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DISTRIBUTION AND METABOLISM OF BARBITAL-^{14}C IN TOLERANT AND NONTOLERANT RATS*

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Abstract—Tolerance to barbital, as evidenced by a significant decrease in sleeping time, was induced in rats by chronic barbital administration. In an effort to elucidate the mechanism of tolerance, barbital-^{14}C distribution and metabolism were compared between tolerant and nontolerant rats. Levels of radioactivity in brain, plasma, and urine did not significantly differ between the two groups. This indicated that tolerance to barbital cannot be ascribed to decreased drug absorption, to increased excretion of the drug, or to a decreased permeability of the blood–brain barrier. Tolerant rats metabolize barbital in a manner qualitatively similar to that of nontolerant rats, as indicated by the recovery of the same three barbital metabolites from urine of either tolerant or control animals. The quantity of barbital metabolites excreted by the tolerant rats was not significantly higher than control values.

TOLERANCE to some of the hypnotic barbiturates has been clearly demonstrated and, for several of these drugs, convincingly attributed to accelerated metabolism of the drug.[1, 2] Studies of barbital, a drug that undergoes little metabolic degradation[3] do not agree that tolerance can be developed. This report deals with experiments undertaken to determine whether tolerance to barbital could be clearly demonstrated in rats and, if so, by what mechanism.

METHODS

Induction of barbital tolerance

Preliminary studies indicated that repeated periods of anesthesia impaired the health of the animals. A regimen was therefore adopted in which tolerance could be induced from the lowest possible dose of barbital given for the shortest possible period of time. Barbital was intraperitoneally administered to male weanling rats of the Holtzman strain, weighing 45 to 55 g, over a 13-day period. The initial dose of 100 mg/kg was increased 10 mg/kg daily to 220 mg/kg. A 3-day rest period, which permitted the excretion of residual amounts of the drug, followed. The rats were fasted during the final 24 hr of the rest period to obtain more consistent responses when a challenging dose of barbital was administered.

The presence or absence of tolerance was determined by intraperitoneally injecting the pretreated rats and a group of control rats with a 200 mg dose of barbital/kg, the minimal sleep-inducing dose for all the rats. Latent periods of each group (time from injection to loss of righting reflex) and sleeping periods of each group (time from

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loss of righting reflex to return of righting reflex) were compared. The significance of differences observed was ascertained by the use of a one-tail "t" test. Either a significant increase in the mean latent period or a significant decrease in the sleeping period of the pretreated rats was to be accepted as evidence of tolerance to barbital.

Drug distribution studies

Barbital-^{14}C (5,5-diethylbarbituric acid-2-^{14}C; purchased from Tracerlab, Inc.), with a specific activity of 1·10 mc/mmole, was used. Purity of the labeled compound was checked by paper chromatographic techniques combined with radiochromatogram scanning. Immediately before injection, unlabeled barbital sodium was mixed with barbital-^{14}C and water to yield a 3 or 6% solution containing sufficient radioactivity so that the animals received 10 μc of radioactivity/kg of body weight in a dose of 150 mg of barbital/kg. On each of 4 to 7 days this solution was administered via the tail vein to four tolerant and four control rats which now weighed 95 to 135 g. Immediately after drug administration, penile ligatures were applied to the rats to prevent urination. At various intervals after injection one tolerant and one control animal were killed by decapitation. Blood was collected in an oxalated evaporating dish, centrifuged 5 min at high speed and a 40-μl sample of plasma removed for assay of radioactivity. Brains were perfused with 0·85% saline via the common carotid artery until the perfusate from the contralateral jugular vein was blood-free. The entire brain except for the frontal lobes was removed and weighed. Two slices weighing approximately 150 mg were removed from the dorsal surface of the cerebral hemispheres and assayed for radioactivity. The urinary bladder was isolated, and all urine from the bladder was removed by means of a syringe equipped with a half-inch 27-gauge needle. Saline washings of each bladder and a 20-μl sample of each urine were assayed for radioactivity.

