zwitterion by treatment with NH_4OH and recrystallized from EtOAc, mp 205–209°.

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Metabolism of Dimethoxymethylphenobarbital in Mice. Relationship between Brain Phenobarbital Levels and Anticonvulsant Activity

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The relationship between the anticonvulsant activity of DMMP and its metabolism to phenobarbital was investigated in mice. Brain, blood, and whole-body phenobarbital levels were determined at various times by gas–liquid chromatography. The brain levels of phenobarbital 3 hr after oral administration of the ED_{50} of DMMP, 42 mg/kg, were 24.6 ± 2.3 μg/g. An equivalent dose of sodium phenobarbital, 31 mg/kg, produced brain phenobarbital levels of 25.4 ± 2.9 μg/g. Blood and whole-body phenobarbital levels paralleled those in the brain. A metabolite of DMMP, *N*-methoxymethylphenobarbital (MMP), was found in the brain when either 120 or 200 mg/kg of DMMP was given alone or when SKF-525A was given prior to DMMP. This intermediate appears to have anticonvulsant activity. These data indicate that in the mouse the activity of DMMP, after a single dose, is a result of its metabolism to phenobarbital.

Several new alkoxymethyl derivatives of barbiturates have recently been synthesized and evaluated as possible anticonvulsants.[1,2] The most potent of these, 1,3-bis(dimethoxymethyl)phenobarbital (DMMP), has been reported to protect mice against induced seizures in a manner similar to phenobarbital but without the initial hypnotic side effects often noted with the parent compound. The oral ED_{50} of DMMP has been reported in the range of 14 mg/kg for protection against maximal electroshock seizures (MES). The pattern of toxicity in mice and rats showed a progressive CNS depression which finally resulted in death due to respiratory paralysis.

Several investigators have demonstrated that N-substituted anticonvulsants are dealkylated *in vivo* and that the dealkylated products are as active or more active as anticonvulsants than the original drug. Craig and Shideman[3] found in rats that mephobarbital (*N*-methylphenobarbital) is metabolized to phenobarbital and that the anticonvulsant potency of mephobarbital is approximately one-third that of the demethylated product. Swinyard, *et al.*,[4] found mephobarbital to be one-half as potent as phenobarbital in mice. Butler[5] studied the metabolism of 1,3-dimethylbarbital in dogs and showed that it was metabolized to barbital. It is possible that DMMP, like other N-substituted barbiturates, is metabolized to phenobarbital *in vivo* and that this metabolite is responsible for the anticonvulsant activity. This series of experiments was designed to determine the concentrations of phenobarbital in blood, brain, and whole body following a single oral dose of DMMP and sodium phenobarbital. The findings indicate that the anticonvulsant activity of DMMP in mice is the result of its metabolism to phenobarbital.

Methods

Peak Time of Anticonvulsant Activity of DMMP and Phenobarbital. National Institutes of Health general purpose albino mice weighing 20–30 g were used throughout the study. The mice were housed in community plastic cages (18 × 11.5 × 6.5 in.), kept in diurnal lighting, and given food and water *ad lib* until used. In general, the MES assay was conducted as described by Toman[6] and Swinyard.[7,8] The day after arrival in the laboratory, each animal was subjected to 50-mA alternating current for 0.2 sec using a stimulus generator (Wahlquist Instrument Co., Salt Lake City, Utah) *via* saline dampened corneal electrodes. This procedure is necessary to establish a base line for the assay.[9] Animals were then used within 3 days of the initial electrical stimulation.

Mice, which had been fasted overnight, were given either sodium phenobarbital or DMMP directly into the stomach *via* a blunt 18 gauge needle introduced transesophageally; the volume administered was 0.01 ml/g of body weight. DMMP, which is insoluble in water, was suspended in 5% acacia. Sodium phenobarbital was dissolved in the same vehicle. In all experiments, control animals received a weight determined volume of 5% acacia only.

The time of peak anticonvulsant effect was determined for orally administered DMMP and sodium phenobarbital by giving a dose which will protect approximately 50% of the mice at 3 hr. This dose was given to a series of mice which were then shocked at 0.5, 1, 2, 3, and 6 hr. A total of 30 mice were used for each time point.

The ED_{50} for both drugs was then determined at the time of peak anticonvulsant effect. Protection was considered positive when the tonic extensor phase of the convulsion

was abolished. At least 12 animals were used to determine the response at each dose tested. The data were analyzed by the method of Litchfield and Wilcoxon.[10] No animals were used for more than one determination.

