MITORIBOSOMES FROM *CANDIDA UTILIS*

Morphological, Physical, and Chemical Characterization of the Monomer Form and of Its Subunits

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ABSTRACT

Highly purified mitochondrial ribosomes (mitoribosomes) have been obtained from the yeast *Candida utilis*. Sedimentation analysis in sucrose gradients made in 5 mM $MgCl_2$, 1 mM Tris, pH 7.4 and 50 mM KCl clearly distinguishes mitoribosomes (72S) from cytoplasmic ribosomes (cytoribosomes) (78S). Mitoribosomes are completely dissociated into 50S and 36S subunits at 10^{-4} M $MgCl_2$ whereas complete dissociation of cytoribosomes into 61S and 37S subunits occurs only at 10^{-6} M $MgCl_2$. Electron microscopy of negatively stained mitoribosomes (72S peak) shows bipartite profiles, about 265 × 210 × 200 A. Characteristic views are interpreted as frontal, dorsal, and lateral projections of the particles, the latter is observed in two enantiomorphic forms. Mitoribosome 50S subunits display rounded profiles bearing a conspicuous knoblike projection, reminiscent of the large bacterial subunit. The 36S subunits show a variety of angular profiles. Mitoribosomal subunits are subject to artifactual dimerization at high Mg^{2+} concentration. Under these conditions, a supplementary 80S peak arises. Electron microscopic observation of the 80S peak reveals closely paired particles of the 50S type. Buoyant density determinations after glutaraldehyde fixation show a single peak at $\rho = 1.48$ for mitoribosomes and 1.53 for cytoribosomes. In the presence of ethylenediaminetetraacetate (EDTA), two species of RNA, 21S and 16S, are obtained from mitoribosomes, while 25S and 17S RNA are obtained from cytoribosomes. It is established that the small and large RNA species are derived from the 36S and 50S subunits, respectively, by extraction of the RNA from each subunit. The G + C content of the RNA is lower for mitoribosomes (33%) than for cytoribosomes (50%). Incubation of *C. utilis* mitochondria with leucine-^{14}C results in the labeling of 72S mitoribosomes. The leucine-^{14}C incorporation is inhibited by chloramphenicol and resistant to cycloheximide. Puromycin strips the incorporated radioactivity from the 72S mitoribosomes, which is consistent with the view that leucine-^{14}C is incorporated into nascent polypeptide chains at the level of mitoribosomes.

INTRODUCTION

A considerable amount of information suggests that mitochondrial ribosomes (mitoribosomes) are present in a variety of cell types as an integral part of the machinery for protein synthesis in mitochondria (see reviews in Swift and Wolstenholme, 1969; Rabinowitz and Swift 1970; Ashwell and Work, 1970 *a*; Borst and Grivell, 1971). Investigations into the functioning of

mitochondrial protein-synthesizing systems must repose upon a complete characterization of the mitoribosome itself To date, however, physicochemical studies of mitoribosomes from unicellular organisms and higher plants and animals indicate an important degree of variability, notably in the size of the monomer and its subunits and in sedimentation values of the ribosomal RNA molecules. While some of the discrepancies can be attributed to differences in techniques, it must be admitted that many reliable data show that a true variability does indeed exist

Among the different organisms studied, the mitoribosomes from several fungi appear to constitute a fairly homogeneous group. For instance, it is clear that some features, such as the size (Borst and Grivell, 1971), tend to distinguish them from mammalian mitoribosomes. In addition, unlike the scattered distribution of ribosomes in mitochondria of mammalian cells (André and Marinozzi, 1965), earlier observations (Vignais et al., 1969) had demonstrated that mitoribosomes in yeast are numerous and display a unique polysome-like arrangement along the inner surface of the inner mitochondrial membrane. This observation, which has been confirmed by Stegeman et al. (1970), points to a high degree of organization of the protein synthesis machinery in yeast mitochondria and suggests this as a suitable system for studying protein synthesis in mitochondria. Therefore, we have endeavored the isolation and purification of mitoribosomes as free as possible from contaminating cytoplasmic ribosomes (cytoribosomes) This paper presents a study of the specific characters in the yeast *Candida utilis* which allow a clear differentiation of isolated mitoribosomes from their cytoplasmic counterparts

Our results indicate that mitoribosomes and cytoribosomes isolated from *C. utilis* differ in physical properties such as the sedimentation coefficient, the buoyant density, and the susceptibility to dissociation into subunits, in chemical properties relevant to the molecular weight and the nucleotide composition of their respective RNA species, and finally in their shape as revealed by the electron microscope. In addition, attention has been paid to the artifactual dimerization of mitoribosomal subunits which may account for accumulation, under certain conditions, of a puzzling 80S particle reported several times (Schmitt, 1969, 1970; Vignais et al, 1969; Morimoto and Halvorson, 1971).

MATERIALS AND METHODS

Preparation and Sedimentation of Ribosomes

C. utilis (strain CBS 1516) was grown for 18 hr at 25°C under forced aeration in fermentor jars containing 10 liters of a medium made of 1% yeast extract, 2% peptone, and 1.5% glucose. When necessary, labeling of nucleic acids by ^{14}C or ^{32}P was achieved by growing the cells in the above medium supplemented with either uracil-^{14}C or inorganic phosphate-^{32}P. Mitochondria were extracted from cells by mechanical treatment and prepared as described by Mattoon and Sherman (1966). All operations were carried out at 2°–4°C. Mitoribosomes were freed from the isolated mitochondria by lysis of the mitochondrial membranes with sodium deoxycholate (DOC).[1]

In standard conditions, which allow the preferential accumulation of the monomer form of ribosomes, 60–100 mg of yeast mitochondria were suspended in 7.5 ml of 10 mM $MgCl_2$ and 10 mM Tris-HCl buffer, pH 7.4. Then 3.0–5.0 ml of 1% DOC were added dropwise (to reach a ratio of DOC to mitochondrial proteins [w/w] equal to 0.5) and the final volume was adjusted to 15 ml with distilled water. After standing for 10 min, the preparation was centrifuged at 20,000 rpm for 20 min (Spinco SW 50 rotor, Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.). The resulting supernatant fluid was spun at 48,000 rpm for 60 min in the same rotor. The ribosomal pellet recovered at this stage was rinsed three times with 5 ml of 5 mM $MgCl_2$, 1 mM Tris-HCl, pH 7 4, 50 mM KCl (standard MTK medium) and finally suspended in 0.4 ml of the same medium. After a clarifying spin at 2,000 *g* for 15 min, a sample fraction of 0.1 ml (corresponding to an optical absorbancy of 8–10 at 260 nm) was layered on a linear 5–20% sucrose gradient made in MTK medium and the tubes were centrifuged for 90 min at 40,000 rpm (Beckman SW 50 rotor). After centrifugation the contents of the tubes were pumped through a 1 mm flow cell of a LKB Uvicord (Laboratorie och Kemikaliska Produkter, Stockholm) connected to a Varian recorder (Varian Associates, Palo Alto, Calif.) to monitor continuously the absorbance at 254 nm.

Postmitochondrial extracts to be used for isolation of cytoribosomes were first freed from contaminant mitochondrial fragments by centrifugation at 20,000 rpm for 20 min in a Beckman Spinco 30 rotor. The supernatant fluid was then centrifuged at 30,000 rpm for 2 hr in the same rotor. The pellet consisting of

[1] *Abbreviations used in this paper:* BSA, bovine serum albumin; DOC, sodium deoxycholate; EDTA, ethylenediaminetetraacetate; SDS, sodium dodecyl sulfate; standard MTK medium, medium made of 5 mM $MgCl_2$, 1 mM Tris-HCl, pH 7.4, 50 mM KCl, TCA, trichloroacetic acid.

crude microsomes was homogenized in MTK medium and treated by DOC as described for the preparation of mitoribosomes, except that the weight ratio of DOC to protein was 2 instead of 0 5. Cytoribosomes were resuspended in MTK medium and their sedimentation pattern in sucrose was determined in parallel to that of mitoribosomes.

The sedimentation coefficients of mitoribosomes and cytoribosomes were calculated by calibration of the gradients with the 81S cytoribosomes isolated from rat liver (Küntzel and Noll, 1967)

Electron Microscopy

Fragments of mitochondrial pellets were fixed in 1% glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.2, rinsed in buffer, and postfixed in 2% osmium tetroxide in the same buffer solution. After dehydration in ethanol and embedding in Epon, thin sections of the isolated mitochondria were stained with a 5% aqueous solution of uranyl acetate followed by lead citrate.

Negative staining was carried out on unfixed ribosomal particles. A small drop of the ribosome suspension was placed with the aid of a platinum wire loop on a 300 mesh grid covered with a carbon-coated Formvar film. The grid was rinsed and excess liquid was withdrawn with filter paper. Without allowing the grid to dry, a drop of a 2% solution of uranyl acetate at pH 4–4.5 was placed on the grid and removed with filter paper after 15–30 sec.

Micrographs were taken with a Siemens Elmiskop IA electron microscope operating at 80 kv. An original magnification of about 60,000 was used for negatively stained material. The magnification was calibrated daily using a grating replica. Measurements of particles were made on photographic prints at a magnification of 300,000 Generally, 50 measurements were averaged for each type of particle

Isopycnic Banding in CsCl

The buoyant densities of mitoribosomes and cytoribosomes were determined by centrifugation on CsCl gradients as described by Perry and Kelley (1966) after fixation of the particles with glutaraldehyde as reported by Baltimore and Huang (1968). To assess more precisely the position of ribosomes in the gradients, the CsCl banding profiles were monitored by both absorbancy and radioactivity. For this purpose, uracil-2-^{14}C (58 mCi/mmole, obtained from the Commissariat à l'Energie Atomique, Saclay, France) was used to label the ribosomes. Labeled ribosomes were obtained from yeast cells grown in the presence of 0.1 mCi of uracil-2-^{14}C per liter of medium and prepared under the above mentioned standard conditions, which allow accumulation of monosomes. Ribosomes were treated with neutralized glutaraldehyde at a final concentration of 6%. The mixture was kept at 4°C for 12 hr before being layered without dialysis onto preformed CsCl gradients. The preformed gradients were obtained essentially as described by Brunk and Leick (1969) by layering in 5 ml Lucite tubes successively 2.75 g of CsCl in 2.5 ml of 10 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.4, and 1.42 g of CsCl in 2 5 ml of the same medium and centrifuging at 35,000 rpm for 30 hr at 4°C (Beckman SW 39 rotor). The equilibrium centrifugation of the glutaraldehyde-fixed ribosomes was achieved by spinning them through the preformed CsCl gradient at 35,000 rpm for 10 hr at 4°C (Beckman SW 39 rotor). After centrifugation, the contents of the tubes were analyzed for absorbancy and collected into fractions of 0.13 ml. Refractive indices were measured on 20 μl samples with an Abbe refractometer at 25°C Densities were calculated from refractive indices using the relationship given by Ifft et al. (1961). Samples of 100 μl were diluted with 3 ml water and counted by liquid scintillation counting (Unilux II, Nuclear Chicago, Des Plaines, Ill).

Isolation of Ribosomal RNA

In some experiments, RNA was directly extracted from mitoribosomes or cytoribosomes, according to Wilson and Quincey (1969). Ribosomes corresponding to 20 mg of ribosomal protein in 0.5 ml of MTK medium were mixed with 7 ml of 2.5% sodium dodecyl sulfate (SDS), 0.3 M NaCl, 0.01 M Tris-HCl, pH 7 4, 0.001 M $ZnCl_2$, and 20 mg of bentonite. 20 ml of water-saturated phenol containing 0.1% of 8-hydroxyquinoline were added and the mixture was shaken at 4°C for 15 min and centrifuged at 2,000 *g* for 5 min. The aqueous layer was pipetted out and the phenol extraction was repeated twice. Traces of phenol remaining in the aqueous layer were extracted by ether and finally ether was removed by bubbling air The RNA preparation was layered on linear 5–20% sucrose gradients and the different species of ribosomal RNA were separated by centrifugation for 5 hr at 40,000 rpm (Beckman Spinco SW 50 rotor)

As an alternative procedure, essentially used to recover the respective RNA species from each mitoribosomal subunit, the subunits were treated with SDS at a final concentration of 2% as described by Küntzel and Noll (1967) and the lysate was immediately centrifuged for 5 hr at 40,000 rpm through linear sucrose gradients in the presence of diethylpyrocarbonate, a reagent known to inhibit ribonuclease (Solymosy et al., 1968). After centrifugation, the RNA profiles were analyzed by monitoring absorbancy and fractions were collected for radioactivity determination.

