CYTOCHEMICAL STUDIES WITH THE ELECTRON MICROSCOPE

I. ADENOSINETRIPHOSPHATASE IN RAT SPERMATOZOA*

LEONARD NELSON

Department of Anatomy, University of Chicago, Ill. (U.S.A.)

Vibratile tails of spermatozoa in active movement describe an undulatory pattern which is primarily a function of the flagellar fine structure; within limits, the sinusoidal wave is relatively independent of the physical properties of the medium (*cf.* ref.[1], Discussion). The flagellum of mammalian sperm contains an inner and an outer cylinder of nine longitudinal fibers each, distributed around two central fibrils[2,3,4]. These fibers are embedded in a matrix which is enclosed in a cortical helix that terminates at the end-piece of the tail.

There has, as yet, been no direct demonstration of a relationship between a specific flagellar structure and the generation of the undulatory wave. The fibers in longitudinal sections of sperm tails viewed in the electron microscope do not appear to spiral, but to run straight[2,4]. This would seem to preclude the production of the two- or three-dimensional waves simply on the mechanical basis of a spiral organization of the axial fibers. The solubility characteristics of the helical cortex argue against its being contractile[5]. Thus, one must surmise that at least some of the longitudinal fibers are capable of contracting.

Contraction in biological systems, in general, is associated with the splitting of adenosinetriphosphate[6]. ENGELHARDT[7] extracted a protein from mammalian sperm which was enzymically active toward adenosinetriphosphate (ATP). Differential centrifugation of homogenized bull sperm yields head, midpiece and tail fractions; Weber-Edsall extracts of these midpieces and tails liberated phosphate from ATP, but not from glycerophosphate, fructose diphosphate or adenylate[8]. A similar enzyme in *Mytilus* sperm exhibited the properties of a "true" ATP-ase in that only half of the labile phosphate of ATP is split off, even on prolonged incubation[9]. The current study is one of a series undertaken to relate the activity of ATP-ase and certain other enzymes to the fine structure of the mammalian sperm flagellum.

The cytochemical technique used to demonstrate the presence of ATP-ase in the flagellar fibers is based on PADYKULA and HERMAN's modification[10] of the GOMORI procedure[11]. The method depends on the deposition, at alkaline pH, of an insoluble calcium salt (referred to here, for the sake of brevity, as calcium phosphate) at the site of enzyme activity[12], and the fact that, after short incubations, enough of the phosphate may be deposited to be detected, without further manipulation (*i.e.*,

* This work was supported by a grant to the author from The Population Council, Inc., by the Commonwealth Fund and by the Wallace C. and Clara A. Abbott Memorial Research Fund of the University of Chicago.

References p. 641.

conversion to cobalt sulfide), in the phase microscope[13]. With the experimental procedures of the present studies, that which appears in the phase microscope as a variation in refraction, is revealed in the electron microscope as a region of increased electron scattering.

MATERIALS AND METHODS

Adult Sprague-Dawley rats were stunned and then bled by severing the major thoracic vessels, One testis was exposed and the cauda epididymis was nicked several times with a razor blade. A small drop of the epididymal fluid was diluted with physiological saline; the spermatozoa were checked for movement under a light microscope. If the sperm were immotile, or only slowly moving, the rat was rejected. Several small squares of metal foil (2 × 2 × 0.05 mm) were dipped into the epididymis and the thin films of fluid (less than 0.1 mm thick) were frozen by immersion in liquid propane at —175°, according to the method of GERSH *et al.*[14]. The frozen specimens were dried *in vacuo*, at about —35°, over a phosphorus pentoxide trap. The sperm samples were then vacuum-infiltrated with a buffer consisting of 0.025 *M* sodium veronal, 0.045 *M* calcium chloride, 0.0025 *M* cysteine (free base)*, and adjusted to pH 9.0 (measured by a glass electrode) by the addition of 1 *N* sodium hydroxide.

