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# GAS CHROMATOGRAPHIC ANALYSIS OF CHLORPROMAZINE AND ITS METABOLITES FORMED BY HEPATIC MICROSOMES—I

## INFLUENCE OF MAGNESIUM

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**Abstract**—The metabolism of chlorpromazine has been studied using rat and rabbit liver microsomal preparations and the Curry extraction and gas chromatographic technique for the compounds involved, namely chlorpromazine, chlorpromazine *N*-oxide, monodemethyl chlorpromazine, didemethyl chlorpromazine, chlorpromazine sulfoxide and monodemethyl chlorpromazine sulfoxide. In both liver systems, chlorpromazine *N*-oxide and monodemethyl chlorpromazine were found to be the major metabolites. The former accounted for 38–62 per cent of the chlorpromazine disappearing under various experimental conditions, while the latter amounted to 28–77 per cent in the same experiments. The 92–104 per cent of the CPZ disappearing could be accounted for by the appearance of the metabolites measured. The addition of $Mg^{2+}$ to the microsomal drug oxidase experimental suspensions caused an increase in the disappearance of chlorpromazine in both the rat and rabbit. In the rat, $Mg^{2+}$ caused little or no increase in chlorpromazine *N*-oxide appearance but increased the formation of monodemethyl chlorpromazine by a factor of 2 to 3. In the rabbit the opposite was true.

DURING the last decade a number of studies have been carried out attempting to elucidate the metabolism of chlorpromazine (CPZ)* in mammals. More than 20 metabolites have already been identified.[1] Given the possible reactions of demethylation, sulfoxidation, *N*-oxidation and hydroxylation, Green and Forrest[2] have calculated from the structure of CPZ that 168 metabolites could occur. The main intracellular site of most of this metabolism has been found to be the endoplasmic reticulum in the form of the microsomal fraction of a mammalian liver homogenate.[3] Until recently all microsomal fractions metabolizing CPZ were found to contain cytochrome P-450, a portion of the microsomal mixed function oxidase enzyme system.[4] In 1969, however, Ziegler *et al.*[5] reported on a mammalian liver enzyme preparation which contained flavoprotein but little or no cytochrome P-450. This preparation metabolized CPZ to CPZNO. Formaldehyde was not formed during this reaction, indicating an absence of demethylation of both CPZ and the *N*-oxide. A short note in 1967 by Beckett and Hewick[6] indicated that CPZNO was the 1 major metabolite produced by male rat liver microsomal preparations when CPZ was the

* Abbreviations used: CPZ = chlorpromazine; MDMCPZ = monodemethyl chlorpromazine; DDMCPZ = didemethyl chlorpromazine; CPZSO = chlorpromazine sulfoxide; MDMCPZSO = monodemethyl chlorpromazine sulfoxide; DDMCPZSO = didemethyl chlorpromazine sulfoxide; CPZNO = chlorpromazine *N*-oxide; NADPH = reduced nicotinamide adenine dinucleotide phosphate; HCHO = formaldehyde.

substrate in the presence of $Mg^{2+}$. The pH of the drug metabolism experiment was not mentioned.

This study reports on the results of experiments comparing disappearance *in vitro* of CPZ (when added to mammalian liver microsomal preparations) with the appearance of its metabolic products. Quantitation of this type has not been reported previously, as far as the authors can ascertain. The Curry extraction technique and gas chromatographic method for phenothiazine determination[7] were adapted to suit microsomal studies and were found to be feasible procedures to quantitate the changes in substrate concentration with the appearance of metabolites. Aliquots of the reaction mixture were taken at various time intervals, extracted and injected into the gas chromatograph. Values for CPZ and all five metabolites could be calculated after each injection. 92–104 per cent of the CPZ disappearing could be accounted for as metabolites. Using either rat or rabbit liver microsomes and CPZ as substrate, the two main metabolites that appeared were CPZNO and MDMCPZ. CPZSO was the third most prevalent metabolite. In addition, the effect of $Mg^{2+}$ on microsomal CPZ metabolism[8] has been confirmed and this cation effect further examined in the two species of hepatic microsomes employed.

