Organic Anions Stabilize the Reactivated Motility of Sperm Flagella and the Latency of Dynein 1 ATPase Activity

BARBARA H. GIBBONS, WEN-JING Y. TANG, and I. R. GIBBONS
Pacific Biomedical Research Center, University of Hawaii, Honolulu, Hawaii 96822

ABSTRACT Substitution of any of a variety of organic anions, including acetate, propionate, lactate, gluconate, and succinate, for chloride in the reactivation medium improves the motility of demembranated sperm of *Tripneustes gratilla*. At the optimum concentration of 0.20 N, all of these anions improve the duration of motility, with lactate and gluconate being the best. The Michaelis constant for beat frequency (K_{mf}) is lower (0.11–0.14 mM at 22°C) in most of the organic anions than it is in Cl^- (0.20 mM), and the minimum ATP concentration required to support oscillatory beating is reduced from 10 μM in chloride to 2 μM in acetate, which together indicate a greater affinity of the axonemal ATPase for $MgATP^{2-}$ in the organic anions media. The maximal beat frequency, f_{max}, is as high as 42 Hz in 0.2 N succinate compared to 31 Hz in Cl^-, whereas the mean bend angle averages 2.8 rad in acetate compared to 2.4 rad in Cl^-; these values give a calculated average velocity of tubule sliding of ~15 μm/s in acetate and succinate, which is ~30% greater than the value of 11 μm/s observed in chloride. The reactivated sperm are sixfold more sensitive to vanadate inhibition in 0.2 M acetate than they are in 0.15 M Cl^-. The specific ATPase activity of soluble dynein 1, which increases more than 15-fold between 0 and 1.0 N Cl^-, undergoes only a twofold activation over the same range of organic anion concentration, and, like the reactivated motility, is up to 50-fold more sensitive to vanadate. This greater apparent mechanochemical efficiency and the increased sensitivity to vanadate inhibition in the organic anions suggest that they, unlike chloride, do not promote the spontaneous dissociation of ADP and PO_4^{3-} from the dynein-ADP-PO_4 kinetic intermediate in the dynein crossbridge cycle.

The use of organic anion media may lead to significant improvements in reactivation of other motile and transport systems.

The development of techniques for the permeabilization and reactivation of organelles that are capable of motility, such as cilia and flagella, proceeded rapidly about 15 years ago when the usefulness of non-ionic detergents such as Triton X-100 and Nonidet P-40 was recognized (19, 38). In these early experiments, Cl^- was chosen as the major supporting anion and a solution containing 0.15 M KCl has figured in many of the subsequent studies of reactivated cells which have yielded important information about the mechanism of microtubule-based motility (3, 5–7, 18, 20–22, 30, 33, 39, 47).

Recently, however, Evans (14) found that the specific activity of latent activity dynein 1 rises barely twofold in concentrations of Na acetate ranging from 0.1–0.6 M, whereas it increases more than 10-fold over the same concentration range of NaCl (25); these results have incited us to investigate the effect of replacing the KCl in our standard reactivating solution with the potassium salt of a variety of organic anions. This modification has led to significant improvement in the quality and longevity of our reactivated sperm preparations. Correlation of the effects of these anions on reactivated motility with their effects on the ATPase activity of dynein 1, and with their effects on the sensitivity to vanadate inhibition, suggests the probable location of the anion-sensitive step in the kinetic pathway of ATP hydrolysis by dynein. A preliminary report of our findings with K acetate has appeared (27).

Our studies may have broad usefulness for the study of reactivated motile systems in cells of various types, and underscore the influence of solution conditions on the sensitivity to inhibition by vanadate.

MATERIALS AND METHODS

Sperm Preparation: Sea urchins of the Hawaiian species *T. gratilla* were induced to shed their sperm by injection of 0.5 M KCl into the body of the animal. The sperm were collected in sea water that contained 0.2 mM EDTA and had been adjusted to pH 8.3, and kept as a stock suspension (3–10 mg protein/ml) at room temperature for up to 4 h.

Reactivated sperm were prepared under potentially symmetric conditions by adding 25 μl of the stock suspension of sperm to 0.3 ml of Triton–Ca^{2+} demembranating solution (10 mM Tris-HCl, pH 8.1, 4 mM $CaCl_2$, 1 mM dithiothreitol, 0.1 mM EGTA, and 0.04% [wt/vol] Triton X-100) at room temperature (22–23.5°C) (22). After a 20–30-s extraction, 5–25 μl of demembranating solution containing sperm were transferred to an observation dish containing 2.5 ml of reactivating solution (10 mM Tris-HCl, 2 mM $MgSO_4$, 0.1 mM EGTA, 1 mM dithiothreitol, 2% [wt/vol] polyethylene glycol [molecular weight = 20,000], 1 mM ATP, and the K^+ salt of Cl^-, acetate, or other anion as described in Results). A pH of 8.25 ± 0.10 was found optimal for reactivating solution containing organic anions and was used throughout this work.

Microscopy and Recording: Sperm swimming at the bottom surface of an observation dish were observed at 22–23°C by dark-field microscopy as described earlier (19). Flagellar beat frequencies were measured with a stroboscopic flash unit. The relative asymmetry of the flagellar waveforms was obtained by recording the circular arcs of the sperm head tracks and measuring their diameters (10, 18, 19). For this we used Polaroid 667 film, an X10 0.22 NA objective, and a low aperture dark-field condenser to increase the sensitivity of this film by raising the background illumination. High magnification flash photographs of moving sperm were taken on 35-mm Kodak 4X negative film with an X40 0.75 NA water-immersion objective and a 1.2–1.4 NA dark-field condenser. For all photography, illumination was provided by an ILC 150 F high intensity xenon pulse lamp (ILC Technology Inc., Sunnyvale, CA) as described by Omoto and Brokaw (40).

Measurement of Flagellar Bend Angles: Images on 35-mm film were projected onto white paper and traced, and tangents were drawn to the inflection point between bends for determination of bend angles (26, 29). Since these measurements only considered the symmetric waveforms observed in 0.1 M succinate, both principal and reverse bends were included in the averages.

