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# RELATIONSHIP BETWEEN THE DURATION OF ANTICONVULSANT ACTIVITY OF CHLORDIAZEPOXIDE AND SYSTEMIC LEVELS OF THE PARENT COMPOUND AND ITS MAJOR METABOLITES IN MICE

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**Abstract**—The duration of the anticonvulsant (antimetrazol) activity of chlordiazepoxide in mice in relation to blood levels and tissue distribution patterns of the parent drug and its major metabolites is presented.

Quantitation of the effect of a 125 mg/kg s.c. injection of metrazol and the degree of protection by a single 20 mg/kg oral dose of chlordiazepoxide, based on measuring the incidence of defined seizure reactions, indicated maximal protection for 4 hr after chlordiazepoxide administration.

The quantitative $^{14}C$ distribution after the oral administration of a single 20 mg/kg dose of chlordiazepoxide-2-$^{14}C$ is indicative of a rapid absorption onset as well as of a gradual increase in the tissue-to-blood $^{14}C$ ratios from 0·5 to 6 hr after administration. In contrast, a 125 mg/kg subcutaneous injection of metrazol given 30 min after the oral administration of chlordiazepoxide appears both to reduce markedly the rate of absorption and to alter the disposition of chlordiazepoxide. This alteration includes a reduction in the blood $^{14}C$ and tissue $^{14}C$ levels immediately after metrazol injection to 4 hr after chlordiazepoxide administration. Although the initial tissue-to-blood $^{14}C$ ratios are similar to those seen in the absence of metrazol, they do not show the marked increment with time.

Differential spectrofluorometric analyses of blood, brain and muscle tissue samples for chlordiazepoxide and its major metabolites show that *N*-desmethylchlordiazepoxide is the major constituent in all three tissues both in the absence and in the presence of metrazol. Besides being present in much higher concentrations, unlike the parent compound and other metabolites, the *N*-desmethyl derivative maintains a maximum level from 30 min to 4 hr in the brain and from 30 min to 6 hr in blood and muscle. Correlation of the levels of chlordiazepoxide and its metabolites in these tissues to its anticonvulsant (antimetrazol) activity indicates that it is the concentration of the *N*-desmethyl metabolite that most closely parallels the pattern of this anticonvulsant activity.

CHLORDIAZEPOXIDE* (7-chloro-2-methylamino-5-phenyl-3H-1,4-benzodiazepine-4-oxide) was the first of several benzodiazepine derivatives shown to have anticonvulsant, taming and muscle relaxant properties in animals[1–4] and to exhibit significant phsycotropic activities in man.[5] Evidence of the metabolism of chlordiazepoxide to "lactam" (7-chloro-1,3-dihydro-5-phenyl-2H-1,4-benzodiazepine-4-oxide)[6] and further to "opened lactam" in dog and man,[7] as well as of its conversion to *N*-desmethylchlordiazepoxide (7-chloro-2-amino-5-phenyl-3H-1,4-benzodiazepine-4-oxide) in the rat and man[8] has been presented. Figure 1 shows the structure of these compounds.

* Marketed as Librium by Hoffmann-La Roche Inc., Nutley, N.J.

The objective of this study is to correlate the anticonvulsant (antimetrazol) activity of chlordiazepoxide in mice to the blood levels and tissue distribution patterns of the parent compound, the *N*-desmethyl metabolite and "lactam".

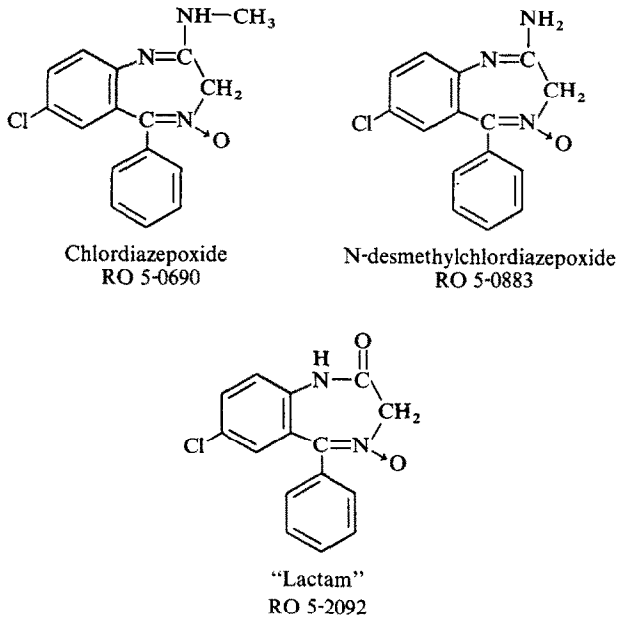


FIG. 1. Structure of chlordiazepoxide and its major metabolites.
Ro 5-0690, 7-chloro-2-methylamino-5-phenyl-3H-1,4-benzodiazepine-4-oxide;
Ro 5-0883, 7-chloro-2-amino-5-phenyl-3H-1,4-benzodiazepine-4-oxide:
Ro 5-2092, 7-chloro-1,3-dihydro-5-phenyl-2H-1,4-benzodiazepine-2-one-4-oxide.

## MATERIALS AND METHODS

*Synthesis and purity of chlordiazepoxide-2-$^{14}C$.* The labeled compound was synthesized from 2-amino-5-chlorbenzophenone oxime and chloroacetylchloride-1-$^{14}C$ by H. H. Kaegi according to the procedure of Sternbach and Reeder.[9] Solutions of labeled chlordiazepoxide, when exposed to light, undergo isomerization with rearrangement of the *N*-oxide function in position 4 to 4,5-epoxide.[10] Therefore, immediately before use, the labeled compound was mixed with an equal quantity of unlabeled chlordiazepoxide and subjected to vacuum sublimation at 200–210°.[6] This sublimation step served to reconvert quantitatively any 4,5-epoxide present to the *N*-oxide. Radiochemical purity was established by thin-layer chromatography using Brinkman precoated $F_{254}$ silica gel plates in solvent systems: A) ethyl acetate–ethanol (95:5) and B) chloroform–methanol (90:10). Chlordiazepoxide standards were carried through all chromatographic procedures. The purified product had a specific activity of 1·99 $\mu$c/mg.

