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Effects of Anesthetic Agents on Synaptosomal GABA Disposal

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In brain slices, halothane was shown to inhibit the metabolic breakdown of GABA (γ-aminobutyric acid), an inhibitory neurotransmitter. This inhibition leads to increased brain GABA content, presumably in the synaptic areas, and to the postulation that halothane anesthesia may arise from an enhanced synaptic inhibition due to this elevated GABA. The ability of many neurotropic agents to inhibit GABA breakdown was studied by assessing synaptosomal "GABA disposal". GABA disposal by intact synaptosomes, which simulate miniature synapses, measures the conversion of [1-^{14}C]GABA to $^{14}CO_2$ and includes the processes of uptake, release, and catabolism of GABA. The most potent inhibitor is chloroform, followed by halothane, enflurane, ether, and thiopental. Pentobarbital, ethanol, paraldehyde, and ketamine are weak inhibitors. Phenobarbital, morphine, and phenytoin are not inhibitory at pharmacologic concentrations. As a whole, anesthetic agents show particular inhibitory action on this metabolic process in this model system where the ID_{10} values (*i.e.*, concentration of a drug necessary to produce 10 per cent inhibition of GABA disposal) correlate well with known pharmacologic potencies, ED_{50} values, or MACs. These observations support the possibility that anesthesia may be related to an inhibition of GABA disposal. (Key words: Alcohol. Anesthetics, intravenous: ketamine; morphine; thiopental. Anesthetics, volatile: chloroform; enflurane; ether; halothane. Anticonvulsants: phenytoin. Brain: gamma-aminobutyric acid; synapses. Hypnotics: paraldehyde; pentobarbital; phenobarbital. Theories of anesthesia.)

HALOTHANE IN ANESTHETIC CONCENTRATIONS causes a dose-related GABA accumulation in rat brain cortex slices.[1–4] This accumulation is not related to uptake, release, or synthesis, but is caused by an inhibition of GABA catabolism.[4] We have hypothesized that the increase in GABA content in brain slices caused by an inhibition of its catabolism, may contribute to the anesthetic action of halothane by inhibition of synaptic transmission.[2–5] A model using brain synaptosomes has been devised to investigate the effects of neurotropic agents, especially anesthetics, on GABA catabolism by synaptic tissue. This study reports effects on synaptosomal GABA catabolism of several anesthetic agents and of some other neurotropic depressants. Liberation of $^{14}CO_2$ from [1-^{14}C]-GABA, defined here as "GABA disposal," was used as an index of GABA catabolism. GABA disposal includes uptake, release, and catabolism of GABA by synaptosomes in contrast to pure catabolism of GABA. We demonstrate here that anesthetic agents, as a group, reduced GABA disposal and may enhance the action of this inhibitory neurotransmitter.

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Materials and Methods

Synaptosomes were prepared from forebrains of male Sprague-Dawley rats according to the sucrose-density centrifugation method.[6] The final sedimented synaptosomes were resuspended in 0.8 ml/brain of a suspension medium containing 100 mM Tris, 450 mM mannitol, 150 mM sucrose, 5 mM KH_2PO_4, 5 mM NaH_2PO_4, 0.1 mM Na_2EDTA, 10 mM NaSuccinate, and HCl to a final *p*H of 7.4.

In preliminary experiments, a linear relationship was obtained between CO_2 production and both the amounts of synaptosomes and the duration of incubation. CO_2 production was also dependent upon the amount of GABA added over a thousandfold range (0.01–10 mM). It was decided to use the following standard conditions to perform routine experiments: 0.05 ml of synaptosomes (at approximately 0.5 mg of protein), 20 μl of [1-^{14}C]GABA (10 μM so that only high affinity active uptake[7,8] is encountered), a 20-min equilibration period and a 1-h incubation at 30° C.

Incubation was carried out in 25-ml flasks fitted with a sidearm and a side vent.[4] After standard conditions were defined, the system was tested by determining the effect of 25 mM and 55 mM KCl, a recognized membrane depolarizer, and of 2 mM Ca^{++}, a recognized membrane stabilizer. Subsequently, separate sets of experiments were performed using various concentrations of the following neurotropic agents: ether (Fisher), halothane (Ayerst), enflurane (Ohio), chloroform (Fisher), thiopental (Abbott), pentobarbital (Abbott), paraldehyde (Mallinkrodt), ketamine (Park-Davis), phenytoin (Sigma), phenobarbital, ethanol, and morphine (Northwestern Memorial Hospital Pharmacy). Between 11 and 40 duplicate observations were made with each drug, the per cent inhibition was calculated and the results plotted as dose-response graphs.

