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ADDITIVE AND NON-ADDITIVE EFFECTS OF MIXTURES OF SHORT-ACTING INTRAVENOUS ANAESTHETIC AGENTS AND THEIR SIGNIFICANCE FOR THEORIES OF ANAESTHESIA

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1 The potency of a series of short-acting anaesthetics was established by measuring the duration of the loss of righting reflex following a single bolus injection into the tail vein of male Wistar rats. The agents were, in order of potency, etomidate, alphaxalone, methohexitone, alphadalone acetate and propanidid.

2 The potency of binary mixtures of these agents was also assessed to see whether the anaesthetic effects of different agents were additive as classical theories of anaesthesia suggest. Mixtures of alphaxalone and alphadalone acetate, alphaxalone and propanidid and methohexitone and propanidid all showed simple additive effects. Mixtures of alphaxalone and etomidate and of alphaxalone and methohexitone showed a greater potency than would be expected if their effects were simply additive. Mixtures of etomidate and methohexitone were not examined.

3 Mixtures of alphaxalone and either methohexitone or pentobarbitone produced a greater depression of synaptic transmission in *in vitro* preparations of guinea-pig olfactory cortex than would have been expected from the sum of the activities of the individual anaesthetics. Other combinations of anaesthetics did not show similar effects although the interaction between alphaxalone and etomidate was not examined.

4 Neither alphaxalone nor pentobarbitone affected the membrane: buffer partition coefficient of the other for a model membrane system.

5 These results are interpreted as evidence against the classical unitary hypotheses of anaesthetic action based on correlations of anaesthetic potency with lipid solubility and as supporting the view that different anaesthetics act on different structures in the neuronal membranes to produce anaesthesia.

Introduction

Many current theories of anaesthesia assume that there is a single, common molecular mechanism by which all anaesthetics produce anaesthesia (e.g. Seeman, 1972; Hill, 1974; Smith, 1974; Trudell, 1977). These theories are generally based on the properties of solutions of anaesthetics in lipids (Miller, Paton, Smith & Smith, 1972) and attribute the effect of anaesthetics either to the number of molecules dissolved in a hydrophobic region (commonly taken to be the neuronal plasma membranes) or to the volume fraction occupied by the molecules of anaesthetic in the hydrophobic region (Mullins, 1954; Miller, Paton, Smith & Smith, 1973). In either case the anaesthetic effect is assumed to be independent of the precise chemical structure of the anaesthetic. If this is correct, then one species of anaesthetic molecule can be substituted for any other and the anaesthetic effects of mixtures of anaesthetics should be strictly additive. In effect, this means that if a given anaesthetic endpoint such as a loss of righting reflex is just achieved by an anaesthetic A at a concentration in the blood a and the same anaesthetic endpoint is achieved by another anaesthetic B at a concentration in the blood of b, then binary mixtures of A and B such as $\frac{1}{2}a + \frac{1}{2}b$, $\frac{3}{4}a + \frac{1}{4}b$, etc. should achieve exactly the same anaesthetic endpoint as either A or B at its full dose.

Simple additivity of anaesthetic effect has been shown for a number of binary mixtures of various volatile anaesthetics: xenon-halothane (Cullen, Eger, Cullen & Gregory, 1969); ethylene-halothane (Miller, Wahrenbrock, Schroeder, Knipstein, Eger & Buechel, 1969); cyclopropane-halothane (Difazio, Brown, Ball, Heckel & Kennedy, 1972); nitrous oxide-argon; nitrous oxide-sulphur hexafluoride; and argon-carbon tetrafluoride (Clarke, Daniels, Harrison, Jordan, Paton, Smith & Smith, 1978).

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However, the volatile anaesthetics as a group do not show subtle changes in anaesthetic potency with relatively small changes in chemical structure (except for a few anomalous agents which possess convulsant properties) nor do their molar volumes differ greatly. Other groups of anaesthetics such as the barbiturates and steroids show a much greater variation in anaesthetic potency with relatively small changes in chemical structure (see for example, Phillipps, 1974). One might reasonably expect these structure-activity relations to reflect the interaction of the anaesthetic molecules with specific binding sites and that different anaesthetics would attach to different sites. This being so, a multi-site hypothesis such as the degenerate perturbation hypothesis proposed by Richards, Martin, Gregory, Keightley, Hesketh, Smith, Warren & Metcalfe (1978) would predict non-additive behaviour for binary mixtures of some anaesthetics, particularly for mixtures of anaesthetics whose individual anaesthetic potency depends on precise chemical features. Thus the unitary hypothesis and multi-site hypotheses of anaesthetic action can be distinguished on the basis of the behaviour of mixtures of anaesthetics. This paper describes the effects of some binary mixtures of short-acting intravenous anaesthetics and further shows that binary mixtures of alphaxalone (a steroid) and methohexitone show a strong synergistic interaction as the multi-site model predicts. Furthermore, a similar synergistic interaction between alphaxalone and either methohexitone or pentobarbitone can be seen with the depressant effects of these anaesthetics on synaptic transmission in the olfactory cortex. A preliminary account has been given to the British Pharmacological Society (Archer, Richards & White, 1977).

