Morphological and Histochemical Studies of the Chromatoid Body in the Grass-snake, *Natrix natrix*

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With two plates (figs. 1 and 2)

Summary

The chromatoid body in the spermatogenesis of the grass-snake, *Natrix natrix*, has been studied by the use of phase-contrast microscopy, vital dyes, and histochemical tests.

It first appears during the growth of the primary spermatocyte and is also seen in the secondary spermatocyte and late spermatid, but is absent at metaphases of both the maturation divisions, in the early spermatid, and during the final stages of spermateleosis. It does not make any visible contribution to the final make-up of the spermatozoon.

In living cells it gives a very low phase change, and is not stained by neutral red or Janus green. The histochemical study reveals that it consists mainly of RNA and of proteins with abundant acidic and basic groups.

It is tentatively suggested that its function is to provide basic proteins for the final maturation of the chromatin in the nucleus of late spermatid.

Introduction

A CHROMATOID body was for the first time described in a reptile by myself in a study of the spermatogenesis of an Indian snake, *Natrix piscator* (Sud, 1955). Painter (1921) had previously figured it in the normal and giant spermatids of a lizard, but he neither labelled it nor mentioned it in the text. Fox (1952) depicted it in the late spermatids of the garter snake, *Thamnophis* sp., but wrongly named it 'acroblast'. I recorded its presence in *Natrix* in the primary spermatocyte and also in the late spermatid, just after the condensation of the nucleus. In the primary spermatocyte it is rounded or ovoid; in the spermatid at the time of its reappearance it is dumbell-shaped and later spherical. At both stages of spermatogenesis it is a large, homogeneous body. In the primary spermatocyte an additional smaller chromatoid body and in the late spermatid two or three very small chromatoid bodies are sometimes present. In the late stages of spermateleosis the chromatoid body moves forward towards the anterior end and disappears.

In *N. piscator* the chromatoid body appeared faint in living cells under phase contrast (Sud, 1955). It was resistant to Bouin fixation. It was not coloured by Sudan black nor darkened by silver and long osmication techniques, but it stained deeply with iron haematoxylin and with acid fuchsine. I concluded that it appeared to arise in the cytoplasm independently of any other inclusion. Similar chromatoid bodies occur in the freshwater turtle,

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Lissemys punctata (Sud, 1956, 1958), and the lizard, *Uromastix hardwickii* (Sud, 1957, 1958).

The fact that the chromatoid body of reptiles appears and disappears during spermatogenesis without making any visible contribution to the final make-up of the spermatozoon remains an enigma. A similar situation exists in mammals, birds, cyclostomes, Crustacea, insects, and arachnids. The accounts given by different authors of the chromatoid body in different species and sometimes even in the same species are diverse. This has led prominent workers, among them Duesberg (1908), the Schreiners (1908), Wilson (1925), and Gresson (1951), to doubt whether all the bodies named as such are of the same nature. To avoid this confusion and a perpetual state of doubt I considered it necessary to undertake a histochemical study of the chromatoid body in different groups. It is hoped that this study combined with electron microscopy may help us to formulate criteria for its identification and to trace its origin and physiological significance. The present communication is the first of the series dealing with this subject.

Material and Methods

Specimens of the grass-snake, *N. natrix* L., were obtained from L. Haig & Co., Beam Brook, Newdigate, Surrey, during different seasons, and kept in a terrarium for periods up to one month. This animal provides ideal material for the study of the chromatoid body because of the large size of this inclusion in its spermatocytes as well as spermatids. In the spermatid of the snake the chromatoid body appears during the late stages of spermateleosis, which occur in the early winter months when the testis is generally devoid of spermatocytes. The spermatocytes are most abundant during late winter and early spring. So, for the study of the chromatoid body in the spermatocytes as well as spermatids, every test had to be done twice with an interval of a couple of months.

The animals were killed by passing an awl through the brain.

For study of the living material, the testes on removal from the animal were

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Fig. 1 (plate). Sections of the testis of *N. natrix*.

A, formaldehyde/calcium followed by AH test; the labelled cell is a primary spermatocyte showing a AH-negative chromatoid body adjacent to the nucleus (*n*), AH positive nucleolus (*nl*).

