ENZYME DISTRIBUTION IN FRAGMENTED BULL SPERMATOZOA

I. ADENYLPYROPHOSPHATASE*

by

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Actomyosin constitutes the predominant part of the "solid" structure of muscle, and has been regarded as the contractile substance[1-4]. One of the outstanding features of this complex protein is its close association with the enzyme, adenosinetriphosphatase. Hydrolysis of adenosine triphosphate liberates energy, and under the influence of the ATP the physical properties of the actomyosin itself are altered[1,3]. Since the role of ATP as the ultimate bearer of the chemical energy utilized in cellular processes is not restricted to muscle, much attention is being directed to the identification in non-muscular motile structures of other actomyosin-like proteins associated with ATP-ase activity. (However, a Mg-activated ATP-ase separable from actomyosin has been described, the insoluble residue, actomyosin, retaining a Ca-activated ATP-ase[5,6,7]).

Bacterial flagella have been likened to monomolecular muscles endowed with the capacity of rhythmic change of form, on the basis of X-ray diffraction, electrophoretic and titrimetric measurements[8-10]. Moreover, addition of ATP to isolated bacterial flagella resulted in a thickening of the axial filaments and in a progressive contraction of these particles[11]. The folding and unfolding of protein molecules, mediated by ATP, has been suggested as the basis of cytoplasmic streaming and ameboid locomotion[12]. A protein isolated by KCl extraction of *Paramecium aurelia* and precipitated by the SZENT-GYÖRGYI method was found to liberate approximately 50% of the labile phosphate from ATP[13]. ATP has also been shown to accelerate the ciliary movement of the frog's pharyngeal mucosa[14]. Intact spermatozoa have also been found to possess ATP-splitting properties, and to contain measurable quantities of ATP as well[15].

Decrease in the ATP content of the semen almost invariably coincides with impairment of the motility of ejaculated bull sperm[16]. Protein fractions extracted from sperm homogenates were also found to possess enzymic activity toward ATP; these facts have led to the conclusion that ATP plays an equally important role in sperm motility as in the contraction of muscle[17]. However, direct evidence as to the possible function of "spermosin" as a contractile substance located in the tails of spermatozoa was lacking[1]. The sperm tails of marine invertebrate organisms, suspended in 0.6 *M* KCl, on dilution with distilled water underwent mild agglutination, while on addition of ATP, the agglutination became more pronounced[18]; this behavior parallels SZENT-GYÖRGYI's

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"precipitation" and "super-precipitation" of actomyosin particles under similar conditions. These phenomena are quite suggestive and it is conceivable that sperm motility may indeed depend upon structural changes in the proteins of the flagellum, rhythmically actuated by the presence or hydrolysis of ATP. If these proteins were to resemble the actomyosin system, they should very likely be associated with ATP-ase activity. Therefore, the investigations reported here were planned in an attempt to ascertain the locus of the ATP-hydrolyzing enzyme in the bull spermatozoon*.

MATERIALS AND METHODS

Within 2 hours after collection by means of an artificial vagina, about 3 to 6 ml of the pooled bull semen was suspended in an equal volume of ice-cold pyrex-redistilled water. The suspension was spun in the cold in a Clay-Adams Angle centrifuge at a relative centrifugal force of 2000 × g. The supernatant was decanted and discarded and the cells, resuspended in 10 ml water, were washed two more times in 3-minute runs at 2000 × g, to assure as complete removal as possible of the seminal fluid which contains a number of ester phosphatases, ATP-ases, and a nucleotidase[16,19,20]. The packed sperm was taken up in 2 ml water and then transferred in a chilled pyrex mortar to the deep freeze until the suspension acquired a slushy consistency, at which time the preparation was thoroughly ground. Fragmentation was found, by microscopic examination of the homogenate, to occur at the neck and also at the junction of the midpiece and tail, with the tail being broken up into smaller particles of variable size.

