STUDIES ON ISOLATED CELL COMPONENTS

XVII. The Distribution of Cytochrome Oxidase Activity in Rat Liver Brei Fractionated in the Zonal Ultracentrifuge

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ABSTRACT

The zonal ultracentrifuge was used to separate the subcellular components of rat liver brei into soluble phase, microsomal, mitochondrial, membranous fragments, and nuclear fractions during a single centrifugation. The centrifuge was run at 10,000 to 30,000 RPM for 15 to 240 minutes, and the rotor contained a 1200 ml sucrose gradient, varying linearly with radius from 17 to 55 per cent sucrose with a "cushion" of 66 per cent sucrose at the rotor edge. The distribution of the mitochondria was determined using cytochrome oxidase as the marker enzyme. An automated assay system for cytochrome oxidase was developed utilizing reduced cytochrome c as substrate, modules of the Technicon Autoanalyzer, and the Beckman DB Spectrophotometer. All of the cytochrome oxidase activity was restricted to a single peak in the gradient, and no activity could be detected in the zones occupied by the microsomes and nuclei. The mitochondrial fraction was isolated from rat liver brei in 0.25 M sucrose by differential centrifugation, and then run in the zonal ultracentrifuge. This fraction behaved in the zonal ultracentrifuge in the same way as mitochondria separated directly from intact brei. Observations of the isolated fractions in the phase contrast microscope indicated that a wide variety of granules was present in the mitochondrial zone in addition to the true mitochondria. Under the conditions employed, the mitochondria were sedimented essentially to their isopycnic position in the gradient at approximately 43.8 per cent sucrose, density 1.20 gm/cc.

INTRODUCTION

Centrifugal fractionation of homogenates prepared from cells and tissues is one of the most widely used tools in contemporary experimental biology. Most of these procedures are based on the differential centrifugation scheme originally proposed by Claude (9), according to which subcellular components are separated into so called soluble-phase, microsomal, mitochondrial, and nuclear fractions. These separations depend upon the simultaneous sedimentation, though at differing rates, of all species of particles present. With complex mixtures such as cellular homogenates,

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repeated centrifugations are required to obtain acceptably purified fractions (4, 12).

The technique of zonal centrifugation in density gradients has greatly improved the obtainable resolution, since all the particles are layered near the top of the tube at the outset and each species of particles is allowed to move a distance from the starting level or zone, proportional to its sedimentation rate and density (3, 8, 11, 13, 29). This approach, utilizing swinging-bucket rotors such as the SW-39, has been hampered by the small volume of the tubes, difficulties in loading the tube with the gradient, difficulties in maintaining gradient stability during acceleration and deceleration, and difficulties in the recovery of the gradient with its separated zones of particles after centrifugation (13). These difficulties have been largely overcome by the development of the zonal ultracentrifuge (5, 6). This device is capable of swinging a 1.6 liter gradient at speeds up to 30,000 RPM with a force in excess of 51,000 g at the rotor edge, and has achieved high resolution separation of subcellular components from gram quantities of tissue during a single centrifugation (6, 23).

As a prelude to the exploitation of this system in studies on cellular physiology, it is essential to compare the composition of the subcellular fractions obtained with the zonal ultracentrifuge with the composition of those obtained by the classical procedures of differential centrifugation. The present paper deals with studies on rat liver mitochondria, and the development of an automated assay system for the marker enzyme of the mitochondria, cytochrome oxidase. The results of this study have been reported briefly elsewhere (30).

MATERIALS AND METHODS

Preparation of Homogenates

Adult male Sprague-Dawley rats were used in this study. The animals were allowed to eat and drink freely until they were killed. They were decapitated, and the livers were perfused with cold Locke's solution prior to homogenization in a manually operated Potter and Elvehjem grinder with a Lucite pestle in cold 8.5 per cent sucrose (2). Large fragments of connective tissue were removed by filtration through several layers of cheesecloth.

