# ELECTRON MICROSCOPIC EXAMINATION

# OF SUBCELLULAR FRACTIONS

## II. Quantitative Analysis of the Mitochondrial Population Isolated from Rat Liver

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### ABSTRACT

Large granule fractions, containing about 80% of the cytochrome oxidase of the tissue, were isolated from rat liver and used to prepare thin pellicles of packed particles which were submitted to quantitative electron microscopic examination. Various parameters describing the mitochondrial population were determined by measuring the size and number of mitochondrial profiles in sections, and the ratio of the inner to the outer membrane area. The mean particle radius and volume were found to be respectively 0.38 $\mu$ and 0.29 $\mu^3$; the average areas per mitochondrion were 2 and 5 $\mu^2$ for the outer and inner membranes respectively. On the basis of the cytochrome oxidase activity recovered in the particulate fractions, the results were extrapolated to the whole liver, and it was concluded that rat liver contains about $5.10^{11}$ mitochondria per gram; this corresponds to a volume of 0.14 ml/g and to an area of 2.5 and 1 $m^2/g$ for the inner and outer membranes respectively. The validity and the accuracy of these determinations is discussed and the results are compared to the information which has been obtained by independent methods or by other investigators.

## INTRODUCTION

Since the original publication of Delesse (18) numerous theoretical studies have dealt with the derivation of quantitative analytical data from measurements made on thin sections of non-homogeneous materials (3, 4, 12, 13, 20, 22, 28, 29, 32–35, 38–42). The methods worked out have been applied at the light microscope level, especially in the field of mineralogy, and have recently been extended to the analysis of electron micrographs of biological specimens (14, 21, 29, 31). The procedures applied so far in the field of electron microscopy have one weakness in common, that they do not allow an estimate of the size distribution of the particles under study. They are further complicated by the difficulty of obtaining statistically representative specimens when the analyzed material displays considerable microscopic and submicroscopic heterogeneity. The present paper describes a method based on the original work of Wicksell (41, 42), which permits the size distribution of particles to be derived from measurements made on micrographs. It is applied here to a study of isolated rat liver mitochondria prepared for electron microscopy by a filtration method which satisfies the criterion of random sampling, and thus allows direct comparison between biochemical and morphological data.

## MATERIALS AND METHODS

Liver tissue from female rats was fractionated by the procedure of de Duve et al. (17), abbreviated to

yield a crude nuclear fraction (N), a large granule fraction (M + L), and a microsome-containing supernatant (P + S). All fractions were analyzed for cytochrome oxidase (2) and protein (30).

Samples of M + L fractions originating from a known weight of fresh liver were fixed with glutaraldehyde, packed by filtration through a Millipore membrane of 0.1 $\mu$ pore size, postfixed with osmium tetroxide, dehydrated, embedded in Epon, and sectioned, according to the procedures described in a preceding paper (7). Ribbons of consecutive ultrathin sections (40–60 m$\mu$), separated each time by a thick section ($\pm$100 $\mu$), were deposited on 200-mesh grids, and a single square of each grid was photographed. This precaution ensured that each recorded transection belonged to a different particle. Each photographed field included the whole thickness of the pellicle, and could therefore be considered as providing a true random sample of the preparation (7). That the space between the mitochondrial pellicle and the covering layer of erythrocytes was essentially void indicates that very few mitochondria became detached from the surface of the pellicle in the course of the manipulation.

The micrographs were taken with a Siemens Elmiskop I electron microscope at 60 kv. The final magnification, of the order of 15,000, was the same for all micrographs of a given preparation, which were enlarged at the same time. It was determined in each case by means of a grating replica (E. F. Fullam, Inc., Schenectady, N. Y.) which was photographed under the same conditions as the preparation. The only source of error then is a difference in position between the grating replica and the specimen grid in the microscope; this necessitates a different current in the objective lens. The ensuing error is small (less than 2% in our conditions), especially since the intermediary lens was used independently of the objective lens. The distortion of the image owing to spherical aberration was checked and found to be less than 2% with the lens currents used. No correction was introduced for compression artifacts, since they also were found to be small, in agreement with the findings of Loud et al. (29) for Epon embedding. Fig. 1 is an example of the micrographs used for quantitative analysis.

The photographs were scanned systematically over the whole thickness of the pellicle with a Zeiss TGZ 3 particle dimension analyzer and the diameter of each recognizable mitochondrial profile was estimated and recorded in the appropriate size class. The classes chosen differed by 1.1-mm increments in diameter; this corresponded to two adjacent classes foreseen in the apparatus, since this was considered the limit of resolution that could be achieved. Some 8–10 micrographs were scanned, which covered a total distance of at least 100 $\mu$ along the surface of the pellicle. This distance, to be designated as the width $W$ of transec-

tion analyzed, was also measured on the micrographs.

The instrument used is constructed for the measurement of circular profiles with a clearly defined contour. As illustrated in Fig. 1, mitochondrial profiles do not rigorously meet these conditions; this raised some difficulties.

In the first place, many profiles are slightly elliptical and some show fairly marked irregularities. In all these cases, the size was estimated by equalizing visually the nonoverlapping areas of the profile and of the measuring diaphragm; the diameter estimated in this way is thus that of the circle having the same surface area as the profile. For independent assessment of the degree of asymmetry of the particles, the major and minor axes were measured with a ruler on a number of profiles chosen at random. As will be indicated in the section on Results, the average eccentricity of the profiles was found to be small and the measurements of surface area made in the manner described may be considered relatively accurate.

As shown in Fig. 1, the most easily recognizable boundary of the profile is the inner membrane or contour of the mitochondrial matrix; the outer membrane is not always clearly seen and often shows a crenated or irregular appearance. When the outer membrane was sharp and close to the matrix, it was taken as the boundary of the profile; in the places where the membrane was apparently absent or obviously detached from the inner membrane, the latter was taken as the boundary. In profiles arising from particles cut far from the equator, the limiting membrane is not sharp, owing to the finite thickness of the sections; it is very likely that the area of the profile was slightly, but systematically, underestimated in this case. Whenever the identification was doubtful, the profile was not measured or counted. There is thus little doubt that a number of mitochondrial profiles were excluded from the analysis because they did not show clear evidence of a double, surrounding membrane or of internal cristae. The profiles missed for this reason were mostly those of near polar sections. Fortunately, the mathematical procedures applied are such that the errors resulting from this technical limitation can be partly corrected for. As usual in large granule fractions isolated from liver, a few mitochondria were swollen and had a matrix of lower than normal density. Swollen mitochondria were counted with the others if they clearly exhibited morphological features which allowed them to be recognized as mitochondria; the main criterion was the presence of characteristic cristae.