Nonvolatile neurotropic agents were added directly to the medium. Gaseous neurotropic agents were flushed through the flasks via inlet and outlet hypodermic needles (#18-gauge) which were withdrawn after gas and temperature equilibration. The concentration of the gaseous anesthetic agent was determined in each experiment with gas chroma-

tography as previously described.[1] Gas mixture contained 50 per cent O_2 and the balance made up with N_2, H_2O vapor, and the anesthetic agent. [1-^{14}C]GABA (49.4 mCi/mmol; New England Nuclear) was added from the sidearm after the equilibration period to a final concentration of 10 μM. After incubation, 0.2 ml of 10 N H_2SO_4 was injected into the medium in the sealed vessel to stop the reaction and to distill the $^{14}CO_2$ into a 2 N NaOH trap in a suspended well. After another 30 min, the well was removed, cut off, and dropped into a scintillation bottle. The sample was counted as described previously.[1] Protein content of the original synaptosomal preparation was determined with phenol reagent.[9]

Least square regression lines relating per cent inhibition (Y) and drug concentration (X) (Y = bX + a) were calculated according to conventional methods.[10] With volatile anesthetics, each point represents a set of duplicate determinations; with nonvolatile drugs, each point represents the mean from several duplicate determinations and its standard error of the mean. The *P* values associated with regression coefficients (b) are the same as for correlation coefficients (r) and are given in the figure legends. The *P* values for the Y-intercepts (a) and the number of each set of determinations (N) are also given. Two-tailed *t* tests were used to obtain *P* values in tests of significance.

Results

Control Rate of GABA Disposal

Under the standard incubation conditions chosen for these experiments, the rate of GABA disposal is 273,000 ± 8,000 cpm/mg protein/hour (N = 226) which is equivalent to 3.56 nmol CO_2/mg synaptosomal protein/hour. Neglecting the saturation of other synaptosomal pools of GABA catabolites, which are probably small since CO_2 production is linear with time, these findings mean that 3.56 nmol GABA were metabolized per hour per mg synaptosomal protein.

Effects of K^+ and Ca^{++} on GABA Disposal

At 25 mM KCl, the inhibition of CO_2 liberation was 78.0 ± 1.2 per cent (N = 6) and, at 55 mM, 87.9 ± 0.5 per cent (N = 11), both significant at $P < 0.001$. Ca^{++} (2 mM), when added to the incubation medium, had very little effect on GABA disposal (4.0 ± 1.9 per cent stimulation at N = 35 and $P < 0.05$). It did not have any effect in drug studies reported below.

Inhibition of Synaptosomal GABA Disposal by Volatile Anesthetics

A dose-related inhibition of GABA disposal was observed for diethyl ether (fig. 1), halothane (fig. 2), and enflurane (fig. 3). In all cases, the Y-intercepts of the regression lines were not different from zero and the regression and correlation coefficients were statistically different from zero. The effect of chloroform on GABA disposal is dose-related but not linear (fig. 4).

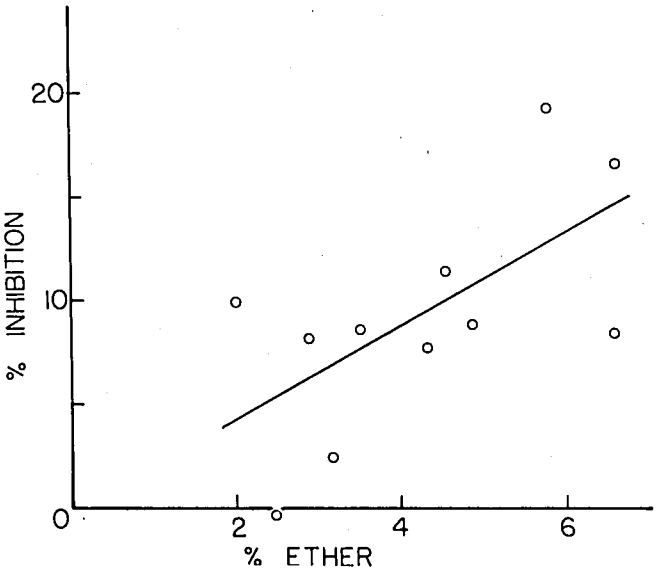

FIG. 1. Inhibition of synaptosomal GABA disposal by diethyl ether. The regression line Y = 2.27X − 0.32 has *P* values of 0.028 for regression and correlation coefficients, and 0.94 for the Y-intercept; r = 0.66 with N = 11.

