Journal of Cellular Biochemistry 19:45-57 (1982)

Heterogeneity of 14S and 30S Dynein ATPase Activities With Respect to Activation by Calmodulin

Jacob J. Blum, A. Hayes, T. Vanaman, and F.H. Schachat

Departments of Physiology (J.J.B., A.H.), Microbiology/Immunology (T.V.), and Anatomy (F.H.S.), Duke University Medical Center, Durham, North Carolina 27710

Demembranated cilia of Tetrahymena pyriformis were extracted with KCl or Tris-EDTA and the crude dyneins from each resolved by sucrose density gradient sedimentation into 14S-K, 30S-K, 14S-E and 30S-E dyneins, respectively. The calmodulin activation ratio (ATPase activity in presence of added calmodulin/ATPase activity in absence of added calmodulin) did not vary across the 30S dynein fractions regardless of the method of extraction nor did it vary across the 14S-E region. However, in going from the "heavy" fractions to the "light" fractions of the 14S-K region, it increased markedly.

The concentration of calmodulin required for half-maximal activation did not differ appreciably in the "light" versus the "heavy" fractions of the 14S-K region, suggesting that the affinity for calmodulin does not vary in these fractions. SDS-polyacrylamide gel electrophoresis studies showed the presence of several polypeptides that varied in a systematic fashion across the 14S-K region and hence may be involved in regulating the sensitivity of 14S-K dynein ATPase activity to added calmodulin.

Key words: dynein ATPase, calmodulin, heterogeneity of composition

We have previously shown that calmodulin is present in the cilia of Tetrahymena pyriformis [1] and that it confers Ca^{++}-sensitivity on the ATPase activities of both 14S and 30S dyneins [2]. The degree of activation of the 14S dynein ATPase depends on the extraction procedure used: The ATPase activity of 14S-K dynein (prepared by extraction of demembranated axonemes with 0.5 M KCl) is variably activated (up to 5-fold) by the addition of calmodulin, whereas 14S-E dynein (prepared by Tris-EDTA extraction) is usually activated less than 2-fold. The reason for the variability in response of the 14S-K dyneins to added calmodulin is unknown, as is the reason for the greater activatability of 14S-K as compared to

Abbreviations used: Tris, tris (hydroxymethyl) amino methane; EGTA, ethylene glycol-bis (β-aminoethyl ether)-N,N'-tetraacetic acid; IMT buffer, 50 mM Tris/50 mM imidazole/7.5 mM $MgCl_2$/0.4 mM EGTA, ph 7.5; SDS, sodium docecyl sulfate.

Received May 13, 1981; revised and accepted November 16, 1981.

0730-2312/82/1901-0045$04.00

14S-E dynein. The ability of calmodulin to activate 30S-E dynein ATPase is about the same as that of 30S-K, and for both a higher concentration of calmodulin is required than is necessary to activate the 14S dyneins [2]. Since the dyneins are complex multicomponent assemblies [2–5], a possible explanation for these variations is the differential extraction of some regulatory component(s) from the dynein arms, but a variable extraction of other regulatory factors is also possible. We therefore decided to examine the response to added calmodulin of individual fractions from the sucrose density gradient instead of pooling the fractions and then measuring the response of the pooled 14S and 30S dyneins. We report here that the sensitivity of 14S-K dynein ATPase activity varies systematically across the peak, whereas those of 14S-E, 30S-E, or 30S-K do not. Gel electrophoresis studies indicated that a possible basis for the systematic variation of the enzymatic properties of the 14S-K dynein-containing fractions is a systematic variation of certain polypeptide species.

METHODS

The preparation of cilia from cultures of T pyriformis (strain HSM) and their demembranation by treatment with 0.5% (v/v) Triton X-100 was performed as described [1]. Crude dynein was prepared by extracting the axonemes with either 0.5 M KCl in IMT/6 buffer (8.33 mM Tris/8.33 mM imidazole/1.25 mM $MgCl_2$/0.067 mM EDTA, pH 7.5) for approximately 18 hr at 4°C or with 1 mM Tris/0.1 mM EDTA, pH 8.0, for 22 hr at 4°C [2]. The crude dyneins were resolved by sedimentation on a linear gradient (5 to 30%, w/v) of sucrose dissolved in IMT/6 buffer [1,2]. In accordance with our preceding nomenclature [2,6] the 14S dyneins obtained from gradients of crude dynein prepared from KCl-extracted axonemes and from Tris-EDTA extracted axonemes will be referred to as 14S-K and 14S-E dyneins, respectively, and similarly for the 30S-K and 30S-E dyneins.

ATPase activity was measured for 20 min at 25°C in a final volume of 1 ml. Reactions were terminated by the addition of 0.1 ml of 30% (w/v) trichloroacetic acid and the inorganic phosphate released was measured by a colorimetric assay [7]. Each reaction mixture contained 0.3 ml of IMT buffer, pH 7.5, 0.1 ml of 12.5 mM $CaCl_2$, from 0.05 to 0.15 ml of the dynein fraction to be assayed, and distilled water to bring the volume to 0.9 ml. Reactions were initiated by adding 0.1 ml of 10 mM ATP. In experiments in which calmodulin was to be added, 20 μg of bovine brain calmodulin, purified as described [2] and dissolved in water, was preincubated with the dynein for 4 min before the addition of ATP to allow the calmodulin to bind to the dynein [2].

