STUDIES ON THE ORIGIN OF YEAST MITOCHONDRIA

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The mitochondria of aerobically grown yeast, in common with those of higher organisms, contain the terminal electron transport, oxidative phosphorylation, and citric acid cycle enzymes of the cell (9). However, unlike higher organisms, the yeast cell is capable of both aerobic and anaerobic growth, and, as was reported some years ago, the *b* and *a* cytochromes are not synthesized in the absence of oxygen but are adaptive enzymes formed in response to the presence of oxygen (2, 7).

This communication compares some of the cytology and enzymology of the yeast *Torulopsis utilis* grown both aerobically and anaerobically. It is shown that, indeed, *none* of the respiratory chain cytochromes normally associated with the mitochondrial matrix are synthesized by *T. utilis* grown under strictly anaerobic conditions and that such cells contain no mitochondria but a new previously undescribed membrane system which contains some of the enzymes normally found in mitochondria. It appears that these membranes are concerned with the morphogenesis of mitochondria.

A complex medium (6) was employed for the cultivation of the cells, and for both aerobic and anaerobic growth the inoculum used was taken from an aerobic shake culture which had reached the stationary phase. For large scale cultivation the cells were grown at 28°C, then at the end of the growth period cooled overnight to 2°C, and subsequently harvested and washed in distilled water, all at about 0°C. It is essential that the anaerobic cells are collected at low temperatures; otherwise adaptive synthesis of the cytochromes takes place on exposure to air during harvesting.

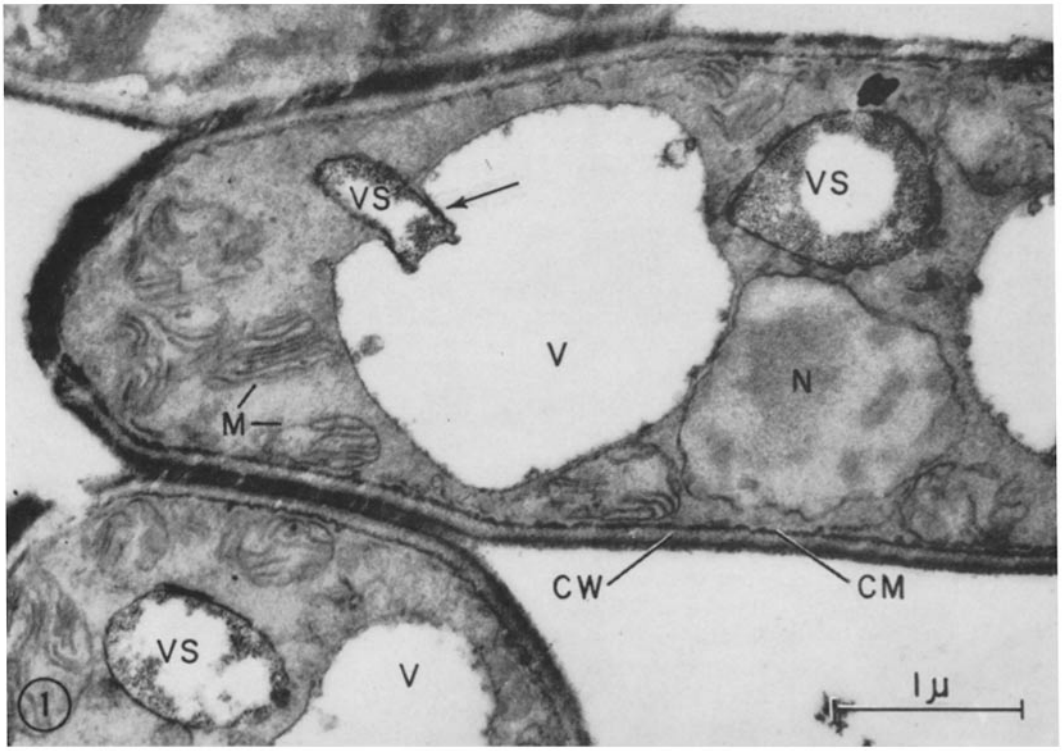
For aerobic growth, batches of about 5 litres of medium were inoculated and the cells grown under forced aeration for 18 to 24 hours, by which time they had reached the early stationary phase.

Anaerobic cells were grown in an air-tight tank containing 40 litres of medium which, after inoculation, was thoroughly flushed with oxygen-free nitrogen; gas was allowed to escape through a water-trap which prevented any back-flow of air. Under anaerobic conditions there was a prolonged lag phase, sometimes up to 30 hours, before the cells moved into a logarithmic growth phase. For experiment the cells were harvested in their early stationary phase and the total growth time, therefore, varied from about 30 to 54 hours. The yields of anaerobic cells were approximately 20 per cent of those obtained by growing *T. utilis* aerobically.

