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CHAPTER

6

Guillotine Freeze Clamping of Rat Brain*

Analysis of Energy Metabolites Along the Freezing Gradient

Bjørn Quistorff

Several techniques have been devised for sampling of brain tissue to study the concentrations of labile metabolites under in vivo conditions. Each one of the methods has advantages and drawbacks, and the question, which one is "best," depends mainly on the aim of the particular experiment. Immersion freezing, first introduced by Stone[29] of either the intact animal or the severed head, has been widely used.[5, 8, 11, 15, 25] The method is convenient since it does not require anesthesia or any special equipment. However, because of the slow progression of the freezing front, only the superficial parts of the brain are frozen fast enough to prevent major autolytic changes.[11, 30] Funnel freezing[6, 21] is a variation of immersion freezing which, however, allows a well controlled physiological state of the animal to be maintained during freezing of the brain. Since there is evidence (in the rat) that circulation in deep cortical structures is upheld to some extent while superficial parts are frozen,[25] the slowness of freezing may be less significant in terms of autolytic changes of energy metabolites.[21] In contrast to immersion freezing, funnel freezing requires immobilization and, at least, a minimum level of anesthesia. With immersion as well as with funnel freezing the relative long fixation times are prohibitive for short time studies except if only superficial parts of the cortex are studied or very small animals are used. Freeze blowing[32, 33] is an extremely fast sampling technique which may be performed with unanesthetized rats. The technique is very suitable for short time studies on whole brain. The drawback is that regional studies are not possible. Guillotine freeze clamping[2, 19, 23] is about as fast as freeze blowing and allows regional studies in two and, to some extent, three dimensions[22, 23]; i.e., in contrast to the methods mentioned above, short time in vivo studies of deep brain structures are possible (see later). As with freeze blowing a drawback is the necessity of rather complicated equipment and sampling procedure.

Immersion freezing with decapitation prior to freezing as well as freeze blowing and guillotine freeze clamping all involve mechanical trauma to the brain. This may introduce a hypermetabolic state which could significantly affect metabolite concentrations in the interval between sampling and fixation by freezing.[10, 17] Finally, microwave irradiation of the brain of the intact animal has been applied as a means of inactivation of enzymatic processes.[12, 27, 28] This technique seems to combine most of the advantages of the different freezing methods: it is convenient and fast and seems to allow full scale regional studies. The problem with this technique, apart from very expensive equipment, would seem to be the rather large difference in heat susceptibility of different enzymes and of different substrates as well, as pointed out by Miller and Shamban.[14] These factors together with the unavoidable acceleration of metabolic processes in the seconds prior to inactivation might explain the less optimal values for labile metabolites found so far with this technique.[14, 32]

This communication will summarize the properties of the guillotine freeze clamping technique, and in order to evaluate the efficiency of the freezing technique applied, some new data will be given on the changes of energy metabolites along the freezing gradient in normal unanesthetized rats. It is suggested that measurements along the freezing gradient provide an alternative way for an accurate time course study of the early events of hypoxia and ischemia, allowing different points in time measured in the same animal.

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METHODS

Sampling and Freezing

A detailed description of the guillotine freeze clamping instrument has been given elsewhere[22] and only the essential principles will be repeated here. The instrument is a mechanical, pneumatically operated system by which the unanesthetized rat may be decapitated and simultaneously a part of the brain (or the liver[22]) cut out and freeze-clamped by precooled aluminum blocks. A diagram outlining the working principle is shown in Figure 6.1. The rat is positioned in a transparent plastic tube which allows for control of the composition of the inhaled gas mixture (Fig. 6.1*A*). Monitoring of blood pressure and measurement of arterial blood gases may be performed with an indwelling tail artery catheter. Figure 6.2 shows an example of the change of arterial P_{O_2} and P_{CO_2} in an unanesthetized rat versus time after switching from room air to 6–8% oxygen.[19] The tube (Fig. 6.1*A*) is divided in three parts. The middle part is an interchangeable (r) of variable width, usually 13 mm, with an inner diameter which fits the head of the rat (27 mm for a rat of 200 g). The rat is restrained in the tube by an adjustable constriction (c) and a stopper (s). During sampling, two cross-sections are made through the head of the rat by means of rotating knives which will pass through the tube on each side of the annulus (along the dotted lines on Fig. 6.1*A*). The time of cutting is 20–30 msec. The annulus with the head slice stays in place after cutting while the other parts of the tube are automatically removed and replaced by two

