The technique of differential centrifugation permits the separation of cytoplasmic particles into fractions on the basis of both the size and the specific gravity of the particles. Customarily, such a separation has been into two fractions: (1) a large granule fraction known to consist mainly of mitochondria, and (2) a small granule (or microsome) fraction composed of particles below the limit of resolution of the light microscope (1, 2). Although marked biochemical and morphological differences have been shown to exist between the mitochondria and microsomes (3), there has been relatively little information concerning the homogeneity of the granules within each fraction. Chantrenne in 1947 (4) emphasized that the isolation of two fractions might be arbitrary and, on the basis of his studies, suggested that there might actually exist a more or less continuous spectrum of size and chemical composition. There have been reports that the microsomes (5, 6) and the mitochondria (7) may be separated into biochemically different fractions. More recently, Novikoff et al. (8) have separated the cytoplasmic particles of rat liver homogenates into as many as eight fractions by differential centrifugation and have found evidence of biochemical heterogeneity both among the isolated microsomes and to a lesser extent among the isolated mitochondria.

In the present study, mitochondria, isolated by differential centrifugation, have been further subjected to two different methods of fractionation. The distribution of pentose nucleic acid and of several enzymatic activities among the subfractions thus obtained has been studied. Results obtained by both methods concur in indicating that the mitochondrial fraction is composed of biochemically heterogeneous particles.

Materials and Methods

Preparation of Mitochondria—Livers of 3 month-old female mice, C3H strain, were perfused with ice-cold saline, followed by cold 0.25 M sucrose. The pooled livers were forced through a perforated steel disk and homogenized in 0.25 M sucrose (9). The mitochondrial fraction, Mw1, isolated by differential centrifugation as previously described (10), was resuspended in 0.25 M sucrose to make a suspension which was approximately 250 per cent
with respect to the original tissue. Care was taken during the mitochon-
drial washings to remove as thoroughly as possible the incompletely sedi-
mented "fluffy" material which appeared above the mitochondrial pellet
and which, on the basis of its morphological and chemical properties, has
been thought to belong to the microsome fraction (11, 12, 3). Microscopic
examination in the present instance has shown that the visible components
of the fluffy coat were a few mitochondria and many very small particles,
some of which were not completely resolved with the phase microscope.

Fractionation of $Mw_2$ by Packing—Polyethylene centrifuge tubes (pre-
pared from polyethylene tubing), 50 mm. in length and 6.35 mm. in inter-
nal diameter, were filled with a known volume of $Mw_2$ and centrifuged for
half an hour at 35,600 r.p.m. (108,000 $\times g$, average) in the SW-39 hori-
zontal rotor of the Spinco model E ultracentrifuge. Firmly packed pellets
showing obvious layering were obtained (Fig. 1). The uppermost layer, T,
was pink and translucent. In other experiments, in which the fluffy coat
was not removed during the mitochondrial washes, Layer T was greatly
increased in width. However, even when the fluffy coat had been removed
as completely as possible, Layer T was always present. The bulk of the
sediment was opaque and showed zones of different colors. Passing down-
wards from Layer T, there was a gray layer (B1), a light tan layer (B2),
and finally, a layer which became progressively browner towards the bot-
tom of the tube (Layer B3). The clear, slightly yellow supernatant fluid
and the various fractions were separated by slicing the centrifuge tube with
a razor blade. The layers thus isolated were resuspended in known vol-
umes of 0.25 M sucrose for analysis.

