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SOME OBSERVATIONS ON THE MECHANISM OF BENZODIAZEPINE–BARBITURATE INTERACTIONS IN THE MOUSE

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1 The prolongation of pentobarbitone sleeping times by five benzodiazepines, administered by prior intraperitoneal injection, was measured in mice. The pentobarbitone was injected either intraperitoneally or intracerebroventricularly. For each benzodiazepine, the prolongation was dose-related and differences in potency between benzodiazepines were not marked.

2 The percentage prolongation of sleeping times produced by most of the benzodiazepines was greater when the pentobarbitone was given intracerebroventricularly and was explained by a preferential addition of CNS depressant effects associated with this route.

3 To test whether the action of intraperitoneally administered pentobarbitone had been influenced by a metabolic component, the effects of nitrazepam on drug metabolism, measured by changes in plasma phenazone levels in the mouse, were studied. Nitrazepam (32 mg/kg, i.p.) produced a 23% reduction in the rate of phenazone metabolism.

4 Nitrazepam was also shown to have produced a transient fall in body temperature. Calculations based on Q_{10} values suggested that this hypothermia accounted, at most, for half the metabolic change measured.

Introduction

The prolongation of barbiturate-induced anaesthesia (sleeping time) by benzodiazepines in laboratory animals has been reported. It has been observed in experiments using different species (Dobkin, 1961; del Pozo & Armas, 1973), different benzodiazepines (Frommel, Fleury, Schmidt-Ginzkey & Béguin, 1960; Hester, Rudzik & Kamdar, 1971) and either pentobarbitone (Jori, Prestini & Pugliatti, 1969), hexobarbitone (Noordhoek, 1968) or thiopentone (Alps, Harry & Southgate, 1973). It has also been reported in man (Tammisto, Elfving, Saikku & Tiitinen, 1967). The prolongation has been assessed in animals either by sleeping time measurements *per se* or by a comparison of the ED_{50} values of barbiturates given with or without benzodiazepine pretreatment (Zbinden, Bagdon, Keith, Phillips & Randall, 1961).

The mechanism of the benzodiazepine-barbiturate interaction has not been fully elucidated. It may involve the addition of central nervous system (CNS) depressant effects; it may involve a metabolic interaction; the rate of penetration of barbiturate into or the rate of its removal from the brain may be altered by benzodiazepine.

In view of the wide variety of experimental variables used in animal studies, and the largely unknown mechanisms of the benzodiazepine-barbiturate interaction, it seemed appropriate to study the effect of a number of clinically used benzodiazepines on pentobarbitone-induced sleeping times in mice under narrowly defined conditions.

The possibility of a metabolic component in the interaction suggested that it be compared both when the pentobarbitone was given intraperitoneally and, in order largely to eliminate hepatic metabolism of pentobarbitone, when administered by intracerebroventricular injection (Stevenson & Turnbull, 1974). To this end, a limited study was also undertaken of the effect of nitrazepam on phenazone metabolism, the latter being a recognized indicator of the activity of hepatic drug metabolizing enzymes (Vessell & Page, 1969) by which barbiturates are also metabolized. In view of the reported hypothermia caused by some benzodiazepines (Gey, 1973) and the possible effect of this hypothermia on drug metabolizing enzymes, changes in body temperature produced by nitrazepam both alone and in combination with phenazone were investigated.

Methods

Male albino mice, Charles River, CDI strain fed on diet 41B and water *ad libitum* were used throughout. The weight range was 16 to 24 g but for individual

experiments the range was not greater than 4 grams. The ambient temperature was maintained throughout at $21 \pm 1°C$. All experiments were started at 14 h 00 min except the phenazone metabolism studies which were started at 10 h 00 minutes. Animals were always divided randomly into experimental groups.

Pentobarbitone sleeping times by intraperitoneal injection

Sleeping time was measured as the time between loss of righting reflex and the moment animals regained this reflex. It was determined to the nearest 5 seconds. Each benzodiazepine 0.5, 2, 8 or 32 mg/kg, suspended in 1.5% sodium carboxymethylcellulose (SCMC), or SCMC was injected intraperitoneally 30 min before intraperitoneal pentobarbitone sodium 45 mg/kg in saline. Groups of 10 mice were used for each of these five treatments. Only one benzodiazepine was examined on any given experimental day.

