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CO_2-INHIBITION OF THE AMPLITUDE OF BENDING OF TRITON-DEMEMBRANATED SEA URCHIN SPERM FLAGELLA

BY C. J. BROKAW

Division of Biology, California Institute of Technology, Pasadena, California 91125

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SUMMARY

Demembranated sea urchin spermatozoa were reactivated in solutions containing $KHCO_3$ and observed in a covered well slide. Although $KHCO_3$ itself causes a small inhibition of flagellar beat frequency, the results confirm previous observations of a direct inhibition of flagellar bend angle by CO_2 with no effect of CO_2 on frequency.

Observation of the effect of pH on the inhibition of bend angle in solutions containing $KHCO_3$ indicates that a given concentration of OH^- has a similar effect to the same concentration of HCO_3^-, as would be expected if CO_2-inhibition results from reaction of CO_2 with protein-NH_3^+ groups to form carbamates.

CO_2 may interfere with a control mechanism which selectively suppresses dynein cross-bridge activity in order to generate rhythmic bending. This control mechanism may incorporate a feedback control involving a measure of flagellar amplitude, which fails to operate successfully when the amplitude is reduced below a critical level.

INTRODUCTION

A reversible inhibition of the movement of reactivated flagella by CO_2, supplied either by flowing CO_2 gas over the surface of a suspension of reactivated spermatozoa or by adding 0·1 M-$NaHCO_3$ to the reactivation solution, has been reported by Brokaw & Simonick (1976). This inhibitory effect is interesting because it appears to be a specific inhibition of the amplitude of the bending waves, rather than the frequency of oscillation, and does not involve direct inhibition of either flagellar ATPase activity, or the ATP-driven active sliding process which is responsible for flagellar bending (Summers & Gibbons, 1971). This paper describes a more quantitative examination of the CO_2-inhibition of flagellar bend angle, using $KHCO_3$ in the reactivation solutions as a source of CO_2, and an observation chamber which restricts the escape of CO_2 to the atmosphere, so that the CO_2 concentration can approach equilibrium with the HCO_3^- ion in the reactivation solution.

METHODS

Demembranated spermatozoa from the sea urchin, *Lytechinus pictus*, were prepared and reactivated as described previously (Brokaw, 1975*a*). Unless noted otherwise, the

ATP and $MgSO_4$ concentrations in the reactivation solutions were 0·2 mM and 2·0 mM, respectively. All experiments were carried out in a room maintained at 16° ± 1 °C, and the microscope stage temperature was also controlled to 16° ± 0·1 °C by circulation of water from a thermostatted bath.

Open preparations were simply a drop of reactivation solution containing demembranated spermatozoa placed on a microscope slide without a coverglass. With these preparations, photographs were taken using a 16×, 0·4 NA objective, to give a magnification on film of 80×. A small plastic shield was mounted on the objective to reduce the circulation of air over the surface of the drop, to minimize cooling of the drop by evaporation.

Closed preparations utilized a well slide made from a 25 × 75 mm piece of 1·5 mm thick aluminium sheet. An 11 mm diameter hole was drilled through the centre of the sheet surrounded by a machined ledge which allowed a 12 mm diameter coverglass to be cemented over the hole with its top surface parallel to and 0·2 mm below the top surface of the metal slide. After placing a drop of reactivation solution in the well, it was covered with a 22 mm square coverglass. A drop size was used such that the coverglass was held close to the surface of the slide, with no air bubble inside the well. This situation was designed to restrict the diffusion of CO_2 between the interior of the drop and the atmosphere, in an attempt to establish a CO_2–HCO_3^- ion equilibrium in the interior of the drop when the reactivation solutions contained $KHCO_3$. Closed preparations were photographed using a 40× oil immersion objective with an iris diaphragm which reduced the NA to 0·6–0·65, to give a magnification on film of 125×. A dark-field condenser was used, to permit multiple-flash exposures of moving sperm flagella.

Illumination was provided by a xenon lamp (Varian Associates, model VIX 150) operated by a pulsed power supply (Chadwick-Helmuth, Monrovia, CA; model 136 and 72). Photographs were taken with a Robot 35 mm camera using Kodak Tri-X film, and developed for 10 min in Acufine at 26 °C. Multiple-flash photomicrographs were taken using a flash frequency adjusted to 4 times the beat frequency of a particular spermatozoon. The flash frequency was controlled with a variable frequency sine wave oscillator (General Radio Co., model 1310B). The output from this oscillator was gated such that only five flashes were obtained while the camera shutter was open. Each time a photograph was taken, an automatic record was made on digital printers of the oscillator period (to 0·01 ms) and of the time (to the nearest second) since mixing demembranated spermatozoa with reactivation solution.

