PITFALLS IN THE USE OF RAPID FREEZING FOR STOPPING BRAIN AND SPINAL CORD METABOLISM IN RAT AND MOUSE

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Abstract—The rate at which the metabolism is stopped by means of freezing in Freon-12 (-150°C) was studied in various areas of the rat and mouse CNS, using changes in temperature and levels of glucose and lactate as parameters for this rate. The rat cerebral cortex was frozen after 0.5 min while the hypothalamus reached 0°C after more than 1.5 min. The skin on the skull was found to be the most important temperature isolator for the cortex. Substrate levels can be studied in this area only if this piece of skin is removed previously.

In the mouse, the cerebral cortex was frozen after 6 s, the hypothalamus after 0.5 min. The lumbar level of the mouse spinal cord was frozen after 15 s, the cervical level only after 47 s. Liquid nitrogen alone cooled the mouse cerebral cortex at least as fast as did Freon at its melting point. A gradual decrease from dorsal to ventral was observed in the glucose level of the molecular layer of the mouse cerebellum. The existence of a freezing front, moving slowly from dorsal to ventral, and its consequences for the measured levels of biologically labile substrates, are discussed.

FOR PRESERVING the *in vivo* levels of many substrates in the CNS, it is essential to stop the metabolism as rapidly as possible by means of quick freezing (e.g. LOWRY, PASSONNEAU, HASSELBERGER and SCHULTZ, 1964). In order to know whether or not the measured levels of biologically labile compounds correspond with the actual *in vivo* levels, it is consequently necessary to know the moment at which metabolism stopped. The present study compares the rates at which metabolism stops in various areas of the rat and mouse CNS, using Freon-12 (CCl_2F_2) at its melting point (-150°C) as the cooling liquid. Moreover, the effect upon the freezing rate of some structures surrounding the brain was studied to examine ways of accelerating freezing. Based upon the work of JONGKIND and BRUNTINK (1970), changes both in temperature and in levels of glucose and lactate were used as parameters for the rate of freezing.

MATERIALS AND METHODS

Animals. For the present study 74 male Wistar rats (50–250 g) and 31 male Swiss random bred mice (20–25 g) were used.

Determination of temperature fall. Whole ether-killed animals or decapitated heads were kept at 38°C in sealed plastic containers in a water bath. A temperature probe (Philips NTC: 2322.62711222—thermometer type) was inserted into the cerebral cortex of rats and mice and also into the hypothalamus of mice, through the foramen occipitale. For reaching the rat hypothalamus the probe was inserted through a hole drilled in the skull via the auditory canal. The tip of the temperature probe was placed in the mouse spinal cord, at the level either of the first or second lumbar vertebra or the fourth or fifth cervical vertebra, using a ventral approach via trepanation of the vertebral body, after which the skin was sutured accurately. Heads or whole animals were frozen in Freon-12 (CCl_2F_2 , LOOSCO) at its melting point (-150°C) while being vigorously stirred. In all experimental conditions the cooling medium was prevented from reaching the tip of the probe via the place of insertion by binding the skin tightly around the probe. For the auditory canal insertion, the uninserted part of the probe was brought through a rubber plug, which was attached to the skin around the canal by suturing. The conductance changes of the temperature probe were observed on an oscilloscope, which allowed the registration of a drop in temperature of 70°C within 0.5 s. The position of the tip

of the probe was checked after each experiment. The time in which the brain temperature reaches 0°C was considered to be a measure of the freezing rate in the CNS (JONGKIND and BRUNTINK, 1970).