## MATERIALS AND METHODS

CPZ, MDMCPZ, DDMCPZ, CPZSO and MDMCPZSO were gifts from Smith, Kline & French Laboratories, Philadelphia, Pa. CPZNO was supplied by Dr. Albert Manian and Dr. Daniel Efron of the Psychopharmacology Service Center, National Institute of Mental Health, Bethesda, Md. NADPH (Type II) was obtained from Sigma Chemical Co., St. Louis, Mo. All other chemicals were reagent grade.

Well-fed, white, female, New Zealand rabbits, weighing approximately 2 kg, were sacrificed by injecting air into the marginal ear vein. The liver was immediately perfused through the inferior vena cava with ice-cold isotonic saline, removed and minced. All the following steps were performed at 0–4°. The minced liver was homogenized in a Potter–Elvehjem homogenizer with a teflon pestle, in twice its weight of 0·25 M sucrose adjusted to pH 7·4 with several drops of 1 M tris. The homogenate was centrifuged at 10,000 *g* for 20 min. All *g* figures recorded were maximal. The supernatant was then centrifuged for 1 hr at 100,000 *g*. Following the removal of the soluble fraction by aspiration, the microsomal pellet was separated from the heavier opalescent glycogen sediment and washed three times with the homogenizing medium, each time at 100,000 *g* for 1 hr. The pellet was carefully separated from any remaining glycogen after centrifugation and finally was reconstituted to about 20 mg protein/ml with homogenizing medium. It was then quick-frozen in dry ice–acetone, stored at −20° and used within 5 days of preparation without any loss of activity. Male, Sprague–Dawley descendant rats (250–300 g) were obtained locally and maintained on standard Purina rat chow. They were decapitated and the livers perfused as above. Microsomes were prepared in the same manner as for rabbits and used within 48 hr. Protein was determined by the biuret method.[9]

The metabolism of CPZ by rabbit microsomes was followed by gas chromatographing an extract of a portion of a 10-ml reaction mixture containing 0·3 m-moles glycine buffer, pH 8·2, 6·0 μmoles neutralized semicarbazide, 6·6 μmoles NADPH, 1·5 μmoles CPZ, 12 mg microsomal protein, and 150 μmoles $MgCl_2$ when added. In the rat experiments, 8 mg of microsomal protein was present in the incubation mixture. The

final pH of the reaction mixture after 20 min incubation without $Mg^{2+}$ was 7·7, while that with $Mg^{2+}$ was 7·6. Incubations were carried out at 37° in open flasks. At 0, 5, 10 and 20 min 1·5-ml aliquots were taken for gas chromatography. Additional 0·5-ml aliquots were taken at the same time intervals and each added to 0·3 ml 7% perchloric acid. After centrifugation at 2000 *g* for 10 min the supernatant was assayed for formaldehyde by the method of Nash.[10] The substrate level in the reaction mixture, to which intact microsomes were added, was so adjusted that about 50 per cent of the CPZ introduced was metabolized in 20 min. In preliminary experiments for rabbit and rat drug oxidation CPZNO was also tested as a substrate with the same reaction mixture. There was no detectable transformation of the *N*-oxide to either CPZ or MDMCPZ, nor was any formaldehyde formed. It is still possible, however that CPZNO might be metabolized if a solubilized microsomal drug oxidase system were utilized instead of one containing intact liver microsomes.

The aliquot for gas chromatography was extracted according to Curry[7] into a final volume of 100 $\mu$l. Five $\mu$l of this extract were injected into a 182·9 cm × 2 mm i.d. glass column packed with 3% OV-25 on chromosorb W-HP 80/100 mesh (Applied Science Laboratories, State College, Pa.). Measurements were carried out with a Varian Aerograph 2100 gas chromatograph, utilizing a flame ionization detector and the following conditions: injector and detector temperatures were 280 and 290°, respectively, nitrogen carrier gas flow at 40 ml/min, and the column oven was initially set at 233° and programmed for a 2·5°/min temperature rise immediately after sample injection. Standard amounts of CPZ and the expected metabolites were injected to determine retention times, standard curves and to calculate the experimental drug values. Areas under the curves were measured with a Series 200 Disc Integrator.

