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TISSUE REDOX RATIO DETERMINATIONS IN TWO AND THREE DIMENSIONS

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PROBLEM

To determine the two and three-dimensional redox structure of the rat brain in vivo.

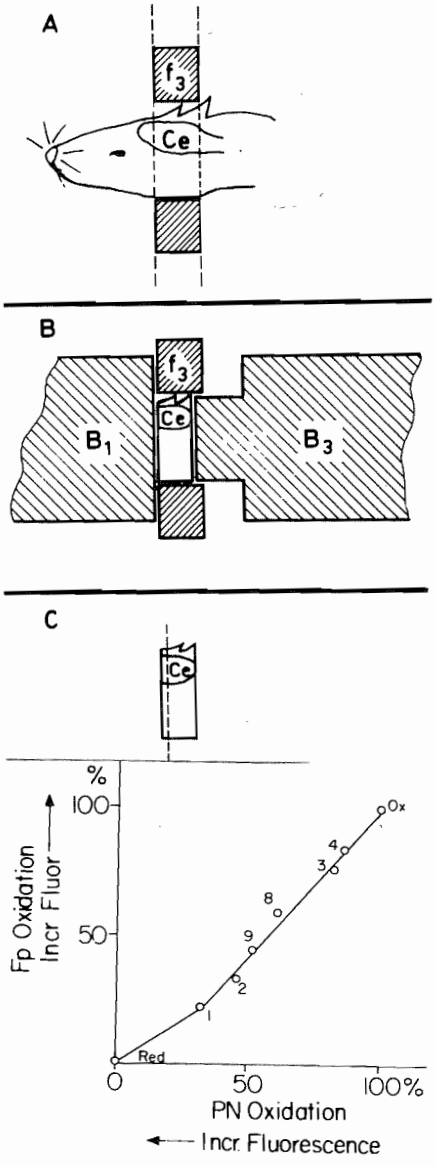

Figure 1. Regional Sampling from Rat Brain with Guillotine - Freeze Clamping Technique. A. The head of the rat is cut according to the dotted lines. B. The liberated head slice is freeze-clamped between aluminum blocks precooled in liquid N_2. C. The brain sample, Ce, at the left of the cross-sections (cf. Fig. 4g) was studied in two dimensions by microfluorometry. The third dimension of the sample was studied by consecutive removal of 320 μ tissue sections by means of a filing system at 77° K.

Figure 2. Correlation between NADH and Flavin Fluorescence. Fluorescence was measured in frozen samples of pigeon heart mitochondria at 77° K trapped in various metabolic states.

The initiating enzymes have been assayed in the liver of developing rats. Serine aminotransferase activity is much greater in the suckling rat than in the fed adult, whereas serine dehydratase activity is lower in the suckling rat than in the adult. (Fig. 1). Gluconeogenesis via pyruvate is blocked by quinolate or mercaptopicotinate (see page 2). These inhibitors blocked gluconeogenesis from 10 mM-serine in perfused livers[1] and isolated hepatocytes[3] from 24 h starved adult rats, but were much less effective with preparations from 18 h starved neonatal rats (see e.g. data with perfused livers using quinolinate shown in Fig. 2). Neonatal rat liver thus appears to be preferentially equipped for glucose formation from serine by the aminotransferase pathway.

The subcellular distribution of the initiating enzymes in neonatal rat liver was investigated using differential centrifugation methods[4]. Fig. 3 shows that serine dehydratase is predominantly cytosolic and serine aminotransferase is predominantly particulate (i.e. sedimented at 26,700 g for 10 min). Lysosome-enriched (from triton-treated rats) and peroximal-enriched fractions were prepared by sucrose density-gradient centrifugation of the particulate fraction[4]. The recovery of the aminotransferase and some marker enzymes (as a percentage of total homogenate activity) in these fractions is shown in Fig. 4. Neither contained any appreciable aminotransferase activity. Altogether these results suggest a mitochondrial location for most of the aminotransferase activity and a cytosolic location for serine dehydratase activity in the suckling rat.

