The Heterogeneous Distribution of Mitochondrial Enzymes in Normal Rat Liver*

R. W. Swick, J. L. Stange, S. L. Nance, and J. F. Thomson

ABSTRACT: Preparations of rat liver mitochondria were distributed by particle size through a linear density gradient in a zonal centrifuge and the activity of 12 different enzymes was measured in each of the fractions recovered. A consistent and significant separation of the midpoints of the distributions of many enzymes from each other and from the total protein was obtained. It has been shown previously that mitochondria found in the peripheral cells of the liver lobule are larger than those in the pericentral cells. The present results suggest, therefore, that differences exist in the intralobular distribution of various enzymes, perhaps resulting in differences in the metabolic capacity of the peripheral and centrolobular cells.

Using a variety of techniques, a number of workers have shown that parenchymal cells in the peripheral region of the lobule, the basic architectural unit of the liver, are different from those in the central zone. Of particular interest to us were the apparent differences in the mitochondria from the two areas of the lobule. From electron micrographs, Novikoff and Shin (1964) concluded that mitochondria in the pericentral cells were small: 0.2–0.3 μ in diameter and up to 2 μ long, while those in the peripheral cells were considerably larger: 0.5–1.0 μ in diameter and up to 4 μ long. Bahr and Zeitler (1962) attempted to quantitate such differences and found two populations of mitochondria, one round, the other oblong, each with its own weight distribution. However, the difference between the two populations was much smaller than that observed by Novikoff and Shin (1964).

It also appears that there may be differences in the enzyme complements of these mitochondria. Shank *et al.* (1959), employing the demanding techniques of quantitative microchemical analysis on cells isolated from the peripheral, midlobular, and central zones, found that glutamate dehydrogenase activity was about 30% higher in the centrolobular area than in the other regions. Several other mitochondrial enzymes showed variation in activity across the lobule; but since they have a soluble counterpart, it was not possible to determine whether or not the heterogeneity was mitochondrial. By a variety of histochemical techniques, cytochrome oxidase (Burstone, 1959) and succinate dehydrogenase (Seligman and Rutenburg, 1951; Schumacher, 1957) can be shown to have a higher activity in the periportal cells, whereas NAD^+- and $NADP^+$-diaphorases[1] and glutamate and 3-hydroxybutyrate dehydrogenase (Novikoff, 1959) appear to be more active in pericentral cells. In a recent study, Pette and Brandau (1966), using an improved gel film method, have largely confirmed the earlier studies. Nonetheless, staining techniques did not permit the localization of the sites of these variations where isozymes were involved. Using the increased sensitivity of slow centrifugation in a zonal gradient centrifuge, we have distributed enriched preparations of rat liver mitochondria according to size and measured the activity of twelve enzymes in the fractions obtained. A significant displacement of the midpoints of the distributions of many enzyme activities from the total protein and from one another was observed. These differences in distribution indicate a variation in the levels of some enzymes in mitochondria with size and strongly suggest the existence of differences in the metabolic capacity of peripheral and centrolobular parenchymal cells.

Methods

Nonfasted adult female rats of the SD/Anl-SPF strain were maintained on Lab-Blox and were killed by decapitation. The livers were quickly removed and chilled in ice for 10 min. A mitochondrial fraction was prepared from 8 g of liver in 0.25 M sucrose–0.01 M K_2HPO_4 by a centrifugation procedure adapted from that of Schneider and Hogeboom (1950). After the second wash, the pellet was suspended in 32–34 ml of 0.25 M sucrose.

Density gradient fractionation of the mitochondria

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[1] Abbreviations used: NAD^+, oxidized nicotinamide–adenine dinucleotide; NADH, reduced NAD^+; $NADP^+$, oxidized nicotinamide–adenine dinucleotide phosphate; ATPase, adenosine triphosphatase.

was carried out with an A-XII zonal rotor (Anderson *et al.*, 1966) in a PR-2 refrigerated centrifuge (International Equipment Co.). With the rotor spinning at about 700 rpm, a total volume of 1160 ml of sucrose density gradient was introduced into the rotor through the edge line by means of two variable-speed peristaltic pumps. The sucrose concentration varied linearly along the radius from 0.29 to 0.88 M. The remainder of the rotor volume was filled with a 170-ml cushion of 1.46 M sucrose.

