THE MITOTIC APPARATUS

Physical Chemical Characterization of the 22S Protein Component and Its Subunits

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ABSTRACT

The major 22S protein of the hexylene glycol-isolated mitotic apparatus has been characterized from spindle isolates and extracts of whole eggs and acetone powders of eggs from the sea urchins *Strongylocentrotus purpuratus*, *Strongylocentrotus droebachiensis*, and *Arbacia punctulata*. The protein is free of nucleotide, lipid, and ATPase activity. Essentially identical in amino acid composition, proteins from these species show a relatively high content of glutamic and aspartic acids and are fairly rich in hydrophobic amino acids. Optical rotatory dispersion studies indicate a helical content of about 20%, a value consistent with the proline content of the protein. The purified proteins have sedimentation rates in the range of 22–24S, diffusion constants of 2.4–2.5F, intrinsic viscosities of 3.7–4.3 ml/g, a partial specific volume of 0.74, and an average molecular weight of 880,000. Electron microscopy indicates a globular molecule with dimensions of approximately 150 by 200 A; such size and symmetry are consistent with hydrodynamic measurements. The 22S protein yields 6–7S, 9–10S, and 13–14S subunits below pH 4 or above pH 11. The 13–14S component has an estimated molecular weight of 600,000–700,000. A 5–6S particle is formed in 8 M urea or 5 M guanidine hydrochloride, while at pH 12 the 6–7S subunit is seen; each particle has a molecular weight of 230,000–240,000. In 8 M urea plus 2% mercaptoethanol or at pH 13, the molecular weight becomes 105,000–120,000; under these conditions the particle sediments at 2.5–3S and 4S, respectively. On the basis of these molecular weights, the 6–7S, 9–10S, 13–14S, and the parent 22S particle should be dimer, tetramer, hexamer, and octamer, respectively, of the 105,000–120,000 molecular weight subunit. The various subunits will reform the 22S particle when returned to neutral buffer, with the exception of the mercaptoethanol-treated urea subunit where breakage of disulfide bonds results in a polydisperse aggregate. The 22S particle itself is not susceptible to sulfhydryl reagents, implying either that the disulfide bonds are inaccessible or that they are unnecessary for maintenance of tertiary structure once the 22S particle has formed from subunits.

INTRODUCTION

The proteins obtainable from mitotic apparatus isolates appear to be as varied as the methods for spindle isolation. Mazia and Dan (1952) produced a single 4S component from 0.5 M KOH-dissolved

isolates obtained by the early peroxide stabilization method whereas Zimmerman (1960) found 3.7S and 8.6S components from Salyrgan-dissolved alcohol-digitonin isolates. Dirksen (1964) obtained low molecular weight heterogeneous material accompanied by a major 14S and a minor 21S component from dithiodiglycol-isolated asters; Sakai (1966) found similar components in dithiodipropanol spindle isolates, with both workers using high salt and mildly alkaline conditions. In addition, Sakai was able to separate a calcium-precipitable 3.5S component from the low molecular weight fraction and reduce it to a 2.5S subunit with sulfite. However, Miki-Noumura (1965) obtained directly a 2.3S material from alkaline-dissolved microfilaments of hexylene glycol mitotic apparatus isolates. Recently, Kane (1965 *b*, 1967) has observed one major 22S particle and a minor 4–5S heterogeneous component from hexylene glycol mitotic apparatus isolates dissolved in high salt at neutral pH.

Considering the varied conditions of isolation and dissolution, it is quite possible that many of these apparently discordant results may be due to the breakdown of a primary structural protein into its constituent subunits. The 21–22S particle, seen as a component common to both dissolved asters (Dirksen, 1964) and spindles (Kane, 1965 *b*, 1967; Sakai, 1966), appears to be a likely candidate for such a protein, particularly in view of its predominance in hexylene glycol isolates solubilized at neutrality where the least amount of denaturation or dissociation would be expected to occur.

The purpose of this study is twofold: first, to characterize the 22S protein obtainable from hexylene glycol mitotic apparatus isolates and whole egg lysates (Kane, 1965 *b*, 1967) or acetone powder extracts of unfertilized eggs (Kane and Hersh, 1959) and, second, to investigate subunit association in order to gain some insight into the quaternary structure of the 22S particle and attempt to correlate the resulting subunit data with the results of previous investigations. Implicit in such a study, of course, is proof that the 22S fraction of the mitotic apparatus is identical with that of the whole egg. This work is performed primarily on protein prepared from acetone powders and whole egg extracts; the use of such protein allows extensive hydrodynamic studies which would be impossible using protein derived from spindle isolates alone.

MATERIALS AND METHODS

Protein Preparation and Purification

Gametes of the sea urchins *Strongylocentrotus purpuratus*, *S. droebachiensis*, and *Arbacia punctulata* were obtained as described by Kane (1967).

Mitotic apparatus isolates were prepared according to Kane (1962 *a*) employing 12% hexylene glycol at pH 6.4. The resulting spindles were washed three times with the hexylene glycol solution and finally dissolved in a volume of 1.2 M KCl, 0.02 M Tris-HCl buffer, pH 7.5, equal to the volume of spindles, i.e., the final concentration was 0.6 M KCl. After clarification at 25,000 *g* for 30 min, the solution was dialyzed for 12 hr against 0.6 M KCl, 0.01 M Tris-HCl buffer, pH 7.5, prior to analytical ultracentrifugation. Except for lysis of the eggs, all steps were carried out at 0°C.

The alternative whole egg procedure involved lysis of membraneless unfertilized eggs in an equal volume of 12% hexylene glycol (Kane, 1967). The resulting suspension was cooled to 0°C, clarified at 25,000 *g* for 30 min, and then dialyzed against 0.6 M KCl, pH 7.5, for 12 hr at 4°C. The 22S protein component was then obtained by preparative ultracentrifugation at 100,000 *g* for 5 hr in the Spinco Model L ultracentrifuge, employing the SW-39 swinging bucket rotor. The rotor was maintained at 4°–6°C by refrigeration.

The acetone powder procedure essentially parallels the method of Kane and Hersh (1959). Acetone powders of the eggs under study were prepared by placing freshly shed eggs in 10 times their volume of 30% ethanol at −20°C for at least 24 hr. The eggs were separated from the alcohol by decantation and centrifugation at temperatures below 0°C. The eggs were then washed three times with 10 times their volume of ice cold *n*-butanol, followed by three washings with the same volume of acetone. Each wash included a 10 min extraction period during which the eggs were stirred occasionally. The resulting dehydrated eggs were air dried in a cold room at 4°C and stored at −20°C.

1 g of acetone powder was extracted for 1 hr at 0°C with 10 ml of 0.1 M KCl, 0.01 M Tris-HCl buffer, pH 7.5. The extraction procedure was begun with a low speed (5,000 *g*) centrifugation to free the eggs of air and then sonicated for 1–2 min to promote solvent penetration. Following the 1 hr extraction, the eggs were sedimented at 10,000 *g* for 10 min and then reextracted with a volume of 0.1 M KCl equal to the volume of supernate obtained from the first extraction. The combined extracts were dialyzed against the extraction buffer for 12–24 hr at 4°C, clarified at 25,000 *g* for 30 min, and finally spun at 100,000 *g* for 5 hr to obtain the crude 22S pellet. 1 g of eggs

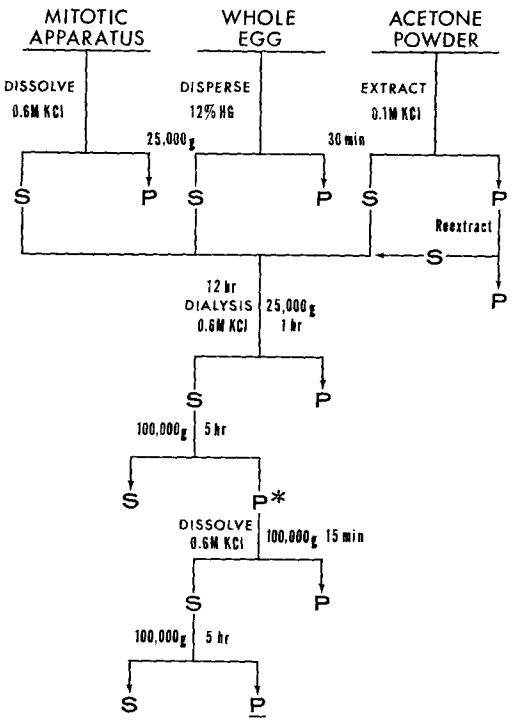

FIGURE 1 Flow diagram for the preparation of 22S protein from mitotic apparatus isolates, whole egg extracts, and acetone powders. All steps were carried out at 0–4°C. The hexylene glycol (*HG*) lysis was done at pH 6.4; all other steps were at pH 7.5. For chromatography on DEAE-cellulose, the first high-speed pellet (*P**) was dissolved in 0.1 M KCl 0.01 M Tris, pH 7.5, and recovered from the eluate by high-speed centrifugation.

(dry weight) yielded, on the average, 30–50 mg of 22S protein.

The crude 22S pellet obtained from either the whole egg procedure or the acetone powder extraction was dissolved in 0.6 M KCl, 0.01 M Tris-HCl, pH 7.5, to a concentration not greater than 15 mg/ml. The resulting cloudy solution was then sedimented at 100,000 *g* for 15 min. The supernate was then sedimented for 5 hr at 100,000 *g*. Normally, this procedure was repeated twice, so that the 22S material was sedimented three times after dissolving the initial pellet.

Fig. 1, a flow diagram, outlines the preparative procedure for obtaining 22S protein from mitotic apparatus isolates, whole egg lysates, and acetone powder extracts, according to the procedure outlined above. The various stages of purification are illustrated by the sedimentation velocity patterns in Fig. 2. Dissolved spindles from *Arbacia punctulata* and whole egg extracts (Kane, 1967), and acetone powders (Fig. 2 *a*) all yield distinct 22S protein peaks, accompanied by varying amounts of inhomogeneous, lower molecular weight material. After 5 hr at 100,000 *g*, a supernate essentially free of 22S protein results (Fig. 2 *b*), while the pellet from this same step consists of at least 95% 22S protein (Fig. 2 *C*). Successive sedimentation results in an essentially homogeneous 22S fraction (Fig. 2 *D*).

If either the 12 hr dialysis step or the resedimentation steps were omitted, the 22S protein contained 5–10% "nucleic acid" (estimated spectrophotometrically) which was easily removable by passage of the protein in 0.1 M KCl, pH 7.5, through a DEAE-cellulose column. The 22S material came through with the solvent front while the contaminating nucleic acids were retained. This apparent simplification of the procedure, although producing homogeneous protein, offered no advantage since approximately 40% of the protein aggregates and is retained on the column.