Base Composition of Ribosomal RNA

^{32}P-labeled RNA was used for base composition analysis and obtained from yeast cells grown in a

medium supplemented with carrier free orthophosphate-^{32}P (2 mCi per liter of medium). Radioactive RNA species were separated by centrifugation on linear 5–20% sucrose gradients. After fractionation of the gradients with optical monitoring, the peaks corresponding to each RNA species were pooled and RNA was precipitated with 3 vol of ethanol and 0 1 vol of 1 M potassium acetate When the amount of ^{32}P-labeled RNA was too small, yeast RNA was added as a carrier. The RNA precipitate was hydrolyzed in 0 3 M KOH for 18 hr at 30°C (Rawson and Stutz, 1969). The mononucleotides were separated by paper chromatography (Duée, 1968). The areas corresponding to cytidine monophosphate (CMP), guanosine monophosphate (GMP), uridine monophosphate (UMP), and adenosine monophosphate (AMP) were located under ultraviolet light and cut out from the paper sheets. Their radioactivity was counted with a thin-window gas-flow counter (Intertechnique type RA15, Paris).

Incorporation of L-Leucine-^{14}C by Mitochondria

Uniformly labeled L-leucine-^{14}C (129 mCi/mmole) was obtained from the CEA (Saclay) The leucine-^{14}C incorporation was assayed essentially as described by Lamb et al. (1968), in a medium with an ATP-regenerating system (as detailed in the legend of Table III). To minimize bacterial contamination, the incubation medium was filtered through a 0.22 μ Millipore membrane (Millipore Corp., Bedford, Mass) Glassware was sterilized by autoclaving. Furthermore, mitochondria were used immediately after isolation. Assays for viable bacteria were made at the end of each incubation by plating 0 2 ml samples of the medium on nutrient agar in four different dilutions and incubating for 3 days at 25°C.

When leucine-^{14}C incorporation was to be directly analyzed in whole mitochondria, the incubation was ended by addition of a mixture of trichloroacetic acid (TCA) and ethanol (7% TCA in 33% ethanol), 5 ml of this mixture being added to 1 ml of incubation medium containing from 2 to 5 mg of mitochondrial protein. After centrifugation, the mitochondrial pellet was homogenized in 2 ml of 1 N NaOH containing 1 mg of unlabeled leucine per ml. After standing for 30 min at 37°C, proteins were again precipitated by 5 ml of the TCA-ethanol mixture, then washed with 5 ml of acetone, 5 ml of a 3 1 (v/v) mixture of ethanol and ether, and 5 ml of ether. The pellet was finally digested by formamide at 180°C and its radioactivity was estimated by liquid scintillation counting.

In some experiments, which were designed to detect nascent ^{14}C polypeptide chains on mitoribosomal particles, mitochondria after incubation with leucine-^{14}C were collected by centrifugation, washed with 0 65 M mannitol, and lysed by DOC The ribosome profile was analyzed by sucrose gradient centrifugation as described above.

RESULTS

Mitoribosomes In Situ

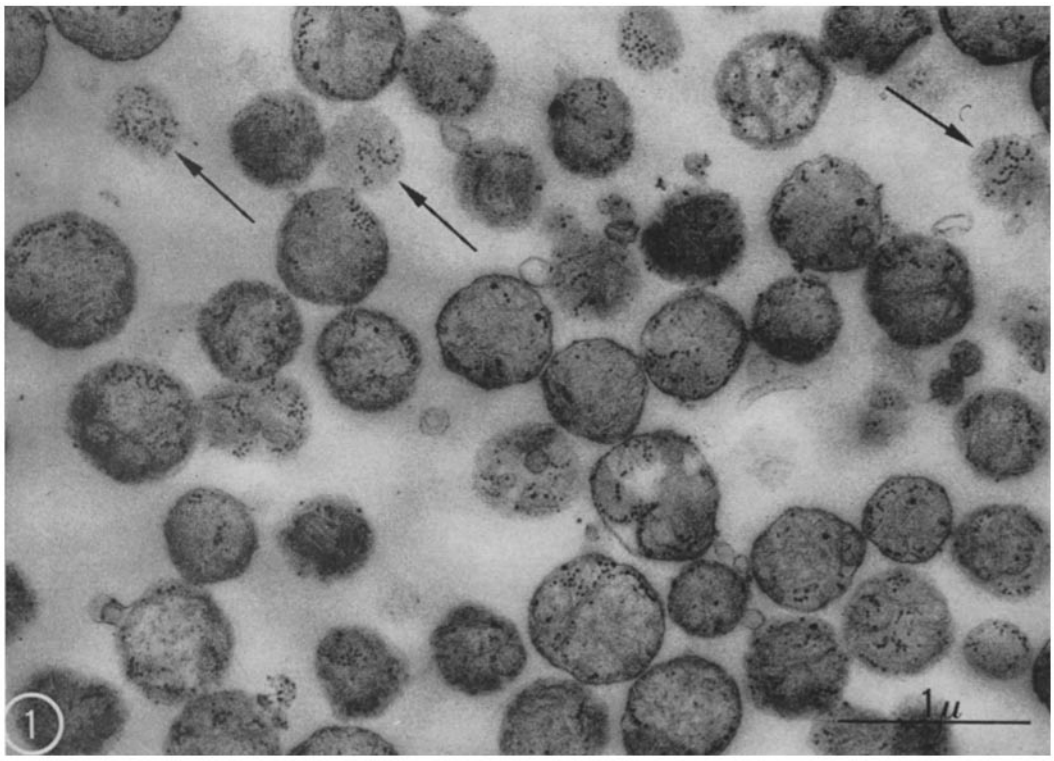
An example of a preparation of isolated mitochondria from *C utilis* viewed in thin section is shown in Fig 1, contamination with cytoplasmic ribosomes or cytoplasmic membranes is very limited if not absent Striking arrays of ribosomal particles are revealed within the mitochondria The particles are numerous and they appear to be preferentially arranged along the surfaces of the inner membrane and the membranes of the cristae facing the matrix In sections grazing the inner membrane, ribosomal particles appear aligned in whorls and curved or circular formations characteristic of polysomes. Alignments containing up to 10 or 12 particles can be observed, others containing 6 to 8 are frequent Occasionally, a faint line evoking messenger RNA, can be discerned between neighboring particles (Fig 2). Diameters of the mitochondrial ribosomes are about 180 A and closely approach those observed for yeast cytoribosomes in sectioned material.

Sedimentation Pattern of Mitoribosomes and Cytoribosomes under Standard Conditions

As shown in experiments presented in Figs 3 and 12, the sedimentation properties of *C. utilis* ribosomes depend on two critical factors: (*a*) the amount of DOC added to the mitochondrial or microsomal fraction to free the ribosomes, (*b*) the Mg^{2+} concentration and, in a more general way, the ratio of the monovalent to the divalent ion concentrations present in the sucrose gradient In this work, *standard conditions* refer to a DOC to protein ratio of 0 5 (w/w) and to linear 5–20% sucrose gradients made in 5 mM $MgCl_2$, 1 mM Tris-HCl, pH 7.4, and 50 mM KCl.

Mitoribosomes and cytoribosomes prepared and centrifuged under standard conditions each exhibit one major component sedimenting at 72S and 78S, respectively (Figs 3 A and C). Criteria for ascribing the 72S peak to the monomer form of mitoribosomes will be discussed later.

Deviation from the standard conditions severely alters the sedimentation properties of mitoribosomes. For instance, enhancing the DOC to protein



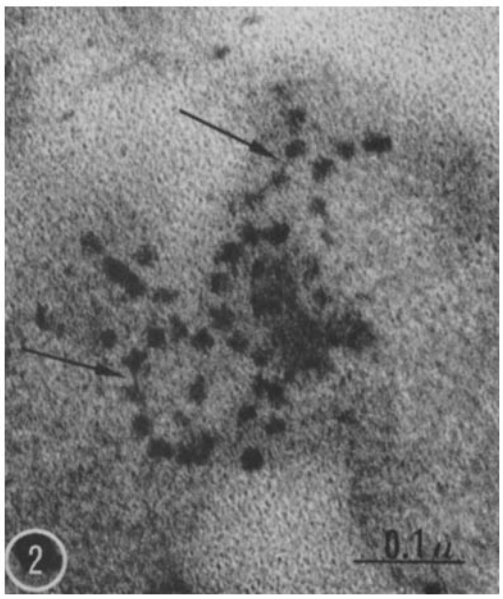


FIGURE 1 Thin section of isolated mitochondria from *C. utilis* showing numerous mitoribosomes arranged along the cristae and inner membrane surfaces. Many mitoribosomes appear in whorls characteristic of polysomes. Arrows point to grazing sections of mitochondria; polysomes are particularly evident here. Contamination by nonmitochondrial material is negligible × 25,000.

FIGURE 2 Mitochondrial polysomes in which a dense line can be observed between neighboring mitoribosomes (arrows). × 145,000.

ratio from 0.5 to 1.0 in the initial step of lysis of mitochondria, without changing the standard concentration of salts in the sucrose gradients, results in a nearly complete disappearance of the 72S peak at the expense of two new peaks sedimenting at 53S and 37S (Fig. 12 C). In contrast, cytoribosomes obtained with a DOC to protein ratio of 2 remain unaltered; they are half disso-

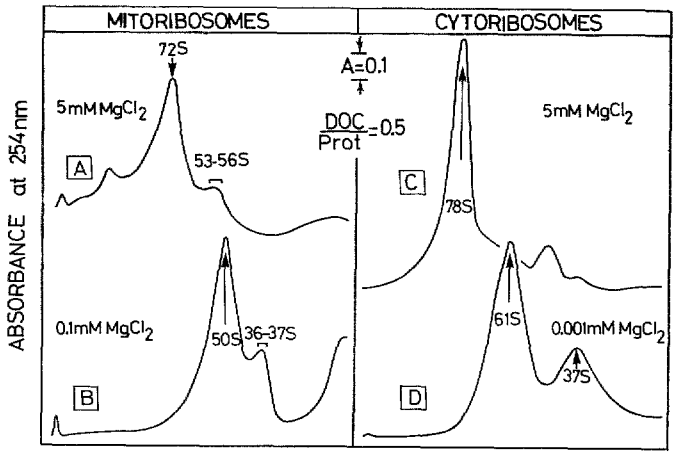


FIGURE 3 Effect of $MgCl_2$ concentration on the sedimentation profile of mitoribosomes and cytoribosomes. Mitoribosomes and cytoribosomes were prepared from mitochondrial and postmitochondrial extracts using a DOC to protein ratio of 0.5. The ribosomes suspended in standard MTK medium were layered on linear 5–20% sucrose gradients containing 1 mM Tris-HCl, pH 7.4, 50 mM KCl, and $MgCl_2$ at the specified concentrations and they were centrifuged for 90 min at 40,000 rpm in the Beckman Spinco SW 50 rotor at 4°C For other conditions see Materials and Methods.

ciated when the DOC to protein ratio reaches a value of 4

Dissociation of mammalian and microbial ribosomes into subunits upon decreasing the $MgCl_2$ concentration in the suspension medium is a well documented phenomenon (for reviews see Tissières et al, 1959, Petermann, 1964, Spirin and Gavrilova, 1969). *C utilis* mitoribosomes prepared with a DOC to protein ratio of 0 5 are completely dissociated into 50S and 36S subunits when the $MgCl_2$ concentration in the sucrose gradient is lowered from 5 to 0 1 mM (Fig 3 B). Cytoribosomes are comparatively much less sensitive to the $MgCl_2$ concentration since their complete dissociation into 61S and 37S subunits occurs only at 10^{-6} M $MgCl_2$ (Fig. 3 D).

Comparative Morphology of Mitoribosomes and Cytoribosomes

MONOMERS. Negatively stained mitoribosomes from the 72S peak prepared under standard conditions, give oval and elongate profiles measuring about 265 × 210 × 200 A (Fig. 4 and Table I). Although a variety of forms are observed, several of them are predominant and characteristic. Some selected examples are shown in Fig 5.

Form *a*, relatively rare, shows an oblong profile in which the division into unequal subunits is clearly suggested. An electron-opaque line is observed perpendicular to the long axis, presumably corresponding to the cleft between large and small subunits previously observed in other ribosomes The subunits visible here have approximately similar widths

Form *b*, which is frequently observed, shows oval profiles with an electron-opaque spot located slightly off-center The contour of these profiles suggests a partial subdivision into unequal subunits, however, images clearly showing the spot do not display the electron-opaque cleft

Form *c* presents a kidney shape with one convex and one concave side The two halves are dissimilar. one is smaller and has angular contours, the other is larger with curved contours. An electron-opaque notch is placed submedially along the concave side, at the place where the two halves join The whole figure is definitely asymmetric and enantiomorphic images can be found with about equal frequency.

