IN VITRO EFFECTS OF THYROXINE ON THE CHROMOSOMES OF HUMAN LYMPHOCYTES

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INTRODUCTION

Thyroxine, as one of the principle hormones of the thyroid gland, plays a primary role in human growth and development, as evidenced by the plethora of molecular processes with which it has been linked. Tapley (1964) reports that it causes an increase in enzyme activity, stimulates amino acid incorporation into the proteins, produces an alteration in the rate of protein leakage from the mitochondrial membrane, and more generally, increases the basal metabolic rate. Although thyroid hormones have been linked with the stimulation of more than fifty key enzymes, the mechanism of thyroxine action still remains obscure.

Siegel and Tobias (1966) have suggested that thyroid hormones mediate their effect directly on the nucleus and associated m-RNA, based on their observations that a rise in the production of nuclear RNA occurs directly after treatment with thyroid hormones and before the corresponding rise in protein synthesis. In addition, the use of I^{125} radiographs indicated that the nucleus may indeed be the primary locus of activity. The relationship between thyroxine activity and nuclear control has also been investigated by Widnell and Tata (1963), who found that protein synthesis increases 30-35 hours after the administration of thyroid hormones, followed by stimulation of protein synthesis and a corresponding rise in general metabolic activity. They also observed that RNA polymerase activity increases within 16 hours of the hormone injection, thereby suggesting that the locus of thyroid hormone action may be in nuclear regulation of protein synthesis.

On the other hand, Sokoloff, Francis, and Campbell (1964), in experimentation with cell-free rat liver preparations in vitro, concluded that thyroxine stimulates amino acid incorporation into microsomal protein independently of any action on RNA polymerase activity or m-RNA synthesis. Using the DNA melting profile as an index of hormone action through its effect on DNA intrastrand bonds, Goldberg and Atchley (1966) found that L-thyroxine at normal and high concentrations had no effect, whereas such compounds as estrone, insulin, epinephrin, and somatotropin, produced positive results.

Thus, this study was undertaken to detect any effects of L-thyroxine at the chromosomal level, not only in relation to cellular metabolism but also concerning effects of the hormone on the chromosomal complement at normal (1.0×10^{-7}M) and higher (1.0×10^{-5}M) concentrations.

MATERIALS AND METHODS

Blood for experimentation was obtained from a human subject, and using sterile technique three to four drops were added to each vial containing 4.9 ml of medium. The medium used was basically TC medium 199 containing phenol red indicator with other constituents such as: fetal calf serum (1ml/vial; Grand Island Biological Company), which serves as a stimulant to proliferation of the cell population; 500 units each of penicillin and streptomycin (Microbiological Associates, Inc.), which reduce the possibility of microbial contamination; 0.02 ml phytohemagglutinin (Difco Laboratories), which acts as a

mitogenic agent; and 100 units of Heparin, which provents coagulation of the blood. Three vials were used for each experimental group, a control group being included with each trial.

During the four day incubation period at 36.5° - 37.0°C, the cultures were observed daily for a change in pH, as evidenced by a color change in the phenol red indicator. If it appeared too acidic (light amber in color), the vial cover was loosened from one-to-two hours to allow excess CO_2 to escape, causing the color to return to normal (red). Three hours before harvesting the cultures, 2 g of colchicine (Nutritional Biochemicals Corporation) were added using a tuberculin syringe, to arrest the dividing cells at metaphase.

The proceedure used for harvesting the lymphocytes was a modification of the method of Moorhead et.al. (1960). The contents of the vials for each experimental group were aspirated into a 15 ml conical centrifuge tube with a fine-tipped pipette and centrifuged at 1200-1500 rpm for 10 minutes. After centrifugation, the supernatent was removed, 8-10 ml of Hank's balanced salt solution (Microbiological Associates, Inc.) added, and the cells resuspended and then centrifuged as above. After removal of all but 0.5 ml of the supernatent Hank's solution, two ml of glass distilled water was added, the cells suspended, and then allowed to stand for 10 minutes to allow time for hemolysis of the red blood cells in this hypotonic solution. After resuspension and centrifugation, followed by removal of the supernatent, including the shadow cells of the hemolyzed RBC, 2 ml of freshly made fixative, consisting of one part glacial acetic acid to three parts

absolute methanol, was added and the tubes allowed to stand undisturbed for thirty minutes. After the waiting period, the cells were re-suspended and centrifuged. The fixative was changed, the cells re-suspended and the suspension recentrifuged until all traces of brown color from the RBC had disappeared.

Slides for examination were immediately prepared by dropping one drop of the cell suspension onto an ice-cold, wet slide placed at a 45^{o} angle to allow the drop to run down the surface. The slide was then passed over a flame until the back was warm to the touch; it was then waved vigorously to quickly complete the drying process. The first slide in each group was examined under phase contrast to evaluate the presence and distribution of metaphase spreads. If they were too scarce, the suspension was recentrifuged and concentrated in a smaller amount of fixative; on the other hand, if they were too concentrated, more fixative was added. At least 10 slides were prepared for each trial of each experimental group.

The Giemsa staining proceedure was employed for further processing the slides for examination. The stain, consisting of 90 ml glass distilled water, 7 ml Giemsa stain, and 5 ml 0.15 M ammonium hydroxide, was prepared just before use and was used for only one passage of eight slides. The proceedure for hydrolyzing was as follows:

1. 1N HCl at 60^{o}C for 10 minutes
2. rinse in distilled water
3. Giemsa stain for 8-15 minutes
4. Acetone I, rinse for 6 dips
5. Acetone II, rinse for 6 dips
6. Acetone-Xylol, 2-5 minutes
7. Xylol I, at least 5 minutes
8. Xylol II, at least 10 minutes

Immediately after staining, the slides were mounted in permount using 24 x 50 mm cover glasses (Corning Glass Works).

