THE ULTRASTRUCTURE OF NUCLEOLI AND CHROMOSOMES DURING THE EARLY STAGES OF LIVER REGENERATION AND THE CHANGES PRODUCED IN THESE STRUCTURES BY X RADIATION

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A method of tissue preparation for electron microscopy is described which allows the observation of more nuclear structure than has previously been reported. Regenerating rat-liver cells were used for this study.

During the early stages of regeneration the nucleolar size was found to be much greater than normal, although nucleolonema fibres and pars amorpha were still present. At 16 to 18 h after operation, however, when there is considerable production of new α-cytomembranes in the tissue, much of the material of the pars amorpha was lost from many cells, and the nucleoli consisted solely of nucleolonema fibres. Later the pars amorpha was re-formed, but just before the start of mitosis at 25 to 26 h after operation, many of the nucleoli became very irregularly shaped. The various mitotic stages seen in liver cells with the electron microscope were recognizably similar to those previously seen in living mammalian cells with the light microscope. In the earliest stages of prophase the chromosomes appeared as strands approximately 1000 Å in diameter, which were made up of thin fibres approximately 100 Å in diameter. By mid-prophase these strands could be seen running diagonally across the chromosomes in a manner suggestive of coiling, but by late prophase this coiling was no longer visible and the chromosomes appeared as homogeneous masses of fibres 100 Å in diameter. The double nature of the chromosomes was, however, evident during metaphase. Telophase nuclei were seen to contain fibrous inclusions approximately 1000 Å in diameter which it is suggested may represent stages in the re-formation of the nucleolus after mitosis.

The effect of X radiation on the ultrastructure of the nuclear components of regenerating liver cells was also studied in these experiments. With small doses (450 r) no ultrastructural changes were found in the chromosomes during cell division, but some changes were visible in the nucleoli just prior to mitosis. Larger doses (2000 r) caused chromosome fragmentation and a loosening of the fibrous chromosome structure. In telophase it was found that when the separation of the two daughter nuclei is inhibited or delayed, the nuclear membrane re-forms around the two partially separated chromosome masses.

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Introduction

Early electron-microscope work on the cell nucleus proved disappointing, as most nuclei appeared to have a homogeneous granular structure. It was originally suggested that osmium fixation was causing a complete dispersal of chromosome structures. However, Fell & Hughes (1949) were able to show by phase contrast examination of individual cells, both in the living state and after osmium fixation, that the general nuclear picture was not noticeably changed by this fixative. Later Gibbons & Bradfield (1956) were able to show that thick methacrylate sections of locust spermatids fixed with osmium and examined with ultra-violet light gave similar nuclear pictures to the living cells. Thin sections of this material, however, showed no chromatin structure when examined in the electron microscope, and the authors concluded that although the chromosome structure was not grossly changed during fixation, the contrast obtainable in the electron microscope was too low to allow them to stand out against the other nuclear material. This conclusion was also reached by Moses in the same year who stained thick sections of osmium-fixed crayfish spermatids by the Feulgen method. These sections showed the *DNA* distribution was not changed during fixation, even though chromosome structure could not be seen in thin sections examined with the electron microscope.

In spite of these difficulties many workers have produced electron microscopical evidence for the structure of various types of chromosomes. Some of the earliest work was done with chromosome material isolated from the interphase nuclei of various types of blood cells by Hovanitz (1947), Yazuzumi (1951), Yazuzumi, Miyao, Yamamoto & Yokoyama (1951) and Yazuzumi & Yamamoto (1953). Resolution in the earlier papers was poor but Yazuzumi (1955) showed that each 'metabolic chromosome' consisted of as many as thirty-two chromofilaments approximately 100 Å in diameter.

Other popular objects for the study of chromosome ultrastructure have been the giant chromosomes of some insect salivary glands. These were first studied with the electron microscope by Beermann & Bahr (1954), who claimed that the chromosomes were made up of threads 0·2 μm in diameter which in turn contained fine fibres 100 Å thick. In the same year Ambrose, Cuckow & Gopal-Ayengar (1954) saw longitudinal fibres 100 to 200 Å in diameter in shadowed material. Gay (1956) claimed that the longitudinal fibres were 200 to 500 Å in diameter, but Lowman (1956) produced pictures from which he concluded that the salivary gland chromosomes were made up of nucleoprotein granules 200 to 300 Å in diameter connected by protein fibres 100 Å in diameter.

Ris (1954) using shadowed 'lampbrush' chromosomes from *Triturus* oocytes found that they contained longitudinal fibres 500 Å in diameter. He found that similar fibres existed in the meiotic prophase chromosomes of other vertebrates, but claimed the diameter of chromosome fibres in interphase was only 250 Å. Evidence for this doubling of the size of chromosome fibres before mitosis has been extended in two later papers by Ris (1956) and Lafontaine & Ris (1958). Chromosome structures of a different nature have been seen in the meiotic prophase nuclei of several species. These have been termed the 'chromosome axial bodies' or 'axial complexes'. Fawcett (1956) claimed that the chromosomes had a central structure consisting of a pair of dense parallel fibres 450 Å in diameter between which was a thin medial fibre 120 Å in diameter. He suggested that

the chromosomes arose by the 'congregation and organization' of the chromatin granules around these fibres. These observations were confirmed by Sotelo & Trugillo-Cenoz (1959) and Moses (1958). The latter author produced evidence to suggest that the two thicker fibres were coiled around one another.

The presence of microfibrils in plant chromosomes was confirmed by Kaufmann & De (1956). These workers claimed that each chromosome contained as many as sixty-four identifiable strands, arranged in intertwined pairs to form a hierarchy of pairs. They gave the diameter of the smallest discernible fibre units as 125 Å, but suggested each consisted of a peripheral region containing highly electron-scattering material disposed around a less dense core. Marquardt, Liese & Hassenkamp (1956) and Shigenaga (1957) also found microfibrils in plant chromosomes, but suggested that their diameter was only 100 Å. The latter worker also demonstrated the presence of two chromatids in each chromosome.

There has been little work done on the structure of mammalian mitotic chromosomes, due, no doubt, to the difficulty of obtaining normal material with a high mitotic rate, but the evidence that has been produced suggests that they, like other types of chromosomes that have been studied, contain microfibrils of the order of 100 Å in diameter. Porter (1954) suggested that the metaphase chromosomes of rat sarcoma cells were condensations of fine filaments 80 Å in diameter, and Wessel & Bernhard (1957) produced photographs showing similar fibres in Ehrlich and Yoshida ascites tumour cells. Dales (1960) also found very fine fibres in mammalian somatic chromosomes. He suggested, however, that the thickness of the fibres varied with the fixative used, and gave figures of 50 to 100 Å for formalin, 50 to 200 Å for osmium and 100 to 400 Å for Carnoy's fluid.

The suggestion of Estable & Sotelo (1954) that the interphase nucleolus was composed of a fibrous component, the nucleolonema, which was surrounded by amorphous material called the 'pars amorpha' has been well confirmed by the electron microscopical studies of many workers including Bernhard, Haguenau & Oberling (1952), Haguenau & Bernhard (1955), Horstmann & Knoop (1957) and Yazuzumi *et al.* (1958). There is, however, some difference of opinion on the ultrastructural elements of the nucleolonema and pars amorpha. Thus Bernhard, Haguenau and Oberling suggested that both these nucleolar structures were made up of granules approximately 100 Å in diameter while Horstmann & Knoop and Yazuzumi *et al.* considered that the nucleolonema fibres at least consisted of fine coiled fibres 30 to 50 Å in diameter. The suggestion of Estable & Sotelo (1954) that the nucleolonema fibres pass through the mitotic process in contact with the chromosomes has not yet received much support from electron microscopical studies, although Sotelo (1959) himself showed that whereas the early prophase nucleoli of mouse spermatogonia contained nucleolonema fibres, these fibres could not be demonstrated at any later stage of mitosis. The only electron microscopical reports on the re-formation of the nucleolus after mitosis were published by Lafontaine (1958). This worker claimed that after mitosis in *Allium* nucleolar material was first visible as granules between the arms of the late anaphase chromosomes. In telophase these granules agglomerated further to form small dense areas. In *Vicia* nucleolar material was not visible until the nuclear membrane had re-formed in telophase. At this stage it appeared as a number of dense areas which varied greatly in size.

The nuclear membrane has been studied with the electron microscope by many workers

including Bahr & Beerman (1954), Afzelius (1955), Beams, Tahmisian, Devine & Anderson (1957), Barnes & Davis (1959) and Watson (1959). Its structure has been found to be remarkably uniform in many tissues of both plants and animals. Recently two papers have been published on the re-formation of this membrane after mitosis. Porter & Machado (1960) suggested that in onion root-tip cells the nuclear membrane was formed from the previously existing double-membraned elements of the endoplasmic reticulum (alpha cytomembranes) which became arranged around the daughter nuclei. Jones (1960), on the other hand, suggested that in foetal rat-liver haemoblasts the nuclear membrane was formed *de novo* after each mitosis in close apposition to the chromosome masses.

In the present study the use of 'Araldite' embedding coupled with phosphotungstic acid (*PTA*) staining has to some extent overcome the problem of nuclear contrast, and it has been possible to follow chromosome and nucleolar structure throughout mitosis. In a study by electron microscope of the mitotic process it is obviously important to correlate observations with the changes that are known to occur in the living cell. So far observations of mitosis have not been made on living liver cells, but Fell & Hughes (1949) published the results of mitotic studies on several other types of mammalian cells grown in tissue culture. In the present paper the results of Fell & Hughes have been taken as a control for the electron microscope results, and comparisons made between electron microscope pictures and the corresponding structures in the living cells.

As it had proved possible to examine the ultrastructure of nucleoli and chromosomes in regenerating rat liver in some detail it was decided to see if ultrastructural modifications occurred in these bodies after doses of X radiation. The cytoplasmic changes caused by X radiation in regenerating liver cells have already been described separately (Davis 1962*b*). The fact that X radiation produces not only mitotic inhibition but also causes chromosome abnormalities in the mitotic figures is well known, and the nucleolus would appear equally as sensitive to ionizing radiations as the chromosomes. A very large amount of work has been done with both light microscope and chemical techniques in attempts to elucidate the processes involved, but so far little work has been published on electron microscopical studies of nuclear structural changes caused by ionizing radiations. This is probably because the results of electron microscopical studies on normal chromosome structure have been somewhat disappointing. Nebel (1959) studied the effect of a dose of 1000r on mouse testicular cells. Many of the cells died, but those that survived did not show any ultrastructural changes from normal. Nebel did, however, claim that many nuclear structures showed increased electron density after irradiation, and suggested that the heterochromatic proximal regions of the meiotic metaphase chromosomes were larger and more prominent. Lacey & Rotblat (1960) also examined mouse spermatic nuclei. They used a dose of 10000r and examined the cells 3 days after irradiation. In normal spermatid nuclei they claimed to see two main components, a nuclear matrix and chromosomes. The chromosomes consisted of a chromosomal matrix of low electron density and microfibrils. It was suggested that these microfibrils were about 25Å thick and coiled, with a gyre width of about 100Å. After irradiation they found that the number of coiled microfibrils of normal size and appearance were reduced, but that some thicker microfibrils were present which measured as much as 200Å in diameter.

Materials and methods

Adult male white Wistar rats were partially hepatectomized under ether anaesthetic by the method of Higgins & Anderson (1931) and the animals used in the study of normal nucleolar and chromosome structure were killed at hourly intervals after operation. In the radiation experiments the animals were irradiated at either 6, 12, 16, 24 or 28 h after operation and killed at hourly intervals after this. Half an hour before irradiation the rats were injected subcutaneously with a dose of Amytal 70 mg/kg. A Maximar 220 kV standard treatment machine was used for irradiation, and the dose rate was 150 r/min. The animals were killed at various intervals after irradiation, and portions of the liver were fixed in 1 % buffered osmium solution as suggested by Palade (1952). In order to increase contrast in the specimens, phosphotungstic acid (*PTA*) was used as an additional electron stain, and the tissue was soaked in a saturated solution of *PTA* in absolute ethanol for 2 h before embedding. The tissue was embedded in 'Araldite' by the method described by Davis (1959). Sections were cut on a Huxley ultramicrotome and examined with a Metropolitan Vickers E.M. 6 electron microscope using an accelerating voltage of 75 kV.

In order to check on the different mitotic stages seen in the electron microscope some material from each of the animals used in this study was fixed in formol saline and stained with haematoxylin and eosin for light-microscope examination. It was found that the various mitotic stages were recognizably similar in both types of preparation but in order to be sure of the possibly controversial stages of prophase and telophase, the following precautions were adopted. Nuclei were only considered in prophase if the nuclear diameter was well above the average of the interphase nuclei, and were only considered to be in telophase if both daughter nuclei were included in the section, and the division of the cytoplasm was incomplete.

Observations

The nucleoli found in rat-liver cells during the early hours of liver regeneration are, in general, larger than those found in normal liver cells, but their structure still conforms exactly with that described by other workers in a variety of animal tissues. They contain dense fibrous elements, the nucleolonema fibres, which are surrounded by areas of less well-organized material, the pars amorpha. During the first 15 h of regeneration at least, the nucleoli are roughly spherical in shape and during this time it is common to find several nucleoli within each nucleus.

The ultrastructure of both nucleolar components has been examined in a large number of high-magnification pictures. In both, the structural units present a variety of patterns. Many of them appear as granules, but some present an elongated form, being as much as ten or fifteen times as long as they are wide, and most of them are four or five times as long as their width. This is the sort of picture one would expect from thin sections of intermingled fibres, and it is therefore suggested that the nucleolus is made up of fibrous components. The fibrous ultrastructure of the nucleolonema fibres is usually less distinct than that of the pars amorpha because the nucleolonema stains very densely with *PTA*. In both cases, however, the fibres would appear to be about 100 Å in diameter (figures 1 and 4, plate 15). Both the nucleolonema fibres and the pars amorpha, when this is visible, appear to retain the same ultrastructure throughout the whole period between operation

and the first mitosis, regardless of the changes in the shape and the density of the nucleolus that occur during this time.

The nucleolar structure remains constant for the first few hours of regeneration, but at about 16 h some of the nucleoli become much more diffuse. In these the nucleolonema fibres still remain distinct, although they are no longer so closely packed, but the pars amorpha is either lost or no longer contrasts with the surrounding nuclear material (figure 2, plate 16). It is of great interest that this diffuse type of nucleolus only occurs in cells that are actively producing new α-cytomembranes. The nucleoli remain distinct in those cells which retain a vacuolated cytoplasm.

By the time glycogen production starts at about 22 h after partial hepatectomy, the nucleoli are once more distinct. From this time until mitosis, however, although many of the nucleoli remain roughly spherical, it is not uncommon to find some with very irregular shapes. An example is shown in figure 3, plate 16. These irregular nucleoli are usually very large and normally no other nucleolus can be seen in the cell. This may be because they are produced by the fusion of smaller nucleoli. In figure 3 it is easy to imagine that the large irregular nucleolus has been formed by the partial fusion of three smaller ones.

In the earliest discernible stage of prophase (figure 5, plate 17) there is an increase in the number of nuclear bodies that stain densely with phosphotungstic acid. These probably represent the heterochromatic granules of Fell & Hughes. High magnification studies of the early prophase chromosome bodies show that many of them assume an elongated form (figure 5) and that they are made up of fine fibres approximately 100 Å in diameter. As prophase continues the nucleus enlarges and the dense 'chromosome' bodies become both more numerous and more prominent (figure 6, plate 18). At this stage these bodies can often be seen to contain very dense fibrous elements approximately 1000 Å in diameter which appear similar in structure to the nucleolonema fibres still present in the nucleolus. At first these dense fibres do not appear to have any particular orientation within the chromosome bodies. Later, however, when these bodies have elongated to the stage when they can definitely be called prophase chromosomes, it is possible in fortuitous longitudinal sections to see the fibrous units arranged transversely in a manner suggestive of coiling (figure 7, plate 17). In late prophase coiling again becomes obvious but in this case the coiling is of greater order involving the whole chromosome as distinct from the fibrous units that make up the chromosome. These units become progressively less clear as prophase continues and they cannot be seen at all in metaphase. Figure 9, plate 19, shows a stage after the nuclear membrane has disintegrated and the nucleolus disappeared. Chromosome coiling is clearly visible as the chromosomes shorten in preparation for metaphase.

In this study it has not been possible to demonstrate clearly chromosome pairing or the existence of two chromatids within each chromosome at any time during prophase. When the chromosomes become arranged on the metaphase plate, however, their double nature can often be seen. Even so, the line of demarcation between the individual units is by no means clear (figures 10, 11, plate 20). It would seem that the ultrastructural elements of the two chromatids remain very closely apposed until they separate in anaphase. Both metaphase chromosomes and early anaphase chromosomes (figure 12, plate 21) appear to have the same ultrastructure. There is no sign of the dense 1000 Å fibrous units of prophase,

and the chromosomes appear to consist of an intermingled mass of fibres approximately 100 Å in diameter. These fibres stain densely with *PTA* and consequently the chromosomes stand out clearly against the background material of the nucleus. In later anaphase the chromosomes begin to fuse until each daughter nucleus consists of an elongated mass of chromosome material in which the individual chromosomes can no longer be distinguished. This mass eventually assumes an ovoid form in early telophase.

As the early telophase mass enlarges, spaces appear among the chromosome material eventually separating it once again into individual units. At the same time the nuclear membrane is re-formed. This re-formation is shown in figure 13, plate 21. It can be seen that the double nature of this structure is evident from its first formation and also that it does not form evenly around the whole chromosome mass. In figure 13 one side of the nucleus shows a clear double membrane while at the other side there is as yet no boundary between the nuclear material and the cytoplasm. It is, of course, possible that in any one picture the lack of a membrane on one side of the nucleus could be due to its running diagonally across the plane of the section. In these studies, however, an incomplete membrane was found frequently in very early telophase, but only rarely in other mitotic stages. During early telophase the nuclear material is still too closely packed for the separate units to show any regularity of shape, but by mid-telophase some areas are clearly organized into elongated bodies not unlike the chromosomes of the earlier mitotic stages. Other areas, however, show no regularity of shape. Figure 14, plate 22, shows a mid-telophase nucleus containing both elongated and irregular chromosome masses. The ultrastructure of the chromosome material at this stage still appears to consist of interwoven fibres approximately 100 Å in diameter which stain densely with *PTA*.