Protein was measured by the Coomassie blue dye binding assay described elsewhere [8], using bovine serum albumin as a standard.

Electrophoresis on SDS-polyacrylamide gels was performed as described earlier [9]. Gels were stained by the silver nitrate method of Oakley et al [10]. Samples of 12.5 μl were applied and the electrophoresis was performed in an electrophoresis apparatus cooled with tap water.

The source of all chemicals is as earlier described [1,2].

RESULTS

Heterogeneity of 14S-K and 30S-K Dyneins

Figure 1A shows a profile of the ATPase activity (measured in the presence of Ca^{++} but the absence of added calmodulin) and of protein concentration in a typical

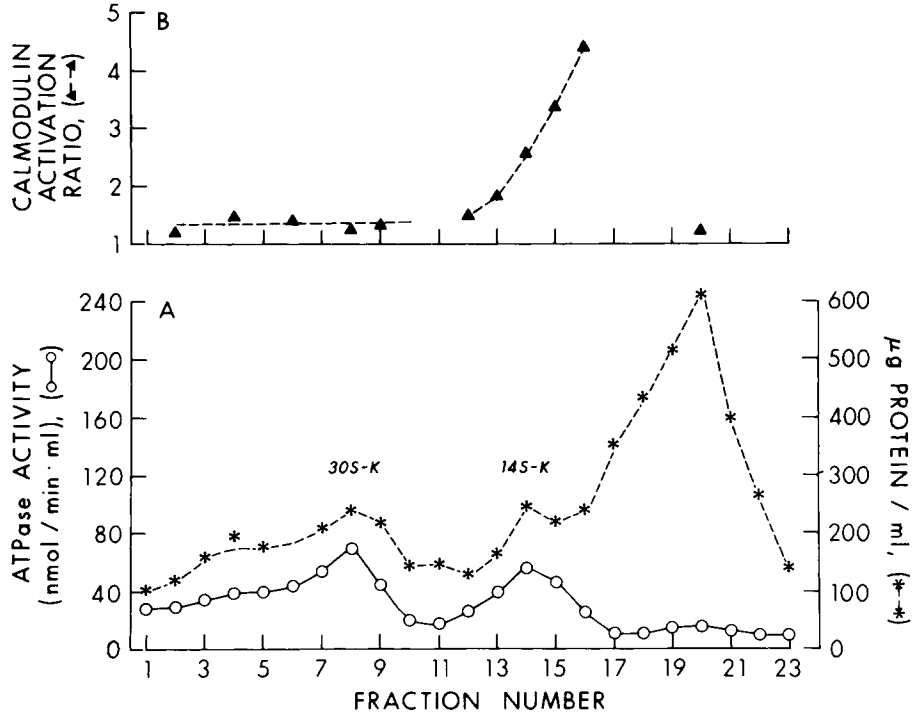


Fig. 1. Sensitivity of 14S-K and 30S-K dynein ATPase activities to calmodulin. Demembranated axonemes were extracted with KCl and the crude dynein resolved by sucrose density gradient centrifugation as described in Methods. The distribution of ATPase activity, measured in the absence of added calmodulin (○———○) and of protein (*- - - - -*) are shown in (A). The calmodulin activation ratio (ATPase activity measured in presence of 20 μg calmodulin/ATPase activity in absence of added calmodulin), ▲---▲, presented for selected fractions in (B).

sucrose density gradient of the crude dynein obtained by extracting axonemes with 0.5 M KCl. Much of the protein solubilized by this procedure is of low S value and appears near the top of the gradient, in fractions 19–21. The 14S-K region of the ATPase activity (fractions 13–16) appears fairly symmetrical, but the 30S-K peak trails off very asymmetrically towards the high density region of the gradient. Figure 1B shows the calmodulin activation ratio, defined [2] as the ratio of ATPase activity measured in the presence of 20 μg added calmodulin (as shown in Fig. 1A) to that measured in the absence of calmodulin, for selected fractions of this gradient. This ratio is about 1.3 across the entire 30S-K region. The calmodulin activation ratio is also low in fraction 13, but rises steeply as one proceeds to the low density region of the 14S-K dynein peak. The extent of increase across the peak is variable: The lowest increase we have observed in other experiments was a rise from about 1.3 to 3; the highest was from about 1.3 to 7. Clearly, the 14S-K fractions are heterogeneous, at least with respect to their sensitivity to added calmodulin. For convenience, we shall refer to the ATPase in the high density region of the 14S-K peak as "heavy" 14S-K dynein, and to that in the low density region of the 14S-K peak as "light" 14S-K dynein.

In one experiment (data not shown) equal portions of crude dyneins obtained by KCl extraction of demembranated axonemes were placed either on a sucrose gradient made as described in Methods (ie, with EGTA and no added Ca^{++}) or on one which contained 1 mM Ca^{++} in excess of the EGTA. As shown earlier [1] there was a shift in the profile of ATPase activity in the densest fractions of the Ca^{++}-containing gradient such that the ATPase activity in fractions 1 to 6 of the Ca^{++}-containing gradient was appreciably higher than in corresponding fractions of the Ca^{++}-free gradient. The calmodulin activation ratio across the 30S-K region, however, ranged between 1.1 and 1.4. The 14S-K region showed a steeper rise in calmodulin activation (from 1.4 in fraction 13 to 6.5 in fraction 16) in the Ca^{++}-free gradient than in the Ca^{++}-containing gradient (which had a calmodulin activation ratio of ~ 5). We do not know whether this smaller apparent increase in calmodulin activation ratio of the light 14S-K particles in the Ca^{++}-free gradient as compared to the Ca^{++}-containing gradient is significant.