When the vacuum was broken, the specimens were transferred into small petri dishes containing the same buffer, removed from the foil and cut into four to eight pieces. The experimental "blocks" were transferred into micro-tubes containing fresh buffer to which had been added crystalline disodium ATP (Sigma) of a final concentration of 0.005 *M* and the pH readjusted to 9.0. Control pieces were placed in micro-tubes in fresh buffer (no ATP) and both groups of specimens incubated at room temperature. At 2½, 5, 10 and 30 min, the reactions were terminated by aspirating the incubation medium from the tubes and washing the specimens several times in 50% ethyl alcohol. Next, the blocks were dehydrated by passing through 70, 95 and 100% ethyl alcohol into alcohol–ether, and embedded in celloidin and then methacrylate (95% butyl: 5% methyl). The double embedding prevents swelling that occurs when this material is embedded in methacrylate only.

Ultrathin sections of both experimental and control methacrylate blocks were cut with a glass knife and floated on a nearly saturated aqueous calcium phosphate solution, since it had been observed that the "histochemical" calcium phosphate very rapidly leached out when the sections were floated on water. The sections were then mounted on single slit Philips silver specimen mounts and viewed with the Philips electron microscope (Model EM 100). Photographs were taken on ADOX KB 14 film with an initial magnification of 5,000 or 10,000 times at the screen. The prints were enlarged 5×.

EXPERIMENTAL

In comparison with the pictures of the ATP-free controls, the images of the specimens incubated in ATP, $CaCl_2$ and cysteine show increased density in sharply defined areas within the flagella (Fig. 1a, 1b). The nine outer axial fibers appear black. Under the conditions of these experiments, the two central fibers are not visible, and the nine smaller fibers of the inner axial bundle cannot be distinguished, but appear to be fused to the central apexes of the corresponding outer fibers. Even after 2½ minutes incubation, the outer longitudinal fibers are clearly differentiated from their surroundings, but appear more densely stained after 5 and 10 min in the ATP, while at 30 min, there is a slight gradient of darkening in the interfibrillar matrix around each fiber. There is little, if any, staining of the helix.

In the ATP-free control (Fig. 2), all the structures visible in the experimentals may also be seen, due to the natural, electron-scattering properties of the flagellar components. However, the fibers are relatively light in appearance.

The procedure had been designed not only to preserve high enzyme activity,

* Cysteine added to augment ATP-ase activity[15] and to suppress alkaline phosphatase[16].

References p. 641.

