THE PRESENCE OF BIOLOGICALLY LABILE COMPOUNDS DURING ISCHEMIA AND THEIR RELATIONSHIP TO THE EEG IN RAT CEREBRAL CORTEX AND HYPOTHALAMUS

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Abstract—Two surgical methods are described in the present paper, allowing for the approximate determination of *in vivo* levels of ATP, lactate, glucose, pyruvate and glycogen in anatomically uninjured cortex and hypothalamus from unanaesthetized rats. It was not possible to obtain such levels for P-creatine in the 2 mm thick samples used in the present investigation. No fundamental difference was observed between the cortical and the hypothalamic levels of these substrates nor in their fluxes.

The substrate fluxes during ischemia were correlated with electrical activity in the rat cortex and hypothalamus, recorded by means of telemetrically transmitted electroencephalograms. The electrical activity declined precipitously at 9.6 s after decapitation in the cortex, and after 12.1 s in the hypothalamus. High levels of glycogen, glucose and ATP were present at this moment, while P-creatine had declined sharply.

USING the conventional quick freezing procedure for mice (e.g. LOWRY, PASSONNEAU, HASSELBERGER and SCHULZ, 1964; LOWRY and PASSONNEAU, 1971) on entire rat heads, it proved impossible in this animal to obtain concentrations approximating to the true *in vivo* levels of biologically labile compounds (JONGKIND and BRUNTINK, 1970; MAKER and LEHRER, 1971; SWAAB, 1971). With regard to the cortex, the skin over the skull was shown to be the most important temperature isolator (SWAAB, 1971). Furthermore, as has been shown by enzyme-, temperature- and substrate-determinations (BRECKENRIDGE and NORMAN, 1965; SOBOTKA, 1971; SWAAB, 1971), a freezing front moving slowly from dorsal to ventral interferes especially with the determination of labile compounds in the most ventral brain area, i.e. the hypothalamus.

In the present study, surgical methods are presented, which allow the approximate determinations of the *in vivo* levels of some biologically labile compounds in anatomically uninjured cortex and hypothalamus from unanaesthetized rats. These methods are based upon removal of the insulating layer overlying the cortex and on a rapid (less than 2 s) post mortem removal of the brain via a trepanation made a week previously. By means of these procedures, the levels and fluxes of certain glycolytic substrates, the metabolic rates (see LOWRY *et al.*, 1964; GATFIELD, LOWRY, SCHULZ and PASSONNEAU, 1966) and the lactate/pyruvate ratio as an indicator of the cytoplasmatic NAD^+/NADH ratio (WILLIAMSON, LUND and KREBS, 1967) were determined.

The portion of the hypothalamus which controls pituitary function contains neurons that also show certain characteristic of endocrine cells (e.g. STUTINSKY, 1970). Moreover, a lower oxygen tension than in the cortex has been observed in this area (CATER, 1966). Since these findings might go together with different metabolic characteristics in the two regions, the values of the compounds in the cortex have been compared to those of the hypothalamus.

Finally, metabolic changes during ischemia were compared with changes in electrical activity of rat cortex and hypothalamus. The electroencephalograms from these brain areas were recorded telemetrically.

MATERIALS AND METHODS

Animals. For the present study 62 male Wistar rats (approx. 200 g) were used. They were kept in individual cages at 25°C and exposed to 12 h light daily (from 7 a.m. to 7 p.m.). The animals received tap water and standard chow *ad libitum*. All rats were killed around 11 a.m.

Operation techniques. Under Hypnorm (a neuroleptic analgetic; Philips-Duphar) anaesthesia (0.1 ml/100 g body wt., subcutaneously), either a piece of skin on the skull was removed (see procedure A) or a trepanation was made (see procedure B).

Procedure A; a piece of skin on the skull was removed from 13 rats (Scalped animals: Sc-group). The animals were decapitated 48 h after operation and the entire heads were immediately frozen in liquid-nitrogen cooled Freon-12 at -150°C (SWAAB, 1971).

