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Analysis of chromatid segregation in CHO cells at mitosis*

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ABSTRACT

In the present paper the results of an analysis performed at M3 on chromatid segregation that occurred at M2 in CHO cells treated and untreated with $K_2Cr_2O_7$ are presented. The percentage of *light* chromosomes (BB×BB chromatid pairs) in a cell at M3 has been estimated and the empirical frequency distributions of light chromosomes in the different experimental groups have been compared with the theoretical ones (sums of binomial distributions with $p=0.5$) by means of the non-parametric Kolmogorov-Smirnov test. In all cases a significant difference has been found, together with a shift to the "left" (i.e. $p<0.5$) of the maximal frequency classes. This behaviour has been explained by assuming the existence of a selective mortality factor acting on cells with light chromosomes prevailing. This cellular mortality of light cells has been attributed by some authors to the presence of the younger one of the two parental DNA strands collecting the damages which occurred during the previous generation. On the contrary cells with *dark* chromosomes prevailing (BT×BB pairs) yield DNA molecules with the older parental strand and do not collect the damages from the previous generation, and in consequence are not submitted to the action of the selective factor. Further, the greater shift leftward registered in the groups of cells treated with chromium has been explained by assuming that the younger of the two parental DNA strands sustained greater damage under our experimental conditions.

Finally our data suggest the existence of a small fraction of cells segregating non-randomly within the randomly segregating population.

Due to the biological relevance of this assumption further investigations are required to confirm it and estimate its extent.

* Reprint requests to Dr. Franca Majone.

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INTRODUCTION

Experiments have been performed to ascertain whether chromatid segregation into daughter cells at mitosis is completely random or if chromatids with older DNA (i. e. with a strand present in the molecule for two generations) tend to segregate together into the same cell (Rosenberg & Kessel, 1968; Comings, 1970; Morris, 1977).

Most of these experiments show a random chromatid segregation at mitosis (Heddle *et al.*, 1967; Comings, 1970; Geard, 1973; Morris, 1977; Potten *et al.*, 1978).

On the contrary some experiments indicate the presence of a non-random segregation, as in mouse embryonic cells *in vitro* (Lark *et al.*, 1966), in turbellarian zygotes (Costello, 1974), in mouse zygotes at the early segregation stages (Odartchenko & Keneklis, 1973), in fungi (Rosenberg & Kessel, 1968) and in meristems of plants (Lark, 1967).

A small cell fraction with non-random segregation but with the remaining cells presenting a random segregation has been observed also in epithelial stem cells (Potten *et al.*, 1978).

Random segregation has been inferred

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by evaluating the fraction of labelled centromeres in cells that have duplicated three times either in the presence of BUdR (Morris, 1977) or after incorporation of tritiated thymidine (Heddle *et al.*, 1967; Geard, 1973). It has been established that the frequency distribution of labelled centromeres at M3 stage is a binomial one with p=0.5 (Heddle *et al.*, 1967; Geard, 1973; Morris, 1977).

In the present work the frequency distribution of labelled centromeres in CHO cells after bromodeoxyuridine incorporation for three replication cycles has been analyzed.

Chromatid segregation at mitosis in CHO cells after treatment with a hexavalent chromium compound inhibiting the DNA synthesis, acting on the DNA molecule as well as on the enzymes of its replication (Levis & Bianchi, 1982) has been finally studied to find out whether segregation is modified by compounds that can interfere with DNA synthesis.

MATERIALS AND METHODS

Cell culture and chromosome preparations

Pseudodiploid Chinese hamster cells (CHO line) were grown as monolayers in Eagle's minimal essential medium with 10% calf serum and were handled as already described (Levis & Majone, 1979; Majone & Levis, 1979; Levis & Majone, 1981). Chromosome preparations were obtained on metaphase cells by staining with Giemsa (Korenberg & Freedlenler, 1974).