The homogenate was made up to 12 ml with water, and after thorough mixing 2 ml of the suspension was drawn off, designated "standard preparation" (S–P) and set aside in the cold. The balance of the suspension (referred to as "subtotal") was separated by differential centrifugation by a modification of the method described by ZITTLE AND O'DELL[21] as diagrammed in Table I.

Air-dried, unstained smears of a few drops each of the final preparations were examined microscopically. Judging from the aggregate appearance of these slides it was estimated that the fractions were of over 90% purity. Contamination of the Heads (H_5) was largely by incompletely fragmented sperm (I). Tails were the major contaminants of the Midpiece (M_1) fraction, with a small admixture of heads. The purity of the Tail (T) fraction was also in the same range, midpieces constituting the chief impurity. Comparison of the nitrogen contents of the fractions with that of the total homogenate (S–P) showed total recoveries to amount to 76–92%. The major portion lost consisted of difficultly separable mixtures of heads and incompletely fragmented sperm which were discarded because the degree of contamination was such as to preclude valid determination of the enzyme distribution. Some of the loss may be attributable to the fact that the washing and subsequent homogenization process reduces portions of the tail to nearly colloidal dimensions. By the addition of fat solvents to the unsedimentable tail suspension, ZITTLE AND O'DELL[21] obtained more complete recovery and thereby found the head, midpiece and tail fractions to be 51, 16 and 33% respectively of the total sperm by dry weight. Using the centrifuge method alone in the studies reported here, yields averaging 46, 18 and 25% were obtainable on the basis of the nitrogen content of the respective fractions as compared to the total nitrogen.

Each of the fractions, in most of the experiments, was suspended in 4 ml extraction medium, which consisted of water or Weber-Edsall solution (0.6 M KCl, buffered with 0.01 M Na_2CO_3 and 0.04 M $NaHCO_3$). The suspensions were extracted in the cold overnight. Apyrase activities of the suspensions, of the extracts and extracted residues separated by centrifugation, were measured after the method of MOOG AND STEINBACH[22]. The reactants were placed in 12 ml tapered pyrex centrifuge tubes; each tube contained a 0.4 ml aliquot of the sample, 0.25 ml veronal buffer (0.1 M, pH 7.5), 0.2 ml ATP** (containing 0.1 mg readily hydrolyzable P) and 0.25 ml $CaCl_2$ (0.05 M), plus 0.1 ml H_2O or 0.1 ml 4 × concentrated Weber-Edsall solution which was added at the time of incubation so that the salt concentration of the reaction mixture was identical in each of the tubes. Total reaction volume was 1.2 ml per tube, the final concentrations then being KCl 0.2 M, ATP $1.33 \cdot 10^{-3}$ M, veronal buffer $2.08 \cdot 10^{-2}$ M, $CaCl_2$ $1.04 \cdot 10^{-2}$ M.

The reaction mixtures, without ATP, were first equilibrated at 37° C for 5 minutes and the reaction was initiated by adding the ATP, rapidly mixing by lateral agitation, and replacing the tube in the thermostat. At the end of 10 minutes, the reaction was terminated by the addition of

* The semen was obtained through the generous cooperation of the Artificial Insemination Project and the Dairy Husbandry Department of the College of Agriculture, University of Nebraska.

** Tetrasodium salt from General Biochemicals, Inc., Chagrin Falls, Ohio; disodium ATP from Pabst Laboratories, Milwaukee, Wisc., neutralized by addition of concentrated NaOH to bromthymol blue endpoint.