All samples to be radioassayed were placed in counting vials and dissolved in 3 ml of a 3 : 1 mixture of methanolic Hyamine hydroxide (Rohm & Haas) and 30% aqueous potassium hydroxide. After all samples had dissolved, 1 ml of glacial acetic acid and 14 ml of scintillation solvent* were added to each vial. After thorough agitation and chilling in the dark, the samples were counted in a liquid scintillation spectrometer (Packard Instrument Co.).

Drug metabolism studies

Barbital undergoes only slight metabolic degradation.[3] High levels of radioactivity were administered to the rats in order to assure the excretion of measurable quantities of radioactive metabolites. A 6% aqueous solution of barbital-^{14}C containing 24·8 μc/ml was intraperitoneally given in a dose of 150 mg/kg to four tolerant and three control rats weighing 135 to 170 g. Each animal was placed in a metabolism cage, and urine samples were collected at various time intervals. After the volume of urine had been recorded, a 50-μl sample was spotted on Whatman No. 1 chromatography paper.

*Xylene	1 part
p-Dioxane	3 parts
Ethyl Cellosolve	3 parts
Napthalene	80·0 g/L
PPO (2,5-diphenyloxazole)	10·0 g/L
POPOP [1,4-bis-2-(5-phenyloxazolyl)benzene]	0·5 g/L

Ten μg of unlabeled barbital and 10 μg of a suspected barbital metabolite, 5-ethyl-barbituric acid, were also spotted on each chromatogram. The chromatograms were developed in a system of butan-1-ol : water : ammonium hydroxide (340 : 57 : 3, v/v). Developed chromatograms were scanned with a gas-flow strip-scanner equipped with a 4-pi counting head. The area under each peak on the scan, caused by a radioactive spot on the chromatogram, was measured with a compensating polar planimeter and the radioactivity calculated from a previously prepared standard curve.

RESULTS

Induction of barbital tolerance

Table 1 summarizes a variety of treatments with barbital that led to tolerance and

TABLE 1. INDUCTION OF BARBITAL TOLERANCE IN RATS

Expt. no.	n	Days pretreated	Pretreatment dose range (mg/kg)	Barbital effect* Latent period (min)	Barbital effect* Sleeping period (min)
1A	7	10	100–190	57 ± 8·95	123 ± 18·18
	9	0		69 ± 5·99	96 ± 5·64
1B	6	12	150 (daily)	78 ± 17·81	80 ± 6·37
	9	0		69 ± 5·99	96 ± 5·64
1C	5	14	120–150	49 ± 11·15‡	272 ± 24·61‡
	6	0		26 ± 3·39‡	461 ± 6·61‡
		36†			
2	18	18	130–260	35 ± 4·04	310 ± 18·45‡
	18	0		32 ± 3·69	355 ± 18·76‡
3	14	12	150–260	31 ± 2·38	198 ± 10·59‡
	17	0		26 ± 3·80	451 ± 14·81‡
4	7	20	100–290	55 ± 3·29	142 ± 16·60‡
	10	0		58 ± 1·61	307 ± 17·74‡
5	18	13	100–220	74 ± 8·18	284 ± 18·37‡
	17	0		62 ± 6·91	387 ± 21·15‡
6	11	13	100–220	26 ± 2·39	319 ± 22·89‡
	13	0		26 ± 1·00	393 ± 14·90‡

n = Number of animals.

* Animals were challenged with barbital, 200 mg/kg, i.p., except in experiment 4 in which 160 mg/kg was administered (values are means ± S.E.).

† The animals did not become tolerant in experiments 1A and 1B. They were continued on barbital treatment until tolerance was achieved (experiment 1C) after pretreatment totalling 36 days.