Chemicals and cell treatments

24 h after seeding, 3×10^{-5} M bromodeoxyuridine (BUdR, Sigma, St. Louis, MO, USA) was added and the cells allowed to incorporate the analogue for three division cycles. A hexavalent chromium compound (potassium dichromate, $K_2Cr_2O_7$, Mallinckrodt, St. Louis, MO, USA) was added at the same time as BUdR. Potassium dichromate was dissolved in sterile twice-distilled water at 10^{-2} M concentration immediately before use, and diluted in prewarmed complete growth medium to a final concentration of 10^{-5} M. The medium used for treatment was changed after 4 h. The cells were collected 38 h or 45 h later. During the last 4 h of treatment, colchicine (0.4 μg/ml, Merck, Darmstadt, Germany) was added.

Observational procedures. The expected labelling pattern.

We have examined the proportion of labelled and unlabelled centromeres in cells dividing for the third time after being treated with BUdR. After exposure to BUdR each pair of sister chromatids at the first metaphase will be BT×TB, where B is the DNA strand containing BUdR and T the DNA strand containing thymine only; at the second metaphase it will be BB×TB. As a consequence, at the second metaphase the two sister chromatids will be labelled differently. The segregation pattern can thus be observed at the third metaphase, where each chromosome will consist either of a BB×BB or of a BT×BB chromatid pair. According to the Korenberg & Freedlender (1974) technique the BT chromatids appear dark and the BB light.

The BB×BB pairs appear at M3 either completely or partially light (Fig. 1) depending on whether the sister chromatid exchanges took place previously or not.

As already mentioned in the literature (Morris, 1977), we considered as belonging to the group of light chromosomes those that appeared to be labelled at the centromere level.

The percentage of partially or completely light chromosomes (BB×BB pairs) has been evaluated from the whole amount of chromosomes present in the cell at the M3 stage. At least 100 cells at M3 stage have been under examination at each sampling.

From the hypothesis of random segregation, the expected frequencies of BB×BB pairs at M3 would follow a binomial distribution B(N, p), where N=number of chromosomes per cell and p=probability of a single BB×BB pairing = 0.5 (which characterizes the "randomness" of the process).

Due to the fact that our experimental material is constituted by *in vitro* stabilized cells (which have different amounts of chromosomes) the resulting theoretical frequency distribution will be a "binomial compound":

$$\sum_{N_1}^{N_2} \nu(N) \cdot B(N, 0.5) \quad \text{(model } B_1\text{)}$$

where

$\nu(N)$ = fraction of cells containing N chromosomes,

N_1, N_2 = minimal and maximal number of chromosomes per cell respectively (in our experiments $N_1=14$, $N_2=24$, Table I).

On the contrary a "nonrandom" segregation would give rise to the following bimodal frequency distribution:

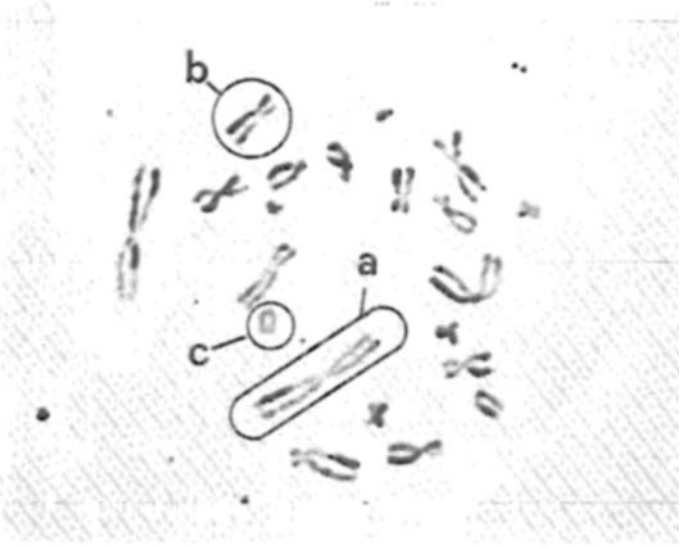

Fig. 1. — Third division metaphase in CHO cell after incorporation of bromodeoxyuridine and processing according to the Korenberg and Freedlender technique. a = BB×BB chromatid pair; b = BT×BB chromatid pair. The BT chromatids appear dark, the BB chromatid appear light. The BB×BB chromatid-pairs can either appear light (c) or only partially light (a) depending on whether the sister chromatid exchanges took place previously or not.