Two concentrations of L-thyroxine were employed for experimentation, 1.0×10^{-7} M and 1.0×10^{-5} M, the prepared solutions being diluted 1:50 by the tissue culture medium. Hormone solutions of concentrations 5.0×10^{-6} M and 5.0×10^{-4} M were prepared by weighing the appropriate amount of the hormone, L-thyroxine (Sodium) Pentahydrate (Sigma Chemical Company), on a semi-micro analytical balance and dissolving it in 10 ml of water. The solutions were then passed through a millipore filter of pore size 0.22μ to insure removal of bacterial contaminants. The solution of lower concentration was prepared by diluting the stronger solution 1:100. Although the solubility of the sodium salt of thyroxine in water is reported to be 2 g./ 100 ml at 25°C (Merck Index), some of the solid material did not go into solution, thereby casting some doubt on the validity of the final hormone concentrations.

The cultured cells were exposed to the two hormone concentrations for three time periods, 72 hours, 48 hours, and 24 hours, producing a total of six experimental groups. First, a pilot control study was carried out for the purpose of learning and perfecting technique. Then, the experiment was repeated three times, and the data from two of these runs were used in computing the results of the study. The solutions were added to the culture vials by means of a sterile tuberculin syringe, with suitable precautions being taken to avoid introduction of contaminants.

One hundred metaphase spreads or more were counted for each

experimental group. The spreads were randomly selected solely on the basis of their desirability for counting. That is, those exhibiting a suitable amount of spreading, with a minimum of overlaps and interference from neighboring spreads were considered suitable. Only the best spreads were selected for photography, and enlargements (8 x10) were later printed by the investigator.

The investigator followed the guidelines for karyotyping set up by the Chicago Conference on the Normal Human Karyotype. (Bergsma, 1966)

RESULTS

The results obtained from analysis of chromosomes are tabulated on Table 1 and summarized in a more general fashion on Table 2. These observations show definite differences in chromosome number between the two groups, especially at a thyroxine concentration of 1.0×10^{-5} M. A very abrupt change from the control value is evident in this group after 24 hour exposure to the hormone, a rise from 2.8% to 10% polyploidy. A slight decrease exists in the 48 hour group, but 72 hour exposure produced an increase to 12.4% polyploid cells. At the lower concentration of 1.0×10^{-7} M, no significant change was noted in the modal chromosome number of 46. Figure 1, p.9 illustrates the difference in effect between the two concentrations. No morphological differences could be detected in the chromosomes between control and experimental diploid spreads, as illustrated in a comparison between Figures 2 a,b

Table 1..Effects of L-thyroxine on the Chromosome Complement of Human Lymphocytes

Experimental Group		Chromosome Number of Cells																																				
		33	36	37	38	39	40	41	42	43	44	45	46	47	48	51	52	53	66	71	72	76	79	80	81	82	83	84	86	87	89	90	91	92	93
Controls							2	2	1	2	8	3	81	2		1									1										2	
1.0×10^{-7} M	24 hours		2			1			1		3	5	90	3																					
	48 Hours			1		1		1	5	2	4	7	75	2												1					1		1		
	72 Hours								1		3	10	81	3	1				1	1			1								1			1	
1.0×10^{-5} M	24 Hours								1	1		12	73		1		1	1											1		1	2	5	1
	48 Hours			1				1	1	3	4	10	90								1						1			1	2		7	
	72 Hours	1		1	1				2	3	5	7	78						1			1		1	1	1			2	1		1	3	2	1

Table 2. Summary of L-thyroxine Effects on the Chromosome Complement of Human Lymphocytes

Experimental Group		Hypodiploid Cells		Diploid Cells		Hyperdiploid Cells		Hypotetraploid Cells		Tetraploid Cells		Hypertetraploid Cells		Total Cells
		No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	
Controls		18	17.1	81	77.1	3	2.9	1	0.95	2	1.9			105
1.0×10^{-7} M	24 Hours	12	11.4	90	85.1	3	2.9							105
	48 Hours	21	20.8	75	74.2	2	2.0	3	3.0					101
	72 Hours	14	13.5	81	77.9	5	4.8	3	2.8	1	0.95			104
1.0×10^{-5} M	24 Hours	14	14.0	73	73.0	3	3.0	4	4.0	5	5.0	1	1.00	100
	48 Hours	20	16.4	90	74.8			5	4.1	7	5.7			122
	72 Hours	20	17.7	78	69.0	1	0.88	11	9.7	2	1.8	1	0.88	113

