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CORRELATION BETWEEN THE DURATION OF THE ANTICONVULSANT ACTIVITY OF DIAZEPAM AND ITS PHYSIOLOGICAL DISPOSITION IN MICE

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Abstract—A correlation between the duration of the anticonvulsant activity of diazepam [7-chloro-1,3-dihydro-1-methyl-5-phenyl-2H-1,4-benzodiazepin-2-one (Ro 5-2807)] and the physiological disposition of the intact drug and three metabolites—(1) the *N*-demethylated derivative [7-chloro-1,3-dihydro-5-phenyl-2H-1,4-benzodiazepin-2-one (Ro 5-2180)], (2) the hydroxylated derivative [7-chloro-1,3-dihydro-3-hydroxy-1-methyl-5-phenyl-2H-1,4-benzodiazepin-2-one (Ro 5-5345)], and (3) oxazepam [7-chloro-1,3-dihydro-3-hydroxy-5-phenyl-2H-1,4-benzodiazepin-2-one (Ro 5-6789)]—in mice is presented.

Maximal protection for 6 hr against a standard 125 mg/kg s.c. convulsant dose of metrazol was afforded by a single 2·5 mg/kg oral dose of diazepam.

Quantitative ^{3}H distribution after the oral administration of a single 2·5 mg/kg dose of diazepam-^{3}H shows rapid absorption and a rapid increase over 1–30 min in the tissue-to-blood ^{3}H ratios of the brain, muscle, heart, fat and carcass. These ratios thereafter increase gradually or remain constant to 4 hr after drug administration. A 125 mg/kg s.c. injection of metrazol given 30 min after the oral administration of diazepam does not significantly affect the tissue-to-blood ^{3}H ratios nor does it alter the ^{3}H disposition, except between 4 and 6 hr, during which time a definite increase in the total ^{3}H concentration of the blood, brain and muscle is observed. This increase is reflected in an increase in the concentration of the parent compound and of the products of its hydroxylation and desmethylation in blood, brain and muscle.

Differential analyses of blood, brain and muscle tissue samples for diazepam and three of its major metabolites (Ro 5-2180, Ro 5-5345 and Ro 5-6789) from 30 min to 24 hr show that, although the concentration of each component at 30 min is similar both in the absence and presence of metrazol, a definite shift in the slope of the fall-off patterns toward a slower rate of disappearance of the parent compound and of its hydroxylated and desmethylated derivatives is evident when the administration of diazepam is followed by a subcutaneous injection of metrazol. The major constituent in all three tissues, both in the absence and presence of metrazol, is the end product of metabolism, namely oxazepam (Ro 5-6789). Besides being present in much higher concentrations, unlike the parent compound and the other metabolites, Ro 5-6789 maintains a fairly constant level in all three tissues from 30 min to 12 hr and then gradually falls to 24 hr. Although the contribution of the parent compound and its hydroxylated derivative to the duration and degree of protection cannot be accurately defined, it appears that the degree of protection to the convulsant action of metrazol is most closely related to the rate of disappearance of the *N*-desmethyl derivative.

CHLORDIAZEPOXIDE* (7-chloro-2-methylamino-5-phenyl-3H-1,4-benzodiazepine-4-oxide) and its analog, diazepam† (7-chloro-1,3-dihydro-1-methyl-5-phenyl-2H-1,4-benzodiazepin-2-one), were subjected to a battery of pharmacological screening tests[1-4] which revealed their taming, muscle relaxant and anticonvulsant activities. Studies on the metabolism of chlordiazepoxide in the rat, dog, man and mouse have been reported,[5-8] as has the metabolic fate of diazepam in the rat, dog and man.[9, 10] In addition, the pharmacological and clinical effects of these compounds have been studied extensively, yet very little has been done to coordinate the pharmacological activity with their pharmacokinetics and metabolism *in vivo*.

It is the purpose of this study to correlate the duration of the anticonvulsant (antimetrazol) activity of diazepam in mice with blood levels and tissue distribution patterns of the parent compound and its major metabolites.

MATERIALS AND METHODS

Synthesis of diazepam-^3H. The compound labeled in the 5-phenyl ring was prepared from randomly labeled ^{3}H-benzoic acid.[10] The synthesis procedure was a modification of the method of Sternbach *et al.*[11-13] Radiochemical purity of the final product was established by two-dimensional (2D) thin-layer chromatography (TLC) on Brinkman precoated F_{254} silica gel plates using System I, heptane–chloroform–ethanol (10:10:1), followed by System II, heptane–chloroform–acetic acid–ethanol (5:5:1:0·3), as the developing solvents. The purified product had a specific activity of 2·25 μc/mg. ^{3}H-diazepam was dispersed in physiological saline by the use of Tween 80 and the technique of Meier *et al.*[14]

Radioactivity measurements. The radioactive content of all samples was determined by liquid scintillation counting techniques on a Nuclear-Chicago Mark I scintillation spectrometer equipped with a ^{133}Ba external standard. Based on channel ratios and a counting efficiency curve, the counts per minute were converted to the sample content in disintegrations per minute. Blood and fecal homogenates were additionally assayed for ^{3}H by the combustion technique of Kelly *et al.*,[15] while urine samples were counted in a medium consisting of 100 g naphthalene, 7 g of 2,5-diphenyloxazole (PPO), 50 mg of 1,4-*bis*-2-(4-methyl-5-phenyloxazolyl) benzene (dimethyl POPOP) per liter of dioxane (Matheson, Coleman & Bell). Segments of thin-layer plates were counted in this phosphor additionally containing a 4% suspension of thixotropic gel (Cab-O-Sil).

Metrazol response in mice. CF-1 mice (Carworth Farms, N.Y.) weighing between 20 and 24 g were used in all tests. Metrazol (pentylenetetrazol) was purchased from Bihuber-Knoll Corp., Orange, N.J. The technique and criteria used to clarify the responses in mice to a standard 125 mg/kg subcutaneous dose of metrazol have been previously described.[8] Essentially, the severity of the convulsant response induced by the metrazol injection was classified on the basis of the relative number of mice undergoing a clonic or tonic seizure and on the per cent mortality occurring within 30 min after metrazol injection.

Effect of diazepam on metrazol-induced convulsions. A single 2·5 mg/kg oral dose of diazepam was given "simultaneously" with, and at intervals of 0·5, 1, 2, 4, 6, 8, 12 and 24 hr prior to a single 125 mg/kg subcutaneous injection of metrazol. Two groups of ten males and two groups of ten female mice were used at each time interval. The ani-

* Its hydrochloride is the active ingredient of Librium, Hoffmann-La Roche Inc., Nutley, New Jersey.
† Active ingredient of Valium, Hoffmann-La Roche Inc., Nutley, New Jersey.

mals were observed for a period of 30 min after metrazol injection and their responses recorded.

