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BREAKAGE IN HUMAN CHROMOSOMES INDUCED BY LOW TEMPERATURE

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I. INTRODUCTION

DURING a study of the human cell line Lu 106 it was observed that a considerable number of heteropycnotic bodies often were visible in the interphase nuclei. It was thought worth-while to investigate whether these heteropycnotic bodies might correspond to chromosome regions at metaphase that could be induced by cold treatment to show negative heteropycnosis, as has been observed in the well-known cases of "nucleic acid starvation" first observed in *Paris* by DARLINGTON and LA COUR (1938), and later in many other plants by different authors. In animals, only one such case is known, viz. that reported by CALLAN (1942) for meiotic *Triton* chromosomes, in which heterochromatic segments appeared as constrictions after cold treatment.

A preliminary experiment indicated that Lu 106 was fairly resistant to cold treatment. A few bottles of this tissue culture, placed in a refrigerator at $+3^\circ$ centigrades, still contained mitoses after 9 days and recovered normal growth after having been brought back into incubator temperature. A great number of experiments with different temperatures and different times of exposure were then made. It was considered especially interesting to extend the observations to a diploid embryonic cell strain.

It was found that even though cold treatment did induce a great number of constrictions in the chromosomes it was not clear whether they were actually comparable to the "starvation" phenomenon. This will be discussed at some length below. A very interesting observation during these experiments was that chromosome breaks were induced by the

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cold treatment, and that these breaks were not scattered randomly over the available chromosome length but appeared in clusters. The induction of chromosome breaks by low temperature has so far been demonstrated only in plants and only in meiotic chromosomes. SAX (1937) thus found chromosome interchange, inversions, fusions and fragments in *Tradescantia* meiosis exposed to low temperature, especially if the cold treatment was followed by a period in a hot chamber. MARQUARDT (1952) showed that a constant temperature of 10° increased the incidence of "chromosome mutations" (fragmentations and rearrangements) four to five times in meiosis of *Oenothera* as compared with normal field conditions, where the temperature was between 16 and 30°. So far, no similar results have been reported with mitotic chromosomes.

The observation that chromosome breaks were induced by cold in human cells in tissue culture was considered to have an interesting bearing on another project of our laboratory, the induction of chromosome breaks by viruses and DNA inhibitors, and it was decided that a detailed analysis should be made of this phenomenon. To obtain a background for the distribution of the chromosome breaks by cold, one experiment was arranged with X-ray irradiation of the same cell line.

II. MATERIAL AND METHODS

Most experiments were made with the cell line Lu 106. This is an epithelium-like cell line, derived from human embryonic lung. It was initiated in 1959 by Dr. CLAES HÖGMAN from a male embryo according to a technique described by HÖGMAN (1960). It was received at our laboratory in December 1963, and the passages 14—27 in Lund were used for the present study.

In selecting diploid human cells, it was thought that cells derived from human embryonic lung would be most suitable for a comparative study. Accordingly, cultures were prepared in April 1964 from the lung of a male foetus. From this culture passages 4 and 5 were used for the present experiments. Even though the tissue origin was the same as that of Lu 106, the histological pattern of the human embryonic cell strain (HEL) was quite different, fibroblast-like, as the Lu 106 had also been for the first 12 passages in 1959 before its transformation into a cell line (personal communication by HÖGMAN).

Lu 106 was maintained on Hank's solution with 0.5 % lactalbumine and 3 % new-born calf serum. HEL was grown on Parker 199 with 20 % calf serum. Both media contained 100 I.U. of penicillin and 0.2 mg

streptomycin per cc. The cultures were grown in 10 cc volumes of medium in milk dilution bottles with screw caps. The day before an experiment was started, suitable cultures were selected under the microscope and fed with new medium.

The cold treatment varied both in temperature and in the duration of time of the treatment. Most experiments were made at $+3°$, but treatments with $\pm 0°$, $+10°$ and $+20°$ were also included. For chromosome studies the period of cooling varied from 1 to 9 days, but some cultures were left longer at low temperature to determine the period of maximum tolerance.

Cultures were taken for chromosome study either directly after the cold treatment, or after a few hours recovery at $37°$. It was found that the best chromosome fixations were obtained if the bottles were slowly warmed in a water bath, so that room temperature was reached after approximately 2 hours of gradual warming. This was made to avoid temperature shock; fixations without prewarming were sometimes also good. Before trypsinization the cultures were treated for one hour with 0.2 gamma colchicine per cc medium. The cells were then trypsinized, and transferred to a centrifuge tube to which 3 times the quantity of distilled water was added. After 20 minutes, the cells were centrifuged and the button fixed for 15 minutes in a mixture of 60 % glacial acetic acid, 10 % 1-n hydrochloric acid and 30 % distilled water. After that the fixative was poured off, a few drops of 2 % orcein in 60 % acetic acid were added and the cells squashed on siliconized slides under microscopic control. Photomicrographs were made with the aid of an Ultraphot and drawings with a camera lucida, the latter at a magnification of 6000 times.

III. GENERAL OBSERVATIONS

In Tables 1 and 2 surveys are given of the cold treatment experiments with Lu 106 and HEL. The following properties were recorded: mitotic index, presence of multiples of the stemline number, the occurrence of endoreduplications (ER), of constrictions and of chromosome breaks. The general appearance of the Lu 106 cell population may be seen from Fig. 1 a, from which the great variation in nuclear size is evident. After cold treatment (Fig. 1 b) this variation increased. A great number of small pycnotic nuclei are present, many of which indicate mitotic breakdowns with the formation of micronuclei.

The mitotic index, MI (% cells in mitosis), was calculated on cells treated with colchicine for one hour. It thus involves some accumula-

TABLE 1. *Mitotic rate, ploidy level, endoreduplication, chromosome breakage and occurrence of chromosome constrictions in Lu 106 after cold treatment.*

1	2	3	4	5	6	7	8	9	10
No.	Temperature	Days of treatment	Hours before fixation	Mitotic index	Per cent polyploidy	Per cent endo-reduplication	Per cent breakage	Estimated constrictions	Mitoses counted in col. 8
1	±0°	1	2/8	7.1	3.5	2.0	11.0	+	100
2	+3°	2	0	1.2	6.0	0	12.5	+	48
3			0	2.1	14.0	10.0	4.7	—	43
4			0/6	6.2	3.5	0.5	15.4	+	65
5			2/8	2.5	5.0	0	11.1	+	45
6			0/12	9.7	9.0	0.5	14.9	+	67
7			0/18	7.0	10.0	1.0	24.6	+	57
8			0/24	5.1	5.5	0	16.9	+	59
9		3	0	1.4	12.0	0.5	11.7	+	60
10			2/8	2.3	8.5	1.0	41.0	+ +	100
11			2/8	—	—	—	33.3	+ +	51
12			2/8	—	—	—	33.3	+ +	63
13			2/12	2.9	2.0	0	20.0	+	50
14		6	2/8	0.3	9.0	2.0	43.6	+ +	39
15		8	0	0.8	28.0	20.0	—	—	—
16	+10°	2	2/8	9.5	3.5	0.5	12.5	+	79
17	+20°	2	0/8	2.7	3.5	0.5	9.9	—	81
18	+37°	—	—	7.0	3.0	0	9.5	—	42
19				4.8	4.5	0	8.3	—	338
20				3.6	3.5	0.5	4.0	—	100
21				3.3	2.0	0	7.1	—	84

Two values in col. 4 mean hours of gradual warming/hours in incubator at 37°; the values of col. 5 are calculated on 1000 cells, except Nos. 14 and 15, which are calculated on 2000 cells each; the values of cols. 6 and 7 are calculated on 200 cells, except Nos. 1, 3 and 14, which were calculated on 100 cells each, and No. 15, on 60 cells; in col. 9 — means constrictions as in the control, + and ++ mean an increasing number of cells with a high incidence of constrictions of the erosion type.

TABLE 2. *Ploidy level, chromosome breakage and occurrence of constrictions in HEL after cold treatment.*

1	2	3	4	5	6	7	8	9
No.	Temperature	Days of treatment	Hours before fixation	Per cent polyploidy	Mitoses counted in col. 5	Per cent breakage	Estimated constrictions	Mitoses counted in col. 7
31	+3°	2	0	11.1	90	30.6	+ +	62
32			2/12	11.3	106	26.1	—	69
33		3	0	21.2	66	39.3	+ + +	28
34			2/8	5.3	112	30.3	+	89
35			2/8	11.8	51	23.1	+	39
36			2/8	11.1	45	21.2	+	33
37			2/12	11.1	63	24.5	+	49
38		6	0/48	0.8	131	5.0	—	100
39	+37°	—	—	5.5	183	2.5	—	157
40				5.3	114	2.7	—	75
41				2.0	100	2.3	—	88
42				0	63	2.2	—	46

tion of c-mitoses. Fixations made immediately after the end of a cold treatment of 2—6 days showed MI:s of 0.8—2.1, thus a clear lowering of the normal mitotic frequency of 4.7 on an average. But 6 hours of recovery was enough to restitute normal MI and after 12 and 18 hours of recovery even higher mitotic frequency was seen. This may indicate that mitoses under way at the start of the cold treatment are slowed down or completely stopped at the same time as fewer mitoses become started. Therefore many cells are ready to complete mitosis or to enter mitosis when the cold-treated cultures are put back into +37°. Cold treatment has been used to attain mitotic synchronization in cultures of microorganisms, and some results in the same direction are known from tissue culture cell lines (NEWTON and WILDY, 1959; MIURA and UTAKOJI, 1961). No influence on the MI was noticed in our cold treatments of 0° for one day, or of 10 and 20° for 2 days.

The mitotic activity of our cultures of HEL was too low even under control conditions to enable us to evaluate the influence of cold on the

TABLE 3. *Survival experiment with long-term cold treatment of Lu 106 and HEL.*

Culture No.	Days in +3°	Lu 106		HEL	
		Dividing cells		Dividing cells	
		Day 27	Day 39	Day 27	Day 39
1	14	±	−	+	+
2	19	±	−	+ +	+ +
3	25	±	−	+	+
4	32		−		−

− no cell propagation
± single colonies formed
+ a great number of cells growing out
+ + a confluent sheet of cells

MI. We tried to use the loosening of the cells from the glass surface for an estimate of the tolerance of the cells towards cold treatment. It was found that the cells of Lu 106 started falling off on day 6—7 in 3° and were completely loosened on day 10—12. The corresponding times for HEL were, about 3 and 5 days, respectively. This may be taken as an indication of more resistance towards cold in the cell line than in the cell strain but has to be supplemented by experiments on cell loosening at other temperatures because of exhaustion of medium. A preliminary experiment with recovery of the cultures after long treatment in 3° actually indicated more endurance in the HEL (Table 3). From HEL good growth could be recovered even after 25 days in +3°, while only a few scattered cells remained in the Lu 106 cultures after 14 days, and these could not be restored to good growth. These observations must be repeated before they can be considered conclusive.

