Effect of Phenobarbital on Cerebral Energy State and Metabolism

Enzymatic Activities


SUMMARY The changes induced by phenobarbital in cerebral enzymatic activities of the Krebs' cycle (citrate synthase, malate dehydrogenase) and electron transfer chain (total NADH-cytochrome c reductase and cytochrome oxidase) were studied. In addition, the activity of lactate dehydrogenase of acetylcholine esterase and of glutamate dehydrogenase was also studied. These enzymatic activities were evaluated in the homogenate in toto and in a crude mitochondrial fraction from rat brain. The modifications in some of these activities indicate that several new metabolic situations occur in brain tissue after phenobarbital treatment.

The enzymatic activities were chosen because of their significance in the energy transduction processes. The enzymatic activities studied were: lactate dehydrogenase (L-lactate: NAD+ oxidoreductase, EC 1.1.1.27) to analyze the glycolytic pathway; citrate synthase (citrate oxaloacetate-lyase, EC 4.1.3.7.) and synthase (citrate oxaloacetate-lyase, EC 4.1.3.7.) and synthase (citrate synthase, malate dehydrogenase) and electron transfer chain (total NADH-cytochrome c reductase and cytochrome oxidase) were studied. In addition, the activities of NADH-cytochrome c reductase and of glutamate dehydrogenase were also studied. These enzymatic activities were evaluated in the homogenate in toto and in a crude mitochondrial fraction from rat brain. The modifications in some of these activities indicate that several new metabolic situations occur in brain tissue after phenobarbital treatment.

The action of barbiturates on oxidative phosphorylation has been mainly ascribed to their ability to act in vitro as uncoupling agents. Subsequent investigations have dealt with the effect of oxygen and thio-barbiturates on mitochondria from the brain and the liver, showing that in vitro phenobarbital inhibits (but does not uncouple) oxidative phosphorylation of liver mitochondria with pyruvate as a substrate. The results obtained with brain mitochondria are in qualitative agreement with those obtained with liver mitochondria, in spite of some discrepancies. In agreement with Cohen, we acknowledge the limitations inherent in the interpretation of many experimental results obtained in vitro. Though there is as yet no generally accepted biochemical mechanism which explains the specific anesthetic action of barbiturates, it has been hypothesized that the complex and broad pharmacological effect of these agents may somehow also affect the activity of mitochondria. As a consequence, the hypothesis of possible actions performed at this level implies that they modulate changes of enzymatic activities involved in energy transduction and brain metabolism. This work was aimed at clarifying the action of phenobarbital at the subcellular level. We therefore studied the effect of phenobarbital treatment (100 mg/kg i.p.) on some cerebral enzymatic activities in the rat.

The enzymatic activities were chosen because of their significance in the energy transduction processes. The enzymatic activities studied were: lactate dehydrogenase (L-lactate: NAD+ oxidoreductase, EC 1.1.1.27) to analyze the glycolytic pathway; citrate synthase (citrate oxaloacetate-lyase, EC 4.1.3.7.) and malate dehydrogenase (L-malate: NAD+ oxidoreductase, EC 1.1.1.37) to analyze the Krebs' cycles; total NADH-cytochrome c reductase (NADH-cytochrome c : oxygen oxidoreductase, EC 1.6.99.3) and cytochrome oxidase (ferrocytochrome c : oxygen oxidoreductase, EC 1.9.3.1) to analyze the electron transport chain. In addition, the activities of acetylcholine esterase (acetylcholine hydrolase, EC 3.1.1.7) and glutamate dehydrogenase (glutamate : NAD-oxidoreductase deaminating, EC 1.4.1.3) were also evaluated, since these enzymes could provide some insight on transmission and amino acid metabolism. All enzymatic activities were measured both in the homogenate in toto and in the crude mitochondrial fraction, since many of them are variously located in the cytoplasm.

Method

This study was carried out on female rats (Sprague-Dawley strain) fed a standard diet of pellets and water ad libitum, and housed 3 per cage under optimal environmental conditions (22°C, 55-60% relative humidity, 12 h day cycle) until the age of 3 months. The animals were given 100 mg·kg⁻¹ of phenobarbital sodium i.p. Controls were given the vehicle only (saline solution) i.p. Blind biochemical evaluations were performed 30 or 60 min after treatment. All animals used for the analytical evaluations were treated between 8:45 and 9:00 a.m., and killed between 9:30 and 10:00 a.m.

