NITROUS OXIDE: EFFECTS ON THE MITOTIC APPARATUS AND CHROMOSOME MOVEMENT IN HeLa CELLS

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ABSTRACT

When HeLa cells were grown in the presence of nitrous oxide (N_2O) under pressure (80 lb/in^2) mitosis was inhibited and the chromosomes displayed a typical colchicine metaphase (c-metaphase) configuration when examined by light microscopy. When the cells were returned to a 37°C incubator, mitosis was resumed and the cells entered G_1 synchronously. Ultrastructural studies of N_2O-blocked cells revealed a bipolar spindle with centriole pairs at each pole. Both chromosomal and interpolar (pole-to-pole) microtubules were also present. Thus, N_2O, unlike most c-mitotic agents, appeared to have little or no effect upon spindle microtubule assembly. However, the failure of chromo somes to become properly aligned onto the metaphase plate indicated an impairment in normal prometaphase movement. The alignment of spindle microtubules was frequently atypical with some chromosomal microtubules extending from kinetochores to the poles, while others extended out at acute angles from the spindle axis. These ultrastructural studies indicated that N_2O blocked cells at a stage in mitosis more advanced than that produced by Colcemid or other c-mitotic agents. Like Colcemid, however, prolonged arrest in mitosis with N_2O led to an increased incidence of multipolar spindles.

INTRODUCTION

Many agents are known to inhibit the mitotic process in mammalian cells. Some such as colchicine, the vinca alkaloids, and cold shock have been extensively studied with regard to their mode of action at the molecular level. However, the precise mechanism of action of some of the gases, viz., xenon, argon, and nitrous oxide, which are known to exert similar effects on the mitotic process, is not well documented. Nitrous oxide (N_2O), when applied at atmospheric pressures, produced c-mitosis in *Pisum sativum* (Ostergren, 1944) but it had to be applied at a higher pressure to obtain a similar result in *Allium cepa* (Ferguson et al., 1950). This work led Rao (1968) to utilize N_2O under pressure (80 lb/in^2) to synchronize HeLa cells by reversible mitotic arrest. The present study was designed to

elucidate the mechanism of N_2O inhibition by ultrastructural analysis of HeLa cells immediately after the treatment and during the subsequent period of recovery. The electron microscope (EM) studies revealed that N_2O, unlike other mitotic inhibitors, had no significant effect on the formation of the spindle microtubules. However, it was found that N_2O treatment interfered with the alignment of chromosomes on the metaphase plate. This study indicated that N_2O blocked cells at a later stage in mitosis than Colcemid.

MATERIALS AND METHODS

HeLa cells were maintained in exponential growth at 37°C as suspension cultures in Eagle's minimal essential medium supplemented with nonessential

amino acids, sodium pyruvate and 5% fetal calf serum. The cell cultures were partially synchronized by 2.5 mM thymidine (TdR) blockade for 16 h. After release, the cells were resuspended in fresh media and plated in 60-mm petri dishes (2.5–3 × 10⁶ cells per dish). At 4 h after plating, when most of the cells were attached to the dish, the medium was removed along with the floating cells and replaced with fresh medium. Immediately after the medium change, the dishes were transferred to the pressure bomb, where the cells were exposed to N₂O under pressure at 80 lb/in² for various periods of time. At the end of the treatment the rounded mitotic cells were selectively harvested for microscopic observations. Within 5 min after the release of the N₂O block, cells were either fixed in glutaraldehyde or transferred to an incubator and allowed to recover. Cell samples were taken at regular intervals during recovery and processed for light and electron microscopy.

For light microscopic preparations, cells were removed from the culture dishes by vigorous pipetting and then spun onto microscope slides in a cyto-centrifuge (Rao and Johnson, 1970). Slides were fixed in glacial acetic acid-ethanol (1:3) mixture and stained with aceto-orcein.

For electron microscopy, cells were spun into a pellet and resuspended in 3% Millonig's phosphate-buffered glutaraldehyde for 1 h. After postfixation in 1% osmium tetroxide, the cells were dehydrated and embedded in Epon 812 according to procedures previously described (Brinkley et al., 1967), Serial sections were cut with a diamond knife and picked up on collodion-coated, slotted grids. The sections were stained in alcoholic uranyl acetate followed by lead citrate and examined with either a Hitachi HU-11C or a Philips EM 300 electron microscope.