Procedure B; from 37 rats the skin was opened by a sagittal incision and the periosteum was scratched off (see Fig. 1A). The skull was opened (according to the dotted line of Fig. 1A) by means of a small circular saw (Meisinger Fig. 231-8). This piece of skull was bluntly released from the dura mater, after which it was replaced again for about 5 min to obtain hemostasis and then removed. A parafilm (American Can Company) roof (Fig. 1B) was placed over the trepanation. The skin was placed over the horizontal part of this roof and the skin edges were pressed against the vertical part of the parafilm roof by means of two polyethylene tubes (Talas, Pt. 55) (see Fig. 1C, D). Two sutures that penetrated the skin and the vertical part of the parafilm roof were tied around the tubes to close the wound (see Fig. 1D). Thus the force of the two sutures was distributed over the whole length of the wound edges by the plastic tubes. Seven days after this operation, the two sutures were cut off and the polyethylene tubes and parafilm roof could easily be removed. The animals were observed continuously during a period of 2.5 h, and only animals that did not show any bleeding and behaved normally were used for the rest of the procedure.

FIG. 1.—Trepanation technique. For explanation see text (Materials and Methods: Procedure B).

One group of 12 rats was killed by removal of the brain at once, by means of a spoon, via the trepanation (Spoon: Sp-group). The brains were frozen either immediately (6 rats) or after 8 s (6 rats) in liquid-nitrogen cooled Freon-12 (-150°C). A second group of 25 rats was killed by decapitation (Decapitation: D-group), after which the brains were removed by means of a spoon via the trepanation and were frozen in liquid-nitrogen cooled Freon at 8 s (7 rats), 15 s (6 rats), 30 s (6 rats) and 60 s (6 rats) after decapitation. During the period between severing the head and freezing the brain the tissues were kept in a nitrogen environment at +38°C.

Substrate measurements. While the tissues were kept submerged in liquid nitrogen, superficial samples (approx. 2 mm thickness) of cerebral parietal cortex and hypothalamus (between the optic chiasm and mammillary bodies) were prepared and stored in sealed plastic containers at -80°C. The frozen samples (weighing about 100 mg) were brought into liquid-nitrogen and weighed at room

temperature within 5 s. Substrates were extracted from the frozen brain areas by the acid-methanol-perchloric acid method (FOLBERGROVÁ, PASSONNEAU, LOWRY and SCHULZ, 1969). The final extracts were stored at -30°C until analysed. In these extracts the substrates were measured fluorimetrically by enzymic methods. Glucose, ATP and P-creatine were determined according to LOWRY *et al.* (1964), lactate according to JONGKIND and BRUNTINK (1970), glycogen following JONGKIND, CORNER and BRUNTINK (1972) and pyruvate determined according to LOWRY *et al.* (1964) using a medium containing in addition 0.25 M-dithiotreitol (Calbiochem).

The total energy reserves have been calculated as $\sim P = 2 \text{ ATP} + \text{ADP} + \text{P-creatine} + 2.9 \text{ glycogen (as glucosyl)} + 2 \text{ glucose}$ (see LOWRY *et al.*, 1964). Since the equilibrium for the reaction catalysed by adenylkinase is maintained during the period (1.8 up to 8 s) over which we calculated the initial metabolic rate (see LOWRY *et al.*, 1964), ADP was not measured but calculated according to GATFIELD *et al.* (1966).

Bovine heart LDH was obtained from Worthington. All other enzymes, substrates and cofactors, if not mentioned otherwise, were obtained from Boehringer.

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

EEG recordings. For the electrocorticogram (ECOG) recordings from 6 rats, two silver ball electrodes, each insulated except for the lower half, were placed under Hypnorm anaesthesia (see before) symmetrically upon the dura of the cerebral hemispheres through closely fitting holes drilled in the skull. For the electrohypothalamogram (EHYG) recordings from 6 rats, a 300 μm unipolar silver wire electrode was stereotaxically placed in the supraoptic nucleus at the right side. These electrodes were diamel-coated except for the tip-surface, which was cut at a 45° angle. A 200 μm silver wire turned around four screws in the skull served as a reference electrode. The distal end of each electrode was attached to a female connector, which was affixed to the skull by means of dental cement.