For electron microscopy the cells were treated in a manner similar to that previously described for *Saccharomyces cerevisiae* (8) but with some important modifications. In order to achieve satisfactory preservation of the cytoplasmic features in *T. utilis*, it was necessary to prolong the period of fixation of the cells. In addition, to increase contrast within the sections, it was found advantageous to include potassium dichromate in the postfixation solution. For both aerobic and anaerobic cells the final fixation procedure adopted was to suspend the cells in 2 per cent potassium permanganate at 0° to 4°C for 3 hours, wash them several times with a solution containing 1 per cent uranyl nitrate and 1 per cent potassium dichromate, and then leave them in this solution in the cold for 3 hours. The fixed cells were subsequently washed with water, dehydrated, embedded in methacrylate, sectioned with a Porter-Blum microtome and photographed, all as described previously (8). In some experiments the cells were embedded in Araldite (3), but no differences were observed between this and methacrylate embedding and the latter has been routinely used.

Fine Structure of Aerobic and Anaerobic Cells

It is evident from Figs. 1 and 2 that the cytology of aerobic *T. utilis* is similar to that of the other yeasts studied so far (1, 5, 8, 10). A prominent nucleus, cell wall, cytoplasmic membrane underlying the wall, mitochondria, granular cytoplasm and vacuole are all characteristic of the yeast cell. In aerobic *T. utilis*, apart from the vacuole (*V*), another vacuole-like structure (*VS*) has been observed in many sections and it does not seem to be directly connected with the vacuole proper (Fig. 1, arrow). It usually contains an abundance of finely granular electron-opaque material, the chemical nature of which is at present unknown.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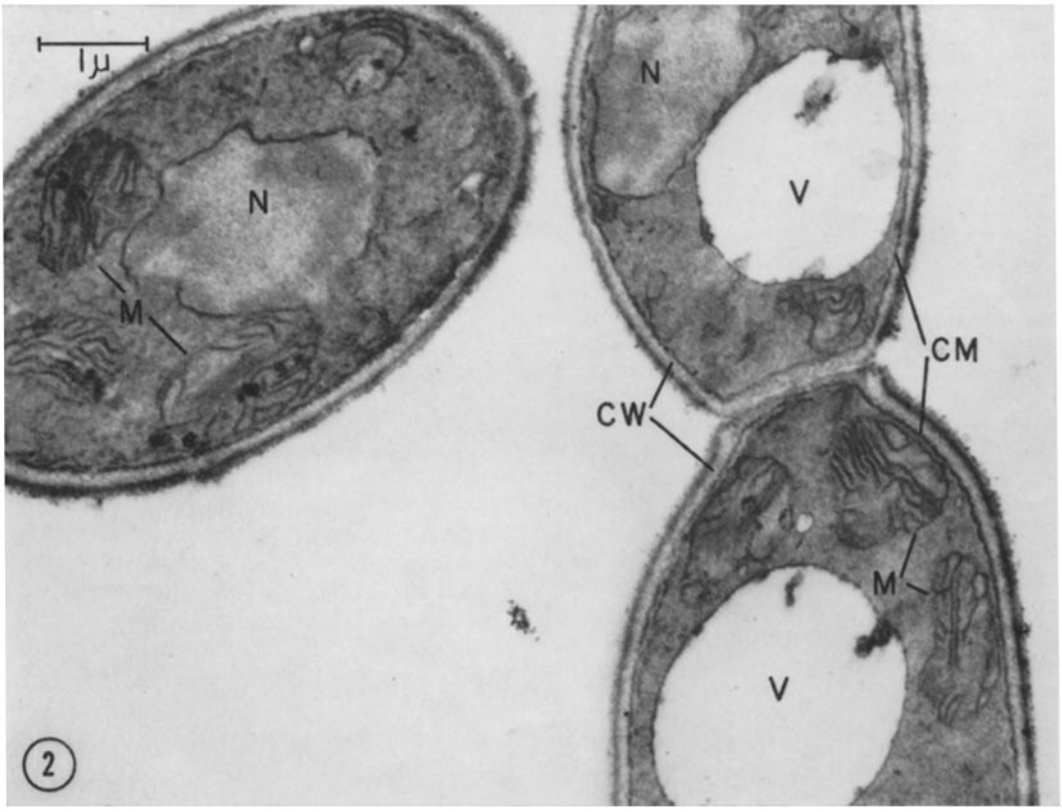
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The mitochondria, which are of particular interest in this study, are generally larger than those observed in *S. cerevisiae*, with a minimum diameter of 0.3 to 0.5 μ and a maximum diameter of 0.6 to 1.0 μ. In sections the mitochondria appear irregular in outline and possess numerous cristae which are often extensively curved.