The distribution of ATPase activity and of protein in a sucrose density gradient of the crude dynein obtained from extraction of axonemes with Tris-EDTA is shown in Figure 2A. A larger amount of activity is solubilized by this procedure as compared to extraction by KCl (see Fig. 1A; the quantity of axonemes used in these two extractions was comparable). As expected from our earlier results, the calmodulin activation ratio of the 30S-E particles (Fig. 2B) was low (~ 1.3) all the way across the 30S-E region. In contrast to what was observed for 14S-K dynein (cf Fig. 1B), there was a very slight but systematic increase in the calmodulin activation

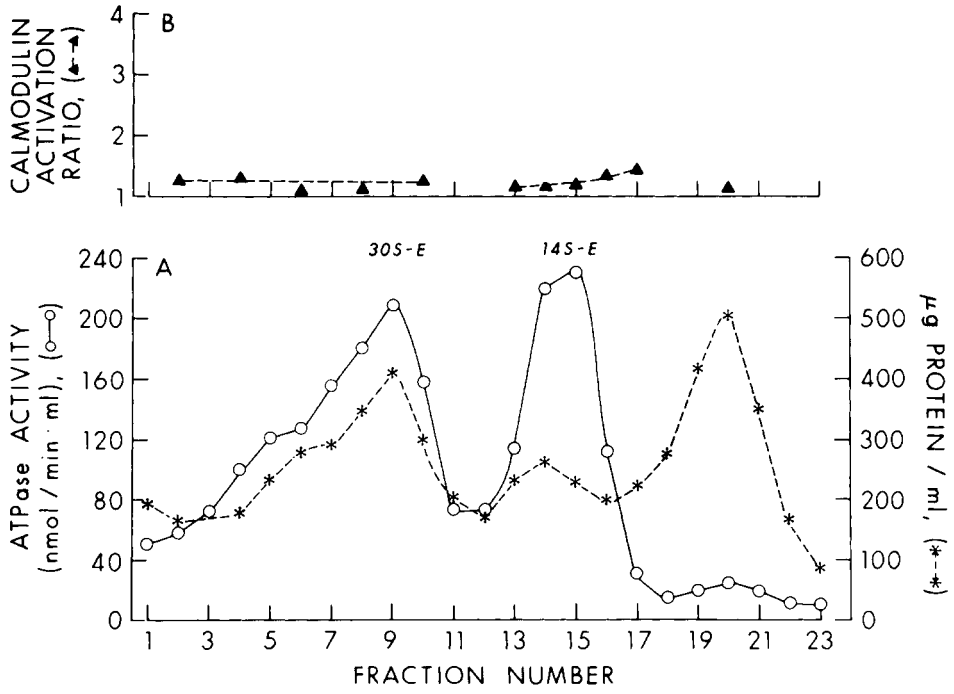


Fig. 2. Sensitivity of 14S-E and 30S-E dynein ATPase activities to calmodulin. Demembranated axonemes were extracted with Tris-EDTA and the crude dynein resolved by sucrose density gradient sedimentation as described in Methods. All other details are as in Figure 1.

ratio across the 14S-E region. In this experiment, this ratio increased from ~ 1.1 to ~ 1.4 in going from fraction 13 to fraction 17; since in other experiments the increase was even less or altogether absent, we do not believe it to be significant.

Nonheterogeneity of 14S-K Dyneins With Respect to Calmodulin Concentration

The discovery (Fig. 1) that the calmodulin activation ratio of 14S-K dynein ATPase varied systematically raised the possibility that this ATPase activity might also vary systematically in sensitivity to calmodulin concentration. The data in Figure 3 show that 14S-K dynein ATPase from fractions 13 and 16 of a preparation similar to that shown in Figure 1 was activated 2.4-fold and 4.6-fold, respectively, by 30 μg of bovine brain calmodulin. For each of these fractions, half-maximal activation required approximately 0.7 μg of calmodulin, consistent with an earlier estimate made on pooled 14S-K dynein [2]. Thus there is no indication that the affinity of calmodulin varies across the 14S-K dynein ATPase region.

Retention of Heterogeneity of 14S-K Dynein ATPase After Recentrifugation

Given that calmodulin sensitivity increases towards the low density side of the 14S-K dynein region in a sucrose density gradient, it was of interest to ascertain whether this heterogeneity of response would be retained if individual fractions from a first sucrose density gradient were subjected to recentrifugation on a second sucrose density gradient. In the experiment shown in Figure 4, 1.5 ml each of frac-