Fractionation in the Zonal Ultracentrifuge

The liver brei was fractionated in zonal ultracentrifuge rotor B-II at speeds of 10,000 to 30,000 RPM for 15 to 240 minutes (6). A 1200 ml sucrose gradient, varying linearly with radius from 17 to 55 per cent (w/w) with a "cushion" of 66 per cent sucrose at the rotor edge, was employed. The tissue sample fractionated in the zonal ultracentrifuge consisted of 12.5 ml of a 25 per cent (w/v) homogenate which contained approximately 3 to 4 gm of liver. All operations, including introduction of the sample layer in a short gradient (8.5 to 17 per cent sucrose, w/w) and the recovery of the gradient with its concentric zones of separated particles, were accomplished while the rotor was rotating at 5,000 RPM. The gradient was collected in 40-ml fractions for subsequent analysis.

Visualization of Material in the Gradient

The presence of subcellular components was determined by continuously analyzing the gradient for ultraviolet absorbance as it emerged from the rotor. The recordings were made directly at 280 mμ (without dilution) on the gradient as it passed through a quartz flow cell with a 0.2 cm light path. The data presented in this paper are presented in terms of the computed absorbance in a 1.0 cm light path.

Microscopic observations were made on the isolated fractions with a phase contrast microscope using a dark medium objective.

Analysis of Sucrose in the Gradient

The concentration of sucrose within the isolated fractions was analyzed refractometrically. These data were then used to calculate the densities of the sucrose solutions at 0°C utilizing a computer program based on an empirical equation describing density as a function of sucrose concentration. This procedure will be described in detail in a subsequent publication. The fractionations in the zonal ultracentrifuge were performed at 5°C. For practical purposes, the densities of sucrose solutions at these two temperatures can be considered to be identical, since the correction factor appears in the fifth decimal place.

Automated Analysis of Cytochrome Oxidase

Either cytochrome oxidase or succinic acid dehydrogenase should be a reliable chemical marker for the mitochondrial fraction of liver tissue, since it is generally considered that these enzymes are localized in the mitochondria (15, 16, 19). The cytochrome oxidase assay was chosen for automation since it appeared to be sensitive enough and to utilize few components, involving basically only tissue sample, diluent, and substrate (10, 25). The method uses reduced cytochrome c as the substrate, and measures the decrease in intensity of its character-
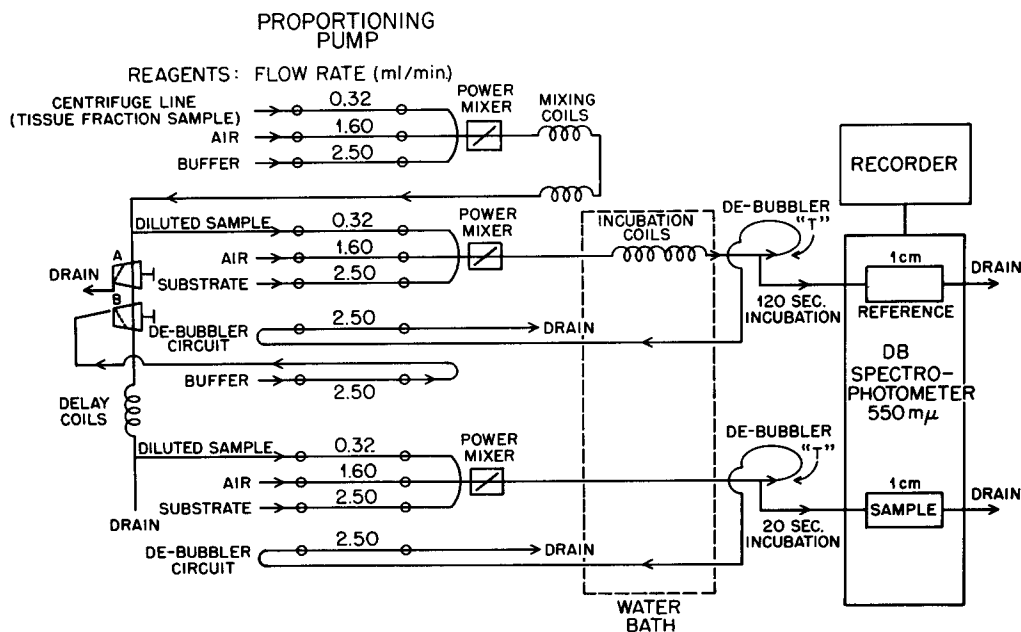
CYTOCHROME OXIDASE

PROPORTIONING
PUMP

REAGENTS: FLOW RATE (ml/min.)