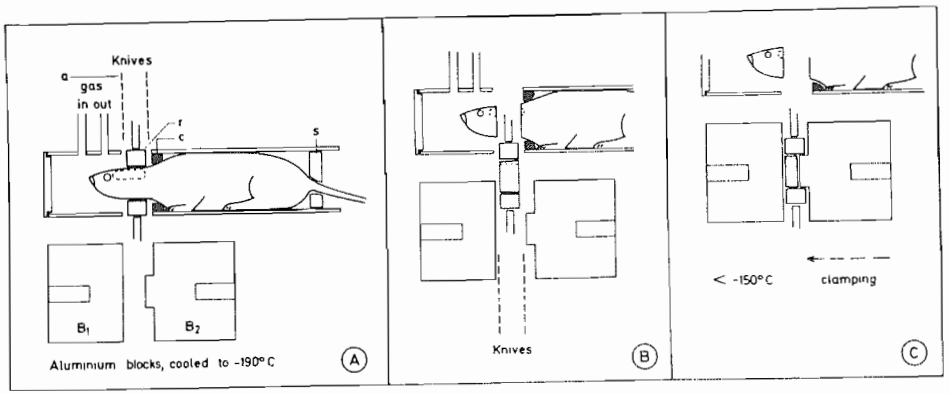

Figure 6.1. The principle of rat brain sampling by guillotine freeze clamping. (See text for details.)

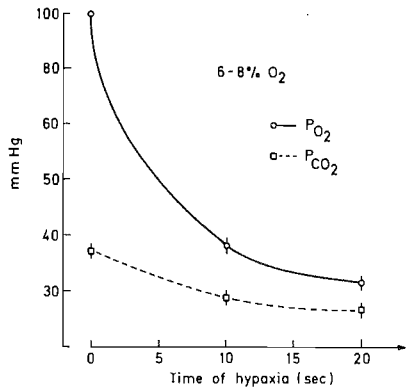

Figure 6.2. Time course for arterial P_{O_2} and P_{CO_2} in unanesthetized rats after switching from room air to nitrogen/oxygen, 93:7, as inhalation gas. The half time for gas exchange in the tube (see Fig. 6.1*A*) was 0.3 sec. (Data taken from Nordberg et al.[19]).

aluminum blocks precooled in liquid nitrogen (Fig. 6.1*B*), between which the head slice in the annulus then is freeze-clamped (Fig. 6.1*C*). The thickness of the slice is reduced from 13 mm to 10.5 mm during freeze clamping. Note that it is the thickness of the annulus (Fig. 6.1*C*) which determines the final thickness of the sample. Since the head of the animal fits rather tight in the annulus, the compression by freeze clamping introduces only a little distortion of the anatomy of the slice[22] (Fig. 6.3). The time from cutting until the closing of the freeze clamping tongs is 60–80 msec, i.e., a total sampling time of less than 0.1 sec. Regional sampling is performed by prepositioning the rat in the tube so that the region of interest is hit by the knife (*a*) (Fig. 6.1*A*), which can be done with an accuracy of ±1 mm.

The final thickness of the freeze-clamped slice is about 10 mm; therefore, even with bilateral freezing, the freezing time for the central part of the sample is much too long (>25 sec, see later) to prevent autolytic changes due to ischemia. Only the part of the sample which has been close to (about 1 mm) the blocks has sufficiently short freezing time to preserve the in vivo metabolic state. However, the freezing gradient obtained in the sample has proven experimentally useful since over a distance of 5 mm there will be a time interval of ischemia of 20–25 sec. In the outer part of the sample, the time resolution is extremely good; e.g., metabolic analysis performed on two slices cut between 0.4–0.5 mm and 0.5–0.6 mm will represent quenching times which are only about 200 msec different. This relation between quenching time and distance from the cooled surface is determined in the present study and used to evaluate the ∼P consumption of the rat brain cortex.[3, 10]

Metabolite Measurements

Male Wistar rats (190–210 g) were used. There were two groups: group I, in which cerebral cortex was sampled for metabolite analysis, and group II, used for thermocouple recordings of the temperature time course of freezing in various parts of the sample.

