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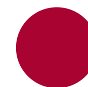

The intralobular distribution of oxidized and reduced pyridine nucleotides in the liver of normal and diabetic rats.

Quistorff, Bjørn; M. Matschinsky, Franz; S. Hintz, Carol; Reichlmeier, Klaus

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THE INTRALOBULAR DISTRIBUTION OF OXIDIZED AND REDUCED PYRIDINE NUCLEOTIDES IN THE LIVER OF NORMAL AND DIABETIC RATS

Franz M. Matschinsky
Carol S. Hintz

Department of Pharmacology
Washington University
St. Louis, Missouri

Diabetes Research Center
University of Pennsylvania
Philadelphia, Pennsylvania

Klaus Reichlmeier

Sandoz Institute of Basic Medical Research
Basel, Switzerland

Bjoern Quistorff
Britton Chance

Johnson Research Foundation
University of Pennsylvania
Philadelphia, Pennsylvania

I. INTRODUCTION

An important design feature of the liver as a biochemical factory is the lobular arrangement of the parenchymal cells (1-3). This is vividly illustrated by an example from the work of Rappaport (3) (Figure 1). The acinus rather than the classical lobule as outlined by Kiernan (1) is the functional unit of the liver in Rappaport's view. He describes the liver aci-

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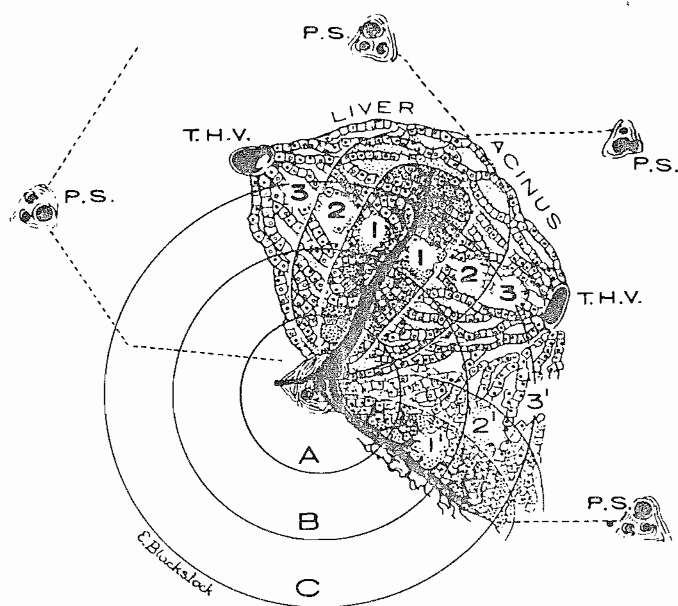

FIGURE I. The blood supply of the simple liver acinus and the zonal arrangement of cells.

The acinus occupies adjacent sectors of neighboring hexagonal fields. Zones 1, 2, and 3, respectively, represent areas supplied with blood of first, second, and third quality with regard to oxygen and nutrients. These zones center about the terminal afferent vascular branches, terminal bile ductules, lymph vessels, and nerves and extend into the triangular portal field from which these branches crop out. Zones 1', 2', and 3' designate corresponding areas in a portion of an adjacent acinar unit. In zones 1 and 1' the afferent vascular twigs empty into the sinusoids. The circles B and C indicate peripheral circulatory areas as commonly described around the "periportal" area A. P.S. = portal space; T.H.V. = terminal hepatic venules. From Rappaport et al. (3)

nus as follows: "The simple liver acinus is a small parenchymal mass, irregular in size and shape, arranged around an axis of a terminal portal venule, hepatic arteriole, bile ductule, lymph vessels and nerves which grow out together from

a small triangular portal field. The simple liver acinus lies between two (or more) terminal hepatic venules (central veins) with which its vascular and biliary axis interdigitates. In a two dimensional view it occupies sectors only of two adjacent hexagonal fields." Blood flow in sinusoids of functional units is apparently concurrent and there may be little opportunity within the unit structures for diffusible materials to short circuit the vascular pathway (4). This arrangement is ideal for the liver functions of storage and supply of fuels. As a result of these structural features lobular concentration gradients of diffusible substances can be expected to exist along the sinusoids and the columns of liver cells. Profiles of diffusible substrates (e.g. FFA and oxygen) and of products (e.g. ketone bodies and CO_2) of liver metabolism are believed to decline and rise, respectively, along the length of the sinusoids. The design of the hepatic microcirculation is complemented by the structural, biochemical, and pharmacological diversity of the parenchymal cells of zones 1, 2, and 3 of the acinus [see (3)]. With staining histochemical methods greater succinic dehydrogenase (5), cytochrome oxidase (6) and lactic dehydrogenase activities (7) were demonstrated in zone 1 as compared to zone 3. Glutamic dehydrogenase (7), β- hydroxy butyric dehydrogenase (7,8) and isocitric dehydrogenase (7) were, however, found to be more active in zone 3 of the acinus. This general picture of enzyme distribution has been confirmed by results obtained with quantitative histochemistry (9,10). But the results of staining histochemistry and quantitative histochemistry do not agree in all instances. For example, differences of opinions still exist about the distribution of glycogen. Whereas most investigators using some version of a staining technique maintain the view that glycogen is distributed unevenly within a lobule (10) there is little evidence for this concept from quantitative studies (11).