Inhibition of Synaptosomal GABA Disposal by Barbiturates

The dose-related inhibition of GABA disposal by thiopental and phenobarbital (fig. 5) was described by regression lines with statistically significant regression and correlation coefficients and with Y-intercepts not significantly different from zero. Similar inhibition by pentobarbital (fig. 5) was best described by a regression line for concentrations greater than 0.3 mM. At lower concentrations, pentobarbital had no effect on GABA disposal.

Inhibition of Synaptosomal GABA Disposal by Other Neurotropic Agents

Inhibition of GABA disposal by paraldehyde, ethanol, and morphine (fig. 6) and ketamine (fig. 7) were all dose-related. The first three had significant regression and correlation coefficients and their Y-intercepts were not significantly different from the origin. Ketamine inhibition was described by a regression line where both the regression and correlation coefficients and the Y-intercept were significantly differently from zero.

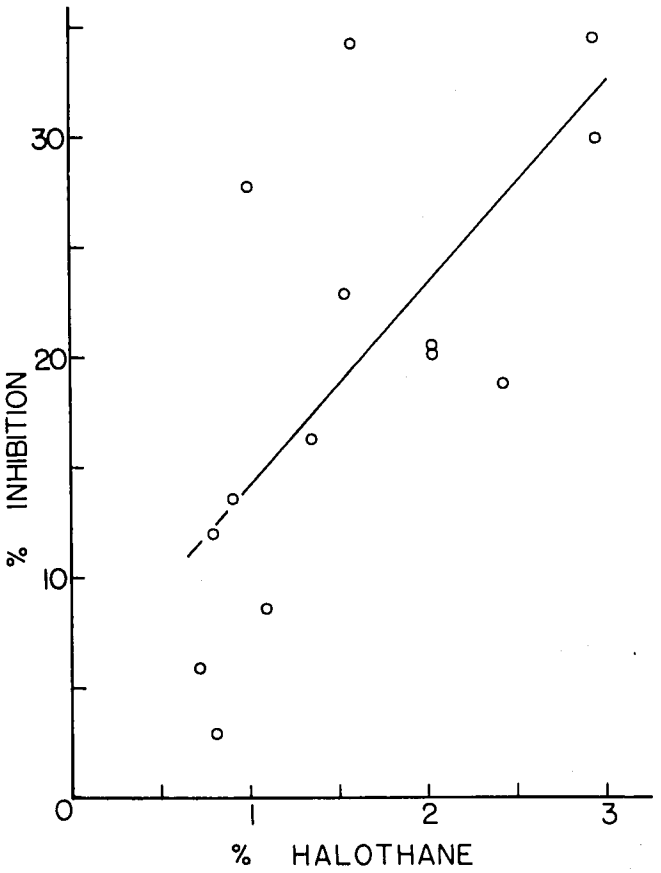

FIG. 2. Inhibition of synaptosomal GABA disposal by halothane. The regression line Y = 8.89X + 5.08 has *P* values of 0.006 for regression and correlation coefficients, and 0.94 for the Y-intercept; r = 0.69 with N = 14.

Phenytoin (diphenylhydantoin, Dilatin®) had no effect on GABA disposal (fig. 8) at less than 1 mM, but at higher concentrations (>1 mM), it had stimulatory effects.

Discussion

GABA Disposal by Synaptosomes

The preceding article[4] shows that halothane inhibits the metabolic breakdown of GABA in rat brain slices. The study presented here attempts to generalize that observation to other anesthetic agents using rat brain synaptosomes as the metabolic model. A variety of neurotropic agents were studied for the inhibition of synaptosomal GABA "disposal" which includes uptake, release, and degradation of GABA to CO_2. A 10 per cent inhibition of this CO_2 liberation reaction, or GABA disposal, is referred to as ID_{10}. Since uptake and release of GABA alter intrasynaptosomal GABA concentration, these two processes can affect the amounts of $^{14}CO_2$ produced from [1-^{14}C]GABA in addition to catabolism. A study of these latter processes by synaptosomes is still in progress.

To verify the responsiveness of this synaptosomal system, we altered K^+ and Ca^{++} concentrations. Potassium depolarization has been used extensively to simulate an excited state in neurochemical and neurophysiological research. We used two concentrations, 25 mM and 55 mM, to assess the K^+ effect. Since high K^+ concentration causes depolarization of the synaptosomal membrane, it also stimulates release of GABA. As a result, the intrasynaptosomal GABA concentration is reduced and a net inhibition of CO_2 liberation should be observed. This was indeed found and the degree of inhibition was more intense at the higher concentration of K^+. Calcium ion also is important for membrane function, but the addition of 2 mM Ca^{++} did not significantly alter the extent of CO_2 production from GABA in either control or drug studies.

