DIRECT COUNTING AND SIZING OF MITOCHONDRIA IN SOLUTION

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ABSTRACT

Resistive particle counting has been developed for the accurate sizing and counting of mitochondria in solution. The normal detection limit with a 30 μ aperture is 0.48 μ diameter, or 0.056 μ^3 particle volume The mean volume of rat liver mitochondria was 0.42 μ^3, or 0.93 μ in diameter. The average value for numbers of particles per milligram of mitochondrial protein was 4.3×10^9, and per gram of rat liver was about 11×10^{10}. These values compare satisfactorily with those derived by light microscopy and electron microscopy. The mean volume for mitochondria from rat heart was 0 60 μ^3 and from rat kidney cortex, 0.23 μ^3. These values agree within 15% of those determined by electron microscopy of whole tissue. Mitochondrial fragility and contaminating subcellular organelles were shown to have little influence on the experimentally determined size distributions The technique may be applied to rapid swelling studies, as well as to estimations of the number and size of mitochondria from animals under different conditions such as liver regeneration and hormonal, pathological, or drug-induced states Mitochondrial DNA, RNA, cytochrome *c*–oxidase, cytochrome ($a + a_3$), and iron were nearly constant per particle over large differences in particle size. Such data may be particularly valuable for biogenesis studies and support the hypothesis that the net amount per particle of certain mitochondrial constituents remains constant during mitochondrial growth and enlargement

INTRODUCTION

For a long time biochemical measurements on mitochondria have been expressed on a protein basis. While this practice is generally useful, it does obscure information which might be valuable for studies on mitochondrial biogenesis (1, 2) and the influence of hormones such as thyroxin (3, 4), or, for example, the hypocholesterolemic drug Clofibrate (ethyl, α-ρ, chlorophenoxyisobutyrate) (5, 6, 7), where it would be most interesting to determine the numbers and size of mitochondria. Conclusions might then be drawn about whether more, smaller mitochondria, or larger mitochondria could be involved during biogenesis and what the changes in net amount of mitochondrial protein (3, 4, 5, 6, 7) represent. Similarly, studies on mitochondrial development in fetal and newborn rats (8, 9, 10) would benefit greatly from information on the size and numbers of mitochondria. Another area which could profit is that of mitochondrial swelling and contraction (11, 12), since it suffers from problems of interpretation about light-scattering results (12, 13).

This paper, then, describes the development and use of a resistive particle counter for the accurate counting and sizing of mitochondria. The principle of resistive particle counting has been known for some 17 yr and has seen extensive use in hospitals for routine counting and sizing of blood cells. It has also been developed for microorganisms (14), as well as many other fields in industry and pollution. However, only limited interest has been shown towards counting particles below 1.0 μ in

diameter. The main reason for this lies in the difficulty of maintaining solution cleanliness for preventing frequent clogging of apertures 30–10 μ in diameter. In addition, electronic background noise becomes a severe problem near the detection limits of the apparatus.

Gebicki and Hunter (15) presented a valuable paper in which they described the use of the Coulter counter to follow mitochondrial swelling and disintegration. This early work clearly demonstrated the feasibility of using a resistive particle counter with mitochondria. However, since their reported detection limit was only 0.4–0.6 μ^3, large numbers of particles below 0.4 μ^3 (0.9 μ diameter) must have escaped detection. Apart from this study and an outline of a method based on it (12), only one major investigation on resistive particle counting of mitochondria has been reported (16). This study by Glas and Bahr was much more rigorous than that of Gebicki and Hunter (15) and provided data on the size and weight characteristics of normal, rat liver mitochondria. Good agreement with other techniques such as quantitative electron microscopy and microcrits was also demonstrated. Even though the detection limits of their apparatus was improved to about 0.2–0.3 μ^3, many small mitochondria must not have been counted and also no biochemical data was given about the contribution of contaminating subcellular particles to the observed size distributions. A report (17) describing use of the method to follow volume changes in isolated chloroplasts on illumination has appeared, while an excellent review (14) by Kubitschek discusses basic principles and some results of counting and sizing bacteria

Some of the results and methodology recorded here have been communicated in preliminary form (18).

EXPERIMENTAL PROCEDURES

Chemicals

Cytochrome *c*, type III, and glucose-6-phosphate (disodium) were obtained from the Sigma Chemical Co. St. Louis, Mo.; Lubrol WX, a nonionic detergent. from I.C.I. Organics, Inc , Stamford, Conn. All other reagents were of A R grade, or the highest purity commercially available.

Apparatus

Several commercial, resistive particle counters are capable of counting and sizing mitochondria. The most readily available are those manufactured by Coulter Electronics Inc., Hialeah, Fla.; and more recently, by Particle Data Inc , Elmhurst, Ill. The method to be described here uses a Coulter Model B (superseded by a more compact Model Z) and may be readily applied to other apparatus. However, the Coulter Model B enjoys wide use in many institutions.

The first requirement for successful sizing and counting of particles in the size range 0.5–2.0μ in diameter is that the apparatus must possess low levels of electronic background noise. Not all models tested by us met this requirement, and it may be necessary to check several before a suitable one is found. The next problem is to work under conditions of lowest electrical interference. This requires use of a noise-free, 110 v line, careful grounding of the vacuum pump to the sample stand and amplifier chassis, as well as a Faraday cage to surround the sample stand. An aluminum, screen wire cage covered with aluminum foil works satisfactorily. It is not necessary to cover the access to the sample beaker, although external, "stand" interference may occasionally be detected by failing to do this. If these precautions fail to cut out interference significantly, then the apparatus should be moved to a different location. It is important that the counter be placed on a bench free from vibration, and far removed from possible sources of electrical interference such as air conditioners, large motors which cut on and off, and high-voltage apparatus. Finally, a factor sometimes overlooked is that of noise, the transducer can amplify harmonics induced in the liquid sample by, for example, conversion near the sample stand. Accoustically induced interference may be eliminated by preventing the free-hanging platinum electrodes from vibrating (personal suggestion of Dr. Robert Leif of the University of Miami, Fla.).

A timer (Model EE, Coulter Electronics Inc.) is most useful for speeding up counting. Counts are made for a precise time, rather than volume, and about three times as many counts can be recorded during the time required for volume counting.

Apertures

For routine sizing and counting of mitochondria a 30 μ aperture is most suitable even though a 19 μ (15) or a 10 μ one may theoretically offer considerably lower detection limits; the 30 μ aperture does not clog frequently and is also considerably cheaper. With careful operation, it has been found that particles as low as 0.45–0.5 μ in diameter may be accurately sized and counted. This is sufficient to include most mitochondria, though for certain cases a 19 μ aperture would be useful for extending the detection limit to 0.35–0.4 μ particle diameter.

Solutions

For routine work, 0.9% (w./v) NaCl (308 mosmolar) is used as electrolyte. This solution must be essentially particle free and it has been found that sodium chloride, injection USP, Abbott Laboratories, North Chicago, Ill., contains negligible particles above 0.45 μ in diameter. Other manufacturers' physiological saline can be used, but in some cases additional filtering through 0.45 μ and 0 22 μ Millipore filters may be required to reduce the levels of contaminating particles to acceptable limits. The advantages of using commercially available saline are speed and low levels of contaminating particles. When filtering solutions through Millipore filters, it is essential to rinse the final containers for the solutions several times with filtrate before collecting the filtrate used for actual counting. In addition, two or three separate filterings may be required to reduce the background to acceptable levels.

Threshold Setting, Calibration, and Determination of Counter Resolution

Independently sized latex particles of either 0.5, 0.8, 1.09, or 1.3 μ in diameter (Coulter Electronics Inc.) can be used, with 0.8 μ particles being routinely employed for threshold setting and calibration in the present study.

The Coulter Counter Model B is allowed to warm up for 30 min with the following settings: internal gain switch at 0; internal matching switch at 32H, recommended to match impedances with the 30 μ aperture and 0 9% NaCl; 1/amplification at $\frac{1}{8}$, 1/current at $\frac{1}{2}$, the maximum possible with a 30 μ aperture; and the internal switch set on "locked." This position enables much more accurate settings for pulse-height analysis by careful manipulation of the two threshold controls.

THRESHOLD SETTING: Threshold settings must be carefully adjusted initially, since successful pulse-height analysis depends upon extremely precise dial settings. If a multichannel pulse-height analyzer (Nuclear-Chicago, Des Plaines, Ill.; Nuclear Data Inc., Palatine, Ill.; or Packard Instrument Co., Inc., Downers Grove, Ill.) is available, then size-distribution analysis becomes more accurate and simpler (14) It is also possible to take the data from a multichannel analyzer and process it either on line (Particle Data Inc.) or off line by a small laboratory computer. However, these options require considerable capital outlay.

The standard procedure for establishing correct threshold settings is described in the Coulter manual It should be followed with great care and once carried out for a new counter, or an older one not previously used for very accurate work, need not be repeated except for occasional weekly checks. A useful test of correct threshold setting is as follows: a suitable dilution of 0.8 μ latex particles (about 50,000 particles per 50 μl) is counted in successive windows of 0 5. That is, the upper threshold is left on 5 (locked position) while the lower one is moved in increments of 0.5 from an initial lower setting of 5, up to 12. The peak for 0 8 μ particles with a 30 μ aperture and the counter settings given above occurs between a threshold setting of 8 and 10 The counts in all these windows should then be summed and must agree with those of a single window obtained from either 5 to 12 on the separate position, or its equivalent on the locked position from windows 5 to 70 If these values do not agree, then the successive windows either overlap—stepwise counts > simple total counts—or fail to overlap—stepwise counts < simple total counts Exactly matching windows may then be obtained by careful adjustment of window zero set screw "A." An example of the procedure is given in Table I. The total number of particles obtained by summing the counts in 14 separate windows from a lower threshold of 5 through 12 was 22,479, in close agreement with the 22,702 obtained singly in the separate position, or the 22,122 obtained singly in the locked position.

CALIBRATION: The data in Table I also illustrate how the calibration factor is obtained. The count in each window is multiplied by the mean window setting to give an apparent particle volume For example, with the lower threshold set at 7, the counts, 373, are multiplied by 7.25, the mean window setting, to yield an apparent volume of 2704. This process is repeated over a range sufficient to cover the singlet peak of the standard particles; that is, it should not be influenced by inclusion of doublet or triplet peaks (14), which, though small, will lead to falsely high calibration factors With 0 8 μ latex particles, the 30 μ aperture, and using the counter settings given above, a typical calibration factor of 0.0284 μ^3 per threshold division is obtained. (Table I).