The existence of intralobular fuel and oxygen gradients and the heterogeneity of the enzymatic complement of hepatic parenchymal cells imply that gradients of the redox and of the phosphate potentials might exist within the lobule. The present studies were undertaken to explore this possibility. The emphasis of this report is on the development and use of quantitative histochemical techniques to measure the intralobular distribution of $NADP^+$, NADPH, NAD^+ and NADH. The levels of these cofactors can be measured by specific highly sensitive methods and serve as indicators of the redox potential (12). These quantitative data are compared with results of redox potential measurements with a biophysical procedure discussed in more detail elsewhere in this symposium.

II. METHODOLOGY

A. Quantitative Histochemical Analysis of Pyridine Nucleotides

About 10 years ago a method was described for measuring oxidized and reduced pyridine nucleotides at the microscopic level (14). This method is now applied systematically to liver tissue, with improvements.

Rats weighing 250-300 g were anesthetized with pentobarbital (30 mg/kg i.p.). The liver was exposed and one lobe was frozen between metal blocks cooled with liquid nitrogen. One of these blocks was made of brass fitted with an array of grooves to hold the frozen piece of liver tissue in place, once the brass block was fixed in the microtome for sectioning (see below). The other block was made of aluminum and had a sharp circular cutting edge, 2mm high and of a diameter smaller than the diameter of the brass block. As a result a 2 mm high circular pellet of liver tissue with a flat surface and with a radius of about 1 cm was obtained fixed to a brass holder.

The liver pellet was trimmed to a small square area (5X5 mm) and cryostat sections were cut parallel to the surface. The temperature was maintained at -25 to -27° C. The sections were kept in order in the holes of an aluminum rack and every fourth section was placed on a glass slide and stained for succinoxidase (3). Sections were freeze-dried save the stained ones. Microscopic samples were dissected from areas of the portal tracts (from zone 1 according to Rappaport's nomenclature) and from areas surrounding the central vein (Rappaport's zone 3). This was done using camera lucida drawings of adjacent sections stained for succinoxidase as a guide (for more detail see result section). The samples were weighed with the quartz fiber balance (15) and transferred to an oil well rack made of Teflon (16), and were suspended in small droplets of 0.03 N NaOH with 1 mM cysteine (for details see Table I). The droplets were covered with mineral oil and were then treated either by heating for 20 min at 70° C (for measuring reduced pyridine nucleotides) or kept at 25° C for no longer than 3 min (for measuring total pyridine nucleotides). NADH, NADH plus NAD^+, NADPH, NADPH plus $NADP^+$ were then measured with the method of enzymatic cycling which is specific for NAD (17) or NADP (15). For that purpose the appropriate reagent was added to the alkaline droplet (for details see Table I). After completion of the cycling step (usually one hour) the reaction was stopped by alkalinization

TABLE I. Enzymatic Analysis of NAD and NADP in Microscopic Liver Samples

Substance Measured	Step (1) Tissue Extraction[a]	Step (2) Cycling[a]	Step (3) Indicator Reaction[a]	Sensitivity (femto moles)
NADH	0.5 µl 1mM cysteine HCl in 0.03 N NaOH; 20 min at 70° C.	1 µl cycling reagent[b]; 1 hr at 25° C; stop cycling with 0.5 µl 0.35 N NaOH and 10 min at 100° C.	50 µl malate reagent[c]; 50 min at 25° C; stop reaction with 50 µl 0.6M PO_4 buffer, pH 11.6 and heating for 20 min at 75° C. NADH measured by alkali enhanced fluorescence.	3-15
total NAD	0.5 µl 1mM cysteine HCl in 0.02 N NaOH; 3 min at 25° C.	5 µl cycling reagent[b]; 1 hr at 25° C; stop reaction with 1 µl 0.5 N NaOH and 10 min 100° C.	1 ml malate reagent[c]; 50 min at 25° C; measure native fluorescence of NADH.	30-210
NADPH	0.5 µl 1mM cysteine HCL in 0.03 N NaOH; 20 min at 70° C.	5 µl cycling reagent[d]; 1 hr at 25° C; stop cycling with 1.5 µl 0.33 N NaOH and 10 min at 100° C.	1 ml 6-P-gluconate reagent[e]; 45 min at 25° C; measure native fluoreacence of NADPH.	25-120
total NADP	0.5 µl 1mM cysteine HCl in 0.02 N NaOH; 3 min st 25° C.	5 µl cycling reagent[d]; 1 hr at 25° C; stop cycling with 1.5 µl 0.33 N NaOH and 10 min 100° C.	1 ml 6-P-gluconate reagent[e]; 45 min at 25° C; measure native fluorescence of NADPH.	25-120

a) Steps (1) and (2) are performed under mineral oil in an oil well rack (16) and step (3) in pyrex glass tubes, 10 X 75 mm.

