edge on a strong permanent magnet for five minutes. Afterward, the supernate was aseptically transferred to a sterile 50 cc. culture tube. This was also placed on a strong magnet for five minutes, and the supernate was aseptically transferred to a sterile glass vessel at 37 deg. C. A sample was aseptically removed, and a routine cell count was performed in a hemocytometer. An estimate was made of the percentage of mononuclear cells present.

CULTURE PREPARATION

Standard 15 cc. glass culture tubes were seeded with a volume of cell suspension sufficient to provide 1×10^7 cells, but only if the mononuclear cells were about 80% or more of the total. If they were a lower proportion of the total, then 0.5×10^7 cells or fewer were seeded. If the

volume of cell suspension required was greater than 3 cc. (equivalent to 1 cc. of original plasma), the suspension was aseptically centrifuged at 70 gravities. Sufficient supernate was then aseptically withdrawn, and replaced with fresh prewarmed NCTC 109, to reduce the plasma concentration of the cell suspension to 15% or less of the total. The cells were then resuspended by gentle agitation.

To the seeded culture tubes was added 1.0 cc. fetal bovine serum (Microbiological Assoc.), 3300 units Penicillin G (Upjohn), 1.7 mg. Streptomycin sulphate (Lilly), and 330 units Mycostatin (Squibb). The antibiotics were added as a solution in 0.1 cc. injectable water. 0.2 cc. of buffered Phytohemagglutinin-M (Difco) was added. Finally, additional prewarmed NCTC 109 was added to yield a final volume of 10-10.5 cc. For each animal, several such cultures were prepared. The tubes were loosely capped, placed in a rack at an angle of about 45 deg., and incubated at 37-38 deg. C. in a 5% CO_2 atmosphere with a relative humidity in excess of 92%. At intervals of 48, 60, and 72 hours, small samples of the cell layers were obtained by aspiration into a sterile Pasteur pipette. These samples were examined as lacto-propionic-orcein squashes to determine the time of appearance of the nearly synchronous first cell divisions.

CELL PRETREATMENT AND FIXATION

When the mitotic index in a sample was seen to exceed about 0.5% (usually at 55-65 hours), 0.15 cc. aqueous solution containing 75 millimicrograms vinblastine sulphate ("Velban" Lilly) was added to the culture to effect metaphase arrest. Incubation was continued for two hours if the sample mitotic index had been high, or for up to six hours if the sample showed a low mitotic index. The cells were harvested by inversion mixing of the culture, decanting the suspension into a conical tipped 12 cc. centrifuge tube, and centrifuging at 70 gravities for five minutes. The supernate was aspirated off and discarded. The cells were washed once in physiological

saline, and suspended in warmed 0.8% sodium citrate dihydrate in distilled water. The suspension was incubated at 37 deg. C. for 8-10 minutes, after which 0.2 cc. of fixative (50% acetic acid, 1% lactic acid, 1% HCl) was added. Mixing was accomplished by inversion; the suspension was centrifuged at 70 gravities for five minutes, and the supernate was aspirated off and discarded. Additional fixative was added dropwise, with mixing by gently blowing through a Pasteur pipette, until a lightly hazy suspension was obtained. This was allowed to stand undisturbed for 20 minutes or more. If further manipulation of the cells was to be delayed for a day or more, the tube was tightly capped and placed in a refrigerator at about 10 deg. C. Prior to further steps, the tube was brought to room temperature and the fixative was replaced with a freshly made solution.

SPECIAL CELL PRETREATMENTS

Attempts were made to enhance the appearance of "secondary constrictions" on the chromosomes obtained from some cultures. Selected cultures from the larger series of replicate attempts were exposed to the action of hyaluronidase (Cal. Biochem.) in concentrations up to 90 units/cc. for periods up to 6 hours immediately before cell harvesting, as suggested by Yerganian (27). Other cultures were washed free of medium, and suspended with the "Velban" in calcium and magnesium free normal saline or Tyrode's solution for periods up to 6 hours. This is a modification of the method of Sasaki and Makino (25). Some cultures, with and without these pretreatments, were fixed according to the method of Saksela and Moorehead (23) instead of the method described above.

CHROMOSOME PREPARATIONS

Semi-permanent squash preparations were made by centrifuging the fixed cells at 70 gravities for five minutes and aspirating off all the

supernatant fixative. The cells were then resuspended in a few drops of 1% natural orcein made in a 1:1 mixture of 50% propionic acid and 50% lactic acid. This stain was best made up as a 2% stock solution which had been refluxed for several hours, cooled, and filtered. Prior to use, the 2% solution was diluted with the mixture of acids to yield a 1% solution. The stain is a modification of that recommended by Dyer (5). A small drop of the stained cell suspension was squashed by thumb pressure on a clean glass slide under a #1 coverslip, and the coverslip edges were sealed with paraffin. Such preparations could be made permanent using the method of Conger and Fairchild (4) by freezing over liquid nitrogen. Alternatively, the stain was occasionally made in 50% acetic acid with 1% HCl; then the freezing was accomplished over "dry ice".

Air-dried preparations were made by placing several small drops of the original fixative cell suspension on a clean #1 coverslip heavily filmed with 5:1 methanol-acetic acid. The coverslip was then quickly but very carefully flooded with additional methanol-acetic acid; care was exercised to maintain the aqueous drops in the center of the coverslip until flooding was complete. The coverslip was drained by touching an edge to filter paper, and it was rapidly dried under warm moving air from a small electric hair dryer. Subsequent staining was by immersion in the 1% lacto-propionic orcein described above. Staining was for periods of 2-6 hours, followed by a rapid rinse in 100% ethanol, dehydration in two changes of 100% ethanol (2-5 minutes each), "clearing" in two changes of toluene (2-5 minutes each), and final mounting in "Permount" (Fisher).

MICROSCOPY

Observations on chromosome number and morphology were made using a Zeiss microscope fitted with an oil-immersion apochromatic objective 100x, N.A. 1.32. Visual work was performed using 16x compensating eyepieces;

photographic work required 8x and 10x Kpl compensating eyepieces. Essentially monochromatic light, contrasting strongly with the orcein stain, was provided by passing high-intensity tungsten light (Koehler method) through a Bausch and Lomb second-order interference filter with a 15 millimicron half-width bandpass centered at 547 millimicrons (green). The entire optical system was frequently checked by observation of selected test diatoms to ensure maintainence of a resolution of 0.25 micron or better.

PHOTOMICROGRAPHY

Photographs of chromosomes were made in an "Orthophot" camera (Silge and Kuhn) on Kodak Panatomic-X #120 roll film, using only the center 1/3 of the object field seen when using oil-immersion optics. Negatives were underexposed about 25% (1/10 to 1/2 second), and overdeveloped about 25% in Kodak Microdol-X. This resulted in an object field density on the negative of 0.6 above film base, and chromosomes of density 0.1-0.2 above film base. The contrast obtained required printing on Kodak Kodabromide F-5 or Agfa Brovira 1, grade 6. Most print enlargements were 4-5 diameters to avoid excessive grain in the final print.

MEASUREMENTS

Individual chromosomes, usually enlarged 2760 or 3550 diameters, were cut from photographs and mounted in order of decreasing length on heavy cardboard. Exact magnifications were determined by comparison with photographs made of a Zeiss stage micrometer. Measurements of chromosomes and arms for absolute size determinations were made on 3550x prints on #5 paper, by "stepping off" length segments with a small bow calipers. Ratios of arms within individual chromosomes, and whole lengths between chromosomes, were obtained. Mean lengths, ratios, ranges, and standard deviations were calculated.

EXAMINATION OF BONE MARROW

An ancillary examination of the bone marrow from a single male mongrel was made. Marrow spicules were obtained from the dog's femur. Three samples were examined: One of squashed material which had been hypotonically swollen and stain-fixed immediately after biopsy; one of material treated as above following 2½ hours exposure to Velban in tissue culture; and one treated as above following 24 hour culture under conditions described previously. Chromosome counts were performed on semi-permanent squashes. The distribution of lengths among 25 X-chromosomes was determined by direct measurement with an ocular micrometer calibrated to 0.5 micron. 200 cells were examined for X-chromosomes in excess of 5 microns.