The mitochondrial suspension (30 ml) was introduced by syringe through the core line; an overlay of 40 ml of 0.25 M glucose was then added to move the mitochondrial zone past the beveled core, so that before acceleration, the rotor contained 30 ml of mitochondrial suspension, 40-ml overlay, 1160-ml gradient, and 100-ml cushion. The centrifuge was then brought up to 4000 rpm. After 50–60 min, the rotor was decelerated to about 700 rpm and the contents were collected from the core line by displacement with additional 1.46 M sucrose. In general, the first fraction represented the glucose overlay, the second the zone initially occupied by the homogenate; the next three were 40 ml each. Thereafter, the volumes of the following 28 fractions were adjusted so that equal increments of mitochondrial diameters, calculated according to Thomson and Klipfel (1957), would be represented in each fraction.

An aliquot of each fraction was reserved for the assay of succinate dehydrogenase, cytochrome oxidase, and ATPase. The remainder was centrifuged for 20 min at 24,000*g*, and the pellets[2] were suspended in 3 ml of a mixture of 5.4 M glycerol, 0.05 M potassium phosphate (pH 7.4), 0.025 M L-alanine, and 0.002 M L-cysteine (Swick *et al.*, 1965). The suspensions were stirred by repeated aspiration into a 20-ml syringe or homogenized with a Teflon pestle. This suspension medium gave preparations in which alanine aminotransferase was stable for the duration of the assays and, in our hands, all of "latent" enzymes showed nearly maximal activity when compared to that obtained with other known methods of "release." Homogenization of the glycerol suspension gave a very uniform preparation, although the activity of 3-hydroxybutyrate dehydrogenase was lower than in suspensions prepared by aspiration.

3-Hydroxybutyrate dehydrogenase (3-hydroxybutyrate:NAD^+ oxidoreductase, EC 1.1.1.30) activity was measured spectrophotometrically by the reduction of NAD^+ according to the procedure of Wise and Lehninger (1962), and 82 ± 6% of the activity in the original homogenate was recovered in the fractions. Malate dehydrogenase (L-malate:NAD^+ oxidoreductase, EC 1.1.1.37) activity was estimated from the oxidation of NADH (Ochoa, 1955) after dilution of the resuspended mitochondria, with a recovery of 123 ± 9%. Isocitrate dehydrogenase (L-isocitrate:$NADP^+$ oxidoreductase, EC 1.1.1.42) activity was determined from the reduction of $NADP^+$ (Plaut and Sung, 1955). The recovery averaged 96 ± 3%. Succinate dehydrogenase [succinate:(acceptor) oxidoreductase, EC 1.3.99.1] activity was assayed manometrically (Schneider and Potter, 1943) and 81 ± 6% of the activity in the original homogenate was recovered in the fractions. Glutamate dehydrogenase [L-glutamate:NAD^+ oxidoreductase (deaminating)] activity was estimated spectrophotometrically from the oxidation of NADH (Beaufay *et al.*, 1959). The recovery was 125 ± 10%. Cytochrome oxidase (ferrocytochrome *c*:O_2 oxidoreductase, EC 1.9.3.1) activity was measured spectrophotometrically (Hogeboom and Schneider, 1952) after dilution of the original fractions. The average recovery was 94 ± 2%. Ornithine transcarbamoylase (carbamoyl phosphate:L-ornithine carbamoyltransferase, EC 2.1.3.3) activity was estimated colorimetrically from the formation of citrulline (Schimke, 1962) during incubation of dilutions of the resuspended mitochondrial pellets. The recovery averaged 101 ± 4%. Aspartate aminotransferase (L-aspartate:2-oxoglutarate aminotransferase, EC 2.6.1.1) activity was measured, after appropriate dilution, by coupling the formation of oxalacetate to the oxidation of NADH with malate dehydrogenase (Karmen, 1955). The recovery was 102 ± 7%. Alanine aminotransferase (L-alanine:2-oxoglutarate aminotransferase, EC 2.6.1.2) activity was determined by the oxidation, in the presence of added lactate dehydrogenase, of NADH by pyruvate formed in an interrupted assay (Swick *et al.*, 1965). The recovery averaged 105 ± 6%. Ornithine–keto acid aminotransferase (L-ornithine:2-oxo acid aminotransferase, EC 2.6.1.13) activity was measured by the colorimetric method of Peraino and Pitot (1963). The recovery from the fractions was 103 ± 6%. Proline oxidase activity was determined by a similar method: 430 μmoles of L-proline, 0.1 μmole of cytochrome *c*, 16 μmoles of *o*-aminobenzaldehyde (dissolved initially in 30% ethanol), and 400 μmoles of potassium phosphate (pH 7.6) were incubated with 0.5 ml of enzyme in a final volume of 2 ml in a shaker bath at 37° for 30 min. The reaction was stopped by the addition of 1 ml of 30% trichloroacetic acid, shaking was continued for 10 min to assure the denaturation of cytochrome *c*, and the color was measured at 440 mμ after centrifugation.[3] The recovery averaged only 63 ± 15%. Magnesium-activated ATPase (ATP phosphohydrolase, EC 3.6.1.3) activity was estimated in the original fractions from the formation of inorganic phosphate by a modification of the method of DuBois and Potter (1943). The recovery was 86%. Protein concentration was measured by the Biuret reaction, with an average recovery in the fractions of 94 ± 2%.

