ISOLATION OF CHROMATIN THREADS FROM THE RESTING NUCLEUS OF LEUKEMIC CELLS*

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Plates 15 to 17

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In the present paper a method is described whereby chromatin threads can be separated from the resting nucleus by mechanical means. The technique is based on the assumption that, if the cell and nuclear membranes are destroyed, the various morphological elements of the cell will segregate in a centrifugal field according to their size and their specific gravity. It has been shown by previous work that the formed elements of cytoplasm can be concentrated and purified in this manner and obtained in quantities adequate for biochemical analysis (1). The chromatin threads which have now been isolated can be stained specifically by the Feulgen technique. Typical arrangement in pairs with similarities in width and beading suggest that these threads may correspond to the chromosome strands as they occur in the nucleus during the resting stage.

The first attempt at isolation of cell nuclei for the purpose of chemical analysis was that of Miescher in 1871 (2). In Miescher's experiments, pus cells were subjected to the action of pepsin by incubation with the enzyme for 24 hours at about 40°C. in acid solutions. The cytoplasm and debris were thus digested away and the free nuclei could be washed and collected on a filter. It was from this and similar material obtained from salmon sperm that Miescher isolated his phosphorus-containing nucleins referred to in the terminology of today as nucleoproteins. Recently, Behrens (3) proposed an elaborate and time-consuming method for the isolation of cell nuclei. In this technique the tissues are desiccated, then ground to a powder, with the aim of reducing the material to microscopic fragments approximately the size of nuclei, and fractionating the fragments in solvents of graded specific gravity. Crossman (4) obtained free nuclei by macerating the tissue in 5 per cent citric acid, and Zittle and O'Dell (5) made use of the method of sonic vibrations for the disintegration of bull sperm. Birds' erythrocytes have been repeatedly used as source material for the preparation of nuclei, for the apparent reason that here, as in the case of pus and sperm, the cells normally are free and their nuclei presumably more easily accessible. Avian erythrocytes were investigated as early as 1869 by Plosz (6). Ackermann (7) prepared nuclei from chicken erythrocytes by laking the cells in distilled water and precipitating the nuclei in 3.6 per cent sodium chloride solution. Warburg (8) used freezing and

* A preliminary account of the separation of chromatin strands from the resting nucleus has been published (17).

thawing for the same purpose and Laskowski (9) has suggested the use of lysolecithin as hemolytic agent. A number of the foregoing techniques involve relatively severe treatment and there is no assurance that the original organization of the nucleus has thereby been preserved.

Materials and Methods

The present work was carried out with leukemic tissues derived from mice and rats, a material that was easily available in the laboratory at the time, but similar material has been obtained from normal tissues, for example, guinea pig or rat liver.

The mouse leukemia known as line I originated from a case of spontaneous lymphatic leukemia in a mouse of strain C58, and has been maintained by consecutive passages in young mice of the same strain. The characteristics of this type of leukemia have been reported elsewhere (10, 11). To obtain tissues for extraction, groups of C58 mice 6 to 8 weeks old were injected with a definite number of line I cells producing a progressive leukemia which killed in almost exactly 6 days. About 2 or 3 hours before death would have occurred, the mice were sacrificed, the spleens were removed aseptically, and stored at $-80°C$. for periods varying from a few days to 7 months.

The average weight of the leukemic spleens was 0.7 gm. as compared with 0.07 gm. for the normal spleen of control mice, microscopic examination indicating that proliferation of leukemic cells was chiefly responsible for the tenfold increase in weight. On section, the spleens of mice dying of line I leukemia are found to be completely infiltrated with leukemic lymphocytes, of which hemocytoblasts are the predominating type. The normal architecture of the spleen is completely destroyed and mitotic divisions are frequent, about 5 to 6 per cent of the cells being in pro-, meta-, or anaphase. The nuclei of non-dividing cells are large in proportion to the amount of surrounding cytoplasm, as in lymphocytes generally. Figs. 1 and 2 represent leukemic cell nuclei obtained by imprint of the cut surface of a leukemic spleen and stained by the Feulgen technique. Figs. 3 and 4 show the same type of cells in mitotic division. The two latter preparations were obtained from the peripheral blood of a leukemic mouse and were stained by the Feulgen and Flemming-crystal violet techniques, respectively.