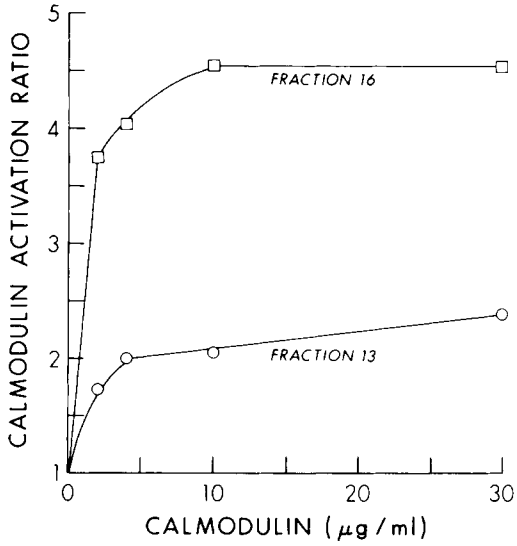


Fig. 3. Effect of calmodulin on ATPase activity of individual 14S-K dynein fractions. Demembranated axonemes were extracted with 0.5 M KCl in IMT/6 buffer, pH 7.5, for 14 hr, and 3 ml portions of the crude dynein were resolved on sucrose gradients containing mM EGTA as described in Methods. The 14S-K dynein ATPase was distributed in fractions 13–16, with maximum activities in fractions 14 and 15 (not shown; see Fig. 1 for a similar gradient). The ATPase activities of fractions 13 and 16 in the absence of added calmodulin were 194 and 136 nmole/min • mg, respectively. The calmodulin activation ratio for fractions 13 ("heavy" 14S-K dynein) and 16 ("light" 14S-K dynein) are shown as a function of the amount of added calmodulin.

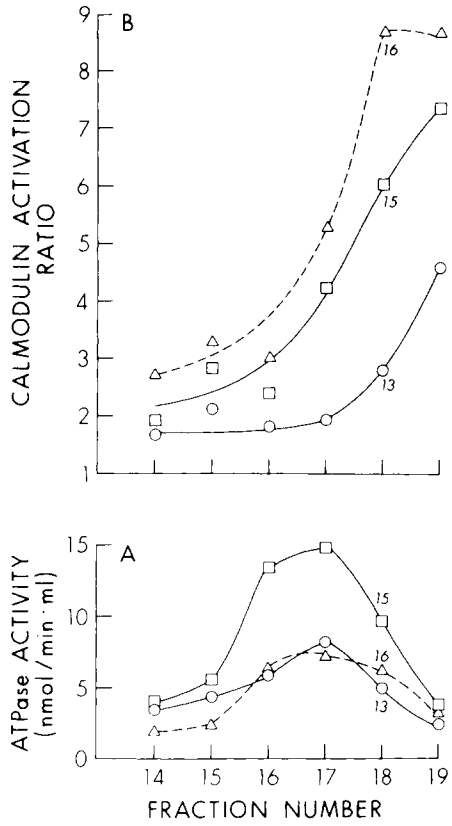
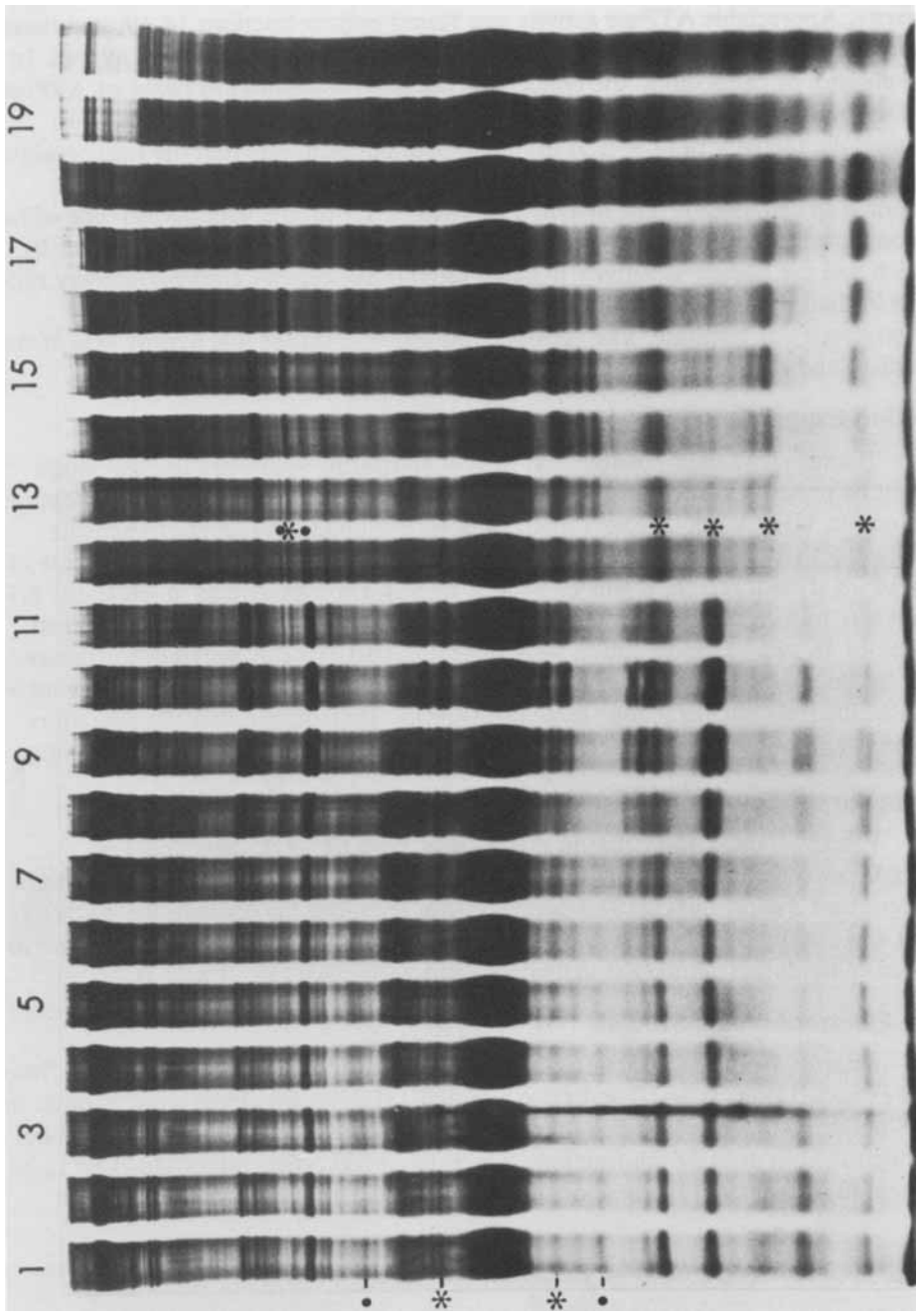