For subunit dissociation studies, the purified 22S protein was transferred from 0.6 M KCl, pH 7.5, to the solvents under investigation by 6–12 hr dialysis, generally in ½ or 1 ml quantities at concentrations of 5–10 mg/ml. For more rapid reactive studies, the protein was mixed with appropriate reagents within the analytical ultracentrifuge cell, just prior to centrifugation.

Since many of the crude protein preparations contained nucleic acid and since only relative protein concentrations were generally needed, the protein-nucleic acid nomogram for spectrophotometric protein determination (Calbiochem, Los Angeles, California) based on the data of Warburg and Christian (1942) was used. Protein concentration by the Lowry method (Lowry et al., 1951, using bovine serum albumin as a standard, was found to be related to the spectrophotometric assay by a factor of 0.8, the Lowry method giving the lower value. When the amino acid composition became known, the extinction coefficient at 280 mμ for the three species studied was found to be 920 ± 10 cm^2/g and gave protein concentrations which were related to the Warburg-Christian data by a factor of 0.95, the extinction coefficient method giving the lower value.

Small traces of nucleotide were estimated by trichloroacetic acid precipitation, followed by ether extraction of the supernate to remove residual trichloroacetic acid. The solution was neutralized, the absorption spectrum was obtained, and the amount of nucleotide was estimated from the absorption at 260 mμ.

Absorption spectra and other spectrophotometric measurements were carried out on either the Zeiss PMQ-II or Beckman DU spectrophotometers using 1 cm quartz cells.

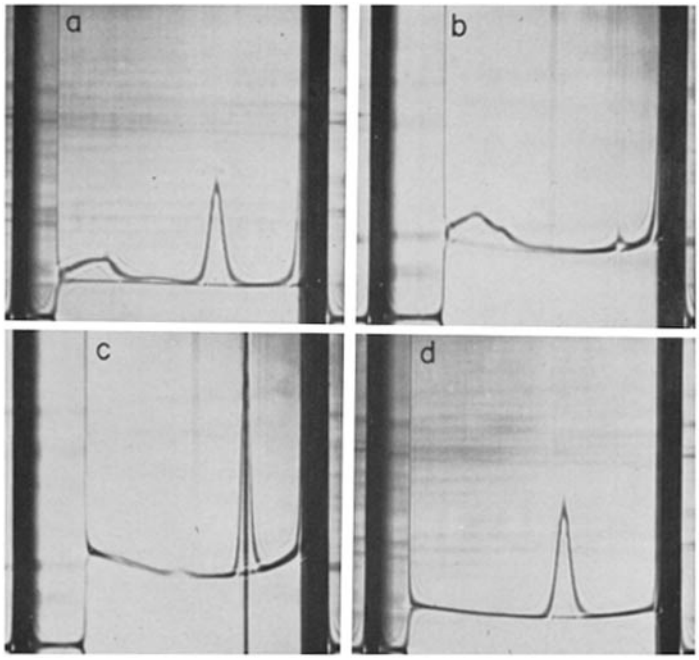

FIGURE 2 Ultracentrifuge patterns of 0.1 M KCl extract of acetone powder (*a*), supernate from first high speed centrifugation of dialysate (*b*) in 0.6 M KCl, pellet from the high speed centrifugation dissolved in 0.6 M KCl (*c*), and purified 22S protein (*d*) in 0.6 M KCl after additional ultracentrifugation step. All solutions were buffered at pH 7.5 with Tris buffer. Photographs were taken 48 min after reaching a speed of 42,040 rpm in (*a*) and (*d*), while (*b*) and (*c*) were taken 24 min after reaching a speed of 59,780 rpm. The bar angle in each case was 60°.

Amino Acid Composition and Partial Specific Volume

In general, 2–4 mg of purified protein in 0.6 M KCl or distilled water was made 6 N in HCl, sealed in a Pyrex tube (the sample being alternately flushed with nitrogen and evacuated 10–12 times), and hydrolyzed for 22 hr at 110°C. The hydrolysate was then evaporated to dryness under vacuum on a rotary evaporator, distilled water was added, and the evaporation was repeated. The resulting dried protein hydrolysate was then analyzed by means of the Beckman Model 120B automatic amino acid analyzer with standard procedures, based on the procedure of Spackman, Stein, and Moore (1958). Owing to the small quantities of material available, no effort was made to run a time curve to estimate the destruction of serine and threonine on hydrolysis.

The tryptophan content was estimated spectrophotometrically by measurement of the extinction of the protein in 0.1 N NaOH at 280 and 294.4 mμ, according to the method of Goodwin and Morton (1946). The tyrosine content evaluated by this method agreed, within experimental error, with that obtained by amino acid analysis.

The partial specific volume of the 22S protein was estimated from its amino acid composition (Schachman, 1957), since the apparent or partial specific volume of a protein approximately equals the sum of its individual amino acids (McMeekin and Marshall, 1952).

Analytical Ultracentrifugation

All work was carried out on the Spinco Model E analytical ultracentrifuge equipped with standard Schlieren optics, employing a phase plate as Schlieren diaphragm. In a limited number of cases, interference optics were also utilized. Unless otherwise specified, all runs were made at 20.0°C, the temperature being controlled with the RTIC unit supplied with the instrument. Photographs were taken on Kodak Metallographic plates and developed in D-19 developer for maximum contrast. Plates were measured on a Nikon Model 16 microcomparator.

Sedimentation rates were determined and corrected to standard conditions by established pro-

cedures (Schachman, 1957). Diffusion constants were determined in the ultracentrifuge by means of a standard valve-type synthetic boundary cell at a speed of 3848 rpm. The constants were evaluated and corrected to standard conditions; sedimentation-diffusion molecular weights were then obtained by application of the Svedberg equation (Schachman, 1957).

Sedimentation-equilibrium molecular weights were determined by the rapid equilibrium method of Van Holde and Baldwin (1958) or by use of the Yphantis (1960) short column technique.

The Archibald method was used in a limited number of cases and was performed and evaluated according to Schachman (1957, 1959). The initial concentration was determined in the customary fashion with a valve-type synthetic boundary cell.

Intrinsic Viscosity

Viscosity determinations were carried out in Ostwald viscometers (E. H. Sargent, & Co., Chicago, Illinois) having a 3 ml capacity and an outflow time of 2 min (distilled water). Measurements were made at 20.0°C or 25.0°C in a thermostatically controlled 10-gal water bath regulated to ±0.05°C. Solutions were rendered dust-free by centrifugation or by Millipore (1.2 μ) filtration under gravity. Specific viscosities at varying concentrations were determined and the intrinsic viscosity was evaluated by means of a plot of specific viscosity versus concentration, the slope of which is the intrinsic viscosity (Schachman, 1957).

Molecular weights were estimated from intrinsic viscosity and sedimentation or diffusion data using the relationship of Scheraga and Mandelkern (1953); length was evaluated from a relationship given by Yang (1961) relating the length of an equivalent ellipsoid to the intrinsic viscosity and molecular weight.

Optical Rotatory Dispersion

Optical rotation measurements were made at varying wavelengths with a Rudolph Model 80 photoelectric spectropolarimeter. Determinations were made in 1.0 dm water-jacketed tubes maintained at 25°C. Appropriate solvent blanks were run under identical conditions. Refractive indices were obtained with a Zeiss Abbe refractometer. The data were plotted and evaluated according to Urnes and Doty (1961) using the relationship of Moffitt and Yang (1956).

Free Thiol Groups

Free sulfhydryl groups were estimated by the *p*-mercuribenzoate spectrophotometric titration method of Boyer (1954), with the procedure being carried out in the spectrophotometer cell by addition of small amounts of titrant with a Hamilton 10 μl syringe. Measurements were made on protein in both the native and denatured states.

A qualitative nitroprusside test was carried out by mixing one drop of protein solution (10 mg/ml) with one drop of 5% sodium nitroprusside, followed by one drop of 27% ammonia. The test was also run in the presence of 8 M urea to test for hidden sulfhydryls. 1 M potassium cyanide was employed to form sulfhydryls from disulfides in order to evaluate free versus hidden disulfides. The sensitivity of the test was found to be about 1 mole of sulfhydryl per 60,000 g of protein.

Adenosinetriphosphatase Activity

In 2.0 ml of a mixture containing 1 mM ATP, 1 mM $MgSO_4$, and 0.05 M Tris-HCl, pH 7.5, between 0.5 and 1.0 mg of phosphate-free protein was incubated for 30 min at 20°C. Inorganic phosphate in the resulting mixture was determined by the method of Fiske and Subbarow (1925).

Carbohydrate

Carbohydrate was estimated by both the anthrone and the orcein colorimetric assays of Sudhof et al. (1955), with glucose as a standard.

Electron Microscopy

A Siemens Elmiskop I electron microscope, equipped with 200 μ condenser aperture and 50 μ objective aperture and operated at 60 kv, was used throughout this study.

Protein samples were negatively stained on carbon-coated Formvar grids according to the methods of Huxley (1963) and Brenner and Horne (1959), using 1–2% neutral PTA or 1% uranyl acetate. Platinum shadowing was carried out on freshly cleaved mica, stripped with a carbon film and mounted on grids, essentially following the method of Hall (1960).

RESULTS

The 22S Particle

General Properties

The 22S protein, after purification by differential sedimentation or separation on DEAE-cellulose, sedimented as a single symmetrical peak (Fig. 2 *d*). Representative data for the variation of sedimentation constant with concentration for mitotic apparatus, whole egg, and acetone powder preparations are given in Fig. 3. The $S^{\circ}_{20,w}$ values determined in 0.6 M KCl, at pH 7.5, differ only slightly for the three species under investigation: *Arbacia punctulata*, 23.7S; *Strongylocentrotus*

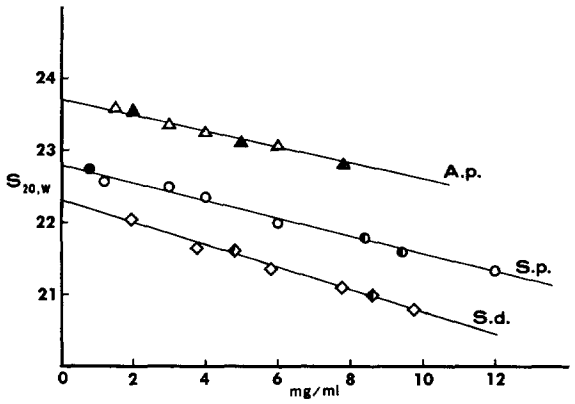

FIGURE 3 Variation of sedimentation rate with concentration for 22S protein from *Arbacia punctulata* (triangles), *Strongylocentrotus purpuratus* (circles), and *S. droebachiensis* (squares). The extrapolated $S^{\circ}_{20,w}$ values are 23.7, 22,8. and 22.3 for these three species, respectively. Open symbols are protein derived from acetone powders, half-filled symbols are from whole egg extracts, and solid symbols are from mitotic apparatus isolates.

purpuratus, 22.8S; and *S. droebachiensis*, 22.3S. No differences among sedimentation rates of proteins derived from mitotic apparatus isolates, whole egg extracts, or acetone powder extracts from a single species can be observed.