Fractionation of $Mw_2$ by Sedimentation in Density Gradient (13)—Cellu-
lloid centrifuge tubes of approximately 5.5 ml. capacity were filled by add-
ing in order the following sucrose solutions: 0.4 ml. of 2.22 M, 0.8 ml. each
of 1.59 M, 1.51 M, 1.42 M, 1.34 M, and 1.26 M, and 0.5 ml. of 0.636 M.
While the boundaries between the sucrose layers were still sharp, 0.4 to 0.5 ml. of
$Mw_2$ was layered at the top of each of three tubes. The tubes were cen-
trifuged for 1 hour at 35,600 r.p.m. in the SW-39 rotor of the ultracentri-
fuge, care being taken to accelerate and decelerate slowly in order to avoid
mixing the layers. Sedimentation in the discontinuous density gradient
resulted in concentrations of particles at each interface between different
sucrose concentrations (Fig. 2). The region in between the interfaces was
clear only when very small volumes of $Mw_2$ were sedimented. With the
amounts used in the present experiments, these regions were generally hazy.
The bulk of the mitochondrial material sedimented at the boundaries 1.34
M-1.42 M and 1.42 M-1.51 M, forming layers of such width that the interval
between them was rather indistinct. A sharp, rust-colored layer was
formed at the boundary 1.59 M-2.22 M. Glycogen particles passed through the 2.22 M sucrose layer and sedimented at the bottom of the tube.

The six mitochondrial zones were separated by sectioning the centrifuge tube with a special cutter (14) at the points indicated in Fig. 2. Sectioning tended to result in somewhat low recoveries, owing to leakage around the knife blade. The corresponding fractions from three tubes were usually combined for analysis.

**Enzymatic Assays and Chemical Determinations—**Uricase activity was
measured spectrophotometrically at 25° (9). Desoxyribonuclease (DNase) activity was determined at 37° by measurement of acid-soluble compounds released from highly polymerized desoxyribonucleic acid (15). Glucose-6-phosphatase (G-6-Pase) was determined at 37° (16) in 0.03 M sodium succinate buffer, pH 6.6.

Succinic dehydrogenase activity was determined at 25° by either of two spectrophotometric methods. In the first (see Potter and Albaum (17)), the reaction mixture was made by the addition, in order, of a suitable dilution of enzyme, water to make a final volume of 3.00 ml., 0.016 M potassium phosphate buffer, pH 7.4, 0.22 × 10\(^{-3}\) M potassium cyanide, 0.26 × 10\(^{-4}\) M cytochrome c (Sigma Chemical Company, approximately 90 per cent in the oxidized form), 0.40 × 10\(^{-3}\) M calcium chloride, and 0.033 M potassium succinate. The blank cuvette contained all components of the reaction mixture except succinate. Observation of the increase in optical density at 550 m\(\mu\) in the experimental cuvette, with enzyme concentrations giving optical density changes of 0.04 or less per minute, showed that the rate of reduction of cytochrome c was linearly proportional to enzyme concentration and was constant over a 5 minute period. No appreciable reduction of cytochrome c occurred in the absence of substrate. Succinic dehydrogenase activity was calculated from the extinction coefficients of oxidized and reduced cytochrome c (18).

Succinic dehydrogenase activity was also determined spectrophotometrically by the reduction of ferricyanide (19). The reaction mixture (volume, 3.00 ml) contained the same concentrations of buffer, calcium, cyanide, and succinate as in the previous method, but enzyme was added to the water after the buffer. Potassium ferricyanide (2.03 × 10\(^{-3}\) M final concentration) was added last. The blank cuvette was prepared by omission of the succinate from the reaction mixture and adjustment of the ferricyanide concentration to 1.52 × 10\(^{-3}\) M. Observation of the decrease in optical density of the experimental cuvette at 420 m\(\mu\), with enzyme concentrations which produced changes in optical density of 0.03 or less per minute, showed that the rate of reduction of ferricyanide was linearly proportional to enzyme concentration and remained constant for periods up to 10 minutes. There was no reduction of ferricyanide in the absence of succinate. The extinction coefficient of ferricyanide at 420 m\(\mu\) was found to be 1.008 × 10\(^{8}\) sq. cm. per mole, and this value was used in calculating the succinic dehydrogenase activities. Ferrocyanide had no appreciable absorption at this wave-length.