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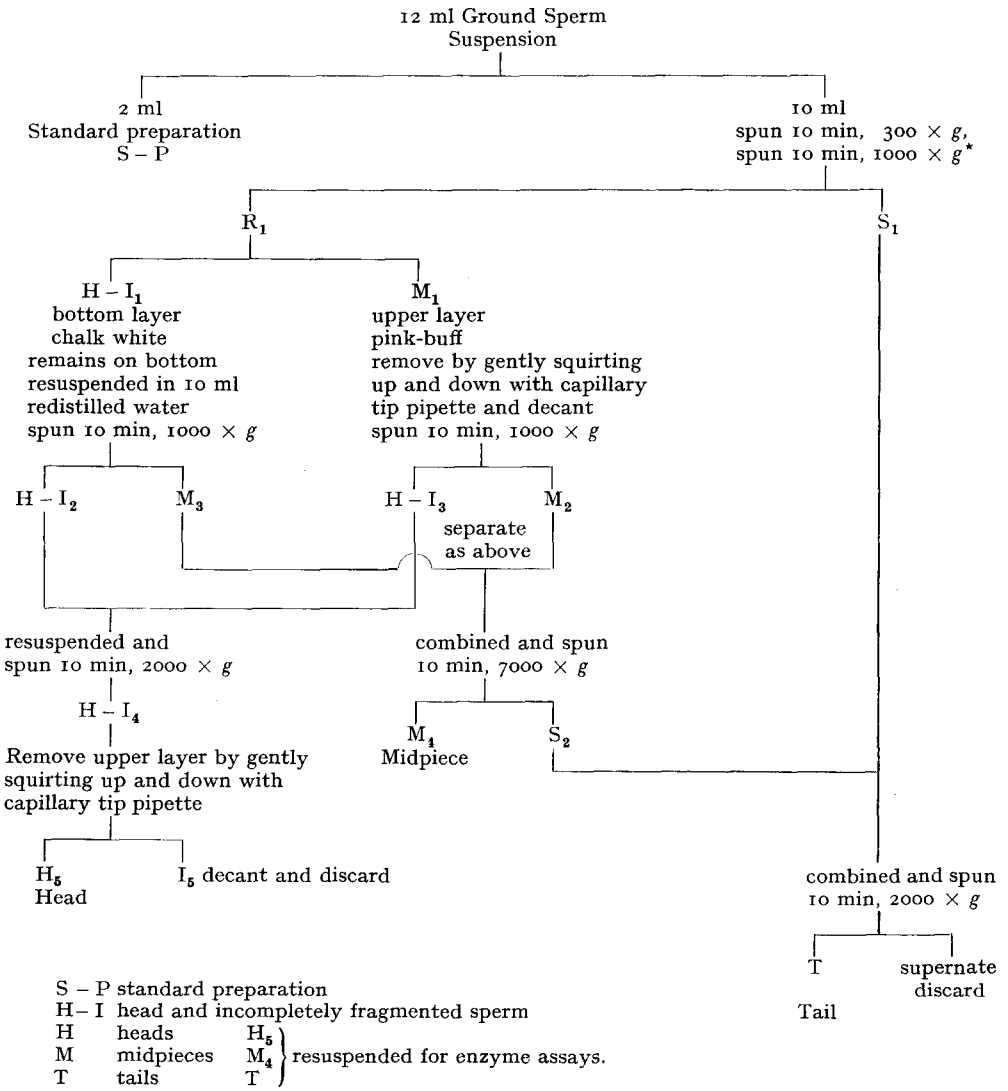
2 ml ice cold 10% trichloroacetic acid, the precipitate centrifuged down and the entire supernatant analyzed for ortho-phosphate by the Gomori modification of the Fiske-Subbarow method[23]. Controls, which also served as reagent blanks, were run simultaneously in duplicate tubes to which the trichloroacetic acid was added prior to the ATP.

Specific activity of apyrase is expressed as micrograms of inorganic P liberated per minute per milligram of nitrogen. The N content of each fraction was measured by duplicate micro Kjeldahl determination[24] on the same quantity of sample used in the corresponding enzyme test.

TABLE I



* This procedure permits the H – I component to sediment first, and the M_1 to settle as a distinct layer on top of it, facilitating the removal of the crude tail supernate S_1. None of the material is discarded at this point.