Methods

The potency of anaesthetics in vivo

To assess the potency of individual anaesthetics and of mixtures of anaesthetics the duration of the loss of righting reflex was measured in normal male Wistar Rats (150–200 g) following single injections of anaesthetics into the tail vein. For these studies it was necessary to ensure a clear anaesthetic end point and this was taken as the point at which an animal fully righted itself after being placed on its back. The duration of the period from injection of anaesthetic to the return of the righting reflex was recorded and considered to be the duration of anaesthesia. In all cases the anaesthetics were injected over a 10 s period and the animals were anaesthetized by the end of the injection. Each injection was kept to the same volume per kg body weight of 1 ml/kg. The agents studied were alphadalone acetate, alphaxalone, etomidate, methohexitone and propanidid.

We chose to study short acting anaesthetics which gave a clear anaesthetic end point as this enabled us to obtain a similar depth and duration of anaesthesia with each agent. For this reason pentobarbitone and thiopentone were excluded from the behavioural studies, thiopentone because it produces a protracted ataxia which outlasts the anaesthesia judged by other criteria, and pentobarbitone because it is not a short-duration anaesthetic with a rapid offset.

All anaesthetics were injected in 0.9% w/v NaCl solution (saline), propanidid and the steroids being rendered soluble by the use of Cremophor EL, the usual commercial vehicle. To make the injection, the appropriate amount of anaesthetic solid or oil (for propanidid) was blended with pure Cremophor EL using chloroform as a solvent. The chloroform was subsequently removed under vacuum and the resulting oil kept overnight under vacuum. Warm saline was then added to make the injection solution.

Electrophysiological studies

These were conducted using slices of guinea-pig olfactory cortex maintained *in vitro*. Full accounts of the methods of preparation and incubation have been given elsewhere (Richards, Russell & Smaje, 1975; Richards, 1981). For the experiments described here, slices of olfactory cortex 350–400 μm thick were taken and incubated at 37°C in the chamber described by Richards & Tegg (1977). This chamber permits the slice to be superfused by a stream of oxygenated artificial cerebrospinal fluid (c.s.f.) over both the cut and pial surfaces.

Stimulation of the slices was effected by a pair of silver wires, which were insulated except at the tip, placed across the anterior end of the olfactory tract close to its exit from the olfactory bulb. The stimulation pulses were taken from an isolated stimulator driven by a Digitimer at 0.1–1 Hz. The evoked field potentials were recorded from the superficial layers of the prepiriform cortex close to the lateral olfactory tract (about 0.2–1 mm lateral to the straight portion of the tract and 4–6 mm distant from the stimulating electrodes). The recording electrodes were made of glass, were 2–4 μm tip diameter and were filled with 0.5 M NaCl. The potentials were recorded monopolarly, the indifferent electrode being a silver-silver chloride wire placed in the fluid of the recording chamber. Conventional methods of amplification and display were used.

The artificial cerebrospinal fluid used to bathe the preparations was saturated with 95% O_2, 5% CO_2 before use and had the following composition (mM): NaCl 134, KCl 5, KH_2PO_4 1.25, $CaCl_2$ 1, $NaHCO_3$ 16 and glucose 10.

Anaesthetics were applied to the preparation either in solution (for the water soluble anaesthetics)

or as liposomes loaded with anaesthetic (for the steroid anaesthetics). Thus alphaxalone, Δ16 alphaxalone and alphadalone acetate were administered as the steroid component of liposomes.

To prepare the liposomes, egg yolk lecithin was dissolved in chloroform-methanol (2:1 v/v) together with the steroid to be applied in a molar ratio of 1:1. The solutions were evaporated to dryness under nitrogen and kept under high vacuum (c. 10^{-3} mmHg) for 2–4 h to remove residual traces of solvent. Artificial c.s.f. (10 ml) was then added to the film and mixed vigorously on a vortex mixer to form a suspension of multilamellar vesicles. The resulting suspension was subjected to ultrasonic dispersion for 2 min with a probe sonicator to suspend the phospholipid as small vesicles. No attempt was made to sonicate till the suspensions were clear. The opalescent suspension was diluted with more artificial c.s.f. until the final steroid concentration was 50 μmol/l artificial c.s.f.

