THE ASSAY OF ANIMAL TISSUES FOR RESPIRATORY ENZYMES

IV. CELL STRUCTURE IN RELATION TO FATTY ACID OXIDATION*

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The "homogenate technique" is at present widely used for the study of enzymatic reactions in various types of biological material. In the original technique (1) an attempt was made to eliminate cell structure and to disperse intracellular constituents so widely that endogenous respiration was eliminated by dilution, while particular phases of metabolism could be restored by the addition of appropriate substrates and cofactors. In 1942, Elliott and Libet (5) made an excellent study which led to the conclusion that "the effects of homogenization in the different media are mainly due to the tendency of isotonic salts or sugars to prevent cell cytolysis, while in hypotonic medium cytolysis occurs." They reported that isotonic preparations of brain cells compared favorably with brain slices for studies on brain metabolism, and they subsequently (6) carried out their studies with such preparations unfortified with coenzymes or with cytochrome. On the other hand, Schneider and Potter (7, 8) pursued the alternative which Elliott and Libet's work suggested and homogenized tissues in distilled water in order to make cytolysis as complete as possible. We propose to refer to this type of preparation as a "water homogenate," while that of Elliott et al. (6) may be referred to as an "isotonic homogenate." On the basis of the cytochrome c content of liver and the amount of cytochrome required to saturate the succinoxidase system (9) an objective technique for estimating the degree of cytolysis was devised (7, 8). To date no other method of obtaining a quantitative measure of cytolysis has been found; microscopic examinations cannot give quantitative data and in fact can give very erroneous impressions. According to the succinoxidase test, water homogenates of rat liver contain about 5 per cent whole cells, while isotonic preparations contain 60 to 80 per cent whole cells. As might be

* This work was aided by a grant from the Jonathan Bowman Fund for Cancer Research.

1 It may be pointed out here that the device used by Potter and Elvehjem in 1936 (1) was similar in principle to a tissue crusher described as early as 1922 (2) and subsequently modified for other purposes (3, 4). The pestles may now be obtained from the Central Scientific Company.
expected, homogenates prepared in 0.25 M/30 phosphate buffer (9), which is hypotonic, yielded a figure that is intermediate, about 30 per cent whole cells. In each case, the per cent of whole cells is of course modified by the tightness of the homogenizer and the duration of the homogenization.

The importance of these considerations was recently emphasized when we attempted to devise an assay for fatty acid oxidation, following the demonstration of this reaction in homogenates (10, 11). The fatty acid oxidase activity was reported to be extremely sensitive both by Muñoz and Leloir (10) and by Lehninger (11). Since many of the findings which they reported could be interpreted in terms of cytolysis, we carried out a number of experiments in which the fatty acid oxidase activity was correlated with a "cytolysis quotient," as determined by means of the succinoxidase test, in order to determine whether cell structure is necessary for fatty acid oxidation.

**EXPERIMENTAL**

Isotonic homogenates were prepared in 0.85 per cent saline, while water homogenates were prepared in distilled water. Livers were removed from decapitated rats and chilled on cracked ice. Homogenization was carried out in cold tubes and media in a cold room, with 9 volumes of media per weight of liver. The isotonic homogenates were centrifuged at 1500 g for 10 minutes and the residue was resuspended in saline equal to one-half the original volume of the homogenate. The suspension was centrifuged down and washed twice more before suspending in one-half the original volume. The washed residue was thus equal to a 20 per cent homogenate on a volume basis. The centrifuging was identical with the procedure used by Schneider to separate nuclei from cytoplasm in water homogenates (unpublished) and was carried out in order to eliminate the endogenous respiration (Lehninger (12)). In some cases, water homogenates were prepared and made isotonic by the addition of 0.5 volume of 2.55 per cent NaCl, about 5 minutes after homogenization.