Pentobarbitone sleeping times by intracerebroventricular (i.c.v.) injection

The method used was based on that of Haley & McCormick (1957); 10 µl volumes of pentobarbitone sodium in saline (0.9% w/v NaCl solution) were injected with a 25 µl Hamilton syringe fitted with a 27 gauge needle of 3.5 mm length and having a 17° bevel. The injections were made into the conscious mouse at a point 2 mm rostrally to the lambdoid suture and 1 mm laterally from the saggital suture on the left side. These co-ordinates were judged visually by reference to the positions of the eyes of the mouse and to an imaginary line drawn between the anterior base of the ears and the longitudinal mid-line. The validity of this technique for injection into the lateral ventricles was confirmed post-mortem in brain sections of 10 mice injected with 10% Indian ink in saline. Further validation of the technique was gained retrospectively by examination of the precision of sleeping times in mice subjected to given drug treatments (see Results section) and from the fact that only 7 out of a total of 106 mice given the selected fixed dose of pentobarbitone by intracereboventricular injection failed to lose their righting reflex.

To determine a suitable fixed dose of pentobarbitone for use with the benzodiazepines, the relationship between the dose of pentobarbitone and duration of anaesthesia was investigated. Pentobarbitone sodium expressed as dose/mouse, was injected in a constant volume of 10 µlitre. Benzodiazepines, in the same doses as used in the intraperitoneal experiments, or SCMC were injected intraperitoneally 30 min before pentobarbitone. Again, only one benzodiazepine was examined on any given experimental day.

Metabolic studies with phenazone

A comparison was made of phenazone plasma levels with respect to time in mice pre-treated with nitrazepam or vehicle. The analysis involved a modification of the gas chromatographic method of Prescott, Adjepon-Yamoah & Roberts (1973). Mice, in groups of three, were pretreated with nitrazepam (32 mg/kg i.p.) in SCMC or SCMC followed 30 min later by an injection of phenazone (50 mg/kg i.p.) in saline. At subsequent 6 min intervals from 12 to 54 min a different group of mice was anaesthetized with 2% halothane in a mixture of 33% O_2 and 67% N_2O and blood was collected from the retro-orbital plexus. The pooled blood obtained from each group of three mice was extracted in the following manner. To a 0.5 ml sample of pooled plasma was added 0.2 ml 5 mol/l NaOH and 1 ml chloroform containing 12.5 µg phenacetin as internal reference standard in a stoppered glass centrifuge tube which was shaken mechanically for 10 minutes. The aqueous phase and interface were removed by aspiration and added to a further 1 ml of chloroform. Shaking was repeated, the aqueous phase and interface aspirated as before and the residual chloroform added to the first chloroform extract. The combined extract was then carefully decanted into a tapered centrifuge tube which was placed in a water bath at 90°C for 10 min to remove the chloroform. The residue was dissolved in 20 µl chloroform and 3 µl aliquots were analysed for phenazone. A Perkin-Elmer gas chromatograph model F33 with a 2 m × 1.75 mm o.d. glass column packed with 80–100 mesh Gas-Chrome Q coated with 0.5% SE 30 and 0.5% carbowax 20 M was used. The temperature of the column was 220°C and that of both the injection port and flame ionization detector was 250°C. The carrier gas was N_2 used at a flow rate of 100 ml/minute.

The whole experiment was repeated on four separate days and the results combined for statistical analysis.

Measurement of body temperature

Oesophageal temperature was measured by the method of Brittain & Spencer (1964), with a thermocouple and Ellab electric thermometer type TE3. Groups of 16 mice were used for each four treatments. At zero time, two of these groups were injected with nitrazepam (32 mg/kg i.p.) in SCMC and the remaining two groups with SCMC. After 30 min, one of the groups of mice pretreated with nitrazepam and one of the groups pretreated with SCMC were injected with phenazone (50 mg/kg i.p.). The remaining two groups were injected with saline. Temperature measurements were made over a period of 3.5 hours.

Materials

The benzodiazepines tested were: chlordiazepoxide (Librium, Roche), diazepam (Valium, Roche), medazepam (Nobrium, Roche), nitrazepam (Mogadon, Roche) and oxazepam (Serenid, Wyeth). Pentobarbitone sodium (Macarthys) and phenazone (BDH Chemicals) solutions in sterile saline (Polyfusor, Boots) were prepared immediately before use. Sodium carboxymethylcellulose (Courlose Gum, P40 grade, British Celanese) solutions were sterilized by autoclaving.