In mid-telophase, however, a new series of structures can be seen that occur at no other time during mitosis. These structures are small areas of material usually completely embedded within the chromosome masses, although there is a distinct boundary between them and the chromosome material. These areas are often spherical although some elongated ones have been seen. Their diameter is fairly constant, being 1000 to 1500 Å and the material of which they consist stains quite densely with *PTA*. This material would appear to have a fibrous ultrastructure, but the fibres are far less distinct than those of the surrounding chromosome masses. These areas can be seen in figure 14 and they are shown at higher magnification in figure 15, plate 23.

As telophase progresses the chromosome material gradually disappears until only three or four elongated regions remain. These structures probably correspond to the diffuse system of irregular lumps and strands that was described at this stage of cell division by Fell & Hughes, and from which these authors suggest the nucleolus is eventually formed. They contain elongated areas of material about 1000 Å in diameter that stain only faintly with *PTA*, but which are outlined by material which stains densely. As shown in figure 19, plate 24, these areas usually run lengthwise along the 'prenucleolar bodies'. They would appear distinct from the areas just described in the telophase chromosomes as they are not clearly delineated from the surrounding material but simply outlined by this.

The disappearance of the nucleolus in prophase and its re-formation in telophase

It can be seen in figure 6 that the nucleolus retains its normal structure during early prophase, but by the end of prophase (figure 8) the nucleolonema fibres have disappeared and the nucleolus remains only as a spherical mass of fibres that probably corresponds to the original pars amorpha. This remaining nucleolar material is lost at about the time the nuclear membrane disintegrates. The reformation of the nucleolus in telophase is complex and appears to involve several stages. The first signs of new nucleolar-like material are found in mid-telophase when densely staining fibrous units similar in structure to the nucleolonema fibres can be seen among the chromosome masses (figure 16, plate 23). These units have a diameter of approximately 1000Å and would appear to have a fibrous ultrastructure. In some cases the ultrastructural fibres would appear to be about the same thickness as those of the surrounding chromosome material, that is to say, about 100Å (figure 17, plate 24). In others they are much thinner and can be no more than 50Å in diameter (figure 18, plate 24).

There is one difficulty in assuming that these bodies are the reforming nucleolonema fibres. This is that they can no longer be seen in many of the slightly later telophase nuclei that contain the 'irregular lumps and strands' of Fell & Hughes. These authors have shown that the final interphase nucleoli are formed from this material, but although it contains fibrous units with similar structure to the nucleolonema fibres, these units do not stain with *PTA* while true nucleolonema fibres stain densely. A few of the prenucleolar bodies do show areas containing dense fibrous units but it is impossible to determine whether these units are condensing to form the prenucleolar body, or are themselves being formed from this body. This matter is discussed later.

In the experiments designed to study the effect of X radiation on cell nuclei the same radiation dose (450 r) was used as in earlier experiments on the effects of radiation on the cytoplasmic structures of regenerating liver cells (Davis 1962*b*). It was found that if the animal was irradiated at either 12 or 16 h after partial hepatectomy the nucleoli retained their sense compact nature in all cells and showed no structural changes at about 16 h after operation. It is at this time in normal regenerating liver that a more diffuse type of nucleolus is found in cells that are producing new α-cytomembranes. Thus, it would appear that a dose of 450r inhibits not only α-cytomembrane production (Davis 1962*b*) but also the concomitant nucleolar changes.

When irradiation occurs 24 h after partial hepatectomy, some of the nucleoli also show some structural modifications. These are illustrated in figure 20, plate 25, and involve a loss of density in the nucleolonema fibres which can no longer be seen with any clarity. The nucleolus, therefore, appears as an almost homogeneous area which exhibits the normal structure of the pars amorpha. It would appear that the normal structure of the nucleoli returns when the effect of radiation wears off, and the cells resume mitosis, because none of the modified nucleoli can be found more than a few hours after irradiation.

In the study of the effects of X radiation on mitotic chromosomes, a dose of 450r was used in the early experiments. This dose is sufficient to inhibit mitosis completely, and no mitotic figures were found for at least 9 h after irradiation. Even when cell division was

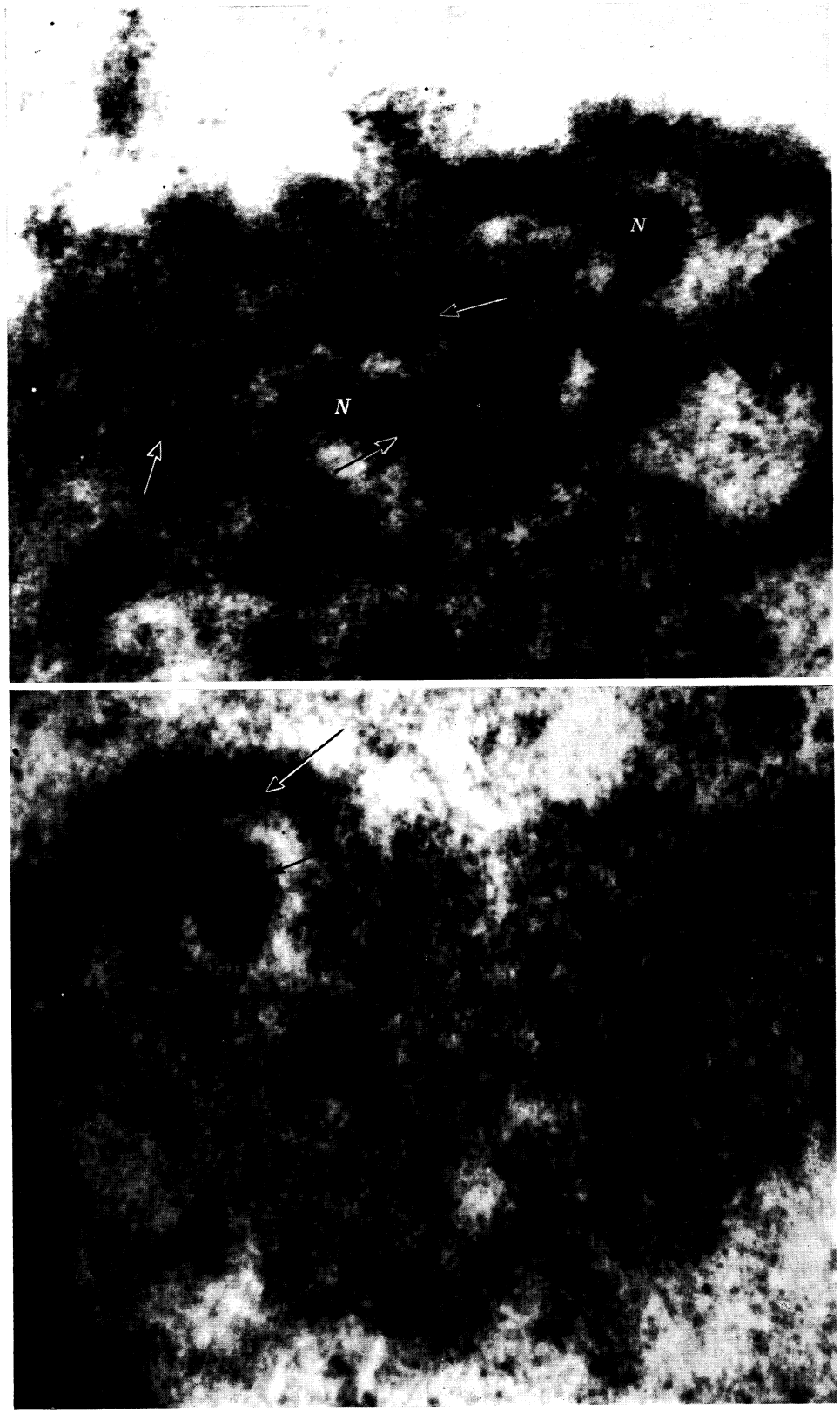

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FIGURE 1. Part of a nucleolus from a regenerating liver cell fixed 12 h after operation. Fine fibres approximately 100 Å in diameter (arrowed) can be seen in both the nucleolonema fibres (*N*) and the surrounding pars amorpha. (Magn. × 67 500.)

FIGURE 4. An area from figure 3 at higher magnification. Both the nucleolonema fibres (arrowed) and the pars amorpha contain fine fibres 100 Å in diameter. (Magn. × 75 000.)

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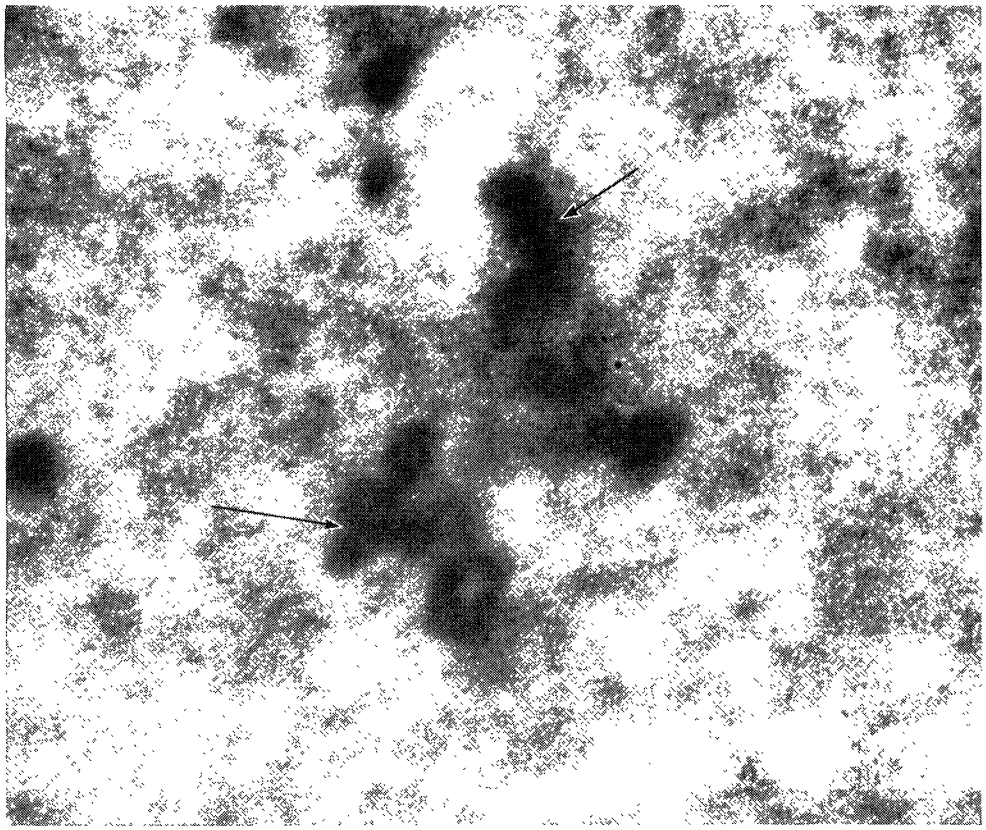

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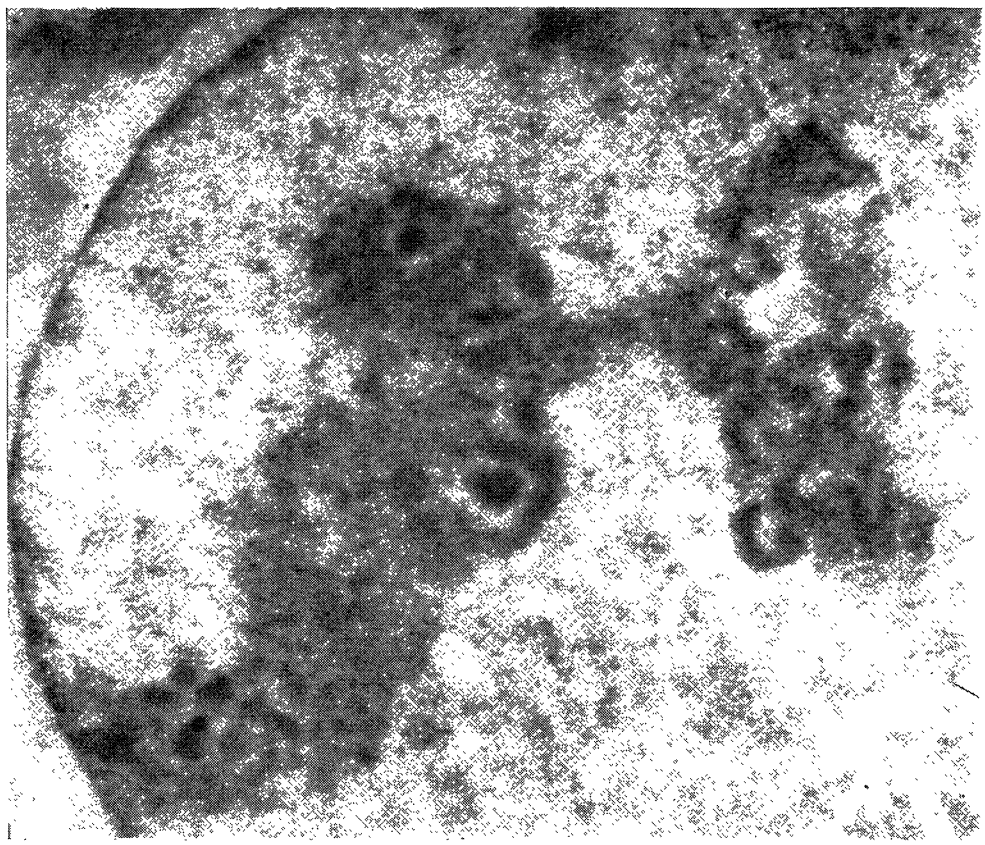

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FIGURE 2. A nucleolus from a regenerating liver cell fixed 18 h after operation. The nucleolonema fibres (arrowed) are still distinct but the pars amorpha has largely disappeared. (Magn. × 41 250.)

FIGURE 3. A large irregularly shaped nucleolus from a regenerating liver cell fixed 20 h after operation. Nucleolonema fibres and pars amorpha are still present. (Magn. × 33 750.)

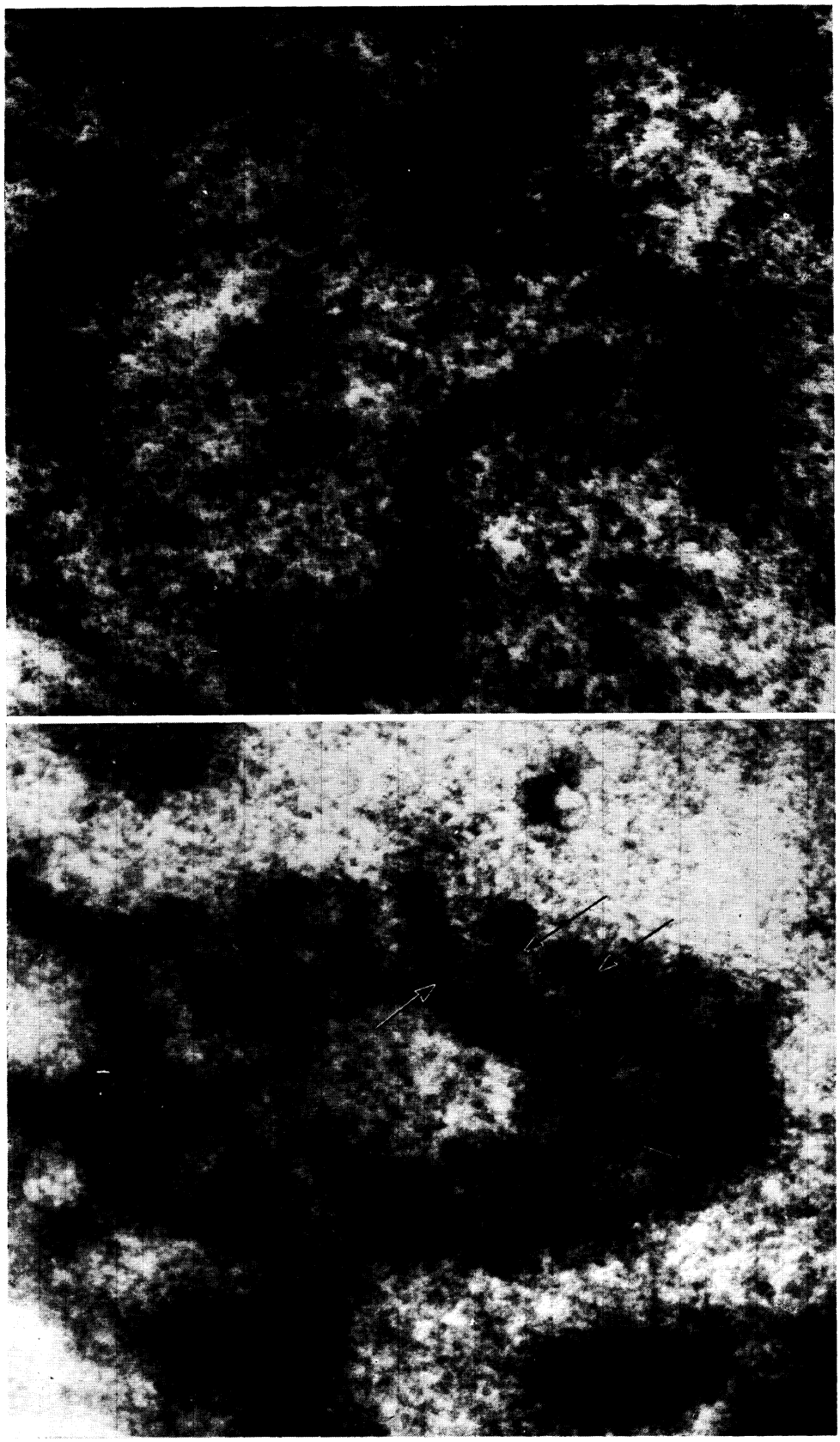

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FIGURE 5. A high magnification photograph of chromatin material from the nucleus of a regenerating liver cell fixed 26 h after partial hepatectomy. The chromatin can be seen to contain fibres approximately 100 Å in diameter, and these appear to be becoming organized into dense fibrous units. (Magn. × 67 500.)