B, formaldehyde/calcium followed by AH test; the labelled cell is a late spermatid with AH-positive chromatoid body, nucleus (*n*), and acrosome (*a*).

C, AH test after pyridine extraction; primary spermatocytes at the periphery of the tubule showing AH-positive chromatoid body adjacent to the nucleus (*n*) containing AH-positive nucleolus (*nl*); chromatoid bodies, not in sharp focus, indicated by arrows.

D, AH test after pyridine extraction; late spermatids showing AH-positive chromatoid body indicated by arrows; nucleus (*n*) and acrosome (*a*) are also AH-positive.

E, Sanfelice / basic fuchsine; note in the spermatocytes basiphil chromatoid body; nucleus (*n*); basiphil nucleolus (*nl*).

F, Sanfelice / basic fuchsine; late spermatids; note the basiphil chromatoid body and nucleus (*n*); acrosome (*a*).

G, Zenker / crystal-violet; two tubules each showing in focus a primary spermatocyte with a basiphil chromatoid body; nucleus (*n*).

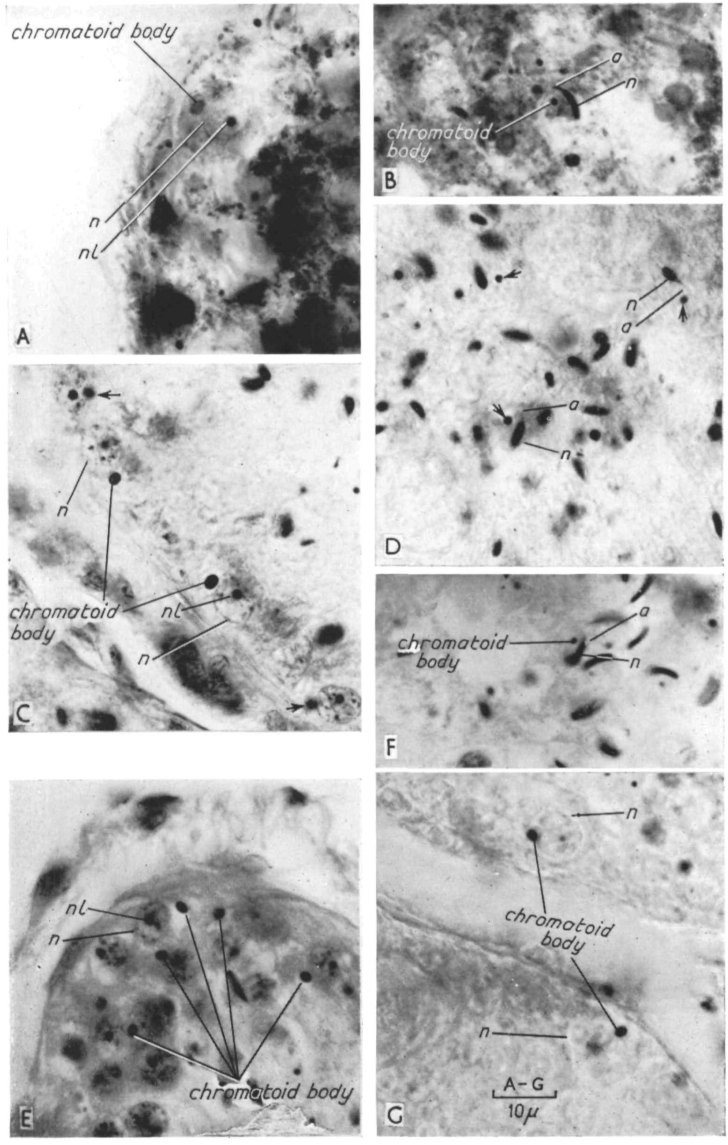
chromatoid body
n
nl
A
chromatoid body
a
n
B
n
a
a
n
D
n
chromatoid body
nl
n
C
chromatoid body
a
n
F
nl
n
chromatoid body
E
n
chromatoid body
n
A-G
10μ
G

