

ISOLATION AND PARTIAL CHARACTERIZATION OF METAPHASE CHROMOSOMES OF A MOUSE ASCITES TUMOR*

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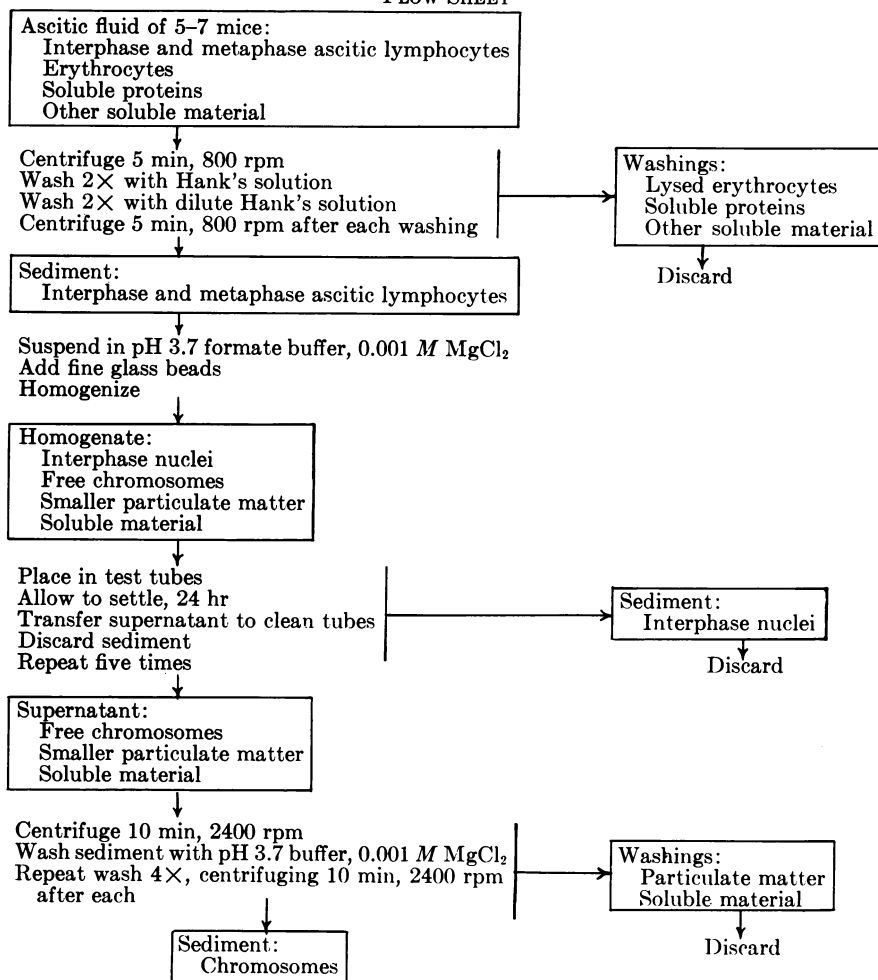
Before understanding more thoroughly the complex relationships between the genetic material and other cellular components, we must examine in detail the chemical and physical properties of the DNA-containing units of the cell. In this report, we describe the isolation and partial characterization of mammalian metaphase chromosomes. The choice of chromosomes in metaphase was influenced by two attributes not found in interphase chromosomes or chromatin. The genetic material is distributed among distinct subunits of recognizable morphology and it is free of heterogeneity resulting from metabolic processes such as RNA and DNA synthesis. The former quality permits unambiguous identification of the isolated material and suggests the possibility of obtaining preparations of the same chromosome.

In recent years, several investigators have reported the isolation of metaphase chromosomes.¹⁻⁴ While the chromosomes isolated thus far have served well in studies of morphology and coiling and in tentative chemical analysis, problems of contamination, chromosome breakage, and insufficient yield have restricted the range of investigations. The technique of mitotic arrest by colchicine is the method commonly used for obtaining large quantities of cells in metaphase from a rapidly dividing population. Two sources of material have been used. Tissue culture of mammalian cell lines was used by Trosko and Brewen,¹ Somers, Cole, and Hsu,² and Lin and Chargaff.³ Chromosomes studied by Chorazy, Bendich, Borenfreund, and Hutchison⁴ were obtained from a mouse ascites tumor. In choosing between these alternatives, our primary considerations were of yield and convenience. We selected an ascites tumor because its use results in large numbers ($10-50 \times 10^6$) of cells in metaphase. In addition, it does not require the maintenance of the carefully controlled sterile conditions necessary for successful tissue culture.