Fig. 4. Effect of calmodulin on individual 14S-K dynein ATPase fractions after recentrifugation on a second sucrose density gradient. Demembranated cilia were extracted with 0.5 M KCl for 14 hr as described in the legend to Figure 3 and the dyneins were resolved on sucrose gradients containing 1 mM EGTA (data not shown). The ATPase activities of fractions 13, 15, and 16 in the absence of added calmodulin were 185, 150, and 92.5 nmole/min • mg, respectively, and the calmodulin activation ratios (+ 20 μg calmodulin/ – calmodulin) were 2.3, 4.7, and 6.0, respectively. Portions (1.5 ml of each fraction) were diluted with an equal volume of IMT/6 buffer (pH 7.5) and layered onto three 10–30% (w/v) sucrose gradients containing 1 mM EGTA, and centrifuged (24,000 RPM, SW 25 rotor, Beckman Model L centrifuge) for 19 hr. Panel A shows the ATPase activities (measured in the presence of 1 mM free Ca^{++}) of fractions 14 through 19 from these gradients. The number adjacent to each line refers to the fraction number in the original gradient. Panel B shows the calmodulin activation ratio for these fractions.

Fig. 5. Gel electrophoresis of fractions from a sucrose density gradient of crude dynein from KCl-extracted axonemes. Samples of 12.5 μl were run on SDS-10% polyacrylamide gels. The fraction numbers are marked across the top of the gel. The protein concentration for these fractions are given in Figure 1A. Asterisks or dots placed to the left of fraction 1 indicate bands whose intensities systematically increase or decrease in going from fractions 1-9 (30S-K region), respectively. Similarly, asterisks or dots placed to the left of fraction 13 refer to components that systematically increase or decrease, respectively, across the 14S-K region (fractions 13–16).


19
17
15
13
11
9
7
5
3
1

tions 13, 15, and 16 of a gradient such as shown in Figure 1 were diluted 1:1 with IMT/6 buffer and layered onto 10–30% (w/v) sucrose gradients containing 1 mM EGTA. After centrifugation, each gradient was collected and assayed for ATPase activity. Appreciable ATPase activity was found only in fractions 14–19, and these were assayed for ATPase activity after addition of 20 μg of calmodulin as well. In the absence of calmodulin, the typical and seemingly symmetrical peaks of ATPase activity were obtained (Fig. 4A). Fraction 13, which had the least sensitivity to calmodulin (activity ratio ~ 2.3) in the original gradient, still had the least sensitivity, but upon recentrifugation, it, too, evidenced considerable heterogeneity of response to calmodulin: The activity ratio was ~ 1.7 on the high density side of the second gradient and rose to ~ 4.5 on the low density side (Fig. 4 B). Fraction 16, which had the highest sensitivity to calmodulin in the orginal gradient (activity ratio ~ 6.0), had the highest sensitivity after recentrifugation and also retained heterogeneity of response: The activity ratio was ~ 3 on the low density side of the gradient and rose to ~ 8.5 on the high density side.

Mixing Experiments

Because it seemed possible that factors conferring sensitivity to calmodulin might be present in fractions (eg, 16) from the low density side of the 14S-K dynein region, or, alternatively, factors interfering with calmodulin's ability to activate ATPase activity might be present in fractions (eg, 14) from the high density side of the 14S-K dynein region, it was of interest to perform experiments in which the ATPase activity was measured when aliquots of the fractions were combined. When fractions 16 and 13, or 16 and 6, were mixed, the activity (measured in the presence and in the absence of added calmodulin) was that expected for the sum of the activities of the individual fractions (data not shown). This implied that the regulatory factors responsible for the effect did not act catalytically under these conditions.