The following analytical methods were employed: pentose nucleic acid phosphorus (PNA P) (20), inorganic phosphorus (21), and total nitrogen (11).
Results

Distribution of PNA P and of G-6-Pase Activity

These two constituents are known to be much more highly concentrated in the microsome fraction of liver than in the mitochondrial fraction (22, 23). Their distribution among the mitochondrial subfractions obtained in the present study is shown in Tables I and II. PNA P was highly concentrated in the uppermost layer (T) of the packed mitochondria and to a lesser extent in the next layer below (B1). The relative concentrations of G-6-Pase activity paralleled those of PNA P almost exactly in all of the fractions isolated by this method. In fractions obtained from the gradient tube, the concentration of PNA P showed a bimodal distribution, being highest in the two lightest and the two heaviest fractions (Layers 1, 2, 5, and 6). It was found in experiments not presented here that G-6-Pase activity showed a similar bimodal distribution in gradient fractionation. In each type of separation, the sum of the PNA P in the fractions in which it was concentrated represented about 60 per cent of the total PNA P of Mw2, suggesting that particles high in PNA P which were found in the upper layers of the packed mitochondria have been separated into two groups in the gradient tube.

Distribution of Succinic Dehydrogenase, Uricase, and DNase Activities

In Packed Mitochondria (Table I)—The main bulk (93 per cent) of the succinic dehydrogenase activity, together with some 75 per cent of the total nitrogen, was found in the two lower layers (B2 and B3). There was a gradual increase in specific activity of this enzyme from top to bottom, and it was present in very low concentration (approximately one-tenth of that of Mw2) in Layer T, while it was significantly concentrated in Layer B3. Uricase activity had an entirely different pattern, with the highest specific activity in Layer B1 and the lowest in Layer B3. 40 per cent of the total uricase activity was found in Layer B1, which contained only 10 per cent of the total nitrogen. DNase activity was significantly concentrated in Layer B3. This activity did not show a progressive increase from top to bottom of the sediment, there being a higher specific activity in Layer B1 than in the layer below.

In Gradient Tube (Table II)—Approximately 93 per cent of the total succinic dehydrogenase activity was associated with Layers 3 and 4, showing a specific activity equal to that of Layer B3 in the above experiment. Uricase activity was highly concentrated in Layer 6 and to a lesser extent in Layer 5. These two layers, which contained about 50 per cent of the total uricase activity, accounted for only 11 per cent of the total nitrogen and 5
per cent of the total succinic dehydrogenase activity. DNase activity showed a bimodal distribution similar in some respects to that of PNA P. The specific activity was highest in Layers 1 and 6 (about 5 times that of

**Table I**

_Distribution of Nitrogen, PNA P, and Enzymatic Activities among Fractions of Mouse Liver Mitochondria Obtained by Packing*

<table>
<thead>
<tr>
<th>Mitochondrial fraction</th>
<th>Total N</th>
<th>Succinic dehydrogenase activity</th>
<th>Uricase activity</th>
<th>DNase activity</th>
<th>G-6-Phosphatase activity</th>
<th>PNA P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Total mg.</td>
<td>Specific mg.</td>
<td>Total µg.</td>
<td>Specific µg.</td>
<td>Total µM per mg. N</td>
</tr>
<tr>
<td>MW2</td>
<td>15.9</td>
<td>6600</td>
<td>414</td>
<td>1.848</td>
<td>0.116</td>
<td>242</td>
</tr>
<tr>
<td>S</td>
<td>0.167</td>
<td>0</td>
<td>0</td>
<td>0.001</td>
<td>0.006</td>
<td>0.67</td>
</tr>
<tr>
<td>T</td>
<td>0.784</td>
<td>38.5</td>
<td>45.7</td>
<td>0.084</td>
<td>0.107</td>
<td>2.62</td>
</tr>
<tr>
<td>B1</td>
<td>1.64</td>
<td>387</td>
<td>246</td>
<td>0.749</td>
<td>0.457</td>
<td>19.8</td>
</tr>
<tr>
<td>B2</td>
<td>8.17</td>
<td>3700</td>
<td>453</td>
<td>0.843</td>
<td>0.104</td>
<td>66.5</td>
</tr>
<tr>
<td>B3</td>
<td>3.89</td>
<td>2535</td>
<td>651</td>
<td>0.187</td>
<td>0.048</td>
<td>152</td>
</tr>
</tbody>
</table>

*The figures in bold-faced type indicate specific activities significantly greater than those of MW2.*

