1 and 2 hr. In contrast, the hypertonic solution increased the amount of drug transferred compared to control values. This increase, however, was statistically significant only in the case of riboflavin.

Measurements of intestinal tissue fluid uptake in the hypotonic and hypertonic buffers were made to test the previously observed relationship between gut fluid uptake and inhibition of drug transfer. Experimental details were reported (2). Essentially, 5-cm. segments of everted rat intestine were incubated for 20 min. in the buffer solution, and fluid uptake was determined as the difference between final and initial tissue weights. The results, in terms of milligrams fluid per gram initial wet weight plus or minus the standard deviation of the mean, were as follows: control buffer (isotonic solution), 52 ± 40 (11); hypotonic buffer, 97 ± 31 (11); and hypertonic buffer, −60 ± 28 (12). The numbers in parentheses represent the numbers of gut segments used. In the case of the hypertonic buffer, a net loss of fluid from the tissue was observed. The results of the gut fluid uptake studies clearly support the apparently general relationship that factors which increase tissue water uptake also inhibit passive intestinal transfer. This has been shown to be the case with glucose, xylose, or K^+ in the buffer medium and now with hypotonic solutions.

Kipnis and Parrish (8) suggested that both hypotonicity and elevated K^+ content of the buffer result in marked expansion of the intracellular volume of rat diaphragm tissue, with concomitant contraction of the extracellular space. The inulin space of the intact tissue, determined after 1 hr. of incubation, was reduced from about 16 ml./100 g. wet weight in control buffer to about 10 ml./100 g. in either high K^+ buffer or hypotonic buffer. Under both conditions, there was a significant increase in total tissue water compared to control levels. Similarly, Fox *et al.* (9), using kidney cortex, and Tews and Harper (10), using rat liver slices, reported a marked decrease in the extracellular space in high K^+ buffer. More recently, Jackson and Cassidy (11) found that glucose-induced fluid uptake by everted sacs of rat intestine was associated with considerable cellular swelling. It is plausible to consider that such swelling occurs at the expense of intercellular space in the mucosal barrier. If this is the case, then intercellular space may play an important role in the transfer of certain compounds across the everted rat intestine.

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Flash Methylation and GLC of Diphenylhydantoin and 5-(*p*-Hydroxyphenyl)-5-phenylhydantoin

Keyphrases ☐ Diphenylhydantoin, 5-(*p*-hydroxyphenyl)-5-phenylhydantoin—determination ☐ Plasma, urine—diphenylhydantoin and metabolites, determination ☐ GLC, flash methylation—analysis

Sir:

The development of rapid, sensitive, and simple assay methods for determining blood and urine levels of diphenylhydantoin and 5-(*p*-hydroxyphenyl)-5-phenylhydantoin (major metabolite of diphenylhydantoin) is most imperative for studying the interactions of diphenylhydantoin with other drugs and for more effective control of seizures in patients. We have been able to simplify the GLC analysis of diphenylhydantoin and 5-(*p*-hydroxyphenyl)-5-phenylhydantoin by converting them to nonpolar, volatile, methylated derivatives in the injection port of a gas chromatograph with trimethylanilinium hydroxide (1, 2).

The use of trimethylanilinium hydroxide as a methylating reagent offers the following advantages over *N,N* - dimethylformamide – bis(trimethylsilyl)acetamide, a reagent commonly used in preparing the trimethylsilyl derivatives of diphenylhydantoin: (*a*) trimethylanilinium hydroxide is a stable reagent that is easily prepared; (*b*) the methylation reaction with diphenylhydantoin and 5-(*p*-hydroxyphenyl)-5-phenylhydantoin occurs instantaneously in the injection port at temperatures of 300°, producing quantitative yields of methylated derivatives; and (*c*) it is not necessary to dry the purified plasma extracts at high temperatures or take time-consuming measures to exclude water from the extract or derivative-forming reagent *N,N*-dimethylformamide–bis(trimethylsilyl)acetamide.

Normal pooled plasma containing known amounts of drugs and internal standards and the patient's plasma, after adding the internal standards, were purified *via*

liquid–liquid extraction methods similar to those developed by Chang and Glazko (3) and Conard *et al.* (4). Two internal standards,[1] 5-(*p*-tolyl)-5-phenylhydantoin (S-1) and 5-(*m*-hydroxyphenyl)-5-phenylhydantoin (S-2), were added to the plasma prior to extraction, carried through the purification procedure, and methylated along with diphenylhydantoin and 5-(*p*-hydroxyphenyl)-5-phenylhydantoin in order to quantitate the drugs and to simplify the extraction procedure by not requiring the transfer of exact volumes during the extraction steps. The standard calibration curves for diphenylhydantoin and free 5-(*p*-hydroxyphenyl)-5-phenylhydantoin were obtained by preparing standard solutions of diphenylhydantoin and 5-(*p*-hydroxyphenyl)-5-phenylhydantoin at five concentrations (2.5, 5.0, 7.5, 10, and 20 mcg./ml. plasma) by dissolving the drugs in 0.1 *N* sodium hydroxide and adding a given volume of this solution to the normal pooled plasma. Prior to extraction, a given volume of internal standards S-1 and S-2 (10 mcg./ml. plasma) dissolved in 0.1 *N* sodium hydroxide was also added to the plasma. Six determinations of diphenylhydantoin and 5-(*p*-hydroxyphenyl)-5-phenylhydantoin were run at each concentration level.

