Quantitative Histochemical Resolution of the Oxidation-Reduction and Phosphate Potentials within the Simple Hepatic Acinus

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Glycerin-3-P, commonly known as α-glycerophosphate and dihydroxyacetone phosphate (DHAP) were measured in well defined microscopic samples of the simple liver acinus allowing a comparison of the glycerin-3-P/dihydroxyacetone-P ratios of Zones 1 and 3 as a measure of the free NAD+/NADH ratio, and we applied established micro-methods to determine the ATP/ADP × P, quotients (7) as a measure of the P-potential in these same microscopic areas of the liver acinus. The results reported here confirm the previously observed (5) apparent uniformity of the intra-acinar oxidation-reduction potential and suggest that a similar homogeneity might exist for the intra-acinar P-potential.

Materials and Methods

Reagents—Enzymes were obtained from Boehringer or Sigma. Alcohol dehydrogenase (Sigma) used for cycling NAD (8) contained less than 5 × 10⁻² mol of βNAD per mol of alcohol dehydrogenase. Lactate dehydrogenase was dialyzed for 48 h against 2 M ammonium acetate before use. Substrates and cofactors were obtained from Sigma.

Tissue Preparation and Microscopic Sampling (5, 9)—Fed male Sprague-Dawley rats (280-300 g) were anesthetized with ether. The abdomen was opened and a small piece of liver was excised and immediately plunged into Freon-12 cooled to the temperature of liquid N₂. In some rats, the liver was removed from the animal and held on a piece of aluminum foil for a desired length of time to cause ischemia before freezing. The samples were mounted and sectioned at -22 to -25 °C. Adjacent sections were freeze-dried for analysis or placed on glass slides to be stained for succinate dehydrogenase. The succinate dehydrogenase staining patterns served as a guide to differentiate Zones 1 and 3 of the simple liver acinus. With a camera lucida attachment to a dissecting microscope, permanent dissecting maps were drawn of the succinate dehydrogenase staining patterns of sections adjacent to slices to be microdissected. The maps were superimposed on the adjacent freeze-dried sections allowing specific dissection of samples from Zones 1 and 3. For each animal, 10 to 20 samples were obtained from each zone usually dissected from one randomly chosen liver slice. Four to 9 rats were analyzed at each time point for each metabolite or cofactor. Samples were weighed on quartz fiber fishpole balances (10). Microdissected liver samples were placed in wells of Teflon racks and were stored at -20 C under vacuum until analysis.

Analytical Procedures—ATP, ADP, Pi, NADH, total NAD, total NADP, α-glycerophosphate, and dihydroxyacetone phosphate were analyzed fluorimetrically by oil well techniques. The desired sensitivities were obtained using enzyme cycling methods for NAD(H) and NADP(H). The principles and the analytical details of most assays are given in previous publications (5, 7, 8, 10). Microassays for α-glycerophosphate and dihydroxyacetone-P are new and are given in detail.

The enzymatic assay of α-glycerophosphate was carried out with glycerin-3-phosphate dehydrogenase, triosephosphate isomerase, and glyceraldehyde-3-phosphate dehydrogenase according to the following equations:

\[
\text{aGOP} + \text{NAD}^+ \xrightarrow{\text{GDPDH}} \text{DHAP} + \text{NADH} + \text{H}^+ \quad (1)
\]

The abbreviations used in equations are: aGOP, α-glycerophosphate or glycerin-3-P; GDPDH, glycerin-3-phosphate dehydrogenase; DHAP, dihydroxyacetone phosphate; TPI, triosephosphate isomerase; GAP, glyceraldehyde-3-P; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; 3-PGA, 3-P-glyceric acid.

The exploration of the hepatic oxidation-reduction state and phosphorylation potential is complicated by the zonal organization of the simple liver acinus (1, 2). The acinar module is attuned to trans-hepatic gradients of oxygen, nutrients, and hormones and metabolites which manifest themselves strikingly in differentials of enzyme activities and densities of cellular organelles, usually described as intralobular functional and structural diversity or heterogeneity.

