^{3}H LABELING PATTERNS OF PERMANENT CELL LINE CHROMOSOMES SHOWING PULVERIZATION OR ACCENTUATED SECONDARY CONSTRICTIONS

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INTRODUCTION

An appearance suggesting extreme fragmentation of chromosomes has been referred to as "pulverization" by Nichols et al. (1). A different appearance, that of accentuated secondary constrictions (or chromomeres), was described by Stubblefield in a Chinese hamster cell line, Don (2). In 1967 zur Hausen (3) described an appearance more often occurring in chromosome No. 1 and referred to as "incomplete condensation." The incompletely condensed segments were shown to synthesize DNA after the normal segments had ceased replication. Subsequently, both Nichols et al. (4) and Kato and Sandberg (5) have reported that pulverized chromosomes exhibit a similar asynchrony.

The present report will provide evidence that pulverization, incomplete condensation, as well as some varieties of enhanced secondary constrictions form a spectrum of changes characterized by their spontaneous occurrence in permanent cell lines of several species and by DNA synthesis in G_2.

MATERIALS AND METHODS

Six permanent cell lines were studied. These included: a HeLa line; a Burkitt lymphoma line, Ogun (6, 7); three leukocyte lines—LK1D, LK63, and SKL-1 (8, 9); a mouse rhabdomyosarcoma line, T811 (O'Neill, F., and A. J. Samuels. Data in preparation); and a Chinese hamster line, Don (2).

With the exception of the Chinese hamster line, all of these lines stem from tumors or from patients with neoplastic disease. Methods for culture and for chromosome analysis have been previously described (10). In terminal labeling experiments with SKL-1, LK1D, Ogun, and T811, cultures were exposed to thymidine-^{3}H (^{3}H TdR, New England Nuclear Corp., Boston, Mass.) at a concentration of 0.3 μc/ml (specific activity, 2 mc/mmole) for various periods of time; and in later experiments with Don and HeLa, the cultures were exposed at a concentration of 0.5 μc/ml (specific activity, 6.7 mc/mmole).

The cultures were then harvested for routine chromosome analysis, as described previously (10), but with repeated washings in fixative. Slides were stained in 2% aceto-orcein containing 65% acetic acid. The slides were screened, and cells to be analyzed were photographed. The slides were then dipped in Kodak NTB-3 liquid emulsion and exposed for 10 days. Subsequently, the filmed slides were developed for 2 min in Kodak D-19 (19°C) developer and fixed for 4 min in Kodak fixer. After being washed in tap water for 20 min, the slides were dried with a hair dryer.

RESULTS

The common feature of the various appearances (pulverization, etc.) is the potential for DNA synthesis out of phase; hence, for brevity, we shall refer to the general phenomenon as SOOP (synthesis out of phase).

All of these cell lines exhibit a small percentage of metaphase figures in which a few or many chromosomes appear to be extensively fragmented (pulverized) (Figs. 3, 6). In some cells the chromosomes show a stretched-out appearance usually accompanied by multiple ladder-like secondary constrictions (Figs. 1–3). The ladder-like secondary-constriction appearance is to be distinguished from the previously described (3, 11) attenuated constrictions observed on chromosomes Nos. 1 and 16 in near-diploid lymphoid lines. Such

