PRESSURE-INDUCED DEPOLYMERIZATION OF SPINDLE MICROTUBULES

I. Changes in Birefringence and Spindle Length

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ABSTRACT

Changes in birefringence retardation (BR) and length of Chaetopterus meiotic metaphase-arrested spindles produced by increased hydrostatic pressure were observed with polarized-light microscopy using a newly developed optical pressure chamber. Increased pressure produced rapid, reversible decreases in spindle BR and length. Pressures of 3,500 psi or higher at 22°C caused complete disappearance of spindle BR within 3 min. Up to 6,000 psi, the rates of both BR decay and spindle shortening increased progressively with increasing pressure. At 6,000 psi or above, the BR decreased rapidly but there was no evidence of spindle shortening. The general observations are consistent with results of earlier classical experiments on effects of pressure on mitosis, and with experiments that used colchicine or low temperature as microtubule-depolymerizing agents. The kinetics of spindle depolymerization and repolymerization showed two phases: an initial phase of rapid decrease or increase in half-spindle microtubule BR; and a second phase of nearly constant BR during which most of the spindle shortening or growth occurs. BR is assumed to be directly related to the number of microtubules in a spindle cross section. It is hypothesized that microtubules in the spindle have different stabilities depending on the attachment or nonattachment of their ends. This hypothesis is used to explain the two phases of spindle depolymerization and repolymerization as well as several other observations.

Most current theories of the mechanisms of mitosis (Inoué and Sato, 1967; McIntosh et al., 1969; Nicklas, 1971; Dietz, 1972; Bajer, 1973) incorporate the concept, as proposed by Inoué (1959, 1964), that spindle fiber microtubules are labile, being in a “dynamic equilibrium” with a cellular pool of tubulin subunits. Spindle microtubules can be reversibly depolymerized, and mitosis blocked, by numerous agents including low temperature and colchicine (Inoué and Sato, 1967). Inoué’s concepts also predict that moderate hydrostatic pressure (less than 6,000 psi), at normal physiological temperatures, will substantially reduce the degree of spindle microtubule assembly.

The influence of pressure on an assembly reaction is inversely related to the degree of association of the subunits at atmospheric pressure, and directly related to the difference between the molar volume of the reactants and the products (Le Chatelier’s principle). The lability of spindle mi-
Microtubules indicates that the microtubule subunits are weakly associated. Thermodynamic analysis of changes in equilibrium spindle birefringence retardation (BR) at metaphase as a function of temperature, in a variety of cell types, has indicated that the spindle microtubule polymerization involves large increases in enthalpy and entropy (Inoué, 1959; Inoué and Sato, 1967; Sato and Bryan, 1968; Stephens, 1973). Such thermodynamic properties are characteristic of the endothermic in vitro assembly of actin (Oosawa and Kasai, 1971), tobacco mosaic virus (TMV) protein (Lauffer, 1971), myosin (Josephs and Harrington, 1968), and bacterial flagellin (Gerber and Noguchi, 1967). The assembly of these proteins involves an unusually large molar volume increase (150–700 ml/mol subunit). By analogy, the assembly of spindle microtubules can be presumed also to involve a large increase in molar volume.

There is some experimental evidence that hydrostatic pressure does affect the assembly of spindle fibers. Pease (1941, 1946), and Marsland (1970 review) and his collaborators (Zimmerman and Marsland, 1964) have gathered evidence from fixed and stained preparations or phase-contrast observations of isolated spindles that pressures from 3,000 to 6,000 psi inhibit mitosis in several types of cells, presumably by disorganizing spindle structures. The general effects of increased pressure were similar to those of lowered temperature. Preliminary electron micrographs (Zimmerman, 1970) indicate that the disorganization of spindle fibers by pressure is accompanied by loss of spindle microtubules.