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RESULTS

The data obtained in the course of these experiments were compiled and results of a typical series of runs are summarized in Tables IIA and IIB, and graphically presented in Figs. 1 and 2. Separate determinations were performed on half a dozen individual water preparations. KCl preparations of at least a dozen other samples were also separately analyzed. The data recorded here represent a total analysis of the apyrase activities of the sperm homogenates and fractions (both H_2O and KCl preparations) prepared from a single specimen of bull semen, and show essentially the same results as those of the other samples. One-half of the original washed, homogenized and fractionated sample having been extracted overnight in water in the cold, the other half of the same semen sample was extracted overnight in cold KCl. The following details may be noted: 1, About one-third of the nitrogen of the total homogenate (or S–P) may be removed by overnight extraction in either water or in the salt solution. The specific apyrase activity of the water-soluble enzyme just equals that of the suspension, while the enzyme

TABLE IIA

APYRASE ACTIVITY OF BULL SPERM FRACTIONS AND STANDARD PREPARATION AS PER CENT TOTAL, AS γP/min, AND AS γP/mg N/min, AT 37° C, INCUBATION TIME 10 min.

Tube contents: ATP = 0.1 mg 7′-P, KCl 0.2 *M*, $CaCl_2$ 0.01 *M*, veronal buffer 0.02 *M* pH 7.5, 0.4 ml sample, total volume 1.2 ml.

Preparation	*Overnight H_2O extraction*		*Overnight KCl extraction*	
	N mg	*Total apyrase activity of suspensions γP/min*	*N mg*	*Total apyrase activity of suspensions γP/min*
Grand total* (S–P × 6)	5.49	26.1	5.22	21.3
Sub-total** (S–P × 5)	4.57	21.75	4.35	17.75
Std. prep.	0.92	4.35	0.87	3.55
Tails	1.20	15.5	1.25	15.2
Midpieces	0.72	3.0	0.72	1.5
Heads	1.59	0.94	1.80	0
Recovery	4.43	23.79	4.64	20.25
% Grand total	81%	91%	88%	95%

Suspensions	*N, % Sub-total*	*Specific apyrase activity*		*N, % Sub-total*	*Specific apyrase activity*	
		% Sub-total	*γP/mg N/min*		*% Sub-total*	*γP/mg N/min*
Tails	26.3	71.3	12.9	28.8	85.5	12.2
Midpieces	16.1	13.8	4.17	16.5	8.5	2.08
Heads	35.5	4.3	0.59	41.3	0	0
Recovery (% Sub-total)	77.9	89.4		86.6	94.0	

* Grand total: obtained by multiplying the activity of S–P by 6, inasmuch as one-sixth of the original was set aside for use as the standard.

** Sub-total: the balance of the homogenate, that is, the Grand total less the two ml of original suspension set aside as S–P.

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TABLE IIB

APYRASE ACTIVITY OF STANDARD PREPARATION AND BULL SPERM FRACTIONS AS PER CENT OF SUSPENSIONS AND AS γP/mg N/min, AT 37° C; INCUBATION TIME, 10 min.

Tube Contents: ATP = 0.1 mg 7'-P, KCl 0.2 *M*, $CaCl_2$ 0.01 *M*, veronal buffer 0.02 *M*, pH 7.5, 0.4 ml sample, total 1.2 ml.

