Isolation and Fractionation of Metaphase Chromosomes from a Normal, Parenchymal Tissue: The Regenerating Rat Liver[1] (35346)

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(Introduced by C. A. Stetson)
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The DNA which forms the genome for mammalian cells is massive when compared with that of cells of lower orders. Its bulk and complexity have made it nearly impossible to subdivide for use in genetic analysis. The packaging of DNA into discrete metaphase chromosomes during cell division offers an opportunity for the separation of units which may contain differing genetic information susceptible to analysis.

Metaphase chromosomes have been isolated from animal cells in tissue culture (1), and in a recent study, from human lymphocytes (2). Fractionation of these chromosomes into groups according to size was accomplished on sucrose gradients (3).

It would be of considerable value to isolate and fractionate the metaphase chromosomes from a parenchymal tissue. This accomplishment would eliminate the need to culture massive numbers of cells and the danger of their acquiring genetic alterations during serial passage. The mammalian hepatocyte is a parenchymal cell of particular interest which can be studied during its evolution to hepatocellular carcinoma resulting from the ingestion of chemical carcinogens (4). Comparison between the genetic components of hepatocytes at each stage in this process might yield information relating to the nature of malignant transformation. The obstacles encountered in such experiments are the low mitotic rates normally exhibited by the liver and the need to apply sufficient force to this solid tissue to disrupt the tightly linked cells without damaging the chromosomes.

We sought to overcome these problems by using the regenerating liver of weanling rats which demonstrates a high mitotic activity, by organ perfusion to weaken intercellular binding and by acidification of the homogenizing fluid to protect the chromosomes upon release. Sprague-Dawley, CFE, male rats, weighing 80 g, were used throughout the study (Carworth Farms, New City, N.Y.). Mitosis was stimulated by 70% hepatectomy and cells were accumulated in metaphase by the administration of Velban (vinblastine sulfate, Lilly) as a split dose of 2 mg/kg (intraperitoneally and subcutaneously) at 22 hr after operation. Histologic examination of these Velban-treated regenerating livers revealed an average of 40% of the hepatocytes in metaphase arrest (a range of 10 to 70%). No other hepatic cell-type was in division at this time.

At 28 hr after operation the livers were perfused with 30 ml/g of tissue of calcium-free Locke's solution (pH 7.3) via the inferior vena cava and portal vein while the rats were under ether anesthesia. The livers were minced in 5-g portions and homogenized in a glass, Douce Homogenizer (clearance of 0.025 cm) in 0.25 *M* sucrose in formate buffer. Formate buffer (FB) = 0.02 *M* formate buffer containing 0.3 m*M* $MgCl_2$, and 0.7 m*M* $CaCl_2$. Two or 3 drops of Tween 80 were added during homogenization to facilitate chromosome separation. This and all subsequent steps were performed at 4°.

The supernatants from 3 successive homogenizations were pooled and spun at 400*g* for 10 min to remove cells and the majority of nuclei. The supernatant from this slow spin was removed and the pellet was vortexed with 5 vol of 0.25 *M* sucrose–FB to release entrapped chromosomes. The resultant sus-

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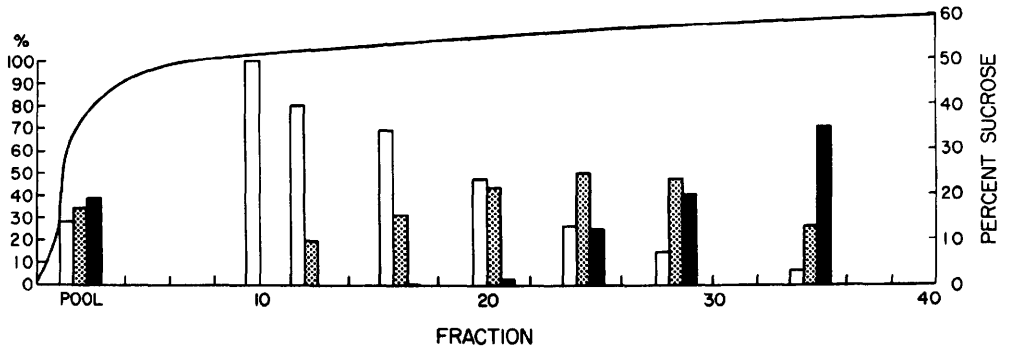

FIG. 1. Distribution of chromosomes in a continuous sucrose gradient: (right ordinate) the percentage of sucrose as indicated by the solid line; (left ordinate) the percentage of chromosomes of a size-group in a fraction; (pool) the distribution of chromosome-size-groups in the original isolate; (clear bar) fraction S; (dotted bar) fraction M; (solid bar) fraction LM/L.

pension was spun at 400*g* for 10 min and its supernatant was added to the pool. This suspension, containing chromosomes and a few nuclei, was centrifuged through 48% sucrose–FB to concentrate the chromosomes and to remove the bulk of debris. This step decreased chromosome aggregation during subsequent fractionation.