TABLE I. — *Chromosomes per cell distribution within the experimental groups.*

N. of chromosomes per cell	14	15	16	17	18	19	20	21	22	23	24
Group	No. of cells										
C_1	0	6	5	17	20	15	19	16	5	0	1
C_2	1	0	15	26	18	9	16	7	2	1	0
T_1	1	3	2	8	14	20	25	11	2	1	0
T_2	0	0	1	10	18	27	20	17	1	1	0

TABLE II. — *Median, lower and upper quartile (L.Q. and U.Q.) and arithmetic mean of the frequency distribution of light chromosome fractions per cell within the experimental groups.*

Group	No. of samples	L. Q.	Median	U. Q.	Mean
C_1	104	0.316	0.412	0.556	0.443
C_2	95	0.353	0.471	0.550	0.457
T_1	87	0.263	0.400	0.571	0.418
T_2	95	0.300	0.400	0.500	0.393

$\sum_{N_1}^{N_2} \frac{1}{2}\nu(N) \cdot \{B(N,p_1)+B(N,1-p_1)\}$ (model B_2)

with $p_1<0.5$ (for this bimodal pattern see also Heddle *et al.*, 1967).

RESULTS

In Table II the medians of the frequency distributions of light chromosomes in cells exposed to BUdR for 38 and 45 h, treated (group T_1 and T_2 respectively) and not treated (groups C_1 and C_2) with $K_2Cr_2O_7$ 10^{-5} M for the first 4 h are given.

In Figures 2A, 2B, 2C, 2D the empirical cumulative frequency distributions of light chromosomes in groups C_1, C_2, T_1, T_2 are plotted (solid line). The dot-dash lines single out the boundaries of the confidence intervals for the different values of the abscissa (which represents the proportion of light chromosomes per cell), at the significance level $p \leq 0.05$.

The theoretical cumulative frequency distributions derived from model B_1 have been also plotted close by (dashed lines).

The empirical and theoretical distribution functions have been compared by means of the non-parametric Kolmogorov-Smirnov test (Hoel, 1971): in all groups a significant difference was evidenced (at the significance level $p \leq 0.05$). The greatest difference was exhibited by groups T_1 and T_2. Comparisons between the empirical distribution functions of the considered groups are summarized in Table III.

TABLE III. — *Comparisons between the empirical frequency distributions of the experimental groups by the Kolmogorov-Smirnov test. Simbols: − = not significant* ($p>0.05$); + = *significant* ($0.01 \leq p < 0.05$); + + = *highly significant* ($p \leq 0.01$).

Group	C_1	C_2	T_1	T_2
C_1	—	—	—	—
C_2	—	—	+ +	+
T_1	—	+ +	—	—
T_2	—	+	—	—

To get a better insight into the chromatid segregation process, let us now consider the frequency distribution curves, referring to discrete intervals with amplitude $\Delta x=0.1$, where x is the fraction of light chromosomes per cell (Figures 3A, 3B, 3C, 3D). Solid lines represent the histograms from the empirical data, while dotted lines join the central values of the theoretical distributions. The residuals, i.e. the differences between empirical and theoretical levels, are drawn with dashed lines.

With the sole exception of group C_2, the empirical patterns clearly differ from the theoretical ones, the former showing a significant shift to the left of the class of maximal density (see also Table II). The behaviour of residuals further evidences the presence of two peaks, one to the left and one to the right of the value 0.5 (with exception of group T_2). The peak to the left (cells with a fraction of light chromosomes less than 50%) dominates over the peak to the right.