As recorded in the instrument, the results could be used directly for the construction of a histogram of profile radii $R$, on an abscissa scale determined by the magnification of the micrographs. Since the magnification varied somewhat from one experiment to the other, whereas the size classes of the analyzer are invariable, the divisions on the histogram were not
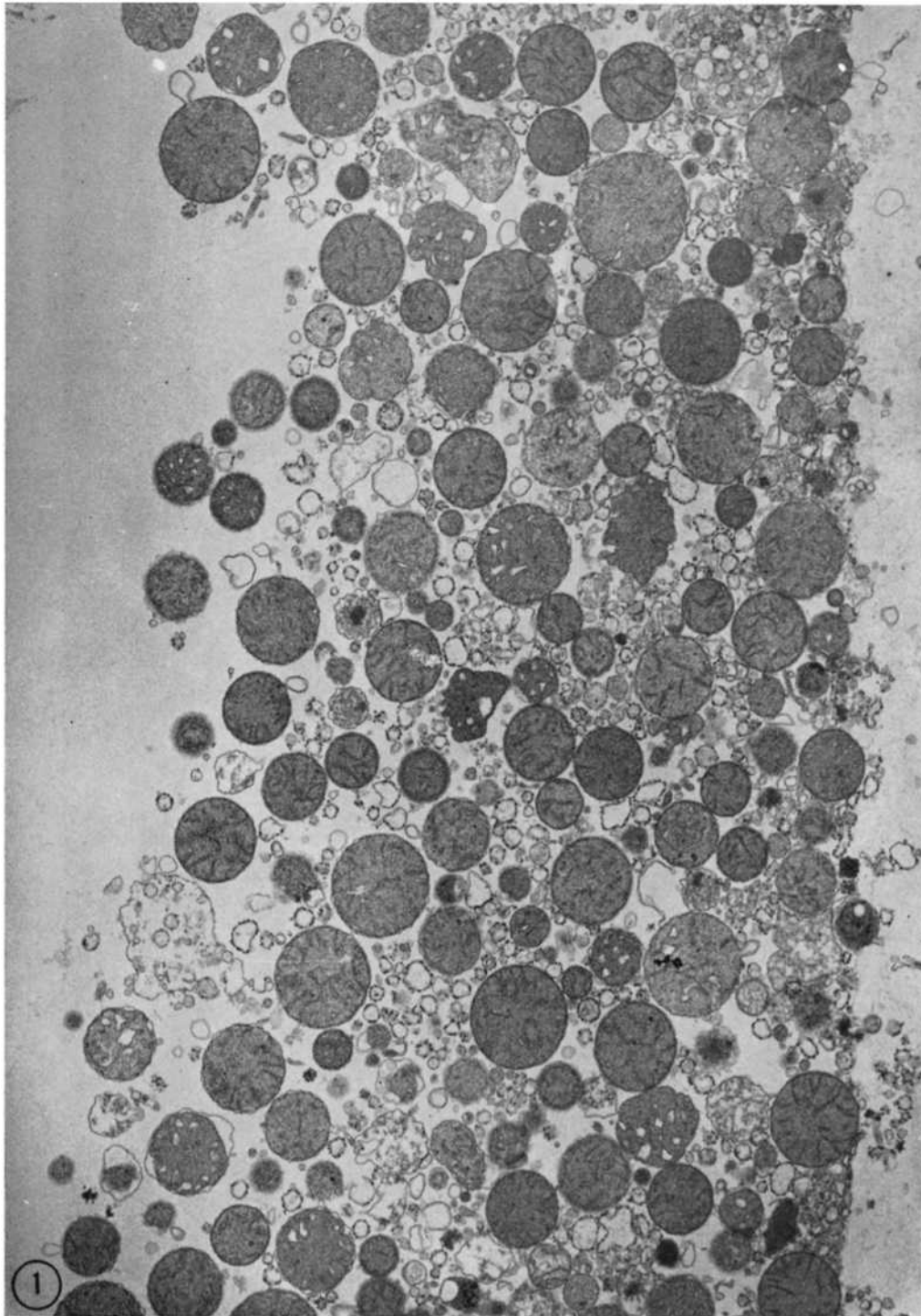
FIGURE 1  Example of micrograph used for quantitative analysis. × *12.000.*

exactly the same for different preparations; they were of the order of 0.04 $\mu$.

A second type of measurement was made on the mitochondrial profiles. An array of parallel lines was superimposed on the micrographs, and the number of intersections of grid lines with the outer membrane of mitochondria and with the membranes of cristae was counted.

## MATHEMATICAL PROCEDURES

The theories on which our mathematical methods are based have been developed by others. We restrict their description to an outline of the principles and to the mathematical procedures which we used in their application to our measurements.

### Theory

Let a population of $n$ spherical particles of variable volume $v$ or radius $r$ be distributed at random within a given solid of volume $V$ through which an infinitely thin section of surface area $A$ is cut; let the radii $R$ of the $N$ circular profiles included in the section be measured; if a profile is not circular, $R$ is the radius of the circle of equivalent area.

(a) According to the principle established by Delesse (18), the fractional surface covered by the profiles in the section is equal, within the limits of sampling fluctuations, to the fractional volume occupied by the particles in the solid:

$$\frac{\sum_{}^{N} \pi R^2}{A} = \frac{\sum_{}^{n} v}{V} \tag{1}$$

which may also be written

$$n\bar{v} = \sum_{}^{n} v = \frac{V}{A} \cdot \sum_{}^{N} \pi R^2 \tag{2}$$

in which $\bar{v}$ is the mean volume of the particles.

In our experiments, the pellicles have a constant area equal to $8.7 \times 10^7 \ \mu^2$. Thus

$$A = W \times T \tag{3}$$

$$\text{and} \quad V = 8.7 \times 10^7 \times T \tag{4}$$

in which $W$ is the total width of the sections scanned and $T$ is the thickness of the pellicle.

From equation 3 and 4 we obtain

$$\frac{V}{A} = \frac{8.7 \times 10^7}{W} \tag{5}$$

and equation 2 becomes

$$n\bar{v} = \frac{8.7 \times 10^7}{W} \cdot \sum_{}^{N} \pi R^2. \tag{6}$$

It will be noted that the pellicle thickness, which varies somewhat owing to unequal local packing, cancels out in the derivation of equation 5 and need not be known. In connection with this, it may also be observed that the perpendicularity of the section with respect to the pellicle plane is not very critical. An error of $10°$ will change the area of the pellicle section only by $1.5\%$.

(b) As first shown by Wicksell (41), it is possible to deduce the frequency distribution of particle radii $r$ from that of the profile radii $R$. Fig. 2 illustrates the principle of Wicksell's procedure as applied to a simple case. The measured profile radii $R$ are plotted in histogram form (Fig. 2 a). From the size and frequency of the radii in the upper class, which obviously represent the actual radii $r$ of the largest particles of the population, one computes the size and frequency of the corresponding class of particle radii (bar I in Fig. 2 d), as well as the contribution of the particles from this class to the other classes of profile radii through nonequatorial sections (darkened area in Fig. 2 a). A new histogram is obtained by subtraction (Fig. 2 b), and the calculation is repeated for the subsequent class of particles, whose equatorial sections now form the upper class of profile radii in the new histogram. This procedure is repeated until all profile radii have been accounted for, a result which is achieved at the third step in the example of Fig. 2. In practice, these repeated subtractions are not carried out explicitly. As shown by Wicksell, the problem can be reduced to the solution of a set of linear equations; the details of the calculations and their theoretical justification can be found in the original paper (41).

Once the frequency distribution of particle radii $r$ has been computed in the above manner, it is a simple matter to construct the corresponding frequency distributions of the surface areas $a$ and the volumes $v$ of the particles and also to calculate such relevant data as the means $\bar{r}$, $\bar{a}$, and $\bar{v}$ and, the medians $r_m$, $a_m$, and $v_m$ as well as the corresponding standard deviations. The shape of these

distributions can also be analyzed and eventually characterized mathematically by fitting the appropriate statistical frequency function. A particularly useful parameter extracted by the analysis is represented by the mean particle volume $\bar{v}$, which, when introduced in equation 2, may serve to calculate the total number of particles $n$.

(c) If a grid is superimposed on an infinitely thin section through a two-dimensional structure, the number of intersections of the grid lines with the structure is proportional to the area of the

urement of the area of the outer membrane, which corresponds to the area of the particles as obtained by the Wicksell procedure, equation 7 may serve to calculate the mean area of the inner mitochondrial membrane.

