BBA 75629

URANYL SALTS AS FIXATIVES FOR ELECTRON MICROSCOPY

STUDY OF THE MEMBRANE ULTRASTRUCTURE AND PHOSPHOLIPID LOSS IN BACILLI

M. T. SILVA, J. M. SANTOS MOTA, J. V. C. MELO AND F. CARVALHO GUERRA
Centro de Microscopia Electrónica, Centro de Estudos de Bioquímica do I.A.C., and Serviço de Bacteriologia e Parasitologia da Faculdade de Medicina, University of Porto, Porto (Portugal)

(Received November 23rd, 1970)

SUMMARY

1. The influence of postfixation with uranyl salts (acetate or nitrate) on the membrane ultrastructure was studied in some bacilli fixed by the procedure of RYTER AND KELLENBERGER (*Z. Naturforsch.*, 13b (1958) 597).

2. Omission of the postfixation, which had no influence on the preservation of the nuclei, resulted in ultrastructural damage to the membranes.

3. The ethanolic treatment during the dehydration step was found to contribute to this damage to an important degree.

4. The chemical estimation of the phospholipid loss during the dehydration supported the ultrastructural findings.

5. Indium trichloride showed a fixative action similar to that of uranyl salts as determined by electron microscopy.

INTRODUCTION

The conventional techniques for the electron microscopic study of biological material after ultramicrotomy involve fixation with OsO_4, dehydration with organic solvents, and embedding in plastic. The first successful adaptation of these techniques to bacterial cells was reported by RYTER AND KELLENBERGER[1] who outlined the so-called R–K procedure. This procedure includes postfixation with uranyl acetate before dehydration, a treatment originally introduced by STRUGGER[2] to give better contrast. It was shown[1,3] that such postfixation has a stabilizing action on the DNA of the bacterial nucleus as well as on other similar DNA's. Further investigations on this stabilizing effect of uranyl acetate on bacterial DNA were reported by SCHREIL[4], KRAHN AND SCHLOTE[5] and FUHS[6]. Postfixation with uranyl acetate has become a routine technique in the preparation of bacterial specimens. However, no detailed information is available concerning its influence on the preservation of bacterial membranes. Some results on this subject concerning several members of genus *Bacillus* and extending results briefly reported elsewhere[7] are presented here.

Indium trichloride has been described as having a fixative action on DNA, possibly by means of a mechanism similar to that of uranyl salts[4]. Therefore, we

decided also to test the fixative action of indium trichloride on bacterial membranes. Some preliminary results are included.

MATERIALS AND METHODS

Bacterial strains and growth conditions

The following bacteria were studied: *Bacillus subtilis* (NCTC Marburg strain No. 3610), *Bacillus cereus* (NCTC No. 7587), *Bacillus megaterium* KM, *Bacillus anthracis* (NCTC Vollum strain No. 7200), and *Bacillus pasteurii* (NCTC No. 4822). The culture medium consisted of 1 % tryptone (Difco), and 0.5 % NaCl (pH 7.2). For *Bacillus pasteurii* the medium was supplemented with 1.5 % urea. In some cultures sodium [^{32}P]phosphate was added (final concentration, 1 mC/l). Bacteria were grown under aerobic conditions (air bubbling or shaking) at optimal temperatures until the exponential phase was reached.

Electron microscopy

Experiments were carried out after fixation by the RYTER AND KELLENBERGER (R–K) OsO_4 procedure[1,3] with, in the case of *B. cereus*, occasional omission of the prefixation step. Postfixation, when used, was carried out before the dehydration stage by a 30 to 120-min treatment, at room temperature, with one of the following solutions: (a) 0.5 % uranyl acetate (E. Merck) in the R–K veronal–acetate buffer[1] (final pH 5.1) or in water (final pH 3.9); (b) 0.5 % uranyl nitrate (May and Baker) in the R–K veronal–acetate buffer (final pH 4.7) or in water (final pH 3.1); (c) 0.5 % indium trichloride (British Drug Houses) in the R–K veronal–acetate buffer (final pH 3.5) or in water (final pH 2.9). In some samples in which postfixation had been omitted, a treatment with uranyl ions was carried out after dehydration by: (d) reaction of the specimens with ethanolic solutions of uranyl acetate (0.5 %) or uranyl nitrate (0.5 %, 5 %, and 20 %) for 2 h at room temperature; (e) as described under (a) but after rehydration of the dehydrated specimens with aqueous ethanol (90 %, 70 %, 50 %, 30 %, 10 min each) and water (twice, 15 min each). As a control for the postfixations, 0.2 M aceto-acetate buffers (pH 2.9 and 5.1) were used in place of the uranyl or indium solutions. No variation in the pH values was observed during the treatment with these buffers.

