ENZYME DISTRIBUTION IN FRAGMENTED BULL SPERMATOZOA

II. SUCCINIC DEHYDROGENASE AND CYTOCHROME OXIDASE*

by

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The enzyme succinic dehydrogenase occupies a pivotal position in intermediary metabolism, whereby oxidation of pyruvate and its precursors is performed by electron mediation through the fumarate-succinate and cytochromes-O_2 systems[1]. Succinic dehydrogenase has been found in whole washed bull sperm by the Thunberg technique; and malonate, as well as cyanide and azide, reversibly inhibits both motility and respiration of the bull sperm[2]. Cytochrome oxidase activity of disintegrated bull sperm fractions was demonstrated manometrically also, using paraphenylenediamine[3]. Direct evidence for the presence of the cytochromes in the sperm of boar, man, ram and bull has been obtained spectroscopically, following vitrification of the spermatozoa in liquid air[4]. The energy requirements for the maintenance of sperm activity can be obtained either from oxidation of intracellular substances such as phospholipids or from glycolytic processes[5]; or from fructolysis of the seminal plasma fructose[6]. Adenosine triphosphate, formed *via* the oxidative phosphorylations, presumably provides the necessary link between fructolysis and motility[7]. Not only is ATP a constituent of the mammalian sperm cell[6,8], but a decrease in the ATP content of ejaculated sperm almost invariably coincides with impairment of motility[9].

Apyrase is located in the flagellar fraction of the bull spermatozoon, as reported in the preceding paper of this series[10]. If the dehydrogenation of succinate is a means of replenishing the energy stores for sperm motility, it would be pertinent to ascertain whether the energy-rich products of cellular oxidation may be produced at or near the site of the motile apparatus. These investigations seek, by assays on the separated morphological components, to determine the distribution of the succinate oxidizing system in the bull spermatozoon.

MATERIAL AND METHODS

Pooled ejaculated bull semen** was washed and homogenized. A portion of the homogenate designated "Standard Preparation (S–P)" was set aside in the cold, and the balance was fractionated by differential centrifugation into heads, midpieces and tail fragments, as previously described[10]. The

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References p. 501.

Standard Preparation and each of the fractions was then suspended in 5 or 10 ml distilled water, depending on the initial quantity of the washed packed sperm. An aliquot of each of these suspensions was saved for the cytochrome oxidase assays—carried out concurrently.

Succinic dehydrogenase

The enzyme assays were carried out on the crude aqueous suspensions according to a spectrophotometric method[11]. The reactants were mixed in corex cuvettes of 1 cm optical path. The reaction mixture, of total volume of 3 ml, consisted of the following components (in final concentrations) added to the cuvette in the indicated sequence: Na succinate (Merck) 0.033 M, 0.1 ml sample, NaCN 0.001 M, cytochrome c (General Biochemicals, Inc.; assuming a molecular weight of 14,000) $1.2 \cdot 10^{-5}$ M, phosphate buffer 0.043 M, pH 7.4 (all the reagents were dissolved in the buffer). The sample was mixed with the succinate in the cuvette by lateral agitation, and after 2 minutes incubation at room temperature, the cyanide was added followed by the cytochrome c. The reaction was initiated (zero time) by inversion of the cuvette to mix the reactants. The cuvette was immediately transferred to the Beckman spectrophotometer (with photomultiplier) where the reduction of cytochrome c was followed by observing the increase in extinction at a wave length of 550 mμ, and a slit width of 0.005 mm. Readings were taken every 30 seconds for 3 minutes, at which time the addition of a few crystals of sodium hydrosulfite to the cuvette completed the reduction of the cytochrome c.