Anions Used: In view of the favorable preliminary results with K^+ acetate (27), we examined a number of other carboxylic anions, using K^+ as the cation in reactivation studies and Na^+, the traditional cation, in the enzyme studies. These included anions of the monovalent acids propionate, lactate, glycolate, gluconate, and glucuronate, and of the dioic acids malonate, succinate, glutarate, glutamate, and tartrate. Of these, glucuronate, glycolate, malonate, glutarate, glutamate, and tartrate were eliminated from detailed study because of unfavorable preliminary results on reactivated sperm motility. Propionate was also not studied in detail, because it appeared to produce effects nearly identical to acetate and had the disadvantage that the K^+ salt was not readily available commercially.

Preparation of Dynein 1: Dynein-1 was obtained by high salt extraction of axonemes from the sperm of *T. gratilla* as described earlier (2, 25). The enzyme was concentrated by precipitation in 70% ammonium sulfate followed by dialysis against 0.45 M Na acetate buffered in 5 mM HEPES, 4 mM $MgAc_2$, 0.1 mM EGTA, and 7 mM β-mercaptoethanol, pH 7.4. The protein concentration in this stock solution was 1–2 mg/ml. The ATPase activity of this preparation was in the latent form and had a specific activity of 0.10–0.16 μmol Pi/min × mg protein. Portions of this material were incubated with 0.1% Triton X-100 for 10 min at room temperature to produce Triton-potentiated dynein 1 (25).

Determination of ATPase Activity and Protein Concentration: Enzyme activity was assayed by adding 5–10 μl of ATPase preparation to 1 ml of assay buffer containing 30 mM Tris-HCl buffer, pH 8.1, 4 mM $MgSO_4$, 0.1 mM EDTA, 1.0 mM ATP, and variable concentrations of the Na^+ salt of the anion being tested for its effect on the activity. Incubations were conducted at 23–24°C for 10 to 60 min, depending on the activity of the enzyme, after which time the reaction was stopped by the addition of 10 μl of 10% SDS. Special precautions were taken to eliminate the potentially substantial effects of organic anions on the colorimetric determination of inorganic phosphate. When measurements of enzyme activity in acetate (or succinate) were compared with Cl^-, assays were run in 1.0 ml of buffer containing 0 to 1.0 N acetate (succinate) or 0 to 1.0 N Cl^-. After the reactions had been stopped by SDS, the volume in each tube was made up to 3 ml, with their anion contents all equalized at 0.33 N Cl^- and 0.33 N acetate (succinate) by adding appropriate volumes of buffer without salt, buffer with 1.0 N NaCl and buffer with 1.0 N Na acetate (succinate), prior to initiation of color development by the Fiske–SubbaRow acid-molybdate reagent supplemented with an extra 0.33 N H_2SO_4 (15). Because lactate strongly suppresses color development in this assay, lactate experiments were performed using the more tolerant Taussky and Schorr assay (46), with the anion concentration equalized at 0.12 N lactate and 0.4 N Cl^- prior to color development. Standard phosphate calibration curves were run for each of the following pairs: Na acetate/NaCl, Na succinate/NaCl, and Na lactate/NaCl.

Gluconate interfered so severely with both methods of assay that it was eliminated from the enzymatic part of the study. Protein concentrations were determined by the method of Lowry et al. (37).

Assay of Vanadate Inhibition: Aliquots of different concentrations of vanadate were incubated in the assay mixture as described above. Results were plotted as percent of uninhibited activity versus vanadate concentration under a given salt condition to obtain the concentration of vanadate giving 50% inhibition of the ATPase activity under each condition. These values were then replotted to show the vanadate sensitivity of the enzyme as a function of salt concentration (see Figs. 5 and 6).

Materials: Reagents purchased as the salts were K^+ gluconate and K^+ glutamate from Sigma Chemical Co., St. Louis, MO; K^+ tartrate from J. T. Baker Chemical Co., Phillipsburg, NJ; and K^+ and Na^+ acetate from Fisher Scientific Co., Pittsburgh, PA. Obtained as the free acids and titrated with reagent grade KOH or NaOH pellets were propionic, glycolic, malonic, succinic, L(+) lactic, and glucuronic acid from Sigma Chemical Co.; and glutaric acid from J. T. Baker Chemical Co. Vanadate was sodium ortho or meta vanadate from Fisher Scientific Co. and was maintained as a stock solution under conditions that minimize polymerization to decavanadate (41), and ATP was from Boehringer-Mannheim Biochemicals, Indianapolis, IN.

RESULTS

Effect of Anion Concentration

The effects of concentration of lactate, acetate, succinate, or gluconate on the longevity and waveform of the reactivated sperm were examined and compared with our earlier results using 0.15 M Cl^-.

The duration of the reactivated motility was optimal in 0.20–0.25 N anion, i.e., 0.10 M for the anions of dioic acids, and 0.20 M for the anions of the monovalent acids. At anion concentrations below 0.20 N, the forward progression of the sperm was slower, and the flagellar waves tended not to propagate all the way to the tip. At concentrations above 0.25 N, the flagellar bend angles were often abnormally low and the forward velocity was diminished. The lowered bend angles were most apparent in succinate (Fig. 1) and were often accompanied by an increase in flagellar beat frequency. In 0.10 and 0.20 M succinate, for example, the beat frequencies were 37.8 ± 1.6 and 41.3 ± 1.2 Hz, while the average bend angles were 2.3 ± 0.2 and 1.5 ± 0.3 rad, respectively. However, the effects of supraoptimal concentrations on waveform and beat frequency differed for different anions, and in 0.40 M K^+ acetate the beat frequency and bend angles were essentially the same as in 0.20 M. This result suggests that the effects on waveform of supraoptimal concentrations of succinate are not due simply to the K^+ concentration being elevated above 0.2 M.

The reversibility of the effects of high succinate was tested by transferring an aliquot of reactivated sperm that had been swimming for 2–3 min in 0.20 M succinate into an observation dish containing 0.10 M succinate. The flagellar waveform reverted within 10 s to that normally seen in 0.10 M succinate, but the duration of the motility did not recover and remained as short as that of flagella left in 0.20 M succinate.