Gaseous Anesthetic Agents

All of the volatile anesthetic agents which we have studied inhibit synaptosomal GABA disposal only at clinically relevant concentrations. If the rat MAC values for individual anesthetic agents[11] are used as an estimate of equipotency, then 1 MAC of ether, halo-

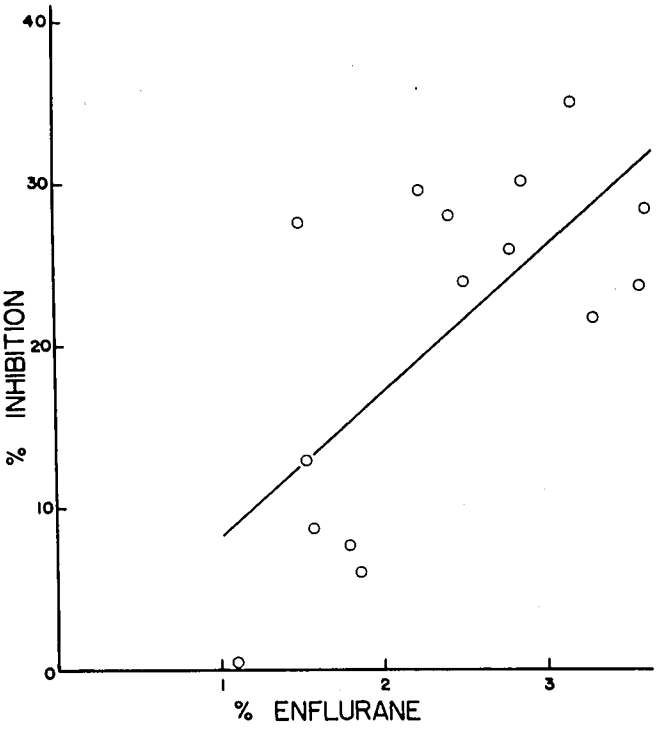

FIG. 3. Inhibition of synaptosomal GABA disposal by enflurane. The regression line Y = 8.98X − 0.71 has *P* values of 0.006 for regression and correlation coefficients, and 0.92 for the Y-intercept; r = 0.68 with N = 15.

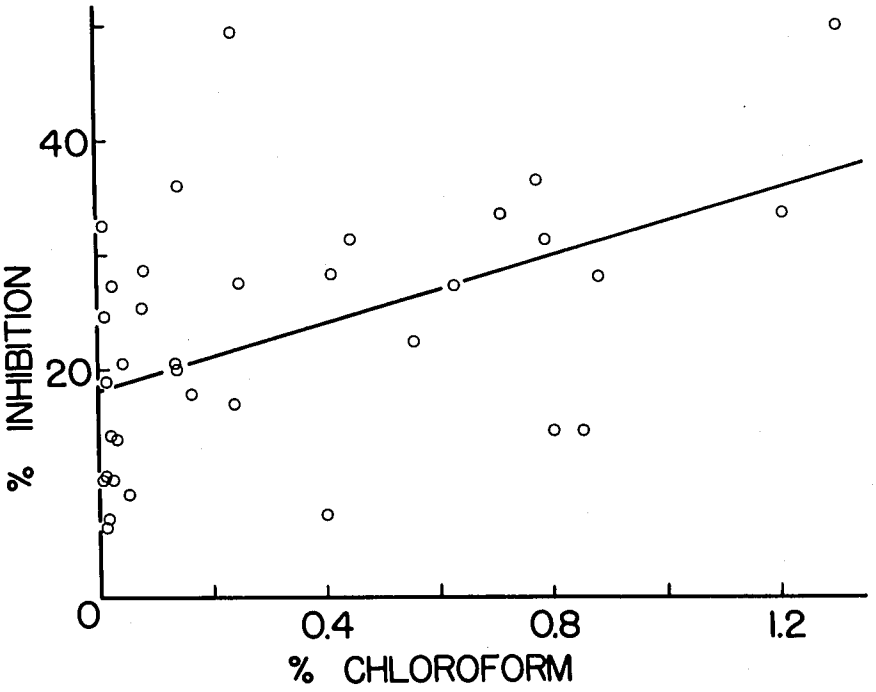

FIG. 4. Inhibition of synaptosomal GABA disposal by chloroform. The regression line Y = 14.6X + 18.2 has *P* values of <0.001 for regression and correlation coefficients and for Y-intercept; N = 35. Attempts to fit a second regression line to the data for low chloroform concentrations where the slope appears to be much steeper fails to reach statistical significance because of the scatter of the data at these low concentrations except if all data less than 0.3 per cent are included. These data generate a line Y = 65.7X + 15.0 which has *P* values of <0.001 for regression and correlation coefficients, and <0.02 for the Y-intercept: N = 22.

thane, enflurane, and chloroform (see below) each produces about 10 per cent inhibition (ID_{10}) of brain synaptosomal GABA disposal (table 1). The physiologic significance of this degree of inhibition has yet to be determined.