The fraction of cells with doubled, quadrupled or higher chromosome number was frequently increased during the treatment (Tables 1 and 2). Since it is unlikely that the short cold treatment in many cases would have had time to let chromosome doubling mechanisms act, it is most likely that we are dealing with a selective phenomenon. Cells with high chromosome numbers are probably favored to enter mitosis. It may also be that mitoses that have started are forced back into resting stage and then re-enter mitosis after having doubled their chromosome number. The increase in mitoses with doubled or higher chromosome

number was observed both in Lu 106 and in the HEL. It was noticed in Lu 106 that the frequency of endoreduplications (ER) was generally somewhat higher in the cold treated cultures, and sometimes strikingly high (20 % after 3 days in 3°). Unfortunately, the factors responsible for this increase in ER are not yet controllable. Lu 106 has always a tendency to ER, an increase sometimes being noted without any treatment of the culture, and often it is increased by a variety of treatments. Thus, in one experiment with Lu 106, in which 0.1 gamma per cc of triethylene melamine (TEM) was given 24 hours before fixation, there was found an incidence of ER of 42 %, and similar levels of ER have been observed occasionally in a Lu 106 line infected with measles virus.

Tables 1 and 2 also contain data on chromosome constrictions and breaks, and there is no doubt that these phenomena are more frequent after cold treatment than in the controls. Before going into a more detailed description of these effects, it will be necessary to pay some attention to the normal chromosome morphology of the cell line Lu 106.

IV. NORMAL CHROMOSOMES OF THE LU 106

The two materials used for this study, the HEL cell strain and the Lu 106 cell line were strikingly different in chromosome constitution. While HEL exhibited the normal male karyotype, Lu 106 had deviated far from its origin. In the present section a few data on the karyotype of Lu 106 will be given.

Fig. 2 shows the chromosomes *in situ* of a colchicine-treated and squashed metaphase plate, and Fig. 3 presents karyotype analyses of three Lu 106 cells. As is characteristic for cell lines, the chromosome number is variable around a stemline number. In Lu 106 the stemline number is in the triploid region. Three samples of chromosome counts are given in Table 4 showing that most cells have 69 or 70 chromosomes. Occasional numbers outside the tabulated region 64—74 may occur. Thus, at one time several numbers between 90 and 95 were counted, possibly indicating the presence of a secondary stemline.

The chromosomes of Lu 106, except three long markers, can all be classed into the morphologic types of normal human chromosomes (Fig. 3). Roughly the chromosomes can be arranged into a triploid human karyotype. In some chromosome groups, however, there is a feeling of uncertainty. Thus, it is more difficult than usual to distinguish between the smallest members of the X, 6—12 group and No. 16. Also,

TABLE 4. *Chromosome numbers of Lu 106.*

Passage No. in Lund	Time	Chromosome number 64	65	66	67	68	69	70	71	72	74	Total cells	Mean
1	Dec. 63	—	1	1	1	8	20	30	8	3	2	74	69.6
14	March 64	1	1	—	1	2	6	4	—	3	—	18	69.0
30	June 64	—	5	8	7	11	47	7	1	3	1	90	68.5[1]
Total:		1	7	9	9	21	73	41	9	9	3	182	69.0

[1] Passage 30 was analyzed by Dr. PERTTI AULA.

similar difficulties exist in the separation of 16 and 17—18. In group 19—20 there are more pronounced size differences than normally; usually three bigger and three smaller chromosomes can be distinguished. All these observations indicate that the Lu 106 has undergone more structural rearrangements than are immediately apparent, even though the main morphology of the human chromosomes is preserved except in the three marker chromosomes.

The three long marker chromosomes (M1, M2 and M3), present in most cells, deviate strongly from normal human chromosomes. Their appearance can be seen from Fig. 2, in which they are marked by arrows, and in Fig. 3, in which they are placed last in the karyotypes. The longest of them, M1, has the proportions of a No. 4—5, but both arms are longer. It often has about the same length as No. 2, but is easily distinguishable from this chromosome by its greater difference in arm length. It regularly has a constriction on its longer arm at about one third the length from the end. The other two markers, M2 and M3, resemble the 13—15 group but are too long to be included in this group. They both have satellites on their short arms and often participate in associations with members of the 13—15 and 21—22 groups.

To get an idea as to the detailed composition of the karyotype, 15 metaphases were analyzed. These cells all belonged to the stemline or adjoining classes, their chromosome numbers being 68—71, plus one or more additional minutes, cells with minutes being rather common. The average composition of the 15 cells analyzed appears in Table 5. A comparison with what would be expected in a triploid male karyotype shows that, except for the addition of the 3 markers, the only deviation is, too low a number of chromosomes in the group Y, 21—22.

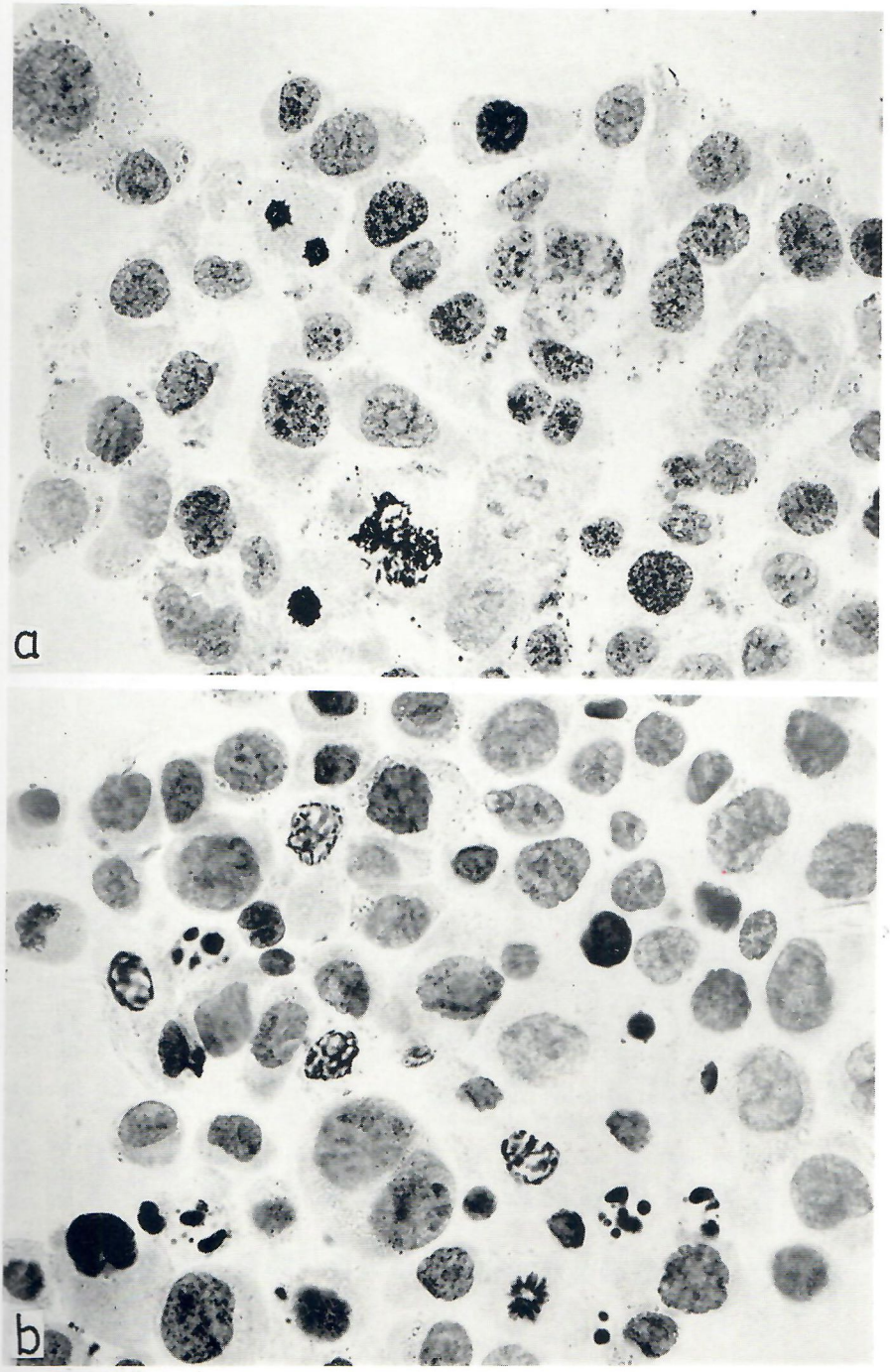

Fig. 1. General appearance of the cell population of Lu 106. a: without cooling; b: after 3 days in +3° C followed by 2 hours of gradual warming and 8 hours in +37°. — ×380.

