RADIATION DAMAGE TO BULL SPERM MOTILITY. II.

PROTON IRRADIATION AND RESPIRATION MEASUREMENTS

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ABSTRACT Diluted bull semen samples were bombarded with a 24 Mev proton beam. Dose response curves for the fraction of cells which survived the bombardment and for the average velocity of the surviving cells were measured. Target theory indicated a cross section of the sensitive volume of 2.1×10^{-10} cm^2. Respiration measurements showed that the oxidative phosphorylation in the sperm remained coupled after the bombardments. The efficiency with which free energy from ATP hydrolysis was converted into mechanical work by the sperm was found to decrease after proton bombardment. The half-value dose for this effect was two and a half times higher than the half-value dose for motility damage. These respiration measurements indicate that the damage due to the bombardment is not to the metabolic system or to the contractile system in the sperm flagellum, but to a control system for the motility. The results of the target theory shows that this control system is localized in a small element of approximately 1600 A diameter. The centriole is tentatively proposed as being this control element.

INTRODUCTION

In the preceding paper (1) the effects of 180 kv X-rays on bull sperm motility were described. Interpretation of the experimental data by means of target theory lead to the conclusion that the site of the radiation damage is a small volume of $\approx 0.75 \times 10^{-15}$ cm^3. This volume is much smaller than that of either the mitochondrial sheath or of the longitudinal fibers in the sperm tail.

To have a better possibility of identifying the "target" of 0.75×10^{-15} cm^3 with some subcellular structure, it is necessary to have an estimate of the shape of the target. This is necessary in view of the fact that most organelles found in sperm flagella have a length to width ratio much larger than one.

An estimate of the length to width ratio of the target can be obtained by means of complementing the X-ray experiments with irradiation by fast protons. Protons

with an energy of 10–20 Mev have an energy loss in water of around 30 Mev/g cm^{-2} (2). Along the path of such a proton, ion pairs are produced at an average spacing of around 100A. This means that for practical considerations the path of such a proton is densely packed with ionizations, and a proton beam can be used for measuring a *cross section* of the radiation sensitive element in the spermatozoa. Comparison of the volume and cross section of the sensitive element gives essentially a shape determination.

Respiratory measurements can give insight into the type of functional damage done by the irradiation. It is known from kinetic experiments (3) that the respiration of a normal bull sperm preparation is ADP limited. The rate limiting factor is therefore in the ATPase system, the major part of which is represented by the contractile elements. If, after irradiation, the sperm respiration is still ADP limited, it can be concluded that the damage is not done to the enzymes responsible for ATP production. Oligomycin was judged to be the most suitable inhibitor to investigate this. It is known that oligomycin inhibits one of the reactions of the coupling of ADP phosphorylation to respiration (4). Therefore, if oligomycin inhibition is effective after irradiation, it can be concluded that the preparation is still ADP limited.

Absolute respiration rates give, together with motility measurements, figures for the efficiency with which the free energy of ATP hydrolysis is converted into mechanical (hydrodynamic) work by the sperm (3, 5, 6). It has been shown (3) that the efficiency of the conversion is constant when the motility of a sperm sample is reduced by limiting the energy supply to the sperm. In the experiments reported in reference 3 the energy supply was reduced by specific inhibition of the flavoproteins in the electron transport chain. Apparently the contractile system was not effected by the inhibition.

A change in the efficiency of energy conversion in the sperm flagella after irradiation can in view of the above be expected to indicate damage to the contractile system. This presumes of course that the coupling of the oxidative phosphorylation in the preparation remains tight.

The present paper describes the results of the motility and respiratory measurements outlined above after irradiation of the sperm with high energy protons.

EXPERIMENTAL METHODS

Sperm Preparations

The semen was obtained from Frisian bulls at the Research Institute for Animal Husbandry at Zeist, Holland. After ejaculation the semen was diluted threefold and cooled to 4°C as described in reference 1. For one experiment the diluted semen was split in six parts, giving one control and five preparations which were bombarded at different doses.