The rat leukemia developed after the injection of dibenzanthracene into the groin of a Wistar rat (12). The peculiarity of the disease is that it will produce local tumors when the leukemic cells are introduced into the subcutaneous tissue of the groin. These tumors, which may reach a considerable size, are composed chiefly of large lymphoid cells with densely basophilic cytoplasm. The nucleus is large, and about 1 per cent of the cells are found to be in mitosis. In preparation for extraction, the tumors were removed, the tissue was forced through a masher which retained much of the stroma, and the resulting pulp was stored at $-80°C$. for periods of 2 to 7 days.

EXPERIMENTAL

As a rule, cell membranes are relatively fragile and are destroyed by mere contact with dilute salt solutions or water. The nuclear membrane, on the other hand, may resist destruction unless some mechanical force is applied (1).

In preliminary experiments, the tissue was first dispersed in water at pH 7.0, a treatment which set free the cytoplasmic constituents and also the nucleus as such. After repeated washing in salt solution, an attempt was made to disintegrate the nuclei under the revolving blades of a Waring blendor.[1] The results were not satisfactory in that, under the rather violent treatment, the content of the nucleus became spun into long fibers of nucleoprotein, insoluble in saline solution, or else the chromatin strands were broken up into short fragments, and the structure of the nuclear elements was consequently lost. In resorting to grinding with sand, it was found that the amount of grinding could be reduced by increasing the proportion of sand. If enough was added, only gentle grinding was necessary to break the nuclear membrane. It was finally realized that preliminary washing of the mass of nuclei could be avoided since, once free in the medium, the various elements would segregate in the centrifuge according to size, and thus could be separated from the other constituents of the cell. The following procedure, which was worked out along these principles, was adopted as a standard technique.

The frozen tissue was thawed but not allowed to reach a temperature above 5°C., and was placed in a mortar in batches of 20 to 30 gm. with an equal weight of beach sand. The mixture was ground gently for 3 minutes and a volume of solvent equal to 6 times the original weight of the tissue was added progressively. Either distilled water or a 0.9 per cent solution of NaCl was used, the reaction of the medium being carefully maintained at pH 7.4 throughout the experiment. Inspection of the suspension at this stage under ordinary or dark field microscope showed practically no intact cells or nuclei, but a swarm of cytoplasmic particles and granules, together with an abundance of strands of varying lengths and approximately 0.5 to 1.0 μ thick. These strands were seen more clearly under dark field illumination, and under these conditions, appeared to have a banded or beaded structure produced apparently by alternating regions of different refractive power.