Distribution of diazepam-^3H. The distribution of total ^3H in blood and tissues was determined in groups of ten animals at 1, 2, 5 and 30 min and at 1, 2, 4, 6, 12 and 24 hr after the oral administration of a single 2·5 mg/kg dose of diazepam-^3H. Two groups of ten mice of both sexes were used at each time interval. In an analogous second set of experiments, a 125 mg/kg subcutaneous dose of metrazol was injected into the animals of each group 30 min after oral pretreatment with diazepam-^3H and the distribution

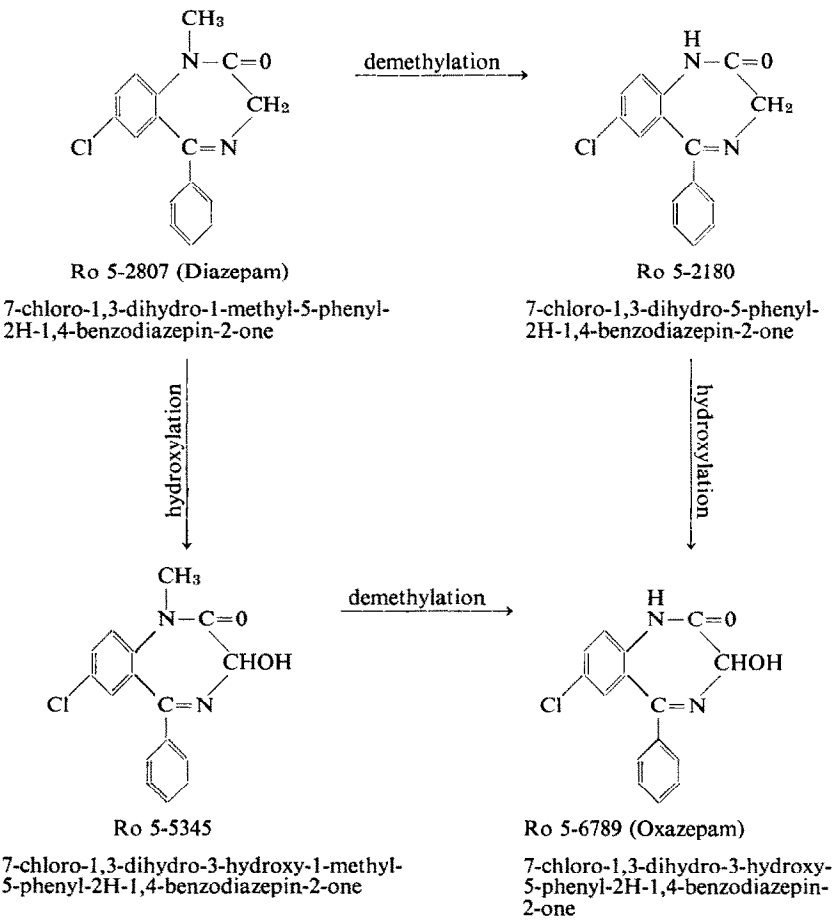


FIG. 1. Major metabolites of diazepam.

of ^3H was studied from 30 min to 24 hr. In both cases, that is in the absence and presence of metrazol, each group comprising ten mice was killed at the appropriate time by decapitation. Heparinized blood was collected from each of the ten animals and equal aliquots were pooled to form a single assay specimen per group or two assay specimens for each time interval (duplicate groups). Individual tissues (see Tables 3 and 4) were excised and pooled, according to their respective groups and

time intervals of sacrifice, for further analysis. Fifty per cent ethanolic homogenates of each pool of tissues were prepared in a Waring Blendor and their ^3H content was determined. Thus, for each tissue and each time period, two analytical values were obtained.

Extraction of diazepam-^3H and its major metabolites from selected tissues. In view of the psychotropic, anticonvulsant and muscle relaxant activity attributed to diazepam, the blood, brain and muscle homogenates were selected to determine the presence and concentration of intact diazepam and its major metabolites. Duplicate aliquots (1–2 g) of each tissue homogenate at each time period were adjusted to pH 6·8 with 1 M phosphate buffer and extracted twice with 10 ml diethyl ether by shaking the tubes gently for 10 min on a mechanical shaker. After centrifugation at 2000 rpm for 15 min, the resultart upper organic layers were combined and the volumes of both the combined ether and aqueous layers recorded. An aliquot of the combined ether layer was evaporated to dryness and counted in order to quantitate the amount of ether-extractable radioactivity, which was expected to consist of the unconjugated "basic components", including the parent compound and the major metabolites illustrated in Fig. 1.

In order to quantitate the "metabolic conjugates" present, the aqueous layer was adjusted to pH 5·3 and incubated with Glusulase (10 μl/ml of aqueous layer) at 37° for 2 hr. Glusulase, a mixture of the enzymes glucuronidase and sulfatase, was obtained from Endo Labs Inc., Garden City, N.Y. After incubation, the aqueous layer was readjusted to pH 7·0 and extracted twice with 10 ml diethyl ether. As described above, the ether extracts were separated, combined and counted for radioactive content.

The aqueous layer was then readjusted to pH 2·0 and extracted twice with 10 ml diethyl ether to quantitate the ether-extractable "acidic components" present in each homogenate. The aqueous phase was finally neutralized and counted to determine the amount of nonextractable radioactivity.

Chromatographic separation and identification of diazepam-^3H and its metabolites. The parent compound and its major metabolites (basic components) present in the combined ether extracts obtained at pH 6·8 were separated by evaporating the extract to dryness under nitrogen, reconstituting the residue in 0·5 ml of a 50% ethanol–ether mixture and subjecting an aliquot of this concentrate to 2D-TLC on Brinkman precoated F_{254} silica gel plates using Systems I and II. Complete separation of the intact drug and its metabolites was achieved in this manner.

In separating the basic components present in the blood, brain and muscle extracts obtained at pH 6·8, 20 μg of unlabeled diazepam and of each metabolite selected to be an internal standard were placed at the origin and also spotted separately (external standards) on the sides of each thin-layer plate. The plate was subsequently developed a distance of 15 cm in each dimension. After development, the plate was scanned under shortwave ultraviolet (u.v.) light and the various u.v.-absorbing component areas were identified on the basis of comparative R_f values of the internal and external standards. Quantitation of the various identified components was achieved by scraping off the u.v.-absorbing areas of silica gel, counting them in a dioxane phosphor containing 4% thixotropic gel, and calculating the percentage of the applied radioactivity migrating with each internal standard. This procedure yielded reproducible values for all the components.

RESULTS AND DISCUSSION

Response to metrazol. The pharmacological responses elicited in 13 groups of ten mice given the standard 125 mg/kg s.c. dose of metrazol alone are presented in Table 1. The relative intensity of the responses is reflected in the percentage of animals undergoing clonic seizures, tonic seizures or death, and in the percentage of animals proceeding from a clonic to the more severe tonic seizure.