RESULTS

CULTURE METHODS

Over 160 different cultures, in duplicate, were attempted using blood from nearly 40 different animals. The cultures were classified as failures, acceptable, or excellent, relative to human cell reference cultures, on the basis of the maximum mitotic index observed in the interval from 48 to 84 hours following culture start. Using Hastings' original method (7), 25 cultures on 10 animals were assessed. 36% were failures (mitotic index below about 0.01%), 8% were excellent (mitotic index 1% or more), while the remainder were acceptable (mitotic index between 0.01% and 1%). Homologous serum, especially fetal bovine serum, was added to the medium in 21 culture attempts. 28% were failures; 19% were excellent. The improvement was not statistically significant; however the mitotic index in the acceptable cultures was generally much higher than in the earlier attempts.

Following a series of 45 test cultures, it was found that approximately 6% autologous plasma in the culture medium was optimal, with values from about 2% to 10% being acceptable. For ease of technical manipulation, subsequent cultures were initiated with any concentration of autologous plasma under about 10%. This limitation of autologous plasma in 48 cultures reduced the failures to 17%, and raised the excellent cultures to 46% of the total. The difference relative to cultures with more than 10% autologous plasma is highly significant ($P \cong 0.01$). In 21 additional cultures, all the preceding changes were made, and in addition the medium was changed from TC 199 to NCTC 109. The closed tube with room air gas-phase system was also changed to incubation in a wet 5% CO_2 atmosphere. The proportion of excellent cultures was not significantly increased (48%), but the failures were reduced to less than 5%. The difference, relative to tightly capped cultures in TC 199, is highly significant ($P \cong 0.01$).

EFFICIENCY OF LYMPHOCYTE SEPARATION

It was found that large numbers of granulocytes in peripheral blood cultures yielded overly acid cultures which often failed to show dividing cells in appreciable numbers. Using the granulocyte removal method described, the absolute yields of lymphocytes from canine blood were about the same as from human blood, generally 20-25% of those present in the original whole blood sample. Removal of granulocytes from canine blood was less efficient than from human blood. Final culture inocula were generally above 50% mononuclear cells, sometimes as high as 85-100%. This corresponds to changing the granulocyte : lymphocyte ratio from 3:1 to 1:6. However, a considerable proportion of the inocula had mononuclear cell concentrations below the 50% level, showing little or no lymphocyte enrichment. By contrast, the method described almost always resulted in 85-100% mononuclear cells when applied to human blood. Of the several separation methods tried, the one described was the only one which reliably produced a significant cell yield and good lymphocyte purity with modest effort.

CHROMOSOME COUNTS

Temporary squash preparations on cells of 32 different animals were made as described. A summary of chromosome counts obtained from preparations varying considerably in quality is given in Table 1. In most male cells, the Y-chromosome usually could be identified as the smallest chromosome; less frequently its metacentric nature was also discernable. The spread of chromosome counts is small, all counts being tightly clustered around the modal number of 78 in both sexes. One animal (#10) had a particularly high percentage of cells containing 80 chromosomes. Unfortunately, it was unavailable for further study.

Permanent preparations of cells from 29 animals were made using all the methods described. A few were made using the method of Rothfels and

TABLE 1. SUMMARY OF CHROMOSOME COUNTS PERFORMED ON SQUASH PREPARATIONS.

DOG	SEX	TYPE	Chromosome number ≤74	75	76	77	78	79	80	~4n?	Sex elements
1	M	Purebred beagle	1			1	5			+	XY
2	M	" "	1				6			+	XY
4	M	Boxer	2				18			+	XY
5	M	"					6			+	XY
6	F	Labrador	1		1	2	24				XX
7	M	"					5			+	XY
8	M	German shepherd					9			+	XY
9	M	" "					4			+	XY
10	F	"Toy" shepherd	2				15		3		XX
11	M	Scotch terrier					5				XY
13	M	Airdale					8			+	XY
16	M	Indeterminate				1	7				XY
17	M	"					7				XY
18	M	"	1				3				XY
19	M	"					5				XY
20	M	"					9			+	XY
21	M	"	1				12			+	XY
22	M	"	1				6			+	XY
23	M	"					4				XY
24	M	"					3				XY
25	M	"	4				32			+	XY
27	F	"					5				XX
28	F	"					6				XX
29	F	"	1				4				XX
30	F	"	1				7		1	+	XX
31	F	"					2				XX
32	F	"					5				XX
33	F	"					5				XX
Lymphatic irradiation and female marrow or spleen cell transplants two months to one year prior to lymphocyte culture:											
35	M	Purebred beagle					6				XY
36	M	" "					3				XY
37	M	Indeterminate					4				XY
24	M	"	1				12				XY

Notes:

1) Dogs 5, 24, and 27 commonly displayed one or more autosomes with distinct short arms.

2) Dog 10 displayed very prominent secondary constrictions on many of its X-chromosomes.

TABLE 2. SUMMARY OF CHROMOSOME COUNTS PERFORMED ON AIR-DRIED PERMANENT PREPARATIONS.

DOG	SEX	TYPE	chromosome number ≤ 70	71	72	73	74	75	76	77	78	79	80	~4n	no count	total cells	sex elements
1	M	Purebred beagle	3							1	19			1	6	30	XY
2	M	" "	3		1			1			17				8	30	XY
3	M	Beagle	3	1		1		1	2		15			2	5	30	XY
4	M	Boxer	2		1						12	1			4	20	XY
5	M	"	3					1		1	18			4	18	45	XY
6	F	Labrador	10	1		1	1				10			1	41	65	XX
7	M	"	4								29			1	6	40	XY
8	M	German shepherd	2								18		1	1	9	30	XY
9	M	" "	12	1		2		1	1		13			1	60	91	XY
10	F	"Toy" shepherd	4		2	1	1	1	2	1	31		3		17	63	XX
11	M	Scotch terrier	9	1		1	1			1	3				28	44	XY
12	F	" "	2			1		1	1	1	19			1	2	28	XX
13	M	Airdale	6	1			1		1		14				7	30	XY
14	F	Cocker spaniel	6		1	1	1			2	13			1	3	28	XX
15	M	" "	3				1	1		2	18				2	27	XY
19	M	Indeterminate									2				2	4	XY
20	M	"	4					1		1	8	1			19	33	XY
21	M	"	7			1					16			1	15	40	XY
23	M	"	3	1					1		5				46	56	XY
25	M	"	2								16			1	4	23	XY
26	M	"	1	1	1	2		1	2		16		1		6	31	XY
27	F	"	5				1		1		23				52	82	XX
29	F	"	8		1			1	1		6			1	39	57	XX
30	F	"	2			1					2				8	13	XX
32	F	"	5		1		1				10			1	39	57	XX
34	F	"	2	1					1	1	20			1	4	30	XX
Lymphatic irradiation and female marrow or spleen cell transplants two months to one year prior to culture:																	
36	M	Purebred beagle	8						1	1	40				not recorded		XY
37	M	Indeterminate	19		1	1					6				23	50	XY
24	M	"	8				1				5				26	40	XY
		TOTALS:	146	8	9	13	9	10	14	12	424	2	5	18	499+	1117+	

Siminovitch (22). The latter has become standard among those who use cell culture for chromosome studies, but it was found largely unsuited to study of canine lymphocyte chromosomes. While a few good preparations were obtained with this method, most demonstrated shrunken cells and a high percentage of broken cells. Cells having enumerable chromosomes constituted only about 40% of all metaphase cells, as compared to nearly 70% using any of the methods described in the preceding section. A summary of chromosome counts obtained from permanent air-dried preparations obtained with all methods is given in Table 2. Counts are less tightly clustered around the modal number of 78 than are the counts obtained from squash preparations. The percentage of broken cells (less than 71 chromosomes) has about doubled. Again, dog #10 showed a surprising number of cells with 80 chromosomes.

Counts performed on both types of preparation showed a significant proportion of tetraploid cells. In the permanent preparations the frequency of tetraploid cells is about 1.5% of all metaphase cells. In certain cultures the frequency of tetraploids approached 10% of all metaphase cells.

Preliminary experiments of the sort described in the Introduction were carried out using animals which had been irradiated and homotransplanted with methods not described here. Successful cultures were obtained infrequently, but karyotypes obtained from them were normal. Consequently, chromosome counts performed on cells from these animals are included in both Table 1 and Table 2.

