DCA and not DPA is complexed with lignin. One group of 2-week-old, greenhouse-grown rice plants was treated with ring-labeled-C^{14} DPA and a comparable group was treated with carbonyl-labeled-C^{14} DPA (specific activity 0.85 mc/g). Lignin was isolated from the two groups of plants 3 weeks after treatment. As in the previous experiment, a high percentage of C^{14} was found in the lignin extracted from plants treated with ring-labeled DPA. The amount recovered was 80-fold greater than that found in the lignin extracted from carbonyl-labeled DPA-treated plants. This clearly established that DCA and not DPA was the compound bound to lignin. Lignin was also isolated from mature field-grown rice straw from groups of plants treated with C^{14}-ring- and C^{14}-carbonyl-labeled DPA. Again, lignin from plants treated with ring-labeled DPA contained the greater amount of radioactivity. The differences were not so great as found in the greenhouse experiment. This, however, is not unexpected, since C^{14} from the propionic acid moiety unquestionably would be available to the organic acid pool and subsequently would be incorporated into lignin.

Our studies demonstrate that most of the aniline moiety from DPA-treated rice leaves is complexed as DCA with soluble carbohydrates and polymeric cell constituents. We were not able to isolate any additional C^{14}-labeled compounds from rice treated with C^{14}-ring-labeled DPA. If others are present, they constitute a small fraction when compared with the amount of C^{14} DCA recovered.

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Mitotic Apparatus: The Selective Extraction of Protein with Mild Acid

Abstract. *The treatment of isolated mitotic apparatus with mild (pH 3) hydrochloric acid results in the extraction of less than 10 percent of its protein, accompanied by the selective morphological disappearance of the microtubules. The same extraction can be shown to dissolve outer doublet microtubules from sperm flagella. A protein with points of similarity to the flagellar microtubule protein is the major component of the extract from mitotic apparatus.*

Since Mazia and Dan (*1*) first isolated the mitotic apparatus, efforts have been made to extract from it molecules which are functional in mitosis (*1–7*). Recent work (*5–7*) has sought to equate various proteins extracted from mitotic apparatus with protein from microtubules, which are the ultrastructural equivalents of the spindle fibers. The mitotic apparatus has been treated with various media which simultaneously cause the disappearance of the microtubules and the solubilization of protein components. In these studies, 40 to 70 percent of the protein from mitotic apparatus has been solubilized under conditions leading to the disappearance of the microtubules. We now describe a method for making microtubules disappear selectively from mitotic apparatus, with extraction of less than 10 percent of the protein; a protein with properties reasonable for protein from microtubules is recovered from the extract. The extraction method, treatment with mild HCl (*p*H 3), is that used to reduce bacterial flagella to their monomer protein, flagellin (*8*). Our use of the method is based on the consideration that bacterial flagella might be a structure of the microtubule family, with chemical properties resembling those of microtubules in higher organisms.

Eggs of *Strongylocentrotus purpuratus* were fertilized and allowed to develop at 15° to 17°C. Mitotic apparatus was isolated from eggs at metaphase of the first cleavage division with molar hexylene glycol at *p*H 6.4 (*9*). Further work was carried out at 1° to 4°C. The isolated unit was washed, by centrifugation and resuspension, four times in isolation medium and twice in 0.01*M* phosphate buffer (*p*H 5.5) to remove hexylene glycol. Figure 1A indicates the appearance of mitotic apparatus at this stage of treatment. The mitotic appara-