The potentials elicited in the prepiriform cortex in response to stimulation of the lateral olfactory tract (l.o.t.) have a characteristic appearance. As in previous papers, the amplitude of the e.p.s.p. at a fixed latency from the stimulus artifact was taken as a measure of the synaptic current flowing following stimulation of the l.o.t. fibres (see Richards *et al.*, 1975).

Partition coefficient measurements

The partition coefficients between liposomes of phosphatidyl choline, phosphatidyl serine and cholesterol (molar ratios of approximately 9:1:5) and artificial c.s.f. were measured for pentobarbitone and alphaxalone with [^{14}C]-pentobarbitone (New England Nuclear and [^{14}C]-alphaxalone (Glaxo) using equilibrium dialysis.

The liposomes were prepared by mixing egg lecithin (116 mg), bovine phosphatidyl serine (12.5 mg) and cholesterol (31 mg) in chloroform-methanol. The mixture was then evaporated to dryness in a stream of nitrogen and kept under vacuum for 4–6 h to remove residual traces of chloroform. The resulting film was resuspended in 3.2 ml artificial c.s.f. containing 0.2 mM pentobarbitone labelled with ^{14}C for the determination of the partition coefficient of pentobarbitone. This mixture was placed on one side of the dialysis membrane and 0.2 mM pentobarbitone solution labelled as before with ^{14}C was placed on the other. The dialysis chamber was gently rotated to promote mixing and samples from each chamber were taken after 30 h and the total amount of pentobarbitone gained by the lipid calculated from the gain of ^{14}C by the chamber containing lipid. Samples of equal volume (100–200 μl) were taken from each side of the dialysis membrane and their radioactivity was measured by blending the sample with scintillation fluid (toluene, 1l; Triton-X-100, 1l; 2,5 diphenyloxazole, 5 g; 1,di [2-(5 phenyloxazolyl)] benzene, 50 mg) and counting in a liquid scintillation spectrometer. From this information the partition coefficient for pentobarbitone was calculated.

To calculate the partition coefficient of alphaxalone a similar procedure was adopted except that liposomes containing [^{14}C]-alphaxalone were used (molar ratios: phosphatidyl choline, 9: phosphatidyl serine, 1: cholesterol, 4.75; [^{14}C]-alphaxalone 0.25) and the loss of counts from the liposomes to the saline were used to estimate the partition coefficient for alphaxalone.

To examine the interaction between alphaxalone and pentobarbitone, the partition coefficients for alphaxalone were estimated using saline containing unlabelled pentobarbitone (0.2 mM) and the partition coefficient for pentobarbitone was estimated as before but with liposomes containing unlabelled alphaxalone in addition to cholesterol. All experiments were conducted at room temperature 22–24°C.

Results

Potency of short-acting anaesthetics in intact rats

The duration of the loss of the righting reflex in normal male Wistar rats following single intravenous injections of various doses of short-acting anaesthetics is shown in Table 1. As explained in the Methods, only anaesthetic agents which gave a clear end point were included in this part of the study. Occasionally, an animal exhibited a partial recovery from the effects of the anaesthetic and lay with its trunk twisted in such a way that the head was righted while the lower body remained supine. In this abnormal posture, the animal would explore the table with its vibrissae. However, the end point for the duration of anaesthesia was always taken as the time at which the animal completely righted itself and could walk.

From the table it is clear that etomidate had the greatest potency, followed by alphaxalone, with alphadalone acetate and methohexitone having similar potency. Propanidid was the least potent of the agents tested.

The anaesthetic potency of mixtures of anaesthetics in rats

Alphaxalone and alphadalone acetate These two substances are of closely similar structure and it is to be expected that they would have a similar mode of action. If this is so, a mixture of half the dose of alphaxalone required to produce, say, 8 min loss of righting reflex added to half the dose of alphadalone

Table 1 The potency of short-acting intravenously administered anaesthetics in male Wistar rats

Anaesthetic	*Dose* (mg/kg)	*Duration of anaesthesia* (min ± s.d.)	n
Alphaxalone	0.75	0.86 ± 0.54	6
	1.5	4.21 ± 0.65	19
	3.0	8.20 ± 1.71	26
Methohexitone	3.0	1.05 ± 0.56*	7
	6.0	4.94 ± 0.89	12
	12.0	8.30 ± 1.51	10
Alphadalone acetate	3.0	0.95 ± 0.10	5
	6.0	3.50 ± 0.29	8
	9.0	6.92 ± 0.66	5
	12.0	8.65 ± 1.39	7
Propanidid	50.0	3.74 ± 0.33	10
Etomidate	1.8	8.64 ± 0.66	9

*Excludes one animal that failed to show loss of righting reflex.
All anaesthetics were administered via the tail vein as a single injection given over 10 s.

acetate required to produce the same duration of anaesthesia (judged by the loss of righting reflex) should cause a loss of righting reflex of 8 min. Such a result is shown in Table 2 and there is, therefore, an additive interaction between the two steroid anaesthetics with respect to their anaesthetic properties.

