Isolation and characterization of dynein ATPase from bull spermatozoa

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Dialysis of demembranated bull spermatozoa against a low-salt buffer resulted in the solubilization of the outer dynein arms and 15–25% of the total ATPase activity. Low-salt extracts contained three high- M_r peptides with M_r values above 300 000. The ATPase activity was associated with two particles sedimenting at 19 S and 12 S. The heavier particle contained two major high- M_r peptides with M_r values above 300 000, one major and one minor intermediate peptides with M_r values of 91 000 and 140 000 respectively and lower- M_r peptides. The 12 S particle contained one high- M_r peptide positioned between the two heavy peptides of the 19 S particle. Even though the peptide compositions of these two particles were different, the enzymic properties of their ATPases were similar. Both particles hydrolysed in the following preference order: ATP > CTP > UTP > ITP > GTP. ATPase activities were not affected by ouabain and oligomycin but were inhibited by vanadate, erythro-9-[3-(2-hydroxynonyl)]adenine and EDTA. Enzyme activities were dependent on the presence of a bivalent cation with the following preference order: Mn^{2+} > Mg^{2+} > Ca^{2+} > Ni^{2+} . Optimal activity was observed between pH 6.5 and 9.5. The K_m for ATP ranged from 40 to 50 μM for both 19 S and 12 S ATPases. These results suggest that the 12 S and 19 S particles are dyneins from outer dynein arms.

INTRODUCTION

The importance of ATP hydrolysis in ciliary and flagellar beating is well established (Gibbons, 1965; Satir, 1968; Brokaw & Benedict, 1968; Summers & Gibbons, 1972; Gibbons & Gibbons, 1973). The protein responsible for this hydrolysis, dynein ATPase, is an Mg^{2+} -dependent enzyme localized on the axoneme. At the ultrastructural level, dynein ATPase is associated with arm-like structures spanning from one doublet microtubule towards the adjacent doublet microtubule (for review see Gibbons, 1981). The importance of these dynein arms in motility was clearly established by several experiments showing a direct correlation between the quantity of dynein arms present on the axoneme and the sliding velocity or beat frequency of the axoneme (Gibbons & Gibbons, 1972; Yano & Miki-Noumura, 1980, 1981).

Dynein arms have been extracted from various sources of axonemes by low-ionic-strength and high-ionic-strength buffers. Their sedimentation coefficients ranged from 10–12 S to 30 S (Gibbons & Rowe, 1965; Gibbons & Fronk, 1979; Huang *et al.*, 1979; Pallini *et al.*, 1982; Johnson & Wall, 1983). The 30 S dynein from *Tetrahymena* cilia has been analysed by scanning transmission electron microscopy and was found to consist of three globular heads each connected by threads extending from a common base (Johnson & Wall, 1983; Goodenough & Heuser, 1984). On the other hand, Witman *et al.* (1983) reported that the 18 S dynein from *Chlamydomonas* had two globular heads linked to a common base and the 12 S dynein only one, whereas the 21 S dynein from sea-urchin spermatozoa was observed as a two-headed particle by Sale *et al.* (1985). In general, the peptide compositions of the various particles are

relatively similar, with two or three heavy chains of apparent M_r above 300 000, intermediate chains of M_r from 70 000 to 130 000 and smaller chains of M_r ranging from 15 000 to 24 000 (Bell *et al.*, 1979; Pfister *et al.*, 1982; Porter & Johnson, 1983).

By contrast with ciliary dyneins from *Tetrahymena* and flagellar dyneins from *Chlamydomonas* and sea-urchin sperm, little is known on flagellar dynein from mammalian spermatozoa. In a comparative study involving ciliary and flagellar dynein of bovine origin, Pallini *et al.* (1982) extracted flagellar ATPases sedimenting at 11–12 S and 19 S. These particles were composed of several high- M_r peptides (up to six), which had a tendency to degrade into smaller peptides. Young & Smithwick (1983) extracted a crude ATPase activity from bull spermatozoa by treating demembranated flagella with 0.6 M-KCl. They found that their crude ATPase was stimulated by Mg^{2+} and Ca^{2+} but inhibited by Zn^{2+} and Cd^{2+} . Other ions such as Na^+ and K^+ had no effect on enzyme activity.