[2] Beyond the fifth or sixth fraction, there was no measurable activity of any of the mitochondrial enzymes in the supernatant solution. Thus, differences in enzyme distribution were not due to differential leakage of the enzymes from the particles during centrifugation or recovery of the fractions. A trace of activity in the first four (supernatant) fractions suggests some leakage from mitochondria suspended in 0.25 M sucrose.

[3] We wish to thank Dr. C. Peraino for making his unpublished methods available to us.

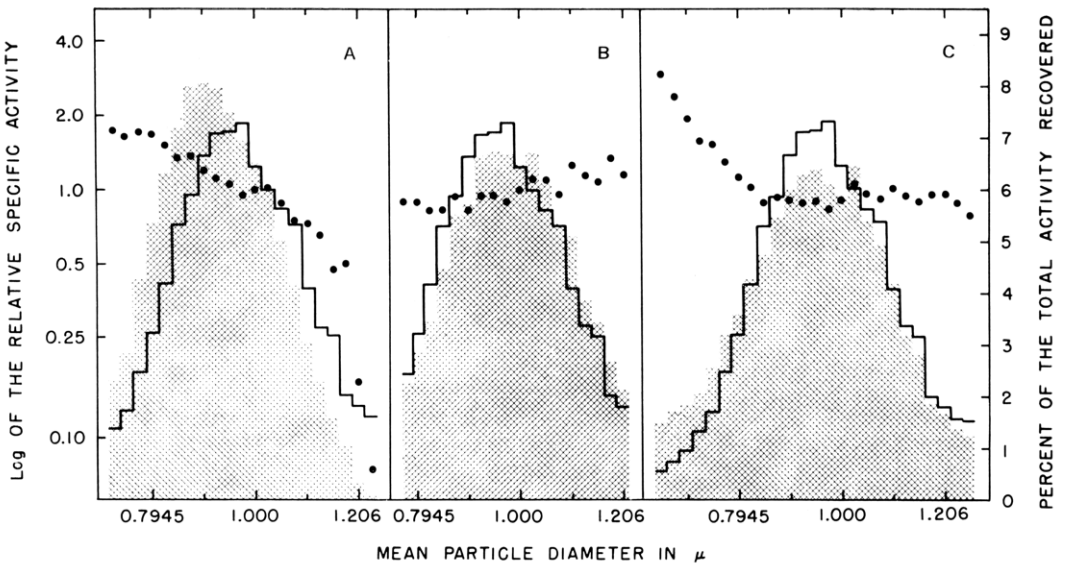

FIGURE 1: Distribution of four mitochondrial enzyme activities in a linear density gradient. (solid line) Glutamate dehydrogenase. (A) (cross hatched) Ornithine–keto acid aminotransferase; (filled circle) ornithine–keto acid aminotransferase:glutamate dehydrogenase. (B) (cross hatched) Aspartate aminotransferase; (filled circle) aspartate aminotransferase:glutamate dehydrogenase. (C) (cross hatched) Isocitrate dehydrogenase; (filled circle) isocitrate dehydrogenase:glutamate dehydrogenase.

Spectrophotometric measurements were made with a Beckman DU spectrophotometer equipped with a Gilford automatic absorbance recorder. The analog output was digitized by a Non-Linear Systems digital voltmeter, No. 4206, and transferred to punch cards by an NLS-output adapter, No. 180, and an IBM summary key punch, No. 526. Calculations were made using a GE-225 digital computer.