FIG. 1

B. N. SUD

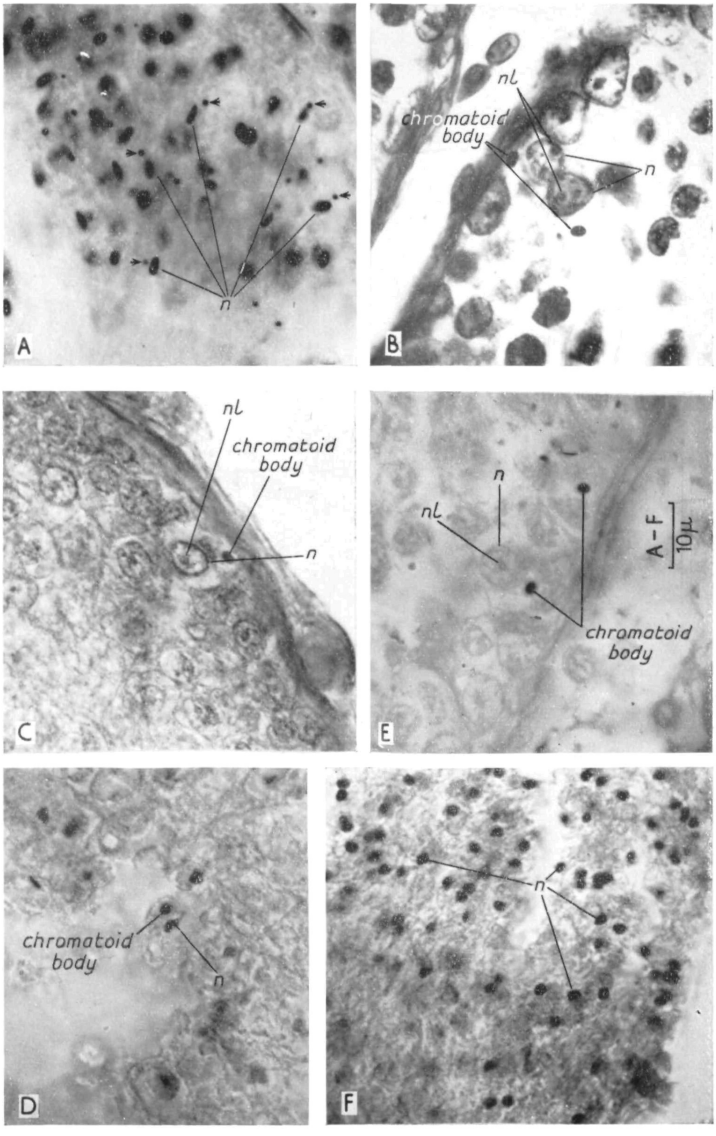
A
n
B
nl
chromatoid body
n
C
nl
chromatoid body
n
D
chromatoid body
n
E
n
nl
A-F
10μ
chromatoid body
F
n

FIG 2.

B. N. SUD

at once transferred to 0·7% sodium chloride solution, to every 100 ml of which 0·2 ml of 10% anhydrous calcium chloride had been added (Baker, 1944). A small piece of testis tubule was teased and mounted in the same solution and studied unstained by phase-contrast microscopy.

Living material was also dyed supravitally with neutral red and with Janus green. In both cases 0·1 ml of a 0·5% aqueous stock solution of the dye was diluted with 5 ml of the saline solution mentioned in the preceding paragraph. A very small piece of testis tubule was dyed in the diluted solution for 5 to 15 min and later teased and studied by direct microscopy.

For fixation, the testes immediately on removal from the animal were divided into small pieces by a sharp razor, and the pieces directly transferred to various fixing fluids. The material was embedded in either paraffin wax, collodion, or gelatine for different histochemical tests, as detailed in the appendix (p. 56).

For deamination a freshly prepared mixture of equal volumes of 0·1 N hydrochloric acid and 0·1 N sodium nitrite was used for 10 min at room temperature (Chayen & others, 1959). More often a stronger deamination mixture, consisting of 2 volumes of concentrated aqueous sodium nitrite solution, 1 volume of glacial acetic acid, and 5 volumes of distilled water, was used for 12 h at room temperature. This mixture of van Slyke was introduced into histochemistry by Monné and Slautterback (1950). The preparations were thoroughly washed after treatment with deaminating fluids. Deaminated and control preparations were subjected to exactly the same treatment.