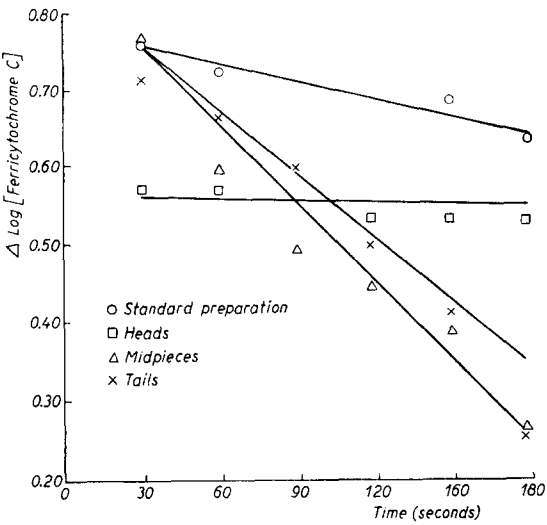

Fig. 1. Reduction of ferricytochrome c by bull sperm fractions and standard preparation. Cuvette contents: Na-succinate $3.3 \cdot 10^{-2}$ M, NaCN 10^{-3} M, cytochrome c $1.2 \cdot 10^{-5}$ M, phosphate buffer $4.3 \cdot 10^{-2}$ M pH 7.4, 0.1 ml sample. Total volume 3.0 ml. Incubated 3 min, 24° C.

Under these conditions, the reduction of cytochrome c by bull sperm homogenates and fractions appears to follow the course of a first order reaction. If the optical density (O. D.) of the reaction mixture at any time is subtracted from that of the completely reduced sample, and the logarithm of this difference is plotted against time, the slope of the line is a measure of the molar drop in concentration of oxidized cytochrome c per unit time, and is proportional to the enzyme activity (see Fig. 1). The concentration of oxidized cytochrome c is calculated according to HORECKER AND HEPPEL[12]. Specific enzyme activity is expressed as Δ log [ferricytochrome c] per minute per milligram of nitrogen (estimated by the microkjeldahl method of MA AND ZUAZAGA[13]); the nitrogen content of the samples ranged from 0.01 to 0.1 mg.

Cytochrome oxidase

Aliquots of the sperm samples prepared for the succinic dehydrogenase assays were concomitantly analyzed for cytochrome oxidase activity by a similar spectrophotometric method[14]. 30 ml of a $1.7 \cdot 10^{-5}$ M solution of cytochrome c (in 0.03 M phosphate buffer, pH 7.4) was reduced by the addition of 0.1 ml freshly prepared 1.2 M $NaHSO_3$. Excess SO_2 was removed by aeration. 2.8 ml of this reduced cytochrome c was pipetted into a corex cuvette and the reaction initiated on addition of 0.1 ml sample and mixing by inversion of the cuvette. Readings were taken immediately and every 30 seconds for 3 minutes. The oxidation of cytochrome c was followed by measuring the rate of decrease in extinction at 550 mμ (slit width 0.005 mm). At the end of the 3 minutes, a few crystals of K-ferricyanide were added to complete the oxidation of the cytochrome c and two minutes later the extinction was determined again. All experiments were conducted at room temperature (24°–25° C.).

RESULTS

All assays were performed within a few hours after the semen was collected. Individual experiments were run separately on a number of different samples obtained on different days. While the absolute values observed vary somewhat from specimen to specimen (which may be a reflection of the variation of "semen quality"), still a high

References p. 501.

degree of consistency can be perceived when the values of the respective fractions of a given day's work are related to one another.

Succinic dehydrogenase

Examination of Fig. 1 reveals that the succinic dehydrogenase activity of the heads is nil and that the activity of the flagellar components, midpieces and tails is considerably greater than that of the total homogenate (Standard preparation). The fact that in two of the cases cited in Table I, there was no enzymic activity in the head fraction and some degree of activity in heads derived from other specimens, may be attributed to variation in the degree of contamination by the other components of the spermatozoa. By far the greatest amount, and in some cases all, of the succinic dehydrogenase activity is to be found in the flagellar components.

TABLE I

SUCCINIC DEHYDROGENASE ACTIVITY OF TAIL, MIDPIECE AND HEAD FRACTIONS AND STANDARD PREPARATION

Reaction mixture, 3 ml: sodium succinate 0.033 *M*, sodium cyanide 0.001 *M*, cytochrome *c* $1.2 \cdot 10^{-5}$ *M*, phosphate buffer (pH 7.4) 0.043 *M*, sample 0.1 ml. Readings every 30 seconds for 3 minutes in Beckman Spectrophotometer with photomultiplier, slit width 0.005 mm, wavelength 550 mμ. Temperature 24–25° C.