Alphaxalone and methohexitone Mixtures of these two substances showed a greater anaesthetic potency than would be predicted on the basis of the potency of either agent alone. Thus a mixture of one half the dose of alphaxalone required to produce a loss of righting reflex of about 8 min added to half the dose of methohexitone required to produce a similar duration of loss of righting reflex produced a loss of righting reflex lasting over 11 min (Table 3). Similar mutual potentiation of the anaesthetic action of these substances was seen with other mixtures such as 25 parts alphaxalone, 75 parts methohexitone, which would also have been expected to produce a loss of righting reflex of about 8 min if the effects of these

Table 2 The duration of the loss of the righting reflex in normal male Wistar rats produced by intravenous injection of mixtures of some short-acting anaesthetics

Anaesthetic combination	*Duration of anaesthesia* (min) Mean ± s.d.	n	P *value*
Alphaxalone 3 mg/kg	8.20 ± 1.71	26	
Alphadalone acetate 12 mg/kg	8.65 ± 1.39	7	
Alphaxalone 1.5 mg/kg + alphadalone acetate 6 mg/kg	8.22 ± 0.82	5	NS
Alphaxalone 1.5 mg/kg	4.35 ± 0.54	8	
Propanidid 50 mg/kg	3.74 ± 0.33	10	
Alphaxalone 0.75 mg/kg + propanidid 25 mg/kg	4.08 ± 0.30	6	NS
Alphaxalone 3 mg/kg	8.20 ± 1.71	26	
Etomidate 1.8 mg/kg	8.64 ± 0.66	9	
Alphaxalone 1.5 mg/kg + etomidate 0.9 mg/kg	10.15 ± 1.84		< 0.05
Methohexitone 6 mg/kg	4.94 ± 0.89	12	
Propanidid 50 mg/kg	3.74 ± 0.33	10	
Methohexitone 3 mg/kg + propanidid 25 mg/kg	3.85 ± 0.35	6	NS

The duration of the loss of righting reflex produced by the binary mixtures of anaesthetics has been compared with that produced by either anaesthetic alone at its full anaesthetic dose. All data were first subjected to F ratio test to assess for differences in variation between populations and then null hypothesis tested by Student's *t* test (two tailed).

Table 3 The duration of the loss of righting reflex in normal male Wistar rats caused by injections of mixtures of alphaxalone and methohexitone

Expt. No.	*Anaesthetic combination*	*Duration of loss of righting reflex* (min) Mean ± s.d.	n	*Comparison*	P *value*
1	Alphaxalone 3 mg/kg	8.20 ± 1.71	26	—	—
2	Methohexitone 12 mg/kg	8.30 ± 1.51	10	1 vs. 2	NS
3	Alphaxalone 2.25 mg/kg + methohexitone 3 mg/kg	9.90 ± 1.43	10	2 vs. 3	<0.02
4	Alphaxalone 1.5 mg/kg + methohexitone 6 mg/kg	11.32 ± 1.48	10	2 vs. 4	<0.001
5	Alphaxalone 0.75 mg/kg + methohexitone 9 mg/kg	10.48 ± 1.17	10	2 vs. 5	<0.001
6	Alphaxalone 1.5 mg/kg	4.21 ± 0.65	19	—	—
7	*Methohexitone 6 mg/kg + Cremaphor EL	4.23 ± 0.75	11	7 vs. 6	NS
8	Alphaxalone 0.75 mg/kg + methohexitone 3 mg/kg	5.45 ± 0.59	6	8 vs. 7	<0.001

*Note the alphaxalone was administered in 20% Cremaphor EL and administration of methohexitone with this vehicle was used to control for the effects of the vehicle in the mixtures.
For statistical methods see notes to Table 2.

agents were additive. In addition, the effect of a mixture of half the dose of alphaxalone required to produce a loss of righting reflex of 4 min with half the dose of methohexitone required to produce a similar duration of loss of righting reflex also showed a longer loss of righting reflex than would have been predicted on the basis of an additive interaction between the two anaesthetic agents (see Table 3).

Other mixtures Those tested are shown in Table 2. In addition to mixtures of alphaxalone and alphadalone acetate, mixtures of alphaxalone and propanidid and methohexitone and propanidid showed simple additive behaviour in respect of their anaesthetic effects. Alphaxalone and etomidate showed a slight synergism that was just statistically significant. Because of the pH difference between the stock solutions of methohexitone and etomidate it was not possible to test this combination.