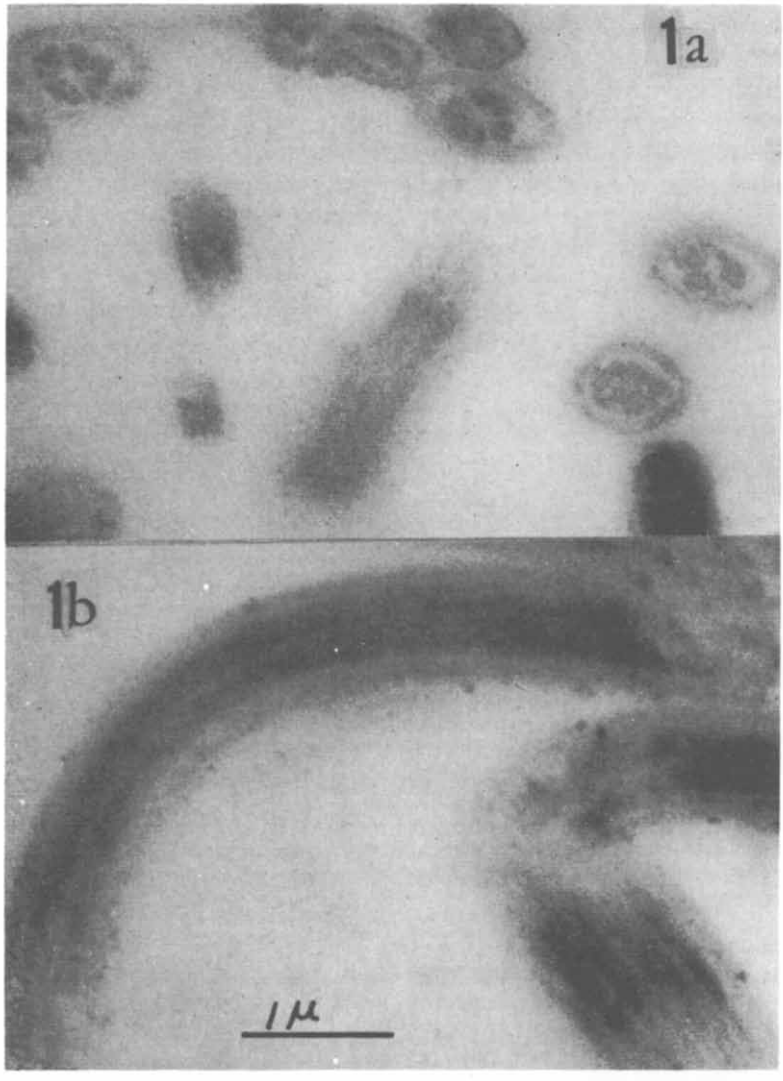

Fig. 1a. Transverse sections of rat epididymal sperm. Incubated in pH 9 buffer containing ATP, $CaCl_2$ and cysteine, 5 min at room temperature. The 9 outer fibers are densely "stained" compared with ATP-free control, (Fig. 2). × 20,000.

Fig. 1b. Longitudinal sections of same specimen as 1a. × 20,000.

but also to minimize possible errors arising through diffusion. Under somewhat similar conditions of incubation, BARTER *et al.*[12], using an interference microscope, concluded that in histochemical preparations at least 97.5% of the phosphate is precipitated as an insoluble calcium salt at the site of dephosphorylation, and once precipitated, does not redissolve in the incubation medium. However, it has been noted that the calcium phosphate precipitate does leach out when the sections are floated on distilled water. The reverse situation was also investigated, namely would

References p. 641.

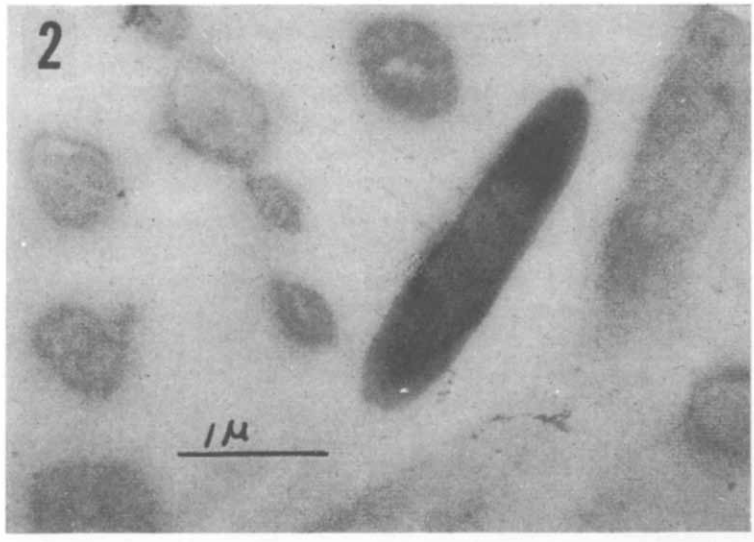

Fig. 2. ATP-free control. Sperm incubated 5 min in pH 9 buffer containing $CaCl_2$ and cysteine, but no ATP. No darkening of any of the flagellar components. × 20,000.