	Rate *Δ log [ferricytochrome c]/minute/fraction*			
Fraction	*S – P*	*T*	*M*	*H*
Sample				
1/15	4.6	13.7	6.9	0.0
1/17	4.5	19.6	9.1	1.9
1/21	8.8	11.5	3.6	0.0
1/24	2.8	45.4	12.1	3.0
1/26	7.7	22.4	12.2	2.7
Ave.	5.7	22.5	8.8	1.5

Note: In samples 1/15, 1/17, 1/21, sodium succinate was added to the diluted sample two minutes prior to the initiation of the experiment; while in samples 1/24 and 1/26, the succinate was added to the preparations immediately after centrifugal fractionation of the homogenate, and these samples were stored at 4° C for 2–3 hours prior to the beginning of the assay.

It is evident from the data presented in Table II (detailed analysis of information in Sample #1 of Table I), that the tail fraction, containing 34% of the total nitrogen accounts for 58% of the total original activity (65% the activity recovered), while the midpieces, containing 12% of the sperm nitrogen, contain 31% of the original activity (35% of the activity recovered); the heads on the other hand, contain no activity although over half of the nitrogen is to be found in this fraction.

If the quantity of suspension added to the rest of the components of the reaction mixture is varied, it is apparent that the rate of reduction of cytochrome *c* is proportional to the enzyme concentration. Examination of Fig. 2 shows that the straight line may be extrapolated back to the origin, indicating that at zero enzyme concentration there is no significant reduction of cytochrome *c*. This suggests that, under the conditions of the experiment, the assay being completed in three minutes, the cyanide does not cause any measurable reduction of ferricytochrome *c*.

References p. 501.

TABLE II

SUCCINIC DEHYDROGENASE ACTIVITY OF TAIL, MIDPIECE AND HEAD FRACTIONS AND STANDARD PREPARATION

Experimental conditions and procedure same as in Table I. (This table reconstructed from data of sample #1 presented in Table I; most complete information was available on recovery of material in this sample.)

Preparation	*N*		*Succinic dehydrogenase activity*		
	mg	*% of total*	*Δ log [ferricytochrome c]/min/mg nitrogen*	*total original activity*	*% of total activity*
Total	11.5	100	2.04	23.3	100
S–P	2.3	20	2.04	4.6	20
Tails	3.89	34	3.50	13.6	58
Mid's	1.45	12	5.00	7.2	31
Heads	6.60	52	0.0	0.0	0
Recovery	11.14	98		20.8	89

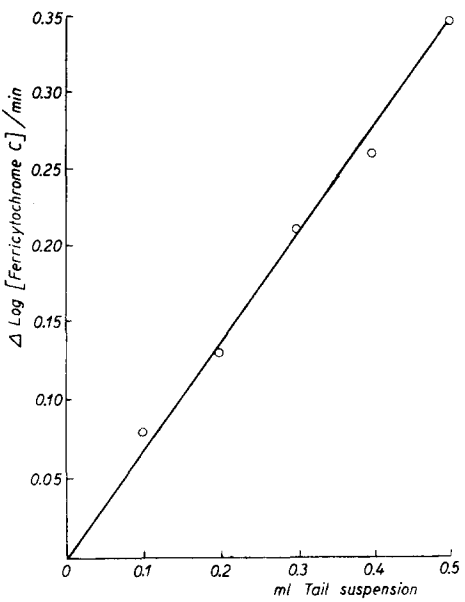

Fig. 2. Effect of varying the concentration of tail suspension. Cuvette contents: Na-succinate $3.3 \cdot 10^{-2}$ *M*, NaCN 10^{-3} *M*, cytochrome *c* $1.2 \cdot 10^{-5}$ *M* phosphate buffer $4.3 \cdot 10^{-2}$ *M* pH 7.4, 0.1 to 0.5 ml sample (0.22 mg N/ml). Total volume 3.0 ml. Incubated 8 min, 25° C.