In the studies reported here I have examined directly the effects of pressure on polymerization of spindle fiber microtubules by using polarization microscopy and a newly developed microscope pressure chamber. Currently, measurements of the BR of spindle fibers provide the best estimate of the degree of polymerization of spindle fiber microtubules in the living cell. Microtubules in the central spindle are aligned essentially parallel to one another, and it is this alignment that produces the spindle BR. Changes in the magnitude and distribution of spindle BR have been correlated with changes in the number and distribution of microtubules seen in electron micrographs (Inoué and Sato, 1967; Rebhub and Sander, 1967; Malawista et al., 1968; Goldman and Rebhub, 1969; Jensen and Bajer, 1973; LaFountain, 1974). Sato et al. (1971) have determined that the Wiener's equation describes accurately the relationship between magnitude of BR and the number of microtubules in a spindle cross section. Also, Stephens (1972) demonstrated that the amount of tubulin extracted from isolated spindles is proportional to the spindle BR in vivo. Consequently, BR seems to be a good indicator of the number of microtubules in the spindle fibers or the concentration of polymerized tubulin in the spindle region.

This paper describes how increases in hydrostatic pressure effect kinetic changes in spindle BR and thus, in number of spindle microtubules, as well as kinetic changes in spindle length. It also provides background material for later studies of the thermodynamics of spindle microtubule assembly and of depolymerization-induced chromosome movement.

**MATERIALS AND METHODS**

**Experimental Material**

To avoid effects of variations in mitotic stages, the first maturation spindle in oocytes of the annelid *Chaetopterus pergamentaceous* was primarily studied. Unless the egg is fertilized or artificially activated, the spindle remains in the metaphase state for 10–20 h.

*Chaetopterus* were collected from a depth of approximately 30 ft in Buzzards Bay near Woods Hole, Mass., between 1 July and the middle of August during 1972 and 1973. Procedures for care of the animals and collection of gametes were essentially those described by Inoué (1952a). The animals were carefully removed from their tubes and placed in individual finger bowls. The sexes were segregated and kept in separate tanks with running seawater. Generally, the animals remained healthy and ripe for at least 1 mo under these conditions.

Gametes were collected from amputated parapodia, the animal being returned to its finger bowl for future use. Sperm was diluted and used directly. Oocytes were washed three times with filtered seawater, dispersed in a syracuse dish, and held on the seawater table to maintain a relatively constant temperature of 17–21°C. One parapodium yielded approximately 0.1 ml of packed eggs.

The first meiotic spindle of the *Chaetopterus* oocyte appears 10–15 min after being shed into seawater at 20°C and reaches full metaphase configuration 10–20 min later. The eggs are very yolky, but the presence of the spindle is easily detectable near the cell cortex as an area free of yolk granules that grows in size as does the spindle. Typically, the spindle is positioned within this clear zone with the smaller aster closely attached to the cell surface. The interfering yolk, some of which is birefringent, must be removed from around the spindle region in order to see clearly the spindle structure or the chromosomes. Flattening the egg to approximately 0.75–0.5 its normal diameter removed most of the yolk.
from above and below the spindle, especially when the
attached spindle pole lay in the plane of flattening. In this
flattened state the eggs appeared to behave normally.

Better image quality could be obtained if the eggs were
first centrifuged at 1,000 g for 5 min on a sucrose
step-gradient using a mixture of 90% 1.1 M sucrose and
10% filtered seawater. After this treatment the spindle,
still attached to the cortex by one pole, was usually
positioned in a clear zone between the oil cap region
and the newly packed yolk region. Not only was a better
polarized-light image of the spindle possible, but after the
eggs were flattened the chromosomes could often be seen
clearly in phase-contrast microscopy.

The best image, however, was obtained when the yolk
was completely removed from the spindle region by
making clear 0.25-μl egg fragments, using the centrifu-
gation methods described by Inoué (1952 a). These
fragments were nearly transparent and the spindles often
were as large and birefringent as in whole eggs. The
fragments were very fragile, however, and thus more
difficult to handle than whole eggs. Also, spindles in
some of the smaller fragments appeared to have both
poles attached with equal strength across the fragment.
Consequently, flattened, or less frequently, stratified and
flattened eggs were used.

In addition, a brief survey was taken of the minimum
pressures required to cause complete spindle depolymeri-
ation at active mitotic or meiotic metaphase in the
following species: Asterias forbesi; Echinarchaeis; Lyte-
chinae pictus; Lytechinus variegatus; and Nephrotoma
saturalis.

