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*RELEASE OF MITOCHONDRIA FROM YEAST CELLS BY THE ACTION OF METAL-CHELATING AGENTS**

BY JOSEPH R. MERKEL† AND WALTER J. NICKERSON

DEPARTMENT OF MICROBIOLOGY, RUTGERS UNIVERSITY

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The reduction of 2,3,5-triphenyltetrazolium chloride (TTC) by resting cells of a yeast, *Candida albicans*, appears to be localized in particulate structures within the cells. The reduced tetrazolium (triphenylformazan, TPF) can readily be seen in structures similar to those identified as mitochondria by Mudd, *et al.*[1, 2] in bakers' yeast and in bacteria.

In the course of our studies[3] on the reduction of TTC by resting cells of *Candida albicans*, it was observed that certain activators of the reduction, particularly potassium cyanide and the disodium salt of ethylenediaminetetraacetic acid (Na_2EDTA), caused a dispersion of the water-insoluble formazan into the medium surrounding the cells. Light microscopic studies revealed that the coloration was due only in small part to the crystallization or colloidization of TPF, and resulted mainly from the release of TPF-stained particles from the yeast cells. The particles that were released were identical with particles observed inside the cell. Indeed, particles could often be observed on the exterior of the cell wall (Fig. 1). In some instances, partly "lysed" cells appeared to have released large clusters of particulate material. It was possible in the following manner to demonstrate the identity of the TPF-stained bodies inside and outside of the cells. Washed resting cells which had been in contact with 0.005 per cent TTC for 5–10 min. were mounted on slides and observed under the light microscope. Illumination from the microscope light hastened the reduction of TTC inside the cells.[4] When numerous TPF stained particles became visible inside the cells and few or none were visible in the surrounding medium, pressure was applied to the cover slip. Since the Na_2EDTA-treated cells were extremely fragile, it was a simple matter to extrude most of the TPF-stained particles into the surrounding medium.

Since the presence of unbound or insoluble crystals and colloidal micelles of TPF interfered somewhat with the identification of the particles, if the cells were left in contact with the TTC for long periods of time, it was necessary to demonstrate the mitochondria-releasing effects of potassium

cyanide and Na_2EDTA in the absence of tetrazolium. It was observed that cells treated either with potassium cyanide (0.005–0.01 *M*) or Na_2EDTA (0.01 *M*) released many particles to the exterior of the cells and into the surrounding medium. However, it is rather difficult to photograph, and reproduce in print, the release of mitochondria when the particles are unstained. In attempts to demonstrate the presence of released mitochondria in the absence of TTC, a very small amount of nile blue sulfate (0.05% dissolved in phosphate buffer) was added to a drop of a dilute suspension of *C. albicans* that had been exposed to 1 per cent Na_2EDTA for 30 min. at 37°C. The basic nile blue staining showed exactly the same picture that had been observed both with the formazan stained material, and with treated cells examined in the absence of a stain. Disodium EDTA was used in preference to cyanide because cyanide itself reduces nile blue and hydrolyzes to give a solution sufficiently alkaline to cause some spontaneous reduction of TTC (see Merkel and Nickerson[3]).

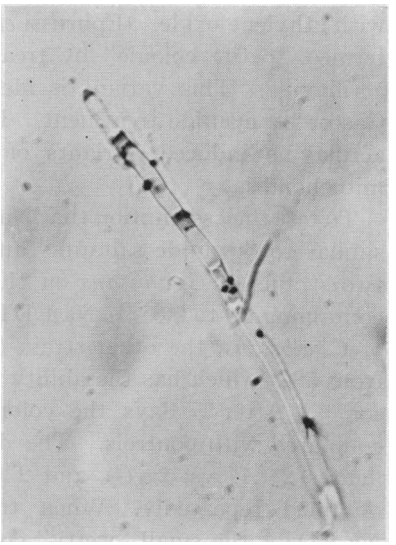

FIGURE 1

C. albicans (806) a filamentous mutant from a 24-hr. culture, collected by centrifugation, washed, and suspended in a solution of TTC and Na_2EDTA for 30 min.

