Biochemical Pharmacology, Vol. 18, pp 2211–2214. Pergamon Press. 1969. Printed in Great Britain

OXIDATION OF SUCCINATE AND PYRUVATE IN RAT BRAIN AND ITS EFFECT ON BARBITURATE ANAESTHESIA

H. W. READING and J. WALLWORK

M.R.C. Unit for Research in Brain Metabolism, Dept. of Pharmacology, Medical School, Teviot Place, Edinburgh, 8, U.K.

(*Received* 24 *March* 1969; *accepted* 29 *April* 1969)

Abstract—Effects on the sleeping times of male rats under amytal anaesthesia by succinate were compared with those of sodium pyruvate, solutions of both salts being introduced directly into the brain by intraventricular injection. The results were also compared with sleeping times in rats given amytal alone. Sodium succinate (1·0 M) reduced the sleeping time by about 70·0 per cent, whereas pyruvate had no effect on duration of anaesthesia. Blood levels of barbiturate were found to be unaffected by injection of succinate and, at the time of arousal, blood amytal concentration was significantly higher in succinate-treated rats than in pyruvate-treated or untreated rats. It was concluded that these results obtained *in vivo* corroborate previous conclusions from *in vitro* experiments on the intracellular site of action of the oxybarbiturates.

EXTENSIVE biochemical studies have been carried out to determine the precise mode and site of action of the barbiturate anaesthetics. Such investigations have been *in vitro*, on isolated phosphorylating mitochondrial systems obtained either from mammalian liver or cerebral tissue.

Early work by Brody and Bain[1] suggested that the mode of action of barbiturate in the CNS was mediated by an uncoupling of oxidative phosphorylation. In contrast, Aldridge and Parker[2] demonstrated an inhibition of respiration by oxybarbiturates without a concomitant uncoupling of oxidative phosphorylation in suspensions of liver mitochondria. These authors further showed that the use of succinate as substrate for mitochondrial oxidation abolished the inhibition of respiration caused by the oxybarbiturates. They suggested that the site of action of the oxybarbiturates lay in the respiratory chain at a stage preceding the entry of succinate. These observations were corroborated by Chance and Hollunger's[3] studies on liver mitochondria whereby they characterised the action of amytal (Na-5-ethyl-5-isoamylbarbiturate) as combining an inhibition of electron transfer between flavin and cytochrome *b*, with an inhibition of energy transfer at the NADH-flavin site of the respiratory chain.

Since none of these studies had involved *in vivo* preparations, it was decided to investigate the effects of succinate and pyruvate on amylobarbitone-induced anaesthesia in the intact brain *in vivo* and to administer the carbohydrate metabolites directly into brain by injection into a lateral ventricle in the rat. It was expected that administration by this route would overcome any effects of transport due to the existence of the blood–brain barrier.

METHODS

Male rats of the Wistar strain weighing 140–160 g were used throughout. They were fasted overnight prior to each experiment which was commenced at approximately the same time each morning to reduce possible errors arising from diurnal variation. Such variations were observed in exploratory experiments at the beginning of the series. The rats were anaesthetised by i.p. injection of sodium amytal in distilled water (135 mg/kg). Fifteen μl of either 1·0M sodium succinate, or 1·0M sodium pyruvate solution was injected into the right lateral ventricle of the rat brain using a specially constructed needle with a depth guard. These injections were made immediately after the onset of anaesthesia, in a manner similar to that described by Noble *et al.*[4] Sleeping times were measured by observation. The onset of sleep was defined as loss of righting reflex and anaesthesia. Determination of the time of arousal was more difficult. Although classically defined as return of the righting reflex, in our preliminary observations some rats showed no righting reflex when left for long periods to recover but would walk when turned on to the abdomen; others would bite the observer's finger placed close to the mouth, whilst still not showing a righting reflex. On the other hand, rats obviously more comatose than the above would show purposeful righting reflexes. It was finally decided to define an end-point to encompass as short a sleeping time as possible, since this would tend to reduce errors arising from factors of experimental variability, which would have increased the standard deviation. The end-point was defined as return of withdrawal reflex to pain and increase in muscle tone together with reappearance of muscle reflexes. Although such an end-point is to some extent subjective, with a little practice it became possible to measure the time of arousal in each group by the same criteria. The employment of control groups in all experiments rendered these observations more reliable. As the results show, the differences in mean sleeping times between the different experimental groups were of such magnitude to render more sophisticated methods of determining arousal time (e.g. E.E.G.) unnecessary.

Estimation of barbiturate levels in blood

On arousal the rats were decapitated and blood collected in heparinized tubes for estimation of barbiturate concentration. A spectrophotometric method was adapted to a microscale and gave reproducible results and obeyed Beer's law over the range of concentrations encountered.

0·6 ml of whole heparinized blood was shaken with 6 ml chloroform to precipitate protein and extract the barbiturate. 5 ml aliquots of the chloroform barbiturate extract were shaken with 1 ml 0·0125N NaOH. The mixtures were centrifuged and 0·35 ml of this alkaline extract (supernatant) were mixed in a microcuvette (1 cm light path) with 0·15 ml borate buffer 0·05M pH 10·0 (50 ml 0·05M Borax, 43 ml 0·2N NaOH, made up to 200 ml).