Pressure Chamber Preparation

A new miniature, temperature-controlled, optical hy-
drostatic pressure chamber, especially designed for work
with cells, was used in these experiments. Pressures as
high as 10,000 psi could be applied within 1 s by using a
Blackhawk hand-operated pressure pump (P-76, Black-
hawk Mfg. Co., Milwaukee, Wis.). Pressure was mea-
sured with a standard Bourdon gauge. The temperature
of the chamber was maintained at 22°C ± 0.25°C by
passing water from a temperature-regulated water res-
ervoir through a water jacket encircling the chamber.
A detailed description of the chamber and associated
equipment is given elsewhere (Salmon and Ellis, 1975).
Generally, a small drop of medium containing about
20–40 eggs was placed within the center of the lower win-
dow of the pressure chamber between two parallel ridges
of silicone grease and covered with a 2 mm × 4 mm cover
slip fragment (see Salmon and Ellis, 1975, for details).
A small amount of fresh filtered seawater was added
immediately to prevent effects of evaporation.

The best image contrast with polarized light was
achieved with specimens at the center of the chamber
windows. Before the chamber was closed, it was placed
on the polarized light microscope and the position of the
eggs examined. If necessary the eggs were repositioned
by gently moving the small cover slip so that a cell with
its spindle attached to the cell surface in the plane of the
chamber window would be near the center of the window.
The chamber was then filled with fresh filtered seawater
(±22°C) and installed in the temperature control stage
of the microscope.

Polarization Microscopy

In the polarized light experiments, BR measurements
and photographs were first taken at atmospheric pres-
sure. Then the pressure was increased to a magnitude
between 2,000 and 9,000 psi and held constant. When the
spindle reached a new equilibrium under pressure, the
pressure was released and the spindle allowed to recover.
After the spindle’s recovery to its normal state at
atmospheric pressure, this procedure was often repeated,
with different magnitudes of pressure being applied to the
same cell. Quantitative data were taken from a total of
31 pressure applications on 10 cells. At the end of several
experiments the cells were fertilized and allowed to
develop. Only rarely did there appear to have been any
detrimental effect from the pressure treatments.

To obtain data for changes in spindle BR and spindle
length a Nikon model S microscope was equipped with
HN22 unlaminated sheet polaroid for analyzer and
polarizer and a Bruce-Kohler rotating mica compensator
(25 nm). With two strain-free Leitz UM 20× long
working-distance objectives, one used as a condenser,
spindle BR could be detected by eye in the chamber over
a 0.75-mm central field to 0.1 nm up to 3,000 psi and to
0.4 nm up to 5,000 psi (Salmon and Ellis, 1975). Even
better performance was possible when the specimen was
located at the exact center of the window or when a
Nikon rectified 10× objective was used as for the 16-mm
cine-time-lapse movies. A 100-W mercury arc lamp
(Illumination Industries Inc., Sunnyvale, Calif., 110)
with both heat-cut and 546-nm interference filters pro-
vided high intensity illumination.

Photographic Methods

Spindle morphology and length were monitored by
taking photographs with either Kodak Plus X 35-mm
film or Kodak 7231 Plus X Neg 16-mm movie film
(Eastman-Kodak Co., Rochester, N.Y.). The 35-mm
photographs were obtained with a Nikon Microflex
camera system with a Zeiss 10X ocular. Exposure times
of between 5 and 10 s produced negatives with satisfac-
tory density and resolution when processed with Kodak
Microdol X developer. The 16-mm movies were taken at
40 frames per minute with the Sage series 300 cine-time-
lapse system. The light intensity given by a constant
compensator setting of about 4 nm resulted in a frame
exposure time of roughly 0.7 s as determined by the Sage
model 293 photometer for the specified film ASA of 80.
The 16-mm negatives were processed and positive prints
produced by Film Service Inc., Boston, Mass.
Measurement of Spindle BR

Spindle BR was measured by one of two techniques: (a) conventional methods employing the Brace-Kohler compensator (Hartshorne and Stuart, 1960, p. 299); or (b) densitometric measurements of the spindle image on 16-mm movie film. For both types of BR measurements, the spindle axis was positioned at 45° with respect to the analyzer and polarizer transmission directions. With the conventional compensator methods, the area of maximum half-spindle BR was brought to extinction by rotating the Brace-Kohler compensator vernier dial. A potentiometer attached to the dial generated an analog voltage to the vernier dial position. Changes in the dial position could easily be read on a voltmeter calibrated in degrees or nanometers (Salmon, 1973). Retardation measurements and time could be recorded accurately every 15-20 s.