THE CANINE KARYOTYPE

Using the methods outlined, photographs were made of well-spread metaphase cells. The chromosomes were cut out and aligned as described. Photographs of a female and a male cell, with sets of serially arranged chromosomes from each cell, are reproduced as Figures 1 and 2, respectively. These are entirely representative of the quality of preparation obtainable with the

methods outlined. No attempt was made to select cells showing the special chromosomal features to be described, and the cells displayed do not show them.

It can be seen that the number of chromosomes in both sexes is 78, of which 76 are acrocentric autosomes with an essentially continuous gradation of sizes. The first autosome pair (#1) is an exception, being noticeably longer than the others. It can be identified easily in every metaphase cell. The sex chromosomes of the female are two large submetacentric chromosomes, while in the male they are one large submetacentric chromosome similar to those of the female, and a very small nearly metacentric element. The latter does not show clearly in Fig. 2, but its morphology is evident in Fig. 7 and Fig. 8.

COMPARISON OF DOG VARIETIES

The 37 animals examined included examples of eight identifiable breeds, of which one was purebred. All had the same modal chromosome number, and all displayed similar karyotypes. No significant differences in respect to size or morphology were apparent among pairs of identifiable chromosomes obtained from any animal. No variety displayed uniquely different chromosomes. The varieties did not differ in possession of details of chromosome morphology described below. The possibility that varieties might differ in respect to the frequency with which these details are displayed, is not excluded.

"GIANT" CHROMOSOMES

Dog #10 had a low but consistent frequency of 80 chromosome cells in all preparations examined. In addition, one of these cells, and a cell fragment, had chromosomes which were approximately twice normal in length, with normal arm widths. The intact cell containing 80 "giant" chromosomes is shown in Fig. 3 at the same magnification as Figs. 1 and 2.

CHROMOSOME MORPHOLOGY AT METAPHASE

In both squashes and air-dried preparations, the X-chromosome was sometimes seen to have a prominent achromatic zone in the long arm close to the centromere. It was very rarely seen when the air-drying method of Rothfels and Siminovitch was used, but appeared with a low frequency using the air-drying method described above. It was seen less often in male cells than female cells, and in most female cells it was seen usually on only one chromosome. Three X-chromosomes with the achromatic zone are shown in the top row of Figure 8.

The Y-chromosome was usually the smallest chromosome in cells from male dogs. In most good preparations it could be seen as a nearly metacentric element. Its morphology was especially evident at very late prophase or very early metaphase, when chromosome contraction was not pronounced. Photographs of the nearly metacentric Y-chromosome are given in the second row of Figure 8.

Chromosome #1, the longest autosome, often showed one or two achromatic zones. The chromosome segment separating these zones often stained more heavily than the remainder of the chromosome. One achromatic zone was nearly terminal, while the other was more nearly medially located. Photographs of #1 chromosomes with one or the other acromatic zone, as well as both together, are shown in the third row of Figure 8.

One or more of the longer autosomes occasionally displayed very short arms in addition to the usual long arms. It was believed that this was a constant feature of a particular autosome. An attempt to identify this chromosome on the basis of a statistical analysis of length measurements is described in a later section. It has been provisionally labelled as autosome "a"; photographs are given in the fourth row of Figure 8.

In certain exceptionally favorable mid-prophase cells, satellite bodies were seen on a middle-sized chromosome. Careful search of a large

number of metaphase cells revealed a very small fraction which displayed a single chromosome pair having satellite bodies on the non-centric terminus of the chromosome arm. An unusually clear demonstration of these satellite bodies at metaphase is the photograph given as Figure 6, and as the last row of Figure 8. The chromosome has been provisionally labelled "s".

Certain other autosomes, notably one about the size of the "s" autosome, also displayed short arms additional to the long arms. This feature was inconstant to a marked degree. Achromatic zones were also seen on autosomes other than #1, but with frequencies too low to permit their use as distinquishing features.

ENHANCEMENT OF SECONDARY CONSTRICTIONS

None of the special methods outlined produced any improvement in clarity or frequency of appearance of the features described in the preceding section. The fixation method of Saksela and Moorehead might have produced an effect. If produced, the effect was generally obscured for reasons to be discussed.

CHROMOSOMES PRIOR TO METAPHASE

The effects of most stathmokinetic agents, including "Velban", are such as to cause over-contraction of chromosomes with prolonged exposure. In an attempt to visualize more clearly internal details of chromosome morphology, cells in mid-prophase and late prophase were observed. The chromosomes were most often severely tangled, and homologies could not be deduced; however, in some cells individual chromosomes could be identified.

The X-chromosome was sometimes seen to have terminal knobs and extended negatively heteropyknotic regions on either side of the centromere. Occasionally in female cells, one X-chromosome was precociously condensed. The Y-chromosome of males often displayed its nearly medial centromere, and it was sometimes precociously condensed.

Most autosomes had patterns of differential staining. Some had pronounced terminal knobs; others had positively heteropyknotic regions medially located. Cells were found in which three or more autosomes had distinct subterminal centromeres, hence autosomes other than "a" must have short arms. In an occasional preparation, the satellites on chromosomes were very clearly displayed, as in the photograph in Figure 5.

TETRAPLOIDY

From Tables 1 and 2 it is clear that a significant percentage of cultures contained tetraploid cells. Actual counts of chromosome number were not performed on most of these cells. Where they were performed, the chromosome number was seen to be 156 (4n) with a sex chromosome complement exactly double that expected on the basis of the animal's phenotypic sex. A photograph of the 156 chromosomes in a tetraploid cell from a male dog is shown as Figure 4. Two X-chromosomes can be seen, and one Y-chromosome. The other Y cannot be identified unequivocally. In cultures exposed to a mitotic poison, the frequency of tetraploid cells ranged from 0.1% to as high as 10% of all cells in mitosis. Interestingly, binucleated cells were also frequent in cultures having an appreciable tetraploid metaphase incidence. The actual frequency of binucleated cells in any given culture was not usually below 0.1%, and often was higher.

CHROMOSOME MEASUREMENTS

Five animals which had yielded very good chromosome preparations were selected for close analysis. Measurements of their chromosomes are summarized in Table 3. The X-chromosome is the longest of the complement, closely followed by autosome #1. The smallest chromosome is the Y-chromosome. Actual chromosome lengths ranged from 0.7 microns to 4.3 microns, with a mean for the longest (the X) of 3.1 microns.

The X-chromosome was found to have an arm ratio (long arm/short arm) of

TABLE 3. SUMMARY OF CHROMOSOME LENGTH MEASUREMENTS AND RATIOS.

	X	Y	#1	"a"	"s"	$\frac{X_l}{X_s}$	$\frac{\#1}{X}$	$\frac{"a"}{X}$	$\frac{"s"}{X}$
Mean length	3.1 microns	0.85 microns	2.9 microns	2.3 microns	1.4 microns	--	--	--	--
Mean ratio	---	---	---	---	---	1.33	0.97	0.696	0.46
Std. deviation	---	---	---	---	---	±0.55	±0.10	±0.045	--
Std. deviation (% mean ratios)	---	---	---	---	---	~41%	~10%	~7%	--
Range	2.3 - 4.3 microns	0.7 - 1.0 microns	2.1 - 4.3 microns	1.9 - 2.4 microns	1.2 - 1.7 microns	1.1-1.8	0.8-1.1	0.63-0.76	0.45-0.49
Number of chromosomes	51	6	67	15	5	39	39	15	5
Number of cells	36	6	36	10	4	39	39	10	4
Number of dogs	4	2	4	3	2	4	4	3	2
Length as % of female haploid set (calculated from 3 cells)	~ 5.6%	~ 1.6%	~ 5.3%	~ 3.9%	~ 2.5%	--	--	--	--

Notes: 1) The length of the Y-chromosome is approximately 29% that of the X.

2) The X-chromosome centromere index is approximately 0.43.

3) The "s"-chromosome satellite region measures approximately 0.4-0.5 microns in length.

4) The "giant" X-chromosomes ranged in length from 6.1 to 6.4 microns, or about twice normal length.

about 1.3, with a large standard deviation. The ratio of lengths of two chromosomes from within the same cell (X and #1) was quite constant.