Sedimentation rates for unpurified preparations (i.e. containing 5–10% nucleic acid contamination) did not differ significantly from those of purified proteins at the same concentration; ribonuclease treatment caused no detectable change in the sedimentation rate. The nucleic acid is easily removed by passage through DEAE-cellulose, by 12 hr dialysis, or by repeated differential centrifugation, indicating that the nucleic acid component is not covalently linked to the protein and that the material is either of low molecular weight or else was degraded to a low molecular weight by a contaminating nuclease since it is removable by dialysis. After purification, the protein contains less than 1 mole of nucleotide per 2×10^6g of protein. The 280/260 absorption ratio was found to be 1.68–1.70 after either DEAE-cellulose treatment or exhaustive dialysis. The ultraviolet absorption spectrum for the protein is given in Fig. 4.

The lack of measurable change in sedimentation rate before and after *n*-butanol and acetone treatment (whole egg 22S versus acetone powder 22S) indicates that the protein is not a lipoprotein and could contain no more than 1% lipid as a contaminant. Also, since the 22S component can be detected in whole egg extracts which have not been subjected to organic solvent treatment (Kane and Hersh, 1959), the 22S particle is clearly not the centrifugal component of a lipoprotein cleaved by solvent treatment.

Amino acid analysis indicates identity of the

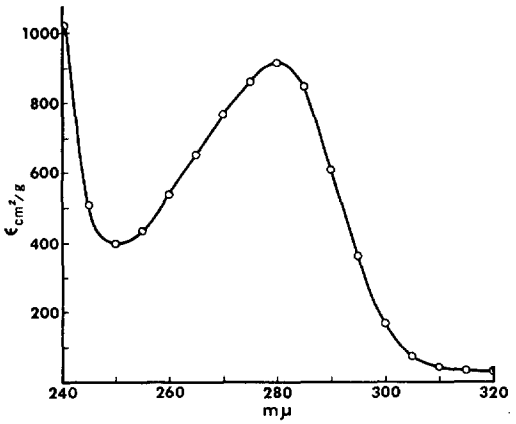

FIGURE 4 Ultraviolet absorption spectrum for purified 22S protein for *S. droebachiensis*. The extinction coefficient at 280 mμ is 920 ± 10 cm^2/g. The spectra for all three species are essentially superimposable.

mitotic apparatus 22S protein and its counterpart derived from unfertilized eggs. Fig. 5 illustrates the relative amino acid composition in moles of amino acid per 100 total moles for *Arbacia punctulata* 22S protein from both acetone powder extracts and mitotic apparatus isolates; the two are identical within experimental error.

The amino acid composition of 22S protein derived from the three urchin species is given in Table I. Each of the results is the average of two determinations performed on different protein preparations and includes spectrophotometrically determined tryptophan. The results are expressed as moles of amino acid per 100,000 g of protein. The experimental error is estimated to be 8%; duplicate samples were within this error. In general, the three species resemble one another

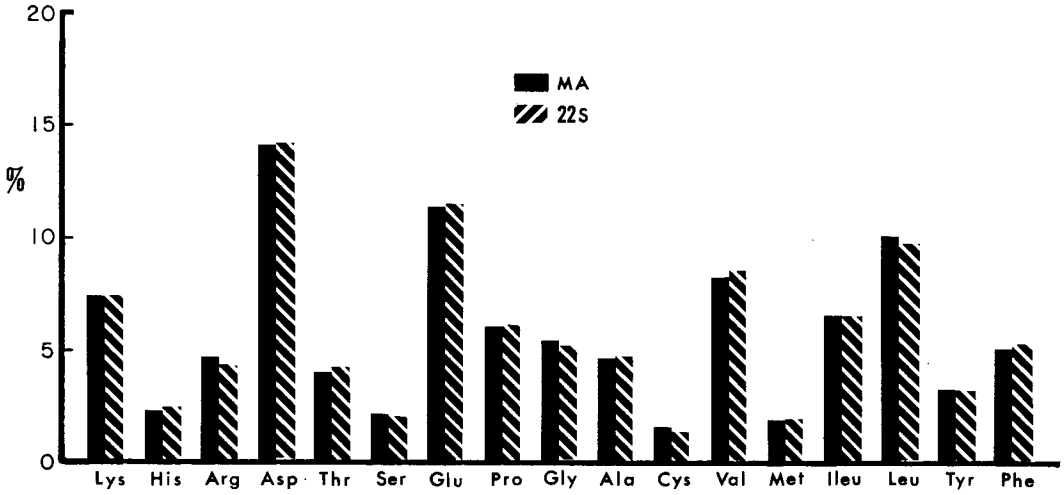

FIGURE 5 Comparative amino acid composition of 22S protein derived from *Arbacia punctulata* mitotic apparatus isolates (MA) and from acetone powder (22S). The values are in relative residue percentages (i.e. residues per 100 total residues). The compositions of the two preparations are the same within experimental error (6–8%). *CYS*, cysteine.

rather closely, the only significant differences occurring in histidine, leucine, serine, and methionine. The latter two deviations may be due to hydrolysis. Glutamic and aspartic acids account for over 25% of the total amino acids present, while the hydrophobic amino acids leucine, isoleucine, valine, and phenylalanine constitute approximately 40% of the remainder.

Carbohydrate analysis of the purified proteins indicated 5–6% carbohydrate by the orcein assay and 7–8% by the anthrone test, both being expressed as glucose. The carbohydrate was not removable with DEAE-cellulose. Amino acid analysis indicated the presence of several per cent glucosamine, though some was probably destroyed by hydrolysis.

The partial specific volume of the protein, estimated from the amino acid content, was found to be 0.740. The calculation did not take into account either tryptophan or the carbohydrate content.

One can predict a maximum of about 30% helix on the basis of the observed 6% proline content (Szent-Györgyi and Cohen, 1957). Optical rotatory dispersion studies on the 22S particle in 0.6 M KCl indicate that the protein is approximately 20% α-helical, assuming a b_0 for 100% helix of -630. The helical content is halved in 8 M urea and becomes zero upon addition of mercaptoethanol to the urea solution. A Moffitt-Yang plot (Urnes and Doty, 1961) of the data for the protein in high salt, 8 M urea, and 8 M urea with mercaptoethanol is illustrated in Fig. 6.

The purified 22S protein from *S. purpuratus* and *S. droebachiensis* acetone powders or from *S. purpuratus* whole egg extract showed no detectable adenosinetriphosphatase activity. The limit of the assay was 0.005 μmoles P_i/hr per mg. The possibility remains, of course, that ATPase activity might be lost through the preparative procedures.

PHYSICAL PARAMETERS AND MOLECULAR WEIGHTS

The translational diffusion constant, measured with a synthetic boundary cell at low speeds or estimated from boundary spreading in sedimentation velocity runs, was found to be comparatively low and showed virtually no concentration dependence. The values for the 22S protein from the *Strongylocentrotus* species were the same within experimental error, while that for *Arbacia punctulata* was approximately 4% higher.

Intrinsic viscosity measurements indicate a relatively symmetric molecule. Fig. 7 illustrates the variation of specific viscosity with concentration; the slope of such a function is the intrinsic viscosity. On the basis of these data, the intrinsic viscosities for the 22S protein in 0.6 M KCl at pH 7.5 are 4.25 and 3.68 ml/g for the *Strongylocentrotus* species, and *Arbacia punctulata*, respectively.

Table II summarizes the sedimentation constant, diffusion constants, and intrinsic viscosities for the three species. The table also includes molecular weights estimated from sedimentation-diffusion data employing the Svedberg equation and from sedimentation-viscosity data utilizing the Scherga-Mandelkern relationship assuming a β-value of 2.16×10^6 (Schachman, 1957), and a $\bar{v}$ of 0.74, obtained from the amino acid composition.

TABLE I

Amino Acid Composition of 22S Protein

The results are expressed in terms of moles per 100,000 g, and each value represents the average of two separate determinations on different samples.

	Strongylocentrotus purpuratus	*Strongylocentrotus droebachiensis*	*Arbacia punctulata*
Lysine	58	58	56
Histidine	*12*	17	18
Arginine	34	32	35
Aspartic acid	109	101	107
Threonine	31	32	31
Serine	12	10	*16*
Glutamic acid	93	92	86
Proline	44	46	46
Glycine	43	44	40
Alanine	38	40	36
Half cystine	9	9	11
Valine	68	64	63
Methionine	22	27	*15*
Isoleucine	47	50	50
Leucine	64	66	*75*
Tyrosine	26	25	25
Phenylalanine	38	42	39
Tryptophan	3	3	4

Molecular weights determined by the rapid sedimentation equilibrium method of Van Holde and Baldwin (1958) and the short column method of Yphantis (1960) showed close agreement with those determined from sedimentation, diffusion, and viscosity data. The sedimentation-equilibrium molecular weight values were not extrapolated to infinite dilution, since runs at several different concentrations yielded nearly identical values. Fig. 8 illustrates a typical sedimentation-equilibrium determination using a 1.7 mm column and schlieren optics, plotted according to Schachman (1957). At concentrations exceeding 10 mg/ml, at ionic strengths less than 0.3, or in preparations several days old, there was evidence of higher aggregation; as a consequence of this, most runs were done at concentrations below 5 mg/ml in 0.6 M KCl, using freshly prepared protein. Determinations at 4°C did not differ from those made at 20°C; after equilibrium was reached, no material accumulated with time at the centrifugal meniscus at the higher temperature and, hence, nearly all runs were made at 20°C for convenience. Table II summarizes representative data from sedimentation-equilibrium determinations and includes two values obtained from the Archibald approach to

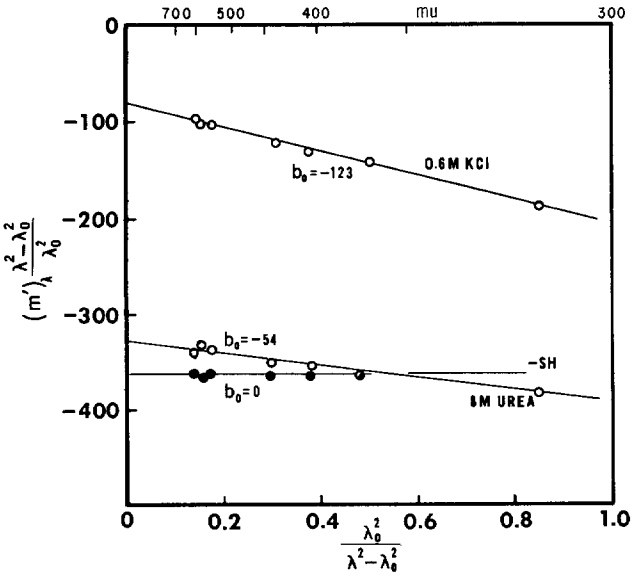

FIGURE 6 Moffitt-Yang plot of optical rotation data for *S. droebachiensis* 22S protein in 0.6 M KCl (pH 7.5), 8 M urea, and 8 M urea plus 2% mercaptoethanol. The data have been plotted assuming a λ_0 of 212 mμ and a mean residue weight of 115 g/mole. The data were not corrected for wavelength dispersion. The slope of such a plot yields b_0 directly while the intercept gives a_0. Assuming a b_0 of -630 for 100% α helix, the protein in 0.6 M KCl is 20% helical, only 9% in 8 M urea, and exists as a random coil in urea plus mercaptoethanol.