Preparation	*Overnight H_2O extraction*			*Overnight KCl extraction*		
	N	*Apyrase activity*		*N*	*Apyrase activity*	
	% *Susp.*	*Total* % *Susp.*	*Specific* γ*P/mg N/min*	% *Susp.*	*Total* % *Susp.*	*Specific* γ*P/mg N/min*
Standard						
Suspension	100	100	4.76	100	100	4.15
Extract	34.5	34.5	4.76	38	74	7.95
Residue	82	21.6	0.83	63	0	0
Tails						
Suspension	100	100	12.9	100	100	12.2
Extract	62.5	75.5	15.6	103	100	11.73
Residue	29.2	23.2	10.4	5.6	0	0
Midpieces						
Suspension	100	100	4.17	100	100	2.08
Extract	12.5	0	0	16.7	50	6.25
Residue	87.5	90	4.25	75	50	1.39
Heads						
Suspension	100	100	0.59	100	0	0
Extract	0	0	0	4.2	0	0
Residue	103	96	0.46	91	0	0

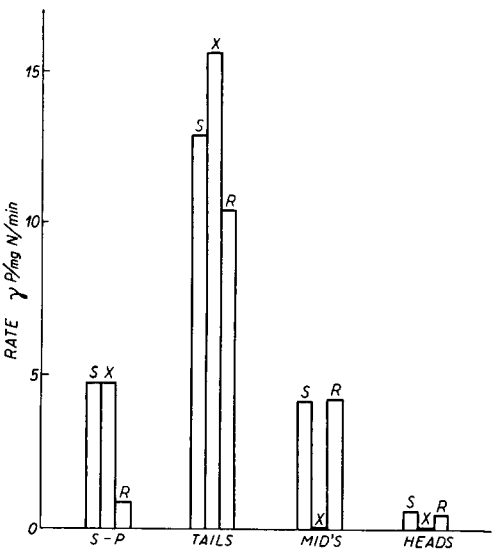

Fig. 1. Specific activity of bull sperm fractions and standard preparation, extracted overnight in distilled water. Tube contents: ATP = 0.1 mg of readily hydrolyzable phosphate, KCl 0.2 *M*, veronal buffer 0.02 *M* pH 7.5, $CaCl_2$ 0.01 *M*, 0.4 ml sample. Total volume 1.2 ml. Incubated 10 min, 37° C. S = suspension; X = extract; R = residue.

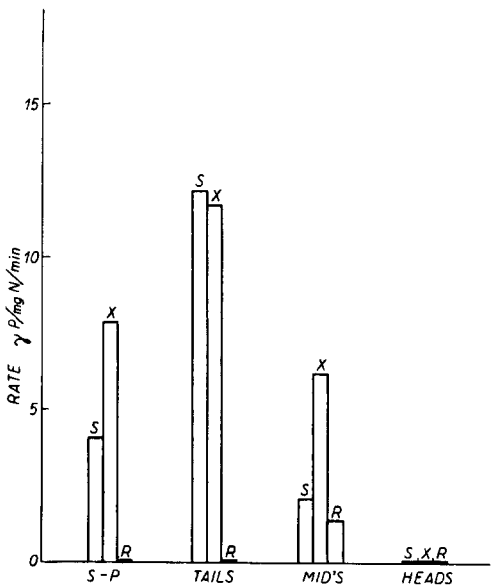

Fig. 2. Specific apyrase activity of bull sperm fractions and standard preparation, extracted overnight in 0.6 *M* KCl. Tube contents: ATP = 0.1 mg of readily hydrolyzable phosphate, KCl 0.2 *M*, veronal buffer 0.02 *M* pH 7.5, $CaCl_2$ 0.01 *M*, 0.4 ml sample. Total volume 1.2 ml. Incubated 10 min, 37° C. S = suspension; X = extract; R = residue.

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obtained by salt extraction is about twice as active as the corresponding suspension. 2, The tail fractions have about three times the specific activity of the total homogenates. Two-thirds of the tail N is H_2O-soluble and contains 75% of the specific activity, while but an insignificant amount of the tail nitrogen is salt-insoluble, all of the enzyme activity appearing in the extract. 3, The specific activity of the water suspension of the midpieces is about 10% less than that of the standard preparation; an eighth of the midpiece N is H_2O-soluble, but this is enzymically inactive, all of the activity remaining with the water-insoluble residue. Only about one-sixth of the midpiece N is soluble in the KCl, and yet this accounts for a specific activity nearly one and a half times that of the S–P suspension; while the salt-insoluble residue has about one-third the specific activity of the S–P. 4, The water preparation of the heads, all the nitrogen of which is insoluble, has relatively little apyrase activity (and that probably due to the presence of contaminants from the other fractions); while all the KCl preparations of the heads are totally inactive enzymically.