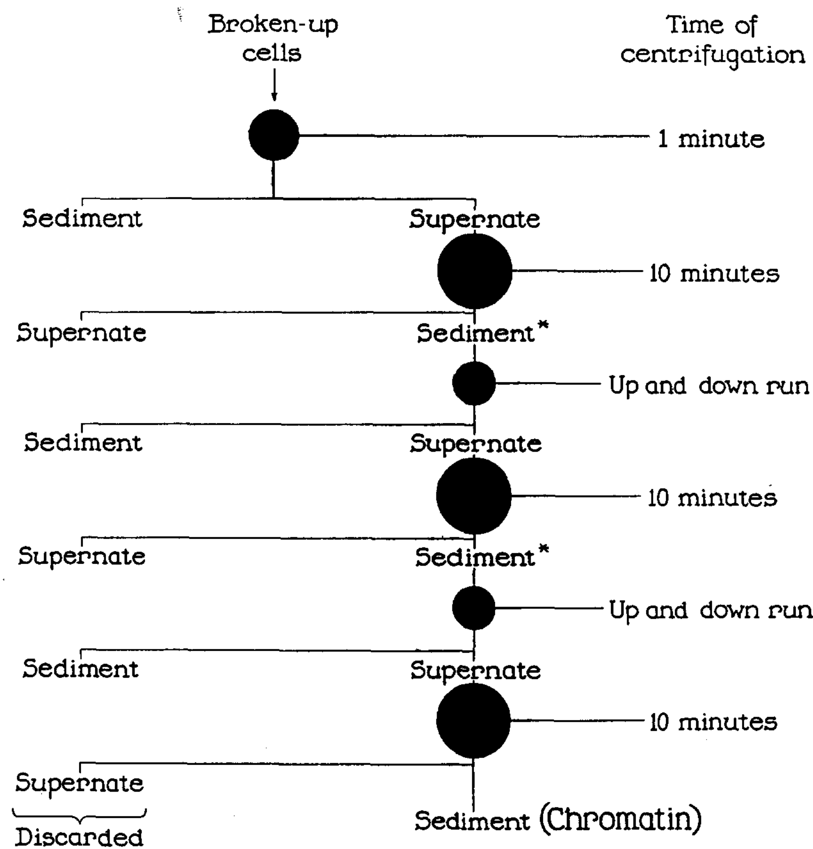
Preliminary Centrifugation.—The tissue suspension prepared as indicated above was centrifuged for 1 minute in an ordinary laboratory centrifuge at 1500 $\times$ gravity.[2] Practically all the sand and tissue debris were removed by this process, the heavy material being covered by a thin white layer composed of cell debris and many strands of the kind described above. The supernate retained all of the granules and most of the thread-like elements.

Differential Centrifugation.—The supernate was then submitted to a "long run" of 10 minutes' centrifugation at 1500 $\times$ gravity. The effect of this centrifugation was to bring down practically all of the thread-like material as an abundant white sediment, while the granules remained in the fluid and were discarded. The centri-

[1] Waring Corporation, New York, N. Y. A container of small size was manufactured at The Rockefeller Institute and fitted with blades of the usual dimensions.

[2] The instrument used throughout was type SB, size 1, centrifuge in conjunction with the horizontal yoke No. 233, International Equipment Company, Boston, Massachusetts.

fugation of 10 minutes though causing sedimentation of the thread-like elements did not make the deposit too compact. The material from 25 gm. tissue was suspended in 35 cc. saline by gentle shaking, and the suspension submitted to an "up-and-down run" in the centrifuge. The purpose of this short run, which consisted



TEXT-FIG. 1

in accelerating the centrifuge to a speed corresponding to 1500 × gravity and then stopping immediately thereafter, was to remove sand and debris which could have been carried over from the first centrifugation. The preparation was again submitted to 10 minutes' centrifugation, resuspension in 35 cc. saline, and a short run, followed by a final run of 10 minutes. For reasons that will be discussed later, the sediment thus obtained will be referred to as "chromatin threads." A summary of the technique just described is given in Text-fig. 1. It can be seen that the entire process of separation and purification of chromatin was completed in about 1 hour. The fact

should be stressed that the manipulations, including extraction and centrifugation, were carried out in a cold room and the temperature of the material was kept within the range of 0–5°C. during the whole experiment. Rapid separation and low temperature are conditions essential in avoiding the spontaneous changes which may affect the chemical composition or shape of the chromatin elements.

Morphology of the Chromatin Threads

The chromatin mass was perfectly white in color. Under the microscope, it was found to be composed almost exclusively of slender filaments of approximately equal width, ranging from 0.5 to 1.0 μ in diameter, and very similar to those encountered in the original extract. On standing, especially in very dilute salt solution, these threads disintegrated progressively and seemed to be replaced by free refractile granules of about the same width. In distilled water, the filaments swelled rapidly and finally disappeared altogether. The morphology of the freshly prepared chromatin threads is illustrated in Figs. 5 to 7. For these photographs, the chromatin threads were spread on a slide, dried, and subsequently stained by the technique of Feulgen (13). All the figures, except Fig. 5, are at the same magnification of 1600 × diameter and they are therefore directly comparable. Fig. 5 is a low power view (500 ×) of the chromatin threads which are illustrated in Fig. 6 at the magnification of × 1600 as just mentioned.