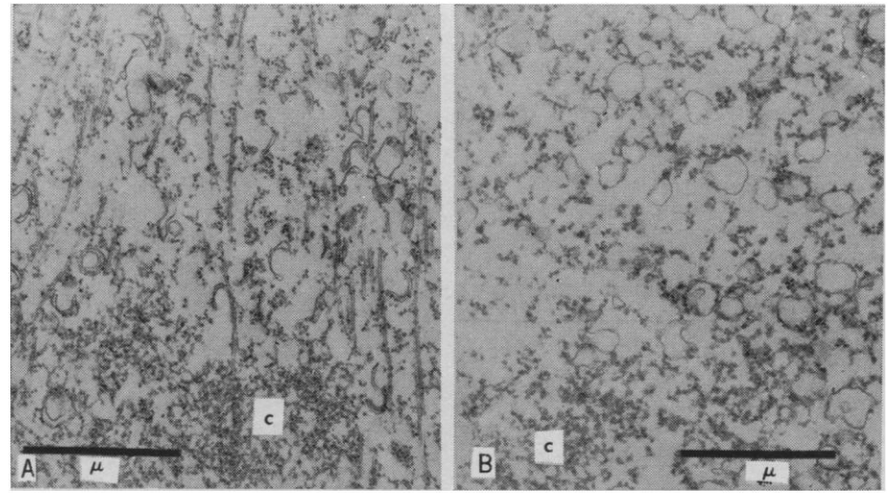

Fig. 1. (A) Isolated mitotic apparatus before acid extraction, fixed in cold 2 percent osmium tetroxide in isotonic acetate buffer (*p*H 6.1) and embedded in Epon 812. The section shown is approximately a sagittal section of mitotic apparatus as determined by previous cutting of thick sections (4 μm) and examination of these by phase microscopy. Microtubules are seen leading from the chromosomes (*c*). Ribosomes and flattened vesicles are present. An amorphous material in which ribosomes and vesicles appear to be embedded is dimly visible. Section stained 30 seconds with 0.1 percent lead citrate. (B) Appearance of isolated mitotic apparatus after 2 hours of acid extraction. Fixation and further processing as in Fig. 1A. No microtubules are visible. Ribosomes, chromosomes (*c*), and vesicles retain their morphological integrity. Ribosomes and chromosomal material appear somewhat swollen and diffuse. Vesicles have partially rounded up.

tus was again centrifuged, resuspended in an equal volume of aqueous HCl (*p*H 3.5), and dialyzed against HCl at *p*H 3.0. By contrast with previous extraction methods which tend to disperse the mitotic apparatus completely, yielding soluble and particulate fractions, this treatment retains the apparatus as a cohesive unit so that morphological changes can be studied *in situ*.

During the first hour of extraction, electron microscopy shows the presence at first of microtubules and later of linear elements tentatively identified as protofilaments. By 75 minutes all linear elements have disappeared. There is little alteration in the morphology of ribosomes, vesicular elements, or chromosomes (Fig. 1B). The fixative used for electron microscopy was 2 percent osmium tetroxide in sodium acetate isotonic with seawater at *p*H 6.1, a fixative that preserves microtubules well (*10*). To assure that the acid conditions prevailing within the mitotic apparatus at the moment of fixation were not responsible for poor preservation of microtubules, we returned some samples of extracted and acid-washed mitotic apparatus to isolation medium for 1 hour before fixation. This did not affect the results.

After 18 hours of acid extraction, the morphology of the mitotic apparatus is still essentially the same, with some continued swelling of the ribosomes and chromosomes. Except for the microtubules, therefore, the morphology is quite stable to this treatment.

Although various transitions resulting in the morphological disappearance of the microtubules during this extraction can be imagined, it is a reasonable hypothesis that their disappearance is due to the solubilization of their molecular components. To support this hypothesis, we have tested the extraction procedure on a preparation of outer doublet microtubules from flagella of *Strongylocentrotus purpuratus* sperm, prepared according to Stephens *et al.* (*11*). Before extraction our microtubule preparations contain some membranous fragments and unidentified amorphous material. Resuspension in HCl (*p*H 3.0) followed by dialysis against this medium for 75 minutes causes the disappearance of the microtubules, leaving in the particulate residue only membranes and amorphous material.

During this procedure about 65 percent of the protein of the preparation is extracted. We can extract approximately the same amount of protein with the mercurial mercuhydrin (10^{-2} mole/liter, in $10^{-2}M$ borate buffer *p*H 9.0), whereas Stephens (*12*) has shown that the mercurial salyrgan at *p*H 8.5 dissolves the microtubule protein, yielding a single component with a sedimentation coefficient of 5.3*S*. The protein we extract with mercuhydrin appears in the analytical ultracentrifuge as a single component having a sedimentation coefficient of 5.5*S*, and is evidently the same protein described by Stephens. Moreover, substantially the same protein is extracted with acid. Acid extraction of a preparation already extracted with mercuhydrin recovers only a few percent of protein; the same is true for mercuhydrin extraction of a preparation that has been extracted with acid. The protein extracted by acid is 80 to 100 percent precipitated by $10^{-2}M$ calcium at neutrality, a property previously reported for microtubule protein from cilia (*13*). We conclude that the dissolution of microtubules by acid treatment, which is suggested in the case of mitotic apparatus by the morphological changes, actually takes place in the case of outer doublet microtubules from flagella.