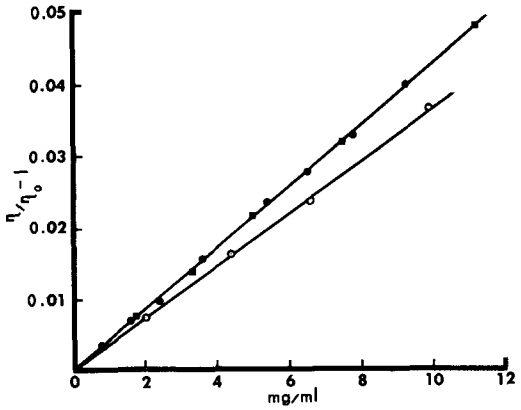

FIGURE 7 Variation of specific viscosity with concentration for 22S protein from *Arbacia punctulata* (open circles), *S. purpuratus* (closed circles), and *S. droebachiensis* (solid squares). The proteins are in 0.6 M KCl at pH 7.5. The intrinsic viscosities for *Arbacia punctulata* and the *Strongylocentrotus* species are 3.7 and 4.3 ml/g, respectively. The determinations were made at 25.0°C.

TABLE II

Molecular Weight of 22S Protein Determined from Sedimentation, Diffusion, and Viscosity Data and by Sedimentation Equilibrium and the Archibald Approach to Sedimentation-Equilibrium Methods

	Strongylocentrotus purpuratus	*Strongylocentrotus droebachiensis*	*Arbacia punctulata*
$S^{\circ}_{20,w}$ (10^{-13})	22.8	22.3	23.7
$D_{20,w}$ (10^{-7})	2.39	2.37	2.47
Intrinsic viscosity (ml/g)	4.25	4.25	3.68
Sedimentation diffusion	895,000	882,000	899,000
Sedimentation viscosity	890,000	870,000	880,000
Sedimentation equilibrium	892,000	855,000	864,000
Short column equilibrium	877,000	885,000	887,000
Archibald method	830,000	900,000	—

sedimentation-equilibrium method. The average experimental errors may be estimated to be in the range of 4–6% for the sedimentation-diffusion and sedimentation-equilibrium data, whereas the rather subjective Archibald method is in the range of 10–15%. On the basis of such figures, the molecular weights of the 22S protein from the three species under investigation agree within experimental error. The average of fourteen determinations gives a molecular weight of 880,000 with a standard deviation of 18,000.

SIZE AND SHAPE OF THE 22S PARTICLE

The frictional ratio for the 22S protein was obtained from the sedimentation and diffusion data for each of the three species and, when coupled with the intrinsic viscosity data, permitted an estimation of the size and shape of the molecule. From the diffusion constants, the frictional coefficient, f, may be calculated, while the molecular weight from the sedimentation data may be used to evaluate the frictional coefficient, f_0, for an equivalent anhydrous sphere. Considering the intrinsic viscosity, the data presented here are consistent only with a prolate ellipsoid of axial ratio no greater than 2.0 and an unusual degree of

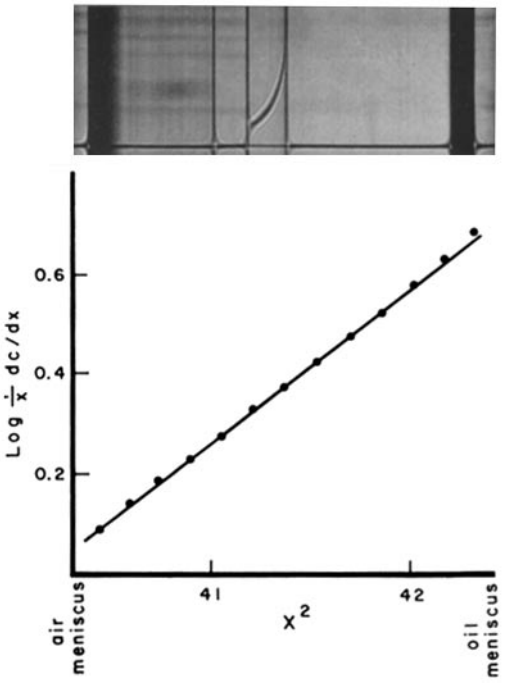

FIGURE 8 Sedimentation - equilibrium molecular weight determination employing short (1.7 mm) columns and Schlieren optics. *Arbacia punctulata* 22S protein in 0.6 M KCl, pH 7.5, 640 min after reaching a speed of 3848 rpm. The concentration was approximately 6 mg/ml and the bar angle was 80°. Homogeneity is indicated by the linearity of the plot, the slope of which gives a molecular weight of 8.9×10^5 for the 22S particle.

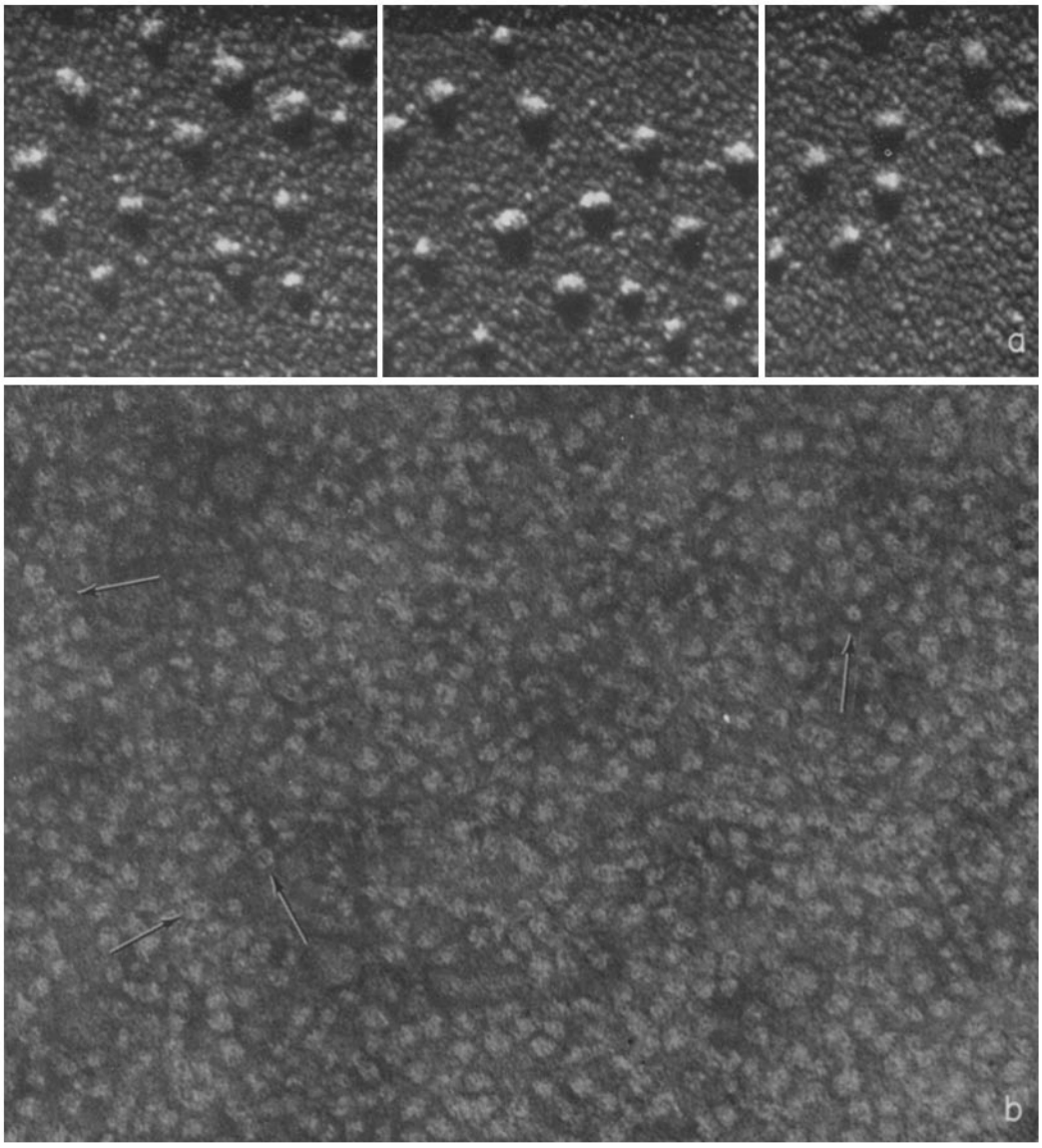

FIGURE 9 *S. purpuratus* 22S protein in 0.1 M ammonium acetate, pH 7.5, shadow cast with platinum (*a*) and negatively stained with 1% uranyl acetate (*b*). The particle is only slightly asymmetric, as can clearly be seen in (*a*), and probably not solid, since areas within the particle appear to be filled with stain (*b*). There is some evidence that the particle is roughly round in cross-section (*b*, arrows). The particle can be approximated as a cylinder of 140–150 A diameter and a length of 190–200 A. ×200,000.

hydration approaching 0.9 g of water per gram of protein, using the Wyman-Ingalls (1943) approximation. Employing the relationship quoted by Yang (1961) and assuming a prolate ellipsoid of axial ratio between 1.0 and 2.0, the estimated length of the molecule falls between 170 and 260 A.

Electron microscope evidence indicates that the 22S protein is a nearly symmetric molecule. Fig. 9 illustrates *S. purpuratus* 22S protein, in 0.1 M ammonium acetate buffer at pH 7.0, both shadow cast with platinum (Fig. 9 *a*) and negatively stained with 1% uranyl acetate (Fig. 9 *b*). These results, and similar results from negative staining

done with 2% potassium phosphotungstate, indicate that the protein has major and minor axes of 180–200 and 140–150 A. Hydrodynamic measurements predicted an axial ratio between 1.0 and 2.0 and a length of 170–260 A. The observed value falls well within these limits. The molecular weight of a cylindrical molecule with a length of 200 A and a diameter of 150 A, assuming a protein density of 1.35 g/ml, would be 1.4×10^6, but this takes into account neither hydration nor the fact that in the negatively stained protein preparations there is some indication that the molecule has a hollow core (Fig. 9 *b*; arrows).

