When fluids were collected from the grafts, and analysed electrophoretically in comparison to normal uterine fluids the results shown in Fig. 3, were obtained. Blastokinin as the criterion for normal endometrial secretory activity was clearly discernable in graft fluid samples taken from pregnant and pseudo-pregnant does and from castrates given exogenous progestrone; it was not detectable in untreated castrates or in those given estrogen supplementation or in anestrous animals.

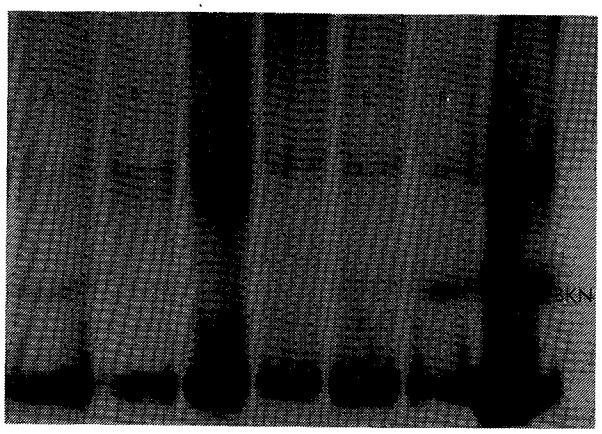

Fig. 3. *Polyacrylamide gel electrophoresis of the secretions of endometrial grafts in rabbit ears. The location of the blastokinin band is designated by BKN.*

A. From an ovariectomized doe, given exogenous progesterone.
B. From an ovariectomized doe, given exogenous estradiol 17β.
C. From an ovariectomized doe, untreated.
D. From an anestrous doe.
E. From a 5-day pseudo-pregnant doe.
F. From a 5-day pregnant doe.
G. Uterine fluids from a 5-day pregnant doe, for comparison.

The authors conclude that endometrium from the rabbit uterus can survive ectopically in the ear of the same animal and in this location can respond to the same hormonal conditions which regulate it in its normal uterine site by typical growth and secretory activity, most notably the synthesis of the uterine protein blastokinin.

In an attempt to perform the ultimate test of normalcy of ectopic endometrium, blastocyst-stage embryos, flushed from the intact horn of a rabbit on day five *post-coitum*, were transferred into the fluid-filled lumena of the ear grafts on the same animals. At the time of writing of this report none of 11 embryos so transferred had grown for the 3 days allowed by the protocol before disection analysis of the graft. This may reflect the incompetence of the grafts to support embryogenesis or merely our failure to recognize and or surmount the technical difficulties involved.

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Legends .....

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EFFECTS OF PHYSOSTIGMINE, NEOSTIGMINE AND METHACHOLINE ON HEXOBARBITAL HYPNOTIC ACTION IN ALBIONO SWISS ICR MALE MICE

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Abstract

Physostigmine (0.35 mg/kg, s.c.) enhanced the hypnotic action of hexobarbital (50 mg/kg, i.p.) and the hypnotic action of barbital (300 mg/kg, i.p.) in male mice when given 20 min. prior to the given barbiturate. These Physostigmine induced enhancements of barbiturate hypnotic action were reversed by atropine sulfare 20 mg/kg, i.p.), given 20 min. prior to the Physostigmine, but not by atropine methyl bromide (20 mg/kg, i.p.), given 20 min. prior to the Physostigmine, analogously. Neostigmine (0.35 mg/kg,

s.c.), or Methacholine (20 mg/kg, s.c.), given 20 min. prior to the hexobarbital (50 mg/kg, i.p.) effects on hexobarbital hypnotic action when given subsequent to atropine methyl bromide (20 mg/kg, i.p.) was not significantly different from that which obtained when that anticholinergic was not given. The Physostigmine enhancement of hexobarbital action, as influenced by the atropine methyl bromide, was not significantly different from that which obtained when that anticholinergic was not given. The Physostigmine enhancement of barbital action, when influenced by the atropine methyl bromide, was less than the extension produced when such influence was absent. The results suggest that the cholinergic agent induced enhancements of barbiturate action observed are effects exerted by those agents on the central nervous system.