Behavior of "Low S" ATPase

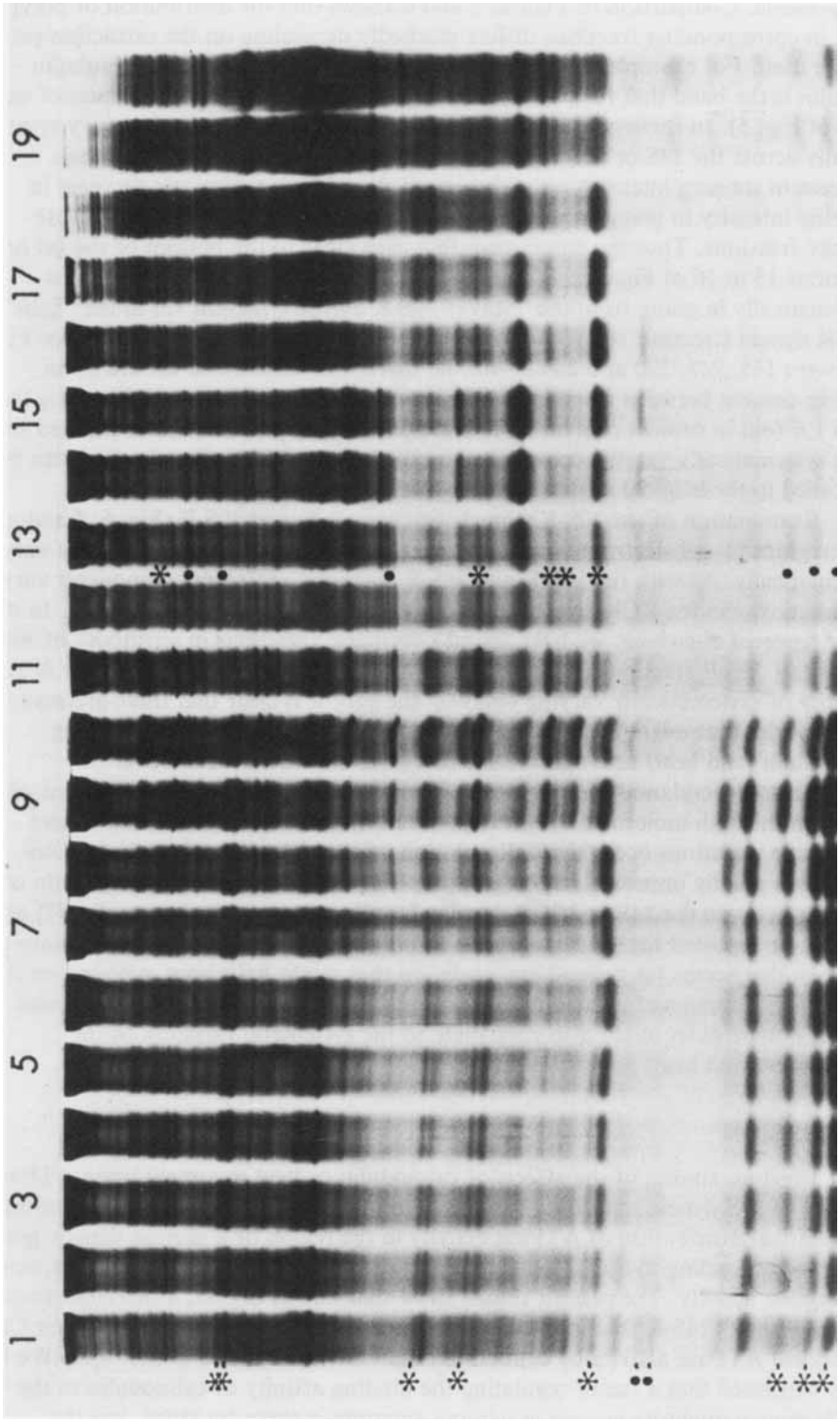
Figures 1 and 2 show that in gradients of both KCl-extracted and Tris-EDTA-extracted crude dynein, a small amount of ATPase activity penetrates into the gradient a short distance (2 fractions) from the meniscus between the crude dynein and the top of the gradient (fraction 22). This ATPase activity is insensitive to added calmodulin (calmodulin activation ratio ~ 1.2; see Figs. 1 and 3).

Gel Electrophoresis Studies

Samples from each fraction of sucrose density gradients of crude dynein from KCl-extracted (Fig. 5) and Tris-EDTA-extracted (Fig. 6) axonemes were resolved on SDS-polyacrylamide gels as described in Methods. At least 50 polypeptides can be distinguished in most of the fractions. Since about 200 polypeptides are likely to be present in axonemes [11], some of the bands in these gels surely contain several

---

Fig. 6. Gel electrophoresis on 7.5% polyacrylamide gels of fractions from a sucrose density gradient of crude dyneins from Tris-EDTA-extracted axonemes. The protein concentrations of fractions 1 through 9 were (in μg/ml) 194, 169, 174, 178, 234, 276, 290, 345, and 409; for fractions 13 through 16, 232, 256, 230, and 198. For further details, see legend to Figure 5.


19
17
15
13
11
9
7
5
3
1

components. Comparison of Figures 5 and 6 shows that the distribution of polypeptides in corresponding fractions differs markedly depending on the extraction procedure used. For example, KCl extraction solubilizes a large amount of tubulin (tubulin is the band that forms a dense region obscuring the central portion of each lane of Fig. 5). In these gels, we have marked several components that vary systematically across the 14S or 30S regions with asterisks, if there was a systematic increase in staining intensity, or with dots, if there was a systematic decrease in staining intensity in going from the higher sucrose density to the lower sucrose density fractions. Thus the component that runs close to the bottom of the gel in fractions 13 to 16 of Figure 5 is marked with an asterisk and clearly increases systematically in going from the "heavy" 14S-K dynein (fraction 13) to the "light" 14S-K dynein (fraction 16). The protein concentrations for these fractions (see Fig. 1A) were 165, 245, 220 and 240 μg/ml, ie, there was virtually no difference in protein content between fractions 14, 15, and 16 and each of them differed by less than 1.6-fold in protein content from fraction 13. In Figure 5 we have marked seven such systematically varying components in the 14S-K region; several others can be discerned in the original negatives.