Other materials were halothane (Fluothane, ICI), chloroform (Macfarlan Smith) and phanacetin (Evans Medical).

Results

Pentobarbitone sleeping times by intraperitoneal injection

The effect of benzodiazepine pretreatment on intraperitoneal pentobarbitone sleeping times is shown in Figure 1. The mean sleeping times in the presence of increasing doses of each benzodiazepine are shown relative to corresponding mean control values in which SCMC was given as pretreatment. None of the mean control sleeping times (range 17.4 to 23.1 min), which had been determined on different experimental days, was shown by t tests to be significantly different from each other ($P > 0.05$).

A dose-related increase in sleeping time was produced by each benzodiazepine. For both nitrazepam and chlordiazepoxide, a significant prolongation of pentobarbitone sleeping times was produced at all dose-levels ($P < 0.05$). Diazepam and oxazepam showed significant prolongation at doses of 2, 8 and 32 mg/kg whilst for medazepam prolongation was only significant at 8 and 32 mg/kg.

At the 32 mg/kg dose level, it was shown that only the difference between the prolongation with diazepam and oxazepam was significant.

Pentobarbitone sleeping times by intracerebroventricular injection

Figure 2 shows the relationship between dose of pentobarbitone sodium and duration of anaesthesia. Above 200 μg/mouse fatalities occurred; at 250 μg/mouse 4 out of 10 mice died due to respiratory arrest. Below 100 μg/mouse loss of righting reflex was not consistently attained. Thus 125 μg/mouse was chosen for the subsequent experiments in order to produce anaesthesia of a consistently short duration.

The effect of intraperitoneal benzodiazepine pretreatment on intracerebroventricular pentobarbitone sleeping times is shown in Figure 3. Again, none of the

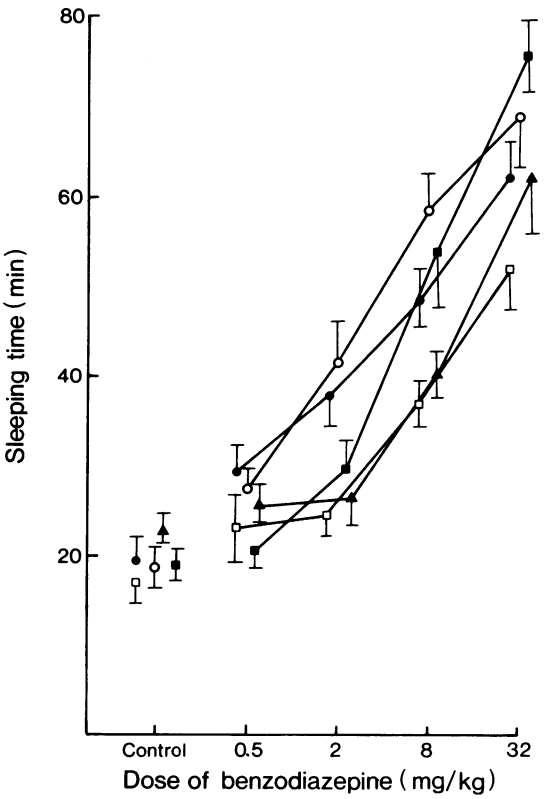

Figure 1 Effect of intraperitoneal benzodiazepine pretreatment on duration of intraperitoneal pentobarbitone sodium-induced sleeping times in mice. Control animals given sodium carboxymethylcellulose pretreatment. Each point represents the mean of ten observations. Vertical lines represent s.e. mean. (●) Chlordiazepoxide; (■) diazepam; (▲) medazepam; (○) nitrazepam and (□) oxazepam.

mean control sleeping times (range 2.4–3.1 min) were significantly different from each other ($P > 0.05$).

Nitrazepam produced a significant prolongation of sleeping time at all dose levels, diazepam at 2, 8 and 32 mg/kg, medazepam at 8 and 32 mg/kg, whilst for chlordiazepoxide and oxazepam the prolongation was only significant at 32 mg/kg.

At the highest dose level the effect produced by diazepam was significantly greater than that of the other benzodiazepines. It was noted at this dose of benzodiazepine that when the sleeping times were ranked numerically from the most potent, the same order was found as in the intraperitoneal sleeping time experiments: diazepam; nitrazepam; medazepam; chlordiazepoxide; oxazepam.