*Characterization of metrazol response.* CF-1 mice (Carworth Farms, N.Y.) weighing between 20 and 24 g were used in all tests. The technique used to determine the convulsant activity of metrazol and the protective effects of chlordiazepoxide was essentially that of Everett and Richards.[11] Initially, experiments were conducted: 1) to characterize

the responses produced by metrazol and to establish criteria for measuring their degree and variation; 2) to select the most appropriate subcutaneous dose of metrazol which would elicit the desired responses; 3) to establish the time at which these reactions occur after metrazol administration; and 4) to check possible sex dependence of these reactions. Doses of 75, 100 and 125 mg/kg of metrazol in the form of a 1% aqueous solution were administered subcutaneously to 4 groups of 10 male and 4 groups of 10 female mice and the animals were observed for 30 min after metrazol administration. On the basis of the severity of the induced responses to metrazol, each animal was classified as undergoing a clonic or tonic convulsion. Clonic reactions were characterized by mild intermittent muscular contractions in contrast to the tonic responses manifested by a relatively prolonged, forceful and violent wrenching convulsion. Control groups were injected with equivalent volumes of distilled water.

These initial tests suggested that the criteria could be well defined and differentiated, and the pharmacological responses could be based on the relative numbers of mice undergoing the two types of seizures and on the per cent mortality occurring within 30 min after metrazol injection. A s.c. injection of 125 mg/kg was found to elicit the best response in mice and was chosen as the standard metrazol dose in all subsequent experiments.

*Effect of chlordiazepoxide on metrazol-induced convulsions.* The following experiments were performed to determine the effect of chlordiazepoxide and its duration of activity on the metrazol-induced convulsions. A single 20 mg/kg oral dose of chlordiazepoxide was given simultaneously with, and at intervals of 0·5, 1, 2, 4, 6, 8, 12, 18 and 24 hr prior to, an injection of the standard 125 mg/kg subcutaneous dose of metrazol. Two groups of 10 males and 2 groups of 10 female mice were used at each time interval. The animals were observed for a period of 30 min and their responses were recorded according to the above criteria.

*Distribution of chlordiazepoxide-2-$^{14}C$.* The distribution of $^{14}C$ in blood, specific tissues and organs was determined in groups of 10 animals at 0·5, 1, 2, 4, 6, 18 and 24 hr after oral administration of a single 20 mg/kg dose of chlordiazepoxide-2-$^{14}C$. Two groups of 10 mice of mixed sexes were used at each time interval to ensure reproducibility of the results.

An analogous second set of experiments was carried out in which, additionally, the standard 125 mg/kg dose of metrazol was injected subcutaneously into the animals of each group 30 min after the administration of chlordiazepoxide-2-$^{14}C$.

In both cases, the 10 mice comprising each group were killed at the appropriate times by decapitation. Heparinized blood was collected from each animal and equal aliquots were pooled to form a single assay specimen per group or two equivalent assay specimens for each time interval (duplicate groups). Individual tissues (see Tables 2, 3) were excised and pooled according to group and time interval of sacrifice for further analysis. Thus, for each tissue and each time point, two individual analytical values were obtained. Homogenates (1:5) in 1·5% KCl of each pool of tissues were prepared in a Waring Blendor and their $^{14}C$ content was determined by radioactivity measurement.

*Radioactivity measurements.* The $^{14}C$ content of the aqueous homogenates was determined by liquid scintillation techniques on a Nuclear-Chicago, Mark I scintillation spectrometer equipped with a $^{133}Ba$ external standard. The counting medium consisted of 100 g naphthalene, 7 g PPO and 50 mg dimethyl POPOP l. of dioxane.

Segments of silica gel from thin-layer plates were counted in the above medium, which also contained a 4·0% suspension of thixotropic gel (Cab-O-sil). Based on channel ratios and a counting efficiency curve, the counts per minute were converted to the sample $^{14}C$ content in disintegrations per minute.

*Differential spectrofluorometric assay of intact chlordiazepoxide and its metabolites in selected tissues.* In view of the action of chlordiazepoxide as an anticonvulsant, muscle relaxant and psychotropic compound, the blood, brain and muscle homogenates were selected to determine the differential concentration patterns of intact chlordiazepoxide and its metabolites, specifically the *N*-desmethyl derivative and "lactam". The differential assay method used was based on the procedure of Schwartz and Postma.[8] Preliminary data on the recovery of the parent compound and its metabolites from mouse tissue homogenates indicated that the initial extraction step with ether at pH 7·0–7·2 was not quantitative when applied to tissue samples other than blood. Therefore the following procedure was used.

A 5·0-ml aliquot (1 g) of tissue homogenate was brought to pH 10·5 in a 40·0-ml graduated centrifuge tube by the addition of 1·0 N NaOH. The aqueous volume was adjusted to 6·5 ml and 19·5 ml chloroform–methanol (2:1) was added. The mixture was shaken gently for 10 min and centrifuged at 1500 rpm for 15 min. The resultant upper aqueous and lower organic layers were separated and their volumes recorded. Quantitative separation of the "lactam" into the upper basic aqueous layer and of chlordiazepoxide and its *N*-desmethyl derivative into the lower organic layer was achieved in this manner.

To estimate the "lactam" content, 10·0 of the basic aqueous phase was readjusted to pH 7·0–7·2, extracted with 10 ml ether and a 5·0-ml aliquot of the ether phase was back-extracted with 4·0 ml of 0·1 N NaOH. As previously described,[8] the 0·1 N NaOH extract was exposed for 1 hr to light from a Pyro-Lux R-57 lamp and its fluorescence was determined at 380 m$\mu$ excitation and 460 m$\mu$ emission.

The *N*-desmethyl metabolite was measured by extracting a 5·0-ml aliquot of the lower organic phase with 3·5 ml of 7 N $H_2SO_4$. The aqueous extract was removed and, after standing for 1 hr, its fluorescence was determined at 370 m$\mu$ excitation and 460 m$\mu$ emission.