## Application

In applying these methods we encountered various difficulties. Some of them are inherent to electron microscopic studies of subcellular particles; others arise from the use of a particulate fraction. Since some of these problems could lead
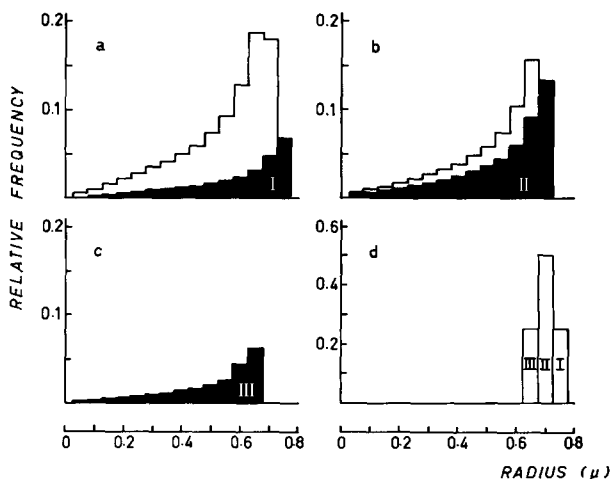
FIGURE 2 Principle of the calculation of the particle sizes from the profile sizes. The histograms $a$, $b$, and $c$ represent frequencies of profile radii, while $d$ represents the frequencies of particle radii in the corresponding population. See text for further explanations.

latter (35). This principle forms the basis of the method used here to measure the ratio of inner-to-outer mitochondrial membrane area.

We consider, in first approximation, that the inner membrane is composed of an envelope apposed to the outer membrane and having the same area as the latter, and of infoldings or cristae. Then if $N_o$ is the number of intersections of the grid lines with the boundary of the mitochondria and if $N_c$ is the number of intersections with the membranes of the cristae (two for each crista), we may write

$$\frac{\text{area of inner membrane}}{\text{area of outer membrane}} = q + 1 \qquad (7)$$

in which

$$q = \frac{N_c}{N_o} \qquad (8)$$

Since we have available an independent meas-

to modifications in the computation procedures, they are discussed here in a systematic manner.

THE SHAPE OF THE PARTICLES IS NOT PERFECTLY SPHERICAL: This difficulty does not invalidate the measurement of the total volume (equation 6) or the determination of the ratio of the membrane areas (equation 7). As to Wicksell's calculations, equations valid for ellipsoids of revolution have also been worked out by this author (42). When ellipsoids of axial ratio smaller than 1.4 are treated as spheres, the error on the parameters of the distribution falls below that to be expected from sampling fluctuations if the analysis is restricted to 4,000 particles, provided that one takes as value for the radius of the profiles the geometric mean of the major and minor axes, as was done here. The radius of the particle will then correspond to the radius of a sphere having the same volume as the ellipsoid. This procedure, termed "spherical reduction" by Wicksell, should be regarded as a mathematical transformation, permitting convenient handling

of the data. It does not imply that the eccentricities of the particles are disregarded and, as will be shown, constitutes an acceptable approximation for isolated mitochondria.

NOT ALL THE PROFILES OF THE PARTICLES UNDER STUDY ARE MEASURED: This difficulty is due to a minor extent to preparative damage that renders some profiles unrecognizable, and to a major extent to the impossibility of identifying the smaller profiles. These include many of the sections passing near the pole of particles of any size, and perhaps a few equatorial sections passing through the smaller particles. As long as the classes where profiles are missed do not include many equatorial profiles, the error made in estimating the distribution of particle sizes by the Wicksell method will be small, since only equatorial profiles are actually taken into account in this procedure (see Fig. 2): the calculation will simply indicate that the number of profiles measured in the smaller classes is less than that expected from the contribution of the larger particles; the calculated distribution of particle radii will nevertheless be correct. The missing nonequatorial profiles can be retrieved by computing the expected frequencies of profile radii from the known particle radii distribution. If the population contains particles whose radius falls within the range where profiles are not all recognized, it is obvious that the data do not supply information on the frequencies of these small particles. Unless some extrapolation is made, the analysis gives a truncated distribution of the particle radii.

It must be pointed out that the missing profiles have to be included in the summations of equations 1, 2, and 6 for a rigorous application of Delesse's principle. Therefore, analysis of the population by Wicksell's procedure should be performed before an attempt is made to apply these equations; this will provide the best estimate of the missing profile frequencies. Fortunately, since the missing profiles are those with the smallest radii and since the squares of the latter actually enter into the equation, errors on this estimate are of relatively minor importance if enough larger profiles exist.[1] This will be shown to be so in the present case.

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[1] When sections are made at random through a sphere, 86.6% of the profiles have a radius larger than one-half the radius of the sphere; the integral of the areas of these profiles represents 97.4% of the volume of the sphere. Thus, if liver mitochondria formed a homo-

THE SECTIONS ARE NOT INFINITELY THIN BY COMPARISON TO THE SIZE OF THE PARTICLES: The average diameter of an isolated rat liver mitochondrion is about 0.8 $\mu$; the sections used in our work have an estimated thickness of about 0.05 $\mu$. If mitochondria are considered as opaque bodies in a transparent medium, the radius of the profiles is thus overestimated, except for sections close to the equator; on the average, the measured profile areas would be 10% larger than the ideal areas in infinitely thin sections (25). However, the near polar sections of the particles which are the main contributors to the error are largely excluded from our measurements. Moreover, when a mitochondrion is cut far from the equator, the outline of the outer membrane is not sharp, and as is pointed out in Material and Methods, the actual area of the profile was somewhat underestimated. Finally, when the outer membrane was not clearly recognizable or was very irregular, the measurement was based on the contour of the matrix; this leads again to some underestimation of the profile area of the whole mitochondria.

We attempted to make approximate estimations of the influence of all these factors. The analysis of the experimental data shows that many profiles are not recognized when their radius is smaller than 0.2 $\mu$. As explained above, the truncated distribution of particle sizes is independent of the frequencies, and thus of the sizes, of the profiles smaller than the truncation level. Almost one-half the error owing to the thickness of the section is eliminated by the truncation. The remaining error seems to be approximately compensated by the other bias in the measurements. Thus, the accuracy of our estimates would probably not be improved by introducing corrections for the section thickness.

THE M + L FRACTION DOES NOT CONTAIN ALL THE MITOCHONDRIA PRESENT IN THE AMOUNT OF LIVER FROM WHICH IT ORIGINATES, AND THE ISOLATION PROCEDURE MAY HAVE ALTERED THE CHARACTERISTICS OF THE PARTICLES: Extrapolation of the results to the whole liver raises more serious problems. Following the accepted practice in this laboratory, we may take advantage of the enzymic determinations by using a correction

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geneous population of spheres with a radius of 0.4 $\mu$, ignoring all profiles of radius smaller than 0.2 $\mu$ would amount to neglecting 2.6% of the profile area.

factor based on the postulates of biochemical homogeneity and single location. As defined by de Duve (15), the former of these postulates assumes that the enzymic activities per unit mass or protein content (specific activities) are the same for all subclasses of particles within a given population, and the latter that individual enzyme species occupy a single intracellular location. Since all subcellular particles of a given type have almost the same density, the first postulate is equivalent to the assumption of a constant enzymic activity per unit volume.

Let $E$ be the activity in the M + L fraction of a reference enzyme obeying the above postulates (cytochrome oxidase[2] in the present instance), expressed in percentage of its total activity in the homogenate; let $w$ be the amount of the M + L fraction included in the whole pellicle, expressed in grams of liver from which it originates; let $n_l$ be the number of particles in 1 g of liver and $\bar{v}_l$ their average volume ($n$ and $\bar{v}$ have the same meaning as before, i.e., they refer to the number and to the average volume in the pellicle). The relationship between $n_l\bar{v}_l$ and $n\bar{v}$ is immediately derived from the postulates above:

$$n_l \, \bar{v}_l = \frac{n\bar{v}}{w} \cdot \frac{100}{E} \tag{9}$$

In addition to the postulates on which it rests, several other conditions must be satisfied for this equation to be valid: (a) $E$ must be a correct expression of the relative enzymic activity of the M + L fraction; this implies both accurate determination and quantitative recovery of this activity in the various fractions analyzed; (b) the mitochondrial volume must be unaltered by preparative artifacts; (c) the method used to prepare the M + L fraction must not have divided the population into two groups differing in mean volume.