Effects of Cremaphor EL Propanidid and the steroids, alphaxalone and alphadalone acetate, are of low solubility in water and are therefore made up in the emulsifying agent Cremaphor EL. This material alone had no anaesthetic properties when administered in saline and when administered with a water-soluble anaesthetic either had no effect on the duration of the loss of righting reflex or reduced it slightly (compare the effects of 6 mg/kg methohexitone in saline with those of 6 mg/kg methohexitone in saline with 10% Cremaphor EL).

The actions of mixtures of anaesthetics on synaptic transmission

Alphaxalone and the barbiturates It is clear from the previous section that mixtures of alphaxalone and methohexitone show a greater anaesthetic potency than either agent administered on its own (Table 3). Furthermore, this synergism was not observed with other combinations of anaesthetics (Table 2). Since both alphaxalone and the barbiturates, including methohexitone, appear to exert their anaesthetic effects in some measure by interfering with synaptic transmission (see Richards, 1972; 1980; Richards & Hesketh, 1975) the actions of mixtures of these anaesthetics on synaptic transmission is clearly of interest. Alphaxalone (50 μM) and methohexitone (100 μM) selectively depressed the postsynaptic components of the evoked potentials elicited by stimulation of the lateral olfactory tract (Figure 1) in agreement with earlier results (Richards, 1972; Richards & Hesketh, 1975). A mixture of 25 μM alphaxalone and 50 μM methohexitone had a far more profound effect on the postsynaptic component of the evoked potential, abolishing it totally. The compound action potential of the lateral olfactory tract was not affected by the mixture. The depression of the postsynaptic potential or e.p.s.p. produced by either alphaxalone of methohexitone was shown to be dose-related.

In the course of these experiments it was noticed that the depressant effect of a given dose of

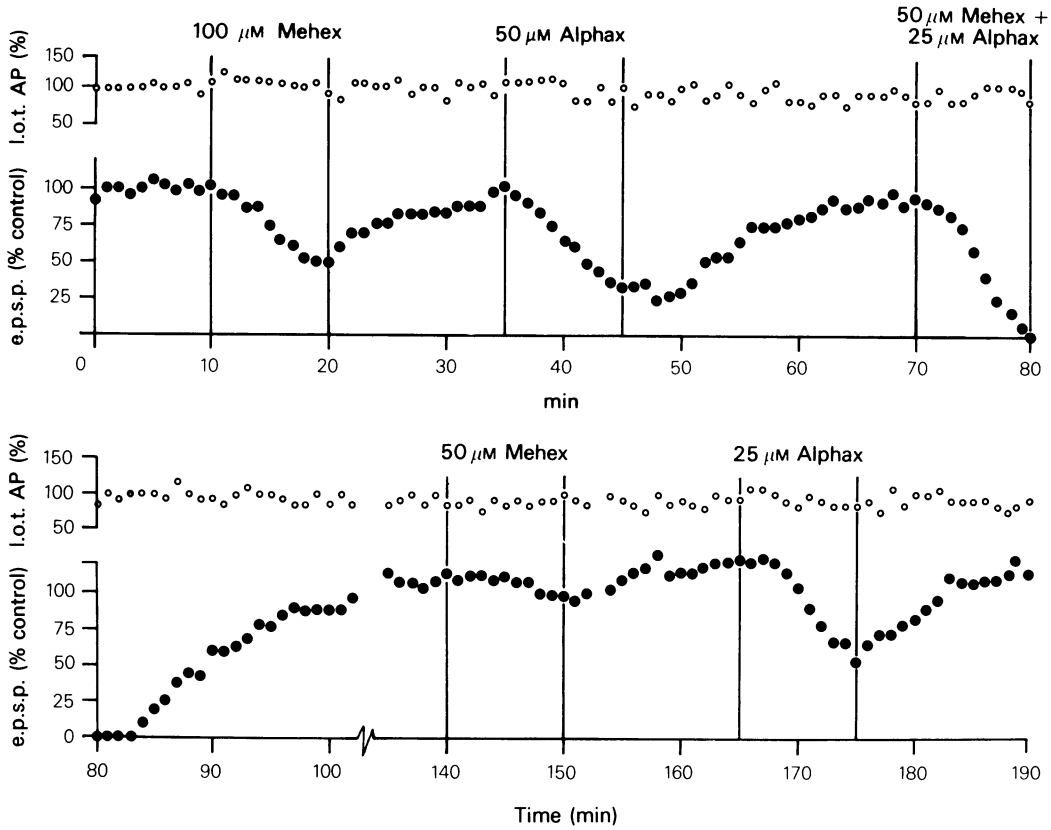

Figure 1 The action of methohexitone (Mehex) and alphaxalone (Alphax) and a mixture of the two on the evoked potentials of the olfactory cortex. All values are expressed as a percentage of the average recorded during the first 10 min of the experiment. Note the break in the abscissa scale at 104 min. (○) Amplitude of the lateral olfactory tract (l.o.t.) action potential (AP); (●) amplitude of the population e.p.s.p. at 1.5 ms from the stimulus artifact.

methohexitone was greater after a preparation had been exposed to alphaxalone for a short period (10–15 min). Therefore, the potentiating effect of alphaxalone on the depressant action of methohexitone outlasted its own depressant effect, in some cases by an hour or more. Similar results were obtained when pentobarbitone was applied after alphaxalone (Figure 2). Repeated applications of the same dose of alphaxalone or barbiturates to a given preparation produced virtually constant degrees of e.p.s.p. depression.