Kane (1962 *a*) has shown that the isolated mitotic apparatus is stable in water without the use of long-chain glycols if the pH is sufficiently low. In 0.1 M ammonium acetate at a pH of 4.5–5.0, the 22S protein forms filamentous aggregates if 0.001 M magnesium is present and if the protein has been freshly prepared. The fibers range from 40 to 60 A in diameter and may also associate in larger bundles (Fig. 10). The filaments are clearly smaller in diameter than the 22S particle from which they arose and such fibrils are very likely formed through some reorganization of subunits. The aggregation is reversible to the 22S particle with increase in pH or ionic strength.

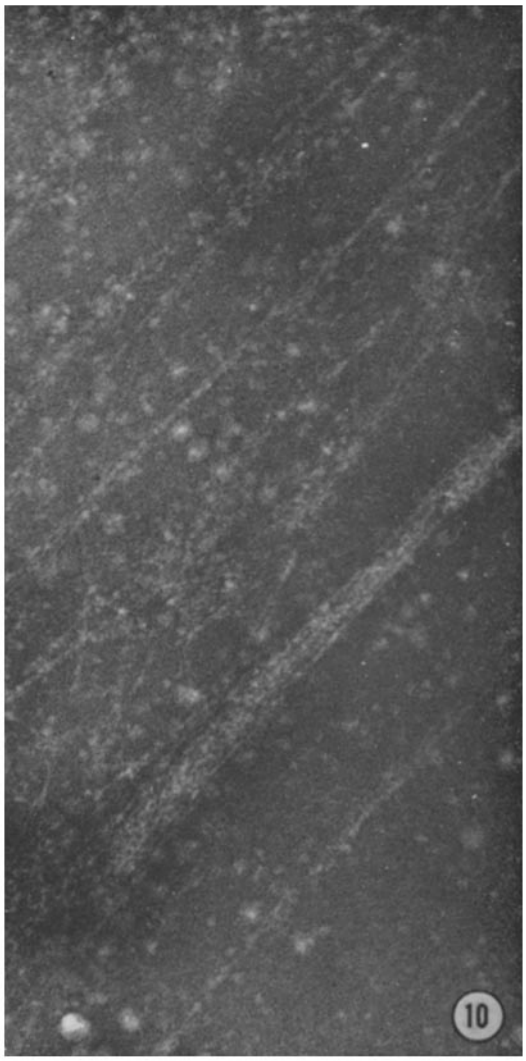

FIGURE 10 *S. purpuratus* 22S protein in 0.1 M ammonium acetate (pH 5.0) and 0.001 M $MgCl_2$, negatively stained with 1% uranyl acetate. Fibers of varying diameter are formed, the smallest being about 40–60 A. The phenomenon is reversible with increase in either pH or ionic strength. ×100,000.

Subunit Formation

Dissociation under Acidic and Basic Conditions

The 22S protein precipitates from solution at pH 4.5 but is soluble 0.5 pH unit above and below this value. At pH 4 or below, in addition to the 22S protein, a 14S subunit becomes evident along with a higher aggregate in the range of 30–35S, and also polydisperse material of even higher sedimentation rate appears. As the pH is decreased, the amount of 22S protein decreases, the amount of 14S subunit increases, and a 10S component forms. The 22S particle persists even in 0.1 N HCl; under this condition, a trace of a 7S component is also evident. Fig. 11 *a* illustrates *Arbacia punctulata* 22S protein in 0.1 N HCl. Here, all the above mentioned subunits and aggregates may be seen in one pattern. Owing to the number of subunits present in such mixtures, with one exception, no attempt was made to determine molecular weights. The 14S component is present in sufficient quantity in 0.1 N HCl to permit a rough estimation of the diffusion constant, yielding a molecular weight of 620,000–690,000.

At pH 5.0 and above, the 22S protein shows only one peak until a pH of 11–11.5 is reached. At this point, *Arbacia punctulata* protein produces 13, 9, and 6–7S subunits, with the 9S component being the predominant. Such a pattern is illustrated in Fig. 11 *b*. In the *Strongylocentrotus* species, this

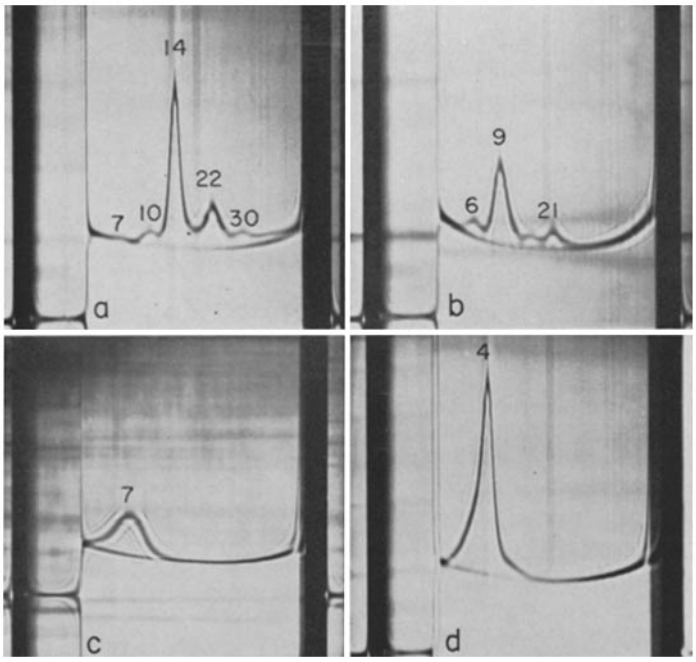

FIGURE 11 Subunit formation at extremes in pH.

a *Arbacia punctulata* 22S protein in 0.1 N HCl and 0.3 M KCl, 24 min after reaching a speed of 59,780 rpm. Bar angle, 60°. Subunits of 7, 10, and 14 Svedbergs, the original 22S protein, and higher aggregates are all evident.

b *Arbacia punctulata* 22S protein in 0.1 M Na_2CO_3 and 0.3 M KCl (pH 11.3), 16 min after reaching a speed of 59,780 rpm. Bar angle, 60°. Subunits 6, 9, and 13 Svedbergs and the 22S particle are present.

c *S. purpuratus* 22S protein in 0.1 M Na_2CO_3 and 0.3 M KCl (pH 11.3), 48 min after reaching a speed of 42,040 rpm. Bar angle, 60°. Only the 6–7S subunit (somewhat heterogeneous) is formed.

d *Arbacia punctulata* 22S protein in 0.1 N KOH and 0.3 M KCl, 48 min after reaching a speed of 59,780 rpm. Bar angle, 60°. At this high pH, a 4S subunit results, although there is some indication of both heavier and lighter components.

same pH range produces essentially all 6–7S material, although with some degree of heterogeneity (Fig. 11 *c*). Raising the pH to 11.5–12.0 produces a single 6–7S peak in *Arbacia punctulata*, indicating that the transition pH for alkaline subunit formation is higher in *Arbacia* than in the *Strongylocentrotus* species. These relationships are true only for freshly prepared protein. Older material produces not only the single 6–7S peak but contains, in addition, the 9S component which persists regardless of pH.

The intrinsic viscosity of *S. purpuratus* at pH 11.5 was found to be 17.5 ml/g (Fig. 12); this value coupled with the sedimentation rate observed at this pH and concentration (6.7S) gives a molecular weight of 254,000 for the particle. The diffusion constant for this material was found to be 2.59×10^{-7}; the resulting molecular weight from sedimentation diffusion is 242,000.

If the 22S protein is made 0.1 M in KOH, the 6–7S subunit results, but when the preparation stands overnight at 0°C a 4S component is formed (Fig. 11 *d*). Short column sedimentation equilibrium yielded a value of 131,000 for the molecular weight, roughly half that of the 6.7S particle. Efforts to reduce either of these alkaline subunits any further through the use of mercaptoethanol failed because of precipitation of the protein. Fig. 13, a plot of $S_{20,w}$ versus pH, summarizes the pH stability of the 22S protein.

Effects of Urea and Guanidine Hydrochloride

In 2 M urea, a polydisperse 12–14S mixture appears in addition to the 22S protein (Fig. 14

a). At a urea concentration of 4 M or above, one major peak with a sedimentation rate of 5–6S results, accompanied by a small, trailing 3.5–4S component (Fig. 14 *b*). Determination of the molecular weight of this major 5–6S component by sedimentation equilibrium yielded a value of 233,000. (The previous assignment of 110,000 as the molecular weight of the 5–6S particle (Stephens, 1965) was an error in calculation).

In 8 M urea plus 2% mercaptoethanol (Fig. 14 *c*), a single 2.5–3S particle is formed. The diffusion constant for this component, sedimenting at 2.7S, was found to be 2.03×10^{-7}; the molecular weight from sedimentation diffusion is thus 120,000. A value of 115,000 is obtained from sedimentation equilibrium, while the Yphantis method yields 103,000, all of these figures being for *S. droebachiensis*. Like the 6–7S high pH subunit, the 5–6S urea subunit may be accompanied by a higher aggregate in old preparations; addition of mercaptoethanol to such a mixture results in only the 2.5–3S subunit. It is very likely that the higher aggregates which occur in such preparations are disulfide-linked, considering their urea and pH stability and susceptibility to mercaptoethanol.

As mentioned above, the 22S particle in 0.6 M KCl was found to have a b_0 of −123 corresponding to about 20% helix. In 8 M urea, where the 5–6S subunit is formed, the b_0 becomes −54, while addition of 2% mercaptoethanol reduces the b_0 to zero. Thus, considerable unfolding occurs in the formation of the 5–6S subunit and a random coil results upon breakage of the disulfide bridges with mercaptoethanol.

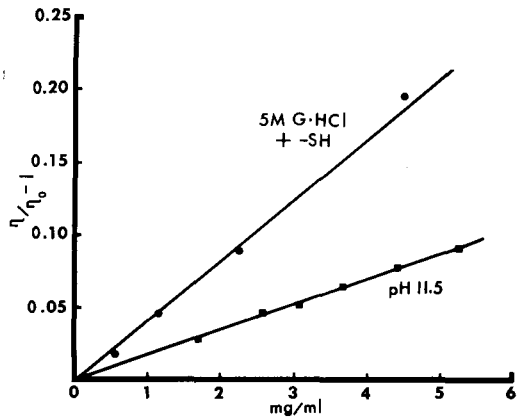

FIGURE 12 Variation of specific viscosity with concentration for *S. purpuratus* 6–7S high pH subunit (squares) and *Arbacia punctulata* 2.5–3S guanidine-mercaptoethanol subunit (circles). The alkaline subunit is in 0.1 M Na_2CO_3 and 0.3 M KCl (pH 11.5); the solvent for the guanidine subunit is 5 M guanidine hydrochloride and 2% mercaptoethanol. The intrinsic viscosity of the alkaline subunit (at 25.0°C) is 17.5 ml/g, whereas that for the guanidine subunit (at 20.0°C) is 40 ml/g.