‡ Mean values differ significantly ($P < 0{\cdot}05$) as determined by a one-tail 't' test.

includes two (1A and 1B) that did not. Experiments 1A and 1B were unsuccessful, but the rats were continued on barbital treatment according to the dosage schedule of experiment 1C. Tolerance finally developed after a total of 36 days of barbital administration. All groups of pretreated rats exhibited significantly shorter sleeping periods with the exception of groups 1A and 1B but only one group (1C) exhibited a significantly longer latent period ($P < 0{\cdot}05$). The animals made tolerant in experiment 5 were used in the 0 to 180-min study of distribution; animals from experiment 6 were used in the 360-min study of distribution and in the metabolism studies.

Drug distribution studies

Levels of radioactivity in brain, plasma, and urine in tolerant and control animals are presented in Fig. 1. At no time interval was there a significant difference between the tolerant rats and controls with respect to concentrations of radioactivity in the brain or in plasma. The total output of radioacitvity in the urine also did not significantly differ between tolerant animals and controls.

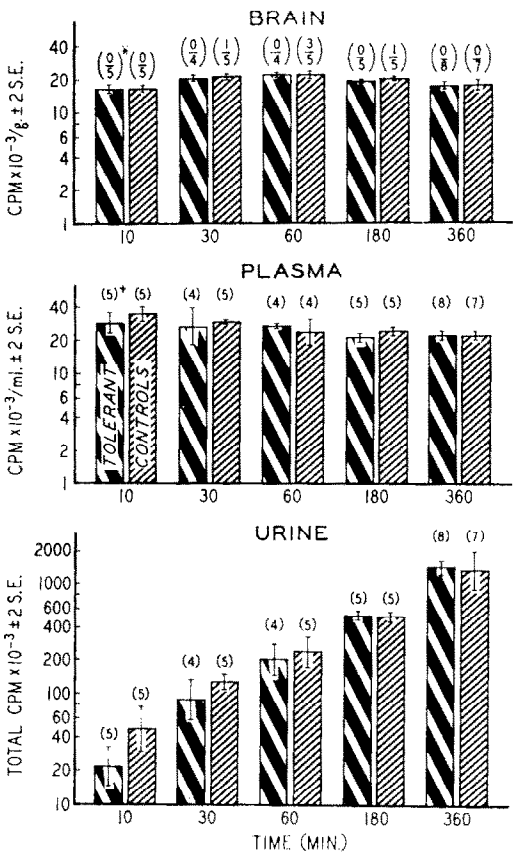

FIG. 1. Radioactivity in brain, plasma, and urine of tolerant and control rats after intravenous administration of barbital-^{14}C (150 mg/kg, 10 μc/kg).

* Ratios in parentheses represent the number of animals asleep when sacrificed per total number of animals.

† Figures in parentheses represent the number of animals.

Barbital metabolism studies

Chromatograms of tolerant rat urine contained four radioactive areas. These zones were also found on chromatograms of control rat urine. It was felt that some of these radioactive zones might represent one or more of the radioactive compounds isolated by Goldschmidt and Wehr[3] who found: A, barbital (accounting for 95 per cent of the dose); B, 5-ethylbarbituric acid (2·5 per cent); C, 5-ethyl-5-(2-hydroxyethyl)barbituric acid (0·8 per cent); D, a conjugate of C (2·0 per cent) in urine of rats given barbital.

On our chromatograms the spot farthest from the origin was identified as unchanged barbital. It contained the greatest amount of radioactivity and had an R_F of 0·75. Several chromatograms were developed with the solvent system employed by Goldschmidt and Wehr. The spot farthest from the origin again contained the most radioactivity but at an R_F of 0·82. Goldschmidt and Wehr reported an R_F value of 0·89 for barbital.

R_F values of the three remaining radioactive compounds are summarized in Table 2.

TABLE 2. MEAN R_F VALUES OF RADIOACTIVE BARBITAL METABOLITES IN URINE OF TOLERANT AND NONTOLERANT RATS*

Spot no.	Metabolite	Tolerant (19)	Control (12)
1	Conjugates(?)	0·03	0·03
2	5-Ethylbarbituric acid	0·13	0·14
3	Unknown	0·39	0·36

* Solvent system: butan-l-ol:water:ammonium hydroxide (340:57:3, v/v). Numbers in parentheses represent the number of chromatograms scanned. Barbital-^{14}C was intraperitoneally administered at a dose of 150 mg/kg (62 μc/kg).