The optical density of the resulting solution was determined in an S.P. 800 recording spectrophotometer (200–300 mμ) against a blank consisting of 0·35 ml 0·0125 NaOH and 0·15 ml pH 10·0 borate buffer. Sample and blank were then both acidified with two drops of 6N H_2SO_4 and the optical density of the sample re-determined. The difference in optical density between the alkali and acid solutions at 239 mμ was

directly proportional to barbiturate concentration. Concentration of barbiturate in blood was calculated from the following expression:—

$$E_{\text{alkaline}}^{239\ m\mu} - E_{\text{acid}}^{239\ m\mu} \quad 6{\cdot}5 = \text{mg amytal/100 mls blood}$$

(Constant is computed from the dilution etc., and the molecular extinction coefficient for amylobarbitone.)

RESULTS AND DISCUSSION

The results are shown in Table 1. It can be seen from Table 1 that a marked reduction in sleeping time was produced by the intraventricular administration of sodium succinate and that such an effect was not observed by giving an equivalent amount of sodium pyruvate by the same method of administration. The differences between sleeping times and blood concentrations on arousal, for succinate-treated rats and those treated with pyruvate or untreated animals have a high degree of statistical significance ($P < 0{\cdot}01$ in all cases). On the other hand, the differences in sleeping time and blood-barbiturate concentration in pyruvate-treated and untreated rats were almost negligible ($P = 0{\cdot}7$ and $0{\cdot}8$ respectively).

TABLE 1. SLEEPING TIMES OF RATS WITH CORRESPONDING AMYTAL CONCENTRATIONS IN BLOOD

Treatment	Sleeping time (min)	Blood amytal concentration on arousal (mg/100ml)
None	100·9 (± 2·21)	5·39 (± 0·81)
Sodium succinate 15 μl. 1·0M intraventricularly	29·4 (± 0·64)	7·34 (± 0·05)
Sodium pyruvate 15 μl. 1·0M intraventricularly	104·3 (± 1·87)	5·48 (± 0·05)

(s.e.m. given in parenthesis)

Previously, Soskin and Taubenhaus[5] attempted to use succinate as an antidote to toxic doses of nembutal in rats. The results obtained showed a low degree of statistical significance, but the route of administration of succinate (intraperitoneally and intravenously) probably contributed largely to this. These authors suggested that further work should be done on the use of succinate in terminating barbiturate anaesthesia. This suggestion does not seem to have received much attention over the years, and the present authors feel that such a procedure would be well worthwhile in cases of acute barbiturate poisoning which do not respond to any of the usual methods of treatment. The succinate would probably have to be administered intracisternally to be effective.

More recently, Kalyanpur *et al.*[6] studied the effect of pre-treatment of rats with vitamins of the B group, steroid hormones and high protein and carbohydrate diets on sleeping times induced by pentobarbital and barbital. Mixed results were obtained but these workers used female rats which are known to respond more readily than

males to barbiturate anaesthesia. Reductions in sleeping time found by Kalyanpur and colleages were not of the same order nor as consistent as those obtained in the present experiments obtained by the intraventricular administration of succinate.

The fact that the concentration of barbiturate in the blood at the time of arousal was significantly greater in the case of succinate-treated animals than in pyruvate-treated or untreated rats, indicates than the intraventricular administration of succinate does not affect the rate of metabolism of the barbiturate but acts at the level of intracellular oxidation in the neuron. This is corroborrated by the close similarity in blood levels of amytal obtained 30 min after the onset of anaesthesia in untreated rats and those obtained on arousal (mean sleeping time = 29·4 min) in succinate-treated rats. The values were 7·33 (s.e.m. $\pm$ 0·17) mg/100 ml in untreated animals, and 7·34 (s.e.m. $\pm$ 0·05) mg/100 ml in succinate-treated rats ($P > 0·9$).

This contrasts with the effects of drugs such as SKF.525A which tend to prolong hypnosis by retarding the rate of metabolism of the barbiturate in liver.

The *in vivo* results obtained in the present experiments confirm the *in vitro* findings[2,3] using isolated liver mitochondria, and extend the conclusions to encompass brain *in vivo*.

The present results strongly support the evidence obtained previously on the pharmacological mode of action of the oxybarbiturates. The fact that succinate shows such marked antagonistic action to amylobarbitone, in contrast to pyruvate, indicates that oxidation of the former substrate by-passes the site of action of the oxybarbiturate in the oxidation chain of the electron transfer particle (Lehninger).[7]

Acknowledgement—The authors wish to thank Dr. H. J. S. Matthew of the Department of Therapeutics, Edinburgh University, for helpful encouragement and advice. They would also like to thank Dr. S. S. Brown of the Department of Clinical Chemistry, Edinburgh University, for advice on the method of estimation of amytal in blood.

One of us (J.W.) received a Gunning Vacation Bursary, 1967, to carry out the work.

REFERENCES

1. T. M. BRODY and J. A. BAIN, *J. Pharmac.* **110**, 148 (1954).
2. W. N. ALDRIDGE and V. H. PARKER, *Biochem. J.* **76**, 47 (1960).
3. B. CHANCE and G. HOLLUNGER, *J. biol. Chem.* **278**, 418 (1963).
4. E. P. NOBLE, R. J. WURTMAN and J. AXELROD, *Life Sci.* **6**, 281 (1967).
5. S. SOSKIN and M. TAUBENHAUS, *J. Pharmac. exp. Ther.* **143**, 49 (1943).
6. S. G. KALYANPUR, S. R. NAIK and U. K. SHETH, *Archs. int. Pharmacodyn. Ther.* **173**, 1 (1968).
7. A. L. Lehninger, *The Mitochondria.* Benjamin, New York (1965).