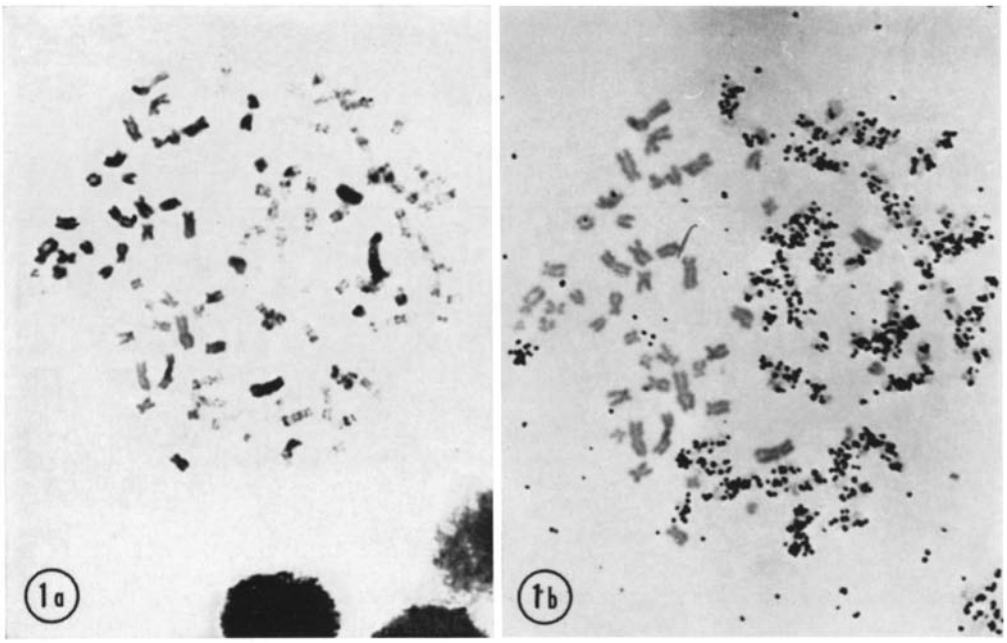

FIGURE 1 *a* and *b* Ladder-like secondary constrictions. Ogun. 4 hr terminal label. × 1600.

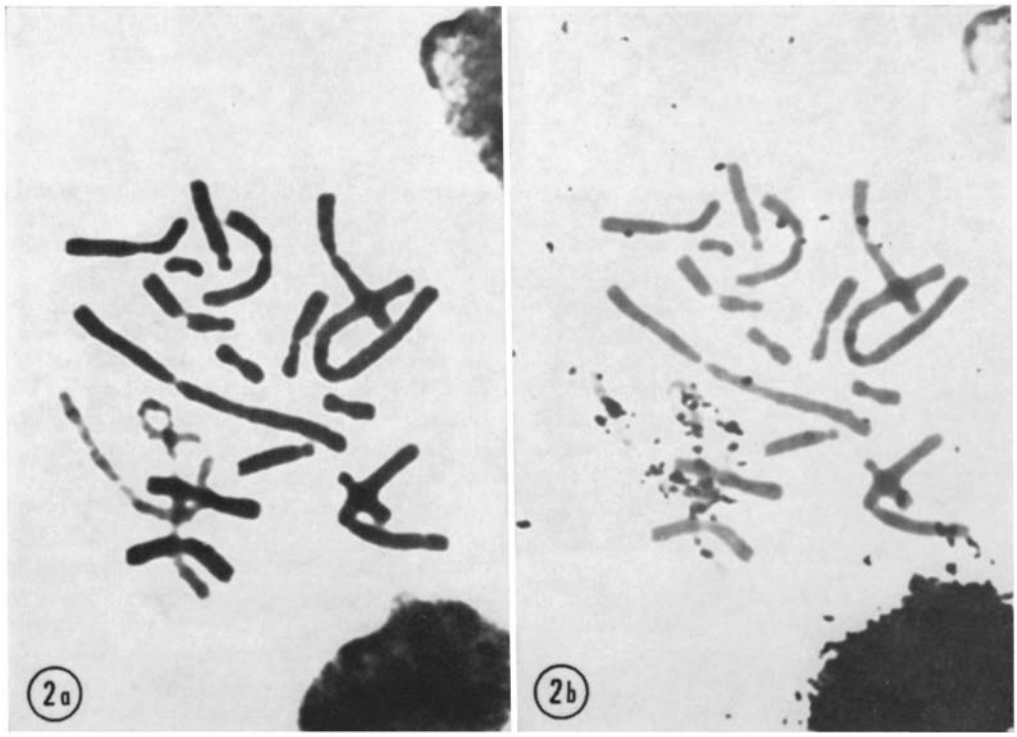

FIGURE 2 *a* and *b* Ladder-like secondary constrictions, Chinese hamster line Don. Diploid cell. 4 hr terminal label. × 1600.