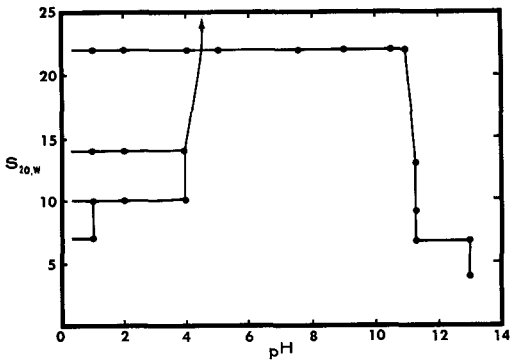

FIGURE 13 Generalized pH stability for 22S protein. The alkaline transition shown here for *S. purpuratus* occurs about 0.5 pH units higher in the case of *Arbacia punctulata*; the high pH transitions are somewhat time dependent.

In 5 M guanidine hydrochloride, a 5–6S particle also forms but, in this case, a smaller 4–4.5S subunit predominates (Fig. 14 *d*). It is likely that this slower component corresponds to the 3.5–4S minor component found in 8 M urea. Addition of sulfite (Fig. 14 *E*) or mercaptoethanol (Fig. 14 *f*) results in the formation of 2.5–3S material. The guanidine hydrochloride-mercaptoethanol subunit (2.9S) has a diffusion constant of 2.57×10^{-7}, yielding a molecular weight of 107,000. The intrinsic viscosity of this particle is 40 ml/g (Fig. 12); this value gives a molecular weight of 112,000 by the Scheraga-Mandelkern (1953) relationship.

Table III summarizes the molecular weight data for the pH, urea, and guanidine hydrochloride subunits. One must bear in mind that only the reduced urea or guanidine hydrochloride subunits appear to be free of higher and lower molecular weight aggregates, and the remaining results may, therefore, deviate somewhat from the true values. From the tabulation, it becomes evident that the 4S alkaline subunit and the 2.5–3S mercaptoethanol-reduced urea and guanidine hydrochloride subunits are probably the same.

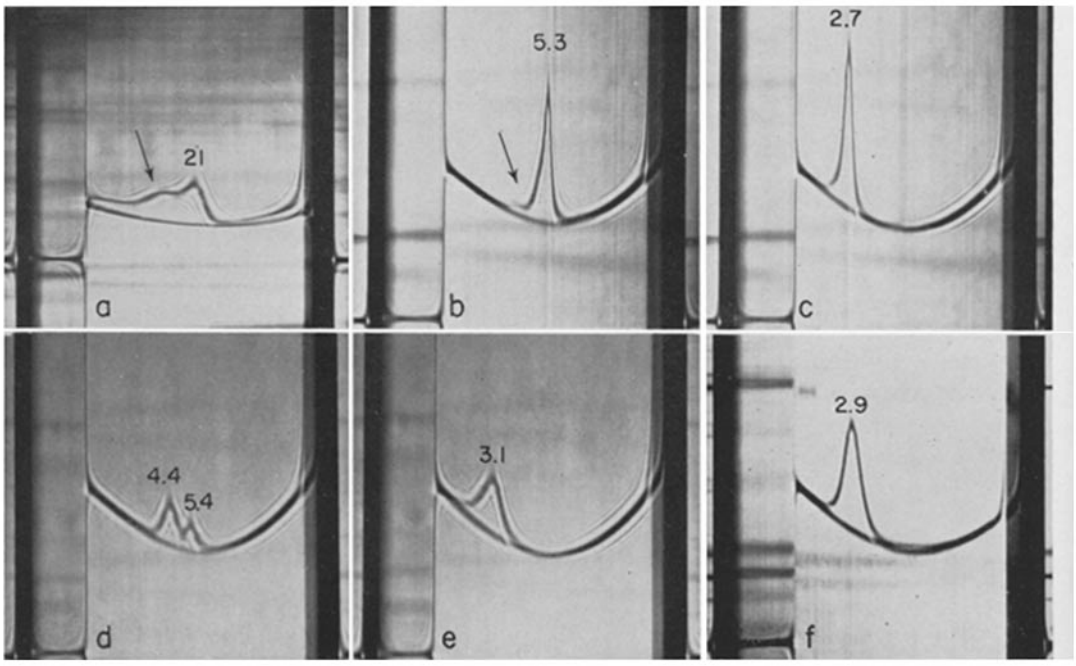

FIGURE 14 Urea and guanidine hydrochloride subunits of the 22S protein.

a *S. purpuratus* 22S protein in 2 M urea and 0.4 M KCl, 48 min after reaching a speed of 42,040 rpm. Bar angle, 60°. The original protein, some polydisperse 12–14S (arrow) material, and both higher and lower aggregates are evident.

b *Arbacia punctulata* 22S protein in 8 M urea, 160 min after reaching a speed of 59,780 rpm. Bar angle, 60°. A 5–6S subunit is formed and a lighter (3.5–4S, arrow) component is also present.

c *Arbacia punctulata* 22S protein in 8 M urea plus 2% mercaptoethanol, 17 min after reaching a speed of 59,780 rpm. Bar angle, 60°. A single 2.5–3S subunit is formed.

d *S. droebachiensis* 22S protein in 5 M guanidine hydrochloride, 128 min after reaching a speed of 59,780 rpm. Bar angle, 60°. As in (*b*), a 5–6S subunit is produced but in this case a smaller (4–5S) subunit is predominant.

e *S. droebachiensis* 22S protein in 5 M guanidine hydrochloride plus 0.01 M sodium sulfite, 128 min after reaching a speed of 59,780 rpm. Bar angle, 60°. An unfolded subunit, probably comparable to (*c*), forms after sulfite reduction.

f *S. purpuratus* 22S protein in 4 M guanidine hydrochloride plus 2% mercaptoethanol, 128 min after reaching a speed of 59,780 rpm. Bar angle, 60°. A 2.5–3S subunit is produced as in (*c*).

The 6–7S alkaline subunit and the 5–6S urea subunit are of essentially the same molecular weight and appear to represent dimers of the 4 or 2.5–3S subunits. The 6–7, 9, and 13S alkaline pH subunits are probably equivalent to the 7S, 10S, and 14S acid subunits, the minor differences in sedimentation rate being due to pH or variation of sedimentation rate with concentration. Considering the stability of the 5–6 or 6–7S dimer, and considering that the 14S acid subunit yielded a molecular weight of 600,000–700,000, it is probable that the subunits associate in pairs, making the 9–10S subunit a tetramer and the 13–14S subunit a hexamer. The 2.5–3S urea-mercaptoethanol and guanidine hydrochloride–mercaptoethanol subunits have an average molecular weight of 112,000; on the basis of the above data on association of dimers, the 22S particle is likely an octamer with a predicted molecular weight of 896,000. The average value actually obtained was 880,000.

REASSOCIATION OF SUBUNITS

As mentioned above, acid treatment of the 22S protein produces predominantly 14S material along with some 10S and a trace of 7S component;

TABLE III

Subunit Molecular Weights Determined by Sedimentation Diffusion, Sedimentation Equilibrium, Short Column Equilibrium, and Sedimentation Viscosity

	Strongylocentrotus sp.		*Arbacia punctulata*				
	2.5-3S (urea)	6-7S (pH 11.5)	2.5-3S (urea)	2.5-3S (G.HCl)	4S (high pH)	5-6S (urea)	14S (low pH)
Sedimentation diffusion	120,000	242,000	112,000	107,000	—	—	620,000-690,000
Sedimentation equilibrium	115,000	—	122,000	—	131,000	233,000	—
Short column equilibrium	103,000	—	106,000	105,000	—	—	—
Sedimentation viscosity	—	254,000	—	112,000	—	—	—

mild basic treatment yields chiefly 9S accompanied by some 6–7 and 13S, and strong basic treatment yields either 6–7 or 4S material, depending upon length of treatment. When such mixtures are returned, by dialysis, to 0.6 M KCl at neutrality, much material comes out of solution but the supernate contains both 14 and 22S components. In the acidic and short-term basic mixtures (Fig. 11 *a*, *b*, and *c*), the 14S material predominates upon return to neutrality; there is no evidence of the original smaller 6–7 or 9–10S subunits (Fig. 15 *a*). In the case of the 0.1 N KOH-treated material (Fig. 11 *d*), the original 4S subunit persists on return to neutral conditions and the amount of reformed 22S exceeds the somewhat heterogeneous 12–14S material (Fig. 15 *b*).

Brief (less than 1 hr) urea treatment produces a 5–6S subunit (Fig. 14 *b*) which, upon dialysis into 0.6 M KCl, returns almost completely to the 22S form (Fig. 15 *c*) with only a small amount of 14S material being evident. Longer urea treatment, followed by dialysis, also yields 22S, but more 14S component, and also higher aggregates, become evident (Fig. 15 *d*). Identical results are obtained from the 5 M guanidine hydrochloride subunits (Fig. 14 *d*), where a smaller subunit predominates. The smallest urea or guanidine hydrochloride subunit, that resulting from mercaptoethanol treatment (Fig. 14 *c* and *f*), produces only a polydisperse mixture upon return to neutral 0.6 M KCl (Fig. 15 *e*).

When the 22S protein at pH 11.5 (Fig. 11 *c*) is made 0.05 M in Salyrgan (mersalyl acid) and then dialyzed into 0.6 M KCl at pH 7.5, a stable 10S subunit, accompanied by some 14S material, is formed (Fig. 15 *f*). Normally, without Salyrgan, 14S and 22S material would have been produced (Fig. 15 *a*). After prolonged dialysis (over 24 hr), nearly all the 10S material precipitates from solution.

The Role of Sulfur in Subunit Association

The following reagents have no measureable effect on the sedimentation rate of the 22S protein in 0.6 M KCl: 0.1 M Salyrgan at pH 7.5; 2% mercaptoethanol; 0.05% dithiothreitol; 0.05 M sodium sulfite; and 2% dithiodiglycol. All but Salyrgan promote the formation of higher aggregates in the range of 30–35S. Dithiodiglycol is the most effective in this respect and produces polydisperse aggregation of even higher sedimentation rate. Such effects might be due to disulfide interchange resulting in cross-linkage between 22S particles under the mediation of sulfhydryl or disulfide reagents. The effect can be reversed by the presence of 0.1–0.2% dithiothreitol, implying reduction of any intermolecular disulfide linkages.

