DISTRIBUTION OF LABELED CHROMATIN

I. M_1 and M_2 Anaphases of Diploid and Tetraploid Cultured Mammalian Cells

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ABSTRACT

The question of whether distribution of chromatids to daughter cells in mitosis is a random or nonrandom process was investigated by study of the distribution of labeled chromatin in anaphase pairs at M_1 and M_2 after a pulse of tritiated thymidine. Diploid and tetraploid rat and diploid human fibroblast-like cells in serial monlayer culture were synchronized by two different methods to "purify" M_1 and M_2 anaphases: metaphase shake, and FUdR block to DNA synthesis followed by exogenous thymidine. Exposed grains of NTB-2 emulsion were counted over M_1 and M_2 anaphase pairs. An analysis (by pair) of diploid M_2 anaphase grain counts showed two discrete populations of daughters with less and with more radioactivity. A similar analysis of diploid M_1 and tetrapolid M_2 anaphases showed a single grain-count distribution. These findings may support a nonrandom model of chromatid segregation for diploid mammalian cells but do not rule out random segregation until sound mathematical models are formulated for expected random grain distributions in M_2 anaphases of cells with differing numbers of chromosomes.

The question of whether the distribution of chromatids to daughter cells in mitosis is a random or nonrandom process was reinvestigated by Lark (1966). There is recent evidence in favor of a nonrandom process, in the sense that chromatids containing "old" polynucleotide templates tend to segregate from chromatids containing "new" templates in diploid cells (Lark, 1966; Lark et al., 1966; Lark, 1967; Rosenberger and Kessel, 1968). There is recent evidence in favor of a random process in diploid cells (Heddle et al., 1967; Cuevas-Sosa, 1968; Callan and Taylor, 1968) and in nondiploid cells (Lark et al., 1966; Lark, 1967).

One experimental approach to determine whether the segregation of chromosomes in mitosis is random or nonrandom involves study of the distribution of labeled chromatin in cultured cells over succeeding generations after an initial pulse of tritiated thymidine (H_3TdR). Following radioautography, exposed emulsion grains are counted over individual pairs of daughter cells at anaphase. The distributions of grain counts from such pairs of cells are complex and become increasingly so with each succeeding generation. The work reported here deals with results obtained at the first anaphase (M_1) and second anaphase (M_2) after incorporation of H_3TdR, where cells were grown in nonradioactive medium between first and second anaphases.

The upper line of Fig. 1 illustrates how all the chromatids are labeled at S_1 by semiconservative replication in the presence of H_3TdR, resulting in an expected equality of label in M_1 anaphase daughter cells. (A labeled chromatid is represented by a dotted line.) Labeled M_1 daughter cells replicating DNA at S_2 in the absence of H_3TdR (lower line of Fig. 1) can produce one of several

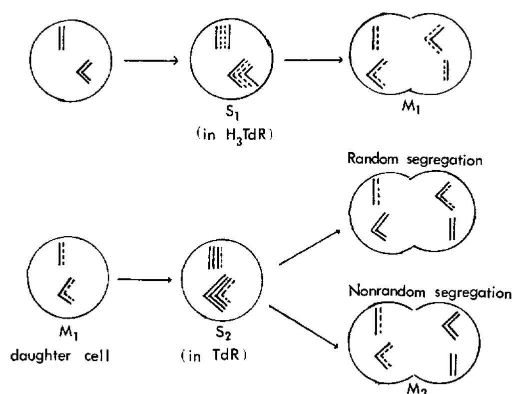

FIGURE 1 The upper diagram illustrates semi-conservative replication of two chromosomes in the presence of a pulse of H_3TdR (S_1) followed by the next mitosis (M_1). M_1 anaphase shows that the two daughter cells must be equally labeled since labeled chromatids must be equally distributed between the two cells if the chromosome complement is equally distributed. (Interrupted lines represent labeled chromatids; polarity is not represented.)

The lower diagram illustrates replication of two chromosomes in one of the daughter cells from M_1 of the upper diagram. Replication is in the presence of TdR (cold). At next mitosis (M_2), analysis of anaphase pairs permits selection of a random or nonrandom model for distribution of the labeled chromatids. Only one possibility for random and one possibility for nonrandom distribution are illustrated.

types of M_2 anaphase grain patterns, the likelihood of each pattern depending on whether the chromosome segregation is random or nonrandom. In this example, which symbolically illustrates only two chromosomes, the random pattern has a radioactive chromatid in each daughter cell; the nonrandom pattern has both radioactive chromatids in one cell and none in the other. In situations with more chromosomes, various degrees between random and completely nonrandom patterns are possible.