According to Cooperstein and Lazarow,[5, 6] cytochrome oxidase prevents the reduction of Janus Green, and supposedly this reaction is responsible for the specific blue staining of the mitochondria. Janus Green B added to washed suspensions of *C. albicans* rapidly underwent the color changes characteristically associated with mitochondrial activity.[5, 6] Cell suspensions of *C. albicans* that had been treated for 16 hrs. with 0.01 *M* KCN reduced Janus Green B much more slowly (30 min.) than did cell suspensions in water, in 0.01 *M* sodium chloride (5–10 min.), or in Na_2EDTA solution. However, when 0.005 *M* KCN was added to the suspensions at the time of addition of Janus Green B, no reduction of the dye occurred for several hours. Microscopic observations of cells treated with Na_2EDTA and KCN indicated that many cells had lost the integrity of their cell walls, but the cytoplasmic particles still retained much of the blue dye.

In view of the confirmatory evidence obtained by the use of nile blue sulfate and Janus Green B, it was concluded that the action of Na_2EDTA and of KCN on *Candida albicans* actually caused a release of mitochondria

from the cytoplasm of the cells. In many respects the reaction is similar to the bacteriolytic action of cyanide causing the premature liberation of phage particles, as observed by Doermann.[7]

If the action of KCN and of Na_2EDTA in the present studies is not merely lytic, the phenomenon leads to an understanding of the "mutagenic" effects of cyanide.[8, 9] Variants of bakers' yeast were obtained by Stier and Castor,[9] after treatment with KCN, which were slow growing and lacked a functional cytochrome system; the small residual respiration possessed by the stable variants was cyanide-insensitive. Whelton and Phaff[10] obtained nearly identical variants of bakers' yeast after treatment with ethylene oxide. Ephrussi *et al.*[11] reported obtaining a stable variant, termed "petite colonie" by treatment of cultures of bakers' yeast with acriflavine. This variant is identical with that obtained by Stier and Castor by cyanide treatment. It has been reported by Mudd *et al.*[2] that acriflavine induced variants of *Saccharomyces cerevisiae* are devoid of mitochondria.

To test the assumption that Na_2EDTA could be used to produce variants similar to "cyanide saltants" and the "petite colonie" variant, we grew two strains of *C. albicans* on glucose-glycine-yeast extract (GGY) plates containing up to 0.01 *M* Na_2EDTA. One strain (582) was a typical strain of *C. albicans*, the other strain (806) was a filamentous mutant (derived from 582) which has the ability to reduce TTC very rapidly (see Nickerson[12]). After 5 days the colonies growing on Na_2EDTA plates were compared with controls. The difference in colony size can be noted in figures 2 (*A*) vs. 2 (*C*), and 2 (*B*) vs. 2 (*D*). These results have been obtained repeatedly. When transferred to a GGY medium lacking Na_2EDTA the small "wefty" colony shown in figure 2 (*D*) was obtained in a larger size but of the same "wefty" appearance, and had only very slight ability to reduce TTC.

When a drop of 0.4 per cent TTC was added to colonies growing on the Na_2EDTA plates as in figures 2 (*C*) and 2 (*D*) and observed under the light microscope, the majority of the TPF-stained particles appeared outside the cells (Fig. 3). The particles were much larger than normally seen and many cells seemed to have been deflated. Nile blue stained the majority of these large bodies a bright pink color, characteristic of lipoidal material.