Calculation of the distribution of the enzymes and protein usually began with the sixth fraction and ended with the 33rd for the following reasons. A small peak of activity was present in the first five fractions, representing enzyme solubilized during preparation of the mitochondria and/or bound to mitochondrial fragments or to contaminating particles such as the microsomes, lysosomes, peroxisomes. The presence of the latter organelles was evident from a small protein peak as well as enzyme activities peculiar to these particles. The protein content and mitochondrial enzyme activities were usually at a minimum in the sixth fraction and were absent or negligible in the 34th. In addition, the ordinate of the plot of the distribution represents the normalized portion of the total activity in each fraction divided by the difference in mean diameter between fractions (Nichols and Bailey, 1949). The divisor could be dropped from the computations when the volume of each fraction recovered was selected to give a constant increment in mean particle diameter. This was most convenient to do beginning with the sixth fraction. This procedure (and the time chosen for the duration of the centrifugation) also resulted in the collection of approximately the same number of samples on each side of the peak, which, presumably, should compensate for any failures in the achievement of linearity in the assays over the range of enzyme activity encountered. Thus, although distributions were occasionally obtained, notably with 3-hydroxybutyrate dehydrogenase and proline oxidase, that were not "normal" distributions, they were usually symmetrical and the estimate of the midpoint reliable (and reproducible).

The midpoints of the distributions were calculated by three independent methods: from the weighted probability transform ("probit") of the cumulative per cent recovered in the 28 fractions (Finney, 1952), by fitting a normal curve to the frequency distribution by the method of least squares,[4] and from the ratio of the total activity of one enzyme to that of another or to total protein in successive fractions (Klein *et al.*, 1964). The midpoints were estimated by the last method in two ways: in one, the dispersions were considered equal and were neglected; in the second, correction was made for differences in dispersion. The theoretical aspects of these computations have been presented by Klein (1966). Each midpoint was then expressed as the mean particle diameter of the mitochondria at that point. The separation, or difference in means, is expressed as $\Delta m\%$, a per cent of the smaller mean diameter. The estimates were made from the central 80–98% of the activity, since determinations of enzyme activity were least accurate in the tail regions of the distributions. The estimates

[4] Dipert (1966), adapted from Struble, G., nonlinear least-squares curve-fitting program, Statistical Laboratory and Computing Center, University of Oregon, Eugene, Ore.

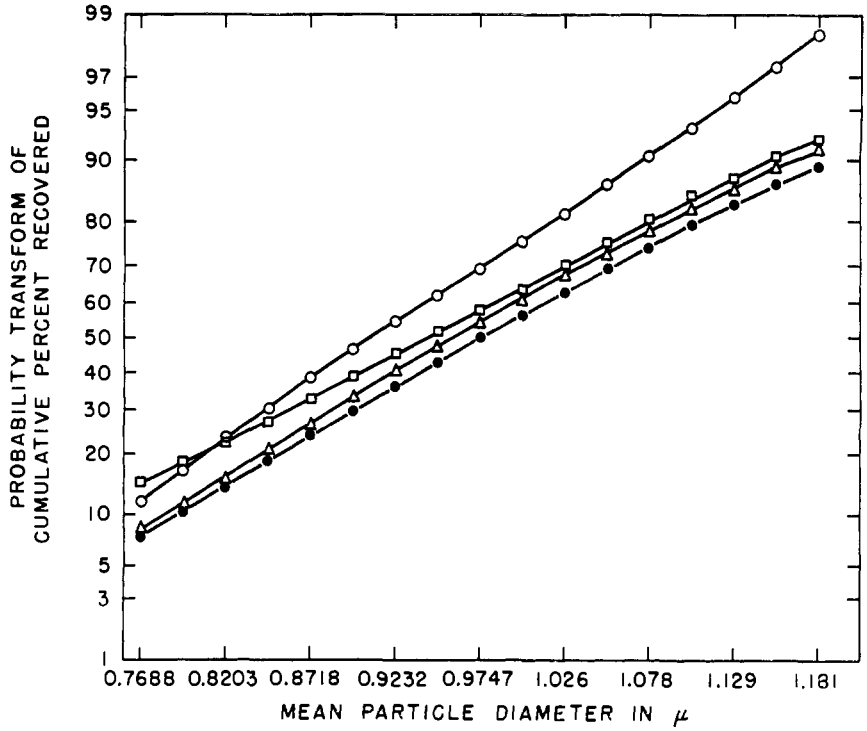

FIGURE 2: Cumulative percentage of four mitochondrial enzyme activities recovered from the linear density gradient. (open circle) Ornithine–keto acid aminotransferase; (open square) isocitrate dehydrogenase; (open triangle) glutamate dehydrogenase; (filled circle) aspartate aminotransferase.