Form *d* showing a roughly circular outline with some straight edges and blunt angles is frequently observed. A pentagonal outline can sometimes be discerned

We interpret forms *a* and *b* as representing, respectively, projections of the frontal and dorsal faces of the particle. The enantiomorphic images of form *c* then represent projections of the lateral faces of the particle viewed from directions at 90°

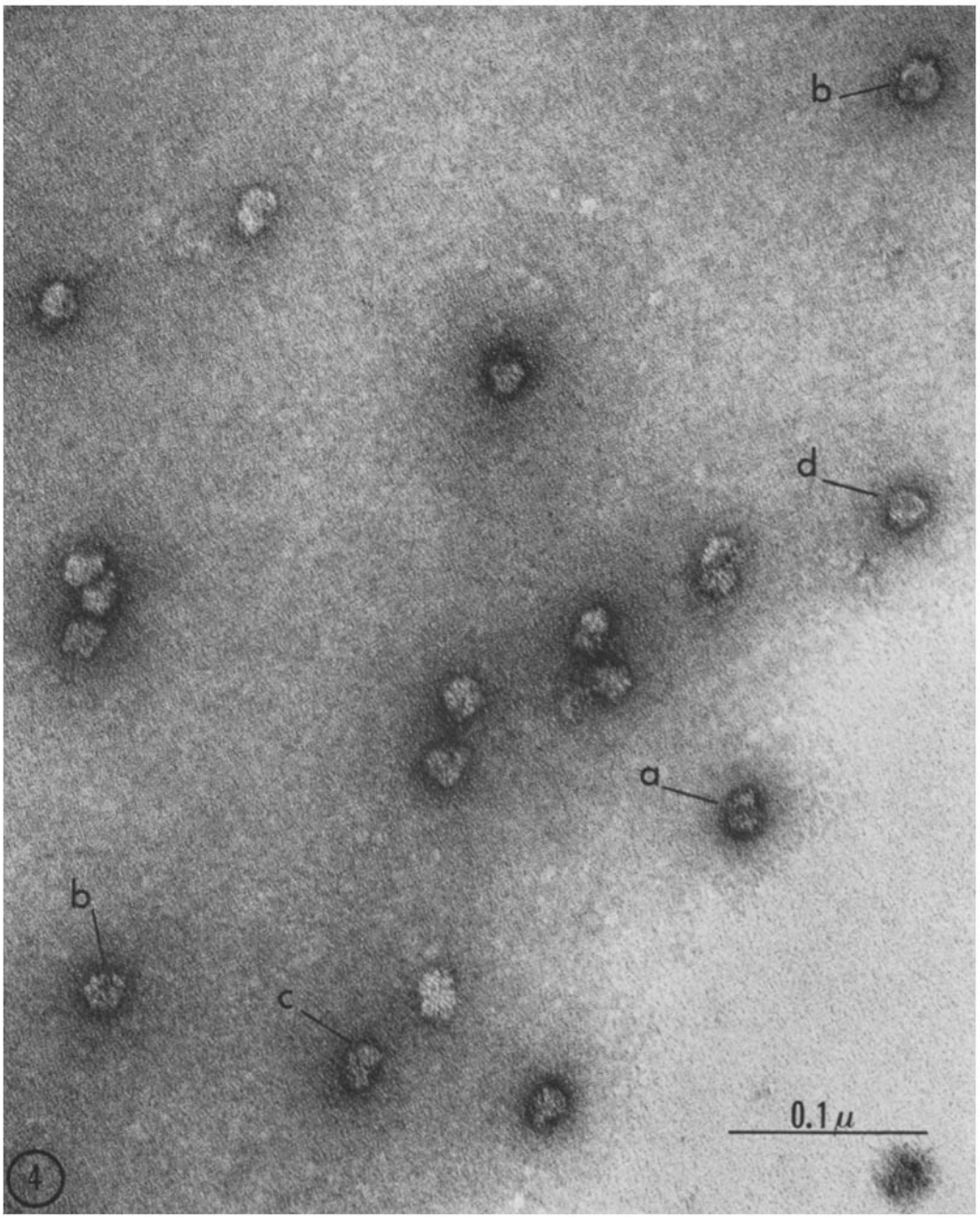


FIGURE 4 General view of a field of negatively stained mitoribosomes (72S) prepared under standard conditions. Forms *a*, *b*, *c*, and *d* (see text) are indicated. × 280,000.

from the preceding ones in the plane perpendicular to the elongation of forms *a* and *b*. Form *c* shows the small subunit asymmetrically positioned on the large, a feature which produces a definite notch on the frontal face. It is likely that a penetration of stain into the notch region can occur and that the notch region corresponds to the cleft observed in form *a*. In form *b*, where a complete

cleft is not seen, the electron-opaque spot appears to mark the boundary between subunits. The frequency of form *b* may suggest a predominant tendency of particles to orient with the dorsal face against the grid surface. Form *d* is interpreted as an end-on view of the mitoribosome in which only one subunit, probably the large, is visible. A variety of images intermediate among these four

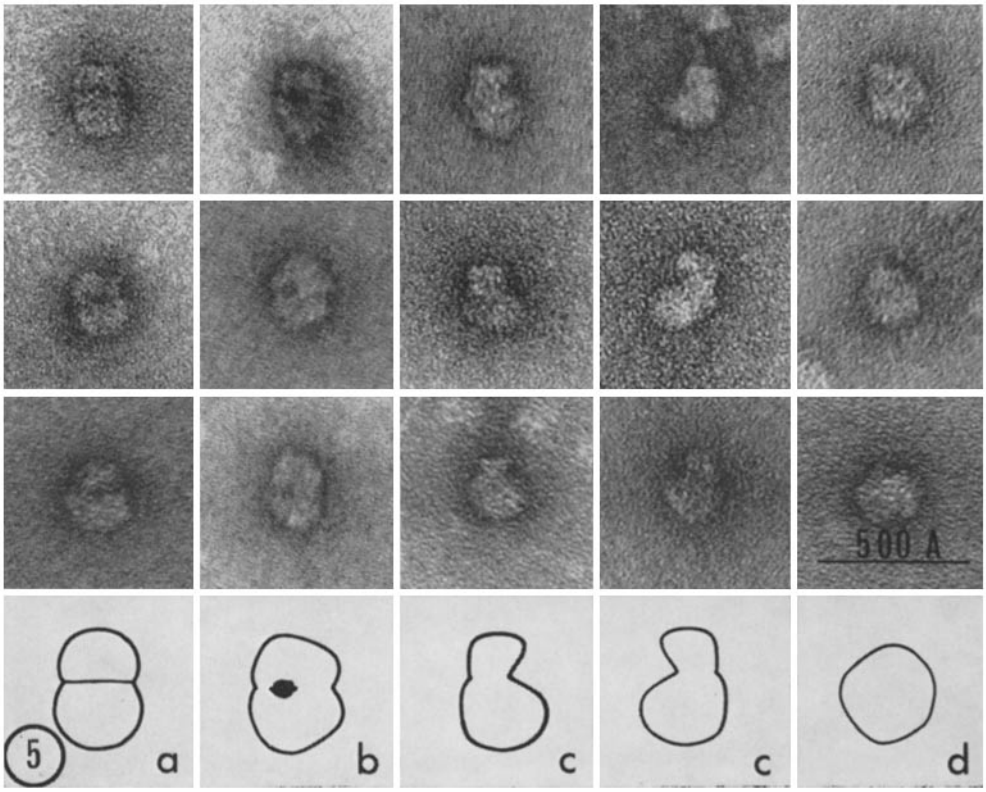
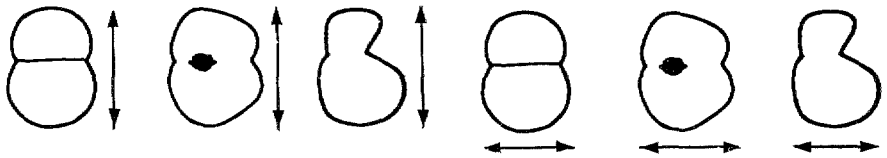


FIGURE 5 Selected images of mitoribosomes. Examples of forms *a*, *b*, *d*, and of the two enantiomorphic images of form *c* are shown. A schematic drawing accompanies each form. × 400,000.

TABLE I

Dimensions of Mitoribosomes and Cytoribosomes (A)

Axis measured						
Mitoribosomes	261 ± 20	266 ± 18	262 ± 18	206 ± 22	214 ± 18	200 ± 15
Cytoribosomes	260 ± 14	262 ± 16	254 ± 17	229 ± 23	222 ± 17	208 ± 17

All measurements were made at a magnification of ×300,000. The error of measurement is expressed as the standard deviation.

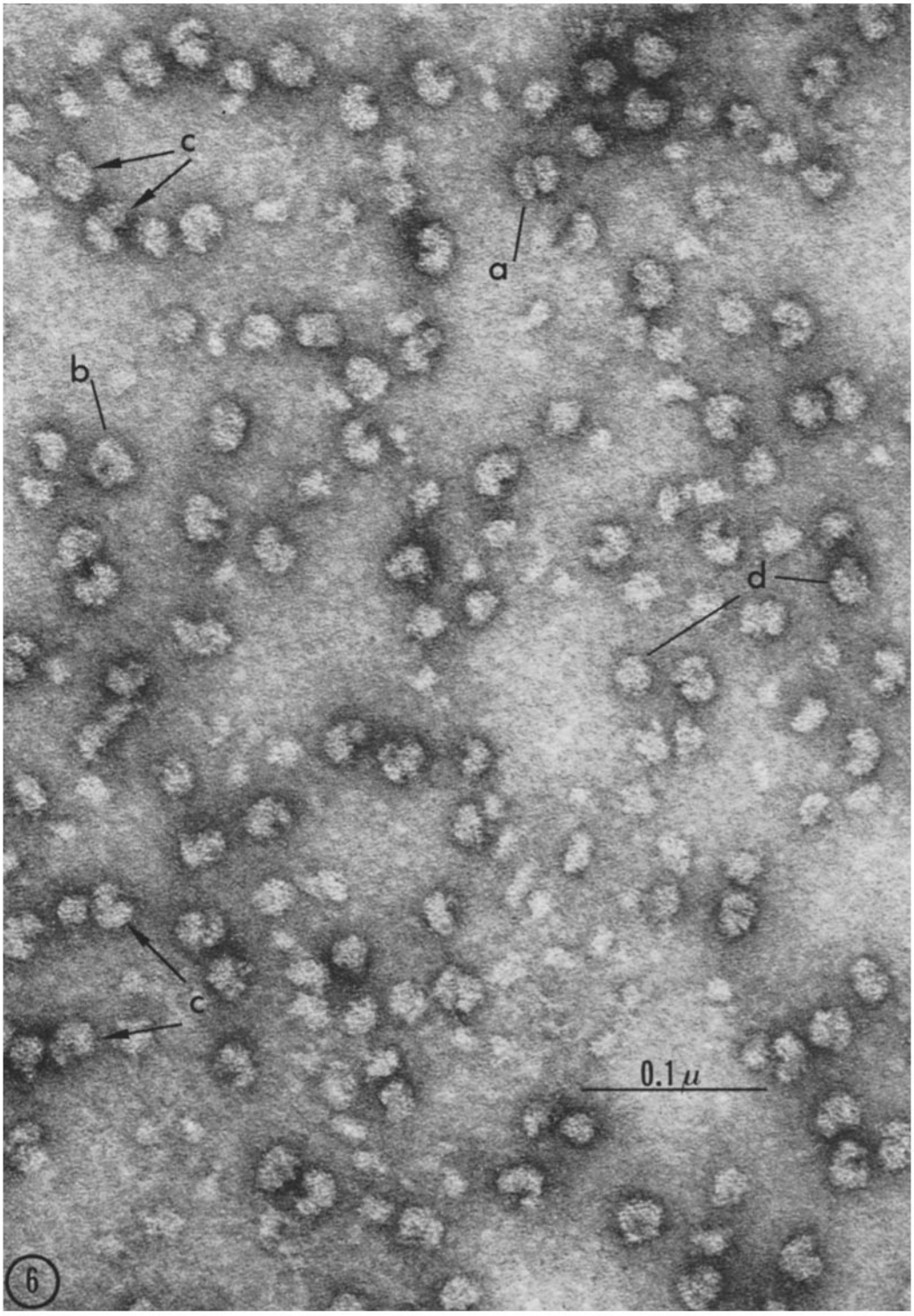


FIGURE 6 General view of a field of negatively stained cytoribosomes (78S). Forms *a*, *b*, and *d* are indicated Two enantiomorphic images of the lateral view *c* are indicated by arrows. Some smaller profiles may represent free subunits. × 280,000.