Accurate BR measurements could be recorded twice as fast with the semiautomatic system described by Inoue et al. (1970). The analog voltage to the compensator position was sampled, when desired, onto a chart recorder along with a continuous time base, thus eliminating the time required to read and record BR and time data manually.

The difference \( \theta \) between the compensator angle for extinction of the spindle and the angle for the extinction of the background light was used to determine retardation in nanometers according to the formula: \( BR_{sp} = BR_{nsp} \sin 2\theta \) (reviewed by Hartshorne and Stuart, 1960, p. 299), where \( BR_{sp} \) is the spindle BR and \( BR_{nsp} \) is the maximum compensator BR (25 nm). For \( \theta \leq 10^\circ \), which is true for the spindle in these experiments, \( \sin 2\theta \approx 2\theta \) (in radian units) within 2% accuracy, so the above equation can be linearized to \( BR_{sp} = 0.035 (BR_{nsp}) (\theta) \).

Spindle measurements obtained from 16-mm time-lapse movie film with methods similar to those described by Swann and Mitchison (1950) gave a better estimate of the kinetics of the rapidly decreasing spindle BR. Measurements taken faster than one every 10 s by the conventional method were found to be unreliable due to fatigue and other human error. Determination of the magnitude of spindle BR from measurements of film density depends on a knowledge of both the film characteristic curve and the relation between the magnitudes of BR and the light intensity. Although a direct calibration curve was not obtained, changes in film density corresponding to changes in spindle BR were in the linear region of the film gamma curve as given by Eastman-Kodak (1972). In this region, changes in film optical density (OD) are proportional to changes in the logarithm of light intensity. The gamma constant, \( \gamma \), of the 7231 movie film is specified as 0.65 \( \pm 0.05 \).

The film densities were measured by projection onto the probe of a Densichron I model 3830A densitometer. The relative size of the probe aperture (3 mm) to the projected spindle is shown in Fig. 1. For each frame examined the maximum image density in the half-spindle nearest the surface was measured; the difference between the two half-spindle densities was generally less than 0.03 OD. For the processed negative, the background light due to the compensator BR produced a film density (OD\(_{bg}\)) of about 0.7. The film density for the region of maximum half-spindle BR (OD\(_{sp}\)) at atmospheric pressure was about 1.05. As the spindle depolymerized under pressure, the OD\(_{sp}\) decreased in value to within 0.02 U of the OD\(_{bg}\) value. This low difference reflects the extreme clarity of the yolk-free spindle zone in the eggs analyzed (Fig. 1). The film density for the background light with zero compensation was about 0.23, or just above the fog level of the film (0.20). Results from densitometric analysis of the positive prints differed from those obtained from the negatives by less than 10%.

Analysis of Film Densitometric Data

Because of the small magnitudes of compensator and spindle BR, and the insignificant amount of light scattering in the spindle region, the expressions relating the background and spindle light intensities to their respective BR are fairly simple (Hartshorne and Stuart, 1960, p. 301). The background light intensity, \( I_{bg} \), outside the egg is related to the compensator BR by:

\[
I_{bg} = I_o \left( \frac{\lambda}{\lambda} \right) (BR_c)^2,
\]

where \( I_o \) is the light intensity passing parallel polarizers, \( BR_c \) is the BR of the compensator in nanometers, and \( \lambda \) is the wavelength of the illuminating light in nanometers. Similarly, the light intensity of the spindle, \( I_{sp} \), is given by:

\[
I_{sp} = I_o (BR_s + BR_c)^2,
\]

where \( \alpha \) is the light attenuation coefficient for the egg cytoplasm in the spindle region and \( BR_{sp} \) is the spindle BR in nanometers. Taking the ratio \( I_{sp}/I_{bg} \), to eliminate variations in lamp intensity, and rearranging the terms gives the ratio of spindle BR to compensator BR:

\[
BR_{sp}/BR_c = \sqrt{I_{bg}/I_{sp}}(BR_s + BR_c).
\]

Note that \( I_{bg} \) is the background light passing through the egg cytoplasm in the spindle region.

