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BRAIN BARBITAL LEVELS AND ANESTHESIA AS INFLUENCED BY PHYSOSTIGMINE AND EPINEPHRINE*

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Abstract—The potentiating effect of physostigmine on barbital anesthesia has been attributed to a modification of the permeability of the blood–brain barrier as a result of cholinesterase inhibition. The rate of penetration of barbital into mouse brain and its possible alteration by pharmacological means were investigated. Individual brain barbital levels were determined by an improved analytical technique and correlated with alterations in the lag in onset of barbital anesthesia.

Barbital with physostigmine produced signs of depression much sooner and much more intense than barbital alone. However, these signs could not be attributed to increased brain barbital concentrations because in animals receiving physostigmine brain barbital levels were not increased over the controls. The early ataxia, loss of righting reflex, produced by physostigmine could be prevented by pretreatment with atropine methyl nitrate without an alteration in brain barbital levels.

Animals receiving barbital with epinephrine displayed immediate signs of depression and became anesthetized much sooner than controls. These animals had brain barbital concentrations above the control from 2 to 20 min. The epinephrine effect, however, cannot be attributed to increased brain barbital levels alone, because at 10 min the controls showed no signs of depression with the same brain barbital levels as the 'enesthetized' epinephrine-treated animals.

There were no apparent differences in behavior of barbital plus norepinephrine-treated mice from that of controls although a small increase in brain barbital levels was noted.

For the experimental conditions chosen it is concluded that: (1) Physostigmine does not alter the rate of penetration of barbital into the brain. The early loss in righting reflex produced by the combination of barbital and physostigmine is probably mediated peripherally. (2) Epinephrine contributes to a greatly increased rate of onset and intensity of 'anesthesia' by some mechanism(s) in addition to increasing the rate of entry of barbital into the brain.

A NUMBER of papers have been presented in support of the concept that the permeability of certain tissues may be related to the activity of the acetycholine-cholinesterase system. An increase in the permeability to sodium, potassium and hemoglobin occurred in erythrocytes when cholinesterase activity was decreased.[1–5] An increased permeability of the hemoencephalic barrier in frogs to acid fuchsin was observed

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when physostigmine was administered along with the dye.[6] Procaine, which does not penetrate the cornea, was found to cause anesthetization of the rabbit cornea when administered in conjunction with physostigmine.[7]

The studies of Butler [8, 9] and of Mark *et al.*[10, 11] have shown that barbital, after intravenous injection, passes slowly into the brain. This slow rate of penetration is responsible for the lag in onset of anesthesia, because the intensity of narcosis can be correlated with the quantity of drug in the brain.

Greig and Mayberry[12] found that the administration of physostigmine in conjunction with barbital to mice decreased the lag in onset of anesthesia by 50 per cent and was accompanied by an increased rate of barbital entrance into the brain. Additional evidence for the relationship of cholinesterase and brain permeability was reported by Greig and Carter.[13] After inhibiting about 90 per cent of the brain cholinesterase with physostigmine, they obtained an increased rate of entry of barbital into guinea pig brain slices.

Our developing familiarity with barbiturate analysis indicated that normal errors could lead to differences as great as those which previous investigators have found between barbital and barbital plus physostigmine-treated animals when using the methods then available.

In the present study we have found that potentiation of barbital anesthesia by physostigmine is not correlated with an increased rate of barbital penetration into the brain. In addition, observations are also presented indicating that atropine methyl nitrate will prevent the loss of righting reflex observed in physostigmine plus barbital-treated animals without significant changes in brain barbital concentration.

As a matter of further interest, the observation by Lamson *et al.*[14] that epinephrine administered together with a barbiturate would reduce the AD_{50} to about one-fourth that required when the barbiturate is given alone prompted an investigation of the effect of epinephrine and related sympathomimetic amines on barbital penetration of the brain.

Since barbital is not metabolized very much,[15, 16] this compound is well suited for the study of brain penetration.

MATERIALS AND METHODS

Animals. Male mice of the Carworth Farms CF No. 1 strain weighing 20–30 g were used. They were allowed free access to food and water until the time of the experiment.

Drugs. Barbital powder (Mallinckrodt) was recrystallized from hot methyl alcohol. The barbital solution was freshly prepared by the addition of 1·1 equiv. of sodium hydroxide to the acid. This solution was then adjusted to pH 7·9 with 6 N hydrochloric acid. The dose of barbital was 0·234 mg/g, which was 1·25 times the median anesthetic dose determined by Butler;[8] this is the same dose used by Greig and Mayberry.[12] The barbital solution was of such concentration (15 mg/ml) that an animal received between 0·2 and 0·4 ml of solution.