Brain and Blood Levels of DMMP and Phenobarbital. Mice were fasted for 12 hr prior to administration of the oral ED_{50} dose of either 31 mg/kg of sodium phenobarbital or 42 mg/kg of DMMP. At various times several mice were sacrificed by decapitation, and whole blood was collected in beakers containing powdered heparin. The brains were immediately removed and homogenized in 5 ml of distilled water. These homogenates were frozen at −20° prior to analysis. Whole blood was analyzed immediately.

In experiments designed to determine the effects of SKF-525A on DMMP anticonvulsant activity, 35 mg/kg of the inhibitor in isotonic saline was administered intraperitoneally 5 min prior to oral administration of the anticonvulsants. Control mice were given SKF-525A and 5% acacia solution in order to determine whether SKF-525A had any anticonvulsant activity or interfered with the analysis of DMMP or phenobarbital. All animals were sacrificed at 2 hr by cervical dislocation, and the brains were homogenized and frozen prior to drug analysis.

Whole-Body Levels of Phenobarbital Following Orally Administered Phenobarbital or DMMP. After the ED_{50} of either DMMP or phenobarbital was administered orally, mice were sacrificed at various intervals by cervical dislocation. The peritoneal cavity was surgically exposed and the gastrointestinal tract removed from esophagus to rectum, sparing the omentum. This was done to eliminate any drug which might not have been absorbed. The remainder of the mouse was homogenized in a Waring Blendor in a volume of water equal to nine times the body weight. An aliquot of the homogenate was filtered through a gauze pad and centrifuged for 10 min at 3000 rpm. The supernatant was frozen prior to analysis for either phenobarbital or DMMP. Phenobarbital and DMMP were added to homogenates of brain and whole mouse and analyzed to assure that breakdown does not occur when stored.

Analysis of Phenobarbital and DMMP. The concentrations of phenobarbital and DMMP in whole blood, brain homogenates, and whole-body homogenates were determined by gas–liquid chromatography. Phenobarbital was extracted from biologic tissue using a modification of the method described by Kupferberg.[11]

Ethylene dichloride (12 ml) was combined with either 0.25 ml of whole blood, 2.5 ml of brain homogenate, or 5 ml of mouse homogenate, and 1 ml of 0.2 *M* sodium phosphate buffer, pH 6.5. Hexobarbital (10 μg) dissolved in methanol was added as an internal standard. The mixture was shaken for 10 min and then centrifuged. The aqueous phase was removed by aspiration, and 10 ml of the organic phase was transferred to a 13-ml ground glass stoppered centrifuge tube. The ethylene dichloride was evaporated under water vacuum on a Buchler Rotary Evapomix.

The residue was dissolved in 3 ml of absolute methanol, and then 2 ml of 0.25 *N* HCl and 6 ml of hexane were added. The mixture was shaken and centrigued and the upper organic phase removed by aspiration. A second 6 ml of hexane was then added. The mixture was again shaken and centrifuged, and the upper hexane layer was aspirated. The hexane washes removed almost all of the lipids, DMMP, and nonpolar compounds.

As much of the acidified methanol phase as possible was transferred to a 37-ml ground glass centrifuge tube containing 10 ml of ethylene dichloride. The mixture was shaken for 10 min and centrifuged. The upper aqueous phase was aspirated and the organic phase transferred to a 13-ml centrifuge tube. The ethylene dichloride was evaporated as before. The residue was dissolved in 50 μl of 0.2 *M* trimethylphenylammonium hydroxide in methanol and 1–3 μl was used for the glc analysis.

All drug determinations were performed using a Hewlett-Packard 7620A gas–liquid chromatograph equipped with dual flame ionization detectors and an electronic integrator. Glass columns (6 ft), 2.0 mm internal diameter, packed with 3% OV-17 (Pierce Chemical Co., Rockford, Ill.) were used.

The glc conditions for measuring phenobarbital were as follows: injector temperature, 300°; detector temperature, 300°; oven temperature, 185°; nitrogen as a carrier, 40 ml/min; hydrogen, 30 ml/min; air, 300 ml/min. The relationship between the ratio of the area of the phenobarbital peak to the area of the hexobarbital peak as a function of phenobarbital concentration was found to be linear between 1 and 15 μg of phenobarbital. The retention time for hexobarbital was 4.4 min and 5.2 min for phenobarbital.