FIGURE 7. A mid-prophase chromosome from a regenerating liver cell fixed 28½ h after partial hepatectomy. Dense fibrous units (arrowed) can be seen running diagonally across the chromosome in a manner suggestive of coiling. These fibrous units are made up of densely staining fibres approximately 100 Å in diameter. (Magn. × 60 000.)

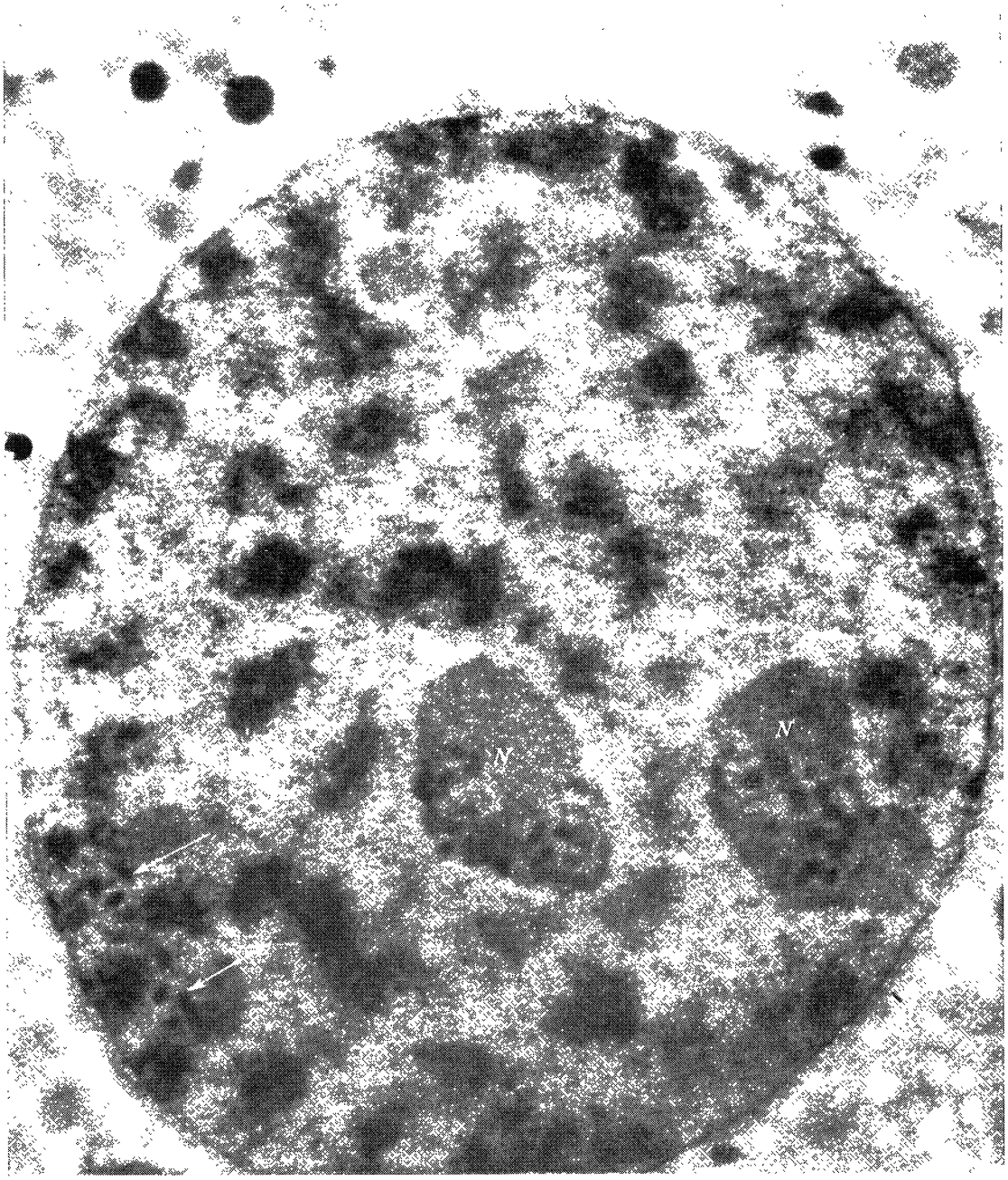
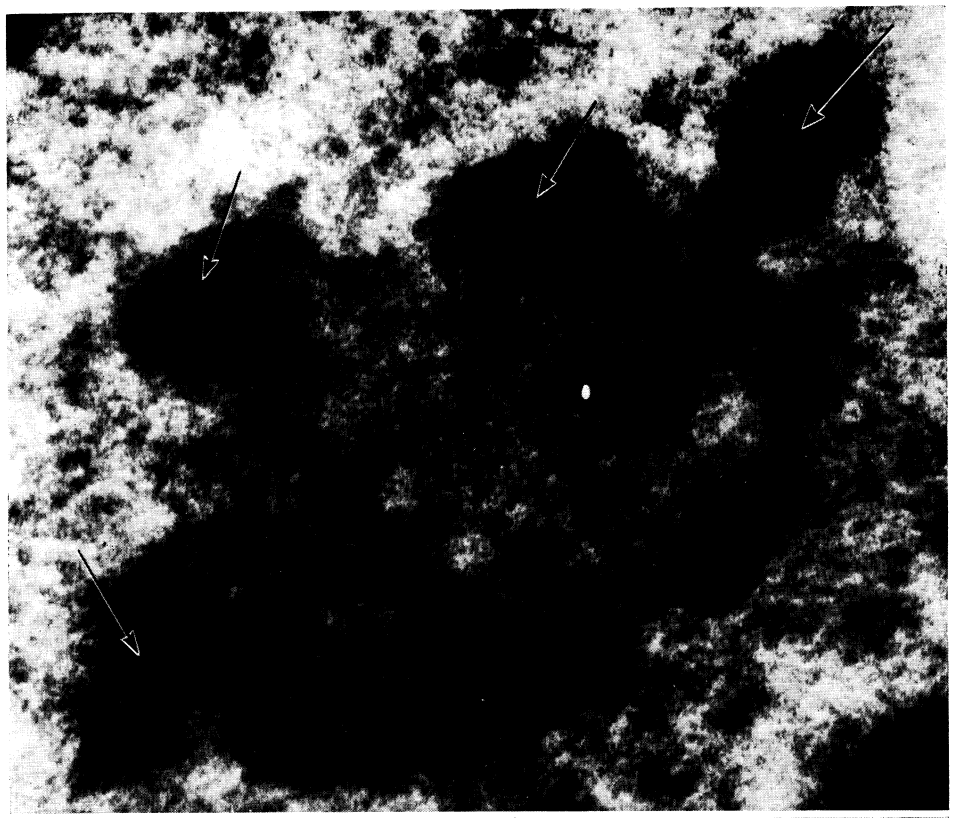


FIGURE 6. An early prophase nucleus from a regenerating liver cell fixed 28½ h after partial hepatectomy. Two nucleoli (*N*) are still present and these contain nucleolonema fibres. Fibrous units with similar structure to these nucleolonema fibres can be seen among the chromosome units (arrowed). (Magn. × 15000.)



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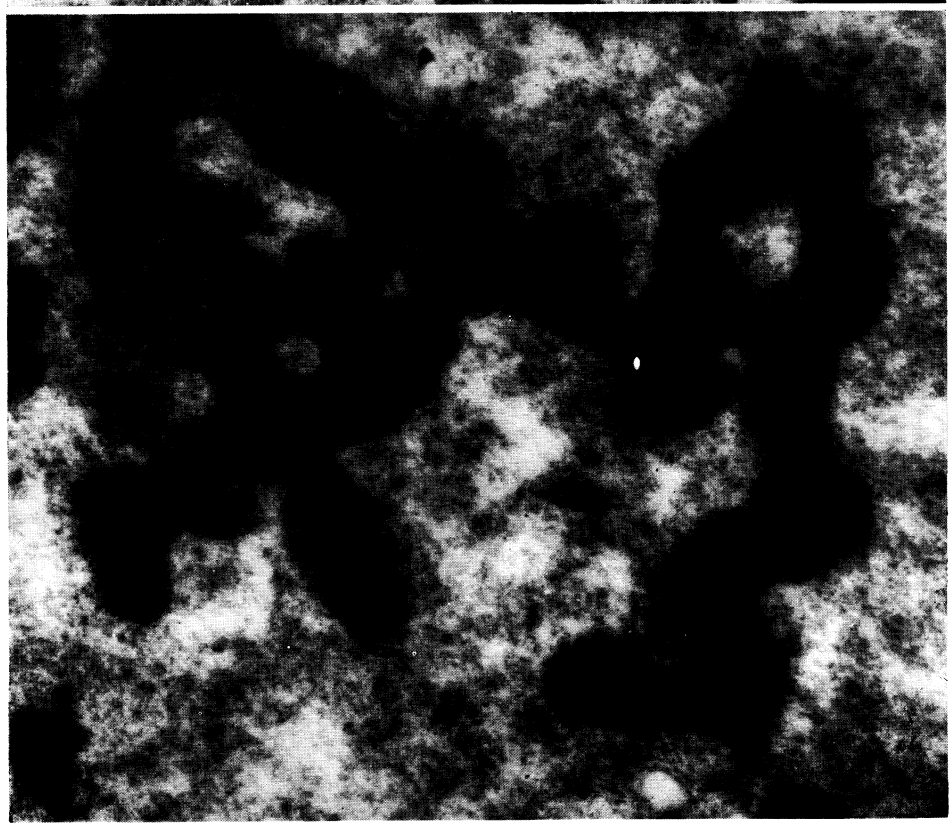

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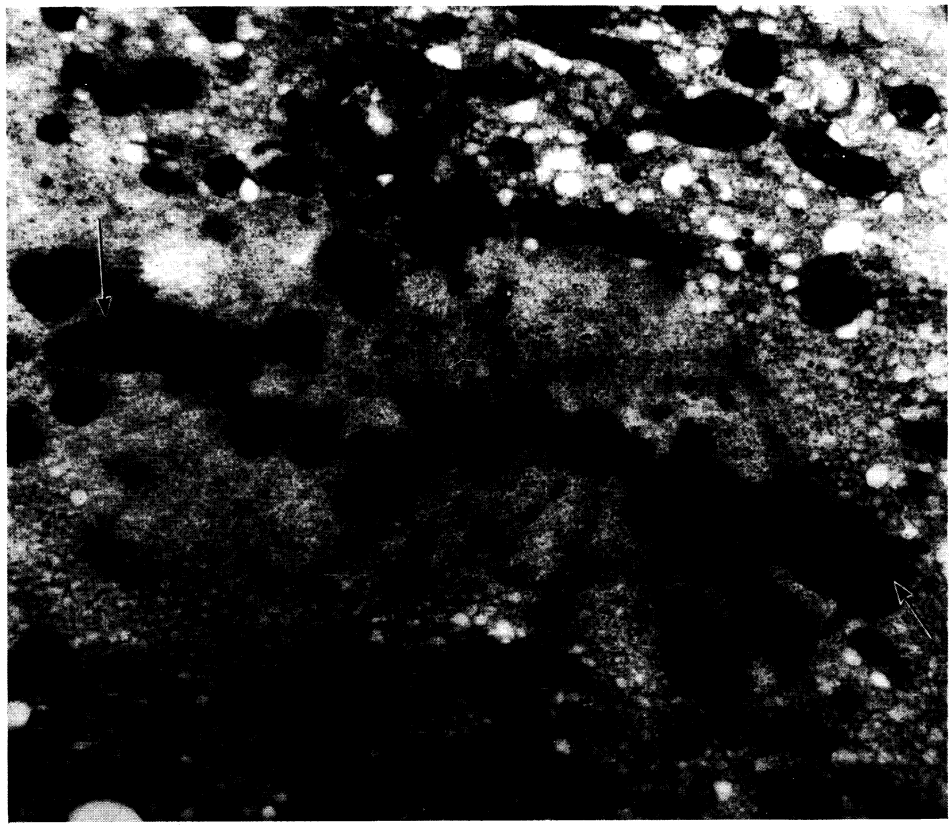
FIGURE 8. A nucleolus from a regenerating liver cell in late prophase. Nucleolonema fibres are no longer present, but parts of four densely staining chromosomes (arrowed) can be seen closely apposed to the nucleolar surface. (Magn. × 48750.)

FIGURE 9. Chromosomes from a regenerating liver nucleus in very late prophase just after the nuclear membrane has disintegrated. Chromosome coiling is clearly visible, but the densely staining fibrous bodies can no longer be seen. (Magn. × 30000.)



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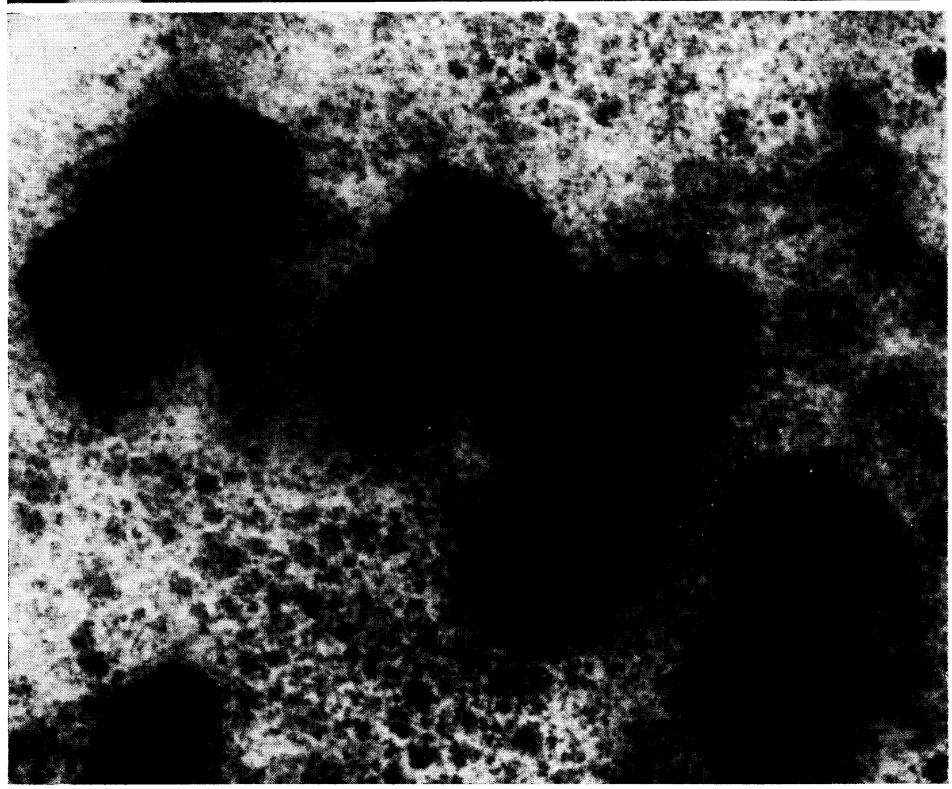

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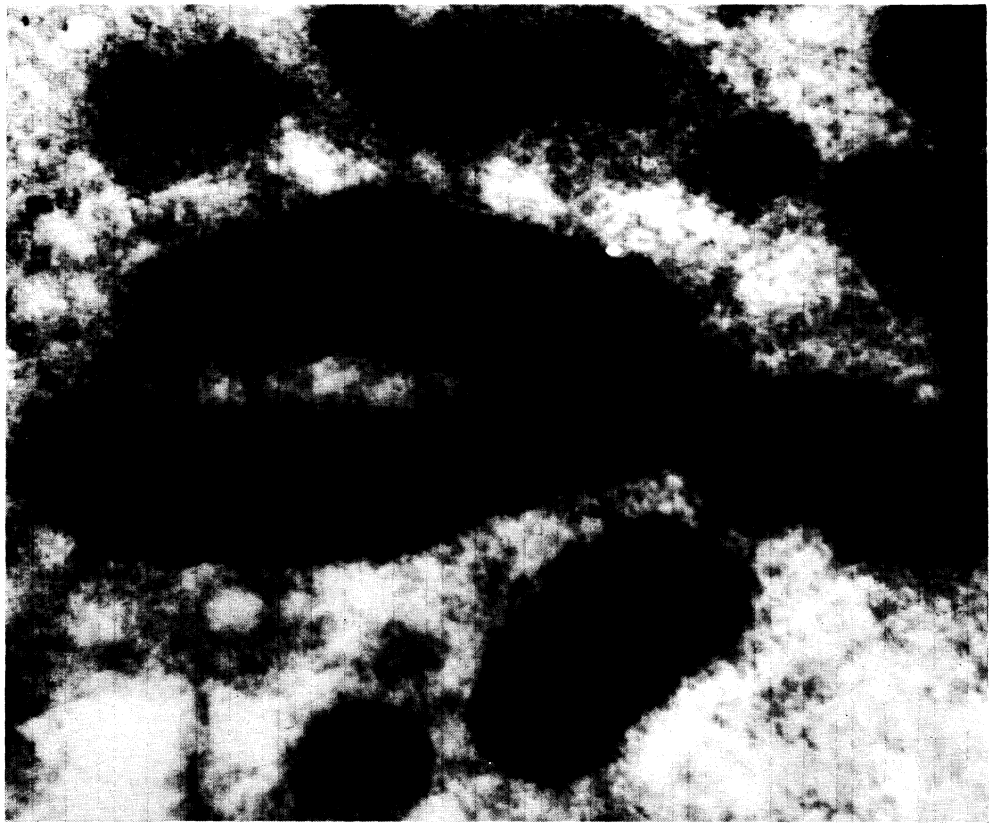
FIGURE 10. Metaphase in a regenerating liver cell. In two places (arrowed) the double nature of the chromosomes is visible. (Magn. × 11 250.)