Biophysical measurements which are based on fluorescence of reduced pyridine nucleotides (NAD(P)ₐₒₑₙ) and oxidized flavine adenine nucleotides (FADₑₒₑₙ) have suggested that parenchymal cells located in the arterial portion of the liver acinus (Zone 1) are physiologically in a more oxidized state than those located in the venous portion (Zone 3) (3, 4). The ratio of oxidized FAD to reduced NAD(P) (FADₑₒₑₙ/NAD(P)ₐₒₑₙ) found to be larger in Zone 1 than in Zone 3. However, previous quantitative histochemical zonal analysis of total NAD⁺/NADH and total NADP⁺/NADPH indicated that the oxidation-reduction potential of the liver acinus might be uniform (5). The interpretation of those microchemical data is open to dispute because of the undefined state of binding of the pyridine nucleotides.

The design of cellular energy metabolism is such that alterations of the oxidation-reduction state find an expression in the phosphorylation potential as well (6). The understanding of the present topic would therefore be significantly advanced if the phosphate potentials (ATP/ADP × P) of Zones 1 and 3 of the hepatic acinus were known.

With this background in mind we developed methods for the estimation of α-glycerophosphate and dihydroxyacetone phosphate in well defined microscopic samples of the simple liver acinus allowing us to compare α-glycerophosphate/dihydroxyacetone-P ratios of Zones 1 and 3 as a measure of the free NAD⁺/NADH ratio, and we applied established micro-methods to determine the ATP/ADP × P, quotients (7) as a measure of the P-potential in these same microscopic areas of the liver acinus. The results reported here confirm the previously observed (5) apparent uniformity of the intra-acinar oxidation-reduction potential and suggest that a similar homogeneity might exist for the intra-acinar P-potential.

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Dihydroxyacetone phosphate was oxidized to dihydroxyacetone-P with NAD'. Dihydroxyacetone-P was assayed using reactions 2 and 3. Arsenate was used to pull the reaction toward the right. Two mol of NADH is formed/mol of a-glycerophosphate and 1/mol of dihydroxyacetone-P.

The weighed samples are placed in oil wells and suspended in 0.15 M phosphate buffer, pH 8.9, containing 0.5 M Tris buffer (pH 8.9), 4 mM mercaptoethanol, 1 mM EDTA, 2 mM sodium arsenate, 50 μM NAD', α-GPDH (20 units/ml), and GAPDH (12 units/ml). Incubate 75 min at 25°C; then add 0.15 μl of 0.3 N NaOH and heat at 75°C for 20 min. Add 5 μl NAD-NADH cycling reagent (100 μM Tris buffer, pH 8.0; 300 mM ethanol; 2 mM mercaptoethanol; 2 mM oxalacetate; 0.02% BSA; ADH, 5 units/ml; MDH, 2 units/ml). Incubate at 25°C for 1 h (10,000 cycles/h). Add 3 μl of 0.3 N NaOH after cycling and heat at 100°C for 10 min.