Many of the basic features of the aerobic cell are clearly recognizable in the anaerobic cell, although considerable differences are also at once apparent. We have examined large numbers of sections from anaerobic cells and so far have not observed organelles suggestive of mitochondrial structure. Instead, the cytoplasm of the anaerobic cells contains numerous membranes the appearance and distribution of which have not been observed previously in yeast (Figs. 3 and 4). These membranes appear to be of two types: one is a multimembrane system resembling the well known myelin forms, consisting of a number of unit membranes closely adjacent to one another; and the other is a membrane system reminiscent of the endoplasmic reticulum of higher organisms. The multimembrane system is sometimes closely associated with an electron-transparent area (Figs. 4 and 5). These areas do not seem to be parts of the vacuole, but may represent lipid globules which are not stained by the fixation procedure. However, although not shown in the figures, the multimembrane system is also frequently observed to be free from any association with the electron-transparent areas of the cell and to lie completely within the electron-opaque cytoplasm. It can occur in the form of either a closed concentric ring (Fig. 5) or an open ring with the membranes branching out into the cytoplasm to simulate the reticular membrane system (Fig. 4). However, examination of Fig. 3 (arrow) also suggests that the reticular membrane system may originate from the nuclear envelope. Thus it seems that both the nuclear envelope and the myelin forms are closely related to the reticular membrane system. At this stage in our investigation the relationship, if any, between the nuclear envelope and the myelin forms is uncertain; they may or may not share a common origin.

Particulate Fractions from Aerobic and Anaerobic Cells

A particulate fraction was isolated from both cell types after disintegration of the cells and fractionation of the extracts exactly as described previously for *S. cerevisiae* (9). In the case of the aerobic cells, this is a true mitochondrial fraction, whereas for the anaerobic cells this fraction consists mainly of fragments of the reticular membrane system.

The isolated mitochondria of aerobic *T. utilis* were found to have the anticipated capacity for electron transport, oxidative phosphorylation, and citric acid cycle activity. P/O ratios approaching theoretical values were obtained with the citric acid cycle substrates which was contrary to our earlier experience with *S. cerevisiae* (9). The mitochondria of *T. utilis* have a complete citric acid cycle, while the strain of baker's yeast used in our earlier experiments had little or no α-ketoglutaric dehydrogenase activity.

By contrast, the particulate fraction from anaerobic *T. utilis* had no aerobic oxidative capacity. However, most significantly, these particles contained both succinic and DPNH dehydrogenase activities (determined with ferricyanide as electron acceptor) which in aerobic cells are exclusively located in the mitochondria. The specific activities of these two dehydrogenases in the anaerobic particles were only about 10 to 20 per cent of those observed in aerobic material.

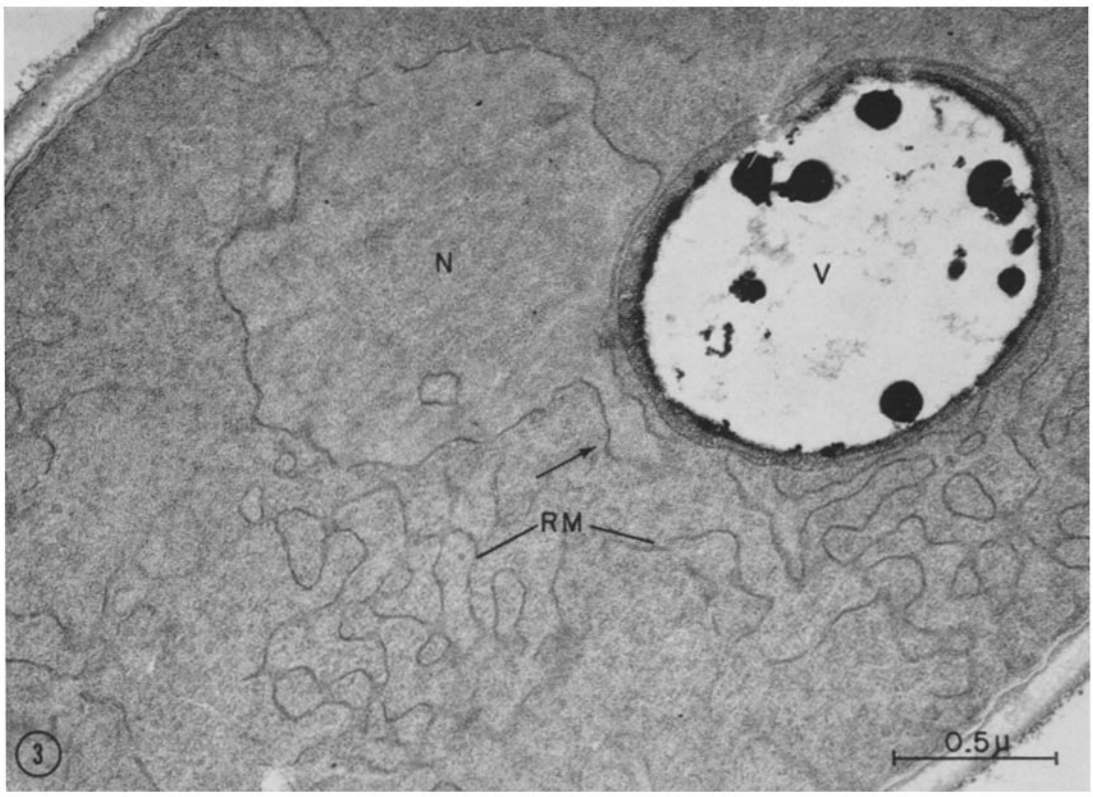
For the observation and spectrophotometric