Spot number one was essentially at the origin of the chromatogram and may have been one of the metabolites found by Goldschmidt and Wehr—namely, the glucuronide of 5-ethyl-5-(2-hydroxyethyl)barbituric acid or other conjugated barbital metabolites. It may merely have represented residual amounts of radioactivity that failed to migrate. The second radioactive compound was probably 5-ethylbarbituric acid. R_F values of this spot on chromatograms of urine from tolerant and control rats were 0·13 and 0·14 respectively. The zone containing reference 5-ethylbarbituric acid was sprayed with 0·5 N NaOH and viewed under a short-wave ultraviolet light.[4] A single quenched spot with an R_F value of 0·12 was observed. The third compound could not be identified.

The quantitative aspects of barbital metabolism are summarized in Table 3. During the 24 hr after administration of barbital-^{14}C, 6·07 per cent of the dose was metabolized in tolerant rats, 3·32 per cent in controls. Chromatograms of urine from tolerant or control animals had the greatest amount of radioactivity, aside from barbital, at the origin. Chromatograms of tolerant rat urine had 3·13 per cent of the administered radioactivity at the origin; chromatograms of control urine had 1·50 per cent of the dose at the origin. In tolerant animals 5-ethylbarbituric acid accounted for 1·72 per cent of the dose, in controls only 0·77 per cent. The unidentified metabolite appeared in similar amounts in urines of tolerant (1·22 per cent) and non tolerant (1·05 per cent) rats. Table 3 also demonstrates the heterogenity of the variances about the sample means. A Mann Whitney "U" test[5] indicated that the percentage of barbital metabolized in the tolerant rats was not significantly higher than the control values ($P > 0·05$).

TABLE 3. URINARY EXCRETION OF BARBITAL METABOLITES BY TOLERANT AND NON-TOLERANT RATS*

Time interval (hr)	n	Metabolite (per cent of dose ± S.E.) Conjugated metabolites (?)	5-Ethylbarbituric acid	Unknown	Total
Tolerant animals (4)					
0–6	4	0·58 ± 0·11	0·58 ± 0·02	0·32 ± 0·01	1·48
6–10	3	0·94 ± 0·36	0·78 ± 0·46	0·31 ± 0·31	2·03
10–12	4	0·23 ± 0·06	0·08 ± 0·02	0·23 ± 0·16	0·54
12–18	4	0·81 ± 0·09	0·14 ± 0·05	0·16 ± 0·07	1·11
18–24	4	0·57 ± 0·04	0·14 ± 0·09	0·20 ± 0·08	0·91
Total		3·13	1·72	1·22	6·07
Control animals (3)					
0–6	3	0·43 ± 0·18	0·36 ± 0·11	0·33 ± 0·08	1·12
6–10	3	0·33 ± 0·06	0·07 ± 0·04	0·20 ± 0·06	0·60
10–12	1	0·04	0·03	0·05	0·12
12–18	3	0·38 ± 0·04	0·17 ± 0·03	0·25 ± 0·04	0·80
18–24	2	0·32 ± 0·05	0·14 ± 0·01	0·22 ± 0·01	0·68
Total		1·50	0·77	1·05	3·32

* Barbital-^{14}C was intraperitoneally administered at a dose of 150 mg/kg (62 μc/kg). Numbers in parentheses represent the number of animals studied; n = number of samples assayed.