The ratio \( I_{sp}/I_{bg} \) is related to the measured film densities in the linear region of the film gamma curve by:

\[
\log(I_{sp}/I_{bg}) = \frac{1}{\gamma} (OD_{sp} - OD_{bg} - \log \alpha),
\]

where \( \log \alpha \) was determined by the measured difference between the background OD and the OD of the spindle region after the spindle had depolymerized and decreased substantially in size. Normally, OD\(_{bg}\) was measured during an experiment for a region just beyond the cell surface at the position of pole attachment and later corrected. The correction term was generally \( \pm 0.02 \) OD.
Measurement of Spindle Length

Measurements of spindle length were made either from enlarged photographs from the 35-mm negatives or from projections of the 16-mm movie film. In each case, a photograph of a stage micrometer provided the calibration.

RESULTS
Preliminary Observations

All the Chaetopterus eggs showed great uniformity in spindle BR and size. At 22°C the maximum half-spindle BR was $3.65 \pm 0.2$ nm (SD) and the average spindle length, $32 \pm 3 \mu$m (SD). Measurements taken from a single spindle were representative of the entire population under observation. Although the spindle can remain in a stable metaphase for many hours, the position of the spindle within the egg was not static. The spindle frequently pivoted about the attached pole at the cell surface, often as much as 150°, at a rate of 10–15° per min. Occasionally, after the spindle had pivoted parallel to the cell surface, it changed attachment from one pole to the other. In addition, the spindle sometimes moved abruptly away from, then back towards, the cell surface. This saltatory-like movement, covering a distance of 5–7 μm, was particularly frequent during depolymerization and repolymerization of the spindle (Fig. 1). The normal, erratic, and rapid spindle movements often made it difficult to take photographs and BR measurements. Consequently, no experimental changes were initiated until the spindle pivoted to a position approximately perpendicular to the cell surface and appeared to stop moving.

General Features of Spindle Response to Pressure

Hydrostatic pressure had a pronounced effect on the assembly of spindle microtubules as shown by the changes in spindle BR and size apparent in Fig. 1. The greater the pressure, the greater the rate of spindle BR decay and, for pressures less than 6,000 psi, the greater the rate of spindle shortening. Pressures of 3,500 psi or higher at 22°C caused the spindle to disappear completely. After the application of lower pressures, spindle BR and length decreased to new equilibrium values that depended on the magnitude of the applied pressure. The specific relationship of equilibrium spindle BR and length to the magnitude of pressure and temperature will be the subject of a following paper (Salmon, 1975 a).

Figure 1 Changes in spindle BR and size after the application (a) and release (b) of 3,000 psi. Also seen are the changes in the curvature of the cell surface during spindle shortening and regrowth, and the abrupt saltatory-like spindle movement away from and closer to the cell surface. Time in minutes is given on each frame with time set equal to zero at the time of pressurization and again at the time of pressure release. Pressure was released 5.5 min after initial pressurization. The prints were obtained from 16-mm Plus X Negative movie film. The compensator setting was constant at 4 nm. The interpolar axis of the spindle is at 45° to the analyzer-polarizer direction and lies parallel to the slow axis of the compensator. The intensity due to the birefringence of the chromosomal and continuous fiber microtubules is brighter than the background. The astral rays perpendicular to the spindle interpolar axis subtract from the compensator birefringence and appear darker than the background. The white circle in (a), frame 0.0, represents the relative size of the densitometer probe aperture to the projected spindle image as discussed in the text. The length of the scale in (b), 15.0 min, is 10 μm.
static pressure are pictured in Fig. 1. At atmospheric pressure the regions of maximum BR are midway between the metaphase plate and the poles of the oblate spindle. Immediately after pressurization the spindle began to shorten and, at a more rapid rate, the half-spindle BR decreased (Fig. 2), the regions of maximum BR moving somewhat closer to the poles. The BR dropped quickly to a low value, then remained constant or slowly decreased during most of the spindle shortening. The spindle became diamond-shaped as its length decreased. After shortening to the new equilibrium length, the regions of maximum BR again became centralized in the half-spindles. If the spindle was very small, it remained diamond shaped. Otherwise, the spindle returned to an oblate shape at equilibrium. During these changes, one aster normally remained attached to the cell surface, the spindle occasionally moving abruptly away from, then closer to the surface as depolymerization progressed.