**Table II**

_Distribution of Nitrogen, PNA P, and Enzymatic Activities among Fractions of Mouse Mitochondria Obtained by Density Gradient Sedimentation*

<table>
<thead>
<tr>
<th>Mitochondrial fraction</th>
<th>Total N</th>
<th>Succinic dehydrogenase activity</th>
<th>Uricase activity</th>
<th>DNase activity</th>
<th>PNA P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Total mg.</td>
<td>Specific mg.</td>
<td>Total µg.</td>
<td>Specific µg.</td>
</tr>
<tr>
<td>MW2</td>
<td>10.25</td>
<td>4505</td>
<td>440</td>
<td>1.030</td>
<td>0.100</td>
</tr>
<tr>
<td>1</td>
<td>0.217</td>
<td>29</td>
<td>134</td>
<td>0.0025</td>
<td>0.012</td>
</tr>
<tr>
<td>2</td>
<td>0.136</td>
<td>102</td>
<td>760</td>
<td>0.0033</td>
<td>0.024</td>
</tr>
<tr>
<td>3</td>
<td>2.22</td>
<td>1581</td>
<td>711</td>
<td>0.025</td>
<td>0.011</td>
</tr>
<tr>
<td>4</td>
<td>4.31</td>
<td>2603</td>
<td>605</td>
<td>0.278</td>
<td>0.065</td>
</tr>
<tr>
<td>5</td>
<td>0.522</td>
<td>144</td>
<td>276</td>
<td>0.145</td>
<td>0.278</td>
</tr>
<tr>
<td>6</td>
<td>0.570</td>
<td>77</td>
<td>135</td>
<td>0.361</td>
<td>0.634</td>
</tr>
</tbody>
</table>

*The figures in bold-faced type indicate specific activities significantly greater than those of MW2.*

MW2). There was an inverse relationship between DNase and succinic dehydrogenase specific activities in the fractions isolated by this method, in contrast to the situation in the packed sediment. In Layers 5 and 6, high
specific activities of uricase and DNase coincided, a phenomenon suggested in Layer B1 of the packed mitochondria.

**Microscopic Examination of Fractions**—Attempts to correlate striking differences in enzymatic activities with distinct morphological properties of the various fractions were not entirely successful. Layer T of the packed mitochondria was found to consist almost exclusively of very small particles, many just at the limit of microscopic resolution. In this it resembled the fluffy coat previously described. There were very few particles which could be identified as mitochondria in this layer, which is felt at present to represent microsomal contamination remaining after the washing of the mitochondria. Layers B3, 3, and 4 presented the appearance of quite homogeneous preparations of medium and large size mitochondria, having only a slight admixture of very small particles. The other fractions were mixtures of granules of various sizes and optical densities. It was felt that subjective estimates of the relative frequencies of the various granules did not have sufficient validity to warrant any correlations between morphology and enzymatic activity.

**DISCUSSION**

While both methods of fractionation employed in this study depended primarily upon differences in specific gravity among the particles, it is probable that certain other factors were also involved. Osmotic effects, which were absent in the packing method, probably played an important rôle in determining the final specific gravity of particles in the sucrose density gradient. For example, uricase activity sedimented near the top of the packed mitochondria, yet appeared among the heaviest components in the gradient tube, suggesting that the uricase-containing particles were particularly sensitive to dehydration when exposed to high sucrose concentrations. On the other hand, occlusion of particles may have prevented some granules from reaching their proper specific gravity level when the mitochondria were subjected to the packing procedure. That this factor did not influence the major layering obtained in this method was shown by replacement of the supernatant fluid over a packed sediment with more MW2 and recentrifugation. Such a procedure resulted only in the widening of the layers previously present, rather than in the formation of a new set of layers lying above the previous ones. Occlusion of particles was probably not a factor during sedimentation in a density gradient, since no packing occurred here.

Exposure of the particles to high sucrose concentrations is a possible disadvantage of the present density gradient method, and attempts are in progress to find materials which will provide adequate specific gravities at lower molar concentrations. However, there was no indication in the
present study that the enzymatic properties of the particles were seriously affected by exposure to the sucrose density gradient. The recoveries of the enzymatic activities were the same as or slightly higher than the recovery of total N (Table II).