<table>
<thead>
<tr>
<th>Step</th>
<th>Protocol</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>First step</strong></td>
<td>α-Glyerin phosphate</td>
</tr>
<tr>
<td><strong>Second step</strong></td>
<td>Add 0.15 μl assay reagent I containing 0.5 M Tris buffer (pH 8.9), 4 mM mercaptoethanol, 1 mM EDTA, 2 mM sodium arsenate, 50 μM NAD', α-GPDH (20 units/ml), and GAPDH (12 units/ml). Incubate 75 min at 25°C; then add 0.15 μl of 0.3 N NaOH and heat at 75°C for 20 min.</td>
</tr>
<tr>
<td><strong>Third step</strong></td>
<td>Add 5 μl NAD-NADH cycling reagent (100 μM Tris buffer, pH 8.0; 300 mM ethanol; 2 mM mercaptoethanol; 2 mM oxalacetate; 0.02% BSA; ADH, 5 units/ml; MDH, 2 units/ml). Incubate at 25°C for 1 h (10,000 cycles/h). Add 3 μl of 0.3 N NaOH after cycling and heat at 100°C for 10 min.</td>
</tr>
<tr>
<td><strong>Fourth step</strong></td>
<td>Transfer total volume from oil wells (8.45 μl) to 50 μl indicator reagent in fluorometer tubes (10 × 75 mm) (50 mM 2-amino-2-methyl-propanal buffer, pH 9.9; 200 μM NAD', 12 units/ml MDH, and 0.4 unit/ml GOT). Incubate at 25°C for 45 min. Add 50 μl of 0.5 M phosphate buffer (pH 12.0) and heat at 60°C for 15 min. Add 1 ml of 7 N NaOH containing 10 mM imidazole and 0.03% H2O2 to each tube, heat at 60°C for 20 min, cool, and read in fluorometer.</td>
</tr>
</tbody>
</table>
Redox and Phosphate Potentials in Simple Hepatic Acinus

Zonal Analysis of Pyridine Nucleotides—The levels of total pyridine nucleotides both of NAD and NADP were similar in Zones 1 and 3 (Table III) and did not change much, if at all, during ischemia. About 10% of the NAD was present in the reduced form. There was an indication (not achieving statistical significance) that the relative fraction of reduced NAD was lower in Zone 3 than in Zone 1 and that the ischemia-induced rise of NADH occurred more readily in Zone 1. The data could suggest that Zone 1 is more sensitive to ischemia than Zone 3. Most of the NADP occurred in the reduced form and there was no zonal difference under any condition. Notable is the tendency of NADPH levels to fall after 160 s of ischemia. However, NADPH seemed to decline equally in both zones.

Zonal Analysis of α-Glycerophosphate and Dihydroxyacetone-P—The levels of α-glycerophosphate and dihydroxyacetone-P in microscopic samples from Zones 1 and 3 of the simple liver acinus in normal and ischemic states are given in Table IV. Calculated values of α-glycerophosphate/dihydroxyacetone-P and free NAD'/free NADH ratios and of oxidation-reduction states (E') in these areas are presented. The results clearly show that the levels of α-glycerophosphate and dihydroxyacetone-P in Zone 1 and Zone 3 are the same in control, 30–60 s ischemia, or 150-s ischemia. Accordingly, the α-glycerophosphate/dihydroxyacetone-P and free NAD'/ NADH ratios and the oxidation-reduction potentials are indistinguishable in the two zones. Ischemia of about 2.5 min

![Graph](image_url)

**FIG. 1. Microassay of glycerein-3-P and dihydroxyacetone phosphate, 0.5–2.5 × 10⁻⁶ mol of dihydroxyacetone-P and 2–10 × 10⁻⁶ mol of α-glycerophosphate are incubated with respective assay reagents in the oil wells for 75 min at 25 °C. NADH formed in these reactions are enzymatically cycled in oil wells for 1 h at 25 °C. The amount of malate formed in this process is measured fluorometrically. Malate formed is proportional to the amounts of α-glycerophosphate or dihydroxyacetone-P used for assay. Details of the methods are given under "Materials and Methods."**

### Table II

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Added (× 10⁻⁶ mol)</th>
<th>Found ± S.E. (× 10⁻⁶ mol)</th>
<th>Recovery or conversion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycerin 3-phosphate</td>
<td>10.3 (7)</td>
<td>9.73 ± 0.24</td>
<td>94.5</td>
</tr>
<tr>
<td></td>
<td>5.16 (8)</td>
<td>5.03 ± 0.16</td>
<td>97.7</td>
</tr>
<tr>
<td></td>
<td>2.56 (8)</td>
<td>2.55 ± 0.08</td>
<td>100.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dihydroxyacetone phosphate</td>
<td>2.16 (5)</td>
<td>2.02 ± 0.06</td>
<td>93.1</td>
</tr>
<tr>
<td></td>
<td>1.08 (5)</td>
<td>1.03 ± 0.04</td>
<td>95.5</td>
</tr>
<tr>
<td></td>
<td>0.54 (6)</td>
<td>0.53 ± 0.02</td>
<td>98.2</td>
</tr>
</tbody>
</table>