Since Lehninger (11, 12) had reported activation by adenosine triphosphate (ATP), we set up reaction mixtures in flasks with no side arms and kept them in cracked ice until the liver preparations were added, according to our previous technique for the study of oxidative phosphorylation (13), in which ATP was in the reaction mixture.

The reaction components were varied, but certain additions were constant. These included 0.3 ml. of 0.1 M Na phosphate at pH 7.5 and 0.3 ml. of 0.1 M Na malonate at pH 7.5. Other additions included 0.85 per cent NaCl, 0.01 M Na octanoate, 0.013 M Na ATP, $4 \times 10^{-4}$ M cytochrome c, 0.1 M MgCl$_2$, liver preparation, and water to make 3.0 ml. The amounts added are shown in the tables.
The oxygen uptake was measured in standard Warburg apparatus at 37°. Equilibration was continued for 6 minutes and readings were taken at 5 minute intervals for 30 minutes. Center cups with 2 N NaOH and filter paper were used.

The "cytolysis quotient" was measured by determining the succinoxidase activity with and without added cytochrome c. It is an indication of the per cent of cells which have been disturbed sufficiently to lose their cytochrome c, and in order to be valid, the intact cells must contain sufficient cytochrome c to saturate the succinoxidase which they contain, and the cytochrome c of the broken cells must completely dissociate from the succinoxidase system. These requirements seem to be met in liver (7-9, and this paper), although the second requirement does not seem to hold for skeletal and cardiac muscle (7, 8). The quotient equals

\[
\frac{(\text{succinoxidase with cytochrome}) - (\text{succinoxidase without cytochrome})}{100 \times \text{succinoxidase with cytochrome}}
\]

Results

In preliminary tests for fatty acid oxidation with various amounts of washed residue from isotonic homogenates of rat liver, we observed vigorous oxidation (100 microliters of O₂ per 10 minutes) of octanoate in the presence of ATP when 1.0 ml. of 20 per cent washed residue was used, but no activity when 0.3 ml. of the preparation was used. There was thus a marked "dilution effect" (1). When the experiment was repeated with the NaCl content of the tissue suspension compensated, it was found that the dilution effect was due entirely to the variation in NaCl. When this was kept constant, the rate of octanoate oxidation was directly proportional to the amount of liver preparation that was added (see Table I). The data also show that cytochrome c was without effect on the rate of octanoate oxidation, while ATP was indispensable. The endogenous respiration of the preparation was zero, and the amount of oxygen uptake, corrected for the equilibration period, was close to 200 microliters. The data are thus in excellent agreement with those of Lehninger (14), who reported that octanoate was oxidized quantitatively to acetoacetic acid (theoretical oxygen uptake, 201.6 microliters) under similar conditions.

The effect of sodium chloride suggested similar data by Elliott et al. (5, 15) and raised the question of the role of tonicity in maintaining activity. Experiments in which the amount of magnesium chloride and sodium chloride were varied (Table II) gave strong indications that hypotonicity was detrimental to the oxidation of fatty acid.

When liver was homogenized in water, the homogenate was completely unable to oxidize octanoate (10, 12). In order to determine further the role of cell structure in fatty acid oxidation, aliquots of rat liver were homogenized...
enzized in water and in saline, with the water homogenate made isotonic within 5 minutes after its preparation, by the addition of hypertonic saline. Aliquots of each homogenate were centrifuged and washed (see “Methods”) and made up to 0.5 volume. The degree of cytolysis in each of the four preparations was estimated by determining the “cytolysis quotient.” The fatty acid oxidation was measured in each of the washed preparations. The

**Table I**

**Oxidation of Octanoate by Washed Isotonic Liver Homogenate**

Each complete flask contained 0.3 ml. of 0.1 M Na phosphate at pH 7.5, 0.3 ml. of 0.1 M Na malonate, 0.2 ml. of 0.1 M MgCl₂, 0.2 ml. of 0.013 M Na adenosine triphosphate, 0.1 ml. of $4 \times 10^{-5}$ M cytochrome c, 0.3 ml. of 0.01 M Na octanoate, plus 20 per cent washed isotonic liver homogenate and saline (0.85 per cent NaCl) as indicated, with sufficient water to make 3.0 ml.