The sub-total (grand total less standard preparation) activity of the H_2O and KCl suspensions combined equals 39.5γ P/minute. The total activities of the tail, midpiece and head suspensions respectively come to 30.7, 4.5 and 0.9γ P/minute/fraction (recovery of 91.5% of the sub-total activity). The lost tails could possibly account for an additional activity of 3.5γ P/minute, this correction being made on the basis of the nitrogen content data of ZITTLE AND O'DELL[21], which would bring the total activity recovered to 39.6γ P/minute. The tails contain 26.8 and 28% of the total nitrogen of the water and salt preparations; the nitrogen of the midpieces was 16.1 and 16.5%, and the head nitrogen was 35.5 and 41.3% respectively of the water and salt preparations. If the percentage composition of the fractions is corrected to the figures obtained by fat solvent precipitation, as cited above, this would bring the activities of the fractions to the following values: tails 86.5%, midpieces 11.4%, heads 2.3% of the total combined activity.

The 0.4 ml aliquot of the Standard Preparation (Table IIA) is equivalent to 0.0265 ml of semen, and liberates 0.29γ P/minute from 100 micrograms of ATP–7'P. MANN's figures for the ATP-ase activity of washed whole sperm (ram) equivalent to 0.04 ml of semen, when recalculated give a value of 0.58 γ P/minute liberated from ATP containing 160 micrograms of 7'P[16]. If the ATP-ase activity of the crude flagellar extracts is expressed as Qp^{25}, values up to 150 may be obtained, at pH 7.5 in veronal buffer. These figures compare with data compiled by BAILEY[26] for myosin two or three times recrystallized from muscle of various species. In bicarbonate buffer, pH 9.1, the Qp ranged from 170 for toad to 1000 for rabbit myosin.

One of the factors which is thought to stabilize actomyosin-ATP-ase against denaturation and inactivation *in vivo* is ATP itself[1]. Therefore, in these studies, quantities of ATP were employed such that not more than 10–20% of the initial 7' acid-labile phosphorus was liberated during the course of the reaction. This assured some measure of protection for the enzyme, and incidentally, at no time could the concentration of the substrate be a limiting factor. When the substrate is present in excess, the velocity of the reaction is proportional to the enzyme concentration, as shown in Fig. 3. When the quantity of phosphorus liberated is plotted as a function of the amount of flagellum extract, a linear relationship is clearly demonstrated between the activity and concentration of the enzyme.

To determine whether a specific apyrase or a general ester phosphatase was involved

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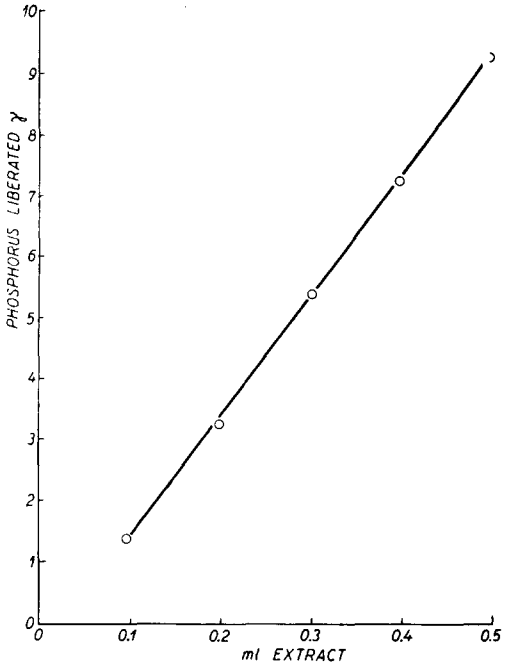

Fig. 3. Total apyrase activity of Weber-Edsall extract of bull sperm flagella. Tube contents: ATP = 0.1 mg readily hydrolyzable phosphate, KCl 0.2 *M*, Veronal buffer 0.02 *M* pH 7.5, $CaCl_2$ 0.01 *M*. Total volume 1.2 ml. Incubated 15 min, 37° C.