Physostigmine sulfate (0·4 μg/g), L-epinephrine bitartrate (0·25 μg/g), norepinephrine bitartrate (0·25 μg/g) and atropine methyl nitrate (5 μg/g) were all prepared fresh in normal saline (100 μg/ml).

The appropriate drug was mixed with the barbital in the syringe just prior to administration and injected intravenously during a period of one-half min.

Determination of brain barbital. At the chosen time, the mouse was decapitated and the brain was rapidly removed and weighed. Those tissues not analyzed immediately were placed in stoppered vials and stored at −20°. Repeated weighing of the stored tissues showed no loss in weight over many weeks.

For quantitative estimation of barbital in extracts of biological material, we have modified the ultraviolet spectrophotometric techniques described by other authors[17–20] and summarized by Bush.[21] The increased sensitivity of the modified method permitted accurate analysis of individual mouse brains, thus eliminating the necessity of pooling samples. In the present work special pains were taken to control and correct for a variety of errors inherent in the method. A brief description follows. The brain was homogenized in a glass homogenizer with 4·0 ml of pH 6·0 phosphate buffer (1·0 M). The homogenate was washed into a glass-stoppered tube with the aid of 2 ml of the buffer and extracted with 20·0 ml of fresh U.S.P. diethyl ether. This was shaken for 2 min, then centrifuged. Approximately 90 per cent of the ether phase was removed with a fine-tipped pipette and delivered to a clean glass-stoppered tube. The extraction was repeated with a fresh batch of 20·0 ml ether and the two ether portions were combined. Mechanical and partition losses of barbital are less than 1 per cent. The ether solution was then equilibrated, by 2 min of shaking, with 10·0 ml of pH 10·0 borate buffer (0·058 M H_3BO_3 + 0·05 M KOH) and centrifuged. The final volume of the aqueous layer was 11·0 ml. Two 4·0-ml portions of the aqueous layer were removed by pipette to two small, wide-mouth bottles. The two bottles were subsequently handled in the same manner, except that to one was added enough 85 per cent phosphoric acid to lower the pH to 6·25. The titration was carried out in the small open bottle with magnetic stirring, using a small glass electrode-calomel electrode cell[22] and a Beckman model GS pH meter. The u.v. absorption spectra were recorded with a Beckman DK-1 spectrophotometer, with pH 6·25 as reference and pH 10·0 as sample. The optical density difference (O.D.D.), 239–268 mμ, was determined and the concentration of barbital estimated from a previously prepared standard curve.

Interference from substances normally present in animal tissues has been shown to be small.[9] In the present method such interference was negligible. The determinations have been made more consistent and accurate by increasing the volumes of ether used for the extraction and by correcting for volume changes due to evaporation of ether. Carefully controlled experiments indicate that volume changes due to ether evaporation can easily introduce errors as high as 10 per cent. Six separate recovery experiments were carried out in the manner described above with recovery of 95–99 per cent after partition coefficient correction (C) (for ether/pH 6·0 buffer, $C = 4{\cdot}2$; for ether/pH 10·0 buffer, $C = 0{\cdot}04$).

RESULTS

Virtually all animals given barbital or physostigmine alone displayed no obvious neurological signs during the early time periods chosen in this study. Animals maintained the righting reflex and responded to tail pinching.

Barbital plus physostigmine. Within 3 or 4 min, these animals displayed incoordination in gait and, in about 5 min, the animals could right themselves only with extreme difficulty. At approximately 10 min, the righting reflex had disappeared.

The animals did not respond to tail pinching, although they did not appear "anesthetized." After decapitation, generalized muscular fasciculations were quite prominent. The behavior of barbital plus physostigmine-treated animals indicated to us a paralysis rather than anesthesia. Prostigmine produced behavioral effects similar to those of physostigmine. Analysis of the date in Table 1 shows that there is no convincing difference in brain barbital levels between barbital plus physostigmine-treated animals and those given barbital alone.