b) The composition of the cycling reagent was 0.2 M Tris-HCl, pH 7.95, 0.03% bovine serum albumin, 3 mM mercaptoethanol, 3 mM oxalacetate, 450 mM ethanol, 41 µg/ml malic dehydrogenase, 445 µg/ml alcohol dehydrogenase [see (17)].

c) The composition of the malate indicator reagent was 50 mM 2-amino-2-methyl-1-propanol-HCl buffer, pH 9.9, 10 mM glutamate, 100 µM NAD^+, 5 µg/ml malic dehydrogenase, 2 µg/ml glutamic-oxalacetic-transaminase [see (15)].

d) The composition of the cycling reagent was 100 mM Tris-acetate buffer, pH 8.0, 5 mM α-ketoglutarate, 1 mM glucose-6-P, 10 mM (NH_4)-acetate, 100 µM ADP, 15 µg/ml G-6-P dehydrogenase, 200 µg/ml glutamic dehudrogenase [see(15)].

e) The composition of the indicator reagent for 6-P-gluconate was 40 mM Tris-HCl, pH 8.37, 0.1 mM EDTA, 30 mM (NH_4)-acetate, 5 mM $MgCl_2$, 30 µM $NADP^+$, 0.5 µg/ml 6-P-gluconate dehydrogenase [see (15)].

For NADH and NADPH measurements the corresponding reduced pyridine nucleotides served as standards. For measurement of total NAD the standard was NAD^+, and for total NADP analysis the standard was NADPH because of the preponderance of NAD^+ and NADPH in tissue, respectively.

and brief boiling and the final product was measured enzymatically [malate for NAD and 6-P-gluconate for NADP (Table I)].

B. The Principle of Low Temperature Fluorescence Scanning

The indirect fluorometric redox potential measurements are based on the following biochemical principle (13,18): oxidized flavoproteins (FP_{ox}) and reduced pyridine nucleotides (PN_{red}) emit fluorescent light at 550 and 450 nm, when irradiated with UV light at 436 or 366 nm, respectively.

The ratio of FP_{ox} fluorescence/PN_{red} fluorescence is, therefore, considered a sensitive indicator of the redox state of certain flavoprotein and pyridine nucleotide-dependent enzyme reactions. It is also believed that the recording of this fluorescence ratio is largely independent of interference by various tissue chromophores, most importantly cytochromes and hemoglobin. The fluorescence is greatly enhanced when the temperature is lowered, i.e. about 10-fold for a delta of 100° C. Therefore, the measurements are made on tissue blocks immersed into liquid nitrogen. Fluorescence of the two redox indicators (FP_{ox} and PN_{red}) is measured with a time sharing fluorometer and high spatial resolution is gained by employing a micro light guide for scanning. This light guide consists of six fibers surrounding a central fiber, the former being used for exitation and the latter for emission measurements. The diameter of these fibers is 80 μ. The spatial resolution of the system is approximately equal to the fiber diameter. Data are acquired with a PDP-11 computer that drives the micro light guide in a rapid scan across the frozen tissue, usually an array of 2,500 points - 50 on each side of a square field. The computer dwells a fraction of a second at each position, enabling the FP_{ox}/PN_{red} measurement to be made and the transfer of the information to the computer memory to be accomplished. The results are then projected on a TV screen and photographed.

III. RESULTS

A. A Simple Procedure for Sample Identification

Identification of the classical microscopic structures in thin unstained freeze-dried liver sections proved to be difficult for us, if not impossible. This is in part due to the distortion of the structure caused by the freeze clamping. Therefore, a simple technique was applied to assist in sample