The chlordiazepoxide content was determined by extracting a second 5·0-ml aliquot of the lower organic phase with 2·0 ml of 0·2 N $H_2SO_4$ instead of 1·5 ml of 0·1 N $H_2SO_4$. Increasing the molarity improved the recovery of the component into the aqueous acid extract. The acid extract was adjusted to pH 6·7–6·8 by the addition of 0·5 ml of 0·5 M phosphate buffer, pH 6·7–6·8, and 1·0 ml of 0·15 M diethanolamine solution; the mixture was hydrolyzed to "lactam" in a boiling water bath for 2 hr. After cooling, 0·4 ml of 5 N NaOH was added, the sample was exposed to light for 1 hr and its fluorescence was determined as for "lactam". All calculations were done according to the method described by Schwartz and Postma.[8] Concentrations in the tissues of dosed animals were based on the recoveries obtained for authentic standards of chlordiazepoxide, *N*-desmethylchlordiazepoxide and "lactam" added to control tissues. Duplicate internal standards for each tissue were carried through the entire assay procedure as the unknowns. Their values were found to be reproducible within 5 per cent, while recoveries for the components in different tissues ranged from 80 to 90 per cent. As a further check on the spectrofluorometric values, the overall recoveries, the efficacy of the extraction procedures and the distribution

of the $^{14}C$ in the various fractions, aliquots of certain extracts were counted. In this fashion, the fluorometric data for chlordiazepoxide, the *N*-desmethyl component and "lactam" could be confirmed.

The identity and completeness of separation of the individual components into specified extracts were further tested by TLC. Aliquots of the tissue extracts were chromatographed together with unlabeled standards and the percentage of the applied radioactivity migrating with the standard on the plate was measured. The detailed fractionation scheme and corresponding tests and assays performed at the various stages of fractionation are presented in Fig. 2. The procedure yielded reproducible and reliable values for all the components.

## RESULTS AND DISCUSSION

*Response to metrazol.* The pharmacologic responses elicited in mice treated with increasing subcutaneous doses of metrazol are presented in Table 1. The relative intensity of the responses is reflected in the percentage of animals undergoing clonic seizures, tonic seizures and death. It is apparent that an increase in the dose of metrazol resulted in an increase in the per cent of tonic seizures and mortality while, within the dose range tested, clonic responses were maximal. No significant differences could be distinguished in the responses elicited in the two sexes.

*Effect of chlordiazepoxide on metrazol response.* The pharmacologic responses to a single 125 mg/kg s.c. injection of metrazol given alone, simultaneously with, and at increasing intervals after oral pretreatment with single 20 mg/kg doses of chlordiazepoxide are shown in Table 2. The protection afforded against the convulsant action of metrazol by pretreatment with chlordiazepoxide is evident from the decrease not only in the percentage of clonic and tonic seizures but also in the complete elimination of mortality within the treated groups for a period of 4 hr after chlordiazepoxide administration. Thereafter a gradual increase in the severity of the reactions elicited by metrazol starts with the advent of clonic seizures, which reach approximately 50 per cent protection values at 6 hr. This is followed by a return to 50 per cent protection values for tonic seizures at 12 hr after chlordiazepoxide administration. No significant protection against either type of seizure is exhibited from 18 to 24 hr after treatment.

Of special interest and unexpected are the results obtained when chlordiazepoxide was administered "simultaneously" with metrazol. When chlordiazepoxide was given only 2–5 sec after the standard metrazol injection, a certain degree of protection was evident by all three criteria but, when given sec before metrazol, chlordiazepoxide afforded a high degree of protection against both tonic seizures and mortality. The significance of the order of chlordiazepoxide administration in these "simultaneously" treated groups was evaluated statistically in relation to the per cent protection afforded against both types of seizures. It was found that a significant difference appeared to exist. In the case of clonic seizures, chlordiazepoxide given before metrazol afforded greater protection at the 95 per cent level of confidence ($P = 0{\cdot}05$) in the two-sided *t*-test, while a 99·9 per cent ($P = 0{\cdot}001$) level of confidence existed in the case of the protection afforded against tonic seizures. Of further interest is the observation that, in the treated mice, a lower percentage of clonic convulsions progressed to the more severe tonic seizure and a lower percentage of the latter resulted in death, suggesting

