ACETYLCYLCHOLINESTERASE IN BULL SPERMATOZOA

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(Received 17th June 1963)

Summary. Acetylcholinesterase occurs in the head, midpiece and tail fractions of sonically disrupted bull epididymal spermatozoa. The enzyme exhibits the characteristic substrate concentration optimum; $1.5 \times 10^{-5}$ m-eserine competitively inhibits the hydrolysis of the acetylcholine. The specific activity of the tail fraction is about five times that of the head fraction; in both fractions, the rate of hydrolysis of butyrylcholine is about 15% and of benzoylcholine is about 5 to 8% that of acetylcholine. The enzyme may be involved in the control of coordination and propagation of the flagellar wave.

INTRODUCTION

Coordination of the propagated flagellar wave is apparently a necessary condition for the successful progression of spermatozoa. Bishop & Hoffmann-Berling (1959) have shown that adenosine triphosphate (ATP) reactivates glycerinated sperm 'models' and that the frequency of the ensuing beat is proportional to the nucleotide concentration; however, in spite of the rhythmic nature of the beat and the fact that oscillation occurs all along the flagellum, the wave is neither coordinated nor propagated and most flagellar 'models' seemingly 'mark time' but do not exhibit effective forward propulsion [the exceptions reported are flagella of Polytona wella (Brokaw, 1961) and of starfish and sea-urchin spermatozoa (Kinoshita, 1959)]. While the physical and chemical changes wrought by the aqueous extraction procedure, which underlie the failure of coordination, remain unknown, Kinoshita observed that reactivation of glycerinated starfish and sea-urchin spermatozoa requires ATP, Mg$^{2+}$ plus some chelating or relaxing agent, such as diaminoethane-tetra-acetic acid (EDTA), or the restoration of the Marsh-Bendall factor to the extracted system. Glycerination also removes inorganic pyrophosphatase from frozen-dried rat spermatozoa although, cytochemically at least, no loss of adenosinetriphosphatase can be detected; loss of pyrophosphatase was tentatively held responsible for lack of forward movement in sperm models (Nelson, 1959). Other organic and water-soluble inorganic constituents are extracted during preparation of the models, and glycerination undoubtedly disrupts the selective permeability of the flagellar surface. If the regulation of flagellation is ultimately determined by ion shifts controlled by depolarization phenomena as suggested by Bishop (1962a) and Tibbs (1960), the permeability mechanism could very well be involved in normal sperm movement.
According to Sekine (1951), cholinesterase activity of boar spermatozoa is of the same order of magnitude as that of brain tissue, while Tibbs finds that trout and perch sperm preparations also split acetylcholine although at a small fraction of that rate. Moreover, the acetylcholinesterase appears to occur primarily in the heads of the freshwater fish spermatozoa. This distribution, while at variance with Mann’s (1954) observation that the enzyme occurs mainly in the tail fraction of ram spermatozoa may be related to fundamental physiological differences as well as differences in submicroscopic morphology of the spermatozoa. On the other hand, spermatozoa of the edible mussel, *Mytilus edulis*, detectably increase their rate of flagellation in $5 \times 10^{-5}$ M-`eserine sulphate, while acetylcholine, in concentrations ranging from $10^{-6}$ to $10^{-3}$ M evokes no apparent change in flagellar activity; crude homogenates of the *Mytilus* spermatozoa break down acetylcholine at a rate of 4 μg/mg protein/hr (Applegate & Nelson, 1962) and a partially purified preparation has an activity of about 50 μg/mg protein/hr.

The present study was undertaken to examine some of the parameters and determine by in-vitro means the localization of bull sperm acetylcholinesterase preliminary to attempts at a cytochemical analysis.