In the light of this data on the intracellular location of the initiating enzymes of the alternative pathways of serine metabolism, metabolic schemes can be drawn up illustrating the possible carbon and nitrogen pathways followed by serine in the course of gluconeogenesis and ureogenesis via the dehydratase pathway (Fig. 5) or the aminotransferase pathway (Fig. 6).

The location of serine aminotransferase in mitochondria has prompted the speculation that it might be involved in mitochondrial pathways of gluconeogenesis in which serine is an intermediate in glucose formation from other precursors[5]. Suggestions for possible intramitochondrial glucogenic precursors that have been made are hydroxyproline[2] and fatty acids[4]. Both of these would be particularly appropriate in terms of the metabolism of the suckling rat[5].

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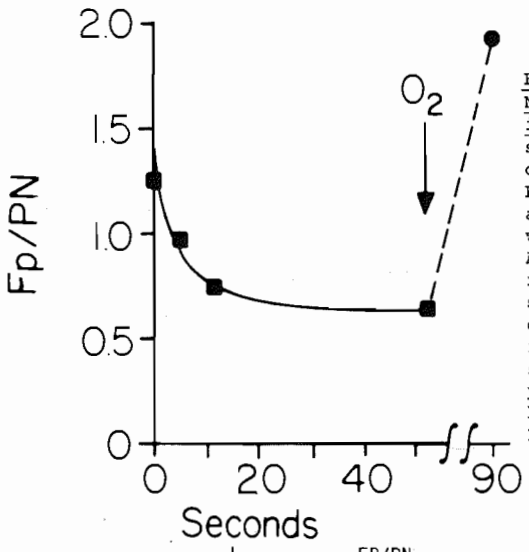
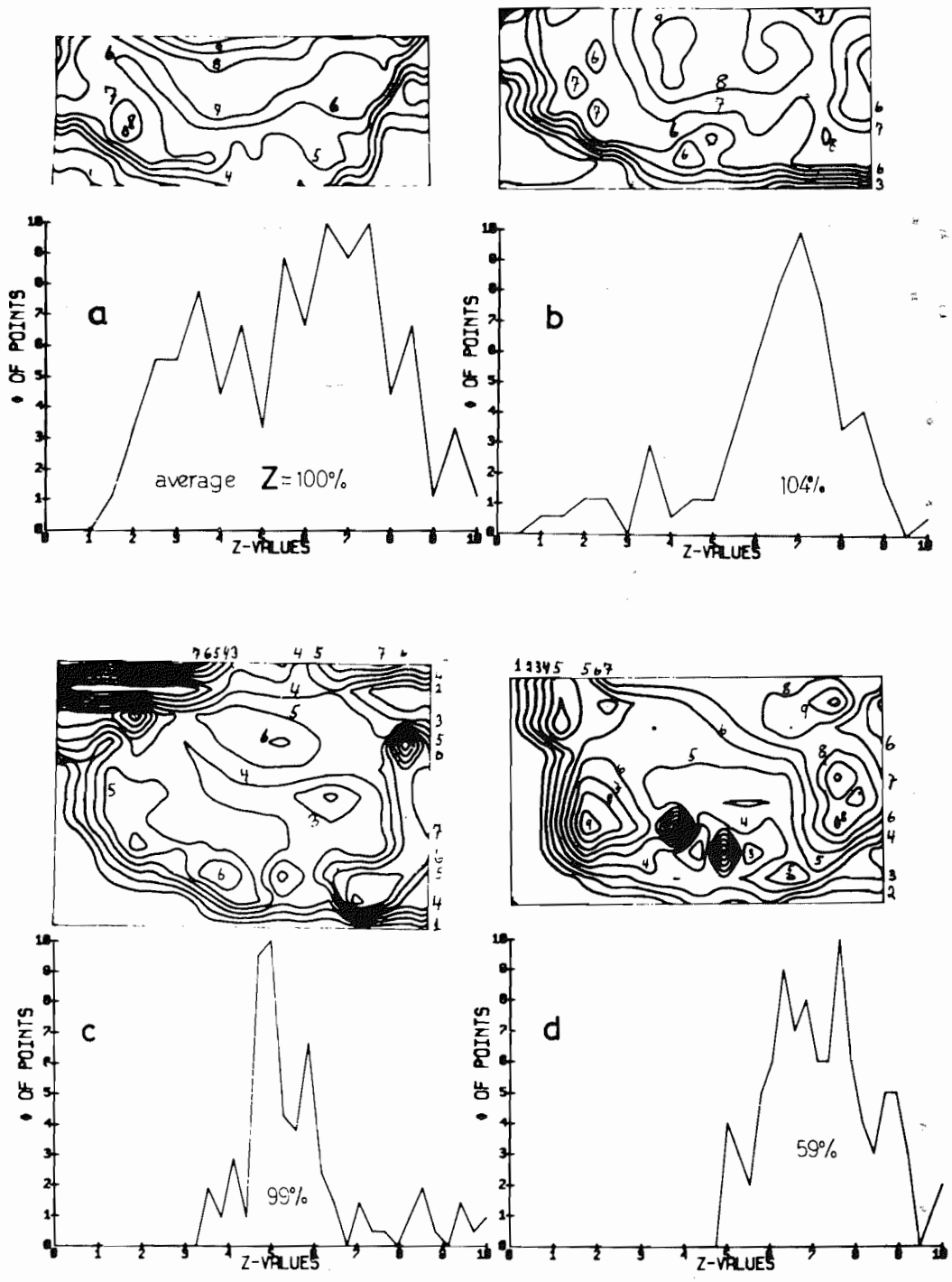