DMMP was extracted from biologic material as follows. Toluene (15 mg) was combined with 1 ml of 0.2 *M* Na_3PO_4 and 2.5 ml of brain homogenate in a 37-ml ground glass centrifuge tube. 1,3-Bis(ethoxymethyl)phenobarbital (DEMP, 10 μg) in methanol was added as an internal standard. The mixture was shaken for 5 min and then centrifuged. Approximately 12 ml of the upper toluene phase was removed and dried under water vacuum. The residue was dissolved in 50 μl of absolute methanol and 1–3 μl of the solution was used for analysis. The glc conditions for DMMP were: injector temperature, 250°; detector temperature, 300°; oven temperature, 215°. Using this procedure, 1–10 μg of DMMP could be quantitatively determined. The retention time for DMMP and DEMP was 4.3 and 5.5 min, respectively.

Results

The ED_{50} for DMMP and sodium phenobarbital 3 hr after oral administration was determined. The calculated ED_{50} for oral sodium phenobarbital was 31 mg/kg (95% confidence limits = 23–42 mg/kg). This figure agrees with previously published data.[5] The ED_{50} for oral DMMP was 42 mg/kg (95% confidence limits = 32–55 mg/kg) at 3 hr. Oral 5% acacia was without anticonvulsant activity in control animals. Protection against MES seizures at various times using the 3-hr ED_{50} dose was then determined. After administration of DMMP (30 min), no protection was observed; 30% at 1 hr and 53% at 2 hr. The activity remained essentially constant for an additional 4 hr.

Sodium phenobarbital protected 42% of the mice against MES seizures within 30 min. Approximately 50% protection was seen at 1 hr and remained constant for an additional 5 hr.

The brain concentrations of phenobarbital following a single oral dose at the ED_{50} of either sodium phenobarbital or DMMP at various times are shown in Figure 1. Following 31 mg/kg of sodium phenobarbital, the concentration in brain quickly reached a maximum of 31.3 ± 3.0 μg/g by 30 min and was 25.8 ± 1.1 μg/g at 2 hr and 25.4 ± 2.9 μg/g at 3 hr. Conversely, only 7.8 ± 2.4 μg/g of phenobarbital was recovered at 30 min after a single oral dose of 42 mg/kg of DMMP. However, the phenobarbital concentration derived from DMMP had increased to 23.3 ± 4.0 μg/g at 2 hr and 24.6 ± 2.3 μg/g at 3 hr. The brain levels of phenobarbital

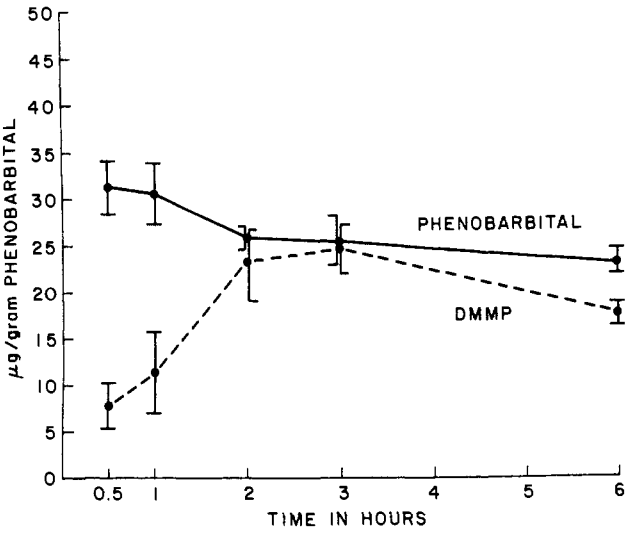

Figure 1. Brain phenobarbital concentrations following the oral administration of either 31 mg/kg of sodium phenobarbital or 42 mg/kg of DMMP at various time intervals. Each point represents the mean of five animals ± the standard error.