RESULTS

Ultrastructure of Spindle and the Arrangement of Chromosomes in N₂O-Blocked Mitoses

After N₂O treatment the chromosome configuration in almost all the mitotic cells resembled configurations produced by c-mitotic agents when viewed in the light microscope (Fig. 1). No typical metaphase or anaphase configurations were observed. After transfer to atmospheric conditions, the chromosomes became aligned on the metaphase plate and a typical bipolar spindle was observed (Fig. 2). Afterwards the cells approached anaphase in synchrony (Fig. 3) and division was completed in approximately 60–80 min. When N₂O-arrested cells were sectioned and observed with the electron microscope, it was immediately

apparent that the cells were not arrested in a typical c-metaphase configuration. Fig. 4 shows a low magnification survey electron micrograph of cells exposed to N₂O for 9 h. When these cells were examined at a higher magnification, both chromosomal and continuous microtubules were apparent. Sections taken parallel to the major spindle axis indicated a typical bipolar spindle with interpolar microtubules and paired centrioles at each pole (Figs. 5 and 6). In fortuitous sections we observed microtubules extending for long distances without association with chromosomes. These were often curved and converged toward the centrioles (Fig. 6). The poles of N₂O-treated cells were generally separated by a distance of 20–25 μm. All microtubules appeared to terminate into an amorphous electron-opaque zone around the centrioles. Although astral fibers were sparse, a few could be seen extending out from the poles.

The most unusual feature of N₂O-treated cells was the distribution of chromosomes on the mitotic spindle. In most cases, chromosomes appeared to be dispersed randomly throughout the spindle, although a few were occasionally seen positioned equidistant between the poles. The kinetochore appeared normal in fine structure (Figs. 7 and 8). In most instances, chromosomal microtubules could be seen associated with the kinetochore. Although some of the microtubules extended in the direction of the poles, many were seen to extend out at widely divergent angles from the major axis of the spindle (Figs. 7 and 8). As shown in these figures, microtubules from a single sister kinetochore were often seen extending out at two different angles. Such arrangements were never seen in normal dividing HeLa cells.

The unusual distribution of chromosomes on the spindle and the absence in some cases, of typical alignment of kinetochore microtubules with continuous microtubules led us to look for cross-bridges between adjacent microtubules. As can be seen in Fig. 9 A–C, crossarms can be observed both in longitudinal and transverse sections. These were obvious only when adjacent microtubules lay in close opposition over long distances. No bridges were apparent in microtubules that extended from the kinetochore at atypical angles to the spindle axis.

Cytoplasmic organelles appeared normal for the most part, except for unusual mitochondria in some cells. As shown in Fig. 10, the mitochondria were often greatly distorted with unusual vesicular cris-
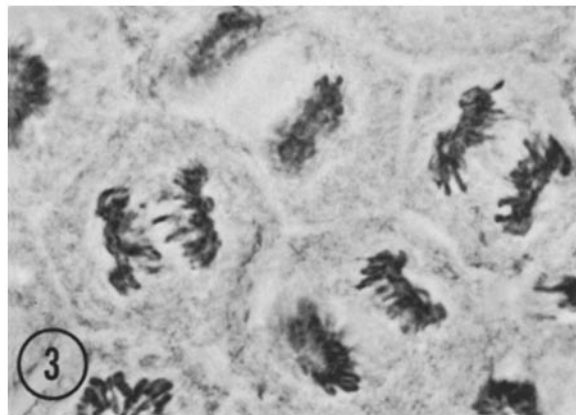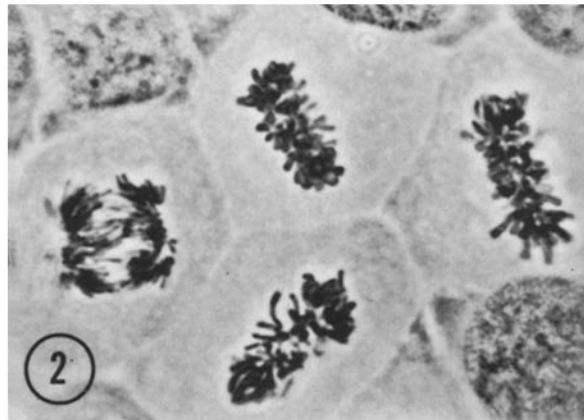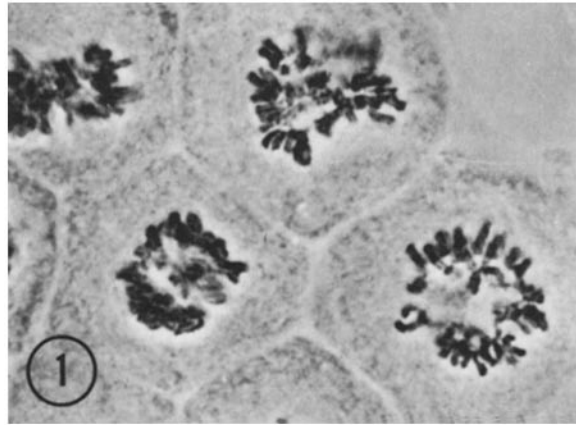
FIGURE 1 HeLa cells treated with N₂O for 9 h. Note typical c-mitotic appearance of chromosomes. × 1,000.

FIGURE 2 Cells which were treated with N₂O as in Fig. 1 but allowed to recover for 30 min. Three cells are in metaphase while one has reached anaphase. × 1,000.