The stained chromatin strands are strikingly similar as regards size and shape, to those seen in the original tissue extract. Some of the strands seem to have been broken at different lengths, while others appear to have been considerably stretched, perhaps at the time of the smear. Not infrequently, the threads were grouped in pairs, a feature which is also characteristic of prophase chromosomes (11). Fig. 7 is of especial interest in that the staining revealed a finer structure characterized by deeply stained granules arranged in succession along the original chromatin thread. To get this effect, the dried smear of chromatin was washed with chloroform prior to staining with the standard technique of Feulgen. In appearance, the beaded strands shown in Fig. 7 have a striking similarity to the filaments seen by Chambers in the living nucleus (14), and those which are often detected in the nuclei of plant cells (15).

Comparative study of the isolated chromatin threads with stained nuclei of the sort from which they were obtained is highly suggestive of a relationship to preformed nuclear structures. A close inspection of the nuclei represented in Fig. 1 shows, especially in the best preserved specimen, a number of delicate strands crossing the nuclear framework which, in their appearance, are not unlike the beaded filaments seen in Fig. 7. It is impossible to judge the length of the chromatin threads in the resting nucleus, but no loose ends are seen in the chromatin strands of nuclei of Fig. 2, which may imply the existence of a continuous filament or end-to-end arrangement of individual segments of chromatin. On the other hand, a fair agreement exists as regards the width of the

chromatin threads. As seen in Figs. 1, 2, 3, 6, and 7, the width of the chromatin thread whether within the framework of the resting nucleus, in the isolated state, or in the form of a metaphase chromosome, is of the same order of magnitude. The metaphase chromosomes of Fig. 4 are thicker and more diffuse and have undoubtedly been swollen by the action of the different staining technique.

Chemical Composition of the Chromatin Threads

It may be recalled that the Feulgen reaction is a test for desoxyribose and that, under the experimental conditions established by Feulgen and his coworkers, it is specific for thymonucleic acid (13). By means of the reaction, it has already been demonstrated that thymonucleic acid is exclusively a nuclear constituent, which appears to be entirely segregated in the chromosomes during mitosis. A positive Feulgen reaction given by both the chromosomes and the chromatin threads obtained in the course of the present work indicates that these structures are not only morphologically alike, but that they are also related chemically, the test demonstrating the presence of thymonucleic acid in both groups of structures.

For direct chemical analysis, the washed chromatin threads were used as such or after dialysis for varying lengths of time at about 4°C. The samples were then desiccated *in vacuo* and in the frozen state. The dry chromatin so obtained was a snow-white, coherent, and glistening material which, to the naked eye, appeared to have a fibrous texture. In terms of dry weight, the chromatin represented 4.5 to 8 per cent of the original leukemic cells.

Extraction of the dry chromatin with alcohol, chloroform, and ether separated a lipoid fraction soluble in chloroform but insoluble in acetone, which amounted to 2.3 per cent of the thread substance. It is not known whether this lipoid fraction is an integral part of chromatin or whether it represents a contaminant from cytoplasm. In view of the high proportion of lipids present in the cytoplasmic constituents which are known to contain as much as 20 to 50 per cent lipids (1), such a contamination would appear possible.

The results of elementary chemical analysis are summarized in Table I. Calculated from these data, the average values for the chemical composition of chromatin threads are as follows: 15.58 per cent nitrogen, 3.72 per cent phosphorus, 45.60 per cent carbon, 6.49 per cent hydrogen, and 1.70 per cent sulfur. Strongly positive Feulgen (13) and Thomas (16) tests, both specific for thymonucleic acid, together with a high nitrogen and phosphorus content indicate that a considerable part of the chromatin thread is represented by desoxyribosenucleoprotein. Thymonucleic acid itself would seem to constitute as much as 40 per cent of the whole chromatin complex, if it is assumed that all of the phosphorus is in the form of nucleic acid of that type. As concerns the protein radical, a positive reaction with the Millon's reagent would suggest that at least a part of the complex belongs to the group of histones.