*Numbers of experiments done are given in parentheses. A 1-2-μl solution containing standard α-glycerophosphate or dihydroxyacetone-P were analyzed in oil wells and results were compared against standard NADH. Details of analytical procedures are given under "Materials and Methods."

### Table III

| Intralobular distribution of pyridine nucleotides in normal and ischemic liver |
|-----------------------------|-----------------------------|-----------------------------|-----------------------------|
| NADH as % of total | NADPH as % of total |
| mmol/kg dry tissue | nmol/kg dry tissue |

**Control**

<table>
<thead>
<tr>
<th>Zone</th>
<th>NADH</th>
<th>NADPH</th>
<th>Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zone 1</td>
<td>0.343 ± 0.043</td>
<td>3.54 ± 0.10</td>
<td>10.0</td>
</tr>
<tr>
<td>Zone 3</td>
<td>0.278 ± 0.028</td>
<td>3.54 ± 0.13</td>
<td>8.0</td>
</tr>
</tbody>
</table>

**30–65 s of ischemia**

<table>
<thead>
<tr>
<th>Zone</th>
<th>NADH</th>
<th>NADPH</th>
<th>Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zone 1</td>
<td>0.409 ± 0.041</td>
<td>3.46 ± 0.16</td>
<td>12.0</td>
</tr>
<tr>
<td>Zone 3</td>
<td>0.287 ± 0.038</td>
<td>3.20 ± 0.15</td>
<td>9.0</td>
</tr>
</tbody>
</table>

**150–160 s of ischemia**

<table>
<thead>
<tr>
<th>Zone</th>
<th>NADH</th>
<th>NADPH</th>
<th>Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zone 1</td>
<td>0.430 ± 0.060</td>
<td>3.20 ± 0.19</td>
<td>12.0</td>
</tr>
<tr>
<td>Zone 3</td>
<td>3.322 ± 0.064</td>
<td>2.76 ± 0.23</td>
<td>4.0</td>
</tr>
</tbody>
</table>

*Numbers of different liver samples analyzed is given in parentheses.
*Significantly different from control, p < 0.05.
*Significantly different from Zone 1, p < 0.05.

### Table IV

<table>
<thead>
<tr>
<th>Glycerein 3-phosphate (α-GOP), dihydroxyacetone phosphate (DHAP), and oxidation-reduction state in simple liver acinus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Period of ischemia</td>
</tr>
<tr>
<td>-------------------</td>
</tr>
<tr>
<td>0 (control)</td>
</tr>
<tr>
<td>0 (control)</td>
</tr>
<tr>
<td>30–60</td>
</tr>
<tr>
<td>150–160</td>
</tr>
</tbody>
</table>

*25–50-μg samples (10 or more) from each zone of each liver were analyzed in oil wells.
*P value was calculated using "t" test method.
*Numbers of different livers analyzed are given in parentheses.
Obviously, the present approach falls short in applying those state of the art methods at the microscopic level. Clearly, though, the present work markedly advances our knowledge similar to reaching a campsite on a climb to a mountain top. The choice of the $\alpha$-glycerophosphate/dihydroxyacetone-P couple for measuring the cytoplasmic free NAD\(^+\)/NADH ratio was a compromise made because of the inordinate obstacle of lactate contamination hampering lactate histochemistry. The bound NAD\(^+\)/NADH ratio was reinvestigated to assess whether it can serve as a rough indicator of intralobular oxidation-reduction state alterations.