FIGURE 3 Cells in late anaphase after 60-min recovery from N₂O treatment. × 1,000.

tae. They were often extended and flattened with the cristae appearing to be twisted into a helix. These unusual forms were not seen in all cells treated with N₂O and were never apparent either in untreated cells or those which were undergoing recovery after release from N₂O. It should be pointed out that almost identical forms of mitochondria can be seen in HeLa cells after treatment
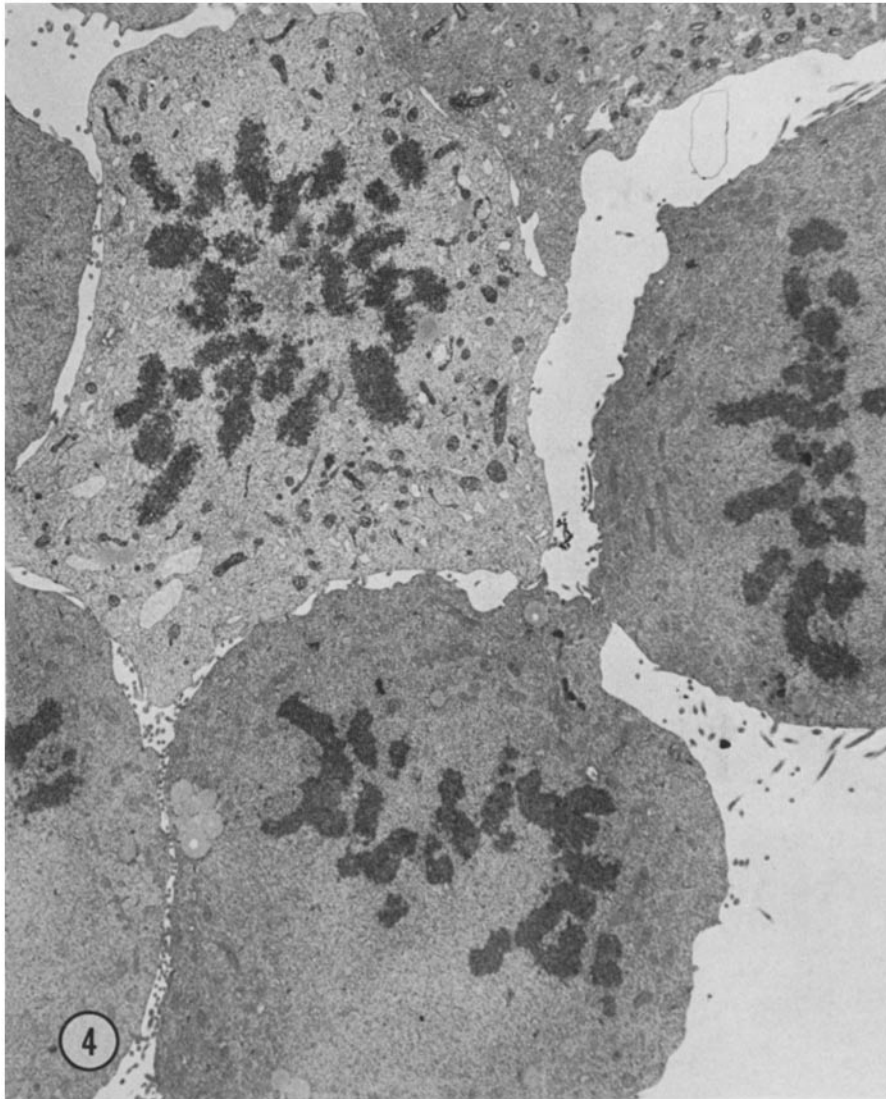
FIGURE 4 Survey electron micrograph of three cells which have been treated with N_2O for 9 h. × 7,500.

with vincristine (George *et al.*, 1965). The significance of these unusual mitochondrial aberrations is not known, but they may suggest impairment in respiratory function by the N_2O.

Reversal of N_2O inhibition was well underway at 30 min after the cells were transferred to the incubator at atmospheric pressure. One of the earliest changes was the shortening of the pole-to-pole distance from 20–25 μm down to 10–12 μm as shown in Fig. 11. Kinetochore microtubules became more closely aligned with interpolar microtubules although a few microtubules were still

seen extending from the spindles at unusual angles up until metaphase. Metaphase appeared normal with chromosomes typically positioned at the equatorial region (Fig. 12). Anaphase, telophase (Fig. 13), and subsequent events of mitosis also appeared normal and were similar to those described for untreated HeLa cells (Robbins and Gonatas, 1964).