On the basis that the longevity, waveform, and apparent vigor indicated by the rapidity of the forward movement were all optimum at an anion concentration of 0.20–0.25 N, even if the beat frequency was not quite maximal at this concentration for certain anions, we chose 0.20 N for the additional experiments described below.

Longevity of Reactivated Motility

All of the anions selected from our initial screening yielded an improvement in longevity compared to Cl^-, with lactate and gluconate being the best. The latter two anions not only stabilized the motility with time, but also minimized the tendency of the sperm to stick together by their heads upon collision. With healthy sperm samples reactivated in 0.20 M gluconate or lactate, 75–90% of the sperm remained freely motile for 1 h with no significant deterioration in waveform. The longevity was nearly as good in 0.20 M acetate or 0.10 M succinate, but in these anions the sperm had a greater tendency to agglutinate and to show failure of bending to propagate all the way to the flagellar tip with time. In 0.20 M succinate, the sperm were nearly all non-motile by 45 min. These results were summarized in Table I.

Effects of the Anions on Waveform

As reported earlier (27), the principal anion in the reactivation solution has a substantial influence upon flagellar waveform. In particular, sperm flagella reactivated in 0.15 M K acetate showed a significant increase in mean bend angle, with a value of 2.8 rad compared to 2.4 rad in 0.15 M KCl.

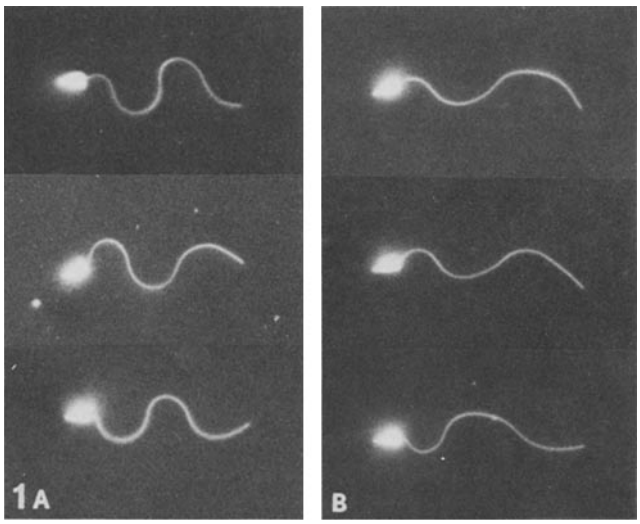

FIGURE 1 Dark-field micrographs showing the effect of different concentrations of K^+ succinate in the reactivation solution on the flagellar waveform. The images are of different sperm. (*A*) 0.10 M K^+ succinate. (*B*) 0.20 M K^+ succinate. × 800.

Lactate had a similar effect to acetate, whereas in either gluconate or succinate the bend angles more closely resembled those seen in Cl^-. The flagellar asymmetry was also influenced by the choice of anion, being generally least in Cl^- and greatest in acetate, although the absolute level of asymmetry varied somewhat with sperm samples from different individual sea urchins. The asymmetry was also affected by the density of the sperm in the demembranating mixture, increasing with increased sperm concentration, especially at values exceeding 0.4 mg sperm protein/ml.

Effects of the Anions on the Kinetics of Reactivated Motility

The flagellar beat frequency was determined as a function of $MgATP^{2-}$ concentration in media containing the various anions and the corresponding values of the Michaelis constant for frequency, K_{mf}, and for the maximum frequency, f_{max}, are shown in Table I. In most of the organic anions tested, f_{max} was higher than in Cl^- medium, with the highest value being attained in succinate. In addition, K_{mf} was lower for most of the anions, indicating a higher affinity for $MgATP^{2-}$ than in Cl^-. Possibly as a consequence of the greater affinity for $MgATP^{2-}$ the organic anion media support beating at substantially lower concentrations of $MgATP^{2-}$ than does Cl^- medium. Preparations remained 100% motile with the bends propagating all the way to the flagellar tip in acetate medium containing as little as 2 μM ATP, whereas 8–10 μM ATP was required for the same level of motility in Cl^- under otherwise identical conditions. At the standard $MgATP^{2-}$ concentration (1.0 mM) that we used in most of this work, the average beat frequencies were 31, 29, and 37 Hz in 0.2 M acetate, 0.2 M lactate, and 0.1 M succinate, respectively, compared to 28 Hz in 0.15 M Cl^- at 22–23°C.

Sensitivity of the Reactivated Motility to Vanadate

Inhibition of the reactivated motility of sea urchin sperm flagella by vanadate in Cl^- medium is sharply dependent on pH and salt concentration (17), with the concentration needed to completely inhibit the sperm ranging from 0.4 μM vanadate in 60 mM Cl^- to 10 μM in 250 mM Cl^-, both at pH 8.1.

In the present work we have found that the sensitivity of the reactivated sperm to vanadate is substantially greater in most of the organic anion media than in Cl^- (Fig. 2 and Table I). The sperm were most sensitive to vanadate in acetate medium, in which their sensitivity was nearly independent of

TABLE I. *Effect of Anion on Properties of Reactivated Flagella and ATPase Activity of Dynein 1, 22°C*

	Reactivated flagella				Dynein 1
Salt	f_{max}*	K_{mf}*	Vanadate concentration for complete inhibition	Longevity‡	Vanadate concentration for 50% inhibition
	Hz	*mM*	*μM*		*μM*
0.15M KCl	31.4 ± 0.2	0.20 ± 0.01	3.0	++	0.31 ± 0.01
0.20M K acetate	32.7 ± 0.6	0.11 ± 0.01	0.5	+++	0.10 ± 0.02
0.20M K lactate	30.3 ± 0.8	0.14 ± 0.01	1.5	++++	0.21 ± 0.04
0.10M K_2 succinate	41.6 ± 1.0	0.13 ± 0.01	0.4	+++	0.11 ± 0.01
0.25M K gluconate	33.4 ± 0.5	0.19 ± 0.01	10.0	++++	—

* Standard deviations for f_{max} and K_{mf} were calculated by the weighted least squares procedure of Wilkinson (48).