Miescher (2) found 1.8 and 1.7 per cent sulfur in pus cell nuclei and in the heads of bull sperm, respectively. The latter value was confirmed recently by Zittle (5). These figures agree with our finding of approximately 1.7 per cent sulfur in the chromatin of leukemic cells. The chlorine present must have been derived mostly from saline used in the preparation of the threads since the values varied considerably, depending on the extent of dialysis or of washing in distilled water.

It can be seen from the data presented in Table I, Experiment 9, that when whole nuclei were present in the preparation to such an extent that they represented about one-third of the chromatin material the values obtained on chemical analysis were lower. The differences were so slight as to suggest that most of the nuclear substance is represented by the chromatin structures.

The observations clearly indicate that the chromatin threads contain large amounts of desoxyribosenucleoproteins. Our analysis of the chromatin

TABLE I

Chemical Composition of Chromatin

Source of material	Experiment No.	Chromatin	N	P	C	H	S	Cl (from saline)
			per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Rat leukemia	4	Complete	15.30	3.79				
	4	Lipoid-free	15.62	3.83	43.88	6.61	1.62	
Mouse leukemia	7	Complete	15.79	3.70	46.00	6.41		0.52
	8	Complete	15.59	3.56	46.94	6.46	1.78	0.08
	9	Nuclei present	14.74	3.16	45.85	6.28	1.62	0.13

material has not progressed much beyond this point, and at the moment little is known regarding the existence and the possible nature of other chemical components.

DISCUSSION

One of the most important problems in cell study is the relation between structure, chemical composition, and function. Research along these lines has been remarkably successful but has been carried out more or less independently, so that we may have gained an accurate knowledge of the morphology of certain cell components while remaining unaware of their chemical constitution; or we may have succeeded in isolating a chemical substance, yet be uncertain as to its place in the architecture of the cell. The attempts to study function and organization together have not on the whole been concerted or systematic.

In recent years, several of the morphological constituents of cytoplasm have been isolated in this laboratory and they are now available for biological and chemical analysis (1, 17). The present investigation constitutes the first attempt to isolate chromatin structures from the living nucleus, and there is no

doubt that the method will lend itself to improvements, especially in the selection of better solvents. From the data presented in the paper, it is suggested that the strands of chromatin which have been isolated have their origin in the resting nucleus and represent either interkinetic chromosomes or fragments of chromatin filaments derived from the chromosomes of the preceding mitotic division. The separation of chromatin filaments from living nuclei indicates that similar elements are present in the nucleus, even if they may not be visible in the living state. In fixed and stained preparations, furthermore, it can be seen that the basophilic framework of the leukemic nucleus is constituted of slender filaments of chromatin (Fig. 1) morphologically similar to the free chromatin strands obtained by mechanical separation (Fig. 7). Apparently the intranuclear filaments of chromatin are readily destroyed when the cell is submitted to the usual means of fixation, and the rearrangement of chromatin within the nucleus gives rise to an alveolar appearance or to the concentration of the substance into chromatin blocks. Such artefacts are illustrated in certain nuclei of Fig. 1.

That the chromatin strands do not represent dividing chromosomes but have their origin in the so called resting nucleus seems certain from the fact that, in the leukemic tumors of the rat, only 0.7 to 1.3 per cent of the cells were found in mitosis, whereas the yield of chromatin was as much as 8 per cent of the dry weight of the tumor. Nevertheless, a striking resemblance obtains between the morphology of the strands of chromatin and that of the metaphase chromosomes which are depicted in Fig. 3. The fact that some of the chromatin strands appear more elongated than the dividing chromosomes could be explained by mechanical stretching or uncoiling during purification. It is possible that, during the resting stage, the chromosomes arrange themselves end to end, forming a long and continuous filament of chromatin in the nucleus (spireme thread). Whatever the mechanism, the fact that chromosome-like structures can be isolated from the resting nucleus supplies direct evidence of a structural continuity between the telophase chromosomes and the prophase chromosomes of the following cell division.