The curve which describes chloroform inhibition of synaptosomal GABA disposal may have two primary slopes, one very steep at low concentrations and the other more gradual at concentrations near the MAC value.[11] The ID_{10} value for chloroform is calculated from the slope of the regression line describing all the chloroform data. The chloroform effect in this test system suggests two different mechanisms. Only the one associated with the lesser slope is compatible with the action of other anesthetic agents (table 1). The nature of the steep slope mechanism is not known.

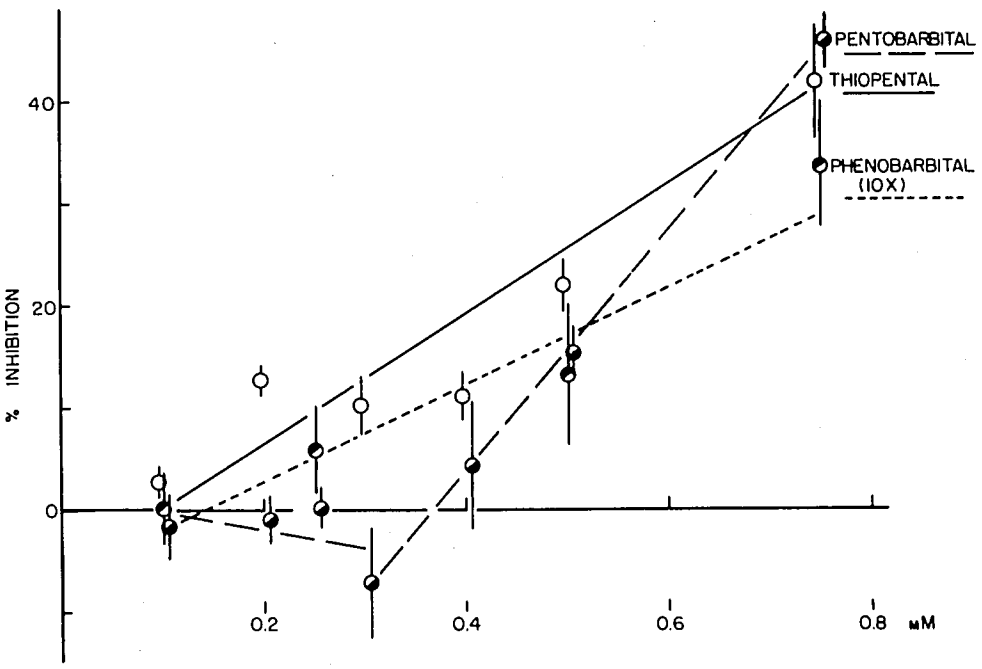

FIG. 5. Inhibition of synaptosomal GABA disposal by barbiturates. Inhibition by thiopental is represented by Y = 64.7X − 6.86 which has *P* values of <0.001 for regression and correlation coefficients, and 0.56 for the Y-intercept; r = 0.75 with N = 5 or 6 for each thiopental concentration. Inhibition by phenobarbital is represented by Y = 5.16X − 7.11 which has *P* values of <0.001 for regression and correlation coefficients, and 0.20 for the Y-intercept; r = 0.83 with N = 3 or 4 for each phenobarbital concentration. Inhibition by pentobarbital begins at >0.3 mM, and is represented by Y = 117.7X − 42.9 which has *P* values of <0.001 for regression and correlation coefficients and for the Y-intercept; r = 0.84 with N = 5 or 6 for each pentobarbital concentration. At concentrations up to 0.3 mM, there appears to be no significant effect.