Prior to the actual experiment the animals of group I went through a "training procedure" with a 10-min period of adaptation in the sampling instrument.[22] Previous experience has shown that this procedure ensures normal values for arterial blood gases and pH as well as body temperature.[2, 19] Therefore, except for body temperature, which was between 37 and 37.8°C, these parameters have not been measured. During sampling the rat was in position in the tube so that the knife (*a*) (Fig. 6.1*A*) went through the midbrain. The samples were treated as follows: The side of the sample, on which the cross-section through the midbrain is exposed, was "cleaned," removing 0.05–0.1 mm by low temperature milling[23, 24] as illustrated in Figures 6.3 and 6.4*A*. The sample was then cut perpendicular to the surface (Fig. 6.4*B*) and five slices of a thickness of 0.8–1.0 mm were cut parallel to the surface which was in contact with the cooling block (Fig. 6.4*C*); i.e., only half of the sample was used. The milling and cutting procedure was performed in a glove box at −20°C with the sample temperature kept below −80°C.[21] From each of the thin slices about 25 mg of parietal cortex was dissected (Fig. 6.4*D*) and extracted with HCl/methanol at −20°C and re-extracted with 0.3 M PCA at

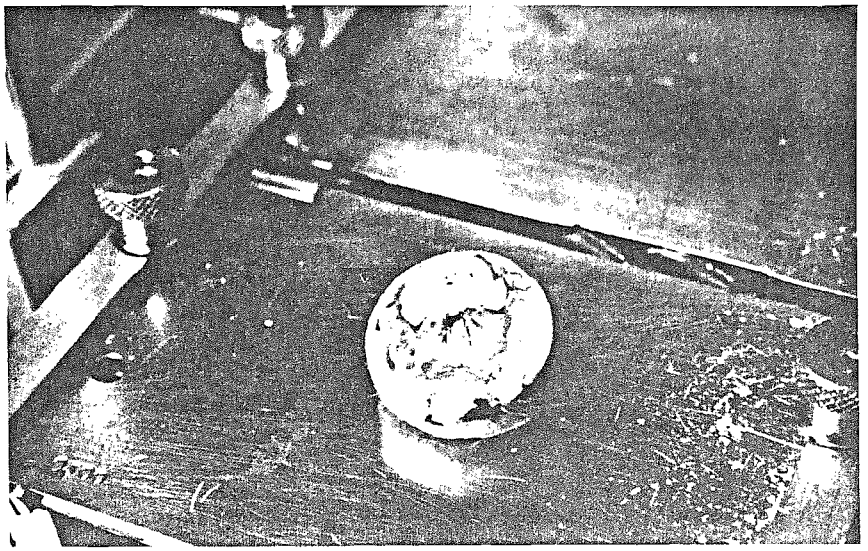

Figure 6.3. Brain sample obtained with the guillotine freeze clamping technique. The surface seen is the cross-section produced by the knife (*a*) in Figure 6.1*A*. About 0.1 mm of the tissue was removed by low temperature milling (Fig. 6.4*A*). The sample is photographed on the milling instrument in the glove box at −20°C. (See text for details.)