Dehydration was performed by 10-min washings in aqueous ethanol (30 %, 50 %, 70 %, and 95 %) followed by four washings, 15 min each, in absolute ethanol. Embedding in Epon was carried out as described by LUFT[8] but usually without infiltration in propylene oxide. Ultrathin sections showing a grey interference colour were obtained with an LKB Ultrotome III fitted with a diamond knife and contrasted with lead citrate[9] for 5 min, or with a saturated aqueous solution of uranyl acetate for 30 min at room temperature, followed by lead citrate for 5 min. Observations were made with a Siemens Elmiskop IA electron microscope, working at 80 kV, with a double condenser and anticontamination system.

Estimation of phospholipid losses

Lipids from the unfixed bacteria or those eventually lost to the OsO_4 fixative, the uranyl postfixative, and the ethanol washings, were extracted as described previously[10]. Lipid extracts were concentrated at 37° under reduced pressure and assayed

for phosphorus[11] or, in experiments with ^{32}P-grown bacteria, for radioactivity using a Tracerlab Compumatic II. To avoid eventual interference of OsO_4 derivatives, in some experiments aliquots of the samples were treated with 6 % H_2O_2 before lipid extraction. A slight increase in the phosphorus values was observed, but it was not found to be significant.

RESULTS

Influence of postfixation on membrane ultrastructure

After the R–K procedure, including the prefixation, followed by a postfixation with uranyl ions (either as acetate or nitrate, in buffer or in water) all the bacteria studied showed an asymmetric and continuous triple-layered cytoplasmic membrane (Figs. 1, CM, and 6) as well as prominent and complex intracytoplasmic membrane systems with the features of the entity usually called the mesosome[12]. They were composed of membranes with the same profile as the cytoplasmic membrane, predominantly assembled in a vesicular configuration (Fig. 1, M). As briefly reported before[13], with *B. cereus* these complex membranous structures were not observed when prefixation was omitted. Instead, small and simple intracytoplasmic membrane systems were found (Fig. 4, S). Postfixation with indium trichloride was compared with the standard uranyl postfixation, using *B. cereus* and *B. subtilis* as test organisms. The results were similar to those obtained with uranyl salts. The density of the mem-

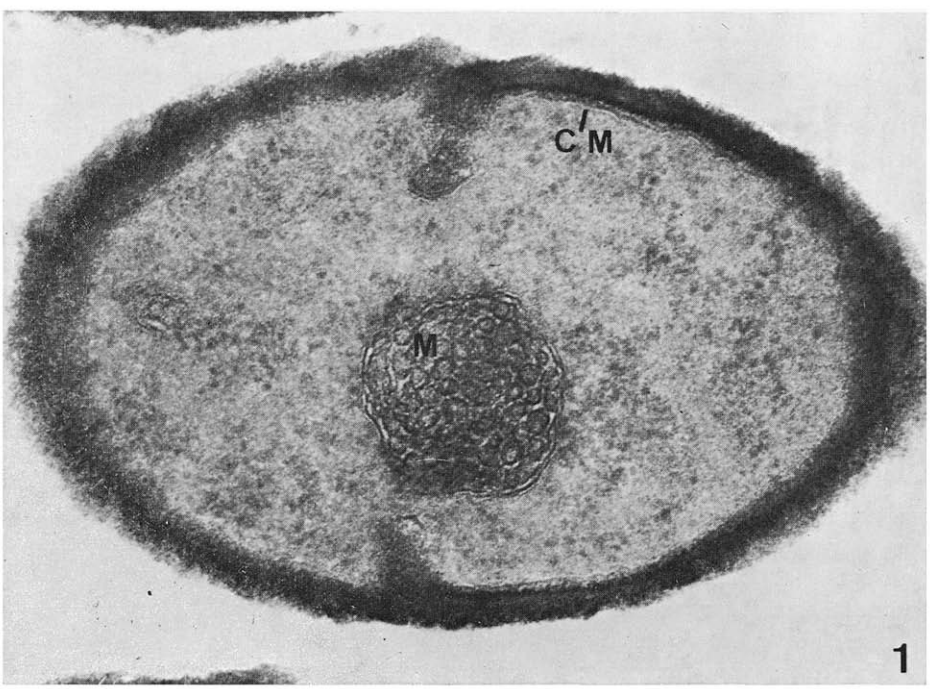
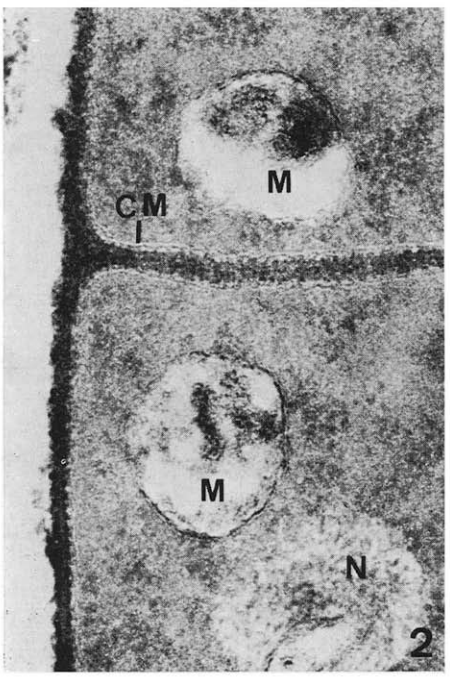