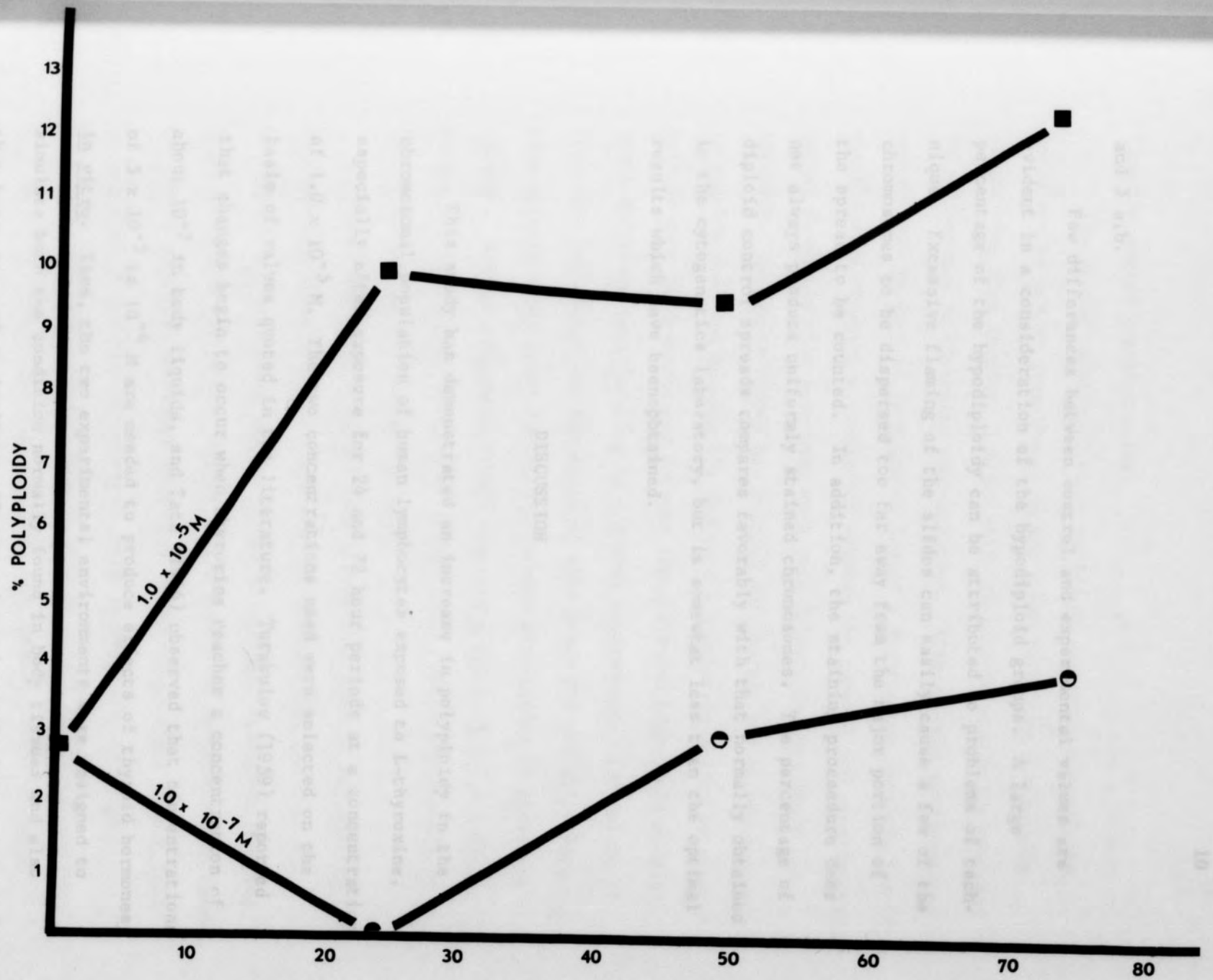
% POLYPLOIDY
13
12
11
10
9
8
7
6
5
4
3
2
1
1.0 x 10⁻⁵ M
1.0 x 10⁻⁷ M
10
20
30
40
50
60
70
80
EXPOSURE TO THYROXINE (HOURS)

Figure 1.

and 3 a,b.

Few differences between control and experimental values are evident in a consideration of the hypodiploid groups. A large percentage of the hypodiploidy can be attributed to problems of technique. Excessive flaming of the slides can easily cause a few of the chromosomes to be dispersed too far away from the major portion of the spread to be counted. In addition, the staining proceedure does not always produce uniformly stained chromosomes. The percentage of diploid control spreads compares favorably with that normally obtained in the cytogenetics laboratory, but is somewhat less than the optimal results which have been obtained.

DISCUSSION

This study has demonstrated an increase in polyploidy in the chromosomal population of human lymphocytes exposed to L-thyroxine, especially after exposure for 24 and 72 hour periods at a concentration of 1.0×10^{-5} M. The two concentrations used were selected on the basis of values quoted in the literature. Turakulov (1959) reported that changes begin to occur when thyroxine reaches a concentration of about 10^{-7} in body liquids, and Tata (1964) observed that concentrations of 5×10^{-5} to 10^{-4} M are needed to produce effects of thyroid hormones *in vitro*. Thus, the two experimental environments were designed to simulate both the condition normally found in body tissues and also that known to produce *in vitro* effects and one hundred times greater

than the normal reported value.

Due to the relatively short period of exposure, the effects noted may be merely transitory; on the other hand, the possibility exists that they indicate a major shift in genetic population mediated by an increase in thyroxine concentration. Although the environmental change from *in vivo* to *in vitro* is indeed a drastic one which in itself could induce changes, a comparison between the control and experimental values indicates that something more than "tissue culture shock" is involved. As Harris (1964) has pointed out, merely the occurrence of polyploidy does not necessarily indicate a population shift; an altered balance must be demonstrated between low-ploid and high-ploid complements which are proliferating in a common environment. The nature of available techniques and the scope of this study did not allow long-term culture which would facilitate further elucidation of thyroxine effects. However, Sandberg (1966) reported a value of 0.7 - 2.0% polyploidy in 2-3 year culture of normal cells, which corresponds to that obtained for the control group.

Levan and Biesele (1958) have stated that genetic variability is a manifestation of certain types of carcinogenesis, and that the development of malignancy can be viewed as cellular selection favoring cells which can escape the control of the host. An environment in which the normal genotype undergoes selective disadvantage might result from an inbalance in certain metabolites, such as hormones, or drugs. Polyploidy and endoreduplication, as well as breaks and translocations, have been observed by Nasjleti, Walden, and Spencer (1965) in human blood cells after administration of the drug N-N'-bis(3-bromoprionyl)

piperiazine. Endoreduplication with somatic pairing has also been demonstrated in cases of leukemia, skin cultures of XYY males, fluid from cancer patients, in mice tumors, and following in vitro irradiation (Friedman, Saenger, and Kreindler, 1964).

How, then, might polyploidy be related to the effects of high concentrations of thyroxine in tissue culture? Polyploidy can arise as the result of a number of breakdowns in the normal mitotic sequence; Hsu and Moorhead (1956) have characterized some of these mechanisms from observations in the HeLa strain. Endoreduplication is a type of polyploidization which arises during interphase, involving DNA replication without cytokinesis, resulting in four-stranded chromosomes or somatic pairing of sister chromosomes. No endoreduplicated figures were found in this study, although a limited amount of somatic pairing was observed in some polyploid spreads. (See Fig. 6, p. 26) Polyploidy can also result from reduplication during metaphase, in which no spindle is formed and nuclear reconstitution occurs without anaphase. Although no evidence exists for thyroxine effects on the spindle apparatus, it might be postulated that the cellular environment of a hyperthyroid state could result in a mitotic breakdown of this type.