For most of these experiments, spermatozoa which were attached by their heads to the glass surface at the bottom of the well slide were photographed. Such spermatozoa are easier to maintain in focus than swimming ones, and with closed preparations they provide a larger and more repesrentative sample. In a typical experiment, recording began about 1 min after mixing a demembranated sperm preparation into reactivation solution. For measurement of the parameters of a sperm sample, the preparation was systematically scanned, and every spermatozoon was photographed if it was attached so that its beat plane was sufficiently parallel to the glass surface, if it was not obscured by other spermatozoa, and if it showed no obvious abnormality such as a break in the flagellum or attachment of part of the flagellum to the glass surface. Recording was continued for about 5 min, or until 15–20 spermatozoa were

Table 1. *Effect of carbonic anhydrase on movement parameters of reactivated sea urchin sperm flagella in the presence of* 0·04 M-$KHCO_3$ *at pH* 8·4

	n	Frequency (Hz)	Bend angle (radians)
Open drop preparations			
Control	29	13·0 ± 0·6	2·47 ± 0·10
+ $KHCO_3$	16	11·7 ± 0·7	2·40 ± 0·07
+ $KHCO_3$ + carbonic anhydrase (10 μg ml^{-1})	10	11·3 ± 0·6	2·19 ± 0·07
Closed drop preparations			
Control	22	13·7 ± 1·0	2·43 ± 0·12
+ $KHCO_3$	29	12·5 ± 0·8	2·21 ± 0·11
+ $KHCO_3$ + carbonic anhydrase (10 μg ml^{-1})	22	12·5 ± 0·8	2·24 ± 0·07

Standard deviations are indicated for the measured values of frequency and bend angle. In both types of preparations, spermatozoa attached to the glass surface at the bottom of the drop were examined. All measurements were made within 4 min after mixing, and all measurements were obtained with the same sperm sample. In cases where n is greater than 20, data from two separate preparations of demembranated spermatozoa were combined.

photographed. In some experiments, a single spermatozoon was selected and photographed at intervals to obtain data on movement parameters as a function of time.

The films were projected with a Kodak Recordak microfilm viewer, and the multiple images of each spermatozoon were examined to confirm that the beat frequency corresponded exactly to $\frac{1}{4}$ the recorded frequency of the flash illuminator. This could be determined precisely by comparing the images from the first and fifth flash. This procedure primarily served to select the proper spermatozoon when the field contained more than one spermatozoon. Bend angles were measured near the middle of the flagellar length, using a straight edge and protractor. The bend angles were measured between the straight regions on either side of a bend, assuming that these straight regions formed tangents to the circular arcs of adjacent bends (cf. Brokaw, 1970). Bend angles were measured independently for principal and reverse bends (Gibbons & Gibbons, 1972), but were averaged for the purposes of this paper.

RESULTS

To test whether closed drop preparations contained CO_2 in equilibrium with HCO_3^-, observation was made of the effect of carbonic anhydrase upon CO_2 concentration, employing a CO_2-sensitive parameter of flagellar movements as an indicator of CO_2 concentration. Carbonic anhydrase, which accelerates the dissociation of H_2CO_3 to give CO_2 and H_2O, should increase the CO_2 concentration in steady-state situations where CO_2 can diffuse away into the atmosphere, but should have no effect on the CO_2 concentration in a closed system after equilibrium between CO_2, H_2CO_3, and HCO_3^- has been established. The results in Table 1, from a representative experiment, show that with closed drop preparations, addition of carbonic anhydrase did not increase the inhibition of flagellar bend angle which is obtained when the reactivation solution contains 0·04 M-$KHCO_3$. Statistical analysis of the results from this and another similar experiment indicates that the probability of not detecting a bend angle decrease of 0·05 radians caused by the addition of carbonic anhydrase is

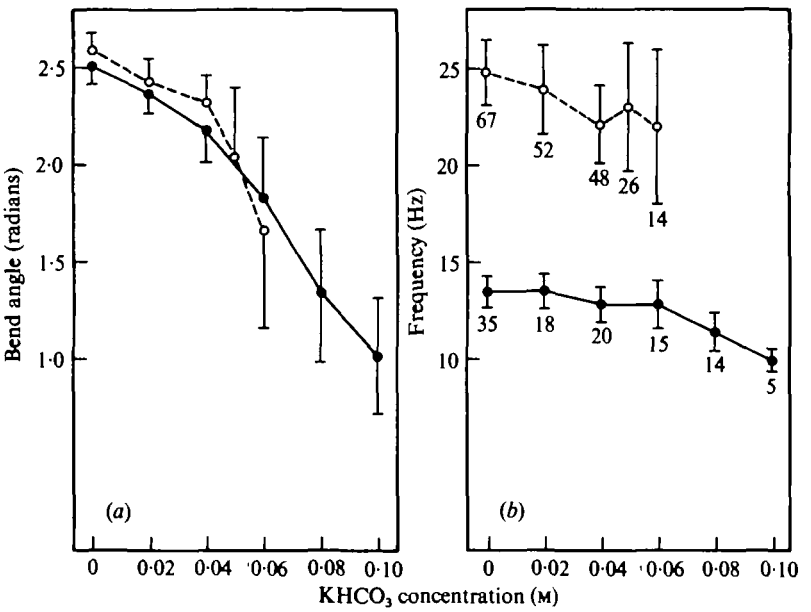


Fig. 1. Measurements of bend angle and beat frequency for reactivated spermatozoa as a function of $KHCO_3$ concentration, at pH 8·4: for one sperm sample, at 0·2 mM-ATP and 2·0 mM-$MgSO_4$ (●); and for two sperm samples, at 2·2 mM-ATP and 4·0 mM-$MgSO_4$ (○). Standard deviations are represented by vertical bars and numbers indicate the number of spermatozoa in the samples, in this and subsequent figures.