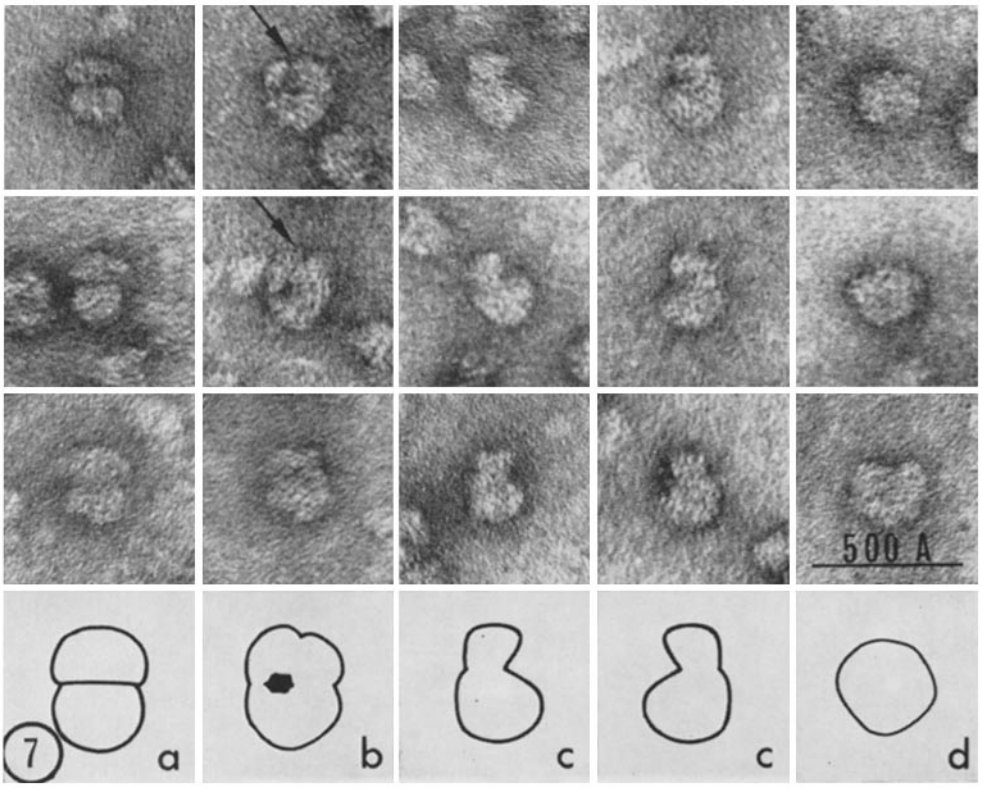


FIGURE 7 Selected images of cytoribosomes Examples of forms *a*, *b*, *d*, and of the two enantiomorphic images of form *c* are shown A partial subdivision in the small subunit is indicated by arrows A schematic drawing accompanies each form. × 400,000.

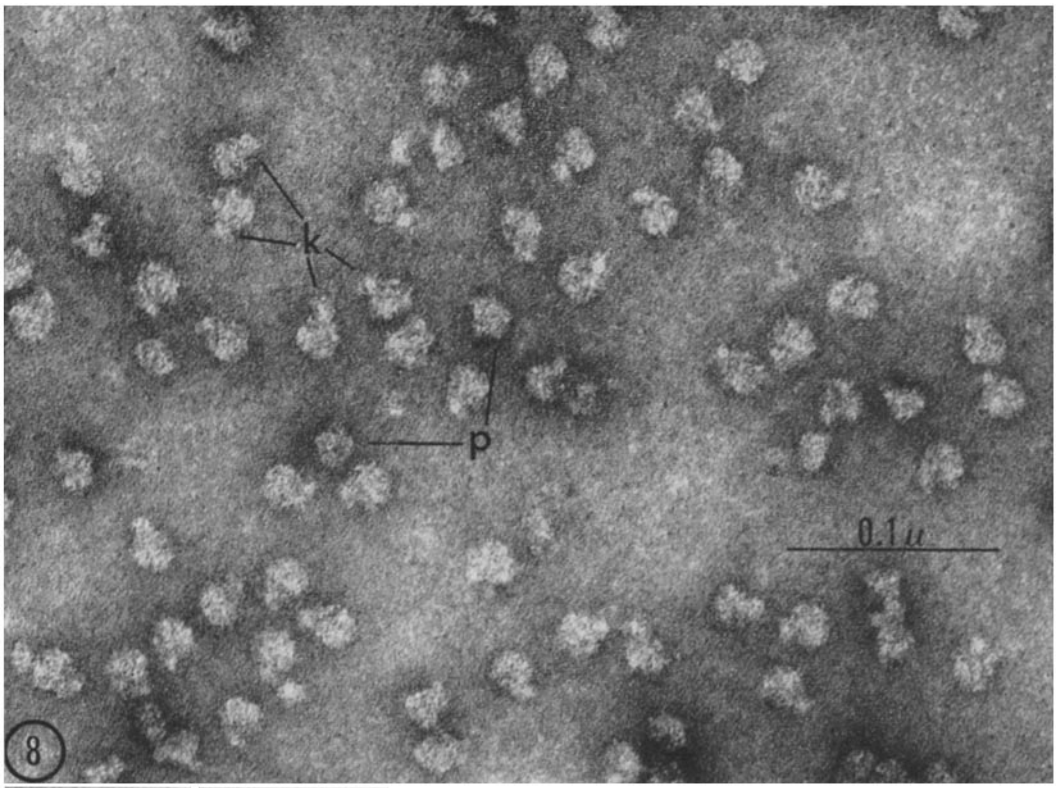
types can be found, indicating a number of possible orientations of the particles as they come to rest on the grid surface

A comparison of cytoribosomes with mitoribosomes reveals some minor differences (Figs. 6 and 7) Form *a*, showing an electron-opaque cleft between the subunits, is also rare in cytoribosomes. In these images, however, the small subunit is often slightly wider than the large and it appears as an overhanging cap. In form *b*, the dense spot is less pronounced and the profile is more irregular than in mitoribosomes. Occasionally, there is an indication of a subdivision in the small subunit (Fig. 7) We have not been able to detect such an image for mitoribosomes

The lateral views *c* are entirely similar to those of mitoribosomes. Again, enantiomorphic images can be found (Fig. 6) Form *d*, representing an end-on view, is also visible.

A comparison of the dimensions of cytoribosomes with those of mitoribosomes discloses little significant difference (Table I) The length along the long axis is close to 260 A, similar to that of mitoribosomes The width, measured in forms *a* and *b*, is slightly greater in cytoribosomes, about 225 A compared to 210 A in mitoribosomes The depth, measured in form *c*, is again similar in the two particles, about 200–208 A

SUBUNITS The large subunit (50S) of mitoribosomes, obtained by monosome dissociation in 0 1 mM $MgCl_2$, displays well-defined and distinctive profiles (Figs 8 and 9). Two principal views are observed. The most frequent shows a rounded profile bearing a knoblike projection. The image is slightly asymmetric in that the knob is slanting on one side. The knob seems of finer texture than the rest of the particle The other view of the large subunit is roughly circular, with some suggestion of flattened sides and a pentagonal outline The dimensions of the subunit are about 190 A in diameter and about 245 A in height, including the knob. The knob has a diameter of about 90 A



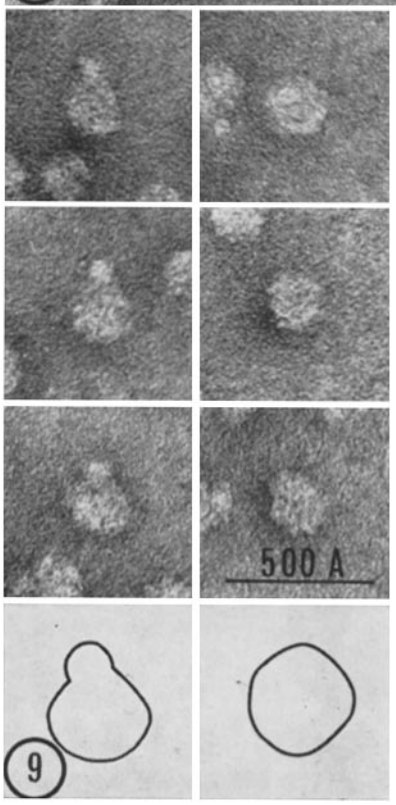
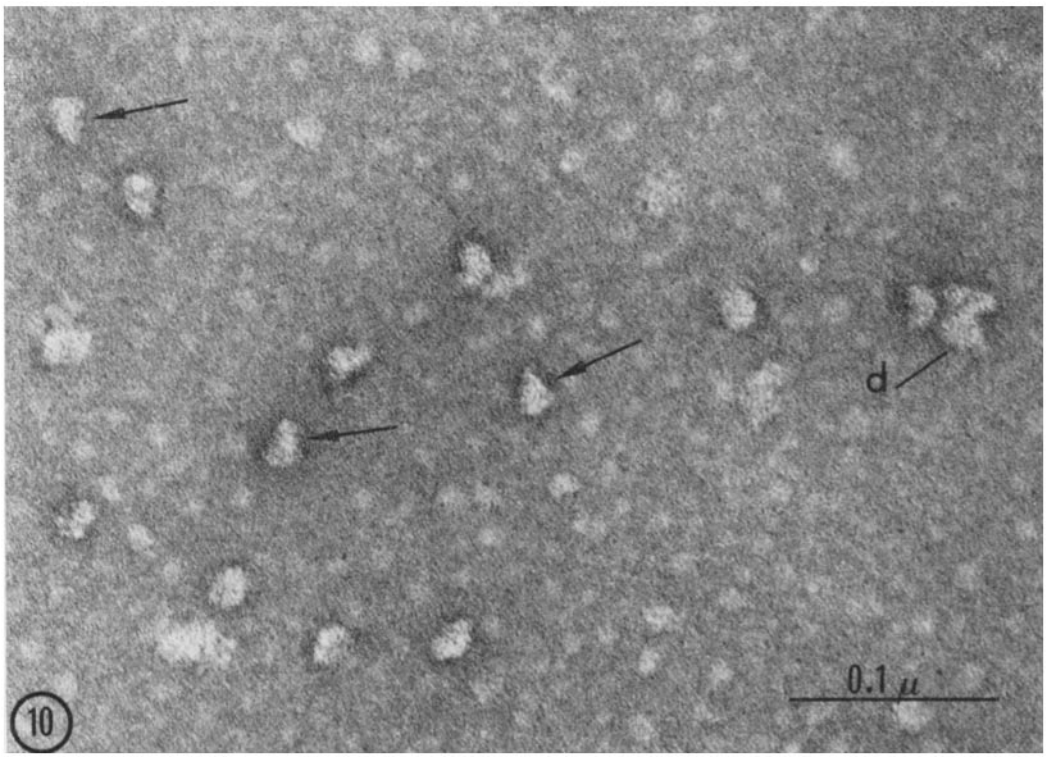


FIGURE 8 General view of a field of large mitoribosomal subunits (50S). Two principal forms are observed: rounded profiles bearing a knoblike projection (*k*) and roughly circular profiles suggesting a pentagonal outline (*p*). × 280,000.

FIGURE 9 Selected images of 50S mitoribosomal subunits showing the two principal forms and schematic drawings of each. The knobbed particles are slightly asymmetrical. × 400,000.



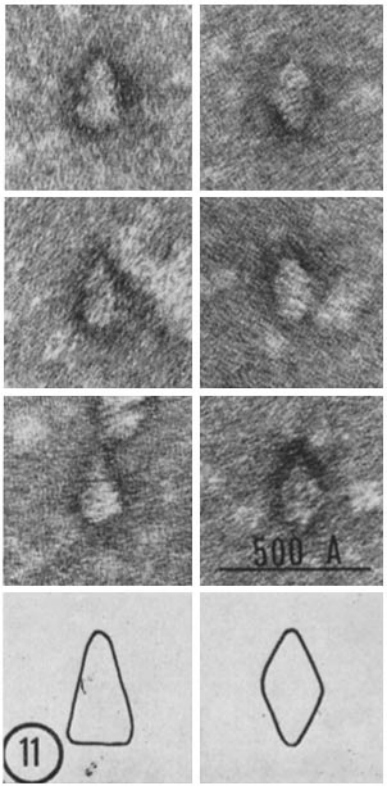


FIGURE 10 General view of a field of small mitoribosomal subunits (36S). Most of the profiles exhibit a triangular or arrowhead shape (arrows). The particle *d* is probably a dimer. × 280,000.

FIGURE 11 Selected images of 36S mitoribosomal subunits showing triangular and tetragonal profiles with their schematic representations × 400,000.