Effect of diazepam on metrazol response. The pharmacologic responses to a single 125 mg/kg subcutaneous injection of metrazol given "simultaneously" with and at

TABLE 1. PHARMACOLOGIC RESPONSES IN MICE AFTER SUBCUTANEOUS INJECTION OF A STANDARD 125 mg/kg DOSE OF METRAZOL*

No. of mice	% Response to metrazol in 30 min			
	Clonic seizures	Tonic seizures	Mortality	Clonic undergoing tonic
130	90·0 (8·1)	85·4 (15·4)	73·9 (17·3)	86·9

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

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

Drug	Time of treatment (hr)	% Protection*			
		Clonic seizures	Tonic seizures	Mortality	Clonic from undergoing tonic
MD	simultaneous†	55·6	100·0	100·0	100·0
DM	simultaneous‡	80·6	100·0	100·0	100·0
D-M	0·5	100·0	100·0	100·0	100·0
	1	91·7	100·0	100·0	100·0
	2	91·1	100·0	100·0	100·0
	4	55·6	100·0	100·0	100·0
	6	47·2	97·1	100·0	100·0
	8	25·0	79·5	79·7	74·0
	12	13·9	76·6	72·9	70·5
	24	5·6	35·6	62·4	29·8

* Per cent protection values are calculated on the basis of a 100% response given by mice receiving the standard 125 mg/kg subcutaneous dose of metrazol. For example, at 1 hr, 3 of 40 (7·5%) mice underwent a clonic convulsion. Based on the above criteria this is equivalent to 8·3% of the mice being affected, or conversely, 91·7% being protected.

† Metrazol (125 mg/kg, s.c.) administered 5–10 sec prior to diazepam (2·5 mg/kg, p.o.).

‡ Diazepam (2·5 mg/kg, p.o.) administered 5–10 sec prior to metrazol.

increasing intervals after oral pretreatment with single 2·5 mg/kg doses of diazepam are shown in Table 2. The protection against the convulsant action of metrazol by pretreatment with diazepam is evident from the reduction not only in the per cent of clonic and tonic seizures but also in the survival of all the pretreated animal groups for a period of 6 hr after diazepam administration. At this point, an increase in the severity of the reactions elicited by metrazol is observed in that protection against clonic seizures has decreased to 47·2 per cent. Subsequently, from 8–24 hr the protection afforded by pretreatment with diazepam declines at a faster rate than during

TABLE 3. ^{3}H DISTRIBUTION IN MICE AFTER ORAL ADMINISTRATION OF A SINGLE 2·5 mg/kg DOSE OF DIAZEPAM-^{3}H

Sample	1 min		2 min		5 min		30 min		1 hr		2 hr		4 hr		6 hr		12 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	(μg-equiv./g)	R	(μg-equiv./g)	R	(μg-equiv./g)	R
Blood	0·04 ±0·00		0·44 ±0·08		0·72 ±0·04		0·63 ±0·01		0·62 ±0·02		0·58 ±0·01		0·50 ±0·03		0·58 ±0·02		0·51 ±0·04		0·37 ±0·02	
Brain	0·03 ±0·01	0·8	0·25 ±0·05	0·6	1·10 ±0·18	1·5	1·14 ±0·04	1·8	1·08 ±0·07	1·7	1·14 ±0·04	2·0	0·94 ±0·60	1·9	0·90 ±0·01	1·6	0·78 ±0·04	1·5	0·50 ±0·02	1·4
Muscle	0·03 ±0·01	0·8	0·23 ±0·04	0·5	0·84 ±0·20	1·2	0·87 ±0·04	1·4	0·92 ±0·06	1·5	0·80 ±0·03	1·4	0·67 ±0·01	1·3	0·71 ±0·00	1·2	0·55 ±0·03	1·1	0·38 ±0·01	1·0
Heart	0·08 ±0·01	2·0	1·12 ±0·20	2·5	2·42 ±0·12	3·4	2·28 ±0·10	3·6	2·23 ±0·49	3·6	1·95 ±0·25	3·4	1·58 ±0·34	3·2	1·47 ±0·17	2·5	1·20 ±0·10	2·4	0·82 ±0·01	2·2
Lung	0·13 ±0·03	3·3	0·95 ±0·13	2·2	1·88 ±0·28	2·6	1·18 ±0·04	1·9	1·21 ±0·13	2·0	1·06 ±0·18	1·8	0·87 ±0·03	1·7	1·04 ±0·02	1·8	0·69 ±0·09	1·4	0·53 ±0·08	1·4
Liver	8·63 ±0·20	215·8	14·92 ±1·88	33·9	8·61 ±0·14	12·0	4·97 ±0·42	7·9	4·84 ±0·32	7·8	4·64 ±0·26	8·0	3·86 ±0·35	7·7	4·44 ±0·21	7·7	3·99 ±0·00	7·8	2·30 ±0·13	6·2
Spleen	0·07 ±0·03	1·8	0·76 ±0·06	1·7	1·11 ±0·12	1·5	1·03 ±0·22	1·6	1·05 ±0·00	1·7	0·95 ±0·10	1·6	0·78 ±0·07	1·6	0·72 ±0·12	1·2	0·55 ±0·11	1·1	0·35 ±0·04	0·9
Kidney	0·25 ±0·04	6·3	1·09 ±0·17	2·5	2·55 ±0·38	3·5	2·09 ±0·09	3·3	2·18 ±0·24	3·5	2·10 ±0·20	3·6	1·65 ±0·04	3·3	1·67 ±0·20	2·9	1·15 ±0·29	2·3	0·89 ±0·08	2·4
Fat	0·19 ±0·07	4·8	0·25 ±0·06	0·6	0·89 ±0·22	1·2	1·53 ±0·09	2·4	1·55 ±0·12	2·5	1·07 ±0·18	1·8	0·59 ±0·04	1·2	0·56 ±0·02	1·0	0·44 ±0·06	0·9	0·29 ±0·08	0·8
Carcass	0·16 ±0·01	4·0	0·29 ±0·04	0·7	0·88 ±0·02	1·2	0·87 ±0·03	1·4	0·93 ±0·03	1·5	0·75 ±0·07	1·3	0·60 ±0·06	1·2	0·71 ±0·01	1·2	0·57 ±0·00	1·1	0·35 ±0·05	0·9

* The ^{3}H concentration (dpm/g) was divided by the specific activity of the dose to obtain μg equivalent/gram (μg-equiv./g).
† Ratio of tissue-to-blood ^{3}H concentration. Values are mean of 2 groups of 10 animals.

the first 6-hr period. This decline (Table 2) is seen not only in an increase in the number of animals undergoing clonic and tonic seizures but also in the per cent of animals proceeding from a clonic to the more severe tonic seizure. However, this decline in protection against seizures is not reflected in an increase in the per cent mortality. The difference in the degree of protection against death (62·4%) and the convulsive responses (5·6–35·6%) 24 hr after pretreatment with diazepam raises new questions as to: (1) whether there are separate and distinct biochemical or physiological mechanisms for the induction, by metrazol, of a convulsive response and death; and (2) the relationship of these mechanisms to the mode of action and specificity of the parent compound and each of its major metabolites.