‡ The scoring indicates that, on average, 75–90% of the sperm remained motile with no significant deterioration in waveform for ~30 (++), 45 (+++), or 60 min (++++).

anion concentration (Fig. 2). They were somewhat less sensitive to vanadate in succinate and in lactate, with a dependence on anion concentration intermediate between that in acetate and that in Cl^-. In 0.2 M gluconate, they were especially insensitive to vanadate with a critical inhibitory concentration of 10 μM, about equal to that in 0.2 M chloride.

Effect of Anion on Dynein ATPase Activity

The ATPase activity of latent dynein 1 was measured in the presence of acetate, lactate, or succinate as a function of anion concentration (Fig. 3). The striking and uniform result of these experiments is that while Cl^- activated the ATPase activity more than 15-fold between 0 and 1.0 N, all the organic anions caused only a relatively small twofold activation over the same range. In all three organic anions, the curves were of similar form, showing a maximum activation between 0.5 and 0.7 N. The apparent ability of acetate to stabilize the latent ATPase of dynein 1 is emphasized by the finding that addition of 0.5 M K^+ acetate to chloride-activated dynein 1 partially restores the activity to the latent level (27).

In the case of dynein 1 which has been irreversibly activated by prior exposure to 0.1% Triton X-100, all of the anions studied give a small further increase in ATPase activity beyond the 10-fold increase induced by Triton treatment (Fig. 4). The extent of this additional activation was greatest in acetate (~70%) and least in lactate (~25%), and reached a maximum of 0.4 to 0.6 N anion, followed by a return to the baseline Triton-activated activity as the anion concentration approached 1.0 N. These results contrast with Cl^-, in which, as reported earlier (25), the ATPase activity increases more than twofold further as the anion concentration is raised to 1.0 N with little or no decline at 1.0 N (Fig. 4).

Sensitivity of Latent Dynein 1 to Vanadate in the Organic Anions

The ATPase activity of latent dynein 1 was measured as a function of vanadate concentration to determine the level that was required to cause 50% inhibition of enzyme activity (Figs. 5 and 6). As is the case with the reactivated sperm motility, the activity of latent dynein 1 was much more sensitive to vanadate in the presence of acetate or succinate than it was in Cl^-, and this sensitivity changes little with concentration in the 0 to 1.0 N range, whereas in Cl^- the sensitivity to vanadate diminished 60-fold when the concentration was increased from 0 to 1.0 N. Lactate behaved unlike the other organic anions tested in its effect on vanadate inhibition, and both the level of sensitivity and its dependence upon concentration were almost exactly the same as in chloride (Fig. 6).

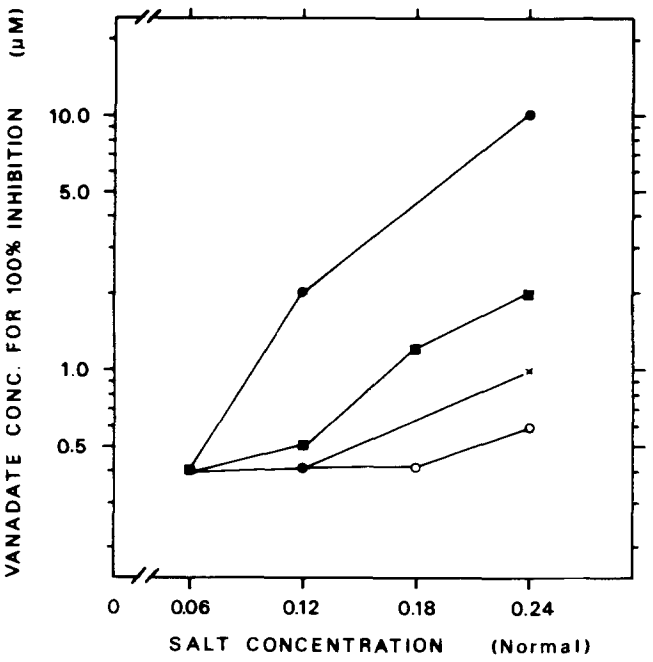

FIGURE 2 Effect of solution anion on the sensitivity of the reactivated motility to inhibition by vanadate. Data are shown as a function of anion concentration. ●, Cl^-; ■, lactate; ×, succinate; ○, acetate.

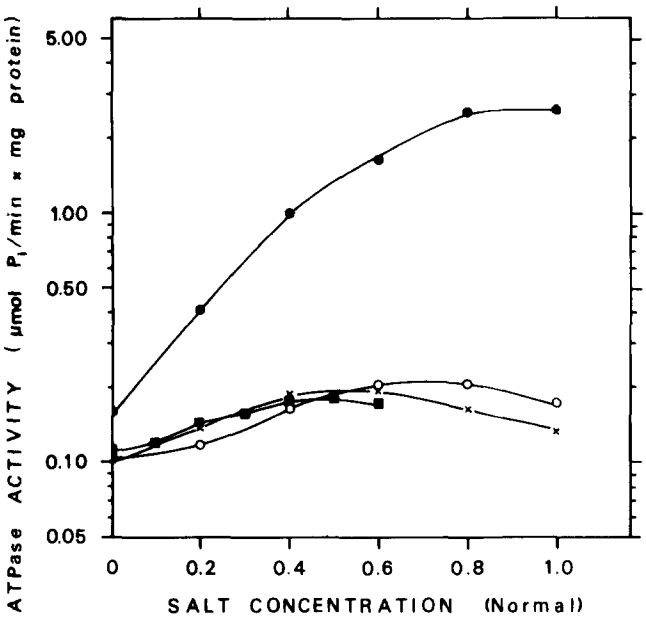

FIGURE 3 Variation of ATPase activity of LAD-1 in different anions as a function of anion concentration. ●, Cl^-; ■, lactate; ×, succinate; and ○, acetate.