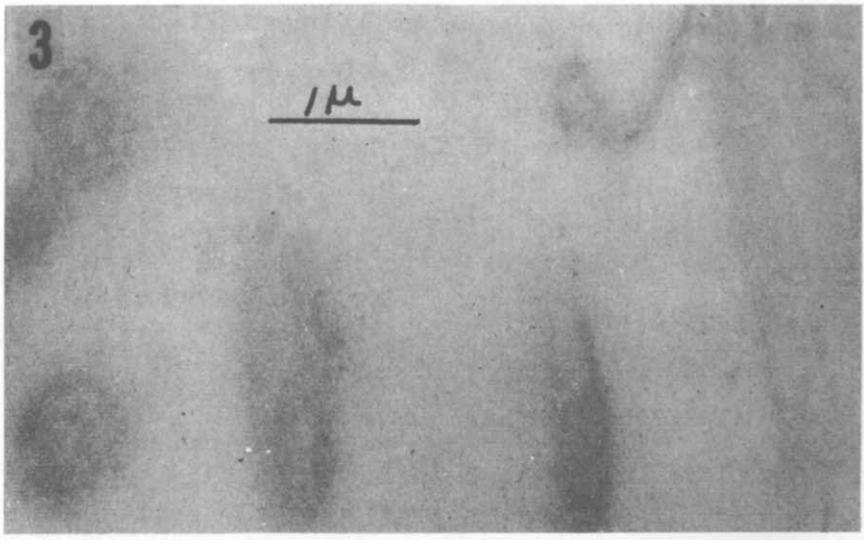

Fig. 3. Sections of rat sperm after soaking in saturated solution of calcium phosphate for 5 min at room temperature. None of the calcium phosphate from the medium has adsorbed onto the fibers. × 20,000.

preformed calcium phosphate in the medium selectively adsorb onto the fiber surfaces? Frozen-dried blocks of the sperm were soaked in a saturated solution of calcium phosphate for up to 30 min, and then dehydrated and embedded in the usual fashion. Sections cut from these preparations appeared light and unstained, virtually identical in appearance to the ATP-free controls (Fig. 3).

Diffusion of the enzyme was tested by soaking the blocks in buffer for 10 min.

References p. 641.

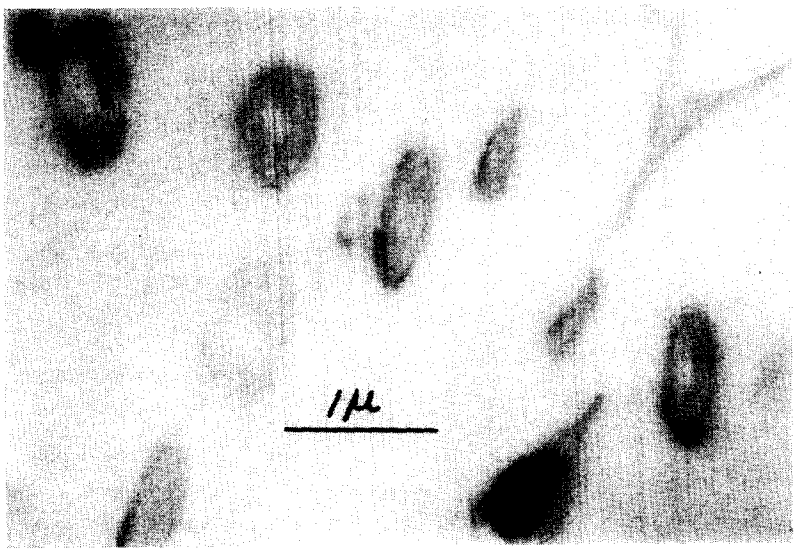

Fig. 4. Calcium-free control. Sperm incubated 10 min in pH 9 buffer containing ATP and cysteine but no $CaCl_2$. Apparently no complex formation of sufficient duration to enhance contrast. × 20,000.