Figure 3. Time course of Oxygen-Nitrogen Transition in Rat Brain in vivo. For the Fp/PN measurement, see Legend to Table I. Each point on the graph represents the average Fp/PN value of the brain sample of a single rat, except the "0" point which is the average of three rats. All rats were conscious and breathing 1 atm O_2. The "0" rats were sampled in that condition, and the others were sampled at the times indicated after changing the inspired gas mixture to N_2. The last point represents a rat which had been reoxygenated after 55 sec of N_2 breathing and sampled at 90 sec.

EXP. NB. CONDITION	FP/PN MIN	MAX	AVERAGE	SPAN	RELATIVE SPAN %	REDUCTION %
1660B/45 O_2	0.69	1.52	1.26	2.2	13	80
1662C/57 PENTO-BARB O_2	0.66	1.97	1.28	3.0	18	79
1659D/70 ROOM AIR	0.56	1.76	0.87	3.1	19	88
1659B/51 90 SEC N_2	0.26	0.80	0.46	3.1	18	97
1659C/72 120 SEC N_2	0.38	0.60	0.35	1.6	9	99

Fp/PN Data from the Cross-section of the Freeze-clamped Brain. The Fp/PN ratio was determined at about 100 spots on each brain cross-section. The measurements were carried out at 77°K and the fluorometer was calibrated from isolated brain mitochondria. The physiological condition of the animals is given in the left-hand column. All rats except those with pentobarbital were unanesthetized. The range of Fp/PN measurements, the average and the span (max/min) are given for each experiment. The columns "Relative Span" (in vivo span x 100/in vitro span) and "Reduction %" represent comarisons with isolated brain mitochondria.