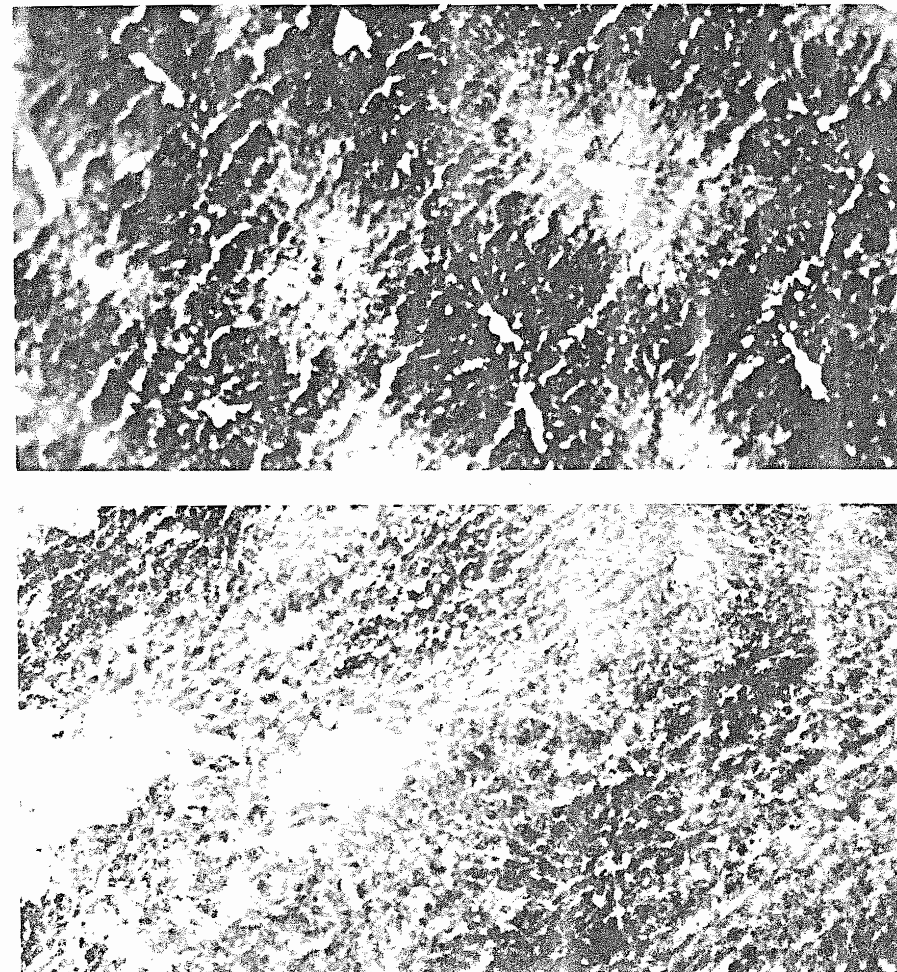

FIGURE 2. Intralobular Distribution of Succinoxidase as Demonstrated by Staining Histochemistry.

A 15 μ thick cryostat section was placed on a glass slide, was air dried and then incubated for 15 min at 37° C in the following medium: 100 mM Na^+ phosphate buffer, pH 7.6; 50 mM Na^+ succinate; 1 mM K^+ cyanide; and 1.4 mM Nitro-Blue Tetrazolium. The sections were then fixed with 10% formalin in 0.9% NaCl. Heavily stained areas are identified as zone 1, lightly stained areas as zone 3. The sample of the lower panel was freeze clamped, that of the upper was not.

FIGURE 3. Typical Dissecting Record

The outline of the stained section and of certain histological landmarks are recorded with heavy black lines. Zone 1 (high succinoxidase activity) is indicated by dark gray shading, zone 3 (low succinoxidase activity) by light gray shading. The heavy dotted lines indicate fissures in the section. The dissecting patterns of an adjacent unstained section are projected and indicated by thin black lines. In this section 12 samples were obtained. The sample weights were between 32 and 75 ng. Some of the data presented in Table II were obtained from the section depicted here. The remainder was taken from the other adjacent unstained section.

identification (19). Every fourth cryostat section was placed on a glass slide and was stained for succinoxidase (7) (Fig 2). The enzyme is concentrated in zone 1 of a simple acinus (see Fig 1). Therefore, heavier formazane deposits identify areas of zone 1 and relatively light areas of zone 3. A mirror image of this result was obtained when the sections were stained

for glutamic dehydrogenase (not shown), consistent with published results (7). A camera lucida drawing was made of the stained section and with the help of this drawing, samples of known composition were dissected from adjacent unstained sections from zones 1 and 3 (Fig 3). With this approach the precise location of each sample is recorded.

TABLE II. REPRODUCIBILITY OF NADH MEASUREMENTS IN MICROSCOPIC LIVER SAMPLES

	Zone 1	Zone 3
	mmole/kg dry tissue	
	.320	.225
	.335	.225
	.317	.224
	.296	.217
	.345	.263
	.265	.174
	.238	.140
	.263	.193
	.250	.189
	.257	.186
	.239	.185
	.263	.211
	.183	.274
	.287	.445
	.324	.210
	.301	.231
	.290	.233
Mean	.281	.225
±SD	±.042	±.065

Two groups of 17 samples each were dissected from zones 1 and 3 of freeze-dried liver sections and were analyzed for NADH. There is a statistically significant difference of 20% of NADH levels between zones 1 and 3, $p \leq 0.001$.