0·1%. The reduction in bend angle which was obtained when carbonic anhydrase was added to an open drop preparation containing $KHCO_3$ confirms the effectiveness of the carbonic anhydrase preparation and, as noted earlier (Brokaw & Simonick, 1976), supports the conclusion that it is the CO_2 molecule which is responsible for the inhibition of bend angle. The closed drop preparations therefore appear to provide a satisfactory means of exposing reactivated spermatozoa to CO_2 concentrations close to those which would be established by equilibrium with HCO_3^-.

The results in Table 1 also show that the beat frequencies measured in the open drop preparations are consistently lower than those measured in the closed drop preparations. This result is frequently obtained. I suspect that it may reflect a temperature difference: the open preparations may be slightly cooler than the stage temperature, because of evaporation, and the closed preparations may be slightly warmer than the stage temperature, because of heat transfer from the body of the observer through the microscope and oil immersion objective.

The effects of $KHCO_3$ concentrations ranging from 0 to 0·10 M on the bend angle and beat frequency of attached spermatozoa are shown in Fig. 1, at two different ATP concentrations. An ATP concentration of 0·2 mM, which was used for most of these experiments, normally gives about half the maximum beat frequency (Brokaw, 1975*a*). At the higher ATP concentration (2·2 mM), there is a greater dispersion of beat frequencies, and most sperm samples show a significant number of erratically beating spermatozoa. Representative photographs from the sample studied at 0·2 mM-ATP are shown in Fig. 5*a–f*. These photographs were selected to have bend angles close to the mean bend angle at each $KHCO_3$ concentration.

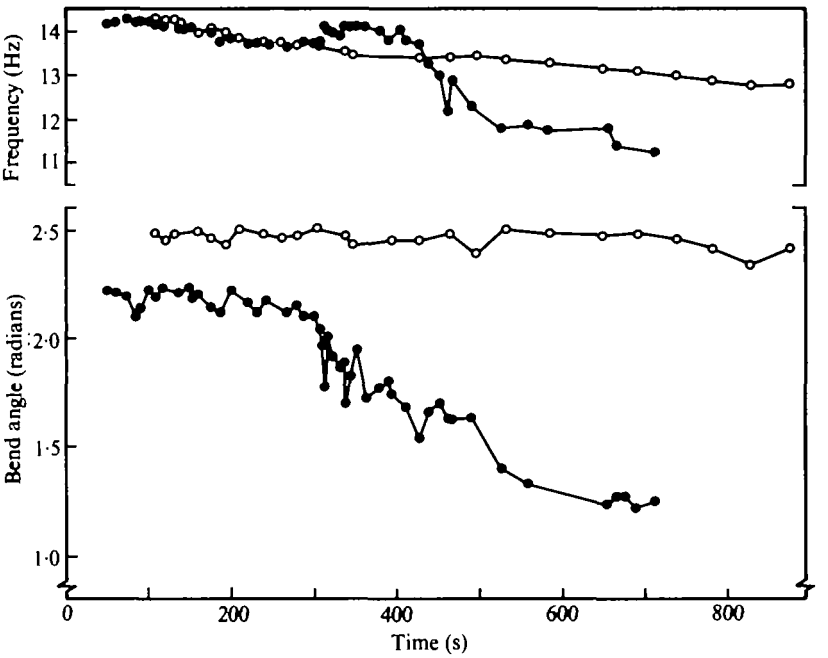


Fig. 2. The time course of the inhibition of bend angle and beat frequency of an attached spermatozoon in a reactivation solution containing 0·04 M-$KHCO_3$ at pH 8·4 is shown by the solid points. Measurements of an attached spermatozoon in a control experiment using reactivation solution without $KHCO_3$, are shown by the open points.

With 0·2 mM-ATP, there is a gradual decrease in bend angle as the $KHCO_3$ concentration is increased. Several factors complicate this result. With 0·08 M and 0·10 M-$KHCO_3$, the preparations contain many non-motile spermatozoa, and the measurements refer to a fraction of the sperm population which is still generating reasonably stable bending waves. The measurements therefore overestimate the mean bend angle at these $KHCO_3$ concentrations. This effect appears to be more pronounced at the higher ATP concentration, where the preparations contain many non-motile spermatozoa even with 0·04 M-$KHCO_3$.