DISCUSSION

As indicated by the shortened sleeping periods of the rats chronically treated with barbital, tolerance to the drug was successfully induced. Similar results have previously been obtained by Hoff and Kauders[6] as well as by Seevers and Tatum,[7] who noted a reduction of sleep time in dogs chronically dosed with barbital. The latter investigators employed a constant dosage schedule of 100 mg/kg daily; the former workers increased the dose 100 mg/kg weekly. Krautwald and Oettel[8] reported that dogs which previously slept for 7 hr after an oral dose of barbital (100 mg/kg), slept for only 3 to 4 hr after 6 weeks of daily barbital administration. Tolerance was lost after drug administration had been interrupted for one week. Recently, Remmer *et al.*[9] reported tolerance in rats given barbital subcutaneously in doses of 150 to 200 mg/kg. Tolerant animals did not sleep for a shorter period than controls but awoke with barbital levels in brain 35 per cent higher than those observed after the first injection of barbital. Eddy[10] also did not find a reduction in sleep time in cats orally dosed for several weeks at 15 to 25 per cent of the average fatal dose. Seevers and Tatum[7] attributed Eddy's findings to residual barbital masking the tolerance formed. Their suggestion may also serve to explain our failure to induce tolerance in our initial experiments, in which the challenging dose of barbital was administered on the day immediately after the last pretreatment dose. It may also explain our subsequent success in detecting tolerance in the trials that were preceded by the 72-hr drug-free period. That Conney *et al.*[11] did not observe a diminution of sleeping time after 4 days of barbital treatment in rats might also be ascribed to residual barbital effects combined with the barbital given on the fifth day of the experiment or to an insufficient length of pretreatment.

If tolerance to barbital had been due to impeded penetration of barbital into brain, one would have expected a prolongation of the latent period in tolerant rats. Barbital latent periods in pretreated animals were, however, significantly longer than controls in only one of nine experiments. Maynert and Van Dyke[12] demonstrated that barbital is uniformly distributed throughout the central nervous system. The lack of significant differences in levels of radioactivity between brain cortices of tolerant and control animals may therefore be interpreted to mean that the amount of barbital entering the brains of tolerant rats did not differ from controls. These data indicate that decreased permeability of the blood–brain barrier is not involved in barbital tolerance. In contrast, recent studies by Lal *et al.*[13] suggest that barbital distribution in brain is not uniform. An alteration in the distribution of barbital within the brains of the tolerant rats could have occurred without necessarily altering total brain content of radioactivity. Such an alteration would not have been detected by our analytical methods.

The four radioactive compounds in the urine of tolerant rats are apparently identical with the four radioactive compounds in the urine of control rats. This indicates that tolerant rats metabolize barbital qualitatively as do controls, thereby eliminating altered mode of drug metabolism as the mechanism of tolerance. Tolerant rats metabolized barbital at a slightly, but not significantly, greater rate than nontolerant rats. This was not surprising in view of the fact that increased rate of drug metabolism has been shown in animals tolerant to certain short-acting barbiturates[1] and that barbital is effective in stimulating the activity of drug-metabolizing enzymes in hepatic microsomes of rats.[14] Metabolism accounted for the degradation of 1·39 mg (6·07 per cent of the dose) of barbital in tolerant rats, as compared with 0·82 mg (3·32 per cent of the dose) in controls. This increase in barbital metabolism, amounting to 2·8 per cent of the dose, was not great enough to cause a significant diminution of barbital brain levels in the tolerant rats (Fig. 1) even though a marked decrease in pharmacological effect was observed (Table 1, experiment 6). Thus, tolerance to barbital is mediated by some mechanism other than accelerated metabolism of the drug.

Changes in barbital absorption, distribution, excretion, and metabolism having been eliminated as mechanisms of barbital tolerance, the possibility remains that tolerance may be ascribed to altered sensitivity of the drug receptor sites—i.e. cellular adaption. Evidence to support this possibility comes from the data collected 60 min after barbital-^{14}C administration. Mean brain levels of radioactivity in tolerant and control animals were identical but the pharmacological effects differed; three of five control rats were asleep, but none of the four tolerant rats was asleep. An alternative possibility is that barbital tolerance is the consequence of compensatory mechansims of a stimulatory nature, triggered by repeated periods of anesthesia, and acting to oppose barbital depression. The convulsions, tremors, and other signs of central nervous system stimulation seen during withdrawal from barbiturate addiction[15] may represent such compensatory manifestations.

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