In the present paper we report that 12 S and 19 S dyneins can be extracted from bull spermatozoa by low-ionic-strength buffer. The 19 S particle is composed of two peptides of M_r above 300 000, whereas the 12 S particle has only one major peptide in the same range. Mg^{2+} -dependent ATPase activities were associated with these high- M_r peptides. The K_m for ATP was in the 40–50 μM range for both the 19 S and 12 S dynein particles.

MATERIAL AND METHODS

Materials

Fresh bull epididymides were obtained from a local slaughterhouse. Phenylmethanesulphonyl fluoride was

Abbreviation used: EHNA, erythro-9-[3-(2-hydroxynonyl)]adenine.

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purchased from Boehringer Mannheim (Montreal, Que., Canada), EHNA was from Burroughs Wellcome (Research Triangle Park, NC, U.S.A.), catalase and bovine serum albumin were from Sigma Chemical Co. (St. Louis, MO, U.S.A.), [γ - ^{32} P]ATP (sp. radioactivity 3000 Ci/mmol) was from Amersham Corp. (Mississauga, Ont., Canada), Epon 812 resin was from J. B. EM. Services (Pointe-Claire, Que., Canada), Ready-Solv EP was from Beckman (Palo Alto, CA, U.S.A.) and ribonuclease-free sucrose was from Schwarz-Mann (London, Ont., Canada). All other chemicals were of at least reagent grade.

Isolation of bull spermatozoa

Spermatozoa were collected from bull cauda epididymides by retroperfusion with paraffin oil. The undiluted sperm suspension was centrifuged at 1500 *g* for 10 min at room temperature. Pelleted spermatozoa were washed twice with 20 vol. of buffer A (containing 17 mM-glucose, 10 mM-EDTA, 5 mM-KCl, 70 mM-sodium citrate and 24 mM-NaHCO₃, pH 8.0) to eliminate epididymal fluids.

Extraction of dyneins

Washed epididymal spermatozoa were demembranated in 20 vol. of buffer B (containing 0.1% Triton X-100, 0.1 mM-phenylmethanesulphonyl fluoride, 200 mM-sucrose, 25 mM-potassium glutamate, 1 mM-dithiothreitol and 35 mM-Tris/HCl, pH 7.9) and centrifuged at 500 *g* for 10 min at 4 °C. The pelleted spermatozoa were then resuspended and the demembranation treatment was repeated. Demembranated spermatozoa were then washed three times with buffer C (buffer B devoid of Triton X-100). Washed demembranated spermatozoa were suspended in 4 vol. of buffer D (containing 0.5 mM-EGTA, 14 mM-2-mercaptoethanol, 0.1 mM-phenylmethanesulphonyl fluoride and 2 mM-Tris/HCl, pH 7.9) and dialysed against the same buffer D for 16 h at 4 °C. The suspension was centrifuged at 15000 *g* for 15 min and the supernatant containing crude dyneins was recovered. Extracted crude dyneins were purified on 12 ml linear 5–25% (w/w) sucrose gradients in buffer D. After 16 h of centrifugation at 35000 rev./min in a Beckman SW 40 rotor at 4 °C, 0.3 ml fractions were collected by puncturing the bottoms of the tubes and analysed for their peptide and ATPase activity contents.

ATPase activity determination

ATPase activity was determined by a modification of a method described elsewhere (Penningroth & Kirschner, 1977). Samples were diluted in buffer B, and 0.1 mM-Mg[γ - ^{32} P]ATP was added in a final volume of 0.25 ml. After a 10 min incubation at 25 °C, the reaction was stopped by the addition of 0.1 ml of 20 mM-silicotungstic

acid in 10 mM-H₂SO₄ immediately followed by 0.1 ml of 2 mM-KH₂PO₄ and 0.1 ml of 5% (w/v) ammonium molybdate in 4 M-H₂SO₄. The phosphomolybdate complex was extracted 3 min later with 1 ml of benzene/2-methylpropan-2-ol (1:1, v/v). After separation of phases by centrifugation, the radioactivity in 0.4 ml of the organic phase was counted by liquid-scintillation spectrometry with the use of 10 ml of Ready-Solv EP scintillation cocktail. For nucleotide-specificity studies, preparations were incubated in buffer B at a nucleotide concentration of 1 mM. The inorganic phosphate generated from hydrolysis of the various nucleotides was determined as described by Hayashi (1976).