At the higher $KHCO_3$ concentrations, the inhibitory effect of $KHCO_3$ can be clearly seen to increase with time; motile spermatozoa can be found when the preparation is first examined, but they become difficult to find after about 5 min. At lower $KHCO_3$ concentrations, there is a slower increase in the inhibition of movement with time. Fig. 2 illustrates the time course of the inhibitory effect of 0·04 M-$KHCO_3$. Selected photographs from this time sequence are shown in Fig. 5*h–n*. Fig. 2 also includes data from a control experiment showing a spermatozoon in the absence of $KHCO_3$. Most of the variability in bend angle shown in the data for the control spermatozoon probably reflects measurement error, resulting from measurement of bends at different positions along the flagellum. Fig. 2 shows that in the presence of $KHCO_3$, an immediate reduction in bend angle to a value below the value obtained in the absence of $KHCO_3$ has occurred by the time of the first photograph. Bend angle and beat frequency both remain nearly constant for about 5 min, after which there is a gradual decline in bend angle. In its later stages, this decline in bend angle is

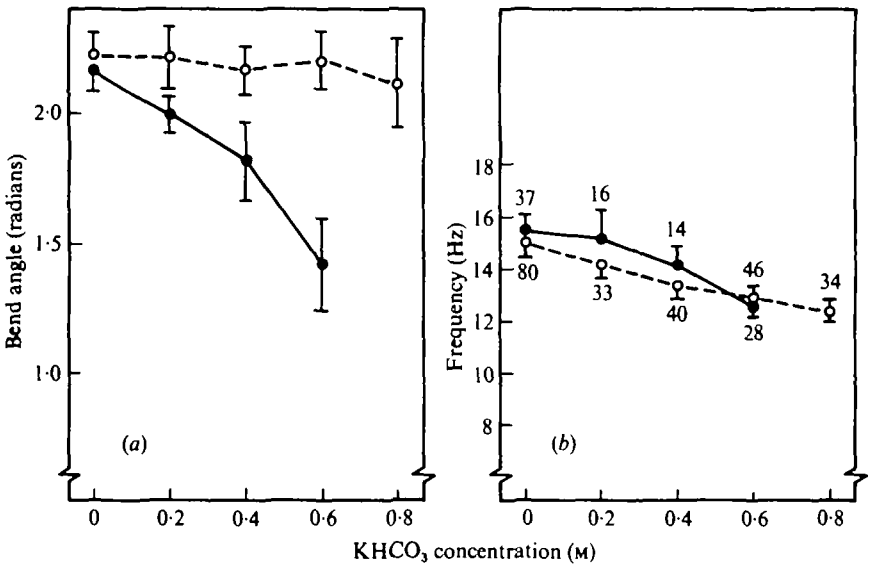


Fig. 3. Measurements of bend angle and beat frequency for reactivated spermatozoa as a function of $KHCO_3$ concentration, at pH 8·4: for spermatozoa swimming in closed drop preparations (●); and for spermatozoa swimming at the upper surface of open drop preparations (○).

accompanied by a decrease in beat frequency, shown in Fig. 2, by a decrease in the symmetry of the bending waves, as shown in Fig. 5, *m*, *n*, and by a noticeable decrease in the regularity of beating. Asymmetrical bending patterns, usually having a lower, and relatively irregular, beat frequency, are commonly found in the later stages of CO_2-inhibition and at higher $KHCO_3$ concentrations; an example from the experiment shown in Fig. 1 is shown in Fig. 5*g*. Spermatozoa beating with these obviously asymmetrical patterns were usually not included in the samples photographed for bend angle measurements. The asymmetrical bending pattern appears to be characterized by a relatively slow development of a bend of near-normal angle near the base of the flagellum, followed by a relatively rapid, decremental propagation of this bend along the flagellum. There is little bending in the opposite direction, and it may be entirely passive. A similar type of asymmetry was observed by Okuno & Hiramoto (1976) when the movement of a flagellum was restricted with a microneedle. In both cases, the axis of the flagellum is more or less straight, in striking contrast to the curved flagellar axis evident in the asymmetrical bending patterns obtained by varying the Ca^{2+} concentration (Brokaw, Josslin & Bobrow, 1974; Brokaw & Simonick, 1976).

The bend angle does not continue to decline gradually to 0. Instead, there is an abrupt cessation of beating, sometimes followed by sporadic attempts to resume beating. The abrupt cessation of beating suggests that there may be a critical amplitude which is required for maintenance of regular oscillation, but this critical amplitude appears to have different values under different conditions. The amplitudes obtained at high $KHCO_3$ concentrations, as shown in Fig. 5*f*, are typically lower than the lowest amplitude obtained before cessation of beating when following the time course of inhibition at lower $KHCO_3$ concentrations. In some cases, there may also be an