TABLE III. Stability of Reduced Pyridine Nucleotides of Normal Liver Under Dissecting Room Conditions

Dissecting Time	NADH		NADPH	
	Zone 1	Zone 3	Zone 1	Zone 3
T_1 (½ hr)	.329±.041	.191±.039	1.64±.15	1.48±.07
T_2 (1 hr)	.343±.053 (104.3)	.218±.029 (114.1)	1.56±.30 (95.4)	1.45±.05 (98.0)
T_3 (3¼ hr)	.285±.024 (86.6)	.181±.017 (94.8)	1.43±.20 (87.4)	1.46±.09 (98.4)

Groups of five samples each were dissected and exposed to dissecting room air (50% humidity and 25° C) for various lengths of time and were then analyzed for NADH or NADPH. The results obtained at the earliest time point (½ hr of air exposure) are used as reference values. It is assumed that there were no losses during that early phase, since the results agree with data obtained by many others with bulk extraction of the tissue (21). Results are given in terms of mmole/kg dry tissue (Mean±SD). For T_2 and T_3 the relative NAD(P)H contents are also recorded as percent of the level found at T_1 (in parentheses).

B. Stability of NADH and NADPH During Sampling

It has been noted previously (14) that reduced pyridine nucleotides might be oxidized during the process of microdissection of dried tissue. To test whether this was so, freeze dried sections were exposed for various lengths of time to 50% humidity at 25° C and were then analyzed for NADH or NADPH (Tables II and III). It has also been observed that extraction of dry tissue with dilute alkali (0.03 N NaOH) was successful only when sample size did not exceed a critical value (20). It became apparent that samples equal to or smaller than 100 ng gave reliable data. The reproducibility was acceptable with standard deviations between 15-30%.

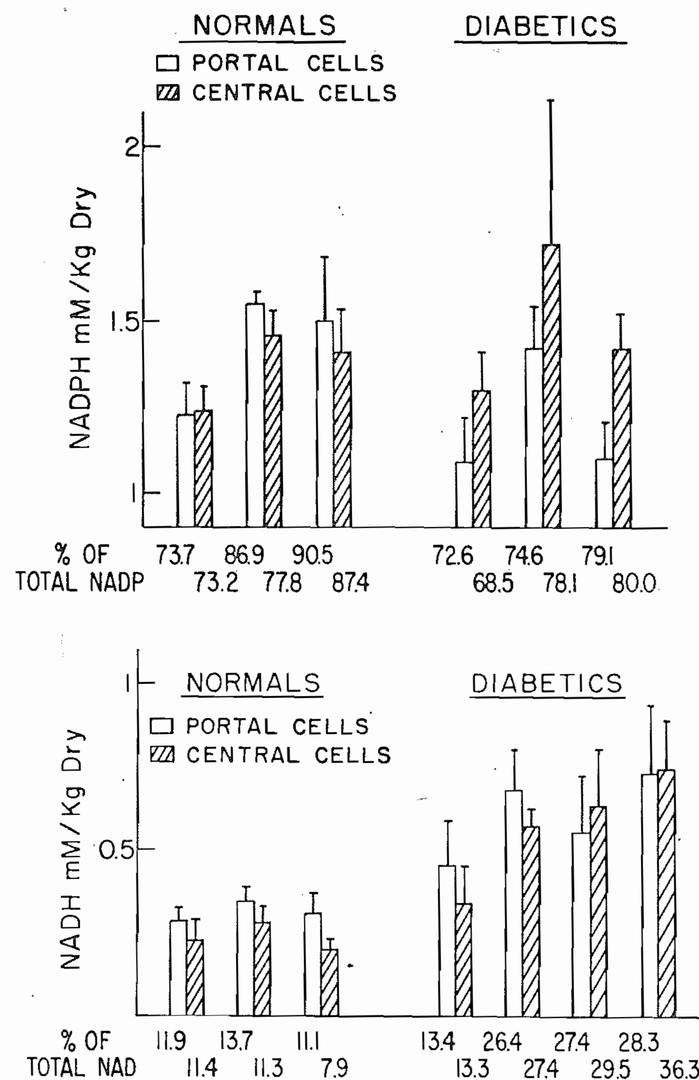

FIGURE 4. Intralobular Distribution of Pyridine Nucleotides in Livers of Normal and Diabetic Rats

The means ±SD of 10 or more samples are recorded in each bar. Diabetes was induced by injecting 70 mg of streptozotocin/kg body weight. The liver tissue was sampled four days later following the demonstration of severe hyperglycemia (19-24 mM). Animals were in the fed state.