It should be pointed out that the two substances which caused the release of mitochondria from the yeast cells are both strong metal-complexing agents and could be functioning by removing metal-linked barriers, thus allowing the mitochondria to escape from the cells. This action seems to rupture the cell wall in many instances. On the basis of the hypothesis that strong metal complexing substances are able to disrupt the cell membranes by removing a metal, several other metal-complexing

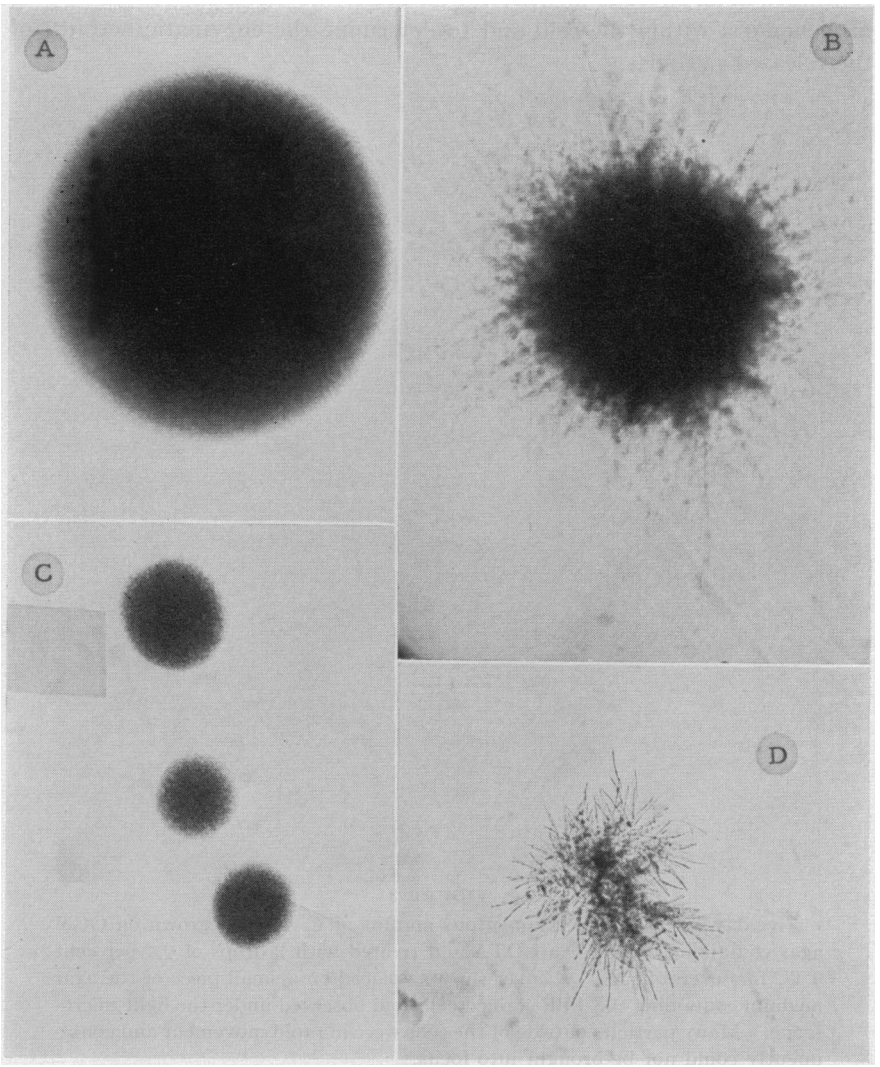

FIGURE 2

(A): Parent *C. albicans* (582) on GGY agar. (B): filamentous mutant of *C. albicans* (806) on same medium. (C): parent *C. albicans* grown on same medium as (A) but containing 0.01 *M* Na_2EDTA. (D): filamentous mutant of *C. albicans* grown on same medium as (A) but containing 0.01 *M* Na_2EDTA.

agents have been tested with success; among these agents were sodium oxalate and sodium diethyldithio-carbamate. These studies are being continued in an effort to identify the nature of the metal barrier restraining mitochondria within the cell, and to determine the enzymatic activity of the released particles.