After restoration of atmospheric pressure, spindle BR and length generally returned to normal. In fact, after 15 repetitive pressurizations of one spindle no irreversible effects could be detected over the experimental period of 5 h. After pressure release, the spindle BR increased rapidly and remained relatively constant during most of the spindle growth (Fig. 2). Initially, BR was maximal in the center of the spindle, sometimes exceeding the atmospheric equilibrium half-spindle value. But as the spindle grew, the maximum BR region moved again towards the centers of the half-spindles. Although the spindle reached equilibrium BR in less than 2 min after raising the pressure, 15-20 min were required for the spindle to return to normal metaphase configuration after releasing the pressure. The saltatory-like spindle movements were particularly noticeable during spindle regrowth, apparently due to the attached aster growing and shortening.

The curvature of the cell surface near the attached spindle pole changed during spindle depolymerization and regrowth. The cell surface normally dimples inwards at the site of the attached pole. After pressurization this dimpling increased at first (Fig. 1 a), then the surface became smoothly rounded as the spindle completely depolymerized. When pressure was released the surface bulged outward as the spindle repolymerized and regrew (Fig. 1 b); the bulge disappeared and the surface again became slightly dimpled as the spindle reached its stable metaphase configuration. These surface changes are suggestive of changes in the force balance between the cell surface and the spindle. As the spindle changes its dimensions, it appears to produce a force between the small attached aster and its other larger aster anchored in the cytoplasm. The slight dimpling of the surface for normal metaphase suggests that the spindle is under a constant level of tension.

The sequence presented in Fig. 1 b typifies recovery of the spindle from all pressures less than 6,000 psi. The increase in spindle BR and length is

![Figure 2](image-url)

**Figure 2** Comparison of the kinetic changes in half-spindle BR and spindle pole-to-pole length during and after a pressurization of 2,000 psi. Notice that the changes in BR occur much faster than the changes in length. The BR was measured by conventional compensator methods. The curves were fitted by eye, and the time in minutes is set equal to zero for the time of pressurization and the time of pressure release.
similar to the pattern in Fig. 2. A slight delay preceded the reappearance of spindle BR and regrowth at pressures greater than 3,500 psi. The delay increased with higher pressures to approximately 30 s after the release of 9,000 psi. After pressures greater than 6,000 psi were released, multipolar spindles frequently reappeared. When first detected, the spindles were about the same length as the original bipolar spindle, indicating that little spindle shortening had occurred. Occasionally, however, a tiny spindle was seen to appear near the cell surface.

**Kinetics of Spindle Shortening and BR Decay as a Function of Pressure**

Representative changes in spindle length as a function of time and magnitude of pressure are presented in Fig. 3. The data were obtained from repetitive experiments on the same spindle. After pressurization, the spindle length approached its equilibrium value as a roughly exponential function. Initial velocities increased progressively with the magnitude of pressure: they were approximately 4.5, 6.5, 9.0, and 17.0 µm/min for pressures of 2,000, 2,500, 3,000, and 4,000 psi, respectively (Fig. 4). Changes in spindle length after pressurizations of 4,000 psi or higher could not be measured accurately due to the weak BR. Also, data for changes in BR after pressurizations higher than 4,000 psi were not reliable due to the weakness of spindle BR and the increased background strain BR in the chamber windows. However, at pressures of 6,000 psi or greater the spindle did not appear to shorten; only its BR decreased.

Representative changes in spindle BR as a function of time and pressure are presented in Fig. 5. Interpretation of the results for pressurizations above 4,000 psi is questionable for the reasons just stated. The majority of spindle BR, however, had clearly disappeared at the times indicated in Fig. 5. For pressures of 4,000 psi and below, the kinetics consist of two phases: an initial phase of rapid BR decay; and a second phase of much slower BR decay. Spindle BR decreased linearly with time during most of the initial phase. The BR decay often slowed abruptly and BR then remained nearly constant or slowly decreased during the second phase when most of the spindle shortening was occurring. The rate of BR decay during the linear phase increased progressively with higher pressures (Fig. 4). The decay rates were roughly 2.0, 3.7, 5.3, and 10.0 nm/min for 2,000, 2,500, 3,000, and 4,000 psi, respectively. The average rate of decay during the second phase did not depend on the magnitude of pressure and had an average value of 0.4 nm/min.