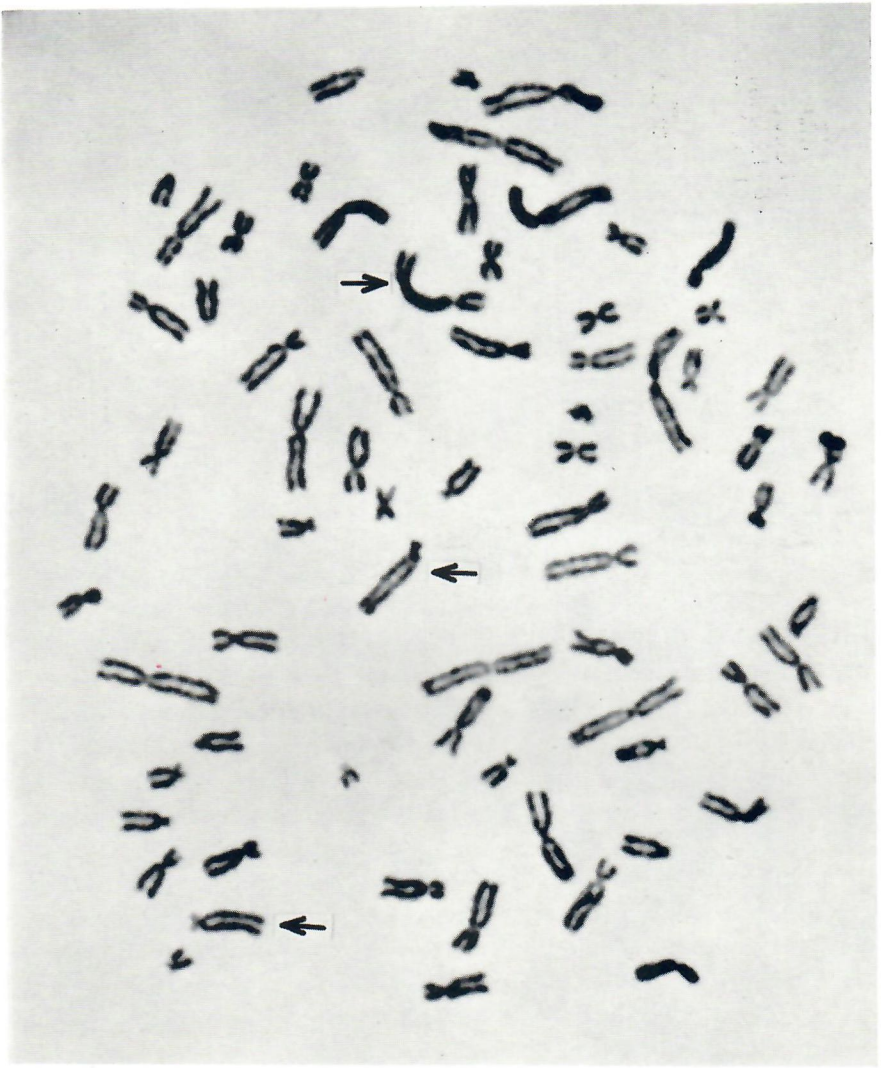

Fig. 2. Normal appearance of the chromosomes of Lu 106. The three marker chromosomes at the arrows, from above to below M1, M2 and M3. — ×2000.

Fig. 3. Three karyotype analyses of normal Lu 106, a: 69; b: 70; c: 74 chromosomes, in c four centric minutes. — ×1900.

TABLE 5. *The karyotype of Lu 106, average of 15 cells.*

Chromosome or chromosome group	Average number	Variation extremities	Expected in 3x male
1	3.1	3—4	3
2	3.0	3	3
3	3.1	3—4	3
4—5	6.0	6	6
X,6—12	23.2	21—25	23
13—15	8.1	7—9	9
16	3.2	3—4	3
17—18	6.1	5—7	6
19—20	6.2	6—7	6
Y,21—22	4.4	3—6	7
M1	1.0	1	0
M2	1.0	1	0
M3	1.1	1—2	0

In order to analyze the distribution of chromosome breaks over the idiogram an estimate of the chromosome length of the stemline set in Lu 106 was obtained. For this purpose we selected one representative cell, in which all chromosomes were under uniform contraction and well spread in one plane. The karyotype of this cell is given in Fig. 3 a. The measurements obtained were then adjusted to the frequencies of different chromosome types characteristic of the average karyotype of the Lu 106.

The absolute length values obtained are recorded in Table 6, column 2. The total chromosome length of the cell was 390 μ. As a comparison, LEVAN and HSU (1959) found an average total length of 157 μ for the normal diploid male cell. This was in leukocytes, which generally have a fairly high degree of contraction. Even so the present Lu 106 cell has very long chromosomes. The measured cell was selected among those having comparatively low chromosome contraction.

To see how the different chromosome classes compare in relative length with the normal diploid karyotype, the chromosome length averages of Table II of the Denver report are listed in Table 6. Our values for Lu 106 adjusted to the same total length are given in the two last columns of the table. Especially when the 3 marker chromosomes are disregarded, the agreement between the Denver values and ours is quite striking, indicating that the proportions of the Lu 106 chromosomes

have not changed greatly. Some of our results with chromosome constrictions and breaks also indicate that the basic structure of the Lu 106 chromosomes agrees with the normal karyotype.

V. CHROMOSOME CONSTRICTIONS INDUCED BY THE COLD TREATMENT

As mentioned in the introduction, this investigation was undertaken with the hope that the reaction of "nucleic acid starvation" might be obtainable by cold treatment. The first results were encouraging. Even though constrictions were infrequently evident in most cells, each experiment was characterized by a varying number of cells showing chromosomes of a strikingly deviating appearance. These cells exhibited a great ruggedness in their chromosome outlines, strongly stained segments alternating with segments having almost no stain and appearing as constrictions, stained and unstained regions following each other in irregular sequence along the chromosome length. This very characteristic appearance is seen from Figs. 4—6 for Lu 106 and in Fig. 7 for HEL. Such cells were not seen in the controls, and were undoubtedly induced by the treatments. They appeared most frequently in treatments at 3°

TABLE 6. *Chromosome length of Lu 106.*

Chromosome type	Lu 106 absolute lengths 0.1 μ	Denver length averages	Lu 106 lengths adjusted to same total length	Lu 106, the same, markers excluded
1	335	1034	972	1039
2	306	968	918	981
3	267	812	1033	1103
4—5	426	1446	1279	1366
X,6—12	1375	4267	4106	4386
13—15	349	1198	1036	1106
16	134	364	375	401
17—18	186	662	549	587
19—20	192	540	558	596
Y,21—22	75	492	204	218
M1	102	—	306	—
M2	86	—	258	—
M3	70	—	189	—

for 3 days after a recovery time of 10 hours at 37°. They showed an apparent correlation with the incidence of breakage, and it was soon clear that the origin of the constrictions is somehow associated with the breakage (Table 1 and 2). As is well known, constrictions are an accompanying feature of chromosome breakage induced by different agents. They were observed in connection with the chromosome breakage induced by phenols in the experiments of LEVAN and TJIO (1948) and were called "erosions" at that time. ÖSTERGREN and WAKONIG (1954) discuss the relations between chromosome breaks and erosions and suggest the term "break-constrictions" for the erosions. It is impossible for us to decide whether in the present case they have anything to do with the "nucleic acid starvation". Since they are irregularly scattered over the karyotype, they could well correspond to the heterochromatic bodies seen at interphase, which undoubtedly are frequent. We did not succeed in finding regularities in the pattern of constrictions, as would have been expected if they represented regions of large heterochromatic segments similar to those in *Paris* and *Trillium* and other materials that have corresponding areas of the chromosomes positively heteropycnotic during interphase and negatively heteropycnotic during mitosis.

For one cell, we made an attempt to analyze the entire chromosome set (Figs. 5—6). The variety of appearance of the different chromosomes was striking. While some chromosomes, as one No. 16, were almost without constrictions, others had a distinct sequence of stained and destained regions. It was observed that in a few instances the very ends of the chromosomes were much narrower than the rest of the chromosome body. If there was a relation between the cold breaks and the observed constrictions, a certain common pattern should be expected between these two phenomena. Indeed, if Fig. 6 is compared with Fig. 14 there does seem to be some similarities in general pattern although it is obvious that no statistically significant data can be derived from only a single cell as the one in Fig. 6.

The same type of erosion constrictions were found also in HEL. In Fig. 7 from HEL some erosions occur, but another effect is more evident in this figure: the exaggeration of secondary constrictions present in normal cells. In Fig. 7, the most apparent constrictions are close to the centromere in the long arm of No. 1 and No. 9. Also the heads of No. 13—15 and 21—22 are narrow. These regions were often marked by constrictions in Lu 106, too, and in addition the constriction on the long arm of M1 was often exaggerated.

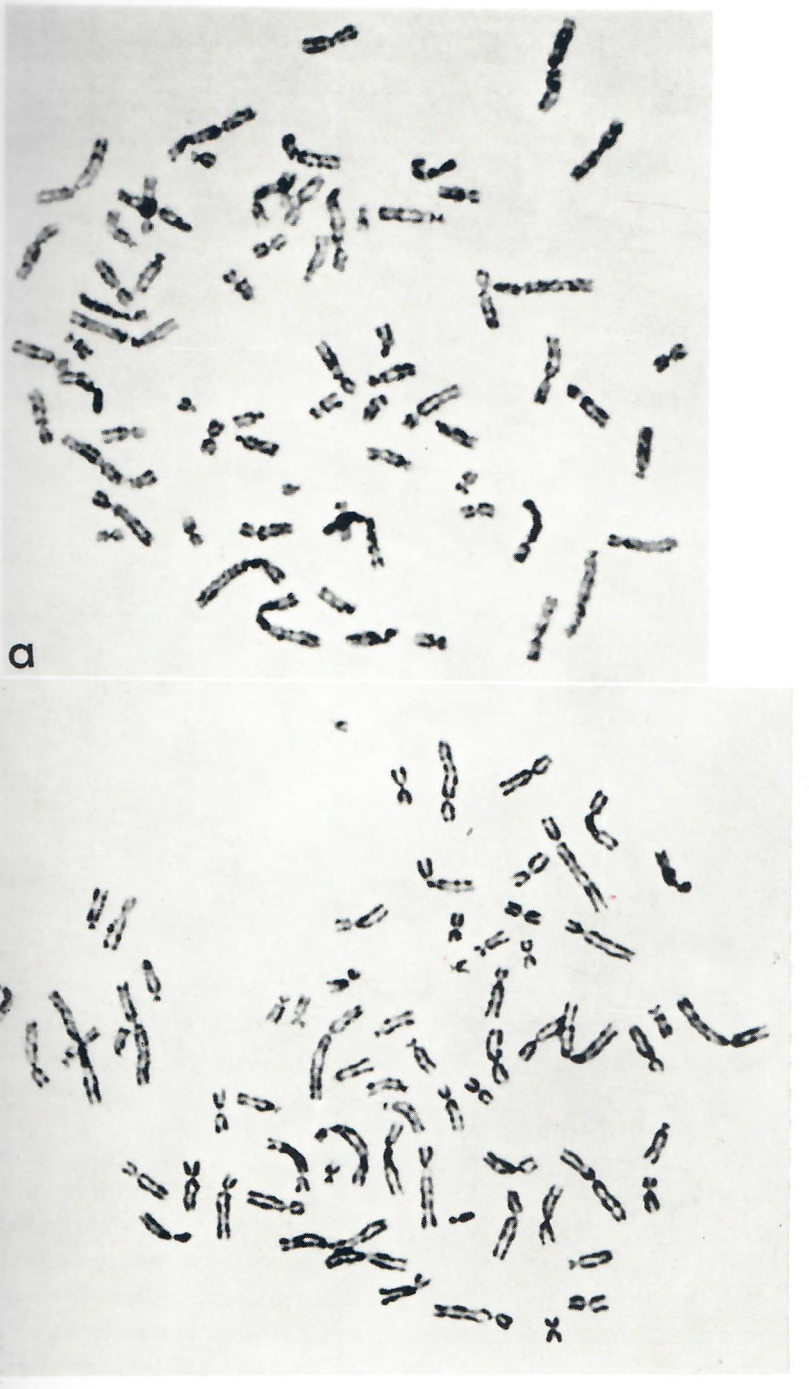

Fig. 4. Lu 106, the appearance of constrictions after cold treatment, a: 2 days at +3° C plus 12 hours at +37°; b: 2 days at +9°, after that slowly warmed during 2 hours, and 8 hours at +37°. — a: ×1700, b: ×1600.