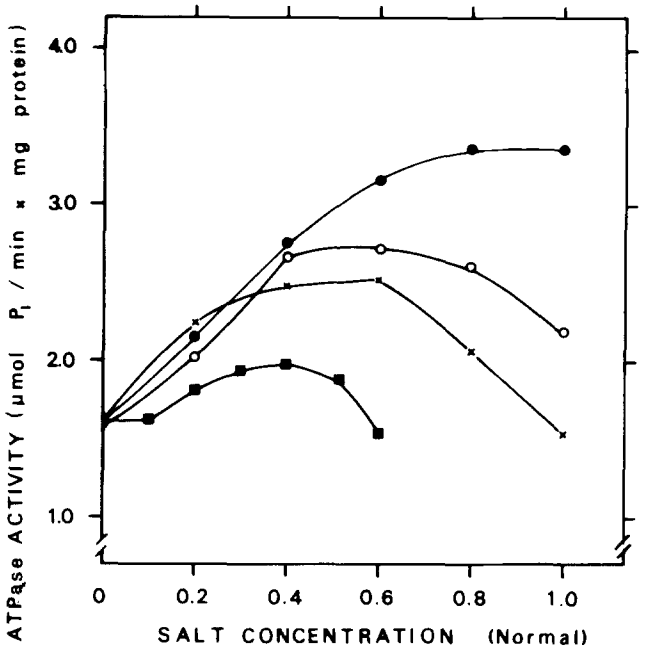

FIGURE 4 Same as Fig. 3, but shown for dynein 1 which has been potentiated by prior exposure to 0.1% Triton X-100 (25).

The anions thus may be classified into three categories according to their effects on the ATPase activity and on the vanadate sensitivity of latent dynein 1. Chloride activates the ATPase activity of dynein 1 at all concentrations, with the enzyme becoming increasingly less sensitive to vanadate as its ATPase activity becomes more activated. Acetate and succinate, on the other hand, neither increase the ATPase activity of dynein 1 substantially at any concentration nor diminish substantially its sensitivity to vanadate inhibition. Lactate is anomalous in that it resembles acetate and succinate in its effect on ATPase activity, but resembles Cl^- in its effect on vanadate sensitivity.

DISCUSSION

Replacement of the chloride in reactivation solution by any of several organic anions stabilizes the motility of reactivated sperm preparations as well as the latency of dynein 1. In addition, the apparent affinity of the reactivated sperm for $MgATP^{2-}$ appears to be higher in most of the organic anions than it is in Cl^-, for the Michaelis constant for beat frequency is reduced from ~0.2 to <0.14 mM in most cases, and the threshold concentration of $MgATP^{2-}$ required to induce beating is reduced from 10 to 2 μM.

By making certain assumptions (23), the average speed of sliding between individual tubules that occurs during oscillatory flagellar beating can be calculated from the bend angle, the beat frequency, and the spacing between adjacent doublet tubules. From the data in this paper and in reference 27, such calculations give sliding velocities of 15 and 14 μm/s for sperm flagella reactivated with 1 mM $MgATP^{2-}$ in acetate and 0.1 M succinate, respectively, both significantly greater than the value of 11 μm/s obtained in optimal concentrations of Cl^-. Although both succinate and acetate yield average sliding velocities that are increased ~30% over those observed for Cl^-, they differ in that this increase is accommodated primarily through an increase in beat frequency for succinate and by an increase in bend angle for acetate. Succinate at 0.2 M supports a still higher flagellar beat frequency than does 0.1 M, but the magnitude of the frequency increase (10%) is insufficient to offset the 50% decrease in average bend angle, indicating that the speed of tubule sliding is substantially diminished.

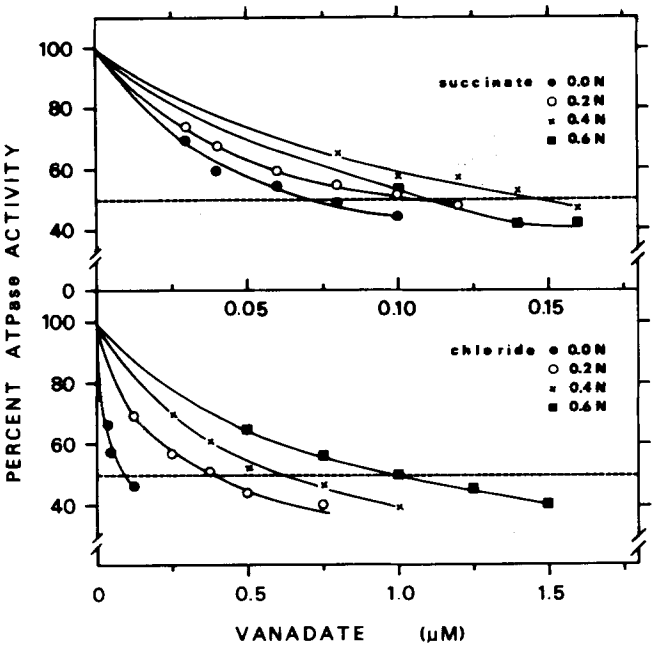

FIGURE 5 Percentage of ATPase activity remaining in the presence of vanadate as a function of the vanadate concentration. (*Top*) Assayed with succinate as the supporting anion. (*Bottom*) With chloride as the supporting anion. Data were used to construct Fig. 6.

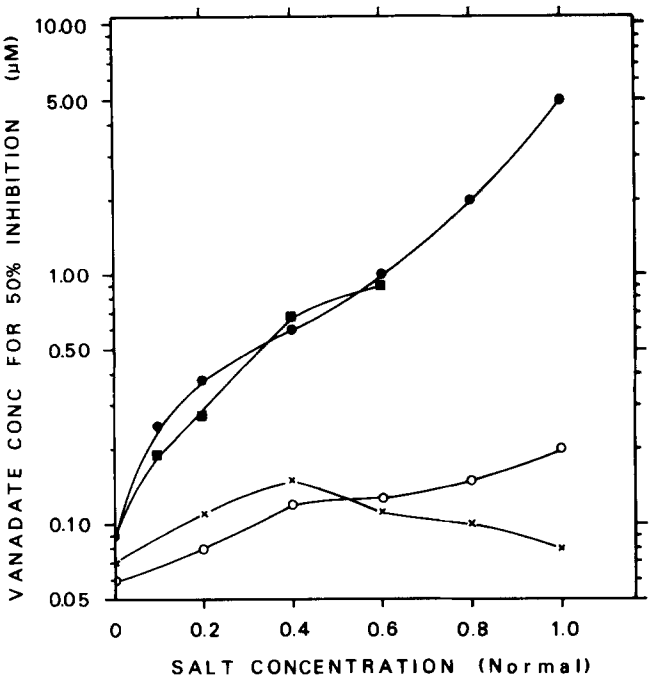

FIGURE 6 Sensitivity of the ATPase activity of LAD-1 to vanadate as a function of the identity and concentration of anion. ●, Cl^-; ■, lactate; ×, succinate; and ○, acetate.