The small subunit (36S) produces slightly elongate profiles which display a variety of both angular and rounded forms (Figs. 10 and 11). One of these, a triangular or "arrowhead" shape, shows a pointed end clearly outlined by the uranyl stain and a less distinct, flattened base Other profiles having four sides and blunt angles can be described as tetragons. Finally, profiles with one convex and one concave or flat side are found. The length of this subunit is about 215 A and its greatest width is about 150 A. The variety of image types observed indicates a great flexibility and a tendency for deformation or poor preservation during drying on the grid surface or another step in the preparation. Nevertheless, from the images obtained, we can tentatively assign a three-dimensional shape of an irregular tetrahedron

The images obtained of the cytoribosomal subunits were less satisfactory than those of mitoribosomal subunits Preparations of large (61S) subunits contained slightly flattened, spherical profiles, and dome-shaped profiles No images were found showing a prominent knoblike projection as was constantly observed in the large mitoribosomal subunits. Images of small cytoribosomal subunits (37S) were unclear and suggested a variety of elongated, angular shapes

Effect of Cations on the Dissociation and Morphology of Ribosomal Particles

Ghysen et al. (1970) have recently reported that the stability of microbial ribosomes depends not only on the Mg^{2+} concentration but also on the ratio of the mono- to the divalent cation concentrations to which they are exposed. The same observation holds for *C utilis* mitoribosomes as illustrated in profiles of Fig. 12 To delineate more sharply the effect of mono- and divalent cations, ribosomes were prepared from *C. utilis* mitochondria with a DOC to protein ratio of 1 (instead of the standard value of 0 5), and the concentra-

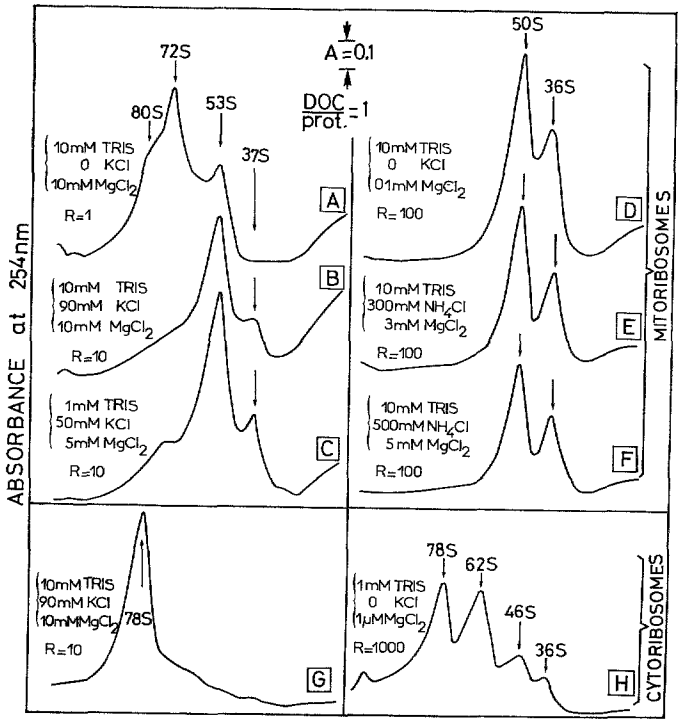


FIGURE 12 Effect of the ratio R of monovalent ion concentration to magnesium ion concentration on the sedimentation pattern of mitoribosomes and cytoribosomes. Mitoribosomes were prepared from mitochondria, using a DOC to protein ratio of 1. The mitoribosome suspension was divided into individual samples in which the ion concentrations (K^+, $Tris^+$, Mg^{2+}) were brought to definite and different values. Each ribosome sample was layered on a 5–20% sucrose gradient made in the same salt medium as that of the ribosome suspension and centrifuged at 40,000 rpm at 4°C (Beckman SW 50 Spinco rotor)

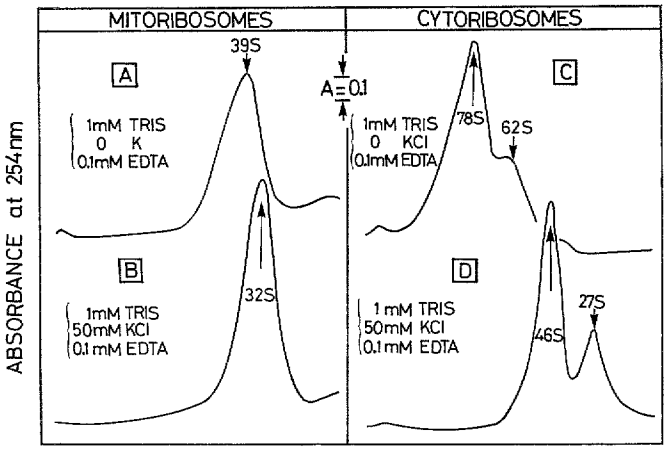


FIGURE 13 Effect of EDTA and KCl concentration on the sedimentation pattern of mitoribosomes and cytoribosomes. The 5–20% linear sucrose gradients contained Tris, KCl, and EDTA at the specified concentrations. Centrifugation conditions were as described in Fig 3.

tion ratio, R, of monovalent cations (K^+ and $Tris^+$) to the divalent cation (Mg^{2+}), in the sucrose gradient was varied from 1 to 1000.

In Fig. 12 C where R = 10, mitoribosomes were partially dissociated into subunits of 53S and 37S. Doubling both the mono- and divalent cation concentrations and maintaining the ratio R at 10 did not alter the sedimentation profile (Fig. 12 B) In contrast, profile A where R equaled 1 was characterized by a major component sedimenting at 72S, a shoulder at 80S (which will be discussed later), and a minor peak at 53S Examination in the electron microscope of the 72S peak reveals monosome particles similar to those prepared under standard conditions, but the profiles appear more irregular and the *b* form is more prominent.

A total dissociation of mitoribosomes into subunits sedimenting at 50S and 36S was obtained by raising R to a value of 100 (Fig. 12 D) A similar profile was obtained by replacing K^+ by NH_4^+ or by Na^+ and keeping the value of R at 100 (Fig 12 E and F) Although the sedimentation coefficient of dissociated subunits in sucrose gradients supplemented with K^+, Na^+, or NH_4^+ was independent of the nature of the cation, observation in the electron microscope revealed slight differences The "NH_4^+ subunits" appear well preserved and show forms indistinguishable from those found for subunits prepared at low $MgCl_2$ concentration, the knob of the 50S subunit is clearly present and the triangular and rounded-off rectangular forms of the 36S subunit are visible The "K^+" and "Na^+ subunits" show more heterogeneity and are less distinct, some images of 50S particles with knobs could be observed in the "K^+ subunits", but the "Na^+ 50S subunits" appear to be more degraded

The mitoribosome profiles in Fig 12 call for two other remarks: (*a*) a slight shift in the sedimentation coefficient of mitoribosome subunits accompanies the transition from partial to total dissociation (53S to 50S, 37S to 36S), no significant differences, however, in either size or form could be discerned in the electron microscope between the 53S and 50S particles or between the 37S and 36S particles, (*b*) the size ratio of the 37S peak to the 53S peak departs from that expected on the basis of an equimolar ratio of small and large subunits, at low R values. This may be due to an artifactual aggregation of homologous mitoribosome subunits, as it will be discussed later.

In contrast to the mitoribosome profiles, the cytoribosome profile obtained with a R value of 10 (Fig. 12 G) did not display any significans dissociation. A partial dissociation of cytoribosomes into particles sedimenting at 62S, 46S, and 36S occurred for a R value of 1000 (Fig. 12 H).

Whereas the concentration ratio of mono- to divalent cations obviously controls the dissociation of *C utilis* mitoribosomes into subunits, the monovalent cation concentration by itself, in the absence of divalent cations, also plays a significant role in the stability of ribosomal subunits, as illustrated with ribosomes depleted of magnesium by treatment with EDTA (Fig 13) The abnormally low sedimentation coefficients exhibited

in the presence of EDTA and KCl may reflect a partial unfolding of ribosome subunits, as observed with bacterial ribosomes (Gavrilova et al., 1966; Gesteland, 1966) and mammalian ribosomes (Petermann and Pavlovec, 1969) depleted of magnesium, or may be due to a release of ribosomal proteins similar to that reported in the case of bacterial (Lerman et al, 1966, Itoh et al., 1968) or mammalian (Reboud et al., 1969; Clegg and Arnstein, 1970) ribosomes treated with high concentrations of a monovalent cation. The great sensitivity of *C. utilis* mitoribosomes to EDTA as compared to the relative stability of cytoribosomes indicates that endogenous magnesium is more critically involved in mitoribosomes than in cytoribosomes to stabilize their structure

The "80S Mitoribosome" as a Dimer of the Large Subunit

Departure from the standard conditions of preparation (decrease of DOC concentration) and sedimentation of mitoribosomes (increase of $MgCl_2$ in the sucrose gradient) may lead to the appearance of a supplementary peak at 80S, as illustrated in Fig. 14. In this experiment, ribosomes were obtained from yeast grown on uracil-^{14}C and were therefore labeled in their RNA components The parallel between the absorbancy and radioactivity profiles indicates that the 80S peak arises from aggregation of ribosomal subunits, and not from sticking of stripped proteins to preexisting ribosomes.

For studying the conditions of accumulation of the 80S particle, any contamination of mitoribosomes by 78S cytoribosomes must be carefully assessed. This was the aim of the experiment shown in Fig 15, in which ^{32}P cytoribosomes had been first added to unlabeled mitochondria and then washed away (cf. legend Fig. 15). The mitoribosomes extracted thereafter exhibited two unlabeled peaks at 80S and 72S (Fig. 15 C). The cosedimentation pattern of ^{32}P cytoribosomes and unlabeled mitoribosomes (Fig. 15 B) was characterized by two absorbancy peaks (78S–80S and 72S) and one radioactivity peak in the 78S–80S region It is therefore obvious that the 80S peak, which incidentally appears on sedimentation profiles of mitoribosomes, is not due to contamination by cytoribosomes but represents genuine mitoribosomal material.

Another condition favoring the accumulation of 80S particles is the treatment of mitochondria

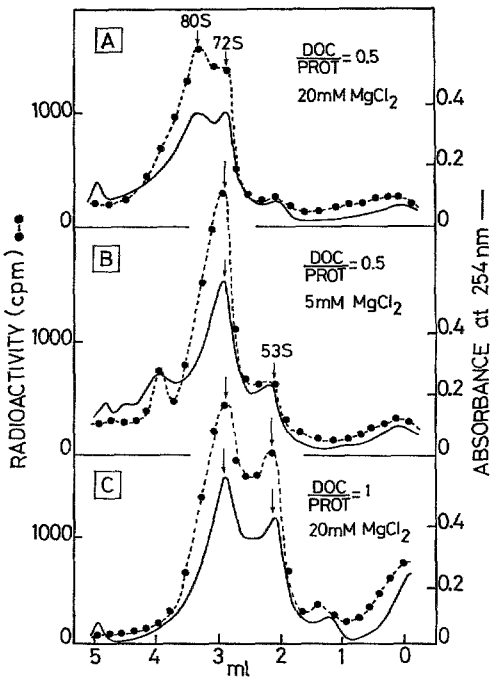


FIGURE 14 Effect of DOC and $MgCl_2$ on the accumulation of 80S mitoribosomal particles. The mitoribosomes were prepared from mitochondria lysed with either 0 5 mg or 1 mg of DOC per mg of mitochondrial protein. The ribosomes suspended in standard MTK medium were layered on linear 5–20% sucrose gradients containing 1 mM Tris-HCl, pH 7.4, 50 mM KCl, and $MgCl_2$ at the specified concentrations. Centrifugation conditions were as described in Fig. 3.

by puromycin before extraction of mitoribosomes.

Electron microscope observations of the material corresponding to the 80S peak show a great number of twin particles, each pair consisting of two roughly spherical profiles closely attached. The measurements of the dimers are about 320 A in length and about 180 A in width. These dimensions are consistent with the hypothesis that the dimers represent attachment of two 50S subunits, the diameter of which is close to 200 A if we exclude the knob. The slightly diminished diameter may signify a loss of superficial proteins.