The partition of chromosomes reported here was effected by centrifugation on continuous or discontinuous sucrose gradients in a B-XV zonal rotor with an effective path length of 7.5 cm (5).

Continuous convex gradients (6) were prepared with 600 ml of 45% sucrose–FB and 1000 ml of 55% sucrose–FB with a cushion of 57.5% sucrose–FB. These and all subsequent solutions for gradients contained 0.5% Tween 80.

The chromosome suspension (2 OD units of material at 450 mμ) was added in 110 ml of 40% sucrose–FB after re-homogenization to reduce clumping; 50 ml of formate buffer was layered above the sample. The gradient was spun for 75 min at 9000 rpm at a temperature of 14°. It was unloaded at 2000 rpm at a rate of 50 ml/min through a Gilford, Model 2000, spectrophotometer adapted with a specially designed flow cell (5). Forty-ml aliquots were taken.

The discontinuous sucrose gradient (sucrose–FB) was prepared by loading the rotor from the edge with the following: 150 ml of 11%; 275 ml of 27.3%; 470 ml of 32%; 470 ml of 37% and 350 ml of 58% sucrose as a cushion. The sample, after re-homogenization, was added in 110 ml of 11.2% sucrose and overlaid with 50 ml of 11.2% sucrose. The gradient was loaded at 2000 rpm and spun at this speed for 75 min from the addition of sample at 10°. The gradient was unloaded at 2000 rpm in 80-ml aliquots. All samples were diluted to a sucrose concentration of 10% with FB and pelleted. Smears of the pellet were fixed with 3:1 methanol:glacial acetic and were stained with 2% aceto-orcein for examination.

The chromosomes of the normal rat cell demonstrate a much smaller range in size than do those of many other species studied (7). For the purposes of this study, the chromosomes were grouped by average length as determined from the normal karyotypes of our rat hepatocyte. Therefore, group S (small) in our classification included chromosomes numbered 15, 17–20 and the Y chromosomes of the classification of Yoshida *et al.* (8); M (medium) chromosomes numbered 8, 9, 11–14, 16; LM (large medium) chromosomes 3–7, 10 and L (large) chromosomes numbered 1 and 2. By this method of classification the percentage of chromosomes in our normal rat hepatocyte was S: 28%; M: 33%; LM/L: 38%. In a typical, purified chromosome pellet obtained from our homogenates, the distribution of chromosome groups was S: 29%; M: 36%; LM/L: 34%.

The separation of chromosomes obtained in a single centrifugation on a typical continu-

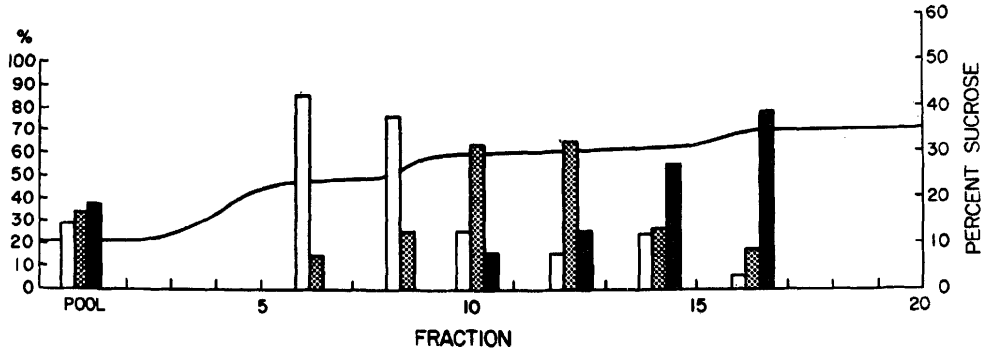

FIG. 2. Distribution of chromosomes in a discontinuous sucrose gradient (see Fig. 1 for legend).

ous gradient is represented graphically (Fig. 1). Fractions which were predominantly composed of S or LM/L were readily obtained by this method. Chromosomes of group M, however, were frequently spread throughout the gradient. In a small number of aliquots, however, relatively pure samples of M could be obtained.

