ANESTHESIA. XLVII. BRAIN PHOSPHOKINASE AND ANESTHETICS *†‡

C. JELLEFF CARR, PH.D., FREDERICK K. BELL, PH.D., AND JOHN C. KRANTZ, JR., PH.D.

Baltimore, Maryland

PREVIOUS investigations in this laboratory on the ATP-dephosphorylating activity of brain homogenates in the presence of central nervous system stimulants and depressants *in vitro* failed to establish a significant relationship between pharmacologic action and enzyme inhibition (1, 2). Brody and Bain (3) produced evidence that certain barbiturates significantly lower the P:O ratio obtained in particulate tissue preparations from rat liver and rabbit brain employing a pyruvate substrate. These same workers subsequently showed that the barbiturates dissociate the process of oxidation and phosphorylation in rat liver and brain mitrochondrial preparations. This uncoupling effect is qualitatively similar to that of dinitrophenol. These observations have been confirmed in this laboratory, and a correlation has been made between hypnotic activity and the ability to uncouple oxidative phosphorylation. In addition, ether in anesthetic concentrations was found to produce a similar effect (4). Levy and Featherstone (5) were unable to demonstrate an effect upon glucose or pyruvate oxidation or upon uncoupling of phosphorylation by the gaseous anesthetics xenon or nitrous oxide.

In a further exploration of the effect of anesthetics upon brain enzyme systems, we have studied the influence of these agents upon the phosphokinase system of rat brain.

In preliminary experiments, we were able to demonstrate the presence of myokinase in rat brain homogenates. Colowick and Kalckar (6) previously described this enzyme as it occurs in rabbit brain. Myokinase is a phosphokinase which catalyzes the transference of phosphate moieties between pairs of molecules. The ribonucleotides adenosine diphosphate (ADP) and adenosine triphosphate (ATP) serve as carriers. Myokinase activity results in a dephosphorylation of ADP to AMP and a synthesis of ATP. The reaction may be expressed as 2 ADP $\rightleftharpoons$ AMP § + ATP. Dephosphorylation of ATP to ADP and PO_4 results from ATPase activity. Enzyme activity may

* From the Department of Pharmacology, University of Maryland School of Medicine, Baltimore, Maryland.

† Accepted for publication September 9, 1954.

‡ Supported by a research grant (H-144 C4) from the National Heart Institute of the National Institutes of Health, U. S. Public Health Service.

§ Adenosine monophosphate.

be measured quantitatively by estimation of the substrates after incubation of tissue homogenates under suitable conditions. This enzyme has been studied by Siekevitz and Potter (7). Eggleston and Hems (8) determined the equilibrium constant for myokinase from pigeon breast muscle. By combining the equilibria of this system and of the ATPase system, we obtained $\frac{[AMP]}{[ADP]} = C_3$, which we have used as a measure of enzymatic action (9).

Recently we investigated the activity of phosphokinases in coronary artery homogenates of the steer. We adapted the experimental method employed in this study to the enzyme system in rat brain. The concentration of enzyme appeared to be five-fold greater in brain than in coronary artery.

Method. Male rats weighing between 150 and 200 Gm. were stunned by a sharp blow, the chest opened, and the animal exsanguinated. The brain was removed immediately, weighed, and a 5 per cent homogenate prepared in cold redistilled water employing a Virtis homogenizer at a speed of 27,000 r.p.m. for one minute, with an outside cup containing ice water. The homogenate was centrifuged for three minutes at 1,200 r.p.m. at 0 C. and a 1 per cent dilution prepared. This homogenate was kept in an ice bath and 1 ml. quantities used in the incubation mixtures. The incubation tubes contained 0.5 ml. of 0.01 M $MgCl_2$, 1 ml. of ADP solution (5 mg.), and 0.5 ml. of phosphate buffer pH 7.0. The incubation was carried out at 38 C. for thirty minutes. At the end of this time, each tube received 1.5 ml. of 1.5 N $HClO_4$ and the tubes were centrifuged at 3,000 r.p.m. at 0 C. for five minutes to remove protein. The supernatant liquid of each tube was removed as completely as possible to another tube immersed in ice water and 1.5 ml. of 1.5 N KOH was added to each tube. They were then allowed to stand overnight at 8 C. The tubes were then centrifuged for ten minutes at 3,000 r.p.m. at 0 C. The supernatant fluid of each tube was drawn off and 5 ml. aliquots were taken for analysis. The analysis of the ribonucleotides was made by the method of Cohn and Carter (10), employing a Dowex 1 resin column. The absorbency of the eluate was measured spectrophotometrically in a Beckman spectrophotometer at 258 mμ with a slit of approximately 0.75 mm. The spectrophotometric readings were converted to micromoles of the respective nucleotide, using the molecular extinction value of 13,800 for AMP, 14,900 for ADP, and 14,400 for ATP. The activity of the rat brain phosphokinase under the conditions of the experiment for 6 normal animals is illustrated in table 1. The results represent average values and served as control determinations for subsequent experiments.