The ATP-ase activity of the supernatant was compared with that of the homogenized, resuspended residue. Ten minutes after the addition of ATP to these preparations, the reactions were terminated with 10% trichloroacetic acid. Enzymically liberated inorganic phosphate was determined by the TAUSSKY AND SHORR[17] method in a Coleman Junior Spectrophotometer. While considerable quantities of phosphate were found in the homogenate, that present in the supernate of the intact sperm was below the limit of sensitivity of the method (2 μg inorganic phosphorus). It is pertinent to note in this context, that while it was customary to initiate the incubations in ATP within 2 min after buffer-infiltration of the frozen-dried specimens, occasionally as much as 15 min intervened. In electron micrographs of these samples, the axial fibers appear as sharply defined as in the standard preparations. It, therefore, appears unlikely that the enzyme could have diffused from other sites to be adsorbed on the fibers.

Should ATP combine with ATP-ase in an enzyme–substrate complex of more than momentary duration, or with some other fibrillar component, it is conceivable that the mass of the fibers would be increased measurably. This could be sufficient to account for the observed contrast without much contribution from the insoluble calcium phosphate. Blocks of the sperm were placed in a buffered substrate medium containing ATP and cysteine, but no calcium. Again, the electron micrograph (Fig. 4) is strikingly similar to that of the ATP-free control in that none of the flagellar structures show any darkening.

Another type of preparation tended to confirm the lack of diffusion of either the enzyme or enzyme-reaction product, and at the same time served to establish some degree of substrate specificity of the reaction. Frozen-dried sperm were incubated in a cysteine-free medium buffered at pH 9, in which glycerophosphate was substituted for ATP. This alkaline phosphatase method likewise depends on the precipitation of calcium phosphate. However, the distribution of the precipitate was distinctly

References p. 641.