FIGURE 1

Section through aerobic cells of *T. utilis* showing the cell wall (*CW*), cytoplasmic membrane (*CM*), nucleus (*N*), mitochondria (*M*), and vacuole (*V*). Note the presence of another vacuole-like structure (*VS*) containing finely granular electron-opaque material. It does not seem to be directly connected with the vacuole, but is separated from it by a membrane (arrow). × 25,000.

FIGURE 2

Section through aerobic cells of *T. utilis*. The cell on the right is seen at a final stage of budding. Labeling as indicated in Fig. 1. × 14,000.


N
V
RM
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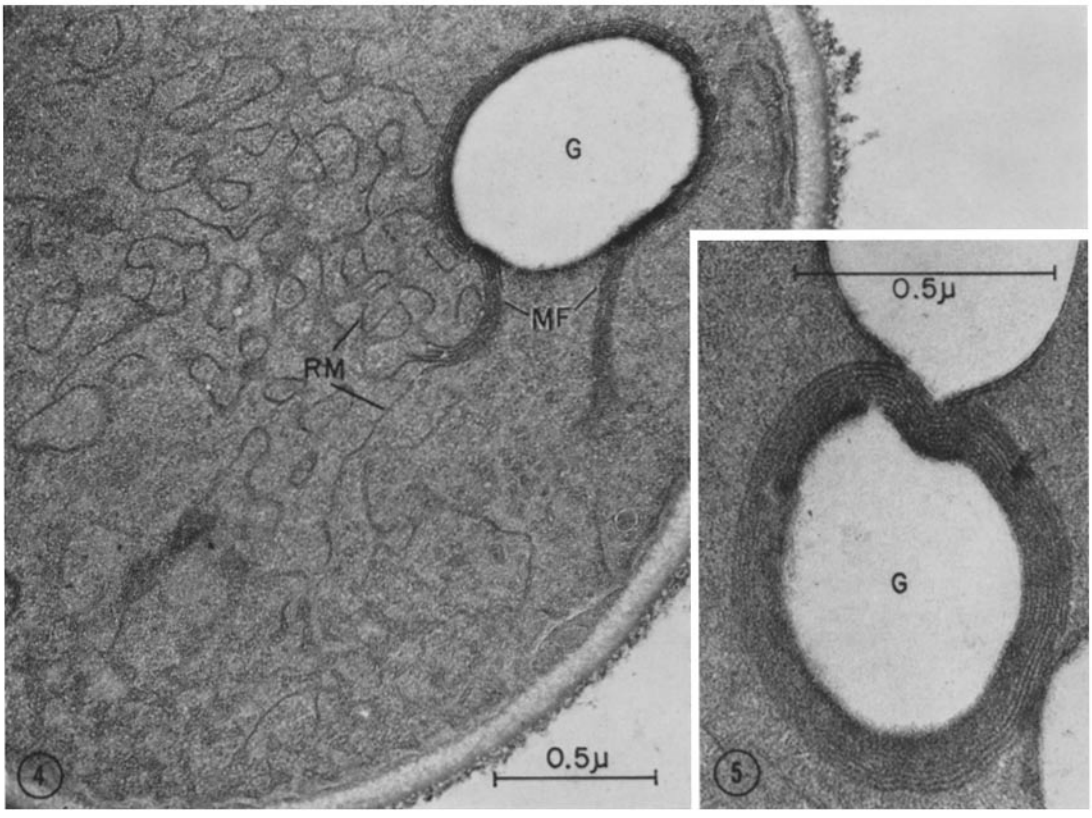
3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5

determination of the cytochromes of the two cell types it has been found convenient to study them in smaller fragments of the isolated particle fractions. These small fragments were prepared by sonication of the particulate material followed by differential centrifugation and collection of the fraction sedimenting between 25,000 and 105,000 *g*. Fig. 6 compares the difference spectra of such particles from aerobic and anaerobic *T. utilis*. Striking differences are evident between the two types of cells. In the aerobic particles the presence of flavin and cytochromes a–a_3, b, c and c_1 is apparent. The flavin is accounted for by the terminal respiratory dehydrogenases of succinate, DPNH, and α-glycerophosphate. These dehydrogenases are all linked to the cytochrome chain, and in the presence of any one of the appropriate substrates the cytochromes are reduced. The molar ratios of the total flavin to the individual cytochromes of this type of yeast particle are of the same general order as those described for electron transport particles derived from beef heart mitochondria (4). Detailed observations on the properties of these particles derived from yeast mitochondria will be described in subsequent reports.