Observations

Morphological studies

The chromatoid body in *N. natrix* first appears during the growth of the primary spermatocyte (figs. 1, C, E, G; 2, B, C). It disappears at metaphase of the first maturation division and is again seen in the secondary spermatocyte. It disappears again at the metaphase of the second maturation division and is not present in the early stages of spermateleosis. It reappears during the condensation and elongation of the spermatid nucleus (figs. 1, B, D, F; 2, A, D), and finally disappears during the ripening of the spermatozoon. It seems to appear and disappear as a whole body, without showing any sign of

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Fig. 2 (plate). A–E, sections of testis, and F, of vas deferens of *N. natrix*.

A, Zenker / crystal violet; late spermatids; note the basiphil chromatoid body (indicated by arrows) and nucleus (*n*).

B, Zenker / azocarmine; spermatocytes; note the acidophil chromatoid body and nucleolus (*nl*); nucleus (*n*).

C, Sakaguchi test; the labelled cell is a primary spermatocyte showing a positive chromatoid body and nucleolus (*nl*); nucleus (*n*).

D, Sakaguchi test; the labelled cell is a late spermatid in the lumen of a tubule; it shows a positive chromatoid body and nucleus (*n*).

E, deamination by van Slyke's reagent / azocarmine; spermatocytes with chromatoid body showing intense acidophilia and negative nucleolus (*nl*); nucleus (*n*).

F, vas deferens after Sakaguchi test; note the intensely positive nuclei (*n*) of spermatozoa.

fragmentation, and does not make any recognizable contribution to the mature spermatozoon.

In the spermatocytes the chromatoid body is of ovoid or subspherical form, measuring up to 3 by $2\frac{1}{2}$ μ; in the spermatids it is always spherical and is up to 1·8 μ in diameter. In both spermatocytes and spermatids it is homogeneous.

In the living cell, under phase contrast, it gives a very low phase-change. In the spermatid it gives a slightly higher phase change than in the spermatocyte, though it is much smaller. Thus there is a higher proportion of solid material in the chromatoid body of the spermatid.

Neutral red and Janus green, used supravitally, failed to dye the chromatoid body in both spermatocytes and spermatids.

Histochemical studies

From the tests listed in the appendix on page 56 it is evident that the chromatoid body does not contain lipid. If there were any solid lipid it would have coloured with Sudan black used at 60° C or given a positive Liebermann's reaction. There does not appear to be any masked lipid either, since Sudan black does not colour the chromatoid body in material treated with such strong splitting agents as cadmium chloride (Clayton, 1959) in Aoyama, or chromic acid (Bradbury & Clayton, 1958) in Flemming's fluid, Lewitsky-saline, or Lewitsky's chromium trioxide / formaldehyde. Negative results by silver and long osmication techniques, and by Rawitz's 'inversion staining' technique also suggest the absence of lipid.

The acid haematein test shows a difference of an unexplained kind between the chromatoid body of the spermatocytes and that of the spermatid. The former gives a negative reaction to the AH test itself but a positive reaction to the pyridine extraction test, while the latter gives a positive reaction to both (fig. 1, A–D).

There is no DNA in the chromatoid body. The intense basiphilia of the body (figs. 1, E–G; 2, A) is due mainly to its high content of RNA, but probably also in part to acidic groups in protein.

The chromatoid body stains readily with all the acid dyes tried (fig. 2, B) and the acidophilia indicates the presence of basic proteins. The intense positive Sakaguchi reaction (Baker, 1947) shows the presence of protein-bound arginine (fig. 2, C, D). Free arginine, if present, would probably be removed from the tissue during fixation and dehydration.

After deamination the chromatoid body gives a negative Sakaguchi reaction but there is no change in its intense acidophilia (fig. 2, E). It is clear that the acid dyes must be reacting with basic groups other than $—NH_2$.