Polysomaty, a telophase failure, arises when no cytokinesis occurs, the daughter nuclei forming a binucleate cell or fusing to produce a tetraploid nucleus with one spindle apparatus. This type of polyploidization is thought to account for liver polyploidy (Wilson and Leduc, 1948). Although mitotic changes are synchronized in a binucleate cell (Harris, 1964; Sandberg, 1966), homolygous chromosome

pairs in polyploid cells sometimes differed, making evaluation of the type of polyploidization involved more difficult.

The occurrence of polyploidy can also result from fusion of two cells, whether directly after cytokinesis or during random encounters of cells, a phenomenon designated as cell hybridization. Some element in the cellular environment causing increased cell adhesiveness could be a mediating factor in this mechanism.

Although the investigator is suggesting that the hyperthyroid state can lead to increased polyploidy in the cell population, increased polyploidy followed by malignancy was reported by Al-Saadi (1965) in studies of iodine deficiency. After several months of experimentation, the number of non-modal cells, consisting mostly of hyperdiploids and tetraploids, increased to 30-44%. The increased mitotic proliferation of the thyroid gland in response to the low iodine environment apparently produced a greater percentage of imperfect divisions and the resulting non-modal cells. However, the data of this experiment do not necessarily conflict with this observation,because the relationship between concentration and effect is not always a direct one.

Thus, it appears that high concentrations of L-thyroxine have an effect on the mitotic process, causing an increase in percentage polyploidy in the cell population of human lymphocytes. However, these data cannot offer any clues to the mechanism or long-range implications of the effects. Further experimentation, such as the use of serial tissue culture techniques, might offer further clarification of this phenomenon.

SUMMARY AND CONCLUSIONS

1. A thyroxine concentration of 1.0×10^{-5} M produced a rise in percentage polyploidy in the cell population of human lymphocytes from 2.8% in the control group to a maximum value of 12.4%, resulting from exposure to the hormone for 72 hours. A lower concentration, 1.0×10^{-7} M did not produce differences of any significance.

2. The mechanism for this polyploidization remains obscure, although a hyperthyroid state could be responsible or play a part in certain irregularities in the mitotic process, such as endoreduplication, polysomaty, cell hybridization, or meta-reduplication.

3. More extensive investigation is needed to determine whether these observed fluctuations in the cell population, apparently mediated by high concentrations of L-thyroxine, are due to temporary responses to the in vitro environment or whether they represent permanent shifts in the low-ploid, high-ploid ratio.

APPENDIX

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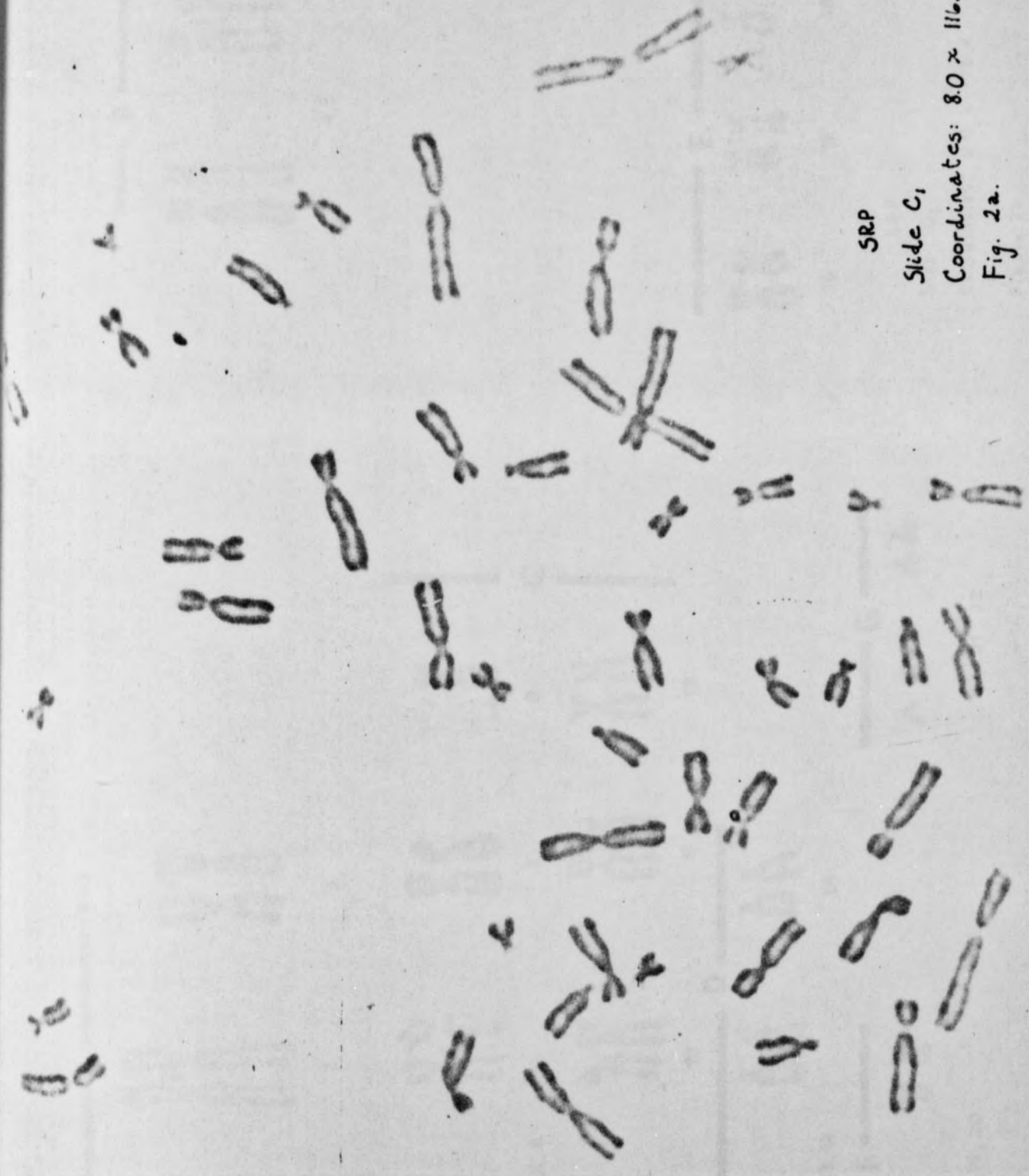