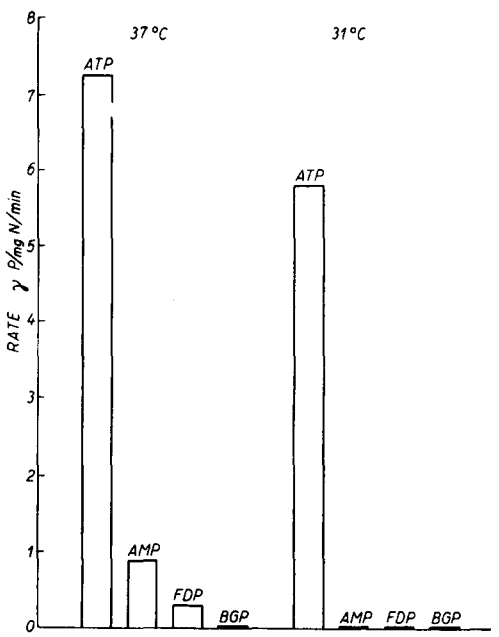

Fig. 4. Apyrase specificity of Weber-Edsall extract of bull sperm flagella. Tube contents: Organic phosphate = 0.1 mg labile P, KCl 0.133 *M*, veronal buffer 0.028 *M* pH 7.5, $CaCl_2$ 0.014 *M*. Total volume 0.9 ml. Incubated 10 min, at temperature indicated. ATP = adenosine triphosphate; AMP = yeast adenylate; FDP = fructose diphosphate; BGP = β-glycerophosphate.

TABLE III

PHOSPHATASE ACTIVITY OF WEBER-EDSALL EXTRACT OF BULL SPERM FLAGELLA

Reaction mixture: 0.9 ml. Organic phosphate = 0.1 mg labile P, KCl 0.133 *M*, veronal buffer 0.028 *M* pH 7.5, $CaCl_2$ 0.014 *M*, 0.2 ml extract. Incubation time: 10 min.

Substrate	*Amount of enzyme mg of protein*	*Temperature °C*	*Rate γP/mg N/min*
ATP	0.25	37	7.25
AMP	0.25	37	0.90*
FDP	0.25	37	0.30*
BGP	0.25	37	0.00
ATP	0.28	31	5.85
AMP	0.28	31	0.00
FDP	0.28	31	0.00
BGP	0.28	31	0.00

ATP = Adenosine triphosphate
AMP = Yeast adenylate
FDP = Fructose-1,6-diphosphate
BGP = Sodium beta-glycerophosphate

* These two figures are very close to the limit of sensitivity of the colorimeter, and so may be of doubtful significance.

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under these conditions, Weber-Edsall extracts of the flagella were tested against several substrates. Besides ATP, these included sodium β-glycerophosphate (General Biochemicals, Inc.), yeast adenylate and fructose diphosphate (Schwarz Laboratories, N.Y.). The reactants were placed in tapered centrifuge tubes; each tube, containing a total volume of 0.9 ml, was incubated for 10 minutes, at which time the reactions were terminated by the addition of ice-cold 10% trichloroacetic acid. The reaction mixture consisted of veronal buffer, pH 7.5 (0.028 *M*), $CaCl_2$ (0.014 *M*), organic phosphate equivalent to 0.1 mg hydrolyzable phosphate, KCl (0.133 *M*) and 0.2 ml flagellum extract. As previously, the whole supernatant, separated by centrifugation from the acid precipitated protein, was analyzed for orthophosphate. Controls were run simultaneously in duplicate tubes to which the trichloroacetic acid was introduced prior to the substrates. The data, summarized in Table III and Fig. 4, indicate that at the lower temperature, the ester phosphatases do not participate in the hydrolysis of the ATP, while at 37° C, their activity, at best, is relatively insignificant in these extracts.