Fig. 1. *B. subtilis* fixed with the R–K OsO_4, including prefixation, and postfixed with uranyl acetate; section contrasted with lead citrate for 5 min. Notice the well-preserved mesosome (M) and the asymmetric cytoplasmic membrane (CM). × 120000.



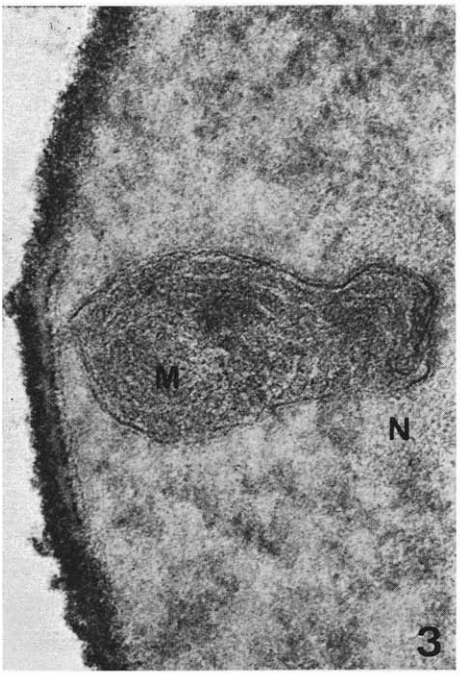


Fig. 2. *B. subtilis* fixed and contrasted as in Fig. 1 but without postfixation. Notice the poorly preserved mesosomes (M), the low density of the outer layer of the cytoplasmic membrane (CM) and the fibrilar nucleus (N). × 120000.

Fig. 3. *B. subtilis* fixed and contrasted as in Fig. 1 but postfixed with indium trichloride. The general preservation of the membranous structures is similar to that shown in Fig. 1. The print was made in hard photographic paper because of the low contrasted membranes. × 120000.

branes was lower than after uranyl postfixation, but the membrane preservation was equally good (Fig. 3). The lower density of the membranes was possibly due to the removal of indium from the specimens by the resin used for embedding[14].

Omission of postfixation resulted in alteration of the membranous structures in all bacteria studied. In most mesosomes, signs of loss of membranous material were detected. These signs were more evident in the vesicules than in the peripheral membrane (Fig. 2). The simple invaginations of the cytoplasmic membrane described above for *B. cereus* when prefixation was omitted, appeared poorly preserved as well (Fig. 5, S). The profile of the cytoplasmic membrane was markedly altered. The alterations consisted mainly of frequent interruptions in the continuity of the triple-layered structure together with a marked reduction in the density of the outer layer (Fig. 7). The alterations in both the cytoplasmic membrane and intracytoplasmic membrane systems were also observed after double staining of the sections with uranyl acetate and lead citrate (Figs. 5 and 8).

Treatment of the unpostfixed specimens with buffers at the extreme pH values of the uranyl and indium postfixatives did not prevent to any detectable extent the alterations in the membranous structures described above.