INTRODUCTION

Prior findings have demonstrated extension of barbiturate and Phenothiazine tranquilizer action induced by anticholinesterase agents under conditions which best support the concept that a central nervous system (CNS) cholinergic action is involved in these anticholinesterase effects (Proctor, 1964; Proctor, et al. 1961; and Proctor, et al. 1964). It has also been shown that pilocarpine and arecoline, two receptor active cholinergic agents with CNS activity, can extend the action of phenothiazine tranquilizers (Pfeiffer, 1957), and that pilocarpine can extend the action of ethyl alcohol (Proctor, et al. 1966). In both of these cases, the extension produced was reversed by the CNS active anticholinergic agent, atropine sulfate, but not the predominately peripherally active anticholinergic agent atropine methyl bromide. These reports engendered our interest in the question as to whether or not Physotigmine, Neostigmine and Methacholine would exert effect on the duration of hexobarbital hypnotic action in a manner which was different when any such effect was influenced by atropine methyl bromide and atropine sulfate, respectively. We have also become interested in whether or not barbital sodium hypnotic action would be affected in a manner similar to that of hexobarbital under such conditions. It was reasoned that test of barbital sodium in this analogous way would afford insight with regard to possible influence of biotransformation on any of the observations made since hexobarbital elimination *in vivo* is considerably involved with biotransformation (Quinn, et al. 1958), while the elimination of barbital sodium *in vivo* is not (Ebert, et al. 1964). The present study was undertaken in order to continue and extend investigations concerned with the modulation of the actions of some central nervous system depressants by cholinergic agents.

METHODS AND MATERIALS

Adult albino Swiss ICR male mice obtained from ARA Sprague-Dawley (Madison, Wisconsin), weighing 20- 26 g, were used as experimental animals. Animals were housed 10/cage in no. 101 keystone plastic cages lined with Pel-E-Cel bedding (Paxton Processing Co., Paxton, Illinois). Under a 12 hr on, 12 hr. off lighting cycle, they were allowed to acclimate to the laboratory environment for 2-5 days prior to use. The laboratory temperature was maintained at 23-24°C. The mice had access to Purina rodent chow and tap water *ad libitum*.

Physostigmine was used as the salicylate, neostigmine as the bromide and methacholine as the chloride. The anticholinergics used were atropine methyl bromide and atropine sulfate. Hexobarbital was used as the sodium salt prepared by dissolving 50 mg of the acid form in 4.2 ml of 0.05N NaOH, and diluating with distilled water to make a concentration of 5.0 mg/ml (pH 10.5-11.5). Barbital was employed as the sodium salt. All drugs were obtained from the Sigma Chemical Co., St. Louis, Mo.

The atropinum agents and the barbiturates were given intraperitoncally. Physostigmine, Neostigmine and Methacholine were administered subcutaneously. Intraperitoneal administrations were given in a volume of 0.01 ml/g body weights; subcutaneous administrations were made at a volume of 0.005 ml/g body weight. Atropine derivatives were given at 40 min. and the cholinergic agents at 20 min. prior to the barbiturate administration, respectively. All injections were made using a 1 ml tuberculin syringe fitted with a 27 gauge, 0.5 in needle. In a given experiment, each animal, whether treated or control, received the same number and volume of injections, by analogous routes of administration and on similar time schedules using physiological saline as a "sham" injection where necessary. For each mouse, the loss of righting reflex time induced by the barbiturate was measured. The loss of righting refles after barbiturate administration was assumed to be present when a mouse, exhibiting immobility in the upright posture, was unable to right itself when unrighted 3 successive times within a 30 sec period of testing for the loss of righting reflex. Return of the righting reflex was deemed to be present when the mouse having exhibited loss of righting reflex, spontaneously righted itself and maintained its righting ability when unrighted 3 times within 30 sec. immediately following such spontaneous righting. The data obtained in this way was used to calculate group mean values which were subjected to student's t-test for assessment of the statistical significance between differences in mean values observed. Probability values at or less than the 5 percent level were accepted as significant in these tests.

RESULTS

The results presented in Table I show the effect of 0.35 mg/kg Physostigmine salicylate on the hypnotic effect of 50 mg/kg hexobarbital sodium, when this anticholinesterase agent was given either alone or subsequently to either 20 mg/kg atropine methyl bromide or 20 mg/kg atropine sulfate. There findings indicate that under the conditions of the experiment Physostigmine salicylate was capable of significantly extending the effect of the hexobarbital when given either alone (Group II) or following prior administration of atropine methyl bromide (Group III) respectively, but not when given subsequently to atropine sulfate administration (Group IV). The mean values providing these extensions were about 54 percent (Group II) and 87 percent (Group III) of control mean value (Group I), respectively. Difference in the mean values for pretreatment with Physostigmine given alone (Group II) from that when given subsequent to atropine methyl bromide (Group III) was not statistically significant.