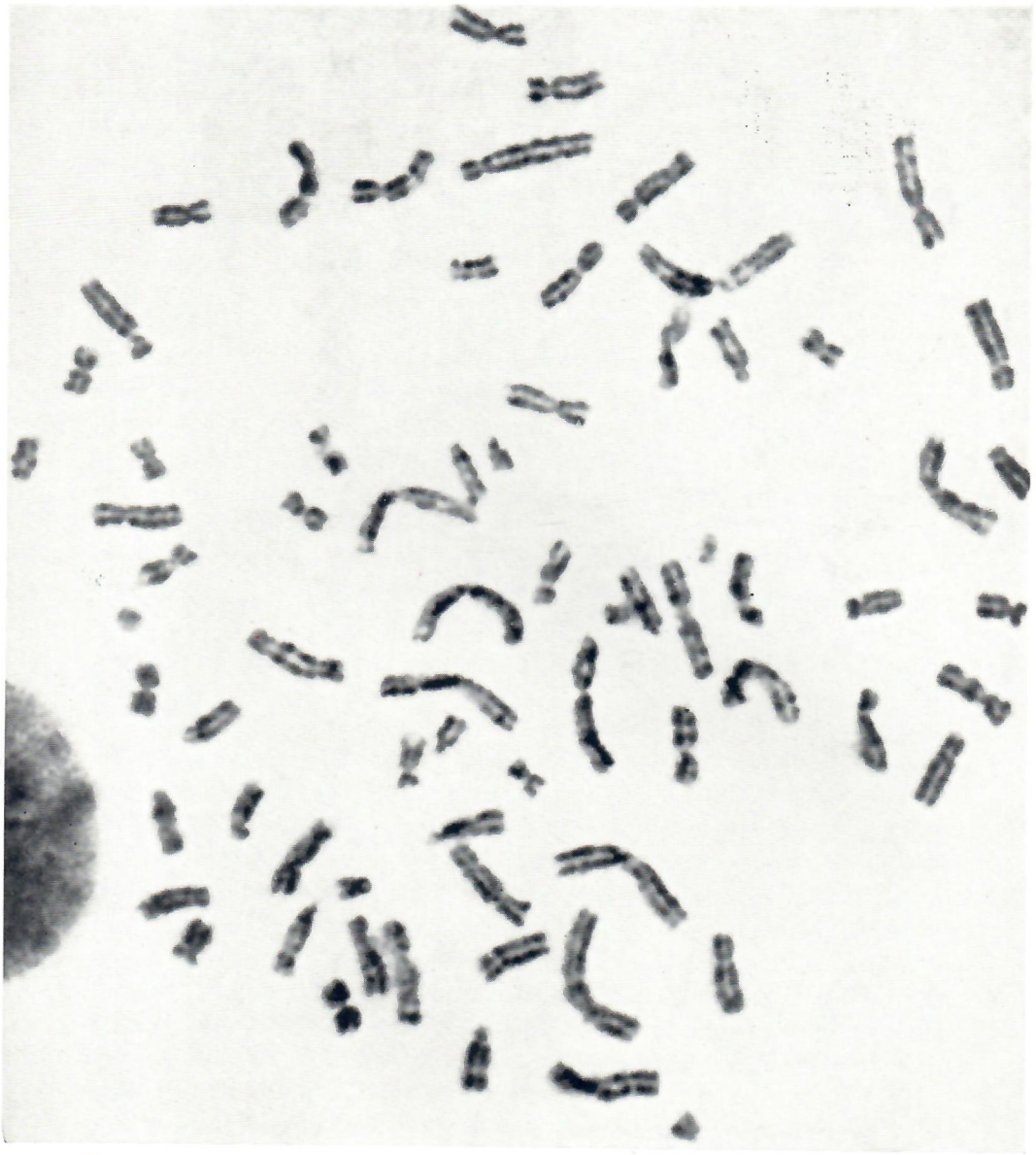

Fig. 5. Lu 106, the appearance of constrictions after cold treatment; 3 days at +3°, warmed slowly during 2 hours, and 6 hours at 37°. — ×2500.

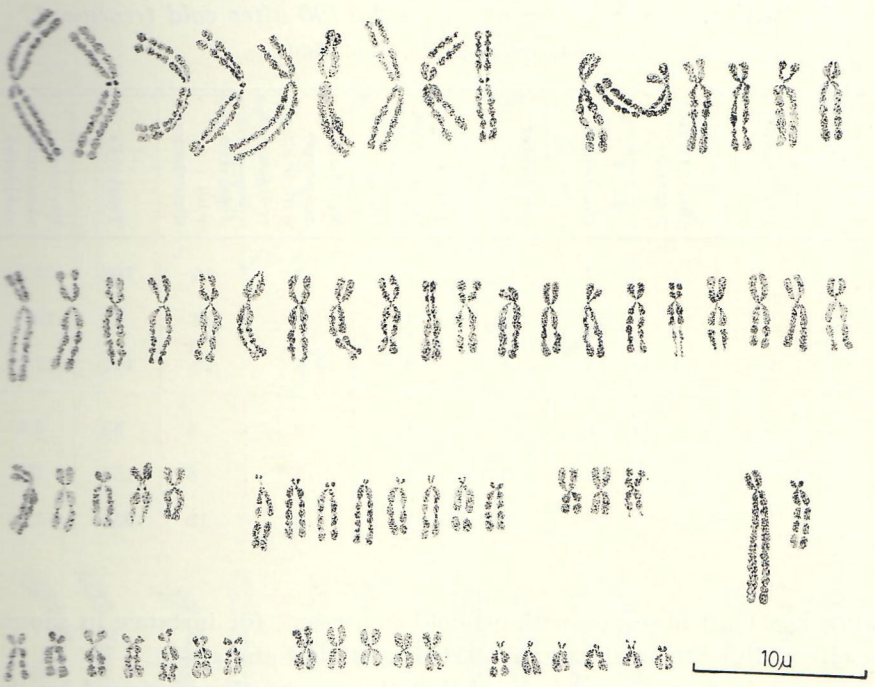

Fig. 6. The chromosome of Fig. 5, arranged into a karyotype. — ×2300.

A phenomenon, which sometimes blurred the pattern of constrictions was the strong spiralization present especially in direct fixations of cold treatments (several instances in Fig. 13). This type of spiralization usually disappeared after a few hours in +37°. As is well-known from many materials it may be very difficult to distinguish spiral gyres from big chromomeres. In some of the cold treatments, the chromosomes were found with a regular sequence of constrictions and chromomeres, that almost suggested a uniform reaction of the chromosomes to the environment resulting in a chromosome phenotype with chromomeres (e.g. one chromosome in Fig. 12 c). This would be a type similar to the instances of chromomeres observed by NICHOLS (1963) in chromosomes of Rous tumors of the rat (l.c., Fig. 5 c, d), or by NOWELL (1964) in a human leukocyte culture treated with mitomycin (l.c. Fig. 2). In a few instances single chromosomes of a cell at metaphase exhibited segments with very low contraction (Fig. 10 d). These undercontracted segments usually showed a chromomeric pattern. A similar chromosome appear-

TABLE 7. *Abnormal anaphases in Lu 106 after cold treatment.*
(treatment as in No. 10 of Table 1)

Treatment	Experiment No.	One acentric fragment	One bridge	One bridge and one acentric	Two or more bridges with or without fragments	Multipolar anaphases with bridges or fragments	Total cells with disturbances	Total cells	Per cent abnormal anaphases
Cold	1	1	7	1	5	2	16	103	15.5
	2	0	8	0	1	4	13	68	19.1
	1+2	1	15	1	6	6	29	171	17.0
Control	1	2	1	0	1	0	4	83	4.8
	2	3	4	0	1	0	8	155	5.2
	1+2	5	5	0	2	0	12	238	5.0

ance has been observed without cold treatment, for instance in mouse ascites cells (TJIO and LEVAN, 1954, Fig. 9 b—d and g—h).

In conclusion, it must be said that the constrictions induced by the cold treatment represent a complex phenomenon. They probably are exaggerations of normally occurring secondary constrictions, break-constrictions, indications of a chomomeric pattern, and possibly also negative heteropycnosis of the "starvation" kind.

VI. CHROMOSOME BREAKS

As mentioned above, certain treatments were especially predisposing to a high incidence of both erosions and breaks. It was found that cold treatments followed by slow warming during 2 hours to room temperature and 8 hours in 37° resulted in many breaks. Since especially in Lu 106 under control conditions the frequency of breaks can be rather high, it is important to have controls parallel to the experimental cultures. In the present experiments the controls had 4—10 % breaks in Lu 106 (Table 1). In Lu 106 the increase in break incidence after cold treatment was double to fourfold, in HEL, where the breakage of the controls was lower, about 2—3 %, the increase was tenfold. The incidence of breaks was routinely checked also in anaphase preparations, in which the cultures were not treated with colchicine or hypotonic.