Gel electrophoresis

Polyacrylamide-gel electrophoresis in the presence of SDS was run in accordance with Pfister *et al.* (1982) with 4–12% or 3.5–5% polyacrylamide gradients as separating gel. After electrophoresis, gels were fixed and silver-stained (Oakley *et al.*, 1980).

Electron microscopy

Spermatozoa were fixed for 1 h with 2% (v/v) glutaraldehyde in 0.1 M-cacodylate/HCl buffer, pH 7.2, and washed by centrifugation three times with the same buffer. Specimens were post-fixed for 1 h in aq. 2% (w/v) OsO₄. They were then dehydrated in increasing concentrations of ethanol followed by propylene oxide and finally embedded in Epon 812 resin. Thin sectioned specimens were double-stained with uranyl acetate and lead citrate. Electron micrographs were taken with a Philips 301 electron microscope at 60 kV.

Protein determination

Protein concentrations were determined by using the method of Bradford (1976), with bovine serum albumin as a standard.

RESULTS

Extraction of bull sperm ATPase by a low-salt buffer

Extraction of demembranated bull spermatozoa with a low-ionic-strength buffer resulted in the solubilization of 15–25% (range for eight different preparations) of the total ATPase activities and 3–5% of the total protein content (Table 1). This treatment also solubilized most of the outer dynein arms on axonemes (only 2.2 ± 0.1 outer arms remaining), whereas no inner dynein arm was extracted (Fig. 1). Occasionally, mild distortion of the axoneme was observed (Fig. 1f), but the fibrous sheet and auxiliary fibres were not significantly affected.

Three major high-*M_r* peptides of *M_r* above 300000

Table 1. Extraction of ATPase activity from demembranated bull spermatozoa

Washed demembranated spermatozoa were extracted with low-salt buffer (buffer D) for 16 h at 4 °C. The results of this experiment are representative of seven others.

	Protein		ATPase activity		Specific activity (nmol/min per mg of protein)
	(mg)	(%)	(nmol/min)	(%)	
Demembranated spermatozoa	350	100	7020	100	20
Low-salt extract	13.8	3.9	1360	19	100

Fig. 1. Electron micrographs of bull sperm flagella

(a) and (b) Intact flagella; (c) and (d) demembranated flagella; (e) and (f) flagella after extraction by low-ionic-strength solutions. Arrows indicate the positions of outer dynein arms, which are present in (a)–(d) but absent in (e) and (f). Bars represent 100 nm (magnification $\times 54000$ to $\times 97000$).

were detected after urea/SDS/polyacrylamide-gel electrophoresis on 4–12% and 3.5–5% gels (Fig. 2). Several other peptides with smaller M_r values were also extracted. When tested against a variety of nucleotides as substrates, the low-salt extract hydrolysed preferentially ATP with a specific activity of $0.07 \mu\text{mol/min per mg}$ of protein (Table 2). Other nucleotides were also hydrolysed but at a lower rate, the lowest being ADP at $0.003 \mu\text{mol/min per mg}$ of protein. No phosphatase activity as measured by *p*-nitrophenyl phosphate hydrolysis at pH 5.0 and 8.0 was detected in the extract.

The ATPase activity present in the extract was associated with two particles sedimenting at 12 S and 19 S (Fig. 3). The 12 S particle had a specific activity of $0.1\text{--}0.3 \mu\text{mol/min per mg}$ of protein, whereas the 19 S particle had a specific activity of $0.5\text{--}1.4 \mu\text{mol/min per mg}$ of protein, depending on the preparation used.

Electrophoretic analysis of the 19 S and 12 S particles

The 19 S and 12 S particles extracted by low-salt buffer were analysed by urea/SDS/polyacrylamide-gel electrophoresis. An electrophoretic scan of proteins from the above sucrose gradient revealed the presence of two major very large peptides in the 19 S particle with M_r values above 300000. Two intermediate peptides with

Fig. 2. Urea/SDS/polyacrylamide-gel electrophoresis of low-salt extracts of demembranated bull spermatozoa

Demembranated bull spermatozoa were extracted with a low-ionic-strength buffer (buffer D). (a) 4–12% polyacrylamide gel gradient; (b) 3.5–5% polyacrylamide gel gradient. S, Bio-Rad standards (1, myosin, M_r 200000; 2, β -galactosidase, M_r 116000; 3, phosphorylase *b*, M_r 92500; 4, bovine serum albumin, M_r 66000; 5, ovalbumin, M_r 45000); L, low-salt extract. The arrows indicate the positions of the three high- M_r peptides.