Location of N_2O Block during Mitosis

The ultrastructure of the mitotic apparatus in N_2O-blocked cells was distinctly different from
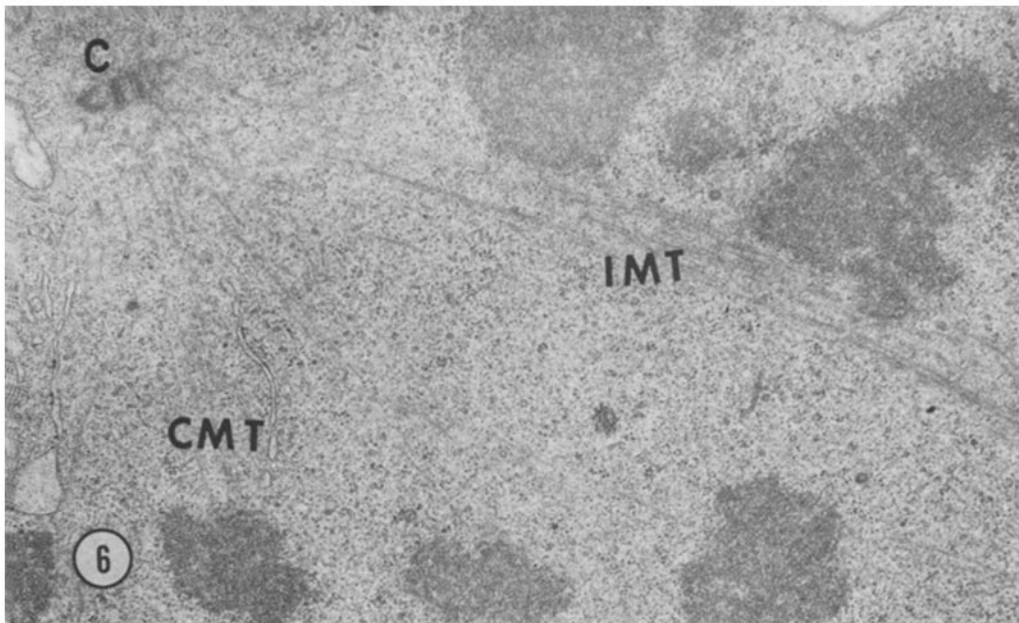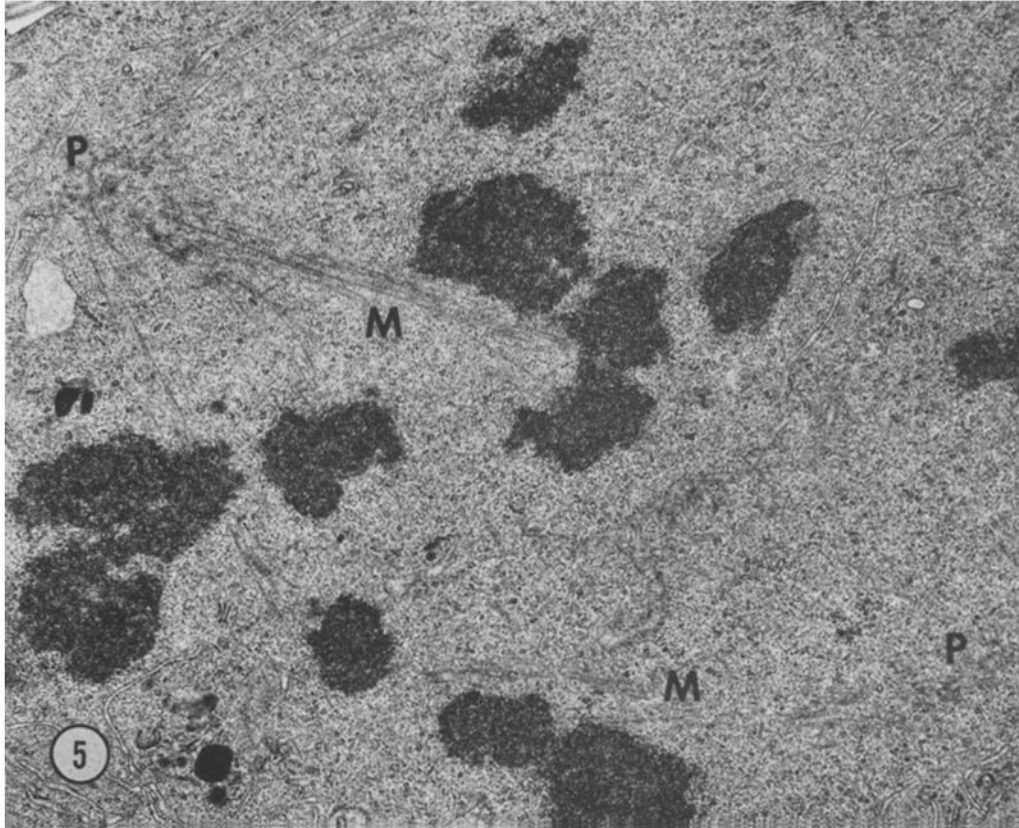
FIGURE 5   A bipolar spindle is seen with microtubules (*M*) converging onto each pole (*P*). × 16,500.