obtained by the three methods were in best agreement (and most valid) when nearly normal distributions were observed. Lack of agreement was seen when nonnormal distributions were obtained or where there was some indication of a small, contaminating population. In the first case, more confidence was placed in the estimate produced by the probit. In the second, fitting a normal curve to a frequency distribution was more reliable, since the effects of the minor component could be minimized by weighting or eliminated by fitting the data to two normal curves.

Results

An example of part of the data obtained in a single centrifugation is illustrated in Figure 1. It can be clearly seen that the distribution of ornithine–keto acid aminotransferase is quite different from that of glutamate dehydrogenase (Figure 1A). The latter enzyme was chosen for comparison because its distribution is almost identical with that of protein. The separation of the two distributions can also be seen in the cumulative probability transform (Figure 2). The specific activity of ornithine–keto acid aminotransferase with respect to glutamate dehydrogenase varied linearly from 1.72 to 0.49, a 3.5-fold range, and ornithine–keto acid aminotransferase was almost absent from the last two fractions indicated (Figure 1A). Figure 1B shows the distribution of aspartate aminotransferase with respect to glutamate dehydrogenase. Here, the midpoint of activity occurred at a larger mean diameter. The difference is not large: $\Delta m\%$ was only 1.73 ± 0.22 by probit analysis but was statistically significant. Similar values were obtained in each experiment which also lends credibility to the reality of the difference. The slope of the specific activity, 1.00 ± 0.14, is also significantly different from 0. The internal consistency of these calculations is shown in Table I where $\Delta m\%$ has been calculated for each pair of enzymes. The estimates obtained with each method are in good agreement. The summation of the $\Delta m\%$'s of the two extreme enzymes with the central one (A + B) also agrees with the estimates of the extent of their separation from each other.

In Figure 1C is shown the distribution of isocitrate dehydrogenase with respect to glutamate dehydrogenase. The curve for most of the activity of the former enzyme can be superimposed on that of the reference enzyme, and the specific activity in this region is relatively constant. However, a small amount of the isocitrate dehydrogenase activity appears to be associated with smaller particles and, relative to glutamate dehydrogenase, is present at a higher concentration than in the larger particles. It is obvious that the midpoint given by probit analysis (Figure 2) does not give an accurate picture of the distribution of this enzyme. This disparity is also shown by the failure of the $\Delta m\%$ calculated by probit analysis to agree with that obtained by the other methods (Table I). One may conclude, therefore, that isocitrate dehydrogenase

TABLE I: The Displacement of the Midpoints of the Distribution of Four Enzyme Activities Expressed as a Percentage of the Mean Particle Diameter of the Midpoint, $\Delta m\%$, with Its Standard Error.[a]

$\Delta m\%$ Calcd	Ornithine–Keto Acid Aminotransferase *vs.* Glutamate Dehydrogenase (A)	Glutamate Dehydrogenase *vs.* Aspartate Aminotransferase (B)	(A + B)	Ornithine–Keto Acid Aminotransferase *vs.* Aspartate Aminotransferase	Isocitrate Dehydrogenase *vs.* Glutamate Dehydrogenase
By probit analysis	5.69 ± 0.18	1.73 ± 0.22	7.42	7.57 ± 0.15	1.94 ± 0.14
By fitting a normal curve to the frequency distribution	4.92 ± 0.15	2.11 ± 0.07	7.03	7.14 ± 0.21	0.46 ± 0.15
From enzyme ratio, neglecting differences in dispersion	5.54 ± 0.37	2.40 ± 0.34	7.76	7.82 ± 0.38	0.18 ± 0.54
From enzyme ratio, correcting for differences in dispersion	4.69 ± 0.48	2.31 ± 0.33	7.00	6.54 ± 0.43	0.35 ± 0.43

[a] Results obtained in one centrifugation.

and glutamate dehydrogenase are probably distributed similarly.