CENTRIFUGE LINE
(TISSUE FRACTION SAMPLE) → 0.32
AIR → 1.60
BUFFER → 2.50

POWER MIXER   MIXING COILS

RECORDER

DILUTED SAMPLE → 0.32
AIR → 1.60
SUBSTRATE → 2.50

POWER MIXER   INCUBATION COILS   DE-BUBBLER "T"

DRAIN

DE-BUBBLER CIRCUIT → 2.50 → DRAIN

BUFFER → 2.50

DELAY COILS

1cm   REFERENCE   DRAIN
120 SEC. INCUBATION

DB SPECTRO-PHOTOMETER 550 mμ

DILUTED SAMPLE → 0.32
AIR → 1.60
SUBSTRATE → 2.50

POWER MIXER   DE-BUBBLER "T"

DRAIN

DE-BUBBLER CIRCUIT → 2.50 → DRAIN

1cm   SAMPLE   DRAIN
20 SEC. INCUBATION

WATER BATH

FIGURE 1   Schematic diagram of the automated assay system for cytochrome oxidase utilizing modules of the Technicon Autoanalyzer and the Beckman DB Spectrophotometer. Flow rates based on specification provided by Technicon Instruments Corp. for the manifold tubing on the proportioning pump of the Autoanalyzer.

istic absorption band at 550 mμ as the cytochrome is oxidized by the enzyme.

The analytical system employed modules from the Technicon Autoanalyzer (24) (proportioning pumps, transmission lines, incubating and mixing coils), small solenoid cells and magnetic fleas within the lines as power mixers, and a recording Beckman DB Spectrophotometer. The system as shown schematically in Fig. 1 was so constructed that aliquots of the same tissue sample passed through both flow cuvettes at the same time (21), but following incubation with the substrate for different periods. The difference between the two streams was recorded in terms of optical density, and represented the slope of the reaction velocity curve for the incubation period. Since each flow cell had the same concentration of tissue sample and of cytochrome c at any moment, the system also provided a continuous bank for turbidity of the various brei fractions and for the slight auto-oxidation of the cytochrome which was observed in some preparations of the substrate.

In actual operation, aliquots of tissue fractions were constantly removed from the gradient stream emerging from the rotor, diluted with phosphate buffer in the presence of a power mixer, segmented by air bubbles, and pumped through a series of

mixing coils. Aliquots taken from the diluted sample stream were injected into the substrate streams on both the reference and sample sides of the system in the presence of power mixers. Air bubbles were added to segment the resulting stream. The aliquot on the reference side was incubated with the substrate for 120 seconds in the incubation coils before reaching the flow cuvette, while an aliquot of the same tissue fraction on the sample side was incubated with the substrate for only 20 seconds in a strip of transmission tubing before reaching the flow cuvette. The simultaneous arrival of both aliquots of the same tissue fraction at the flow cuvettes was achieved by placing a delay coil and extra transmission tubing in the line between the tap-off points for the reference and sample sides of the system, because in flow systems time en route is proportional to the length of tubing. The bubbles used to segment the streams were removed by a de-bubbler T and de-bubbler circuit just before the fluid entered the flow cuvettes. The decrease of the characteristic absorption band of reduced cytochrome c as a function of oxidase activity was thus represented as a positive deflection of the recorder.