Evidence for the artifactual dimerization of mitoribosomal subunits at high $MgCl_2$ concentration is provided by the experiment described in Fig. 16 and carried out with subunits labeled with uracil-^{14}C. In particular, it is seen that the 50S subunit gives rise to two new species of particles sedimenting at 69S and 80S when centrifuged

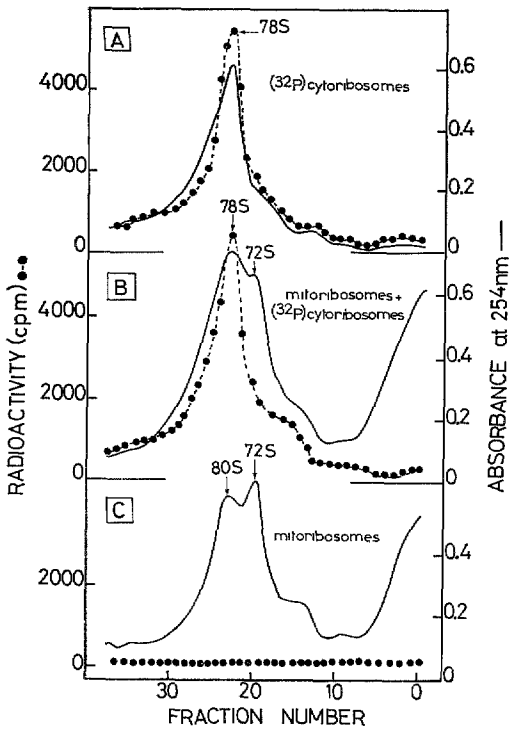


FIGURE 15 Cosedimentations of ^{32}P-labeled cytoribosomes with unlabeled mitoribosomes through sucrose gradients. ^{32}P cytoribosomes were isolated from yeast cells grown in a $^{32}P_i$-supplemented medium (A) On the other hand, mitochondria were prepared from unlabeled yeast and mixed with the ^{32}P cytoribosomes in the proportion of about 4:1 as measured by absorbancy at 260 mm (B). To eliminate the contaminant cytoribosomes, the "contaminated" mitochondria were washed three times by sedimentation at 6,000 *g* for 15 mn and resuspended once with 0.6 M mannitol, 2% bovine serum albumin (BSA), and 1 mM EDTA, and twice with 0.6 M mannitol and 2% BSA (C) Mitoribosomes were extracted from contaminated (B) and "decontaminated" (C) mitochondria by using a DOC to protein ratio of 0.5 and layered on a 5–20% linear sucrose gradient made in 10 mM Tris-HCl, pH 7.4, and 10 mM $MgCl_2$. Conditions of centrifugation as in Fig. 3.

through a sucrose gradient supplemented with 20 mM $MgCl_2$. In a similar way, the 36S subunit aggregates into 58S particles Electron microscope observation of the 80S component reveals closely paired particles of the 50S type (Fig. 17) and establishes that the 80S particle corresponds to a 50S dimer. These paired particles are similar in form and dimensions (about 316 × 165 A) to those described above. It must be noted, however, that the knobs, which are regularly found on the isolated 50S subunit, are never visible in the pairs Release of some surface proteins from 80S dimer particles without alteration of their RNA-^{14}C material may result in the slower 69S particles, apparently more radioactive than the 80S particles.

The above mentioned data on dimerization of mitoribosomal subunits are in agreement with reports that high concentrations of $MgCl_2$ promote the dimerization of small subunits of hepatic ribosomes (Tashiro and Siekevitz, 1965 *a*) and of large subunits of *Escherichia coli* (Huxley and Zubay, 1960).

Buoyant Densities of Mitoribosomes and Cytoribosomes

The physical properties of intra- and extramitochondrial ribosomes from *C. utilis* were further characterized by determining their buoyant densities after fixation with glutaraldehyde. As shown in Fig. 18 mitoribosomes exhibited a single density band at $\rho = 1.48$ and cytoribosomes a distinct peak at $\rho = 1.53$. In two other experiments the density values of mitoribosomes were found to be 1.48 and 1.49 and those of cytoribosomes 1.53 and 1.54. The lower density of mitoribosomes cannot be accounted for by contamination with mitochondrial membrane fragments since isolated mitoribosomes are characterized by a high ratio (1.82–1.83) of 260 to 280 nm absorbancy and since the fractions appear homogeneous in the electron microscope.

An approximate value of the weight per cent of RNA in ribosomes was calculated by the formula of Perry and Kelley (1966). Taking 1.90 and 1.25 for the density of RNA and protein, respectively, and neglecting the differences in density of mitoribosomal and cytoribosomal RNA which may arise from differences in base composition, it was estimated that the 78S cytoribosome contains about 55% RNA whereas the 72S mitoribosome contains only 46% RNA.

RNA Species of Mitoribosomes and Cytoribosomes

As shown in Fig 19, RNA components extracted from *C. utilis* mitoribosomes and cytoribosomes can be easily differentiated by their sedimentation properties in a sucrose gradient. In a $MgCl_2$-supplemented sucrose gradient, taking as reference the 29S and 18S RNA of rat

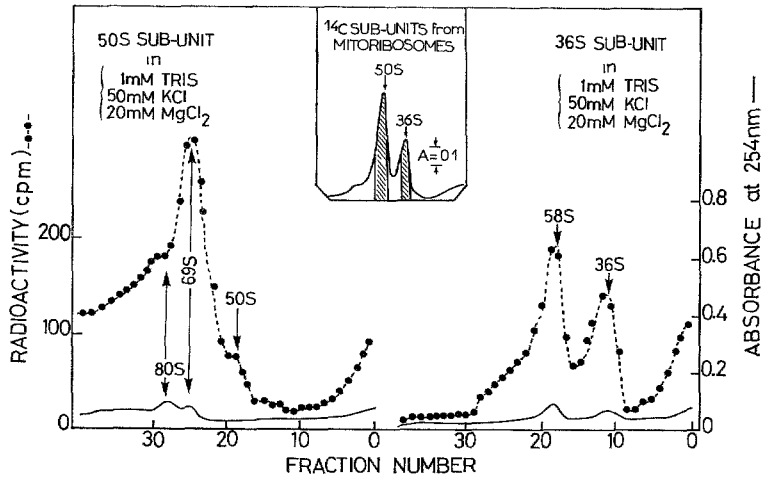


FIGURE 16 Effects of high $MgCl_2$ concentration on the sedimentation profile of 50S and 36S mitoribosomal subunits. Mitoribosomal subunits labeled by uracil-^{14}C were prepared by dissociation of ^{14}C mitoribosomes in 50 mM KCl, 1 mM Tris-HCl, pH 7.4, and 0 1 mM $MgCl_2$, and separated by centrifugation through linear 5–20% sucrose gradients made in the above saline medium. The pooled fractions (36S and 50S subunits) were mixed with an equal volume of 20 mM Tris-HCl and 20 mM $MgCl_2$, and pelleted by high speed centrifugation The particles were resuspended in 20 mM $MgCl_2$, 50 mM KCL, and 1 mM Tris-HCl, pH 7 4, and centrifuged through linear 5–20% sucrose gradients made in the same saline medium.

liver cytoribosomes (Küntzel and Noll, 1967, Spirin and Gavrilova, 1969), the following sedimentation coefficients were obtained: 23S and 19S for mitoribosomal RNA, 27S and 17 5S for cytoribosomal RNA. In $MgCl_2$-supplemented sucrose gradients, mitoribosomal RNA displays a supplementary peak at 28S and cytoribosomal RNA a shoulder at 32S It is likely that these faster sedimenting RNA result from a Mg^{2+}-induced aggregation of lower molecular weight RNA. When $MgCl_2$ in the sucrose gradient was replaced by EDTA, lower sedimentation coefficients were obtained 21S and 16S for mitoribosomal RNA, 25S and 17S for cytoribosomal RNA. The marked increase of the sedimentation coefficients of mitoribosomal and cytoribosomal RNA upon addition of $MgCl_2$ (except in the case of the 17S cytoribosomal RNA) may be due to a transition towards a more compact configuration as postulated by Loening (1969).

The empirical equation $M = KS^{\alpha}$ (Gierer, 1958; Spirin, 1961) was used to calculate the molecular weights M of *C. utilis* RNA from the sedimentation coefficients S K and α were calculated from sedimentation data of mammalian cytoribosomal RNA (Table II of Spirin and Gavrilova, 1969) and found to be, $K = 5\,8 \times 10^{3}$ and $\alpha = 1\,70$ for $MgCl_2$-supplemented gradient, $K = 3.3 \times 10^{3}$ and $\alpha = 1\,95$ for EDTA-supplemented gradient. The molecular weights of *C. utilis* RNA derived from sedimentation data in $MgCl_2$-supplemented gradients were, $1\,58 \times 10^{6}$ and $0\,76 \times 10^{6}$ for the 27S and 17 5S cytoribosomal RNA, $1\,26 \times 10^{6}$ and $0\,83 \times 10^{6}$ for the 23S and 19S mitoribosomal RNA. The sedimentation data in EDTA-supplemented gradients yielded molecular weight values of $1\,67 \times 10^{6}$ and 0.80×10^{6} for the 25S and 17S cytoribosomal RNA, 1.21×10^{6} and 0.71×10^{6} for the 21S and 16S mitoribosomal RNA Differences in the calculated values of molecular weight (especially in the case of the small mitoribosomal RNA) may arise from RNA-RNA interactions which are known to be enhanced by $MgCl_2$ (Sporn and Dingman, 1963; Watson, 1964, Stanley and Bock, 1965).

To decide whether the 21S and 16S mitoribosomal RNA species belong to the 50S and 36S subunits, respectively, the RNA-^{14}C component of each mitoribosomal subunit labeled with uracil-^{14}C was extracted as detailed in the legend of Fig. 20, and characterized by its sedimentation profile. As shown in Fig. 20, the RNA-^{14}C obtained from the 50S particles sediments at 22S–

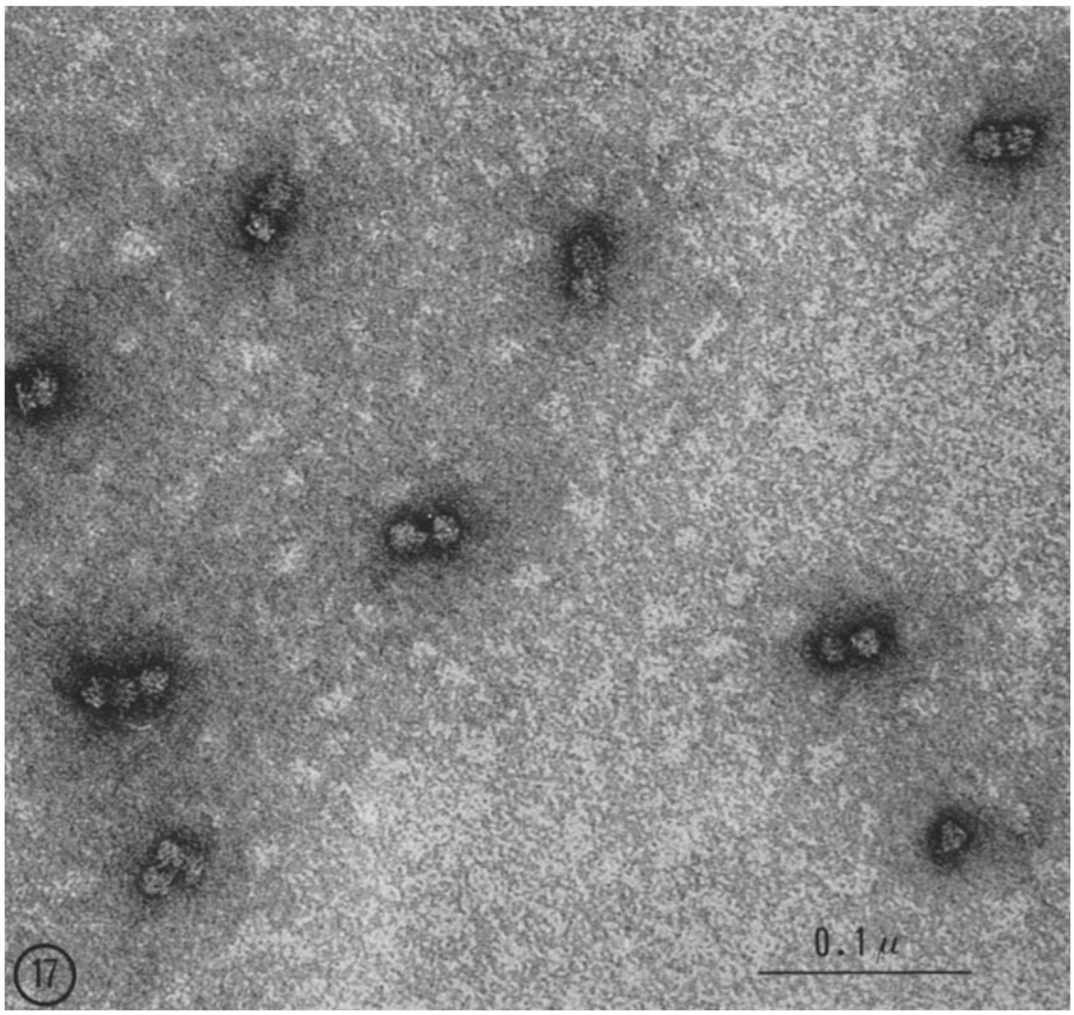


FIGURE 17 General view of a preparation from the 80S peak obtained by resuspension of 50S subunits in 20 mM $MgCl_2$ The majority of the material consists of dimers formed by closely paired particles of the 50S type which, however, lack the knob. × 280,000

23S whereas that obtained from the 36S particles sediments at 18S Consequently, it is inferred that the 50S and 36S mitoribosomal particles are two distinct subunits obtained by dissociation of the 72S monosome.