Other anaesthetic mixtures The actions of a further number of mixtures of anaesthetics on synaptic transmission in the olfactory cortex were also investigated. The blocking effect of Δ16-alphaxalone on the depressant action of alphaxalone has been described elsewhere (Richards & Hesketh, 1975). In view of these results, the actions of Δ16-alphaxalone (50 μM) on the depressant effects of pentobarbitone (0.3 mM) and phenobarbitone (0.4 mM) were also examined. In neither case did Δ16-alphaxalone diminish or enhance the depressant effects of these barbiturates though in all cases (4 experiments) the time course of the depressant effect of the barbiturate appeared somewhat slower when Δ16-alphaxalone was present in the incubation medium.

Rather surprisingly for a general anaesthetic, propanidid had a local anaesthetic action on synaptic transmission. The depression of the synaptic waves accompanied a decrease in the amplitude of the presynaptic action potential and an increase in its duration (Richards, unpublished). However, mixtures of methohexitone and propanidid showed simple additive effects in their depressant actions in the evoked synaptic potentials of the olfactory cortex (Figure 3). It will be recalled (Table 2) that mixtures of these two agents had additive anaesthetic effects *in vivo*.

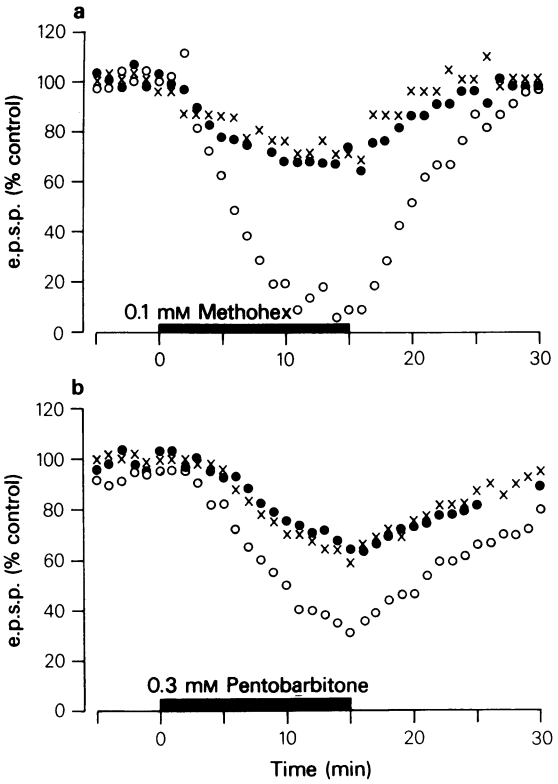

Figure 2 The action of methohexitone 0.1 mM (a) and pentobarbitone 0.3 mM (b) on the population e.p.s.p. of the olfactory cortex. In each case the preparations were first challenged by the barbiturate alone at the stated dose (×) and then were exposed to artificial c.s.f. containing alphaxalone (not shown). Ten (b) or 15 (a) min after the exposure to alphaxalone had been terminated and the population e.p.s.p. had returned to its original amplitude, the preparations were again challenged with the barbiturate alone at the stated concentration (○). This procedure was then repeated after a further hour had elapsed (●). In both experiments the depressant action of the barbiturate was greatly augmented following prior treatment with alphaxalone.

Partition coefficient measurements

The results of the studies of the anaesthetic potency of mixtures of methohexitone and alphaxalone in intact rats and on the depression of synaptic transmission in slices of olfactory cortex suggested that administration or application of alphaxalone had somehow affected the sensitivity of central synapses to the depressant effects of barbiturates. As anaesthetics are believed to interact with membrane lipids, one possible explanation of the synergism between alphaxalone and the barbiturates is that alphaxalone increases the partition coefficient for the barbiturates between the lipid of the synaptic membranes and the extracellular fluid, or that the barbiturates increased the partition coefficient for alphaxalone. This possibility was tested by measuring the partition coefficient for pentobarbitone between liposomes and artificial c.s.f. in the presence and absence of alphaxalone as part of the steroid component of the liposomes and by measuring the partition coefficient for alphaxalone between liposomes and artificial c.s.f. in the presence and absence of pentobarbitone (0.2 mM). The results are given in Table 4 from which it can be seen that neither pentobarbitone nor alphaxalone modified the membrane-buffer partition coefficient for the other anaesthetic. Pentobarbitone was chosen for this part of the study simply because [^{14}C]-pentobarbitone was readily available while [^{14}C]-methohexitone was not. Furthermore, alphaxalone potentiated the depressant actions of both methohexitone and pentobarbitone on synaptic transmission.