For the pH optima experiments, the reaction mixture was that described above for the metabolism experiments, except that only the pH of the buffers was varied. The concentration of tris or glycine in the incubation mixture was always 0·03 M.

In the metabolite recovery experiments, various amounts of the expected metabolites were added to the incubation mixture (lacking microsomes) described above. The microsomes were first boiled for 10 min, homogenized in an all-glass Duall homogenizer and then added to the incubation mixture. The extraction and gas chromatography then proceeded as described above.

In the reaction mixture used for determining CPZ metabolism and containing intact rat liver microsomes, the 8 mg of microsomal protein added was found to contain 9·04 $\mu$g $Mg^{2+}$. This was made up to 10 ml in the reaction mixture which contained 150 $\mu$moles (or 3645 $\mu$g) of added $Mg^{2+}$, or about 400 times as much as is found endogenously in the microsomes. Rabbit microsomes were found to contain 0·69 $\mu$g $Mg^{2+}$/mg protein. $Mg^{2+}$ was determined using a Model 303 Atomic Absorption Spectrophotometer (Perkin–Elmer Corp., Norwalk, Conn.). The procedure followed was the standard method furnished with the instrument by Perkin–Elmer. The washed microsomal preparations were sonified for 1 min by means of a Branson Sonifier set to the highest energy level and then aspirated directly into the flame of the instrument. Appropriate $Mg^{2+}$ standards were treated in the same manner.

## RESULTS

A typical chromatogram of CPZ and its metabolites from an extract of a 20-min incubation mixture is shown in Fig. 1. Retention times of the various metabolites did

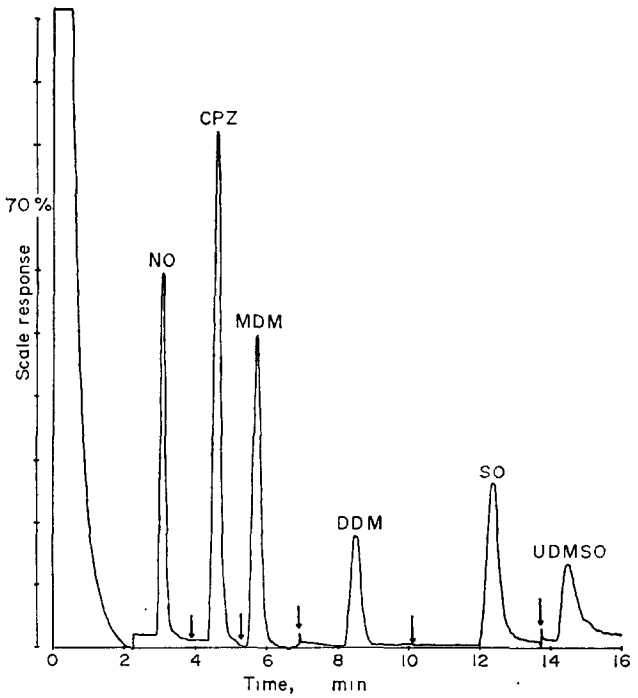


FIG. 1. Gas chromatographic trace of chlorpromazine and metabolites from an aliquot taken after a 20 min incubation with rabbit liver microsomes in the presence of $Mg^{2+}$. Conditions as described in the Methods section. Arrows indicate attenuation changes. Abbreviations are explained in footnote on first page of text.

not vary more than 5 sec from those of the standard compounds. DDMCPZSO would have a longer retention time than MDMCPZSO if it were present.

Since compounds are separated by their polarity on an OV-25 column, the more polar compounds are found to have the longer retention times. However, this is not the case with CPZNO since it undergoes a Cope elimination reaction, as described by Craig *et al.*[11] when subjected to gas chromatography. The product formed is 10-allyl-2-chlorophenothiazine, a relatively non-polar compound with a retention time less than that of CPZ. A pure sample of CPZNO was chromatographed and only 5 per cent of the amount injected was reduced to CPZ, while 95 per cent could be recovered as the allyl compound.