FIGURE 6   A pair of centrioles (*c*) is seen at one pole. Both chromosomal microtubules (*CMT*) and interpolar microtubules (*IMT*) are apparent. × 34,000.
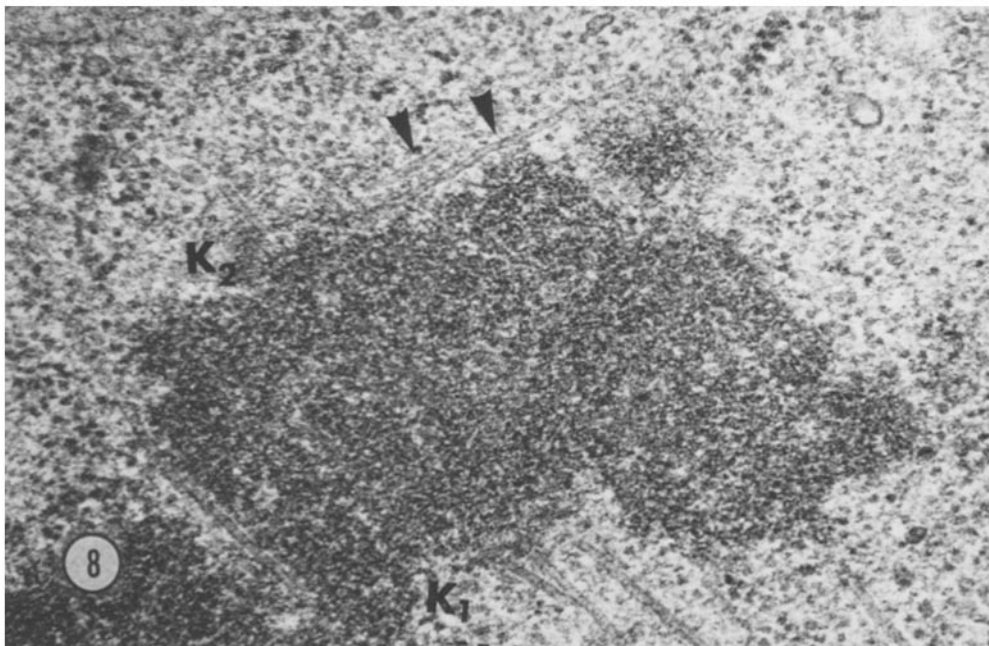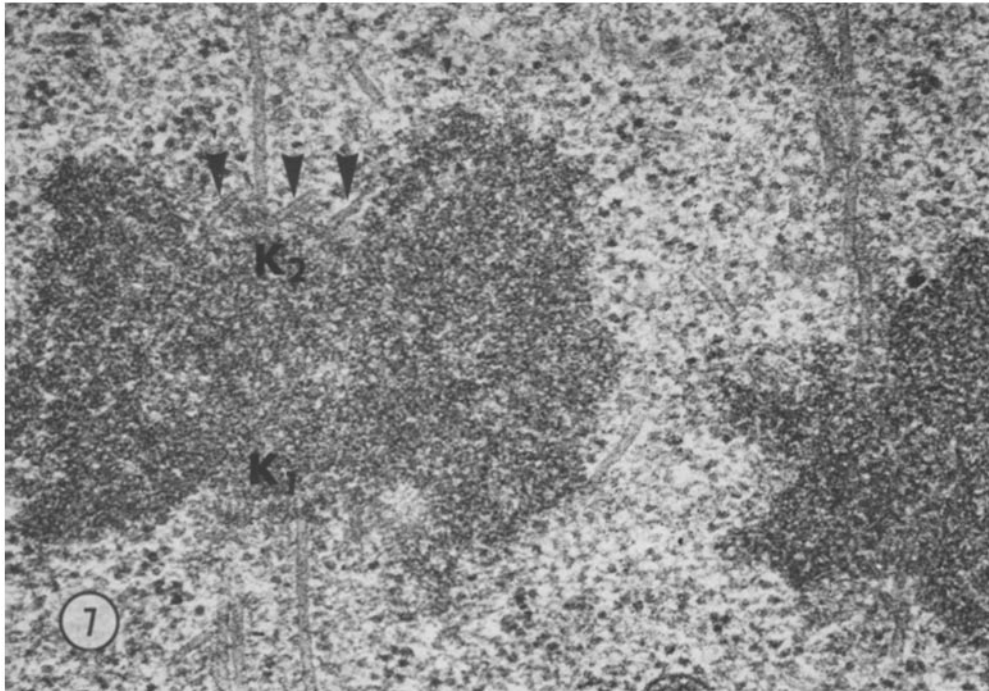
FIGURE 7 Higher magnification showing microtubules associated with each daughter kinetochore (K_1 and K_2). Note microtubules extending out at acute angles from K_2 (arrowheads). $\times$ 65,500.