Of significance are the results obtained when diazepam was administered just before or immediately after metrazol. When given 5–10 sec after the standard subcutaneous dose (125 mg/kg) of metrazol, a certain degree of protection was evident, while when given seconds before metrazol (Table 2), diazepam afforded a higher degree of protection against clonic seizures. The significance of the order of diazepam administration in these "simultaneously" treated groups was evaluated statistically in relation to the per cent protection against clonic seizures. It was found that diazepam offered protection at the 95 per cent level of confidence ($P = 0{\cdot}05$) against clonic seizures.

Distribution of diazepam-^3H. The tissue distribution of ^3H between 1 min and 24 hr after a single 2·5 mg/kg oral dose of diazepam-^3H together with the ratios of tissue-to-blood ^3H are given in Table 3. The corresponding data when diazepam-^3H administration was followed after 30 min by the standard subcutaneous injection of 125 mg/kg of metrazol are given in Table 4.

After the administration of diazepam-^3H alone (Table 3), the highest blood ^3H concentrations are observed between 5 min and 1 hr. Thereafter, a gradual decline in level occurs to 12 hr, followed by a more rapid fall-off to 24 hr post administration. The ratios of tissue-to-blood ^3H concentration are greater than one in all tissues. With the exception of the brain, muscle and heart, peak ratios are evident in all tissues as early as 1 min after oral dosing. The concentration of ^3H in the brain and muscle in the first 2 min after dosing are approximately 50–75 per cent of those of blood.

Comparison of the data in Table 3 with those found after the subcutaneous injection of metrazol 30 min after oral administration of diazepam-^3H (presented in Table 4) indicates some changes in the overall disposition of the latter. The blood ^3H levels are lower in the presence of metrazol. Between 4 and 6 hr, ^3H levels in certain tissues such as the blood, brain, muscle, heart, liver, kidney and fat increase (Table 4), while others including the lung, spleen and carcass remain relatively unchanged. Of particular interest is the increase between 4 and 6 hr in the ^3H concentration of the blood, brain and muscle, which returns to and even surpasses the values obtained in the absence of metrazol (Table 3). This observation, which may be interpreted as changes in tissue compartment size or cell permeability, was also observed and reported in a previous study by Coutinho *et al.*[8] The mechanism by which metrazol may produce these changes remains unexplained.

The ^3H content of the gastrointestinal tract (GIT), urine and feces after the administration of diazepam-^3H alone and when followed by metrazol injection is given in Table 5. No major differences in the ^3H patterns are observed. Absorption from the

TABLE 4. ^3H DISTRIBUTION IN MICE AFTER A 125 mg/kg SUBCUTANEOUS DOSE OF METRAZOL GIVEN 30 min AFTER A SINGLE 2·5 mg/kg ORAL DOSE OF DIAZEPAM-^3H

Sample	30 min		1 hr		2 hr		4 hr		6 hr		12 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	0·60 ±0·00		0·60 ±0·11		0·63 ±0·00		0·44 ±0·00		0·62 ±0·04		0·50 ±0·00		0·35 ±0·01	
Brain	1·24 ±0·08	2·1	1·19 ±0·06	2·0	1·20 ±0·03	1·9	0·87 ±0·09	2·0	1·03 ±0·08	1·7	0·82 ±0·02	1·6	0·43 ±0·08	1·2
Muscle	0·96 ±0·08	1·6	0·89 ±0·05	1·5	0·81 ±0·02	1·3	0·60 ±0·07	1·4	0·74 ±0·08	1·2	0·54 ±0·02	1·1	0·34 ±0·03	1·0
Heart	2·34 ±0·22	3·9	1·83 ±0·03	3·1	1·82 ±0·27	2·9	1·05 ±0·09	2·4	1·12 ±0·12	1·8	1·04 ±0·04	2·1	0·62 ±0·06	1·8
Lung	1·14 ±0·10	1·9	1·12 ±0·14	1·9	1·02 ±0·00	1·6	0·89 ±0·06	2·0	0·94 ±0·05	1·5	0·76 ±0·06	1·5	0·42 ±0·02	1·2
Liver	5·16 ±0·04	8·6	4·04 ±0·15	6·7	4·08 ±0·01	6·5	3·59 ±0·54	8·2	3·67 ±0·23	5·9	3·21 ±0·11	6·4	1·95 ±0·04	5·6
Spleen	1·06 ±0·08	1·8	0·98 ±0·04	1·6	1·00 ±0·14	1·6	0·69 ±0·05	1·6	0·67 ±0·01	1·1	0·57 ±0·02	1·1	0·38 ±0·10	1·1
Kidney	2·07 ±0·23	3·5	1·85 ±0·45	3·1	1·92 ±0·06	3·0	1·23 ±0·13	2·8	1·44 ±0·09	2·3	1·29 ±0·13	2·6	0·72 ±0·06	2·1
Fat	1·55 ±0·21	2·6	1·44 ±0·13	2·4	1·02 ±0·01	1·6	0·58 ±0·09	1·3	0·70 ±0·10	1·1	0·49 ±0·04	1·0	0·24 ±0·01	0·7
Carcass	0·93 ±0·16	1·6	0·87 ±0·22	1·5	0·78 ±0·08	1·2	0·54 ±0·08	1·2	0·56 ±0·07	0·9	0·61 ±0·07	1·2	0·31 ±0·01	0·9

* The ^3H concentration (dpm/g) was divided by the specific activity of the dose to obtain μg equivalent/gram (μg-equiv./g).
† Ratio of tissue-to-blood ^3H concentration. Values are mean of 2 groups of 10 animals.