The cuvets in which the semen was contained during the irradiation in the cyclotron, held each approximately 0.6 cm^3. Of this, 0.1 cm^3 was used for the motility measurement and 0.3 cm^3 for the respiratory experiments.

For the motility measurements the 0.1 cm^3 of diluted semen was once more diluted 8- to 20-fold. For each sample the dilution ratio was chosen such that a concentration of moving cells which was optimal for the motility measurement was obtained. All dilutions were done with the medium described in references 1 and 3.

Proton Bombardment

The 30 Mev sector focused Philips cyclotron of the Free University of Amsterdam was used, through the kindness of Dr. J. Rethmeier. The specimen holder which was mounted on the pipe for the external proton beam has been described before (7).

For the bombardment the preparations were contained in a thin rectangular cuvet shown in Fig. 1. The proton beam was collimated to correspond exactly to the thin (3 mm) vertical layer of sperm, which offers a surface of 1.5×1.5 cm^2 perpendicular to the beam.

A proton energy of 24 Mev was used. The beam intensity, measured with a Keithley model 300 electrometer (Keithley Instrument Inc., Cleveland, Ohio) and recorded on a strip chart recorder (7), could be regulated at that energy very stably at approximately 10^{-10} A. Within the dose range used actual bombardments at that beam intensity took from 6 to 300 sec.

It was calculated from tables of proton stopping power in water and glass (2) that the energy of the protons during the traverse of the sperm preparation decreased almost linearly from 22 to 13 Mev. In that energy range the stopping power increases from 24 to 37 Mev/g cm^{-2}, or approximately 50%.

During the proton bombardment the semen samples were kept at 4°C, and at atmospheric pressure.

Motility measurements were done exactly as described in the preceding paper.

Respiration Measurements

For respiratory measurements the 0.3 cm^3 semen samples were suspended to a total volume of 2 cm^3 in the egg yolk medium in a thermostated cuvet at 37°C. Respiration was measured and recorded by means of the Clark oxygen electrode (Yellow Spring Instrument Co., Yellow Spring, Ohio), as described before (3).

Inhibitors could be introduced into the measuring cuvet by means of a Hamilton syringe (Hamilton Co., Whittier, California) without interrupting the respiration measurement. The oligomycin used was obtained from Sigma Pharmaceutical Co., St. Louis, Mo. It contained 85% oligomycin B and 15% oligomycin A.

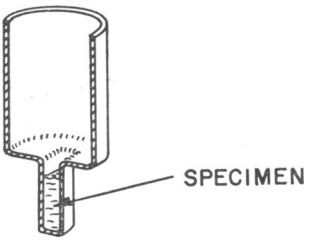

FIGURE 1 Cut away view of cuvets holding the specimen during proton bombardment. The cylindrical top part of the cuvet is for ease of handling it, and for an O ring seal in the holder.

RESULTS

Dose Response Curves for Motility

Fig. 2 shows the fraction of cells which survived the proton bombardment as a function of dose for four ejaculates. The dose is expressed as the total charge passing through the sample as measured on the electrometer. One half of the cells have been rendered motionless at a dose of 1.8×10^{-9} coul, or 0.8×10^{-9} coul/cm^2.

The decrease in average velocity of the surviving cells as a function of proton dose is shown in Fig. 3. The different ejaculates each seem to follow a different curve. In the section on target theory, this will be discussed further.