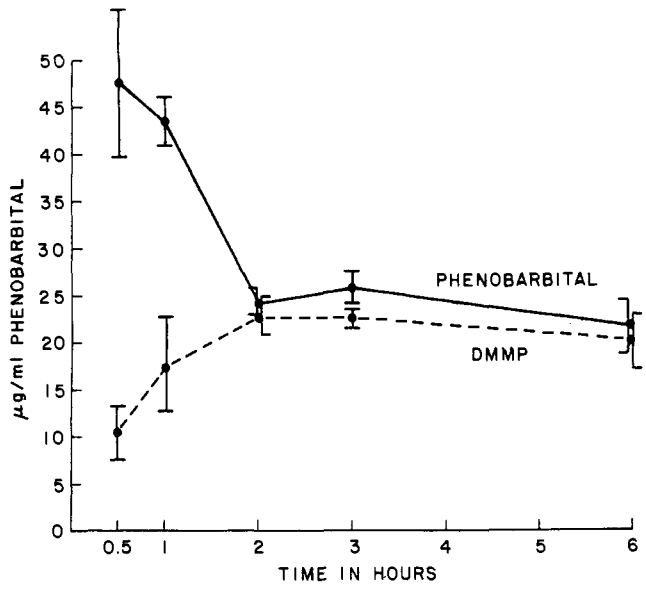

Figure 2. Blood phenobarbital concentrations following the oral administration of either 31 mg/kg of sodium phenobarbital or 42 mg/kg of DMMP at various time intervals. Each point represents the mean of five animals ± the standard error.

derived from the two drugs at 2 and 3 hr are not statistically different.

The concentrations of phenobarbital in blood and in whole mouse following oral administration of test drugs at their respective ED_{50}'s are shown in Figure 2. The phenobarbital in blood closely paralleled the brain concentrations. The mean values in whole mouse followed the same general pattern (Figure 3). The percentage of phenobarbital recovered from whole mouse over time was calculated for both experimental groups. Urine collected after the drugs were given until sacrifice was added to the homogenate so that any excreted phenobarbital was included in the determination. Thirty minutes after sodium phenobarbital administration, 89.9 ± 3.2% of the dose given was recovered as phenobarbital, and at 3 hr 84.1 ± 4.1% was found. Recovery of phenobarbital from animals receiving DMMP increased from 27.6 ± 6.8% at 30 min to 76.2 ± 6.2% at 3 hr. There was no statistical difference in the per cent of phenobarbital recovered from animals given DMMP or sodium phenobarbital at 3 or 6 hr. As evidenced by these results, it seemed likely that DMMP was being metabolized to phenobarbital by liver microsomes.

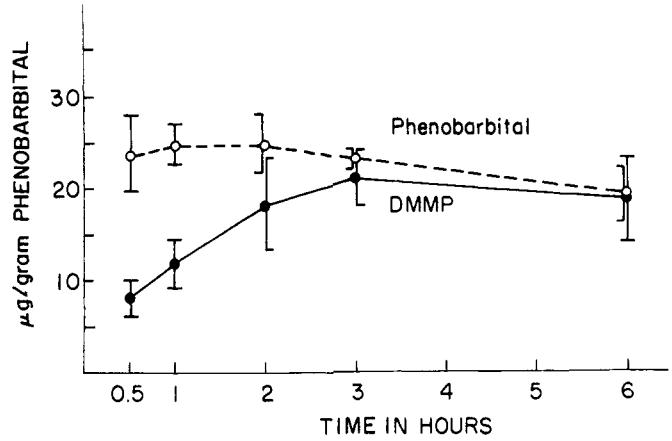

Figure 3. Whole mouse phenobarbital concentrations following the oral administration of either 31 mg/kg of sodium phenobarbital or 42 mg/kg of DMMP at various time intervals. Each point represents the mean of five animals ± the standard error.

In order to determine the anticonvulsant activity of DMMP alone, blockade of DMMP metabolism with SKF-525A was attempted. SKF-525A was found to be devoid of anticonvulsant activity and did not change the ED_{50} of phenobarbital. This finding confirms that of Swinyard, *et al.*,[4] who found that SKF-525A prolonged but did not change activity of phenobarbital. Mice given an ED_{50} dose of DMMP, 42 mg/kg, and SKF-525A were not protected against MES seizures. When given 120 and 200 mg/kg of DMMP along with SKF-525A, 58 and 83% of mice, respectively, were protected against seizures. Protection occurred even though the brain levels of phenobarbital were lower after treatment with SKF-525A than when the ED_{50} of DMMP was given alone. Brain concentrations of phenobarbital were 46.7 ± 3.5 and 48.3 ± 13.0 μg/g following 120 and 200 mg/kg of DMMP, whereas 9.3 ± 3.2 and 11.8 ± 2.4 μg/g were found when DMMP was given in combination with SKF-525A.

The animals were sacrificed 2 hr after the administration of DMMP rather than 3 hr. This time was chosen in order to maximize the effect of the metabolic inhibitor. Quantifiable amounts of DMMP were not found at any time interval, at any dose of DMMP, or following the administration of SKF-525A. The lower quantitative limit of the glc analysis of DMMP is 1 μg/g.