The results of zonal analysis of the NAD\(^+\)/NADH ratios both of the bound and free forms and of the calculated \(E^0\) values of the control liver acinus in Zones 1 and 3 are similar to those reported by Veech et al. (15) and Bücher (11) for the freeze-clamped control livers as a whole. Thus, the $\alpha$-glycerophosphate/dihydroxyacetone-P ratio appears to be an oxidation-reduction indicator comparable to the lactate/pyruvate ratio. Bound NADH of microscopic samples increases with ischemia, thus showing some potential for a histochemical oxidation-reduction state indicator.

The direct zonal analysis of the hepatic ATP/ADP × P\(_i\) ratios can be assumed to provide data closely approximating those calculated from the glyceraldehyde-3-P and 3-P-glycerate kinase reactions (see above equation) (14). This was shown by Veech et al. (16) for fed and starved rats maintained on a high carbohydrate diet. However, greater divergence between the directly measured and the indirectly calculated values was seen by Greenbaum et al. (17) in rats given a high fat diet or made diabetic with alloxan. Those exceptions warrant caution in overinterpreting the quantitative histochemical results. Nevertheless, the level of ATP in the two zones is comparable with results of global analysis in freeze-clamped liver (18) and is slightly greater than that from 48-h fasted or perfused rat livers (19). The levels of ADP and P\(_i\) in our preparation are slightly higher than those reported for freeze-clamped tissue and could reflect a brief delay in freezing or could be due to using alkalai to extract the tissue. This could explain why the calculated phosphate potentials in our control samples are somewhat lower than those obtained by macroanalysis. Despite this, prolonged ischemia results in the appropriate changes in the levels of ATP, ADP, and P\(_i\), without a zonal gradient.

While our data agree well with other biochemical determinations, the results are contrary to the data from low temperature fluorescent scanning (3, 4). The discrepancy is almost certainly methodologic. The depth of the scan using the fluorescent probe may be greater than 50 \(\mu\)m while our free hand sections are routinely sampled at 13–15 \(\mu\)m. Mapping techniques using microdissection allow venous lakes and other bloody areas to be excluded while an automated scanning probe may record variable degrees of fluorescent quenching by blood and other chromophores. A possible gradient of total FAD declining in centroacinar direction and incongruent with NAD pools could feign the oxidation-reduction ratio gradient as measured by fluorescence scanning. That such a gradient of total FAD could exist is not unlikely in view of the lower mitochondrial count of hepatocytes in Zone 3 (1, 2). Finally, it is thought that surface fluorescence measurements respond primarily to the mitochondrial NADH and FAD, whereas the present measurements provide chiefly an indication of the cytoplasmic adenosine nucleotide and pyridine nucleotide systems. Quantitative histochemical zonal analysis has to be extended to include the measurement of indicators of the mitochondrial oxidation-reduction and phosphate potentials in order to provide a more adequate basis for comparison.

**Phylosiological Implications of Intralobular Constancy of**
Oxidation-Reduction and Phosphate Potentials—The ratios of α-glycerophosphate/dihydroxyacetone-P in Zone 1 and Zone 3 of simple liver acinus indicate constancy of the free NAD+/NADH ratio in these areas. The absence of any difference in the levels of bound NADH and NAD+ in these hepatic areas in barbiturate anesthetized animals was reported earlier (5) and was confirmed here with samples from etherized rats. This similarly suggests intralobular constancy of the hepatic oxidation-reduction state. Even though an oxygen gradient across Zones 1 and 3 in simple liver acinus was reported to be present (20, 21), a difference in the oxidation-reduction state between these zones could be expected only if one area were substantially depleted of oxygen since the $K_a$ for oxygen of cytochrome oxidase is very low (22). The present result of an unaltered oxidation-reduction state in liver acinus until after 60 s of ischemia suggests a substantial oxygen reserve of the tissue. The effects of 150 s of ischemia are manifested equally in Zones 1 and 3 of liver acinus. The α-glycerophosphate/dihydroxyacetone-P and free NAD+/NADH ratios and also the $E^*$ values change in a typical fashion with similar rates in both zones.