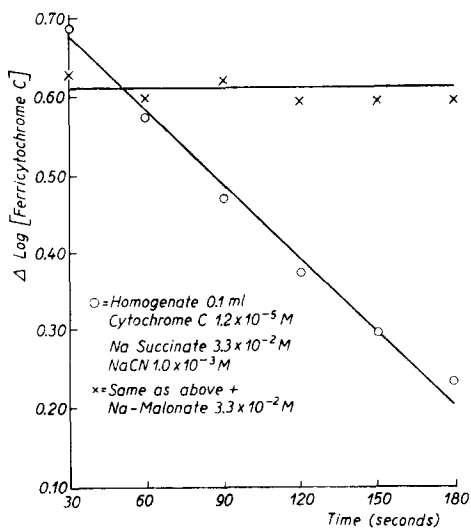

Fig. 3. Effect of malonate on reduction of ferricytochrome *c* by standard preparation: Na-succinate $3.3 \cdot 10^{-2}$ *M*, NaCN 10^{-3} *M*, cytochrome *c* $1.2 \cdot 10^{-5}$ *M*, phosphate buffer $4.3 \cdot 10^{-2}$ *M* pH 7.4, 0.1 ml sample. Total volume 3.0 ml. Incubated 3 min, 25° C. ○ — control: no additions. × — plus Na-malonate $3.3 \cdot 10^{-2}$ *M*.

This spectrophotometric method measures the rate of reduction of cytochrome *c* with succinate acting as the electron donor, and with the dissociation of the hydrogen to the phosphate buffer of the medium. Competitive inhibition by malonate has been regarded as a specific indication that succinic dehydrogenase is one of the constituents

References p. 501.

of the system. Fig. 3 illustrates the effect of the addition of malonate in a final concentration of 0.003 M to a standard preparation. In this concentration, equimolar with that of the succinate, the inhibition is complete.

Cytochrome oxidase

The oxidation of ferrocytochrome *c* likewise appeared to follow the course of a first order reaction. The optical density of the completely oxidized sample was subtracted from the O.D. at a given time. In this case, the slope of the line is a measure of the molar decrease in concentration of ferrocytochrome *c* per unit time, and is proportional to the oxidase activity. Enzyme activity is expressed as Δ log [ferrocytochrome *c*] per minute per milligram of nitrogen.

From the graph (Fig. 4) and the data assembled in Tables III and IV, it is apparent that most, if not all, of the oxidase activity resides in the tail and midpiece fractions. While the concentration of the enzyme on a nitrogen basis is nearly equal (5.20 and 6.21 activity units per mg N respectively), the total enzyme content of the tail fraction, 20.2 activity units, is somewhat over twice the midpiece content of 9.0 units (see Table IV). Thus the tail fraction accounts for about 75% and the midpiece fraction accounts for nearly 25% of the recovered cytochrome oxidase activity. Comparison with the

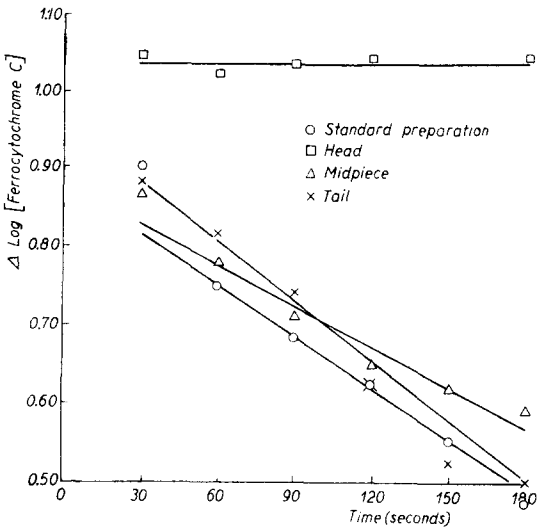

Fig. 4. Oxidation of ferrocytochrome *c* by bull sperm fractions and standard preparation. Cuvette contents: cytochrome *c* $1.6 \cdot 10^{-5}$ M, phosphate buffer $2.9 \cdot 10^{-2}$ M pH 7.4, 0.1 ml sample. Total volume 2.9 ml. Incubated 3 min, 24° C.

TABLE III

CYTOCHROME OXIDASE ACTIVITY OF TAIL, MIDPIECE AND HEAD FRACTIONS AND STANDARD PREPARATIONS

Reaction mixture, 2.9 ml: cytochrome *c* $1.6 \cdot 10^{-5}$ M in phosphate buffer (pH 7.4) 0.029 M, sample 0.1 ml. Readings every 30 seconds for 3 minutes in Beckman Spectrophotometer with photomultiplier, slit width 0.005 mm, wavelength 550 mμ. Temperature 24–25° C.