Table 2. Nucleotide-specificity of the low-salt-extract hydrolytic activity and 19 S-particle dynein

Low-salt extract and 19 S-particle dynein were incubated with 1 mM-nucleotides, and the inorganic phosphate formed was measured as described by Hayashi (1976).

Substrate	Specific activity ($\mu\text{mol/min per mg}$ of protein)	
	Low-salt extract	19 S dynein
ATP	0.070	1.20
CTP	0.013	0.29
UTP	0.012	0.11
ITP	0.006	0.09
GTP	0.004	0.03
ADP	0.003	0

apparent M_r values of 140000 and 91000 and smaller peptides were also present (Figs. 4 and 5). The 91000- M_r peptide was always more prominent than the other intermediate peptide. The electrophoretic pattern of the 12 S particle was different, with no bands matching those

Fig. 3. Sucrose-density-gradient centrifugation of low-salt extracts of demembrated bull spermatozoa

Low-salt extracts were layered on a linear 5–25% sucrose gradient and centrifuged at 35000 rev./min in a Beckman SW 40 rotor for 16 h. Arrows indicate the positions of catalase (11.3 S) and of thyroglobulin (19 S). ATPase activity is expressed as nmol of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ hydrolysed/min.

of the 19 S particle, with the possible exception of contaminating bands also appearing distally towards the top of the gradient. The 12 S particle contained one major high- M_r peptide positioned between the two heavy peptides of the 19 S particles, a second (but minor) high- M_r peptide smaller than the two large 19 S peptides and other contaminating lower- M_r peptides.

Enzymology of the 19 S and 12 S dynein ATPase

To ascertain that the ATPase activity associated with the 19 S particle had properties generally attributed to dynein ATPase, such as those from sea-urchin spermatozoa, a series of inhibitors were added to the 19 S particle and ATP hydrolysis was monitored. Ouabain and oligomycin at concentrations up to $50\text{ }\mu\text{M}$ had no effect on 19 S-particle ATPase activity, indicating that the ATPase present was not the $\text{Na}^+ + \text{K}^+$ -dependent ATPase nor the mitochondrial ATPase (Table 3). On the other hand, $10\text{ }\mu\text{M}$ -vanadate and 1 mM -EHNA, two known inhibitors of dynein ATPase (Gibbons *et al.*, 1978; Bouchard *et al.*, 1981; Penningroth *et al.*, 1982), decreased the enzyme activity by 57% and 48% respectively. Similar inhibitory specificity was observed with the ATPase activity associated with the 12 S particle (results not shown). The ATPase associated with the 19 S particle was entirely dependent on the presence of Mg^{2+} for activity, as chelation by EDTA completely blocked the hydrolysis of ATP (Table 3). Mn^{2+} was even better than Mg^{2+} in stimulating the 19 S-associated ATPase activity, whereas Ca^{2+} and Ni^{2+} were less effective (Fig. 6). The K_m for MgATP was in the $40\text{--}50\text{ }\mu\text{M}$ range for the 19 S-associated ATPase (Fig. 7) as well as for the 12 S-associated ATPase (results not shown). The enzymic activity associated with the 19 S particle preferentially hydrolysed ATP with a specific activity of $1.2\text{ }\mu\text{mol/min per mg}$ of protein (Table 2). The second best nucleotide substrate was CTP with a specific activity

Fig. 4. Urea/SDS/polyacrylamide-gel electrophoresis 4–12% gels of fractions from the sucrose-density-gradient centrifugation of low-salt extracts of demembrated bull spermatozoa

The numbers below each slot correspond to fraction numbers of the 5–25% sucrose gradient (Fig. 3). S, Bio-Rad standards as in Fig. 2. Large arrows indicate the positions of high- M_r peptides and small arrows those of intermediate peptides.