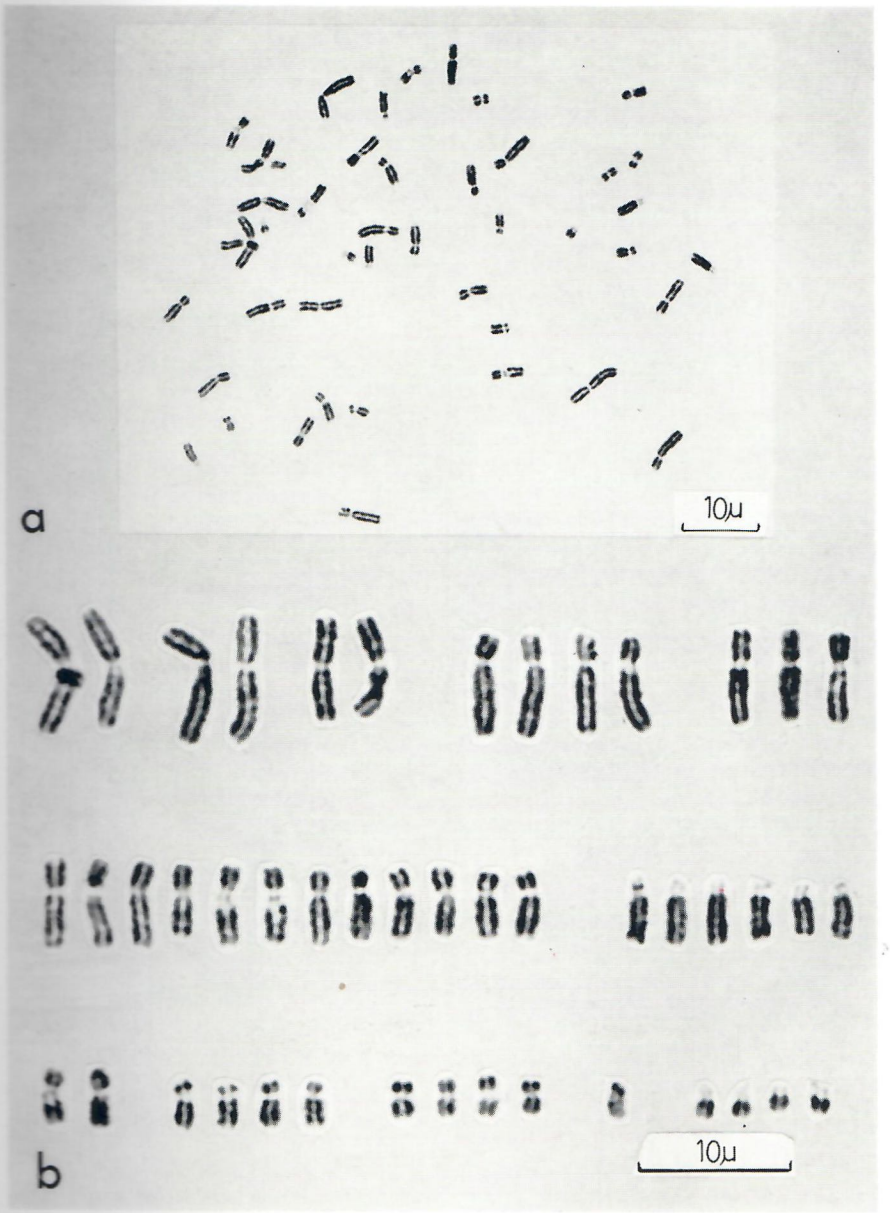

Fig. 7. HEL, one cell showing exaggerated secondary constrictions and a certain degree of erosions after treatment for 3 days in +3°, warmed slowly to +37° and fixed 8 hours later, a: chromosomes *in situ*; b: arranged into a karyotype. — ×1000 (a), 2000 (b).

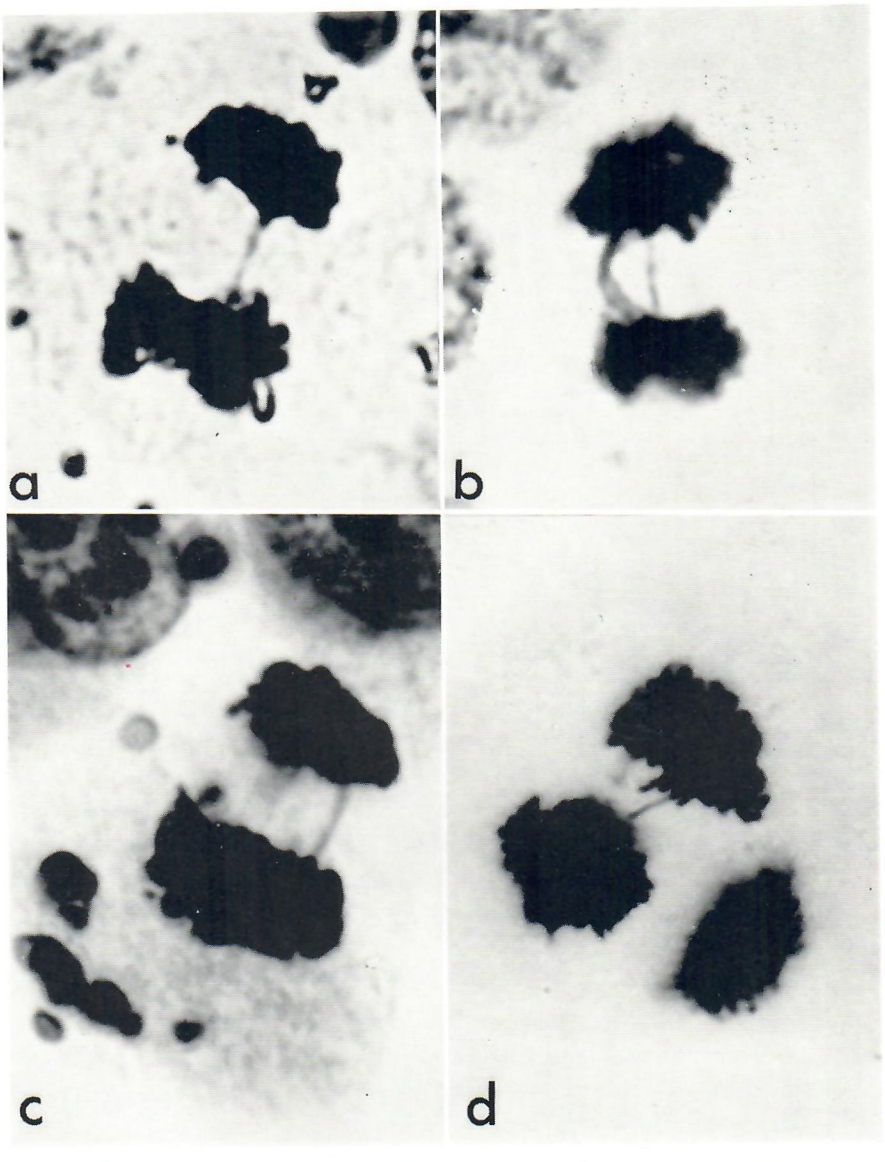

Fig. 8. Lu 106, anaphase disturbances after cold treatment. — ×950.

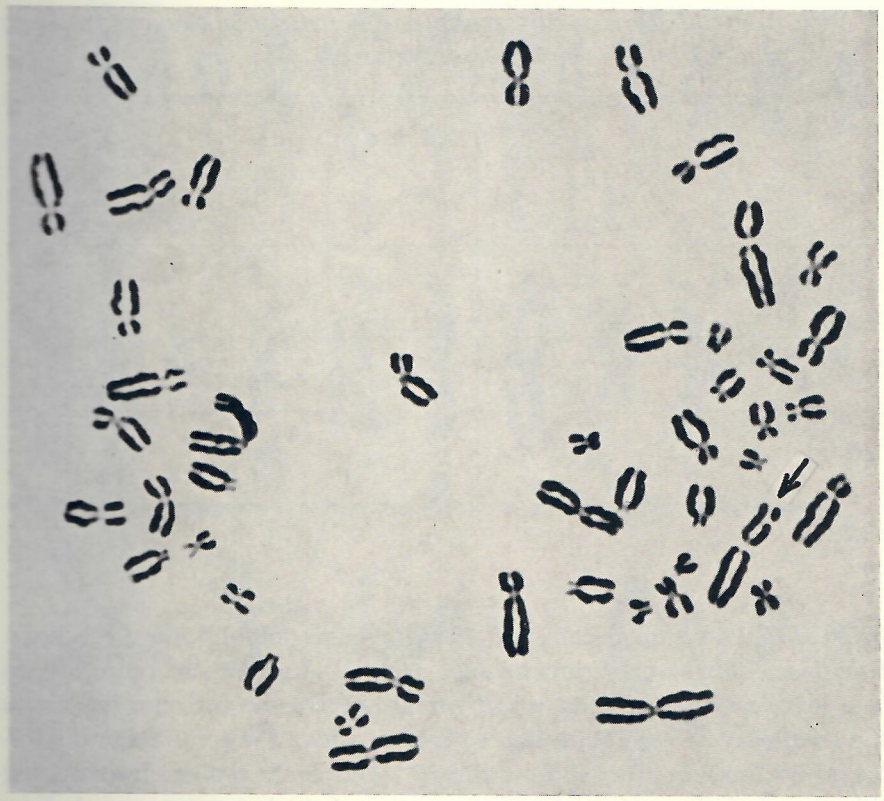

Fig. 9. HEL, metaphase with chromatid break (arrow) after cold treatment. — ×2000.

Table 7 and Fig. 8 illustrate the anaphase experiments. The frequency of anaphase disturbances, bridges and acentric fragments, was about 5 % in the control and 3 to 4 times as many in the treated series. One instance of a HEL cell after cold treatment with a chromatid break is given in Fig. 9.

In order to compare the structural changes induced by cold treatment with those of a standard mutagenic agent, and thus get an opportunity of characterizing the present experiments in relation to standard mutagenic experiments, we arranged an X-irradiation experiment with Lu 106. The irradiation exposed the cultures to a total of 100 r, over a time period of 12 seconds. This dose was chosen on the basis of the experiences of PUCK (1960) that 40 r will induce an average of one break per cell in human diploid cells in culture, and that the mean lethal dose is

TABLE 8. *Frequencies of different types of structural changes in X-ray and cold experiments.*

Cell	Experiment		Open chromosome breaks	Dicentrics	Acentrics	Rings	Chromatid interchanges	Chromatid intrachange	Total abnormal chromosomes and rearrangements
Lu 106	X-rays[1]	Abs	113	5	3	—	11	2	134
		%	84.3	3.7	2.2	—	8.2	1.5	49.9
	Cold	Abs	184	22	14	1	4	—	225
		%	81.8	9.8	6.2	0.4	1.8		100.0
HEL	Cold	Abs	134	2	3	1	2	—	142
		%	94.4	1.4	2.1	0.7	1.4		100.0

[1] Upper row, all abnormalities observed; lower row, percentage of total abnormalities.

50 r for diploid human cells and 100 r for heteroploid human cell lines. Our dose, 100 r, would thus be expected to induce two or more breaks in a large number of cells, which should give some information about the frequency of recombination of broken ends. The treatment was followed by fixation after 10 hours in 37°. Our main reason in arranging this experiment was to test whether cells in which two or more breaks are induced during presynthesis period, G1, and in which the broken ends recombine into dicentrics or rings may reach metaphase in 10 hours. Most of our cold treatments were given a 10 hours recovery time after their cold exposure. The rather frequent occurrence of dicentrics in the cold experiments of Lu 106 was somewhat puzzling, and it was thought that the X-ray experiment might help understanding this. Later on, however, it was realized that a low grade activity is probably going on continually during the cold treatment. Through the gradual warming these mitoses accelerate, and it is possible that cells that have undergone DNA synthesis during the cold treatment may reach metaphase during recovery time. Among others, our reasons for this conclusion are the increase in cell number and the gradual color change of the medium during treatment indicative of metabolic activity. Definite evidence of DNA synthesis during the cold treatment might be obtained by thymidine autoradiography. Such experiments are planned.