All three conditions must be met if the absolute number $n_l$ of particles per gram liver is estimated (by making $\bar{v}_l = \bar{v}$).

Only the first two must be satisfied when the total volume occupied by the mitochondria in the liver, $n_l\bar{v}_l$, is computed from their total volume in the pellicle, $n\bar{v}$.

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[2] Since cytochrome oxidase belongs to the inner mitochondrial membrane, the postulate of homogeneity implies that the area of the inner membrane with its infoldings is proportional to the volume of the particles.

## Computation Procedures

In early stages of this work, Wicksell's procedure was used as such. This required conversion of the experimental histogram of profile radii to a smooth frequency distribution curve, which was then redivided to form a 15 class histogram, necessary for application of the coefficients of equation 18 of Wicksell's paper (41). After greater familiarity with the method was achieved, a computer program, applicable to any number of classes and allowing direct handling of the experimental results, was developed on the basis of Wicksell's equations 16 bis.

## RESULTS

### Shape of Mitochondria

From measurement of longer (a) and shorter (b) axes on 224 profiles chosen at random, we have calculated the corresponding profile eccentricities, defined as $\sqrt{a^2 - b^2}/a$. These values were used to establish the distribution of the mitochondrial eccentricities by means of Wicksell's procedure for prolate ellipsoids (42). The mean eccentricity was found to be 0.4 with a standard deviation smaller than 0.1; it made very little difference whether the eccentricity was assumed to be independent of the longer axis, the shorter axis, or the volume of the particles. An eccentricity of 0.4 corresponds to an axial ratio of 1.1, well below the limit of 1.4 set by Wicksell (see above). It therefore seemed legitimate to use in the calculations the method of spherical reduction proposed by this author.

### Size Distribution of Mitochondria

The histograms shown in the upper half of Fig. 3 summarize the results of direct measurements of profile radii, made in each case on some 1,000 profiles, on four different preparations. When Wicksell's procedure was applied to these histograms for derivation of the distribution of true mitochondrial radii, negative values were obtained for the frequency of radii smaller than 0.2 $\mu$; this indicated that the number of profile radii in the lower classes of the experimental histograms was smaller than that to be expected from the sole contribution of nonequatorial sections through particles of larger size. This difficulty was anticipated, since, as mentioned above, many of the smaller profiles could not be identi-

fied with certainty as belonging to mitochondria and were for this reason neglected in the scanning of the micrographs. As a minimum correction to avoid nonsense, it was assumed that the neglected profiles were all nonequatorial and the computer was programmed to treat all negative values as zero. The distributions of mitochondrial radii calculated in this manner are shown in the lower half of Fig. 3. By definition, these histograms comprise no classes of radii smaller than 0.2 $\mu$, since this would require equatorial sections to be included in the corresponding classes of profile radii. The shaded areas in the upper histograms represent the neglected profiles.

chondrial volumes that can be constructed from the former. In these computations, the particles in each size class have all been treated as having the mean radius characteristic of the class.

The subsequent steps of the computations are easily followed on Table I. Under total profile area we give the sum of all the surfaces actually measured, as they can be derived from the experimental histograms of Fig. 3. Addition of the missing profiles (shaded areas of the histograms of Fig. 3) leads to the corrected total profile area. It is seen that this correction amounts to only a few per cent. The total volumes of the mitochondria in the pellicle and in the liver are then derived
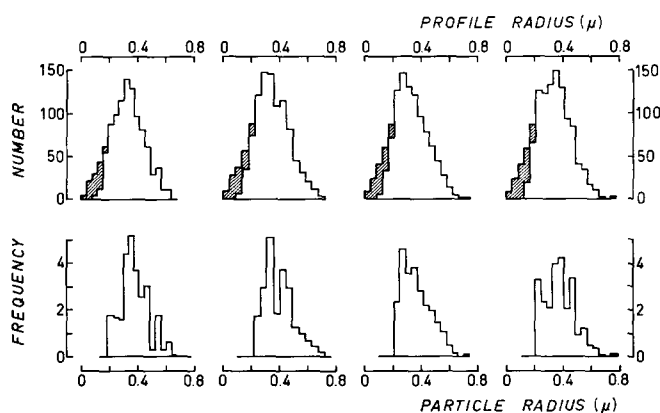


FIGURE 3 Size distributions of mitochondria in the four experiments. The upper diagrams represent the distribution of profile radii while the lower diagrams give the distribution of the particle radii computed according to Wicksell (41). In the upper diagrams the shaded area represents the correction for the near polar sections which were not identified in the counting procedure.

## Number and Average Volume of Mitochondria

In Table I are listed other experimental data obtained on the four preparations as well as the various values which could be computed from the results by application of the equations given in Mathematical Procedures; $\bar{v}$ and $\bar{v}_l$ are assumed to be equal.

On an average, the M + L fractions contained 78.1% of the cytochrome oxidase activity found on the homogenates. When corrected for the recovery, which varied between 88.7 and 98.2% (average, 93.8%), this amounted to 83.3% of the sum of the activities recovered in the three fractions. Most of the remainder (14.3%) was in the nuclear fraction (N), with only 2.4% in the microsome-containing supernatant P + S.

The parameters characterizing the size distribution of the mitochondria are those that can be obtained directly from the histograms of mitochondrial radii or from the histogram of mito-

from the corrected total profile surface area by means of equations 6 and 9. Divided by the mean volume ($\bar{v}$ and $\bar{v}_l$ are assumed to be equal), they give the numbers $n$ and $n_l$ of mitochondria in the pellicle and in the liver.

## Areas of Mitochondrial Membranes

In Table II are listed the inner-to-outer membrane area ratios, as they have been measured by the grid method and calculated by equations 7 and 8. The average areas of the outer mitochondrial membrane were computed from histograms of mitochondrial area constructed from the distributions of particle radii. The numbers $n_l$ of Table I were used for computing the total areas per gram of liver.

## Statistical Evaluation

The statistical evaluation of our data raises some difficulties, even though the variance of the population is known, because the relationship between the number of profiles counted and the

TABLE I

Results Pertaining to the Radius, Volume, and Number of Mitochondria

| | Preparation | | | | Average |
|---|---|---|---|---|---|
| | I | II | III | IV | |
| Cytochrome-oxidase | | | | | |
| % of the homogenate | 77.3 | 80.0 | 78.4 | 76.6 | 78.1 |
| % of total recovered activities | 87.1 | 81.5 | 83.6 | 81.2 | 83.3 |
| Amount $w$ of fraction filtered, mg liver | 2.46 | 2.50 | 2.50 | 2.50 | 2.49 |
| Total width $W$ of sections scanned, $\mu$ | 105.5 | 120.4 | 136.3 | 129.9 | 123.0 |
| Number of profiles measured | 959 | 951 | 966 | 1030 | 977.0 |
| Median particle radius $r_m$, $\mu$ | 0.367 | 0.384 | 0.353 | 0.375 | 0.370 |
| Mean particle radius $\bar{r}$, $\mu$ | 0.379 | 0.399 | 0.370 | 0.381 | 0.382 |
| Standard deviation of radius, $\mu$ | 0.098 | 0.102 | 0.103 | 0.106 | 0.102 |
| Mean particle volume $\bar{v}$, $\mu^3$ | 0.274 | 0.321 | 0.265 | 0.287 | 0.287 |
| Total profile area, $\mu^2$ | | | | | |
| uncorrected for missing profiles | 380 | 418 | 383 | 432 | 403 |
| corrected for missing profiles | 383 | 423 | 388 | 443 | 409 |
| Total volume of mitochondria in pellicle, $\mu^3 \times 10^8$ | 3.16 | 3.05 | 2.47 | 2.96 | 2.91 |
| Total volume of mitochondria in liver, ml/g of liver | 0.148 | 0.150 | 0.118 | 0.146 | 0.141 |
| Number $n_l$ of mitochondria in $10^{-12}$ g liver | 0.538 | 0.467 | 0.446 | 0.508 | 0.490 |