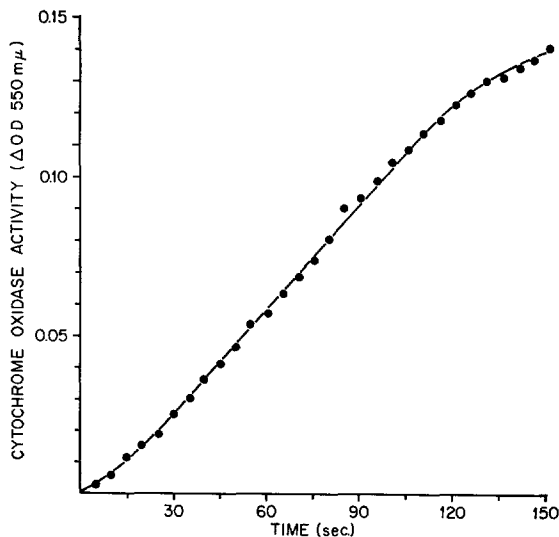The total amount of enzyme activity (the total area under the recording) may be refered to the

FIGURE 2 Oxidation of 3 ml of reduced cytochrome c(conc 0.25 × 10⁻⁴ M) in a cuvette on the reference side of the Beckman DB Spectrophotometer following the addition of 0.04 ml of a mitochondria preparation. The blank consisted of reduced cytochrome c on the sample side of the spectrophotometer, and the loss of the substrate's characteristic absorption band was monitored as a positive deflection on the recorder.

amount of nitrogen or protein in the sample. The introduction of known dilutions of the initial tissue brei in place of the tissue samples obtained from the rotor after centrifugal fractionation allowed a determination of total recovery. Dilutions in the system were calculated on the basis of tube specifications furnished by Technicon Instruments Corp., Chauncey, New York. The actual dilution of the tissue sample in the system was determined by using the absorbance of a known amount of nucleotide at 280 mμ in place of the tissue sample.

The absorbance of completely oxidized cytochrome c could be recorded in the system by introducing potassium ferricyanide into the reference line only. For this operation, stop-cock A was turned so that the tissue sample line led directly to drain. The ferricyanide which entered the system through the tissue sample line was thus allowed to pass through the reference side only, and total oxidation of the substrate registered as a positive deflection on the recorder. Stop-cock B was turned to allow buffer to be pumped into the sample side of the system to maintain the same dilution of the substrate.

Fig. 2 is a recording of the decrease in optical density of 3 ml of reduced cytochrome c after addition of 0.04 ml of mitochondrial suspension. The velocity of the reaction appeared to be constant during the incubation period of 20 to 120 seconds used in our automated analysis. Cytochrome oxidase activity measured with the automated assay system was directly proportional to the concentration of mitochondria (Fig. 3). The mitochondrial preparations used in these tests were obtained by means of zonal ultracentrifuge fractionation of rat liver brei, and the dilutions were made with 43 per cent (w/w) sucrose.

The substrate was prepared from cytochrome c

(Sigma Chemical Co., St. Louis, Missouri) which was reduced by adding sodium dithionite. The rotor has a volume of 1,625 ml, and for the automated analysis of this volume about 300 to 400 ml of 0.25 × 10⁻⁴ M reduced cytochrome c was used. An aliquot volume of 100 ml was taken and sufficient crystalline sodium dithionite was added to give the desired ratio of OD_{550}/OD_{565} of 7–8 (25). The appropriate amount necessary to bring the total volume to this ratio was then added, the whole volume aerated for 10 minutes, and cooled until used. The cytochrome c reduced in such a manner had no excess dithionite and gave us better results than the cytochrome reduced with palladium and hydrogen. The dilution of the tissue in the system was such that the zonal ultracentrifuge fraction with the highest activity would produce a complete oxidation of the reduced cytochrome c in 5 minutes at 25°C. The complete oxidation of the cytochrome was recorded in the experiment by replacing the tissue sample on the reference side with 0.1 M potassium ferricyanide.

In the analysis, 0.03 M phosphate buffer, pH 7.4, was the diluent, the tissue sample dilution was 1:50, the flow rate through each flow cell was 4.43 ml/minute, and the final cytochrome c concentration was 0.22 × 10⁻⁴ M.