The nuclei always had the fibrilar appearance which is considered an index of

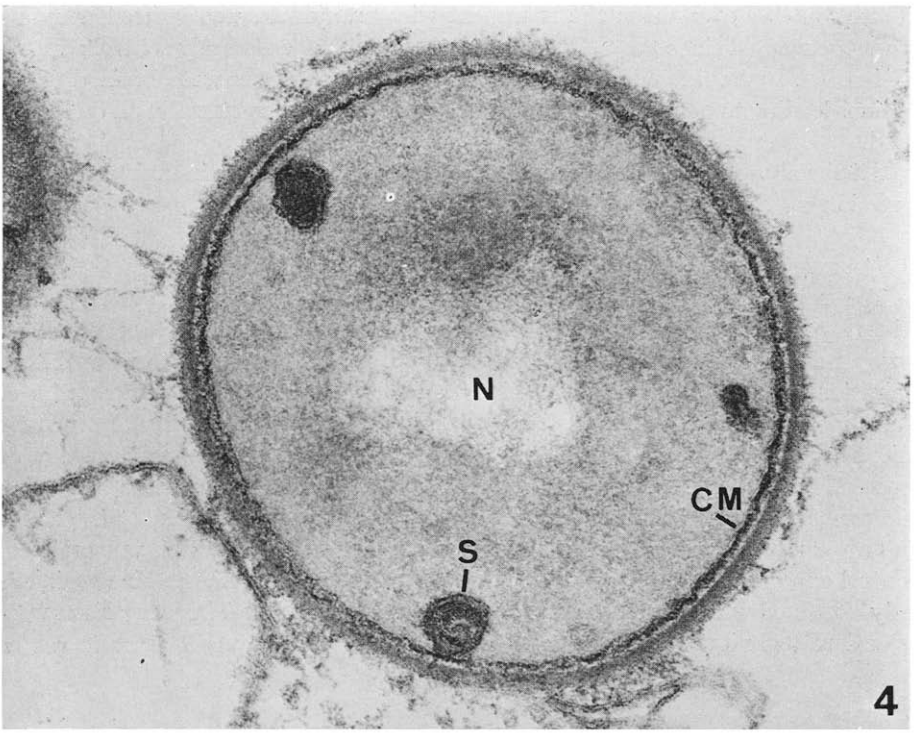


Fig. 4. *B. cereus* fixed with the R–K OsO_4 without prefixation and postfixed with uranyl acetate; section contrasted with lead citrate for 5 min. S, small intracytoplasmic membrane system; CM, cytoplasmic membrane; N, nucleus. × 90000.

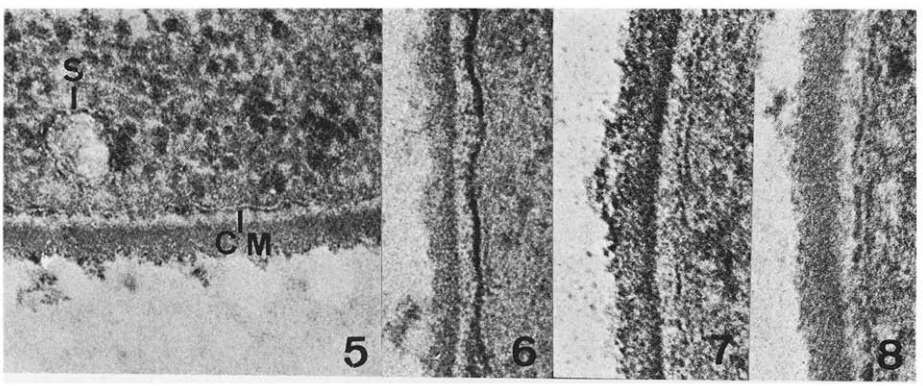


Fig. 5. *B. cereus* fixed as in Fig. 4 but without postfixation and section contrasted with uranyl acetate for 30 min followed by lead citrate for 5 min. Notice the poorly preserved intracytoplasmic membrane system (S) and the discontinuities in the cytoplasmic membrane (CM). × 114000.

Figs. 6–8. Higher magnifications (× 200000) showing the cytoplasmic membrane profile of *B. cereus* after the R–K OsO_4 fixation with or without postfixation. Fig. 6. Postfixation with uranyl acetate; section contrasted with lead citrate for 5 min. Fig. 7. Without postfixation; section contrasted with lead citrate for 5 min. Fig. 8. Without postfixation; section contrasted with uranyl acetate for 30 min, followed by lead citrate for 5 min.

good fixation[1,4,15] even when the treatment with uranyl salts was omitted, a finding which was expected since the OsO_4 fixation was done under carefully controlled R–K conditions[4,15]. It should be stressed that good fixation of nuclei was obtained under conditions leading to poor preservation of the membranous structures in all the bacilli studied.