Discussion

In this paper we set out to verify the unitary hypothesis of anaesthetic action by testing the prediction that the anaesthetic effects of mixtures of anaesthetics should be strictly additive (see Smith, 1974). The feature of the unitary hypothesis that gives rise to this prediction is the implicit assumption that anaesthesia occurs either when a critical number of anaesthetic molecules occupy the neuronal membranes (The Meyer-Overton hypothesis; see Meyer, 1937) or when a critical volume fraction of anaesthetic molecules is exceeded in the neuronal membranes (the critical volume hypothesis: Mullins, 1954; Miller *et al.*, 1973). According to these hypotheses, anaesthesia depends either on the number, or the volume fraction of anaesthetic molecules present in the neuronal membranes but not on their chemical nature. Accordingly, one type of anaesthetic molecule should be able to substitute for another, with perhaps a minor correction for differences in molecular volume. Consequently, mixtures of two anaesthetics should show additive behaviour. If, however, two anaesthetics act at different sites by virtue of their differing chemical constitutions, then the effects of mixtures are less predictable. It may be that a binary mixture of two such anaesthetics would show additive behaviour because their effects, though different, added together to produce anaesthesia simply because their dose-response curves were parallel. A second possibility is that mixtures of anaesthetics may show mutual antagonism with each other because of competition for available binding sites and the anaesthetic effect would therefore be less than that expected from the sum of the potencies of either

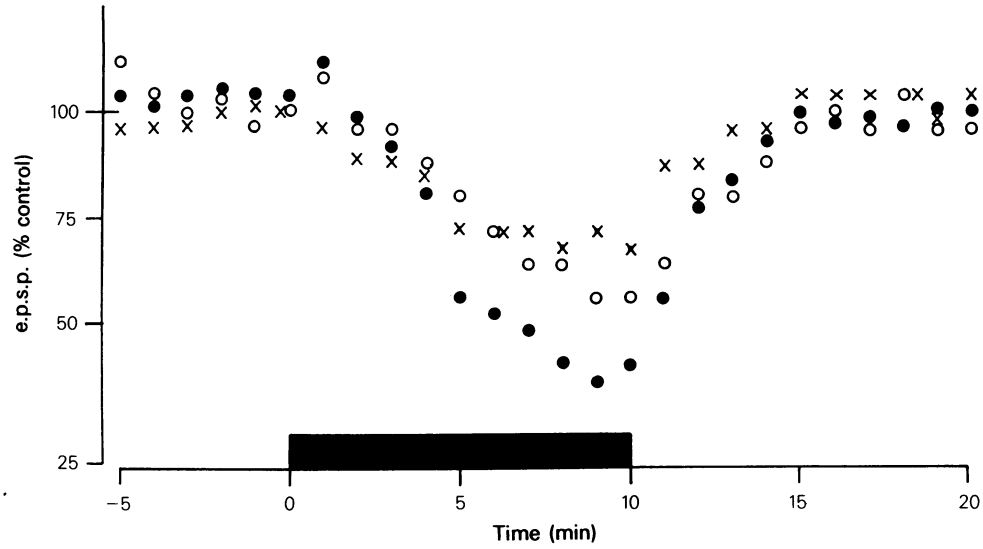

Figure 3 The action of methohexitone 0.1 mM (×), propanidid 0.3 mM (●) and a mixture of 0.05 mM methohexitone and 0.15 mM propanidid (○) on the evoked population e.p.s.p. of the olfactory cortex. Ordinate scale is expressed as a percentage of the average value of the e.p.s.p. during the 5 min preceding the first application of drug (methohexitone 0.1 mM). The black bar along the abscissa scale indicates the period during which the drugs were superfused in successive applications.

component alone. A third possibility is that the potency of a mixture of two anaesthetics could be greater than that of either agent alone because of allosteric effects on the binding sites; the effects could therefore be synergistic. While additive behaviour of anaesthetic mixtures is consistent with the unitary hypothesis, it does not prove it; however, any deviation from additivity disproves the unitary hypothesis. Furthermore, since anaesthesia is supposed to result from the presence of a specific number of molecules in the neuronal membranes, the dose-response curves of different anaesthetics need not be parallel for the prediction to be valid.