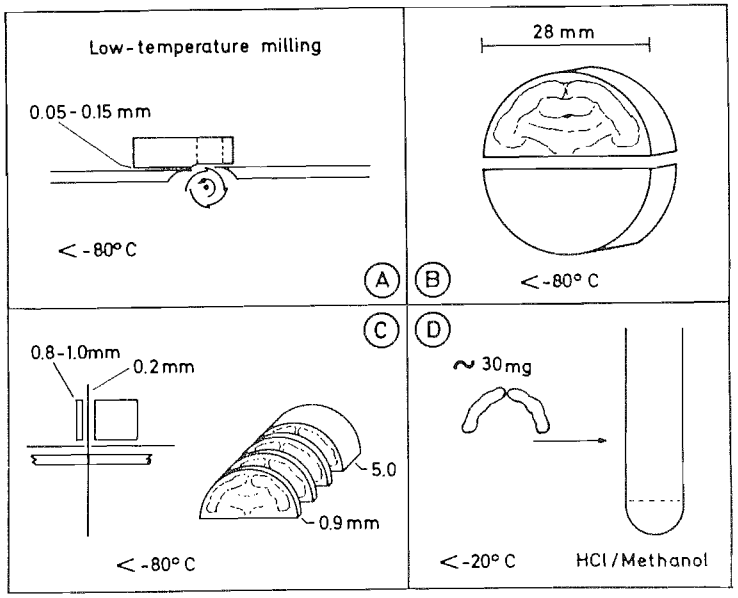

Figure 6.4. Steps in the procedure for regional sampling from unanesthetized rats. Note in *C* that only half of the sample is used. (See text for details.)

0°C, essentially as described by Lowry and Passonneau.[9] Metabolite concentrations were measured on the neutralized tissue extracts by standard enzymatic procedures: ATP and phosphocreatine (PCr) spectrophotometrically[10] and ADP, AMP, lactate, and pyruvate fluorometrically.[9]

Thermocouple Recordings

The animals of group II used for thermocouple recordings were anesthetized with sodium pentothal, 75 mg/kg. The anesthetized animal was placed in the sampling instrument and the thermocouples (hypodermic needles, 0.7 mm in diameter, EL-Lab Inc., Copenhagen) were inserted into the brain through the skull and left in a vertical position (Fig. 6.5*A*). The entire thermojunction at the tip at the needle was well below the skull. Before sampling, the brain temperature was brought to 37°C by heating with a spotlight. Upon cutting and freeze clamping the thermocouple remained in position in the brain and temperature was recorded as a function of time after cutting on a strip chart recorder, 1 cm/sec. When freezing was completed, the sample was brought to −20°C in a glove box. At this temperature the thermocouple was easily pulled out, leaving a visible channel in the frozen tissue block. The distance from the surface to the middle of the channel was determined by measuring the thickness of the tissue layer that had to be milled off before a longitudinal cross-section of the channel was obtained (Fig. 6.5*B*).

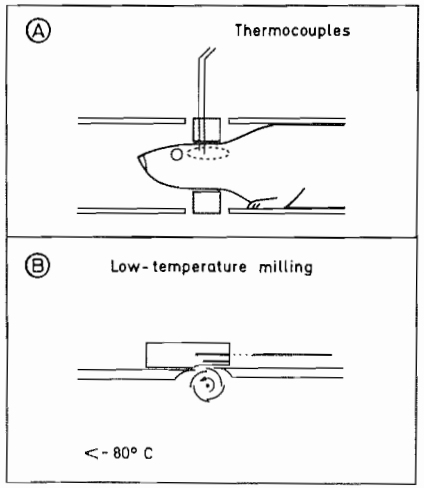

Figure 6.5. Principle of recording of the time course of freezing during sampling with the guillotine freeze clamping instrument.

RESULTS

Time-Temperature-Distance Relation During Freezing

It may be deduced from the equation describing one-dimensional heat diffusion in an idealized system that the time t required for a given temperature drop ΔT to occur in the distance x from a heat sink is proportional to the square of the distance times a constant k.[32] Figure 6.6 shows a number of time-temperature recordings at different distance x from the tissue-metal interface. From these data, a k value for cooling from 37 to 0°C may be calculated to an average of 1.44. However, the heat diffusion in the present cooling system is not accurately described by the simple relation mentioned above, since a nonlinear relation is obtained between *log* t and x. Measuring time of freezing (defined as hardening of the tissue), Veech and Hawkins found a k value of 2.36 cooling isolated cubes of brain tissue.[32] However, since the temperature was actually not measured, the k value may represent a temperature interval substantially larger than 0–37°C.