RESOLUTION: One of the requirements for accurate sizing is good resolution. This question has been carefully considered by Kubitschek (14) in his excellent review, and is a combination of several factors: pulse amplitudes should be several times background noise; large particles should be relatively infrequent since they may perturb the pulse-height analyzer; counting rates should be low enough to make coincidence a negligible problem, and last, flow rates, aperture lengths, and the amplifier response time must be matched to give output pulses of the correct amplitude

Practically, the resolution of a counter with a given aperture tube may be readily determined with 1–2 μ latex particles The measured distribution of the singlet counts should be unimodal and nearly symmetrical, and not show any skewing as discussed by Harvey (19). In order to determine the resolution of

TABLE I

Use of Stepwise Counting to Calibrate the Counter and Establish Correct Threshold Settings

0 8 μ latex particles were diluted in 0 9% NaCl and counted in 0.5 increments on the locked position at the counter settings described in the text. Then total counts were taken for an 8.9 sec counting time (29.6 μl solution), both on the locked and separate positions between windows 5 and 12.

Lower threshold	Upper threshold	Background counts	Observed counts	Corrected for bcakground	Apparent vol
5	5	36	126	90	473
5.5	"	13	93	80	460
6	"	7	85	78	488
6.5	"	9	129	120	810
7	"	5	378	373	2,704
7.5	"	2	947	945	7,324
8	"	2	2,389	2,387	19,693
8.5	"	3	3,747	3,744	32,760
9	"	1	4,240	4,239	39,211
9.5	"	2	3,809	3,807	37,118
10	"	1	2,864	2,863	29,346
10.5	"	2	1,927	1,925	20,694
11	"	0	1,150	1,150	12,938
11.5	"	0	678	678	7,967
				Σ = 22,479	Σ = 211,983

$\overset{\text{Upper } 12}{\text{Lower } 5}\sum$ Separate = 22,702; $\overset{70}{5}\sum$ Locked = 22,122

$$\text{Mean threshold setting for 0.8 } \mu \text{ particles} = \frac{211,983}{22,474} = 9.43$$

$$\therefore \text{ Calibration factor} = \frac{\text{vol of 0 8 } \mu \text{ particles in } \mu^3}{9\,43}\ \mu^3/\text{threshold division}$$

$$= \frac{0\,2681}{9.43}\ \mu^3/\text{threshold division}$$

$$= 0.0284\ \mu^3/\text{threshold division}$$

the apparatus used in the present study, and to try to improve it, a 500 μl manometer was severed at its lowest point to allow variable flow rates through the aperture (personal suggestion of Dr. Kubitschek, and discussed in his review). A typical distribution for 1.305 μ latex particles at a given flow rate is shown in Fig. 1. The resolution, defined as half the width of the distribution at half the maximum height (14), was 8.0% of the distribution mean. It was only slightly improved by lowering the aperture flow rate, going to 5.6% and 5.5% for ½ and ⅓ the standard flow rates, respectively. Such a resolution is quite acceptable, but will decrease to about 20% with smaller particles, such as 0.5 μ, which are only just detected above background electronic noise If a given apparatus does not possess a satisfactory resolution, then either a longer path-length aperture or decreasing the flow rate, or a different amplifier, may improve the results. Changing apertures may improve the resolution markedly, for example, one particular 30 μ aperture gave 10.5% resolution with considerable skewing; a second aperture reduced the resolution to 6.4% with an almost completely symmetrical peak. Since adequate resolution for the 30 μ aperture was obtained at the normal flow rate of about 50 μl per 15 sec, this was maintained for all the data reported in the present investigations.

Dilution of Mitochondrial Suspensions for Counting

The most suitable concentration of mitochondria for sizing and counting lies between 0.2 and 0 5 μg protein per ml. For rat liver mitochondria, 1:100,000 and 1:200,000 dilutions of a stock suspension of 50 mg protein per ml were made in 0.9% NaCl. Plastic beakers were used for the dilutions, since glass ones failed to give satisfactory reproducibility for counting serial dilutions. The final dilution was made in the

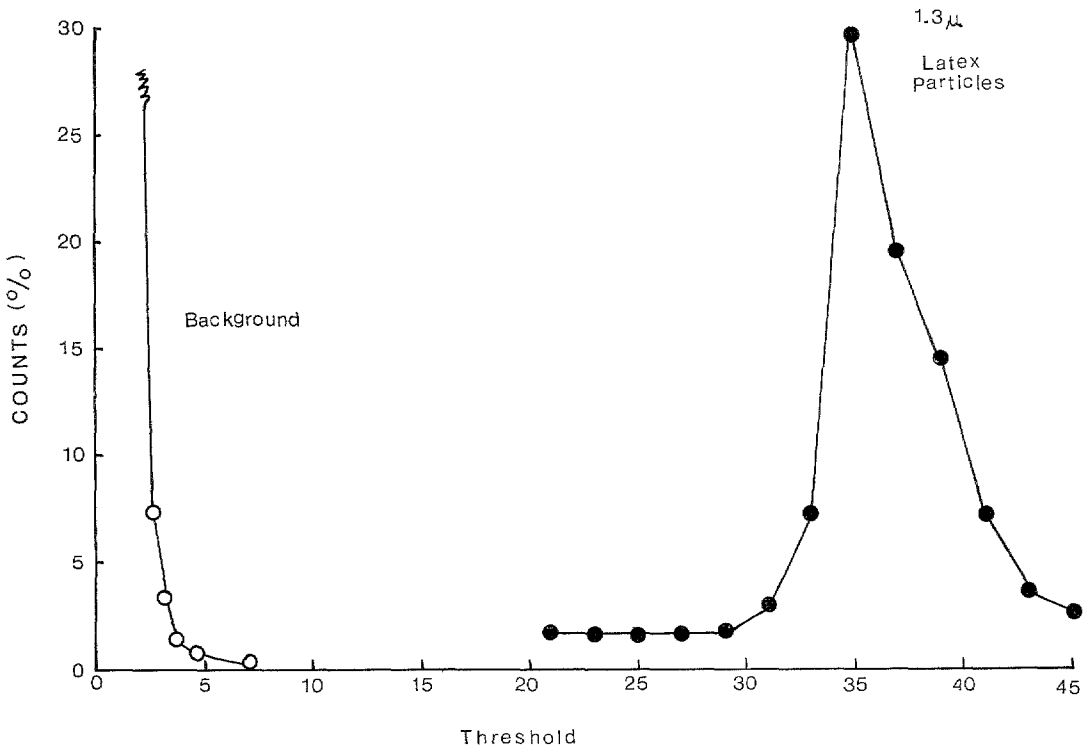

FIGURE 1 Resolution of Coulter counter with a 30 aperture and flow rate of 50 μl per 15 sec. Standard latex particles, 1.3 μ in diameter, were suspended in 0 9% NaCl at a concentration of about 1500 particles per 50 μl. Stepwise counting was initiated well below the singlet peak at a threshold of 35. Background counts under similar conditions are also shown. Apparatus settings are described in the text.

clear plastic, disposable vials (Accuvette, No. B3157-7, Coulter Diagnostics Inc., Hialeah, Fla).

Before diluting the stock mitochondrial suspensions, all containers and pipettes were rinsed several times with the particle-free saline to remove any contaminating particles which might clog the orifice or contribute to the background counts.

Counter Settings for Mitochondrial Sizing and Treatment of Data

In order to obtain sufficient accuracy for sizing mitochondria, 20 separate windows were selected for pulse-height analysis in terms of the arbitrary threshold scales of the Coulter counter These are given in Table II. The distribution of windows was skewed to yield the best resolution, especially near the detection limit of the counter. Here, the background may represent a significant fraction, being about 50% of the total counts in window 1. For routine work, eight separate counts on the same solution were made in windows 1 and 2. For windows 3–20, three separate counts were taken since the background counts fall dramatically and usually comprise less than 5% of the total counts in each window. The size range covered by the 20 windows is from about 0.06 μ^3 (0.48 μ) to 6 μ^3 (2.24 μ).

To calculate the total numbers of particles and their average volume, the raw background and test data were entered into a Hewlett-Packard Programmable calculator (No. 9100 A). The program was set up to subtract the mean of the background count in each window from the mean test count The resultant corrected particle count was multiplied by the mean threshold setting for that particular window, to obtain the apparent particle volume. This process was then repeated for each window, with the calculator automatically summing both the corrected particle counts and the apparent volume. Finally, division of the latter number by the first gave the mean particle volume as an arbitrary threshold number. To obtain the true particle volume, this average threshold number was multiplied by the calibration factor whose derivation with standard latex particles was discussed earlier (Table I). The whole calculation requires about 10 min, or about 15–20 min using an ordinary desk calculator.

Standard procedure in sizing mitochondria is to

TABLE II

Routine Counter Settings Using a 30 μ Aperture for Mitochondrial Sizing Chosen To Give Adequate Resolution over the Whole Volume Range of 0.06–6μ³ (0.48–2 3 μ diameter)

The matching switch is set to 32-H and the 1/current knob is left on ½.

Window No	Internal switch	1/Amplification	Lower threshold	Upper threshold
1	Locked	⅛	2	5
2	↓	↓	2 5	↓
3	↓	↓	3	↓
4	↓	↓	3 5	↓
5	↓	↓	4	10
6	↓	↓	5	↓
7	↓	↓	6	20
8	↓	↓	8	↓
9	↓	↓	10	↓
10	↓	↓	12	↓
11	↓	↓	14	↓
12	↓	↓	16	↓
13	↓	↓	18	↓
14	↓	↓	20	↓
15	↓	↓	22	↓
16	↓	↓	24	↓
17	↓	↓	26	40
18	↓	↓	30	100
19	Separate	¼	20	50
20	"	"	50	100

determine the average volume and numbers of particles per milligram of protein from results of the 1:100,000 and 1:200,000 dilutions. For windows 1 and 2, and eight separate counts, counting precision is reflected as a standard deviation of about 3–6%. Precision then increases and standard deviations are usually between 1 and 2% of the observed counts by window 6. Standard deviations, which combine both counting precision and animal variation from different mitochondrial preparations, are given in Table III and discussed in the Results section.