The automated analysis was disturbed by poor mixing of the highly concentrated sucrose of the density gradient with the buffer and substrate solutions in the transmission lines, resulting in a smearing of activity and some fluctuation in the recording base line. Power mixers composed of a solenoid and a magnetic flea within the lines were added at the junction points of tissue sample with buffer diluent and substrate streams as indicated in Fig. 1 and gave much better mixing, but some smearing on the
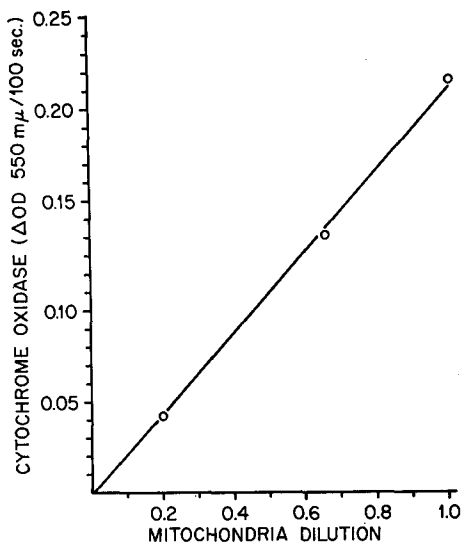
FIGURE 3 The relationship between the concentration of rat liver mitochondria and cytochrome oxidase activity measured with the automated assay system. The mitochondrial preparation was obtained by zonal ultracentrifuge fractionation of rat liver brei, and the dilutions were made with 43 per cent (w/w) sucrose.

nuclear side of the mitochondria peak still persisted.

Assays were made *in stream* on the gradient as it was recovered from the rotor. A small fraction of the gradient (0.32 ml/minute) was diverted into the analyzing system by means of the proportioning pump. The flow rate of the gradient emerging from the rotor could be computed by reference to the time scale of the recorder. The actual distance of material from the axis of the rotor could then be calculated from the total volume of fluid pumped out of the rotor.

EXPERIMENTAL RESULTS

Rat liver homogenates were fractionated in the zonal ultracentrifuge over a wide range of centrifugal fields, 10,000 to 30,000 RPM for 15 to 240 minutes. Under these conditions, separations of subcellular components are achieved by sedimentation to the isopycnic position for the larger particles, and by sedimentation rate for the smaller particles.

The results of an experiment in which rat liver brei was subjected to 10,000 RPM for 60 minutes are shown in Fig. 4. This treatment separated the components of the original brei into soluble-
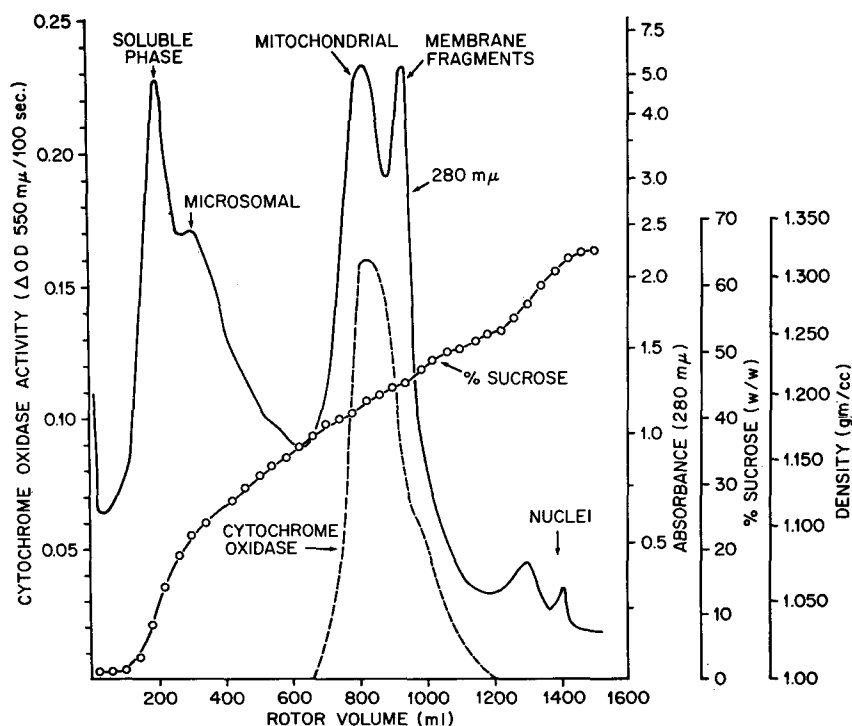