TABLE 5. ABSORPTION, EXCRETION AND RECOVERY OF ^3H AFTER ORAL ADMINISTRATION OF A SINGLE 2·5 mg/kg DOSE OF DIAZEPAM-^3H ALONE (D) AND AFTER A 125 mg/kg S.C. INJECTION OF METRAZOL GIVEN 30 min AFTER DIAZEPAM (DM)

Specimen	Drug	% ^3H of dose administered									
		1 min	2 min	5 min	30 min	1 hr	2 hr	4 hr	6 hr	12 hr	24 hr
GIT*	D	79·94 ±6·43	69·94 ±7·06	60·73 ±4·72	53·82 ±1·96	47·77 ±0·98	49·01 ±6·05	42·73 ±2·63	35·88 ±3·58	27·93 ±4·75	21·55 ±0·14
	DM				55·64 ±3·80	53·42 ±4·09	49·61 ±2·22	52·35 ±3·94	40·30 ±2·94	26·12 ±1·10	21·39 ±1·42
Urine	D							14·39 ±1·43	19·77 ±1·55	30·63 ±1·46	38·10 ±5·67
	DM							11·92 ±2·28	18·54 ±0·59	28·92 ±1·26	42·16 ±2·33
Feces	D							1·04 ±0·21	1·37 ±0·21	4·46 ±0·72	9·28 ±1·13
	DM							0·04 ±0·02	0·20 ±0·16	2·69 ±1·46	7·24 ±1·45
Tissues	D	20·05 ±3·42	30·19 ±3·67	40·51 ±6·11	45·02 ±2·03	47·06 ±0·63	37·92 ±4·34	30·50 ±0·51	32·80 ±1·44	25·94 ±1·43	15·48 ±2·31
	DM				45·80 ±2·21	38·47 ±3·02	36·45 ±1·29	26·00 ±2·16	28·73 ±1·96	25·64 ±0·26	13·21 ±0·21
Recovery	D	99·99 ±3·01	100·18 ±3·43	101·24 ±1·39	98·83 ±0·07	94·83 ±1·61	86·92 ±1·71	88·65 ±1·92	89·81 ±0·37	88·95 ±3·88	84·40 ±2·17
	DM				101·42 ±1·61	91·89 ±3·06	86·06 ±1·84	90·36 ±1·28	87·76 ±2·69	83·38 ±4·07	83·98 ±5·40

* GIT = gastrointestinal tract.

TABLE 6. PER CENT OF THE VARIOUS ETHER-EXTRACTABLE COMPONENTS COMPRISING THE TOTAL ^3H PER gram OF BLOOD, BRAIN AND MUSCLE AFTER A SINGLE 2·5 mg/kg ORAL DOSE OF DIAZEPAM-^3H TO MICE

Classification and conditions of component extraction	Sample	% Components/g of tissue									
		1 min	2 min	5 min	30 min	1 hr	2 hr	4 hr	6 hr	12 hr	24 hr
Basic components extracted at pH 6·8	Blood	90·3± 4·6	92·1±3·4	88·9±3·6	78·6±3·8	81·1±3·4	75·6±8·6	71·5±6·4	67·3±9·9	56·8±0·9	49·8±3·1
	Brain	53·1± 9·9	96·6±2·0	94·5±4·6	92·6±2·0	90·1±2·0	91·5±6·5	86·0±2·9	87·3±4·6	83·9±4·9	75·2±5·7
	Muscle	74·0±12·7	97·5±2·8	92·5±4·5	89·7±4·9	86·2±4·2	86·1±6·0	85·2±5·1	84·0±3·9	78·1±4·4	73·8±5·8
Conjugates extracted at pH 7·0 after Glusulase	Blood	5·1± 0·6	3·2±0·4	4·2±0·9	6·5±1·2	8·3±3·6	8·7±1·2	8·7±2·3	8·2±0·9	8·0±0·7	7·6±1·1
	Brain	0·3± 0·6	0·6±0·4	1·3±0·8	1·2±0·5	0·7±0·3	0·9±0·2	1·0±0·1	0·8±0·2	0·8±0·1	0·8±0·2
	Muscle	0·7± 1·0	0·7±0·3	0·6±0·1	1·8±0·3	1·3±0·4	1·6±0·1	1·7±0·1	1·7±0·3	1·8±0·1	2·0±0·5
treatment at pH 5·3											
Acidic components extracted at pH 2·0	Blood	5·5± 0·9	0·7±0·3	0·7±0·2	3·6±1·8	4·2±1·7	4·6±1·2	5·0±1·1	4·7±1·1	5·5±0·6	6·0±0·8
	Brain	1·4± 1·3	0·2±0·1	0·4±0·1	0·4±0·2	0·3±0·1	0·4±0·2	0·4±0·3	0·4±0·2	0·5±0·2	0·4±0·2
	Muscle	1·0± 1·5	0·2±0·1	0·1±0·0	0·5±0·2	0·3±0·1	0·6±0·1	0·7±0·2	0·7±0·2	0·9±0·2	1·0±0·2
Nonextractable components in aqueous residue	Blood	0·0± —	1·3±0·9	3·9±0·7	9·0±0·9	6·8±1·9	8·4±3·5	16·0±6·3	13·6±3·9	17·3±5·7	29·0±8·5
	Brain	42·6±17·2	2·7±1·4	1·5±0·2	1·4±0·7	1·6±0·9	2·1±0·6	3·4±0·4	4·3±0·9	6·0±2·0	9·5±0·6
	Muscle	24·2±16·2	1·7±0·8	0·9±0·2	2·7±0·8	2·8±1·1	3·9±0·7	5·7±1·8	6·8±1·9	8·0±2·1	15·3±4·9

GIT during the first 5 min is fairly rapid, approximately 40 per cent of the orally administered ^{3}H dose having left the GIT. From 5 min to 2 hr, there appears to be a decline in the rate of disappearance of intestinal ^{3}H in both groups. At 4 hr after diazepam administration, a 10 per cent difference in the ^{3}H content of the GIT is observed between the two groups. Unlike the major differences observed and reported in previous studies on the ^{14}C absorption from the GIT of chlordiazepoxide-^{14}C in the absence and presence of metrazol,[8] the significance of the ^{3}H difference in the GIT content of its analog, diazepam, is not as clearly evident. In view of the biological half-life of metrazol, which is reported to be approximately 2·5 hr,[16] and the effect of metrazol on processes which control the ^{3}H balance in the GIT, such as enterohepatic circulation and reabsorption, some consideration, however speculative, should be given to the relationship of these effects to the difference in the ^{3}H content of GIT observed between the two groups at 4 hr after the administration of diazepam-^{3}H and the increase in total ^{3}H concentration observed between 4 and 6 hr in several tissues, including the blood, brain and muscle tissues (Table 4).