**METHODS**

Bull epididymal sperm suspensions were washed three times in Ca$^{2+}$-free Krebs-Ringer solution, placed in an ice bath and then subjected to ultrasonic vibration for 45 sec in a Model LS-75 Branson Ultrasonic Sonifier at a frequency of 20 kilocycles. The heads, midpieces and tail fragments were separated in a refrigerated centrifuge in Krebs-Ringer solution by a method described by Nelson (1954). Acetylcholine remaining after incubation was measured by the Hestrin (1949) alkaline hydroxylamine method in most of the experiments reported below. Protein was determined by the Lowry method (Lowry, Rosebrough, Farr & Randall, 1951). Concentrated sperm fractions resuspended in Krebs-Ringer-phosphate buffer were incubated in an equal volume of varying concentrations of acetylcholine chloride in $10^{-3}$ M-sodium acetate for 2 hr at 25° C. Specific activities of the fractions (Table 1) indicate that the highest activity occurs in the tail fraction, although heads and midpieces possess substantial activity as well. For comparison, ATP hydrolysis is included, measured concurrently with the head fraction cholinesterase activity.

**RESULTS**

While the head fraction exhibits negligible ‘ATP-ase’ activity (about 4% of the tail fraction) as previously reported for ejaculated bull spermatozoa (Nelson, 1954), there is considerably higher cholinesterase activity, the heads averaging about 20% of the tail enzyme activity. Some of this activity may conceivably be due to fraction contamination, but comparison with ATP-ase activity indicates that at least a portion of the acetylcholine breakdown may legitimately be ascribed to the head fraction unless the heads adsorb cholinesterase from the medium more avidly than they do ATP-ase.
Acetylcholinesterase in bull spermatozoa

In order further to characterize the sperm enzyme, the preparations were tested for inhibitory response to eserine, for exhibition of the substrate concentration optimum peculiar to this class of enzymes, and for substrate specificity.

Table 1

<table>
<thead>
<tr>
<th>Expt. No.</th>
<th>Heads</th>
<th>Midpieces</th>
<th>Tails</th>
<th>Whole sonicate</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-24</td>
<td>11</td>
<td>13</td>
<td>74</td>
<td>-</td>
</tr>
<tr>
<td>5-2</td>
<td>17</td>
<td>14</td>
<td>45</td>
<td>-</td>
</tr>
<tr>
<td>5-8</td>
<td>23</td>
<td>34</td>
<td>109</td>
<td>32</td>
</tr>
<tr>
<td>5-8 ('ATP-ase')</td>
<td>10</td>
<td>72</td>
<td>252</td>
<td>-</td>
</tr>
</tbody>
</table>

Cholinesterase specific activity measured as ug acetylcholine breakdown per mg protein per hr after 2 hr incubation in 2.5 mm-acetylcholine chloride, 0.00625 m-phosphate buffer, pH 7.4; 'adenosinetriphosphatase' specific activity measured as ug inorganic phosphate liberated per mg of protein per hr after 10 min incubation in 1 mm-ATP, 0.01 m-tris buffer, pH 8.6, containing 10^-4 m-MgCl2. Temperature 25°C.

Text-fig. 1. (a) Lineweaver-Burk plot of bull sperm-tail suspension in acetylthiocholine and acetylthiocholine plus eserine. Ordinate: reciprocal of rate of hydrolysis of acetylthiocholine (1/ν = 1/µg/mg protein/hr); abscissa: reciprocal of substrate concentration (1/S = 1/m). • = Varying concentrations of acetylthiocholine; × = same concentration of acetylthiocholine plus 1.5 x 10^-5 m-eserine sulphate. (Thirty min incubation in 0.05 m-tris buffer pH 7.4, 0.025 m-MgCl2, 0.5 m-NaCl, 0.002 m-acetylthiocholine iodide, 25°C). Results show competitive inhibition by eserine.

(b) Substrate optimum. Bull sperm-tail suspension in Krebs-Ringer-phosphate buffer pH 7.4, 0.0625 m. Ordinate: hydrolysis of acetylcholine in µg/mg protein/hr; abscissa: concentration of acetylcholine chloride in mm. The optimum enzyme activity is over the range of concentration from 2.5 to 20 mM-acetylcholine.