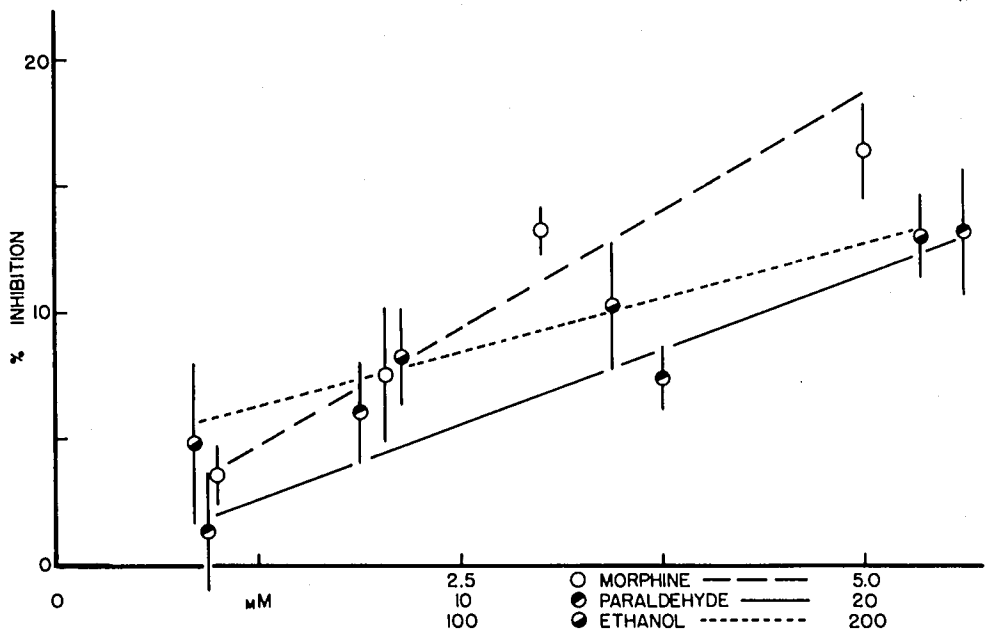

FIG. 6. Inhibition of synaptosomal GABA disposal by paraldehyde, ethanol, and morphine. The concentrations of these drugs are individually indicated. Inhibition by paraldehyde is represented by Y = 0.594X − 0.29 which has *P* values of <0.001 for regression and correlation coefficients, and 0.88 for the Y-intercept; r = 0.71 with N = 4 for each paraldehyde concentration. Inhibition by ethanol is represented by Y = 0.0429X + 4.19 which has *P* values of 0.017 for regression and correlation coefficients, and 0.040 for the Y-intercept with N = 4 or 5 for each ethanol concentration. Inhibition by morphine is represented by Y = 3.73X + 0.14 which has *P* values of <0.001 for regression and correlation coefficients, and 0.94 for the Y-intercept; r = 0.92 with N = 3 or 4 for each morphine concentration.

BARBITURATES

Brain content of thiopental during anesthesia varies with time and dose after intravenous injection,[12] and has been reported to be 0.24 μmol/g.[13] The concentration of thipoental required for 10 per cent inhibition of GABA disposal by synaptosomes in this study is 0.26 mM. This is within the range of brain concentrations reported to produce anesthesia and may infer a GABA mechanism.

Sleep induced by intravenous pentobarbital is accompanied by a brain content of 0.0071 μmol/g.[14] Levels in the brain tenfold higher accompany sleep induced by intraperitoneal pentobarbital.[15] In any case, these values are far below the ID_{10} values of 0.45 mM in the synaptosomal system and imply that the relation between the action of pentobarbital and GABA may be questionable. Recent electrophysiological evidence does suggest a positive association of GABA to pentobarbital action.[16]

Brain concentrations of 0.11–0.12 μmol/g phenobarbital in mice or rats, respectively,[15,17] occur with sleep, and are far lower than the ID_{10} value of 3.3 mM found in this study. Therefore, the action of phenobarbital probably is not mediated through a GABA mechanism. Of the three barbiturates studied, only thiopental showed a strong positive correlation between its pharmacologic potency and GABA disposal inhibition.