The pooled data from the five experiments are illustrated in Figure 3, and the average mean particle diameters of the midpoints of the distributions and the standard errors of the estimates are presented in Table II. The 12 enzymes appear to fall into four groups. Ornithine–keto acid aminotransferase and cytochrome oxidase may or may not be members of one group, and isocitrate dehydrogenase seems to fall between two groups. However, in all but one experi-

TABLE II: Mean Particle Diameter of the Midpoint of the Distribution of 12 Mitochondrial Enzyme Activities and Total Protein in a Linear Density Gradient.[a]

Enzyme	Mean Particle Diameter of the Midpoint of the Distribn and Its SE (μ)
Ornithine–keto acid aminotransferase	0.8922 ± 0.0022
Cytochrome oxidase	0.9018 ± 0.0023
Proline oxidase	0.9155 ± 0.0041
Succinate dehydrogenase	0.9186 ± 0.0033
3-Hydroxybutyrate dehydrogenase	0.9213 ± 0.0020
Isocitrate dehydrogenase	0.9267 ± 0.0020
ATPase	0.9320 ± 0.0011
Glutamate dehydrogenase	0.9338 ± 0.0024
Alanine aminotransferase	0.9363 ± 0.0020
Protein	0.9380 ± 0.0016
Malate dehydrogenase	0.9412 ± 0.0016
Ornithine transcarbamoylase	0.9557 ± 0.0023
Aspartate aminotransferase	0.9567 ± 0.0031

[a] Data were pooled from five experiments (ATPase from only one). SE = standard error.

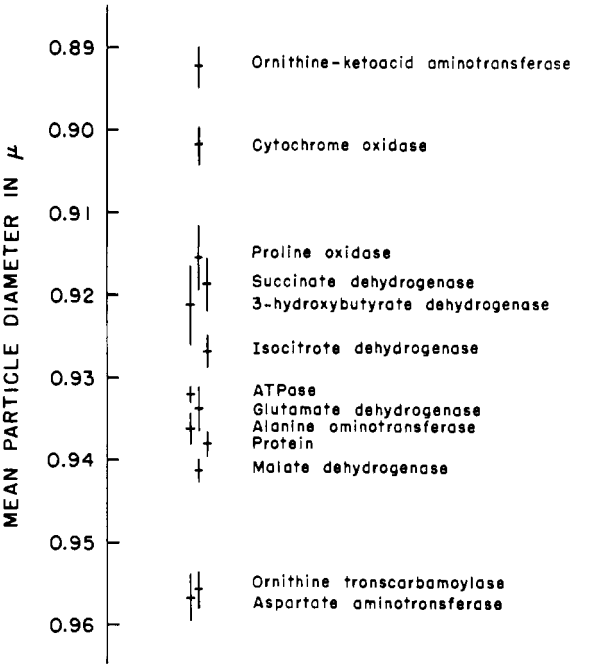

FIGURE 3: Mean particle diameters of the midpoints of mitochondrial enzyme activity and total protein distributions. Data were pooled from five experiments. The horizontal line represents the mean particle diameter of the midpoint in microns. The vertical line indicates its standard error. The value for ATPase was obtained in a single experiment.

ment, the midpoint of the distribution of this enzyme was insignificantly different from the cluster with the larger mean particle diameter. Otherwise, the mean particle diameters of the midpoints of the members of the groups are significantly ($p < 0.001$) different from those of the members of the others. Here, the analysis considered 98% of the total activity recovered in the 28 fractions. The value for ATPase is provisional since the distribution of its activity was only determined in one experiment. One might have expected it to fall near the other membrane-bound enzymes, *e.g.*, succinate dehydrogenase.