The counting procedure for a complete size distribution involves some 70 individual counts for the 20 windows and usually takes 20–25 min. This requires using the Model EE timer mentioned earlier, at a setting 10, which corresponds to a counting time of 8.9 sec. The normal flow rate with a 30 μ aperture for 50 μl is about 15 sec. Thus, each count using the timer involves 8.9/15 × 50 μl, or about 30 μl. If the user does not possess a timer, then volume counting with a 50 μl manometer takes about 1 hr to complete the whole size distribution. On the other hand, it is possible to use a multichannel analyzer coupled to a small laboratory computer and obtain a size distribution within 30 sec (Particle Data Inc.). However, this approach necessitates considerable capital outlay.

Animals and Preparation of Mitochondria

Male, albino, Sprague-Dawley rats were obtained from Carworth, Div., Becton, Dickinson and Co., New City, N.Y., or Charles River Breeding Labs., Inc., Wilmington, Mass ; or of Wistar strain, from Manor Research in Puerto Rico (now taken over by Purina Lab. Animals, Vincent Town, N.J.). The animals weighed from 200 to 350 g.

Liver mitochondria (20) and kidney cortex mitochondria (21) were isolated by differential centrifugation from tissue homogenates in 0.25 M sucrose and stored on ice at 50 mg protein per ml Heavy and light mitochondrial fractions (20) were combined except in special cases to be described later.

Rat heart mitochondria were isolated after proteolytic disruption of the heart muscle (22) in a mannitol-sucrose-ethylenediaminetetraacetate medium. The final protein concentration was usually 25–35 mg per ml. All mitochondrial preparations were routinely checked for structural integrity by the criterion of respiratory control. This was determined polarographically with sodium succinate as substrate in the medium described by Gear and Lehninger (23) Most preparations of rat liver mitochondria possessed respiratory-control ratios greater than 3.5. It was found that poor reproducibility of volumes and counts with the Coulter counter sometimes correlated with very low respiratory-control ratios. Such preparations were discarded.

Density-Gradient Centrifugation

This was used to reduce levels of contaminating cytoplasmic particles which might influence mitochondrial size distributions.

The following discontinuous gradient in Spinco SW27 tubes was set up: 2 ml of 2 M sucrose as "cushion," then 3 ml of 1.6 M, 7 ml of 1.5 M, 13 ml of 1.3 M, 7 ml of 1.2 M, 3 ml of 1.1 M, and, after standing for 3 hr, 1–2 ml of mitochondria (50 mg per ml) in 0 25 M sucrose. The pH of the sucrose was previously adjusted to 7.4 with Tris base The gradients were then centrifuged at 4°C for 90 min at 27,000 rpm (131,000 g_{max}). Fractions were collected in order from the bottom by sucking with a peristaltic pump connected to a long, stainless-steel tube introduced through the gradient to the base of the tube. The first 8 ml were discarded. The main band of mitochondria was found in the next 5 ml and normally consisted of about 75% of the total protein in the gradient. The mean sucrose density in this fraction was 1.18. Further, less dense fractions were all discarded for the present study. Collection was carried out at 4°C in a cold

TABLE III

Reproducibility of Particle Counting over the Complete Size Range (from 0.06 to 6.32 μ^3, or 0.49 to 2.29 μ)

The average values from five separate mitochondrial preparations (two livers combined for each preparation) are given together with their standard deviations. The stock suspension contained 50 mg protein per ml, and the standard counting time of 8.9 sec corresponds to 29.6 μl solution passing through the detecting orifice.

		Dilution	
		1 100,000	1 200,000
Total particles by summing 20 separate windows		49,023 ± 4015	24,730 ± 1037
Ratio of 1:100,000 to 1:200,000	Total particles	1.98 ± 0.19	
	Average for all 20 windows taken separately	2.15 ± 0.18	

room, and was monitored spectrophotometrically at 280 nm by a flow-through cell. The mitochondrial fraction was slowly diluted to yield a sucrose concentration of 0.25 M, and then centrifuged for 10 min at 17,000 *g*. The resultant pellet was carfully suspended in a small volume of 0.25 M sucrose and used for Coulter counter analysis and estimation of levels of contaminating enzymes.

Enzymes

CYTOCHROME *C* OXIDASE: This was monitored spectrophotometrically at 25°C by following the oxidation of reduced cytochrome *c* at 550 nm according to the procedure described by Gear (20). Lubrol WX, a nonionic detergent, was added at a level of 0.2 mg protein to activate fully the enzyme in the initial homogenate and purified mitochondrial fraction. Activity is expressed as a first-order reaction constant, k^{-1} (24).

CATALASE: The disappearance of hydrogen peroxide was followed spectrophotometrically at 240 nm (25) at 25°C An extinction coefficient (26) of 0 36 mM^{-1} cm^{-1} was used and the results are expressed as micromoles of substrate decomposed per minute per milligram of protein.

ACID PHOSPHATASE. The hydrolysis of *p*-nitrophenylphosphate was taken as a measure of acid phosphatase activity (27). Data are expressed as micromoles of *p*-nitrophenol formed per minute per milligram of protein at 37°C.

GLUCOSE-6-PHOSPHATASE: The procedure of Harper (28) was followed which involves determining the amount of phosphate formed on hydrolysis of glucose-6-phosphate, as micromoles per minute per milligram of mitochondrial protein at 37°C.

Estimation of Cytochromes ($a + a_3$), b, c_1, and c

The cytochrome determinations on liver, kidney, and heart mitochondria were carried out with a Cary 14 spectrophotometer according to the technique of Williams (29), using more recently reported extinction coefficients for the appropriate wavelength pairs (30).

Spectrophotometric Monitoring of Mitochondrial Volume Changes

Optical density changes in diluted mitochondrial suspensions (0.25 mg protein per ml), were followed at 25°C at 520 nm (12). The exact media used for the various experiments are described later.

Protein Estimation

The UV absorption method of Murphy and Kies (31) was followed, with bovine serum albumin being used as standard.

RESULTS

The data in Table III represent the averages from five separate mitochondrial preparations for 1–100,000 and 1–200,000 dilutions of stock mitochondrial suspensions at 50 mg protein per ml Standard deviations, which give an idea of both the counting precision and variation in particle numbers from animal to animal, are also given. The close agreement with the expected ratio of

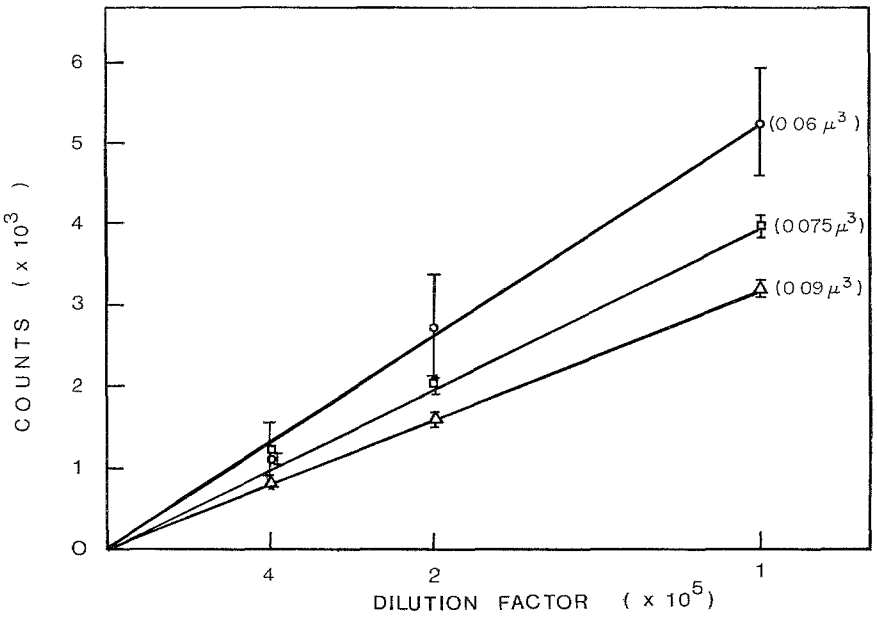

FIGURE 2 Linearity of particle counting on serial dilution of mitochondrial suspensions near the detection limit of the Coulter counter with a 30 μ aperture. Mitochondrial suspensions, 50 mg protein per ml, were diluted in 0.9% NaCl to 100,000; 1 to 200,000; and 1 to 400,000 times in 0.9% NaCl. Counts were made for 8 9 sec. Average of eight separate counts was made for the windows with mean volume 0.06 μ^3 and 0.75 μ^3; and four for the window of 0.09 μ^3. The plotted values have been corrected for background counts obtained by an identical procedure. Standard deviations derived from the total counts, which include background, are also given.

2.00 for counts on serial dilution demonstrates the validity of the counting procedures.

The routine detection limit of particles using a 30 μ aperture is about 0.06 μ^3. The data in Fig. 2 illustrate that satisfactory linearity of counts in the three lowest windows was obtained on serial dilution; consequently, coincidence corrections were not applied in the present study. As discussed in the methods section, it is advisable to record at least six separate counts in windows 1 and 2 (Table II), since the electronic background noise contributes a significant fraction of the total counts. It can be seen that standard deviations of about 6% of the observed counts in the first window (0.06 μ^3) decrease rapidly to 3% for window 2, (0.075 μ^3) and 2% by window 3 (0.09 μ^3). These standard deviations reflect counting precision on a given mitochondrial suspension and are derived from the total oberved counts which includes the background.

Stability of Counting and Sizing

The problem of particle fragility in counting and sizing mitochondria in 0.9% (w/v) (0.154 M) NaCl must be considered. Two questions may be asked First, how stable is volume with time and, second, does significant particle disintegration occur during the period of a size-distribution study?

The following types of experiments were directed to these questions. The data illustrated in Fig. 3 confirm that even after 30 sec, the shortest possible measuring time from particles being in 0 25 M sucrose to their being in 0.9% NaCl, there was little subsequent change in numbers. If significant particle disintegration occurred, a decrease in the numbers of large particles, with an increase in small particles, would be expected. This was not seen, although an initial increase in numbers, 2 min after dilution, followed by a decrease, may be noted for the largest particles. However, this behavior was not shown by all mitochondrial preparations, some being more stable than others.