One crucial factor in this experimental approach is that the M_1 and M_2 cells studied should be as homogeneous as possible. To this end, experiments were designed to synchronize (phase) diploid and tetraploid rat and diploid human cells in serial culture; the experimental approach just described was applied and analyzed. Two synchronization methods were compared and found to give similar results.

MATERIALS AND METHODS

An established line of rat, fibroblast-like diploid cells (2n = 42), PR 105, was maintained in serial culture for between 70 and 250 population doublings. The mean cell cycle time was about 18 hr and G2 4 hr. From this mass culture an established clonal line of tetraploid cells (4n = 84) was studied at approximately 100 population doublings. The karyotype was stable and revealed no abnormal chromosomes. A line of human fibroblast-like diploid cells, PR 100, from female embryonic lung was maintained in serial culture for between 20 and 50 population doublings. The mean cell cycle time was about 20 hr and G2 4 hr. Culture medium was that of Dulbecco and Vogt supplemented with 10–15% fetal calf serum. All cell types were synchronized with 5 fluoro 2′ deoxyuridine (FUdR) followed by exogenous thymidine (TdR) according to methods already described (Priest et al., 1967a; 1967b). M_1 anaphases were studied following 0.1 μg/ml FUdR for 16 hr and then 0.1 μCi/ml H_3TdR for 1 hr to reverse the FUdR block to DNA synthesis and label the chromosomes. M_2 anaphases were studied following 0.5 μCi/ml H_3TdR for 1 hr, an 8 hr chase with 6×10^{-6} M TdR; then FUdR for 16 hr and TdR reversal. Radioautographs were prepared of M_1 and M_2 anaphases trypsinized between 7 and 9 hr after TdR reversal of FUdR block, and without colchicine (Figs. 2 *a,b*). The FUdR-TdR synchronization schedule is illustrated in Fig. 3.

Rat diploid cells were also synchronized by the metaphase shake method (Robbins and Marcus, 1964), without colchicine. Mitoses at M_1 were shaken off following 0.05 μCi/ml H_3TdR for 1 hr and chase for the length of G2 with Ca-free medium containing TdR. Mitoses at M_2 were shaken off following 0.2 μCi/ml H_3TdR for 1 hr and then TdR for the length of G2 plus one cell cycle, the last 4 hr in Ca-free medium. The metaphase shake schedule is illustrated in Fig. 4.

Good synchrony at M_2 cannot be achieved by simply following cells synchronized for M_1 through to M_2. Therefore synchrony was introduced separately for M_1 and M_2 as summarized in Figs. 3 and 4. The amount of label applied to M_1 cells was less than the amount applied to M_2 cells, and thus M_2 counts would not be expected to be halved. It was necessary to adjust the radioactivity for M_1 and M_2 in order to make the number of grains within countable range for both M_1 and M_2 pairs.

After each synchronization method, suspended M_1 and M_2 cells were centrifuged, rinsed in balanced salt solution, fixed in 1 part glacial acetic acid to 3 parts absolute methanol, and air dried on microscope slides which were dipped in 2 parts NTB-2 emulsion to 3 parts water. The emulsion was exposed for 3, 7, or 14 days. Cells were stained lightly with Giemsa at pH 6.4, and exposed grains of emulsion were