Anesthetized Animals. Six rats were anesthetized with chloroform and the anesthesia maintained for ten minutes. At the end of this period, the brain was removed and a homogenate prepared as de-

scribed previously. The analysis of enzyme activity was conducted in triplicate. Six rats received pentobarbital sodium intraperitoneally in doses of 3 mg. per 100 Gm. of body weight. Anesthesia followed promptly and was allowed to continue for ten minutes. At the end of this period, the brain was removed and a homogenate was prepared. The results of these experiments are outlined in table 1.

In Vitro Experiments. There is reason to believe that tissue removed from an animal under the influence of a drug may not necessarily represent the state of the tissue as a result of drug action. This may be especially true of enzyme systems where an equilibrium may be reestablished upon dilution of the tissue in the preparation of homogenates. Therefore experiments were conducted employing theoretical anesthetic concentrations of chloroform and pentobarbital

TABLE 1

AVERAGE NUCLEOTIDE DISTRIBUTION AFTER INCUBATION

	No. of Experiments	Ave. Brain Wt. (Cm.)	Micro-mols				C_1*
			AMP	ADP	ATP	Totals	
Normal activity	6	1.67	4.10	2.54	1.39	8.03	1.62
$CHCl_3$ anesthesia	6	1.75	4.21	2.50	1.32	8.03	1.68
Pentobarbital sodium anesthesia	6	1.69	3.97	2.52	1.41	7.90	1.59
Brain susp. + 50 mg. % $CHCl_3$	6	1.71	3.98	2.63	1.46	8.07	1.52
Brain susp. + 0.5 mg. % pentobarbital sodium	6	1.71	4.09	2.58	1.46	8.13	1.59
Average		1.71	4.06	2.56	1.41	8.03	1.60

* $\frac{[AMP]}{[ADP]} = C_1$.

sodium *in vitro*. The concentrations selected were maximal anesthetic concentrations based upon the assumption of a uniform distribution of the anesthetic between the blood and tissues, namely, 50 mg. of $CHCl_3$ per 100 ml. and 0.5 mg. of pentobarbital sodium per 100 ml. In these experiments, suitable dilutions of weighed amounts of chloroform or pentobarbital sodium were added to the incubation mixtures of a freshly prepared brain homogenate and the other reactants. All experiments were carried out in triplicate. The results are summarized in table 1.

Discussion and Summary. It is clear from numerous studies of enzyme systems of brain tissue that the anesthetic drugs exert their effect as a result of many actions. Certain of these influences upon enzymes have been referred to and an additional enzyme pathway studied. Not any of these investigations offers a completely adequate

explanation of the finer mechanism of action of anesthetic drugs, and further explorations in this field are necessary.

An inspection of the results set forth in the table reveals that the phosphokinase activity of brain homogenates as measured under the conditions of these experiments is not influenced by chloroform or pentobarbital sodium anesthesia. In addition, chloroform or pentobarbital sodium in theoretical anesthetic concentrations *in vitro* do not influence this enzyme significantly. Refined separation techniques may yield an enzyme preparation more exquisitely sensitive to drug action. Exploratory investigations of this character are planned.

REFERENCES

1. Lu, G., and Krantz, J. C., Jr.: Anesthesia: In Vitro Studies of Influence on Adenosine Triphosphate (ATP) Dephosphorylation by Central Depressants and Stimulants, Anesthesiology 14: 348 (July) 1953.
2. Bain, J. A.: Enzymatic Aspects of Barbiturate Action, Fed. Proc. 11: 653 (June) 1952.
3. Brody, T. M., and Bain, J. A.: Effect of Barbiturates on Oxidative Phosphorylation, Proc. Soc. Exper. Biol. & Med. 77: 50 (May) 1951.
4. Hulme, N. A., and Krantz, J. C., Jr.: Anesthesia: Effect of Ethyl Ether on Oxidative-Phosphorylation in Brain, In press.
5. Levy, L., and Featherstone, R. M.: Effect of Xenon and Nitrous Oxide on In Vitro Guinea Pig Brain Respiration and Oxidative Phosphorylation, J. Pharmacol. & Exper. Therap. 110: 221 (Feb.) 1954.
6. Colowick, S. P., and Kalckar, H. M.: Role of Myokinase in Transphosphorylations; Enzymic Phosphorylation of Hexoses by Adenyl Pyrophosphate, J. Biol. Chem. 148: 117 (April) 1943.
7. Siekevitz, P., and Potter, V. R.: Adenylate Kinase of Rat Liver Mitochondria, J. Biol. Chem. 200: 187 (Jan.) 1953.
8. Eggleston, L. V., and Hems, R.: Separation of Adenosine Phosphates by Paper Chromatography and Equilibrium Constant of Myokinase System, Biochem. J. 52: 156, 1952.
9. Carr, C. J.; Bell, F. K.; Hurst, J. Neil, and Krantz, J. C., Jr.: Myokinase Activity of Coronary Arteries, Circul. Res. 2: 516 (Nov.) 1954.
10. Cohn, W. E., and Carter, C. E.: Separation of Adenosine Polyphosphates by Ion Exchange and Paper Chromatography, J. Am. Chem. Soc. 72: 4273 (Sept.) 1950.