An important similarity between chromosomes, as seen in stained preparations, and the free chromatin strands is also found in their chemical constitution. Both contain large amounts of desoxyribose nucleic acid, a substance highly characteristic of chromosomes. The phosphorus content of the chromatin thread suggests that as much as 40 per cent of the whole structure may be represented by thymonucleic acid. The occurrence of nucleoproteins in preponderant amount in the nucleus was undoubtedly responsible for the discovery of these organic substances and for the early elucidation of their chemical nature (2). Yet the very abundance of nucleoproteins in chromatin can easily overshadow the presence of other chemical constituents which may be expected to exist if the physiological complexity of the chromosome is to be accounted

for. Search for other chemical constituents, possibly present only in minute amounts, may be facilitated by the fact that unlimited quantities of chromatin can be made available now by the use of the new technique. Of practical importance in this respect is the fact that the material can be isolated by mechanical means exclusively, and in a relatively short time, thus reducing the liability of changes that these elements may undergo spontaneously.

SUMMARY

1. A method for the separation of chromatin threads from the resting nucleus of leukemic cells has been described.

2. The isolation of the chromatin strands was accomplished by purely mechanical means, and the separation, including extraction and purification in the centrifuge, required only about 1 hour.

3. Evidence is presented to show that the chromatin strands are morphologically related to the chromosomes, if not identical.

4. It seems probable that, by the present technique, chromatin strands can be obtained from different mammalian tissues for direct microscopical examination and biochemical analysis.

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EXPLANATION OF PLATES

The material for the following photographs was derived from instances of mouse leukemia, line I. All the preparations were stained by the Feulgen technique, except Fig. 4 which was treated with Flemming's fixative and stained with crystal violet. All photographs were made at the same magnification of 1600, except Fig. 5 whose magnification was 500.