TABLE II

Results Pertaining to the Areas of Mitochondrial Membranes

| | Preparation | | | | Average |
|---|---|---|---|---|---|
| | I | II | III | IV | |
| Mean particle outer area, $\mu^2$ | 1.92 | 2.13 | 1.86 | 1.96 | 1.97 |
| Ratio of inner to outer membrane area | 2.7 | 2.4 | 2.5 | 2.6 | 2.6 |
| Surface to volume ratio, $\mu^{-1}$ | 7.01 | 6.64 | 7.00 | 6.83 | 6.87 |
| Total mitochondrial membrane area, $m^2/g$ of liver | | | | | |
| outer membrane | 1.03 | 0.99 | 0.83 | 1.00 | 0.96 |
| inner membrane | 2.78 | 2.38 | 2.08 | 2.60 | 2.46 |

number of degrees of freedom is uncertain. Wicksell (42) assumed that these numbers can be taken as equal for large samples; he implies in fact that measuring profile diameters supplies as much information as measuring particle diameters. Although this is certainly not rigorously correct, it is probably an acceptable approximation, especially if we exclude from the profiles those which are not effectively used in the computation because their radii fall below the truncation level. This leaves at least 800 profiles in each of our experiments. Since the standard deviation of particle radius is about 0.1 $\mu$, its standard error will be of the order of $0.1/\sqrt{800} = 0.0035$ $\mu$. By this criterion, the values of mean radius obtained in the four experiments are significantly different ($P < 0.001$).

Another and perhaps more direct way of assessing the significance of differences between experiments is to compare the profile distributions, which represent the actual experimental results. Using for this purpose the test of Kolmogorov Smyrnov (26), we have also found that the four profile distributions are have significantly different
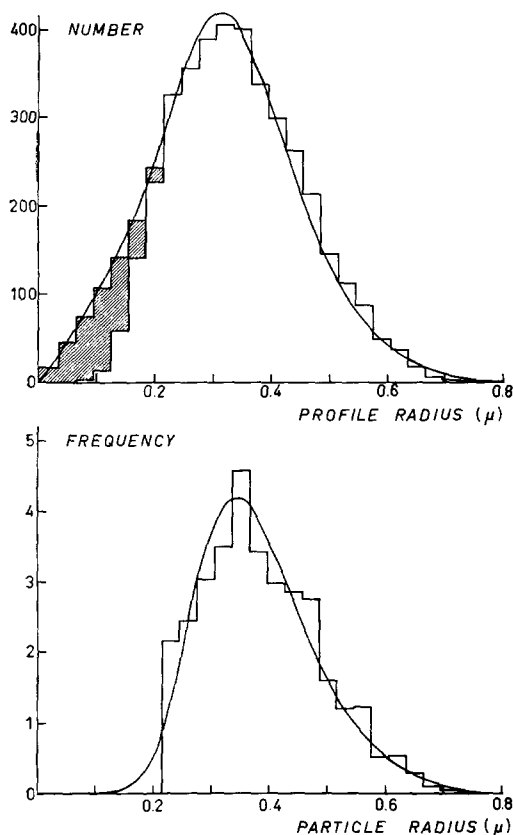
FIGURE 4 The open part of the upper diagram represents the compound histogram derived from the four experiments as explained in the text. From this histogram, the particle size distribution shown in the lower diagram was computed. As in Fig. 3, the shaded area in the profile histogram corresponds to the missed profiles. The continuous curve in the lower diagram represents the log-normal function fitted to the particle size distribution; that in the upper diagram gives the corresponding theoretical profile distribution.

($P$ < 0.01). A substantial part of the difference arises from the frequencies of the smaller profiles below the truncation level of 0.2 $\mu$, probably because they were more easily recognized in some preparations than in others. However, exclusion of the smaller profiles from the calculation led to mean profile radii which, though falling within a narrower range, were still significantly different ($P$ < 0.01).

Finally, we have calculated the variation coefficient of the total particle volume. It is given in first approximation by the variation coefficient of the number of profiles measured per unit area of

section. Since this number obeys a Poisson distribution, with 1000 measurements, the total mitochondrial volume is known with a precision of about $1/\sqrt{1000}$, or 3.5%. The differences found between the four experiments are much larger and obviously statistically significant. Thus according to the three tests applied, it appears that the four preparations examined cannot be considered samples of the same parent population.

## Pooled Results

Although significantly different, the results of the four experiments may nevertheless be pooled to provide a better representation of the mitochondrial population in an average M + L fraction. This is best done on the profile radii. Direct summation of the data being impossible owing to slight differences in final magnification of the micrographs, and therefore in size classes, a preliminary homogenization of the results had to be performed. To do this, we divided each experimental histogram into equal size classes of 0.03 $\mu$ and measured the mean ordinate of the fractional areas covered by each division to obtain comparable frequencies. The compound histogram of profile radii was obtained from the sum of the computed frequencies (upper half of Fig. 4). These, in turn, served to calculate the corresponding histogram of particle radii (lower half of Fig. 4). In addition, the total volume occupied by the mitochondria in each size class was calculated from the compound histogram of particle radii. The normalized results of this computation are represented in Fig. 5 which gives the distribution of total mitochondrial volume as a function of particle size. The main parameters estimated from the pooled results are listed in Table III. They are not different from the averages given in Tables I and II.

## Log–Normal Fit

Following the example of Bahr and coworkers (6, 24) we have also attempted to fit a log–normal frequency distribution to the compound histogram of mitochondrial radii, by using a method of maximum likelihood which took into account the truncation of the histogram at the 0.2 $\mu$ level. By reversing Wicksell's method, the distribution of profile radii corresponding to the fitted log–normal function was also calculated. The computed curves are represented in Fig. 4, and the corresponding parameters are listed in Table III.
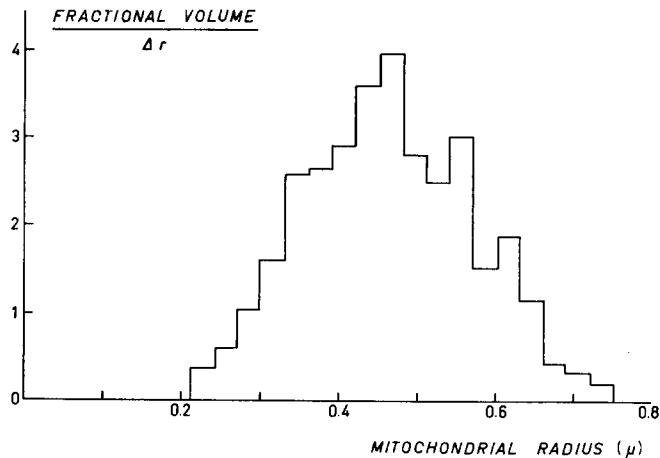
FIGURE 5 Distribution of the total mitochondrial volume as a function of particle size. The histogram is derived directly from the lower diagram of Fig. 4.