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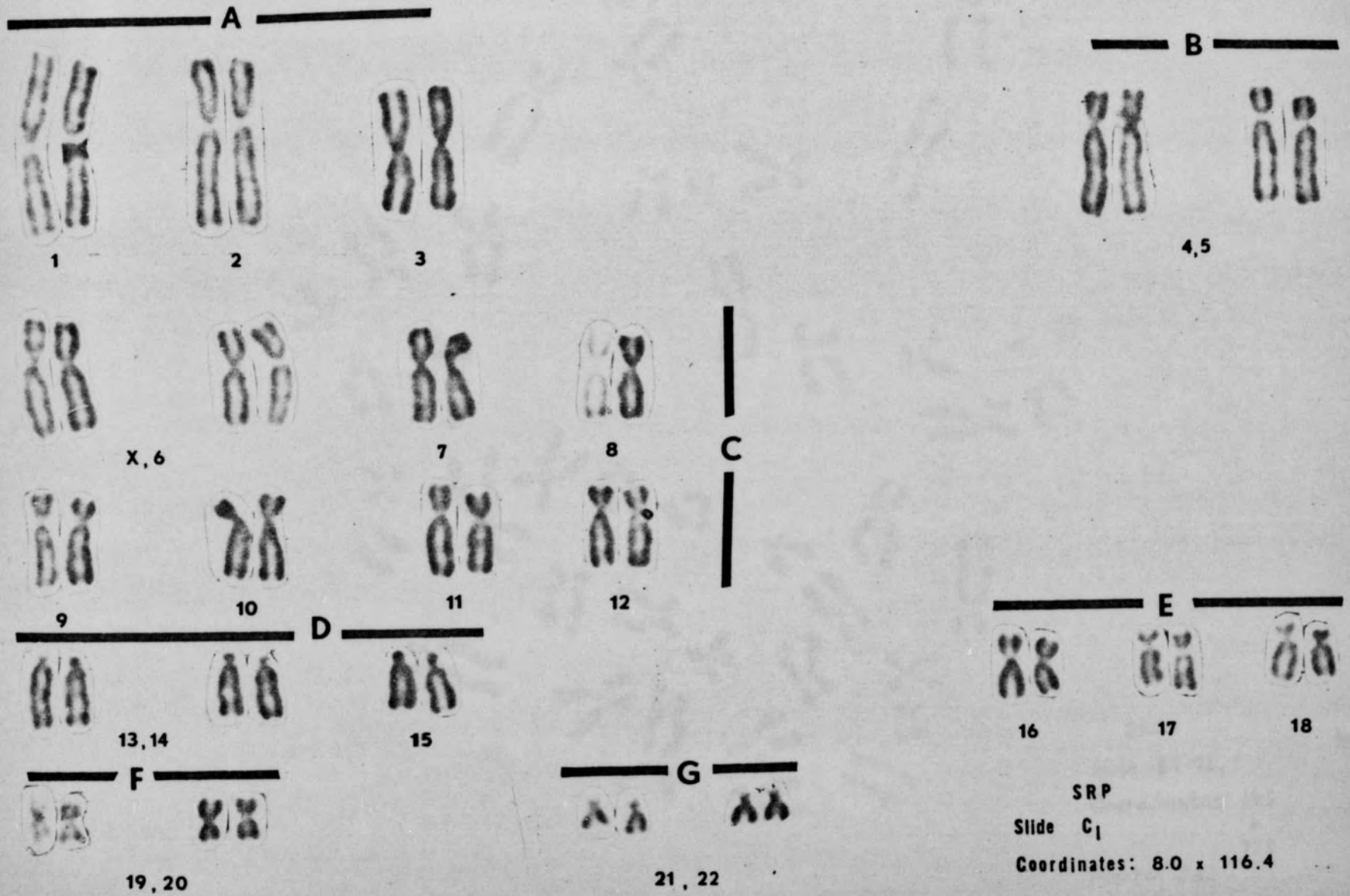

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Figure 2b.