The encephalograms were transmitted telemetrically by a subminiature transmitter based upon SKUTT, BESCHLE, MOULTON and KOELLA (1967). This transmitter was plugged into the female plug just prior to the experiment, which was carried out one week after the operation. The signal was received by a slightly modified FM-tuner (Philips-type RH 691). It was passed either through a Tektronix preamplifier (type RM 122) with the bandpass set at 0.2–250 Hz or a Grass preamplifier (type P15) with the bandpass set at 0.3–100 Hz and displayed on a dual beam oscilloscope (Tektronix type 502 A). The signal was stored on magnetic tape (Philips Ana-log 7) and also written out on a polygraph (Schwarzer-type E502), with the bandpass set at ± 1 –30 Hz. Recordings were made before, during and immediately after decapitation.

After death a current of 2–8 mA was sent through the hypothalamic electrode for 10–30 s. The electrode tip was localized using the combined data on (1) the disposition of silver granules in unstained cryostat sections (20 μm) and (2) the glia around the electrode track (Boer and Swaab, in preparation) that was observed after thionin staining (see SWAAB, 1970).

RESULTS

Operation techniques. The animals withstood both surgical procedures perfectly. The few animals which showed either a fall in body weight after the operation, or paralysis or bleeding after removal of the plastic roof were excluded from consideration.

After some practice it proved possible to remove instantly an anatomically intact piece of brain containing both the cerebral hemispheres and the hypothalamus. In the group from which the brains had been removed in this way after decapitation (D), however, the tissues could not be got into Freon more quickly than with an average of 8.1 s (S.E.M.: 0.23) after death. In contrast, brains from the animals killed by immediate removal of the brains (Sp group) could be frozen within 1.8 s (S.E.M.: 0.13, minimal value 1.2 s).

Substrate measurements. The substrate levels in cortex and hypothalamus after the various periods of ischemia are given in Figs. 2–7. Except for the lower hypothalamic glycogen level ($P < 0.02$), the initial values for the hypothalamus do not differ significantly from those for the cortex.

FIG. 2.

FIG. 3.

FIG. 4.

FIG. 5.

FIG. 6.

FIG. 7.

FIGS. 2-7.—Effect of ischemia on various substrate levels in cortex and hypothalamus of rats killed either by removal of the brain by means of a spoon (Sp), or by decapitation (D), and compared with the substrate levels in the cortex of rats from which a piece of skin on the skull was removed (scalped animals: Sc).

The initial substrate fluxus (between 1.8 and 8 s) showed important differences between the various compounds. The P-creatine level decreased by 63 per cent in the cortex ($P < 0.001$) and 56 per cent in the hypothalamus ($P < 0.005$). ATP decreased in this period 36 per cent in the cortex ($P < 0.001$) and 30 per cent in the hypothalamus ($P < 0.01$). Lactate increased 61 per cent in the cortex and 57 per cent in the hypothalamus (both $P < 0.02$). The changes in glucose, pyruvate and glycogen on the other hand did not show significant changes during the period mentioned above (glucose in hypothalamus, $0.10 < P < 0.20$; pyruvate in both areas, $0.20 < P < 0.30$; for the other values, $P > 0.70$).

Since the pyruvate level during anoxia remained within about the same range, whereas the lactate level rose markedly, the lactate/pyruvate ratio increased from the initial levels, 22 (S.E.M.: 4.2) and 16 (S.E.M.: 4.7); in cortex and hypothalamus respectively, up to 89 (S.E.M.: 14.9) and 124 (S.E.M.: 26.8) after 60 s of ischemia. In the cortex of the Sc group this ratio was found to be 17 (S.E.M.: 2.9). No significant ($P > 0.10$) differences were found between the initial cortex and hypothalamus values, nor between this cortex value and the ratio in Sc animals.

FIG. 8.—Total energy reserves in the cortex and hypothalamus of rats killed by removal of the brains (Sp) or by decapitation (D), and the total energy reserve in the cortex of rats from which a piece of skin on the skull was first removed (Sc). The values were obtained as described in the text (see Materials and Methods).