The product of the concentration of moving cells and their average velocity is

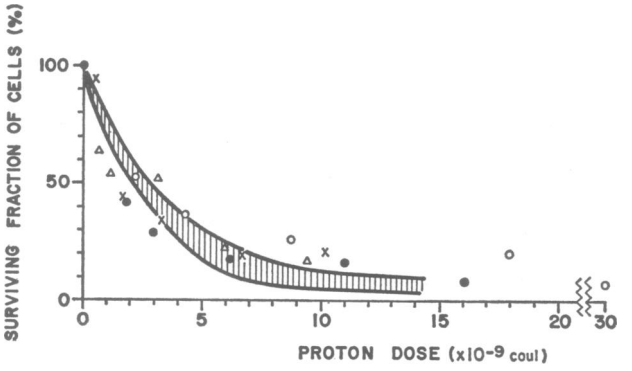

FIGURE 2 "Survival fraction" of proton bombarded sperm as a function of dose. Each symbol represents a different ejaculate. In Figs. 2, 3, 4, 6, 7, and 9 identical symbols refer to the same ejaculate. The computed dose response curves fall within the shaded band. Since the curves for the individual ejaculates are but little different they are not shown separately.

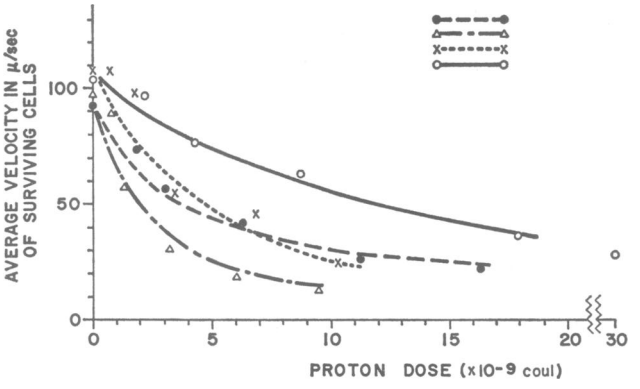

FIGURE 3 Average velocity of the sperm surviving proton bombardment as a function of dose. The lines drawn in the figure represent dose response curves computed from target theory.

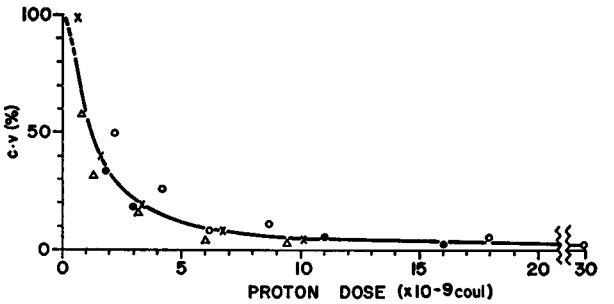

FIGURE 4 Product of concentration (c) and average velocity (v) of surviving cells, as a function of proton dose. ($c \cdot v$) is expressed as fraction of the value for the control samples.

proportional to the hydrodynamic work performed by the sperm. This quantity, plotted as a function of dose, is shown in Fig. 4. A smooth curve is obtained which shows only minor difference between the ejaculates.

It was not possible to carry out the bombardment at the cyclotron at exactly preset amounts of charge delivered. Pooling of data at a certain dose, as was done in the X-ray experiment (1), was therefore not possible. Since the statistics of the individual motility measurements are insufficient, no meaningful velocity distributions were obtained.

Respiration Measurements

The respiration was measured on all samples on which motility figures were reported. After warming-up the samples to 37°C, the respiration was recorded during approximately 10 min. Oligomycin (20 μM) was then introduced in the cuvet, and the inhibited respiration measured during approximately 10 min. A subsequent inhibition was performed by means of 0.5 mM KCN. It was assumed that all respiration was completely cut-off after the addition of KCN. Any residual oxygen uptake recorded after the cyanide inhibition was taken to be due to drift effects in the measuring system. The uninhibited—and the oligomycin inhibited rates were corrected for the observed drift after KCN inhibition.

Fig. 5 shows tracings of two typical curves obtained, one from a control sample, one from an irradiated sample of the same ejaculate. It can be seen in Fig. 5 that the rates after KCN inhibition are quite small.

The respiration rate (before inhibition) of the irradiated samples is plotted in Fig. 6; the rates are expressed as a fraction of the rate observed in the control samples. Fig. 6 shows that the decrease in respiratory rate is less than the decrease in "amount of motility" shown in Fig. 4. The implications of this are discussed in the section "Energy conversion in irradiated sperm".