The incidence of structural deviations belonging to different morpho-

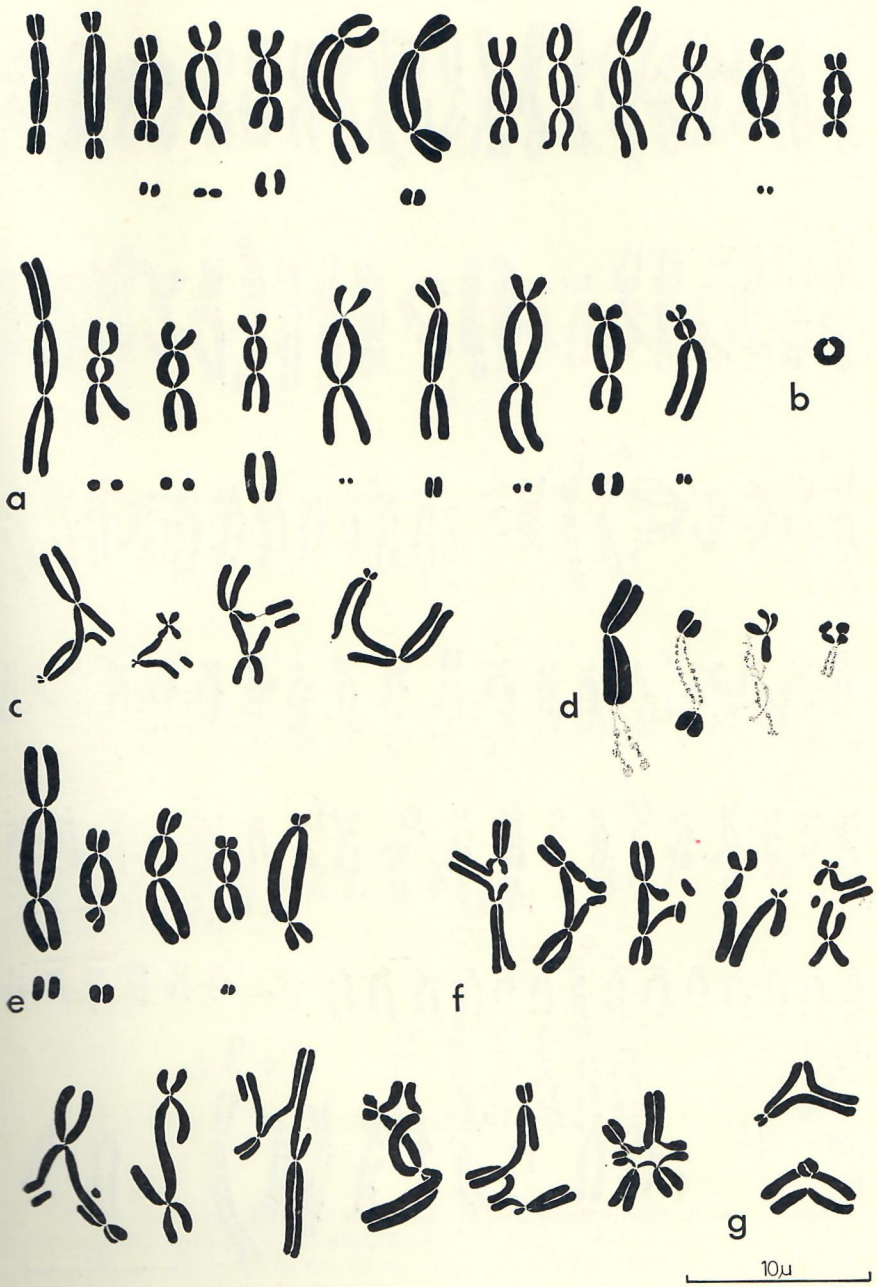

Fig. 10. Lu 106, chromosome and chromatid interchanges etc. after cold treatment (a—d), or X-irradiation (e—g); a, e: dicentrics and acentrics; b: ring chromosome; c, f: chromatid interchanges; g: possible chromatid intrachanges; d: differentially contracted chromosomes. — ×2400.

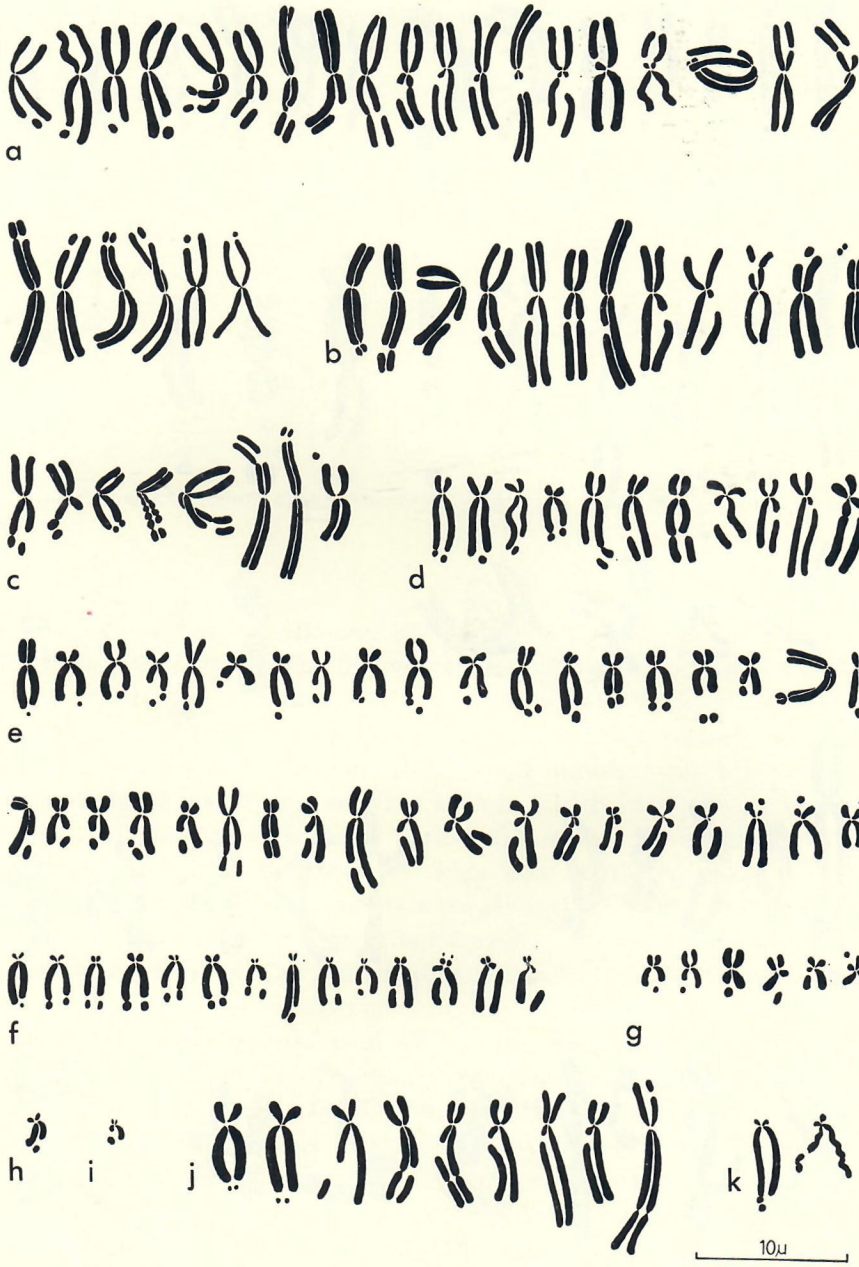

Fig. 11. Lu 106; all isochromatid breaks in certain cold experiments, the chromosomes arranged according to type in the following groups: a: No. 1; b: 2; c: 3; d: 4—5; e: X, 6—12; f: 13—15; g: 16; h: 17—18; i: Y, 21—22; j: M1; k: M2; in each group the chromosomes have been arranged according to location of the break, starting from the end of the long arm and proceeding to the end of the short arm. — ×2000.

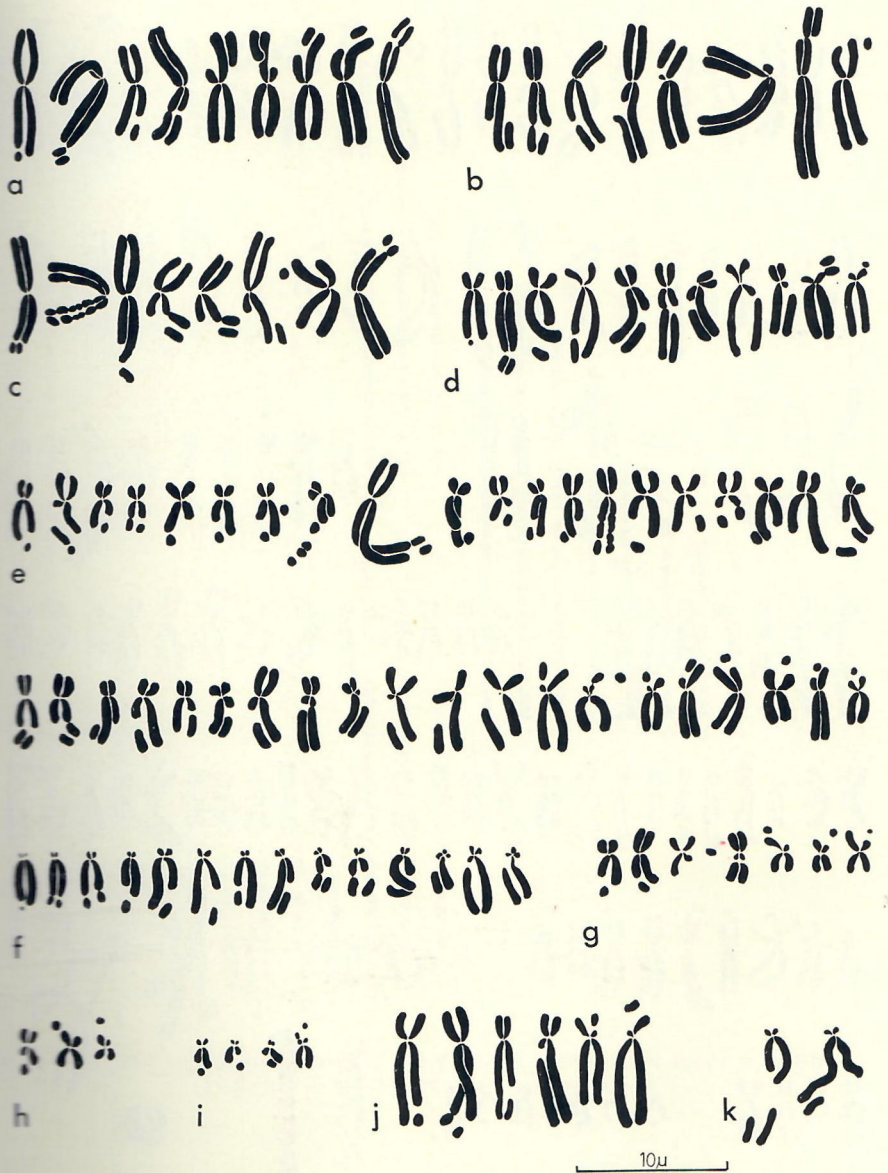

Fig. 12. Lu 106; all isochromatid breaks of one X-ray experiment. Same arrangement of chromosome types as in preceding figure: a: No. 1; b: 2; c: 3; d: 4—5; e: X, 6—12; f: 13—15; g: 16; h: 19—20; i: Y, 21—22; j: M1; k: M2. — ×2000.