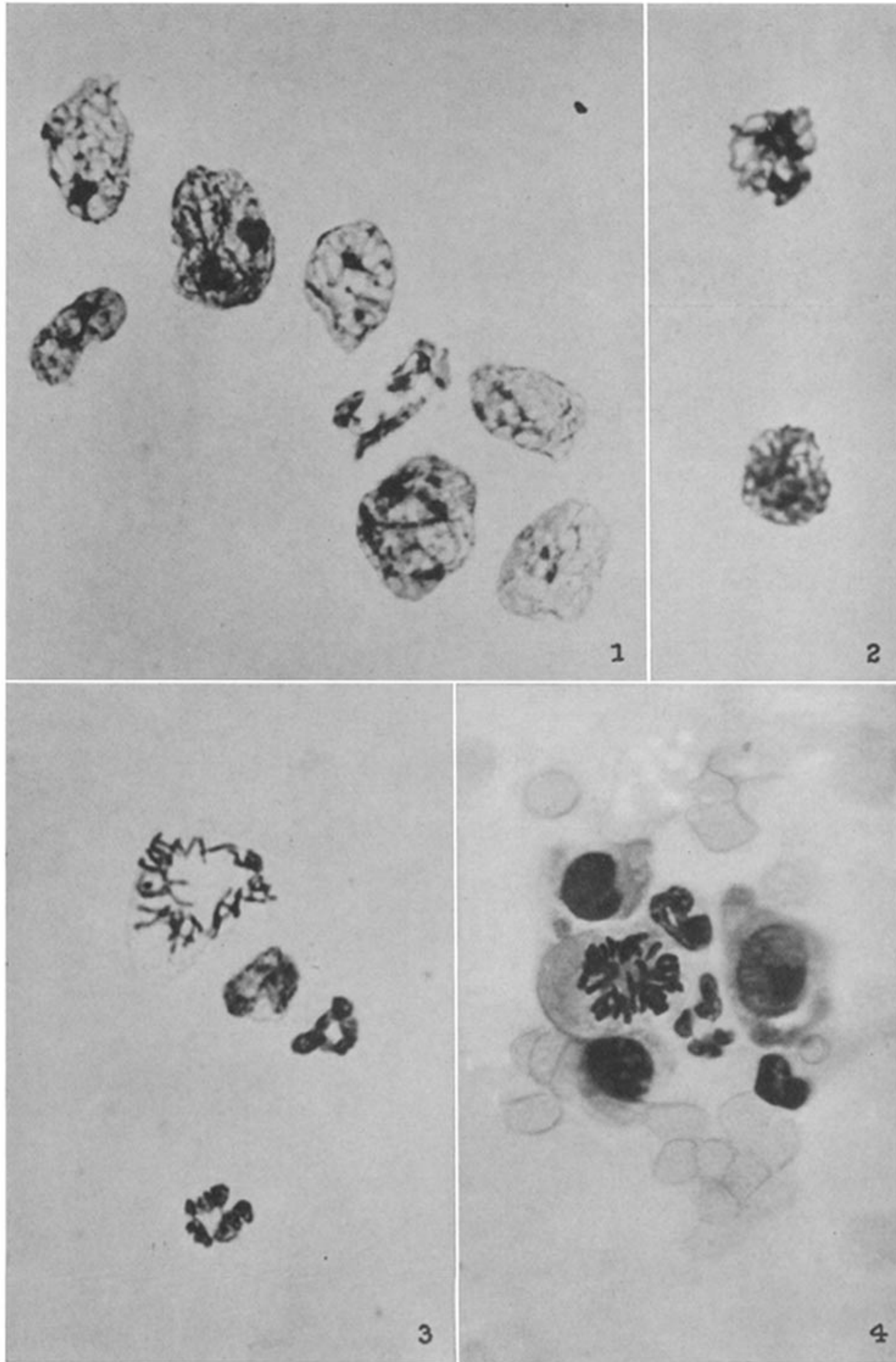
PLATE 15

FIG. 1. Imprint from the cut surface of a leukemic spleen. Nuclei of leukemic cells showing chromatin structures during the resting stage.

FIG. 2. Smear of peripheral blood. Nuclei of leukemic cells showing ribbon-like arrangement of chromatin.

FIG. 3. Smear of peripheral blood. Metaphase chromosomes of dividing leukemic cells.