	Rate Δ log [ferrocytochrome *c*]/minute/fraction			
Fraction	*S – P*	*T*	*M*	*H*
Sample #				
1/15	6.98	20.2	9.0	0.25
1/17	5.9	15.6	7.1	0.0
1/21	8.3	26.3	3.5	0.0
1/24	5.2	9.7	2.5	0.0
1/26	2.65	9.8	2.4	0.0
Ave.	5.8	16.3	4.9	0.05

References p. 501.

TABLE IV

CYTOCHROME OXIDASE ACTIVITY OF TAIL, MIDPIECE AND HEAD FRACTIONS AND STANDARD PREPARATION

Experimental conditions and procedure same as in Table III. (This table reconstructed from data of sample #1 presented in Table III; most complete information available on recovery of material in this sample.)

Preparation	*N*		*Cytochrome oxidase activity*		
	mg	*% of total*	*Δ log [ferrocytochrome c]/ min/mg nitrogen*	*total original activity*	*% of total activity*
Total	11.5	100	3.04	34.90	100
S–P	2.3	20	3.04	6.98	20
Tails	3.89	34	5.20	20.2	58
Mid's	1.45	12	6.21	9.0	26
Heads	6.60	52	0.04	0.25	0.7
Recovery	11.14	98		29.45	84.7

succinic dehydrogenase activity in Table I (69% and 27% of the recovered activity for tail and midpiece fractions respectively) reveals a very close parallelism in the distribution of these two enzymes in the bull spermatozoon.

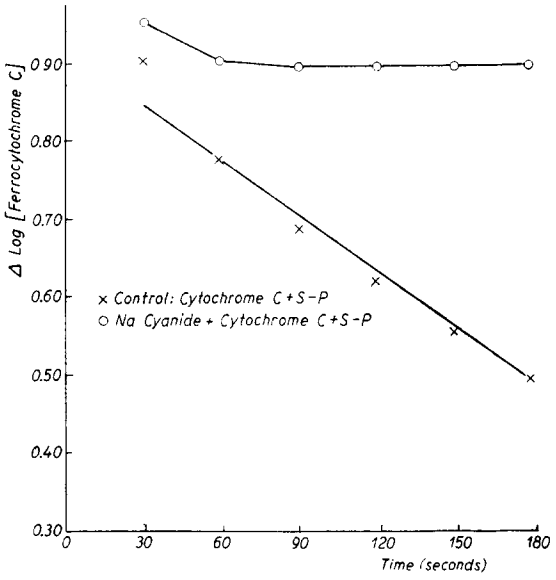

Fig. 5. Effect of cyanide on oxidation of ferrocytochrome *c* by standard preparation: cytochrome *c* $1.6 \cdot 10^{-5}$ *M*, phosphate buffer $2.9 \cdot 10^{-2}$ *M*, pH 7.4, 0.1 ml sample. Total volume 2.9 ml. Incubated 3 min, 24° C. × — control: no additions. ○ — plus NaCN 10^{-3} *M*.

An abundance of evidence has accumulated in the literature indicative of the cytochrome involvement in sperm respiration, so that while it cannot be assumed conclusively that any respiration of biological material inhibited by cyanide is cytochrome-mediated (*cf.* BROWN AND GODDARD[15]), still cyanide inhibition may be taken as corroborative evidence. The spectrophotometric method has here been employed to measure the enzymic oxidation of ferrocytochrome *c*, no other cytochrome-oxidizing agents having been added to the reaction mixture except at the termination of each experiment. Fig. 5 illustrates the complete inhibition of the reaction on addition of 10^{-3} *M* NaCN to standard sperm homogenate suspensions.

DISCUSSION

It is clear from the results presented here that the succinate oxidizing enzyme system of the bull spermatozoon is concentrated in the flagellar components in contrast

References p. 501.

to the deficiency of the head fraction in this respect. Why the activity of the heads with respect to succinic dehydrogenase and cytochrome oxidase was so low, was not ascertained. But, by virtue of the distribution of these enzymes, the intracellular systems responsible for the oxidation of succinate coincide morphologically with the apparatus which, converting chemical into mechanical energy, is responsible for sperm motility[10].