At first sight, the fit between the computed log–normal function and the pooled histogram appears reasonably good. However, by applying a $\chi^2$ test to the predicted and observed values of profile radii (upper part of Fig. 4), above the truncation level, we have found them to be significantly different ($P < 0.01$); this suggests that a single log–normal function may not adequately represent the mitochondrial population of the liver. The discrepancy does not arise from the pooling of results obtained on possibly dissimilar preparations, for the same conclusion was arrived at when the results of each individual experiment were treated in this fashion ($P < 0.05$). Such deviations are much less evident when the distribution is graphed in cumulative fashion on log–probit paper, as was done by Bahr and coworkers (6, 24). As shown by Table III, the values of the main parameters are the same, whether they are calculated with or without log–normal fit.

## DISCUSSION

### Applicability and Validity of the Technique

Although it has been used here with isolated particle fractions, the technique described in this work can theoretically be applied equally well to intact tissue sections. The choice of one material or the other is a matter to be decided in each case. Our own approach has been dictated by the desire (a) to combine quantitative morphology with the numerous resources, already exploited in this laboratory, of quantitative biochemistry, and (b) to overcome the serious difficulty arising from the heterogeneity of intact tissues, by taking

TABLE III

Characteristics of the Mitochondrial Population Derived from the Pooled Results

| Parameter | Without log-normal fit | With log-normal fit |
|---|---|---|
| Median particle radius, $\mu$ | 0.371 | 0.368 |
| Mean particle radius, $\mu$ | 0.385 | 0.381 |
| Standard deviation of radius, $\mu$ | 0.102 | |
| Standard deviation of $\log_{10}$ (radius) | | 0.118 |
| Mean particle area, $\mu^2$ | 2.00 | 1.97 |
| Mean particle volume, $\mu^3$ | 0.292 | 0.289 |

advantage of the randomizing effect or homogenization, associated with a preparative technique allowing random sampling. These advantages have to be weighed against the losses and morphological alterations caused to subcellular particles by tissue disruption and fractionation in a foreign medium. In the present application to mitochondria, the choice of isolated fractions may not appear compelling. But it becomes more so when it comes to evaluating the size and frequency of rarer cytoplasmic particles that are very heterogeneously distributed within cells, such as peroxisomes, lysosomes, or autophagic vacuoles. The recent work of Deter et al.[3] gives a good example of this type of application. We decided to make

[3] Deter, R. L., P. Baudhuin, and C. de Duve. 1967. Participation of lysosomes in cellular autophagy induced in rat liver by glucagon. *J. Cell Biol.*, 35: C11.

rat liver mitochondria the first test object of the technique because considerable information, some of it mutually conflicting, has already been published on the dimensional parameters of these particles. Thus it was possible to evaluate critically the technique itself, and perhaps at the same time to resolve some of the discrepancies occurring in the literature.

As a biometric tool, our method rests on the same principles utilized by other workers (14, 21, 29, 31), except that it utilizes the procedure of Wicksell (41, 42) for derivation of the size distribution of particles from measurements of their profiles in thin sections, and thereby provides a complete description of a population of particles, including their absolute number. Another morphological technique allowing an estimate of this number, that described by Weibel and coworkers (38–40), also relies on planimetry of profiles in thin sections, but uses a different mathematical derivation for conversion of profile to particle frequency. With $N_i$ being the number of profiles per unit area of section, and $V_i$ the fractional area occupied by the profiles in the section, the number $n_i$ of particles per unit volume is given, according to Weibel et al. (40), by the formula

$$n_i = \frac{N_i^{3/2}}{\beta V_i^{1/2}} D \qquad (10)$$

in which $\beta$ is a shape coefficient, equal to 1.382 for spheres, and $D$ is a distribution coefficient equal to

$$D = \left(\frac{m_3}{m_1^3}\right)^{1/2} \qquad (11)$$

where $m_n$ is the $n$th moment about the origin for the distribution of particle radii.

Compared with the Weibel procedure, the method described in this paper has the following advantages:

(a) It gives an actual size distribution of the particles, information which is of interest in itself, and which also allows direct calculation of the coefficient $D$. With our material, we have obtained $D = 1.11$, a value very close to the value of 1.1 recommended by Weibel et al. (38, 40) as being adequate for most particle populations.

(b) It allows automatic retrieval of many of the missing profiles (shaded areas in Figs 3 and 4) and is thereby less exposed to the intrinsic limita-

tion, common to both techniques, set by the difficulty of identifying the smaller profiles. For this reason, the frequency $N_i$ of profiles, though probably still underestimated, is less so with our method than with that of Weibel et al.

(c) It is less dependent on the accuracy of $N_i$, which comes into our calculation only indirectly, whereas it enters explicitly, raised at power $3/2$, in equation 10.

For these reasons, the Weibel procedure, in addition to giving less information on the particle population, may be expected to yield a lower value than ours for the particle frequency $n_i$. With our material, the deficit was found to be of the order of 20%. When the profiles retrieved by the Wicksell procedure were taken into account, equation 10 gave results identical with ours.

There is, however, one weakness to the Wicksell procedure. As can be seen from the schematic drawing of Fig. 2, the number of profiles remaining at each step and taken to belong to equatorial sections is relatively small. Thus, an excess or deficit of even a few profile leads to an over- or underestimation of the contribution of nonequatorial sections to the subsequent class of profile radii, which in turn results in an under- or overestimation of the residual profile radii, in particular of those attributed to equatorial sections in the adjacent class. In this manner, oscillations of the kind most clearly seen in the first computed histogram of Fig. 3 are easily generated. Thus the method is more sensitive to sampling fluctuations than might be expected. We believe this fact to be mainly responsible for the irregularities occurring in the computed histograms of Fig. 3 and 4. It should not greatly affect the statistical parameters of the distribution derived from the histograms.

As already mentioned, our technique is limited, though less so than other morphological methods, by the possibility of recognizing the smaller profiles. According to our experience, the limit below which identification becomes difficult is about 0.2 $\mu$ in radius. This limit is lower for equatorial than for polar sections; it depends also on the degree of preservation and on the distinctiveness of the particle structure. In this respect, tissue sections may provide more favorable conditions than isolated fractions.

It could be argued that, as long as the structural integrity of the tissue is sacrificed, one might as well take advantage of this fact and use a direct

counting technique. However, the counting techniques that have been described so far are all subject to limitations that make them at least as unsatisfactory as the indirect methods, and frequently more so. Counting particles in hemocytometer cells under the phase-contrast microscope (1, 36) appears unreliable, even for particles as large as mitochondria (5, 24). Up to now, counting with the Coulter counter does not seem to be applicable to spheres smaller than 0.15 $\mu^3$ (radius smaller than 0.33 $\mu$) (24). The direct particle count in sprayed droplets described by Williams and Backus (43) and modified by Bahr et al. (5), although not subject to any theoretical size limitation, in practice could not be used for mitochondria weighing less than 0.03 ng (radius smaller than 0.26 $\mu$) (6, 24). The reason for this is that, like all other direct counting techniques, it does not allow any accurate identification of the particles and is therefore applicable only to pure fractions, a condition not likely to be frequently fulfilled. With mitochondrial fractions, any such technique becomes unreliable in the size ranges where contaminants, such as lysosomes and peroxisomes, contribute significantly to the total number of particles. There is thus a real justification for a counting technique applicable to sections in which particle profiles can be accurately identified.