FIGURE 8 Microtubules (arrowheads) are seen extending from kinetochore (K_2) at an angle perpendicular to the spindle axis .$\times$ 44,000.
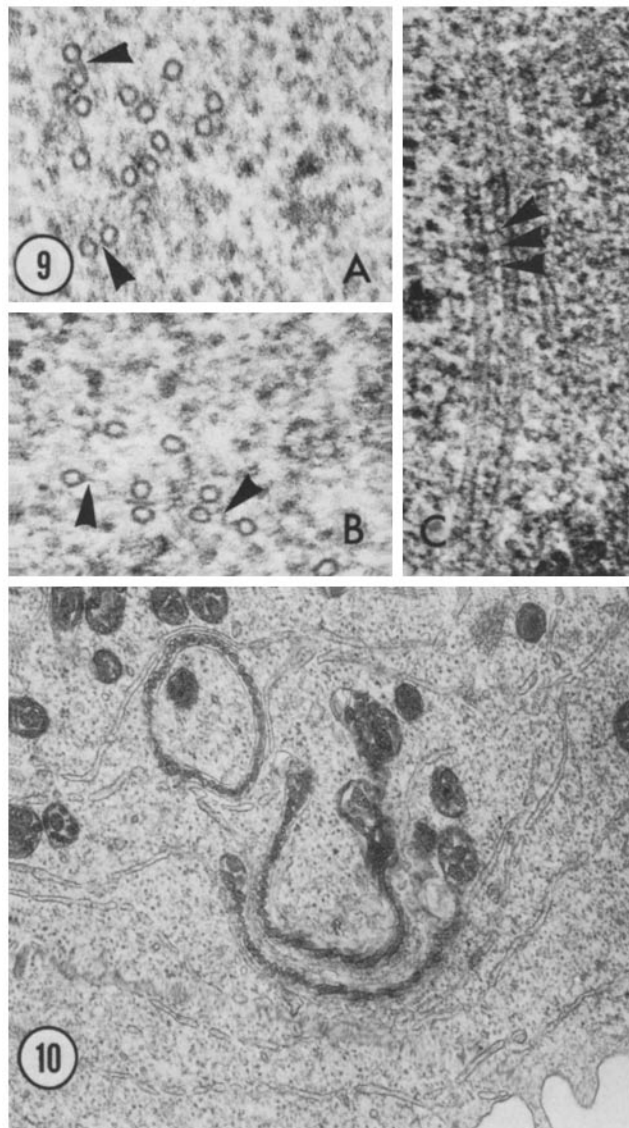
FIGURE 9 A–C   Transverse and longitudinal sections of microtubules from N₂O-arrested cells showing typical crossarms (arrowheads) between adjacent microtubules. × 105,000.

FIGURE 10   Unusual mitochondria are frequently seen in N₂O-arrested cells. × 36,750.

that of Colcemid-blocked cells. In Colcemid-treated cells the migration of centrioles and the formation of pole-to-pole (interpolar) microtubules are selectively inhibited (Brinkley et al., 1967). In the presence of N₂O, centriole pairs appeared to be positioned at opposite poles of the cell and interpolar microtubules were present; however, most of the chromosomes did not migrate to the metaphase plate. The relative position of centrioles in

N₂O-treated cells as compared to those treated with Colcemid suggests that N₂O blocks cells at a later stage in mitosis than Colcemid.