The dose-related prolongation of the sleeping time confirms and extends the results previously reported in the rat (Stevenson & Turnbull, 1974).

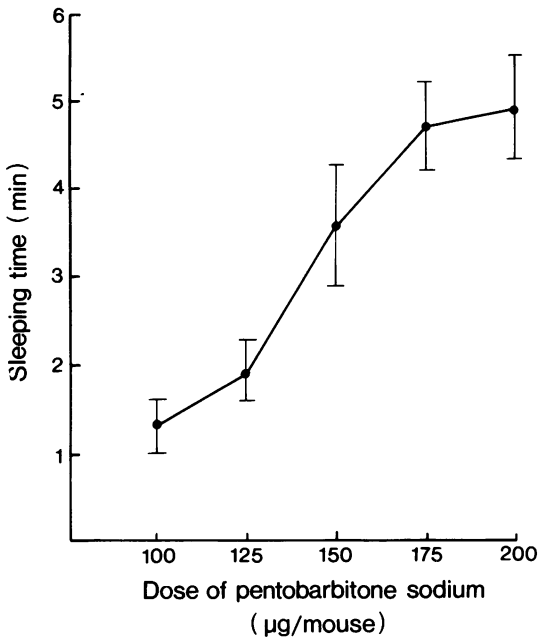

Figure 2 The relationship between dose of intracerebroventricularly administered pentobarbitone sodium and duration of anaesthesia. Each point represents the mean of at least 12 observations. Vertical lines represent s.e. mean.

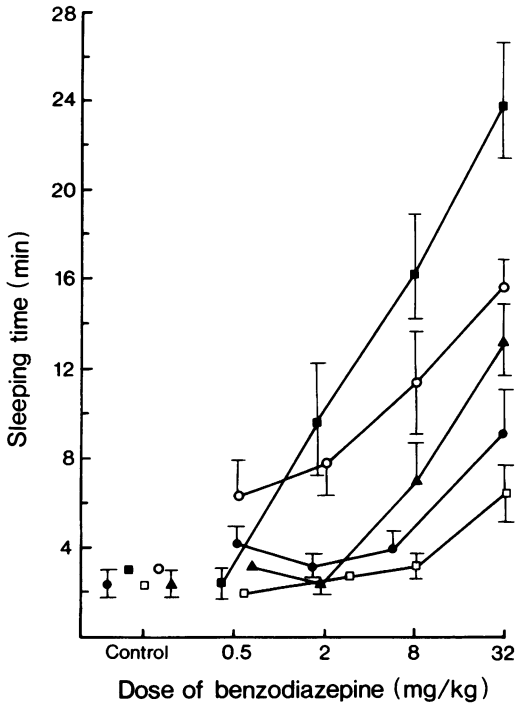

Figure 3 Effect of intraperitoneal benzodiazepine pretreatment on duration of intracerebroventricularly administered pentobarbitone sodium-induced sleeping times in mice. Control animals given sodium carboxymethylcellulose pretreatment. Each point represents the mean of ten observations. Vertical lines represent s.e. mean. (●) Chlordiazepoxide; (■) diazepam; (▲) medazepam; (○) nitrazepam and (□) oxazepam.

Metabolic studies with phenazone

Figure 4 shows the decline in plasma levels of phenazone in mice pretreated (i.p.) with either nitrazepam (32 mg/kg) or SCMC. Each point is the mean of 4 determinations, the 4 determinations being made of different days. The lines were fitted by regression analysis, the errors of the slopes being calculated from the value of $Sb/_b$ where Sb is the standard error of the regression coefficient (b) of the plot. These errors are 4% and 7.5% after nitrazepam and SCMC pretreatment, respectively. A test for parallelism (Diem & Lentner, 1970) indicated that the regression lines were not parallel.

The half life $T_{\frac{1}{2}}$ of phenazone in the presence of SCMC or nitrazepam was 22.6 and 29.2 min respectively, the corresponding elimination rate constants (K_e) being 3.07×10^{-2} and 2.37×10^{-2}/minute. The constants were calculated from $K_e = 0.693/T_{\frac{1}{2}}$ (Riggs, 1963). Thus nitrazepam caused a 23% decrease in the rate of phenazone metabolism.