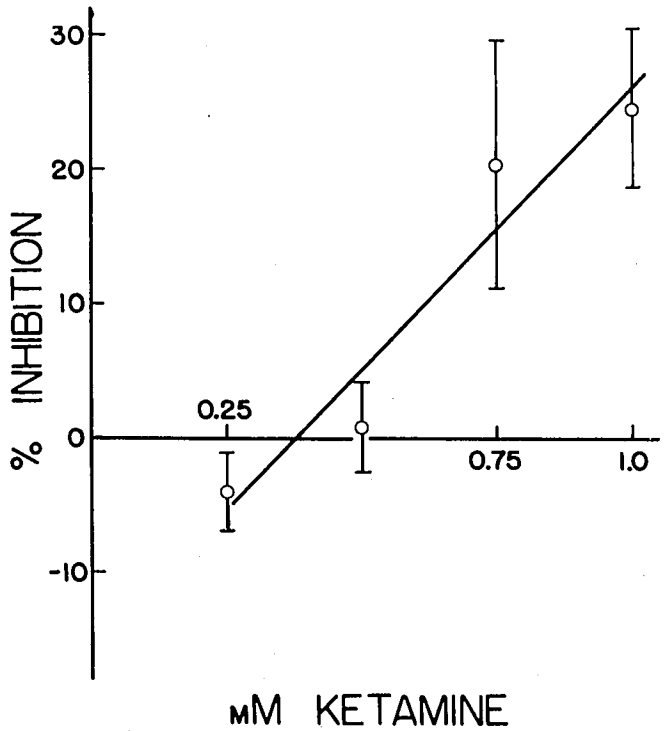

FIG. 7. Inhibition of synaptosomal GABA disposal by ketamine. The regression line Y = 42.1X − 15.9 has *P* values of <0.001 for regression and correlation coefficients, and 0.037 for the Y-intercept; r = 0.65 with N = 5 for each ketamine concentration.

OTHER NEURODEPRESSANTS

Paraldehyde is usually administered orally. It is rapidly absorbed and its concentration in the brain is approximately 60 per cent of that in blood.[18] LD_{50} in rats corresponds to a 4.2–5.3 mM (average 4.7 mM)

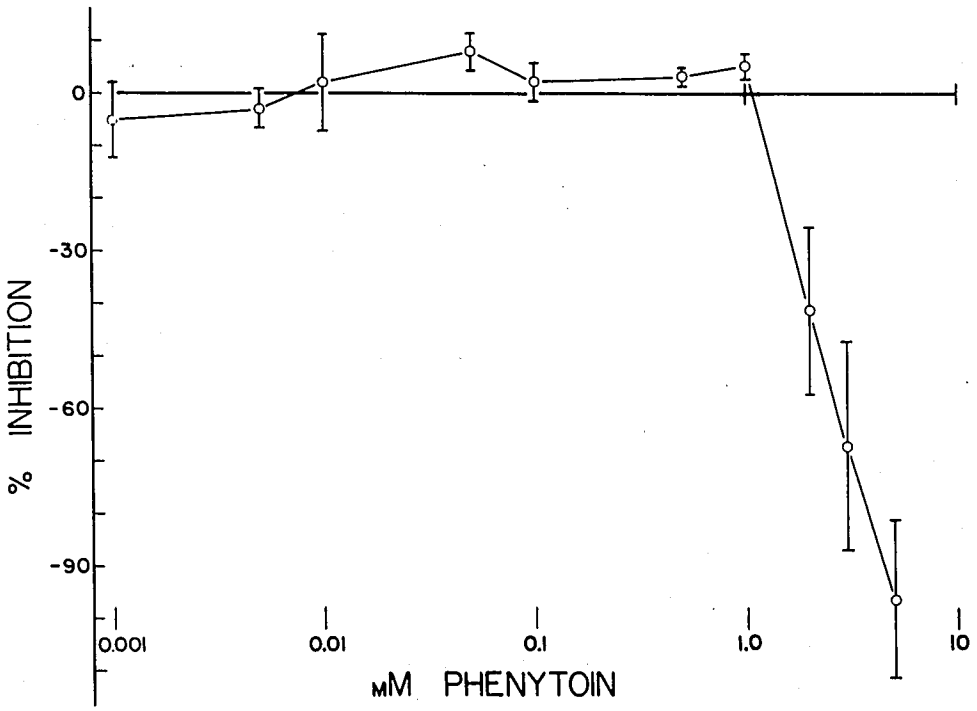

FIG. 8. Inhibition of synaptosomal GABA disposal by phenytoin. N = 6–10 for various phenytoin concentrations. There is no significant inhibition of GABA disposal at pharmacologic concentrations of phenytoin.

brain concentration of paraldehyde.[18] ED_{50} must be less than 4.7 mM. The concentration of paraldehyde required for 10 per cent inhibition of synaptosomal GABA disposal is 17.3 mM which is several times larger than the estimated ED_{50} value. Therefore, paraldehyde may or may not act through a GABA mechanism.

For alcohol intoxication, ED_{50} occurs at 125 mg/100 ml. At a blood/brain partition coefficient[19] of 1.17:1, the corresponding brain concentration of ethanol is 0.106 g/100 ml (0.13 per cent v/v; 28.2 mM). ID_{10} for ethanol required 13.5 mM in this study, and is 4.8 times larger than the estimated ED_{50} for alcoholic intoxication. Ethanol intoxication may or may not involve a GABA mechanism.