The results of these rapid-counting experiments were confirmed by carrying out a complete size-distribution analysis and repeating it on the same solution 1 hr after the initial counting was begun. In Table IV, it may be seen that a small swelling of about 8% occurred over 1 hr, while the total

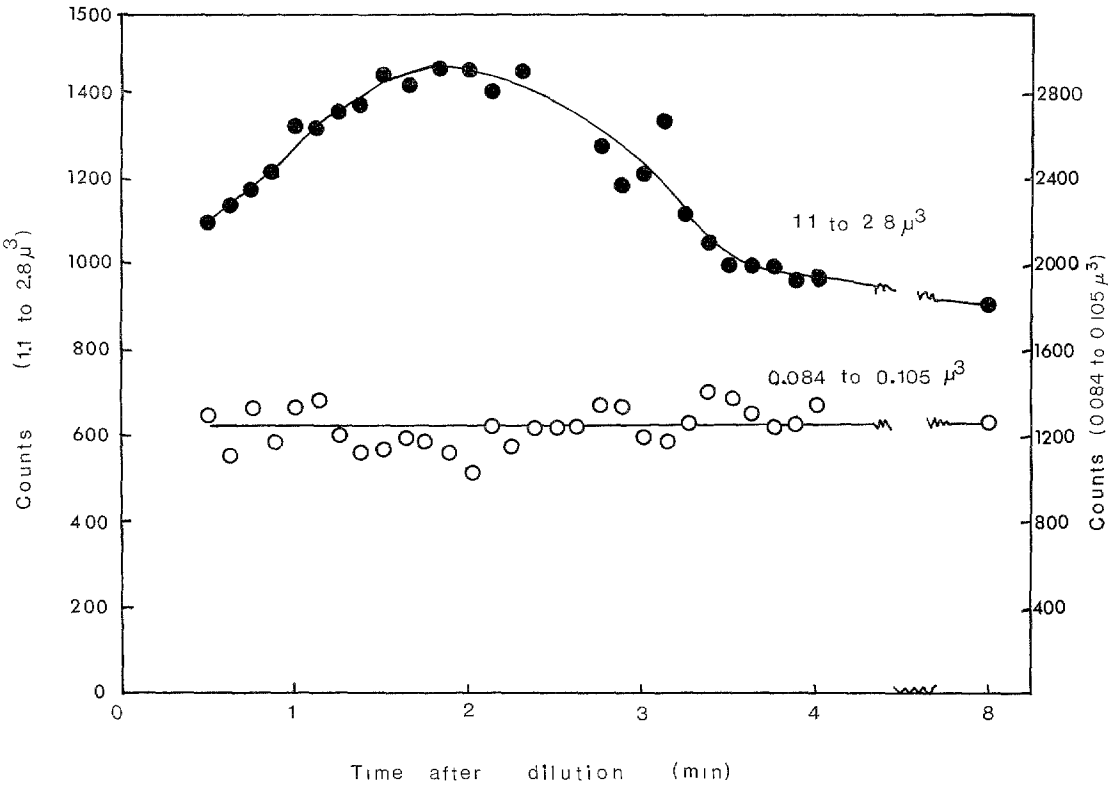

FIGURE 3 Rapid counting of mitochondria immediately after mixing in 0.9% NaCl. Rat liver mitochondria (50 mg per ml) were rapidly diluted 1 to 100,000 and then counted for 3.15 sec, about every 10 sec, up to 8 min after initial mixing. Two separate runs were carried out first, a window was selected to monitor very large mitochondria 1.1 to 2.8 μ^3; the second, to count small particles 0.084 to 0.105 μ^3.

TABLE IV

Long-Term Stability of Mitochondria Diluted in 0.9% NaCl

A complete size-distribution analysis was carried out on a 1 to 100,000 dilution of a stock suspension of mitochondria. It was then repeated after standing for 1 hr.

Time	Average particle vol (μ^3)	Total Nos of particles detected (No /mg protein)
Initial run	0.38	3.43×10^9
1 hr later	0.41	3.00×10^9

number of particles decreased by about 12%. The latter could result from some particle disintegration into fragments below the detection limit of the counter.

Light scattering has been often used to monitor changes in mitochondrial volume (12) To substantiate the results obtained with the Coulter counter, light scattering in 0.9% NaCl was compared with that seen in 0.25 M sucrose, the medium used for differential centrifugation and storage of the stock mitochondrial suspension at 0°–4°C. The traces shown in Fig. 4 demonstrate little swelling, even at 10 sec after mixing 0.5 mg mitochondrial protein in either 3.0 ml of 0.25 M sucrose or 0.9% NaCl solution The optical density at 25°C decreased over 34 min from only 0.41 to 0.36 for the NaCl-treated mitochondria It was slightly more for sucrose, in contrast with the extremely rapid, and massive, phosphate-induced swelling (32). As an additional check of mitochondrial fragility when diluted in 0.9% NaCl, a centrifugation recovery experiment was carried out Mitochondria were separately diluted in 0.9% NaCl and 0.25 M sucrose, and then kept for 1 hr at 0°C. The suspensions were then centrifuged for 5 min at 12,000 *g*, a force sufficient to sediment mitochondria. The resultant pellets were suspended in 0.25 M sucrose, and protein determinations were

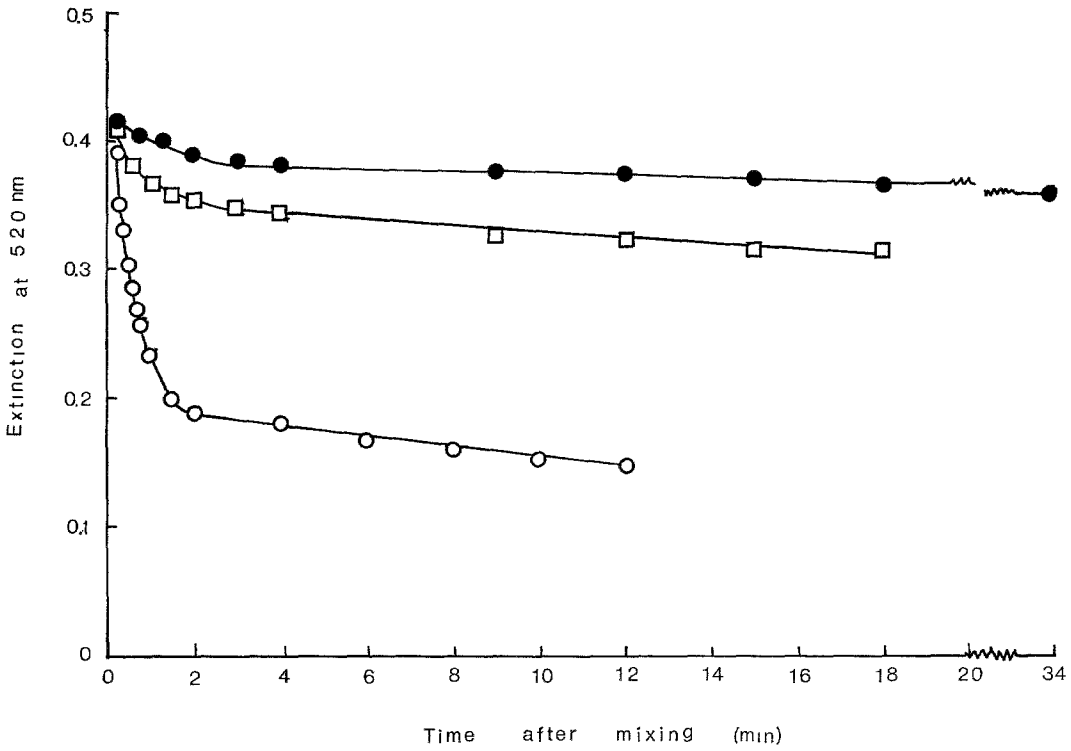

FIGURE 4 Stability of dilute suspensions of mitochondria: spectrophotometric monitoring of volume. Changes after mixing in 0.25 M sucrose (□·), 0.9% NaCl (●), or a 10 mM Na phosphate, pH 7.4 20 mM Tris-Cl, pH 7.4, and 0.125 M KCl (○). Rat liver mitochondria rapidly mixed in the respective media at a concentration of 0.167 mg per ml. Optical density changes for a 1 cm light path were then followed for up to 34 min at 25°C.

identical for both the sucrose or NaCl-treated mitochondria. Consequently, no measurable protein loss, or lysis, occurred when mitochondria were suspended in dilute NaCl solutions.

The conclusion to be drawn from these experiments on mitochondrial fragility is that, up to 1 hr after dilution in 0.9% NaCl, the suspensions are quite stable for both sizing and counting. Little or no particle disintegration could be detected. It should be reemphasized, however, that the initial preparations were all checked for structural intactness by the criterion of respiratory control (23).

Contribution of Contaminating Particles to Mitochondrial Size Distributions

This is an important consideration, since the Coulter counter, by its nature, fails to distinguish biochemically or cytologically the various types of particles passing through its orifice. The following experiments were then directed to help investigate this problem. A mitochondrial suspension was layered on top of a discontinuous sucrose gradient and centrifuged to reduce particle contamination. The exact procedure is described in the methods section After 90 min centrifugation, by which time essentially isopychnic separation had occurred, the main band of purified mitochondria (about 75% of the total protein) was collected. The shoulders of the main band, representing particles equilibrating below 1.5 M sucrose, and above 1.3 M sucrose, were discarded. The size distribution of particles from the density gradient was then compared with the original mitochondrial suspension prepared by differential centrifugation, as were levels of the marker enzymes, glucose-6-phosphatase for endoplasmic reticulum, acid phosphatase for lysosomes, and catalase for microbodies or peroxisomes.

The data in Table V show that there was only a slight decrease in the percentage of total particles

in windows 1 through 4 after density-gradient centrifugation. That is, the smallest detected particles in the size range 0.06–0.12 μ^3, or 0.48–0 61 μ in diameter, were not removed on purification. Most probably, they represent small mitochondria and not lysosomes, microbodies, or endoplasmic reticulum The latter conclusion is supported from separate experiments by the data given in Table VI, which demonstrate a significant lowering of contaminating enzymes, especially for catalase, a microbody marker, by 5.7 ×; and for glucose-6-phosphatase, an endoplasmic reticulum marker, by 2.0 ×. The results in Table V also show that the mean particle volume was increased by only 8%, from 0 46 to 0.50 μ^3 after the numerous manipulations and large tonicity changes necessitated by density-gradient centrifugation.