Text-fig. 1(a) is a Lineweaver-Burk (1934) plot of the reciprocals of rate of enzyme action versus reciprocals of substrate concentration both in the presence and absence of 1.5 x 10^-5 m-eserine sulphate. This clearly illustrates the classical
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competitive type of enzyme inhibition and suggests that the sperm enzyme may be regarded as a cholinesterase. Further, according to Nachmansohn & Wilson (1955): "for classifying an esterase as acetylcholinesterase it is sufficient in most cases to determine whether there is an optimum substrate concentration and

Text-fig. 2. (a) and (b) Substrate specificity. Sperm-tail (a) and sperm-head (b) cholinesterase: assay medium contains 2.0 mM-choline ester in 10^{-3} M-sodium acetate, sperm fractions in Krebs-Ringer-phosphate buffer pH 7.4, 0.0625 M. Ordinates: amount of choline ester hydrolysed per milligramme of sperm-tail or sperm-head protein (average 1.5 mg protein in each sample). Abscissa: time of incubation in hr; ○ = acetylcholine 2.0 mM; □ = butyrylcholine 2.0 mM; × = benzoylcholine 2.0 mM. Hydrolysis of butyrylcholine was about 13%, and benzoylcholine about 5 to 8% of the rate of hydrolysis of acetylcholine.

(c) Relation of cholinesterase activity to enzyme concentration. Varying amounts of sperm tails in 2.5 mM-acetylcholine, other conditions same as in preceding Text-figs. Ordinate: rate of hydrolysis of 2.5 mM-acetylcholine in µM of acetylcholine per hr; abscissa: relative enzyme concentration. One hundred is equivalent to 4.2 mg sperm-tail protein. ○ = rate of enzyme activity at varying enzyme concentrations; ○ = specific activity as µM-acetylcholine hydrolysed per mg sperm-tail protein per hr. Note the relatively high specific activity at lower enzyme concentrations.

(d) Relation of amount of acetylcholine hydrolysed to duration of incubation period. Conditions same as above except constant initial acetylcholine concentration and constant enzyme concentration. Ordinate: µM-acetylcholine hydrolysed per mg of sperm-tail protein; abscissa: time of incubation in hr; ○ = rate of enzyme activity at various time intervals; ○ = specific activity as µM-acetylcholine split per mg sperm-tail protein per hr.
whether the hydrolysis of butyrylcholine is very low compared to that of acetylcholine. The hydrolytic power is still more decreased with benzoylcholine as substrate. Text-fig. 1(b) shows the variation in enzyme activity with respect to different concentrations of acetylcholine. The sharp optimum and rather abrupt decline in activity at 25 mM concentration meet the criterion set forth above. The specificity of the bull sperm enzyme in both tail and head fractions is graphically represented in Text-figs. 2(a) and 2(b). In both cases, the hydrolysis of acetylcholine far exceeds that of the butyryl and benzoyl esters of choline. The peculiarities of the enzyme–substrate relationship are further demonstrated in Text-fig. 2(c) where the rate of enzyme action appears to be exponential with respect to relative enzyme concentration (solid line). If this is expressed in terms of specific activity (interrupted line) it is apparent that, at a substrate concentration of 2.5 mM, the lower the enzyme content of the assay system, within certain limits, the higher is the rate of hydrolysis (per milligramme of tail suspension protein). Similarly, the initial rate of hydrolysis is apparently considerably higher (though this levels off asymptotically on longer incubation), ranging from over 1.8 to 0.4 µ-mole acetylcholine per milligramme protein per hour (dashed line, Text-fig. 2(d)) at the nearly optimum initial substrate concentration, the rate of acetylcholine breakdown dropping off as the enzyme depletes the substrate.