At the early stages of this work, three criteria were established for the evaluation of subsequent isolation procedures. First, the morphology of the isolated chromosomes had to be the same as the morphology of chromosomes in standard metaphase spreads. Second, the preparation had to be free of gel formation as well as contamination from cells, nuclei, smaller organelles, and soluble cytoplasmic components. Third, the yield of chromosomes had to be large enough for chemical analysis and physical studies (milligram amounts). We present here a procedure for the isolation of chromosomes which satisfies these criteria.

ISOLATION OF CHROMOSOMES

Methods and Materials.—Isolation procedure: Female Heston mice (age 60-80 days) were injected intraperitoneally (IP) with 1×10^6 cells of lymphoma L2⁵,⁶ ascites tumor suspended in 0.1 ml TC-199 medium (Difco). Six days following the administration of the tumor, 0.25 ml of a 0.9% saline solution containing 100 μ g colchicine (Calbiochem) per ml was injected IP. After 18-24 hr the mice were anesthetized with ether and sacrificed by cervical dislocation. The peri-

TABLE 1
FLOW SHEET

toneal cavity was opened and ascitic fluid (approximately 2 ml) drained into 2 ml Hank's balanced salt solution (Difco) in a precooled beaker. In subsequent steps the material was maintained at 0-4°C. Two 1-ml washings of the peritoneal cavity with Hank's solution were added to the removed fluid. The combined fluid and washings from 5-7 mice were centrifuged at 800 rpm (80 *g*) for 5 min in a clinical centrifuge (Table 1). The pellet, containing metaphase and interphase tumor cells as well as erythrocytes, was washed with 10 ml Hank's solution, centrifuging as above. The resulting pellet was washed two times with 10 ml Hank's solution which had been diluted to 1/4 physiological (original) strength. These last washings resulted in the osmotic lysis and removal of the contaminating erythrocytes. Ascitic lymphocytes were killed and swollen by this procedure, but not lysed. The pellet of the second hypotonic washing was taken up in 110 ml pH 3.7 formate buffer (0.01 ionic strength)⁷ containing 0.001 *M* MgCl₂. This solution will hereafter be referred to as the pH 3.7 buffer. Four grams of fine glass beads (VirTis no. H3640 fine homogenizing beads) were added. The suspended cells were homogenized with a VirTis model 23 mixer at 9000 rpm (2/3 maximum voltage) for 12 min. The homogenizing vessel was immersed in an ice bath during this procedure. Following homogenization, the suspension which contained disrupted metaphase cells and intact interphase nuclei was placed in 18 × 150-mm test tubes and stored 24

hr. Most (85–90%) of the nuclei settled in this period, while a like percentage of the freed chromosomes remained in the supernatant fraction. The supernatants were transferred to a clean set of tubes, and allowed again to settle for 24 hr. This procedure was repeated a total of five times, after which the final supernatant fraction contained suspended chromosomes, smaller particulate matter, and soluble cytoplasmic material. It was essentially free of clumped chromosomes, nuclei, and cells which had not been disrupted. Chromosomes were separated from the smaller particulate matter and soluble material by centrifugation in the clinical centrifuge at 2400 rpm (720 *g*) for 10 min. The pellet, containing chromosomes, was washed four times with a pH 3.7 buffer, centrifuging in each case at 2400 rpm for 10 min. The final pellet contained intact metaphase chromosomes with little contamination, as detailed below. The dry weight yield was approximately 300–400 μg of chromosomes per mouse.

Preparation of slides: Slides of fixed and stained material were made at various points during the isolation procedure (Figs. 1–5). Modifications of the procedure of Moorhead, Nowell, Mellman, Battips, and Hungerford⁸ were used in the fixing of material and the technique of air-drying the slides. A volume of cell suspension, raw homogenate, or isolated chromosomes, taken at the appropriate time during the isolation procedure, was centrifuged at 2400 rpm for 10 min in the clinical centrifuge. After discarding the supernatant, the pelleted material was taken up in acetic acid:methanol, 2:3 (v:v) fixative and again centrifuged. The pelleted material was suspended in a volume of fixative adjusted so that an adequate density of material would result on the completed slide. One or two drops of the suspended material were placed on a cold, wet, clean glass slide which was then gently heated over an alcohol lamp until the fixative had evaporated. The slides were stained with Giemsa stain for 15 min and then mounted. This procedure was uniformly successful in the preparation of slides of unbroken interphase nuclei, chromosome spreads of metaphase cells, and free chromosomes (Figs. 1, 2, 3, 5). When chromosomes were not entirely freed from nonchromosomal material, as occurred frequently during development of this procedure, this was reflected on slides prepared in the above manner (Fig. 4).