Substrate measurements (macro-procedure). For this experiment 24 rats (140 g) were divided at random into two groups. Under Hypnorm (a neuroleptic analgetic, Philips-Duphar) anaesthesia ($0.1\text{ ml}/100\text{ g}$ body wt. subcutaneously), a piece of skin on the skull was removed from 12 rats. Twelve others were subjected to a sham operation, making the same incision in the skin, after which the skin was closed by suturing. From each group nine animals were decapitated 48 h after operation. Immediately after decapitation the entire heads were frozen in Freon-12 (-150°C). Three brain areas (cerebral cortex, cerebellum and hypothalamus) were chiselled free from the skull, while the tissues were kept submerged in liquid nitrogen. The samples were stored in sealed plastic containers at -80°C . The frozen samples (weighing about 100 mg) were brought into liquid nitrogen (AGA) and then weighed at room temperature within 5 s. Glucose and lactate were extracted from the frozen brain areas by the acid methanol method (FOLBERGROVÁ, PASSONNEAU, LOWRY and SCHULTZ, 1969). The final extracts were stored at -30°C until analysed. Glucose was measured according to LOWRY *et al.* (1964) and lactate was determined according to JONGKIND and BRUNTINK (1970). Three operated and three sham-operated animals were kept in individual cages for a time after surgery for observation.

Substrate measurements (micro-procedure). Immediately after decapitation six mouse heads were frozen in liquid nitrogen-cooled Freon-12 (-150°C). The frozen cerebellum was removed (see above) and paramedian frozen-dried cryostat sections ($16\text{ }\mu\text{m}$) were used for the rest of the procedure. Molecular layer samples (about $0.6\text{ }\mu\text{g}$) were dissected at room temperature at three different levels from the same section (see FIG. 6) and weighed on quartz fibre balances. Glucose was determined in these samples using the oil-well technique (MATSHINSKY, PASSONNEAU and LOWRY, 1968). The sources of the chemicals used in the present study are the same as those that had been used by the cited authors.

Statistics. Differences in temperature and substrate levels were tested using the Student *t*-test (DE JONGE, 1963). A value for $P < 0.05$ was considered to be significant.

RESULTS

Determination of temperature fall. Figure 1 shows the position of the tip of the temperature probes in the cerebral cortex and the hypothalamus, projected onto paramedian sections of rat and mouse brain. The probes were located within 1.5 mm of the median plane. Each temperature measurement in the following experiments represents the mean of five separate determinations in different animals or tissues. As can be observed from Fig. 2a, rat (140 g) cerebral cortex reached 0°C in 0.5 min after the intact head (N) was immersed in Freon-12 (-150°C). This time was not significantly lowered by completely shaving the heads (B) ($0.10 > P > 0.05$). Removal of a piece of skin above the skull (S), however, resulted in a much faster drop in cortical temperature (to 0°C in 3.8 s; $P < 0.02$ as compared to normal or bald heads). There was a slightly faster drop in temperature after removal of the skull (C) which was not significant, however ($P > 0.20$). The rapid drop in cortical temperature after removal of the skin above the skull was also observed in 50 g rats (2.8 s) and 250 g rats (5.6 s) (Fig. 2b).

FIG. 1.—Positions of the tip of the temperature probe in the cerebral cortex and hypothalamus of rat (left) and mouse (right) (shaded areas). The vertical lines indicate the scale in mm.

FIG. 2.—Temperature changes after immersion in Freon (-150°C) in the cerebral cortex of decapitated heads of rats (140 g) (2a) and 50–250 g (2b) rats. N: normal heads, B: bald heads, S: piece of skin on the skull removed, C: skull removed. Every point represents the mean of five animals, the horizontal lines indicate the S.E.M.

A very slow freezing was observed in the hypothalamus of rats (130 g) (Fig. 3): it required more than 1.5 min for this area to reach 0°C . Removal of the mandible and tongue shortened this time significantly, to 52.7 s ($P < 0.01$). Moreover, when the skin on the skull was removed, this area reached 0°C even faster ($P < 0.05$), although this still took over 0.5 min. The geometrical centres of blocks of rats (250 g) brain with 8 mm edges reached 0°C after 12.4 s (Fig. 3). The cerebral cortex of decapitated mouse heads reached 0°C in 6.4 s using Freon-12 as the cooling medium (Fig. 4). Using only liquid nitrogen as the cooling medium, the 0° , -10° and -20°C values did not change significantly ($P > 0.10$), whereas liquid nitrogen-cooled heads reached -30°C faster when compared to Freon-immersed heads ($P < 0.05$). The hypothala-

FIG. 3.—Temperature changes after immersion in Freon (-150°C) in the hypothalamus of decapitated rat (130 g) heads. N: normal heads, M: mandible and tongue removed, MS: mandible, tongue and a piece of skin on the skull removed. B: block of ($8 \times 8 \times 8$ mm) rat (250 g) brain tissue. Every point represents the mean of five animals, the horizontal lines indicate the S.E.M.