TABLE 1. *Effect of Physostigmine, given subsequent to Atropine Methyl Bromine or Atropine Sulfate, on 50 mg/kg Hexobarbital Hypontic action.*

Group of 10 mice	Drugs and dosages given prior to 50 mg/kg hexobarbital sodium, I.P. (a)	Loss of righting reflex time (min.) (Mean ± S.E.) (b)
I.	0.01 ml/gm 0.9% NaCl (I.P.) 0.005 ml/gm 0.9% NaCl (S.C.)	16.3 ± 2.7
II.	0.01 ml/gm 0.9% NaCl (I.P.) 0.35 mg/kg Physostigmine salicylate (S.C.)	25.1 ± 2.5
III.	20 mg/kg Atropine Me Br (I.P.) 0.35 mg/kg Physostigmine salicylate (S.C.)	30.5 ± 5.46
IV.	20 mg/kg Atropine So_4 (I.P.) 0.35 mg/kg Physostigmine salicylate (S.C.)	18.9 ± 1.98

(a) Atropine agents or I.P. saline were given at 40 min. and the Physostigmine or S.C. saline at 20 min prior to the hexobarbital, respectively

(b) P values from comparison of mean responses (calculated by t-test): Group I compared with Group II, $p < 0.05$; Group I compared with Group III, $p < 0.01$; Groups III and IV, $p < 0.01$; Group I and Group IV, $p > 0.1$; Groups II and III, $p > 0.1$

The findings entered in Table II indicate the results obtained when an experiment was performed to investigate the effect of neostigmine bromide on hexobarbital hypnotic action when given subsequently to either atropine methyl bromide or atropine sulfate, respectively. Given subsequent to 20 mg/kg atropine methyl bromide, or 20 mg/kg atropine sulfate, 0.35 mg/kg neostigmine bromide did not cause a significant extension of the hexobarbital action (Group III or Group IV) in a comparison with action of that barbiturate in control animals (Group I).

TABLE II. *Effect of Neostigmine, given subsequent to Atropine Methyl Bromide or Atropine Sulfate, on 50 mg/kg Hexobarbital Hypnotic action*

Group of 10 mice	Drugs and dosages given prior to 50 mg/kg hexobarbital sodium, I.P. (a)	Loss of righting reflex time (min.) (Mean ± S.E.) (b)
I.	0.01 ml/gm 0.9% NaCl (I.P.) 0.005 ml/gm 0.9% NaCl (S.C.)	16.3 ± 2.78
II.	0.01 ml/gm 0.9% NaCl (I.P.) 0.35 mg/kg Neostigmine Br (S.C.)	17.0 ± 1.85
III.	20 mg/kg Atropine Me Br. (I.P.) 0.35 mg/kg Neostigmine Br. (S.C.)	20.1 ± 1.06
IV.	20 mg/kg Atropine So_4 (I.P.) 0.35 mg/kg Neostigmine Br. (S.C.)	17.3 ± 1.58

(a) Atropine agents or I.P. saline were given at 40 min. and the neostigmine or S.C. saline at 20 min. prior to the hexobarbital, respectively

(b) P values from comparison of mean responses (calculated by t-test): Group I compared with either Group II, III or IV, $p > 0.1$

Table III shows data obtained when an experiment was performed to investigate the effect of methacholine chloride on hexobarbital hypnotic action when given subsequent to either atropine methyl bromide or atropine sulfate, respectively. Given subsequent to 20 mg/kg atropine methyl bromide, 8 mg/kg of methacholine chloride caused a decreased of the hexobarbital action (Group III) in a comparison with action of that barbiturate in control animals (Group I). This decrease was reflected in a mean value which was about 21 percent of control mean value. However when given after the administration of 20 mg/kg atropine sulfate, the dose of methacholine caused greater reduction of hexobarbital action (Group IV).