At high doses of DMMP or following metabolic blockade, a peak appeared on the chromatogram which had not been present before. Its retention time was 9.6 min. Mass spectral analysis of this peak indicated a molecular weight of 290. Authentic *N*-methoxymethyl-*N'*-methylphenobarbital gave identical fragmentation patterns and molecular ion. This compound is the product of *N*-methoxymethylphenobarbital following on column methylation with trimethylphenylammonium hydroxide. The peak was not seen when the brain extracts were injected without methylating agent or in controls. Unfortunately, it is not possible to quantitate this compound at the present time.

Discussion

Dimethoxymethylphenobarbital (DMMP) and sodium phenobarbital were given to mice at dosages which af-

forded the same protection against a standardized electrical convulsive stimulus. At a time when activity for both compounds was the same, at 2 and 3 hr, the brain levels of phenobarbital were the same. Whole blood and total-body phenobarbital levels paralleled those in the brain. The equivalency of the brain phenobarbital levels correlated well with the fact that the ratio of the ED_{50} for both compounds was the same as the ratio of their molecular weights (31/42 mg/kg = 254/320 = 0.93).

If some of the activity after a single oral dose was due to DMMP or a metabolite or metabolites other than phenobarbital at a time when the brain contained the same amount of phenobarbital, then a greater degree of activity should be seen following DMMP administration. An alternate view would be that if active metabolites are present, then less phenobarbital should be present in the brain following DMMP administration than after giving an equivalent amount of phenobarbital. The alternate viewpoint is seen when either 120 or 200 mg/kg of DMMP was given in combination with SKF-525A. The brain levels of phenobarbital were less than one-half those found when the ED_{50}, 42 mg/kg, of DMMP was given alone. Although the brain phenobarbital levels decreased, protection against MES seizures was observed, 58% at 120 mg/kg. *N*-Methoxymethylphenobarbital (MMP) was found in the brains of mice when this dose of DMMP was given along with the SKF-525A, indicating that the intermediate probably has activity. MMP was also found when large amounts of DMMP were given alone.

The importance of MMP as an anticonvulsant has yet to be fully determined. Its activity must be evaluated at a time when no phenobarbital has been produced through metabolism. The rate of conversion of DMMP to MMP as well as the metabolism of MMP to phenobarbital must also be established in man as well as in animals. Only then can the interrelationships of the various compounds to activity be determined. The MMP brain levels appear to increase with increasing amounts of administered DMMP, whereas DMMP levels remain less than 1 μg/g, the lower limit of the glc analysis. These facts indicate that the metabolism of DMMP to MMP is faster than the metabolism of MMP to phenobarbital. Butler[5] found that the metabolism of 1,3-dimethylbarbital to monomethylbarbital was several times faster than the latter's conversion to barbital.

In conclusion, these data indicate that in mouse the activity of DMMP following the oral administration of an ED_{50} dose of the drug is due to its metabolism to phenobarbital. However, other metabolite(s) may play an important role when the drug is given chronically.

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Anticonvulsants. 3. Phenobarbital and Mephobarbital Derivatives

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Several phenobarbital and mephobarbital derivatives were found to possess potent anticonvulsant activity and yet were either devoid of the marked hypnotic effects associated with the parent compounds or displayed very weak hypnotic activity. Particularly active compounds were the Mannich-type derivatives 2a,b and 3, the bis(hexamethylenetetramine salt) of 1,3-bis(bromomethyl)phenobarbital (**6**), and 1-methyl-3-methoxymethylphenobarbital (**10**).

We reported previously[1] that alkoxymethyl derivatives of phenobarbital possess marked anticonvulsant activity against both maximal electroshock and pentylenetetrazole-induced seizures and yet are devoid of the hypnotic effects associated with the parent compound. We also reported[2] that the bis(acyloxymethyl) derivatives of phenobarbital are active against maximal electroshock seizures as well as pentylenetetrazole, while the bis(halomethyl) derivatives of phenobarbital are effective anticonvulsants against pentylenetetrazole only. At the same time these compounds, unlike phenobarbital, are completely devoid of hypnotic activity. We became interested in finding out whether the salts and other derivatives of phenobarbital and mephobarbital described below would display anticonvulsant properties.

Chemistry. The compounds described herein were synthesized as indicated on Scheme I. The Mannich derivatives were isolated as alcoholates **2a,b** or as the hydrochloride salt **3**.

The bis(hexamethylenetetramine salt) of 1,3-bis(bromomethyl)phenobarbital (**6**) was obtained from the previously described 1,3-bis(methoxymethyl)phenobarbital[1] (**4**) *via* the intermediate 1,3-bis(bromomethyl)phenobarbital[2] (**5**).