Applying immersion freezing, the distance-temperature relation has been measured in the brain of the intact animal or the severed head in a number of investigations.[1, 5, 21, 25, 30] In these studies, a roughly linear relationship is found, with a speed of progression of the freezing front of the order of 0.2 mm/sec, depending on the experimental conditions, e.g., the coolant used, the size of the animal, and whether or not the skin of the head was removed prior to freezing. It is difficult to compare the present results with these

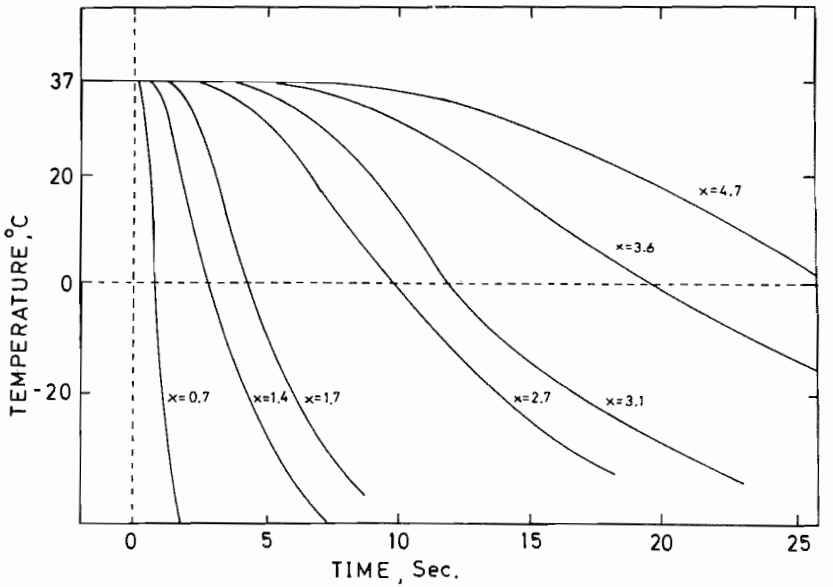

Figure 6.6. Representative time-temperature recordings in the brain sample at different distance from the tissue-metal interface. The recording thermocouple was positioned in the brain during sampling (Fig. 6.5*A*).

data because of the crucial effect on heat diffusion of different freezing geometry.[13] It is important to note in Figure 6.6 that the temperature in a given small part of the sample remains relatively high for a substantial fraction of the time needed to cool from 37 to, e.g., 0°C. Consequently, in this time interval, the tissue will not only be exposed to ischemia but also to varying degree of hypothermia.

Metabolite Concentrations Along the Freezing Gradient

Table 6.1 shows the concentration of adenylates, PCr, lactate, and pyruvate. The metabolites were measured in five consecutive sections cut parallel with the surface of the cooling blocks (Fig. 6.4*A*). Slice 1, nearest to the cooling block, had the most rapid quenching time, while slice 5, cut approximately from the middle of the sample, has been exposed to a maximum of time of ischemia. The changes in metabolite concentrations clearly indicate this (Table 6.1). The changes of PCr, lactate, and AMP are most rapid, while ATP and ADP are maintained close to normal values somewhat longer. These changes are qualitatively in agreement with previous reports on ischemia changes in the mouse,[10] in the gerbil,[7, 8] and in the rat.[20] A quantitative comparison will, however, require a conversion of the distance scale of the present results to a time scale (see Discussion).