According to biological assumptions that will be discussed in the next section, we attributed this behaviour to the presence in our samples of a fraction α of cells with random segregation (model B_1) and of a fraction β with nonrandom segregation (model B_2). We further assumed the existence of a selective mortality factor acting on cells with light chromosomes prevailing (for the sake of simplicity we regarded it as proportional to the fraction x of light chromosomes per cell, with a proportionality constant ε). This factor would account for the dominance of the peak to the left over that to the right in our empirical frequency distribution curves. In mathematical terms, the hypothe-

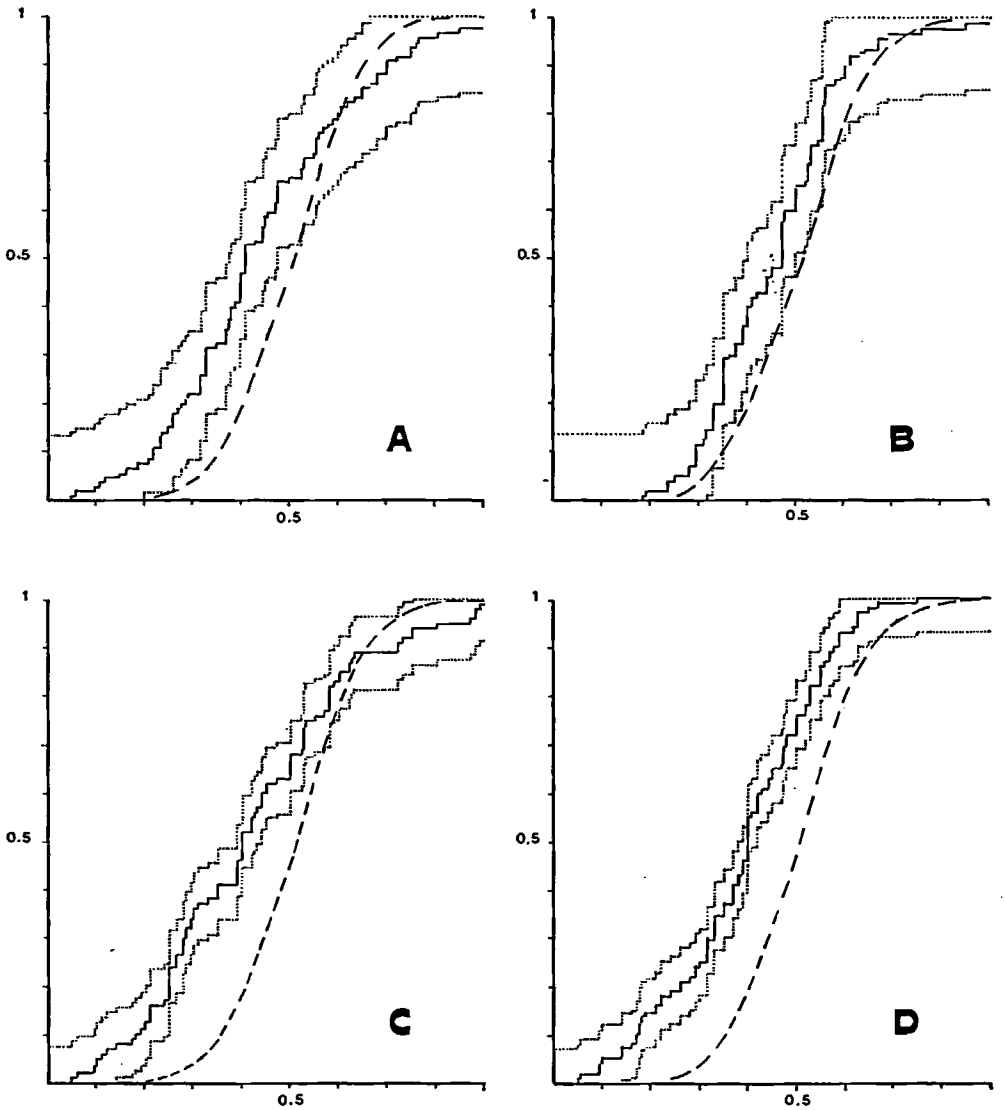

Fig. 2. — A. Empirical cumulative distribution function of the fraction $x (0 \leq x \leq 1)$ of light chromosomes per cell, relative to group C_1 (solid line), and confidence intervals ($p \leq 0.05$) (dotted lines). Theoretical cumulative distribution function relative to group C_1 and according to the compound binomial model B_1 (dashed line). B. As in A, but relative to group C_2. C. As in A, but relative to group T_1. D. As in A, but relative to group T_2.

sized frequency distribution function of light chromosomes is the following:

$$f(x)=(1-\varepsilon.x)\cdot\{f_1(x \mid B_1)+f_2(x \mid B_2)\},$$

where $f_1(x \mid B_1)$ and $f_2(x \mid B_2)$ are the frequency distribution functions of light chromosomes according to models B_1 and B_2 respectively.