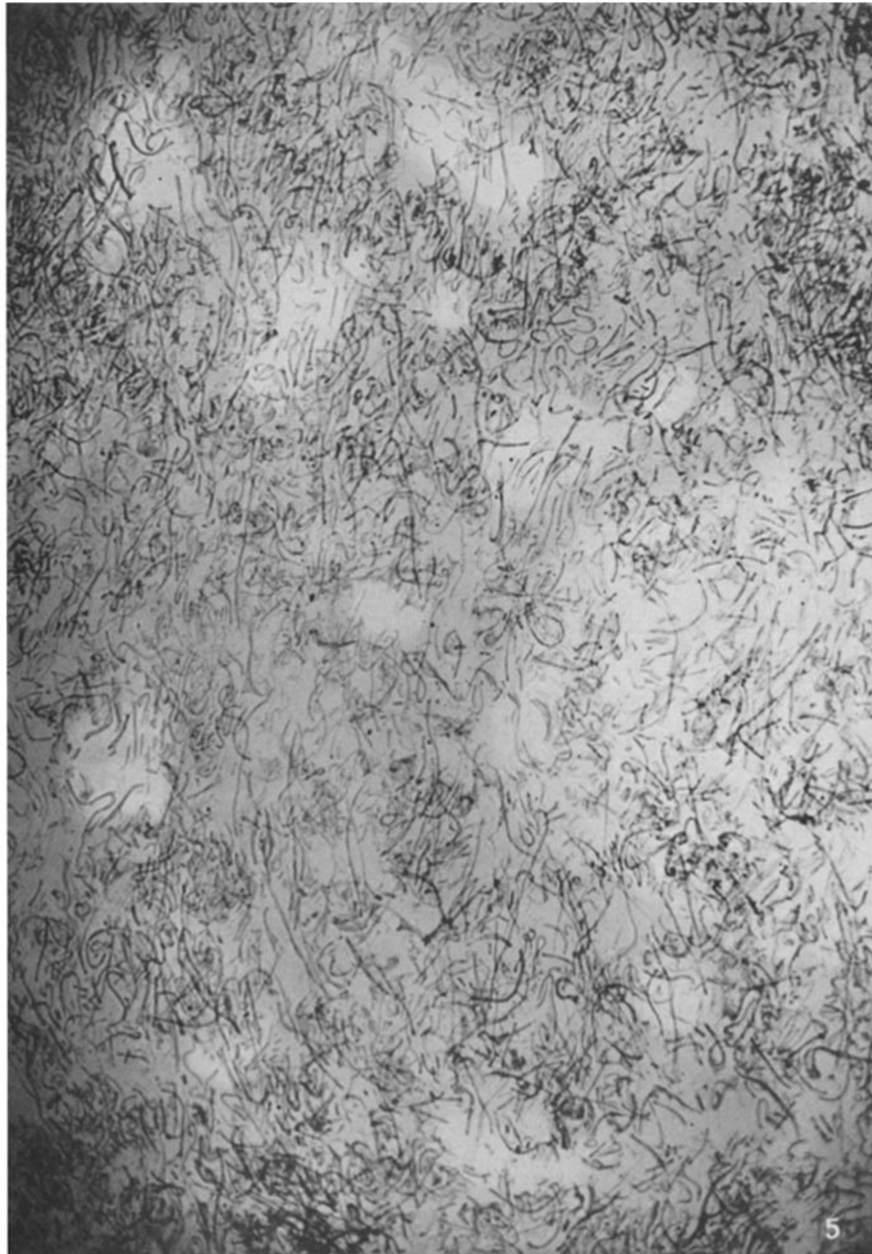
FIG. 4. Same as Fig. 3. The chromosomes appear swollen, an effect probably due to the difference in the staining technique.



(Claude and Potter: Isolation of chromatin threads)

PLATE 16

FIG. 5. Smear of chromatin threads isolated by the technique described in the paper.

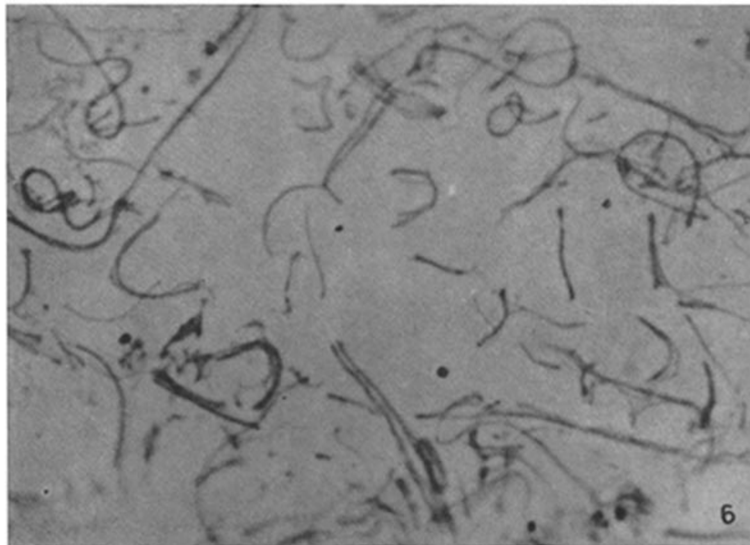


(Claude and Potter: Isolation of chromatin threads)

Plate 17

Fig. 6. Same as Fig. 5, but at a higher magnification. The photograph shows paired strands of chromatin.

Fig. 7. Smear of chromatin threads. The dried film was washed in chloroform before staining with the Feulgen technique. The strands of chromatin have taken on a beaded appearance; several of the chromatin threads are paired.



(Claude and Potter: Isolation of chromatin threads)