DISCUSSION

With respect to its enzymic attributes in part, the motile structure of the spermatozoon bears some resemblance to that of the actomyosin of muscle. The analogy of the axial filaments of the sperm flagellum to a contractile structure like a muscle fibril had been suggested at the beginning of the century on the basis of purely morphological considerations (*cf.* 27). Recent electron photomicrograph studies have revealed a series of continuous cortical helices surrounding the axial fibrils[28,29]. BRETSCHNEIDER AND VAN ITERSON[28] suggest, as the basis of sperm motility, a possible antagonism between the fibrils and the helices. Should even part of the "contractile protein" reside within the cortical helices, its peculiar molecular arrangement may not only account for the undulatory motion of the sperm tail, but may also be responsible for the negative response to ATP of the protein threads prepared from sperm tails[18].

Thus, while it has been demonstrated that the apyrase of the bull spermatozoon is concentrated within the flagellum, it is evident that more direct methods will be required to establish the association of this enzyme with a mechanism capable of converting the chemical energy of ATP into mechanical energy.

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SUMMARY

1. Washed, homogenized bull sperm was separated into head, midpiece and tail components by differential centrifugation.

2. The average yields on a nitrogen basis were 46, 18 and 25% for the respective fractions.

3. Specific activity of the apyrase of the tail fraction was about 3 times that of the midpiece fraction, and of the standard preparation (total homogenate), while the heads showed practically no apyrase activity.

4. A linear relationship between apyrase activity and enzyme concentration was observed.

5. Phosphate liberation is of the adenylpyrophosphatase type, the flagellum extracts being relatively inactive toward glycerophosphate, yeast adenylate and fructose diphosphate.

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RÉSUMÉ

1. Du sperme de taureau lavé et homogénéisé a été fractionné en têtes, pièces intermédiaires et flagelles par centrifugation différentielle.

2. Les rendements moyens, calculés d'après l'azote, sont de 46, 18 et 25% pour les fractions respectives.

3. L'activité spécifique de l'apyrase de la fraction flagellaire est environ 3 fois plus élevée que celle de la fraction intermédiaire, et que celle d'une préparation standard (homogénat total), les têtes ne présentant pratiquement aucune activité apyrasique.

4. Il existe une relation linéaire entre l'activité apyrasique et la concentration en enzyme.

5. La libération de phosphate est du type adénylpyrophosphatase, les extraits flagellaires étant relativement peu actifs vis à vis du glycérophosphate, de l'adénylate de levure et du fructose diphosphate.

ZUSAMMENFASSUNG

1. Gewaschenes, homogenisiertes Bullensperma wird durch Differentialzentrifugation in die Kopf-, Mittelstück- und Schwanzfraktion zerlegt.

2. Die durchschnittlichen Ausbeuten der jeweiligen Fraktionen bezogen auf den Gesamtstickstoff betragen 46, 18 und 25%.

3. Die spezifische Apyraseaktivität der Schwanzfraktion ist dreimal so gross wie die der Mittelstückfraktion und des Standardpräparates (Gesamthomogenat), während die Kopffraktion praktisch keine Apyraseaktivität zeigt.

4. Apyraseaktivität und Enzymkonzentration sind einander proportional.

5. Die Phosphataseaktivität ist vom Typ der Adenylpyrophosphatase. Glycerinphosphat, Hefeadenylat und Fruktosediphosphat werden von dem Extrakt der Flagellen nicht gespalten.

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