Fig. 7 shows the percentage inhibition of the respiration effected by the oligomycin as a function of the proton dose. In the (not irradiated) control samples ap-

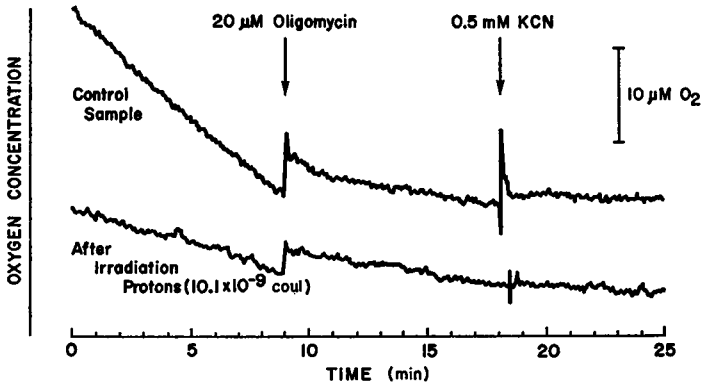

FIGURE 5 Tracings of records made with the oxygen electrode of respiration of sperm before and after proton bombardment. The effects of inhibition by oligomycin and KCN are also shown.

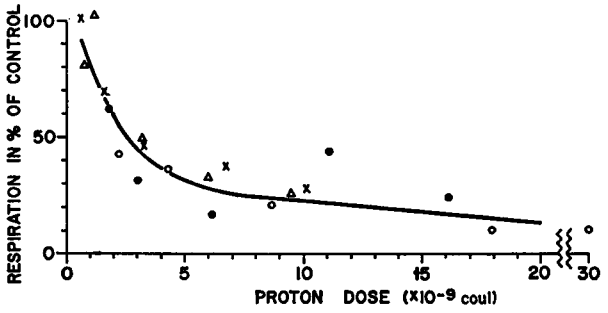

FIGURE 6 Respiration rate, relative to the control samples of proton irradiated sperm as a function of proton dose.

proximately 75% inhibition is obtained. This can be taken as an indication that the oxidative phosphorylation in the control sample was tightly coupled. Mohri and Ernster (8) have reported tight coupling in bull sperm preparations, in agreement with the present observations.

It can be observed in Fig. 7 that an indication for a decrease in the effect of oligomycin becomes noticeable only at doses $>15 \times 10^{-9}$ coul.

Abnormal Cells

In a few preparations it was observed that a relatively large number of abnormally moving cells (see references 9 and 10; described in more detail in the preceding paper) was present.

Visual estimates were made of the percentage normally and abnormally moving cells in these preparations before and after proton irradiation. The procedure was analogous to that described in the preceding paper.

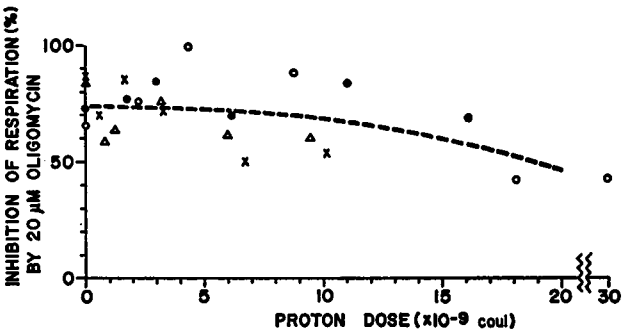

FIGURE 7 Effectiveness of oligomycin inhibition of sperm samples as a function of proton dose.