Concerning the total initial energy reserves (Fig. 8) no significant difference was observed ($0.10 < P < 0.20$) in the Sp group at 1.8 s in either the cortex (18.3 mmol/kg wet wt.) or in the hypothalamus (15.5 mmol/kg wet wt.), while moreover the difference between the Sp cortex (18.3) and the Sc cortex (16.0) was not significant ($0.10 < P < 0.20$). The fluxes in the energy reserves during ischemia are shown in Fig. 8 for both cortex and hypothalamus. No important difference was observed between the initial metabolic rates (calculated in terms of high energy phosphate utilization during the period of 1.8–8 s) for cortex ($26.8 \sim P$ mmol/min) and hypothalamus ($23.1 \sim P$ mmol/min), nor was any difference seen between these two regions throughout the rest of the course of these curves.

EEG recordings

Electrocorticogram. Prior to decapitation a normal waking EEG was observed, showing a relatively low amplitude and fast frequencies. The ECoG recorded less than 1 s following decapitation showed the same pattern, although the amplitude was usually slightly smaller. At 5.7 s (S.E.M.: 0.22) a marked decrease in electrical activity suddenly took place, while by 9.6 s (S.E.M.: 0.65) after decapitation no electrical activity that could be discerned from the base line remained.

Electrohypothalamogram. The electrode tips were situated in or near the supra-optic nucleus (Boer and Swaab, in preparation). Prior to decapitation two prominent waking patterns could be discerned: a low amplitude-fast frequency EEG during the normal waking state (Fig. 9: 2a) and a synchronized 7–10 Hz EEG of higher voltage during behaviourally highly alert states (Fig. 9: 2b). This synchronized EEG was induced by arousing stimuli such as handling and loud noises. It also occurred during the rats investigation of unfamiliar objects and during introduction of the animal into the decapitator. Such an EEG pattern resembled closely the synchronized electrical activity observed following decapitation. The signal from the severed head went on with the same amplitude for 5.6 s (S.E.M.: 0.24), after which a precipitous decrease

FIG. 9.—EEGs after decapitation (D) in the unanaesthetized rat. 1a: *electrocorticogram* before, and 1b; after decapitation. 2a–b: *electrohypothalamograms* before, and 2c: after decapitation. Note the resemblance between the EEG pattern during a behaviourally highly alert state (2b) and the pattern immediately following decapitation (2c). In addition, the figure shows: jaw movement artifacts (O) after decapitation, the instant of onset of a rapid decline in voltage (Δ), and the point after which EEG activity can no longer be discerned from the baseline (Δ).

could be observed. By 12.1 s (S.E.M.: 0.47) no signal could be recognized above the baseline.

All recordings after decapitation showed some artifacts caused by jaw movements. Movement artifacts were also caused by introduction of the animal into the decapitator and by decapitation itself, as a result of frequency drifting of the transmitter and overloading of the recording equipment. The jaw movements artifacts were not obviously related to the EEG survival time, as is shown in Fig. 9: 1b, since they were seen for 20 s following decapitation.

DISCUSSION

After conversion of the brain into a closed system (LOWRY *et al.*, 1964) regional brain chemistry can be studied by means of microchemical methods even down to the single neuron level (PASSONNEAU and LOWRY, 1971). Moreover, this procedure may possibly avoid certain of the problems, e.g. a lower metabolic rate (HOLOWACH-THURSTON and MCDUGAL, 1969), of *in vitro* systems (GATFIELD *et al.*, 1966).

Various questions concerning the chemistry of the rat hypothalamus, e.g. the role of TPP-ase in the supraoptic nucleus, might be tackled by this closed system procedure (SWAAB, 1970; JONGKIND, 1971; SWAAB, 1972). For these kind of studies, however, it is essential to stop the brain metabolism very shortly after death (for review see MAKER and LEHRER, 1971). Although microwave irradiation (SCHMIDT, SCHMIDT and ROBISON, 1971) is a promising technique for the future, quick freezing has up to now been the method of choice for stopping the brain metabolism (e.g. LOWRY and PASSONNEAU, 1971). The slow freezing rate in rat brain (SWAAB, 1971) made application of the conventional freezing procedure impossible, and the *in situ* freezing technique of the Lund group (e.g. KAASIK, NILSSON and SIESJÖ, 1970; MESSETER and SIESJÖ, 1971) could for the same reason not be applied for the study of a ventral brain area like the hypothalamus. The brain extractor, as developed by E. H. Veech allows a very rapid (less than 1 s) isolation of rat brain without preceding operative procedures (VEECH, HARRIS, VELOSO and VEECH, 1972). This technique could not be applied in our case, however, since the brain tissue is extruded through a probe. Our requirement was anatomically intact brain e.g. in order to make localization of the various hypothalamic areas possible (see SWAAB, 1972).