The recoveries of added metabolites from a reaction mixture containing boiled microsomes are shown in Table 1. All values reported in the metabolic experiments presented in Tables 2 and 3 have been corrected for these recoveries. We have recently been able to increase the *N*-oxide extraction recovery to 55–60 percent by increasing the isoamyl alcohol content to 2·5% in heptane in the first part of the extraction procedure.[7] Metabolic experiments were also done using this extraction solvent as well as the original one with 1·5% isoamyl alcohol on two aliquots of the same reaction mixture. The results were in substantial agreement with those reported in Tables 2 and 3 and with each other. Further attempts to increase this recovery, using many other solvents, have not been successful. Control extractions and chromatography of

the incubation mixture with liver microsomes but without CPZ did not yield ghost peaks that would interfere with the evaluation of the experimental peaks (see Discussion).

TABLE 1. RECOVERY OF CHLORPROMAZINE AND ITS METABOLITES

| Compound* | Recovery† |
|---|---|
| CPZNO | 32·6 ± 6·0 (16)‡ |
| CPZ | 84·3 ± 10·1 (17) |
| MDMCPZ | 73·2 ± 9·7 (13) |
| DDMCPZ | 70·9 ± 12·4 (9) |
| CPZSO | 96·4 ± 8·4 (14) |
| MDMCPZSO | 89·4 ± 6·3 (13) |

* Known amounts of each compound were added to a reaction mixture and extracted and chromatographed as described in the Methods section. The microsomes were boiled for 10 min before addition to the reaction mixture.

† Per cent recovered ± Standard Deviation.

‡ Number of experiments.

The results of the metabolic experiments using rabbit liver microsomes are presented in Table 2. The amounts of CPZ metabolized and CPZNO produced are fairly linear with respect to time in the absence or presence of $Mg^{2+}$. However, the demethylation reaction (appearance of MDMCPZ) slows down between 10 and 20 min and there is little or no $Mg^{2+}$ stimulation during this period. The three remaining CPZ metabolites recorded are produced in small amounts. Control incubations were performed to determine conversions of CPZ in the absence of NADPH. Sulfoxidation was the only transformation noted and CPZSO was found to the extent of 0·3 nmoles/mg protein/10 min in the absence of $Mg^{2+}$ and 0·6 nmoles/mg protein/10 min with added $Mg^{2+}$. The CPZSO values in Table 2 have been corrected for such sulfoxidation after which no definite $Mg^{2+}$ effect could be noted. Large variations in individual CPZSO values are obvious as seen in the extensive standard deviations.

In general, there is good correlation between the amount of CPZ metabolized and the sum of the amount of products formed (see Discussion), considering the number of steps involved in each determination. After incubating with rabbit microsomes for 10 min the amounts of CPZNO and MDMCPZ produced in the system without $Mg^{2+}$ (7·9 and 14·6 nmoles/mg protein) amount to 41·5 and 77 per cent of the 19·0 nmoles CPZ metabolized/mg protein. In this system stimulated with $Mg^{2+}$, the disappearance of CPZ (40·3 nmoles/mg protein) is about double that of the unstimulated loss; the CPZNO level then amounts to 51 per cent of the metabolized CPZ and the MDMCPZ falls to 46 per cent.

Also to be noted in Table 2 is a large discrepancy between the amount of MDMCPZ formed and the amount of formaldehyde measured after 10 and 20 min in the presence