FIGURE 4 Distribution of cytochrome oxidase activity in rat liver brei subjected to 10,000 RPM for 60 minutes in the zonal ultracentrifuge.

phase, microsomal, mitochondrial, membranous fragments, and nuclear fractions. At this relatively low speed the microsomes were just barely separated from the soluble-phase and ribosomal material in the "sample zone" of the gradient, while the nuclei were thrown out into the cushion of 66 per cent sucrose near the edge of the rotor. In most zonal centrifuge fractionations of rat liver brei the nuclei were sedimented out to form a single peak in the "cushion" of 66 per cent sucrose. However, occasionally, as was the case in this run, the nuclei were thrown out to form multiple peaks in the sucrose cushion. The mitochondria appeared to be distributed rather sharply about a peak at 42.7 per cent sucrose, density

appearing granules. The mitochondrial zone contained large numbers of rod-like mitochondria, a few large round mitochondria, and large numbers of small granules. The membrane fragments zone contained large pieces of membranous material, a few mitochondria, and large numbers of smaller granules. The nuclear fraction in the 66 per cent sucrose cushion appeared to contain a population of nuclei, apparently free of any significant contamination by other cellular components. These observations have been confirmed by preliminary studies with the electron microscope which will be published in detail elsewhere.

To compare the results obtained in the present study with those obtained in classical fractionation
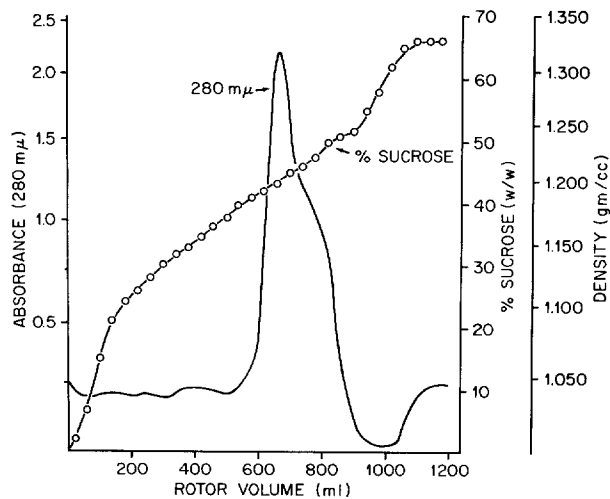

FIGURE 5 Distribution of mitochondrial fraction, originally obtained by differential centrifugation, in the sucrose gradient of the zonal ultracentrifuge after being subjected to 20,700 RPM for 60 minutes.

1.192 gm/cc. All of the cytochrome c oxidase activity was restricted to this region of the gradient, and no activity could be detected in the microsomal and nuclear fractions. In several experiments cytochrome oxidase activity was assayed manually according to the procedure of Cooperstein and Lazarow (10). The distribution pattern of oxidase activity in the gradient measured in this way was the same as with the automated assay.

The isolated fractions were examined morphologically in the phase contrast microscope. Photomicrographs are not included in this report, because it was not possible to obtain clear pictures of mitochondria at high magnification with the equipment available, due to the Brownian movement of the smaller particles. The region in the gradient just in front of the mitochondrial fraction was occupied by large numbers of small, dense-

procedures, mitochondria were first purified from rat liver brei in 0.25 M sucrose according to the differential centrifugation scheme of Schneider and Hogeboom (22), and this preparation was then subjected to 20,700 RPM for 60 minutes in the zonal ultracentrifuge (Fig. 5). The mitochondria were sedimented out to form a sharp peak around 43.6 per cent sucrose, density 1.202 gm/cc. The shoulder on the mitochondrial peak represents the membranous fragments fraction.