Discussion

Although earlier studies with density gradient centrifugation had suggested a lack of concordance of distribution patterns of succinate dehydrogenase and cytochrome oxidase (Thomson and Klipfel, 1957), the extent of the heterogeneous distribution of mitochondrial enzymes has not previously been demonstrated by physical means. Indeed, it probably would have been overlooked in the beginning of the present study but for the marked displacement of ornithine–keto acid aminotransferase. Even so, these small differences were made clearly visible only by virtue of the analysis of numerous, sequential fractions in which small but progressive changes in specific activity occurred. A few enzymes, certain properties, and fundamental functions resulting from the action of several enzymes have been studied in as many as four fractions of mitochondria obtained by careful differential centrifugation. For example, Monakhov (1964) reported that the capacity to produce glycolysis-stimulating substances and to couple oxidation to phosphorylation were most prominent in the middle two fractions of four nearly equal portions of liver mitochondria so obtained. The capacity for mechanochemical activity was maximal here, too, while ATPase activity was greatest in the fraction obtained with the highest speed of centrifugation. Lusena and Depocas (1966) found that mitochondria sedimented at low speed were more sensitive to freeze thawing than were higher speed fractions. Frisell *et al.* (1965) separated mitochondria into heavy and light fractions, and found twice as much sarcosine dehydrogenase in the heavy fraction, while four other enzymes were evenly distributed. Our results with ATPase are not in agreement with those of Monakhov (1964), and we have been unable to obtain satisfactory assays of sarcosine dehydrogenase (Hoskins and Mackenzie, 1961).

It is possible that this heterogeneity reflects the existence of mitochondria of different sizes and enzyme complement in the different kinds of cells within the liver. This is unlikely. Although the parenchymal cells represent only 60–70% of the cells in the liver (Abercrombie and Harkness, 1951), they constitute some 95% of the total volume (Harkness, 1952). If we assume, then, that our profile of activity across the range of sizes of mitochondria represents the profile of mitochondrial sizes in parenchymal cells across the liver lobule, our results again differ from those obtained by histochemical means. We found cytochrome oxidase and succinate dehydrogenase activity more concentrated in smaller mitochondria, while Burstone (1959) found cytochrome oxidase activity and others (Seligman and Rutenburg, 1951; Schumacher, 1957; Novikoff, 1959) reported succinate dehydrogenase activity highest in peripheral cells, *i.e.*, in large mitochondria. We found glutamate dehydrogenase uniformly distributed, whereas Pette and Brandau (1966) and Novikoff (1959) reported higher activity in the centrolobular cells, *i.e.*, small mitochondria. Other differences exist, and all are without explanation at the present.

There appeared to be some association of enzymes catalyzing proximate or adjacent reactions in mitochondria of a given size. For example, ornithine transcarbamoylase and aspartate aminotransferase are both intimately involved in urea synthesis and their activity, relative to each other, was constant across the gradient. The distribution of malate dehydrogenase, glutamate dehydrogenase, alanine aminotransferase, and probably isocitrate dehydrogenase activity were the same (and the same as protein), and all are involved in reactions in or immediately adjacent to the tricarboxylic acid cycle. On the other hand, ornithine–keto acid aminotransferase and ornithine transcarbamoylase were greatly dissimilar in their distribution while they too catalyze adjacent reactions. Since only catalytic amounts of ornithine need be maintained for the operation of the urea cycle, ornithine–keto acid aminotransferase need not parallel ornithine transcarbamoylase activity. The relatively high concentration of the former enzyme, in smaller mitochondria, with respect to the latter, suggests another role for the enzyme such as that proposed by Katunuma *et al.* (1965).

Finally, in terms of the nature of the mitochondrial population, the heterogeneous distribution of enzymes seen in the present work may be interpreted in two ways. It is possible that we are studying a single population of mitochondria whose size ranges from 0.6 to 1.2 μ, and whose individual particle diameters are coincidentally related to their location within the lobule and to the fine control of their enzyme complement by a gradient in substrate levels across the lobule. Indeed, in the computer analysis of the enzyme and protein data, the best fit was obtained for single, normally distributed populations. An alternative is that we are dealing with two populations of mitochondria, each with a size distribution which greatly overlaps the other, and each with a unique enzyme complement.[5]

Let us consider further the latter possibility. Bahr and Zeitler (1962) discerned two populations of mitochondria of nearly equal size, each with a weight distribution, differing in mean particle weights by a factor of 1.5, which would correspond to a difference in mean

[5] That there might be two populations each with a heterogeneous distribution of enzymes is a third possibility, one that would be extremely difficult to untangle.

particle diameters of 1.15 or 15%. For the combined populations Glas and Bahr (1966) reported a mean particle diameter of 0.94 μ, which agrees with our estimates (based on sedimentation characteristics) of the midpoint of the protein distribution, 0.938 μ. This would set the mean particle diameter of their small mitochondria at 0.88 μ, and of the large particles at 1.00 μ. Klein and Kunze-Falkner (1965) found that when two normal populations were combined, the resulting midpoint (by probit) would obey the equation, $m_0 = (m_1n_1 + m_2n_2)/(n_1 + n_2)$, where m_0 is the computed midpoint, m_1 and m_2 are the midpoints of the two populations, and n_1 and n_2 the proportions of the two populations ($n_1 + n_2 = 1$). Therefore, if we substitute the observed mean particle diameter of the midpoint, m_0, and 0.88 and 1.00 for m_1 and m_2, respectively, we can calculate n_1 and n_2, the fractions of the total activity in the particles of the presumed mean diameters of our two populations. The observed and rounded ratios are shown in Table III.