The drugs and internal standards were added to 2.0 ml. of normal pooled plasma, adjusted to an acid pH (5–6), and extracted first with cyclohexane and then with isobutyl alcohol–chloroform (1:4). The latter organic layer was then extracted with 1.0 *N* sodium hydroxide and separated; the alkaline aqueous layer was acidified and extracted with ethyl acetate. The ethyl acetate was removed under nitrogen in a constant-temperature bath at 75°. To the residue was added 40 μl. of 0.1 *M* trimethylanilinium hydroxide solution (1), and 1–2 μl. was removed and injected on the gas chromatograph. To determine total 5-(*p*-hydroxyphenyl)-5-phenylhydantoin, the plasma must be hydrolyzed with 3.0 ml. of hydrochloric acid at 100° for 1 hr. (3) to cleave the conjugated sugar form of 5-(*p*-hydroxyphenyl)-5-phenylhydantoin, which occurs in plasma and urine primarily as the glucuronide. The hydrolyzed plasma, after neutralization with a saturated aqueous solution of sodium hydroxide, may then be treated similarly to the nonhydrolyzed plasma by liquid–liquid extraction, methylation, and injection on the gas chromatograph.

Gas chromatographic injections were made on a Varian 2100 dual-channel instrument equipped with four hydrogen-flame detectors. The 1.83-m. (6-ft.) long U-shaped glass columns (2 mm. i.d.) were packed with 3% OV-17 on Gas Chrom Q, 100–120 mesh. Operating temperatures were: injector port, 300°; column, 200°; and detector, 300°. Flow rates (milliliters per minute) were: nitrogen, 65; air, 300; and hydrogen, 50. Under these conditions, the retention times (minutes) for the methylated products were: diphenylhydantoin, 7.25; S-1, 10.5; S-2, 16.5; and 5-(*p*-hydroxyphenyl)-5-phenylhydantoin, 21.25.

The standard calibration curves were derived by measuring the peak heights and determining the mean peak height ratio of the six determinations at each concentration level [diphenylhydantoin/S-1 and 5-(*p*-hydroxyphenyl)-5-phenylhydantoin/S-2]. The mean peak height ratio of diphenylhydantoin/S-1 was plotted *versus* the weight ratio of diphenylhydantoin/S-1 for the diphenylhydantoin standard calibration curve; the mean peak height ratio of 5-(*p*-hydroxyphenyl)-5-phenylhydantoin/S-2 for free 5-(*p*-hydroxyphenyl)-5-phenylhydantoin (nonhydrolyzed) and total 5-(*p*-hydroxyphenyl)-5-phenylhydantoin (hydrolyzed) was plotted *versus* the weight ratio of 5-(*p*-hydroxyphenyl)-5-phenylhydantoin/S-2. The least-squares method was employed to obtain the best fit for the five concentration levels. The concentration levels were linear over the range studied. Therefore, plasma levels can be determined for diphenylhydantoin, free 5-(*p*-hydroxyphenyl)-5-phenylhydantoin, and total 5-(*p*-hydroxyphenyl)-5-phenylhydantoin by analyzing hydrolyzed and nonhydrolyzed samples.

To determine the concentration levels of unknowns, the peak height ratio is obtained from the chromatogram and the weight ratio is derived from the calibration curve. The micrograms per milliliter of drug, for example, of 5-(*p*-hydroxyphenyl)-5-phenylhydantoin may be calculated as follows:

$$\text{weight ratio} = \frac{\text{5-(p-hydroxyphenyl)-5-phenylhydantoin (mcg./ml. plasma)}}{\text{S-2 (mcg./ml. plasma)}} \qquad \text{(Eq. 1)}$$

$$\text{5-(p-hydroxyphenyl)-5-phenylhydantoin (mcg./ml.)} = [\text{S-2 (mcg./ml. plasma)}]\,(\text{weight ratio from graph}) \qquad \text{(Eq. 2)}$$

Methylation of diphenylhydantoin with a similar reagent, tetramethylammonium hydroxide, was reported by MacGee (5) to form the 1,3-dimethyl-5,5-diphenylhydantoin product. Reaction of diphenylhydantoin with trimethylanilinium hydroxide, considered by Brochmann-Hanssen and Olawuyi Oke (1) to be a better methylating reagent than tetramethylammonium hydroxide, would be expected to form the same 1,3-dimethyl-5,5-diphenylhydantoin derivative. In methylation studies involving phenolic opium alkaloids, Brochmann-Hanssen and Olawuyi Oke (1) reported that morphine, codamine, laudanine, and reticuline gave complete methylation of the phenolic hydroxyl groups with trimethylanilinium hydroxide but not with tetramethylammonium hydroxide. Therefore, it would appear that the flash heater methylation of 5-(*p*-hydroxyphenyl)-5-phenylhydantoin at 300° in the injection port of a gas chromatograph with trimethylanilinium hydroxide would methylate the phenolic hydroxyl group as well as the 1,3 nitrogens of the hydantoin ring, forming 1,3-dimethyl-5(*p*-methoxyphenyl)-5-phenylhydantoin.