The results of both methods, considered as a whole, indicate that different particles within the mitochondrial fraction varied considerably in their specific biochemical properties. In Table III are presented the ratios of the specific properties of the isolated fractions to the corresponding specific properties of the whole mitochondrial fraction. By one method of fractionation or the other, each of the components studied could be concentrated at the expense of all of the others, with the single exception that it

<table>
<thead>
<tr>
<th>Mitochondrial fraction</th>
<th>Succinic dehydrogenase activity</th>
<th>Uricase activity</th>
<th>DNase activity</th>
<th>PNA P</th>
</tr>
</thead>
<tbody>
<tr>
<td>T</td>
<td>0.11</td>
<td>0.92</td>
<td>0.22</td>
<td>3.34</td>
</tr>
<tr>
<td>B1</td>
<td>0.59</td>
<td>3.94</td>
<td>0.90</td>
<td>1.72</td>
</tr>
<tr>
<td>B2</td>
<td>1.09</td>
<td>0.90</td>
<td>0.54</td>
<td>0.82</td>
</tr>
<tr>
<td>B3</td>
<td>1.57</td>
<td>0.41</td>
<td>2.57</td>
<td>0.69</td>
</tr>
<tr>
<td>1</td>
<td>0.30</td>
<td>0.12</td>
<td>4.95</td>
<td>2.87</td>
</tr>
<tr>
<td>2</td>
<td>1.70</td>
<td>0.24</td>
<td>1.67</td>
<td>2.86</td>
</tr>
<tr>
<td>3</td>
<td>1.62</td>
<td>0.11</td>
<td>0.22</td>
<td>0.81</td>
</tr>
<tr>
<td>4</td>
<td>1.37</td>
<td>0.65</td>
<td>0.32</td>
<td>1.05</td>
</tr>
<tr>
<td>5</td>
<td>0.63</td>
<td>2.78</td>
<td>2.34</td>
<td>1.70</td>
</tr>
<tr>
<td>6</td>
<td>0.31</td>
<td>6.34</td>
<td>5.22</td>
<td>2.01</td>
</tr>
</tbody>
</table>

has thus far been impossible to concentrate uricase activity without at the same time concentrating PNA P to some extent. Such a situation of multiple inverse relationships seems best explained by the assumption of heterogeneity among the particles making up the original mitochondrial population. So far, no particles have been isolated which were entirely lacking in any of the components investigated. Whether this is the result of deficiencies in the methods of fractionation or is due to the fact that the particles differ only quantitatively from one another is not yet known. With regard to the question of whether the mitochondrial fractions isolated by either method of fractionation might vary with respect to substrate accessibility or permeability in addition to or instead of differing in total enzyme content, it may be said that there is no evidence at present that the activities of any of the enzymes as measured here are materially influenced by such phenomena (9, 15, 24).

The present study seems to be in general agreement with a previous re-
port (8) in indicating the probable existence of a class of particles exhibiting high specific activity of uricase, low specific activity of succinic dehydrogenase, and moderately increased concentration of PNA. However, we have been unable to find any clear cut correlation between the frequency of any morphological type of granule and high uricase activity.

SUMMARY

The mitochondrial fraction isolated from mouse liver homogenates in 0.25 M sucrose by differential centrifugation was further fractionated by packing under high centrifugal force and by sedimentation in a specific gravity gradient. The fractions isolated after these procedures were studied for their activities of succinic dehydrogenase, uricase, deoxyribonuclease, and glucose-6-phosphatase and for their content of total nitrogen and pentose nucleic acid. The distribution of these components was such as to indicate marked biochemical heterogeneity among the particles making up the original mitochondrial population.

BIBLIOGRAPHY

INTRACELLULAR DISTRIBUTION OF ENZYMES: XII. BIOCHEMICAL HETEROGENEITY OF MITOCHONDRIA
Edward L. Kuff and Walter C. Schneider


Access the most updated version of this article at http://www.jbc.org/content/206/2/677.citation

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/206/2/677.citation.full.html#ref-list-1