As a result of fractionation in the zonal ultracentrifuge the larger cytoplasmic granules such as the mitochondria are sedimented out essentially to their isopycnic or density-equilibrium position in the sucrose gradient, at approximately 43.6 to 43.8 per cent sucrose, density 1.20 gm/cc. This point is illustrated in Fig. 6 which shows the position of the mitochondrial peak in the sucrose

gradient as a function of the centrifugal force applied to the particles. The centrifugal fields represented covered a range of 10,000 to 30,000 RPM for 15 to 240 minutes. The total force applied in a centrifugal field is proportional to the square of angular velocity multiplied by the time of centrifugation, and the data are therefore expressed in terms of $\omega^2 t$. The centrifugation during acceleration and deceleration is included and is expressed in terms of equivalent sedimentation time at the plateau speed (4).
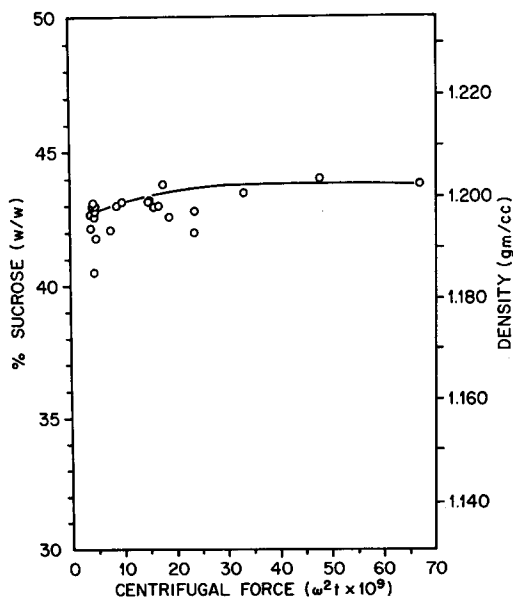

FIGURE 6 Approach to density equilibrium position in the sucrose gradient of mitochondria from rat liver brei subjected to 10,000 to 30,000 RPM for 15 to 240 minutes in the zonal ultracentrifuge. Centrifugal force expressed in terms of angular velocity squared multiplied by the time of centrifugation $-\omega^2 t$.

DISCUSSION

The experiments just described illustrate the kind of work in tissue fractionation that can be accomplished using the zonal ultracentrifuge. Subcellular components can now be visualized as a continuous spectrum of particles which can be separated on the basis of sedimentation rate and density. In addition to these analytical features, the system is also a preparative one, since the subcellular components are readily separated in large quantities and are available for subsequent chemical and morphological analysis.

To exploit the zonal ultracentrifuge system completely, it is desirable to perform as large a number of different chemical or enzymatic assays as possible, and only automated assay systems can make this approach feasible.

The automated assay system for cytochrome oxidase appears to be a practical modification of the original manual procedure (10, 25), and is of particular value when used in conjunction with zonal centrifuge fractionation of tissue homogenates, since the equivalent of a large number of individual assays is readily performed without sacrificing any of the resolution obtained in the rotor. Furthermore, the sensitivity of the automated assay appears to be comparable with that of the original manual procedure, since similar distribution patterns of oxidase activity in zonal centrifuge fractionated liver breis were observed using both assay systems. If it were necessary to obtain maximum assay values with the system as we used it, one could do so by preincubating the tissue fraction with deoxycholate or detergents (26, 28).

The standard manual assay for cytochrome oxidase employs a single-beam spectrophotometer to monitor the disappearance of the characteristic absorption band of reduced cytochrome c at 550 mμ. The first order velocity constant for the oxidation can be calculated from these data and is considered to be the most satisfactory method for expressing enzyme activity (25). It is possible to monitor the oxidation as a positive deflection on the recorder using a double-beam spectrophotometer by running the reaction in the reference cuvette. One is thus able to exploit the most sensitive range of the optical equipment, an important feature in following weak reactions for a short incubation period. The automated assay system built around the double-beam spectrophotometer measures the difference between reactions run for different time intervals (20 and 120 seconds, in the present case), and thus records directly the slope of the reaction velocity curve for the incubation period. In addition, the automated system provides a continuous blank for the turbidity of the brei fractions and for auto-oxidation of the reduced cytochrome c substrate. It is thus apparent that automated enzymatic assays are most satisfactorily performed in systems based on double-beam optical equipment (21) which record directly the difference between reactions run for various time intervals.