In Table 3, in the column headed $\frac{\text{"a"}}{X}$, an attempt has been made to identify a specific autosome, provisionally designated "a". In each of ten cells containing an obvious autosome with short arms additional to the long arms, a ratio of lengths between the X and #1 was obtained, and also between the autosome in question and the X. The low variability of the "a":X ratio relative to the #1:X ratio was taken as evidence that the same autosome "a" was being measured in each cell. In the fourth row of Figure 8 are photographs of the pair of "a" autosomes from a single cell.

A set of measurements similar to those above was obtained for the satellited chromosome designated "s". The number of chromosomes measured was too small to yield a meaningful standard deviation, but the total range was itself a small fraction of the mean. This provided evidence that the same chromosome was satellited in each cell.

BONE MARROW EXAMINATION

Relative to questions raised during the course of the work described above, which will be discussed, the bone marrow of a single male dog was examined. With respect to the deleterious effects of "Velban" on chromosome morphology, the cells of the bone marrow proved more sensitive than cultured lymphocytes. When "Velban" was applied to marrow cell suspensions in concentrations lower than normal for lymphocytes, for periods of time shorter than used for lymphocytes, metaphase chromosomes appeared as fuzzy separated chromatids. This effect was seen only rarely in lymphocytes cultured as described. 24-hour marrow culture did not appreciable raise the marrow's normal mitotic index, nor did it aid in decreasing the cells' sensitivity to "Velban". For these reasons, direct squash of bone marrow cells proved most satisfactory.

Of 25 cells whose chromosomes were examined in detail, 22 displayed the normal male karyotype with 78 chromosomes. One cell had 80 chromosomes; the remainder had been fragmented by squashing, and had many fewer than 78 chromosomes. 25 X-chromosomes measured 2.5 to 4.5 microns in length, with a mean of 3.2 microns. 200 X-chromosomes were checked for unusual length; none were over 5 microns. Those near this figure were in very early metaphase, and had a distinctive appearance different from that of the "giant" chromosomes described previously.

DISCUSSION

CULTURE PREPARATION AND METHOD

Andersen (2) has found that the dog has a concentration of granulocytes in peripheral blood which is roughly twice that found in humans. There is no correspondingly large increase in the lymphocyte concentration. In canine peripheral blood cultures, the presence of large numbers of granulocytes was correlated with high acidity as evidenced by changes of the pH indicator within the media. They also tended to form adherent sheets of cells on the walls of the culture vessel, trapping many of the other cells. This sheet of cells tended to form a clot-like mass during cell harvesting, and made subsequent slide preparation difficult. For these reasons, as well as to obtain a "pure" cell type for culture, peripheral blood samples were treated to remove granulocytes as thoroughly as possible. The method described, essentially that of Hastings, et al (7), proved to be the best for canine cells of several which were tried. Certain other methods gave a higher cell yield, but lymphocyte purity was more inconstant and yield lower than with Hastings' method.

Numerous attempts to use the Phytohemagglutinin method of red cell removal were unsatisfactory. High concentrations of the material were necessary for canine blood. The red cell separation was not as good, nor were the white cell rich plasma yields as high, as when the method was applied to human blood. There are numerous reports in the literature of poor results with this method when it is applied to certain species other than man. To these species the dog must be added.

The addition of fetal bovine serum to the medium did not produce a significant change in cell transformation when used in cultures containing more than 15% autologous plasma. However, in cultures having less than 15% autologous plasma, deletion of fetal bovine serum produced a typical adverse

effect. There was a high cell death rate, a low rate of cell transformation, and a delayed and much reduced mitotic peak. For this reason, and because of its content of fetuin and other growth factors, fetal bovine serum was considered a necessary component of canine lymphocyte culture media.

Limitation of autologous plasma to less than 10% of the medium produced dramatic results. When higher levels were used, there was frequently a much reduced cell transformation rate, and a lower mitotic index, than when autologous plasma levels were kept below 10%. The reason for this phenomenon was not clear. However, inspection of centrifuged dog plasma showed it to be more turbid than human blood, and especially so shortly after meals. Microscopically, and with histochemical methods, large quantities of colloidal fats could be demonstrated in dog plasma. The colloid was often sufficiently concentrated in the plasma to impart a distinct turbidity to the final culture medium. In turbid cultures standing undisturbed for one or more days, a layer of fats often accumulated at the liquid surface. When animals had been fasted for 24 hours or more, all these effects were greatly reduced, and the cultures were found to tolerate higher concentrations of autologous plasma. It is believed therefore that one of the substances in dog plasma which is inhibitory to peripheral blood cultures is associated with colloidal fat.

The use of a CO_2 atmosphere provided more satisfactory pH control in the medium than was obtained with the conventional closed tube system. Residual concentrations of granulocytes after the removal step were often rather high in canine cultures. In tightly capped tubes they caused a rapid reduction of the medium pH to unacceptable levels.

Medium NCTC 109 gave better results than the conventional TC 199, not only with canine cultures, but with human cultures as well. The effect is probably related to the greater complexity and completeness of NCTC 109. It also contains a high concentration of Tween 80. This may be advantageous in increasing the dispersion of the chylomicra which are present in quantity in

canine plasma.

CHROMOSOME PREPARATION

To effect metaphase arrest in mitotic cells, "Velban" was used in place of the more common colchicine or Colcemid. It has been found effectual in producing metaphase arrest when the concentration is 1/100 or less of that necessary when colchicine is used. Further, it appears that the over-contraction produced in chromosomes by extended exposure to "Velban" is less severe than the effect produced by colchicine.

It was found that any fixative containing appreciable concentrations of alcohols was unsuitable for canine lymphocytes. Cells exposed to the usual 1:3 acetic-methanol were shown to shrink appreciably after a short time in the fixative. Worse, they became quite "sticky", and would adhere to each other and even to the walls of siliconized containers. The consequent loss of cells was excessive. When the cells were fixed in aqueous acid fluids, the stickiness was greatly reduced. The chromosome swelling often associated with fixation in acetic acid was reduced by the addition of HCl and lactic acid. For squashes, the lactic acid proved advantageous because of its softening effect.

In the chromosome spreading method described, the stickiness caused by alcohols is used to advantage. Cells carried onto the coverslip in the aqueous fixative adhere quickly and firmly to the glass upon flooding of the coverslip with the alcoholic fluid. Additionally, although air-drying is possible when aqueous fixatives are used alone, the dilution and replacement of the aqueous phase by an alcoholic phase permits rapid air-drying.

The lack of apparent effect when using Saksela and Moorehead's method for accentuation of secondary constrictions may be an artifact. Excessive loss of cells in their alcohol containing fixative, as well as cell clumping and consequent poor chromosome spreading and staining, could easily obscure

the infrequent appearance of secondary constrictions. The fixative described in this paper is similar to that of Saksela and Moorehead, in that both contain about 50% acetic acid as the principle component. The achromatic zones on certain chromosomes which are reported in this paper may therefore be the same as might have been produced by Saksela and Moorehead's fixative.

THE CANINE KARYOTYPE

The karyotypes of 37 dogs, including eight breeds, were compared. All showed the basic modal chromosome number of 78, and all had similar chromosome complements. With respect to the specifically identified chromosomes (X,Y,#1, "a"), no differences could be found among the eight breeds, or among the mongrels. The satellited chromosome was considered to be rare for technical reasons related to metaphase contraction and inadequacies of the preparative method. The constancy of the canine karyotype in the eight breeds examined is not surprising in view of the ease with which fertile dog hybrids are formed. It is possible that an unusual karyotype may be found in an uncommon breed, perhaps one of the very small or very large varieties. These animals are generally maintained by breeders and fanciers, and are isolated reproductively from other breeds through human intervention. Fixation of genetic, and possibly chromosomal, differences would be more probable in these isolated breeds than in the dog population at large. Investigation of this question is in progress.

In this connection, the mongrel karyotypes obtained in this study are important. Each mongrel is a hybrid resulting from the union of at least two different breeds. The 22 mongrel karyotypes thus represent samples of the karyotypes of 44 dogs, none of which contributed anything unusual to the hybrid karyotype. However, from the animals' configurations, it was evident that breeds as unusual as the Mexican Hairless or the St. Bernard were not represented.

CHROMOSOME MORPHOLOGY

Excellent chromosome preparations and high resolution microscopy are requisite for routine display of the morphology of the Y-chromosome of the dog. With an average overall length of 0.9 micron, each arm of the Y is less than 0.5 micron long. The width of the arms is equal to or greater than 0.2 micron. Geometric considerations indicate that even when the arms are maximally separated, the arm tips are separated by a distance close to the resolution limits of ordinary light microscopy. The preparation schedule described has yielded the necessary high quality on a routine basis. It is therefore certain that the assumptions of Reiter, et al, and of Moore and Lambert, are correct with respect to the metacentric nature of the Y.