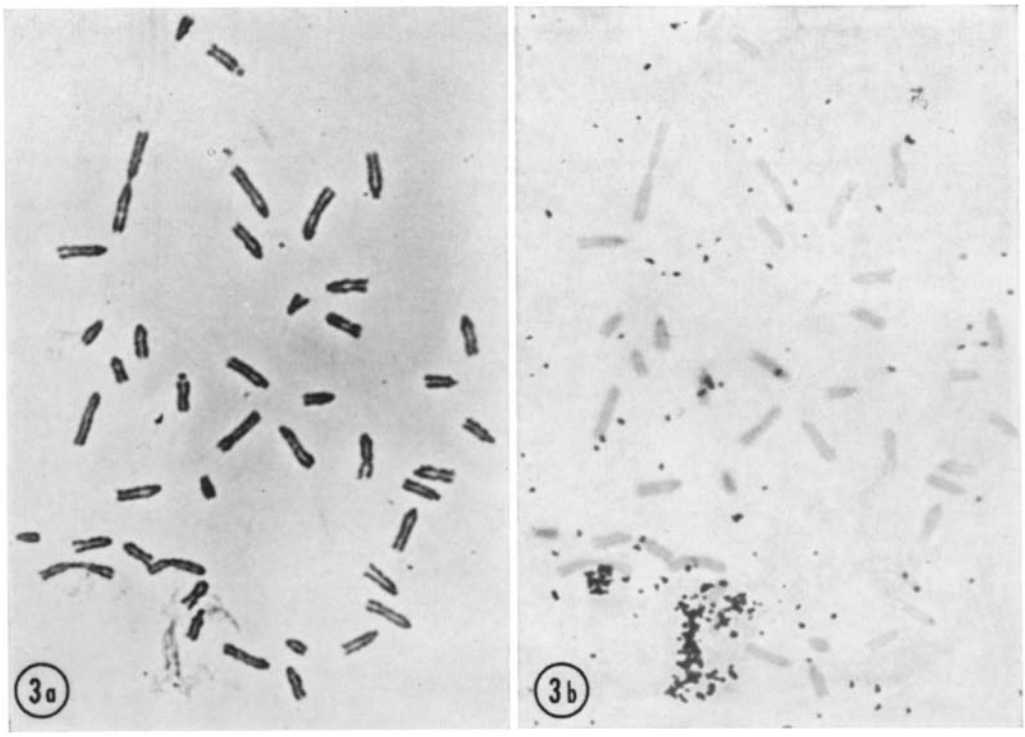

FIGURE 3 *a* and *b* Pulverization and stretched-out chromosomes showing SOOP effect. T811, mouse rhabdomyosarcoma line. 2 hr terminal label. × 1800.

TABLE I

Incidence of SOOP Chromatin in Successive Passage of a HeLa Cell Line

Passage No.	Incidence	No. of metaphase figures scored
	%	
13	1.1	3759
14	4.8	250
23	2.5	1267

(Ladder-like secondary constrictions generally form a small percentage of these totals but show a similar variation.) Passage numbers from point of subculture in our laboratory.

single, attenuated constrictions do not show G_2 labeling.

It is our impression that the incidence of the SOOP phenomenon varies considerably among different cell lines and even from passage to passage in the same line (see Table I). In a given cell, SOOP chromosomes usually show similar abnormal morphology, i.e. either all pulverized or all with ladder-like secondary constrictions. However, there are many cells in which both appearances are seen, thus suggesting that pulverization and ladder-like secondary constrictions represent a spectrum of changes resulting from a common mechanism (Fig. 4). The apparent fragments sometimes resemble minute chromosomes (12) or the fragments which might be expected from multiple, closely spaced isochromatid breaks. In many cells the SOOP chromatin tends to be confined to a focal area of the metaphase figure.

Both types of abnormal chromosomes or chromatin tend to show labeling with tritiated thymidine during the G_2 period from 30 min to 4½ hr before metaphase (see Table II). Whether the unaffected chromosomes show label depends upon the length of the G_2 period and the length of the terminal label. As noted for Ogun, at 4½ hr the label is presumably available also toward the end of S, and some unaffected chromosomes show label. At 2 hr, for Ogun, the label is only available during G_2; hence, no unaffected chromosomes