TABLE IV. Intralobular Distribution of NADH and of the Sum of NAD^+ plus NADH in Normal and Diabetic Rat Liver

	NADH		Total NAD	
	Zone 1	Zone 3	Zone 1	Zone 3
		NORMAL LIVER		
Tissue Level	.31±.05 (41)	.24±.06 (49)	2.54±.40 (46)	2.28±.43 (49)
NADH as % of Total	12.1	10.4	--	--
Zonal Diff. % of Zone 1	-23.3		-10.5	
p-value	≤0.001		≤0.001	
		DIABETIC LIVER		
Tissue Level	.64±.21 (66)	.58±.22 (56)	2.85±.46 (47)	2.23±.36 (48)
NADH as % of Total	22.3	25.8	--	--
Zonal Diff. % of Zone 1	-9.6		-21.7	
p-value	≤0.001		≤0.001	

Three animals were analyzed in each group (i.e. Normals vs. Diabetics). Tissue levels of pyridine nucleotides are given in terms of mmole/kg dry tissue. Means±SD are recorded. The total number of samples analyzed is given in parentheses. The statistical analysis for individual livers showed similarly high significance of differences as recorded here for the comparison of all samples analyzed.

TABLE V. Intralobular Distribution of NADPH and of the Sum of $NADP^+$ Plus NADPH in Normal and Diabetic Rat Liver

	NADPH		Total NADP	
	Zone 1	Zone 3	Zone 1	Zone 3
		NORMAL LIVER		
Tissue Level	1.44±.18 (36)	1.39±.13 (35)	1.70±.19 (45)	1.72±.18 (47)
NADPH as % of Total	84.5	79.1	--	--
Zonal Diff. % of Zone 1	-3.6		-0.9	
p-value	≤0.001		0.9	
		DIABETIC LIVER		
Tissue Level	1.20±.19 (30)	1.47±.31 (31)	1.59±.28 (43)	1.94±.34 (45)
NADPH as % of Total	76.0	76.0	--	--
Zonal Diff. % of Zone 1	+18.1		+18.1	
p-value	≤0.001		≤0.001	

For more details see legend to Table IV.

C. Intralobular Distribution of Pyridine Nucleotides in Normal Livers (Fig 4, Tables IV and V)

The pyridine nucleotides are distributed nearly evenly throughout the liver lobule. But in the NAD system a very shallow gradient of total NAD with a tendency to fall in the direction of the central vein appears to exist. The difference between levels of zones 1 and 3 is not more than 10%. The NADH profile follows this trend and the porto-central dif-

ference is 23%. These differences are significant statistically. From 8-14% of the NAD is reduced. In the case of the NADP system there are no concentration differences between zones 1 and 3 and as much as 73-91% of the NADP is in the reduced form. The absolute levels and the degree of reduction found here with a micro analytical method are similar to those obtained in classical macroscale studies of pyridine nucleotides in liver (21).

D. Intralobular Distribution of Pyridine Nucleotides in the Liver of Severely Diabetic Rats (Fig 4, Tables IV and V)

The total NAD content and its intralobular distribution are not affected by severe insulin deficiency of 4 days duration induced by streptozotocin. However, the NAD system is more reduced in diabetics than in controls. The NADH levels doubled in liver tissue of diabetics. It also seems that the distribution of $NADP^+$ and of NADPH were altered somewhat by streptozotocin treatment. The levels of total NADP and of NADPH of zone 3 were 18% higher than the corresponding values found in zone 1. These differences had statistical significance. The degree of reduction of the NADP system was, however, not changed.

These results with diabetic tissue must be interpreted cautiously, because streptozotocin, which is used here to distroy the pancreatic β-cells, might have affected the pyridine nucleotide levels independently of its diabetogenic action. The data illustrate, however, that the approach enables the biochemist to detect small circumscribed alterations of the pyridine nucleotide system at the microscopic level. The preferential rise of NADP in zone 3 in the diabetic state may have pathophysiological significance.

E. Heterogeneity of the Redox State of Freeze Trapped Liver as Observed with Low Temperature Fluorescence Scanning (Fig 5)

Results obtained by application of the light guide scanner to the surface of freeze-clamped liver at a series of parallel planes 50 μ apart are shown here to illustrate the biophysical approach to the problem. The data consist of about 40,000 data points taken over a square 6 mm on a side. The light areas correspond to high FP_{ox}/PN_{red} ratios (oxidized zones) and the dark areas to low ratios (reduced zones). The extent of heterogeneity is large and might correspond to different

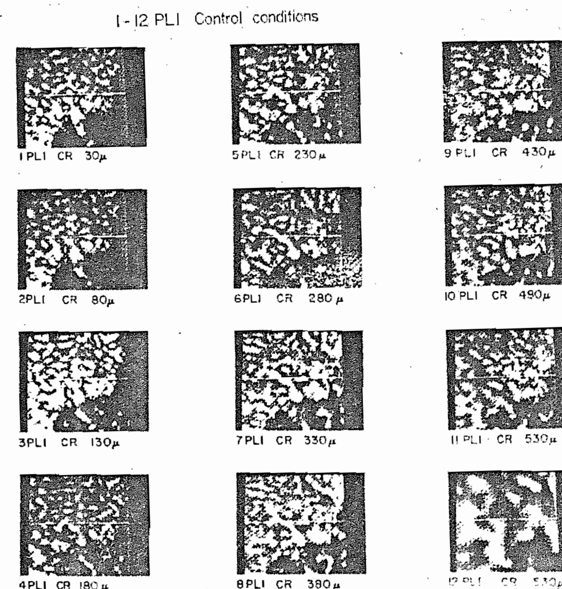

FIGURE 5. Heterogeneity of the Redox State of Freeze Trapped Perfused Rat Liver.