<table>
<thead>
<tr>
<th>Additions</th>
<th>Average oxygen uptake per 5 min.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Washed homogenate</td>
</tr>
<tr>
<td>ml.</td>
<td>ml.</td>
</tr>
<tr>
<td>0.3</td>
<td>0.7</td>
</tr>
<tr>
<td>0.6</td>
<td>0.4</td>
</tr>
<tr>
<td>1.0</td>
<td></td>
</tr>
</tbody>
</table>

* When either adenosine triphosphate or octanoate was omitted, no oxygen was taken up. In all flasks the oxygen uptake ceased rather abruptly when about 150 microliters of O₂ had been taken up. See the text.

**Table II**

**Effect of Tonicity on Octanoate Oxidation**

Conditions as in Table I, except 0.3 ml. of washed 20 per cent isotonic liver homogenate in all flasks, with NaCl and MgCl₂ varied. The amount of NaCl present includes that in the homogenate. Oxygen uptake per best 10 minutes.

<table>
<thead>
<tr>
<th>0.14 M NaCl per flask</th>
<th>0.1 M MgCl₂ per flask</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 ml.</td>
<td>0 ml.</td>
</tr>
<tr>
<td>0.2 ml.</td>
<td>0.2 ml.</td>
</tr>
<tr>
<td>0.4 ml.</td>
<td>0.4 ml.</td>
</tr>
<tr>
<td>0.6 ml.</td>
<td>0.6 ml.</td>
</tr>
<tr>
<td>0.3 ml.</td>
<td>12.5 microliters O₂</td>
</tr>
<tr>
<td>0.9 ml.</td>
<td>35.6 microliters O₂</td>
</tr>
<tr>
<td>1.3 ml.</td>
<td>33.3 microliters O₂</td>
</tr>
</tbody>
</table>

results are shown in Table III. In the case of the washed water homogenate, no oxygen was taken up when octanoate was the substrate, and the cytolysis quotient was high, indicating almost complete cell disruption, at least to the extent that cytochrome c could diffuse away from succinoxidase. There was considerable loss in succinoxidase, due to the association of this enzyme with particles too small to be completely sedimented under these
However, considerable succinoxidase was in the form of particles which could be sedimented; these particles did not include whole cells, on the basis of the "cytolysis quotient." Furthermore, Schneider (unpublished data) has shown that cytoplasmic fractions free from nuclear nucleic acid contain granules of succinoxidase which can be sedimented under isotonic conditions. In the case of the isotonic homogenate, the "cytolysis quotient" indicated that about 75 per cent of the material was still intact enough to retain cytochrome c in association with succinoxidase, and in this preparation octanoate was vigorously oxidized. The data strongly suggest that octanoate was oxidized only by those cells which were unruptured, since the granules obtained by washing the water homogenates were completely inactive.

The difficulties in assaying for fatty acid oxidation are thus apparent. Unless a cytolysis quotient is determined and the Q_{O_2} on fatty acid corrected back to 100 per cent whole cells, no quantitative data can be secured. It is difficult to prove that such a correction would be valid. However, it is of interest to see how great the Q_{O_2} on fatty acid is, even when uncorrected, since no such attempts have been made by previous investigators. From the data in Table III, assuming a water content of 70 per cent in fresh

*The "cytolysis quotient" is based upon the succinate oxidation with and without added cytochrome. Its significance is not altogether certain, but its objectivity is emphasized here by the fact that the succinate oxidation data in bold-face type are in nearly perfect agreement.

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*The data do not, of course, eliminate the possibility that 100 per cent of the cells were whole but had lost enough cytochrome c to make the succinoxidase activity 75 per cent of the maximum.