The data suggest that the cytochrome oxidase reaction apparently follows zero order kinetics under our assay conditions. Similar results have been reported by Hess and Pope (18) using a concentration of substrate (2.5 × 10⁻⁵ M) similar to ours, and very low levels of enzyme which produced a change in optical density of 0.01 to 0.1 units per minute. When the assays are performed in the presence of larger amounts of enzyme, the reaction appears to follow first order kinetics (10, 25, 27), as would be expected from the theories of enzyme kinetics. It would thus appear that the automated assays could be performed under conditions which produce either zero or first order kinetics. In the case of the latter, under conditions where the amount of substrate used is small relative to the total concentration of substrate, the instrumentation, in recording the difference between reactions run for different time intervals, would be measuring a direct function of the first order velocity constant for the oxidation.

Cytochrome oxidase is known to be built into the structural elements of rat liver mitochondria (16, 17, 20). In spite of this fact, tissue fractionation performed according to the classical procedures of differential centrifugation invariably shows that a significant fraction (5 to 25 per cent) of the cytochrome oxidase activity is associated with fractions other than the mitochondrial, such as the microsomal and nuclear fractions (12, 14, 15, 19). These chemical results have been interpreted as showing that the other fractions were contaminated with mitochondria. This conclusion is supported by morphological evidence showing that about 8 per cent of the "mitochondria" of rat liver breis can be counted in the nuclear fraction obtained by using conventional Hogeboom and Schneider differential centrifugation techniques (1). In the present study utilizing the zonal ultracentrifuge, all the cytochrome oxidase activity was found to be associated with the mitochondrial fraction. In spite of the fact that the assay system employed was very sensitive, no activity could be detected in the regions of the gradient occupied by the microsomal and nuclear fractions.

The composition of the mitochondrial fraction obtained in the zonal ultracentrifuge appears to be similar to that obtained by the classical procedures of differential centrifugation. Thus, the mitochondrial fraction obtained by differential centrifugation (22) exhibited the same sedimentation characteristics in the zonal ultracentrifuge as the mitochondrial fraction separated directly from intact brei. The resolution obtained in the zonal ultracentrifuge, however, appears to be superior, as can be seen in the absence of significant contamination of the microsomal and nuclear fractions by mitochondria, using cytochrome oxidase as the enzymatic marker.

However, one must always remember that particles are distributed in the sucrose density gradient in the zonal ultracentrifuge on the basis of purely physical parameters, sedimentation velocity, and density. Consequently, several populations of particles may be present in a particular zone by virtue of their characteristics under the conditions of centrifugation. The observations made with the phase contrast microscope in this study suggest that a wide variety of particles may be present in the mitochondrial zone. Preliminary observations in the electron microscope, which will be reported in detail at a later time, support this conclusion. In addition, the mitochondrial region of the gradient has been shown to contain a very high level of acid phenyl phosphatase activities, part of which may be associated with granules other than the mitochondria (23). It is thus clear that although all the mitochondria are in one place, it does not necessarily mean that all the particles there are mitochondria.

Under the conditions employed with zonal ultracentrifuge rotor B-II, the larger subcellular components are essentially sedimented to their isopycnic position in the sucrose gradient. The data obtained in the present study suggest that the isopycnic density of rat liver mitochondria is approximately 1.20 gm/cc. Previous work using mitochondrial fractions obtained by differential centrifugation suggests that the isopycnic position in sucrose gradients is between 1.19 to 1.22 g/cc (8, 13, 29). It must be recognized that the granules are subjected to an osmotic gradient during such experiments. These methods thus measure the density of particles at equilibrium with a solution of the same density, and do not indicate what the density might be in solutions of lower osmotic pressure or in intact cells.

To date it has not been possible to obtain pure preparations of the larger cytoplasmic granules using the zonal ultracentrifuge. The separations

of these particles in this system are based largely upon sedimentation to the isopycnic position in sucrose gradients, and the properties of the various populations of particles under these conditions are too similar to allow more satisfactory separations. The use of different types of gradients (8, 11, 13) may make it possible to improve the resolution obtainable in gradient fractionations. In addition, new low-speed rotors, now in the early stages of development in this laboratory (7), should make it possible to separate the larger

cytoplasmic granules on the basis of differences in sedimentation rate.

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