The anaerobic particle fractions, however, contain no spectrophotometrically detectable cytochromes. The detection of certain cytochromes in anaerobic yeast reported by earlier workers (2, 7) was probably due to the fact that the cells were either not grown under strictly anaerobic conditions or were not harvested and washed at 0°C. Our experiments indicate that cells grown in the presence of traces of oxygen lack cytochrome a–a_3 but contain small amounts of the b and c cytochromes. Nevertheless, even under these conditions of growth, where traces of cytochrome are detectable, the reticular membrane system is found in the cytoplasm and there are still no mitochondria present.

It may be concluded from these studies that aerobic *T. utilis* is essentially similar, both cytologically and enzymically, to the aerobic yeasts previously studied (8, 9). By contrast, the same yeast grown strictly anaerobically contains no cytochromes and forms no mitochondria, but a new type of membrane system. However, the presence in this membrane system of two primary dehydrogenases of the terminal respiratory chain, which in the aerobic cells are located exclusively in the mitochondria, establishes a relationship between this system and the mitochondria of the aerobic cell. Indeed, current experiments in our laboratory show that on aeration of resting cell suspensions of anaerobically grown *T. utilis*, the reticular membranes appear to line up in a parallel array and subsequently fuse and infold to form primitive mitochondria containing a few cristae. During this process the cytochromes are also synthesized. Details of this work will be described in further reports. Experiments with *S. cerevisiae* show that this organism, like *T. utilis*, does not possess mitochondria or terminal electron transport cytochromes when grown strictly anaerobically, and that primitive mitochondria form on aeration of resting cell suspensions of anaerobic *S. cerevisiae*.

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FIGURE 3

Section through part of an anaerobic cell of *T. utilis* showing a vacuole (*V*), the nucleus (*N*), and reticular membranes (*RM*) throughout the cytoplasm. It appears that some of these membranes originate from the nuclear envelope (arrow). × 50,000.

FIGURE 4

Section through part of an anaerobic cell of *T. utilis* showing a multimembrane system (*MF*) partly enclosing an electron-transparent area (*G*) and opening into the reticular membrane system (*RM*). × 50,000.

FIGURE 5

Section through part of an anaerobic cell of *T. utilis* showing the multimembrane system in closed concentric rings around an electron-transparent area (*G*). These membranes resemble the well known myelin forms. × 80,000.

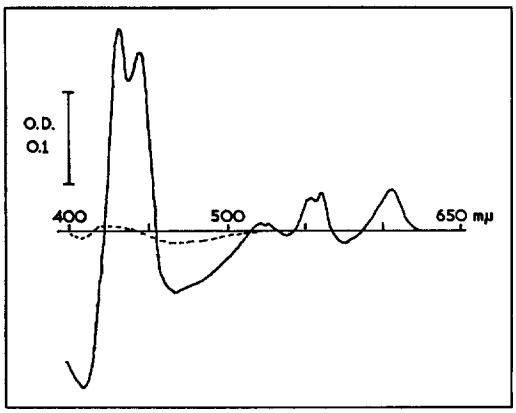

FIGURE 6

Difference spectra of particle fractions prepared from aerobic and anaerobic *T. utilis*. Solid line—aerobic cells; broken line—anaerobic cells.

The spectra were taken with a Cary, model 14, recording spectrophotometer. The cuvettes, with a 1 cm light path, contained 6 mg of particle protein, 9 mg of sodium deoxycholate, and 0.1 mmoles of potassium phosphate buffer, pH 7.4, in a total volume of 1.0 ml. The cytochromes were reduced with sodium hydrosulphite.

The presence of cytochromes ($a - a_3$), b, and ($c + c_1$) in the particles derived from the aerobic cells is indicated by the absorption maxima at 605, 564, and 552 mμ, respectively.