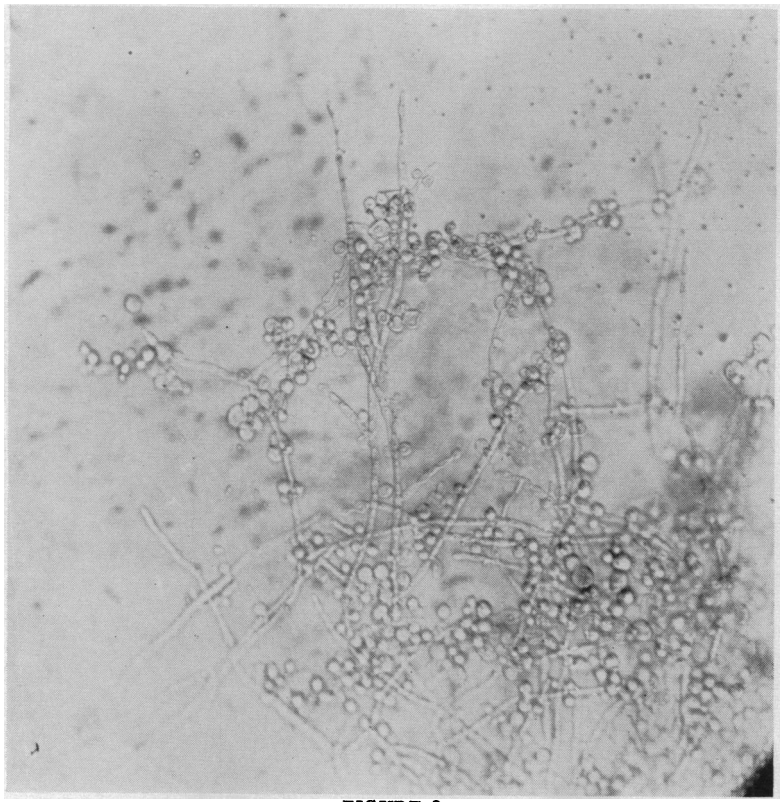

FIGURE 3

Five-day culture of the filamentous mutant of *C. albicans* grown on GGY agar containing 0.01 *M* Na_2EDTA and treated with 2 drops of 0.4 per cent TTC for several hours. A cover slip was placed on a small piece of the agar medium containing the TPF-stained cells and observed under the light microscope. Many particles outside of the cells were in rapid movement and consequently could not be brought into focus.

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† Waksman-Merck Postdoctoral Fellow in the Natural Sciences.

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THE ROLE OF THE TRICARBOXYLIC ACID CYCLE IN AMINO ACID SYNTHESIS IN ESCHERICHIA COLI

By R. B. ROBERTS, D. B. COWIE, R. BRITTEN, E. BOLTON, AND P. H. ABELSON

DEPARTMENT OF TERRESTRIAL MAGNETISM, CARNEGIE INSTITUTION OF WASHINGTON, WASHINGTON, D. C.

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For the past three years we have been using a variety of C^{14} compounds in studies of amino acid synthesis in *Escherichia coli*. Some of the results obtained with $C^{14}O_2$ have been published[1] and a report of other experiments using C^{14}-acetate is in manuscript.[2] In addition to these tracers C^{14}-labeled glucose and C^{14}-labeled amino acids have been used. As a result we have accumulated a wide variety of data concerning the utilization of various carbon compounds in amino acid synthesis. A large part of the results bear on the synthesis of aspartic acid and glutamic acid and can be interpreted in terms of the Krebs cycle. This paper describes the operation of the cycle and the following paper[9] shows the utilization of the aspartic and glutamic acids as precursors for the synthesis of two families of amino acids. The interpretation of the data shows that the Krebs cycle is of importance in amino acid synthesis, accounting for more than 50 per cent of the protein carbon, but relatively unimportant as a mechanism for oxidizing glucose.

Methods.—Cultures of *E. coli*, strain B, were grown with aeration at 37°C. in mineral media containing appropriate carbon sources, one source being labeled with C^{14}. After one hour's exponential growth, during which the bacterial mass doubled, the cells were harvested and their protein was