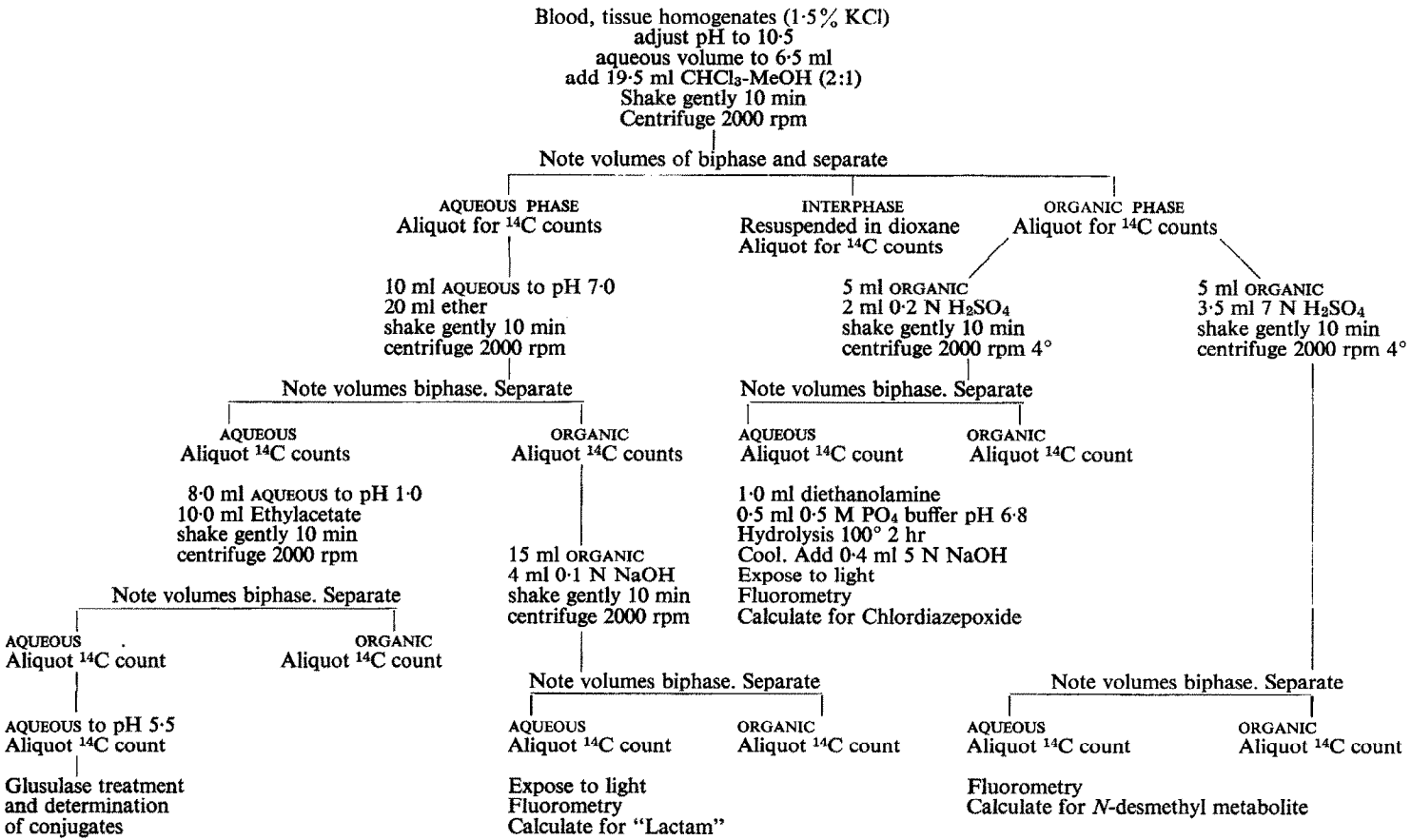


FIG. 2. Schematic diagram of extraction procedure.

that pre-treatment with chlordiazepoxide reduced the severity of the convulsive response.

*Distribution of chlordiazepoxide-2-$^{14}C$.* The tissue distribution of $^{14}C$ between 0·5 and 24 hr after a single 20 mg/kg oral dose of chlordiazepoxide, together with the

TABLE 1. PHARMACOLOGIC RESPONSES IN MICE AFTER S.C. ADMINISTRATION OF VARYING DOSES OF METRAZOL

| No. of mice | Sex | Dose (mg/kg) | Per cent responses to metrazol within 30 min: Clonic seizures* | Tonic seizures* | Mortality* | Clonic undergoing tonic | Tonic dying |
|---|---|---|---|---|---|---|---|
| 40 | ♂ | 75 | 80·0 (7·4) | 7·5 (24·7) | 1·3 (27·5) | 0·0 | 0·0 |
| 40 | ♀ | | 77·5 (7·9) | 2·5 (28·2) | 0·0((30·0) | 0·0 | 0·0 |
| 40 | ♂ | 100 | 85·0 (5·2) | 67·5((13·9) | 60·0 (23·0) | 70·1 | 88·8 |
| 40 | ♀ | | 82·5 (8·5) | 65·0 (15·0) | 45·0 (18·6) | 66·6 | 69·2 |
| 40 | ♂ | 125 | 85·2 (6·1) | 82·5 (10·3) | 72·5 (16·4) | 93·1 | 84·8 |
| 40 | ♀ | | 82·5 (7·7) | 82·5 (12·3) | 77·5 (15·3) | 86·6 | 90·0 |

* Average time (in minutes) required for response is in parentheses.

TABLE 2. PER CENT PROTECTION FROM METRAZOL (M)-INDUCED RESPONSES AFTER TREATMENT WITH CHLORDIAZEPOXIDE (C)

| Drug | Time of treatment (hr) | Per cent protection*: Clonic seizures | Tonic seizures | Mortality | Clonic from undergoing tonic | Tonic from dying |
|---|---|---|---|---|---|---|
| MC | simultaneous† | 25·1 | 49·5 | 63·7 | 66·0 | 28·2 |
| CM | simultaneous‡ | 41·5 | 78·4 | 100·0 | 81·5 | 100·0 |
| C-M | 0·5 | 75·3 | 100·0 | 100·0 | 100·0 | 100·0 |
| | 1 | 71·1 | 100·0 | 100·0 | 100·0 | 100·0 |
| | 2 | 67·0 | 100·0 | 100·0 | 100·0 | 100·0 |
| | 4 | 58·6 | 74·2 | 100·0 | 69·6 | 100·0 |
| | 6 | 46·0 | 66·0 | 63·7 | 65·2 | 63·6 |
| | 8 | 37·7 | 61·9 | 58·1 | 57·0 | 42·4 |
| | 12 | 23·0 | 57·1 | 35·6 | 32·9 | 11·5 |
| | 18 | 14·6 | 12·4 | 15·0 | 15·2 | 10·3 |
| | 24 | 8·4 | 12·4 | 15·0 | 9·3 | 14·9 |

* Per cent protection values are based on the mean per cent response given by mice receiving the standard 125 mg/kg s.c. dose of metrazol (see Table 1).

† Metrazol (125 mg/kg, s.c.) administered 2–5 sec prior to chlordiazepoxide (20 mg/kg, p.o.).

‡ Chlordiazepoxide (20 mg/kg, p.o.) administered 2–5 sec prior to metrazol.

ratios of tissue-to-blood $^{14}C$ concentrations is given in Table 3. The corresponding data obtained when chlordiazepoxide administration was followed after 30 min by the standard subcutaneous injection of 125 mg/kg of metrazol are given in Table 4.

When chlordiazepoxide is administered alone (Table 3), the highest blood $^{14}C$ concentrations are observed between 0·5 and 2 hr. Thereafter a gradual decline in levels occurs to 6 hr, followed by a more rapid fall-off to 24 hr after administration. The ratios of tissue-to-blood $^{14}C$ concentrations initially approximate 1·0 or are