Body temperature after intraperitoneal injections of nitrazepam and phenazone

The body temperature of both groups of mice injected with 32 mg/kg nitrazepam reached its lowest value at 50 min (Figure 5). These falls were short-lived. The maximum fall was measured in those mice receiving a combination of nitrazepam and phenazone and was only 1°C. A comparison of the temperature fall at 50 min in groups of mice receiving nitrazepam with the temperature of the corresponding control groups, showed that only in the case of combined nitrazepam and phenazone treatment, was the fall significant ($P < 0.05$). The body temperature of mice receiving phenazone without nitrazepam showed no change.

Discussion

While the fact of an interaction between barbiturates and benzodiazepines is indisputable, the mechanism is little understood. The present study confirms the ability of the benzodiazepines to prolong intraperitoneal pentobarbitone sleeping time in the mouse.

This interaction has been explained in terms of an addition of barbiturate-induced CNS depression to

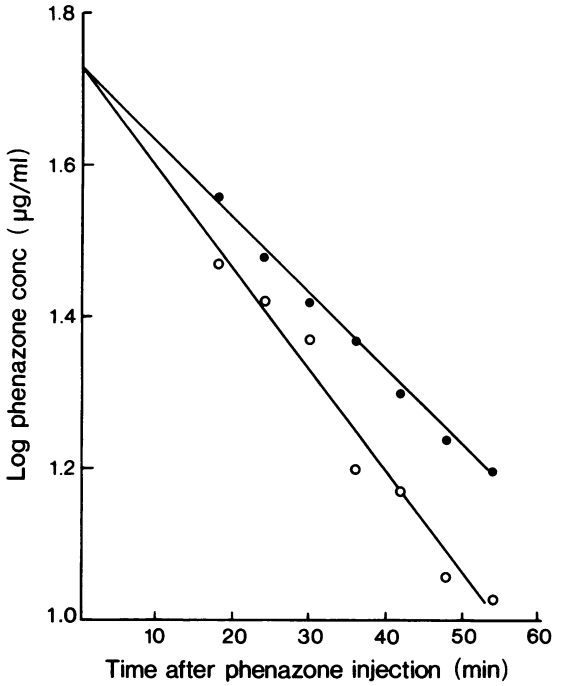

Figure 4 The effect of nitrazepam on phenazone plasma concentration. Nitrazepam 32 mg/kg (●) or sodium carboxymethylcellulose (○) injected intraperitoneally 30 min before phenazone (50 mg/kg, i.p.). Each point is the mean of 4 determinations; lines calculated by regression analysis.

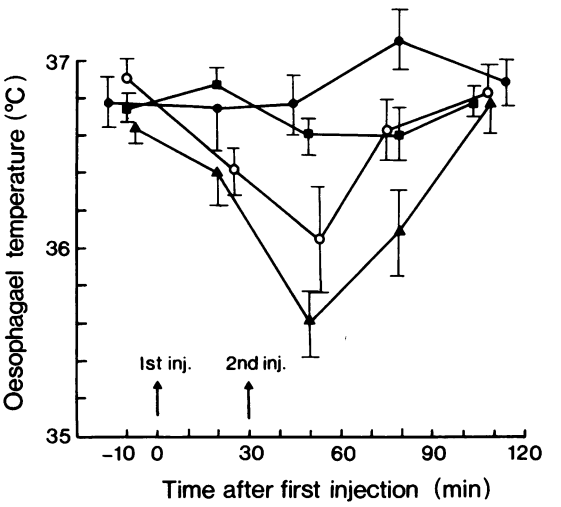

Figure 5 Differences in oesophageal temperature in mice injected with (●) sodium carboxymethylcellulose (SCMC) followed by saline; (■) SCMC followed by phenazone 50 mg/kg; (▲) nitrazepam, 32 mg/kg, followed by phenazone, 50 mg/kg, and (○) nitrazepam, 32 mg/kg, followed by saline. In each treatment the first injection was 30 min before the second. Each point represents the mean of 16 determinations. Vertical lines represent s.e. mean.

the more selective depression produced by benzodiazepines (Stevenson and Turnbull, 1974). It has also been suggested that the interaction may involve potentiation rather than simple addition (Dobkin, 1961; Heckmatt, Houston, Clow, Stephenson, Dodd, Lealman & Logan, 1976). The only study to measure potentiation however was that of Alps *et al.* (1973) who employed the isobologram plot (Loewe, 1957) in an investigation into the interaction of diazepam and lorazepam with thiopentone.