The important result of these experiments is that values for the volume and numbers of mitochondria determined by the Coulter counter are probably not significantly influenced by nonmitochondrial contamination. This is particularly true for very small mitochondria in the size range 0 48–0.61 μ in diameter

TABLE V

Effect of Density-Gradient Purification on the Distribution of Particles and Their Mean Volume

Values represent the average of three separate experiments.

	Normal mitochondrial preparation	Density-gradient purified mitochondria
Percentage of particles detected from 0 06 to 0.12 μ^3	26.3	24.4
Mean particle vol, μ^3	0.46	0.50

TABLE VI

Specific Activities of Contaminating Enzymes in Mitochondria Purified by Density-Gradient Centrifugation (μmoles/min per mg per protein)

Enzyme	Normal mitochondrial preparation	Density-gradient purified mitochondria	Purification factor
Glucose-6-phosphatase	0 0087	0 0043	2.0
Acid phosphatase	0.033	0.025	1.3
Catalase	475	84	5.7

Influence of Variations in the Differential Centrifugation Procedure on Particle Size Distribution

A question which arose during the present study was whether slight variations in the technique of differential centrifugation could alter particle size distributions Mitochondria are separated from other cellular components in two critical centrifugation steps: first, from nuclei and cell debris at low speed; and second from the microsomal fraction at a higher speed. For example, the extent to which a centrifuge tube is filled will change the average forces within that tube, and thus the average size of sedimenting particles. This would apply to both critical centrifugation steps and will influence the relative concentrations of extremely large and extremely small particles in the final mitochondrial suspension.

These considerations were tested in an experiment where one centrifuge tube was filled half full (20 ml) and the other full (40 ml) for the second critical separation, in which mitochondria are sedimented whereas endoplasmic reticulum is not. Based on the procedure of Gear (20), the tubes were centrifuged for 5 min at 11,000 rpm in the SS-34 rotor of a Sorvall RC-2 centrifuge. This speed gives about 10,000 *g* for full tubes, based on the average radius of centrifugation, or 50,000 *g*-min for a 5 min spin For the half-full tubes, the *g*-min increases to 60,000.

The data in Table VII show how the simple difference in tube filling can have a significant effect on both the total numbers of particles detected and their average volume. Of particular interest is the fact that about 50% more particles were detected in the size range from 0.49 to 0.63μ in the half-full tubes. This result is intriguing since it agrees moderately well with calculations based on the primary sedimentation equation (33), and suitable values for viscosity, solute and solvent density. That is, one-half of all particles in the size range from 0.44 to 0.68 μ in diameter should theoretically *not* sediment in a full tube, whereas all particles larger than 0.44 μ should do so for a half-full tube

Also shown in Table VII are values for the acid phosphatase content of the two preparations The

TABLE VII

Effect on Particle Size Distribution of Filling a Centrifuge Tube Half Full, or Full, during the Critical Separation of the Mitochondrial Fraction from the Microsomal Fraction

The tubes were either half filled (20 ml) or full (40 ml) and centrifuged for 5 min at 11,000 rpm. The g_{av_g} for the half-full tube is about 12,000 g; for the full tube, 10,000 g. Following this difference, the resulting washing procedure was identical for both tubes, and as described in the methods section.

Parameter	Full tube	Half-full tube
Mean particle vol, μ^3	0.483	0 398
Mean particle diameter, μ	0.975	0 913
Total No. of particle/mg protein detected from 0.49 to 2.3 μ	3.46×10^9	3.90×10^9
No. of particles/mg protein detected between 0 49 and 0 63 μ	0.97×10^9	1.45×10^9
Acid phosphatase activity. (μmoles p-nitrophenyl-phosphate hydrolyzed/min per mg protein)	0.017	0.021

half-full tube was more contaminated and had a lower respiratory control. This is not unexpected since de Duve's group (34) used high g forces to prepare their "light" mitochondrial fraction (250,000 g-min), which consisted mainly of lysosomes. The forces for their "heavy" mitochondrial fraction were 33,000 g-min, which corresponds more closely to the 50,000 g-min used in the present study.

The important point to be derived from these observations, then, is that the extent of tube filling during the critical separation of mitochondria away from other cellular constituents strongly influences the size distributions determined by the Coulter counter. Consequently, it is necessary to standardize tube filling in order to allow a valid analysis of particle size and numbers in a particular experimental situation Also, it should be remembered that results are derived only from a particular mitochondrial fraction, prepared under specified conditions Caution must be used in extending conclusions to *all* mitochondria, a point strongly emphasized by de Duve (35), since many mitochondria, of necessity, are lost in the nuclear and microsomal fractions.

Characterization of Heavy and Light Mitochondria

The experiments just described were carried out on suspensions in which the heavy and light mitochondrial fractions had been combined during the differential centrifugation procedure (20). Earlier research (36, 37) clearly demonstrated a degree of heterogeneity in a mitochondrial population, when separated according to size and density. This was especially evident for cytochrome ($a + a_3$), cytochrome c–oxidase, and iron. The smaller particles, or light mitochondrial fraction or activity, possessed about 1.3 $\times$ the specific content found in the larger particles, or heavy mitochondrial fraction. These findings were later confirmed by careful analysis of enzyme distribution among mitochondria separated by rate zonal centrifugation (38), particularly of cytochrome c–oxidase, which was concentrated among the smallest particles

In view of these observations, it was decided to examine the size distribution of the heavy and light mitochondrial fractions, and test the hypothesis that certain mitochondrial components might be constant on a *particle* basis, while being different on a protein basis. The results in Table VIII summarize the values determined by the Coulter counter. The differences in particle volume, 0.55 μ^3 compared with 0.30 μ^3 as well as the numbers of particles per milligram of protein, $3\,95 \times 10^9$ vs. 5.14×10^9, are quite marked for the two fractions. It is satisfying to note that the values for particle diameter by electron microscopy, derived from earlier research (37), agree moderately well with the Coulter counter data. The lack of better agreement may result from differences in rat strain, centrifuges, and sampling. The mitochondrial content of certain constituents on both a protein basis as well as a particle basis is shown in Table IX. The data are derived from this and earlier research (36, 37). It is most in-

triguing that on a *particle* basis the contents for the two mitochondrial fractions are much more nearly equal than on a protein basis. The implications of these relationships will be considered in the discussion.

Size Distributions and Cytochrome Content of Rat Kidney, Heart, and Liver Mitochondria

All the results described above were obtained with rat liver mitochondrial suspensions. In order to confirm that the techniques just developed could apply equally well to other tissues, mitochondria from kidney cortex and heart muscle were counted and sized in the Coulter counter. The results in Table X also include those derived by electron microscopy The agreement between the two completely different techniques is remarkably good. The large size of heart mitochondria, 0 60 μ^3, is interesting compared with liver, 0 42 μ^3, while kidney mitochondria, 0 23 μ are quite small.

TABLE VIII

Characteristics of Heavy and Light Mitochondrial Fractions Determined by the Coulter Counter

Parameter	Heavy mitochondrial fraction	Light mitochondrial fraction
Vol, μ^3	0 55	0.30
Diameter, μ	1.02	0.82
Diameter measured by electron microscopy, μ	0 99	0 72
Nos. of particles detected per mg protein, $\times 10^9$	3.95	5.14

The data in Table XI illustrate the importance of the units in comparing the content of components of mitochondria in different tissues. For example, the cytochrome *c* content of kidney and liver mitochondria is 0.48 and 0 19 nmoles per mg protein, respectively on a particle basis this becomes 0 45 and 0 44 nmoles $\times 10\mu^9$ per particle, or almost identical

Comparison of Coulter Counter Data with Those Obtained by Electron Microscopy and Light Microscopy

It is useful to know how the present results compare with previously published data based on other techniques. In some of the preceding sections it has already been noted that particle volumes estimated from electron micrographs of mitochondria in tissue sections, or in suspension, agree well with those derived from the Coulter counter The information now given in Table XII shows that the agreement with electron microscopy and light microscopy for the number of particles per milligram of protein is quite good. The rather low value of Gebicki and Hunter (15) is undoubtedly caused by the poor detection limit of their particle-counting apparatus. It is uncertain why the present value of 4 3 $\times 10^9$ particles per mg protein is about one-half that of Glas and Bahr (16), based on quantitative electron microscopy since they also obtained a similar value with a Coulter counter. In addition, their published value for particle volume (0 43 μ^3) agrees very well with that of 0 42 μ^3 found in this study. However, examination of one of Glas and Bahr's (16) figures, Fig 7, suggests that a mean particle volume occurred at a threshold of about 10, which, according to their

TABLE IX

A Comparison of Certain Mitochondrial Constituents Expressed on a Protein or Particle Basis for Heavy and Light Mitochondrial Fractions

Parameter	Specific content (per mg of protein)		Particle content (units per particle $\times 10^{-9}$)	
	Heavy mitochondria	Light mitochondria	Heavy mitochondria	Light mitochondria
Nos. of particles	3.95×10^9	5.14×10^9	—	—
DNA, μg	0 155	0 245	0 0392	0 0477
RNA, μg	9 0	13 8	2 28	2 68
Cytochrome ($a + a_3$), nmoles	0 49	0.66	0.124	0.128
Cytochrome *c* oxidase, k^{-1}	45	70	11.4	13.6
Total iron, nmoles	4.1	5.3	1 04	1.03

TABLE X

Comparison of the Size and Numbers of Rat Heart, Liver, and Kidney Mitochondria

The number of separate mitochondrial preparations is given in parentheses, with standard deviations where possible.

Tissue	Mean particle vol (μ^3) (Coulter counter)	Mean particle vol (μ^3) (Electron microscope)	Nos of particles per mg protein
Heart	0.60 ± 0.14 (3)	0 63*	$4.7 \pm 1\ 0 \times 10^9$
Liver	0.42 ± 0 11 (12)	0.38‡	$4.3 \pm 1.2 \times 10^9$
Kidney	0.23 (2)	0.20*	10.6×10^9

* Derived from reference 41.

‡ Calculated from reference 37.