FIGURE 11. A transverse section of four metaphase chromosomes. The chromatids are still very closely apposed but the double nature of the chromosomes can be recognized by their 'waisted' appearance. (Magn. × 48 750.)



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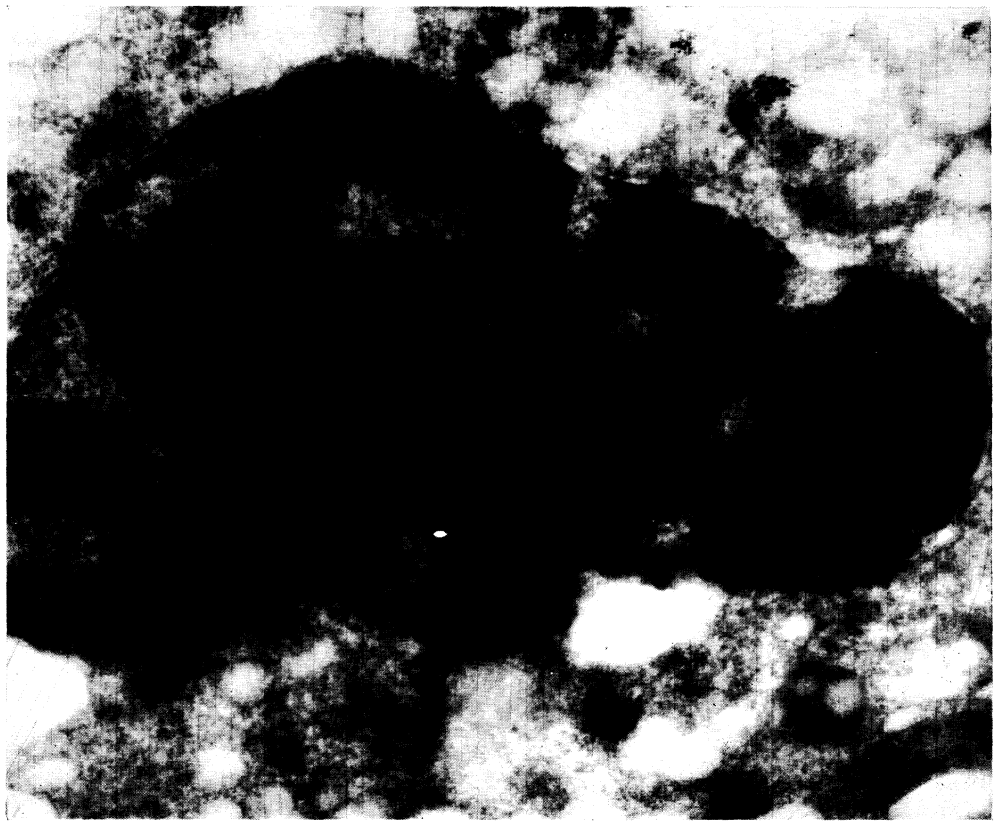

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FIGURE 12. Anaphase chromosomes from regenerating rat liver. Each chromosome can be seen to consist of densely staining fibres approximately 100 Å in diameter. (Magn. × 56 250.)

FIGURE 13. An early telophase nucleus from regenerating liver. The chromosome material is once more separated into distinct units, and the re-formation of the nuclear membrane is almost complete. This re-formation is not uniform around the whole nucleus. To the top of the picture the membrane is clearly double, but at the bottom there is, as yet, no barrier between the chromosome material and the cytoplasm. It can be seen that the chromosomes still consist of densely staining fibres approximately 100 Å in diameter. (Magn. × 33 750.)

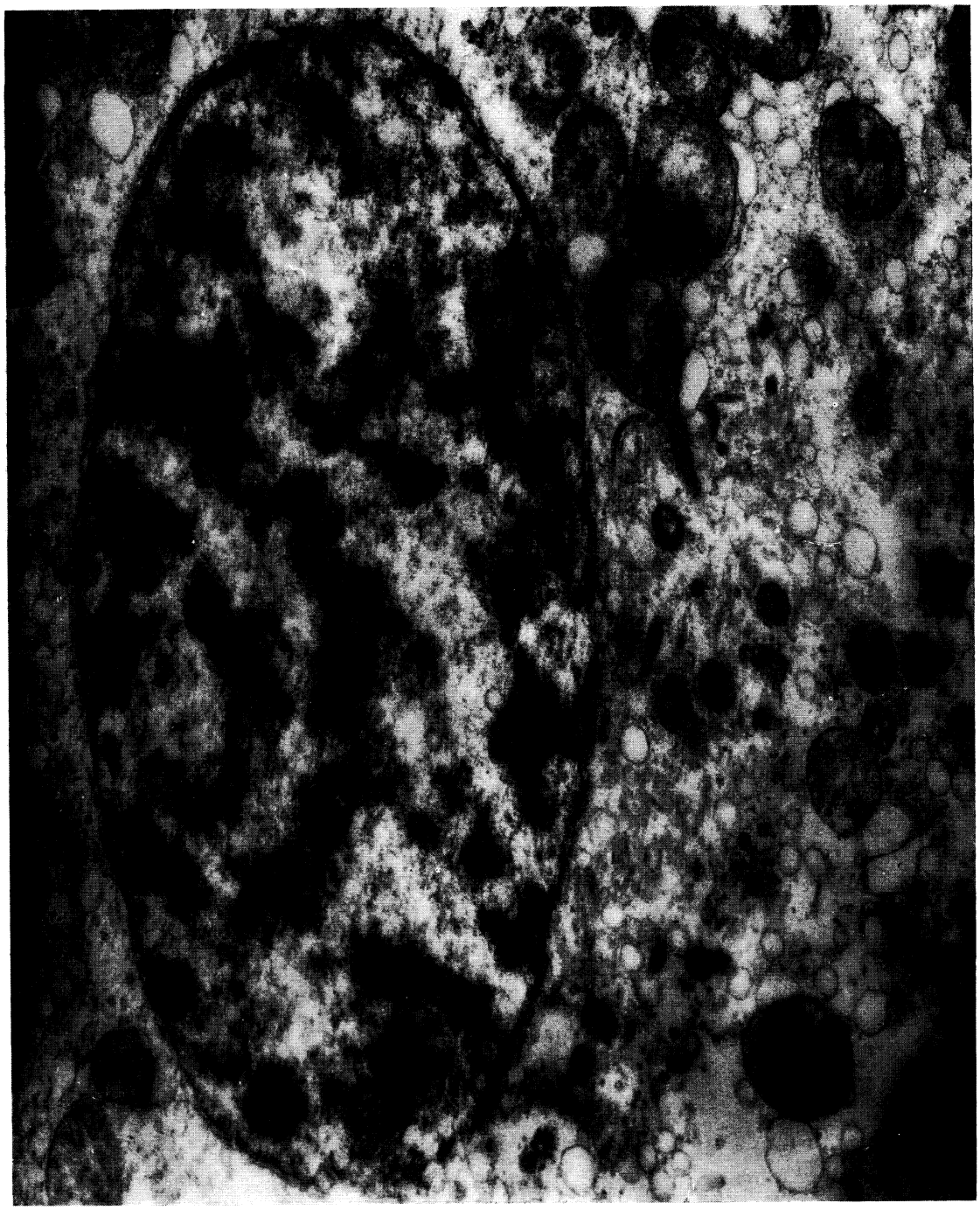
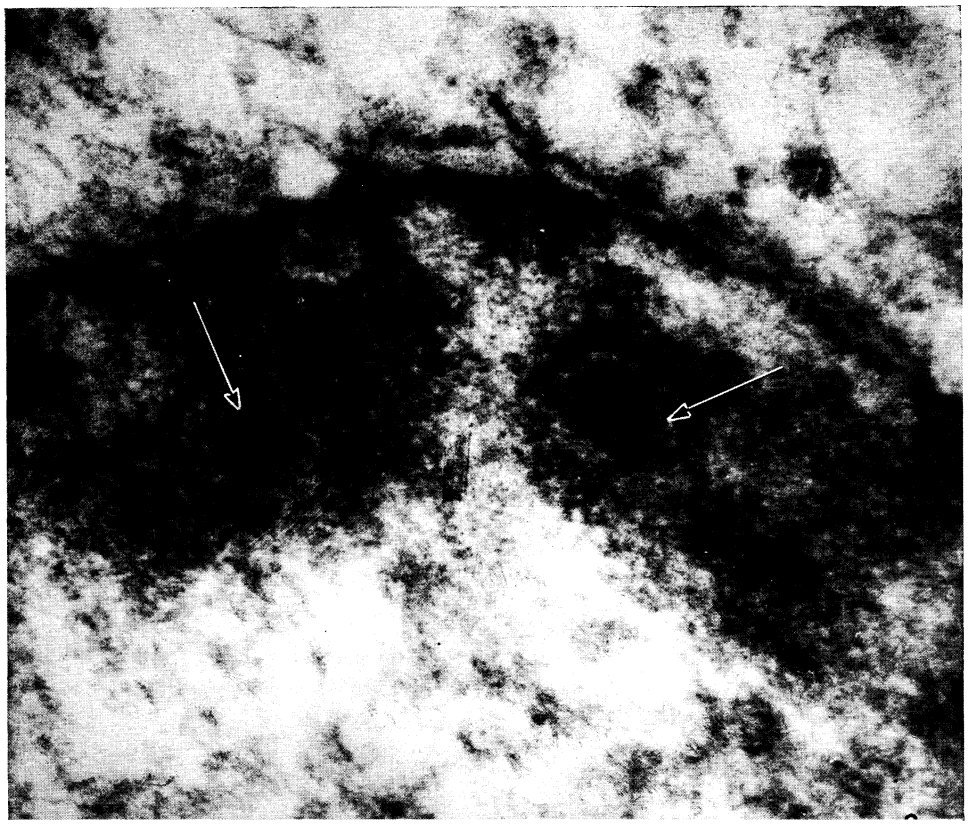


FIGURE 14. A mid-telophase nucleus from a regenerating liver cell. The re-formation of the nuclear membrane is complete, and the chromosome material is once more divided into distinct units. (Magn. × 26 250.)



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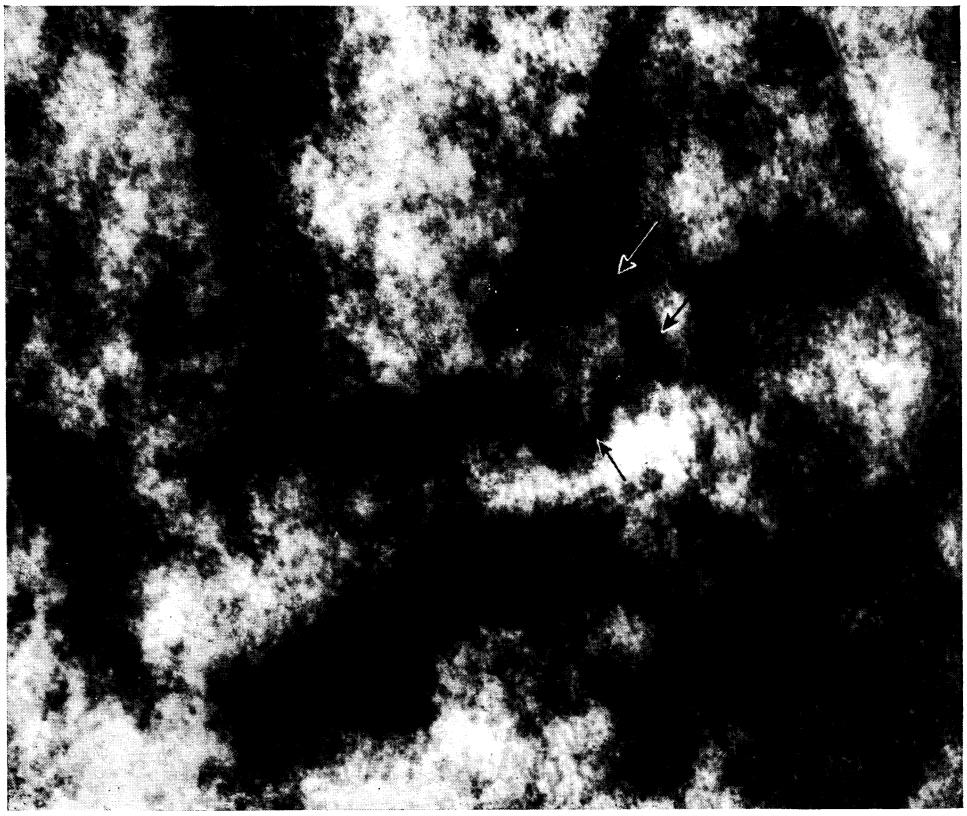

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FIGURE 15. This figure shows two of the areas of material embedded in the telophase chromosomes at higher magnification (arrowed). They would appear to have a fibrous structure, but this is less distinct than that of the surrounding chromosome material. (Magn. × 75000.)

FIGURE 16. An area of chromosome material from a mid-telophase nucleus. Among the chromosomes can be seen densely staining fibrous units approximately 1000Å in diameter (arrowed). These fibrous units have a structure similar to the nucleolonema fibres seen in interphase. (Magn. × 43500.)

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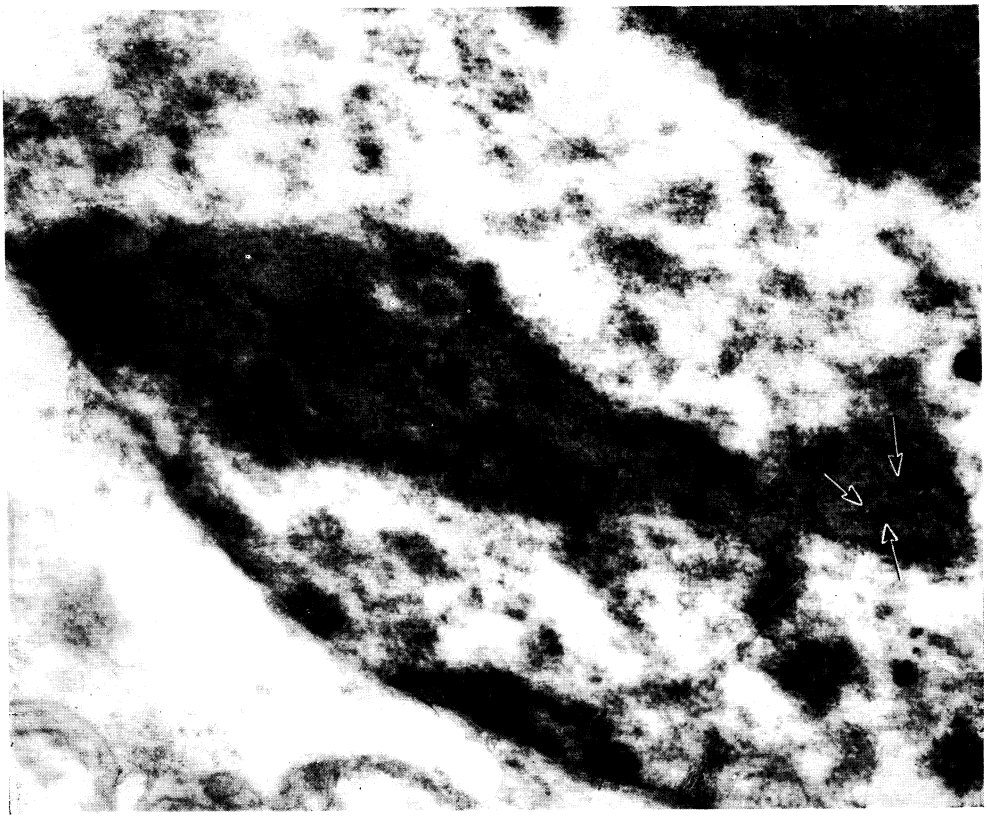

FIGURES 17 and 18. These figures show fibrous units from mid-telophase nuclei at higher magnification. In figure 17 the densely staining fibres that make up the unit (arrowed) would appear to have the same diameter (100 Å) as those of the surrounding chromosome material. In figure 18, however, the fibres that make up the 1000 Å units (single arrow), are much finer than those of the surrounding chromosome material (double arrow). (Magn. × 90 000.)

FIGURE 19. This figure shows an area of prenucleolar material from a late-telophase nucleus. No nucleolonema fibres are visible, but elongated lightly stained areas of similar diameter (arrowed) can be seen. (Magn. × 33 750.)