TABLE III. *Effect of Methacholine, given subsequent to Atropine Methyl Bromide or Atropine Sulfate, on 50 mg/kg Hexobarbital Hypontic action.*

Group of 10 mice	Drugs and dosages given prior to 50 mg/kg hexobarbital sodium, I.P. (a)	Loss of righting reflex time (min.) (Mean ± S.E.) (b)
I.	0.01 ml/gm 0.9% NaCl (I.P.) 0.01 ml/gm 0.9% NaCl (S.C.)	16.3 ± 2.78
II.	0.01 ml/gm 0.9% NaCl (I.P.) 8 mg/kg Methacholine Cl (S.C.)	16.9 ± 3.32
III.	20 mg/kg Atropine Me Br. (I.P.) 8 mg/kg Methacholine Cl (S.C.)	12.8 ± 1.16
IV.	20 mg/kg Atropine So_4 (I.P.) 8 mg/kg Methacholine Cl (S.C.)	11.9 ± 1.58

(a) Atropine agents or I.P. saline were given at 40 min. and the Methacholine or S.C. saline at 20 min. prior to the hexobarbital, respectively

(b) P values from comparison of mean responses (calculated by t-test): Group I compared with either Groups II, III, or IV, $p > 0.1$

Table IV presents data obtained when the effect of Physostigmine salicylate on the hypnotic action of barbital sodium was given alone and given following administration of either atropine methyl bromide or atropine sulfate, respectively. Given pursuant to 20 mg/kg atropine methyl bromide 0.35 mg/kg of physostigmine caused extension of the barbital sodium action (Group III), producing a mean loss of righting reflex time induced by 300 mg/kg barbital sodium which was about 170 percent of the control mean value (Group I). Conversely when given subsequent to 20 mg/kg of atropine sulfate, the same dose of physostigmine did not significantly extend the barbital sodium action (Group IV), in a comparison with the barbital action in control mice. Enhancement of barbital action caused by the physostigmine when given pursuant to atropine methyl bromide (Group III) was 60 percent greater than enhancement produced when given alone (Group II).

TABLE IV. *Effect of Physotigmine, given subsequent to Atropine Methyl Bromide or Atropine Sulfate, on 300 mg/kg Barbital Hypontic action.*

Group of 10 mice	Drugs and dosages given prior to 300 mg/kg barbital sodium I.P. (a)	Loss of righting reflex time (min.) (Mean ± S.E.) (b)
I.	0.01 ml/gm 0.9% NaCl (I.P.) 0.005 mg/kg 0.9% NaCl (S.C.)	128.8 ± 7.0
II.	0.01 mg/gm 0.9% NaCl (I.P.) 0.35 mg/kg Physostigmine (S.C.)	216.8 ± 26.15
III.	20 mg/kg Atropine Me Br. (I.P.) 0.35 mg/kg Physostigmine (S.C.)	347.8 ± 36.01
IV.	20 mg/kg Atropine So_4 (I.P.) 0.35 mg/kg Physostigmine (S.C.)	151.2 ± 11.86

(a) Atropinium agents or I.P. saline were given 40 min. and the physostigmine or S.C. saline at 20 min. prior to the barbital, respectively

(b) P values from comparison of mean responses (calculated by t-test):
Group I compared with either Group II or Group III, $p < 0.01$;
Group IV compared with either Group II or Group II, $p < 0.05$ and $p < 0.01$, respectively; Groups II and III, $p < 0.05$; Groups I and IV, $p > 0.1$

TABLE V. *Effect of Neostigmine, given subsequent to Atropine Methyl Bromide or Atropine Sulfate, on 300 mg/kg Barbital Hypontic action.*

Group of 10 mice	Drugs and dosages given prior to 300 mg/kg barbital sodium I.P. (a)	Loss of righting reflex time (min.) (Mean ± S.E.) (b)
I.	0.01 ml 0.9% NaCl (I.P.) 0.005 ml 0.9% NaCl (S.C.)	128.8 ± 7.0
II.	0.01 ml 0.9% NaCl (I.P.) 0.35 mg/kg Neostigmine Br (S.C.)	117.2 ± 3.87
III.	20 mg/kg Atropine Me Br. (I.P.) 0.35 mg/kg Neostigmine Br. (S.C.)	142.8 ± 12.81
IV.	20 mg/kg Atropine So_4 (I.P.) 0.35 mg/kg Neostigmine Br. (S.C.)	122.9 ± 2.18

(a) Atropine agents or I.P. saline were given 40 min. and the Neostigmine or S.C. saline at 20 min. prior to the barbital, respectively

(b) P value from comparison of mean response (calculated by t-test):
Group I compared with either Groups II, III or Group IV, $p > 0.1$

The results presented in Table V show when an experiment was performed to investigate the effect of neostigmine bromide on barbital hypnotic action when given subsequently to either atropine methyl bromide or atropin sulfate, respectfully. Given subsequent to 20mg/kg atropine sulfate, or in the absence of atropinium agents, 0.35mg/kg neostigmine bromide did not cause a significant extension of the barbital action (Group II, III, or Group IV) in a comparison with action of that barbiturate in control animals (Group I).