Base Composition of Ribosomal RNA

Data in Table II show that the (G + C)/(A + U) ratio is close to 0 5 for the 16S and 21S mitoribosomal RNA and to 1 for the 25S and 17S cytoribosomal RNA from *C. utilis* Similar differences have been found between the cytoribosomal and the mitochondrial RNA in *Saccharomyces cerevisiae* (Fauman et al , 1969, Morimoto and Halvorson, 1971), *Neurospora crassa* (Küntzel and Noll, 1967, Rifkin et al , 1967), *Tetrahymena pyriformis* (Chi and Suyama, 1970), *Aspergillus nidulans* (Edelman et al , 1970, 1971), and also in higher organisms (Vesco and Penman, 1969, Bartoov et al., 1970, Dubin and Montenecourt, 1970, Montenecourt et al , 1970).

Chloramphenicol-Sensitive Protein Synthesis at the Level of Mitoribosomes

Mitochondrial and cytoplasmic protein synthesis can be differentiated by their sensitivity to

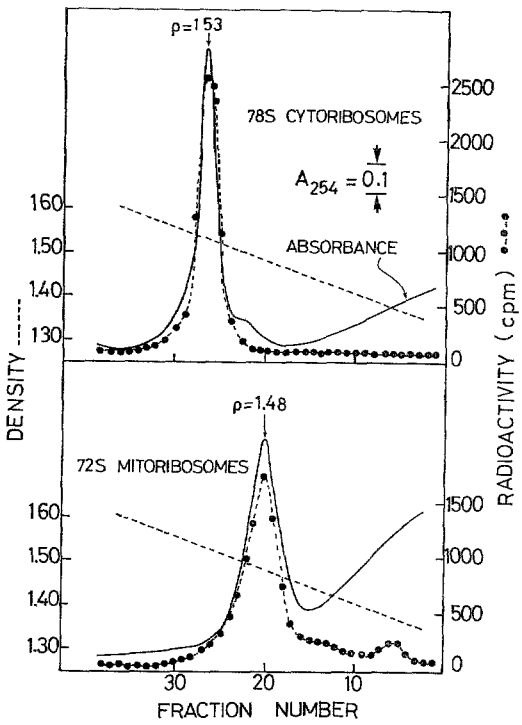


FIGURE 18 CsCl density gradient centrifugation of cytoribosomes and mitoribosomes. ^{14}C cytoribosomes and ^{14}C mitoribosomes fixed by glutaraldehyde were layered on preformed gradients and centrifuged for 10 hr at 35,000 rpm in the Beckman Spinco SW 30 rotor at 4°C as described in Materials and Methods. Densities were calculated from the refractive indices.

chloramphenicol and cycloheximide. Whereas chloramphenicol is a potent inhibitor of protein synthesis in isolated mitochondria, but has no effect on the activity of the cytoplasmic ribosomes in eukaryotes (Mager, 1960; Wheeldon and Lehninger, 1966; Kroon and Jansen, 1968), the reverse is true for cycloheximide (Beattie et al., 1967; Lamb et al, 1968, Linnane, 1968; Loeb and Hubby, 1968) On the other hand, acriflavine, which inhibits protein synthesis in mitochondria but not in bacteria, has been proposed to monitor the bacterial contamination (Kroon et al., 1968). Data in Table III show the extent of leucine-^{14}C incorporation into the proteins of *C. utilis* mitochondria in the presence of the above mentioned antibiotics As expected, the leucine-^{14}C incorporation is highly sensitive to chloramphenicol and unaltered by cycloheximide The partial resistance to acriflavine is accounted for by an inescapable bacterial contamination. Actually the contamination was found to be less than 5×10^3 bacteria per ml of incubation medium.

When mitoribosomes are extracted from mitochondria immediately after incubation with leucine-^{14}C and analyzed by sedimentation on a sucrose gradient, they exhibit a major peak of radioactivity at 72S (Fig. 21), which reflects the binding to monoribosomes of ^{14}C nascent polypeptide chains Chloramphenicol prevents the leucine-^{14}C incorporation. On the other hand, addition of puromycin, subsequent to the incuba-

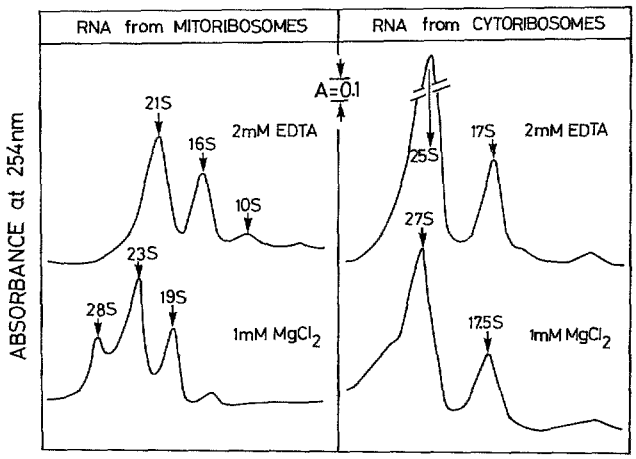


FIGURE 19 Sucrose gradient centrifugation of RNA extracted from mitoribosomes or from cytoribosomes. RNA was extracted from isolated mitoribosomes and cytoribosomes as described in Materials and Methods and layered on linear 5–20% sucrose gradients made in 1 mM Tris-HCl, pH 7.4 and, either 2 mM EDTA or 1 mM $MgCl_2$, as specified in the figure. Centrifugation was performed at 40,000 rpm for 5 hr in the Beckman Spinco SW 50 rotor at 4°C.

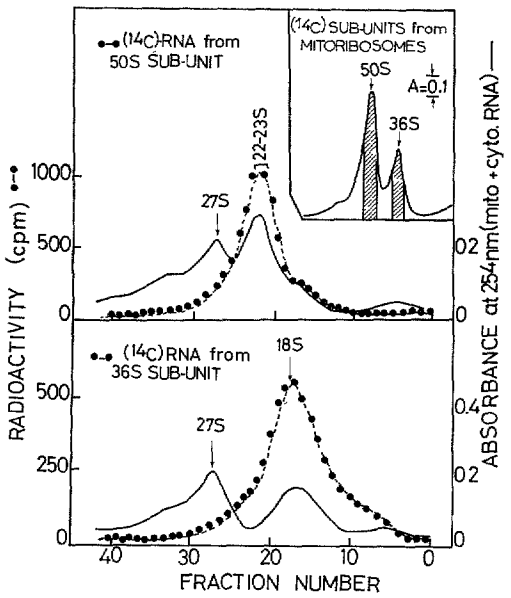


FIGURE 20. Sedimentation patterns of RNA extracted from mitoribosomal 50S and 36S subunits. Mitoribosomes labeled by uracil-^{14}C were dissociated into subunits by suspension in 10 mM Tris, 300 mM NH_4Cl, and 3 mM $MgCl_2$. Mercaptoethanol was added to a final concentration of 0.1 M to lower the ribonuclease activity. After centrifugation through linear sucrose gradients made in the same saline medium, fractions corresponding to each ribosomal subunit were pooled and mixed with bentonite (about 1 mg/ml) and polyvinylsulfate (20 μg/ml). They were diluted with an equal volume of ethanol and 2 vol of 10 mM Tris-HCl, pH 7.4 and 10 mM $MgCl_2$, then centrifuged for 30 min at 48,000 rpm in a Beckman SW 50 Spinco rotor at 4°C. The RNA-^{14}C was immediately extracted from the mitoribosome subunits. The pellet in each tube was resuspended in 200 μl of a mixture of 10 mM $MgCl_2$, 10 mM Tris, pH 7.4, and 5% diethylpyrocarbonate and lysed by 2% SDS for 1 min at 20°C. After clarification by a centrifugation of 2 min in an Eppendorf centrifuge (Netheler Hinz, Hamburg, Germany), the lysate, containing the ^{14}C mitoribosomal RNA, was mixed with unlabeled cytoplasmic RNA (corresponding to an absorbancy of about 5 in a volume of 100 μl), layered on top of a 5–20% linear sucrose gradient made in 1 mM Tris, pH 7.4 and 1 mM $MgCl_2$ and centrifuged for 5 hr at 40,000 rpm in a Beckman SW 50 rotor at 4°C. The absorbancy profile corresponds to the mixture of cytoribosomal and mitoribosomal RNA whereas the radioactivity profile only corresponds to the mitoribosomal RNA.

tion of mitochondria with leucine-^{14}C, results in a release of radioactive polypeptides recovered on top of the sucrose gradient after centrifugation.

TABLE II

Base Composition of RNA Extracted from Mitoribosomes and Cytoribosomes from Candida utilis

		Cytoribosomal RNA		Mitoribosomal RNA	
	Exp	17S	25S	16S	21S
UMP	1	25.7	24.4	32.3	29.7
	2	25.0	25.0	33.6	32.5
AMP	1	24.8	23.7	34.1	34.2
	2	25.2	24.7	34.9	34.1
GMP	1	28.2	30.3	19.8	20.8
	2	27.7	30.0	18.8	20.6
CMP	1	21.3	21.6	13.8	15.3
	2	22.1	20.3	12.7	12.8

For the study of the base composition of ribosomal RNA, *C. utilis* was grown in a phosphate-^{32}P-supplemented medium. The ^{32}P-labeled RNA species were extracted from mitoribosomes and from cytoribosomes as described in Materials and Methods and separated by centrifugation in a 5–20% linear sucrose gradient made in 1 mM Tris-HCl, pH 7.4, 50 mM KCl, and 2 mM EDTA. The sucrose fractions corresponding to the absorbancy peaks were collected and pooled. The RNA present in these fractions was precipitated by adding 0.1 vol of 1 M potassium acetate and 3 vol of 95% ethanol in the presence of 0.5 mg of unlabeled yeast RNA, and hydrolyzed into nucleotides. The nucleotide content of RNA is expressed as per cent of total phosphorus-^{32}P.

DISCUSSION

Chemical Characterization of Candida utilis Mitoribosomes

Although in many reports, isolated mitoribosomes have been convincingly proven to differ from their cytoplasmic counterparts by physical properties such as the sedimentation coefficient and the susceptibility to dissociation into subunits, their chemical characterization in terms of specific RNA species has not often been critically examined (see review in Rabinowitz and Swift, 1970). In fact, it usually is implicitly admitted that the high molecular weight RNA components extracted from whole mitochondria are mitoribosomal RNA. Although this statement is most likely valid, it may lead to erroneous conclusions. For instance, the question has recently been posed (Malkin,

TABLE III

Effect of Chloramphenicol, Cycloheximide, and Acriflavine on Leucine-^{14}C Incorporation by Whole Yeast Mitochondria

Exp		Incorporated leucine-^{14}C	Inhibition
		cpm	%
1	Control	6640	—
	Chloramphenicol	100	99
	Acriflavine	1280	81
2	Control	7800	—
	Cycloheximide	7950	0

Mitochondria (1.5 mg protein) in 1 ml of 100 mM mannitol, 5 mM KH_2PO_4, 6 mM $MgCl_2$, 40 mM Tris, pH 7.2, and 1 mM EDTA were preincubated for 2 min either with 1 6 mM chloramphenicol, 0.5 mM cycloheximide, or 6 μg acriflavine/mg protein. Incubation at 25°C was started by the addition of 0.2 μCi leucine-^{14}C, 1 mM ATP, 25 μg oligomycin, 5 mM phosphoenolpyruvate, and 25 μg pyruvate kinase It was ended after 10 min by addition of 7 ml of 7% TCA in 95% ethanol The radioactivity of the sediment was determined as described in Materials and Methods

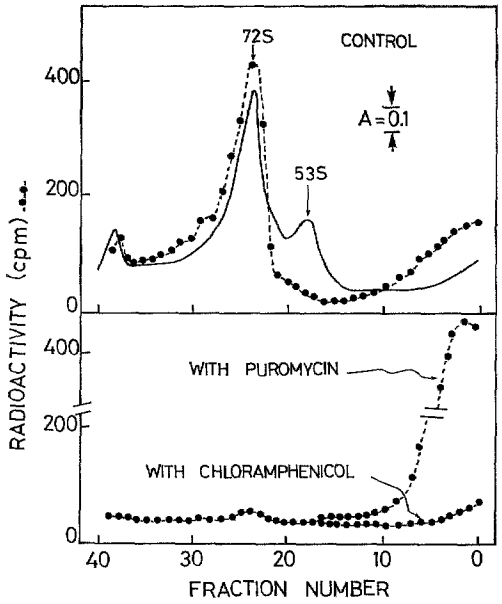


FIGURE 21 Sedimentation analysis of mitoribosomes as protein synthesizing particles. effect of puromycin and chloramphenicol. Mitochondria (80 mg protein) were incubated at 25°C in the leucine-^{14}C medium (0.8 μCi/ml) described in Table III. After a 5 min incubation period, the suspension was divided into two fractions. The first was incubated for another 5 min at 25° C with 0.4 mM puromycin, the other was the control. A parallel incubation for 10 min at 25°C was carried out with 80 mg mitochondria, in the same leucine-^{14}C medium supplemented with 1.6 mM chloramphenicol. After incubation, the three different mitochondrial suspensions were centrifuged, washed with 100 ml of cold 0.65 M mannitol, and lysed by deoxycholate to prepare mitoribosomes (DOC/protein ratio = 0.5). Centrifugation of mitoribosomes on 5–20% linear sucrose gradients made in 1 mM Tris-HCl, pH 7.4, 50 mM KCl, and 5 mM $MgCl_2$ was carried out as described in Fig. 3.