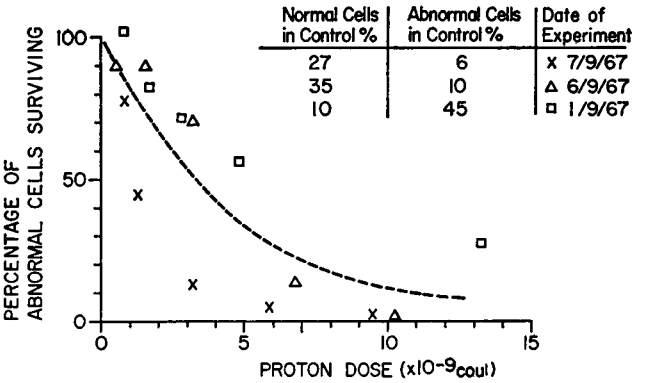

FIGURE 8 Survival fraction of abnormally moving cells against proton dose. Each point represents the average of a visual estimation by two observers on three slides each.

Fig. 8 presents the data on the abnormal cells obtained from the visual estimates. The three experiments shown in Fig. 8 give a value for the proton dose at which the number of abnormal cells has dropped to half of the control value of $(3 \pm 1.5) \times 10^{-9}$ coul (average and standard deviation). This is not significantly different from the measured value of 1.8×10^{-9} coul for the normally moving cells. It can be concluded that the damage to motility by the proton bombardment is not the conversion of normal into abnormal cells. This agrees with our observations on X-ray irradiated cells (1).

APPLICATION OF TARGET THEORY

The dose response curves for the surviving fraction and for the average velocity in Figs. 2 and 3 show the same form as the dose response curves in the X-ray experiment (1). No indications for a threshold or for inflexions are seen in the curves for

either experiment. From this we have concluded that the type of damage due to the radiation was the same in both experiments.

In analogy with the interpretation of the X-ray experiment, the effect of n proton hits on a sperm with velocity v_o has been taken as the reduction of the velocity to v with

$$v = v_o - \sqrt{n} \cdot \beta \qquad (1)$$

where β represents the "sensitivity" of the sperm. Dose response curves for the average velocity and the survival fraction were computed according to equations 4 and 5 of reference 1. For each of the four ejaculates represented in Figs. 2 and 3 a separate fit was performed. Table I shows the results of these computations. The curves found to give the best fit have been drawn in Figs. 2 and 3.

It can be observed from Table 1 that the values for β range from 50 to 100 μ/sec. On the basis of the present data it cannot be decided whether this is due to experimental uncertainties or to a real variation in sensitivity between the ejaculates. This question falls outside of the scope of the present paper, however. Extensive redesign of the present experimental set up would be necessary to measure the precise dose response curves at low dosage ($< 3 \times 10^{-9}$ coul), required to elucidate this point.

In the X-ray experiment an average value of β was determined from pooled data of all experiments and no individual curve fits were performed. The found value $\beta = 60$ μ/sec is well within the range of value of 50 to 100 μ/sec obtained in the proton experiment.

The normalization in hits/charge density of the computed dose response curves yields an average and standard deviation:

$$1 \text{ hit equivalent to } (0.7^5 \pm 0.2^5) \times 10^{-9} \text{ coul/cm}^2. \qquad (2)$$

The cross section of the target which is sensitive to the radiation can directly be derived from equation 2. At a proton charge of 1.6×10^{-19} coul, a target which is

TABLE I

PARAMETERS OF CURVES COMPUTED WITH TARGET THEORY, WHICH ARE BEST FITTING TO THE MOTILITY MEASUREMENTS SHOWN IN FIGS. 2 AND 3

Symbol	β	Normalization: 1 hit equivalent to	
	μ/sec	coul	coul/cm^2
△	100	2.8×10^{-9}	1.24×10^{-9}
×	80	1.6×10^{-9}	0.72×10^{-9}
●	80	1.5×10^{-9}	0.67×10^{-9}
○	50	0.7×10^{-9}	0.32×10^{-9}

hit on the average once at a charge density of $0.7^5 \times 10^{-9}$ coul/cm^2 has a cross section of

$$2.1 \times 10^{-10} \text{ cm}^2. \tag{3}$$

If the cross section had a circular shape the diameter would be $\approx$ 1600 A.