A serial display of cross sections 50 μ apart, from 30-530 μ depth, is presented. A 6X6 mm scan area was used which resulted in 3,600 points per scan. The single elements are 0.01 mm^2. The FP_{ox}/PN_{red} ratios (CR) are recorded, white representing a relatively oxidized and black a relatively reduced area.

metabolic states in various portions of the liver. The spatial pattern of the metabolic inhomogeneity appears to follow the architecture of the lobule. Preliminary microscopic controls suggest that the relatively oxidized areas correspond to the portal triads and zone 1, whereas the more reduced areas correspond to zone 3 of the acinus. Virtually identical results were obtained with livers freeze-clamped in vivo using fed rats briefly anesthetized with pentobarbital.

IV. DISCUSSION

The two sets of data, i.e. the results obtained by direct chemical analysis of well-defined microscopic liver structures on one hand and those recorded by indirect biophysical methods on the other, appear contradictory. The biophysical results

seem to suggest that redox potential gradients exist within the liver lobule with a higher degree of reduction in zone 3 as compared to zone 1. The chemical analytical results suggest that the redox potential is equal throughout the liver lobule.

In order to resolve the dilemma, the advantages and the potential shortcomings of the two approaches have to be compared and assessed.

The results of the microchemical determinations are more easily evaluated than those obtained by the biophysical approach. The specific nature of the enzymatic fluorometric assay, the fortunate outcome of the storage experiments and the favorable comparison with corresponding whole tissue data accumulated by other investigators indicate that the measurements are reliable. Most importantly, reduced pyridine nucleotides seem to be reasonably stable during the process of sampling and seem to survive the mild extraction process used here. The small, but clearly demonstrable gradient of NAD is consistant with numerous histochemical data (3,5,6-10) which suggest that zone 1 of the hepatic acinus is metabolically more active than zone 3. The constancy of the NAD^+/NADH and $NADP^+$/NADPH ratios across the lobule is not unreasonable physiologically, since under normal conditions the oxygen tension might be maintained sufficiently high to assure saturation of cytochrome oxidase in all 3 metabolic zones of the liver acinus. However, the interpretation of the data is clouded by the uncertainties about the degree of binding of pyridine nucleotides to cellular components (22-24). The present data represent, therefore, merely a rough estimate of the redox potential of zones 1 and 3 and more sophisticated approaches have to be used to obtain information on the intralobular distributions of the free fractions of pyridine nucleotides. Such measurements would seem feasible.

Great efforts have been made to validate the biophysical procedure for measuring redox potentials by surface fluorescence (13,18,25). The low temperature fluorescence scanner of FP_{ox}/PN_{red} ratios has been designed to overcome some of the difficulties encountered in the past (13,18). It is assumed that the ratio method is largely independent of the fluorochrome concentrations and that it is less susceptible to interference by hemoglobin or tissue pigments than selected monitoring of reduced pyridine nucleotides or oxidized flavoproteins. The method is extremely sensitive to small opposite level changes of the two members of the redox couple FP_{ox} and PN_{red}. But this increased sensitivity also increases the susceptibility to interference. The technique is attractive because

data acquisition is comprehensive and rapid. The results gathered to date indicating the presence of a redox potential gradient across the liver lobule have considerable biochemical implications. The most plausible explanation might be that the cells in zone 1 and 3 are biochemically different and that peculiar features of the energy metabolism of these two cell populations determine the redox potential differences.

It is not possible at this stage of the investigations to resolve the discrepancies of results obtained with the two methods. The opposite results in liver are particularly puzzling since in another tissue the two methods provided comparable data (13). It was found that the redox potential gradient that characterizes the border zone between infarcted and normal tissue of the rabbit heart manifests itself equally well with both techniques. One possible explanation for the opposite outcome in liver is that the results of the quantitative histochemical approach may have become biased due to limiting the sampling to zones 1 and 3 so that the heterogeneity of the redox potential, manifest in the comprehensive picture obtained by the fluorescence scanner, may have been missed. It is, therefore, essential to carry out parallel studies with both techniques on the same tissue blocks focusing on the same lobules and the same groups of cells. Such point by point comparisons of results seems feasible.

The topic is of importance for many questions of the physiology, pathophysiology and toxicology of the liver and deserves vigorous research.