TABLE 3. $^{14}C$ DISTRIBUTION IN MICE AFTER ORAL ADMINISTRATION OF A SINGLE 10 mg/kg DOSE OF CHLORDIAZEPOXIDE-2-$^{14}C$

| Specimen | 0·5 hr | | 1 hr | | 2 hr | | 4 hr | | 6 hr | | 18 hr | | 24 hr | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | (μg equiv./g)* | R† | (μg equiv./g) | R | (μg equiv./g) | R | (μg equiv./g) | R | (μg equiv./g) | R | (μg equiv./g) | R | (μg equiv./g) | R |
| Blood | 7·40 (± 0·73) | | 7·30 (± 0·85) | | 7·40 (± 0·59) | | 5·40 (± 0·30) | | 4·10 (± 0·30) | | 1·20 (± 0·10) | | 0·57 (± 0·13) | |
| Brain | 6·40 (± 0·51) | 0·9 | 6·20 (± 0·27) | 0·9 | 6·60 (± 0·30) | 0·9 | 5·00 (± 0·30) | 0·9 | 3·70 (± 0·21) | 0·9 | 0·92 (± 0·12) | 0·8 | 0·49 (± 0·09) | 0·9 |
| Muscle | 9·20 (± 0·17) | 1·2 | 9·40 (± 0·10) | 1·3 | 9·00 (± 0·10) | 1·2 | 7·50 (± 0·22) | 1·4 | 6·00 (± 0·20) | 1·5 | 1·48 (± 0·02) | 1·2 | 0·77 (± 0·03) | 1·4 |
| Heart | 6·27 (± 0·60) | 0·9 | 5·85 (± 0·25) | 0·8 | 7·67 (± 0·17) | 1·0 | 8·09 (± 0·41) | 1·5 | 7·27 (± 0·17) | 1·8 | 0·28 (± 0·08) | 0·2 | n.m.‡ | |
| Lung | 6·92 (± 0·09) | 0·9 | 6·94 (± 0·18) | 1·0 | 9·20 (± 0·30) | 1·2 | 7·35 (± 0·25) | 1·4 | 6·75 (± 0·15) | 1·7 | 0·36 (± 0·10) | 0·3 | 0·21 (± 0·05) | 0·4 |
| Liver | 33·20 (± 2·00) | 4·5 | 35·80 (± 1·40) | 4·9 | 40·50 (± 1·68) | 5·5 | 32·20 (± 1·92) | 6·0 | 28·40 (± 1·10) | 6·9 | 3·30 (± 0·60) | 2·8 | 2·70 (± 0·40) | 4·7 |
| Spleen | 6·35 (± 0·55) | 0·9 | 12·91 (± 0·59) | 1·8 | 17·20 (± 1·10) | 2·3 | 11·79 (± 0·61) | 2·2 | 8·80 (± 0·60) | 2·2 | 0·63 (± 0·20) | 0·5 | 0·66 (± 0·26) | 1·2 |
| Kidney | 17·80 (± 1·30) | 2·4 | 14·80 (± 1·60) | 2·0 | 15·10 (± 1·40) | 2·0 | 15·20 (± 1·20) | 2·8 | 12·10 (± 1·00) | 3·0 | 0·76 (± 0·07) | 0·6 | 0·77 (± 0·08) | 1·4 |
| Fat | 7·67 (± 0·43) | 1·0 | 7·18 (± 0·52) | 1·0 | 9·88 (± 0·63) | 1·3 | 7·88 (± 0·22) | 1·5 | 6·73 (± 0·27) | 1·6 | 0·37 (± 0·03) | 0·3 | 0·55 (± 0·07) | 1·0 |
| Carcass | 7·40 (± 0·62) | 1·0 | 7·24 (± 0·23) | 1·0 | 8·64 (± 0·47) | 1·2 | 7·19 (± 0·53) | 1·3 | 5·96 (± 0·34) | 1·5 | 0·63 (± 0·17) | 0·5 | 0·56 (± 0·04) | 1·0 |

* The $^{14}C$ concentration (dpm/g) was divided by the specific activity of the dose to obtain μg-equiv./g.

† Ratio of tissue-to-blood $^{14}C$ concentration. Values are mean of 2 groups of 10 mice.

‡ Not measurable.

TABLE 4. $^{14}C$ DISTRIBUTION IN MICE AFTER A 125 mg/kg s.c. DOSE OF METRAZOL GIVEN 0·5 hr AFTER A SINGLE 10 mg/kg DOSE OF CHLORDIAZEPOXIDE-2-$^{14}C$

| | 0·5 hr | | 1 hr | | 2 hr | | 4 hr | | 6 hr | | 18 hr | | 24 hr | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Specimen | (μg equiv./g)* | R* | (μg equiv./g) | R | (μg equiv./g) | R | (μg equiv./g) | R | (μg equiv./g) | R | (μg equiv./g) | R | (μg equiv./g) | R |
| Blood | 6·16 (± 0·16) | | 5·79 (± 0·21) | | 5·84 (± 0·36) | | 5·55 (± 0·45) | | 6·89 (± 0·39) | | 0·85 (± 0·25) | | 0·47 (± 0·06) | |
| Brain | 5·77 (± 0·12) | 0·9 | 5·43 (± 0·18) | 0·9 | 5·44 (± 0·32) | 0·9 | 5·35 (± 0·15) | 1·0 | 5·65 (± 0·20) | 0·8 | 0·53 (± 0·09) | 0·6 | 0·19 (± 0·19) | 0·4 |
| Muscle | 6·12 (± 0·28) | 1·0 | 5·40 (± 0·10) | 0·9 | 5·32 (± 0·11) | 0·9 | 5·05 (± 0·16) | 0·9 | 6·13 (± 0·19) | 0·9 | 0·60 (± 0·07) | 0·7 | 0·30 (± 0·10) | 0·6 |
| Heart | 13·00 (± 0·80) | 2·1 | 11·65 (± 0·35) | 2·0 | 9·88 (± 0·33) | 1·7 | 8·56 (± 0·08) | 1·5 | 12·45 (± 0·15) | 1·8 | 1·01 (± 0·31) | 1·2 | 0·53 (± 0·15) | 1·1 |
| Lung | 18·50 (± 0·50) | 3·0 | 13·65 (± 0·65) | 2·3 | 12·75 (± 2·35) | 2·2 | 12·69 (± 1·11) | 2·3 | 17·75 (± 2·25) | 2·6 | 1·38 (± 0·14) | 1·6 | 0·69 (± 0·14) | 1·5 |
| Liver | 29·74 (± 1·07) | 4·8 | 25·75 (± 0·95) | 4·5 | 26·33 (± 0·23) | 4·5 | 22·09 (± 0·29) | 4·0 | 30·62 (± 0·98) | 4·4 | 4·66 (± 0·90) | 5·5 | 2·65 (± 1·29) | 5·6 |
| Spleen | 9·00 (±0·06) | 1·5 | 10·80 (± 0·80) | 1·9 | 7·17 (± 0·23) | 1·2 | 7·07 (± 0·13) | 1·3 | 9·50 (± 0·16) | 1·4 | 1·24 (± 0·36) | 1·5 | 0·62 (± 0·22) | 1·3 |
| Kidney | 14·70 (± 1·10) | 2·4 | 13·73 (± 0·03) | 2·4 | 15·30 (± 0·40) | 2·6 | 9·57 (± 0·33) | 1·7 | 14·20 (± 1·00) | 2·1 | 1·77 (± 0·67) | 2·1 | 1·13 (± 0·37) | 2·4 |
| Fat | 7·87 (± 0·87) | 1·3 | 6·55 (± 0·31) | 1·1 | 6·39 (± 0·50) | 1·1 | 5·28 (± 0·34) | 1·0 | 6·88 (± 0·16) | 1·0 | 0·72 (± 0·20) | 0·9 | 0·55 (± 0·00) | 1·2 |
| Carcass | 7·44 (± 0·28) | 1·2 | 6·30 (± 0·20) | 1·1 | 6·82 (± 0·15) | 1·2 | 5·05 (± 0·58) | 0·9 | 6·98 (± 0·22) | 1·0 | 0·61 (± 0·04) | 0·7 | 0·35 (± 0·05) | 0·7 |