TABLE 2. METABOLISM OF CHLORPROMAZINE (CPZ) BY RABBIT LIVER MICROSOMES*

| Metabolite | After 5 min | | | After 10 min | | | After 20 min | | |
|---|---|---|---|---|---|---|---|---|---|
| | Without Mg | With Mg | P Value | Without Mg | With Mg | P Value | Without Mg | With Mg | P Value |
| CPZ (loss) | 9·6 ± 2·7 (4) | 22·2 ± 2·9 (5) | < 0·001 | 19·0 ± 9·8 (8) | 40·3 ± 6·2 (8) | < 0·001 | 37·4 ± 11·4 (8) | 71·3 ± 7·9 (9) | < 0·001 |
| CPZNO | 2·8 ± 0·9 (5) | 6·8 ± 2·2 (5) | < 0·005 | 7·9 ± 3·2 (8) | 20·5 ± 5·7 (7) | < 0·001 | 12·6 ± 3·2 (7) | 39·0 ± 8·7 (8) | < 0·001 |
| MDMCPZ | 7·6 ± 1·8 (5) | 10·6 ± 0·9 (5) | < 0·010 | 14·6 ± 3·7 (8) | 18·6 ± 3·1 (8) | < 0·05 | 21·9 ± 1·5 (8) | 22·6 ± 3·6 (9) | N.S. |
| HCHO | 8·2 ± 2·1 (5) | 13·6 ± 2·9 (5) | < 0·025 | 16·4 ± 3·9 (9) | 26·4 ± 3·2 (9) | < 0·001 | 29·0 ± 5·3 (9) | 42·6 ± 5·2 (9) | < 0·001 |
| DDMCPZ | — | — | — | 0·4 ± 0·2 (8) | 0·6 ± 0·3 (8) | N.S. | 1·1 ± 0·4 (8) | 1·4 ± 0·5 (9) | N.S. |
| CPZSO | — | — | — | 1·2 ± 0·8 (8) | 1·7 ± 1·4 (8) | N.S. | 2·1 ± 1·6 (8) | 2·3 ± 2·0 (9) | N.S. |
| MDMCPZSO | — | — | — | 0·3 ± 0·1 (2) | 0·6 ± 0·1 (2) | N.S. | 0·6 ± 0·4 (2) | 1·1 ± 0·0 (2) | N.S. |

* The reaction mixture is as described in the Methods section. All values represent nanomoles of product formed per milligram protein ± standard deviation. Numbers in parentheses indicate the number of experiments. Abbreviations are explained in footnote on first page of text. N.S. = Not significant. — Indicates experiment not done.

of $Mg^{2+}$. A statistically significant ($P < 0.005$) difference between the observed values (18·6 vs. 26·4 and 22·6 vs. 42·6 nmoles/mg protein) is present. Without $Mg^{2+}$ there is no such difference at 10 min (14·6 vs. 16·4 nmoles/mg protein), but at 20 min there is again a significant difference between the 21·9 nmoles of MDMCPZ and 29·0 nmoles of formaldehyde which appear. The latter, however, is not nearly as great as the doubling which occurs when $Mg^{2+}$ is added. When testing the rat microsomal preparations (Table 3) good agreement is found between MDMCPZ and formaldehyde production except for a significant excess of the latter after 10 min and then only in the absence of $Mg^{2+}$.

The pattern of CPZ metabolism by rat liver microsomes is shown in Table 3. After 10 min, CPZNO and MDMCPZ appearance account for 61·5 and 28·4 per cent respectively, of the substrate disappearing in the absence of $Mg^{2+}$ and 38 and 45·5 per cent respectively, in the presence of added $Mg^{2+}$. In addition to the $Mg^{2+}$ effect on *N*-oxide formation and CPZ demethylation, $Mg^{2+}$ also stimulated the disappearance of CPZ by a factor of 2, from 31·3 to 60·2 nmoles/mg protein after 10 min. This same factor of 2 is also evident after 5 min incubation. Not only is the rate of CPZ disappearance greater in the rat, but the rate of CPZNO production (19·2 nmoles/mg protein/10 min) is more than double that noted with rabbit microsomes. However, CPZNO formation is only slightly stimulated by $Mg^{2+}$, in contrast to the figures for rabbit microsomes. The rate of MDMCPZ appearance (8·9 nmoles/mg protein/10 min) is lower in the rat, but is increased 2 to 3-fold by $Mg^{2+}$ addition. CPZSO is produced by the rat liver microsomal fraction at a rate of 3·7 nmoles/mg protein/10 min in the absence of $Mg^{2+}$ and 6·2 nmoles in its presence. In this case, $Mg^{2+}$ effects a significant 67 per cent rise in CPZSO production. These figures have already been corrected for a non-enzymatic sulfoxidation of 0·3 and 0·6 nmoles/mg protein/10 min without and with $Mg^{2+}$. All other metabolites appear in minor amounts. In Table 2 it can be seen that the rate of CPZSO formation for the rabbit is only 1/3 as high as in the rat and does not increase significantly when $Mg^{2+}$ is present.

All experiments which have been described were carried out with liver microsomes which had been flash-frozen immediately after preparation. These microsomes were used only once after being thawed out. Similar experiments were performed with freshly prepared microsomes and the resulting values for CPZ disappearance and metabolite formation were the same as those obtained with the frozen preparations.