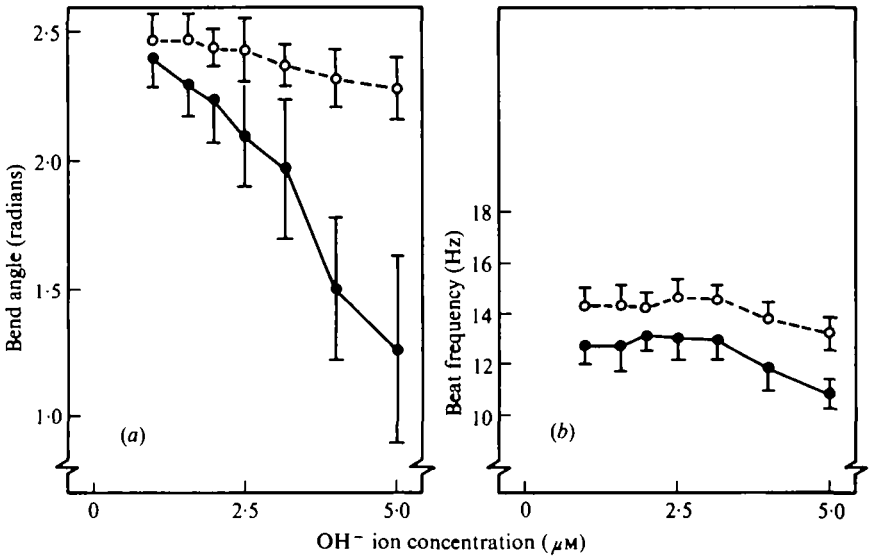


Fig. 4. Measurements of bend angle and beat frequency for reactivated spermatozoa as a function of pH, plotted as OH^- ion concentration: for spermatozoa in reactivation solution containing 0·04 M-$KHCO_3$ (●); and for spermatozoa in standard reactivation solution without $KHCO_3$ (○). Two samples were measured at pH 8·0, 8·2, 8·4 and 8·6; a third sample was measured at pH 8·0, 8·3, 8·5, and 8·7. Data were adjusted to match the results at pH 8·0.

abrupt transition from a normal type of bending pattern to the asymmetrical type of bending pattern. Fig. 5*o–q* shows this behaviour in a spermatozoon at 0·04 M-$KHCO_3$, which switched abruptly from the pattern shown in Fig. 5*p* to the pattern shown in Fig. 5*q*. The early transition to asymmetrical bending shown by this spermatozoon is atypical; most spermatozoa at 0·04 M-$KHCO_3$ follow a time course more similar to the one shown in Fig. 2. Measurements for Figs. 1, 3, and 4 were all made during the first 5 min of exposure to $KHCO_3$.

The second type of complicating factor is the occurrence of changes in beat frequency as well as bend angle. In earlier work, direct application of CO_2 gas initially caused a decrease in bend angle, with no change in beat frequency (Brokaw & Simonick, 1976). When CO_2 is supplied by adding $KHCO_3$ to the reactivation solution, there is an additional inhibitory effect on beat frequency, which appears to be a direct effect of $KHCO_3$ rather than an effect of CO_2. Evidence for this conclusion can be obtained by comparing the effects observed in closed preparations where the spermatozoa are exposed to both $KHCO_3$ and CO_2 with the effects observed in open drop preparations where the exposure to $KHCO_3$ should be identical but the exposure to CO_2 should be much less. A comparison of this type was made in the experiment summarized in Table 1, and Fig. 3 shows results from a more extensive experiment in which bend angles and beat frequencies were measured at the upper surface of the open drop preparations. Swimming spermatozoa were measured for this experiment, since relatively few spermatozoa attach to the air interface at the surface of an open drop. The results show that the inhibition of bend angle almost disappears at the surface of an open drop, as expected if this effect is caused by CO_2. The beat frequency decrease is approximately the same in both situations, as expected if this effect is caused directly by $KHCO_3$. The inhibitory effects observed with low concentrations

of $KHCO_3$ are therefore consistent with the earlier conclusion that the CO_2 molecule has a specific inhibitory effect on the amplitude of flagellar oscillation, measured by the bend angle, and does not alter the frequency of oscillation. At the higher $KHCO_3$ concentrations there appears to be a somewhat larger effect on beat frequency in the closed drop preparations than in the open drop preparations. This may be a reflexion of the same inhibition of beat frequency which is seen during the later stages of the time course of CO_2-inhibition, as shown in Fig. 2.

The results in Figs. 1 and 3 also show that the effect of $KHCO_3$ concentration on bend angle is similar for swimming and attached spermatozoa, in contrast to the effect of small increases in viscosity, which cause the bend angle of attached spermatozoa to increase but have little effect on the bend angle of swimming spermatozoa (Brokaw & Gibbons, 1975; Brokaw & Simonick, 1977).

Additional experiments were carried out in which beat frequencies of spermatozoa in open drop preparations were measured at $KHCO_3$ concentrations up to 0·1 M and compared with preparations containing up to 0·1 M additional KCl. Approximately half of the frequency effect obtained by adding $KHCO_3$ to the reactivation solution could be obtained by adding a similar concentration of KCl.