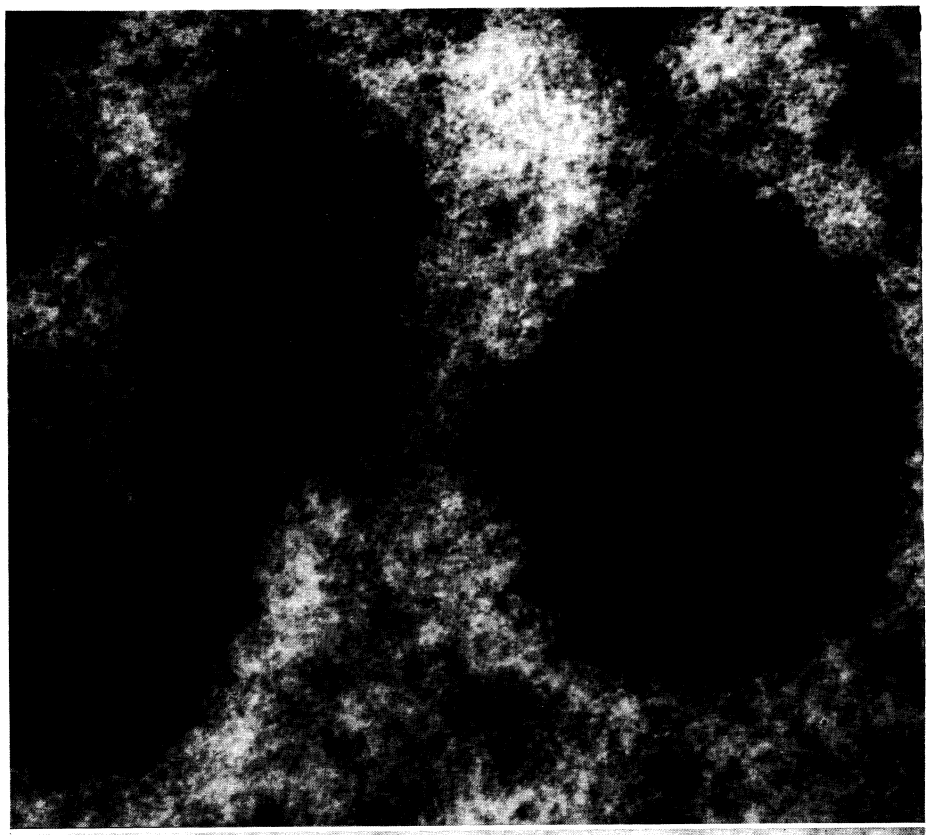

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FIGURE 20. Two nucleoli from a regenerating liver cell irradiated with a dose of 450 r at 24 h after operation, and fixed 4 h later. The densely staining nucleolonema fibres are no longer clearly visible. (Magn. × 30000.)

FIGURE 21. This figure shows two anaphase chromosomes from a regenerating liver cell irradiated with a dose of 2000 r at 28½ h after operation and fixed 30 min later. They still consist of fibres approximately 100 Å in diameter, but the packing of their fibres, especially at the surface of the chromosomes, is much looser than normal and this gives the chromosomes a rather ragged appearance. (Magn. × 60000.)

resumed, the mitotic rate was so low that it was rare to find a dividing cell in the electron microscope and for this reason it was not possible to study the long-term effect of radiation on chromosome structure. A second series of experiments was therefore undertaken in which the animals were irradiated at 28 h after operation, when the highest mitotic rate is found, and killed 15 or 30 min later. In this way it was possible to examine the immediate effects of irradiation upon the chromosomes. It was found that after a dose of 450r no ultrastructural changes could be demonstrated in the chromosome material of any of the cell nuclei. It was therefore decided to repeat the experiments using a dose of 2000r, and it was found that after a dose of this magnitude, ultrastructural nuclear changes are visible. These changes are most obvious in metaphase and anaphase chromosomes which often show signs of fragmentation. The chromosome structure still appears to consist of an intermingled mass of fibres about 100Å in diameter (figure 21, plate 25), but the packing of the fibres at the edges of the chromosomes is much looser than usual, and this gives the chromosomes a rather ragged appearance. This is best demonstrated by comparing figure 21 with figure 12. This loosening effect of 2000r on chromosome structure is less obvious in telophase. It must be remembered, however, that the telophase chromosome material is normally rather loose and irregular, and an exact comparison between irradiated and non-irradiated telophase material is therefore difficult. Although the telophase chromosome ultrastructure is not noticeably changed, however, it is obvious that 2000r has caused considerable mitotic derangement, and many of the telophase nuclei are very irregularly shaped. In some cases the two daughter nuclei have been unable to separate completely and the nuclear membrane has reformed around the whole chromosome mass.

Discussion

The finding of distinct changes in nucleolar morphology at the time new α-cytomembranes are being produced in regnerating rat liver is of great interest. Not only has the nucleolus been considered to be involved in the formation of ribonucleoprotein and its transfer from the nucleus to the cytoplasm, but both Haguenau & Bernhard (1955) and Gay (1956) have suggested that this transfer is achieved by means of the α-cytomembranes.

Caspersson (1950), has, however, shown that the nucleoli of cells engaged in active protein production are much larger than normal, and this conflicts with the electron microscopical finding that the nucleoli are less distinct than normal. There is, however, one possibility that could explain this discrepancy. As a rule, the liver nuclei seen during the first 15 h of regeneration are distinctly larger than those in normal liver cells. This increase in size probably represents preparation for protein production and the increased size would probably be retained during sustained periods of moderate protein production. It has, however, been shown (Davis 1962*a*) that α-cytomembrane production in any individual regenerating liver cell occurs in one burst lasting at most a few hours and probably much less. If the material of the pars amorpha is involved in this production, it could be exhausted by the very rapidity of the process and take some hours to re-form.

The results obtained from the electron microscope study of mitosis in regenerating rat liver are encouraging. Not only has it been possible to recognize various mitotic stages in the electron microscope that can also be seen in material from the same animals fixed for the light microscope, but the process of mitosis would appear very similar to that described

in living mammalian cells by Fell & Hughes (1949). These authors stated that the first signs of prophase are an increase in the number and a diminution in the size of the heterochromatic granules. Figures 5 and 6 show a very similar state of affairs. Fell & Hughes also report that in late prophase the chromosomes are often finely banded structures and that some of them radiate from the nucleoli. Further similarities between the two sets of observations are that the double nature of the chromosomes is not visible until early metaphase, and that in late telophase the main chromosome material is replaced by a diffuse system of irregular lumps and strands. Fell & Hughes claim that the nucleolus is formed from this material. These similarities between the mitotic pictures seen in the living cell, and those obtained with the electron microscope, indicate that some of the previous difficulties have been overcome and that osmium fixation followed by *PTA* staining and embedding in 'Araldite' is a suitable technique for the preparation of cell nuclei for study by an electron microscope.

The present study has confirmed the findings of Porter (1954), Wessel & Bernhard (1957) and Dales (1960) to the extent that the liver chromosomes at most stages of mitosis appear to be made up of dense fibres approximately 100Å in diameter. Two types of structure have, however, been seen that have not previously been reported in electron microscopical studies. First there are the dense fibrous bodies approximately 1000Å in diameter that are found in early- and mid-prophase chromosome material. It is at this time that the chromosomes are most clearly seen to be divided into chromatids and half chromatids in studies by a light microscope and it seems likely that the dense fibrous bodies represent one of these two types of structures. It has not been possible to determine the number of these bodies present in any chromosome examined but figure 7, plate 17, suggests that they are coiled within the chromosome.

The second series of new structures observed in this study consists of the areas of material seen within the mid-telophase chromosomes, but which are clearly delineated from these structures. It is difficult to correlate these areas with any previous observation, although vacuoles have been reported in telophase chromosomes in several studies by a light microscope. The areas seen in the electron microscope may correspond to these vacuoles although they do contain definite stainable material even if it is somewhat diffuse. Other possibilities are that they represent stages in the formation of the dense fibrous units after mitosis or transverse sections of these bodies that have become partially surrounded by chromosome material.

The information obtained from this study on the re-formation of the nuclear membrane in telophase would appear to conflict with the suggestions of Porter & Machado (1960), and support those of Jones (1960). Regenerating liver cells contain at various times large numbers of α-cytomembranes but most of these are lost by the onset of prophase, and by telophase many of the cells have completely vacuolated cytoplasms. There are thus no double-membraned elements of the endoplasmic reticulum that could take part in the re-formation of the nuclear membrane, and it must be concluded that this structure is indeed formed *de novo*.

Estable & Sotelo (1954) suggested that the nucleolonema fibres were carried through mitosis on the chromosomes, but the nature of this transport is still obscure. The present study of regenerating rat liver has not been able to take the matter much further, although

it has been shown that at the time the nucleolonema fibres disappear in prophase chromosomes are often closely attached to the nucleolar surface. The re-formation of nucleolonema-like fibres can be seen in the cell nuclei during mid-telophase but they often show no definite structural relationship to the chromosome masses. At later stages there are added complications as no typical nucleolonema fibres can be seen in the prenucleolar bodies, although it is known that the final interphase nucleolus is formed from these. There would appear to be two possibilities; either the nucleolonema fibres are first formed among the chromosome masses in mid-telophase, and later coalesce to form the prenucleolar bodies; or these bodies arise from parts of the chromosome masses, and the nucleolonema fibres are formed in them *de novo*. If the first suggestion is correct, then the nucleolonema fibres must lose their affinity for *PTA* when they form prenucleolar bodies and later regain it in the interphase nucleus. If the second suggestion is true, then the fibrous-like bodies seen in mid-telephase cannot be nucleolar components.

It is not possible to correlate the present observation on X-ray damage to nuclei with the previous electron microscopical radiation studies of Nebel (1959) and Lacey & Rotblat (1960). Nebel's observation that after irradiation nuclear profiles are easier to read, does not appear to apply to rat-liver cells. Lacey & Rotblat observed a thickening of the chromosome fibres after irradiation, but in the present study, although the chromosome material is obviously damaged by a dose of 2000 r, the diameter of the chromosome fibres is not altered and remained at about 100 Å. It may be that the changes seen by Lacey & Rotblat only occur after very high doses (they used 10 000 r). It must also be remembered that these workers examined the tissue 3 days after irradiation while the regenerating liver chromosomes used in the present study were examined not more than 30 min after irradiation.

The author would like to express his appreciation to Professor J. S. Mitchell, F.R.S., and Dr D. B. Cater for helpful and stimulating discussion during the preparation of this paper, and also to Mr E. G. T. Morley, in charge of the Metropolitan Vickers E.M. 6 electron microscope at the Cavendish Laboratory. I am also indebted to the Medical Research Council for a grant held during the course of this work.

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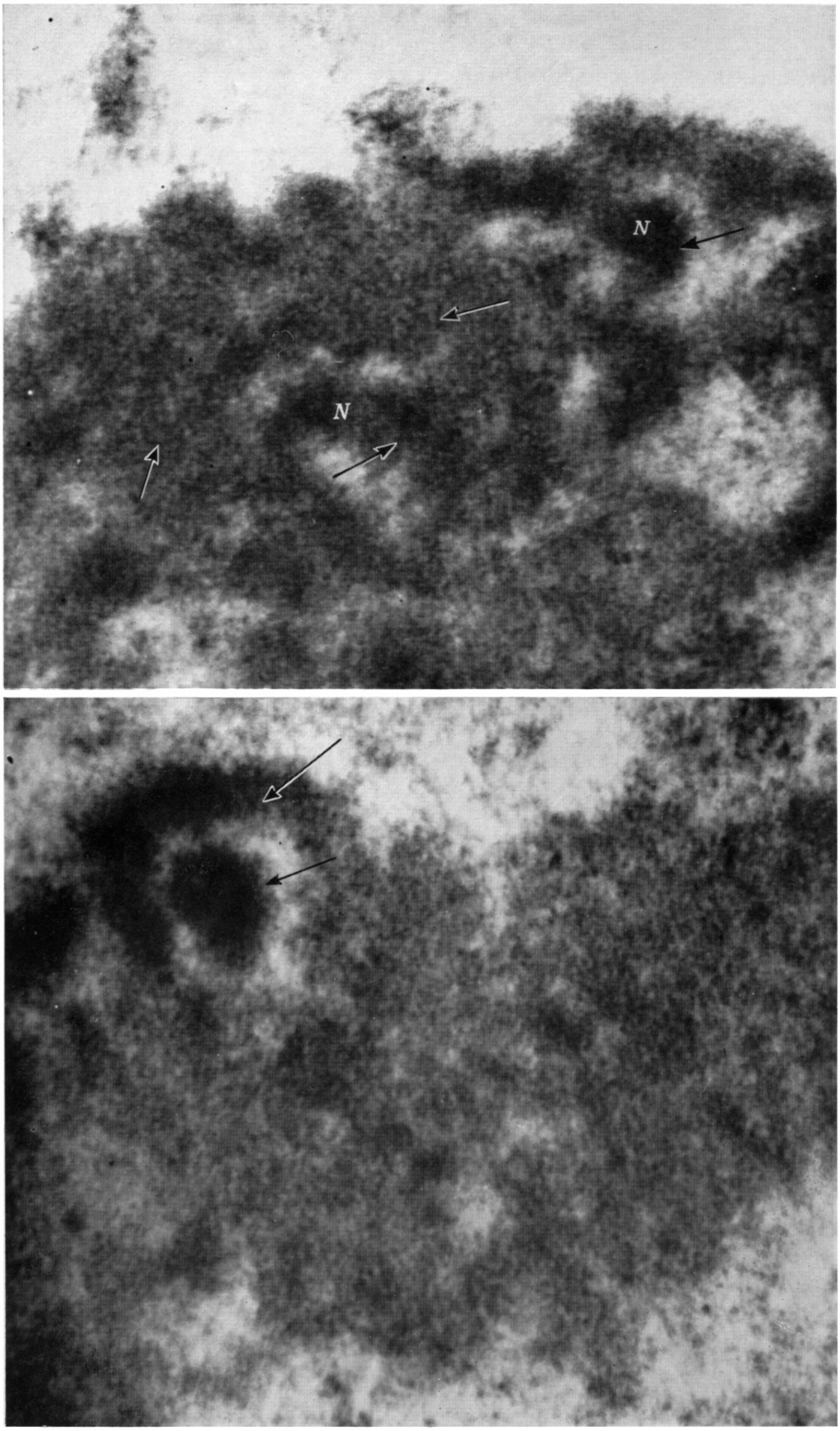

FIGURE 1. Part of a nucleolus from a regenerating liver cell fixed 12 h after operation. Fine fibres approximately 100 Å in diameter (arrowed) can be seen in both the nucleolonema fibres (*N*) and the surrounding pars amorpha. (Magn. × 67 500.)

FIGURE 4. An area from figure 3 at higher magnification. Both the nucleolonema fibres (arrowed) and the pars amorpha contain fine fibres 100 Å in diameter. (Magn. × 75 000.)

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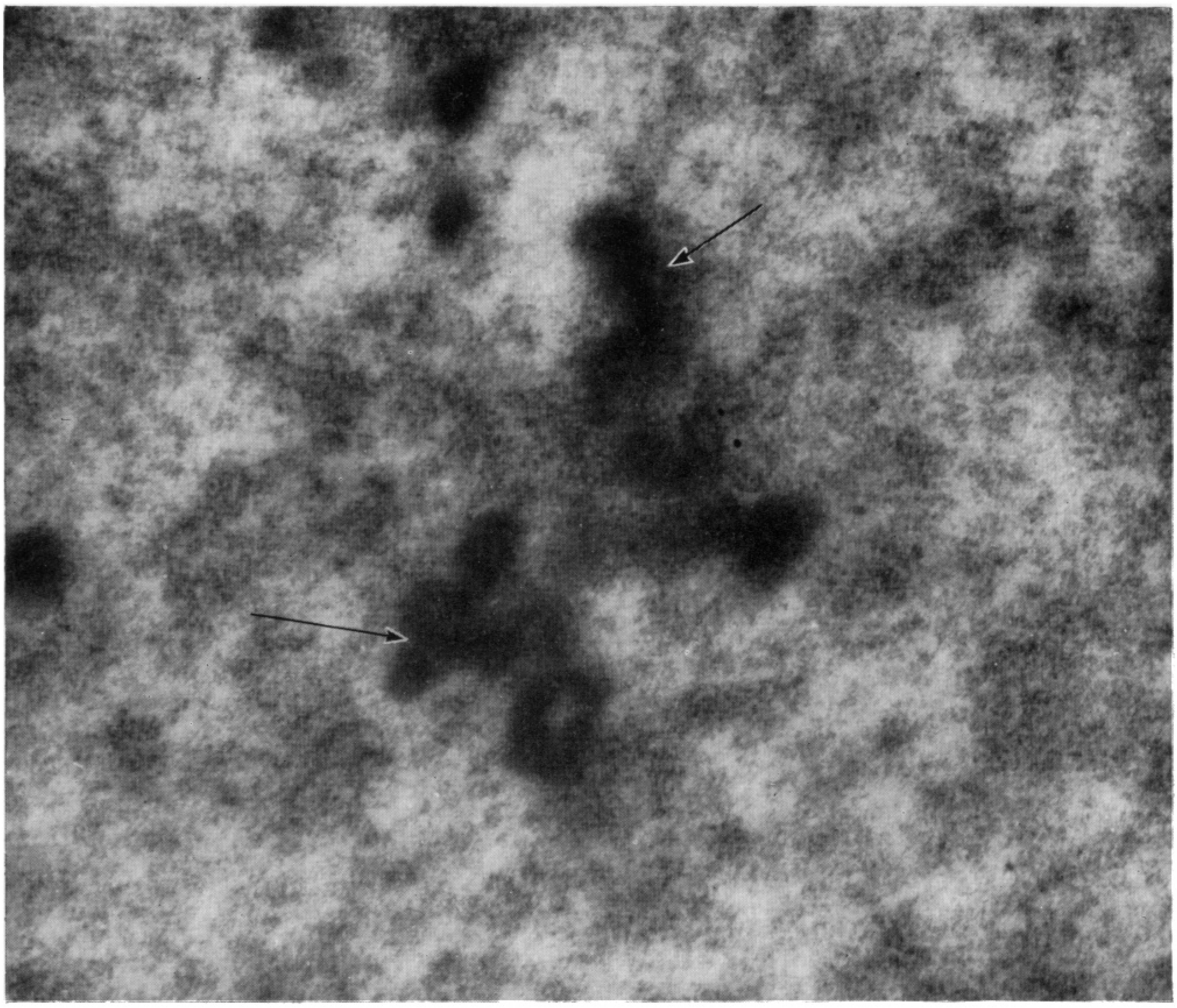