ENERGY CONVERSION IN IRRADIATED SPERM

The respiration of the proton irradiated sperm retained its oligomycin sensitivity for doses up to approximately 7×10^{-9} coul/cm^2. This means that radiation damage to the enzyme system which couples the oxidative phosphorylation occurs only at doses which are ten times larger than those at which the number of motile cells is reduced to half (approximately 0.8×10^{-9} coul/cm^2). At doses $< 7 \times 10^{-10}$ coul/cm^2 the P/O ratio of the oxydative phosphorylation is therefore intact. We can also conclude that the respiration at these lower doses is still ADP limited.

Apparently the reduced motility of the irradiated sperm is not caused by a reduced ATP supply. This, combined with the conclusion that the P/O ratio in the irradiated sperm is not reduced makes it meaningful to consider the efficiency of the conversion of free energy from ATP hydrolysis into hydrodynamic work by the sperm.

It has been shown (3) that the work performed by a moving sperm can in first order approximation taken to be proportional to its forward velocity. The total work W by all moving sperm in a 1 cm^3 sample can thus be written

$$W = \alpha \cdot (c\, v) \text{ ergs/sec} \tag{4}$$

where c is the concentration of moving sperm and v the average velocity in cm/sec. The constant α can be calculated from the characteristics of the flagellar movement (3, 10). On the basis of recent observations of the movement of bull sperm (9) the value should be $\alpha = 2 \times 10^{-4}$ ergs/sperm per cm/sec (11).

With a free energy of ATP hydrolysis of 10 kcal/mol (12), and with six ATP produced per molecule of oxygen, the free energy ΔF is related to the respiration rate R by

$$\Delta F = 4 \times 10^{-12} \times R \text{ ergs/sec} \tag{5}$$

where R is expressed in number of oxygen molecules consumed per cm^3/sec.

The quantities $(c \cdot v)$ and R in equations 4 and 5 have both been measured absolutely in our experiments. Figs. 4 and 6 show these quantities for irradiated samples relative to the control values. For our present considerations of the efficiency, the ratio of the absolute values, $(c \cdot v)/R$, is plotted in Fig. 9 for all experimental data.

The average shown in Fig. 9 for the control samples is 0.48×10^{-2} cm/sec/10^6

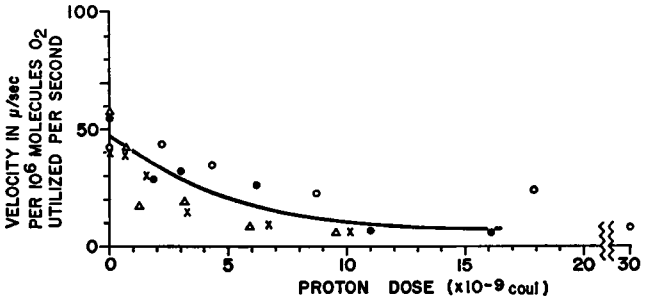

FIGURE 9 "Efficiency" of the sperm motility as a function of the proton dose received by the sample. The efficiency is expressed as the average distance travelled by a sperm per million molecules of O_2 consumed.

molecules O_2. This figure, together with equations 4 and 5 gives an efficiency of conversion of ΔF into work for the control samples of 23%. This value is higher than the value reported in reference 3, which after correction for α on the basis of the newest motility data (9), would be 12%. The value for the efficiency in reference 3 was obtained with preparations in which no fructolysis was present, however. One of us (R. R.) has reported recently (11) that at a fructose concentration of 250 $\mu g/cm^3$, as in our preparations, the fructolysis produces approximately 50% as much ATP in bull sperm as does the respiration. This means that the total ΔF produced in our control samples should be raised by approximately 50% above the value in equation 5, to $6 \times 10^{-12} R$ ergs/sec. Consequently the effective efficiency of our control samples is reduced by one-third, and the resulting figure of approximately 15% is in good agreement with the results from reference 3.