### Table III

*Inability of Hypotonic Liver Homogenates to Oxidize Octanoate*

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Cytochrome</th>
<th>Succinate oxidation</th>
<th>Cytolysis quotient*</th>
<th>Octanoate oxidation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+</td>
<td>72.4</td>
<td>96.0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>3.0</td>
<td>96.5</td>
<td>0</td>
</tr>
<tr>
<td>Washed residue from hypotonic</td>
<td>+</td>
<td>28.8</td>
<td>24.4</td>
<td>66.5</td>
</tr>
<tr>
<td>homogenate</td>
<td>-</td>
<td>53.6</td>
<td>25.8</td>
<td>60.6</td>
</tr>
<tr>
<td>Whole isotonic homogenate</td>
<td>+</td>
<td>71.0</td>
<td>96.0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>53.6</td>
<td>24.4</td>
<td>66.5</td>
</tr>
</tbody>
</table>

* The "cytolysis quotient" is based upon the succinate oxidation with and without added cytochrome. Its significance is not altogether certain, but its objectivity is emphasized here by the fact that the succinate oxidation data in bold-face type are in nearly perfect agreement.

2 The data do not, of course, eliminate the possibility that 100 per cent of the cells were whole but had lost enough cytochrome c to make the succinoxidase activity 75 per cent of the maximum.
liver (16), the uncorrected octanoate $Q_O$ would be 13.3 in comparison with the succinate $Q_O$ of 72.4. However, if this figure were corrected for the degree of cytolysis and the losses due to washing, the figure would be $13.3 \times 72.4 / 35.6$, or 27.0, which is about one-third as large as the succinate $Q_O$, and is 3 to 4 times as high as the $Q_O$ of a liver slice with glucose (16). It must also be borne in mind that the observed rate is not necessarily the potential rate, since no activators other than ATP were added to the system.

The data in Table III show that active octanoate oxidation did not occur in preparations which had been washed after hypotonic treatment, and the cytolysis quotients in these inactive preparations invariably indicated loss of cell structure. Furthermore, the data in Table II show that hypotonicity was detrimental, again suggesting the need for cell structure. But if the enzyme system per se were inhibited by hypotonicity, this data would not be conclusive, and if the system included soluble proteins, these would surely be washed away from the laked cells, while they would probably not escape from the whole cells. The use of unwashed water homogenates is complicated by the endogenous respiration, and also by the presence of calcium ions. Experiments in which washed isotonic homogenates were frozen and thawed showed that the cells were laked, on the basis of the cytolysis quotients, and concomitantly the ability to oxidize octanoate was lost. However, this is not conclusive, since freezing might damage the octanoate system inasmuch as it has been shown to damage the oxidative phosphorylation previously studied (13).

What appears to be unimpeachable evidence was obtained by carefully washing aliquots of an isotonic homogenate, and then suspending one of the final residues in distilled water to lake the washed cells, while the other aliquot was suspended in saline as before. The laked cells were added to Warburg flasks containing enough sodium chloride to make the final reaction mixture optimum for both laked cells and whole cells. The laked preparations gave results which were nearly always inferior to the washed cells, both in rate and duration of oxygen uptake, and the data were not satisfactory until potassium ions were included in the final reaction mixture. This was done in order to compensate for losses which may have occurred during the washing in isotonic sodium chloride, to compensate for the decrease in $K^+$ concentration due to dispersal of cell contents upon lysis (17), and because Lehninger's reaction mixture (12) had included $K^+$ ions. Data from a single experiment are given in Table IV to show the effect of both $K^+$ and cytochrome $c$ in both whole cell and laked cell preparations which have been washed, with cytolysis quotients determined on the basis of the succinoxidase system. This experiment has been repeated a number of times in this exact pattern, with the same excellent
agreement between the rates of octanoate oxidation in laked preparations as compared with the whole cell preparations, when both cytochrome c and K+ ions are present in the reaction mixture. It is noteworthy that cytochrome c is shown to stimulate octanoate oxidation when laked cells are used but not when whole cells are used, thus explaining the data in Table I. The data in Table IV are apparently the first demonstration of the participation of cytochrome c in the ATP-activated oxidation of octanoate. We have encountered a number of special situations, to be reported elsewhere, in which washed cells will oxidize octanoate but laked aliquots are inactive.