Discussion of Isolation Method.—Homogenizing conditions: In preliminary investigations, it was observed that homogenization of cell suspensions at neutral pH led to breakage of cells, but metaphase chromosomes were always bound to a material which resembled a gel-like matrix. Disruption of cells in a more acidic medium was attempted. Studies in the pH range 3.2–4.1 demonstrated the great sensitivity of cell breakage and chromosome aggregation to small variations in pH. At pH 3.2, both interphase nuclei and metaphase cells were broken, but chromosomes were again bound to nonchromosomal material (Fig. 4). At pH 4.1, no interphase cells and few metaphase cells were disrupted. Of the few chromosomes

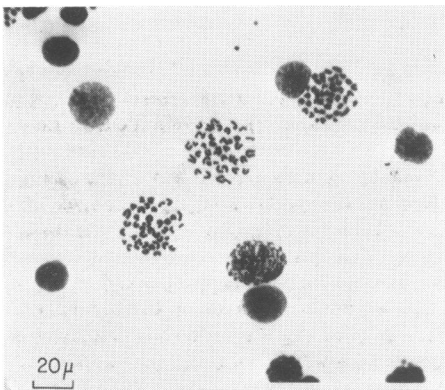


FIG. 1.—Typical metaphase figures of lymphoma L2 cells. Note the size and morphology of the chromosomes. Giemsa stain.

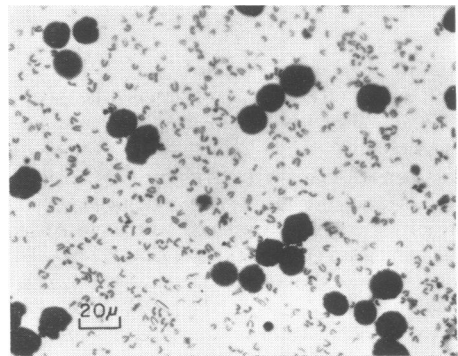


FIG. 2.—Interphase nuclei and free chromosomes of homogenized cells. Note the integrity of chromosomal structure and the separation of chromosomes from one another and from nuclei. Giemsa stain.

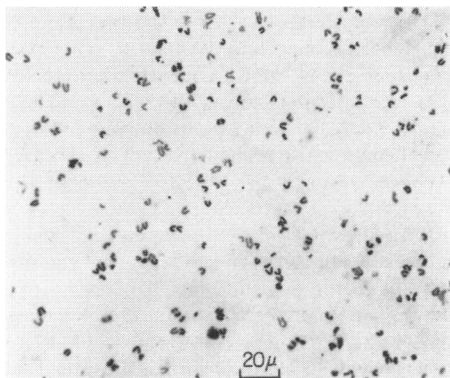


FIG. 3.—Final preparation of chromosomes. Note the random distribution of chromosomes, and their morphological characteristics. Giemsa stain.

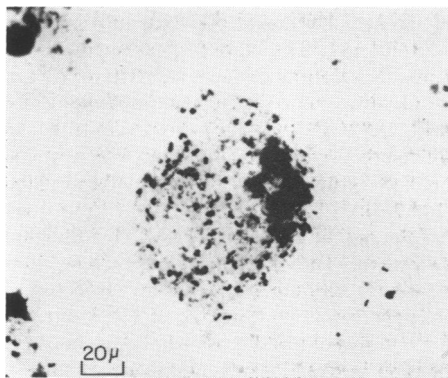


FIG. 4.—Chromosomes, nuclei, and other material resulting from homogenization of cells at pH 3.2. Homogenization at this pH resulted in association of chromosomes with a gel-like matrix. Giemsa stain.

released, most were in a gel, but some were completely free of contaminating material. In the region of pH 3.7–3.8, most of the metaphase cells were broken and numerous unbroken interphase nuclei were observed. The great majority of the chromosomes appeared to be free of cytoplasmic material (Fig. 2).