Fig. 5. Urea/SDS/polyacrylamide-gel electrophoresis on 3.5-5% gels of fractions from the sucrose-density-gradient centrifugation of low-salt extracts of demembrated bull spermatozoa

The numbers below each slot correspond to fraction numbers of the 5-25% sucrose gradient (Fig. 3). S, Bio-Rad standards as in Fig. 2 except for the omission of ovalbumin. Large arrows indicate the positions of high- M_r peptides and small arrows those of intermediate peptides.

of $0.34 \mu\text{mol/min per mg}$ of protein. Other nucleotides were hydrolysed but at a much lower rate, and ADP was not degraded at all. The optimum pH was broad for the 19 S-associated ATPase, being between 6.5 and 9.5

Table 3. Effects of various inhibitors on dynein ATPase activity associated with the 19 S particle

The concentration of MgATP used in the assay mixture was 0.1 mM . Inhibitors were added to the enzyme before the addition of MgATP.

Inhibitor	ATPase activity (%)
None (control)	100
Oligomycin ($50 \mu\text{M}$)	103
Ouabain ($50 \mu\text{M}$)	108
Oligomycin ($50 \mu\text{M}$) + ouabain ($50 \mu\text{M}$)	97
EHNA (1 mM)	52
Vanadate ($10 \mu\text{M}$)	43
EDTA (1 mM)	0

Fig. 6. Effects of bivalent cations on 19 S-particle dynein ATPase

Enzyme activity is expressed as nmol of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ hydrolysed/min.

Fig. 7. Determination of K_m of 19 S-particle dynein ATPase

A fixed amount of 19 S-particle dynein was incubated with $0.01\text{--}0.1 \text{ mM}$ -MgATP. A K_m of $43 \mu\text{M}$ was obtained from these data.

(Fig. 8). The 12 S-associated ATPase had similar nucleotide-specificity and pH-activity curve (results not shown).

DISCUSSION

Dyneins have been extracted and extensively studied from three main sources of axoneme: *Tetrahymena* cilia, sea-urchin flagella and *Chlamydomonas* flagella. Sedimentation coefficients varied from 10-12 S to 30 S (Gibbons & Rowe, 1965; Gibbons & Fronk, 1979; Huang *et al.*, 1979). This 30 S value for *Tetrahymena* dynein was later lowered to 21-22 S (Mitchell & Warner, 1981; Johnson *et al.*, 1984). The sedimentation coefficients of 12 S and 19 S obtained for the particles extracted by low-salt solutions from bull sperm axoneme are within the range observed for similar particles. In fact, they are very similar to the 12 S and 18 S reported for dyneins extracted from *Chlamydomonas* flagella (Huang *et al.*, 1979; Pfister *et al.*, 1982).

Fig. 8. Effect of pH on 19 S-particle dynein ATPase

Bull sperm 19 S dynein was incubated at different pH values in a 35 mM-Tris/maleate/borate buffer. Enzyme activity is expressed as nmol of [γ - ^{32} P]ATP hydrolysed/min.

The 12 S and 19 S particles contained peptides with M_r values above 300000. The 19 S particle had two major peptides in this category, whereas the 12 S particle had one with M_r between those of the peptides of the 19 S particle. Two intermediate peptides of M_r 91000 and 140000 were constantly associated with the 19 S particle. However, in contrast with similar particles reported in the literature, where the staining intensities of the intermediate peptides were similar or proportional to their mass (Bell *et al.*, 1979; Pfister *et al.*, 1982; Porter & Johnson, 1983), the 91000- M_r intermediate peptide from bull sperm always had a much higher staining intensity than the 140000- M_r peptide irrespective of the staining method used (Coomassie Blue or silver). This may suggest that there is less than one copy of the 140000- M_r peptide per particle and/or it may represent a degradation product of one of the higher- M_r peptides. Other smaller peptides are also present in the 19 S particle, but, because of their low staining intensity, their definite association with the 19 S particle must await further studies.