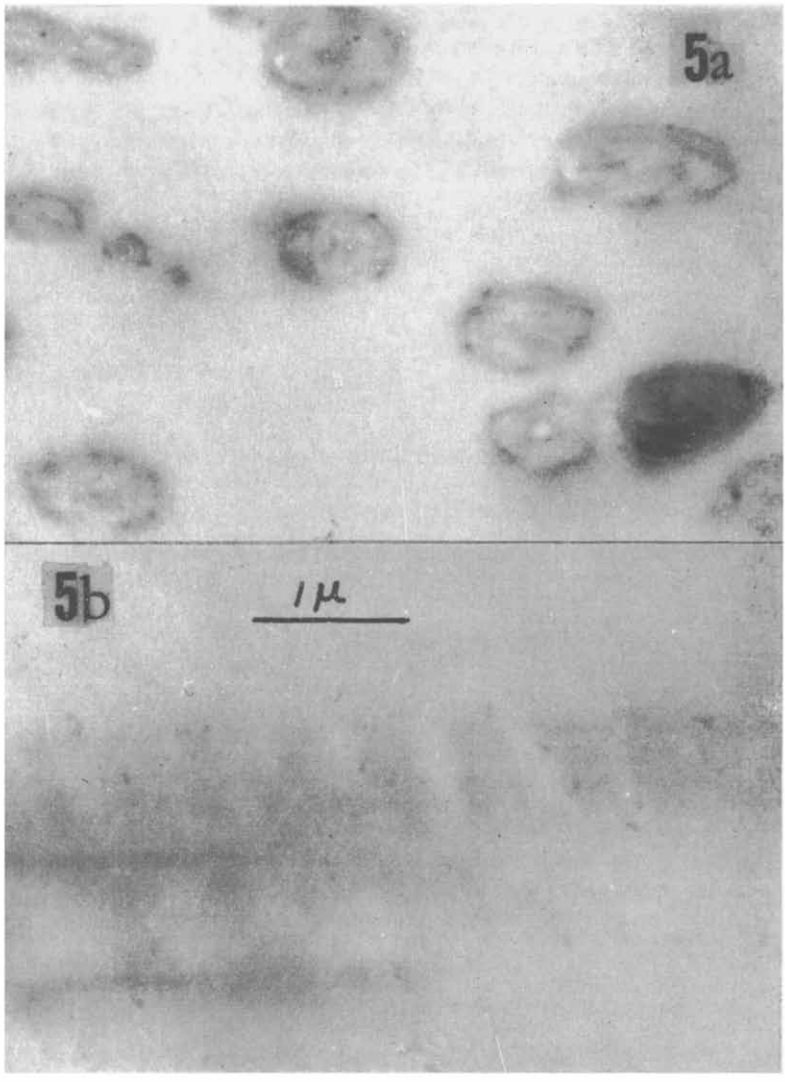

Fig. 5b. Rat sperm incubated in pH 9 buffered glycerophosphate, 5 min. Note relative darkening of the cortical helix, appearing as oblique bands in longitudinal sections. × 20,000.

Fig. 5a. Transverse sections of same specimen as in 5b. × 20,000.

different (Fig. 5a, 5b). There is apparently little activity within the fibrils, while the spiral sheath shows increased density. The intensity of the reaction was somewhat less than that of ATP-ase, suggesting a possible difference in the concentration of these enzymes.

DISCUSSION

If sperm motility is dependent upon energy liberation from such compounds as ATP, the energy-conversion system should be intimately associated with appropriate

References p. 641.

structures within the flagellum. The longitudinal fibers are the submicroscopic organelles which are strategically situated, and which, even if they have been implicated only by indirection as the contractile components, possess the necessary attribute of catalyzing the dephosphorylation of ATP. Since the undulatory wave passes along much of the flagellum with unreduced amplitude, the dissipated energy must continually be replaced[18], and so the enzymes capable of liberating the energy and of replenishing the energy-rich substances should, perhaps, be coextensive along the length of the flagellum. By means of suitable cytochemical reactions, it has been possible to demonstrate in the electron microscope the presence of at least one of the enzymes which meets these requirements. This confirms an earlier observation[8] that, in the fragmented bull spermatozoon, the ATP-dephosphorylating enzyme is concentrated in the flagellum, and is apparently distinct from other types of phosphoesterases.

In the electron micrographs, not only is it evident that ATP-ase is present in each fiber of the outer axial cylinder, but also that the enzyme occurs along the whole length of the fiber. In addition, the electron micrographs indicate that, in rat sperm, the ATP-ase distribution is distinct from that of alkaline phosphatase.

When the substrate is glycerophosphate, calcium phosphate is deposited at different sites from those when ATP is split. At once, this points to the specific nature of the fibrillar enzyme, the lack of diffusibility of the enzymes (at the 100 A level), and the fact that calcium phosphate deposition in the fibers is not the fortuitous result of the diffusion and adsorption of the enzyme reaction product. Moreover, the glycerophosphatase distribution at certain regions of the flagellar surface (coinciding with some component of the helix) is of interest in terms of speculation on its possible role in fructose transport, in view of the high degree of permeability of the flagellum of intact sperm[19].

Since each of the nine larger flagellar fibers which comprise the outer fiber cylinder appears to contain the ATP-ase, and the enzyme activity is detectable the length of the flagellum, this virtually confirms the dependence of flagellar contraction (or relaxation) on ATP-splitting. This distribution could subserve the enzymic basis for the undulatory wave, resulting from the sequential propagated localized contractions of each of the large fibers[4]. Such an organization, presumably, would provide sufficient time for recovery in each fiber, and permit initiation of subsequent contraction waves, before the preceding waves have completed their transit down the flagellum. Therefore, it may not be necessary to invoke a spring-like antagonistic action of the cortical helix to transverse stretching and compression as the wave passes[2, 20], in view of the fact that a tail helix is apparently lacking in fowl sperm[21], unless the evolution of the helix was of adaptive significance for mammalian sperm. These conclusions are in accord with the mechanism proposed by BRADFIELD[4], and also with HOFFMANN-BERLING's observations that ATP initiates spontaneous rhythmic contractions in glycerol-extracted sperm tails[22].