TABLE 1. EFFECT OF PHYSOSTIGMINE AND EPINEPHRINE ON BRAIN LEVELS OF BARBITAL AT VARIOUS TIME INTERVALS

Treatment* time (min after injection)	Barbital† S.E.	Barbital + physostigmine	Difference from control (%)	Barbital + epinephrine	Difference from control (%)
2	100 ± 3 (18)‡	94 ± 8 (10)	− 6·0	160 ± 10 (8)	+ 60§
5	149 ± 7 (15)*	130 ± 6 (7)	− 13·0	188 ± 8 (6)	+ 33§
10	170 ± 4 (25)‡	172 ± 5 (22)	+ 1·2	225 ± 8 (6)	+ 32§
20	204 ± 9 (14)§	204 ± 7 (11)	0	238 ± 8 (5)	+ 17§

* All drugs injected intravenously as described under Materials and Methods.
† Expressed as μg/g wet wt. of brain.
‡ Number of animals is in parentheses.
§ Significant by Student's *t* test; P = 0·01.

Barbital plus atropine methyl nitrate. Since no increased brain barbital levels were found in physostigmine plus barbital-treated animals as compared to controls, experiments were performed to ascertain if atropine methyl nitrate would modify the physostigmine effect. Atropine methyl nitrate would not be expected to penetrate the central nervous system and, therefore, would block only the peripheral cholinesterase inhibition produced by physostigmine. Animals pretreated with atropine methyl nitrate for 5 min followed by barbital plus physostigmine, displayed no evident neurological symptoms (loss of righting reflex, response to tail pinching) during the time interval chosen. The data of Table 2 indicate that brain barbital levels 10 min later were not significantly different from those of the controls.

Barbital plus epinephrine. These animals displayed obvious neurological signs almost immediately. There was muscular incoordination and ataxia, and the righting reflex was lost within a few minutes and the animals appeared anesthetized. The data presented in Table 1 indicate a definite increase in brain barbital concentration over controls at all time intervals.

In view of the dramatic effects of epinephrine on barbital anesthesia, related sympathomimetic amines were also studied. Although norepinephrine increased brain barbital levels slightly at the 10 min period (Table 2), no apparent differences in the behavior of barbital plus norepinephrine-treated mice from that of the controls were observed. In a small series of animals, vasopressin (1 unit/10 g) acted similarly to norepinephrine. Thus, the dramatic effect of epinephrine is not likely related to its hypertensive action.

Furthermore, it was found that epinine (10 times the dose of epinephrine) and methyl epinephrine (30 times the dose of epinephrine) in conjunction with barbital

produced no obvious anesthetic effect. However, marked piloerection was observed in these animals. Phenylephrine (8 times the dose of epinephrine) produced effects similar to those following epinephrine.

TABLE 2. EFFECT OF VARIOUS DRUGS ON BRAIN BARBITAL LEVELS

Treatment	Brain barbital concentrations (μg/g wet wt of brain 10 min after i.v. injection)
Experiment 1	
Barbital control (7)*	162 ± 7·0
Barbital + physostigmine (6)	170 ± 6·5
Barbital + atropine methyl nitrate (12)	160 ± 3·5
Barbital + atropine methyl nitrate + physostigmine (10)	175 ± 5·8
Experiment 2	
Barbital control (10)	162 ± 7·0
Barbital + norepinephrine (10)	177 ± 9·8

* Number of animals is in parentheses.

DISCUSSION

These studies confirm and extend earlier observations that coadministration of barbital with physostigmine decreases the lag in onset of barbital "anesthesia" by producing signs of depression much sooner and much more intense than barbital alone.[12] The potentiating effect of physostigmine has been attributed to an increase in the permeability of the blood–brain barrier to barbital.[12] This interpretation has been used by others to explain physostigmine's potentiation of hexobarbital,[23, 24] although in many instances no brain barbiturate levels were determined for this drug.[23, 24] Furthermore, hexobarbital does not normally display a lag in onset of anesthesia.[8]

The current studies indicate that physostigmine's "potentiating" action cannot be attributed to increased brain barbital levels, because in animals receiving these drugs brain barbital levels were not increased over controls. The analytical procedure used in this study yields 95–98 per cent recovery of brain barbital, with most of this actually in the cuvettes, whereas the method of Butler,[9] utilized by most investigators, yields about 90 per cent recovery, but only 32 per cent of the brain barbital from three combined brains is in the final aqueous extract (an adequate amount only if large doses are given). Furthermore, the pooling of three brains also tends to hide the individual variation which can be as much as 50 per cent.