1971) as to whether the species of RNA extracted from crude mitochondrial preparations of rat liver and sedimenting at 20S and 22S are not degradation products of contaminant cytoribosomal 28S RNA. In the present work, mitoribosomal RNA has been extracted directly from purified mitoribosomes We have furthermore identified the RNA species contained in the large and small subunits of these mitoribosomes; the low molecular weight RNA (16S–19S) is specific of the 36S mitoribosomal subunit and the high molecular weight RNA (21S–23S) of the 50S subunit Since both subunits are obtained by dissociation of the 72S mitoribosome, it is concluded that the 72S ribosome is the monomer form of the mitoribosome. This view is corroborated by the finding that nascent polypeptide chains are recovered with the 72S particles after centrifugation on sucrose gradients.

Size and Shape of Mitoribosomes

A survey of data concerning the sedimentation coefficients of mitoribosomes from various organisms points to a similarity in size between mitoribosomes from several fungi and bacterial ribosomes (70S, Tissières et al., 1959). Among the more relevant values, the following may be quoted, 73S in *N. crassa* (Küntzel and Noll, 1967; Küntzel, 1969), 72S in *S. cerevisiae* (Schmitt, 1970), 74S in *S. carlsbergensis* (Grivell et al , 1971), 67S in *A nidulans* (Edelman et al , 1970; 67S was also found for *E coli* ribosomes in this latter study) These values are close to that found in the present work on *C utilis* (72S). On the other hand, somewhat diverse values have been reported, 81S in *Neurospora* (Rifkin et al , 1967) and 75S–80S in several yeasts (Vignais et al , 1969, Schmitt, 1969, Stegeman et al , 1970, Morimoto and Halvorson, 1971) Taking the 72S–74S value as representative

of the fungal group, these mitoribosomes stand intermediate in size between those of *T. pyriformis* which sediment at 80S (Chi and Suyama, 1970) and those of higher organisms In the latter, values of 60S have been found in *Xenopus laevis* (Swanson and Dawid, 1970), 50S–55S in chick, rat, and other mammals (O'Brien and Kalf, 1967; Ashwell and Work, 1970 *b*, Rabbitts and Work, 1971, O'Brien, 1971) and 55S–61S in HeLa cells (Brega and Vesco, 1971, Attardi and Ojala, 1971, Houssais, 1971). Higher values, however, have also been reported in mammalian cells. Perlman and Penman (1970) have described a 95S particle from HeLa mitochondria which is active in protein synthesis and contains 12S and 21S RNA, this particle may be considered as a native monomeric structure. Eladari et al (1971) have isolated ribosomal particles from rat liver mitochondria having a sedimentation coefficient of 83S compared to 80S for cytoribosomes

Electron microscope data point to the similarity in size of mitoribosomes and cytoribosomes from *C utilis*. For comparison, molecular weights of mitoribosomes and cytoribosomes have been roughly estimated from their content in RNA and the molecular weights of the RNA Since, as discussed above, RNA-RNA interactions are minimized by EDTA, RNA molecular weight values used for this calculation were taken from the data of sedimentation in EDTA-supplemented sucrose gradients Assuming that there is only one molecule of RNA per ribosomal subunit, the molecular weights of the 50S and 36S mitoribosomal subunits are tentatively approximated at 2.47×10^6 and 1.69×10^6, respectively, and that of the 72S mitoribosomal monomer at 4.16×10^6 The same type of calculation applied to the cytoribosome results in a molecular weight of 4.49×10^6 for the 78S cytoribosomal monomer. It is evident that such calculations should only be taken as estimates

The morphological features of yeast mitoribosomes have also been described in the present study. The monosome appears as an asymmetric, elongate particle formed of two unequal subunits A number of characteristic forms (*a*, *b*, *c*, *d*) were observed in negatively stained preparations. Recently, Nonomura et al (1971) have established that all principal profiles observed in rat liver ribosomes could be interconverted by tilting the specimen and could be accounted for by a single three-dimensional model We have made use of their conclusion and have interpreted forms *a*, *b*, *c*, and *d* by the model shown in Fig. 22 The mitoribosome depicted shows a roughly spherical large subunit, slightly flattened along the plane adjacent to the small subunit and presenting some straightened edges in its lateral profile The small subunit is shown as a prolate body positioned toward one side of the flattened surface of the large subunit An off-center depression along the plane joining the two subunits, corresponding to the dense spot observed, is prominent on the dorsal face and less apparent on the frontal face

Comparison of our images and model with those of a variety of other ribosomes including the cytoribosomes of yeast discloses an over-all general resemblance. An electron-opaque cleft dividing the particle into unequal subunits has often been described, namely in bacterial ribosomes (Huxley and Zubay, 1960, Bruskov and Kiselev, 1968 *a*), in rat liver cytoribosomes (Nonomura et al, 1971), and in chloroplast ribosomes (Miller et al, 1966, Bruskov and Odintsova, 1968). An electron-opaque spot, noted by Bruskov and Kiselev (1968 *a* and *b*) and by Nonomura et al. (1971), is interpreted by these authors as corresponding to an internal channel traversing the particle at the level of the plane between the subunits The dense spot which we have observed in an off-center position in both mito- and cytoribosomes of yeast presumably conforms to a similar internal channel The displaced position of the small subunit on the large is clearly shown by Nonomura et al (1971); again, lateral views of yeast cyto- and mitoribosomes display very similar images.

Despite these clear resemblances between mitoribosomes and other ribosomes, some minor features distinguish mitoribosomes from cytosomes In particular, the partition of the small subunit, shown by Shelton and Kuff (1966) and Nonomura et al (1971) in mammalian cytoribosomes and occasionally observed in *C. utilis* cytoribosomes, appears to be absent in mitoribosomes In addition, the small subunit in cytoribosomes is wider than the large subunit, a feature not seen in mitoribosomes. Measurements comparing mito- and cytoribosomes also show a slightly greater width, in forms *a* and *b*, of cytoribosomes.

It is at the level of isolated mitoribosomal subunits, however, that striking differences from cytoribosomes, along with clear similarities to bacterial ribosomes, are found The 50S mitoribosomal subunit, bearing a distinctive knob, is clearly reminiscent of the 50S bacterial subunit

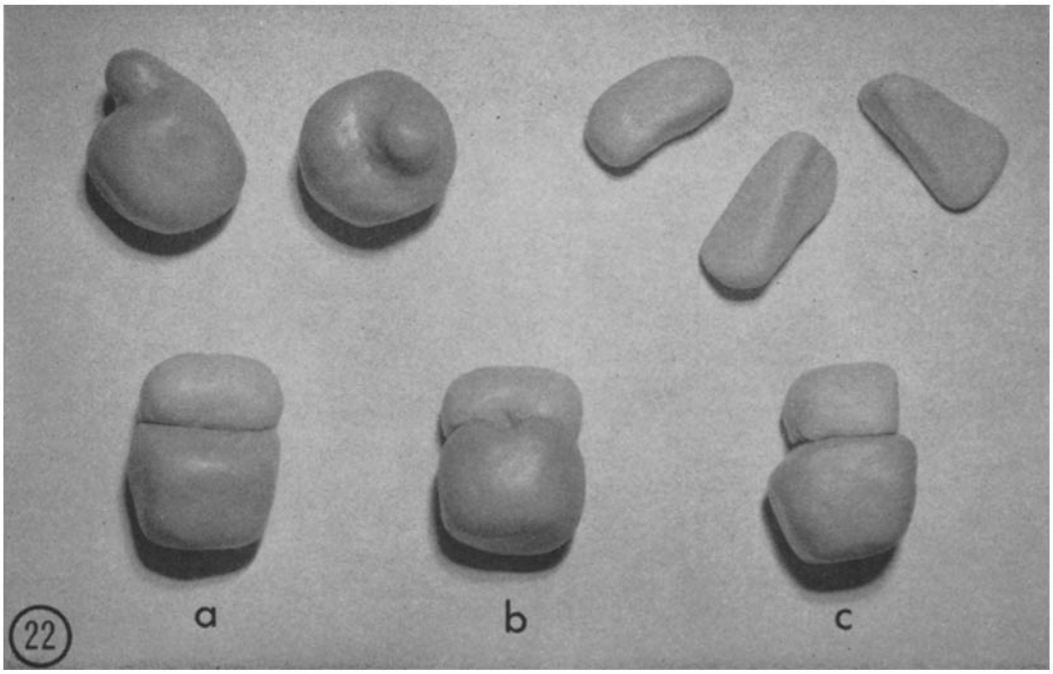


FIGURE 22 Photograph of three-dimensional models of mitoribosomal monomers (72S) and subunits (36S and 50S). The 36S subunit (upper right) is shown as an irregular tetrahedron, producing triangular and tetragonal profiles and as a prolate body, providing a rounded-off rectangular image. The 50S subunit (upper left) is shown as a rounded particle bearing a knob. In an end view, the particle shows a roughly pentagonal outline. The monomer is shown in frontal (*a*), dorsal (*b*), and lateral (*c*) views, from left to right. The lateral view shows the small subunit positioned toward one side of the flattened surface of the large subunit, producing the notch visible on the frontal face. The dorsal view shows a depression along the plane joining the subunits, corresponding to the dense spot observed in form *b*.

which also bears a projection, termed "nose" by Lubin (1968). Our model of the 50S subunit approaches that proposed by this author and views similar to his crescent, slit, and nose views can be found in our preparations, although the nose or knob view is predominant. At variance, the large subunit of cytoribosomes has been shown to have a dome or skiff shape (Bruskov and Kiselev, 1968 *b*; Nonomura et al., 1971) but no knoblike projection has so far been shown.

Images similar to the triangular and tetragonal views of the 36S subunits were shown by Kleinschmidt (1970) of bacterial small subunits and similar images are seen in the study by Lubin (1968). These preparations all show more heterogeneity in the small subunits and the absence of a definite partition compared to those shown by Nonomura et al. (1971) of cytoribosomes. Whether such differences reflect varying degrees of preservation or actual morphological dissimilarities cannot be decided. In any case, it was noted that the characteristic views of mitoribosomal subunits were found following monosome dissociation by either low Mg^{2+} or high NH_4^+ concentrations

Artifactual Dimerization of Subunits

To our knowledge, the artifactual aggregation of mitoribosomal subunits has never been assessed in the evaluation of the sedimentation coefficient of mitoribosomes. Evidence has been presented in this paper that the dimerization of the large mitoribosomal subunits into 80S particles spontaneously occurs in the presence of $MgCl_2$.

Schmitt (1970, 1971) has recently described in *S. cerevisiae* a special class of 80S cytoribosomes bound to mitochondrial membranes and more resistant to dissociation than free cytoribosomes

Their properties are clearly different from the 80S mitoribosomal particles described here

72S Mitoribosomes as Functional Units in Mitochondrial Protein Synthesis

The conclusion that the 72S nucleoprotein particles isolated from *C utilis* mitochondria are the functional units involved in protein synthesis is based on several lines of evidence: (*a*) Purified mitochondria are able to carry out protein synthesis which is sensitive to chloramphenicol and insensitive to cycloheximide, a result which points to a specific mitochondrial protein synthesis system. (*b*) Sedimentation of the mitoribosomal material prepared from mitochondria preincubated with leucine-^{14}C shows a peak of nascent ^{14}C peptide which cosediments with the 72S monomer form of mitoribosomes The bound ^{14}C peptide material is released upon addition of puromycin, in agreement with the statement that the ^{14}C peptide was originally attached to a ribosomal structure (*c*) Dissociation of mitoribosomes into subunits leads to the release of the ^{14}C peptide as soluble material. It is inferred that the 72S particles to which the ^{14}C peptide chains are attached are monosomes, a situation which may be compared to that of guinea pig liver cytoribosomes, as reported by Tashiro and Siekevitz (1965 *b*) (*d*) The structural organization of mitoribosomes within mitochondria, as polysomes attached to the inner face of the inner membrane, is reminiscent of the cytoplasmic polyribosome organization on ergastoplasmic membranes The localization of mitoribosomes on the mitochondrial membranes together with the observation that the protein material which is synthesized by mitochondria is water insoluble (Wheeldon and Lehninger, 1966), are in accordance with the suggestion that the mitochondrial ribosomes are involved in the synthesis or the renewal of one or some mitochondrial membrane proteins.

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