In rats from which a piece of skin over the skull has been removed (Sc group), the freezing rate of the cortex corresponds to that in the mouse (SWAAB, 1971). Since the glucose, glycogen and lactate levels in these rats, as determined in the present study, agree with those in mice (LOWRY *et al.*, 1964; GATFIELD *et al.*, 1966; for a review see MAKER and LEHRER, 1971), and the pyruvate and ATP levels correspond to those obtained by the rapid freezing method of VEECH *et al.* (1972) and although the ATP values may be slightly lower than the true *in vivo* levels (see FERRENDELLI, GAY, SEDGWICK and CHANG, 1972), our operative technique seems to offer a suitable tool for the study of these biologically labile substrates in the rat cortex. Although the more laborious trepanation technique thus becomes unnecessary for studies on these substrates in the cortex, the initial substrate levels in this brain area obtained by the trepanation procedure agreed with those mentioned before. The advantage obtained by the trepanation procedure, in terms of shortening of the period of ischemia in the ventral brain area, is obvious when the hypothalamic glucose and lactate levels obtained by the conventional freezing procedure of whole rat heads (0.21 and 5.31

mmol/kg wet wt. respectively: see SWAAB, 1971) are compared with those obtained by the trepanation procedure (1.44 and 1.96 mmol/kg wet wt. respectively).

In contrast to the above-mentioned substrate levels, the P-creatine values cannot be considered as approximating to the true *in vivo* levels in rat (cf. VEECH *et al.*, 1972). Our low values cannot be explained by loss of this compound during the extraction nor by condensation of water vapour onto the samples during the weighing procedure. Therefore, the time necessary to stop the brain metabolism, in combination with the extremely rapid disappearance of P-creatine during ischemia, must be the explanation for these low values. In this connection, the time (1.8 s) necessary to get the brain tissue into the Freon becomes important, and in addition the sample thickness must play a role. Under the freezing conditions described in the present paper, the cold front spreads in the brain tissue at approx. 1 mm per 3 s (SWAAB, 1972). It will thus take around 8 s (1.8 plus 6 s) before the central part of our sample will be frozen. This problem of slow spreading will be less serious when only structures in the extreme periphery (e.g. the hypothalamic supraoptic nucleus) of the brain are studied. A third factor leading to relatively high P-creatine levels seems to be the freezing of tissues while the blood circulation is intact since after immersion of whole rats higher levels were found than after decapitation (VEECH *et al.*, 1972). This procedure cannot be applied, however, in the study of $\sim$ P fluxes during shortlasting ischemia.

Although the various groups of animals used in the present study (Sp, D and Sc) are not entirely comparable with regard to either the kind of operation, the post-operative period or the way of killing, the substrate levels of the Sc animals and those of the Sp animals (at 1.8 s of ischemia), as well as the substrate levels of the two groups (Sp and D) at 8 s of ischemia, agree quite well. Preliminary studies on rat cortex and hypothalamus have shown, moreover, that the length of the postoperative period (3, 5 or 7 days) is not of importance for the substrate levels after 8 s of ischemia. Whether or not the difference in glycogen levels between the various experimental groups (Sp, D and Sc) is a consistent one has still to be investigated.

No significant difference in initial substrate levels (Sp, 1.8 s) was observed between cortex and hypothalamus, with the exception of glycogen, which in all experimental groups was higher in the cortex than in the hypothalamus. This is an unexpected finding, since histochemical data point to a higher glycogen content in the hypothalamus (SHIMIZU and KUMAMOTO, 1952). Since the methods of handling the tissues in that study were not suitable for the acquisition of the approximate *in vivo* values, however, the histochemical localization of glycogen has to be repeated in rapidly frozen tissue.