In light of the fact that disulfide bridges appear to be necessary for proper folding of subunits to allow formation of the 22S particle, as evidenced by the observation that mercaptoethanol-treated urea subunits will not recombine to the 22S form, it seems curious that the 22S protein remains intact after either mercaptoethanol or dithiothreitol treatment, particularly in light of the effectiveness of the latter reagent (Cleland, 1964). From amino acid analysis there are 9–10 potential half-cystines per 100,000 g of protein but *p*-

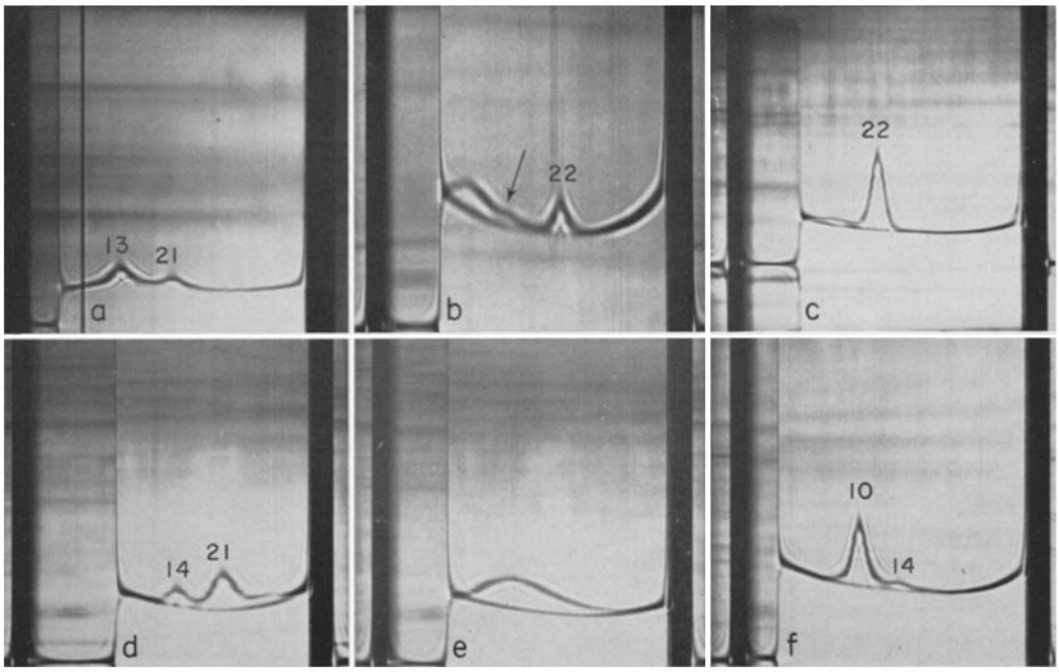

FIGURE 15 Recombination of protein subunits.

a *S. purpuratus* 6–7S alkaline subunit (Fig. 11 *c*) transferred from 0.1 M Na_2CO_3 and 0.3 M KCl (pH 11.5) to 0.6 M KCl, pH 7.5, by dialysis; 32 min after reaching a speed of 42,040 rpm. Bar angle, 60°. The 22S protein reforms but 13S material is predominant.

b *Arbacia punctulata* 4S alkaline subunit (Fig. 11 *d*) transferred from 0.1 M KOH and 0.3 M KCl to 0.6 M KCl, pH 7.5; 24 min after reaching a speed of 59,780 rpm. Bar angle, 50°. The 22S particle reforms, less 13–14S material (arrow) is present than in (*a*), but the subunit itself persists.

c *S. purpuratus* 5–6S urea subunit (Fig. 14 *b*) dialysed into 0.6 M KCl, pH 7.5, after 1 hr urea treatment at 20°C, 20 min after reaching a speed of 42,040 rpm. Bar angle, 60°. The 22S protein has reformed; only a small amount of lower molecular weight aggregate accompanies it.

d Same as (*c*) except the urea treatment was for 6 hr; 32 min after reaching a speed of 42,040 rpm. Bar angle, 50°. The 22S protein again reforms, but much 14S material and some higher aggregates are present.

e *S. droebachiensis* 2.5–3S urea-mercaptoethanol subunit (as in Fig. 14 *c*) dialysed into 0.6 M KCl, pH 7.5, from 8 M urea plus 2% mercaptoethanol; 32 min after reaching a speed of 42,040 rpm. Bar angle, 50°. Breakage of disulfide bridges does not permit reaggregation to the 22S particle.

f *S. droebachiensis* 6–7S alkaline subunit treated with 0.05 M Salyrgan and dialysed into 0.6 M KCl, pH 7.5, 24 min after reaching a speed of 59,780 rpm. Bar angle, 60°. Without Salyrgan, a pattern such as (*a*) results, but in this case only 10S material and a trace of 13–14S component are seen.

mercuribenzoate titration reveals less than one sulfhydryl per 100,000 g of 22S protein or 8 M urea subunit, implying that the remainder exist as disulfide bridges. Treatment of the 22S protein with cyanide produces no detectable sulfhydryls when the nitroprusside test is used; urea-treated protein gives a highly positive test, indicating that the disulfides were hidden or masked in the 22S form but became accessible to cyanide reduction in the dissociated form. Hidden or protected disulfide bonds may well explain the stability or the 22S particle in mercaptoethanol or dithiothreitol.

DISCUSSION

The 22S Particle

The major 22S protein component of the mitotic apparatus, purified from hexylene glycol mitotic apparatus isolates and extracts of whole

eggs and acetone powders of eggs, is a relatively symmetric particle with a mean molecular weight of 880,000, dimensions of roughly 150 by 200 A, and α-helical content of 20%. The protein is homogeneous on the basis of sedimentation velocity behavior; sedimentation-equilibrium studies show lower molecular weight material to be absent and indicate higher aggregates of the 22S particle only at relatively high protein concentrations or at ionic strengths below 0.3.

When prepared as outlined above, the protein is free of nucleotide or nucleic acid. Zimmerman (1960) and Sakai (1966) both report nucleic acid to be present in protein fractions obtained from alcohol-digitonin and dithiodipropanol isolates, respectively. Mazia (1955) reported that both protein and nucleoprotein components could be obtained from digitonin isolates. The 22S protein of this study contains 3–5% nucleic acid if either dialysis or DEAE-cellulose treatments are eliminated, but the protein is free of 260 mμ absorbing material if either precaution is taken. The question of whether the nucleic acid is an integral part of the protein remains unanswered. It may be a contaminant or else a loosely bound but functionally necessary component in the mitotic apparatus structure.

The carbohydrate associated with the 22S particle is apparently tightly bound since neither dialysis nor DEAE-cellulose treatment removes it. Its function, if any, is unknown.

The amino acid analysis of *S. purpuratus* 22S protein differs somewhat from that of the whole spindle preparation of Roslansky (Mazia, 1955). Many of the amino acids occur in roughly the same proportions but several very significant differences exist between others, notably alanine, arginine, glutamic acid, glycine, serine, threonine, and tyrosine. One must bear in mind, however, that neither analysis was corrected for destruction of serine and threonine on hydrolysis and that the Roslansky assay was done by starch column chromatography with a crude spindle preparation, not a purified protein. Considering these facts, the differences seem less serious. It might be pointed out that, though the early whole spindle analysis somewhat resembled the muscle protein actin, the 22S protein shows no obvious similarities to either rabbit actin or myosin (Kominz et al. 1954).

It is conceivable that the 22S protein might be a ribosomal contaminant since ribosomes are quite abundant in the interstitial areas of the mitotic apparatus (Kane, 1962 *b*), and a ribosomal protein might conceivably arise through the breakdown of these particles. This point is settled by the amino acid composition. Ribosomal proteins throughout the animal kingdom show similar and characteristic amino acid compositions (Peterman, 1965). The 22S protein is significantly unlike any reported. On the basis of density-gradient centrifugation, Sakai (1966) arrived at a similar conclusion concerning the 22S particle observed as a minor component from dithiodipropanol isolates.

Kolodny and Roslansky (1966) have carried out optical rotatory dispersion studies on hexylene glycol–isolated mitotic apparatus preparations from *S. droebachiensis*. Although the dispersion was somewhat anomalous owing to the presence of nucleic acids, these workers estimate an α-helix content of about 30% for the constituent proteins. This value is in reasonable agreement with the values reported here for the purified 22S component from the same species: no more than 30% from the proline content and about 20% from optical rotatory dispersion.

Neither hexylene glycol spindle isolates nor the 22S protein shows any ATPase activity. No activity was found in the early preparations of alcohol-digitonin isolates (Mazia, 1955), but more recently Miki (1963) has detected ATPase activity in preparations treated only briefly with alcohol. Dithiodiglycol spindles (Mazia et al. 1961 *a*) and asters (Dirksen, 1964) have both been shown to possess low ATPase activity. Miki (1963) has demonstrated that an ATPase is localized in the spindle region of the intact cell, but it has not been demonstrated that this ATPase activity is associated with the fibrous structural component. The low enzymatic activity found in the alcohol-digitonin and dithiodiglycol spindles thus may be due to a nonfibrous matrix component carried along with these isolates. It is possible, however, that ATPase activity might be affected by the preparatory procedures for hexylene glycol spindles, whole egg extracts, and acetone powders. A totally different procedure for the preparation of 22S protein, not involving organic solvents, may shed some light on the question.

Mazia et al. (1961 *b*) have shown spindle isolates to be stabilized by the presence of dithiodiglycol, while Kane (1962 *a*, 1965 *a*) has demonstrated that many glycols not containing the disulfide linkage serve the same purpose. However, the dithiodiglycol isolates are generally more

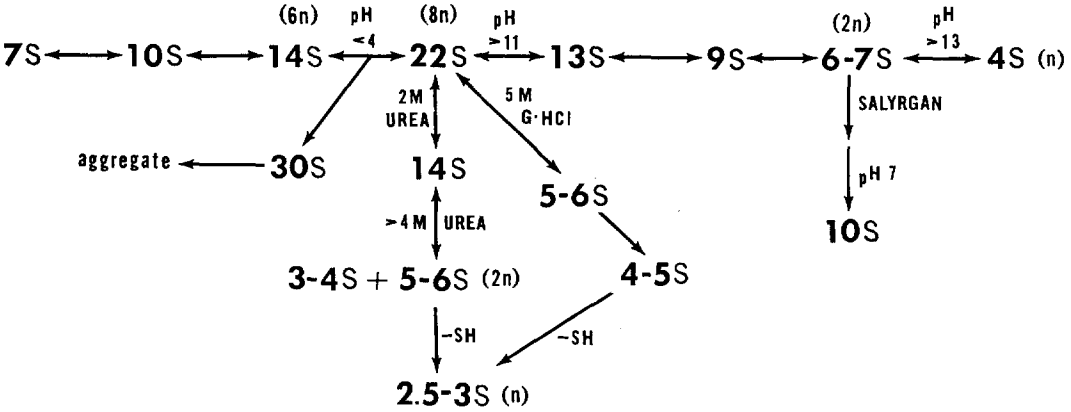

FIGURE 16 Summary of acid, base, urea, and guanidine hydrochloride dissociation products of the 22S protein. Many of these steps, though reversible, often result in the precipitation of much material. Where molecular weight determinations were carried out, the molecular weight is indicated in terms of multiples of "*n*," the average weight of the smallest subunit thus far obtained (112,000).

difficult to dissolve in neutral salt than their hexylene glycol counterparts and both become insoluble on standing (Dirksen, 1964; Kane and Forer, 1965), requiring the use of either high pH or sulfhydryl reagents for solubilization. In this study, the 22S protein, even in high salt, will form aggregates when either sulfhydryl or disulfide compounds are present. High concentrations of mercaptoethanol (2%) or dithiothreitol (0.2%) will reverse such an effect. It appears that the presence of small amounts of such reagents catalyze disulfide interchange between the 22S particles, i.e., intermolecular cross-linkage. Thus it is quite conceivable that the presence of dithiodiglycol could promote the formation of disulfide linkages, and spindles isolated from such a medium may become rapidly cross-linked. The same action may take place in hexylene glycol isolates but the effect would be minimized and would depend upon sulfhydryl compounds naturally present in such isolates and also upon atmospheric oxidation.