The values found in slice 1 are close to control values usually found with immersion freezing[2, 15] for cerebral cortex and freeze blowing for whole brain,[14, 32] except for lactate and ATP, which in the present study are found about 40% lower and 20% higher, respectively. Compared with control data obtained with funnel freezing of superficial cortex,[20, 21] PCr and lactate in slice 1 is about 20% and 40% lower, respectively, while ADP and ATP are somewhat higher. The significance of the high ATP

is unclear at present. Figure 6.7 gives the proportionate changes[7] of ATP/ADP, L/P, and (ATP · AMP)/ADP[2] as a function of distance from the cooled surface. The ATP/ADP ratio decreases by a factor of 4 while the lactate/pyruvate ratio is increased almost 7-fold. The ratio of the adenylate kinase reaction shows an abrupt increase and then a decline towards equilibrium, however, remaining significantly elevated (Fig. 6.7). Similar changes in the myokinase ratio during ischemia in the gerbil has been observed recently[7] and were tentatively ascribed to a compartmentation of the adenine nucleotides, especially ADP (see also Veech[31]).

Table 6.1
Metabolite Concentrations Along the Freezing Gradient[a]

Slice number	1	2	3	4	5
Distance from surface to midpoint of slice (mm)	0.5[b]	1.7	2.8	3.9	5.0
			μmol/g		
PCr	3.79	2.61[d]	2.29[d]	1.57[d]	1.04[d]
	(0.14)	(0.15)	(0.10)	(0.08)	(0.10)
ATP	3.32	3.01	3.02[c]	2.64[c]	2.11[d]
	(0.20)	(0.11)	(0.10)	(0.09)	(0.12)
ADP	0.396	0.534	0.560[c]	0.696[d]	0.925[d]
	(0.041)	(0.120)	(0.060)	(0.039	(0.085)
AMP	0.044	0.125[c]	0.152[d]	0.281[d]	0.493[d]
	(0.005)	(0.032)	(0.018)	(0.024)	(0.077)
ΣAXP	3.82	3.73	3.76	3.62	3.55
	(0.23)	(0.31)	(0.17)	(0.13)	(0.08)
Lactate	0.720	1.03	2.14	3.07	3.61
Pyruvate	0.079	0.069	0.056	0.080	0.061

[a] The figures are means of five experiments with SEM given in parenthesis, except for lactate and pyruvate, which are means of three experiments.
[b] Significant difference relative to the values of the "0.5 mm slice" were evaluated by paired data *t* test.
[c] $P < 0.05$.
[d] $P < 0.01$.

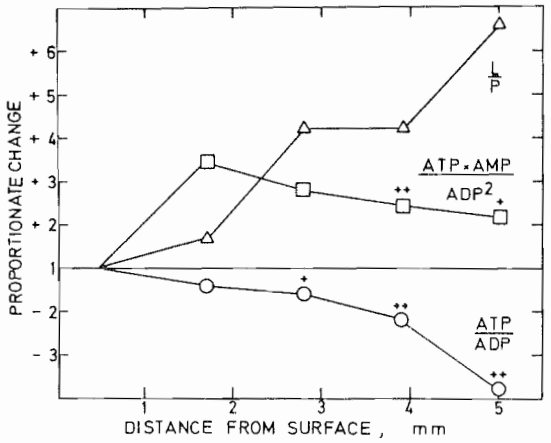

Figure 6.7. Changes of metabolic state along the freezing gradient. Metabolites were measured in five consecutive sections cut parallel with the tissue-metal interface. The figures were derived from the data on which Table 6.1 is based. The values of slice 1, i.e. ATP/ADP = 7.9 ± 0.6 (SEM), (ATP · AMP)/ADP[2] = 0.70 ± 0.09, L/P = 9, are taken to unity, while the values of slice 2 through 5 are given in proportion to this. The proportionate change was calculated as $x/control$ for $x >$ control and $control/-x$ for $x <$ control.[7] Significant differences with respect to the control (slice 1) were evaluated by paired data *t* test. + denotes significance $P < 0.05$ and ++ $P < 0.01$.

DISCUSSION

It was found above that heat transfer from the brain tissue under the present experimental conditions could not be described by simple one-dimensional heat diffusion to a heat sink (for a theoretical treatment of such an idealized case, see Wollenberger et al.[34]). The following factors might have influenced the heat transfer and made the function more complex:

1. The quality of the contact between tissue and metal is unknown, i.e., the effective area of heat transfer. It is our experience that there is a vast difference in speed of freezing, depending on whether the metal blocks are pressed gently or firmly against the tissue.
2. Since the heat diffusion constant of water changes significantly with temperature, this is probably also the case for brain tissue. However, no data are available at present.
3. The temperature of the cooling blocks does not remain constant during cooling (about −150°C 10 sec after clamping).