Morphine analgesia has been accompanied by brain levels of 0.15 μmol/g in rats.[20] The ID_{10} value in this study for morphine is 2.65 mM which is far higher than the cited analgesic level. This observation precludes GABA involvement in morphine analgesia.

Ketamine concentration in rat brain was 96–122 μg/g (0.40–0.51 μmol/g) during central depression.[21] The return of righting reflex occurred at approximately 30 μg/g (0.13 μmol/g) brain. The ID_{10} concentration from this study of 0.62 mM is 4.8 times higher than the concentration at return of righing reflexes, but close to the higher value reported for central depression. A GABA mechanism may be involved. A recent study of ketamine-anesthetized rats showed elevated GABA content in synaptosomes.[22]

Anticonvulsant concentration of phenytoin in rat brain[23,24] varies between 17–29 μg/g (63–107 nmol/g; 0.06–0.11 mM). Phenytoin concentration in this range (fig. 8) has no effect on synaptosomal GABA disposal. The intense stimulation of phenytoin at concentrations above 1 mM is dose-dependent. Since this concentration range is very high, it has no clinical relevance. The relationship between phenytoin and GABA disposal, if there is any, remains obscure and is probably not involved in its mechanism of action.

TABLE 1. Summary of Drug Effects on GABA Disposal:Comparison of 10 Per Cent Inhibition of Synaptosomal GABA Disposal (ID_{10}) to Pharmacologic Potency (ED_{50}) of Each Drug

Drug	ID_{10}	ED_{50} or MAC	$\frac{ID_{10}}{ED_{50}}$
Ether	4.5 per cent	3.2 per cent[11]	1.4
Halothane	0.55 per cent	1.0 per cent[11]	0.55
Enflurane	1.2 per cent	1.7 per cent[11]	0.71
Chloroform	0.68 per cent*	0.8 per cent[11]	0.85
Thiopental	0.26 mM	0.24 mM[13]	1.1
Pentobarbital	0.45 mM	0.07 mM[14]	6.4
Phenobarbital	3.3 mM	0.11 mM[15,17]	30
Paraldehyde	17 mM	4.7 mM[18]	3.6
Ketamine	0.62 mM	0.13 mM[21]	4.8
Ethanol	140 mM	28 mM[19]	5.0
Morphine	2.7 mM	0.15 mM[20]	18
Phenytoin	—	0.085 mM[23,24]	—

ID_{10} values are calculated from the regression lines. For chloroform, the ID_{10} is calculated from the slope. For pentobarbital, the ID_{10} is calculated for concentrations larger than 0.3 mM. MAC values are for rats except that of enflurane which is for humans.

Drug Effects in General

The comparison of ED_{50} with $1D_{10}$ (ID_{10}/ED_{50} in table 1) reveals four groups of drugs. Diethyl ether, halothane, and enflurane form the basis for equating ID_{10} to MAC. Thiopental also fits into this category. The observation that these four anesthetic agents inhibit GABA disposal supports the hypothesis[2,3,5,25] that GABA accumulation may be a contributing factor in the production of the anesthetic state. Phenobarbital and morphine required far higher concentrations to achieve 10 per cent inhibition of synaptosomal GABA disposal than their corresponding ED_{50} values, and phenytoin showed no inhibitory action. These neurotropic drugs obviously do not act via a GABA mechanism and they are not anesthetic agents. Pentobarbital, paraldehyde, ethanol, and ketamine require several times their ED_{50} concentrations for ID_{10}. These drugs have definite central nervous system depressant actions, but they are not pharmacologically grouped with the general anesthetic agents. A GABA mechanism may or may not be involved in their central nervous system action. Chloroform constitutes an anomaly. It definitely has a dual effect. The effect described by the lesser slope is congruent with the action of the classical general anesthetic agents. The effect described by the steep slope remains to be defined (see above). These data, as a whole, support a correlation between the inhibition of synaptosomal GABA disposal (ID_{10}) and anesthetic action (MAC or ED_{50}). Further delineation of this action of anesthetic agents is needed. In particular, alterations in uptake and release of GABA by the synaptosomes (thereby changing the intrasynaptosomal GABA concentration and its rate of degradation) should be dissociated from alterations in the metabolic or enzymatic breakdown of GABA. For the latter, thiopental appears to inhibit synaptosomal GABA-transaminase at pharmacological relevant concentrations.[25]

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