Further studies of patients taking only diphenylhydantoin and diphenylhydantoin in combination with other drugs are currently in progress in these laboratories, and the results will be reported at a later date.

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Micelle Formation and Its Significance in Interpretation of NMR Spectra of Phenothiazine Derivatives

Keyphrases ☐ Phenothiazine derivatives—micelle formation ☐ Micelle formation, phenothiazine derivatives—effect on NMR spectra ☐ NMR spectroscopy—micelle formation effect

Sir:

On occasion, we have observed some unusual behavior when evaluating NMR spectra of the salts of phenothiazine derivatives. The spectra of the compounds run in D_2O consisted of a series of broad peaks with little or no resolution and fine structure, which made it impossible to determine J constants. The broadening effect suggested the possibility of a ferromagnetic impurity in the compounds. Accordingly, the phenothiazines were purified as described by Craig *et al.* (1). The spectra were rerun, but the broadening effect previously observed persisted. This effect is illustrated in Fig. 1, using chlorpromazine hydrochloride as the model compound. At this point, the possibility of micelle formation was considered. Micelle formation was reported previously for phenothiazine derivatives (2).

In the previous study, it was noted that a compound in the micellar state showed broad resolution bands; however, when the temperature was raised the micelle was disrupted and the NMR bands became sharper and more distinguishable (3). We obtained similar results when chlorpromazine HCl was run in D_2O at 95°. Figure 1 shows this behavior. The spectrum at the elevated temperature showed increased resolution and sharp absorption peaks, which permitted the determination of J constants. Evidently, the strong association in the micellar state completely masked the NMR spectrum of the compound. A series of phenothiazine derivatives, including promazine HCl, prochlorperazine HCl, trifluoperazine HCl, trifluoromeprazine HCl, and vespazine HCl, were run in the same manner, and all exhibited similar behavior. These studies suggest that the broadening effect observed in the NMR spectra of the compounds in D_2O may be attributable to the formation of micelles, rather than to the presence of ferromagnetic impurities. We feel that this observation should be of general interest to other investigators and should save considerable time and effort when NMR studies of a similar or related nature are involved.

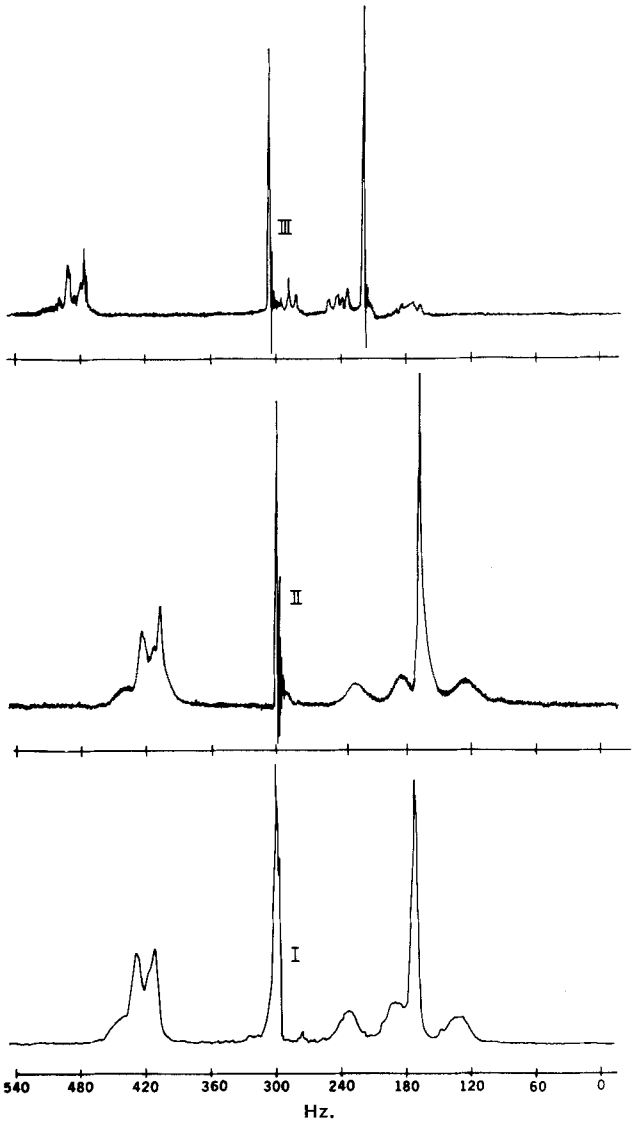

Figure 1—*NMR spectra for 10% solutions of chlorpromazine HCl in D_2O. Key: I, solution containing added amount of known ferromagnetic impurity; II, solution prepared with purified chemical; and III, solution from II run at 95°.*

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