FIG. 4.—Temperature changes in various areas of the mouse CNS. C(LN₂): cerebral cortex, immersion in liquid nitrogen alone (the rest of the observations were obtained by immersion in liquid nitrogen-cooled Freon-12). C(F): cerebral cortex, immersion in Freon-12. L₁₋₂: spinal cord at the level of the first or second lumbar vertebra. H: hypothalamus. C₄₋₅: spinal cord at the level of the fourth or fifth cervical vertebra. Every point represents the mean of five animals, the horizontal lines indicate the S.E.M.

mus required 33 s to reach 0°C in decapitated mouse heads. A considerable difference in the time at which the spinal cords of whole mice reached 0°C was found between the lumbar level (15.4 s) and the cervical level (46.7 s).

Substrate measurements (macro-procedure). The animals from which the skin on the skull was removed (S), as well as the controls (N), withstood the surgery perfectly for more than 6 months at present, although no antibiotics were given and the animals were placed after operation in the normal cages with sawdust. The skull remained exposed for 6 weeks, after which it became gradually covered with skin and hair. Glucose and lactate levels in the cerebral cortex, hypothalamus and cerebellum of sham-operated and operated rats are given in Fig. 5. Removal of a piece of skin on the skull resulted in a significant increase in glucose and decrease in lactate levels in

FIG. 5.—Levels (S.E.M.) of glucose and lactate (in mmol/kg wet wt.) in the cerebral cortex, the cerebellum and the hypothalamus of nine sham-operated control rats (N) and in nine rats from which the skin on the skull had been removed (S).

the cortex as well as in the cerebellum ($P < 0.001$). The glucose and lactate levels of the hypothalamus, on the other hand, were not significantly influenced by this operation ($P > 0.10$). Moreover, as compared to the cortex, significantly higher lactate and lower glucose levels were found in the hypothalamus of both normal and operated animals ($P < 0.01$).

Substrate measurements (micro-procedure). The glucose levels in the molecular layer of the cerebellum showed a gradual decrease from dorsal to ventral (Fig. 6). In the most dorsally situated samples a glucose level of 7.6 mmol/kg dry wt. was found, whereas significantly less than this value (3.6 mmol/kg) was found in samples situated 1.4 mm ventrally ($P < 0.05$). A still lower glucose level (2.1 mmol/kg) was found in the most ventral samples ($P < 0.01$ as compared to the values most dorsally), which were situated 2.4 mm from the dorsal ones.

GLUCOSE LEVEL (mmol/kg d.w.) IN THE MOLECULAR
LAYER OF MOUSE CEREBELLUM

FIG. 6.—Glucose levels (in mmol/kg dry wt.) in the molecular layer of samples, dissected at different distances from dorsal in the mouse ($n = 6$) cerebellum. Left values: mean distances (S.E.M.) of samples from the dorsal ones. Right values: glucose levels (S.E.M.).

DISCUSSION

After decapitation the brain is transformed into a closed system, in which energy stores are expended (e.g. LOWRY, 1966). Important changes have been demonstrated within a few seconds of ischaemia in the brain levels of: substrates of the glycolytic pathway, phosphocreatine, ATP, inorganic phosphate (LOWRY *et al.*, 1964; FOLBERGROVÁ, LOWRY and PASSONNEAU, 1970), cyclic AMP (BRECKENRIDGE, 1970), intermediates of the citric acid cycle (GOLDBERG, PASSONNEAU and LOWRY, 1966) and the pentose pathway (KAUFFMAN, BROWN, PASSONNEAU and LOWRY, 1969). Consequently, for the measurement of *in vivo* levels of any of these substrates it is essential to stop brain metabolism immediately after decapitation. Although it is often claimed that Freon-12 or isopentane at their melting points accomplish freezing faster than does liquid nitrogen (e.g. MOLINE and GLENNER, 1964; LOWRY and PASSONNEAU, 1971), the freezing rate to -20°C of mouse cortex was found to be the same for Freon (-150°C) and liquid nitrogen. An even quicker cooling was in fact found from -20° to -30°C when liquid nitrogen alone was used as a cooling medium. For the remainder of the present study, nevertheless, Freon (-150°C) was used as the freezing liquid, in order to be able to compare the published data obtained for CNS substrate levels by means of this procedure with our present temperature and substrate measurements.