TABLE XI

The Cytochrome Content of Heart, Kidney, and Liver Mitochondria Expressed Both on a Particle and Protein Basis

Cytochrome	Protein content (nmoles per mg protein) Heart	Kidney	Liver	Particle content (nmoles per particle $\times 10^{-10}$) Heart	Kidney	Liver
$a + a_3$	0.72	0.44	0.28	1.53	0.41	0.65
b	0.26	0 18	0 13	0.55	0.17	0 30
c_1	0.18	0.12	0.12	0 38	0.11	0.28
c	0 40	0.48	0 19	0.85	0.45	0 44

TABLE XII

Comparison of Mitochondrial Size and Numbers as Determined by the Coulter Counter, Light and Electron Microscopes

The values were calculated where possible from the literature, using appropriate mitochondrial yields, and dry weight to protein relationships

Technique	Reference	Vol (μ^3)	Nos. per mg protein ($\times 10^9$)	Protein per particle (mg $\times 10^{-10}$)	Nos per g of liver ($\times 10^{10}$)
Light microscopy	(40)	—	7.1	1.4	33
Electron microscopy	(37)	0 38	—	—	—
	(42)	—	9.4	1.1	—
Coulter counter	(15)	1 0	2	5	
	(16)	0 43	9 1	1 1	—
	Present study	0 42	4.3	2 2	11

calibration factor of 0.0974 μ^3 per threshold step, yields a mean particle volume of 0.974 μ^3. This is more than twice their published value of 0.43 μ^3 for mean mitochondrial volume. It was also reported that small mitochondria only began to be counted above a threshold of 6, or a volume of $6 \times 0.0974 = 0.57\ \mu^3$; thus, it is difficult to derive their stated results on volume from the counting procedures they describe.

The numbers of mitochondria per gram of liver (wet weight) were determined to be about 11×10^{10}, based on an average mitochondrial yield of 24.8 mg protein per g of liver. This is one-third of that recorded by a light microscopy study (39, 40), 33×10^{10}. The latter value represents the sum of mitochondria, as detected by the phase-contrast microscope, in all the various subcellular fractions. In the present study the yield of mitochondria in the mitochondrial fraction, based on recovery of total cytochrome *c*–oxidase activity, varied between 50 and 80%. Thus, the value of 11×10^{10} particles per g of liver could rise twofold if cor-

rected for the percentage of cytochrome *c*–oxidase recovered in the mitochondrial fraction.

Use of the Particle Counter To Follow Rapid, Mitochondrial Volume Changes

Mitochondria behave as osmometers capable of changing size in response to a variety of conditions (11, 12), such as energy-linked ion transport, exposure to certain antibiotics, or simple variations in osmotic strength. In order to test the feasibility of using the Coulter counter to follow the rate of these processes, mitochondria were allowed to swell as a result of energy-dependent phosphate transport (32). The Model EE timer was set for its shortest counting interval of 3.13 sec, and, taking into account the reset time, actual measurements could be made about once in each 10 sec. The procedure was as follows: the stock mitochondrial suspension in 0.25 M sucrose at 50 mg of protein per ml was initially diluted 1 to 1000 in 0.9% NaCl. This was earlier shown to cause very little volume change (Figs. 3, 4). The suspension of mitochondria in 0.9% saline was then further diluted 1 to 100 in the swelling medium (32) of: 0.125 M KCl; 0.02 M Tris-Cl, pH 7.4; and 0 01 M Na_2 HPO_4, pH 7.4; which had been filtered at least twice through 0 45 μ Millipore filters to reduce background contamination. The time interval between making the second dilution and obtaining the first particle count was usually between 20 and 30 sec

Results from such a procedure are illustrated in Fig. 5. A large increase in the numbers of large particles, 1.1–2.8 μ^3, may be contrasted with a decline in the numbers of very small particles, 0.28–0.34 μ^3. After a lag phase of about 1 min, the rate of volume change was nearly linear from 2 to 6 min, and by 8 min no further swelling was observed.

A second experiment with a different preparation of mitochondria was carried out to contrast the rate of swelling measured directly by the Coulter counter with that seen indirectly by light-scattering (12). The data expressed in Fig. 6 show that, although the two techniques agree qualitatively, the relative rates are quite different. Thus, about 76% of total swelling was complete within 1 min when measured by light scattering, in sharp contrast with only 17% with the Coulter counter. As seen with the previous experiment (Fig. 4), a nearly uniform increase in particle numbers from 1.1 to 2.8 μ^3 occurred up to 5 min after mixing in the swelling medium.

These results then demonstrate that the Coulter counter can easily be used to follow actual rates of mitochondrial swelling This is technically rather difficult to carry out using the manual procedures just described, and the mean particle volume as a function of time cannot be measured since it

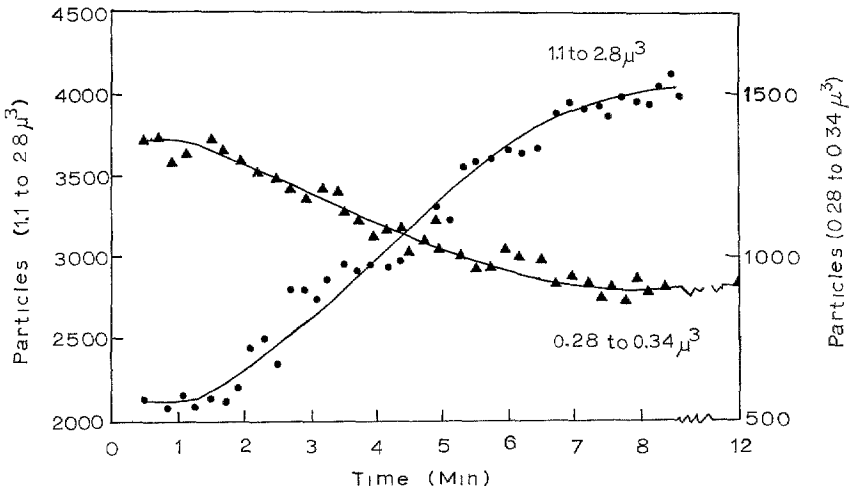

FIGURE 5 Rapid mitochondrial volume changes during energy-dependent, phosphate-induced swelling. Rapid counting (for 3.15 sec, about every 10 sec) was commenced as soon as possible after 1 to 100,000 dilution of mitochondria (50 mg per ml) in phosphate-containing medium: 10 mM Na phosphate, pH 7.4, 20 mM Tris-Cl, pH 7 4 and 0.125 M KCl. Two size ranges were followed in consecutive experiments; large particles 1.1–2.8 μ^3, then smaller ones 0.28–0.34 μ^3.

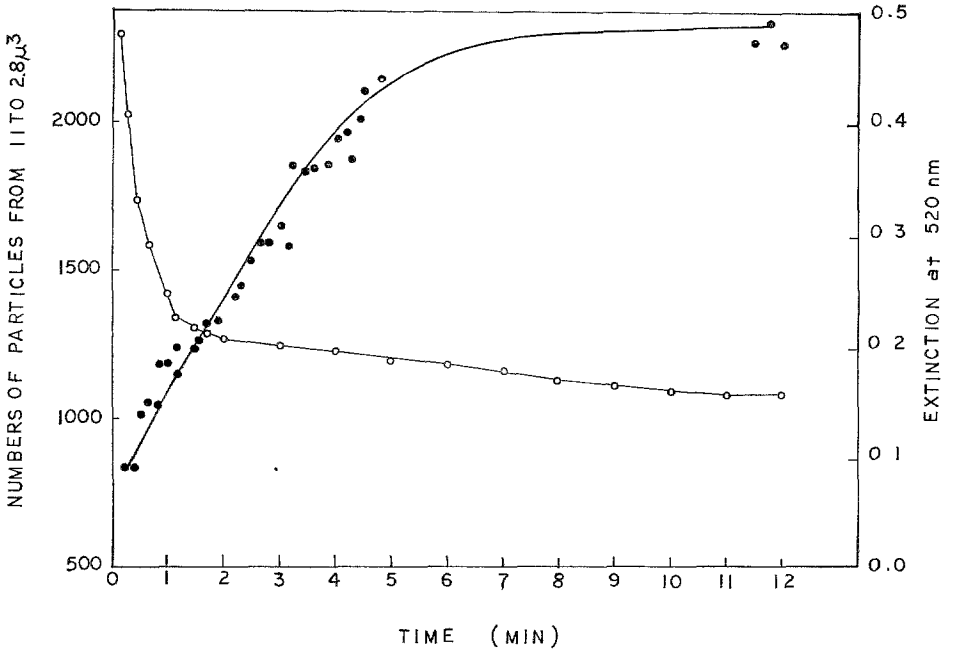

FIGURE 6 Comparison of resistive particle counting (●) and light scattering at 520 nm (○) as techniques to monitor mitochondrial volume changes. Conditions as in Fig. 5 for particle counting, and Fig. 4 for light-scattering changes.

usually requires 20 min for a complete size distribution analysis. Only the numbers of particles in a given size range can be monitored. These technical problems may be overcome by using a multichannel analyzer coupled to an on line, laboratory computer, such as the system of Particle Data Inc.

It is interesting to note that light scattering and resistive particle counting gave quite different rate profiles for mitochondrial swelling (Fig 6). The latter technique measures total particle volume, while severe doubt has been cast on exactly what the changes in light scattering really reflect (13). Thus, no direct relationship exists between extinction and mitochondrial water content A brief report on light-induced swelling of chloroplasts also demonstrated lack of correlation between light-scattering and Coulter counter data (17). The problem of what resistive particle counting measures in comparison with other techniques, such as light scattering, rapid-fixation electron microscopy, and packed volume analysis, will be considered further in the discussion.

DISCUSSION

The data collected in this paper demonstrate that a resistive particle counter, the Coulter Counter Model B, can be routinely used with a 30 μ aperture to count and size mitochondria down to a lower detection limit of 0.06 μ^3, or 0.48 μ in diameter. Careful procedures were developed for maximal apparatus performance. These included accurate threshold setting, calibration with standard latex particles, and serial dilution of mitochondrial suspensions in 0.9% NaCl. In addition the important question of particle resolution was considered. It was shown how varying the flow rate through the aperture could improve resolution, and that the apparatus used in the present study gave a symmetrical and narrow resolution with standard latex particles.