Separation of chromosomes from other material: In our preparation, we desired a large quantity of material, retention of chromosomal morphology, and a high degree of purity. In developing the procedure, no attempt was made to preserve presumptive enzymatic activity or to prevent minor damage to the detailed macromolecular structure. Our requirement for milligram quantities of chromosomes ruled out separation of the raw homogenate by centrifugation. When this was attempted, interphase nuclei sedimented only slightly faster than metaphase chromosomes. When the homogenate was left to settle, however, chromosomes remained suspended due to Brownian motion, while most interphase nuclei settled in 24 hr. Modifications of this procedure are possible and probably necessary before embarking on a study of chromosomes with respect to enzymatic activity and detailed macromolecular structure.

PROPERTIES OF CHROMOSOMES

Methods.—Microscopical observation and photography: Day-to-day observations were made with a Tiyoda light microscope. Pictures were taken using a Zeiss light microscope with phase contrast.

Dry weight determination: A suspension of chromosomes containing 3–5 mg in 5–10 ml pH 3.7 buffer was centrifuged at 2400 rpm for 15 min. The supernatant was discarded and replaced with methanol. After suspension of the chromosomes in this solvent, they were placed in a weighing jar and dried overnight in a vacuum oven at 100°C prior to weighing.

Measurement of concentration: The chromosomes were dispersed by alkaline treatment before measurement of absorbance. Three and a half ml of 0.1 *N* NaOH were added to 0.5 ml of chromosome suspension which had been removed from a larger sample of known volume. The optical density of this basic solution was measured in a Cary 14 spectrophotometer at 260 m μ using an appropriate mixture of pH 3.7 buffer and 0.1 *N* NaOH as blank. The remainder of the suspension was dried and weighed as above, and the extinction coefficient of base-treated chromosomes was calculated.

Chemical analyses: RNA was determined by the orcinol colorimetric test.⁹ Basic hydrolysis

of RNA and removal of DNA and protein with 20% perchloric acid followed the procedure of Schmidt and Thannhauser¹⁰ as modified by Wannemacher, Banks, and Wunner.¹¹ One part 1.5 *N* NaOH was added to 4 parts of a chromosome suspension of known concentration. RNA was hydrolyzed by incubation overnight at 37°C. Upon addition of 0.5 ml cold 60% perchloric acid, DNA and protein were precipitated. Centrifugation at 2400 rpm for 10 min was followed by removal of the supernatant which was assayed for RNA by the orcinol test. Yeast RNA¹² (Worthington) of spectrophotometrically determined concentration ($\epsilon_{259}^{1\%} = 206$)¹² was used as a standard.

In assaying for DNA, the Dische diphenylamine reaction¹³ was used with samples prepared in two ways. In one, diphenylamine reagent was added directly to the untreated chromosome suspension. All nine samples were subjected to the assay under these conditions. To demonstrate that there was no blockage of DNA during this reaction, we applied the method of Schneider¹⁴ for removal of protein prior to addition of the Dische reagent. To 3 parts of the chromosome suspension in question was added 1 part 20% trichloroacetic acid. The resulting mixture was kept at 90°C for 15 min in a water bath. After cooling and pelleting the proteinaceous precipitate by centrifugation, the supernatant was removed and assayed for DNA by the above method. In both cases, we used calf thymus DNA (Nutritional Biochemicals) of spectrophotometrically determined concentration ($\epsilon_{260}^{1\%} = 201$) as a standard.

Protein was measured by the method of Lowry, Rosebrough, Farr, and Randall¹⁵ on chromosome samples of known concentration. Before determination of protein by this method, aliquots of the chromosome suspension were diluted with 2 vol of 0.1 *N* NaOH. Commercially prepared calf thymus histone (Worthington HLY) was used as a standard. Standard solutions of known concentration were made by dissolving weighed samples of vacuum-dried histone in known volumes of 0.1 *N* NaOH.

Results and Discussion.—Morphology: The generally accepted criterion for the recognition and characterization of metaphase chromosomes is the morphological one. Chromosomes isolated by the procedure outlined above fulfill this criterion in most respects. In Figure 1 are shown spreads of chromosomes from cells of a population of ascites tumor cells used as a source for the isolated material. These spreads were prepared in a manner similar to the standard one used for identification and characterization of metaphase chromosomes in cytogenetic studies.^{8, 16} At the magnification used in Figure 1, the sizes and shapes of the spread chromosomes are indistinguishable from those of the isolated chromosomes shown in Figure 3. Examination of large numbers of isolated chromosomes indicated little or no breakage in this material. Close similarities between isolated chromosomes and those of standard spreads also obtain at higher magnifications, as in Figure 5. The fine fibrous network which extends from all regions of the spread chromosomes is observed in the isolated chromosomes as well, but to a lesser extent. Small differences in size reflect different degrees of coiling. Staining properties of chromosomes were not examined because of the availability of large quantities for direct chemical analysis. The presence of DNA, RNA, and protein in chromosomes conforms to the qualitative picture which histological methods have provided.