The magnitude of the normalized initial rate of BR decay was highly correlated \( r = +.996 \) with the normalized initial rate of spindle shortening. Both rates increased roughly exponentially with pressure, approximately doubling for every 1,000 psi (Fig. 4), and showed a volume change of activation of -250 ml/mol. When the normalized rate of BR decay was plotted against the normalized shortening rate for pressures of 4,000 psi and
DISCUSSION

The in vivo spindle BR studies reported here demonstrate that hydrostatic pressure can produce rapid, reversible depolymerization of spindle fiber microtubules. The depolymerization of the mi-
crotubules accounts for the disorganization of the spindle fibers with pressure as observed earlier in fixed and stained or stabilized preparations (Pease, 1941, 1946; Zimmerman and Marsland, 1964). The spindle appears susceptible to complete depolymerization by pressures between 3,000 and 7,000 psi near 22°C, depending on cell type (Table I). Higher pressures are required to retard significantly synthesis of DNA, RNA, or proteins (Zimmerman, 1970; Landau, 1970).

The pressure-induced depolymerization of spindle microtubules appears, in general, to be qualitatively accounted for by Inoué’s equilibrium theory of spindle assembly: that the birefringent spindle microtubules are composed of weakly associated subunits that are in a labile equilibrium with a pool of dissociated subunits. As required by the model, progressively less pressure is necessary to produce complete spindle depolymerization for lower equilibrium temperatures, and more pressure is necessary when 45% D2O seawater is substituted (Table I). Higher temperatures and addition of D2O have already been shown to increase the magnitude of pressure required to inhibit mitosis and cell cleavage (Marsland, 1965; Marsland and Zimmerman, 1963, 1965; Marsland and Asterita, 1966).

Pressures less than 6,000 psi cause the microtubules to decrease in number, as indicated by the loss of BR, and to shorten, as indicated by the overall shortening of the spindle and changes in the pattern of half-spindle BR. When microtubules are rapidly depolymerized by pressures greater than 6,000 psi, spindle shortening does not accompany BR decay. These general observations are identical to those reported earlier for application of colchicine and low temperature to the meiotic metaphase-arrested spindle of *Chaetopterus* (Inoué, 1952 b, 1964) and other spindle types (Inoué and Sato, 1967; Inoué and Fuseler, 1970; Fuseler, 1973). Colchicine is known to depolymerize labile microtubules through competitive binding of the microtubule subunits (Olmsted and Borisy, 1973). Low temperature, like increasing pressure, is postulated to produce depolymerization by altering the equilibrium constant, driving the reaction towards the subunit state (Inoué and Sato, 1967). The labile microtubules in axonemes of the protozoan *Echinospaera*, and other cytoplasmic microtubules responsible for maintaining cell shape, also respond similarly to high pressure, low temperature, and colchicine (Tilney et al., 1966; Tilney and Porter, 1967; Tilney and Gibbins, 1969).

The kinetic data reported here show that the number of microtubules in the half-spindle changes much more rapidly than the length of the half-spindle or the spindle’s interpolar distance (Figs. 1–4). The majority of the spindle’s shortening or growth occurs with little change in microtubule number. As pressure increases, there is a progressive increase in the initial decay rate of the microtubule number accompanied by a proportional increase in the spindle shortening rate (Figs. 5, 6).

To explain these results, I would propose the hypothesis that the length of the longest microtubules controls the spindle pole-to-pole and half-spindle lengths, and that the stability of a microtubule depends on the attachment or nonattachment of its ends. Ends that do not attach on the kinetochore nor end in the pole region I will call “free ends.” Ends that do terminate at a kinetochore or in a pole region I will term “attached ends.” Presumably, polymerization and depolymerization occur more rapidly at free ends than at attached ends. Microtubules may be capable of exchanging subunits along their entire lengths (Inoué, 1964; Inoué and Sato, 1967; Dietz, 1972; Stephens, 1973), but the subunits within the microtubule are more strongly bonded than at the microtubule free ends.

### Table I

| Organism (cell type)          | Temperatures | Pressure |
|------------------------------|--------------|----------|
| *Chaetopterus pergamentaceous* (oocyte) | 14.7°C | 1,500* |
|                             | 22°C         | 3,500*  |
|                             | 22°C; 45% D2O | 4,500-5,000* |
| *Urechis caupo* (oocyte)     | RT           | 3,000-6,000* |
| *Lytechinus variegatus*      | RT           | 4,000*  |
| *Lytechinus pictus* (oocyte) | RT           | 5,000-7,000§ |
| *Tradescantia* (pollen mother cells) | RT  | 5,000-7,000§ |
| *Echinophiochus* (oocyte)    | 22°C         | 6,000*  |
| *Arbacia punctulata* (oocyte) | 20°C       | 6,000*  |
| *Asterias forbesi* (oocyte)  | 18°C         | 6,500*  |
| *Nephrotomia suturalis* (spermatocytes) | 20°C | 7,000*  |

RT, room temperature.