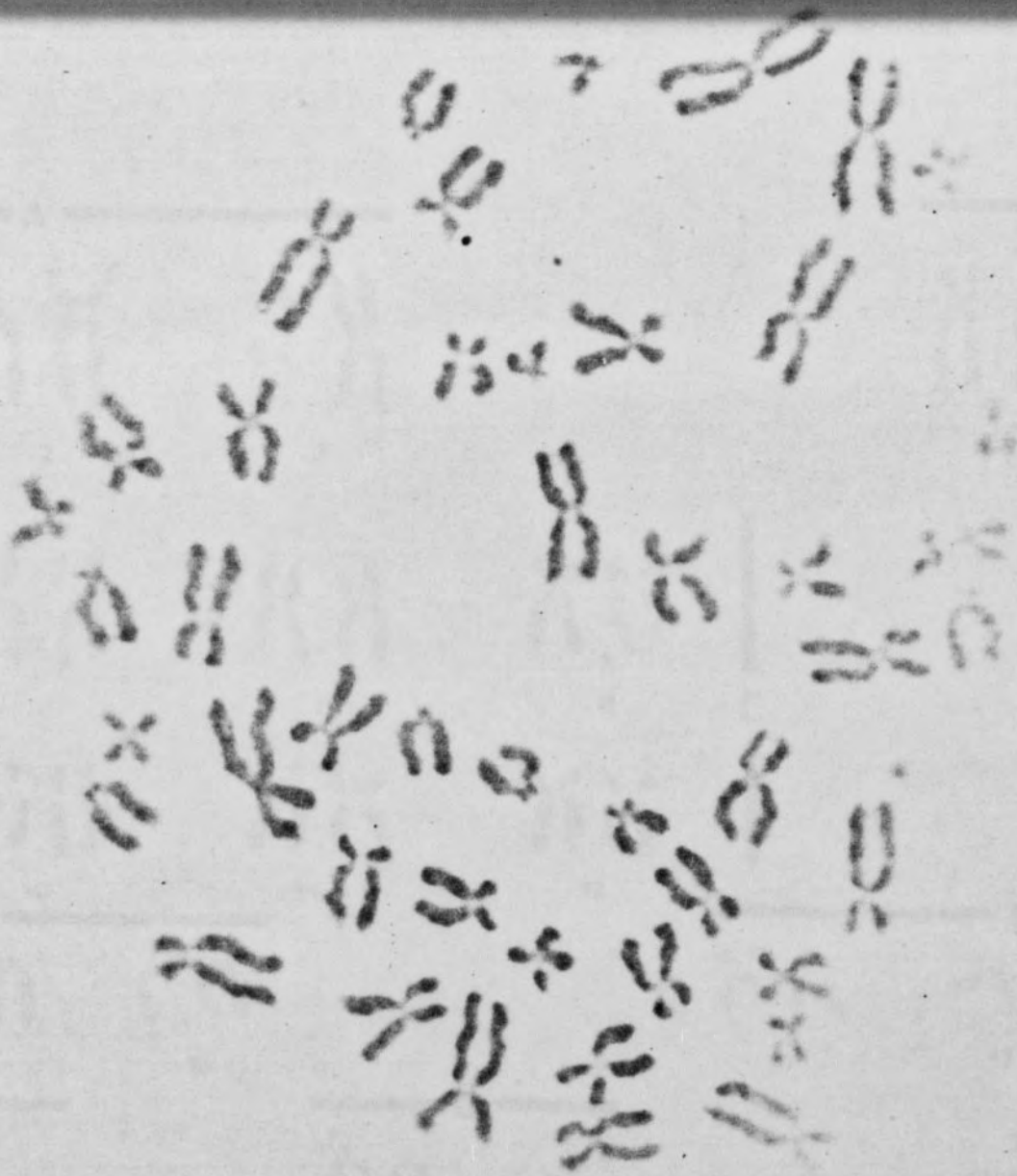

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Figure 3a.

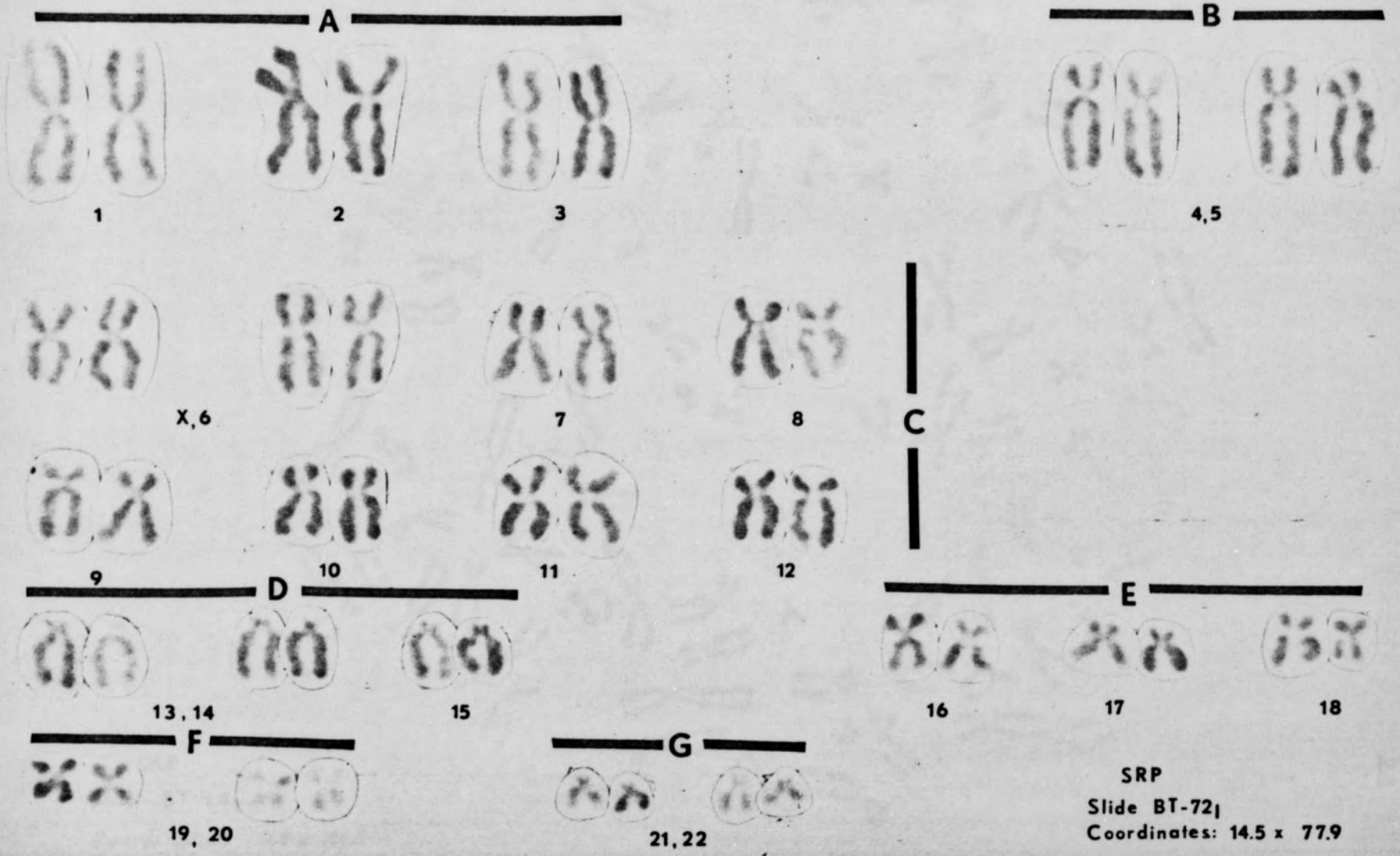

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Figure 3b.