The infrequently observed, but very prominent, achromatic zone on the canine X-chromosome has special interest in light of the old observations of Rath, Malone, Painter, and Makino. Their association of the X-chromosome with the nucleolus may have a basis in fact. Secondary constrictions are known to be frequent sites for the nucleolar organizing region (13, p.372). The prominence of the achromatic zone on the X suggests this association.

On the other hand, the nucleolar organizer is often associated with the satellites present on some chromosomes. The demonstration of a satellited chromosome within the autosomal complement of the dog would imply the presence of a nucleolar organizer on that chromosome. The question of the location of the nucleolar organizer could not be answered. In most prophase cells, the nucleolus could not be visualized, even with carmine or the various Romanovsky stains.

The probable autosomal short arms remarked upon by Awa, et al, and by Moore and Lambert, are real. This study indicates the possibility of using these features for identification of some autosomes. However, the attempt to characterize one such autosome is subject to a number of counter-arguments. Perhaps the most damaging arises from the fact that the autosome

in question is placed within the first 6-10 pairs after #1 on the basis of length. These first pairs are all much alike in length, and they may be identical. If this is true, then any one of them could display short arms and be indistinguishable from the autosome labelled "a". It is thought that the low variability in the length of the designated chromosome relative to an intracellular standard (the X) would indicate that only one specific chromosome was being measured. If two or more different chromosomes were being confused and identified as the same, the variability in length relative to the standard should become fairly large. From Table 3, it may be seen that when "a", #1, and the X, are all measured in the same cell, the ratio of "a" to X is less than the ratio of #1 to X. The variability in the arm ratio of the X is not a suitable standard, as the comparison has been made between cells, not all of which were at the same mitotic stage.

TETRAPLOIDY

In peripheral blood cultures, cell division is a stimulated process and is therefore nearly synchronous at first division. The wave of first divisions is quite sharply peaked in a period of a few hours. In canine lymphocyte cultures, the peak occurs 60-65 hours after stimulation, sometimes as early as 48 hours after start. Tetraploids with the metaphase separation of two chromatids are seen at this first division. Aberrant division *in vitro* would require two division cycles to give this picture, and thus cannot account for it. The frequency of tetraploids is too high to be accounted for by the tiny fraction of cells in whole blood which might have been in mitosis at the time of sampling, before stimulation. Tetraploids must therefore arise in canine lymphocyte cultures without prior nuclear division. The natural frequency of tetraploids in these cultures, without a mitotic poison, has ranged from 0.1% to about 1%. This corresponds closely to the observed frequency of binucleated cells, which is seldom much lower than about 0.1%.

In their turn, binucleated cells in culture cannot be accounted for by the small numbers which exist in normal peripheral blood. In the peripheral blood of normal dogs, the natural frequency of binucleated lymphocytes is very low, about 0.001% or less. The binucleated cells seen in these cultures must therefore have arisen in vitro prior to the first wave of divisions.

It may be that the binucleated cells arise by cell fusion. Synchronous division of the two nuclei would yield an appearance of true tetraploidy, especially in squashes of hypotonically swollen cells, or in air-dried preparations. Mazia (13, pp.388-390) has stated that in certain species with binucleated cells common in vivo, synchronous division of the two nuclei is the rule rather than the exception. This hypothesis that the tetraploids are a product of binucleated cells created by fusion of two cells finds added support in the finding of a single late prophase cell with two groups of chromosomes, each about the diploid number.

The process of cell fusion may find a useful application in investigations on the cellular immune response. If the hypothesis advanced above is correct, binucleated cells should have the nuclear and cytoplasmic contents of two different cells. This author has successfully cultured cells from two different dogs simultaneously in the same medium. The tetraploids produced should include some with the contents of two genetically dissimilar cells. Such cells may prove to have unusual and informative immunological capabilities if there is such capability at all. The binucleated cells in canine lymphocyte cultures are sufficiently common to make such investigations feasible.

80 CHROMOSOME CELLS

Several animals displayed cells with 80 chromosomes, especially dog #10. Most such cells were indubitably intact; random acquisition of one or more chromosomes through procedural error seems ruled out. This is also the case for the cell with "giant" chromosomes, as all those chromosomes were

oversize. It seems possible that some dogs may be mosaics, possessed of a cell line with an atypical karyotype as well as the normal cell line.

"GIANT" CHROMOSOMES

The cell with "giant" chromosomes is particularly interesting. A few cell fragments seen by Hare (6) in 1964 also displayed "giant" chromosomes. In the experiment described, and in that of Hare, a considerable proportion of granulocytes was present in the culture. The possibility that the unusual cells might be representative of cells other than lymphocytes prompted an examination of canine bone marrow. 200 bone marrow cells were examined for "giant" chromosomes; none were found, and therefore their frequency must be below about 0.005 if they occur normally at all.

There is reason to believe the cells with "giant" chromosomes obtained from only 1 of 37 dogs (2 cells out of a total of almost 2000) are not a normal finding. In The Sandoz Atlas of Hematology (pp. 20, 23, and Fig. 35C) (24), giant chromosomes are described as occasional findings in pathological giant pronormoblasts from a case of human "hemolytic jaundice". The text considers these oversize chromosomes to be rare, and to be found only in pathological conditions. This, and the rarity of the cells in the dog, suggest the finding of "giant" chromosomes in canine peripheral blood cultures is a rare aberrant event, not typical of any cell line or variety of dog.

SUMMARY

1. An improved culture method satisfactory for the routine culture of canine peripheral blood lymphocytes has been presented.
2. A new method for producing high quality air-dried chromosome preparations has been described.
3. Confirmation of the canine modal chromosome number of 78 has been obtained for eight identifiable breeds, one of which was purebred, and for nineteen mongrels.
4. Karyotypes are presented which are typical of male and female mongrel dogs. These confirm the work of other authors.
5. Details of chromosome morphology have been presented for five different chromosomes. The X has a prominent achromatic zone, or secondary constriction, on its long arm. #1 autosome has two such zones. A short autosome has satellite bodies at the terminus of the long arm. A longer autosome, or several, has distinct short arms. All these characters are seen with a low frequency at metaphase. The Y chromosome is definitely nearly metacentric.
6. Chromosome lengths and arm ratios are presented. Chromosomes range in length from 0.7 to 4.3 microns. The arm ratio of the X is 1.33.
7. The appearance of tetraploid cells in cultures of canine lymphocytes is discussed.
8. An unusual cell with 80 "giant" chromosomes is presented and discussed.

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ADDENDUM

In mid-1964, Gustavsson reported the karyotype of the dog (1). A dextran sedimentation procedure was used to obtain white cell rich plasma. All the autologous plasma was replaced by fetal calf serum for culture of cells. Fixation was in a fixative similar to that reported above, and cells were examined as squash preparations. The karyotype reported is essentially that obtained by other workers, and does not include the detail reported in this paper. It is the only report of a canine karyotype obtained from peripheral blood which is known to this author.

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FIGURE LEGENDS

Fig. 1 : Karyotype of female dog #10 (indeterminate type), with the metaphase cell from which it was derived. Air-dried; 2760x.

Fig. 2 : Karyotype of male dog #5 (Boxer type), with the metaphase cell from which it was derived. L-P-orcein squash; 2760x.

Fig. 3 : 80 "giant" chromosomes in cell from female dog #10. The magnification is the same as in Figs. 1 and 2. Air-dried; 2760x.