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BIBLIOGRAPHY

1. AGAR, H. D., and DOUGLAS, H. C., *J. Bact.*, 1957, **73**, 365.
2. EPHRUSSI, B., *Harvey Lectures*, 1950, **46**, 45.
3. GLAUERT, A. M., and GLAUERT, R. H., *J. Biophysic. and Biochem. Cytol.*, 1958, **4**, 191.
4. GREEN, D. E., and ODA, T., *J. Biochem.* (Tokyo), 1961, **49**, 742.
5. HASHIMOTO, T., CONTI, S. F., and NAYLOR, H. B., *J. Bact.*, 1959, **77**, 344.
6. LINDENMAYER, A., and ESTABROOK, R. W., *Arch. Biochem. and Biophysics*, 1958, **78**, 66.
7. SLONIMSKI, P. P., *Actualites Biochimiques*, Paris, Masson et Cie, 1953, No. 17.
8. VITOLS, E., NORTH, R. J., and LINNANE, A. W., *J. Biophysic. and Biochem. Cytol.*, 1961, **9**, 689.
9. VITOLS, E., and LINNANE, A. W., *J. Biophysic. and Biochem. Cytol.*, 1961, **9**, 701.
10. YOTSUYANAGI, Y., *Compt. rend. Acad. sc.*, 1959, **248**, 274.

ISOLATION OF CYTOPLASMIC PITUITARY GRANULES BY COLUMN CHROMATOGRAPHY

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Differential centrifugation has been used by several workers for the separation of submicroscopic cytoplasmic particulates from homogenates of the anterior pituitary gland of the rat (5, 3, 4). Hartley *et al.* (2) successfully isolated highly purified basophilic granules (200 mμ maximal diameter) using the techniques of differential and isopycnic gradient centrifugations and microfiltration. Gonadotropic activity was associated with these granules.

This preliminary communication reports the isolation of pituitary granules in highly purified form by use of columns of Celite. Riley *et al.* (6, 7) used a similar method for the isolation of melanin granules and virus particles.

MATERIAL AND METHODS

Source of Pituitary Glands

Normal male rats of the Holtzman strain (obtained from the Endocrine Laboratories, Madison, Wisconsin) were used as the source of the glands. The animals were killed by cervical dislocation, and the anterior lobes were collected and kept on ice until a sufficient number was obtained. A 5 per cent homogenate was made by mashing the glands in a sharp pointed ground-glass homogenizer using 0.25 M sucrose plus 7.3 per cent polyvinylpyrrolidone (PVP) adjusted to pH 7.3 as the homogenizing medium.

Differential Centrifugation and Microfiltration

The whole homogenate (WH) was centrifuged in the multispeed head of a Model PR-2 International refrigerated centrifuge at 275 *g* for 10 minutes to obtain a pellet (NF) of red blood cells, nuclei, and unbroken cells. The supernatant (S_1) which contained pituitary granules, mitochondria, and microsomal material was filtered through a Millipore filter with 5.0 μ diameter pores in a Swinny adapter

fitted on a 5 ml syringe. This filtered S_1 (FS_1) was then layered on a column of Celite.

Preparation of Column

A 10 per cent (w/v) slurry of Celite No. 545 (Johns-Manville Co., Chicago) in 0.25 M sucrose was allowed to settle and the fines decanted; this procedure was repeated 4 times over a period of 2 hours. A 1.5 × 20 cm column (Fig. 1) was used, care being taken to avoid uneven packing. The height of the Celite in the column varied from 12.6 to 13.0 cm. The columns were washed with 0.25 M sucrose for an additional 2 hours. The pH of the eluted sucrose was 6.8.

Operation of Column

One ml of FS_1 was carefully layered on the top of the Celite column. The flow rate was routinely adjusted to 7 drops per minute. Twenty minutes after the start of layering, 0.6 ml fractions were collected in centrifuge tubes (Spinco No. 5528). Cloudy material was collected 25 to 30 minutes after the start of layering, and this cloudiness usually persisted for 6 tubes. Sedimentable material, however, was always present in several tubes after the cloudiness ceased.