**DISCUSSION**

One may reasonably conclude from these observations not only that mammalian sperm cells contain a respectable and seemingly conventional specific acetylcholinesterase, but also that this enzyme occurs in quite high concentration. The presence of the enzyme in the three bull sperm cell fractions, head, midpiece and tail, is somewhat unexpected in the light of Mann's (1954) report, which suggests that the enzyme is largely confined to the 'tail' fragments of ram sperm homogenates, as well as Tibbs's (1960) finding that most of the enzyme occurred in the heads of trout and perch spermatozoa. Three possible sources of these discrepancies immediately come to mind: (1) species differences, (2) differences between epididymal and ejaculated spermatozoa, and (3) methods of preparation. Morphologically, the species differences are quite remarkable, the midpieces of mammalian spermatozoa occupying a considerable portion of the flagellar length, demarcated by anterior and posterior distal centriole, while both freshwater fish and marine invertebrate spermatozoa have distinctly circumscribed midpiece components tightly clustered at the base of the head. This could possibly account for most of the activity if during the homogenization process the midpiece accompanied the head fraction of the fish spermatozoa. On the other hand, the vastly different rate of esteratic activity of the *Mytilus* spermatozoa and the fish sperm homogenates (about 30- to 80-fold) appears to indicate that more than morphological differences exist between these species. In the mammalian spermatozoa, the degree of maturation may possibly account for the relatively high activity of both head and midpiece fractions of the bull epididymal spermatozoa; the fate of the cytoplasmic droplet in the sonicates is at present unknown. The Mickle disintegrator used to fractionate ram spermatozoa could conceivably dislodge any basal body organelle from which the
flagellum may originate and thus account for differences in enzyme distribution between bull and ram spermatozoa. Moreover, while the sonic disruption of bull spermatozoa appeared so successful, the use of ultrasound seemed drastically to inactivate the *Mytilus* sperm cholinesterase and a Potter homogenizer had to be employed in that case. While it may be fruitless to speculate that the technique employed in the fish sperm studies was partially responsible for what may have been a fairly high degree of enzyme inactivation, the technical differences of specimen preparation may be worth noting.

Tibbs (1962) has stated that “glycerol and digitonin extraction would destroy a control mechanism based on permeability changes resulting from acetylcholine release and breakdown”. The rate of acetylcholine breakdown needed to maintain the continuous flagellation of spermatozoa, once wave propagation has been initiated (if cholinesterase is not implicated here also) and for the duration of the motile life of the spermatozoa, has not been estimated. On the other hand, the velocity of propagation of the ‘conduction wave’ can be calculated from wave length and frequency data. According to Bishop (1962b) this amounts to approximately 600 to 700 µ/sec for bull spermatozoa and 800 to 1000 µ/sec for sea urchin spermatozoa. The conduction velocity of *Rana catesbeiana* non-myelinated nerve fibres of only slightly greater diameter is about 300 times that calculated for bull sperm flagellum; but if one examines a somewhat more analogous structure such as the carpopodite extensor muscle fibre of *Carcinus*, the comparison is more favourable (Spector, 1956). The muscle fibre is from 200 to 1200 times the diameter of the flagellum and the conduction velocity is of the order of 400 times that of the bull spermatozoa, so that if wave propagation is indeed a function of the surface membrane the similarity of this parameter, although fortuitous, is certainly striking. Verification of the submicroscopic distribution, however, must await the application of cytochemical techniques at the electron microscope level.

The axial filaments within the developing rat spermatid exhibit active movements (Austin & Sapsford, 1952), the filaments appearing to be attached to centriolar structures and to no other organelles. Sustained motility by isolated flagella, both those which occur naturally in the ejaculates of certain bulls and those produced by mechanical fragmentation, indicates the apparently essential nature of the centriolar apparatus for this activity, for isolated flagella lacking the anterior portions are non-motile. However, the relationship of the cholinesterase activity to the centriole can only be surmised, since there is as yet no direct information concerning the presence of this enzyme in the centriole itself or in its derivatives in spermatozoa. Nevertheless, the evidence suggests that, quantitatively at least, cholinesterase may play a significant role in the motility of mammalian and marine invertebrate spermatozoa. Having established this possibility, it is now necessary to determine whether the preparative procedure, by disrupting the morphological continuity of the ‘conductile’ system, interferes with the coordinatory or propagatory mechanism. Nachmannsohn & Wilson (1955) consider acetylcholinesterase to be highly stable and not readily brought into solution, since it is difficult to separate from the tissue debris of a homogenate (although the purified enzyme is quite soluble). It therefore seems unlikely that glycerination achieves its effect by directly
modifying the cholinesterase activity of the sperm cell, or that the enzyme may be eluted from the sperm tails thereby contaminating the head and mid-piece fractions.

ACKNOWLEDGMENTS

This work was done while the author held USPHS Career Research Development Award No. GM K3-15, 193, and was supported by grant No. M 6251 from the Population Council, Inc., and USPHS grant No. GM-06815-3.

The author gratefully acknowledges the preliminary studies on *Mytilus* spermatozoa carried out by Arthur Applegate which made the present work possible.

The technical assistance of Miss Sally Seebeck and Mrs Pat Martinez is also gratefully acknowledged.

REFERENCES