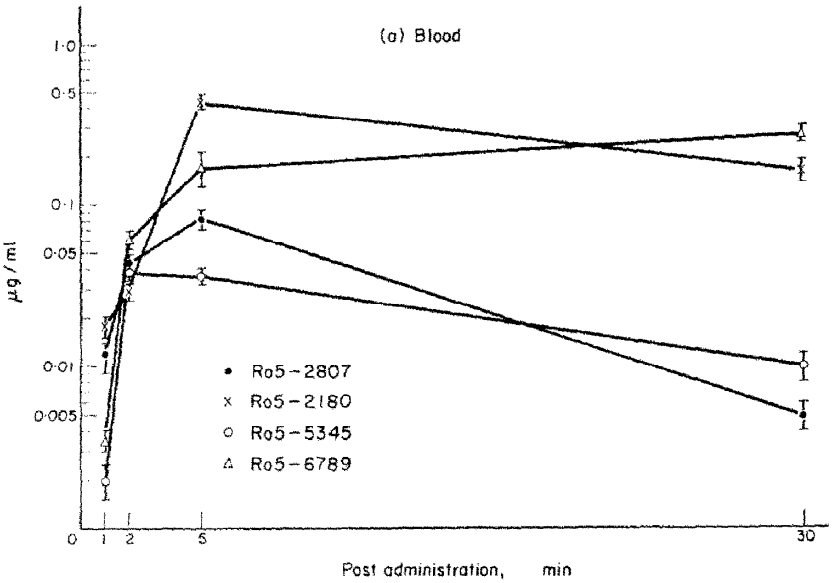
Concentration of diazepam-^{3}H and its major metabolites from blood, brain and muscle. The percentages of the various ether-extractable components comprising the total ^{3}H per gram of blood, brain and muscle at each time period, both in absence and presence of metrazol, are given in Tables 6 and 7. In general, the "basic components" constitute the major percentage of the total ^{3}H per gram of tissue. In the absence of metrazol (Table 6), the concentration of these basic components in blood decrease from 90 per cent at 1 min after dosing to approximately 50 per cent at 24 hr. This is matched with a corresponding increase in the percentage of polar nonextractable components at the same time intervals. The decrease of the basic components content in the circulatory system is indicative of a reduction with time in the concentration of intact diazepam or of its major metabolites or of both. It is not seen to occur as markedly in the brain or muscle tissue. This difference in the rate of ^{3}H depletion between the blood on one hand and the brain and muscle tissues on the other is also seen in the presence of metrazol (Table 7). Thus the differential extraction of blood, brain and muscle presented in Tables 6 and 7 identifies the ^{3}H content of the various tissues and the tissue-to-blood ^{3}H ratio trends given in Tables 2 and 3 as being attributable in the major part to the presence of intact diazepam or of its major metabolites or of both (Fig. 1) rather than to the presence of the other ether-extractable components. The percentage and level of the metabolic "conjugates" and "acidic components" both in the absence and presence of metrazol remain fairly constant in all three tissues over the 24-hr period.

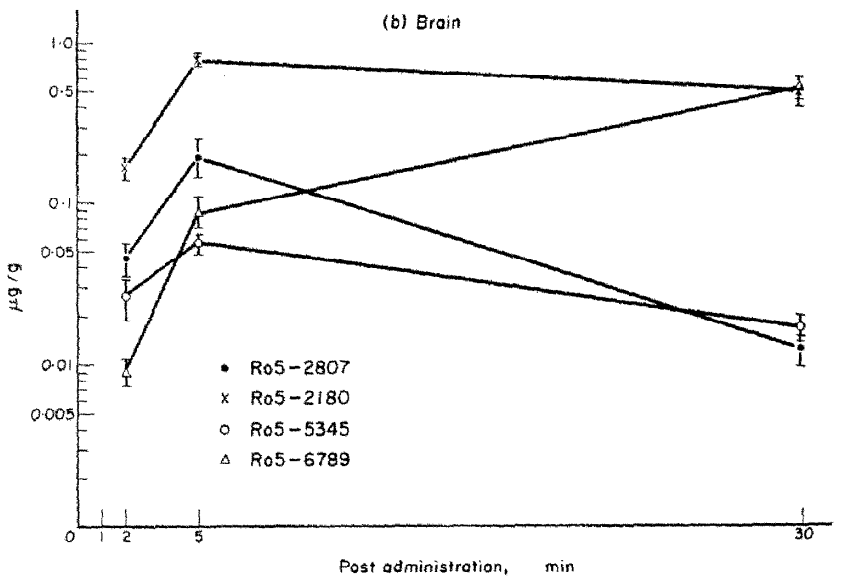
Comparative results of the qualitative and quantitative analyses of the basic components extracts of blood, brain and muscle in the absence and presence of metrazol are shown in Figs. 2 and 3. Diazepam and the major metabolites given in Fig. 1 accounted for 91–97 per cent of the total basic components activity at all the various time intervals, indicating the absence of any other major biotransformation product.

It is apparent from Fig. 2 that the metabolic pattern of diazepam in blood, brain and muscle is very similar and proceeds very rapidly. Measurable levels of the parent compound and its metabolites are obtained in all three tissues as early as 1 min (Fig. 2) after the oral administration of diazepam alone. The concentration of intact diazepam, as well as of the products of its demethylation (Ro 5-2180) and hydroxylation (Ro 5-5345), intermediates in the formation of oxazepam (Ro 5-6789), reaches peak

TABLE 7. PER CENT OF THE VARIOUS ETHER-EXTRACTABLE COMPONENTS COMPRISING THE TOTAL ^3H PER gram OF BLOOD, BRAIN AND MUSCLE AFTER A 125 mg/kg S.C. DOSE OF METRAZOL GIVEN 30 min AFTER A SINGLE 2·5 mg/kg P.O. DOSE OF DIAZEPAM-^3H

Classification and conditions of component extraction	Sample	% Components/g of tissue						
		30 min	1 hr	2 hr	4 hr	6 hr	12 hr	24 hr
Basic components extracted at pH 6·8	Blood	80·4 ± 7·3	79·4 ± 2·4	73·9 ± 1·8	74·8 ± 6·1	70·7 ± 6·3	66·4 ± 1·5	48·3 ± 4·9
	Brain	93·7 ± 4·1	91·6 ± 7·5	94·2 ± 7·5	90·5 ± 6·7	91·8 ± 9·9	92·8 ± 2·1	86·8 ± 1·6
	Muscle	89·1 ± 3·9	87·6 ± 6·1	91·8 ± 1·7	84·4 ± 10·8	88·5 ± 3·5	88·0 ± 2·6	79·7 ± 3·2
Conjugates extracted at pH 7·0 after Glusulase treatment at pH 5·3	Blood	6·4 ± 1·8	6·1 ± 2·0	8·2 ± 1·4	10·0 ± 1·0	8·8 ± 2·8	7·4 ± 1·3	7·3 ± 1·1
	Brain	0·9 ± 1·4	1·0 ± 0·2	0·8 ± 0·2	1·0 ± 0·5	0·9 ± 0·4	1·2 ± 0·3	1·4 ± 0·4
	Muscle	1·5 ± 0·2	1·4 ± 0·1	1·8 ± 0·1	1·6 ± 0·5	1·7 ± 0·5	2·3 ± 0·3	2·5 ± 0·4
Acidic components extracted at pH 2·0	Blood	4·5 ± 0·9	4·0 ± 2·3	6·4 ± 2·1	3·3 ± 0·2	3·4 ± 0·9	5·3 ± 1·3	7·0 ± 2·0
	Brain	0·5 ± 0·4	0·2 ± 0·0	0·3 ± 0·0	0·3 ± 0·0	0·3 ± 0·1	0·3 ± 0·1	0·5 ± 0·2
	Muscle	0·5 ± 0·1	0·5 ± 0·0	0·7 ± 0·2	0·6 ± 0·1	0·7 ± 0·1	0·8 ± 0·2	1·1 ± 0·1
Nonextractable components in aqueous residue	Blood	6·5 ± 1·0	10·1 ± 2·5	12·0 ± 3·3	13·3 ± 0·8	14·2 ± 0·8	20·2 ± 5·8	40·3 ± 9·6
	Brain	2·8 ± 1·4	0·9 ± 0·5	1·6 ± 0·3	3·5 ± 0·8	3·8 ± 1·1	5·2 ± 0·9	11·5 ± 1·2
	Muscle	3·6 ± 1·8	2·7 ± 1·1	4·1 ± 0·6	4·7 ± 1·0	7·1 ± 3·7	9·5 ± 2·9	14·7 ± 0·2