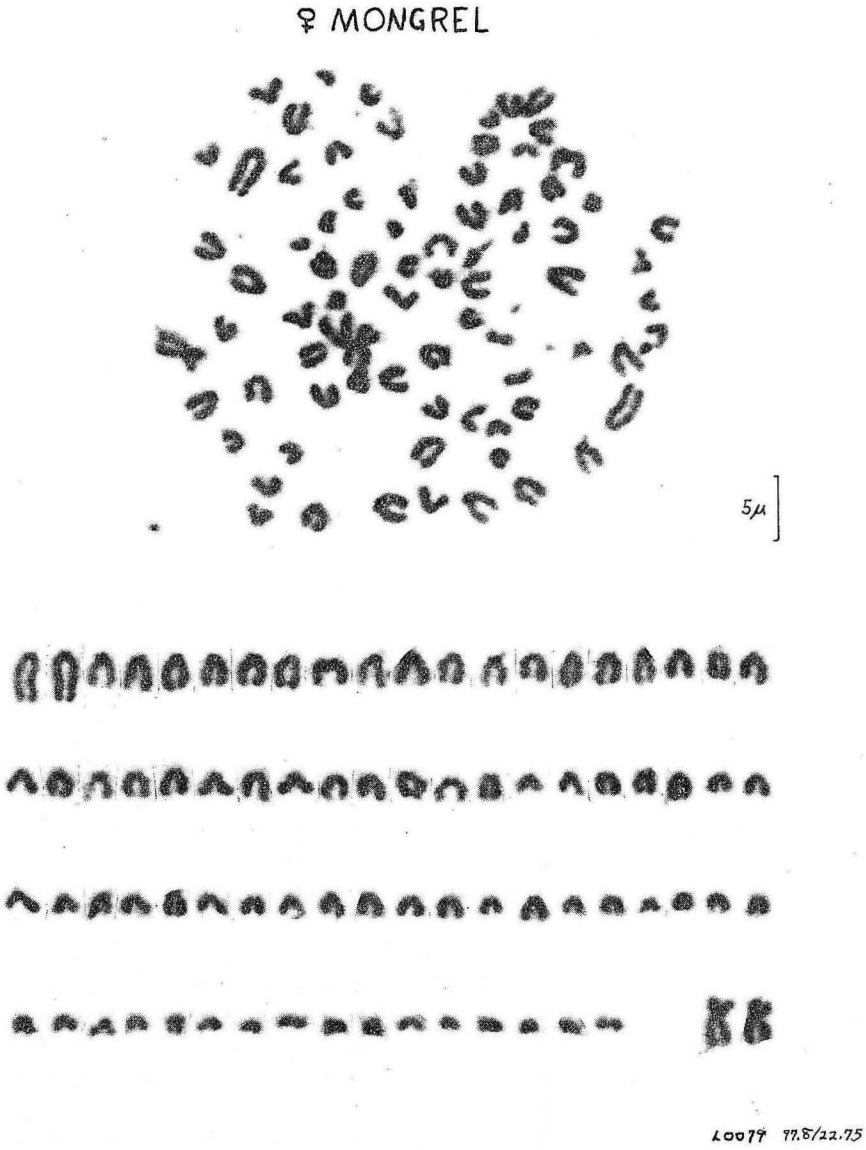
Fig. 4 : Tetraploid metaphase from male dog #5. Two X-chromosomes are circled; the arrow points to one Y-chromosome. 156 chromosomes (twice the diploid number) are visible. L-P-orcein squash; 2760x.

Fig. 5 : Prophase from male dog #5. The arrow indicates satellite bodies on a medium-length chromosome. L-P-orcein squash; 3550x.

Fig. 6 : Part of metaphase cell from female dog #34. The circle indicates satellite bodies. Air-dried; approx. 4000x.

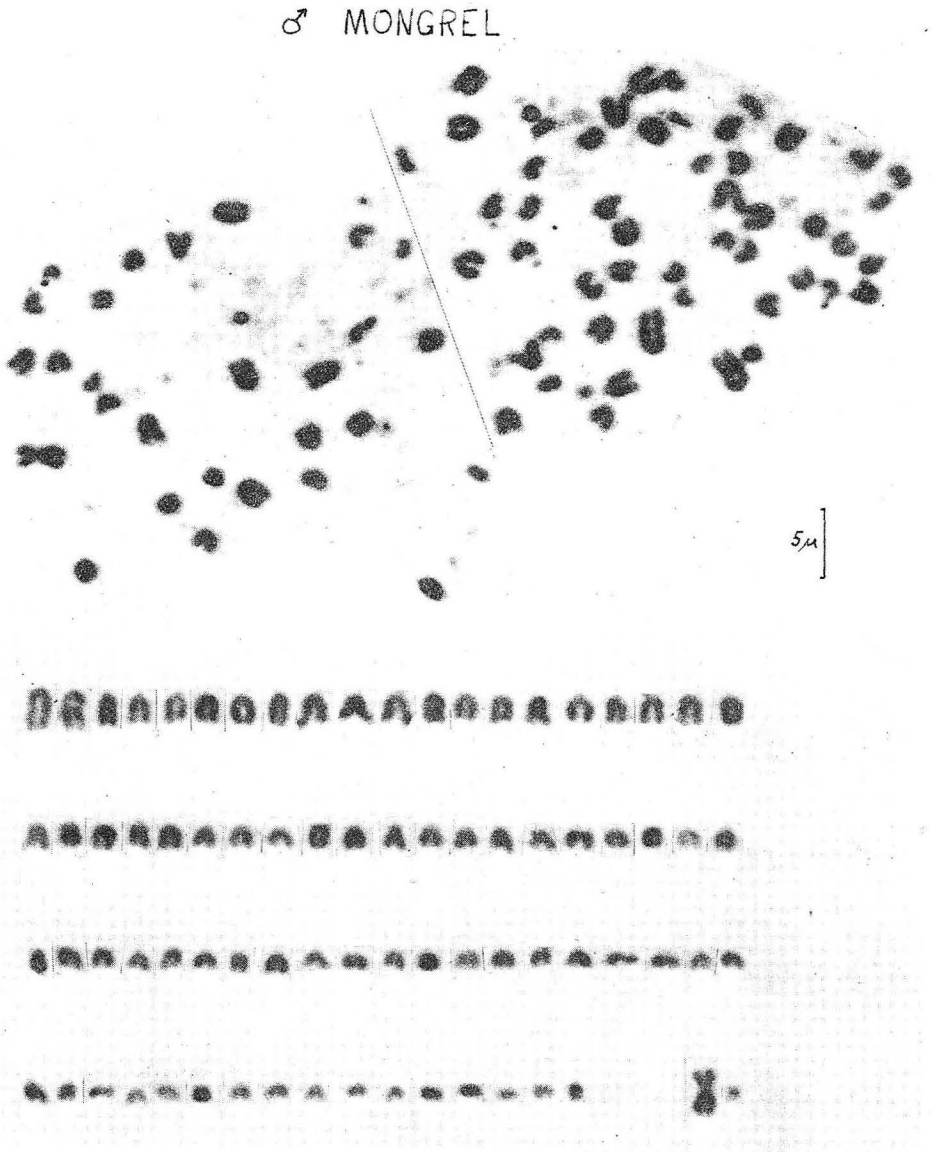
Fig. 7 : Metaphase cell from male dog #25. The Y-chromosome is circled, and appears as the only small metacentric element. Air-dried; 3550x.

Fig. 8 : Morphological detail of five distinctive canine chromosomes. At the right of each row of photographs is a diagrammatic interpretation. In the diagrams, arrows indicate centromere position; light areas indicate achromatic zones. Detailed description in the text. From L-P-orcein squashes and from air-dried preparations; all about 3550x.