* For definition, see footnote to Table 3.

TABLE 5. ABSORPTION, EXCRETION AND RECOVERY OF $^{14}C$ AFTER ORAL ADMINISTRATION OF A SINGLE 10 mg/kg DOSE OF CHLORDIAZEPOXIDE-2-$^{14}C$ ALONE (C) AND AFTER A 125 mg/kg s.c. INJECTION OF METRAZOL GIVEN 0·5 hr AFTER THE CHLORDIAZEPOXIDE (CM)

| Specimen | | Per cent $^{14}C$ of dose administered | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | 0·5 hr | 1 hr | 2 hr | 4 hr | 6 hr | 18 hr | 24 hr |
| GIT* | C | 59·21 (± 8·40) | 47·50 (± 4·60) | 37·29 (± 3·90) | 32·75 (± 3·21) | 29·60 (± 3·10) | 19·70 (± 4·20) | 12·20 (± 3·10) |
| | CM | 65·84 (± 6·43) | 65·10 (± 3·10) | 60·17 (± 2·83) | 58·08 (± 5·97) | 31·26 (± 4·36) | 19·65 (± 4·06) | 11·84 (± 2·00) |
| Urine | C | 0·20 (± 0·10) | 0·80 (± 0·20) | 4·70 (± 1·20) | 11·10 (± 2·82) | 23·09 (± 3·60) | 60·80 (± 4·80) | 68·90 (± 5·90) |
| | CM | | | | 5·10 (± 0·20) | 12·17 (± 0·61) | 56·50 (± 5·10) | 65·94 (± 5·44) |
| Feces | C | 0·01 (± 0·01) | 0·02 (± 0·01) | 0·37 (± 0·07) | 0·53 (± 0·09) | 1·76 (± 0·07) | 2·10 (± 0·19) | 2·77 (± 0·21) |
| | CM | | | | | 0·18 (± 0·02) | 1·15 (± 0·05) | 1·21 (± 0·10) |
| Tissues | C | 43·60 (± 2·10) | 48·57 (± 2·81) | 50·24 (± 3·20) | 47·01 (± 2·60) | 36·99 (± 1·90) | 4·13 (± 0·64) | 3·69 (± 0·88) |
| | CM | 37·01 (± 3·10) | 32·78 (± 3·90) | 34·71 (± 5·40) | 26·86 (± 3·72) | 35·98 (± 2·50) | 4·78 (± 0·90) | 3·05 (± 0·77) |
| Recovery | C | 103·02 | 96·89 | 92·60 | 91·39 | 91·44 | 86·73 | 87·56 |
| | CM | 102·85 | 97·88 | 94·88 | 90·04 | 86·59 | 82·08 | 82·04 |

* GIT = gastrointestinal tract.

greater than 1·0 in all tissues, increasing to peak values around 6 hr after chlordiazepoxide. The $^{14}C$ concentrations in brain and muscle parallel those of blood, their ratios ranging from 0·8 to 0·9 and 1·2 to 1·4 respectively. The most pronounced increase in this ratio over the 0·5–6 hr period is seen in the spleen, followed by the heart, lung, fat, carcass, muscle and brain.

Comparison of the data in Tables 3 and 4 indicates that the subcutaneous injection of metrazol 30 min after oral administration of $^{14}C$-chlordiazepoxide altered the overall disposition of the latter in several respects. The most pronounced and definite changes appear to include the following. The blood $^{14}C$ levels as well as the over-all tissue $^{14}C$ concentrations are lower in the presence of metrazol. In contrast to the pattern obtained with chlordiazepoxide alone, the $^{14}C$ concentrations in general show a distinct reversible drop between 30 min, i.e. immediately after metrazol injection, and 4 hr. Between 4 and 6 hr, the blood and tissue levels return to and even surpass the values obtained in the absence of metrazol.

The tissue distribution of $^{14}C$ in each case appears to be altered. The metrazol-induced overall reduction of tissue $^{14}C$ levels appears to be confined mostly to the muscle. $^{14}C$ levels in certain tissues such as the brain, fat and carcass are only slightly changed, while others, including lung and heart, show increased $^{14}C$ levels. The initial ratios of blood-to-tissue $^{14}C$ are similar to those seen in absence of metrazol, but do not show the marked increments with time of those noted after administration of chlordiazepoxide alone. These observations may be interpreted as changes of tissue compartment sizes or cell permeability, although the mechanism by which metrazol may produce these changes remains unexplained.