On the basis of the experimentally determined temperature-time-distance relation (Fig. 6.6), an attempt will be made in the following to calculate the ~P consumption[10] in the cerebral cortex as a function of distance from the tissue-metal interface accounting for the effect of the progressive hypothermia during cooling. It is assumed that the cortex is homogenous with respect to its response to ischemia and hypothermia. It is further assumed that ~P consumption in the time interval between decapitation and metabolic arrest by freezing is constant and relates to temperature as does oxygen uptake ($CMRO_2$) of the intact brain in vivo. Concerning the latter, there are rather different findings in the literature (for a recent review see Siesjö[26]). However, the most extensive study on rat cerebral cortex under conditions similar to those of the present experiments is that of Hägerdal et al.[4] In this study, a linear relation was found

$$C\dot{M}RO_2 = \alpha T - \beta \qquad (1)$$

where T is temperature and α and β constants determined to 0.23 and 3.91, respectively. $CMRO_2 = 4.6$ μmol/min·g at 37°C. For the calculation of ~P, equation (1) is adopted, substituting ~P for ⅙·$CMRO_2$. We have

$$T = f(x, t) \qquad (2)$$

and

$$\sim P = g(T) \qquad (3)$$

where T is temperature in a given small tissue volume at the distance x from the tissue-metal interface at the time t after decapitation. Since (1) may be substituted for equation (3) and since equation (2) has been experimentally determined for a number of x values $x_1, x_2, \cdots, x_i$ (cf., Fig. 6.6), it is possible to calculate ~P for these x values individually as

$$\sim P_{(x_i)} = \int_0^{t_i} g(T(x_i, t))\, dt \qquad (4)$$

with $T = 37$°C at $t = 0$ sec and $T = 17$°C at $t = t_i$ min. The expression (4) is calculated for the different x values, e.g., for $x = x_1$

$$\sim P_{(x_1)} = \sim P_{(T=37^\circ C)} \cdot \frac{I_1}{I_2} \cdot t_1 \qquad (5)$$

where I_1 is the area under the time-temperature curve for $x = x_1$ (Fig. 6.6) from 0 to t_1 and I_2 is the area of the corresponding rectangle $t_1 \cdot \Delta T$. ($\Delta T = 37 - 17$°C). The ratio I_1/I_2 was obtained by cutting out and weighing the two areas. Equation (1) assumes that ~P consumption is 0 below 17°C[4]. It was tried, however, to substitute $\sim P = ke^{\alpha T}$ for equation (1) in the expression (4), ($\alpha = \ln 2/10$ and $k = 0.35$). There was less than 5% difference with the two calculations of ~P.

The result of the calculations according to (5) is presented in Figure 6.8 where ~P is given as a function of distance from the surface. In order to allow a comparison with $\Delta \sim P$, which may be calculated from the data of Table 6.1, the position of each of the five slices on which the metabolite

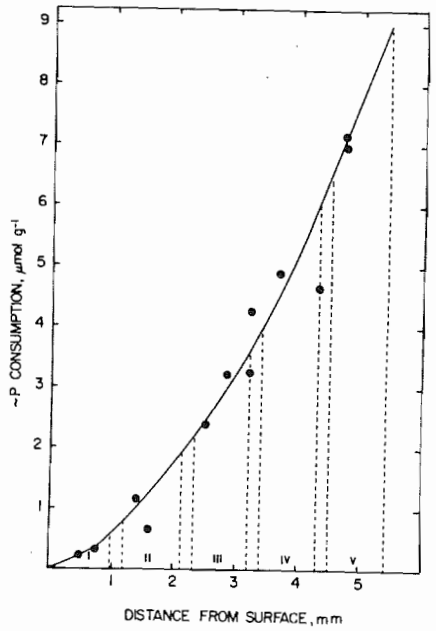

Figure 6.8. ~*P* utilization along the freezing gradient. The ~*P* was calculated from the time-temperature-distance recordings of Figure 6.6, as described in the text. The vertical dotted lines indicated the relative position of the five slices, 1 through 5, on which metabolite concentrations were measured (cf., Table 6.1).