The pH optimum for the *N*-oxidation reaction is 8·5 using 0·03 M glycine buffer and 7·6 in the presence of 0·03 M tris buffer as shown in Fig. 2. Figure 3 demonstrates the pH optimum for the monodemethylation of CPZ in 0·03 M tris or glycine buffer. It is broad and extends from pH 7·2 to 8·2. Even though it was obvious that the pH 8·2 glycine buffer was inadequate (the final pH of the reaction mixture was 7·6–7·7), this buffer was used. It was not known to inhibit drug oxidation and we felt that other buffers like tris might. In addition, we wished to work at a pH close to the physiological value even though we knew the optimum pH for CPZNO formation in glycine buffer was 8·5. In the case of MDMCPZ, the rate of formation of this metabolite was at its maximum, because of the broad pH optimum found.

## DISCUSSION

It is apparent from Tables 2 and 3 that there is fairly good agreement between the amount of CPZ metabolized and the amount of metabolites appearing, either in the

TABLE 3. METABOLISM OF CHLORPROMAZINE (CPZ) BY RAT LIVER MICROSOMES*

| Metabolite | After 5 min | | | After 10 min | | | After 20 min | | |
|---|---|---|---|---|---|---|---|---|---|
| | Without Mg | With Mg | P Value | Without Mg | With Mg | P Value | Without Mg | With Mg | P Value |
| CPZ (loss) | 16·5 ± 2·4 (4) | 33·0 ± 2·8 (5) | < 0·001 | 31·3 ± 2·8 (5) | 60·2 ± 7·2 (5) | < 0·001 | 69·0 ± 3·5 (5) | 94·1 ± 10·6 (5) | < 0·005 |
| CPZNO | 9·4 ± 0·7 (4) | 11·4 ± 0·7 (5) | < 0·005 | 19·2 ± 1·2 (5) | 23·0 ± 2·0 (5) | < 0·01 | 43·8 ± 2·4 (5) | 38·3 ± 3·5 (5) | < 0·025 |
| MDMCPZ | 4·4 ± 0·3 (4) | 15·3 ± 2·1 (5) | < 0·001 | 8·9 ± 1·5 (5) | 27·3 ± 4·1 (5) | < 0·001 | 20·4 ± 3·1 (5) | 40·6 ± 7·9 (5) | < 0·001 |
| HCHO | 5·7 ± 2·3 (4) | 14·6 ± 2·4 (5) | < 0·001 | 13·2 ± 2·4 (5) | 28·5 ± 4·5 (5) | < 0·001 | 24·0 ± 2·2 (5) | 44·3 ± 6·9 (5) | < 0·001 |
| DDMCPZ | — | — | — | 0·1 ± 0·1 (5) | 0·2 ± 0·0 (5) | < 0·005 | 0·2 ± 0·0 (5) | 0·4 ± 0·1 (5) | < 0·025 |
| CPZSO | — | — | — | 3·7 ± 0·8 (5) | 6·2 ± 0·7 (5) | < 0·001 | 5·7 ± 0·8 (5) | 9·5 ± 1·3 (5) | < 0·001 |
| MDMCPZSO | — | — | — | 0·2 ± 0·1 (5) | 0·4 ± 0·2 (5) | N.S. | 0·2 ± 0·2 (5) | 0·8 ± 0·2 (5) | < 0·001 |

* See footnote to Table 2.

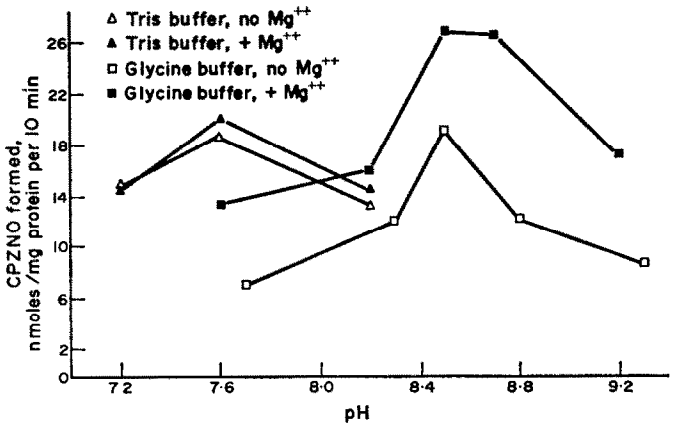


FIG. 2. pH Optima for the *N*-oxidation of chlorpromazine in Tris or glycine buffer. Incubation and extraction conditions are as described in the Methods section.