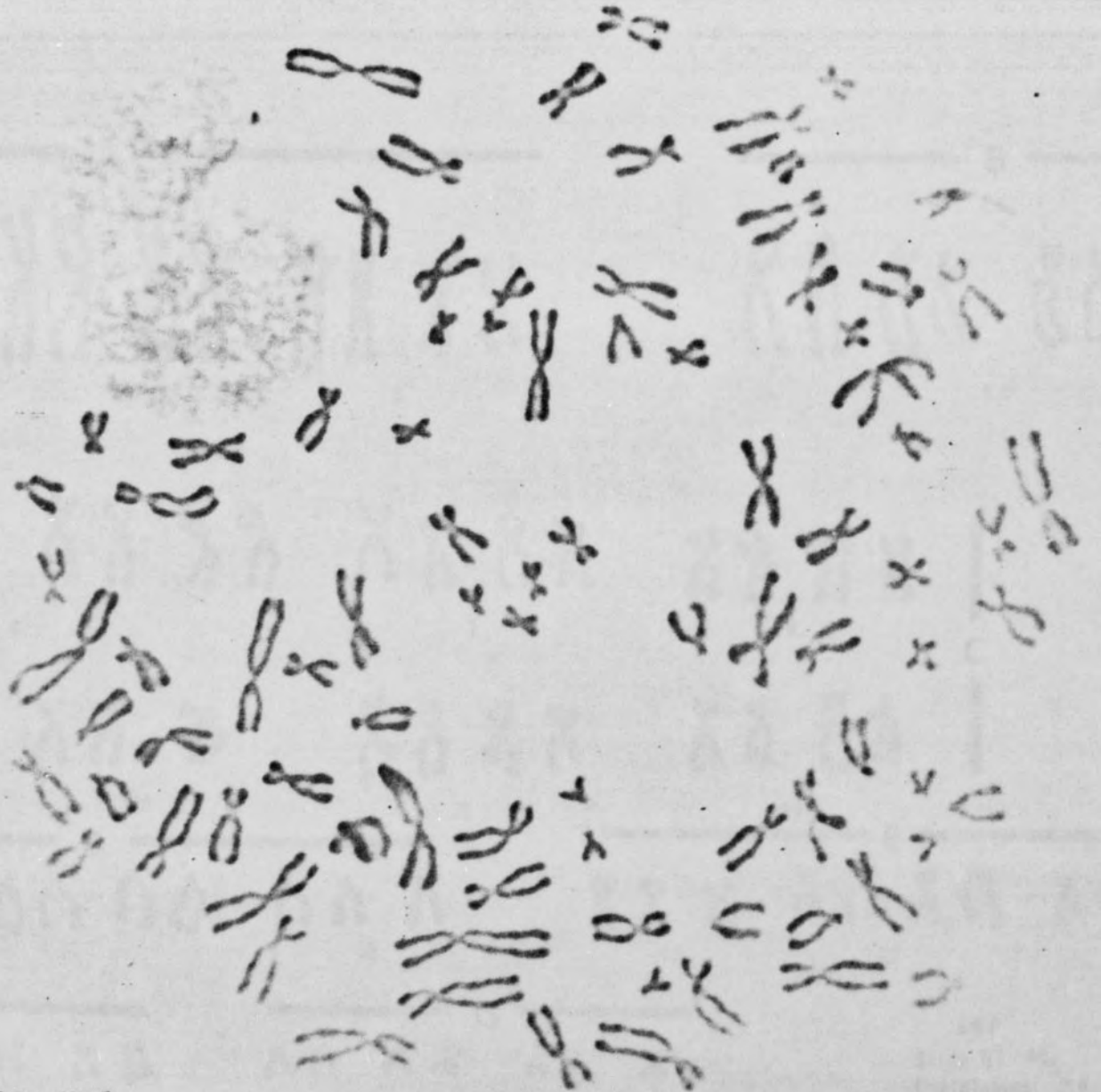

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Figure 4b.