measurements were performed are indicated by vertical dotted lines on Figure 6.8, allowing 0.2 mm between each slice, corresponding to the thickness of the saw blade. $\Delta \sim P$ was calculated, according to Lowry et al.[10], as the difference in ~*P* content of slice 1 and slices 2 through 5. The equivalent to these data, when derived from Figure 6.8, would be, e.g., for slice 2

$$\Delta \sim P_{\text{slice } 2} = \int_{1.2}^{2.1} h(x)\,dx - \int_{0.1}^{1.0} h(x)\,dx \quad (6)$$

representing Figure 6.8 as $\sim P = h(x)$.

Table 6.2 compares the two sets of calculations: the $\Delta \sim P$ derived from the metabolite measurements is increased about 100% in slice 2 compared with the value predicted from Figure 6.8. Slice 3 is 30% elevated and slice 4 is 15% elevated, while the predicted value is obtained in slice 5, suggesting that an increase of metabolic rate has occurred, which gradually decreases with distance from the site of the decapitation trauma.[17, 18]

This conclusion, however, presupposes a constant rate of ∾*P* utilization during the first 20–25 sec of ischemia,[10] an assumption which has recently been challenged by Nilsson et al.,[16, 20] who found rates of ∾*P* utilization of 30, 24, and 17 μmol/min · g for 5, 10, and 20 sec of ischemia, respectively. In order to compare the data of Table 6.2 with those results, the integral time of ischemia for the particular slice must be calculated. In analogy with the derivation of Figure 6.8, a graph of time of ischemia, corrected for the effect of hypothermia, versus distance from the surface may be derived. From this graph, the average time of ischemia for the slices 2 through 5 may be calculated to 2.8, 6.2, 11.2, and 17.3 sec, respectively, subtracting the time of ischemia for slice 1. Finally, dividing the figures for Δ ∾*P* (line one, Table 6.2) by the time of ischemia for the particular slice give rates of ∾*P* utilization of 43, 33, 30, and 26 μmol/min · g for slice 2 through 5, respectively. It should be noted that the only assumption made in the above calculation is that ∾*P* utilization is affected by hypothermia as stated in equation (1).

The data of Nilsson et al.[16] and the present results point to roughly the same nonlinear time course of ∾*P* utilization. Thus, the relative high value found in slice 2 in the present study (corresponding to 2.8

Table 6.2
$\Delta \sim P$ Consumption Along the Freezing Gradient

Slice number	2	3	4	5
		μmol/g		
Calculated from the analytical data of Table 6.1	2.0	3.4	5.6	7.5
Calculated from the time-temperature-distance relation, Figures 6.6 and 6.8	1.0	2.6	4.9	7.5

sec) does not necessarily indicate that a local increase in metabolic rate has occurred close to the decapitation trauma. Rather the similarity of the two sets of data suggests a general stimulation of the entire cortex, the degree of which may depend upon the method used to introduce ischemia and the psychological state of the animal at the time of the introduction of ischemia. Table 6.2 suggests an acceleration of metabolic rate by a factor of 2, however, a precise quantitative evaluation is not possible because of the nonlinear time course of ∾*P* utilization.[26]

In conclusion, the guillotine freeze clamping technique provides a way of rather precise regional sampling of brain tissue from the rat, allowing for 3-dimensional metabolic studies in vivo.[23] The present results, together with previously published data,[2, 19, 23] suggest that the part of the sample close to the cooling blocks (slice 1) has been frozen fast enough to preserve the in vivo metabolic state in spite of the increased metabolic rate which may have been caused by decapitation. The width of this zone of sufficiently fast quenching may not be determined accurately on basis of the present results. However, in a previous study[23] it was shown that significant change in metabolic state of the cortex did not occur within a distance of approximately 0.9 mm from the tissue-metal interface.

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