The negative result with the alkaline fast green reagent of Alfert and Geschwind (1953) suggests the absence of histones. Protamines, if present, would be removed during treatment with hot trichloracetic acid for the necessary removal of nucleic acids for this technique (Alfert, 1956). The negative

result with alkaline fast green suggests that the arginine occurs mostly as a component of protamine rather than histone.

There is little or no tyrosine in the chromatoid body. Tests for carbohydrates, alkaline phosphatase, and calcium also give negative results.

The evidence suggests that the chromatoid body consists mainly of RNA and of proteins with abundant acidic and basic groups.

Discussion

Protamines consisting predominantly of arginine residues replace the histones of chromatin during spermatogenesis. Fig. 2, F shows the strong reaction of the nuclei of the ripe spermatoza of *Natrix* with the Sakaguchi reaction. It appears that in the salmon this change takes place rather abruptly at a late stage of spermateleosis, when the nucleus begins to elongate (Alfert, 1956).

In *Natrix* the chromatoid body reappears during spermateleosis at the time when the characteristic change in the nucleoprotein of the spermatid probably takes place. Further, the chromatoid body in *Natrix* is characterized by the presence of basic proteins rich in arginine and by the absence of histones. Thus the protein constituents of the sperm nucleus and of the chromatoid body are remarkably similar.

The histochemical reactions of the chromatoid body are very similar to those of the nucleolus (see appendix). The abundance of RNA and proteins in the chromatoid body may perhaps indicate that it acts as a centre for protein synthesis. It may be tentatively suggested that it is the function of this body to provide basic proteins for the final maturation of the chromatin in the nucleus of late spermatids.

I am grateful to Dr. J. R. Baker, F.R.S., for teaching me histochemistry and supervising this investigation, and to Professor Sir A. C. Hardy, F.R.S., for providing me research facilities in his Department. I wish to thank Mrs. B. M. Luke and Miss Elizabeth Collins for their valuable technical help. I am greatly indebted to the Vice-Chancellor, University of the Panjab (India), for the grant of study-leave.

APPENDIX

A summary of the histochemistry of the chromatoid body and nucleolus in Natrix natrix

Tests applied					*Results obtained*		
					Chromatoid body		
Test	*Fixation*	*Embedding medium*	*Thickness of section in μ*	*Reference*	*In spermatocytes*	*In spermatids*	*Nucleolus in spermatocytes*
Natural colour					Colourless		
Neutral red	living tissue				O	O	O
Janus green	living tissue				O	O	O
Sudan black (RT; 60° C)	F/S+PC	G	10	Baker, 1944, 1949	O	O	O
Sudan black (RT; 60° C)	F/Ca+PC	G	10	Baker, 1946	O	O	O
Sudan black (RT; 60° C)	WB+PE	G	10	Baker, 1946	O	O	O
Sudan black (RT; 60° C)	Aoy+PC	G	10	Baker (unpublished)	O	O	O
Sudan black (RT; 60° C)	Cr/F+PC	G	10	Lewitsky, 1931	O	O	O
Sudan black (RT; 60° C)	Flm+PC	G	10	Bradbury & Clayton, 1958	O	O	O
Sudan black (RT; 60° C)	LS	G	10	Baker, 1956*a*	O	O	O
Acid haematein	F/Ca+PC	G	10	Baker, 1946	O	+++	+ to +++
Acid haematein with pyridine extraction	WB+PE	G	10	Baker, 1946	+++	+++	O to ++
Acid haematein	Aoy+PC	G	10	Baker (unpublished)	O	+++	O
Acid haematein	Cr/F+PC	G	10	Chayen & others, 1959	+++	+++	+++
Acid haematein	Flm+PC	G	10	Bradbury & Clayton, 1958	O	+++	O to +++
Liebermann	F/Ca; F/Ca+PC	G	10	Lison, 1953	O	O	O
Silver impregnation	Aoy	P	6	Aoyama, 1929	O	O	O
Weigl (Mann–Kopsch)	Mann PO	P	2	Weigl, 1910	O	O	O
Kolatchev	Champy PO	P	2	Kolatchev, 1916	O	O	O
Baker (Hermann–Kopsch)	Hermann PO	P	2	Baker, 1957	O	O	O
Rawitz	Flm	P	6	Baker, 1959; Przełęcka, 1959	O	O	O
Toluidine blue for metachromasy	Z	P	10	Baker (unpublished)	O	O	O
Periodic acid / Schiff	Z	P	6	McManus, 1948; Pearse, 1954	O	O	O
Feulgen	Z; Cl	P	8; 16	Feulgen & Rossenbeck, 1924	O	O	O
Pyronin / methyl green	Z 3 h	P	7; 16	Jordan & Baker, 1955	+++	+++	++
P/MG after trichloracetic acid	Z 3 h	P	7; 16	Pearse, 1954	++	++	+
P/MG after ribonuclease	Z 3 h	P	7; 16	Bradbury, 1956	O	O	O
Basic fuchsine (0·5% aq., RT) for basiphilia	S; Flm	P	6	—	+++	+++	+++
Safranine (0·5% aq., RT) for basiphilia	Z 3 h; Flm	P	6	—	+++	+++	+++