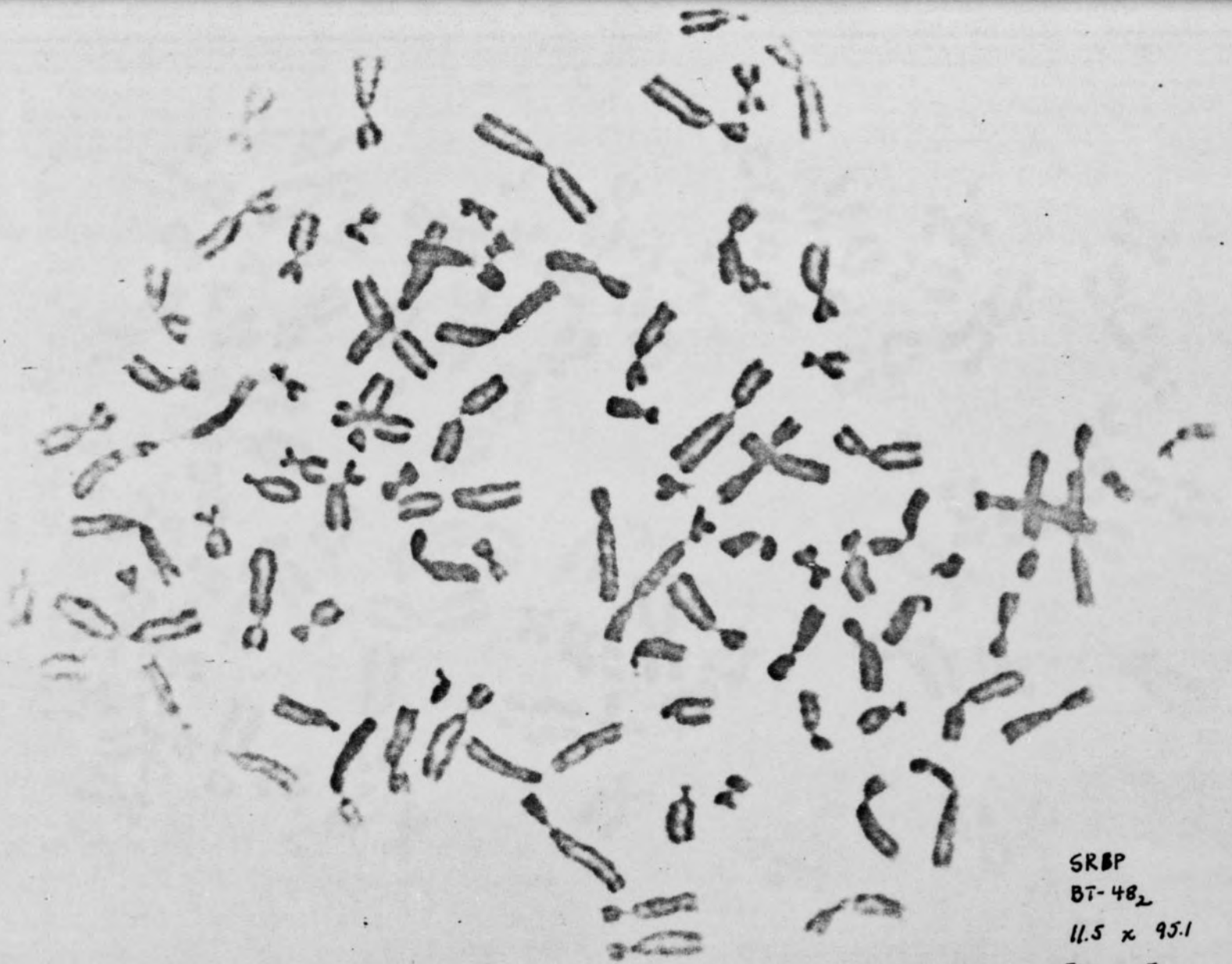

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Figure 5.

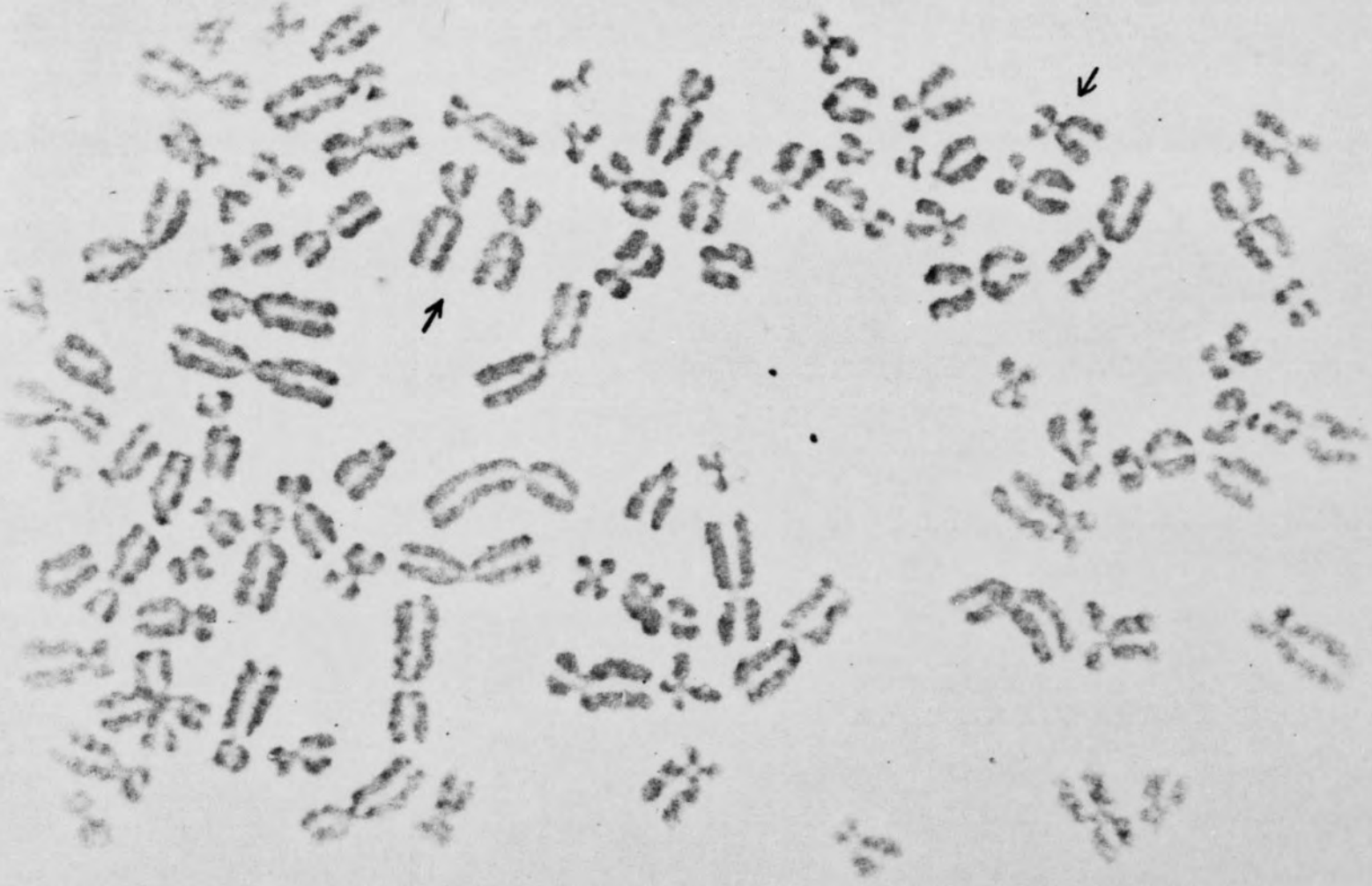

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Figure 6.