Concentration measurements: In preliminary work, attempts were made to establish the concentration of chromosomes by evaluating the extinction coefficient of chromosome suspensions in pH 3.7 buffer. The extinc-

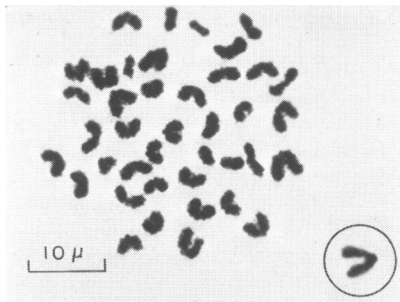


FIG. 5.—A spread of chromosomes with an insert of a typical isolated chromosome. Giemsa stain.

TABLE 2
CHEMICAL ANALYSIS OF METAPHASE CHROMOSOMES

Sample no.	Dry weight chromo- somes from OD ₂₆₀ ($\mu\text{g}/\text{ml}$)	Protein		RNA		DNA		DNA-TCA sample, % dry weight
		$\mu\text{g}/\text{ml}$	% Dry weight	$\mu\text{g}/\text{ml}$	% Dry weight	$\mu\text{g}/\text{ml}$	% Dry weight	
1	522	368	70.5	65.1	12.5	76.3	14.6	...
2	580	384	66.1	78.2	13.5	75.8	13.1	...
3	489	337	68.8	57.9	11.8	65.0	13.3	...
4	416	284	68.3	58.0	13.9	53.7	12.9	...
5	400	276	69.0	55.0	13.7	55.9	14.0	...
6	446	314	70.4	61.4	13.8	56.4	12.6	...
7	508	332	65.4	72.5	14.3	66.6	13.1	12.3
8	506	325	64.3	70.9	14.0	73.1	14.4	14.4
9	632	451	71.3	91.0	14.4	82.9	13.1	12.1
Average %		68.3 \pm 2.5		13.5 \pm 0.9		13.5 \pm 0.7		

tion coefficients calculated from measurements made in a Cary 14 spectrophotometer without the scattered transmission attachment were not reproducible. It was observed that addition of base caused visible light scattering of a chromosome suspension to disappear. The spectrum of the base-treated chromosomes was also found to have a negligible light-scattering correction in the ultraviolet range. Moreover, it was reproducible. Measurements of $\epsilon_{260}^{1\%}$ were thus taken after chromosome suspensions had been made alkaline. Three such measurements showed $\epsilon_{260}^{1\%} = 64.9 \pm 0.7$. This corresponds to an $\text{OD}_{260} = 1.0$ for a chromosome suspension containing $154.0 \mu\text{g}/\text{ml}$ dry weight material. This value was used to determine the concentration of chromosome suspensions used in chemical analysis and other studies.

Chemical analysis: Analysis of the RNA, DNA, and protein content of metaphase chromosomes from L2 ascites tumor cells reveals 13.5 per cent RNA, 13.5 per cent DNA, and 68.3 per cent protein (Table 2). The discrepancy between the sum of these figures and 100 per cent may be caused by error in the analysis procedure, error in the extinction coefficient, or the presence of material other than nucleic acid and protein in the chromosomes. A comparison of our results with data on the composition of interphase nuclei is of interest. Umaña, Updike, Randall, and Dounce¹⁷ found from 5.7 to 12.9 per cent DNA in populations of interphase mammalian nuclei isolated from various species and organs. Data compiled by Allfrey, Daly, and Mirsky¹⁸ show that the protein content of nuclei isolated in nonaqueous media ranges from 71.8 to 80.0 per cent of the dry weight. In the tissues studied by Allfrey *et al.*, the ratio of protein to DNA fell between 2.7 and 7.0, depending on the source. The absolute amount of RNA and its concentration with respect to the DNA content of interphase nuclei is highly variable, and thus comparisons between our data and results on interphase nuclei would be quite meaningless. Normalized to 100 per cent yield, L2 ascites tumor metaphase chromosomes contain 14.1 per cent DNA and 71.7 per cent protein. The protein:DNA ratio is 5.1. These values for DNA and protein are comparable to those reported for interphase nuclei if we consider that interphase cells have not, on the average, completed DNA synthesis. This comparison suggests that chromosomes may serve to transfer to the daughter cells not only DNA and closely associated histone, but most of the macromolecular components of nuclei. From this point of view, metaphase chro-

mosomes should contain most of the enzymatic activity observed in interphase nuclei. Incorporation of nuclear ribosomes into the chromosome structure is also a possibility.