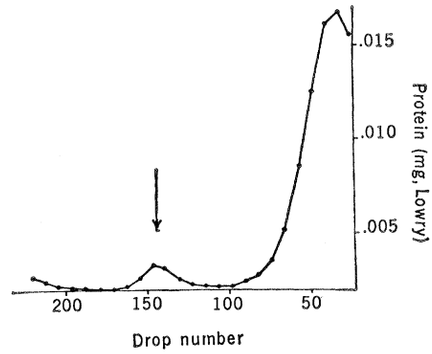

Fig. 2. Pattern of sedimentation of mitotic apparatus acid extract in a 5 percent to 20 percent sucrose gradient, after 75-minute extraction. Top of tube is drawn to right. Centrifugation was for 6 hours at 31,000 rev/min at 5°C in a Spinco 39-L rotor. Fractions were collected by dripping from the bottom of the tube through a silicone-coated capillary, at constant speed maintained manually by controlling pressure with a syringe barrel. A linear rise in the baseline due to a Lowry-positive reaction given by the gradient alone has been subtracted out.

Protein determinations (*14*) show that after 75 minutes of acid extraction an average of 8 percent of the protein of mitotic apparatus has passed into solution. (Soluble protein is here defined as the supernatant after centrifugation for 2 hours at 30,000*g*.) Preliminary analysis of the extract (still *p*H 3) in the analytical ultracentrifuge shows two components having sedimentation coefficients of $S_{20,w} = 21S$ and $S_{20,w} = 4S$, in addition to some material which remains at the meniscus. The values for sedimentation coefficients must be regarded as quite approximate, however, because the amount of material available for analysis is small. The sedimentation pattern of Lowry-positive material from the extract in a 5 to 20 percent sucrose gradient at *p*H 3 is shown in Fig. 2. Only two fractions are distinguishable, a small amount of fast-sedimenting material which evidently corresponds to the 21*S* boundary seen in the analytical ultracentrifuge, and a peak comprising the slow-sedimenting material, which accounts for 80 to 95 percent of Lowry-positive material in the extract. (If acid extraction is protracted, for example, 60 hours, the fast-sedimenting material sometimes increases to as much as 50 percent of the material present.)

The fast-sedimenting material appears to consist partly or entirely of the 22*S* protein which is a major component of mitotic apparatus isolated by the hexylene glycol method (*5*). Using a sucrose gradient at *p*H 3, we have compared the sedimentation of the fast-sedimenting material with that of the 22*S* protein, which we extracted from unfertilized eggs (*15*). The sedimentation position of the undissociated component of the 22*S* protein from unfertilized eggs is indicated in Fig. 2 by an arrow. However, this protein dissociates for the most part under these conditions to give a 7*S* material [see also Stephens (*15*)], whereas no 7*S* peak is found in our acid extracts. This may happen because the protein, when extracted directly into acid from mitotic apparatus, retains from the isolation medium bound hexylene glycol which alters its properties.

Immunochemical tests confirm the identity of the fast-sedimenting material with the 22*S* protein. Acid extracts containing substantial amounts of fast-sedimenting material give a reaction of immunochemical identity with the 22*S* protein when tested by immunodiffusion (*16*) against an antiserum (*17*) to the 22*S* protein. When the fast-sedimenting material is largely removed by centrifugation monitored by sucrose gradient analysis, the cross-reactivity also is greatly diminished.

On the other hand, the slow-sedimenting fraction appears to be distinct from 22*S* protein. Its sedimentation is too slow for any acid-dissociated subunits (*15*) of the 22*S* protein; when dialyzed against 0.6*M* KCl in 0.005*M*

phosphate, *p*H 7.3, it does not reform any heavier units as acid-dissociated 22*S* protein does (*15*). Moreover, the immunochemical experiment indicates that cross-reactivity with antiserum against 22*S* protein resides in the fast-sedimenting fraction rather than in the slow-sedimenting fraction. Finally, a major part of the slow-sedimenting fraction is precipitable by calcium at neutrality, whereas the 22*S* protein, as well as the fast-sedimenting peak in the acid extract, are soluble under these conditions.