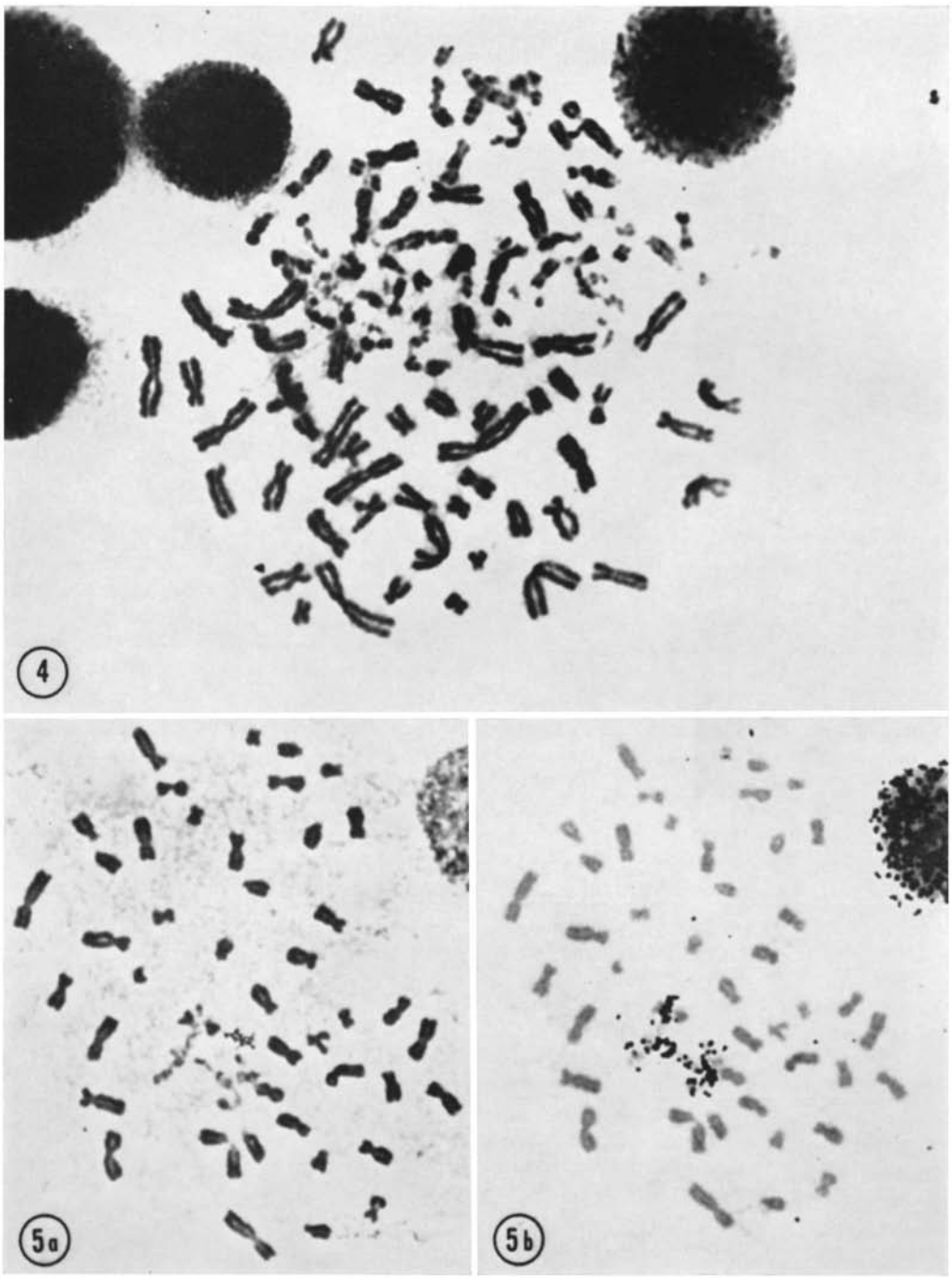

FIGURE 4 Pulverization and ladder-like secondary constrictions in the same cell. Ogun. × 2300.

FIGURE 5 *a* and *b* The chromosome missing in this metaphase is one of the No. 1' s; hence, the fragment associated with the intact No. 1 is presumably one of the arms of the missing No. 1. Note the G chromosomes near the pulverized material. LK1D. 2 hr terminal label. × 1800.