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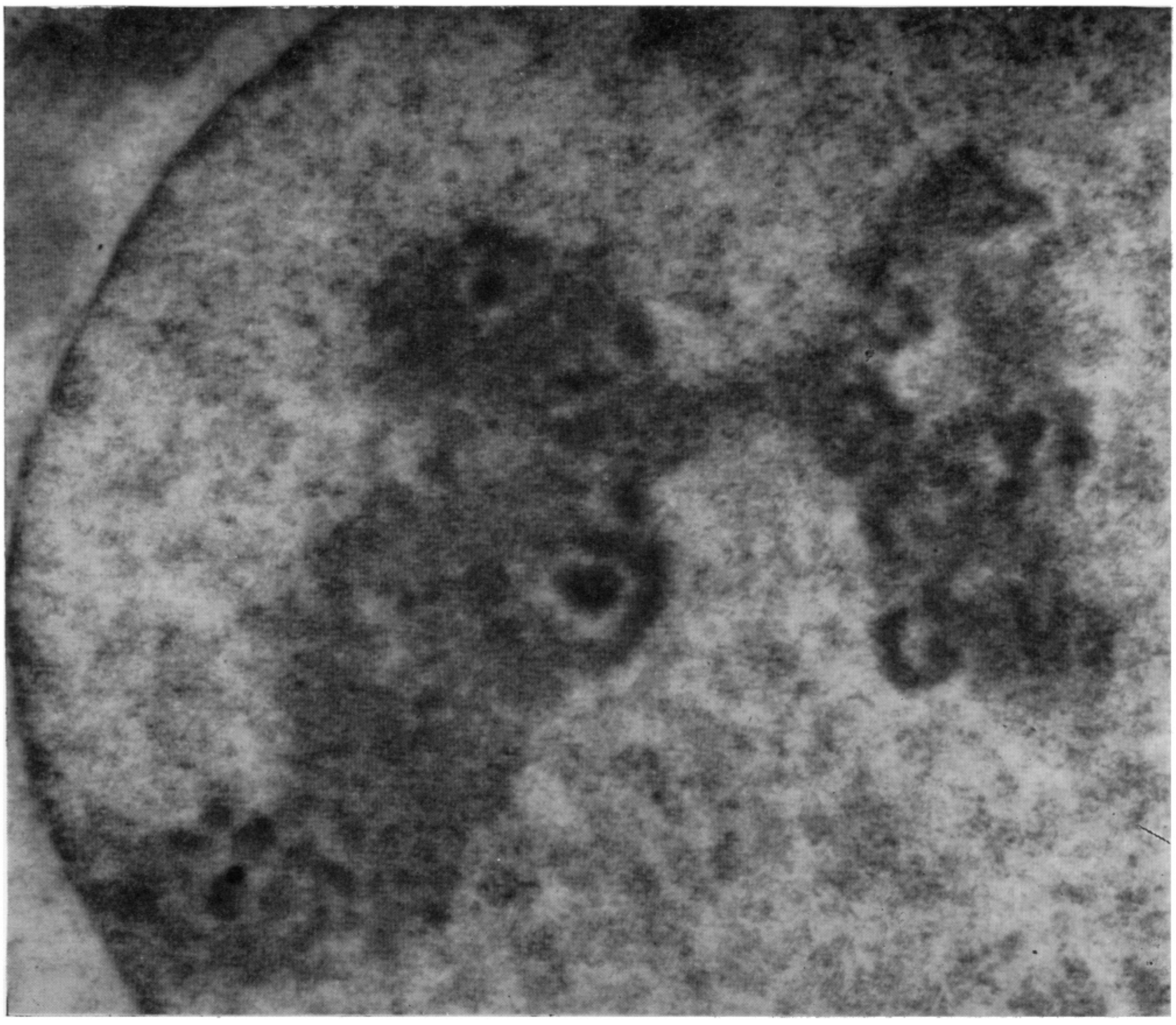

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FIGURE 2. A nucleolus from a regenerating liver cell fixed 18 h after operation. The nucleolonema fibres (arrowed) are still distinct but the pars amorpha has largely disappeared. (Magn. × 41 250.)

FIGURE 3. A large irregularly shaped nucleolus from a regenerating liver cell fixed 20 h after operation. Nucleolonema fibres and pars amorpha are still present. (Magn. × 33 750.)

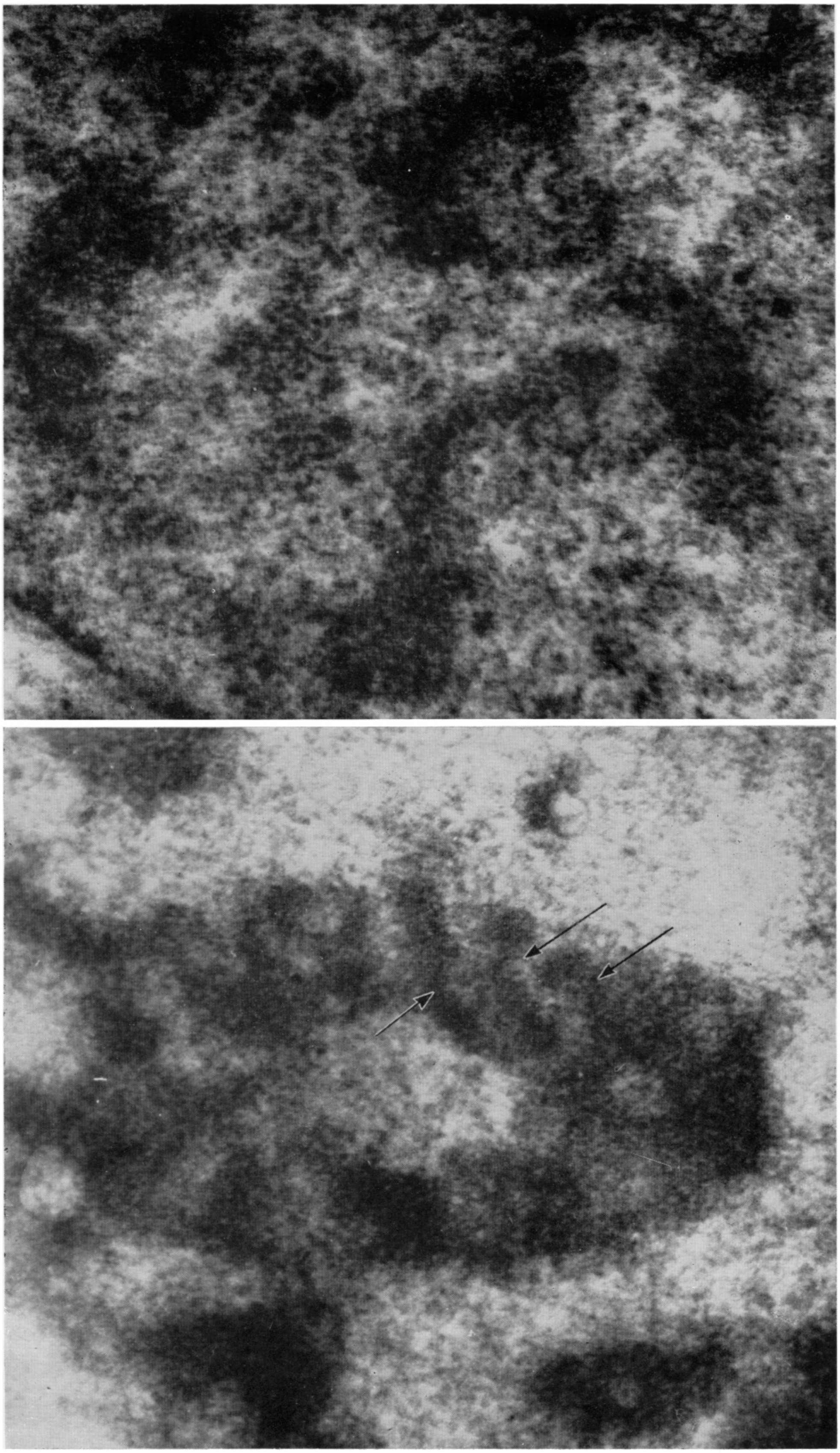


FIGURE 5. A high magnification photograph of chromatin material from the nucleus of a regenerating liver cell fixed 26 h after partial hepatectomy. The chromatin can be seen to contain fibres approximately 100 Å in diameter, and these appear to be becoming organized into dense fibrous units. (Magn. × 67 500.)

FIGURE 7. A mid-prophase chromosome from a regenerating liver cell fixed 28½ h after partial hepatectomy. Dense fibrous units (arrowed) can be seen running diagonally across the chromosome in a manner suggestive of coiling. These fibrous units are made up of densely staining fibres approximately 100 Å in diameter. (Magn. × 60 000.)

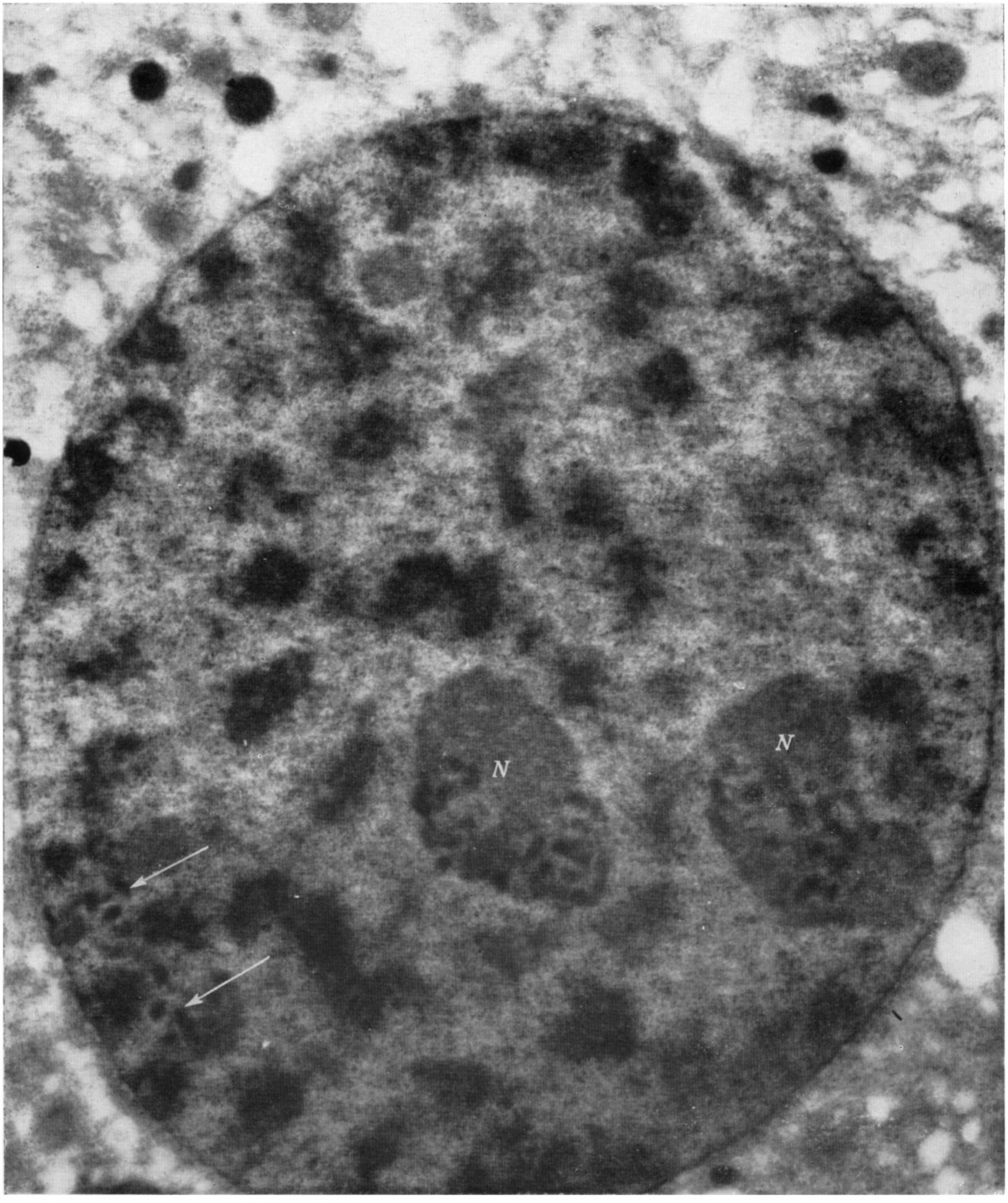


FIGURE 6. An early prophase nucleus from a regenerating liver cell fixed $28\frac{1}{2}$ h after partial hepatectomy. Two nucleoli (*N*) are still present and these contain nucleolonema fibres. Fibrous units with similar structure to these nucleolonema fibres can be seen among the chromosome units (arrowed). (Magn. × 15 000.)

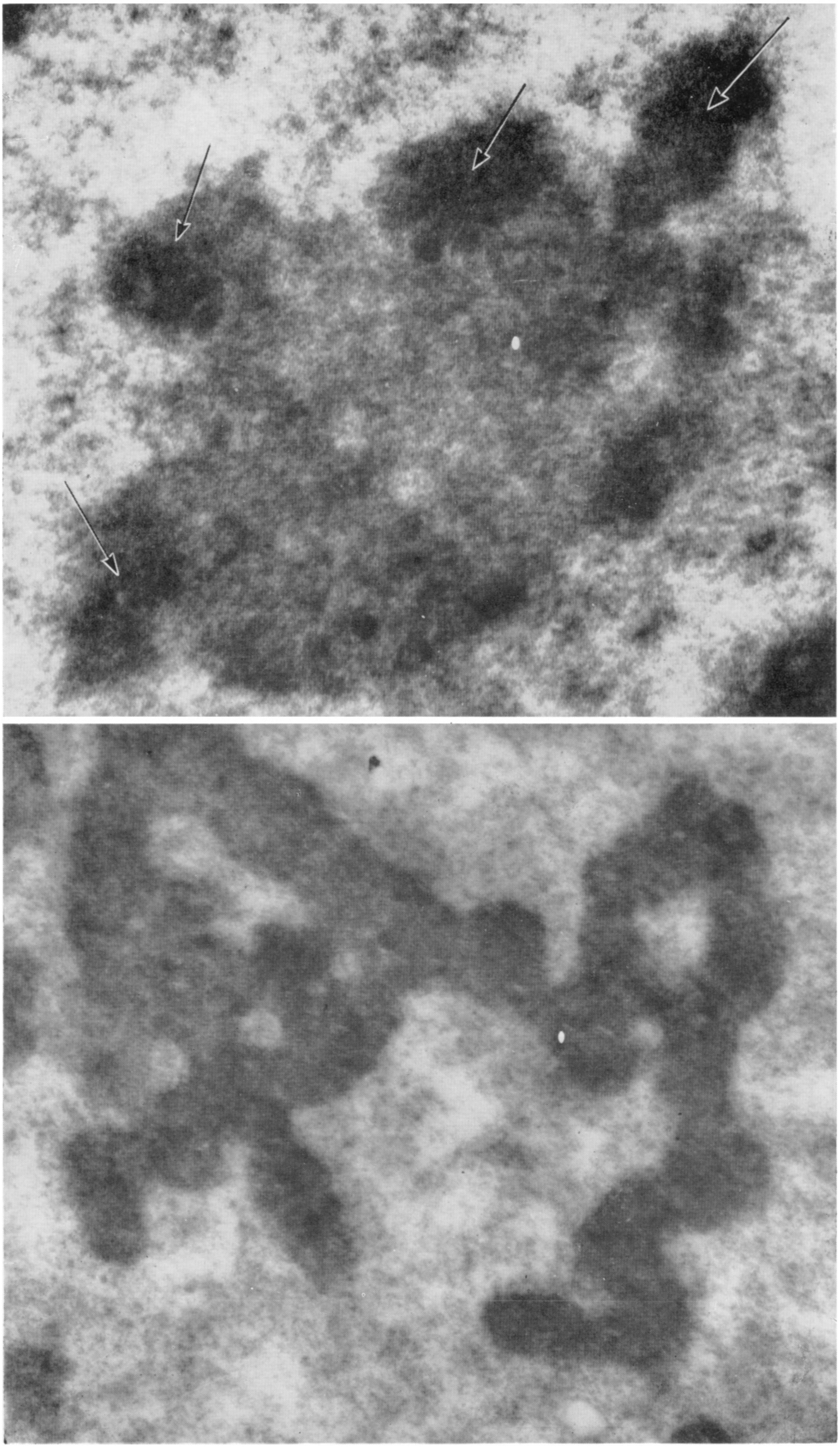
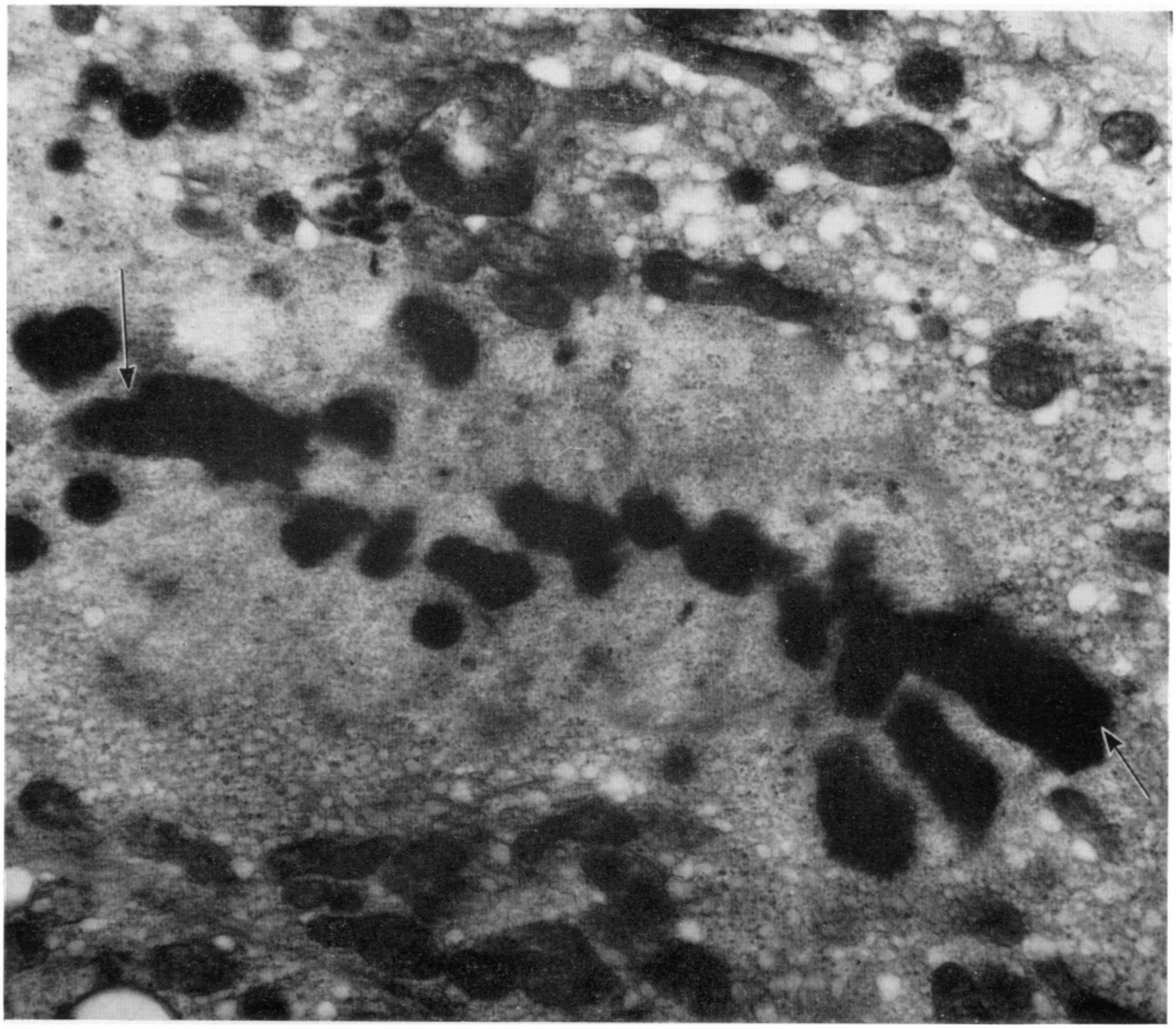