It was not possible to stop the brain metabolism in the adult rat quickly enough for the maintenance of *in vivo* levels of biologically labile substrates. It took about 45 s to cool the cerebral cortices of intact heads of adult rats to 0°C (JONGKIND and BRUNTINK, 1970), while the removal of the brain from the skull of these rats before freezing took approx. 20 s (SWAAB, unpublished). Moreover, anaesthesia interferes seriously with brain metabolism (LOWRY *et al.*, 1964; GATFIELD, LOWRY, SCHULTZ and PASSONNEAU, 1966; GOLDBERG *et al.*, 1966; KAUFFMAN *et al.*, 1969; FOLBERGROVÁ *et al.*, 1970). For the study of normal brain metabolism it is therefore not valid to shorten the time of ischaemia by isolating the brain under anaesthesia.

The reason for this low rate of freezing and some possible ways of accelerating the process, were therefore studied in the rat. In addition, the freezing rate was studied

in various areas of the mouse CNS in order to allow interpretation of certain data in the literature concerning substrate levels in these areas. As is apparent from the temperature measurements in the rat cerebral cortex (Fig. 2), it is neither the hair nor the skull, but the skin which is the most important temperature isolator. Since a fast freezing rate was found in rats of 50 to 250 g following removal of a piece of skin on the skull, and since these animals tolerate the surgery excellently, this procedure is most useful for the determination of biologically labile substrates in superficial dorsal areas. Brain areas that are situated more ventrally, however, show a freezing rate which is too slow to permit such studies. Removal of mandible, tongue and a piece of skin on the skull did not change this situation enough to permit these substrate studies in the hypothalamus (Fig. 3). Although the freezing rates observed in mice were much faster as compared to the adult rat, large differences were also observed in the time required for the various CNS areas to reach 0°C (Fig. 4).

Combination of the various temperature and substrate data during freezing pointed to the existence of a freezing front, moving from dorsal to basal with a mean speed of 1 mm per 5–7 s. This was suggested in the first place by the finding that the hypothalamus in rat was frozen 52 s later than the cerebral cortex, their distance apart being about 7.5 mm. For the mouse these values were 27 s and 5 mm respectively. Approximately the same time–distance ratio could be calculated from the data of TAKAHASHI and APRISON (1964). In addition, a gradient was observed in glucose and lactate concentrations, pointing to a gradual inhibition of the metabolism from dorsal to ventral (Fig. 5). Moreover, an important influence upon the freezing rate was found for the skin on the skull (Figs. 2 and 5). Finally, 0°C was reached in the geometrical centres of $8 \times 8 \times 8$ mm blocks after 12.4 s, which gives a mean spreading of the cold of 1 mm per 3 s. In order to test this freezing front hypothesis, glucose levels were measured in the cerebellar molecular layer, a structure situated over a large distance from dorsal to ventral. The gradual decrease in glucose levels found from dorsal to ventral in the molecular layer further suggests a freezing front moving slowly in this direction.

The difference in freezing rates between ventral and dorsal brain areas may have important consequences for the interpretation of data concerning substrate levels of biologically labile compounds. Whole brain values of these substrates give only the mean between levels in dorsal areas that freeze quickly and ventral areas that freeze slowly. For the same reason, one has to guard against comparison of regional substrate levels, which have been measured in areas that may well have been frozen at different times. Moreover, corrections for differences in freezing time may be necessary if substrates are to be studied in layered structures. Various chemical problems concerning the hypothalamus can be solved only by the measurement of biologically labile compounds (SWAAB, 1970; JONGKIND, 1971). The slow freezing of the basal brain region makes measurement of these compounds impossible in the hypothalamus *in situ*. For this reason a procedure has been developed for isolating rat brain within 7 s after decapitation, via a trepanation made some days previously (SWAAB, in preparation).

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