TABLE II

Incidence of Labeling of Pulverized Chromosomes and Chromosomes with Multiple Secondary Constrictions Using Different Pulses of Thymidine-^3H

Cell lines	Ploidy	Total No. cells with pulverization	Pulverized chromosomes labeled only	Both norm and pulverized labeled	Exposure or pulse time	No. pulverized cells without label
Ogun	≥4N	20	2	0	30 min	18 (90%)
Ogun, LK1D, SKL-1	≥4N	20	9	0	2 hr	11 (62.8%)
	2N	37	13	0	2 hr	24
Ogun	≥4N	7	6	1	4½ hr	0 (0%)
HeLa	≥4N	25	5	0	1 hr	20 (80%)
T811	≥4N	7	2	1	2 hr	4 (35%)
	2N	7	3	3	2 hr	1
Chinese hamster	≥4N	15	3		1½ hr	12 (79%)
	2N	23	4	1	1½ hr	18

show label. Apparently the G_2 period is shorter for T811 since at least some unaffected chromosomes show label at 2 hr.

As the terminal labeling period is shortened there is a progressive decline in the percentage of affected cells with label. Thus for Ogun, at 4½ hr 100% of the affected cells showed labeling of SOOP chromosomes; at 2 hr, only 37%, and at 30 min, only 10% showed labeling. At 1 hr terminal label for HeLa cells, the value is 20%. When labeling periods are as short as 30 min, it would appear that some SOOP chromosomes are labeling at least into prophase and possibly into metaphase.

The incidence of cells with SOOP material is much higher in polyploid (near-4N) cells than in diploid or near-diploid cells in the same cell line (see Table II). We have not been able to identify any polyploid cells in which a precise diploid complement was left intact. As the number of SOOP chromosomes increases, it becomes more difficult to distinguish diploid cells from polyploid cells. However, as shown in Table IV, it is often the case that only one or two chromosomes are affected in polyploid cells. This is also true of diploid or near-diploid cells. Both LK1D and SKL-1 have well-defined karyotypes (11). Thus, it is possible, by a process of elimination, to determine which homologs are most often affected in these cells. As may be seen from Table III, chromosome No. 1, and particularly one arm of No. 1, probably the long arm, is preferentially affected to an overwhelming degree. 84 diploid or near-diploid cells with SOOP chromatin were surveyed. In 38% of these, chromosome No. 1 was affected. In 85% of the cases involving chromosome No. 1, only one of the arms was affected. In agreement with zur Hausen (3), the affected arm was, at least in many cases, probably the long arm since a trace of remaining attenuated constriction could be seen (Fig. 5).

Allowing for the number of chromosomes at risk in each group, one may see that the next most common chromosome to be affected is the small acrocentric chromosome (Nos. 21 and 22). Thus chromosome No. 1 is preferentially affected while all other groups except the G and possibly the D chromosomes are preferentially spared. While this incidence for the G group is only chance expectation, it is also true that G chromosomes were often seen in association with SOOP chromatin with much greater than random expectation (see Table V). In addition, there were 29 cells in which the origin of the SOOP chromatin could not be determined since these cells contained 46 normal homologs in addition to the SOOP material.

In addition to lines in which cells were labeled, we have observed spontaneous pulverization or ladder-like secondary constrictions in a number of other permanent cell lines including RPMI 2650 (13), RPMI 7216 and 7466 (14) and, in EB1, EB2, and SL1 (10). We have never observed the phenomenon in W138 or in any of the many diploid human fibroblast strains we have examined. We have, however, observed pulverization and multiple chromosome breaks in one peripheral lymphocyte culture (out of about 150 such cultures done in our laboratory).

Thus, it would appear that the SOOP phenomenon tends to be largely restricted to permanent cell lines and perhaps occurs, albeit usually in low incidence, in all such lines.

DISCUSSION

It appears from the data presented that pulverization as described by Nichols et al. (1, 4) and incomplete condensation as described by zur Hausen (3) are two extremes of a basically similar phenomenon, and that the extended chromosomes described by Stubblefield (2) probably present the same effect. However incomplete condensation seems an undesirable designation since it could as plausibly refer to single, attenuated secondary constrictions (11) which do not show label in G_2.