Examination of the patterns of the $^{14}C$ content of the gastrointestinal tract (GIT) (Table 5) after administration of chlordiazepoxide-$^{14}C$ alone and when followed by metrazol injection suggests that an effect of metrazol on the absorption (or on other processes or on both, which control the $^{14}C$ balance in the GIT, such as enterohepatic circulation) may be responsible for changes of the $^{14}C$ patterns in the blood and tissues. In both experiments, 35–41 per cent of the orally administered $^{14}C$ dose had left the GIT within the first half hour. However, while in the absence of metrazol the intestinal $^{14}C$ continues to decline at a gradual rate, metrazol injection practically stops the disappearance of intestinal $^{14}C$ for a period of approximately 3 hr. This reduced absorption could be attributed to the activity of metrazol, whose biological half-life is reported to be approximately 2·5 hr in rats.[12] The respective urinary excretion patterns appear to be consistent with such an interpretation.

Comparative results of the qualitative and quantitative analysis of blood, brain and muscle for chlordiazepoxide and its major metabolites in the absence and presence of metrazol are shown in Table 6. In addition to the parent compound, its *N*-desmethyl derivative and "lactam", a fourth component was detected in all three tissue samples. Based on its partitioning characteristics and the point in the extraction procedure at which it was obtained (Fig. 2), the fourth substance has been tentatively regarded as a highly polar, possibly conjugated constituent.

Additional effects of metrazol on the disposition of $^{14}C$-chlordiazepoxide become evident on comparison of the differential concentration patterns of intact chlordiazepoxide, the *N*-desmethyl derivative and "lactam". The *N*-desmethyl derivative is the major constituent in all three tissues throughout the entire experimental period, both in the presence and in the absence of metrazol. Its concentrations between 0·5 and 6

TABLE 6. CONCENTRATION OF CHLORDIAZEPOXIDE AND METABOLITES IN MOUSE BLOOD, BRAIN AND MUSCLE AFTER ORAL ADMINISTRATION OF CHLORDIAZEPOXIDE-2-$^{14}$C ALONE (C) AND WHEN A S.C. INJECTION OF METRAZOL WAS GIVEN 0·5 hr AFTER THE CHLORDIAZEPOXIDE (CM)

| Metabolic Components | | Micrograms per gram after chlordiazepoxide administration | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | 0·5 hr | 1 hr | 2 hr | 4 hr | 6 hr | 18 hr | 24 hr |
| Blood | | | | | | | | |
| Chlordiazepoxide | C | 1·12 (± 0·08) | 0·99 (± 0·06) | 0·90 (± 0·10) | 0·74 (± 0·10) | n.m.* | n.m. | n.m. |
| | CM | 1·27 (± 0·15) | 0·44 (± 0·20) | 1·16 (± 0·16) | 1·40 (± 0·14) | 1·10 (± 0·12) | n.m. | n.m. |
| *N*-desmethyl-chl. | C | 5·01 (± 0·39) | 4·60 (± 0·20) | 3·98 (± 0·35) | 2·80 (± 0·20) | 2·45 (± 0·33) | 0·20 (± 0·20) | n.m. |
| | CM | 4·65 (± 1·45) | 4·20 (± 0·10) | 4·00 (± 0·22) | 3·85 (± 0·15) | 4·33 (± 0·13) | n.m. | n.m. |
| Lactam | C | 0·46 (± 0·06) | 0·50 (± 0·10) | 0·52 (± 0·12) | 0·57 (± 0·03) | 0·57 (± 0·02) | 0·78 (± 0·09) | 0·85 (± 0·10) |
| | CM | 0·19 (± 0·04) | 0·20 (± 0·06) | 0·20 (± 0·05) | 0·24 (± 0·10) | 0·34 (± 0·06) | 0·10 (± 0·05) | n.m. |
| Polar-conjugate | C | 0·93 (± 0·07) | 1·17 (± 0·08) | 1·46 (± 0·16) | 1·51 (± 0·14) | 0·99 (± 0·16) | n.m. | n.m. |
| | CM | 0·55 (± 0·10) | 0·55 (± 0·09) | 0·65 (± 0·05) | 0·68 (± 0·00) | 0·98 (± 0·12) | n.m. | n.m. |
| Brain | | | | | | | | |
| Chlordiazepoxide | C | 0·90 (± 0·11) | 0·70 (± 0·15) | 0·45 (± 0·10) | n.m. | n.m. | n.m. | n.m. |
| | CM | 1·31 (± 0·12) | 0·24 (± 0·06) | 0·56 (± 0·13) | 1·15 (± 0·15) | 1·10 (± 0·08) | n.m. | n.m. |
| *N*-desmethyl-chl. | C | 4·80 (± 0·20) | 4·80 (± 0·18) | 4·50 (± 0·25) | 3·42 (± 0·22) | 2·40 (± 0·15) | 0·38 (± 0·00) | n.m. |
| | CM | 3·80 (± 0·15) | 3·90 (± 0·10) | 4·28 (± 0·58) | 4·00 (± 0·20) | 3·65 (± 0·05) | n.m. | n.m. |
| Lactam | C | 0·60 (± 0·08) | 0·76 (± 0·20) | 1·10 (± 0·15) | 0·79 (± 0·11) | 0·58 (± 0·10) | n.m. | n.m. |
| | CM | 0·14 (± 0·00) | 0·15 (± 0·02) | 0·14 (± 0·03) | 0·16 (± 0·05) | 0·18 (± 0·06) | n.m. | n.m. |
| Polar conjugate | C | 0·62 (± 0·08) | 0·70 (± 0·20) | 1·28 (± 0·07) | 0·99 (± 0·16) | 0·90 (± 0·05) | n.m. | n.m. |
| | CM | 0·25 (± 0·03) | 0·24 (± 0·02) | 0·26 (± 0·03) | 0·28 (± 0·01) | 0·29 (± 0·02) | n.m. | n.m. |
| Muscle | | | | | | | | |
| Chlordiazepoxide | C | 1·70 (± 0·25) | 1·45 (± 0·20) | 1·20 (± 0·00) | 0·70 (± 0·25) | n.m. | n.m. | n.m. |
| | CM | 0·80 (± 0·15) | 1·05 (± 0·05) | 0·99 (± 0·10) | 0·52 (± 0·08) | 0·43 (± 0·12) | n.m. | n.m. |
| *N*-desmethyl-chl. | C | 6·60 (± 0·30) | 6·30 (± 0·20) | 6·00 (± 0·20) | 4·15 (± 0·95) | 2·80 (± 0·30) | n.m. | n.m. |
| | CM | 4·20 (± 0·10) | 3·50 (± 0·10) | 3·30 (± 0·20) | 3·99 (± 0·07) | 4·39 (± 0·36) | n.m. | n.m. |
| Lactam | C | 1·08 (± 0·22) | 1·05 (± 0·15) | 0·98 (± 0·08) | 0·70 (± 0·15) | 0·56 (± 0·06) | n.m. | n.m. |
| | CM | 0·20 (± 0·03) | 0·23 (± 0·03) | 0·23 (± 0·04) | 0·31 (± 0·01) | 0·34 (± 0·04) | n.m. | n.m. |
| Polar conjugate | C | 0·19 (± 0·01) | 0·17 (± 0·02) | 0·18 (± 0·02) | 0·19 (± 0·03) | 0·18 (± 0·01) | n.m. | n.m. |
| | CM | 0·53 (± 0·03) | 0·52 (± 0·04) | 0·54 (± 0·01) | 0·64 (± 0·03) | 0·62 (± 0·04) | n.m. | n.m. |