ZN-4561

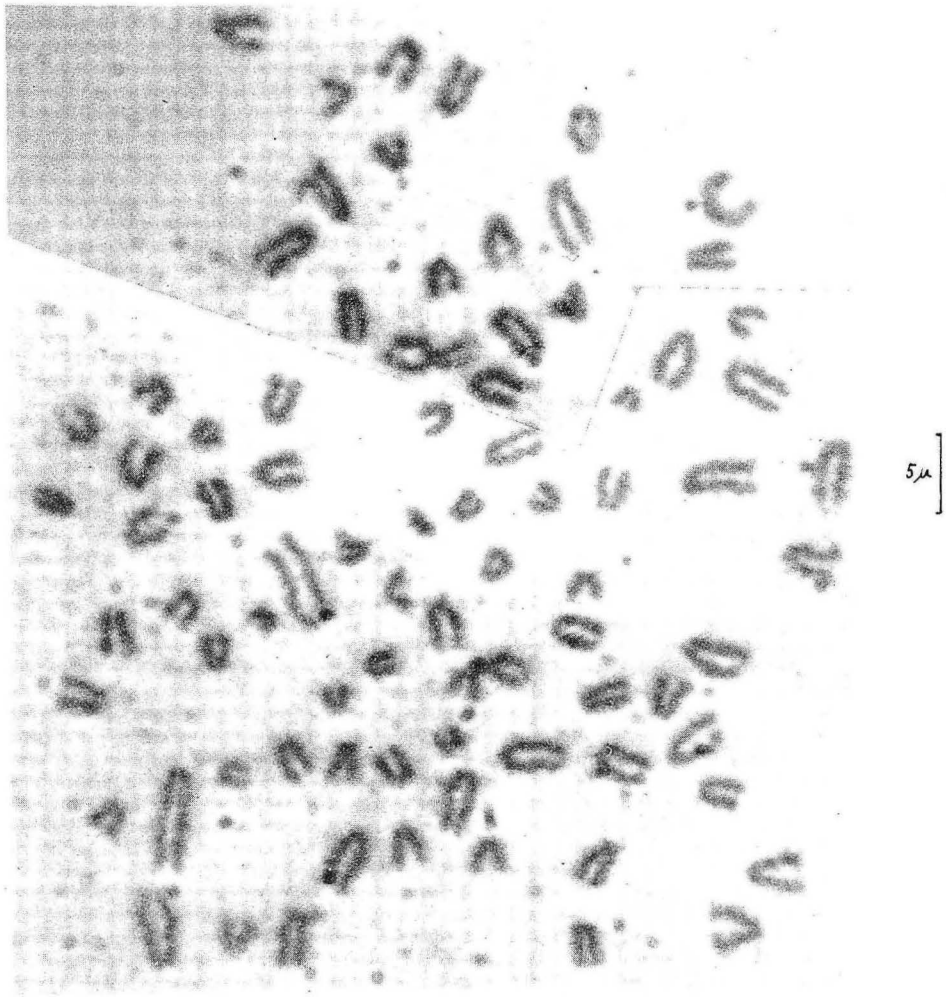
Fig. 1.



ZN-4562

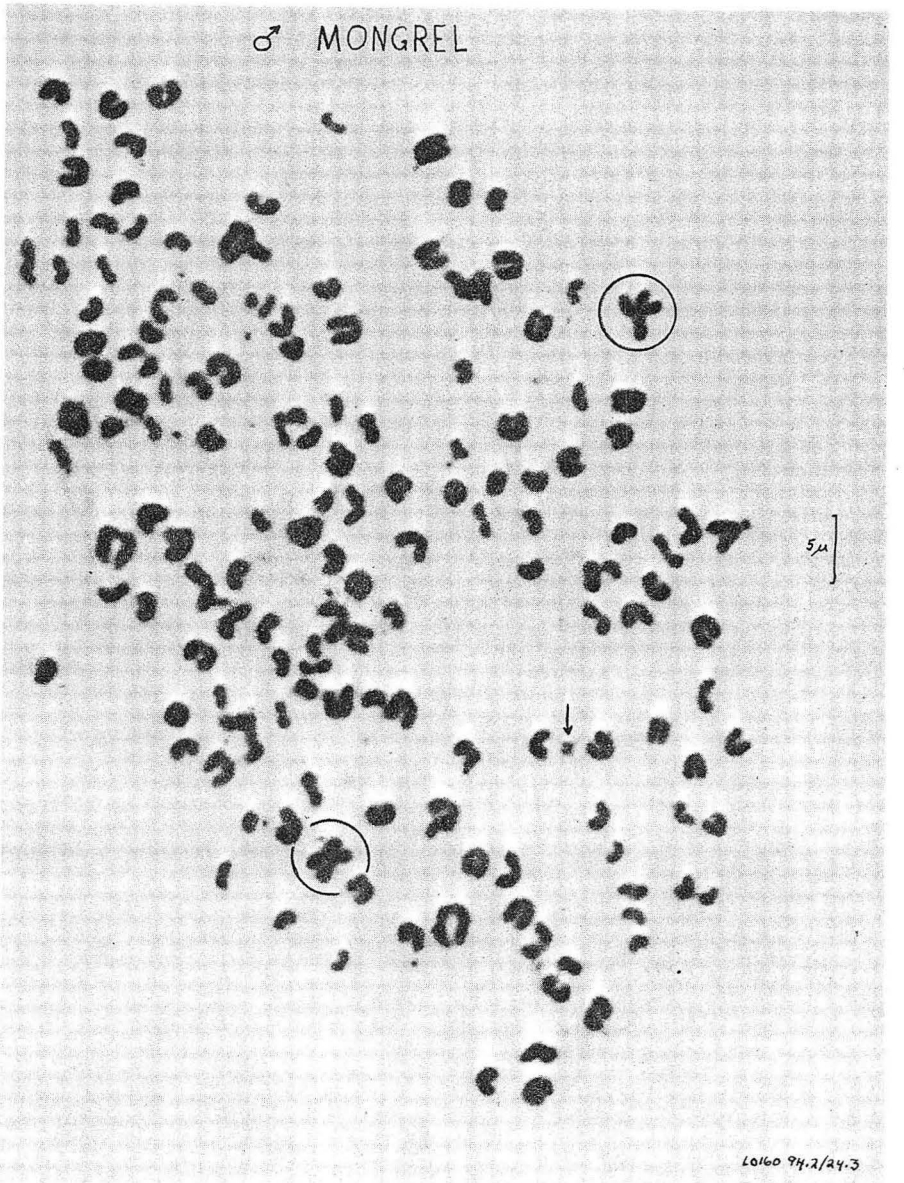
Fig. 2.

♀ MONGREL



ZN-4563

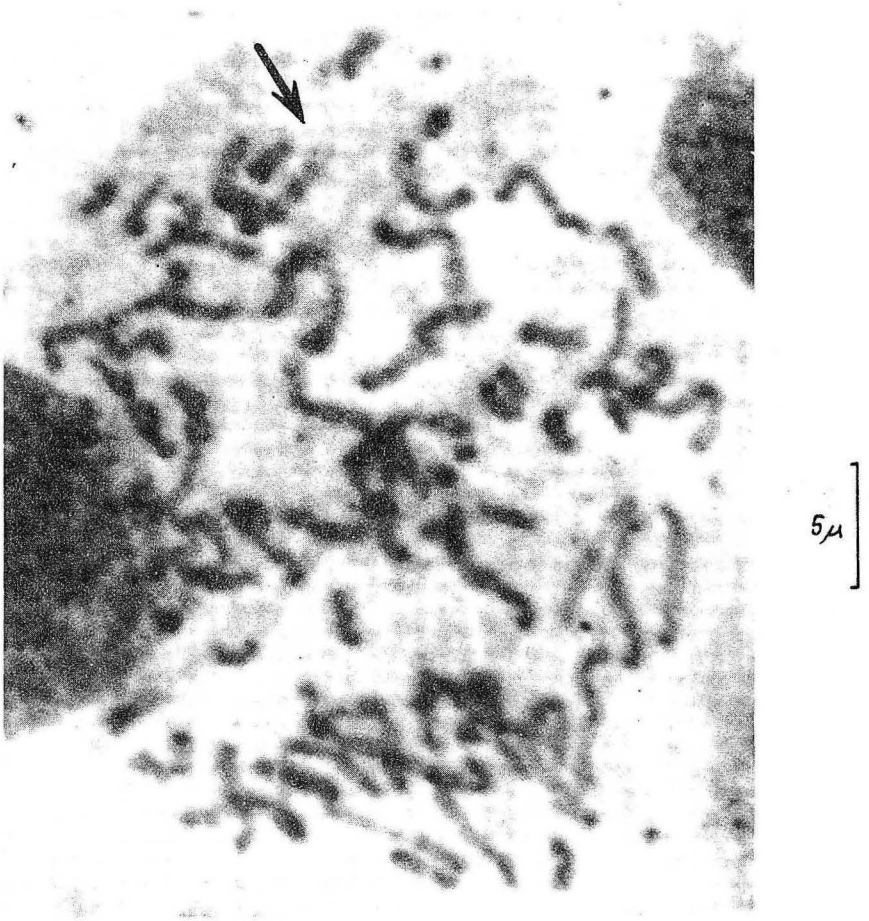
Fig. 3.