Methyl green (1% aq. RT) for basiphilia	Z 3 h	P	6	—	O	O	O
Methyl green (1% aq. 50° C) for basiphilia	Z 3 h	P	6	—	+	+	+
Newton's crystal violet	Z 3 h	P	14	Baker & Jordan, 1953	+++	+++	O to +
Newton's crystal violet	S	P	14	Baker & Jordan, 1953	++	++	+ to ++
Nissl stain (cresyl violet)	Z 3 h	P	6	Fernstrom, 1958	+++	+++	+++
Iron haematoxylin	Z 3 h; Flm	P	6	—	+++	+++	+++
Acid fuchsine (1% aq., RT) for acidophilia	Z 3 h	P	8	—	+++	+++	+++
Eosin (1% aq., RT) for acidophila	Z 3 h	P	6	—	++	++	++
Metzner	Alt; Flm; H+PC	P	6; 3	Metzner & Krause, 1928	+++	+++	+++
Azocarmine (2% aq., acidified, 50° C)	Z 3 h; Cl	P	6	Gatenby & Beams, 1950	+++	+++	+++
Deamination by van Slyke reagent / azocarmine (2% aq., acidified, 50° C)	Z 3 h; Cl	P	6	Monné & Slautterback, 1950	+++	+++	O
Xylidine red (0·1% in 1% acetic acid, RT)	Z 3 h; Cl	P	6	—	++	++	++
Deamination by van Slyke reagent / xylidine (0·1% in 1% acetic acid, RT)	Z 3 h; Cl	P	6	Monné & Slautterback, 1950	++	++	O
Sakaguchi	Z	P	10	Baker, 1947	+++	+++	+ to ++
Deamination by van Slyke reagent / Sakaguchi	Z	P	10	Monné & Slautterback, 1950; Baker, 1947	O	O	O
Hg/nitrite	F/S	C	24	Baker, 1956*b*	O	O	O
Coupled tetrazonium reaction	F/S	P	16	Danielli, 1947, 1950; Pearse, 1954	O	O	O
Alkaline fast green method	F	P	16	Alfert & Geschwind, 1953	O	O	O
Gomori's for alkaline phosphatase	Alc/Acet	P	8	Gomori, 1952	O	O	O
Cobalt method for calcium	F/M/P	P	10	As in alkaline phosphatase test used by Danielli (1946)	O	O	O

KEY: Alc/Acet = absolute alcohol and acetone in equal volumes; Alt = Altmann's fluid; Aoy = Aoyama's fluid; aq. = aqueous; C = collodion; Cl = Clarke's (Carnoy's) fluid; Cr/F = chromium trioxide/formaldehyde; F = formaldehyde; F/Ca = formaldehyde/calcium; Flm = Flemming's fluid; F/M/P = formaldehyde/methanol/pyridine; F/S = formaldehyde/saline; G = gelatine; H = Helly's fluid; LS = Lewitsky-saline; P = paraffin; PC = postchromed; PO = postosmicated; RT = room temperature; S = Sanfelice's fluid; WB+PE = weak Bouin+pyridine extraction; Z = Zenker's fluid; 3 h = 3 hours; +++ = strong reaction; ++ = moderate reaction; + = weak reaction; O = negative reaction.