In qualitative terms, the characteristic differences between chloride and the organic anions on the functional properties and vanadate sensitivity of reactivated sperm appear generally to parallel the corresponding differences for the properties and vanadate sensitivity of soluble latent dynein ATPase. This being the case, it is reasonable to interpret these differences in terms of what is known of the steady-state and pre-steady state kinetic mechanisms of dynein ATPase. The demonstration of a pre-steady state phosphate burst by Evans (14) with the more detailed pre-steady state studies of Johnson (34, 35) have shown that the rate-limiting step of ATP hydrolysis by soluble dynein is the rate of breakdown of the dynein-ADP-PO_4 ternary complex (k_3), with the Michaelis constant being approximately equal to the ratio of k_3 to the rate of association of the dynein–ATP complex (k_1) (i.e., $K_m \cong k_3/k_1$). More recent kinetic data suggest that the release of PO_4 from the dynein-ADP-PO_4 complex precedes that of ADP, and that breakdown of the dynein–ADP complex may constitute the actual rate-limiting step in the overall ATPase rate of soluble dynein (Dr. K. A. Johnson, personal communication). When applied to our findings, these results suggest that the increase in steady-state ATPase with increasing chloride is due to destabilization of the dynein-ADP complex with concomitant increase in k_4. The relatively small effect of organic anions on steady-state ATPase can then be interpreted as indicating that, over the range up to 1 N, they have little or no destabilizing effect on the dynein-ADP intermediate, so that the rate of ATP turnover, and, presumably also the lifetime of the dynein-ADP intermediate, remain little

changed from their values in the near absence of salt.

This hypothesis is supported both by the steady-state studies of Gibbons and Fronk (24, 25), which have shown that the activation of dynein 1 ATPase by chloride is accompanied by an approximately proportionate increase in the value of the Michaelis constant, K_m, and by the different effects of chloride and of most organic anions on the sensitivity of soluble dynein ATPase to inhibition by vanadate reported here. There is fairly substantial evidence that vanadate inhibition occurs through the PO_4^{3-} moiety in the dynein-ADP-PO_4 intermediate being replaced by the VO_4^{3-} anion, forming a relatively stable dynein-ADP-VO_4 complex that acts as a dead-end kinetic block (28, 36, 44). This being the case, any factor that decreases the lifetime of the dynein–ADP intermediate will ipso facto decrease the sensitivity of the ATPase reaction to vanadate. In the present case, increasing the chloride concentration from 0 to 0.6 N produces an approximate 10-fold increase in ATPase activity, and an approximate 10-fold decrease in sensitivity to vanadate, whereas acetate and succinate over the 0–1 N range produce an approximate twofold increase in ATPase activity and a twofold decrease in vanadate sensitivity, consistent with the lifetime of the dynein–ADP intermediate playing a major role in both the overall ATPase rate and the sensitivity to vanadate. It is notable that with other activators of latent dynein ATPase, such as Triton X-100, there is also a substantial increase in K_m and decrease in sensitivity to vanadate accompanying the activation of ATPase activity (24, 25, 28).

The anomalous result obtained here with lactate, which decreases the sensitivity to vanadate while giving little change in ATPase reaction velocity, may be due to secondary effects, such as complexation of vanadate by the lactate anion.

The above hypothesis to explain the different effects of chloride and organic anions on properties of soluble dynein ATPase can be extended to their effects on reactivated sperm motility. In reactivated sperm, ATP hydrolysis is thought to occur through a mechanochemical crossbridge cycle in which the individual steps resemble those of soluble dynein ATPase, but with the additional factors that the initial binding of ATP to the dynein crossbridge causes it to detach very rapidly from the adjacent tubule, and that, following ATP hydrolysis, the reattachment of the crossbridge containing the dynein-ADP-PO_4 intermediate to the next site on the adjacent tubule causes rapid dissociation of the ADP and phosphate from the dynein (20, 35, 42, 43). It is presumably the difference between this rapid reattachment-induced rate of product release and the slower spontaneous rate that provides the basis for the coupling of ATP hydrolysis to the detachment/reattachment crossbridge cycle and the performance of mechanochemical work. For reactivated sperm in chloride-based media, it has been found experimentally that 70–75% of the total ATP hydrolysis is tightly coupled to the flagellar motility (8, 19). It is not at present possible to make a quantitative association between the parameters of flagellar beating and the rate constants of dynein ATPase, but it seems likely that the higher value of the Michaelis constant for beat frequency (K_{mf}) observed in chloride media compared to organic anion media, as well as the higher threshold $MgATP^{2-}$ concentration needed to sustain oscillatory beating and the decreased speed of tubule sliding in 1 mM $MgATP^{2-}$, may all reflect a faster rate of spontaneous dissociation of the products from the dynein-ADP-PO_4 step in the crossbridge cycle in chloride media. This would be analogous to the effect occurring at the equivalent step of the reaction in soluble dynein. Since such spontaneous product release from the crossbridge represents a bypass of the presumed normal energy coupling step, an increase in its rate would be expected to diminish mechanochemical efficiency.