(a) Blood
1·0
0·5
0·1
0·05
0·01
0·005
µg / ml
• Ro5-2807
x Ro5-2180
o Ro5-5345
Δ Ro5-6789
0
1
2
5
30
Post administration, min


(b) Brain
1·0
0·5
0·1
0·05
0·01
0·005
µg / g
• Ro5-2807
x Ro5-2180
o Ro5-5345
Δ Ro5-6789
0
1
2
5
30
Post administration, min

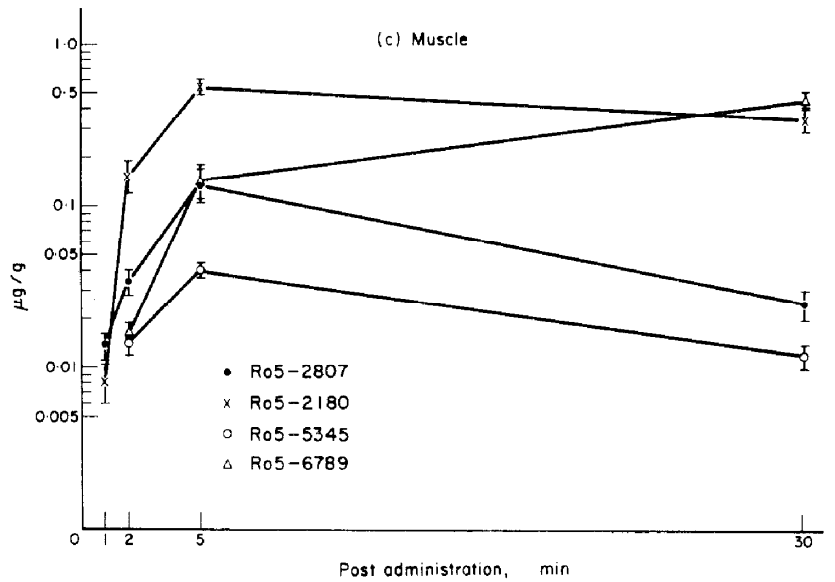
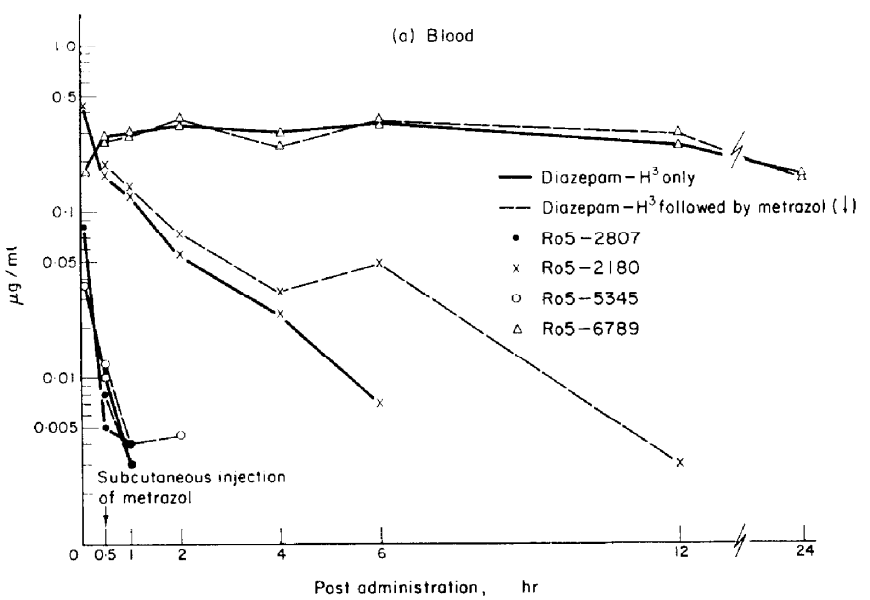


FIG. 2. Concentration of diazepam and metabolites between 0 and 30 min in mouse blood (a), brain (b) and muscle (c) after oral administration of diazepam-^{3}H.