**Table IV**

Octanoate Oxidation in Washed Rat Liver Cells Following Lysis

Succinate oxidation was measured as previously described (7), with enzyme preparations equivalent to 20 mg. of fresh liver. Octanoate oxidation was measured with reaction mixtures which contained phosphate, malonate, octanoate, adenosine triphosphate, and cytochrome, as in Table I, plus 0.2 ml. of 0.2 M MgCl₂ and water to make 3.0 ml. in the final volume. The washed cells were added as 0.5 ml. of a 20 per cent suspension in saline, and the laked cells were added as 1.0 ml. of a 10 per cent suspension in distilled water. Sodium chloride was added as 0.2 ml. of 0.42 M solution, and K⁺ ions were added as 0.1 ml. of 0.42 M of KCl substituted for an equivalent amount of NaCl. Oxygen uptake reported on the basis of the best two 5 minute readings.

<table>
<thead>
<tr>
<th>Washed cells</th>
<th>K⁺</th>
<th>Cytochrome</th>
<th>Succinate oxidation</th>
<th>Cytolysis quotient</th>
<th>Octanoate oxidation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>−</td>
<td>+</td>
<td>24.2</td>
<td>44</td>
<td>37.8</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>−</td>
<td>43.5</td>
<td></td>
<td>43.5</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td>42.7</td>
</tr>
<tr>
<td>Laked</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>−</td>
<td>−</td>
<td>0</td>
<td>100</td>
<td>48.3</td>
</tr>
<tr>
<td></td>
<td>−</td>
<td>+</td>
<td></td>
<td></td>
<td>8.1</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>−</td>
<td>46.6</td>
<td></td>
<td>31.0</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td>8.7</td>
</tr>
</tbody>
</table>

with or without added K⁺ ions. In other cases, the laked cells oxidize octanoate nearly as well in the absence of added K⁺ as in its presence. In general, there must be some activity with Na⁺ ions only, in order for K⁺ ions to exert an effect. It seems not unlikely that the action of potassium is indirect, and since Lehninger has reported that DPN³ is a component of the system (12), it may be a deficient component in some of the inactive preparations encountered. With the technique for demonstrating octanoate oxidation in laked cell preparations thoroughly established, it was possible to test some of the questions raised in the preceding paragraph.

³ Diphosphopyridine nucleotide.
It was found that the enzyme system was sensitive to hypotonic media and that data analogous to that in Table II were obtained with the laked washed cell preparations, which were in addition somewhat more sensitive to hypertonic media than were the whole cell preparations. Furthermore, the laked cell preparations lost their activity upon freezing (in liquid air), showing that this type of experiment could not be used to test the rôle of cell structure in octanoate oxidation. The conclusion that fatty acid oxidation does not require cell structure is thus based upon the data in Table IV.

Microscopic examination of the preparations was carried out with about 9 volumes of Loeffler's methylene blue (alcoholic) to 1 volume of preparation. Nuclei are stained dark blue, nucleoli very dark blue, and cytoplasmic granules light blue. In unwashed homogenates almost no conclusions could be drawn, due to the masking of whole cells by adhering cytoplasmic granules. Even in the washed isotonic preparations this effect was noticeable. However, in the latter it was easier to discern chains of liver cells, rather uniform in size, about 3 to 4 times as long as they were broad, though the cell membranes did not stand out as smooth boundaries. On the other hand, some whole cells could be seen even in water homogenates. The microscopic examination provided no reliable guide as to the extent of cytolysis.