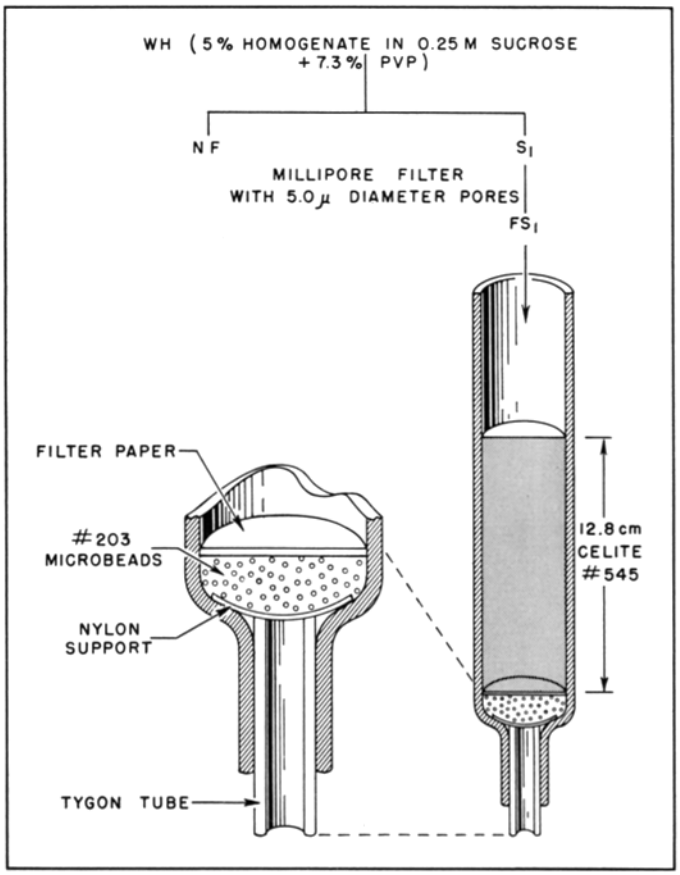


FIGURE 1

Procedure for obtaining the starting material, FS_1, and a representation of the column on which the granules were separated.

Preparation of Material for Electron Microscopy

Material from appropriate tubes was centrifuged at 100,000 *g* in a SW-39L Type rotor with swinging buckets in a Spinco Model L ultracentrifuge. Pellets were fixed in 1 per cent OsO_4 in 0.25 M sucrose overnight in the cold. Rapid dehydration was done in a series of methyl alcohol solutions of increasing concentrations. The pellets were embedded in a 5:1 *N*-butyl-methyl methacrylate (2 per cent benzoyl peroxide) and incubated at 50°C for 24 to 36 hours. Sections of the pellets were studied with a Phillips EM-75B electron microscope.

RESULTS AND DISCUSSION

An electron micrograph from the anterior pituitary gland of a male rat is shown in Fig. 2. Two cell types can be readily identified on the basis of the maximum diameter of their granules, as reported by Farquhar (1). A basophil (*b*) with granules of 200 mμ maximal diameter in the cytoplasm and several acidophils (*a*) which have granules of 350 mμ maximal diameter are present.

One ml of FS_1, which represents the starting material, was centrifuged at 100,000 *g* for 1 hour to obtain a FS_1 pellet. An electron micrograph of a thin section through this pellet is shown in Fig. 3. It contained acidophilic and basophilic granules, mitochondria, and microsomal material, but was essentially free of nuclei and red blood cells. When this fraction was layered on the column and developed at a flow rate of 7 drops per minute, cloudy material came off the column in fractions 2 to 6. Highly concentrated granules were recovered from fractions 3, 4, and 5 by high speed centrifugation. An electron micrograph of a section of a pellet from fraction 4 is shown in Fig. 4. Significant microsomal contamination was present in this pellet.

It was found that centrifugation of fractions 3, 4, and 5 at 8,000 *g* for 20 minutes yielded small white pellets. These pellets were examined with the electron microscope. Fig. 5 shows an electron micrograph of a thin section through a pellet obtained from fraction 5 at low speed. It contained granules which were essentially free of contaminating material. The granules shown in Fig. 5 range from 90 to 420 mμ. Approximately 70 per cent of these granules have diameters which measure greater than 230 mμ, indicating that they are acidophilic granules. The supernatant from this pellet obtained at low speed, when centrifuged at 100,000 *g* for 15 minutes, also yielded a very small pellet. Fig. 6 shows an electron micrograph of a thin section through this pellet. It contained material tentatively identified as microsomal in character, although some granules from basophilic cells were also present. The granules in Fig. 6 have diameters which range from less than 50 up to 210 mμ. Approximately 70 per cent of these granules measure 50 mμ or less.

The basis for the separation of the granules is difficult to determine at this time. It appears that the Celite, since it is relatively inert (diatomaceous earth), is acting primarily as a filter. Adsorption may play a role, however, since it has been found that changing the pH of the Celite slurry will greatly affect the elution pattern. Further work is being done in an attempt to answer this question.

The biochemistry of the granules is currently under investigation, and several bioassays are being done to determine the hormonal activities associated with them.

FIGURE 2

An electron micrograph showing a gonadotropic basophil (*b*) with cytoplasmic granules which are 200 mμ in diameter. Several acidophils (*a*) which have granules 350 mμ in diameter are shown. Normal male rat pituitary. × 4500.

FIGURE 3

An electron micrograph of a thin section through the FS_1 pellet. This represents the starting material, *i.e.*, the material which was layered on the column. × 8500.