* Not measurable = n.m.

hr after chlordiazepoxide administration are practically identical in brain and blood and are not significantly affected by the presence of metrazol. Its concentrations exceed those of intact chlordiazepoxide 3 to 10-fold and maintain relative constant values during the 6-hr period. In muscle, the levels of this metabolite are equally dominant; however, they exhibit a gradual decline with time in the absence of metrazol and are significantly lowered when metrazol is injected.

In contrast, levels of intact chlordiazepoxide are low in blood, brain and muscle and fall off to unmeasurable values within 4 hr when chlordiazepoxide is administered alone. The presence of metrazol markedly changed this pattern; a sharp reversible drop was observed in brain and blood at 1 hr, followed by a moderate rise at 4 hr and a final fall-off after 6 hr. In muscle, this pattern was absent; the only metrazol-induced change was that of a reduction of the levels and a shifted fall-off pattern.

The "polar conjugate" and "lactam" are present in all tissues throughout the experimental period. Their levels are noticeably lower in both the absence and presence of metrazol.

It is apparent that along with the pharmacological counteraction of the convulsant effect of metrazol by chlordiazepoxide, metrazol exerts a distinct effect on the metabolic disposition of its antagonist. The drastic nature of this drug interaction at the drug metabolic level is surprising and represents a by-product of considerable significance to the study, yet beyond its primary objective. Therefore an interpretation of these findings and an explanation of the mechanism by which metrazol alters chlordiazepoxide disposition will require further experiments specifically designed to answer these questions and to test specific hypotheses.

In attempting to correlate drug level patterns of chlordiazepoxide or of its metabolites or of both to the pattern of the anticonvulsant activity of chlordiazepoxide, the pattern of the anticonvulsant activity of chlordiazepoxide, the patterns, as modified by the administration of metrazol, the convulsant, have to be considered primarily. As shown in Table 2, onset of the anticonvulsant activity is almost immediate. Protection is maximal for a period from 0·5 to 2 hr and declines to a 50 per cent value by approximately 6–8 hr after chlordiazepoxide administration. Among the individual chlordiazepoxide-derived components in brain and muscle, the concentration curve of the *N*-desmethyl metabolite, rather than that of intact chlordiazepoxide or of "lactam", appears to parallel most closely this anticonvulsant activity pattern. Besides being present in much higher concentration than the other components, the *N*-desmethyl derivative maintains a maximal level from 30 min to 4 hr in brain and from 30 min to 6 hr in blood and muscle. The patterns of intact drug and "lactam" are not only considerably lower, but their fall-off, specifically the distinct discontinuity of chlordiazepoxide seen in the metrazol-treated animals, shows no parallelism to the anticonvulsant activity. It should be noted that the *N*-desmethyl derivative patterns in brain and blood, as the parameter most closely related to the activity, are practically not affected by the metrazol. It is further to be emphasized that the pattern of the desmethyl metabolite blood level is a close indicator of the pattern which exists in brain.

That the *N*-desmethyl metabolite may be the active anticonvulsant agent raises new questions which need to be answered by additional experiments. The major problem derives from the observation that the anticonvulsant (antimetrazol) activity of chlordiazepoxide occurs within a few seconds of its administration. If this is indeed

true and our hypothesis holds, the active *N*-desmethyl metabolite would have to be formed almost instantaneously and in adequate concentration. Analysis of differential tissue levels during the early 0–30 min period after oral administration of chlordiazepoxide, a period not covered in the present study, is to be investigated to resolve this point.

## REFERENCES

1. L. O. RANDALL, *Dis. nerv. Syst. suppl.* 3, **21**, 7 (1960).
2. L. O. RANDALL, *Dis. nerv. Syst. suppl.* 7, **22**, 1 (1961).
3. L. O. RANDALL, W. SCHALLEK, G. A. HEISE, E. F. KEITH and R. E. BAGDON, *J. Pharmac. exp. Ther.* **129**, 163 (1960).
4. L. O. RANDALL, G. A. HEISE, W. SCHALLEK, R. E. BAGDON, R. BANZIGER, A. BORIS, R. A. MOE and W. B. ABRAMS, *Curr. ther. Res.* **3**, 405 (1961).
5. L. H. STERNBACH, L. O. RANDALL and S. R. GUSTAFSON, *Phsychopharmacological Agents* (Ed. VON M. GORDON), vol. 1, p. 137. Academic Press, New York (1964).
6. B. A. KOECHLIN and L. D'ARCONTE, *Analyt. Biochem.* **5**, 195 (1963).
7. B. A. KOECHLIN, M. A. SCHWARTZ, G. KROL and W. OBERHAENSLI, *J. Pharmac. exp. Ther.* **148**, 339 (1965).
8. M. A. SCHWARTZ and E. POSTMA, *J. pharm. Sci.* **55**, 1358 (1966).
9. L. H. STERNBACH and E. REEDER, *J. Org. Chem.* **26**, 1111 (1961).
10. L. H. STERNBACH, B. A. KOECHLIN and E. REEDER, *J. org. Chem.* **27**, 4671 (1962).
11. G. M. EVERETT and R. K. RICHARDS, *J. Pharmac. exp. Ther.* **81**, 402 (1944).
12. D. W. ESPLIN and D. M. WOODBURY, *J. Pharmac. exp. Ther.* **118**, 129 (1956).