A data fitting was performed according to the iterative non-linear least squares procedure of Newton-Raphson-Lavenberg (Davies & Whitting, 1972), whose results are summarized in Table IV.

DISCUSSION

Heddle *et al.* (1967) analyzed the chromatid segregation at mitosis by counting the fraction of labelled centromeres at M3 stage in cells of *Vicia faba* and in an aneuploid cell line of *Potorous tridactylis* after incorporation of tritiated thymidine to obtain an empirical unimodal density function of binomial type, but with a mean value less than 0.5. According to them the existence of only one peak should prove the "randomness" of the phenomenon, because otherwise a bimodal density funtion should have been obtained, with peaks corresponding to the fractions of centromeres segregating together. The authors attributed the shift toward the left of the mean value to the adopted auto-radiographic technique, which would have underestimated the amount of labelled centromeres.

Even in our experiments the shift leftward of the mean frequency value is noticed (Table II; Figures 3A-3D). The frequency distribution functions, even though unimodal, significantly differ from the "compound binomial" ones centered at $p=0.5$, as confirmed by the Kolmogorov-Smirnov test on the cumulative frequency distribution functions (see also Figures 2A-2D). However we would not attribute this fact to a systematic error, because of the presence of tails, in our empirical distribution functions, higher than the theoretical levels.

Our assumption that the obtained frequency distribution functions be the sum of an unimodal, compound-binomial distribution centred at $p=0.5$, and of two compound-binomial distributions centred respectively at $p_1<0.5$ and at $p_2=1-p_1$, is based on the hypothesis that our cell population segregate "randomly" on the whole, but with a fraction of cells presenting a nonrandom chromatid segregation.

This fact could be in agreement with the results obtained from staminal epithelial cells (Potten *et al.*, 1978). With reference to the latter ,it was suggested that nonrandom segregation could reflect a protection from possible alterations intervening at the DNA replication stage: yielding in the same cell DNA molecules with the older one of the parental strands, and in the sister cell the DNA molecules with the younger strand would be equivalent to creating a set of "immortal" cells, perpetuating through subsequent cellular divisions, together with a set of "mortal" cells, collecting the damages of the previous generation (Cairns, 1975).

Even neglecting the assumption of non-random segregation, the significant shifting toward the left of the maximal frequency class, also noticed by Heddle *et al.* (1967), can be considered as due to the existence of a reduction factor, acting proportionally to the fraction of light chromosomes per cell. The latter should be responsible for the reduction of the peak to the right of 0.5 in our density distribution functions.

We could consider it as a selective mortality in cells with greater amounts of light chromosomes.

Light chromosomes contain indeed DNA molecules with the younger one of the parental strands, so that cells yielding these chromosomes should also collect pos-