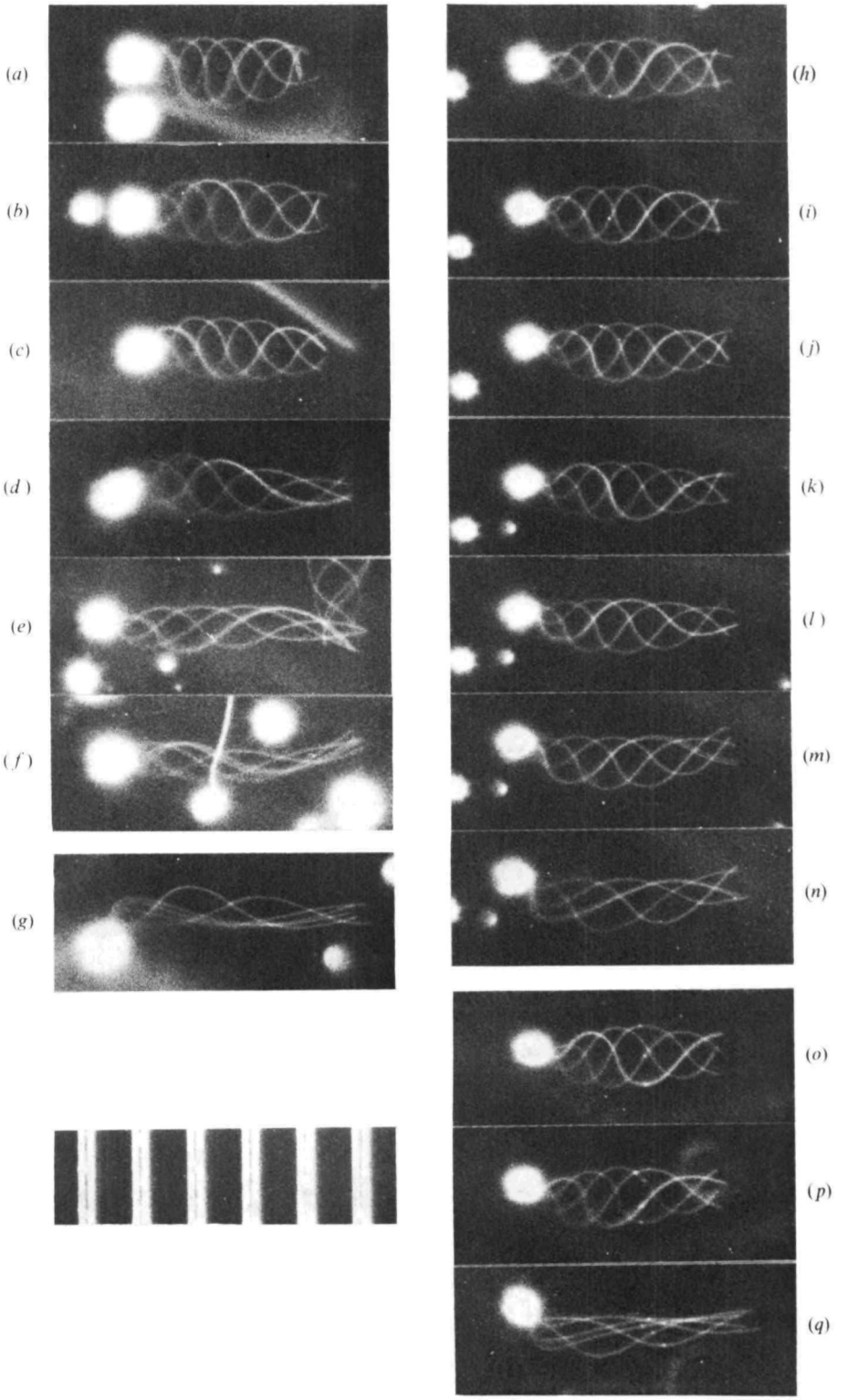
Measurements of bend angle and beat frequency as a function of pH are shown in Fig. 4. There is only a small effect of pH on these movement parameters in the absence of $KHCO_3$, as noted previously (Holwill, 1969; Gibbons & Gibbons, 1972). In the presence of 0·04 M-$KHCO_3$, the effect of pH on the beat frequency is unchanged, but there is a large effect of pH on the bend angle. This provides additional support for interpretation of the inhibition of beat frequency as a direct effect of $KHCO_3$ which is unrelated to the effect of CO_2 on the bend angle. The data in Fig. 4 have been plotted as an exponential function of pH, so that the abscissa is proportional to the concentration of OH^-. The points in Fig. 1 at 0·04 M-$KHCO_3$, with 0·2 mM ATP, and in Fig. 4 at pH 8·4 (2·5 μM OH^-), correspond to the same conditions. The slopes on either side of these points in Figs. 1 and 4 are similar, especially if allowance is made for the effect of pH on bend angle in the absence of $KHCO_3$. $KHCO_3$ and OH^- concentrations therefore have quantitatively similar inhibitory effects on bend angle.