The results of a single centrifugation on a typical discontinuous gradient are presented (Fig. 2). Once again, fractions containing 50% groups S and LM/L were obtained readily. In this gradient, fractions containing chromosomes which were predominantly of group M were often obtained. In both types of sucrose gradient some aggregation occurs, apparently during centrifugation, causing a mixing of chromosomes seen in occasional fractions near the bottom of the gradient.

The morphology of the chromosomes obtained by gradient fractionation was close to that seen in rat hepatocyte karyotypes (Fig. 3).

The methods described make possible the

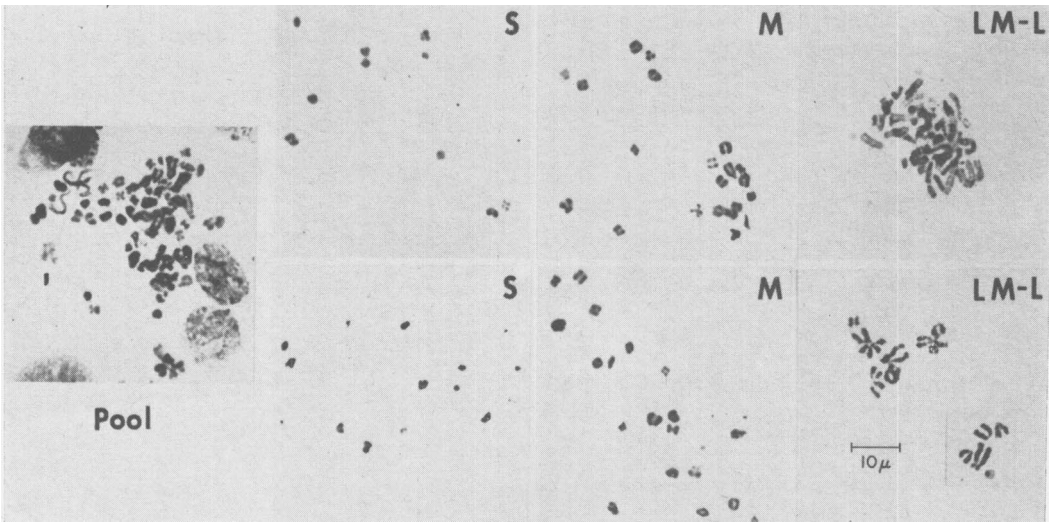

FIG. 3. Photomicrographs of the original chromosomal isolate and of various chromosomal fractions obtained from continuous and discontinuous sucrose gradients (pool) the whole homogenate containing chromosomes of all sizes and nuclei; (S) small chromosome fraction; (M) medium chromosome fraction; (LM/L) large chromosome fraction. The aggregation of large chromosomes seen here occurs frequently during the preparation of slides when large chromosomes are present in a high percentage. The structure of these chromosomes remains intact and is comparable to that of large chromosomes which remain separate (see insert).

isolation and fractionation of the metaphase chromosomes from normal dividing hepatocytes in quantities permitting analysis of their macromolecular components. The results achieved using this solid tissue are comparable to those previously reported for single cell-types.

Although the isolation of chromosomes from a parenchymal tissue offered additional obstacles, they were overcome by extensive perfusion, use of an acidic buffer and extremely gentle homogenization. The major difficulty in group fractionation arises from the subtle gradation and small range in chromosome size in the rat compared to those previously studied. Other gradients and recentrifugation of enriched chromosome pools are being used to solve this problem.

Even at this stage of development, the technique produces pools composed of more than 50% chromosomes of one group. The identification of sites of transcription of specific RNA by use of molecular hybridization with the DNA obtained from specific chromosome groups is then possible. The chromosomes from livers during various stages of chemical carcinogenesis can be compared with those of normal liver. We have already isolated chromosomes from the hyperplastic hepatic nodules induced by ingestion of *N*-2-fluorenylacetamide, in preparation for these studies.

Summary. A method of isolating large numbers of metaphase chromosomes from normal, regenerating rat liver is described. The chromosomes were separated into size groups in a large-capacity zonal rotor (B-XV) on sucrose density gradients.

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