In spite of the great difference in metabolic rate observed between adult rat brain (see Results) and adult mouse brain (LOWRY *et al.*, 1964) on the one hand, and newborn mice (HOLOWACH-THURSTON and MCDUGAL, 1969) or chick embryos (JONGKIND *et al.*, 1972) on the other, a striking similarity exists in their total energy reserves. That these values are not valid throughout the whole animal kingdom, however, appears from the very high (50–70 mmol $\sim$ P/kg wet wt.) energy reserves observed in some poikilothermic vertebrates (MCDUGAL, HOLOWACH, HOWE, JONES and THOMAS, 1968).

Although our values for the initial energy reserves are slightly lower than the actual *in vivo* situation, as has been discussed before, this will probably not interfere with the comparison of the fluxes in cortex and hypothalamus, since the initial rate of $\sim$ P utilization in brain is maintained for about 15 s (LOWRY *et al.*, 1964).

Energy rich phosphates are supposed to be the source of energy for active ion transport, and are consequently essential for the maintenance of electrical activity of nervous tissue (e.g. McILWAIN, 1969; OKADA and McDUGAL, 1971). Since various types of anoxia result in a rapid loss of electrical activity (e.g. BAUMGARTNER, CREUTZFELDT and JUNG, 1961; NAQUET and FERNANDES-GUARDIOLA, 1961), the metabolic changes during ischemia in the cortex and hypothalamus were compared to the post-decapitation electrical activity in these areas. To make the use of unanaesthetized and uncurarized rats possible (cf. KING, SCHOEPPLE, LOWRY, PASSONNEAU and WILSON, 1967), and to shorten the time of postdecapital loss of the signal (see JONGKIND, 1971; JONGKIND *et al.*, 1972), a telemetric technique was applied. This technique seems, moreover, to be promising for the study of electrophysiological phenomena in the hypothalamus of freely moving animals (see SWAAB, 1972; Boer and Swaab, in preparation). At the time when EEG activity had disappeared following decapitation, the ATP level was still very high, while P-creatine had declined sharply. This parallel between the disappearance of P-creatine and electric activity—which has been reported previously, e.g. in chick embryos (JONGKIND, 1971; JONGKIND *et al.*, 1972) and anaesthetized cats (SCHMAHL, BETZ, DETTINGER and HOHORST, 1966)—points to a specific role of P-creatine for the maintenance of electrical activity. P-creatine might be connected by creatine kinase to a negligible amount of ATP which in turn is coupled to ion transport (cf. JONGKIND, 1971). The idea that much of the $\sim P$ is not directly available for processes maintaining electrical activity would thus explain the phenomenon that EEG activity had already disappeared while the energy reserves were still very high (cf. JONGKIND, 1971; MAKER and LEHRER, 1971; JONGKIND *et al.*, 1972). Various authors have supposed an increased brain metabolism after decapitation, attributed to a massive neuronal discharge concurrent with this way of killing (for references see MAKER and LEHRER, 1971). EEG recordings in chick embryos (JONGKIND *et al.*, 1972) and mice (KING *et al.*, 1967) could not adduce an argument for such a discharge, however, since the same electrical activity was observed shortly after decapitation as before. Although our ECoG recordings in the present study also showed the same phenomenon, the postdecapital synchronization observed in the hypothalamic recordings is not inconsistent with the idea of stimulation, since this pattern is normally characteristic of states of increased alertness (cf. IRMIŠ, MADLAFOUSEK and HLIŇÁK, 1970).

In spite of the specialized endocrine function of the hypothalamus and its low oxygen tension, only a few slight differences were observed as compared to the cortical area. Although the resemblances between these two areas thus seem to support the idea of others (e.g. SAWYER, 1970) that neuroendocrine cells possess all the features of 'ordinary' neurons too, the metabolic data on the rather roughly isolated hypothalamus in the present study would have to be confirmed in histologically pure neuroendocrine tissue (see e.g. SWAAB, JONGKIND and DE RIJKE-ARKENBOUT, 1971). The trepanation technique, in combination with presently available ultramicrochemical substrate determination techniques (LOWRY and PASSONNEAU, 1971), would allow this kind of study to be carried out in the hypothalamus.

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