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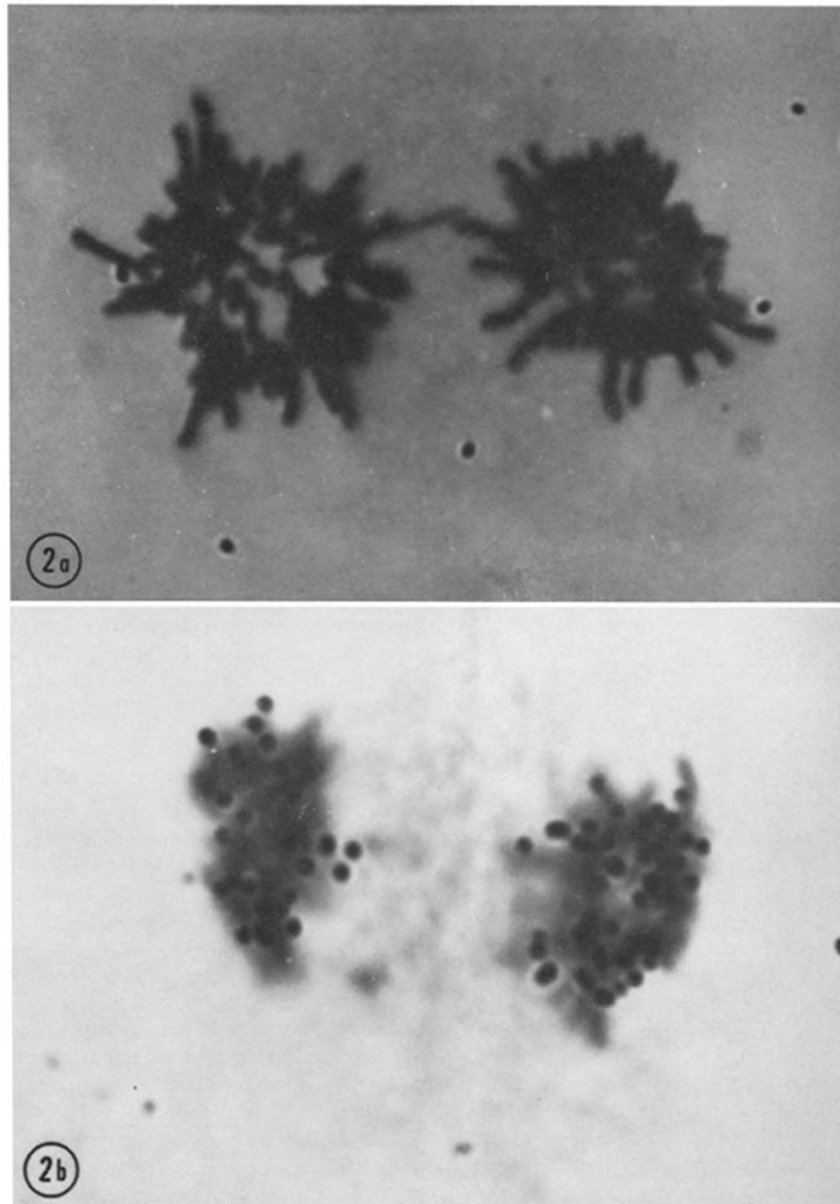

FIGURE 2 *a* An unlabeled rat 2n anaphase figure prepared on a microscope slide by the air drying technique. The slide was then dipped in NTB-2 emulsion, exposed for 5 days, developed, and stained with Giemsa at pH 6.4. The cytoplasm is lightly stained by this technique. In this figure, chromosomes from the two daughter cells are still in contact. Therefore, this anaphase would not be suitable for grain count if any portion of the labeled chromatin could not be discriminated between the two halves. Similarly, the figure would not be suitable if the daughter cells were sufficiently separated to prevent positive identification as an anaphase. 100 × oil immersion objective. × 1200.

FIGURE 2 *b* A labeled M_2 rat 2n anaphase radioautograph suitable for counting. In this situation, the count is not equal between the two daughter cells. Giemsa stain; 100 × oil immersion objective. × 1200.

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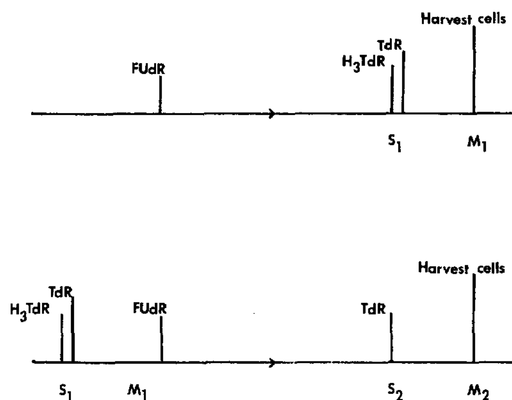

FIGURE 3 This illustration is the plan for FUdR-TdR synchronization to obtain labeled M_1 and M_2 anaphases as defined in Fig. 1. In the case of M_1 cells, the FUdR block to DNA synthesis is reversed by the addition of H_3TdR and cells are selectively labeled at the beginning of S. A 1-hr pulse of H_3TdR is followed by TdR. Anaphases are harvested after the length of S_1 plus $(G2)_1$ counting from the time of onset of label, which is also the onset of S_1. Metaphase-arresting agents are not used. In the case of M_2 cells, the pulse of H_3TdR is applied to asynchronous cells. When the labeled cells are expected to be through M_1 and into the next G1, FUdR block to DNA synthesis is applied. Reversal of this block with TdR starts S_2 of the cells previously labeled. Anaphases are harvested after the length of S_2 plus $(G2)_2$ counting from the time of introduction of TdR.