TABLE III

Homologs Pulverized in Diploid and Near Diploid Cells; 84 Cells of LK1D and SKL-1 Analyzed

Chromosome No.	No. of cells	Chromosome No.	No. of cells
No. 1 long arm	26	6–12 + X	13
		13–15	10
Both No. 1 long arms	1	16–18	5
All of No. 1	4	21–22	7
All of both No. 1's	1	Undetermined, i.e. cell already had	29
No. 2	2	46 normal homologs	

TABLE IV

Approximate Number of Pulverized Chromosomes in 85 Polyploid Cells (Ogun)

No. of pulverized chromosomes	No. of cells with 1, 2, 3, etc. pulverized chromosomes
1	16
2	8
3	2
4	1
6	2
8	1
10	1
11	1
Greater than 11	53

TABLE V

Association of Chromosomes with Area of Pulverization (LK1D): Nonrandom for G Chromosomes

Chromosome No. or group	Observed*	Expected*	χ^2	P
1	17	19.7	0.40	$0.70 > P > 0.50$
2	7	18.4	7.68	$P < 0.01$
3	13	15.5	0.43	$0.70 > P > 0.50$
B (M)	24	40.3	7.93	$P > 0.01$
C	94	87.2	0.86	$0.50 > P > 0.30$
D	24	22.7	0.08	$0.80 > P > 0.70$
16	10	7.0	1.32	$0.30 > P > 0.20$
17, 18	8	12.6	1.77	$0.20 > P > 0.10$
F	16	10.3	3.29	$0.10 > P > 0.05$
G	26	5.5	78.21	$P < 0.01$

Total points: 239 d.f‡ = 1

* Observed and expected points by relative length of homolog or group.

‡ d.f. = degrees of freedom.

Groups of pulverized fragments were scored if they occupied an area no greater than 20% of the metaphase plate. The most peripheral fragments were outlined by a polygon. If a homolog lay within or overlapped the polygon, it was given 3 points. A homolog close by (no further than a G homolog length away) was given 2 points, and one at no greater distance than a D homolog, 1 point.

If calculations are based on the number of chromosomes at risk in each category (homolog pair or group) the results are random for all categories except 17–18 and G which give $P < 0.01$, and No. 1 with $P < 0.05$.

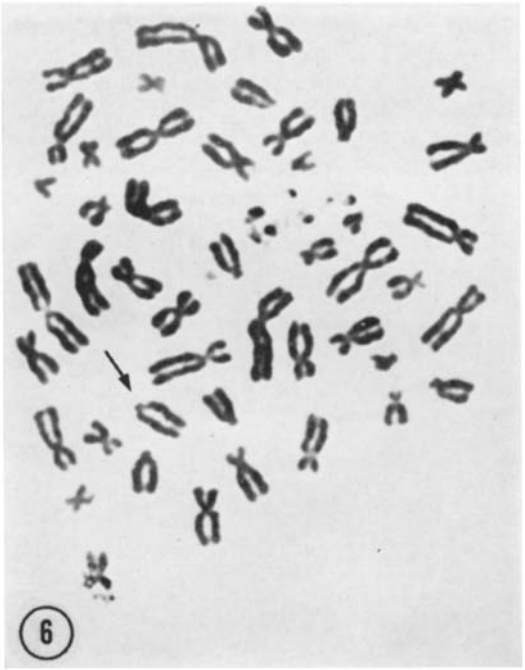

FIGURE 6 Acrocentric chromosomes adjacent to pulverized chromatin. The missing fragment is an arm of one of the No. 1 homologs, and the opposite intact arm (arrow) is considerably displaced indicating that pulverization is associated with chromosome breakage. LK1D. × 1800.

The suitability of the word pulverization has been placed in doubt by those who suggest that the chromosomes are not broken but simply greatly extended. This problem is not yet resolved. On the one hand, in the extreme case, the chromosomes certainly appear to be fragmented, and the evidence is precisely the same as in other experimental situations in cells in which chromosomes have fewer breaks, unhesitatingly scored as isochromatid breaks. Where one arm of a No. 1 chromosome is pulverized, the intact arm is not infrequently displaced from the SOOP chromatin (Fig. 6). On the other hand, the chromosomes with ladder-like secondary constriction do not appear broken, and it is difficult to rule out the possibility that apparent fragments are in fact joined by thinned-out chromosome strands too highly extended to be readily demonstrable. Observations of living cells in anaphase could determine whether pulverized material is left behind or passes in equal amounts to both poles. zur Hausen's observations suggest that such material is left behind (3), and we might, therefore, infer again that these chromosomes are in fact broken.