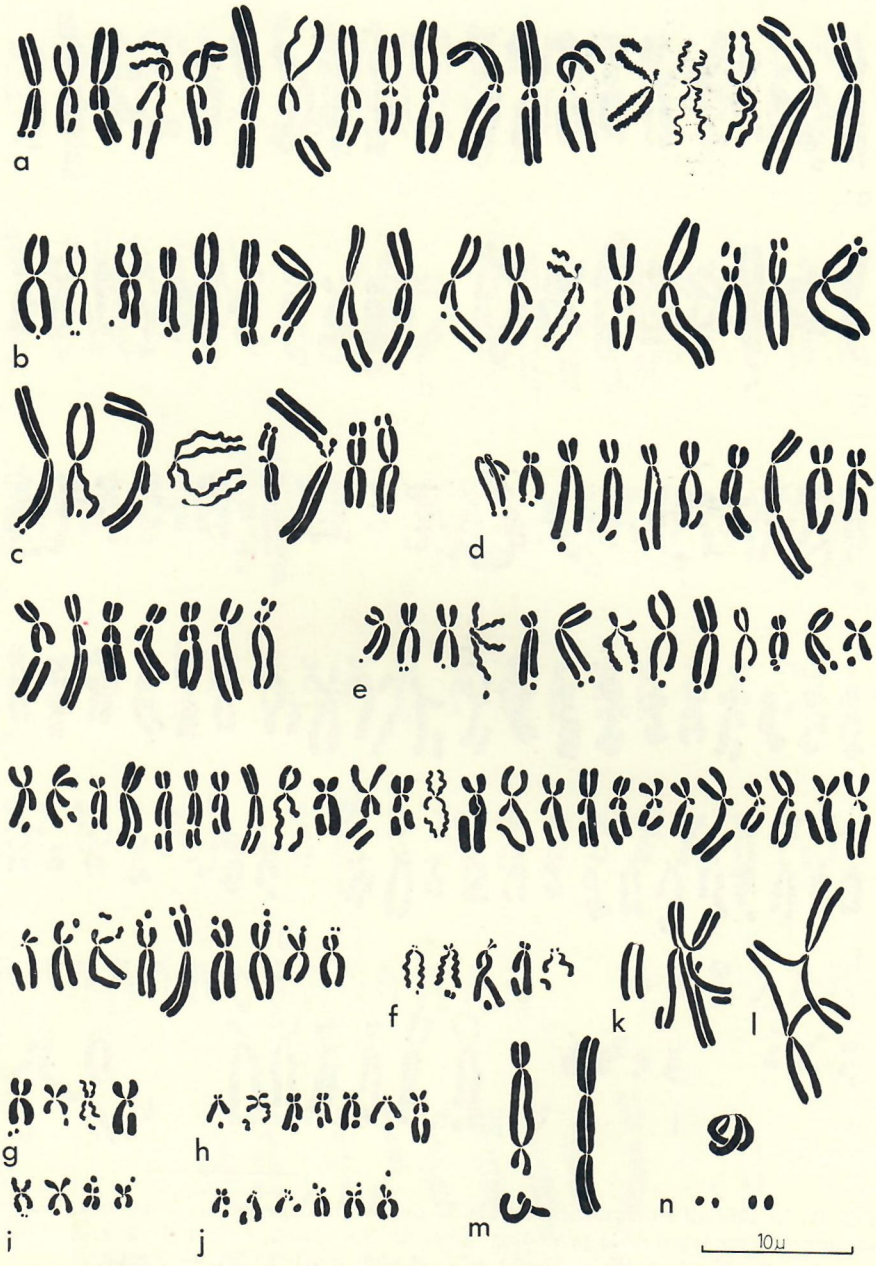

Fig. 13. HEL; isochromatid breaks (a—j) and interchanges (k—n) from cold experiments. a: No. 1; b: 2; c: 3; d: 4—5; e: X, 6—12; f: 13—15; g: 16; h: 17—18; i: 19—20; j: Y, 21—22; k—l: chromatid interchanges; m: dicentrics and acentrics; n: ring and acentrics. — ×2000.

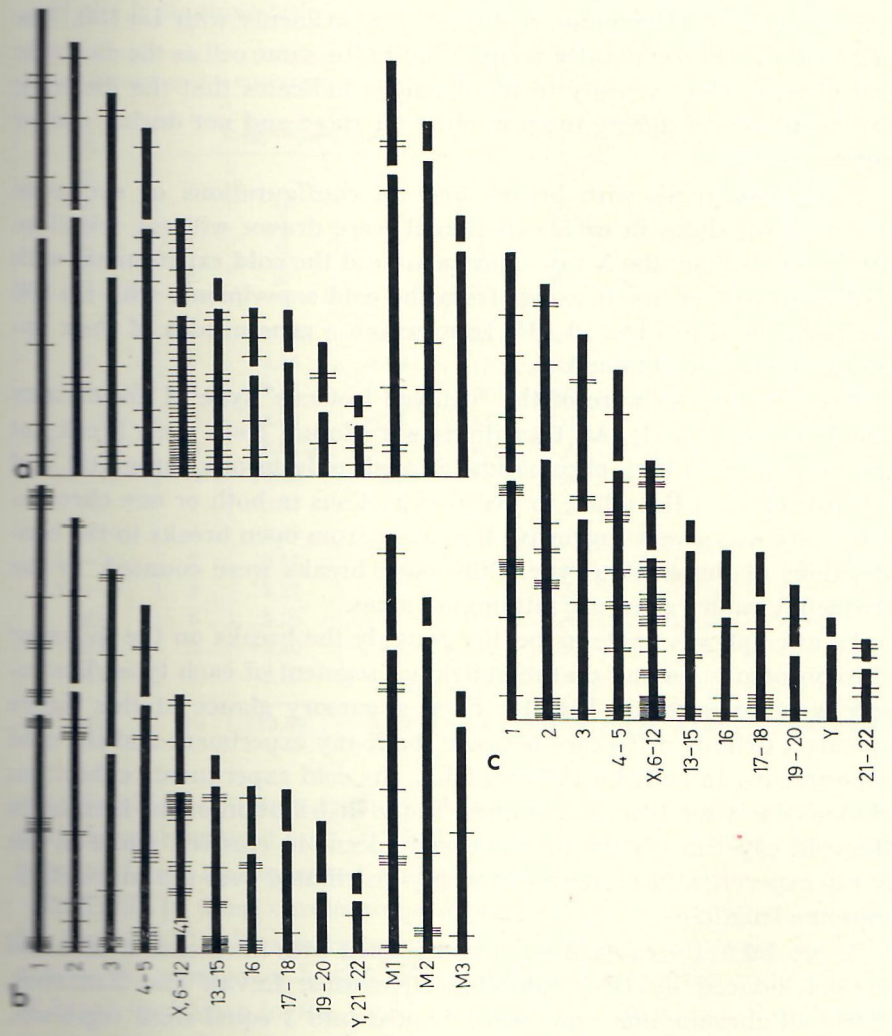

Fig. 14. Chromosome breaks plotted on the different chromosome types. a, b: Lu 106; c: HEL; a: X-irradiation; b, c: cold treatment.

logic classes found in the 3 experimental series are surveyed in Table 8. Open breaks predominated in all 3 series, varying in frequency between 82 and 94 % and were as frequent in the X-ray series as in the cold treatments of Lu 106. The X-ray series differred from the cold series by having more chromatid exchanges, 10 % as compared with 1—2 % in the cold experiments. Dicentrics and acentrics occurred in all three se-

ries but were most common in the cold experiments with Lu 106. The fact that an acentric usually was present in the same cell as the dicentric and often in close vicinity to the dicentric indicates that the dicentric had been formed during the preceding G1 stage and not during earlier mitotic cycles.

All chromosomes with breaks and all configurations of exchange from certain slides in each experiment were drawn without selection. All drawings from the X-ray experiment and the cold experiments with HEL, and part of the drawings from the cold experiments with Lu 106 are reproduced in Figs. 10—13, from which a general idea of their appearance may be obtained.

The open breaks were of the "delayed isolocus" type of ÖSTERGREN and WAKONIG (1954). All transitions were found from open breaks at the same level in both chromatids, a break only in one chromatid and a constriction in the other, to just constrictions in both or one chromatid. There was a very suggestive transition from open breaks to the constrictions of the erosion type. Only open breaks were counted, so the frequency values given are minimum values.

An attempt was made to localize roughly the breaks on the different chromosome types and on the individual segment of each type. The result is summarized in Fig. 14. Even a cursory glance at this figure reveals a striking difference between the X-ray experiment and the cold experiments. In both Lu 106 and HEL, the cold experiments exhibit an obvious deviation from randomness in the distribution of the breaks. In the cold experiments the breaks clearly occur in clusters, while in the X-ray experiment they are more evenly distributed over the entire chromosome length.