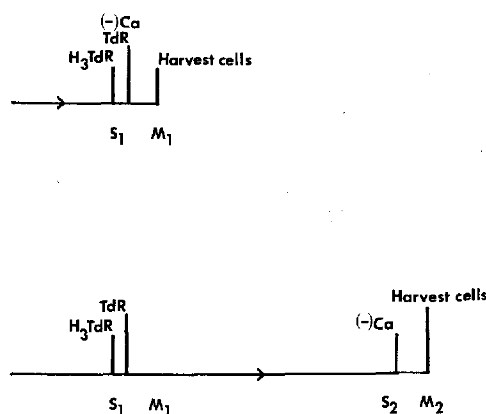

FIGURE 4 This illustration is the plan for metaphase shake synchronization to obtain labeled M_1 and M_2 anaphases as defined in Fig. 1. In the case of M_1 cells, the H_3TdR pulse to asynchronous cells is chased with TdR in low calcium medium. Mitoses are shaken off after the length of G2. Metaphase-arresting agents are not used. In the case of M_2 cells, the H_3TdR pulse to asynchronous cells is followed by TdR for the length of G2 plus one mean cell cycle. Ca-free medium containing TdR is substituted for regular TdR medium 4 hr prior to shaking off M_2 anaphases.

counted over chromosomes in anaphase daughter cell pairs. Each cell was counted two times. A total of 176 M_1 pairs and 227 M_2 pairs were studied. Pairs with total grain counts below 8 or over 110 were not included. Counts above 55 per cell were difficult to perform with accuracy. Background did not exceed three grains per cell.

In these studies several possible types of error included: fading of latent image; background; miscounting, for reasons such as individual bias, failure to distinguish individual grains. (*a*) Fading of latent image was considered minimal under the conditions of these experiments because the majority of slides were exposed for only 3–7 days. Furthermore, M_1 and M_2 slides were exposed for approximately the same number of days. Grain counts increased with exposure time but the increase was not a linear function of exposure time because of selection of the anaphases counted, since pairs with total counts below 8 or above 110 were excluded. In any case, fading of latent image would be unlikely to differ between anaphase pair members. (*b*) The effect of background was also considered to be minimal, since it did not exceed three grains over an area near to and comparable in size to one member of an anaphase pair, and did not differ between the two members of the pair. Background also did not differ between M_1 and M_2 cells analyzed. (*c*) Miscounting was avoided by two counts on each cell; use of a digital counter so that the running total was unknown to the person counting; counting by three different individuals, some pairs counted by one person, some pairs by another; destaining of heavily stained anaphases so that a clear contrast was obtained between grain and chromatin background by light microscopy. An additional 44 M_1 and 44 M_2 diploid rat pairs were analyzed by one person without knowledge of the source of the specimen.

RESULTS

Duplicate Counts

Each cell was counted twice, with the mean of these counts being taken as the best estimate of the true count for that cell. The absolute difference between repeated counts had a mean of 1.07 and variance of 1.98. Therefore, counting errors contributed a negligible amount to variability in the data.

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Comparison of Grain Counts Performed with and without Knowledge of the Source of the Specimen

In Table I, grain counts performed with and without knowledge of specimen source are compared. There was no significant difference, by Student's *t* test, between the two methods of counting.

M_1 Diploid Anaphase Pairs—Comparison to Poisson Variate

Because of the forced equality of radioactive material in members of each M_1 daughter cell pair (Fig. 1), measures of radioactivity released by the members of any M_1 pair should represent two independent observations on a single poisson variate. A standard test to determine whether several observations come for a single poisson distribution (i.e., from poisson distributions having the same parameter) is the so-called variance test, first introduced by R. A. Fisher (1958). This analysis was carried out for 176 M_1 diploid pairs, with the results shown in Table II. In this table, the last column indicates the probability of obtaining an X_N^2-value at least as large as that obtained if the null hypothesis of equality of means within pairs is true. It is apparent from the results shown in Table II that the observations display less disparity between members of a pair than one would expect in independent observations from a poisson distribution. The explanation of this apparent anomaly is presently unclear.