TABLE III: The Ratio of the Fractions of the Total Enzyme Activity in Two Hypothetical Populations of Mitochondria with Mean Particle Diameters of 0.88 and 1.00 μ.

Enzyme	Calcd Ratio Small: Large	Rounded Ratio Small: Large
Ornithine–keto acid aminotransferase	90:10	9:1
Cytochrome oxidase	82:18	4 or 5:1
Proline oxidase	70:30	2:1
Succinate dehydrogenase	68:32	2:1
3-Hydroxybutyrate dehydrogenase	65:35	2:1
Isocitrate dehydrogenase	61:39	1:1
Glutamate dehydrogenase	55:45	1:1
Alanine aminotransferase	53:47	1:1
Protein	51:49	1:1
Malate dehydrogenase	49:51	1:1
Ornithine transcarbamoylase	37:63	1:2
Aspartate aminotransferase	35:65	1:2

The ratios fall nicely into several distinct groups with small whole-number values.

It is interesting that three enzymes which are dependent on their intimate association with the mitochondrial membrane for activity (succinate dehydrogenase, 3-hydroxybutyrate dehydrogenase, and proline oxidase) have a very similar distribution. These "bound" enzymes appear to be present in some fixed proportion to each other. One might surmise that in addition to their enzymic function, they are necessary constituents of a rigidly ordered membrane structure. The dry matter in the average mitochondrion is 26.5% (Glas and Bahr, 1966), of which 85% (Frisell *et al.*, 1965), or 21.5% of the total mass, is protein. Of the protein, 33% (Criddle *et al.*, 1962), or 7% of the total particle, is structural or membrane protein. If we assume that the distribution of the bound enzymes (which was 2:1 in small:large mitochondria) represents the distribution of the membrane, then the concentration of the membrane in small mitochondria is twice that in the large or 9.3 *vs.* 4.7%, respectively. The difference in diameter of the two sizes of 15% is sufficient to produce this difference in concentration of structural membrane. This leads to the hypothesis, therefore, that the membrane component contributes a constant mass of material to the particle regardless of its size, *i.e.*, larger mitochondria are larger by virtue of a larger matrix.[6] This space might be occupied by increased amounts of such enzymes as aspartate aminotransferase and ornithine transcarbamoylase (hence, the ratio of these two of 1:2 in small:large mitochondria). Such enzymes as malate dehydrogenase and glutamate dehydrogenase appear in constant proportion to the total protein of the mitochondria regardless of size. The very high fraction of ornithine–keto acid aminotransferase in small particles, its near absence in very large particles, and the closeness of the midpoint of its activity to that presumed for the small mitochondria lead to the possibility that ornithine–keto acid aminotransferase might be present in only one of the two populations.

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[6] It is possible that the difference in mean diameter of the presumed two populations of mitochondria may be somewhat greater than we have estimated. The calculation of mitochondrial diameters from sedimentation behavior is based on the assumption that all mitochondria have the same density and osmotic properties (Thomson and Klipfel, 1957). If in fact all mitochondria have the same absolute mass of membrane material, then the smaller mitochondria would have a higher density because of the greater proportion of membrane mass; consequently, their sedimentation rate would be faster than anticipated on the basis of their diameters alone. Data on the density of mitochondria as a function of their volume are lacking. However, calculations based on the observations of Tedeschi (1961) on the volume of the osmotic dead space, together with the assumption of a fixed content of membrane protein regardless of size, suggest that the density differential ($\rho_{particles} - \rho_{medium}$) in 0.25 M sucrose is no more than 5% greater for the 0.88-μ than for the 1.0-μ particles. Since on the basis of particle size alone the sedimentation rate for the latter should be 30% faster $(1.0/0.88)^2$, the assumption of constant density introduces a relatively small error: the value 0.88 μ would be reduced to 0.85 μ.

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