The finding that thus far the SOOP phenomenon is found in many permanent lines in which a deliberate search has been made raises the question as to whether such lines are suitable test objects for the experimental induction of pulverization. This is especially true when one considers the varying incidence from passage to passage.

The thesis that pulverized chromosomes are not really broken is an inference from the theory that the G_2 DNA synthesis simply reflects a delayed onset of synthesis in S. Thus the SOOP chromosomes should be spoken of as being in S rather than in G_2. While to some extent this is a question of semantics, it seems fair to point out that the cell cycle designations G_1, S, and G_2 refer not to individual chromosomes but rather to the entire nucleus. Moreover, where only one arm of chromosome No. 1 is affected, we would have to assume that the short arm is in G_2 while the long arm is in S. Nevertheless, preliminary pulse-chase experiments of our own do suggest that DNA synthesis in SOOP chromatin is either delayed in onset or slowed in rate. Alternative to the delayed onset theory is the possibility that SOOP represents repair synthesis in chromosomes which had already started, and perhaps in some instances even completed, normal DNA synthesis on time. However, Zakharov and Egolina (15) have shown that the sequence of DNA synthesis is presumably normal in hamster chromosomes with ladder-like secondary constrictions, i.e. the euchromatin segments label early, while the heterochromatin segments label late. With a repair mechanism, the sequence of repair should follow the sequence of damage. If anything, however, the sequence of damage may be the reverse of the sequence of normal synthesis. There is a suggestion that the damage begins in the heterochromatin areas (cf. the secondary constriction region on chromosome No. 1) and spreads to the euchromatin. This predilection for heterochromatin is also remarked by Zakharov and Egolina who state that . . . "when few chromosomes show inhibition of spiralization, they are predominantly chromosomes with large heterochromatic portions" (15). It should be emphasized, however, that the SOOP effect is not restricted to heterochromatin.

Some experimentally induced effects hitherto referred to as pulverization may differ from the spontaneous variety. In preliminary experiments with diploid human fibroblast lines, we have found

that *herpes zoster* always affects *all* the chromosomes in a dividing cell. Moreover, the virus inhibits DNA synthesis, so G_2 synthesis is not observed. It has been suggested that pulverization tends to occur totally in one nucleus of a binucleate cell (16). However, this mechanism must be very uncommon in our material, as is shown by the numerous polyploid cells in which only a small number of chromosomes are affected (Table IV) and by the paucity of cells which include SOOP material as well as a diploid set of chromosomes. Nor are cells of this latter type conclusive evidence of cell fusion, since two or more cells may simply adhere to one another both in culture and in chromosome preparations.

On the other hand, that there is some relation between pulverization and polyploidy is clearly indicated by the markedly higher incidence of the phenomenon in polyploid cells (Table II). In this connection, it may be noted that in some human cell lines all varieties of chromosome abnormalities occur in higher incidence among polyploid cells (10). The finding of SOOP chromatin in addition to 46 normal homologs (Table III) also suggests a relationship with some mechanism, probably nondisjunction, which adds chromosomes. It has also been suggested that small amounts of pulverization may be secondary to micronuclei formed by one or a small number of chromosomes (16). However, in those cases in which only one arm of a No. 1 chromosome is affected, this would seem to imply that only one arm had formed a micronucleus, while the other arm was part of the major nucleus. This implication seems implausible.

In a previous report (11) it was postulated that many cytogenetic abnormalities in cancer cells and/or in permanent cell lines may be related to the nucleolus or to nucleolar-associated heterochromatin. If indeed the small acrocentric chromosomes (G group) are related to the nucleolus and also to the secondary constriction on chromosome No. 1 (17) then, pulverization is also related to the nucleolus. However, the preferential effect on chromosome No. 1 and on the G chromosomes has thus far only been demonstrated in two of the leukocyte lines. Aneuploid lines such as HeLa are much more difficult to analyze. It might be that different homologs are preferentially affected in different cell lines. Gripenberg (18) has reported a human cell strain in which pulverization was seen in a ring chromosome which probably arose from a 17–18 chromosome

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