The data for vanadate inhibition of reactivated sperm motility support this hypothesis in that an increase in chloride concentration from 0.06 to 0.24 N decreases vanadate sensitivity ~20-fold, whereas the same change in acetate or succinate has only a twofold effect. This difference is consistent with chloride promoting the spontaneous release of products from the detached crossbridge-ADP-PO_4 intermediate and so reducing the opportunity for the PO_4^{3-} moiety to exchange with vanadate. An alternative explanation for the vanadate data involving a stabilization of the dynein-ADP-VO_4 complex by organic anion appears less likely, for it fails to explain the vanadate sensitivity being nearly independent of anion concentration in acetate, or to explain the apparently greater mechanochemical efficiency observed in vanadate-free organic anion media.

Hamel et al. have shown that a variety of organic anions stabilize the colchicine binding activity and polymerizability of tubulin (32). These included 2-(*N*-morpholino)ethane sulfonic acid, glutamate, PIPES, glutarate, and glucose-1-phosphate. The stabilizing effects of glutamate have been exploited to aid in the large-scale purification of tubulin (31).

A further instance of protein stabilization by organic anions has been shown in X-ray diffraction studies of skinned rabbit psoas fibers. Comparing the effects of propionate and Cl^- on relaxed muscle fibers, Brenner et al. found that whereas the lattice expanded linearly between 50 and 200 mM KCl, it remained nearly unchanged in 20–80 mM K propionate (4). The lattice stability is believed to be maintained by loose attachments of the myosin heads to the thin filaments in the relaxed state, with these attachments being unaffected by K propionate over the range studied but progressively destabilized by increasing concentrations of KCl up to 200 mM.

Our results indicate that chloride has deleterious effects on the motility of sperm flagella and that use of any of several organic anions is to be preferred. This finding may have general applicability to other reactivated systems (9). However, although choice of anion may greatly affect the sensitivity of a demembranated system to the inhibitor vanadate, it is not clear that this effect can be invoked to explain the relatively low sensitivity of many motile cytoplasmic microtubule systems to vanadate in vivo. The high sensitivity of ciliary movement to microinjected vanadate (11, 45) suggests that the low vanadate sensitivity of other motile microtubule systems lies in the inherent properties of the contractile systems involved (1, 12, 13, 16).

The authors thank Mrs. Aileen M. Feldman for her expert technical assistance, and Mrs. Alice Lee-Eiford for helpful comments on the manuscript. We also thank Dr. Ken Johnson for stimulating discussion.

This work was supported by grants HD 06565 and GM 30401 from the National Institutes of Health.

Received for publication 8 April 1985, and in revised form 28 May 1985.

REFERENCES

1. Beckerle, M. C., and K. R. Porter. 1982. Inhibitors of dynein activity block intracellular transport in erythrophores. *Nature (Lond.)*. 295:701–703.