FIGURE 8. A nucleolus from a regenerating liver cell in late prophase. Nucleolonema fibres are no longer present, but parts of four densely staining chromosomes (arrowed) can be seen closely apposed to the nucleolar surface. (Magn. $\times 48750$.)

FIGURE 9. Chromosomes from a regenerating liver nucleus in very late prophase just after the nuclear membrane has disintegrated. Chromosome coiling is clearly visible, but the densely staining fibrous bodies can no longer be seen. (Magn. $\times 30000$.)



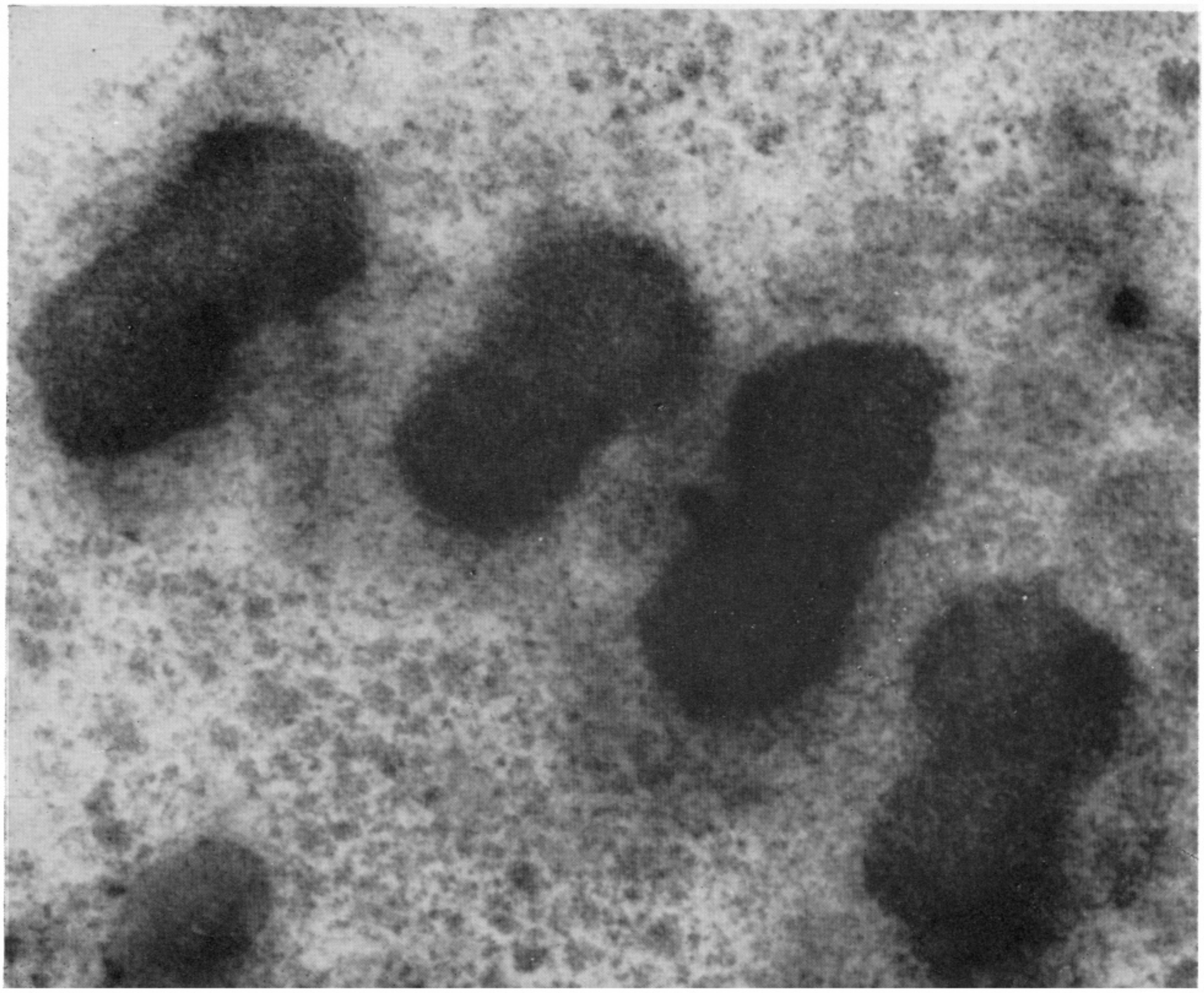


FIGURE 10. Metaphase in a regenerating liver cell. In two places (arrowed) the double nature of the chromosomes is visible. (Magn. × 11 250.)

FIGURE 11. A transverse section of four metaphase chromosomes. The chromatids are still very closely apposed but the double nature of the chromosomes can be recognized by their 'waisted' appearance. (Magn. × 48 750.)

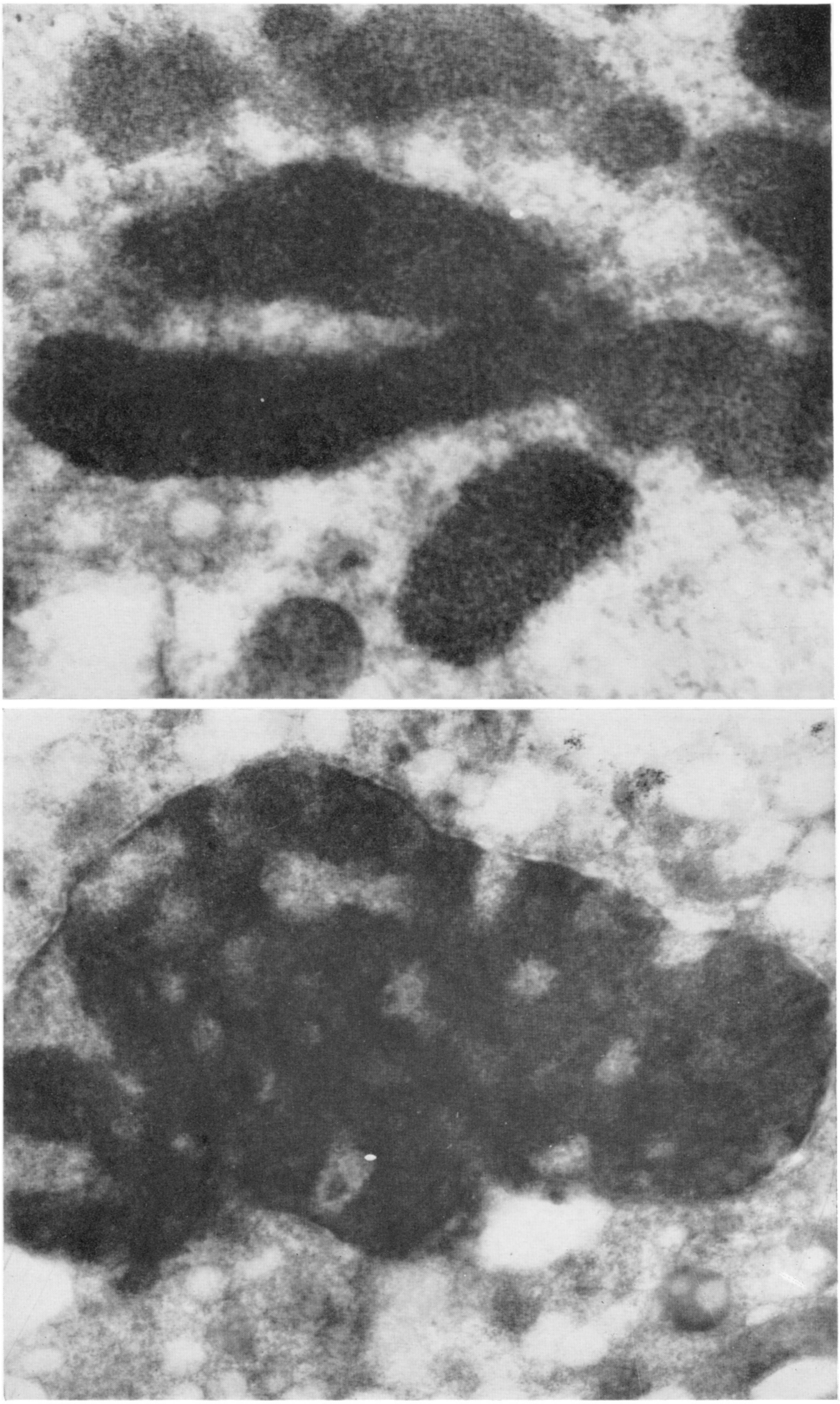


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FIGURE 12. Anaphase chromosomes from regenerating rat liver. Each chromosome can be seen to consist of densely staining fibres approximately 100 Å in diameter. (Magn. × 56 250.)

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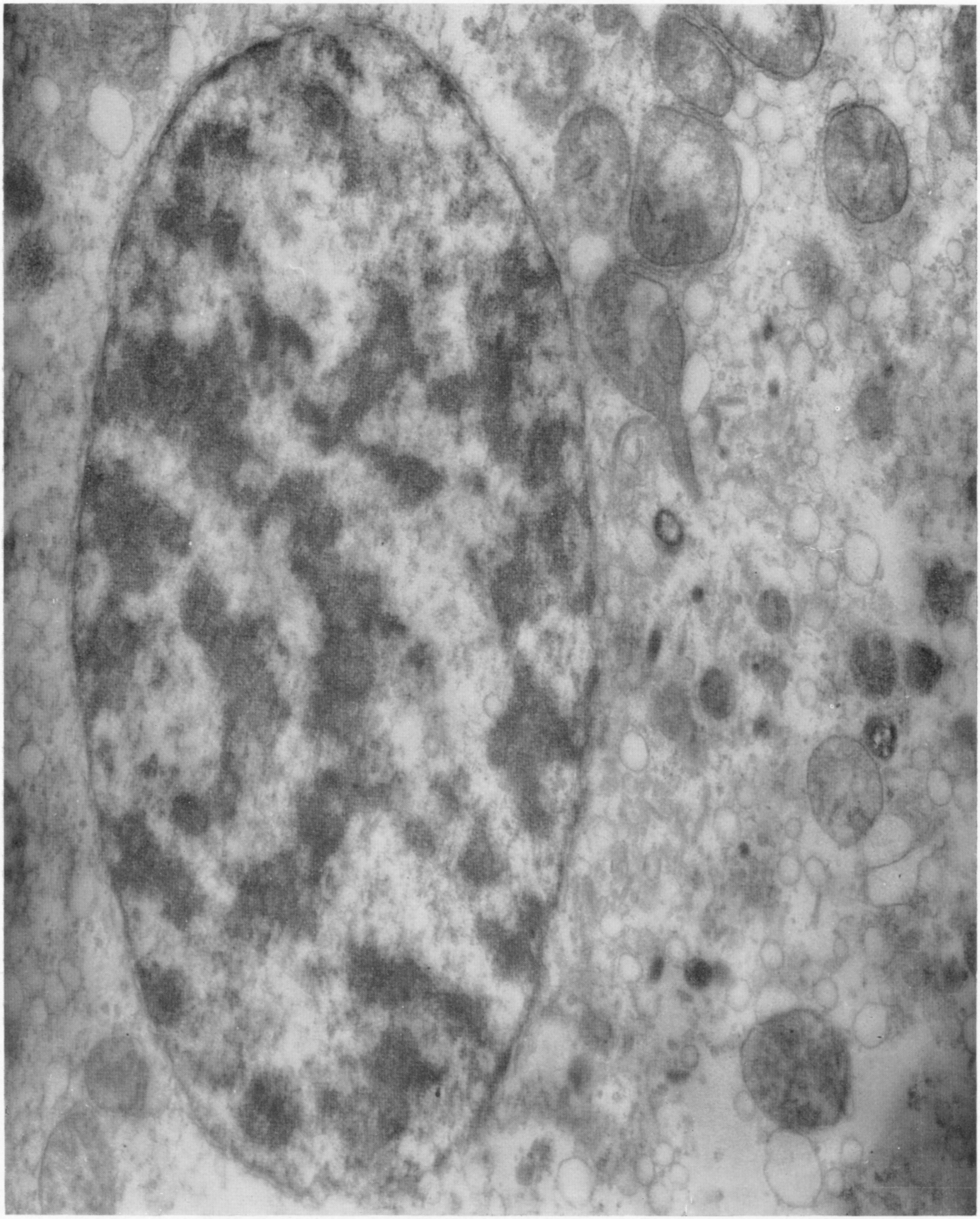


FIGURE 14. A mid-telophase nucleus from a regenerating liver cell. The re-formation of the nuclear membrane is complete, and the chromosome material is once more divided into distinct units. (Magn. × 26 250.)