A technique of this kind, applicable to isolated fractions has been described recently by Clementi et al. (14). It consists of embedding with the particles a known concentration of polystyrene beads similar in size to the particles under study. With populations having exactly the same size distribution, the relative frequency of profiles down to any size limit gives a direct measure of the number of particles. Obviously, the accuracy of this technique depends on knowledge of the size distribution of the particles to be counted and on the precision with which this distribution is mimicked by the reference beads. It also requires random mixing of the two populations in the embedded material, as well as special qualities on the part of the reference beads.

From the technical point of view, our method relies essentially on planimetry of profiles. As is explained in Materials and Methods, we have found it convenient to use a particle dimension analyzer for this purpose. The profiles were sufficiently close to circular to make this possible and the recorded results could be used directly for computation by spherical reduction according to Wicksell's procedure (42). Other planimetric methods should lead to equally valid determinations of the fractional volume of particles in cells (21, 29, 39) or in subcellular fractions (31); some of these methods have a more general applicability, since they are not restricted to near circular profiles. As long as these are not too irregular, the size distribution of the particles can still be estimated by one of the procedures of Wicksell (41, 42).

## Validity of Results

As indicated by our statistical analysis, the four preparations examined differ significantly from each other. Although nonreproducible artifacts could be involved, this variability is more likely to be related to individual differences in the mitochondrial composition of the liver. This is not particularly surprising in view of the wide variations that are encountered from one animal to the other in the hepatic level of mitochondrial enzymes and in their specific activity in purified fractions. The pooled results which we have obtained thus provide us only with some kind of estimate of an average mitochondrial population, the precision of which is best assessed by the standard deviation of the means.

In Table IV are listed the data that are available in the literature concerning liver mitochondria, together with the corresponding ones derived from our own investigations. For derivation of the mean dry mass of mitochondria from their mean volume, we multiply the latter by 1.10, the measured average density of the particles in 0.25 M sucrose, and by 0.347, the ratio of their dry to their wet weight (exclusive of the sucrose present in the sucrose space) computed from the results of density gradient centrifugation experiments (9, 16).

The midpoint radius of the total volume distribution has been estimated from Fig. 5. It is taken to apply also to the distribution of total dry mass (assuming a constant average density in all size classes) and to that of mitochondrial enzymes (assuming biochemical homogeneity). It serves to calculate the median sedimentation coefficient of mitochondrial volume, mass or enzymes in 0.25 M sucrose at 0°, by means of the Svedberg equation for spherical particles, with 1.10 for the particle density, as mentioned above.

Conversion of total mitochondrial volume to relative specific activity of cytochrome oxidase is

TABLE IV

*Comparison with Other Data*

| Parameter | This work Mean $\pm$ SD | | From literature* | |
|---|---|---|---|---|
| Mean volume, $\mu^3$ | 0.287 $\pm$ 0.025 | | 0.430 (24) | 0.806 (29) |
| Standard deviation of $\log_{10}$ (volume) | 0.354 | | 0.278 (24) | |
| Mean dry mass, ng | 0.110 $\pm$ 0.010 | 0.121 (6) | 0.136 (24) | |
| Standard deviation of $\log_{10}$ (dry mass) | 0.354‡ | 0.212 (6) | 0.249 (24) | |
| Midpoint radius of distribution of total volume, protein, or enzymes, $\mu$ | 0.465 | 0.445 (19) | 0.446–0.478 (37) | |
| Median sedimentation coefficient of total volume, protein, or enzymes, $10^{-9}$ sec | 1.30 | 1.28 (19) | 1.27–1.47 (37) | |
| Surface to volume ratio, $\mu^{-1}$ | 6.87 $\pm$ 0.17 | | | 7.14 (29) |
| Total volume, $cm^3/g$ liver | 0.141 $\pm$ 0.015 | | | |
| Total volume, $cm^3/cm^3$ parenchymal cytoplasm | | | | 0.185 (29) |
| Total number per ng liver | 0.490 $\pm$ 0.041 | 0.330 (1) | 0.119 (36) | |
| Relative specific cytochrome oxidase activity | 4.65 $\pm$ 0.50 | 4§ | | |

* In parentheses, reference number.
‡ Taken to be equal to the standard deviation of $\lg_{10}$ (volume).
§ Combined results from this laboratory (see, for example, references 10, 17).

done as follows. We first compute the total mitochondrial dry mass as explained above (54 mg/g liver). Of this amount 80%, or 43 mg, is taken to consist of protein on the basis of the nitrogen determination of Glas and Bahr (24), which has been confirmed in this laboratory. Since the livers of our animals contain about 200 mg of protein per gram, we conclude that the specific cytochrome oxidase activity of pure mitochondria is 200/43 or 4.65 times that of a whole liver homogenate. For this value as for that of dry mass, we have assumed the relative standard deviation to be the same as that of the determination from which it has been derived.

Considering first the results obtained by other workers on isolated fractions, we note that our calculated value for the mean dry mass agrees within 10% (one standard deviation) with that determined experimentally by Bahr and Zeitler (6). Actually, the agreement may be even better, since the value of Bahr and Zeitler is obtained by fitting a log–normal function to a distribution truncated at the level of 0.03 ng or 0.26 $\mu$ in radius. They thereby exclude from their analysis any excess of smaller particles over their extrapo-

lated frequency. That such an excess may exist is suggested by our results (Fig. 4). This reason probably also explains the smaller standard deviation arrived at by the authors. The same considerations apply to the more recent value of mean dry mass given by Glas and Bahr (24), with the additional point that these authors separate the mitochondria at a speed lower than those used by Bahr and Zeitler (6) and in this work. Thus their measurements apply to a somewhat biased sample of the mitochondrial population, deprived of its smaller members by the preparative procedure and characterized for this reason by a higher mean dry mass.

The mean mitochondrial volume given by Glas and Bahr (24) is considerably greater than our own estimate, and does not agree with their value of dry mass. The agreement claimed by the authors is spurious, since it rests on the use of two erroneous conversion factors: 1.18 (attributed to Anderson) for the mitochondrial density and 0.263 (determined by the silicone technique of Glas and Bahr, reference 23), for the ratio of dry-to-wet mitochondrial weight. It is easy to see that these factors cannot both be correct, since a particle containing

73.7% of water and having a wet density of 1.18 has a dry density of 1.68, a most unlikely value for a structure made of mostly of proteins and lipids. The value of 1.18 represents the equilibrium density of mitochondria in a sucrose gradient and applies to particles almost completely dehydrated osmotically; as already mentioned, their density in 0.25 M sucrose is lower, of the order of 1.10 (10). Furthermore, as shown elsewhere (11), the method developed by Glas and Bahr (23) for the determination of the water content must lead to an overestimation of this content. Our own estimate of 0.347 (16) corresponds to a dry density of 1.315, a very plausible figure. It appears from these considerations that either the mean volume or the

indicates strongly that our measurements are not vitiated by serious artifacts, and also shows that the postulate of biochemical homogeneity, on which our conversion of mass to enzyme distribution is based, represents a valid approximation. In contrast, the distributions of enzyme frequency as a function of particle radius reported by Swick et al. (37) are more Gaussian in shape and are characterized by a distinctly smaller standard deviation than those derived from our determinations and those of Deter and de Duve (19). They also differ somewhat from one mitochondrial enzyme to the other and thus contradict the postulate of biochemical homogeneity.