Examination of the 30S-K (Fig. 5, fractions 1–9) and 30S-E (Fig. 6, fractions 1–9) regions by gel electrophoresis also reveals a number of components that vary systematically. As with the 14S-K and 14S-E fractions, different components vary systematically in the KCl-extracted versus the Tris-EDTA–extracted systems. In data to be reported elsewhere, we have found systematic variations in sensitivity of 30S-K but not of 30S-E fractions to thermal activation of ATPase activity. In view of the number of systematically varying bands in the gels, it is clear that there are many polypeptides that might be involved in determining the systematically varying calmodulin (and heat) activation ratios that have been described above.

The polyacrylamide gels shown in Figures 5 and 6 are so densely packed with bands in the high molecular weight region that it is difficult to see whether any systematic variations occur there. Resolution of the high molecular weight components is greatly improved in 5% gels (Fig. 7). The differences in heavy chain components between the 14S and 30 S dyneins have been commented on earlier [2] and will not be repeated here. Several bands in both the 14S and the 30S region vary to a degree that seems far beyond any variation that might have been expected on the basis of the amount of protein placed on the gels, so that several high molecular weight polypeptides might also contribute to the systematic variation of the calmodulin (and heat) activation ratios.

DISCUSSION

Previous studies of the effects of calmodulin or heat treatment upon ATPase activity of 14S dynein have been performed on fractions pooled from the seemingly symmetrical distribution of ATPase activity in the region of a sucrose density gradient corresponding to an S_{20} value of approximately 14 in the fraction containing the highest activity. The present results show that this is, in fact, a heterogeneous region. "Light" 14S-K fractions are always more sensitive to activation of their Ca^{++}-dependent ATPase activity by calmodulin than the "heavy" 14S-K fractions. We initially suspected that a factor regulating the binding affinity of calmodulin to the 14S-K dynein might be present in varying amounts in these fractions, but the evidence presented in Figure 3 appears to rule out this notion. Another hypothesis