Several experimental findings suggest that the particles isolated from bull spermatozoa are dyneins. First, these particles have sedimentation coefficients and peptides with M_r values above 300000 compatible with those of other axonemal dyneins (Bell *et al.*, 1979; Pfister *et al.*, 1982; Porter & Johnson, 1983). Secondly, the 19 S and 12 S particles hydrolyse a series of nucleotides in the following priority order: ATP > CTP > UTP > ITP > GTP, the hydrolysis rate of ATP being at least 4-fold faster than that of CTP. Thirdly, this ATPase activity was not inhibited by ouabain and oligomycin. Fourthly, even though EHNA and vanadate are not specific dynein ATPase inhibitors (Schliwa *et al.*, 1984; Souness *et al.*, 1985), they block the ATPase activities of the 19 S and 12 S particles as they do for other axonemal dyneins (Gibbons *et al.*, 1978; Bouchard *et al.*, 1981;

Penningroth *et al.*, 1982). Fifthly, low-salt extraction of the axoneme results in the preferential release of outer dynein arms from bull sperm axonemes. The possibility that the 12 S and 19 S particles originate from sperm heads was rejected, since sperm tails isolated by the method of Calvin (1976) also release 12 S and 19 S particles with similar physical and enzymic properties to those reported there (results not shown). The possibility that the above particles originated from auxiliary fibres and the fibrous sheath was also rejected, since these two structures were not affected by the extraction procedure and they do not contain high- M_r peptides in the range reported here (Olson *et al.*, 1976; Olson & Sammons, 1980).

When compared with sea-urchin dynein, bull sperm dyneins are less sensitive to vanadate and EHNA, as they required 4 times more EHNA (Penningroth *et al.*, 1982) and 10 times more vanadate (Gibbons *et al.*, 1978) to inhibit bull dynein ATPase activity by 50%. Bull dynein is also different from sea-urchin sperm dynein with respect to latency of activation. Detergent, mild heat and proteolytic treatments had no stimulatory or only marginal effects on ATPase activity (results not shown). This lack of activation could be explained by the fact that bull dyneins may already be activated. As pointed out by Johnson *et al.* (1984), activation by the above-mentioned treatments is not a property shared by all dynein preparations.

Young & Smithwick (1983) reported the extraction of flagellar ATPase from bull spermatozoa by high-ionic-strength buffer. They observed that dialysed crude extracts had no ATPase activity unless bivalent cations were added. At 2 mM concentrations, the best stimulatory ion was Ca^{2+} , followed by Mg^{2+} and Mn^{2+} . With our dynein preparation purified through a 5–25% sucrose gradient a different order of stimulation was observed, with Mn^{2+} being the best ion followed by Mg^{2+} , Ca^{2+} and Ni^{2+} . These discrepancies may be explained, first, by the fact that Young & Smithwick (1983) did not purify their extract, which consequently must have contained ATPases other than dynein, and, secondly, that they were dealing with a partially degraded preparation, since high-salt treatment releases proteinases from the axoneme (the present work). The optimum pH range for bull dynein ATPase was broad (between 6.5 and 9.5), a property shared by other dyneins such as the 21 S dynein from sea-urchin spermatozoa (Gibbons & Fronk, 1979). Similarly, the K_m of 40–50 μM for ATP and the specific activities of the two bull dyneins were also of the same order of magnitude as those reported for other dyneins (Ogawa & Mohri, 1972; Gibbons & Fronk, 1979; Pfister *et al.*, 1982).

In spite of the differences in their peptide compositions, the two particles isolated from bull axoneme have comparable enzymic properties. They share similar substrate specificity, optimum pH, K_m , activation curves by cations and inhibitions by vanadate and EHNA. However, there is a 4-fold difference in the specific activities of the two dyneins. This difference could be explained in part by the higher degree of exogenous protein contamination in the low- M_r range in the 12 S dynein peak as it sedimented closer to the surface of the gradient. On the other hand, 12 S dynein from *Chlamydomonas* flagella has also a specific activity 3–4-fold lower than that of the 18 S dynein (Pfister *et al.*, 1982).

Considering the similarities between bull sperm and *Chlamydomonas* dyneins as well as the known structures of *Chlamydomonas*, *Tetrahymena* and sea-urchin dyneins, these results may suggest that the 19 S dynein has two globular heads whereas the 12 S dynein has only one. Preliminary results obtained with scanning transmission electron microscopy support this hypothesis (Marchese-Ragona *et al.*, 1984).

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