Effect of N₂O Treatment on the Number of Poles of the Mitotic Spindle

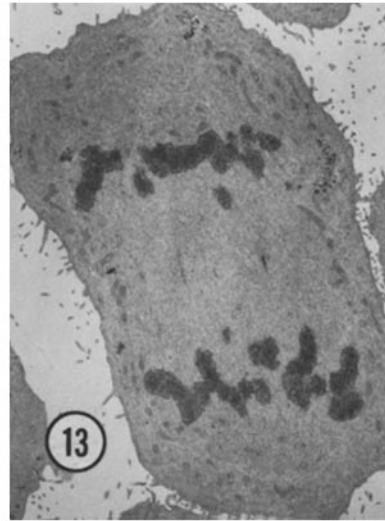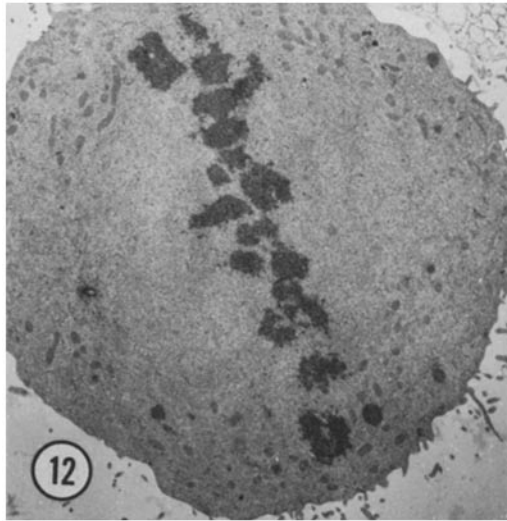During the reversal of the N₂O block, we observed a high incidence of multipolar spindles
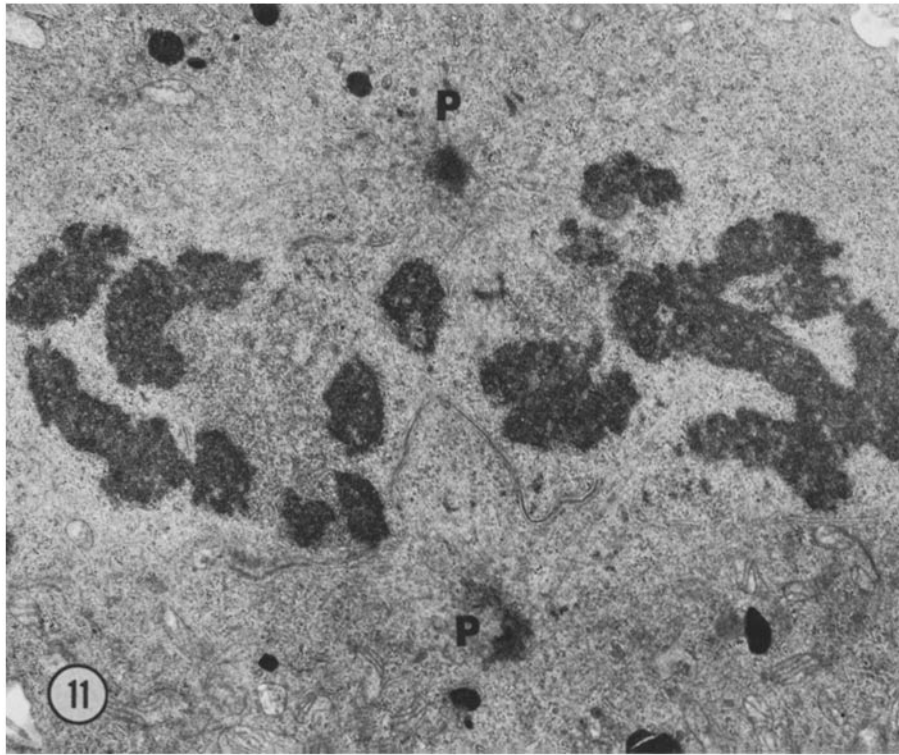
FIGURE 11   30 min after reversal from N₂O arrest. Note position of poles (P). Chromosomes are becoming aligned onto the metaphase plate. × 15,000.

FIGURE 12   Survey micrograph of typical metaphase after 60-min reversal from N₂O arrest. × 6,000.

FIGURE 13   Typical telophase cell from culture which had been reversed for 60 min after N₂O treatment. × 6,000.

among the dividing cells. In order to further investigate the effects of N_2O on the poles of the mitotic apparatus, the following experiment was performed to discover whether there was any correlation between the duration of N_2O treatment and the incidence of multipolarity among the dividing cells. HeLa cells were synchronized in S phase by the reversal of excess thymidine (2.5 mM) double-block technique (Rao and Engleberg, 1966). About 7 h after the reversal of the second TdR block, cells were plated in a number of 60-mm plastic dishes. One dish was kept in an incubator at 37°C to serve as a control while the rest of them were subjected to N_2O treatment for varying periods of time. Dishes were taken out of the N_2O chamber at 4, 12, 16, and 36 h of N_2O (80 lb/in² at 37°C) treatment and then transferred to an incubator at 37°C to reverse the mitotic block. Cells were collected from the dishes by vigorous pipetting and slides were made using a cytocentrifuge (Rao and Johnson, 1970) at 30, 60, and 90 min of incubation. The shorter the duration of N_2O block the faster was the rate of reversal. At 60 min after the reversal of a 4-h N_2O block, most of the mitotic cells were in anaphase or telophase. For a mitotic population to reach a similar state of reversal it required 90

min after a 12- or 16-h block and 3 h after a 36-h block. The cells were fixed and stained for light microscopy. Only the cells in metaphase, anaphase, and telophase were scored for polarity. The numbers of cells with bi-, tri-, or tetrapolar spindles were expressed as a percentage of the total number of cells scored. These data plotted as a histogram (Fig. 14) clearly indicate that the longer the cell is blocked in mitosis the greater the chance of it developing a tri- or tetrapolar spindle. Blocking the cells in S phase by excess thymidine treatment for a period of 14 h did not increase the incidence of multipolar divisions over the control values.

DISCUSSION

Although the molecular basis of mitotic arrest by N_2O and similar narcotic gases remains to be elucidated, it is apparent from these results that N_2O blocks mitosis in a manner entirely different from that of other c-mitotic agents. Colchicine and colchicine-like compounds (i.e., Colcemid, vinblastine and vincristine sulfate, podophyllin) arrest mitosis by inhibiting spindle formation (for reviews, see Eigsti and Dustin, 1955; Biesele, 1958; Mazia, 1961; Kihlman, 1966; and Brinkley and Stubblefield, 1970). Colchicine binds to microtubule proteins (tubulin) and prevents the assembly of spindle microtubules (Taylor, 1965; Borisy and Taylor, 1967 a, 1967 b). At minimal doses, Colcemid selectively inhibits the formation of interpolar microtubules in mammalian cells and prevents the separation of centriole pairs to appropriate poles at prophase (Brinkley et al., 1967 and Brinkley and Stubblefield, 1970).