The mechanism underlying the initiation and coordination of the contraction wave remains to be explored. At present, studies are in progress on the flagellar distribution of succinic dehydrogenase, one of the enzymes probably involved in the oxidative regeneration of ATP.

References p. 641.

ACKNOWLEDGEMENTS

The author wishes to express his thanks to Mrs. F. MANELIS and to Mrs. E. VILKAS for their technical assistance, and also to Dr. I. GERSH for his discussions and criticism during the course of this work.

SUMMARY

The cytochemical localization of adenosinetriphosphatase in the frozen-dried preparation of rat epididymal sperm is described. ATP-ase is confined to the nine longitudinal fibers of the outer axial fiber bundle of the flagellum. By means of appropriate controls it has been possible to show that neither the enzyme nor the enzyme reaction product, calcium phosphate, diffuses during the brief incubations.

Specimens incubated in ATP-free buffer containing calcium chloride, in Ca-free buffer containing ATP, as well as blocks of sperm soaked in saturated aqueous calcium phosphate, show no darkening of the fibers in contrast to specimens incubated in the complete medium. In none of these preparations is there any darkening of the cortical helix; however, when glycerophosphate is substituted for ATP, regions of the helix do show signs of deposition of calcium-phosphate, whereas the fibers do not show any increase in density.

REFERENCES

[1] LORD ROTHSCHILD, *Mammalian Germ Cells, Ciba Foundation Symposia*, Little, Brown and Co., Boston, 1953, p. 122.
[2] C. E. CHALLICE, *J. Roy. Microscop. Soc.*, 73 (1953) 115.
[3] M. L. WATSON, *Biochim. Biophys. Acta*, 10 (1953) 1.
[4] J. R. G. BRADFIELD, *Fibrous Proteins and Their Biological Significance, Symposia Soc. Exptl. Biol.* 9 (1955) 306.
[5] J. R. G. BRADFIELD, *Quart. J. Microscop. Sci.*, 94 (1953) 351.
[6] H. H. WEBER, *Fibrous Proteins and Their Biological Significance, Symposia Soc. Exptl. Biol.*, 9 (1955) 271.
[7] V. A. ENGELHARDT, *Advances in Enzymol.*, 6 (1946) 147.
[8] L. NELSON, *Biochim. Biophys. Acta*, 14 (1954) 312.
[9] L. NELSON, *Biol. Bull.*, 109 (1955) 295.
[10] H. A. PADYKULA AND E. HERMAN, *J. Histochem. and Cytochem.*, 3 (1955) 161.
[11] G. GOMORI, *J. Cell. Comp. Physiol.*, 17 (1941) 71.
[12] R. BARTER, J. F. DANIELLI AND H. G. DAVIES, *Proc. Roy. Soc. B.*, 144 (1955) 412.
[13] N. M. HANCOX AND E. NICHOLAS, *Acta Anat.*, 26 (1956) 302.
[14] I. GERSH, I. ISENBERG, J. STEPHENSON AND W. BONDAREFF, *Anat. Record*, 128 (1957) 91.
[15] T. P. SINGER AND E. S. G. BARRON, *Proc. Soc. Exptl. Biol. Med.*, 56 (1944) 120.
[16] J. J. GORDON, *Biochem. J.*, 51 (1952) 97.
[17] H. H. TAUSSKY and E. SHORR, *J. Biol. Chem.*, 202 (1953) 675.
[18] J. GRAY, *Ciliary Movement*, Cambridge Univ. Press, Cambridge, 1928.
[19] T. MANN, *The Biochemistry of Semen*, Methuen & Co., Ltd., London, 1954.
[20] L. H. BRETSCHNEIDER AND W. VAN ITERSON, *Proc. Koninkl. Ned. Akad. Wetenschap.*, 50 (1947) 88.
[21] A. J. HODGE, *Australian J. Sci. Research, Ser. B*, 2 (1949) 368.
[22] H. HOFFMANN-BERLING, *Biochim. Biophys. Acta*, 14 (1954) 182.

Received July 19th, 1957