Both the measurements of total NADH and of the phosphate potential (Tables III and V) provide an indication that Zone 1 is more susceptible to ischemia than Zone 3. This could be due to a greater metabolic rate of hepatocytes in Zone 1 of the acinus, which finds other experimental support. Studies of Zone 1 sinusoids suggest they contain a lesser volume fraction of blood but greater surface to volume ratio than Zone 3 sinusoids (1, 2).

The actual numerical values and the intralobular constancy of hepatic oxidation-reduction and P-potentials under basal conditions indicates that thermodynamically speaking the hepatic cells, both of Zone 1 and 3, are in or close to an equilibrium state, and that nutrient and oxygen supply is in excess of energy demands even at the venous end of the capillary bed. It also seems that the intralobular constancy of the pool sizes and of the concentration ratios of the adenylates and the pyridine nucleotides makes it unlikely that zonal patterns of these metabolic coupling agents could explain the purported metabolic heterogeneity of intralobular zones (23, 24).

Pathological Implications of Intralobular Constancy of P-Potential and Oxidation-Reduction State—The present data could be uniquely relevant for the pathogenesis of alcoholic liver disease. The pathogenetic factors that are responsible for the centrolobular origin of the alcoholic disease as manifest in fatty liver, hepatitis, and cirrhosis are largely unknown (25, 26). However, there is evidence that the intralobular oxygen gradient could be of major importance for our understanding of the selective toxicity of ethanol for hepatocytes of Zone 3 (27, 28). This is illustrated by the finding that in dogs centrolobular necrosis and fibrosis occur after ligation of the hepatic artery, which provides about 40% of the oxygen supply to the liver in this species (29, 30). A recent study with the isolated perfused rat liver illustrates that circumscribed oxygen deficiency of Zone 3 caused by low perfusion flow can lead to circumscribed centrolobular cell damage (31). This suggests that under pathological conditions, the oxygen buffer of the blood may have too low a capacity. Since in the alcoholic, the oxygen consumption of the liver tissue can be as much as doubled, there may be a reduction in oxygen tension in Zone 3 sufficient to impair cell function or even cause cell death (27, 28). Various contributing factors have been discussed; for example, involvement of thyroid hormones and catecholamines and of anemia frequently seen in alcoholics. It is noteworthy that centrolobular liver necrosis and fibrosis have been reported to occur in 50–60% of humans that died of thyrotoxicosis prior to the introduction of modern therapies (32, 33). The hypermetabolic state known to exist in liver tissue in alcoholics could therefore be at least a contributing factor in the pathogenesis of alcoholic liver disease.

The other consistent finding in animals that are chronically fed a liquid diet containing 35% of the caloric intake in the form of ethanol is an impressive fall of the phosphorylation potential (ATP/ADP × P) from about 400 $\text{m}^{-1}$ to 250 $\text{m}^{-1}$ (34, 35). It seems to be crucial for our understanding of this failing energy metabolism to study its temporal development and its intralobular location in experimental animals and, of course, find out whether the impairment of the phosphorylation potential exists in the alcoholic man’s liver and if so to explore its ontogeny and possible intrahepatic selectivity. The effects of ethanol on levels of reduced and oxidized pyridine nucleotides in liver of experimental animals are similarly impressive (36). Both acute and chronic ethanol treatment causes a dramatic fall of the oxidation-reduction ratios of total pyridine nucleotides and of the calculated ratios of free nucleotides. Here again, it seems crucial for our understanding of pathogenesis to explore the temporal development of these alterations and their intralobular locations. In view of our own data of intralobular homogeneity of the phosphorylation potential and the oxidation-reduction potential in normal liver tissue, it seems important to investigate whether ethanol affects the oxidation-reduction and/or phosphate potentials of the hepatic Zone 3 selectively.

REFERENCES

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