The role of the RNA is as yet poorly understood, and it would be premature to consider the equal amounts of RNA and DNA as particularly significant. During mitosis in cells of both higher plants and animals, chromosomes take on a charge of RNA during prophase and release it during telophase.¹⁹⁻²² Origin of this RNA has been ascribed to various sources.²³ Recently, evidence has appeared for a histone-bound RNA of interphase cells which might be found in metaphase chromosomes.²⁴ It is likely that in metaphase chromosomes, as in interphase nuclei, the RNA-DNA ratio is highly variable, being dependent on the organ and species. Studies on chromosomes isolated from different sources as well as detailed investigation of the RNA from L2 ascites tumor chromosomes should suggest answers to the questions raised here.

The spark emission spectrum of a 0.1 per cent solution of the chromosomes in the 3:2 methanol-acetic acid fixative (see *Methods: Preparation of slides*) was measured by the analysis laboratory of the Lawrence Radiation Laboratory (Table 3). Copper was measured with a flame absorption photometer because of contamination from the copper electrodes of the spark emission instrument. With the exception of copper and magnesium, the analysis of the blank and the chromosomes was identical within the measurable accuracy. The amounts of copper and magnesium were both greater in the chromosome solution by a factor of 5 with respect to the minimum detectable amount of metal in the blank. This still represents an extremely low level of metal content in the chromosomes.

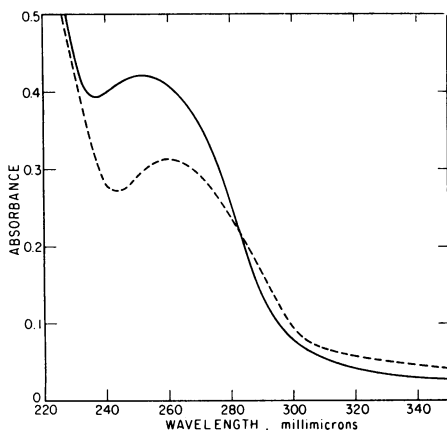


FIG. 6.—Ultraviolet absorption spectra of chromosomes suspended in pH 3.7 buffer, 0.001 *M* MgCl₂ (---) and dispersed in 0.1 *N* NaOH (—). The path length is 1 cm, and concentration of chromosomes is 62 μg/ml.

Physical properties: Figure 6 shows the absorption spectrum of metaphase chromosomes at pH 3.7 in the Cary 14 with scattered transmission attachment. There is a large hyperchromic effect upon transfer of the chromosomes to 0.1 *N* NaOH as well as a shift of the absorption peak to lower wavelengths. Both are tentatively attributed to the denaturation of the protein and the DNA in this solvent. The shape of the spectrum is similar to that of other complexes of protein and nucleic acid such as virus particles.^{25, 26}

The chromosomes have no sharp temperature transition in the pH 3.7 buffer in the temperature range from 20 to 100°C, as observed by monitoring the ab-

sorbance at 260 μ of a chromosome suspension as a function of temperature. If the chromosomes are treated with 4.7 *M* guanidinium chloride, the metaphase structure disappears, and the histone and the nucleic acid are believed to be dissociated. A sharp temperature transition occurs in this solution at 60°C, indicating that the DNA has its native structure in the isolated chromosomes.

Summary.—A method for the isolation of metaphase chromosomes from colchicine-arrested cells of L2 mouse ascites tumor has been developed. The unique feature of the isolation procedure is homogenization of cells at pH 3.7. The chromosomes, isolated in milligram quantities, are morphologically intact. There is little gel formation or contamination from cells, nuclei, smaller organelles, or soluble components. Chemical analysis of isolated chromosomes shows 13.5 per cent RNA, 13.5 per cent DNA, and 68.3 per cent protein. The ultraviolet absorption spectrum and metal content of chromosomes are also presented.

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