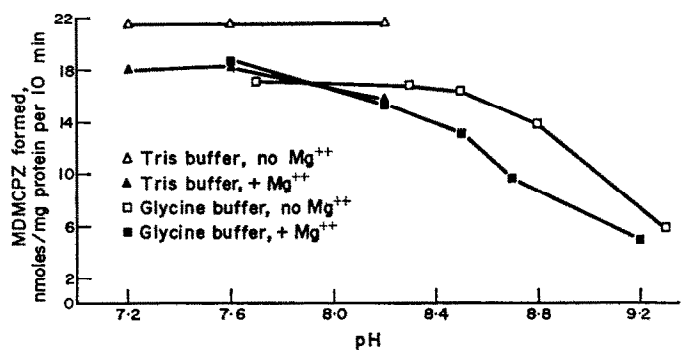


FIG. 3. pH Optima for the monodemethylation of chlorpromazine in Tris or glycine buffer. Incubation and extraction conditions are as described in the Methods section.

absence or presence of added $Mg^{2+}$, with one exception. Following the reaction with rabbit liver microsomes after 10 min in the absence of $Mg^{2+}$, there is a discrepancy between the amount of CPZ metabolized and the metabolites formed. This may be because of the appearance of a "ghost" peak in the CPZ area of the gas chromatogram early in the course of some experiments, leading to smaller differences between the 0 and 10 min values. Non-parametric statistics[12] applied to these 10 min values have indicated that the increase in products formed over CPZ disappearing was not significant at $P = 0{\cdot}05$. This is also obvious if one notes that the standard deviation for CPZ disappearance at 10 min is much larger than the standard deviations of the products formed. After 20 min of incubation this large variability is reduced and the amount of metabolites produced is equivalent to 92–104 per cent of the CPZ disappearing. Such equivalence is also evident at all times in similar rat experiments.

There appears to be no question that the two main metabolites of CPZ formed by rat and rabbit liver microsomes are CPZNO and MDMCPZ. The amounts of these compounds that are made by both types of microsomes under our conditions differ

and can be attributed to species difference in the metabolism of the drug. Beckett and Hewick[6] have indicated that *N*-oxidation was the major metabolic route in rat liver since the amount of *N*-oxide appearing was 34 per cent of the CPZ added, and that other routes, including monodemethylation, were relatively minor.

It had been determined some years ago that formaldehyde appeared during the liver microsomal metabolism of methylated amines.[13] We present here a comparison between demethylated products appearing and formaldehyde generated. Since DDMCPZ and MDMCPZSO amounted to about 3 per cent of the total demethylated products, only the demethylation figures involving MDMCPZ formation will be considered. After incubating 20 min with the rabbit microsomes, the formaldehyde formed was 32 and 88 per cent higher than the MDMCPZ formed in the absence and presence of $Mg^{2+}$ respectively. These figures are statistically significant at the $P = 0{\cdot}001$ level. For the rat microsomes, however, the differences between the formaldehyde formed and the MDMCPZ appearing are insignificant after 10 and 20 min incubations except for the 10 min incubation without $Mg^{2+}$. Since lipid peroxidation is known to occur in the liver microsomal system *in vitro* and leads to the formation of malonaldehyde,[14] tests were performed to determine (a) whether or not the aldehyde was formed under our experimental conditions and (b) whether or not malonaldehyde gave a positive color reaction in the Nash formaldehyde test. It was ascertained that (a) malonaldehyde did not appear under our conditions and (b) in any case, malonaldehyde gave no color in the Nash test. The excess of formaldehyde formed, over demethylation products found, may be due to other aldehydes but essentially this problem remains unsolved. This same type of result was also noticed by Wilson[15] who found that formaldehyde production was excessive in relation to the demethylation of aminopyrine. The lack of agreement between MDMCPZ and formaldehyde appearing should be considered therefore by investigators who assume that formaldehyde found by the Nash reaction is equivalent to demethylation products formed in the metabolism of CPZ by rabbit liver microsomes.