TABLE I

*Analysis of Grains in the Less Radioactive Member of M_1 and M_2 Diploid Pairs**

Method of counting	M_1		M_2	
	No.	Mean	No.	Mean
With knowledge of source of specimens	176	46.3 ± 4.2	227	39.4 ± 7.2
Without knowledge of source of specimens	44	45.2 ± 3.9	44	39.5 ± 6.8

* Lesser count/total count for anaphase, expressed as per cent.

TABLE II

*Variance Analysis for Identity of Label in M_1 Cell Pairs**

Cell type	Synchronization	N	X_N^2	Probability‡
Rat diploid	FUdR-TdR	75	18.243	1.0
Human diploid	FUdR-TdR	63	35.440	0.995
Rat diploid	Metaphase shake	38	14.159	1.0
All	All	176	67.843	1.0

* If x_1 and x_2 represent the two counts for a pair, then the estimate of the poisson parameter, λ, is given by $\hat{\lambda} = \frac{x_1 + x_2}{2}$, and the statistic $X_1^2 = \sum_{i=1}^{2} \frac{(x_i - \hat{\lambda})^2}{\hat{\lambda}}$ is approximately distributed as a chi-square variate with one degree of freedom when λ is moderately large. In the M_1 data collected for this study, the minimum λ estimate is about 8, while over 85% of the estimates are greater than 10. Therefore, the approximation should be adequate. After substituting the mean value of the observations for λ, this statistic reduces to $X_1^2 = \frac{(x_1 - x_2)^2}{x_1 + x_2}$ for any given pair of observations.
Since the counts on every anaphase pair are independent of the counts on every other pair, the statistics for all pairs can be added to give a chi-square statistic with N degrees of freedom, X_N^2, where N is the number of pairs available.
‡ A P-value of 1.0 represents $P > 0.9995$.

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Table III

*Analysis of Grains in the Less Radioactive Member of M_1 and M_2 Pairs**

Method of synchronization	Cell type	M_1 Pairs No.	M_1 Pairs Mean	M_2 pairs No.	M_2 pairs Mean
FUdR-TdR	Rat 2n	75	47.3 ± 3.1	89	40.6 ± 6.3
Metaphase shake	Rat 2n	38	46.1 ± 4.1	88	39.4 ± 7.1
FUdR-TdR	Human 2n	63	45.2 ± 5.0	50	37.6 ± 5.9
FUdR-TdR	Rat 4n	100	45.4 ± 3.9	100	45.2 ± 4.5

* Lesser count/total count for anaphase, expressed as per cent.

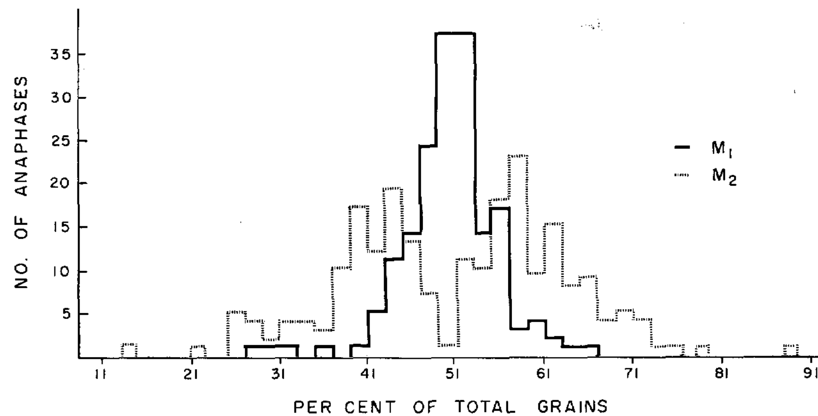

Figure 5 Per cents of total grains in one member selected randomly from each M_1 and M_2 diploid anaphase pair are plotted against number of anaphase pairs. A single mode around 50% is seen for M_1 cells. A double mode at about 42 and 58% is seen for M_2 cells. Note that the number of anaphase pairs differs for M_1 and M_2 (176 for M_1 and 227 for M_2).

Mean Per Cent of Grains in the Less Radioactive Member of Diploid and Tetraploid M_1 and M_2 Pairs

There was no significant difference, by Student's t test, between the two methods of synchronization for rat diploid cells (Table III). The data for human diploid cells and rat tetraploid cells are also presented in Table III. For diploid cells but not for tetraploid cells the mean per cents are significantly lower (by Student's t test) for the lesser member of M_2 pairs, as compared to the lesser member of M_1 pairs when grain counts are compared.