That physostigmine is producing its effect(s) by some mechanism(s) other than increasing blood–brain permeability is also suggested from the following observations: (1) the increase in neurological signs at the early time period when brain barbital levels were not significant is not consistent with the observations of others that increased neurological signs occur with increasing brain barbiturate concentrations;[8–11] (2) pretreatment of animals with atropine methyl nitrate, which does not penetrate the brain, decreases the potentiating effect of physostigmine without affecting brain levels of barbital; and (3) the potentiation of hexobarbital,[23, 24] pentobarbital[25] and thiamylal,[26] compounds which normally attain maximal brain levels instantaneously,[8] also implies the existence of some other mechanism(s).

The reports of other investigators with various other systems offer little support to the concept that cholinesterase activity is related to brain permeability. Cholinesterase inhibition did not increase ^{42}K or ^{24}Na transport from blood to cerebrospinal fluid,[27, 28] the penetration of sulfonamides,[29] morphine,[30, 31] 2 PAM-^{14}C, ^{14}C-aminobutyric acid into brain.[32, 33] That physostigmine is exerting its effect(s) by some peripheral mechanism is also suggested from the findings of Harris *et al.*,[34, 35] who observed physostigmine activation of the previously inactive narcotic analgesic pentazocine in the tail-flick test. Furthermore, physostigmine alone in large doses depressed the tail-flick response in the absence of any indication that brain cholinesterase had been inhibited. Atropine methyl nitrate was used to protect the animals from the peripheral effects of physostigmine. Thus it would appear that physostigmine's potentiation of pentazocine may involve some peripheral paralysis to that seen with barbital plus physostigmine.

Furthermore, a reduced induction time for barbital "anesthesia" does not necessarily indicate an increased rate of barbital penetration into the brain, as shown in the studies of Child *et al.*,[36] who found that pretreatment with a number of compounds (reserpine, 5-hydroxytryptamine, chlorpromazine, benactyzine) would reduce the induction time of barbital "anesthesia" without increasing the rate of barbital penetration into the brain.

Finally, it is conceivable that physostigmine may be exerting its effect by altering the distribution of barbital in various regions of the brain. This hypothesis has not been tested but seems unlikely in view of the ability of atropine methyl nitrate to prevent the physostigmine effect.

The current studies confirm earlier observations on the potentiation of barbiturate anesthesia by epinephrine. The epinephrine effect was immediate and persisted for the longest time interval used in this study (20 min). Only in doses 10–40 times that of epinephrine were similar effects noted with related compounds such as norepinephrine, vasopressin, isoproterenol, methyl epinephrine, epinine or phenylephrine. These observations are in agreement with those of Lamson *et al.*,[14] who found that norepinephrine, tyramine, methoxyphenamine and pitressin did not potentiate barbiturates.

Although the data presented in Table 1 indicate that epinephrine increased brain barbital levels above controls, one cannot attribute the observed physiological responses to increased brain barbital concentrations alone. Examination of Table 1 reveals that the brain barbital concentrations of epinephrine-treated mice at 3 min are approximately the same as those of controls at 10 min. The epinephrine plus barbital-treated animals are quite depressed, while animals given barbital alone are alert at 10 min with the same barbital brain concentration.

Other studies indicate that epinephrine may affect the permeability of the blood–brain barrier as well as of other barriers. Epinephrine and norepinephrine were found to increase the concentration of amphetamine in rat brain[37] and the penetration of glucose, sucrose, raffinose and polyglycol into saliva.[38]

Although a number of investigators have shown that epinephrine can produce anesthesia when injected directly into the central nervous systems,[39] it is felt that the effect of epinephrine observed in our experiments is not a direct one on the central nervous system. This is supported by the observation that epinephrine and its metabolites cross the blood–brain barrier very slowly.[40] However, significant amounts were found in the hypothalamus. Methyl epinephrine, which would be expected to penetrate

the blood–brain barrier more readily, was virtually inactive in potentiating barbital anesthesia.

Indirect support that epinephrine potentiates barbiturates is demonstrated by the study of Lasagna,[41] who reported that potassium chloride would potentiate barbiturates. Potassium chloride has been shown to cause liberation of epinephrine from the adrenal medulla.[42] The potassium chloride effect on barbiturate anesthesia can be blocked by adrenergic blocking agents, indicating that the potassium effect is mediated through epinephrine.[43] Recently, Malhotra *et al.*[44] observed that epinephrine's potentiation of pentobarbital could be prevented with the adrenergic blocking agent, dibenzyline.

Finally, in view of epinephrine's dynamic effects, i.e. release of ACTH, corticoids, insulin, thyroid hormones, glucose and other substances, further information concerning the mechanism of epinephrine's potentiation of barbiturates will require the use of a number of experimental techniques and systems.

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