A wide variety of binary mixtures of gaseous and volatile anaesthetics have been shown to possess simple additive anaesthetic properties (see Introduction). Nevertheless, a few exceptions have been found; mixtures of cyclopropane with either ethylene or nitrous oxide have been reported to show slight antagonistic effects (Difazio *et al.*, 1972) as have mixtures of argon and sulphur hexafluoride in mice (Clarke *et al.*, 1978). Clarke *et al.* (1978) and Richards (1978) have already thrown doubt on the conclusion reached by Difazio *et al.* (1972) because of the errors involved in estimating the anaesthetic potency of the gases concerned. Clarke *et al.* (1978)

Table 4 Partition coefficient of pentobarbitone and alphaxalone between artificial lipid bilayers and buffered artificial c.s.f. at pH 7.6 and 25°C

Anaesthetic	*Bilayer: Buffer Partition coefficient* mean ± s.d.	*Other conditions*	n	P *value*
Pentobarbitone	45.7 ± 2.8	—	3	NS
Pentobarbitone	46.5 ± 6.0	Alphaxalone in liposomes	6	
Alphaxalone	1065 ± 44	—	4	NS
Alphaxalone	1082 ± 66	Pentobarbitone (0.2 mM) in buffer	3	

Liposomes were composed of egg lecithin (phosphatidyl choline PC), bovine phosphatidyl serine (PS) and cholesterol in a molar ratio of approx 9:1:5. When alphaxalone was added either to measure its partition coefficient or as unlabelled material the molar composition of the liposomes was 9PC: 1 PS: 4.75 cholesterol: 0.25 alphaxalone.

took great care in estimating the potency of the agents used in their study and clearly showed that a mixture of argon and sulphur hexafluoride had a non-additive effect on the rolling response of mice. However, they did note that this combination had additive effects in newts and suggested that the non-additive effects with mice might be attributed to respiratory complications, as both gases had to be administered under pressure. Thus the studies on the anaesthetic effects of mixtures of gases and vapours have failed to show any clear example of non-additive behaviour. However, these data are not conclusive proof of the identity of action of these agents, a point that is reinforced by the additive behaviour of methohexitone and propanidid, two agents with well marked differences in their mode of action on synaptic transmission. A more convincing example of non-additive behaviour is the antagonism of the depressant actions of alphaxalone on synaptic transmission by its non-anaesthetic homologue Δ16-alphaxalone (Richards & Hesketh, 1975) but a similar antagonism between these agents has not been found *in vivo*.

As Tables 2 and 3 indicate, binary mixtures of alphaxalone and etomidate and of alphaxalone and methohexitone show a strong synergistic anaesthetic effect while mixtures of a variety of other intravenous anaesthetics showed additive behaviour. These results are not consistent with the unitary hypothesis of anaesthetic action in its simplest form. However, they could be reconciled with the unitary hypothesis if either component of the mixtures increased the partition coefficient of the other between the extracellular fluid and the nerve cell membranes and thereby increased the total membrane concentration of anaesthetic. Similarly, if either component inhibited the metabolic inactivation of the other in the liver, it is conceivable that administration of, for example, a 50:50 binary mixture could produce anaesthesia of longer duration than that produced by either component alone at its full dose. These two possibilities will now be considered for mixtures of alphaxalone and barbiturates, the agents for which we have greatest information.

The first of these possible explanations is very unlikely as neither alphaxalone nor pentobarbitone affected the partition of the other between saline and artificial lipid bilayers (Table 4), and similar results may be anticipated for methohexitone and alphaxalone. The second possibility is the most plausible as both methohexitone and alphaxalone are substantially metabolized by the liver to produce water soluble conjugates which are without significant anaesthetic potency (Welles, McMahon & Doran, 1963; Child, Gibson, Harnaby & Hart, 1972). Nevertheless, the weight of evidence is against this view. The action of short-acting anaesthetic agents given by a single injection is terminated not chiefly by metabolism but by redistribution within the body mass (Papper & Kitz, 1963; Card, McCulloch & Pratt, 1972). Moreover, a similar synergistic action on synaptic transmission between alphaxalone and methohexitone or pentobarbitone could be shown *in vitro* in slices of brain tissue (Figures 1 and 2) in which metabolic destruction of either anaesthetic is very unlikely.

The simplest explanation for our observations is that alphaxalone and the barbiturates act at different sites in the neuronal membranes to cause anaesthesia. The differing lesions have cooperative effects in disrupting neural function. In consequence, the observations described here give strength to the view that different anaesthetics act at different sites, a hypothesis that was originally proposed by Metcalfe, Hoult & Colley (1974) and has been recently elaborated by Richards (1978, 1980) and by Richards *et al.* (1978). This hypothesis has been termed the degenerate perturbation hypothesis in acknowledgement of the loss of the simplicity inherent in the unitary hypothesis.

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