DISCUSSION

These observations provide further evidence for a specific effect of the CO_2 molecule on the amplitude of flagellar bending. In a closed preparation, a strong inhibition is

Fig. 5. (*a–g*) Multiple-flash photomicrographs of reactivated spermatozoa at various $KHCO_3$ concentrations in the experiment summarized in Fig. 1, at 0·2 mM-ATP. The $KHCO_3$ concentration, flash frequency/4, and measured bend angle for each photograph are: (*a*) 0, 13·8 Hz, 2·50 radians; (*b*) 0·02 M, 14·5 Hz, 2·36 radians; (*c*) 0·04 M, 12·5 Hz, 2·16 radians; (*d*) 0·06 M, 13·7 Hz, 1·75 radians; (*e*) 0·08 M, 11·5 Hz, 1·38 radians; (*f*) 0·10 M, 9·9 Hz, 1·0 radian; (*g*) 0·06 M, 10·3 Hz; (*h–n*) Photographs of one spermatozoon in a reactivation solution containing 0·04 M-$KHCO_3$, as a function of time. Measurements on this spermatozoon are given in Fig. 2. The times for each photograph are: (*h*) 71 s; (*i*) 185 s; (*j*) 286 s; (*k*) 306 s; (*l*) 309 s; (*m*) 378 s; (*n*) 557 s; (*o–q*) Photographs of another spermatozoon in a reactivation solution containing 0·04 M-$KHCO_3$. The times and flash frequency/4 for each photograph are: (*o*) 100 s, 11·9 Hz; (*p*) 174 s, 11·6 Hz; (*q*) 176 s, 11·6 Hz.

The scale intervals are 0·01 mm. Brighter images in each photograph are produced by the superposition of the images from the first and fifth flash.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obtained with 0·10 M-$KHCO_3$ at pH 8·4, although some movement is seen in the first few minutes. A more immediate inhibition is obtained with 0·1 M-$KHCO_3$ at pH 8·6. In a closed system, the partial pressure of CO_2 in equilibrium with 0·1 M-HCO_3^- at pH 8·6, 16 °C, and an ionic strength of 0·2 M can be calculated to be 0·008 atm (cf. Edsall & Wyman, 1958) (1 atm = 101·325 kPa). Since a gas mixture containing 1–2% CO_2 at atmospheric pressure was found to be required for complete inhibition at pH 8·6 (Brokaw & Simonick, 1976), the observations with CO_2 gas in an open system and $KHCO_3$ in a closed system are consistent.

CO_2 is known to react with amino groups of proteins to form carbamates (cf. Edsall & Wyman, 1958). The reactive forms for this reaction are CO_2 and R–NH_2. However, if the important amino groups are ones which have pKs higher than 9, such as the ϵ-amino groups of lysine residues, they will be predominantly in the R–NH_3^+ form at the pHs of these experiments. The overall reaction to form the stable carbamate anion will then be:

$$R\text{–}NH_3^+ + HCO_3^- + OH^- \rightleftharpoons R\text{–}NH\text{–}COO^- + 2H_2O.$$

This reaction will therefore be driven to the right equally strongly by HCO_3^- and by OH^-, as was found in the measurements of the effect of $KHCO_3$ concentration and pH on flagellar bend angle. The effect of pH on $KHCO_3$-inhibition therefore indicates that if a reaction of CO_2 with amino groups is responsible for the observed inhibition of bend angle, the relevant amino groups must be ones with pKs greater than 9. Addition of CO_2 will cause a significant change in the charge on these amino groups, which may be expected to have significant effects on protein function.

This interpretation of the effects of CO_2 is consistent with the reversibility of the inhibitory effects obtained both with CO_2 gas and with $NaHCO_3$ (Brokaw & Simonick, 1976), but does not explain the complexities revealed by observation of the time course of the inhibition. There are two distinct phases in the inhibitory effect of CO_2: an early phase in which bend angle is inhibited, with no inhibition of beat frequency, and a later phase which normally involves further reduction in bend angle, a transition to asymmetrical bending, and some reduction in beat frequency. There is also usually a noticeable decrease in the regularity of beating, and often an abrupt cessation of bending or an abrupt transition to asymmetrical bending. These characteristics have been seen also in studies of the inhibitory effects of antisera against flagellar dynein (Ogawa, Asai & Brokaw, 1977). In neither case was it ever possible to obtain a gradual decrease in bend angle to 0 with no other changes. On the other hand, the beat frequency of these flagella can be decreased gradually down to 0, with no loss of regular beating (Brokaw, 1975*a*). These observations might be interpreted as evidence for mechanisms for flagellar oscillation involving a feedback control of active sliding by bending and requiring a critical amount of bending for successful activation of sliding. The asymmetrical bending observed in these experiments might then reflect a situation in which the necessary amount of bending is generated in only one direction. According to this interpretation, only the initial effect of CO_2 to inhibit bend angle without inhibiting frequency would be a direct effect of CO_2; the additional inhibitory effects seen during later stages of the time course, and with high concentrations of $KHCO_3$, would be secondary effects resulting from the inhibition of bend angle.