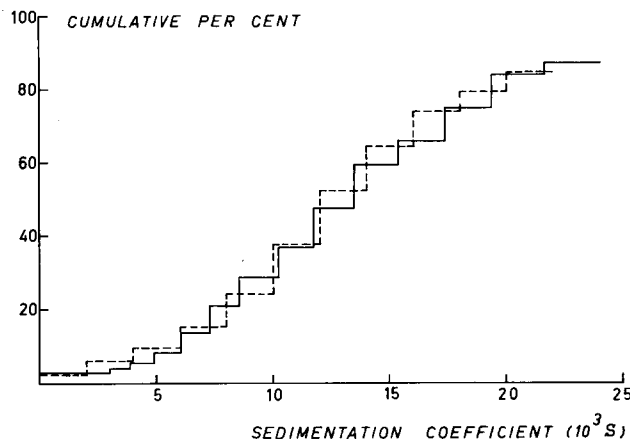In the discussion of their paper, Swick et al. (37)



FIGURE 6 Cumulative distribution of the sedimentation coefficients of cytochrome oxidase. The experimental results obtained by Deter and de Duve (19) are represented by the broken line (median sedimentation coefficient, 12,800S in 0.25 M sucrose). The continuous line is derived from the size distribution of mitochondria observed in this work (median sedimentation coefficient, 13,000S in 0.25 M sucrose). The content of the first class, on which no information is supplied by the morphological analysis, was set equal to the cytochrome oxidase activity of the P + S fraction (2.4%).

mean dry weight value given by Glas and Bahr (24) must be incorrect. In our opinion, the mean volume value is more suspect since it is based on the use of the Coulter counter, which, as mentioned by the authors themselves, was used at the limit of its resolving capacity and which ceases to be reliable below a particle volume of 0.15 $\mu^3$. Therefore, we tend to attach more significance to the agreement between the mean dry weight values of Bahr and coworkers (6, 24) and our own, than to the discrepancy between the mean volumes.

As shown in Table IV, there is also good agreement between our calculated values for the midpoint radius of the volume, mass, or enzyme distribution and for the corresponding median sedimentation coefficient, and those determined experimentally by Deter and de Duve (19) and Swick et al. (37). As shown in Fig. 6, the agreement between our data and those of Deter and de Duve (19) extends over the entire distribution; this

state that their results agree with the value of mean mitochondrial volume of Glas and Bahr (24), which we have criticized above as being overestimated. They write "Glas and Bahr (1966) reported a mean particle diameter of 0.94 $\mu$, which agrees with our estimates (based on sedimentation characteristics) of the midpoint of the protein distribution, 0.938 $\mu$." This statement is misleading in two respects. First, 0.94 $\mu$ is not reported by Glas and Bahr (24) as a mean diameter; it is the diameter of a particle having the mean volume reported by the authors, which is not the same thing. Second, the midpoint diameter of the protein distribution, assumed to be equal to that of the volume distribution, characterizes a particle which is necessarily bigger than the mean volume, since the number of particles of smaller volume included in the left-hand half of the distribution must be greater than that of particles of larger volume included in the right-hand half, whichever the shape of the

distribution. The midpoint diameter calculated from the log–normal volume frequency function given by Glas and Bahr (24) is 1.02 $\mu$; that obtained from our results is 0.93 $\mu$ (Table IV). Thus, the results of Swick et al. (37) agree much better with our mean volume estimate than with that of Glas and Bahr (24).

Summarizing this part of the discussion, we find that, except for one serious discrepancy, which we believe to have accounted for satisfactorily, our data agree reasonably well with those obtained by other authors on isolated rat liver mitochondria. Our mean values tend to be somewhat smaller, and our standard deviations somewhat higher, than the corresponding values in the literature, possibly because our determinations extend further into the lower size range than those of others. As already pointed out, all techniques become unsatisfactory below a radius of 0.2–0.3 $\mu$. Thus, one would expect frequency distribution curves to be distorted in the lower size range in a manner suggesting a deficit of smaller particles. This tendency is not seen in our histograms, whereas the dry mass measurements of Bahr and Zeitler (6) have actually provided evidence of the existence of a second population of smaller particles. These are assumed to be nonmitochondrial, but the possibility that the liver may contain a population of small mitochondria, originating, for instance, from the Kupffer cells cannot be discounted at the present stage. It must be remembered that a small but significant fraction (2.4%) of the cytochrome oxidase activity remains in the P + S supernatant. If this activity is associated with small intact mitochondria, their total number would be appreciable.

The literature contains little information that can help to test the validity of the manner in which our results are extrapolated to whole liver. Our estimated number of mitochondria is much greater than the values obtained by direct counts under the phase-contrast microscope (Table IV), but the latter method is so unreliable that no significance can be attached to this difference. On the other hand, our estimate of the total volume occupied by the mitochondria appears not to be incompatible with the value obtained by Loud et al. (29) on intact tissue sections. Unfortunately, the two values are not directly comparable, since that of Loud et al. (29) applies to the cytoplasm of parenchymal cells. However, taking 1.05 for the density of liver (8), we find that the two values

agree if it is assumed that the cytoplasm of parenchymal cells occupies about 80% of the liver volume and that the volume occupied by the mitochondria of nonparenchymal cells is small enough to be neglected. These assumptions are not unreasonable. Our estimate of the surface-to-volume ratio also agrees with that of Loud et al. (29), but there is marked disagreement between the mean volume given by these authors and our value. The reason for this discrepancy is not clear, since Loud et al. (29) do not give the details of their calculations, but it is obvious that their results are not coherent. Spherical particles forming a homogeneous population with an average surface-to-volume ratio of 7.14 $\mu^{-1}$ should have a radius of $3/7.14 = 0.42$ $\mu$, and therefore a volume of 0.31 $\mu^3$. If they are not spherical (the authors' calculations apply to right cylinders), their volume can be only smaller, not 2.6 times larger. Furthermore, multiplying the mean mitochondrial volume given the authors by their value for the number of mitochondria per $\mu^3$ of cytoplasm, 0.292, leads to a fractional volume of 0.235 which contradicts the value of 0.185 found by the authors by application of the Delesse principle.

As shown in Table IV, the relative specific cytochrome oxidase activity of pure mitochondria predicted from our data is higher than the average value obtained experimentally in this laboratory on the purest mitochondrial subfractions. However, such fractions still contain some contaminants, and their relative specific cytochrome oxidase activity varies within a wide range, at least between 3 and 5. Since our predicted values are also quite variable, the difference is certainly not significant.

From a theoretical point of view, the main factor that could seriously invalidate our estimate of the total mitochondrial volume would be the selective separation with the nuclear fraction of a group of mitochondria differing considerably in their cytochrome oxidase content from those present in the M + L fraction. It can be estimated from the manner in which the nuclear fraction is separated that, if their selection is due to their higher rate of sedimentation, the mitochondria in this hypothetical group should have a radius of 1.2 $\mu$ or more. There is no evidence of the existence of a special population of large mitochondria in rat liver and it must be noted that the M + L fraction contains practically no particle with a radius greater than 0.8 $\mu$ (Figs. 3 and 4). It seems more likely, there-

fore, that the mitochondria in the nuclear fraction are a fairly random sample of the total population and are included in this fraction by an agglutination artifact. If such is the case, the losses to the nuclear fraction should not greatly affect our estimate of the total volume of mitochondria, or that of their total number. However, as has already been pointed out, the small amount of mitochondrial material present in the P + S fraction, although of little importance for the estimate of total mitochondrial volume, if owing to small mitochondria, could represent a larger number of particles than that obtained by extrapolation, which assumes randomization of sizes in this fraction as well.

From the practical point of view, there remains the possibility that the mitochondria suffer a change in size as the result of isolation. Indeed, as can be seen in Fig. 1, many particles appear somewhat condensed and a few are highly swollen. Appreciation of the incidence these artifacts may have on the accuracy of our determination will have to await more accurate measurements on intact tissue sections than have been made so far. Another difficulty, which could be corrected by

the choice of a more stable reference enzyme, arises from the losses in cytochrome oxidase activity suffered upon fractionation (6%, on an average). Since our results are normalized, these losses vitiate our conclusions only to the extent that they occur unequally in different fractions.

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