DISCUSSION

Whether Leloir and Muñoz or Lehninger have obtained oxidation of higher fatty acids independent of cell structure cannot be stated definitely at this time, since they have not determined "cytolysis quotients" by the succinoxidase test or by some equally objective measurement. All of the published data seem to emphasize the importance of avoiding conditions which would logically be expected to produce cytolysis, and in some of the data the omission of cytochrome c from the reaction mixture, or the lack of an increased oxygen uptake when cytochrome was added, strongly suggests that whole cells were being used. The data presented in Table IV, on the other hand, seem to provide clear cut proof that octanoate oxidation, at least, is possible in the absence of cell structure. The chief point to be emphasized is that the cells must not be laked prior to the washing procedure in order to obtain activity in the present reaction mixture.

One of the striking new developments in Lehninger's work is the ATP activation of fatty acid oxidation. We have completely confirmed this observation, and although our experimental techniques deliberately deviated considerably from Lehninger's, we adhered quite closely to the principles which his work established and used most of the components of the reaction mixture at the concentrations he found to be optimum. Thus it
seems likely that, whether or not the previous work was actually done with cell-free preparations, fatty acid oxidation would have been obtained if the washed preparations had been laked before they were tested.

The ATP activation of octanoate oxidation in preparations of "whole" (i.e., unlaked according to the cytolysis quotient) cells was rather unexpected, since phosphorylated coenzymes and intermediates have generally been found to penetrate whole cells very poorly in comparison with the non-phosphorylated derivatives; cf., for example, the comparison of thiamine with cocarboxylase in brain cell suspensions (18), in which thiamine was superior to cocarboxylase in minced tissue while the reverse was true in more finely ground ("broken cell") suspensions. It is unclear whether the latter would have been classified as whole or laked by the succinoxidase technique. More recently, isotonic homogenates have given marked responses to the addition of phosphorylated cofactors and intermediates (19), although the preparations were not washed and therefore must also have contained some broken cells. Nevertheless the water homogenates seemed superior since, when properly fortified, they gave somewhat greater activity than the isotonic homogenates, possibly "because there is no permeability barrier between the [added] coenzymes and enzymes in disrupted cells" (19). It seems possible that the permeability of the washed cells has been altered by the washing to the extent that the ATP molecule can get in, while the much larger cytochrome molecule cannot get out.

The significance of the present study is not simply whether fatty acids can be oxidized in the absence of cell structure, particularly since the essential means of securing this result were fairly well established by the work of Leloir and Muñoz and of Lehninger. However, it may be anticipated that other metabolic reactions which have hitherto required intact cells may be attacked with the object of demonstrating the reaction in cell-free extracts. The present study may serve to show how the homogenate technique may be employed to gain this end and to help establish the conclusion.

Although assays for fatty acid oxidase are not yet possible on a strictly quantitative direct basis, considerable information can be obtained by means of the washed preparations if satisfactory succinoxidase assays with and without cytochrome c can be obtained on the original homogenate as well as on the washed preparation.

**SUMMARY**

1. The variation in the extent of cell rupture in "isotonic," "hypotonic," and "water" homogenates is emphasized, and the inadequacy of microscopic examination is pointed out.

2. An objective test, which measures the loss of cytochrome c from cells
in liver homogenates, is utilized to determine a "cytolysis quotient" in the various types of homogenates.

3. The activation of fatty acid (C₃) oxidation by adenosine triphosphate in the presence of washed cells prepared from isotonic rat liver homogenates has been confirmed.

4. Washed rat liver cells which were laked with distilled water gave fatty acid oxidase activity equal to that of isotonic control suspensions, when the reaction media contained K⁺ ions and cytochrome c in addition to the components used with the suspensions.

5. Water homogenates made isotonic and washed with isotonic saline gave residues which would oxidize succinate but not octanoate under the conditions which permitted oxidation in the laked washed cell preparation.

6. Due to the above considerations, the assay of fatty acid oxidase activity cannot be made by direct means as yet.

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BIBLIOGRAPHY

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