As we did in the cases of breaks associated with the measles virus and breaks induced by DNA inhibitors (NICHOLS, LEVAN and KIHLMAN, 1964) all chromosome arms were divided into 3 equal-sized segments, numbered 1—3 and 4—6 from the end of the long arm to the centromere, and from the centromere to the end of the short arm, respectively. The number of breaks in each segment was compared with the number expected for this segment on the assumption of random distribution. In Table 9 the values are collected for entire chromosome types, and in Table 10 also chromosomal subunits have been considered. χ^2 has been calculated, when the expected numbers exceeded 5. In Table 9 pronounced differences are seen between the three materials. The X-ray material shows only slight deviation from expectation, just one χ^2 is high (deficit of breaks in chromosomes 17—18). In both cold experiments

TABLE 9. *Distribution of chromosome breaks on the different chromosome types.*

Chromosome type	Lu 106						HEL		
	X-ray experiment			Cold experiments					
	Found	Expected	χ^2	Found	Expected	χ^2	Found	Expected	χ^2
1	9	9.70	0.05	33	15.79	18.75	19	11.58	4.75
2	8	8.86	0.08	15	14.43	0.02	17	10.84	3.49
3	8	7.73	0.01	9	12.59	1.02	8	9.10	0.13
4—5	11	12.33	0.14	16	20.08	0.83	17	16.20	0.04
X,6—12	41	39.81	0.04	62	64.82	0.12	45	47.80	0.16
13—15	14	10.10	1.50	19	16.45	0.39	5	13.42	5.28
16	7	3.88	—	8	6.32	0.45	4	4.08	—
17—18	0	5.39	5.39	1	8.77	6.88	7	7.42	0.02
19—20	3	5.56	1.17	0	9.05	9.05	4	6.05	0.69
Y,21—22	4	2.17	—	1	3.54	—	6	5.51	0.04
M1	6	2.95	—	13	4.81	13.95	—	—	—
M2	2	2.49	—	5	4.05	—	—	—	—
M3	0	2.03	—	2	3.30	—	—	—	—
Total	113	113.00	8.38	184	184.00	51.46	132	132.00	14.60

considerable deviations from randomness occur. In both series, these deviations are caused by 3—4 chromosome classes with poor agreement between found and expected, while most classes agree well.

In Table 10 those chromosome regions which are mainly responsible for the high χ^2 values have been listed as excesses or deficits. Unfortunately, the number of breaks was insufficient to provide safe samples of break incidence in the small chromosomes, therefore, the chromosome arms and segments in the table are all from the longer chromosomes of the complement. It should be observed that safe deviations from expectation are found in X, 6—12 region 1 in all three series (excess), and in chromosome 1 long arm (excess), in X, 6—12 region 4 (deficit) in the two cold series.

If all chromosome segments with obvious deviations from randomness are tabulated regardless of whether they have statistical significance or not, the survey of Table 11 is obtained. In this table, observations of NICHOLS, LEVAN and KIHLMAN (1964) on the DNA inhibitors deoxyadenosine (AdR) and cytosine arabinoside (CA) as well as on

TABLE 10. χ^2 *test for randomness in distribution of breaks.*

Cell	Treatment	Unit recorded	$\Sigma\chi^2$	df	P	Itemization of χ^2:s >5			
						Excess		Deficit	
						Unit	χ^2	Unit	χ^2
Lu 106	X-rays	Chromosomes	8.38	7	<0.05			17—18	5.39
		Chromosome arms	6.30	5	<0.3				
		Chromosome segments	7.30	2	<0.05	X,6—12:1	6.28		
	Cold	Chromosomes	51.46	9	<0.001	1 M1	18.75 13.95	17—18 19—20	6.88 9.05
		Chromosome arms	51.83	11	<0.001	1 la 1 sa X,6—12 la	9.28 9.48 5.92	X,6—12 sa 17—18 la	15.34 5.03
		Chromosome segments	94.43	11	<0.001	X,6—12:1 13—15:1	51.59 20.40	X,6—12:4 X,6—12:5	5.68 5.68
HEL		Chromosomes	14.60	8	<0.1			13—15	5.28
		Chromosome arms	29.49	8	<0.001	1 la 2 la	10.37 8.33		
		Chromosome segments	17.85	5	<0.01	X,6—12:1	8.34	X,6—12:4	5.33

In the itemization-unit columns la and sa mean long arm and short arm, respectively; numbers following the : sign refer to segments of chromosomes.

measles (NICHOLS, 1963) are included, even though these observations were made on other cytologic systems, leukocytes in short-term culture. In the table, presence of clusters of breaks is indicated and also secondary constrictions observed by SAKSELA and MOORHEAD (1962) and SASAKI and MAKINO (1963). Few features common to all six materials are noticeable. Segment 1 of chromosomes X, 6—12 has an excess of breaks in all materials, and segment 5 of the same chromosome group has a deficit in all series except the X-ray series. It seems that two cold-treated materials show more agreement, and so do the two series of DNA inhibitors. Also these four series are more similar to each other than to the X-ray and the measles series. The latter two seem to represent each its individual type. Both in the cold treatments and in the

TABLE 11. *Deviations from expected break frequencies in different chromosome segments.*

Chromosome type	Segment No.	Lu 106, X-rays	Lu 106, cold	HEL, cold	AdR	CA	Measles
1	1		⊞		□		
	2		⊞	⊞	−	−	
	③		⊞	⊞		⊞	
	4		+		□		
	5				−		
	6		⊞				
2	1			⊞	⊞	+	
	2			+		−	+
	③		⊞		−	+	+
	4						⊞
	6				+		+
3	1		+				
	2					−	
	3				+		
4—5	1		□		⊞	□	
	2					⊟	
	③				−		−
	4				□		
	5					⊞	
	6				□	⊞	

Chromosome type	Segment No.	Lu 106, X-rays	Lu 106, cold	HEL, cold	AdR	CA	Measles
X,6—12	1	+	⊞	⊞	⊞	⊞	+
	2		−			−	
	③		□	□		+	+
	4		−	−		−	−
	5		−	−	−	−	−
	6		−		+	+	−
13—15	①	+	⊞		−	⊞	□
	②	−			□		□
	③			−	−		
16	1	□	+	+	⊞	⊞	
17—18	1			⊞		⊞	
19—20	1					⊞	
M1	①	+	⊞				
	3		+				
	4	+					
M2	1		+				

+=greater number of breaks than expected
−=lower number of breaks than expected
□=cluster of breaks in segment
○=presence of constriction in segment

DNA inhibitors, clusters of breaks occur, and sometimes show similarity in location. There is a vague impression that clusters of breaks are sometimes coinciding with the presence of secondary constrictions. This was especially so in the cases of the constriction in segment 3 of chromosome 1 and segment 1 of M1. This may be seen in connection with the observation that cold treatment often seemed to exaggerate secondary constrictions (Fig. 7) and that the erosion constrictions sometimes were especially pronounced in the same regions.

VII. DISCUSSION

The most interesting observation of the present investigation is the increase in number of chromosome breaks in tissue cultures exposed to low temperature. This has been observed earlier in meiotic chromosomes of plants, but as far as we know, no such observations have been made on mitotic chromosomes in plants or animals. The general impression of a mutagenic action of the cold treatment is strengthened by the presence of chromosome constrictions of the erosion type, or so-called break-constrictions, in those cold-treated cultures in which most breaks were found.

It is easier to visualize the production of chromosome breaks with agents as X-irradiation and radiomimetic chemicals than it is with an agent with seemingly such a low specificity of action as low temperature. It is also somewhat unexpected that cold treatment induces localized breaks, which of course may mean non-randomness in the restitution of randomly induced breaks as suggested long ago by SAX (1940) and recently discussed by HSU (1963) in relation to non-random X-ray breaks in a Chinese hamster chromosome. Some deoxyribosides as arabinosylcytosin that are known to influence specific chemical reactions in the synthesis of DNA precursors may more easily be thought to give rise to faulty chromosome reproduction and ensuing discontinuities of the chromonema. Quite specific biochemical conclusions have been drawn by HSU and SOMERS (1961) and SOMERS and HSU (1962) to explain the preferential induction in specific chromosome regions of breaks in a Chinese hamster cell line by bromodeoxyuridine and hydroxylamine. In the experiments of NICHOLS, LEVAN and KIHLMAN (1964) with deoxyadenosine and cytosine arabinoside in human leukocytes a non-random distribution was noticed, but it was found that individual cells could represent essentially different patterns with many breaks in the centromeric regions of some cells, and in the ends of others. In most cells no favoring of specific regions could be discerned. This may indicate that small differences in the stage affected may have decisive significance, and that perhaps the replication pattern of different chromosomes and chromosome segments plays a role.

In human leukocyte chromosomes COHEN (1964) found that a cytostatic substance, streptonigrin, induced a preferential breakage in the centromeric regions in only two of the three analyzed chromosomes, viz. in No. 1 and 2, but not in No. 3. In addition, the secondary constriction area of the long arm of No. 1, close to the centromere, had an excess of breaks. Both in the experiments of HSU and SOMERS and in those of

COHEN, X-rays differred strongly from the chemical agents in giving random distribution of the breaks, just as in the present experiments.

It seems natural to assume that a lowering of the temperature to 3° in the environment of a cell accustomed to a temperature of 37° would cause severe disturbances of the entire metabolic system. Generally a temperature permitting some metabolic processes would be expected to have a more deleterious influence than a temperature effectively stopping metabolism completely. A shortage of some metabolites and an accumulation in abnormal quantities of others would follow, and it is reasonable that some of these disturbances would be able to act on vulnerable stages of the chromosome reproduction causing breakage. Depending on what metabolic processes are affected, even such a non-specific influence as low temperature may result in highly specific final effects, as localized chromosome breaks.

It is impossible to tell definitively what stages of the interphasic chromosome cycle are affected. The considerable incidence of dicentrics in some experiments, makes it necessary to assume that at least some part of the G1 is influenced. The great majority of abnormalities was open breaks of the isolocus type, which might be induced both during G1, S and G2. Real chromatid rearrangements were rare in the cold treatments, but this may, as in the measles breaks, depend on a low tendency to recombination of broken ends. Considering that the highest frequency of breaks was found in cold treatments that had been permitted to recover for 10 hours at 37°, it seems probable that the most sensitive stage would be the end of G1 and the S period.

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SUMMARY

The influence of low temperature on human chromosomes was analyzed in two tissue culture materials, a human cell line, Lu 106, and a human cell strain, HEL, both derived from male embryonic lung tissue.

It was shown that several different cold treatments resulted in an increase of the incidence of chromosome breaks and of chromosome constrictions of the erosion type (break-constrictions). Most efficient treatment was +3 centigrades for 3 days, followed by gradual warming during 2 hours to room temperature, and a period of 8 additional hours at +37 centigrades. This treatment resulted in 20—40 % cells with breaks, while in the controls corresponding frequency was 4—10 % in Lu 106, and 2—3 % in HEL. There was an evident correlation between the presence of chromosome breaks and of break-constrictions.

All chromosome breaks of certain experiments were plotted on the human idiogram, and it was found that the cold-induced breaks showed a non-random distribution over available chromosome length, forming clusters in specific chromosome segments, while the breaks of an X-ray experiment with Lu 106 were randomly scattered.

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