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DISCUSSION

J. KATZ: I would like to ask a question of Dr. Matschinsky. Did you measure the activity of succinate dehydrogenase or just the levels of reduced pyridine nucleotides; the DPNH and TPNH. Maybe I didn't get you.

F. MATSCHINSKY: You didn't get me. The slide which I showed you was a stained section used to guide us in the microdissection. I have not the slightest idea whether this represents the actual activity of succinate dehydrogenase.

J. KATZ: You show a very sharp boundary on the slide between the periportal succinic dehydrogenase zone (which is blue), and the unstained perivenous zone. Nolte and Pette (J. Histochem. Cytochem. 20, 507, 1972) have measured succinate dehydrogenase in the periportal and perivenous zones of the liver lobule and report the activity of the dehydrogenase in the periportal zone to be 1.5 times that in the perivenous area. This is not much of a difference. I see a discrepancy between the histochemical distribution, which is nearly all or nothing, and the enzyme assays which only show a moderate difference.

F. MATSCHINSKY: I have been wondering about this discrepancy myself. In this study, the staining histochemistry was used merely as an indicator of the location of the sample. We have complementary staining results with glutamate dehydrogenase which show the opposite distribution. It is well known that glutamate dehydrogenase is higher in the central than the portal area. From these studies we are sure that the samples we get are of portal or central origin. That is the point I wanted to make. I don't want to get into the value or lack of value of staining histochemistry. That is a very muddy area as you very well know.

J. LOWENSTEIN: It seems to me that Dr. Chance's evidence for microheterogeneity in the redox state of pyridine and flavin nucleotides might be explained in terms of oscillations of metabolite concentrations in individual cells. The oscillations in different cells would have to be non-synchronized or at most partially synchronized. Would you comment Dr. Chance?

B. CHANCE: Fascinating idea and indeed verified by microfluorometry of individual yeast cells. But there is a clear synchronizing signal in yeast, and in slime mold as well. In the brain cortex the neurons are synchronized in spreading depression. Thus most of the metabolic oscillations known are self-synchronizing, but of course this is how they are observed.

J. LOWENSTEIN: Conditions that prevail in mammalian cells are different and would tend to run the synchrony. For example in muscle not all cells are contracting at the same moment and in brain not all cells are firing at the same time and that would tend to lead to asynchronous oscillations.

B. CHANCE: Yes, an asynchronous oscillation in a tissue would be difficult to observe by any other approach than the histogram display that we use. Perhaps synchronizers, such as substrates, functionally active, would decrease the apparent heterogeneity. Again, it's a good idea.

S. BESSMAN: I have a question that is just a technical one. The size of a liver lobule is a millimeter or 2 millimeters thick. If you freeze-clamp it, and if you are going to retain the architecture without making a hash of it, then I think it is going to take more time which permits changes in the concentration of intermediates than one would think would occur with freeze-clamping. In other words, the question is how can you get away from the problem of heat exchange and still retain the architecture of the system.

B. CHANCE: Well, a cold block (-196°) is laid upon the liver lobe and the freezing gradient moves at about 12 microns per millisecond initially and slower after about 50 μ. Within 1 millimeter travel you have fairly valid freezing since 1 or 2 seconds is satisfactory for maintaining aerobiosis in the tissue. Now, if you do a redox scan through such a tissue, of course the initial 50 μ layer may be disturbed. In fact you saw that in the heart redox scan. From 200 microns onwards the profiles are very clear and consecutive. Ice-crystal formation could be another disturbance in these sections but they are usually too small to affect the mitochondrial signals.

COMPARTMENTATION OF GLYCOLYSIS IN ESCHERICHIA COLI

Vivian Moses

Department of Plant Biology and Microbiology
Queen Mary College, University of London
London, England

I. INTRODUCTION

One cannot fail to be impressed and intrigued by the complexity of metabolism, particularly when one realises how small is the volume in which so much takes place. It has been estimated that an individual growing bacterial cell, in a volume of perhaps 10^{-12}ml., may contain at any one time a million protein molecules catalysing a variety of chemical interactions approaching a thousand in number. Instinctively one feels that such a system is "highly organised" but the use of this term is not really very informative unless an attempt is made to define the many sorts of organisation which may be applicable. At the very least two main categories present themselves for consideration. One is to suppose that the whole complex of biochemical events is interrelated through a comprehensive network of mutual interactions so that a perturbation in one area will have its ramifications throughout the system as a readjustment takes place to meet it. The other, and it is not exclusive of the first, imagines a hierarchy of organisational states in which local interactions and equilibrations may take place without immediately involving the whole system. The consequences of the local adjustments would, of course, have their repercussions on neighbouring activities but only, perhaps, at a limited number of points of interaction. This second organisational state is one of the possible forms of compartmentalised arrangement; but merely recognising the existence of the organisation does not *ipso facto* reveal anything about its physical or chemical basis.

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