All the above phenomena were observed wether infiltration in propylene oxide was used or not.

Participation of the dehydration step in membrane damage

The results described above for uranyl salts suggest that these salts act as fixatives by reducing the damage to the membranous material which occurs during the preparative steps following OsO_4 fixation. In order to estimate to what extent dehydration with ethanol was responsible for this damage, experiments were designed in which the dehydrated specimens (in absolute ethanol) were treated with ethanolic solutions of uranyl salts. As the activity of these solutions may be lower than that of aqueous solutions[6], parallel experiments were carried out using the normal treatment with aqueous uranyl acetate on rehydrated specimens. In both instances the membranes appeared to be damaged in the same way as when postfixation was omitted. In control experiments the dehydration–rehydration scheme was tested in specimens already postfixed with uranyl acetate. No differences were found in comparison with the standard technique.

TABLE I

LOSS OF LIPID PHOSPHORUS FROM ^{32}P-GROWN *B. cereus* DURING FIXATION WITH THE R–K OsO_4, POSTFIXATION WITH URANYL ACETATE, AND DEHYDRATION WITH ETHANOL

Values were calculated as a percentage of the total lipid-phosphorus content of identical aliquots of untreated cells.

	Lipid-P lost to*		
	R–K OsO_4 fixative	*Uranyl acetate postfixative*	*Ethanol washings*
With uranyl acetate postfixation	0.6 (0.5)	0.1 (0.2)	1.5 (1.3)
Without postfixation	0.6 (0.5)	—	21.8 (19.9)

* Determined by chemical assay of phosphorus (mean of five independent determinations) or by measurement of the radioactivity (in parentheses; mean of three independent determinations) in the lipid extracts.

These results indicate that the damage observed in membranes when postfixation was not used occurred to an important degree, during the dehydration step. The quantitation of this conclusion, and of the fixative action of uranyl salts, was approached through the study of the loss of membranous material to the ethanol washings during dehydration. Since phospholipids are known to be located almost exclusively in the membranes (see ref. 16) this loss was estimated by means of the assay of the lipid phosphorus. The results reported in Table I show that postfixation with uranyl acetate reduces the loss of phospholipid material from about 20 % to about 1.5 %.

DISCUSSION

As RIEMERSMA[17] pointed out, the purpose of fixative procedures is to stabilize cellular organization to such an extent that during the subsequent drastic preparative steps the ultrastructural relations are preserved and the chemical constituents are not lost. Accordingly, in the present study the fixative action of uranyl salts was demonstrated by both morphological and chemical data.

The fixative action of uranyl acetate on eukaryotic cell membranes has previously been concluded from comparative morphological studies on retinal cells[18]. These studies showed that omission of the uranyl postfixation after OsO_4 or glutaraldehyde–OsO_4 resulted in a discontinuous and less evidently triple-layered membrane profile. Similar morphological results were later published by TERZAKIS[19] who demonstrated that the use of uranyl acetate before dehydration of Plasmodium sporozoites markedly improved the preservation of the membranes. Treatment with uranyl acetate before dehydration has been used for eukaryotic cells by several authors, but the improved images obtained have usually been considered the result of improved contrast. Consequently, this treatment has been generally described as staining instead of postfixation. Our present ultrastructural results give evidence supporting a fixative action of uranyl salts on bacterial membranes, but a contrast effect obviously coexists, as in the case of OsO_4. The improvement induced by the use of uranyl postfixation before dehydration would be primarily due to the stabilization of materials which otherwise are to be lost.

Our results showing loss of lipid phosphorus during dehydration support the ultrastructural data and substantiate the conclusion that postfixation with uranyl salts greatly reduces the loss of membranous material during dehydration. Similar results have already been reported for rat brain tissue[20]. BIAVA AND SHELLEY[21] also reported the reduction of cardiolipin loss during dehydration by the use of uranyl acetate in the preparation of animal tissues, but no quantitative data were presented. Other published works on lipid-phosphorus loss during dehydration of eukaryotic cells[22–28] and chloroplasts[29] deal with unpostfixed preparations. With one exception[24] all the reported values are lower than our own for unpostfixed bacterial specimens. However, it should be stressed that our OsO_4 fixative contained Ca^{2+} which was not used in the above papers. If both calcium and uranyl ions are omitted, the loss of lipid phosphorus from *B. cereus* specimens was found to be as high as 70 % (unpublished observations). If uranyl postfixation is used after the fixation without Ca^{2+}, that value is reduced to 1.7 %, which again clearly points to the important fixative action of uranyl salts on the membranes of bacilli.