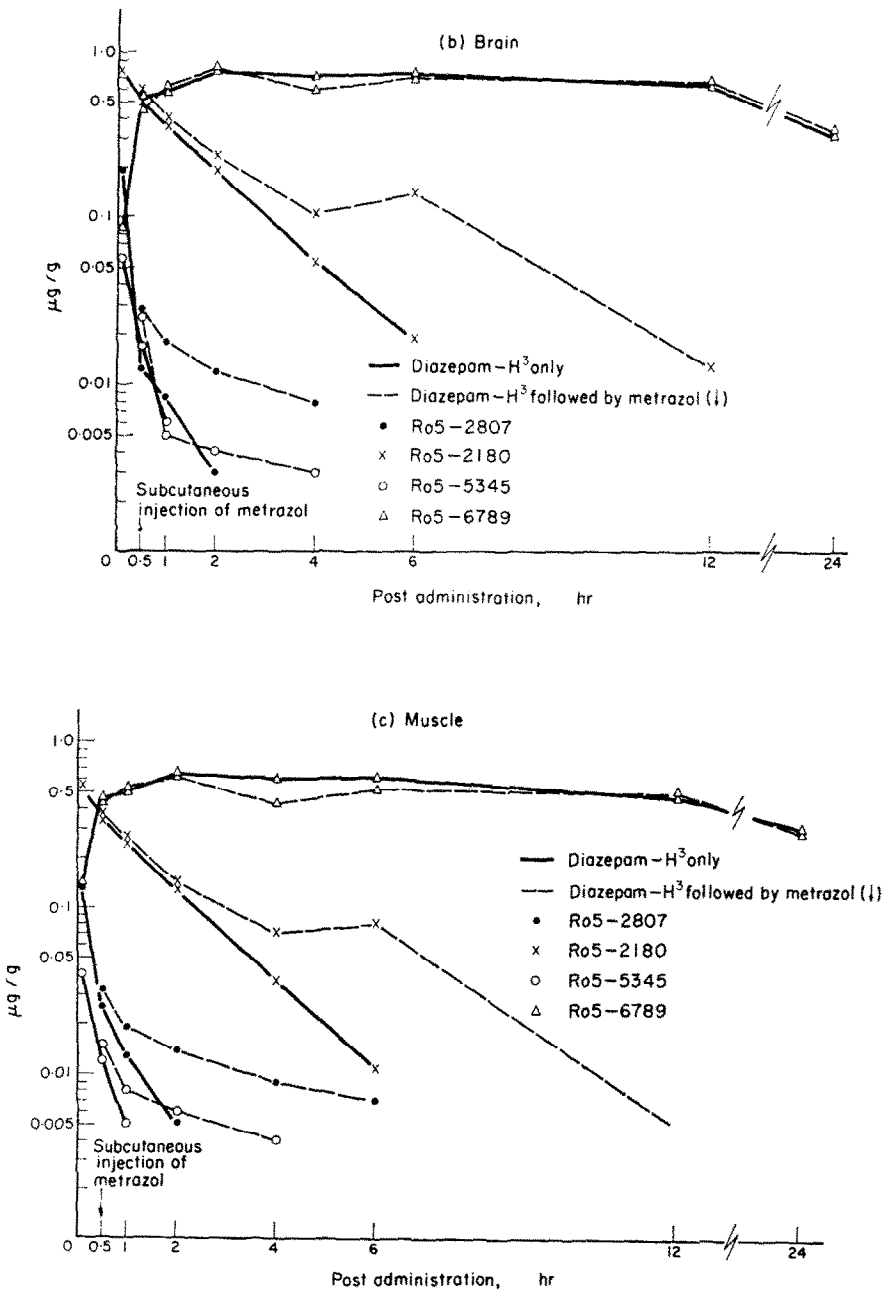


FIG. 3. Concentration of diazepam and metabolites in mouse blood (a), brain (b) and muscle (c) after oral administration of diazepam-^{3}H alone and when an s.c. injection of metrazol was given 0·5 hr after the diazepam.

values in all three tissues at 5 min. Subsequently (Fig. 3), levels of diazepam and Ro 5-5345 fall rapidly to unmeasurable values at 2 and 4 hr, respectively, while the concentration of Ro 5-2180 decreases gradually to unmeasurable levels between 6 and 12 hr after dosing. The concentration of Ro 5-6789 reaches a maximum 30 min after oral administration of the parent compound, at which point its level exceeds that of the other biotransformation product and maintains a relatively constant and predominant value in all three tissues for 12 hr. Thereafter a gradual fall in level occurs to 24 hr after drug administration.

The effect of metrazol on the disposition of diazepam-^{3}H becomes evident on comparison of the differential patterns of the intact drug and its metabolic components from 30 min after drug administration (Fig. 3). In the presence of metrazol, the rate of disappearance of the parent compound, Ro 5-2180 and Ro 5-5345 from all three tissues decreases, while that of Ro 5-6789, the end product of N_1-demethylation and C_3-hydroxylation, is not significantly affected. In both cases, namely in the absence or presence of metrazol, N_1-demethylation and C_3-hydroxylation occur rapidly compared to the process of elimination, by conjugation or clearance, of the end product, Ro 5-6789. Furthermore, formation of Ro 5-2180 by the N_1-demethylation of Ro 5-2807 is occurring relatively faster compared to Ro 5-2180 elimination by excretion or hydroxylation to Ro 5-6789. In contrast to the fall-off patterns obtained when diazepam is given alone, a definite shift in the slope of the fall-off curves toward a slower rate of disappearance (Fig. 3) of Ro 5-2807, Ro 5-2180 and Ro 5-5345 is evident when the administration of diazepam is followed by a subcutaneous injection of metrazol.

In attempting to correlate the drug level patterns of diazepam or its metabolites or of both with the anticonvulsant activity of diazepam, the physiologic disposition patterns of diazepam and its metabolites, as modified by the convulsant metrazol, are of primary concern.

As shown in Table 2, the anticonvulsant activity of diazepam is evident in the "simultaneously" treated groups within seconds of its oral administration. Of significance at this time are the extremely low concentrations of the parent compound or of its metabolites or of both in all three tissues that are responsible for protection against the metrazol-induced responses. Maximum protection from clonic seizures is obtained from 30 min to 2 hr and declines to a 50 per cent value between 4 and 6 hr (Table 2). Additionally, protection from the more severe tonic seizures and from death is maintained at 100 per cent up to 6 hr. From 30 min to 6 hr, all four components, namely the parent compound, Ro 5-2180, Ro 5-5345 and Ro 5-6789 are present in all three tissues (Figs. 2 and 3). Thus, the protection against the convulsant responses elicited by metrazol during this period could be attributed to the presence of any of these components. However, the relationship of the concentration of each component in blood, brain and muscle to the anticonvulsant effect gives us an insight into the relative contribution of each component to the protection afforded against metrazol. From Fig. 3, which represents the concentration of each component over the experimental period, we see that the contribution of intact diazepam and Ro 5-5345 to the per cent protection from 30 min to 4 hr (Table 2) cannot be accurately defined. That the protection decreases after 6–12 hr (Table 2) along with the disappearance of Ro 5-2180 (Fig. 3), a period when the levels of Ro 5-6789 remain unchanged, is obvious. In addition, the decrease in concentration of Ro 5-2180 appears to be

associated with an increase in the severity of the convulsive response as seen in the rapid increase in the number of mice proceeding from a clonic seizure to the more severe tonic seizure (Table 2) between 6 and 24 hr. Thus it seems valid to conclude that, on a microgram per milliliter basis, Ro 5-2180 contributes to a greater extent to the protective effect than does Ro 5-6789. That Ro 5-6789 contributes to the duration of the protective effect is evidenced by the fact that both the concentration of Ro 5-6789 and a certain degree of the protective effect persist as long as 24 hr after drug administration, at which time neither the parent compound nor its hydroxylated or demethylated derivatives are present.

In summary, we have shown that the duration of the anticonvulsant (antimetrazol) activity of diazepam in mice appears to be associated with the concentration and presence of both Ro 5-2180 and Ro 5-6789.

Since both Ro 5-2180 and Ro 5-6789 have been shown to possess antimetrazol activity,[4] these results suggest that the degree of this protective effect is more closely related to the presence of the *N*-demethylated derivative (Ro 5-2180) in these tissues rather than to the other biotransformation products. It must be pointed out that the conclusions drawn from the data presented serve only to relate the drug level patterns of diazepam and its major metabolite to their antimetrazol activity. The results obtained should not be interpreted to exclude the possibility that the pharmacologic effectiveness of diazepam may result from the triggering or alteration, by the parent compound or its biotransformation products, of some biochemical mechanisms or parameters which may or may not be dependent on the quantitative persistence of drug and which have yet to be investigated.

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