On the basis of the above we have taken the data in Fig. 9 as meaningful for indicating the change in efficiency of our preparations due to the proton irradiation. It can be seen that this efficiency drops to half the control value at a proton dose of approximately 4.5×10^{-9} coul $= 2 \times 10^{-9}$ coul/cm^2. The drop in efficiency has apparently to be understood as representing ATP-ase activity due to radiation, which is not connected with contractility. The characteristic dose for this effect (2×10^{-9} coul/cm^2) is, however, two and a half times higher than the characteristic dose for the reduction of motility (0.8×10^{-9} coul/cm^2). The damage to the contractile system as manifested in the reduced "efficiency" can therefore not be the primary effect which causes the reduction in motility.

A comparison of the dose response curves for the motility in the proton experiment with those of the X-ray experiment shows that a dose of 0.8×10^{-9} coul/cm^2 is roughly equivalent to 10 kr. It can therefore be assumed that in the X-ray experiments in the range 0–10 kr the efficiency of the contractile elements was hardly affected. This confirms the finding that the sensitive target identified with the target theory is not the contractile system.

DISCUSSION

In the following discussion the results of the present proton experiment as well as those of the X-ray experiment of the preceding paper shall be related.

Application of target theory to the dose response curves for motility has shown that with X-ray as well as with proton radiation the sensitive target is destroyed with relatively few hits. On the basis of this, a target consisting of many small sub units, divided over the whole sperm flagellum (for example protein molecules in the matrix) can be ruled out.

The volume of the target was found to be $\approx 0.75 \times 10^{-15}$ cm^3, and its cross section 2.1×10^{-10} cm^2. This indicates that the element which contains the target does not have one dimension much larger than the two others, but that it is essentially of spherical shape. Table II shows the volume and the cross section of the main organelles identified by electron microscopy in the bull sperm flagellum. The figures for the cross section are given for incidence of radiation normal to their longest dimension. The correction to the cross section for not normal incidence is smaller than a factor of two and is neglected here. It can be seen that the absolute values for volume and cross section, as well as the volume to cross section ratios rule out the identification of our target with any of the organelles in Table II.

The respiration measurements have shown that the reduced motility after irradiation is not caused by reduced energy supply and not by a damaged contractile system. The primary effect is therefore in a control system in the sperm and this control system should be localized in an approximately globular element of 1000-2000 A diameter.

Analysis of the wave in sperm flagella has shown that the wave is initiated in the area of the head-flagellum junction (11, 13). The "target" from our radiation experiments should therefore probably be situated at the proximal end of the flagellum. The *centriole* found at the proximal junction of sperm flagella has indeed the proper globular shape and a diameter of approximately 2000 A (14, 15).

To confirm an identification of the centriole with the radiation sensitive target it

TABLE II

VOLUME AND CROSS SECTION OF ORGANELLES IDENTIFIED IN THE BULL SPERM FLAGELLUM AND OF THE TARGET SENSITIVE TO THE IRRADIATION

Organelle	Volume	Cross section
	cm^3	cm^2
Target	0.7×10^{-15}	2.1×10^{-10}
Mitochondrial sheath	6×10^{-12}	1×10^{-7}
Coarse fibers	4.5×10^{-13}	7×10^{-8}
Doublet central fibers	2.5×10^{-14}	1.5×10^{-8}
Matrix	1.5×10^{-12}	7×10^{-8}

would be necessary to reproduce the motility reduction by specifically damaging the centriole. Recently Goldstein and Brokaw (16) have reported the results of such experiments. By means of a focused laser beam a spot of 2 μ diameter was burned out on the flagella of sea urchin sperm. It was observed that the flagellar wave would travel distally up to the area damaged by the laser beam. In case the junction of the flagellum was burned out by the laser beam all wave motion would stop, however. This experiment tends to confirm that the flagellar motion is controlled from the area of the head-flagellum junction.

As a final conclusion we would like to propose that a control system for sperm flagellar motion is located in the centriole.

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