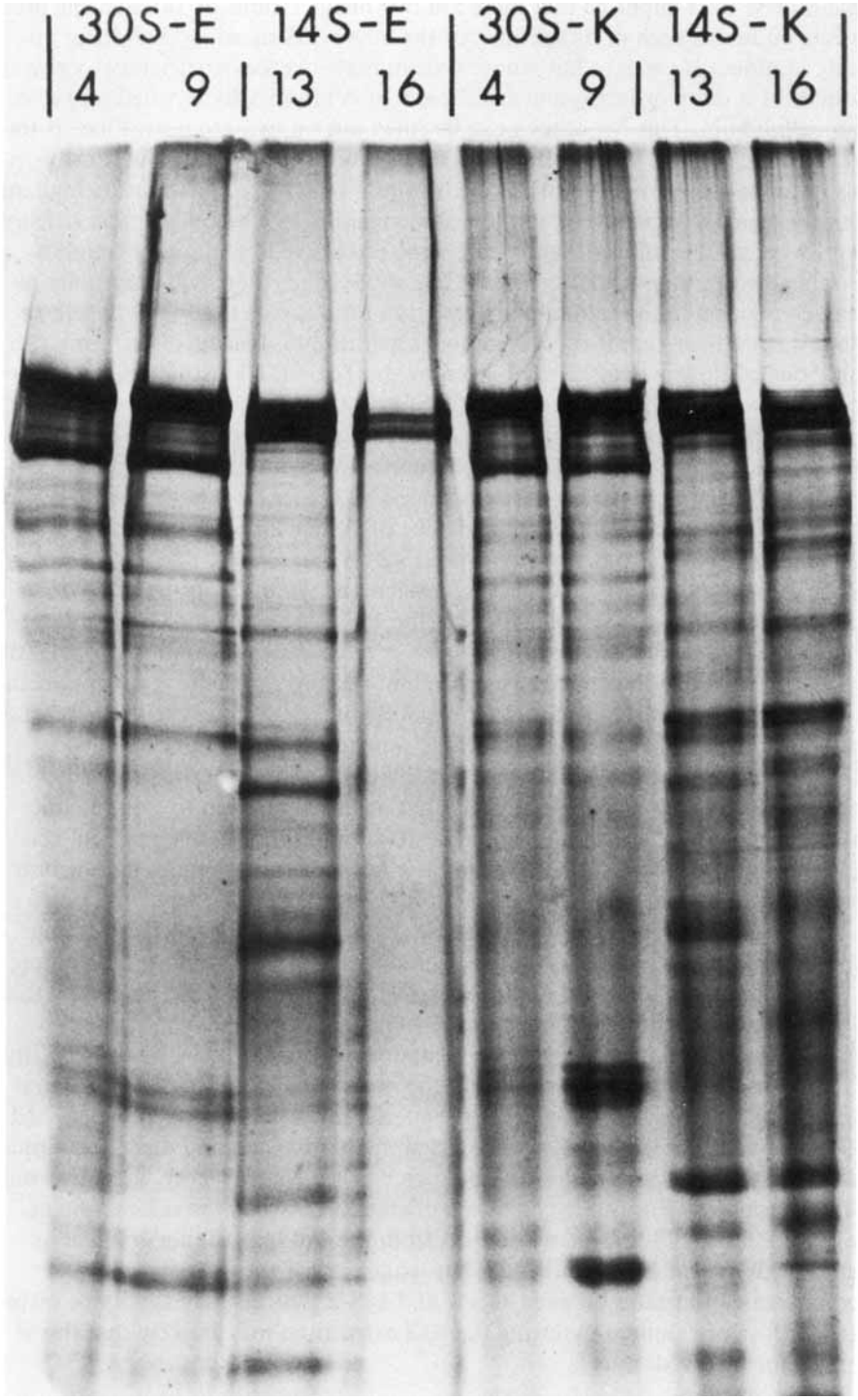


Fig. 7. Gel electrophoresis of fractions 4, 9, 13, and 16 from the preparations used for Figures 5 and 6 but run on 5% polyacrylamide gels.

to explain these data might be that there are two distinct kinds of 14S dyneins present in cilia (either as such or as subunits of the larger 30S dyneins) that differ appreciably in molecular weight but whose distributions overlap sufficiently to give the appearance of a more or less symmetrical peak of ATPase activity when measured without calmodulin. This hypothesis can be ruled out on two grounds. First, if the hypothesis were correct, the 14S-K dynein taken from the high and low density regions of an initial sucrose density gradient would tend to sediment to the high and low density regions, respectively, upon recentrifugation in a second sucrose density gradient, whereas they move to about the same peak position and have roughly comparable distributions about the peak (Fig. 4). Second, there was essentially no systematic variation in the calmodulin activation ratio across the 14S-E dynein region, but it is unlikely that if there were two separate 14S dyneins of differing size that only one of these would be solubilized by the Tris-EDTA extraction procedure whereas both would be solubilized by the KCl extraction. It therefore seems reasonable to attribute the systematic rise in calmodulin activation ratio across the 14S-K region to a systematic difference of some component or components across the fractions. This interpretation is strengthened by the finding that resedimentation of individual fractions may increase the degree of systematic variation of the calmodulin activation ratio (Fig. 4).

The present data show that there is a systematic variation in sensitivity to added calmodulin of the ATPase activity in the 14S-dynein region. This does not necessarily imply that the 14S dynein complex per se varies systematically across the peak. It is also possible that there is a systematic variation of some regulatory component(s) across this region. The gels shown in Figures 5–8 reveal the presence of several polypeptides that vary in a systematic way across this region, and hence could be responsible for the variation in sensitivity of ATPase activity to added calmodulin. Experiments on 14S-K dynein that has been purified (by, eg, affinity chromatography on a calmodulin-Sepharose 4B resin [2]) before the gradient centrifugation step will be necessary to decide whether these polypeptides are intrinsic components of the 14S-K complexes or simply cosediment with them.

The finding that extraction of axonemes by KCl yields 14S and 30S dynein fractions with lower total ATPase activities and lower specific ATPase activities (cf Figs. 1 and 2) as compared with extraction by Tris-EDTA raises the possibility that the low calmodulin activity ratio seen in the 14S-E region might simply reflect a greater retention of calmodulin by these complexes as compared with the 14S-K dyneins. If so, one would have expected that the presence of calcium during the gradient centrifugation step of KCl-extracted material might have markedly increased the ATPase activity of the 14S-K dynein fractions so obtained and made them much less sensitive to added calmodulin. In one such experiment, however, there was only a 20% increase in ATPase activity and a moderate decrease in the calmodulin activation ratio in the 14S-K dynein obtained from the Ca^{++}-containing gradient as compared with the Ca^{++}-free gradient. Thus while part of the difference in calmodulin activation ratio between 14S-K and 14S-E dyneins may simply be owing to a greater loss of calmodulin during the KCl extraction, it is unlikely that this is the sole factor involved.

ACKNOWLEDGMENTS

This work was supported by grant PCM78-03866 from the National Science Foundation (to JJB), NIH grant NS 10123 (to TV), and NIH grant NS 15269 (to FHS).

REFERENCES

1. Jamieson GA Jr, Vanaman TC, Blum JJ: Proc Nat Acad Sci USA 76:6471, 1979.
2. Blum JJ, Hayes A, Jamieson GA Jr, Vanaman TC: J Cell Biol 87:386, 1980.
3. Bell CW, Fronk E, Gibbons IR: J Supramol Struct 11:311, 1979.
4. Warner FD, Mitchell DR, Perkins CR: J Mol Biol 114:367, 1977.
5. Piperno G, Luck DJL: J Biol Chem 254:3084, 1979.
6. Blum JJ, Hays A, Jamieson GA Jr, Vanaman TC: Arch Biochem Biophys 210:361, 1981.
7. Taussky HH, Shorr E: J Biol Chem 202:675, 1953.
8. Blum JJ, Hayes A: J Supramol Struct 11:117, 1979.
9. Laemmli UK: Nature (London) 227:680, 1970.
10. Oakley BR, Kirsch DR, Morris NR: Anal Biochem 105:361, 1980.
11. Huang B, Piperno G, Luck DJL: J Biol Chem 254:3091, 1979.