Because a large number of flagellar proteins might potentially be altered by reaction

with CO_2 to form carbamates, even a positive identification of the involvement of this reaction in the CO_2-inhibition of movement would not be very helpful in identifying the target of CO_2. The important feature of the observed effect of CO_2 is that it is possible to specifically inhibit the amplitude of flagellar bending without altering the frequency. Other work has shown that changes in ATP concentration can alter the frequency with little or no change in bend angle (Gibbons & Gibbons, 1972; Brokaw & Josslin, 1973; Brokaw, 1975*a*). Flagella appear to have control mechanisms which independently regulate the bend angle and the beat frequency (Gibbons, 1974). CO_2 might act directly on the control mechanism which regulates bend angle; but it is also possible that CO_2 has a more general effect on the mechanism which generates bending, and that under conditions of CO_2-inhibition the frequency-regulating mechanism is dominant over the bend angle-regulating mechanism, so that a constant frequency is maintained.

A reduction in bend angle will decrease the viscous moment which has to be overcome by active moment generated within the flagellum. Since the frequency-regulating mechanism of these flagella normally allows the beat frequency to change in response to changes in external viscosity (cf. Brokaw, 1975*a*), a direct effect of CO_2 on a bend angle-regulating mechanism, causing a decrease in bend angle, would be expected to cause an increase in frequency, unless there is also a reduction in active moment. One mechanism proposed for the control of active moment generation by flagella (Brokaw, 1971, 1972*a*) will satisfy this condition. In this control mechanism, the active shear moment is proportional to the curvature of the flagellum. A decrease in bend angle will cause a decrease in curvature and therefore a decrease in active shear moment, if the wavelength is constant. Computer simulations of a flagellar model containing this form of control mechanism have shown that the bend angle can be decreased, with little change in frequency, by increasing the elastic resistances of the flagellar model (Brokaw, 1972*b*). This control mechanism would therefore be consistent with a CO_2-sensitive mechanism for regulating bend angle, with the frequency determined by the balance of active and viscous moments. However, this control mechanism does not predict a critical bend angle.

Alternatively CO_2 could act directly to inhibit the generation of active shear moment by the dynein cross-bridges in flagella. CO_2 could act by reducing the number of available attachment sites for the dynein cross-bridges, in a manner similar to the control of the availability of cross-bridge attachment sites on actin by Ca^{2+} ion in muscle. This could decrease the active shear moment, without altering the kinetic properties of those cross-bridges which can find attachment sites. The observation that 0·1 M-$NaHCO_3$ inhibits flagellar bending but does not prevent the ATP-driven extrusion of tubules from trypsin-digested flagella (Brokaw & Simonick, 1976) does not completely exclude this possibility, since the forces required for tubule extrusion in this experimental situation are unknown, and may be small compared to the forces required for flagellar bending. However, a possibility which is more consistent with this observation is that CO_2 interferes with a control mechanism which selectively inactivates cross-bridges during the normal bending cycle. If all the dynein cross-bridges in a flagellum are simultaneously active, those on one side of the flagellum will act in opposition to those on the other side, and no bending, and no net active shear moment, will be generated. A mechanism which inactivates cross-bridges on one side of the flagellum will be required in order to generate bending; one such mechanism,

based on shear-rate sensitivity of the generation of active shear moment by cross-bridges, has been discussed in detail (Brokaw, 1975*b*, 1976). CO_2 might interfere with this type of control mechanism to produce a situation where all the dynein cross-bridges are simultaneously active, so that no bending can be generated, but tubule extrusion can be readily observed after trypsin digestion. Measurements of the stiffness of CO_2-inhibited axonemes might discriminate between these two possibilities.

A distinction between an effect of CO_2 on a bend angle-regulating mechanism and an effect of CO_2 on a mechanism controlling active shear moment might be made by examining the effects of CO_2 on reactivated spermatozoa of *Colobocentrotus*. The flagella of these spermatozoa appear to have a particularly strong amplitude-regulating mechanism (Ogawa, Asai & Brokaw, 1977). Removal of up to half the dynein cross-bridges by KCl extraction (Gibbons & Gibbons, 1973) or inhibition with antiserum against a tryptic fragment of dynein (Gibbons, Ogawa & Gibbons, 1976) causes the frequency of *Colobocentrotus* sperm flagella to decrease with little change in amplitude. If CO_2 acts on an amplitude-regulatory mechanism, *Colobocentrotus* spermatozoa might be expected to be less sensitive to CO_2 and/or to show an inhibition of bend angle similar to *Lytechinus* spermatozoa. If CO_2 inhibits the generation of active shear moment, the strong amplitude-regulating mechanism in *Colobocentrotus* spermatozoa might cause them to respond by changing their frequency, rather than their bend angle.

At this point, the most plausible, but still very speculative, interpretation of the effect of CO_2 may be that CO_2 reacts with protein-NH_3^- groups to form carbamate anion groups, thus causing significant local changes in protein charge. There could be specific interference with a control mechanism which selectively suppresses dynein cross-bridge activity in order to generate rhythmic bending and propagated bending waves. This control mechanism may incorporate a feedback control which depends on some measure of flagellar amplitude, such that it fails to operate successfully when the amplitude is reduced below a critical level.

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