ACKNOWLEDGMENTS

We thank Dr. Dinah Abram, Dr. P. F. Elbers and Prof. L. L. M. van Deenen for their helpful suggestions in the course of this work, and Prof. B. Afzelius for reading the manuscript.

This work was supported in part by Grants from Fundação Calouste Gulbenkian and Instituto de Alta Cultura.

REFERENCES

1 A. RYTER AND E. KELLENBERGER, *Z. Naturforsch.*, 13b (1958) 597.
2 S. STRUGGER, *Naturwissenschaften*, 43 (1956) 357.
3 E. KELLENBERGER, A. RYTER AND J. SÉCHAUD, *J. Biophys. Biochem. Cytol.*, 4 (1958) 671.
4 W. H. SCHREIL, *J. Cell Biol.*, 22 (1964) 1.
5 K. KRAHN AND F. SCHLOTE, *Arch. Mikrobiol.*, 34 (1959) 412.
6 G. W. FUHS, *Arch. Mikrobiol.*, 49 (1964) 383.
7 M. T. SILVA, *6th Intern. Congr. Electron Microscopy, Kyoto, 1966*, p. 275.
8 J. H. LUFT, *J. Biophys. Biochem. Cytol.*, 9 (1961) 409.
9 J. H. VENABLE AND R. A. COGGESHALL, *J. Cell Biol.*, 25 (1965) 407.
10 J. M. SANTOS MOTA, J. A. F. OP DEN KAMP, H. M. VERHEIJ AND L. L. M. VAN DEENEN, *J. Bacteriol.*, 104 (1970) 611.
11 C. H. FISKE AND Y. SUBBAROW, *J. Biol. Chem.*, 66 (1925) 375.
12 P. C. FITZ-JAMES, *J. Biophys. Biochem. Cytol.*, 8 (1960) 507.
13 M. T. SILVA, *Proc. 1st Ann. Meeting Port. Soc. Electron Microscopy, 1966*, Abstr. 11.
14 M. L. WATSON AND W. G. ALDRIDGE, *J. Biophys. Biochem. Cytol.*, 11 (1961) 257.
15 E. KELLENBERGER AND A. RYTER, in B. SIEGEL, *Modern Developments in Electron Microscopy*, Academic Press, New York and London, 1964, p. 315.
16 L. ROTHFIELD AND A. FINKELSTEIN, *Ann. Rev. Biochem.*, 37 (1968) 463.
17 J. C. RIEMERSMA, *Biochim. Biophys. Acta*, 152 (1968) 718.
18 M. M. MAGALHÃES AND M. T. SILVA, *Proc. 1st Meeting Port. Soc. Electron Microscopy*, 1966, Abstr. 5.
19 J. A. TERZAKIS, *J. Ultrastruct. Res.*, 22 (1968) 168.
20 M. T. SILVA, F. CARVALHO GUERRA AND M. M. MAGALHÃES, *Experientia*, 24 (1968) 1074.
21 C. G. BIAVA AND S. SHELLEY, *J. Cell Biol.*, 39 (1968) 15a.
22 C. T. ASWORTH, J. S. LEONARD, E. H. EIGENBRODT AND F. J. WRIGHTSMAN, *J. Cell Biol.*, 31 (1966) 301.
23 E. D. KORN AND R. A. WEISMAN, *Biochim. Biophys. Acta*, 116 (1966) 309.
24 T. E. MORGAN AND G. L. HUBER, *J. Cell Biol.*, 32 (1967) 757
25 O. STEIN AND Y. STEIN, *J. Cell Biol.*, 33 (1967) 319.
26 H. CARLIER, *J. Microscopie*, 7 (1968) 825.
27 G. B. DERMER, *J. Ultrastruct. Res.*, 22 (1968) 312.
28 D. R. SAUNDERS, *J. Cell Biol.*, 37 (1968) 183.
29 A. OGUM, W. W. THOMSON AND J. B. MUDD, *J. Lipid Res.*, 9 (1968) 416.