* This report.

† Pease, 1941.

‡ Pease, 1946.

§ Zimmerman and Marsland, 1964.
It appears that only 10%-40% of the microtubules in continuous spindle fibers extend from pole to pole. Most extend from one pole towards the other, ending and overlapping in the metaphase plate region (Manton et al., 1969; Brinkley and Cartwright, 1971a; McIntosh and Landis, 1971; McIntosh and Vanderslice, 1972). In chromosomal fibers, a higher percentage of microtubules appears to extend all the way from the kinetochore to the pole (Alienspach and Roth, 1967; Brinkley and Stubblefield, 1970; Nicklas, 1971; Brinkley and Cartwright, 1971a). After pressurization, the large number of continuous fiber microtubules that have a free end in either of the half-spindle regions will depolymerize rapidly, producing the majority of the BR decay (Fig. 4). The majority of spindle shortening subsequently takes place as subunits are substracted more slowly from microtubules with two attached ends, i.e. interpolar microtubules or microtubules that extend from the kinetochores to the poles.

Returning to atmospheric pressure reverses the process. Initially, most microtubules have a free end. The fibers repolymerize and grow rapidly until they enter the polar region. Thus, the BR rises rapidly before much elongation occurs (Fig. 2). The spindle then slowly elongates, with nearly constant microtubule number, as the interpolar and kinetochore-to-pole microtubules slowly polymerize and grow longer. The delay in the reappearance of the spindle after release of pressures higher than needed to cause complete spindle disappearance can be attributed to the time required to renucleate the microtubule growth at the orienting centers, i.e. the kinetochores and the poles.

It has frequently been reported that astral and continuous fiber microtubules are more susceptible to depolymerization than chromosomal fiber microtubules (Inoué, 1952a; Brinkley et al., 1967; Brinkley and Stubblefield, 1970; Brinkley and Cartwright, 1971b; Stephens, 1972). Since the astral microtubules may be assumed to have a high percentage of free ends, the observed sensitivity can also be explained by the proposed hypothesis. The shortening of the Chaetopterus spindle towards the cell surface as the spindle depolymerizes is a direct consequence of the firm anchorage of one spindle aster to the cell surface and the loose attachment of the other aster in the cytoplasm or on the opposite cell surface. This close anchorage to the surface probably engages many normally free ends of the aster microtubules and would account for the aster's unusual stability during pressurization.

Note that the hypothesis presented here does not require the assumption of different types of microtubules (different types of microtubule subunits) to account for stability differences; stability is determined only by the location of microtubule ends. The constant proportionality of the normalized initial BR decay rate and normalized spindle shortening rate (Figs. 5, 6) over the pressure range examined indicates that both free-end and attached-end microtubules have the same molar volume change of activation for subunit dissociation. The difference in the rates is the result of differences in other physiological factors that affect subunit bonding and that appear to depend on the location of the microtubule ends.

The failure of the spindle to shorten in response to pressures greater than 6,000 psi, high doses of colchicine (Inoué, 1952a), or to very cold temperatures (Inoué, 1952b, 1964) demonstrates that the spindle interpolar forces and the forces between the poles and the kinetochores are transmitted by the microtubules. These high depolymerization stresses probably cause the microtubules to disassemble rapidly in a disorganized fashion, possibly disintegrating all along their lengths. As a result, their mechanical integrity is lost (Inoué and Sato, 1967).

The sensitivity of the spindle to depolymerization by pressure is consistent with a large positive molar volume difference between the polymerized and depolymerized states. This difference may be attributed to the release of many moles of constricted bound water from the subunits as they associate into the microtubules (Lauffer, 1971 review). This concept, along with the quantitative effects of pressure on the equilibrium spindle assembly, particularly in respect to Inoué's equilibrium model, will be considered in more detail in a following paper (Salmon, 1975a). The role of a polymerization-depolymerization cycle in force production for interpolar elongation and chromosome movement will also be considered more fully in another paper (Salmon, 1975b).

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