ZN-4564

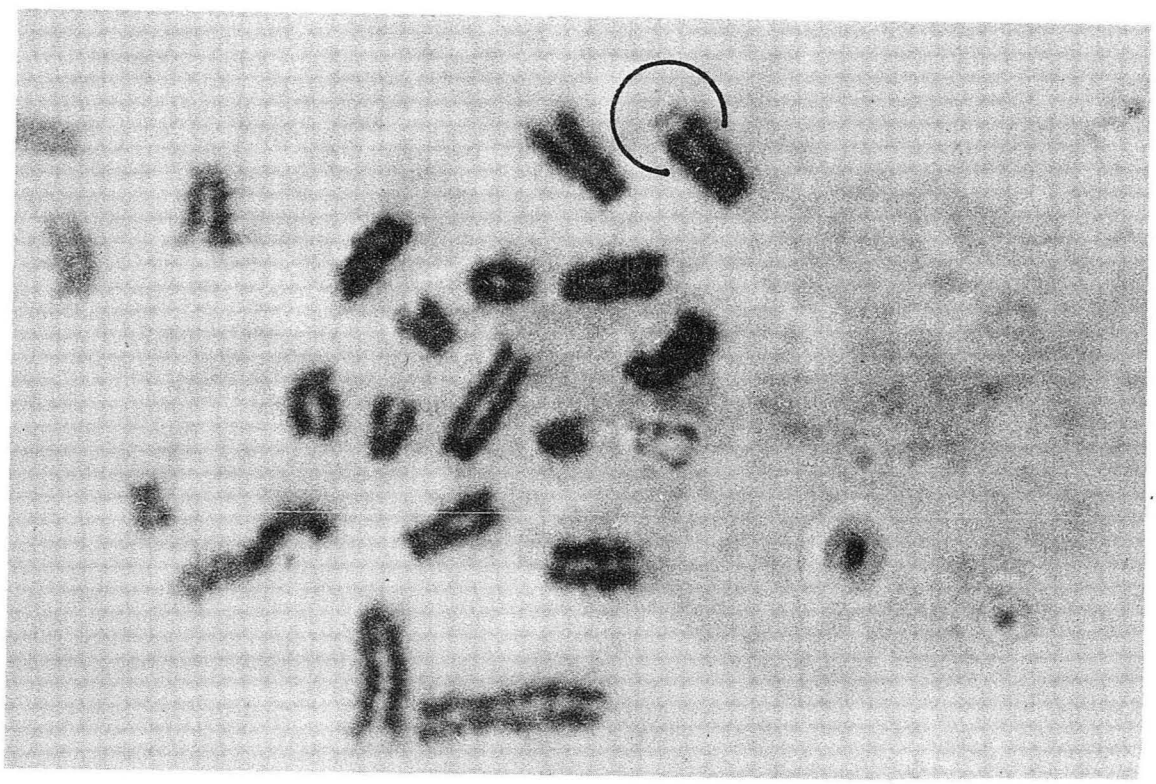
Fig. 4.

♂ MONGREL



ZN-4565

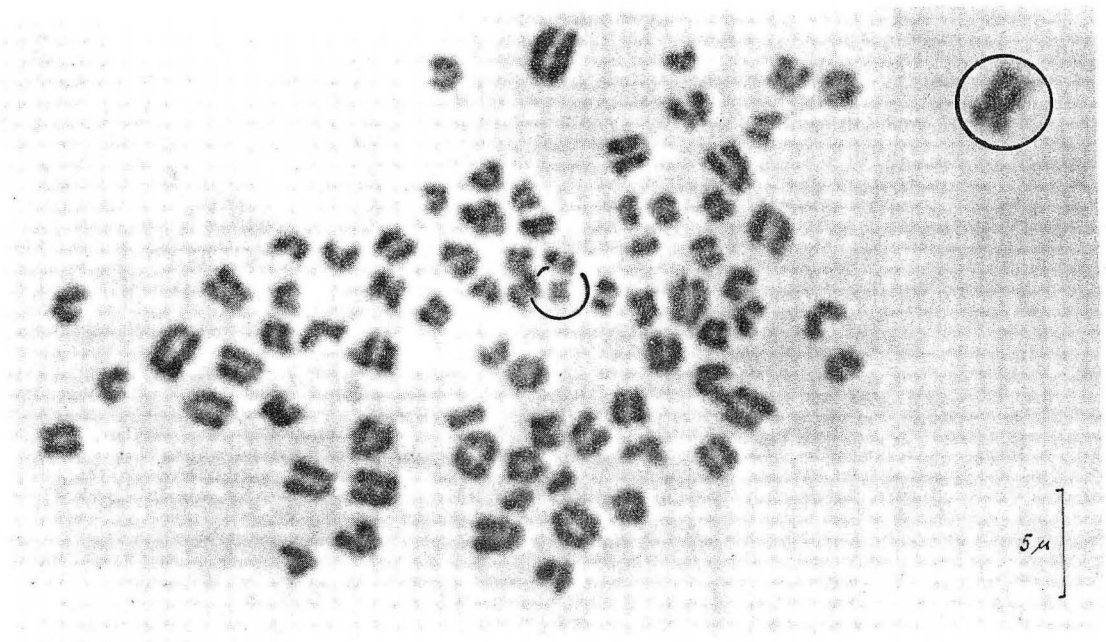
Fig. 5.



ZN-4567

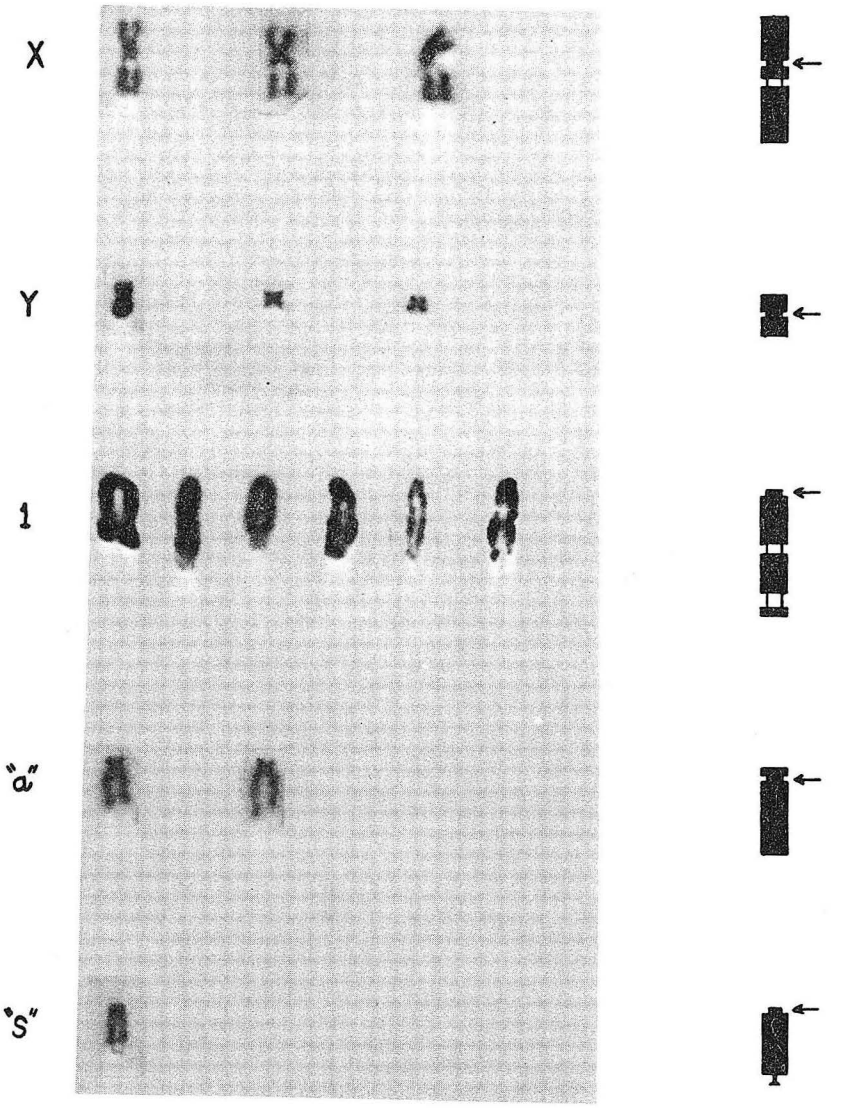
Fig. 6.

♂ MONGREL



ZN-4566

Fig. 7.



ZN-4568

Fig. 8.

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