a
average Z=100%
OF POINTS
Z-VALUES
b
104%
OF POINTS
Z-VALUES
c
99%
OF POINTS
Z-VALUES
d
59%
OF POINTS
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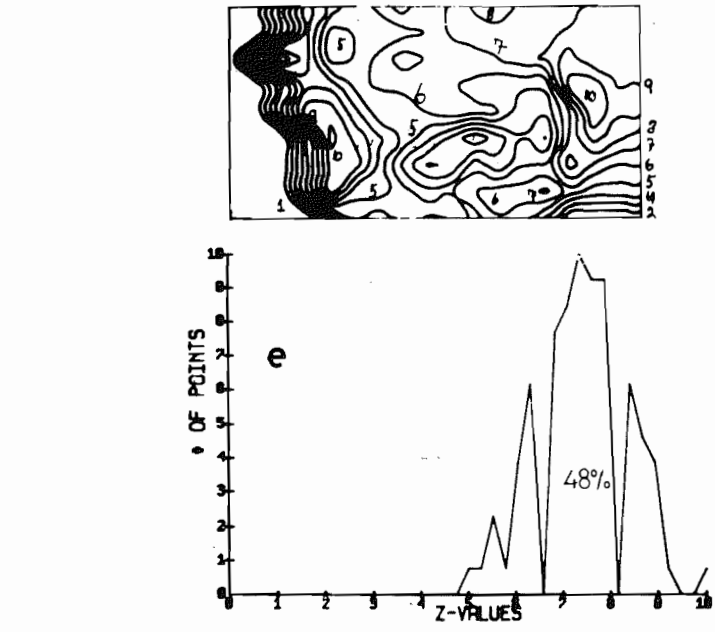

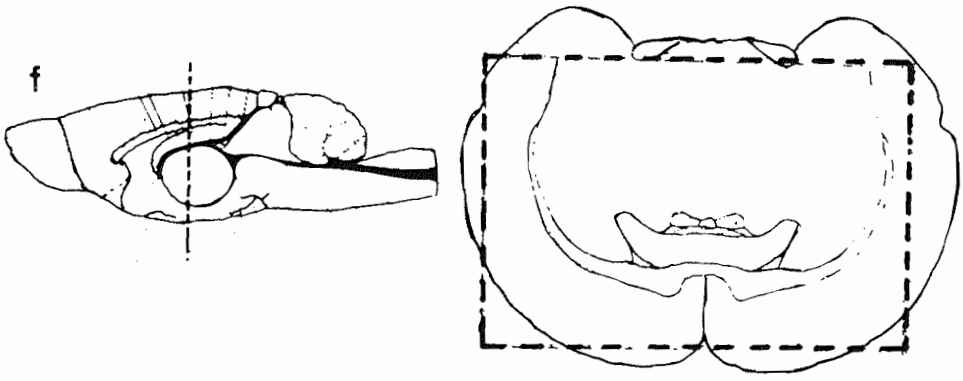

Figure 4. Fp/PN Measurements on Serial Sections from Normoxic Rat Brain. For the Fp/PN determination, see Legend to Table I. Plates a - e represent data from serial sections of Sample #70 (cf Table I). The serial section was performed as in Fig. 1c. See text for details. f shows the level of the brain at which the cut was made in the process of freeze-clamping. The dotted line corresponds to the left-hand dotted line of Fig. 1a. g shows the cross-section corresponding to the dotted line of 4f above. The rectangle indicates the area studied with the microfluorometer.

METHODS

In vitro experiments. Pigeon heart and brain mitochondria were prepared according to (1) and (2). The procedure for obtaining different oxidation-reduction levels in mitochondria has been described elsewhere (3).

In vivo experiments. Fed female Wistar rats weighing 200-220 g were used. Different regions of the brain were made accessible to low temperature surface fluorometry by the guillotine - freeze clamping method of Quistorff (4) (Fig. 1). All brains were cut at the level of the mesencephalon (Fig. 4f) resulting in a freeze-clamped cross-section, as shown in Fig. 4g.

Surface fluorescence measurements. The brain or mitochondrial sample was placed in a quartz Dewar clamped to an aluminum block which was partially submerged in liquid N_2. The aluminum block was movable in two dimensions in a plane perpendicular to the axis of the fluorometer beam. The fluorescence was measured through the wall of the Dewar. The beam size on the tissue surface was adjusted to a circle of 1 mm diameter, giving about 100 read-out points from the brain cross-section (cf Fig. 4g). The microfluorometer applied to this study was a modification of the instrument developed and previously described by Chance et al. (5). Excitation of NADH and flavin was done via 366 ± 5 nm and 463 ± 3 nm interference filters respectively. The corresponding fluorescence signals were measured via 460 ± 10 nm and 540 ± 20 nm Wratten color filters.