Mean Per Cent of Grains in One Member Selected Randomly from Each M_1 Pair and Each M_2 Pair

When the higher or lower member of M_1 diploid pairs (176) was selected randomly, the mean per cent of grains in one member was 50.2 ± 5.6. The mean per cent of grains in one random member of M_2 diploid pairs (227) was 51.1 ± 12.7. Both means closely approached 50, showing that the selection of higher or lower member was indeed random. Fig. 5 presents mean per cent of grains in one random member for M_1 and M_2 diploid pairs plotted against number of anaphase pairs. M_1 pairs show one mode at about 50%, and M_2 pairs show two modes at about 42 and 58%. Fig. 6 presents mean per cent of grains in one random member of M_1 and M_2 tetraploid pairs plotted against number of anaphase pairs. Both M_1 and M_2 distributions show a single mode in rat tetraploid pairs.

DISCUSSION

The present studies do not distinguish individual chromosomes and therefore do not exclude the possibility that various-sized chromosomes could differ in regard to: (*a*) radioactive content relative

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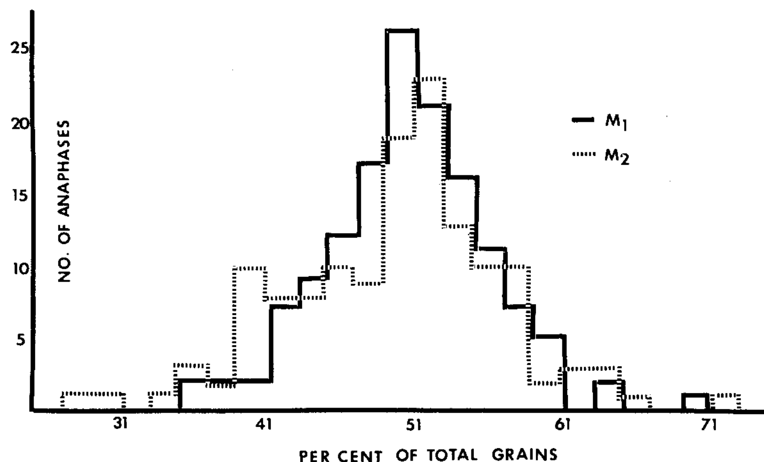

FIGURE 6 Per cents of total grains in one member selected randomly from each M_1 and M_2 tetraploid anaphase pair are plotted against number of anaphase pairs. A single mode around 50% is seen for both M_1 and M_2 pairs.

to total length, (*b*) segregation behavior, and (*c*) other characteristics affecting the distribution of thymidine label at mitosis. For instance, if newly replicated DNA strands tend to segregate together in the same daughter cell because of a membrane mechanism for replication, one result might be preferential nonrandom segregation of late replicating chromosomes. To date, studies of individual chromosomes have failed to confirm nonrandom segregation (Heddle et al., 1967; Cuevas-Sosa, 1968). However, when analysis is restricted to particular chromosome regions, many chromosomes simply do not produce enough grains to differentiate labeled, from partially labeled, from unlabeled regions. An arbitrary division of grain count number, below which is to be considered "unlabeled" and above which is to be considered "labeled", is unsatisfactory. Selection of a few chromosomes for analysis from each cell is unsatisfactory.

It is apparent that exchange of labeled chromatin (by sister chromatid exchanges) superimposed on a distribution of label between daughter cells that is unequal to begin with would tend to equalize the distribution. The implication is that if sister chromatid exchanges were not occurring (and they do occur), the difference between M_2 pair members reported in this paper would be even greater.

If the method of grain count analysis of anaphases, following a pulse label of H_3TdR, is used to study chromatid segregation certain rules of methodology must be followed. (*a*) The number of cell divisions between pulse label and the division under analysis should be known exactly; the divisions under analysis should be "pure." To these purposes some method of synchronization may be essential but must be shown not to influence the grain distribution. (*b*) M_1 diploid data should be available as control counts.

Confirmation of nonrandom chromatid segregation, by using the method of grain count analysis of anaphases, awaits mathematical and computer models now being formulated, for M_2 anaphase grain distributions expected on the basis of various types of chromatid segregation in cells with various chromosome numbers. A bimodal distribution composed of M_2 anaphase daughter cells with high and low grain counts is unequivocal and reproducible in the rat and human diploid cells studied in this report. The observed result is a by-pair inequality of labeled chromatin at M_2 anaphase following a pulse of H_3TdR.

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