The predominant concentration of the cytochrome-oxidizing system in the tails and midpieces confirms the manometric observations of ZITTLE AND ZITIN[3]. This distribution is probably correlated with differences in functions of the spermatozoan structures. Cytochemical studies on ram spermatozoa show that the heads are not mere metabolically inert sacs of chromatin[16], although analysis of fish sperm nuclei indicates that nucleoprotamine is the only protein present[17]. However, in the case of nuclei of other cell types, the deficiency in cytochrome oxidase and succinic dehydrogenase has been interpreted as meaning that the entire Krebs cycle is lacking, and hence this important step for the generation of high energy phosphate cannot be operating[18]. Furthermore, "there is little evidence that the nucleus contributes significantly to the current activities of the cell" according to MAZIA[19]. Under the conditions of the experiments reported here and in the previous paper of this series[10] it appears that the head of the sperm is enzymically inactive toward adenosinetriphosphate, succinate and ferrocytochrome *c*. The association of the cytochrome system and succinic dehydrogenase with the midpieces and tail fractions, parts of the energy-requiring "organ" of locomotion, is not unexpected. Such an association of the cytochromes with the most active components of an organism has repeatedly been emphasized. A sheath of mitochondrial origin comprises the bulk of the midpiece, the helical coils apparently corresponding to the mitochondrial sheath surrounding the axial fiber which runs down the entire length of the tail[20]. The work of SCHNEIDER AND HOGEBOOM[21] demonstrating the greatest portion of cytochrome oxidase and succinoxidase in the mitochondrial fraction of liver and kidney, is thus extended to the corresponding components of the spermatozoon. The possibility has been established for the formation of ATP at the site of the structural system peculiarly adapted for the channeling of the chemical energy into motile activity.

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SUMMARY

1. Homogenized bull sperm was separated into head, midpiece and tail fractions by differential centrifugation.

2. On a nitrogen basis the yields were 52 %, 12 % and 34 % for the respective fractions.

3. Succinic dehydrogenase activity of the tails amounted to 69 % and of the midpieces about 27 % of the total recovered activity.

4. Malonate completely inhibits the enzyme activity.

5. Oxidation of cytochrome *c* parallels the distribution of the succinic dehydrogenase (75 % in the tail fraction, 25 % in the midpiece fraction).

6. Cyanide completely inhibits cytochrome *c* oxidation.

7. The head fraction was relatively inert with respect to the two enzymes.

8. The enzyme distribution is related to apyrase distribution in the spermatozoon.

References p. 501.

RÉSUMÉ

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

2. D'après les teneurs en azote, les rendements sont respectivement de 52 %, 12 % et 34 %.

3. L'activité succinodéshydrasique des flagelles représente 69 %, et celle des pièces intermédiaires, environ 27 % de l'activité totale obtenue.

4. La malonate inhibe complètement l'activité enzymatique.

5. L'oxydation du cytochrome *c* varie parallèlement à la distribution de la succinodéshydrase (75 % dans les flagelles, 25 % dans les pièces intermédiaires).

6. Le cyanure inhibe totalement l'oxydation du cytochrome *c*.

7. L'activité des deux enzymes dans les têtes est très faible.

8. La distribution des enzymes est en rapport avec la distribution de l'apyrase dans le spermatozoïde.

ZUSAMMENFASSUNG

1. Stierspermahomogenat wurde durch differentielles Zentrifugieren in eine Kopf-, eine Mittelteil- und eine Schwanzfraktion zerlegt.

2. Als Ausbeuten der jeweiligen Fraktionen wurden, auf den Gesamtstickstoff bezogen, 52 %, 12 % und 34 % gefunden.

3. Die Succinodehydraseaktivität der Schwanzfraktion wurde auf 69 %, die der Mittelteilfraktion auf 27 % der Gesamtaktivität festgelegt.

4. Malonat inhibiert die Aktivität dieses Enzymes vollkommen.

5. Oxydation des Cytochroms *c* stimmt mit der Verteilung der Succinodehydrase völlig überein (75 % in der Schwanzfraktion, 25 % in der Mittelteilfraktion).

6. Cyanid inhibiert die Oxydation des Cytochroms *c* vollkommen.

7. Die Kopffraktion erwies sich, in Bezug auf diese beiden enzymatischen Aktivitäten, als wenig wirksam.

8. Die Verteilung dieser Enzyme in den Spermatozoen stimmt mit der Verteilung der Apyrase überein.

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