FIGURE 4

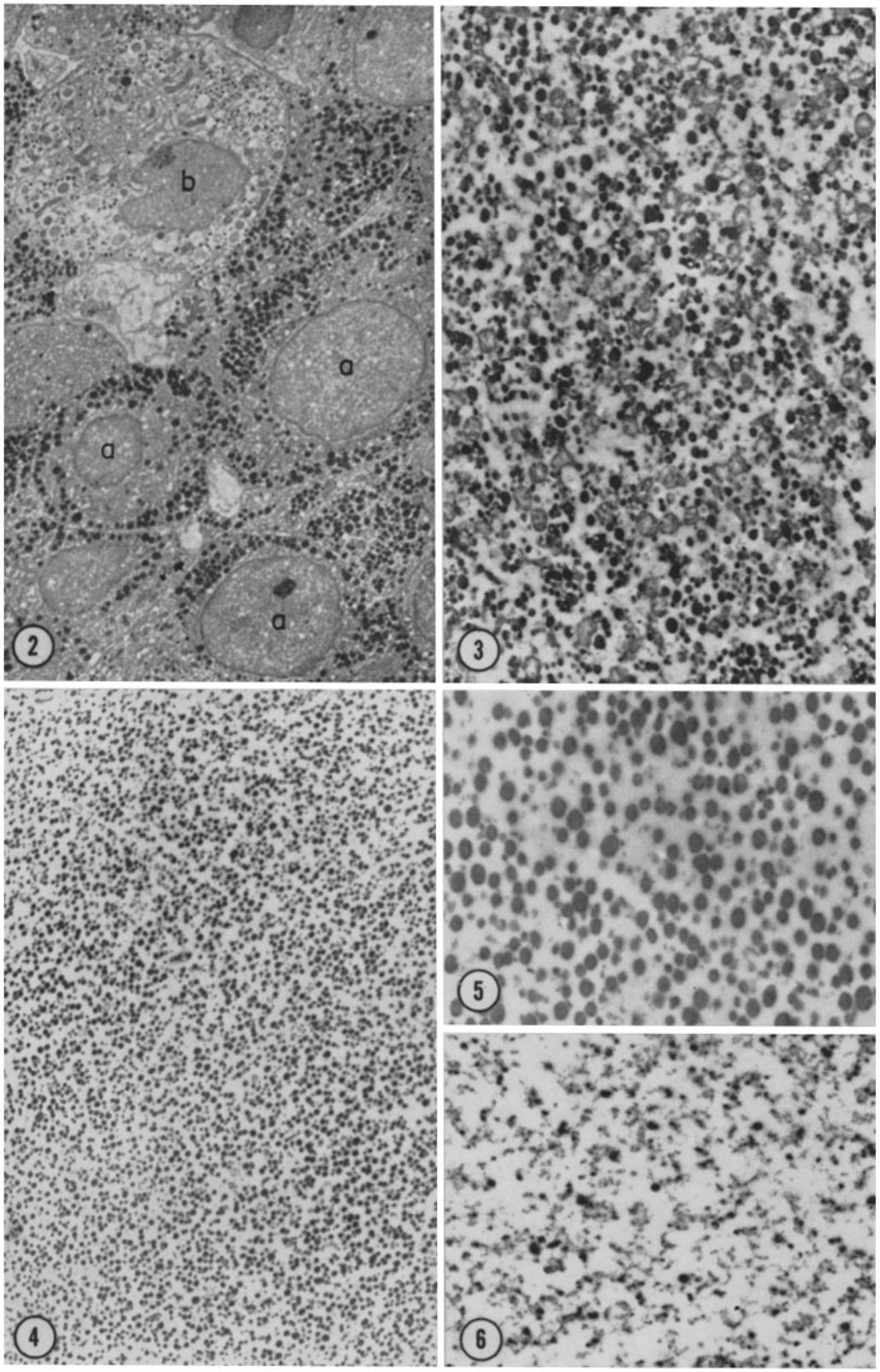
An electron micrograph of thin section through a pellet from fraction 4. Many granules are present although some areas show microsomal contamination. × 3500.

FIGURE 5

An electron micrograph of a thin section from a pellet obtained from fraction 5 at low speed. This pellet contains a high population of acidophilic granules. × 11,000.

FIGURE 6

An electron micrograph of a thin section taken from the high speed pellet of fraction 5. This pellet consists essentially of basophilic granules and microsomal material. × 8500.


b
a
a
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2
3
4
5
6

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REFERENCES

1. FARQUHAR, M. G., and RINEHART, J. F., *Endocrinology*, 1954, **54,** 516.
2. HARTLEY, M. W., MCSHAN, W. H., and RIS, H., *J. Biophysic. and Biochem. Cytol.*, 1960, **7,** 209.
3. HERLANT, M., *Ann. Endocrinol.*, 1952, **13,** 611.
4. HESS, M., *Anat. Rec.*, 1958, **130,** 315.
5. MCSHAN, W. H., and MEYER, R. K., *Endocrinology*, 1952, **50,** 294.
6. RILEY, V. T., HESSELBACH, M. L., FIALA, S., WOODS, M. W., and BURK, D., *Science*, 1949, **109,** 361.
7. RILEY, V. T., *Science*, 1948, **107,** 573.