The presence of bipolar spindles with both interpolar and kinetochore microtubules in cells treated with N_2O suggests that mitotic arrest by this treatment is not due to inhibition of microtubule assembly. However, the absence of complete alignment of chromosomes on the metaphase plate suggests that the treatment prevents normal prometaphase movement. The unusual appearance of microtubules extending out at various angles from the kinetochores of many chromosomes indicates some impairment in the alignment of kinetochore microtubules with centrioles and interpolar tubules. Moreover, this would imply that some interaction between these two types of microtubules is necessary for normal prometaphase movement. Whether or not such interaction facilitates sliding of antiparallel microtubules, as
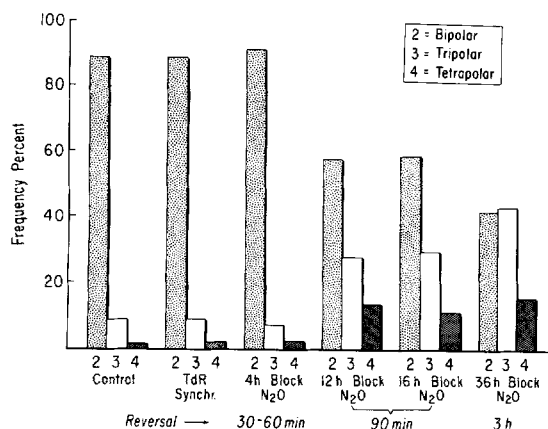

Figure 14 Effects of duration of N_2O treatment on spindle polarity. The three vertical bars indicate the frequency (percent) of anaphase cells with bi-, tri-, and tetrapolar spindles. Note that cells synchronized with TdR as well as those exposed to N_2O for only 4 h were essentially the same as control. N_2O blockage for 12 h or more led to a pronounced increase in the number of tri- and tetrapolar spindles. Tripolar spindles were more frequent than tetrapolar spindles.

suggested by McIntosh and co-workers (1969, 1971), is yet to be determined. For sliding or shearing to take place according to their model, close lateral association of all tubules would be necessary. The presence of crossarms between some parallel microtubules in N_2O-treated cells suggested that this treatment had little effect on these structures. Similar crossarms are seen between microtubules in a variety of mitotic cells (Wilson, 1969; Hepler et al., 1970; Brinkley and Cartwright, 1971). Although their function is not yet known, they could be the hypothetical force transducers as proposed by McIntosh et al., (1969).

From these observations, we conclude that N_2O treatment under pressure had little or no effect on microtubule assembly either at the poles (interpolar microtubules) or at the kinetochore. Thus, microtubule assembly at prometaphase and the interaction of spindle microtubules to bring about chromosome movement appear to be two separate events which can be uncoupled by the application of N_2O.

Although we have no information on the molecular interaction of N_2O with the spindle, we can assume that its effect is not due to simple anoxia. Thus, Rao (1968) has shown that when nitrogen is applied to HeLa cells under identical conditions, no increase in mitotic index is observed. Similarly, it is unlikely that pressure alone, especially at the order of magnitude (80 lb/in^2) used in these experiments, could have any effect on the microtubules. Ferguson et al. (1950) concluded that pressure was necessary in order to achieve the necessary concentration of N_2O within the cell to permit effective thermodynamic activity of the gas. As pointed out earlier, however, Ostergren (1944) observed c-mitosis in *P. sativum* at ordinary pressures.

The unusual structure of mitochondria in some N_2O-treated cells suggests that this treatment may have some effect upon cellular respiration. This feature remains unexplained and merits further study.

The fact that the formation of the spindle is not affected by N_2O treatment indicates that the mode of mitotic arrest by N_2O is different from that of Colcemid or any other c-mitotic agent. The separation of centriole pairs and the establishment of poles in N_2O-blocked mitotic cells suggest that the cell is actually nearer to true metaphase than when blocked with Colcemid.

The high incidence of multipolar spindles after

prolonged mitotic arrest with N_2O (Fig. 14) is consistent with results obtained by other investigators using widely different antimitotic agents. As Mazia (1961) has pointed out, the incidence of multipolarity appears to be greater after prolonged arrest or delay in mitosis. As he first pointed out, this is apparently due to the maturation of additional poles or "mitotic centers" which become functional upon resumption of mitosis. Stubblefield (1967) arrived at a similar conclusion from time lapse and electron microscopic analysis of Chinese hamster cells treated for prolonged periods with Colcemid. He concluded that the appearance of additional poles was due to maturation and separation of daughter centrioles. The fact that we observed more tripolar than tetrapolar spindles is somewhat unexpected in view of Stubblefield's interpretation. Thus, according to his view, maturation of both daughters would be expected to lead to a greater proportion of tetrapolar spindles. Moreover, one would expect to find a single centriole at the poles, whereas we have consistently found pairs of centrioles at the poles of multipolar spindles. This problem is the subject of a separate study in our laboratory.

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