Miki-Noumura (1965) found 40 A microfilaments in the supernate of hexylene glycol mitotic apparatus isolates treated with neutral 0.5 M KCl. At mildly acid pH, in the presence of magnesium, the 22S protein of the present study forms fibers with diameters of 40–60 A (Fig. 10); these presumably arise through a rearrangement of subunits, since the diameter of the fiber is less than that of the parent 22S particle. Miki-Noumura has suggested that the tubular elements of the mitotic apparatus break down longitudinally, giving rise to microfilaments. The 40–60 A fibers reported here may likewise represent an intermediate stage in tubule formation, but it is possible, of course, that in either or both cases the filaments represent protein aggregates completely unrelated to microtubules.

Recently, Malkin, Mangan, and Gross (1965) have reported a crystalline 27S protein associated with yolk granules in eggs of several species of sea urchins. The 27S particle had a molecular weight of 894,000 versus 880,000 for the 22S protein of this study. Upon negative staining, the 27S particle was morphologically similar to, if not identical with, the 22S protein. Slight differences in sedimentation rate and ultraviolet absorption spectra were evident but these discrepancies may only reflect differences in preparation, purification, and methods of measurement. Using centrifuged half eggs. Malkin et al. found that the 27S protein was localized primarily in the yolk granules; similar experiments by the author have shown that the 22S particle is likewise associated with the granular fraction. The 22S particle, however, cannot be crystallized by the ammonium sulfate fractionation method employed by Malkin et al. to prepare crystals of the 27S protein; crystals of identical morphology result but are found to consist chiefly of ammonium sulfate containing only a few per cent protein. It appears as if these two proteins may be identical, but conclusive proof of identity awaits data on the amino acid composition and subunit association for the 27S protein.

In regard to the number of ultracentrifugal

components and their characteristics, the results of this and the preceding study (Kane, 1967) are at variance with those of previous workers, the only similarity being the presence of a 20–22S particle as a minor constituent of dissolved asters (Dirksen, 1964) or mitotic apparatus isolates (Sakai, 1966). However, the 22S particle yields a wide variety of subunits which have properties closely resembling many of the protein fractions previously observed.

Subunit Association

Above pH 11 and below pH 4, the 22S protein dissociates into 6–7, 9–10, and 13–14S subunits. The molecular weights of the 6–7 and 14S particles have been estimated at 240,000 and 600,000–700,000, respectively. In 0.1 N KOH, a 4S material is formed, having a molecular weight of 130,000 but containing higher aggregates. At urea concentrations greater than 4 M, a 5–6S subunit of molecular weight 230,000 is obtained; upon addition of mercaptoethanol, the sedimentation rate becomes 2.5–3S and the molecular weight is halved. The 5–6 and 6–7S subunits thus represent dimers of the 2.5–3S or 4S subunits, suggesting that the 9–10, 13–14, and 22–24S particles are tetramer, hexamer, and octamer, respectively, of the 110,000–115,000 molecular weight subunits. The dimer, though quite stable as such, is apparently not disulfide-linked since the smaller subunit ("monomer") exists in 8 M urea or guanidine hydrochloride along with the dimer, while the 4S particle readily forms in 0.1 N KOH without the use of sulfhydryl reagents. Addition of mercaptoethanol to the 5–6S dimer apparently cleaves intramolecular disulfide bridges, permitting complete unfolding and concomitant dissociation into the monomer unit. The term "monomer" is not meant to imply a single polypeptide chain; there may still be smaller subunits than those found in this study.

The subunits formed by either acidic or basic treatment produce 22S protein when restored to neutral buffer but considerable 13–14S material, apparently not reassociable, is present. The urea or guanidine hydrochloride–formed 5–6S subunit almost completely reforms the 22S particle, if the treatment is brief. The 2.5–3S urea-mercaptoethanol subunit produces a polydisperse mixture, presumably because of random reformation of disulfide bonds, upon transfer to neutral conditions. However, the integrity of the 22S protein itself is not affected by sulfhydryl reagents at neutral pH, suggesting that disulfide bridges are either inaccessible to the reagent or are unnecessary for the maintenance of tertiary structure once the 22S particle has formed from subunits. These various subunit relationships are summarized graphically in Fig. 16.

Some interesting correlations exist between the results of this study and those of previous workers. Though circumstantial, these facts may well explain many of the conflicting results.

Mazia and Dan (1952) and Mazia (1955) dissolved either oxidation-stabilized or alcohol-digitonin spindles in 0.5 N NaOH and obtained a single 4S component. The 22S protein of this study yields a 4S subunit on treatment with 0.1 N KOH (Fig. 11 *d*) and this component is not completely reassociable to higher aggregates (Fig. 15 *d*). Thus both studies show similar subunits after strong alkaline treatment.

Zimmerman (1960), upon dissolving alcohol-digitonin spindles in alkaline 0.1 M Salyrgan, found heterogeneous 3.7 and 8.6S components; the latter component vanished after dialysis. The 22S protein, when treated with 0.05 M Salyrgan at pH 11.5, produces a 10S material not reassociable to either the 14 or 22S particles (Fig. 15 *f*); upon extended dialysis, the material precipitates from solution. The minor 8.6S component of Zimmerman would be expected to have a higher sedimentation rate when pure, and might well correspond to the 10S particle of this study. Sulfhydryl hydrogen bonding may be involved in the formation of the 22S particle from the 10S subunit since Salyrgan, which binds sulfhydryl groups, prevents the reassociation.

Dirksen (1964) dissolved dithiodiglycol asters in alkaline 0.5 M KCl and found, after return to neutral conditions, a 14S particle accompanied by much heterogeneous low molecular weight material and a trace of a 21S component. The 22S protein, after alkaline treatment, produces chiefly 13–14S material after dialysis into neutral buffer (Fig. 15 *a*). Under the conditions used by Dirksen, one would expect a 14S subunit to result from any 22S protein present.

The 2.5 and 3.5S sulfite subunits obtained by Sakai (1966) were thought to be derived from 13S material also present in dissolved dithiodipropanol spindle isolates. The 13S material could very well have arisen from 22S protein, a trace of which was observed in such preparations. Sakai obtained molecular weights of 34,700 and 68,700

for the 2.5 and 3.5S particles, respectively, using the Archibald technique, and he points out that the Archibald molecular weight of 315,000 obtained by Zimmerman (1960) for the somewhat heterogeneous 3.7S particle (presumably corresponding to the 3.5S sulfite subunit) was much too high and was likely a result of the coexistence of higher molecular weight particles. The converse argument, however, can be applied to Sakai's data and the validity of his conclusions likewise depends upon demonstration of homogeneity. An average molecular weight of 112,000 was found for the 2.5–3S reduced urea or guanidine hydrochloride subunits in the present study; the particles were homogeneous on the basis of sedimentation velocity and sedimentation equilibrium. Without resorting to reducing agents, Miki-Noumura (1965) produced a 2.3S component from alkaline-dissolved microfilaments of hexylene glycol isolates but did not carry out a detailed study on the particle. The relationship of this 2.3S alkaline subunit to the 2.5S sulfite-cleaved subunit of Sakai and the reduced urea or guanidine hydrochloride 2.5–3S subunit reported here cannot be established with the data available.

Thus it appears that the 4S component of Mazia (1955), the 8.6S material of Zimmerman (1960), and the 13–14S particles of Dirksen (1964) and Sakai (1966) correspond to subunits obtainable from the 22S protein when this protein is treated in a manner similar to the spindle isolates employed in these various studies. The 2.5S and 3.5S sulfite components of Sakai or the 3.7S Salyrgan material obtained by Zimmerman may be the same as the urea or guanidine hydrochloride subunits reported here, but of modified form, unable to reassociate at neutrality, or they may more likely correspond to some component in the 4–5S heterogeneous mixture reported by Kane (1967) to accompany the major 22S protein. The relationship of any of these subunits to the structure and function of the mitotic apparatus microtubule is, as yet, unclear.

The Relationship of the 22S Particle to Microtubules

Considering factors of size and geometry, it is unlikely that the 22S particle per se is a segment of the classical 180–300 A microtubule. It is clearly too large to be a subunit of the tubule wall but is too small to be a cross-sectional element since the particle is, at most, a globule of 150 × 200 A. Though it appears to have a hollow core or center, it does not appear to be a well-defined cylinder. Also, the 22S particle is made up of eight subunits whereas microtubules from sperm tails (Pease, 1963) and plant cell walls (Ledbetter and Porter, 1964) have been shown to be composed of 10 and 13 subunits, respectively.

The 22S particle could represent, however, a reaggregation of microtubular subunits. It may be significant that the 22S protein, under acid conditions and in the presence of magnesium, forms 40–60 A microfilaments similar in appearance to those obtained by Miki-Noumura (1965) by dissociation of the mitotic apparatus. Microtubules of sperm tails (Pease, 1963) and the mitotic apparatus (Barnicot, 1966) are made up of longitudinal fibers of 35–40 A globular subunits with a center-to-center spacing somewhat greater than this dimension. Conceivably, the 22S protein could represent an inactive aggregate of such globular subunits which, under proper conditions, could reaggregate into microfibrils and thence into microtubules.

One final alternative remains, however. The low molecular weight 3.7, 2.3, and 3.5S particles observed by Zimmerman (1960), Miki-Noumura (1965), and Sakai (1966), respectively, do not appear to be obviously related to the 22S particle but more likely correspond to the 4–5S material observed in dissolved hexylene glycol isolates by Kane (1967). It may be one of these particles which represents the 35–40 A globular subunit of the microtubule, and the 22S particle may be a second component of the mitotic apparatus, possibly a matrix material intimately associated with the microtubules.

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