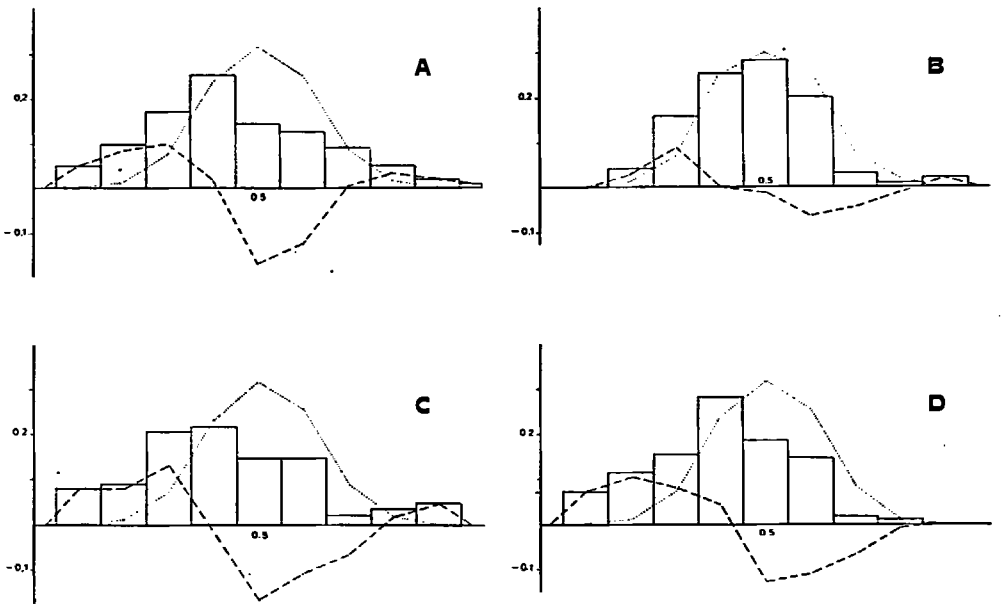

Fig. 3. — A. Empirical density distribution function of the fraction of light chromosomes per cell, relative to group C_1 (the abscissa has been subdivided into intervals of equal amplitude 0.1, with exception of the first and the last one, whose amplitude is 0.05). (Solid line). Theoretical levels according to the compound binomial model B_1, calculated at the centres of the abscissa intervals (dotted line). Curve of residuals (empirical values - theoretical values) (dashed line). B. As in A, but relative to group C_2. C. As in A. but relative to group T_1. D. As in A. but relative to group T_2.

sible damages intervened during the previous generation, while cells with greater amounts of dark chromosomes, keeping on containing the older one of the parental strands, should not register possible damages intervened at the previous replication. Light chromosomes further contain BUdR, which is mutagen (Hsu & Somes, 1961), in both DNA strands, while dark chromosomes (i.e. the BT × BB pairs) yield a parental strand without BUdR. It is thus possible that a prolonged BUdR treatment gives rise to a greater mortality in cells with dominantly light chromosomes, thus explaining their lower amount.

This effect should become even more noticeable in cells treated with a compound such as potassium dichromate. It was suggested indeed (Rainaldi *et al.*, 1982) that in acute treatments Cr (III), produced by Cr(IV) reduction, interferes mainly with the accuracy of DNA polymerase, whereas in chronic treatment the main target for Cr(III) would be the DNA itself. The induced inaccuracy of DNA replication could result from the interaction of Cr(III) with the DNA polymerase molecule, as described in the case of other carcinogenic metals or with precursor nucleotides (Levis & Bianchi, 1982). It is thus thinkable that an acute dichromate treatment, as in our case, should damage the DNA strand of new synthesis rather than the parental one.

TABLE IV. — *Best fitting parameters* ε, α, β, *residual variance and percent of explained variance for the frequency distribution function* $f(x)=(1-\varepsilon . x).\{\alpha.f_1(x \mid B_1)+\beta.f_2(x \mid B_2)\}$. *The peaks of* $f_1(x \mid B_1)$ *were assumed to be located at* x=0.3 *and at* x=0.7.

Group	ε	α	β	Residual variance	% Explained variance
C_1	0.846	0.778	0.482	7.28×10^{-4}	87.83
C_2	0.941	1.524	0.200	5.56×10^{-4}	95.44
T_1	1.	0.807	0.588	1.39×10^{-3}	77.94
T_2	1.216	1.450	0.529	8.11×10^{-4}	91.15

The shift leftward of the class of maximal density noticed in groups C_1, C_2 of cells exposed for 38 h and 45 h to BUdR remains and is indeed more pronounced in groups T_1, T_2 of cells treated with chromium. The shift is particularly strong in the group T_2 of cells exposed for 48 h to BUdR.

The results indicate that mortality is higher in cells treated with chromium than in those of the control groups. This is particularly evident in cells reaching the M3 stage late, which shows that the worst damage accumulates in cells that are slown down in their cellular cycle by the presence of chromium, as previously observed (Majone, 1977; Majone & Levis, 1979).

This preliminary analysis on chromatid segregation at mitosis indicates that the problem of random-nonrandom segregation is worth further examination.

In particular, the question should be examined, whether the presence of a small fraction of cells presenting a non-random segregation at mitosis within a population with random segregation is or is not a general fact.

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