Because precipitability by calcium is a characteristic both of microtubule protein of cilia and flagella (*13* and above) and of the protein obtained by Sakai from mitotic apparatus (*4*) and proposed by Kiefer *et al.* to be the microtubule protein (*6*), we have determined the solubility of the acid extract after neutralization and addition of 0.01*M* calcium chloride in various buffers. Under these conditions 50 to 75 percent of the extract (4 to 6 percent of the original mitotic apparatus) precipitates rapidly. Sucrose gradient analysis of the calcium-soluble fraction and of the insoluble fraction after redissolution in acid shows that the calcium-insoluble fraction is derived solely from the slow-sedimenting material, whereas the rapidly sedimenting material is soluble in calcium.

Preliminary sedimentation analysis of the calcium precipitate from mitotic apparatus (dissolved in acid) suggests that this material comes from both the 4*S* material of the acid extract and the material that remains near the meniscus. Similarly, the acid extract of outer doublet microtubules of sperm flagella shows two components on analytical ultracentrifugation while still in acid. One has a sedimentation coefficient of 4.6*S*, and one sediments much more slowly. Both components are insoluble in calcium, and our experiments with sequential acid and mercuhydrin extraction indicate that both components are forms of the microtubule protein. Therefore, the sedimentation profiles in acid of the calcium-precipitable protein from mitotic apparatus and the microtubule protein from sperm tail are comparable.

Our preliminary data indicate that in both cases the relative amounts of the two components depend on the amount of calcium ion present in the acid. Thus, the addition of ethylenediaminetetraacetic acid to the acid in which the calcium precipitate is redissolved causes the material to appear almost exclusively as the lighter component, whereas addition of calcium in concentrations as low as $10^{-4}M$ to the acid used to extract the sperm tail microtubules causes the lighter component to be absent.

The data suggest that the calcium-precipitable protein from mitotic apparatus is the microtubule protein and has properties similar to those of the protein of outer doublet microtubules from sperm flagella. Extraction with HCl at *p*H 3 dissolves the microtubule protein from sperm tail; it causes the disappearance of the microtubules from isolated mitotic apparatus. The major component of the mitotic apparatus extract resembles the sperm tail microtubule protein in its precipitation with calcium and, approximately, in its sedimentation pattern in acid.

The calcium-precipitable protein also has points of resemblance to proteins previously extracted from mitotic apparatus. The protein obtained by Sakai (*4*) is also insoluble in calcium. It has been recovered in two different forms, either with a sedimentation coefficient of 3.5*S*, or as a material that remains near the meniscus (*3*, *4*). We have not yet studied the sedimentation of this protein under the acid conditions used in our work. Sakai's protein constitutes a much higher percentage of mitotic apparatus than ours does (*6*), but it was obtained from mitotic apparatus isolated by a different method, so this does not exclude identity of the proteins.

The extracts of mitotic apparatus isolated in hexylene glycol obtained (*5*, *15*) in 0.6*M* potassium chloride show as a minor component a heterogeneous material with sedimentation coefficient of approximately 4*S*. This material constitutes about the same percentage of mitotic apparatus as our calcium-precipitate does (4 to 6 percent). Stephens (*12*) has recently reported that this protein has an amino acid composition very similar to that of actin, as does microtubule protein from sperm flagella. Finally, Borisy and Taylor (*7*) have calculated that 5 to 15 percent of mitotic apparatus isolated by the hexylene glycol method is a protein that binds colchicine and is proposed to be the microtubule protein, a percentage which agrees fairly well with the percentage of calcium-precipitated protein which we recover.

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Antigenic Specificities Related to the Cold Agglutinin Activity of Gamma M Globulins

Abstract. *Certain antiserums prepared against isolated cold agglutinins demonstrate specificity for gamma M globulins with this activity and not for similar gamma M globulins lacking such activity. The cold agglutinin specific antigens fall into several major and minor groups. Their exact relation to the presumed antibody-combining sites remains to be determined.*

The delineation of a wide variety of antigenic determinants among the immunoglobulins has aided greatly in the classification of these proteins with respect to major subdivisions of both the heavy and light chains (*1–3*). Chemical studies, particularly those involving sequence analyses, have brought forward the structural basis for many such differences (*4*). However, no antigenic determinant which relates to the antibody specificity of the immunoglobulins has been observed. Part of the difficulty stems from the very apparent heterogeneity of most antibodies, but even with certain very homogeneous antibodies no such relationship has been apparent. Instead, each antibody shows "individual antigenic specificity" (*5*). Studies with antibodies to A substance in humans (*5*, *6*) and with antibodies to proteus in rabbits (*7*) have revealed only "individual antigenic specificity" with no cross specificity among these