2. Bell, C. W., C. L. Fraser, W. S. Sale, W.-J. Y. Tang, and I. R. Gibbons. 1982. Preparation and purification of dynein. *In* Methods in Cell Biology. L. Wilson, editor. Vol. 24A, Academic Press Inc. New York. 373–397.
3. Bessen, M., R. B. Fay, and G. B. Witman. 1980. Calcium control of waveform in isolated flagellar axonemes of *Chlamydomonas*. *J. Cell Biol.* 86:446–455.
4. Brenner, B., L. C. Yu, and R. J. Podolsky. 1984. X-ray diffraction evidence for cross-bridge formation in relaxed muscle fibers at various ionic strengths. *Biophys. J.* 46:299–306.
5. Brokaw, C. J. 1975. Effects of viscosity and ATP concentration on the movement of reactivated sea urchin sperm flagella. *J. Exp. Biol.* 62:701–719.
6. Brokaw, C. J. 1979. Calcium-induced asymmetrical beating of Triton-demembranated sea urchin sperm flagella. *J. Cell. Biol.* 82:401–411.
7. Brokaw, C. J. 1982. Activation and reactivation of *Ciona* spermatozoa. *Cell Motility*-1: 185–189. (*Suppl.*)
8. Brokaw, C. J., and B. Benedict. 1968. Mechanochemical coupling in flagella. I. Movement-dependent dephosphorylation of ATP by glycerinated spermatozoa. *Arch. Biochem. Biophys.* 125:770–778.
9. Brokaw, C. J., and S. N. Nagayama. 1985. Modulation of the asymmetry of sea urchin sperm flagellar bending by calmodulin. *J. Cell Biol.* 100:1875–1883.
10. Brokaw, C. J., R. Josslin, and L. Bobrow. 1974. Calcium ion regulation of flagellar beat symmetry in reactivated sea urchin spermatozoa. *Biochem. Biophys. Res. Commun.* 58:795–800.
11. Buckley, I., and M. Stewart. 1983. Ciliary but not saltatory movements are inhibited by vanadate microinjected into living cultured cells. *Cell Motility.* 3:167–184.
12. Cande, W. Z., and S. M. Wolniak. 1978. Chromosome movement in lysed mitotic cells is inhibited by vanadate. *J. Cell Biol.* 79:573–580.
13. Clark, T. G., and J. L. Rosenbaum. 1982. Pigment particle translocation in detergent-permeabilized melanophores of *Fundulus heteroclitus*. *Proc. Natl. Acad. Sci. USA.* 79:4655–4659.
14. Evans, J. A. 1982. A kinetic study of latent and Triton-potentiated dynein-1 adenosine triphosphatase. PhD. thesis, University of Hawaii.
15. Fiske, C. H., and Y. Subbarow. 1925. The colorimetric determination of phosphorus. *J. Biol. Chem.* 66:375–400.
16. Forman, D. S. 1982. Vanadate inhibits saltatory organelle movement in a permeabilized cell model. *Exp. Cell Res.* 141:139–147.
17. Gibbons, B. H. 1982. Reactivation of sperm flagella: properties of microtubule-mediated motility. *In* Methods in Cell Biology. L. Wilson, editor. Academic Press Inc. New York. 253–271.
18. Gibbons, B. H. 1982. Effects of organic solvents on flagellar asymmetry and quiescence in sea urchin sperm. *J. Cell Sci.* 54:115–135.
19. Gibbons, B. H., and I. R. Gibbons. 1972. Flagellar movement and adenosine triphosphatase activity in sea urchin sperm extracted with Triton X-100. *J. Cell Biol.* 54:75–97.
20. Gibbons, B. H., and I. R. Gibbons. 1974. Properties of flagellar "rigor waves" produced by abrupt removal of adenosine triphosphate from actively swimming sea urchin sperm. *J. Cell Biol.* 63:970–985.
21. Gibbons, B. H., and I. R. Gibbons. 1979. Relationship between the latent adenosine triphosphatase state of dynein 1 and its ability to recombine functionally with KCl-extracted sea urchin sperm flagella. *J. Biol. Chem.* 254:197–201.
22. Gibbons, B. H., and I. R. Gibbons. 1980. Calcium-induced quiescence in reactivated sea urchin sperm. *J. Cell Biol.* 84:13–27.
23. Gibbons, I. R. 1974. Mechanisms of flagellar motility. *In* The Functional Anatomy of the Spermatozoan. B. A. Afzelius, editor. Pergamon, Oxford. 127–140.
24. Gibbons, I. R., and E. Fronk. 1972. Some properties of bound and soluble dynein from sea urchin sperm flagella. *J. Cell Biol.* 54:365–381.
25. Gibbons, I. R., and E. Fronk. 1979. A latent adenosine triphosphatase form of dynein 1 from sea urchin sperm flagella. *J. Biol. Chem.* 254:187–196.
26. Gibbons, I. R., and B. H. Gibbons. 1980. Transient flagellar waveforms during intermittent swimming in sea urchin sperm. I. Wave parameters. *J. Muscle Res. Cell Motil.* 1:31–59.
27. Gibbons, I. R., J. A. Evans, and B. H. Gibbons. 1982. Acetate anions stabilize the latency of dynein 1 ATPase and increase the velocity of tubule sliding in reactivated sperm flagella. *Cell Motility.* 1:181–184. (*Suppl.*)
28. Gibbons, I. R., M. P. Cosson, J. A. Evans, B. H. Gibbons, B. Houck, K. H. Martinson, W. S. Sale, and W.-J. Tang. 1978. Potent inhibition of dynein adenosine-triphosphatase and of the motility of cilia and sperm flagella by vanadate. *Proc. Natl. Acad. Sci. USA.* 75:2220–2224.
29. Goldstein, S. F. 1976. Form of developing bends in reactivated sperm flagella. *J. Exp. Biol.* 64:173–184.
30. Goodenough, U. W. 1983. Motile detergent-extracted cells of *Tetrahymena* and *Chlamydomonas*. *J. Cell Biol.* 96:1610–1621.
31. Hamel, E., and C. M. Lin. 1981. Glutamate-induced polymerization of tubulin: characteristics of the reaction and application to the large-scale purification of tubulin. *Arch. Biochem. Biophys.* 209:29–40.
32. Hamel, E., A. A. del Campo, M. C. Lowe, P. G. Waxman, and C. M. Lin. 1982. Effects of organic acids on tubulin polymerization and associated guanosine 5′-triphosphate hydrolysis. *Biochemistry.* 21:503–509.
33. Hyams, J. S., and G. G. Borisy. 1978. Isolated flagellar apparatus of *Chlamydomonas*: characterization of forward swimming and alteration of waveform and reversal of motion by calcium ions in vitro. *J. Cell Sci.* 33:235–253.
34. Johnson, K. A. 1983. The pathway of ATP hydrolysis by dynein. Kinetics of a pre-steady state phosphate burst. *J. Biol. Chem.* 258:13825–13832.
35. Johnson, K. A. 1985. Pathway of the microtubule-dynein ATPases and the structure of dynein: a comparison with actomyosin. *Annual Review of Biophysics and Biophysical Chemistry.* 14:161–188.
36. Kobayashi, T., T. Martensen, J. Nath, and M. Flavin. 1978. Inhibition of dynein ATPase by vanadate and its possible use as a probe for the role of dynein in cytoplasmic motility. *Biochem. Biophys. Res. Commun.* 81:1313–1318.
37. Lowry, O. H., N. J. Rosenbrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193:265–275.
38. Naitoh, Y., and H. Kaneko. 1972. Reactivated Triton-extracted models of Paramecium: modification of ciliary movement by Ca^{++} ions. *Science (Wash. DC).* 176:523–524.
39. Okuno, M. 1980. Inhibition and relaxation of sea urchin sperm flagella by vanadate. *J. Cell Biol.* 85:712–715.
40. Omoto, C. K., and C. J. Brokaw. 1982. Structure and behavior of the sperm terminal filament. *J. Cell Sci.* 58:385–409.
41. Pope, M. T., and B. W. Dale. 1968. Isopoly-vanadates, -niobates, and -tantalates. *Q. Rev. Chem. Soc. Lond.* 22:527–548.
42. Porter, M. E., and K. A. Johnson. 1983. Transient state kinetic analysis of the ATP-induced dissociation of the dynein-microtubule complex. *J. Biol. Chem.* 258:6582–6587.
43. Sale, W. S., and I. R. Gibbons. 1980. Study of the mechanism of vanadate inhibition of the dynein cross-bridge cycle in sea urchin sperm flagella. *J. Cell Biol.* 82:291–298.
44. Shimizu, T., and K. A. Johnson. 1983. Pre-steady state kinetic analysis of vanadate-induced inhibition of the dynein ATPase. *J. Biol. Chem.* 258:13833–13840.
45. Stommel, E. W., R. E. Stephens, and D. L. Alkon. 1980. Motile statocyst cilia transmit rather than directly transduce mechanical stimuli. *J. Cell Biol.* 87:652–662.
46. Taussky, H. H., and E. Schorr. 1952. A microcolorimetric method for the determination of inorganic phosphorous. *J. Biol. Chem.* 202:675–685.
47. Wais-Steider, J., and P. Satir. 1979. Effect of vanadate on gill cilia: switching mechanism in ciliary beat. *J. Supramol. Struct.* 11:339–347.
48. Wilkinson, G. N. 1961. Statistical estimations in enzyme kinetics. *Biochem. J.* 80:324–332.