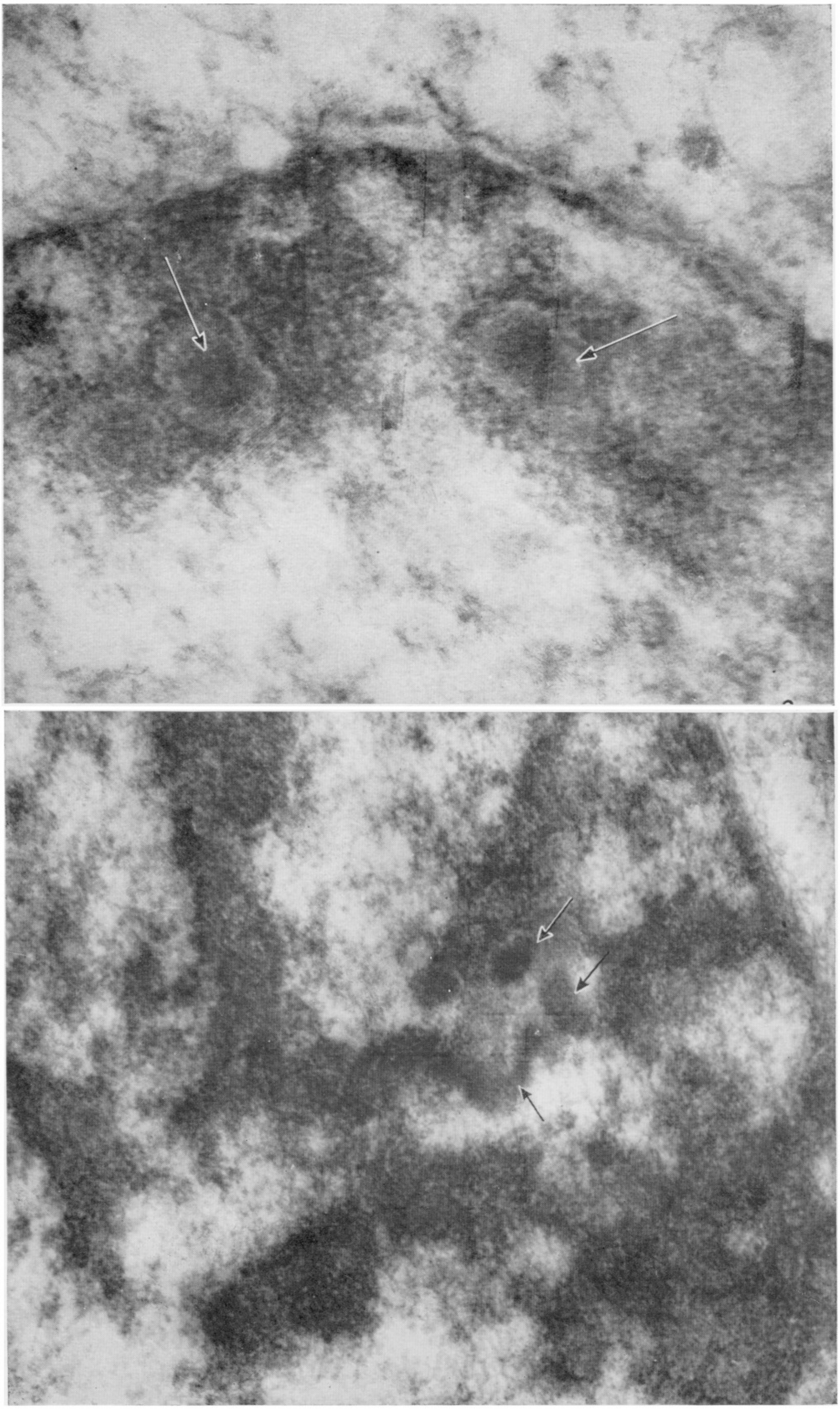
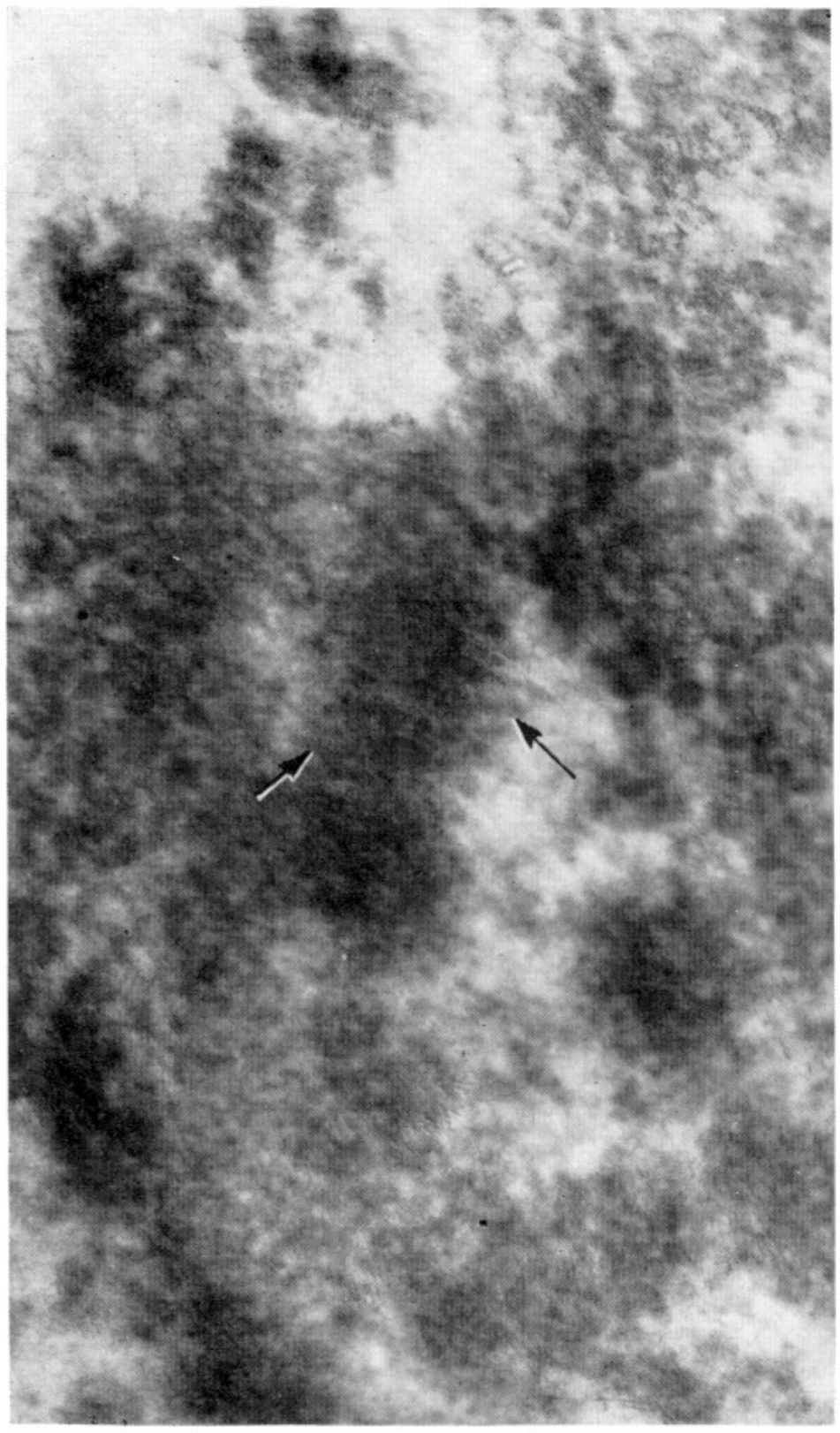


FIGURE 15. This figure shows two of the areas of material embedded in the telophase chromosomes at higher magnification (arrowed). They would appear to have a fibrous structure, but this is less distinct than that of the surrounding chromosome material. (Magn. × 75 000.)

FIGURE 16. An area of chromosome material from a mid-telophase nucleus. Among the chromosomes can be seen densely staining fibrous units approximately 1000 Å in diameter (arrowed). These fibrous units have a structure similar to the nucleolonema fibres seen in interphase. (Magn. × 43 500.)



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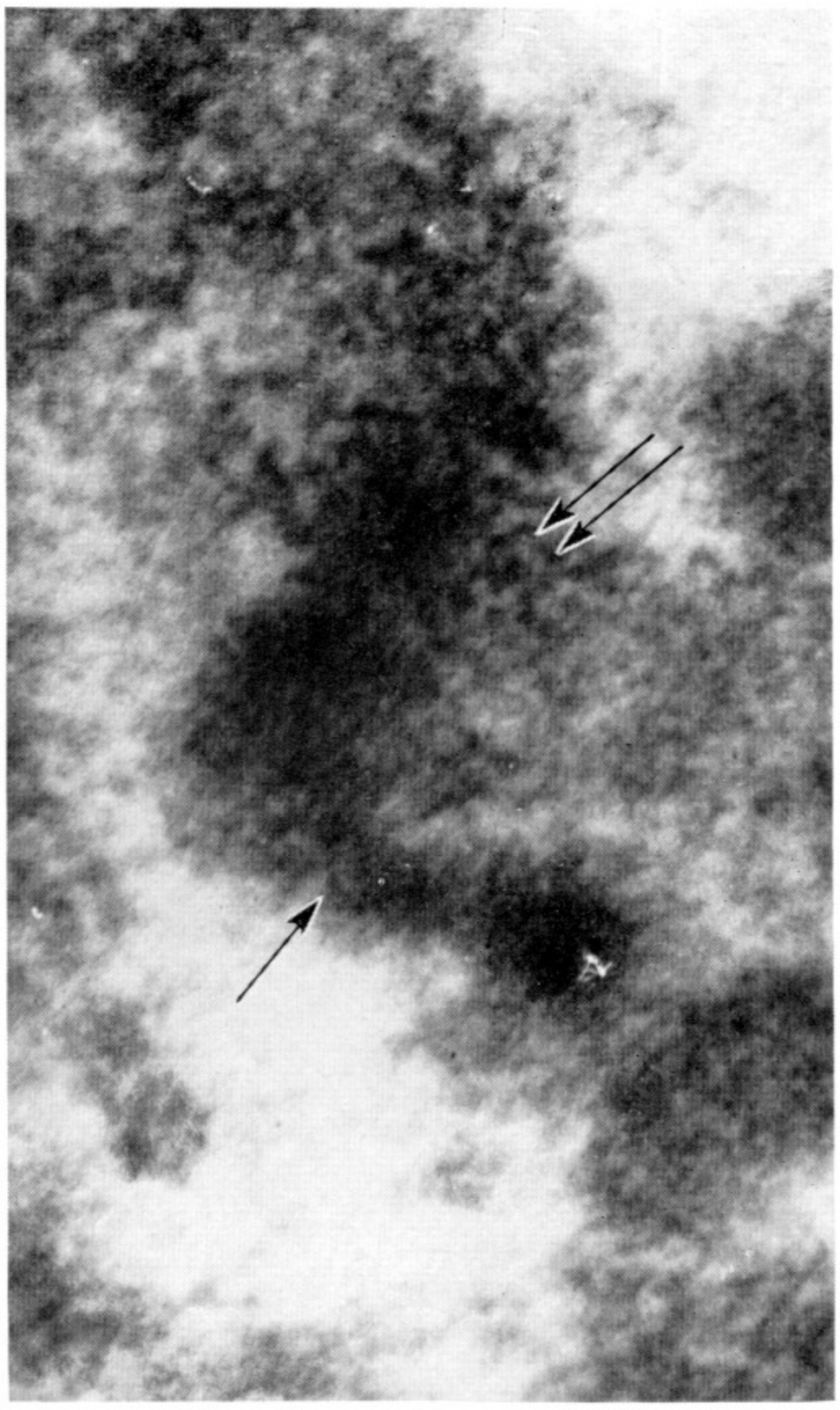
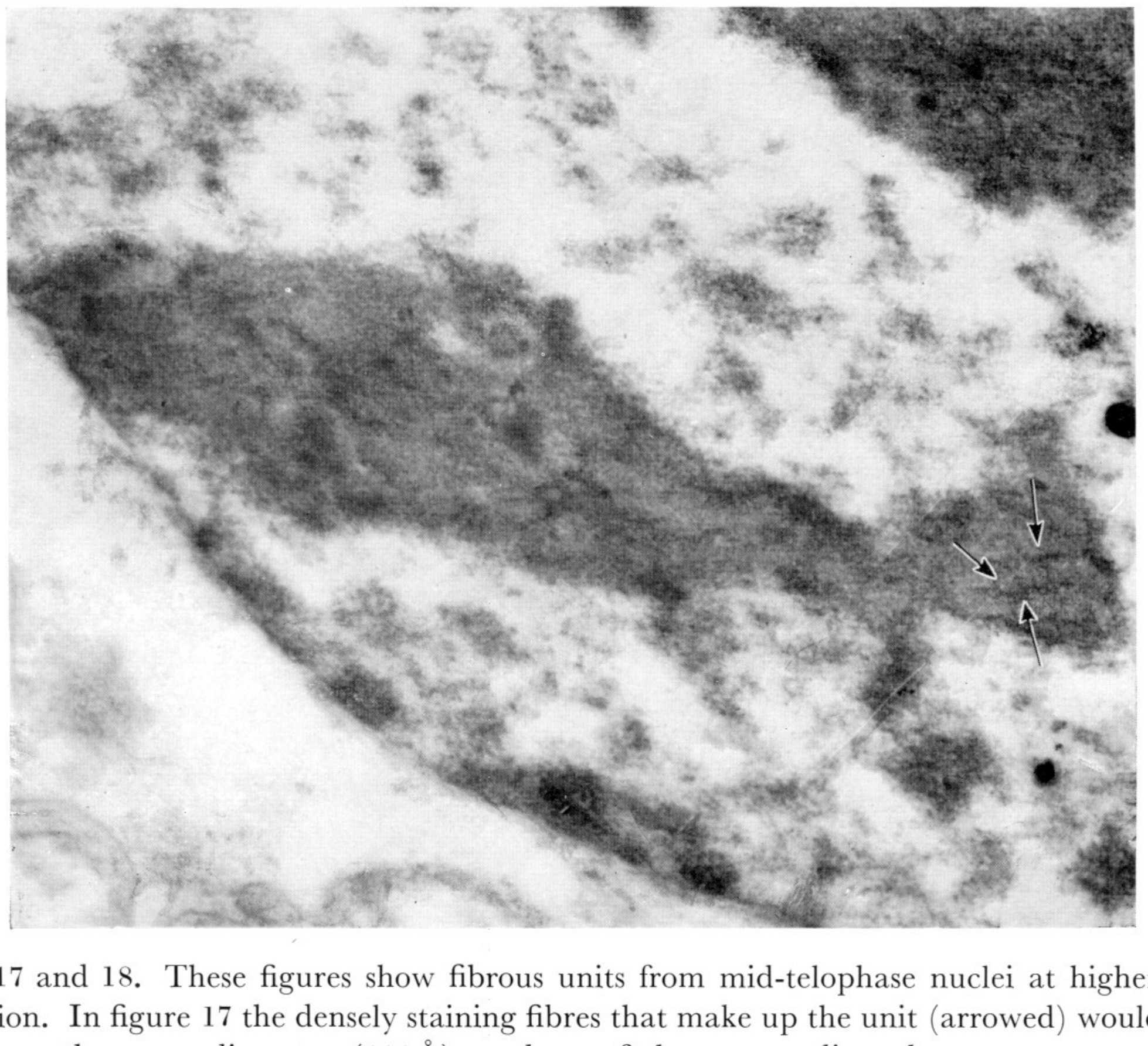


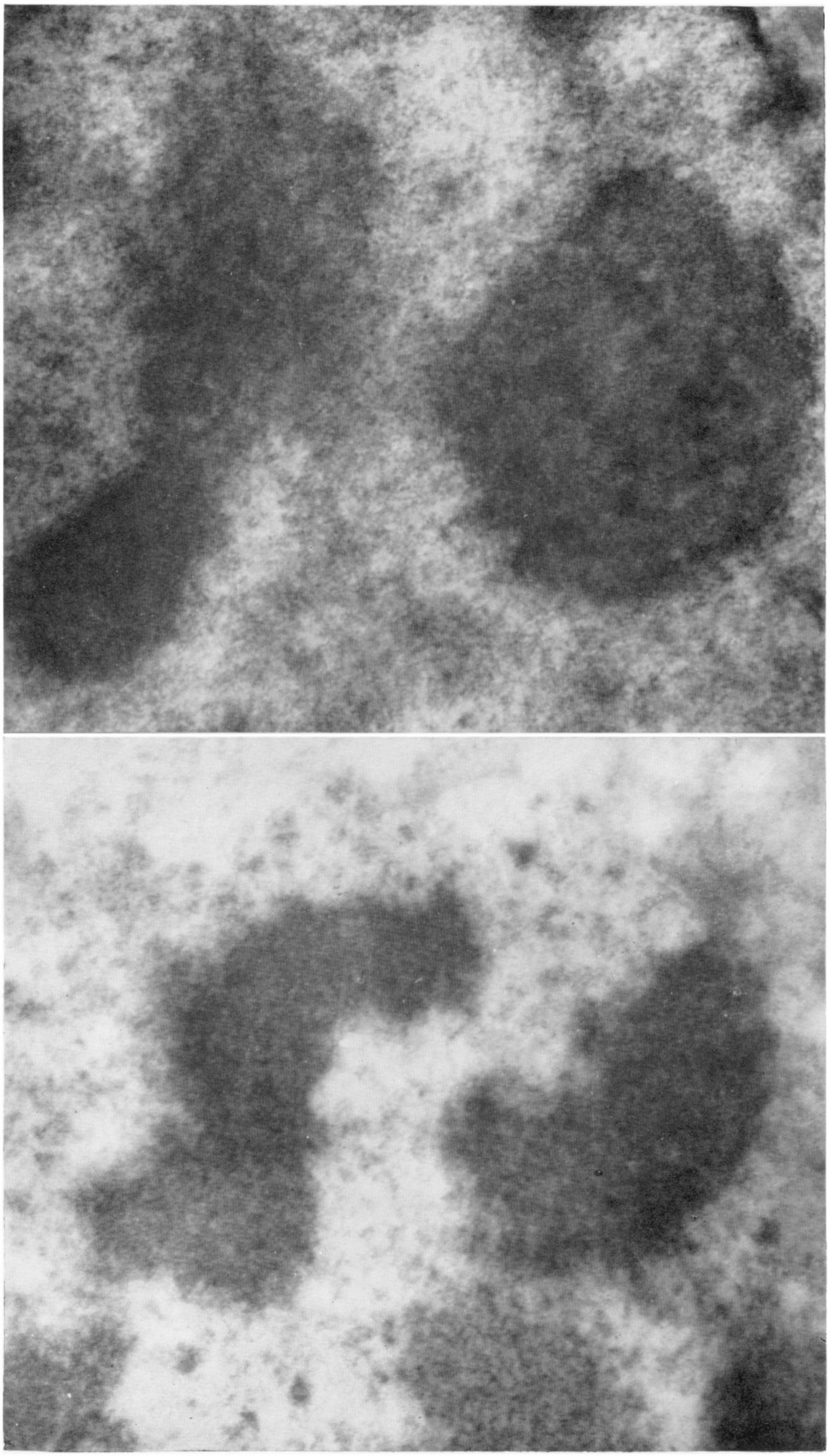
18



19

GURES 17 and 18. These figures show fibrous units from mid-telophase nuclei at higher magnification. In figure 17 the densely staining fibres that make up the unit (arrowed) would appear to have the same diameter (100 Å) as those of the surrounding chromosome material. In figure 18, however, the fibres that make up the 1000 Å units (single arrow), are much finer than those of the surrounding chromosome material (double arrow). (Magn. × 90 000.)

FIGURE 19. This figure shows an area of prenucleolar material from a late-telophase nucleus. No nucleolonema fibres are visible, but elongated lightly stained areas of similar diameter (arrowed) can be seen. (Magn. × 33 750.)



20

21

FIGURE 20. Two nucleoli from a regenerating liver cell irradiated with a dose of 450 r at 24 h after operation, and fixed 4 h later. The densely staining nucleolonema fibres are no longer clearly visible. (Magn. $\times 30\,000$.)

FIGURE 21. This figure shows two anaphase chromosomes from a regenerating liver cell irradiated with a dose of 2000 r at $28\frac{1}{2}$ h after operation and fixed 30 min later. They still consist of fibres approximately 100 Å in diameter, but the packing of their fibres, especially at the surface of the chromosomes, is much looser than normal and this gives the chromosomes a rather ragged appearance. (Magn. $\times 60\,000$.)