RESULTS

Validity of the Fp/PN ratio as a parameter for the tissue redox state. The ratio of flavin to NADH fluorescence was chosen as a measure of the redox state of the mitochondrial compartment of a tissue. The arguments for the validity of this procedure follow. a) The correlation of the fluorescence of flavoprotein and of NADH over a variety of redox states is close in mitochondrial suspensions (Fig. 2). Similar results are obtained in suspensions of hepatocytes and yeast cells (3), suggesting that either the bound NADH of the mitochondrial space is more fluorescent than that of the cytosolic space, or that the two spaces maintain approximate equilibrium in the redox transitions so far tested. b) The strongly fluorescent oxidized form of the flavin of the lipoate dehydrogenase and component of the pyruvate and α-ketoglutarate dehydrogenase has a redox potential of about -300 mV and appears to be in rapid near-equilibrium with mitochondrial NADH (3); the reduced forms of these flavins have a very weak fluorescence. c) The Fp/PN ratio is independent of the concentration of mitochondria, in contrast to each of the two parameters forming the ratio. d) The influence of hemoglobin is minimized in the Fp/PN ratio; less than 10% variation of the ratio is observed when the hemoglobin concentration is varied between 0 and 100 μM. e) The Fp/PN ratio is a sensitive indicator of the redox state; calibrated with isolated mitochondria, it is 5.0 in the oxidized and 0.3 in the reduced state.

Fp/PN determination at 77° K in the freeze-clamped rat brain. According to this

calibration, Table I gives the redox ratio of brains from rats in a variety of physiological states, with average values as well as maximum and minimum values measured. The greatest redox range was seen in the pentobarbital-anesthetized brain, which also had a very high average redox ratio. The average Fp/PN was lower in"room air" than in "O_2", whereas the range was greater in the former, indicating that under normoxic conditions some parts of the brain are anoxic. In the "N_2" rats the average Fp/PN decreased, accompanied by a narrowing of the range. Compared with substrate-depleted isolated mitochondria, even the "O_2" brain is 80% reduced. In Fig. 3 a discontinuous time course of anoxia in the brain of a conscious rat is shown; the Fp/PN ratio indicates the redox level and each point represents a different animal. A 50% overshoot is seen following reoxygenation; this phenomenon has been observed before both in vivo and in vitro, and is attributed to the ATP debt in recovery from anoxia.

Fp/PN data in three dimensions on the rat brain, shown as contour plots. Fp/PN values were indicated on the location of their origin on a photograph of the surface of the freeze-clamped brain sample. The Fp/PN range was divided into ten intervals and points in the same interval were connected to form the contour plots of Fig. 4, a-e. Beneath each contour plot the same set of data is given in histograms. The units on the axis of the histograms and the position of the histogram along the x-axis are arbitrary. The relative average Fp/PN value for the entire cross-section is given on each histogram.

Conclusions. 1) The histograms of Fig. 4, a-e, show a decreasing average redox ratio paralleled by a narrowing of the range of the Fp/PN values; the same pattern is seen in Table I, comparing the O_2 and N_2 brains. As the freeze-sampling of the tissue involves a certain period of ischemia in which metabolism is not yet stopped by freezing, one would expect a result like that found in the serial section experiment. Thus, relative anoxia seems to have occurred in sections d and e (960-1600 μ/depth), and it is therefore concluded that the metabolic state in the tissue within a distance of 1 mm is not affected by the sampling technique.

2) The heterogeneity of the redox level in the brain is obvious in Fig. 4, a-e, and as indicated in the data of Table I, the variation within the same brain is almost as great as the changes in the average values obtained by a shift in the physiological state of the rat. This may lead to the conclusion that a number of physiological states exist at the same time in the normoxic brain of the conscious animal.

3) In most of the contour plots of Figure 4, a-e, a confluence of lines is seen, indicating that the isometabolic volumes of these areas are smaller than the resolution of the fluorometer (about 100,000 cells).

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