REFERENCES

ALFERT, M., 1956. J. biophys. biochem. Cytol., **2,** 109.
—— & GESCHWIND, I. I., 1953. Proc. Nat. Acad. Sci., Wash. **39,** 991.
AOYAMA, F., 1929. Z. wiss Mikr., **46,** 489.
BAKER, J. R., 1944. Quart. J. micr. Sci., **85,** 1.
—— 1946. Ibid., **87,** 441.
—— 1947. Ibid., **88,** 115.
—— 1949. Ibid., **90,** 293.
—— 1956*a*. Ibid., **97,** 621.
—— 1956*b*. Ibid., 161.
—— 1957. Ibid., **98,** 425.
—— 1959. J. Roy. micr. Soc., **77,** 116.
—— & JORDAN, B. M., 1953. Quart. J. micr. Sci., **94,** 237.
BRADBURY, S., 1956. Ibid., **97,** 302.
—— & CLAYTON, B.-P., 1958. Nature, **181,** 1347.
CHAYEN, J., GAHAN, P. B., & LA COUR, L. F., 1959. Quart. J. micr. Sci., **100,** 279.
CLAYTON, B.-P., 1959. Ibid., 269.
DANIELLI, J. F., 1946. J. exp. Biol., **22,** 110.
—— 1947. Symp. Soc. exp. Biol., **1,** 101.
—— 1950. Cold Spr. Harb. Symp. quant. Biol., **14,** 32.
DUESBERG, J., 1908. Arch. Zellforsch, **1,** 399.
FERNSTROM, R. C., 1958. Stain Tech., **33,** 175.
FEULGEN, R., & ROSSENBECK, H., 1924. Z. phys. Chem., **135,** 203.
FOX, W., 1952. J. Morph., **90,** 481.
GATENBY, J. B., & BEAMS, H. W., 1950. *The microtomist's vade-mecum* (*Bolles Lee*). London (Churchill).
GOMORI, G., 1952. *Microscopic histochemistry*. Chicago (University Press).
GRESSON, R. A. R., 1951. La Cellule, **54,** 79.
JORDAN, B. M., & BAKER, J. R., 1955. Quart. J. micr. Sci., **96,** 177.
KOLATCHEV, A., 1916. Arch. russes d'Anat. d'Hist. et d'Emb., **1,** 383.
LEWITSKY, G. A., 1931. Bull. appl. Bot. Genet. Plant Breeding, **27,** 175.
LISON, L., 1953. *Histochimie et cytochimie animales*. Paris (Gauthier-Villars).
MCMANUS, J. F. A., 1948. Stain Tech., **23,** 99.
METZNER, R., & KRAUSE, R., 1928. In *Abderhalden's Handbuch der biologischen Arbeitsmethoden*. Abt. V, Teil 2, 1 Hälfte, 325. Quoted from Chou, J. T. Y., 1957, Quart. J. micr. Sci., **98,** 47.
MONNÉ, L., & SLAUTTERBACK, D. B., 1950. Ark. Zool., **1,** 455.
PAINTER, T. S., 1921. J. exp. Zool., **34,** 281.
PEARSE, A. G. E., 1954. *Histochemistry, theoretical and applied*. London (Churchill).
PRZEŁĘCKA, A., 1959. Quart. J. micr. Sci., **100,** 231.
SCHREINER, A., & SCHREINER, K. E., 1908. Arch. Zellforsch., **1,** 152.
SUD, B. N., 1955. Res. Bull. Panjab Univ. India, **75,** 101.
—— 1956. Ibid., **86,** 31.
—— 1957. Ibid., **116,** 345.
—— 1958. Proc. Indian Sci. Congr. Asso. Part III, 384.
WEIGL, R., 1910. Bull. Internat. Acad. Sci. Cracovie, Ser. B, 691.
WILSON, E. B., 1925. *The cell in development and heredity*. New York (Macmillan).