Discussion

The experiments performed appear to demonstrate that under certain conditions Physostigmine can enhance the hypnotic action of hexobarbital and barbital in the mouse. While under similar conditions, the effects of neostismine and methacholine on the hypnotic action of hexobarbital was not extended. The cholinergic agent, physostigmine enhancement of barbiturate action is apparently exerted when the agent is administered subsequent to prior administration of atropine methyl bromide but not when given following prior administration of atropine sulfate. The pattern of the influence of these two anticholinergic agents on the physostigmine modulation of barbiturate action would make it appear that this response to this cholinergic agent is more related to a CNS action than to a peripheral action of this drugs. The basis for this tentative conclusion derives from the fact that its effect was not yielded when influenced by atropine sulfate, but was evident when influenced by atropine methyl bromide, the anticholinergic agent having been given under conditions so as to expect CNS anticholinergic activity from the former but not latter (Pfeiffer, et al. 1958; Proctor, et al. 1964; Stein, 1963). A similar pattern governing the influence of those two anticholinergics on pilocarpine extension of ethahol action, (Proctor, et al. 1966), and on pilocarpine and aercoline extension of phenothiazine tranquilizer action (Pfeiffer, et al. 1958), and paraoxon extension of chloral hydrate (Kayaalp, et al. 1965) has been shown to exist. In the second instance and in our own work reported here, the cholinergic agent enhancement of CNS depressant action observed was produced with more potency by physostigmine than by neostigmine. Physostigmine has greater muscarinic potency than does neostigmine, and neostigmine has greater nicotinic potency than does physostigmine (Waser, 1960). These differences in potency at muscarinic and nicotinic sites, considered in conjunction with the fact that the cholinergic agent extension of barbiturate action seen in our results is more potently produced by physostigmine than by neostigmine, may indicate that this response is more related to muscarinic actions of those agents than it is to their nicotinic actions.

Our findings presented here do not rule out a peripheral acting component of the action of physostigmine in extending hexobarbital effect. Our results are most supportive of the proposition that a portion of the mechanism involved in this extension is located in the CNS. In the case of the physostigmine induced extension of hexobarbital action, which we observed, the extension was not significantly reversed when the physostigmine was given following prior administration of atropine methyl bromide, but was reduced to control level of hexobarbital action when given pursuant to atropine sulfate. The extending action of the physostigmine on hexobarbital hypnosis when given alone was not significantly greater than when it was given subsequent to atropine methyl bromide. A somewhat similar pattern of results was yielded when physostigmine effect on barbital hypnotic action was investigated. Given alone, the physostigmine extended the barbital action well above control level. This enhancement was not only not blocked by atropine methyl bromide it was slightly greater under influence of the anticholinergic agent than without it. Conversely atropine sulfate reversed this physostigmine enhancement of barbital action. These findings were indicative of a muscarinic modulation of barbiturate action in the CNS which is atropinium agent sensitive. Causing extensions of hexobarbital and barbital action when given alone, this receptor-active muscarinic agent physostigmine yield such responses when influenced by atropine methyl bromide, but not when influenced by atropine sulfate, under conditions so as to expect CNS anticholinergic activity from latter agent but not the former one (Pfeiffer, et al. 1957; Proctor, et al. 1964; and Stein, 1963). If peripheral activity of the physostigmine made a marked contribution to its mechanism of action producing extension of barbiturate effect then one would expect to see significant decrease in that extension produced by atropine methyl bromide as well as by atropine sulfate if the physostigmine effect in this respect was atropinium sensitive. Our results do not support such a possibility. Since pattern of anticholinergic agent influence on physostigmine induced extension of barbiturate action was the same in the case of barbital as it was in the case of hexobarbital, we have provided evidence supportive of a postulate which postulates that CNS activity of a muscarinic agent can modulate barbiturate action by extending that action, irrespective of its peripheral factor influences such as its effects on barbiturate pharmocokinetics and biotransformation (Tsujimoto, et al. 1974). Basis for this postulate is provided by the great similarity in the pattern of anticholinergic agent influence on physostigmine effect which we obtained whether hexobarbital, a barbiturate is markedly eliminated by biotransformation (Quinn, et al. 1958), or barbital, which is virtually not biotransformed (Ebert, 1964), was the barbiturate action extended by the physostigmine.

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