Pentobarbitone is inactivated almost exclusively by liver microsomal enzymes (Cooper & Brodie, 1957). Drugs, classically SKF 525A, which inhibit these enzymes can prolong barbiturate-induced anaesthesia without themselves causing anaesthesia (Cook, Macko & Fellows, 1954). In the present study, benzodiazepines, even at low doses, doubled the pentobarbitone sleeping time without themselves causing loss of righting reflex. Thus there could be a metabolic component contributing to this interaction. The intracerebroventricular administration of pentobarbitone after benzodiazepine was an attempt to eliminate any metabolic interactions because pentobarbitone is not metabolized in mouse brain (Sauberman, Gallagher & Hedley-Whyte, 1974). The benzodiazepine prolongation in mice was dose-related, diazepam being the most potent compound tested. If, in general, the mechanism of the benzodiazepine-barbiturate interaction includes a significant metabolic component then the prolongation of intraperitoneal pentobarbitone sleeping time should be greater than when pentobarbitone was given by the intracerebroventricular route. However, when those results are considered and compared with their respective controls it is clear that there is a greater percentage prolongation following intracerebroventricular injection than following intraperitoneal injection. These data are not inconsistent with a metabolic interaction between the benzodiazepines and intraperitoneal pentobarbitone. However, the intracerebroventricular results require a different explanation.

Injection of the chosen volume of pentobarbitone solution into cerebral ventricles by the technique of Haley & McCormick (1957) causes the majority of the injection to flood the ventricular system, although some solution is immediately lost to the periphery along the needle tract. Additional loss of solution into the saggital sinus may occur through arachnoid villi ruptured by the technique (Shaw, 1974). As pentobarbitone is very lipid soluble at physiological pH, diffusion will rapidly occur from ventricular system

into neuronal tissue along its concentration gradient. This was shown in rats (Stevenson & Turnbull, 1970) with ^{14}C-labelled pentobarbitone, the subsequent fall in drug concentration from neuronal tissue by diffusion and removal into blood producing an anaesthesia of short duration. A detailed study of [^{14}C]-pentobarbitone injected intravenously in mice has shown an uneven brain distribution of this compound (Saubermann *et al.*, 1974). This distribution changes not only absolutely but also relatively with time and is related to blood flow. It might be expected that brain distribution of pentobarbitone would be different when given by the two routes used in the present study. It is possible, therefore, that a central additive effect may exist between pentobarbitone given by intrecerebroventricular injection and benzodiazepine in a brain area which receives a lower pentobarbitone concentration by the intraperitoneal route. This could explain the greater effect of benzodiazepines on centrally administered pentobarbitone.

Barbiturates are metabolized by liver microsomal enzymes (Cooper & Brodie, 1957); phenazone half-lives are a good measure of the activity of these enzymes (Vessell & Page, 1969). Thus an investigation of the effect of nitrazepam, one of the most potent of the benzodiazepines tested in the present work, on the half-life of phenazone was carried out. The results show a 23% decrease in the rate of phenazone metabolism and a concomitant increase in half-life in mice treated with nitrazepam.

Prazepam a newly-developed long-acting benzodiazepine, has been shown to cause an increase in phenazone half-lives in man (Vesell, Passananti, Viau, Epps & Di Carlo, 1972). Although the present study has shown the effect of acute nitrazepam pretreatment on phenazone metabolism, it should be recognized that the effect of benzodiazepines may be different on chronic administration. There is evidence that after chronic administration some benzodiazepines may cause induction of drug metabolizing enzymes (Hoogland, Miya & Bousquet, 1966; Vorne & Idänpään-Heikkilä, 1975).

The present work showed a maximum fall in body temperature of 1°C after nitrazepam and the corresponding change in the rate of metabolism attributable to this hypothermia was calculated from Q_{10} values (Heintzen, 1958). For most drugs, Q_{10} values are between 2 and 2.5 (Prosser, 1973); assuming a value of 2.5, the change in the rate of metabolism is 9.6% for the 1°C fall in body temperature. This value represents the maximum metabolic change attributable to the hypothermia as the temperature fall was not sustained. These findings suggest that, at most, half the change in phenazone metabolism brought about by nitrazepam may be due to induced hypothermia and the remainder is probably due to metabolic interaction.

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