If one is to test adequately the effect of $Mg^{2+}$ on the microsomal mixed function oxidase, it is necessary to remove most of the $Mg^{2+}$ found in the original liver homogenates. This was carefully done as described in the Methods section. Whether or not the $Mg^{2+}$ exerts its effect on the microsomal membrane and allows for better access of substrate to the enzyme or exerts its effect on the enzyme itself will probably not be known until a solubilized P-450 mixed function oxidase is tested. Preliminary experiments performed by us with such an oxidase indicate that there may be a $Mg^{2+}$ effect on the enzyme itself. These tests were performed with an oxidase prepared by sonication of liver microsomes followed by centrifugation at 200,000 *g* for 1 hr at 0° and use of the clear supernatant as the enzyme source. Lu *et al.*[16] have recently tested their reconstituted solubilized microsomal drug hydroxylase and found $Mg^{2+}$ to have no effect. It is possible, however, that the detergent or chemicals used in these enzyme purifications may have altered the protein configuration as to make it unreactive to $Mg^{2+}$. On the other hand, the supernatant from our sonified preparation may still contain sufficient particulate membrane upon which the $Mg^{2+}$ might have an effect.

An interesting observation emerges when the $Mg^{2+}$ effect on the appearance of CPZNO and MDMCPZ is compared in the rat and the rabbit. In Table 2 for the rabbit it can be seen that CPZNO formation is clearly stimulated 2 to 3-fold both at

10 and 20 min, whereas the appearance of MDMCPZ is mildly stimulated at 10 min and not at all at 20 min. In the case of the rat there is little or no increase in the CPZNO level and at least a doubling of the amount of MDMCPZ that forms.

Several complicated explanations could be devised to explain these findings and would depend on such factors as (a) $Mg^{2+}$ stimulation of the cytochrome P-450 portion of the microsomal drug oxidase, or (b) the presence of an entirely different microsomal enzyme containing cytochrome P-450. This enzyme would metabolize CPZ to CPZNO only, with the demethylation and formaldehyde formation probably being carried out by the microsomal cytochrome P-450 oxidase. The work of Ziegler *et al.*[5] and Machinist *et al.*[17] would favor the 2 enzyme assumption for CPZ metabolism. As stated earlier, Ziegler *et al.*[5] has isolated an enzyme complex which is practically devoid of cytochrome P-450 and oxidizes CPZ only to CPZNO. Machinist *et al.*[17] were able to enzymatically dealkylate a number of *N*,*N*-dialkylarylamine *N*-oxides in the absence of oxygen, pyridine nucleotides or other cofactors, using pork liver microsomes. We, however, could not show any demethylation or reduction of CPZNO under our conditions with rabbit liver microsomes. We have been able to repeat the isolation of the *N*-oxidation enzyme of Ziegler *et al.*[5] and essentially confirm their findings.

The pH optimum (8·5) for the *N*-oxidation of CPZ in glycine buffer agrees well with that found by Harinath and Odell[18] on a microsomal preparation that was purified about 900-fold.

LaDu *et al.*[13] showed that the demethylase reaction had a very broad pH optimum, from 6·8 to 7·5. This is in good agreement with our data in which we have shown that above pH 7·6 the demethylation reaction begins to decrease.

A recent report of Fok and Ziegler[19] presents data on the *N*-oxidation and demethylation of CPZ by rat liver microsomes. The reaction mixture contained $Mg^{2+}$ and the pH was 8·4. Their system produced 55 nmoles of CPZNO per mg protein per 10 min and 17 nmoles of formaldehyde (or MDMCPZ) per protein per 10 min. This data would be in substantial agreement with ours, considering that our working pH of 7·6 has decreased CPZNO formation and increased MDMCPZ production. As shown by our pH optima curves, at the higher pH of 8·4, we would have at least doubled CPZNO production, and decreased MDMCPZ formation by about 33 per cent.

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