Although resistive particle counting is attractive for its speed and ability to sample enormous numbers of particles as compared with light (40) or electron microscopy (16, 42) of mitochondria, it has several potential drawbacks. These include detection limit, stability of particles in dilute electrolyte solution, contribution of contaminating particles to the observed size distribution, and the accuracy in absoute units of the parameters measured, which are particle volume and numbers. The results of the present research show that many of these drawbacks are of little significance in the counting and sizing of mitochondria. The

detection limit (0.06 μ^3) with a 30 μ aperture has been greatly improved over that of earlier research (15, 16). It is now suitable for studies on mitochondrial biogenesis where a newly divided mitochondrion (42, 43), or one enlarging up from some particle below the detection limit of the electron microscope (44), might logically be expected to be at least one-half the mean mitochondrial volume of 0 42 μ^3 (Table X).

A potentially serious question in extending the detection limit of resistive particle counters for studies on mitochondrial biogenesis is the possibility that the measured size distribution may be influenced by contaminating subcellular particles.

This problem was considered by Gebicki and Hunter (15) who cleaned up their crude mitochondrial suspensions through a simple sucrose gradient. The percentage of total counts for particles below 1 μ^3 decreased dramatically after their washing procedure in a sucrose gradient from 0.33 to 0.44 M. The mean particle size increased correspondingly to greater than 1 μ^3. However, as these authors failed to detect any particles below 0.4 μ^3 (0.91 μ diameter) by their experimental procedures, large numbers of mitochondria must have escaped detection. The general study carried out by Glas and Bahr (16) was more rigorous in this respect Several variants on the procedures used for differential centrifugation were carried out, and contamination was monitored by electron microscopy. Unfortunately, no enzymic markers for contamination were used, nor was their technique capable of resolving particles below about 0.3 μ^3. Consequently, it was difficult in these earlier studies to resolve the problem of contaminating particles in the over-all mitochondrial suspension.

The data in Tables V and VI showed, however, that this is probably not too serious, since mitochondria purified by density-gradient centrifugation possessed mean volumes and numbers of particles in the size range 0 06–0.12 μ^3, very similar to those found with the normal mitochondrial preparation. Even so, as with all work (33) on subcellular fractionations, only *fractions* are studied; generalities should be extended to include *all* mitochondria only with great caution. Thus, numerous, very large mitochondria will necessarily be lost in the nuclear fraction, while very small mitochondria will separate with the microsomal fraction during differential centrifugation This drawback is best tackled by several approaches. First, mitochondrial yield should be carefully followed by means of specific marker enzymes in the various subcellular fractions. Second, improved particle separations should be attempted by means of more careful differential centrifugation, or rate-zonal centrifugation which promises to be a good technique for giving clean mitochondrial fractions in high yield (38). Third, data on particle size and numbers obtained from fractions should, where possible, be confirmed by other techniques such as light and electron microscopy of whole tissue. These considerations become particularly significant when particles lying on the route of mitochondrial biogenesis may normally sediment outside the mitochondrial fraction.

A considerable advantage in determining mitochondrial numbers and size is that relationships may be revealed which would otherwise remain obscured. Thus, data were presented supporting the hypothesis that certain mitochondrial constituents may be constant on a particle basis (Table IX). Allard et al. (39, 40) originally suggested the mitochondrion as a unit of measurement, but, since then, essentially all information on mitochondrial constituents has been on a protein basis The reason for this is that light or electron microscopy is very time consuming and suffers from severe sampling artifacts. The hypothesis that some mitochondrial constituents (Table IX) are constant per particle—notably DNA, RNA, cytochrome *c*–oxidase, cytochrome ($a + a_3$), and iron—has an intriguing relevance to mitochondrial biogenesis. It is possible that a primary mitochondrion possesses a definite complement of certain components which remain unchanged within the particle as it enlarges to become a mature mitochondrion. In keeping with this idea is the well-known observation that a considerable number of mitochondrial proteins, cytochrome *c* being an excellent example (45, 46), are synthesized on the endoplasmic reticulum for later export and incorporation into an intact mitochondrion (47, 48). The net effect of this would be to dilute out preexisting components on a protein basis as particle size increases. This is precisely what was seen for some constituents; cytochrome *c* was constant per milligram of protein (36, 37) between heavy and light mitochondria, whereas cytochrome ($a + a_3$) was much more concentrated in the light, or small, mitochondria, on a protein basis.

It could be argued that membrane constituents would naturally have a high specific content on a protein basis for small particles, since volume (protein) varies as the radius^3, whereas area

(membrane) varies as the radius^2. However, this consideration is probably not valid since succinate-cytochrome *c*–reductase, and cytochrome *c* were present at the same levels in either heavy or light mitochondria (36, 37). Both these constituents are membrane linked and not found in the mitochondrial matrix (49). Consequently, it is reasonable to suggest that certain membrane constituents are diluted during membrane expansion as mitochondria enlarge, while others such as cytochrome *c*, or succinate-*c*–reductase are continuously being synthesized. The concept of a unit membrane of constant composition is therefore difficult to maintain. It is more likely that membrane constituents, in this case specific proteins, can be inserted into a previously existing membrane (50, 51, 52).

The results discussed in this paper have demonstrated that valuable information and new insights concerning mitochondrial biogenesis may be revealed by the direct sizing and counting of mitochondria in solution. The techniques developed require a relatively inexpensive and commonly available apparatus, the Coulter Counter Model B, with minor modifications. However, it is extremely desirable to extend the detection limits of the present system and especially the speed with which a complete size distribution may be obtained. To this end it is proposed to extend the system with a 128 multichannel analyzer coupled directly to a small laboratory computer with a high-speed print-out. The system developed by Particle Data Inc. seems quite suitable, though any combination of appropriate apparatus would do. High-speed sizing would enable accurate studies to be made on the rate of mitochondrial swelling and would not suffer from the uncertainties of following volume changes by spectrophotometry (12, 13). The results in Figs. 5 and 6 clearly illustrate the ease with which rate measurements may be made.

As described earlier, a report by Packer, Deamer, and Crofts (17) noted a lack of correlation between light-scattering changes and Coulter counter measurements for light-induced swelling of chloroplasts. This agrees with the differences (Fig. 6) found in the present study for the phosphate-induced swelling of liver mitochondria. The important early study by Bartley and Enser (13), which compared light-scattering changes with mitochondrial water content, also failed to reveal any clear relationship between these two parameters. Several useful investigations on changes in mitochondrial structure and volume induced by varying the metabolic state (53, 54) or tonicity (55, 56) have been reported. These have employed rapid fixation for electron microscopy and packed volume measurements for comparison with light scattering, fluorescence, ion movements, and oxygen consumption. In none of these studies, because of experimental limitations, was it possible to relate directly the actual *rate* of structural changes, as measured by electron microscopy and packed volume, with the other parameters which could be continuously monitored on recorders. However, some of the major observations are relevant to the present investigations

Hackenbrock (53) clearly demonstrated that gross internal changes in mitochondrial morphology as determined by electron microscopy could be induced by a state 2, or state 4, to state 3 transition; or, in his terminology, an "orthodox" to "condensed" transition. These dramatic internal changes were not paralleled by any consistent ones in light scattering. A recent report by Hackenbrock, Rehn, Weinbach, and LeMasters (54), using isolated whole cells, confirms that internal structural changes may occur under conditions approaching those in vivo. These authors also attempted to correlate rate of mitochondrial ultrastructural changes with that of 90° light scattering of whole cells. They were able to show a parallelism between the rate of whole cell, optical-density changes and the maximal appearance of the condensed mitochondrial configurations. The $T\ ½$ for these changes was 25 sec, in sharp contrast with a $T\ ½$ of less than 6 sec for individual mitochondria within one cell. However, in view of Hackenbrock's earlier results (53) on isolated mitochondrial suspensions where light-scattering could be either increased or decreased, depending on how the ultrastructural change was initiated, it is perhaps premature to correlate light-scattering changes of whole cells with mitochondrial ultrastructure.

Finally, some studies relating mitochondrial packed volume with light scattering (55, 56) failed to demonstrate a consistent relationship. Stoner and Sirak (55) clearly showed that variation in medium tonicity elicited differing responses. On the other hand, although Packer, Wrigglesworth, Fortes, and Pressman (56) did report a direct relationship between packed volume and light scattering, they did not extend their conditions over as wide a range as Stoner and Sirak (55). A useful result of the work of Packer et al. (56) was the correlation between mitochondrial packed volume and that of the inner membrane compartment, or

sucrose-inaccessible space, as measured by rapid glutaraldehyde fixation. Consequently, it would be very useful to be able to relate volume changes, as measured by resistive particle counting, with changes in total mitochondrial volume, as distinct from those of the inner-membrane compartment. The resistive particle counter may well not be influenced by inner membrane plus matrix changes independent of the outer membrane, since it probably only detects total particle volume (14, 19). However, this cannot be certain and is presently the subject of further experimentation which will involve the computerised particle-analysis system mentioned earlier.

Sensing zone methods (57), therefore, have great promise for analyzing fine particles. One of them (58), using nucleopore membrane filters, offers significantly improved detection limits, but may be completely impracticable for counting mitochondrial suspensions because of aperture clogging. It is most likely that future developments will come more from improved electronics and data handling rather than from any major change in basic technique.