At the set time the animals were sacrificed by decapitation, their brains were removed from the skull within 15 sec, and transferred to a box precooled to −5°C. The 0.32 M sucrose washed and weighed brains were homogenized in 0.32 M sucrose for 30 sec (precooled Potter-Braun S homogenizer) with a Teflon pestle. The homogenate obtained was diluted with 0.32 M sucrose (10% w/v) and an aliquot of each sample was taken for the assay of enzymatic activities. The remaining homogenate was submitted to fractioning to isolate the crude mitochondrial fraction. Three centrifugations (Sorvall RC-5 Supercenrifuge) at 900 × g were performed to remove nuclei and contaminating materials. A crude mitochondrial fraction was obtained after 2 centrifugations at 14000 × g. Mitochondrial pellets were resuspended in 0.32 M sucrose. Protein content was evaluated in both the homogenate and the mitochondrial preparation samples. The following enzymatic activities were measured: malate dehydrogenase; total NADH-
cytochrome c reductase; cytochrome oxidase. The activities of lactate dehydrogenase and acetylcholine esterase were evaluated only in homogenate samples, while those of citrate synthase and glutamate dehydrogenase were measured only in the mitochondrial preparation samples. Enzymatic activities were recorded (Beckman 25 Spectrophotometer Recorder) and calculated using the straight portion of the reaction curves. Results were expressed as specific activities (μmoles/min/mg protein) and statistically analyzed with the Dunnat test.

**Results**

Table 1 reports the data on cerebral enzymatic activities studied in the homogenate in toto. The enzymatic activities affected by phenobarbital were total NADH-cytochrome c reductase (which was increased) and cytochrome oxidase (which was decreased) at 1 h but not at 30 min post-treatment. These results might indicate a higher activity of the cytoplasmic form of the cytochrome c reductase enzyme system, and a partial inhibition (due to phenobarbital) of cell respiration by the cytoplasmic enzymatic components. No changes were found in acetylcholine esterase activity.

Table 2 reports the data on enzymatic activities studied in the brain crude mitochondrial fraction. The only enzymatic activity affected by phenobarbital was malate dehydrogenase, the activity of which was decreased both at 30 and 60 min. This finding might suggest that phenobarbital acts on the terminal steps of the Krebs' cycle. The enzymatic activities of the electron transfer chain were basically unmodified. The activity of glutamate dehydrogenase was not significantly increased.

**Discussion**

It is noteworthy that the evaluated activity of the individual enzymes tested indicates their potential activity but not their actual intracellular activity. This is because, as in many subcellular biochemical studies, the enzymatic activities were assayed in an environment free of cellular normal regulation. Even with this limitation, this investigation has confirmed the data obtained by studying the modifications of cerebral metabolites concentration after phenobarbital treatment. It is important to compare these enzymatic data with those yielded by studies on cerebral substrates and intermediates, following phenobarbital treatment. Under these conditions the cerebral energy state (as evaluated from the concentration of ATP, ADP and AMP) was unmodified, and the brain creatine phosphate content was increased. This finding can be related to the present observation that the enzymatic activities of the electron transfer chain at the mitochondrial level were working regularly. The in vitro decrease in CMRO₂ by phenobarbital may be unrelated to, and have no connection with, the inability of mitochondrial systems to utilize oxygen. In agreement with Cohen, we believe that in this field the data obtained from in vitro phenobarbital treatment do not yield reliable results. The absence of lactate changes can be explained enzymatically, as phenobarbital treatment does not affect the activity of lactate dehydrogenase. The suggestion that the presence or absence of elevated lactate levels in brain relates directly to the activity of LDH alone is an oversimplification of the metabolic controls on this metabolite. Changes in brain lactate can be induced by alterations in tissue pH, cytoplasmic redox potential and in the availability of pyruvate, independent of changes in LDH activity.

The present data are also negative: the activities of lactate dehydrogenase, acetylcholine esterase, citrate synthase and glutamate dehydrogenase were unaffected by drug treatment. Changes in the largely mitochondrial enzymes, cytochrome oxidase and NADH-cytochrome c reductase, were detected in whole brain homogenates (at 1 h but not at 30 min post-treatment). The importance of those changes in understanding mitochondrial function is obscure because no differences in the activities of these en-
zymes could be demonstrated by measurements obtained with a partially purified mitochondrial fraction.

The finding of reduced malate dehydrogenase activity in the mitochondrial fraction after barbiturate treatment is of interest and is in agreement with the data obtained from studies using sodium thiopental, showing the accumulation of malate, the increased concentration of glutamine and aspartate, and the unchanged concentrations of citrate and glutamate. The possible significance of the decrease on TCA cycle flux or the effective regulation of the cytoplasmic redox potential via the malate-aspartate shuttle is only tentatively outlined in this study.

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