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REFERENCES

1. SCHATZ, G. 1970. *In* Membranes of Mitochondria and Chloroplasts. E. Racker, editor. Van Nostrand Reinhold Company, New York. 251.
2. ASHWELL, M., and T. S. WORK. 1970. The biogenesis of mitochondria. *Annu. Rev. Biochem* **39**:251.
3. HOCH, F. 1971. Energy Transformations in Mammals: Regulatory Mechanisms. E. J. Masoro, editor. W. B. Saunders Company, Philadelphia 83.
4. TATA, J. R 1966. The regulation of mitochondrial structure and function by thyroid hormones under physiological conditions. *In* Regulation of Metabolic Processes in Mitochondria. J. M. Tager, S. Papa, E. Quagliariello, and E. C. Slater, editors. American Elsevier Publishing Co , Inc , New York. 489.
5. KRITCHEVSKY, D., S. A. TEPPER, P. SALLATA, J. R. KABAKJIAN, and V. J. CRISTOFALO. 1969. Effect of the ethyl ester and sodium salt of α-*p*-chlorophenoxyisobutyric. acid on cholesterol oxidation by rat liver mitochondria. *Proc. Soc. Exp. Biol. Med.* **132**:76.
6. BURCH, R. W., and G. L. CURRAN. 1969. Hepatic aceto-actyl-CO-A deacylase in rats fed ethyl chlorphenoxyisobutyrate (CPIB). *J. Lipid Res.* **10**:668
7. KURUP, C K. R , H. N. AITHAL, and T RAMASARMA. 1970. Increase in hepatic mitochondria on administration of ethyl α-*p*-chlorophenoxyisobutyrate to the rat. *Biochem. J.* **116**:773.
8. JAKOVCIC, S , J. HADDOCK, G S. GETZ, and M RABINOWITZ. 1971. Mitochondrial development in liver of foetal and newborn rats *Biochem. J.* **121**:341.
9. POLLAK, J K , and E. A. MUNN. 1970. The isolation by isopycnic density-gradient centrifugation of two mitochondrial populations from livers of embryonic and fed and starved adult rats. *Biochem J.* **117**:913.
10. POLLAK, J. K., and M. WOOG. 1971. Changes in the proportions of two mitochondrial populations during the development of embryonic chick liver. *Biochem. J.* **123**:347.
11. PACKER, L. 1967. Energy-linked low amplitude mitochondrial swelling. *Methods Enzymol.* **10**: 685.
12. HUNTER, F. E., and E. E. SMITH. 1967 Measurement of mitochondrial swelling and shrinking —high amplitude. *Methods Enzymol.* **10**:689.
13. BARTLEY, W., and M. B. ENSER. 1964. The swelling and contraction of isolated rat-liver mitochondria. *Biochem. J.* **93**:322.
14. KUBITSCHEK, H. E. 1969. Counting and sizing micro-organisms with the Coulter counter *In* Methods in Microbiology. J. R. Norris and D. W. Ribbons, editors. Academic Press Inc , New York. 593.
15. GEBICKI, J. M., and F. E. HUNTER. 1964. Determination of swelling and disintegration of mitochondria with an electronic particle counter. *J. Biol. Chem.* **239**:631.

16. GLAS, U., and G. F. BAHR. 1966. Quantitative study of mitochondria in rat liver, dry mass, wet mass, volume and concentration of solids. *J. Cell Biol.* **29**:507.
17. PACKER, L., D. W. DEAMER, and A. R. CROFTS. 1966. Conformational changes in chloroplasts. *Brookhaven Symp. Biol.* **19**:281.
18. GEAR, A. R. L. 1971. Changes in particle volume and numbers during mitochondrial biogenesis in regenerating liver. *Fed. Proc.* **30**:1225.
19. HARVEY, R. J. 1968. Measurement of cell volumes by electric sensing zone instruments. *In* Methods in Cell Physiology. P. M. Prescott, editor. Academic Press Inc., New York. **3**:1.
20. GEAR, A. R. L. 1965. Some features of mitochondria and fluffy layer in regenerating rat liver. *Biochem. J.* **95**:118.
21. JOHNSON, D., and H. LARDY. 1967. Isolation of liver or kidney mitochondria. *Methods Enzymol.* **10**:94.
22. SMITH, A. L. 1967. Preparation, properties, and conditions for assay of mitochondria: Slaughterhouse material, small-scale. *Methods Enzymol.* **10**:81.
23. GEAR, A. R. L., and A. L. LEHNINGER 1968. Rapid respiration–independent binding of alkali metal cations by rat liver mitochondria. *J. Biol. Chem.* **243**:3953.
24. MINNAERT, K. 1961. The kinetics of cytochrome c–cytochrome oxidase–oxygen. *Biochim. Biophys. Acta.* **50**:23.
25. LÜCK, H. 1965. Catalase. *In* Methods of Enzymatic Analysis H. U. Bergmeyer, editor. Academic Press Inc., New York. 885.
26. BEERS, R. F., and I. W. SIZER. 1952. Spectrophotometric method for measuring the breakdown of hydrogen peroxide by catalase. *J. Biol. Chem.* **195**:133.
27. LINHARDT, K., and K. WALTER. 1965. Determination in serum with *p*-nitrophenylphosphate. *In* Methods of Enzymatic Analysis. H. U. Bergmeyer, editor. Academic Press Inc., New York. 783.
28. HARPER, A. E. 1965. Glucose-6-phosphatase. *In* Methods of Enzymatic Analysis. H. U. Bergmeyer, editor. Academic Press Inc., New York. 788
29. WILLIAMS, J. N. 1964. A method for the simultaneous quantitative estimation of cytochromes, a, b, c_1, and c in mitochondria. *Arch. Biochem. Biophys.* **107**:537.
30. VANNESTE, W. H. 1966. Molecular proportion of the fixed cytochrome components of the respiratory chain of Keilin-Hartree particles and beef heart mitochondria. *Biochim. Biophys. Acta.* **113**:175.
31. MURPHY, J. B., and M. W. KIES. 1960. Note on spectrophotometric determination of proteins in dilute solutions. *Biochim. Biophys. Acta.* **45**: 382.
32. NEUBERT, D., G. V. FOSTER, and A. L. LEHNINGER. 1962. Effect of temperature on uptake and extrusion of water by isolated rat-liver mitochondria. *Biochim. Biophys. Acta* **60**: 498.
33. DE DUVE, C, and J. BERTHET. 1959. The use of differential centrifugation in the study of tissue enzymes. *Int. Rev. Cytol.* **3**:228.
34. DE DUVE, C., B. C. PRESSMAN, R. GIANETTO, R. WATTIAUX, and F. APPELMANS. 1955. Tissue fractionation studies. 6. Intracellular distribution patterns of enzymes in rat-liver tissue. *Biochem. J.* **60**:604.
35. DE DUVE, C., R. WATTIAUX, and P. BAUDHUIN. 1962. Distribution of enzymes between subcellular fractions in animal tissues. *Adv. Enzymol.* **24**:291.
36. GEAR, A. R. L. 1965. Observations on iron uptake, iron metabolism, cytochrome c content, cytochrome a content and cytochrome c–oxidase activity in regenerating rat liver. *Biochem. J.* **97**:538.
37. GEAR, A. R. L. 1965. A comparative study of the structural, enzymic and chemical make-up of normal and regenerating rat liver. D Phil. Thesis. Bodlean Library, Oxford.
38. SWICK, R. W., J. L. STANGE, S. L. NACE, and J F. THOMPSON. 1967. The heterogeneous distribution of mitochondrial enzymes in normal rat liver. *Biochemistry.* **6**:737.
39. ALLARD, C., R. MATHIEU, G. DE LAMIRANDE, and A. CANTERO. 1952. Mitochondrial population in mammalian cells, L. description of a counting technique and preliminary results on rat liver in different physiological and pathological conditions. *Cancer Res.* **12**:407.
40. ALLARD, C., G. DE LAMIRANDE, and A. CANTERO. 1952. Mitochondrial population of the average rat liver cell during regeneration. Use of the mitochondrion as a unit of measurement. *Cancer Res.* **12**:580.
41. PORTER, K. R., and M. A. BONNEVILLE. 1968. *In* Fine Structure of Cells and Tissues. Lea and Febiger, Philadelphia. 101, 153.
42. BAHR, G. F., and E. ZEITLER. 1962. Study of mitochondria in rat liver, quantitative electron microscopy. *J. Cell Biol.* **15**:489.
43. LUCK, D. J. L. 1963. DNA in Mitochondria of *Neurospora crassa. Proc. Natl. Acad. Sci. U. S. A.* **52**:931.
44. GREENWALT, J. W., and D. O. HALL. 1964. Structural studies on the biogenesis of mitochondria in *Neurospora crassa. J. Cell Biol.* **23**: 38 A. (Abstr.)
45. GONZALEZ-CADAVID, N. F., and P. W. CAMPBELL. Subcellular distribution of cytochrome c in rat

liver. Methods for its extraction and purification. *Biochem. J.* **105**:427.

46. GONZALEZ-CADAVID, N. F., and P. W. CAMPBELL. 1967. The biosynthesis of cytochrome c. Sequence of incorporation in vivo of (^{14}C) lysine into cytochrome c and total proteins of rat-liver subcellular fractions. *Biochem. J.* **105**:443.
47. KADENBACH, B. 1966. Synthesis of mitochondrial proteins. Demonstration of a transfer of proteins from microsomes into mitochondria. *Biochim. Biophys. Acta.* **134**:430.
48. KADENBACH, B. 1968. *In* Biochemical Aspects of the Biogenesis of Mitochondria. E. C. Slater, J. M. Tager, S. Papa, and E. Quagliariello, editors. Adriatica Editrice, Bari. 415.
49. LARDY, H. A., and S M. FERGUSON. 1969. Oxidative phosphorylation in mitochondria. *Annu Rev. Biochem.* **38**:1004.
50. DALLNER, G , P. SIEKEVITZ, and G. E. PALADE. 1966 Biogenesis of endoplasmic reticulum membranes. I. Structural and chemical differentiation in developing rat hepatocyte. *J. Cell Biol.* **30**:73.
51. DALLNER, G., P SIEKEVITZ, and G. E. PALADE. 1966. Biogenesis of endoplasmic reticulum membranes. II. Synthesis of constitutive microsomal enzymes in developing rat hepatocyte. *J . Cell Biol.* **30**:97
52. GEAR, A. R. L. 1970. Inner and outer membrane enzymes of mitochondria during liver regeneration *Biochem J.* **120**:577.
53. HACKENBROCK, C. R 1966. Ultrastructural bases for metabolically linked mechanical activity in mitochondria. I. Reversible ultrastructural changes with change in metabolic steady state in isolated liver mitochondria. *J Cell Biol.* **30**:269.
54. HACKENBROCK, C. R., T. G. REHN, E C WEINBACH, and J. J. LEMASTERS. 1971. Oxidative phosphorylation and ultrastructural transformation in mitochondria in the intact ascites tumor cell. *J. Cell Biol* **51**:123.
55. STONER, C. D., and H. D. SIRAK. 1969. Osmotically-induced alterations in volume and ultrastructure of mitochondria isolated from rat liver and bovine heart. *J Cell Biol.* **43**:521.
56. PACKER, L , J. M. WRIGGLESWORTH, P. A. G. FORTES, and B. C. PRESSMAN. 1968. Expansion of the inner membrane compartment and its relation to mitochondrial volume and ion transport. *J. Cell Biol.* **39**:382.
57. BERG, R. H 1965. Sensing zone methods in fine particle analysis *Mater. Res Stand* **5**:119.
58 DEBLOIS, R. W., and C. P. BEAN. 1970. Counting and sizing of submicron particles by the resistive pulse technique. *Rev Sci Instrum.* **41**:909.