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DISTRIBUTION AND HEPATIC METABOLISM OF PREGNANOLONE IN THE RAT*

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Abstract—Studies in the rat of pregnanolone (3α-hydroxy-5β-pregnane-20-one), a pharmacologically active metabolite of progesterone, indicated rapid transformation to more polar metabolites. Ten min after intravenous administration, high concentrations were found in the liver, intestine and fat. Over 6 hr, amounts in the intestine increased whereas levels in all other tissues declined rapidly. Only 8 per cent of the injected dose appeared in the urine. Altering the activity of the hepatic mixed-function oxidase system had little effect on the duration of pregnanolone-induced hypnosis as compared to significantly decreased duration of progesterone or pentobarbital hypnosis. It was proposed that redistribution rather than hepatic metabolism was the principle mechanism for termination of the hypnotic effect of pregnanolone and that the major route of metabolism was the excretion of conjugates of pregnanolone and its hydroxylated metabolites into the intestine.

PREGNANOLONE (epipregnanolone; 3α-hydroxy-5β-pregnane-20-one) is a metabolite of progesterone with potent hypnotic, thermogenic, hemolytic, uterotropic, and other pharmacologic properties.[1–5] After intravenous administration, its hypnotic effect is immediate, lasts 15–30 min, and terminates abruptly.[2] Our previous study[6] showed that even in newborn rats it was rapidly metabolized, primarily to more polar compounds and to sulfate and glucuronic acid esters of pregnanolone and certain other metabolites.

The goals of the present investigation were to define further the distribution, excretion and metabolism of pregnanolone, and to attempt to elucidate the role of hepatic biotransformation in terminating its hypnotic effect.

Our approach was to determine the distribution of pregnanolone and its metabolites in various tissues, to study the metabolites in detail by thin-layer and gas–liquid chromatography, and to examine the influence of induction and inhibition of the mixed-function oxidase system on the hypnotic response *in vivo* of rats to pregnanolone and other hypnotic agents.

MATERIALS AND METHODS

Animals. Male Sprague–Dawley rats, raised in our animal colony, were used when they were 40–60 days of age, except in one experiment when 18-day-old animals were employed to minimize the isotope expenditure.

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Chemicals. Pregnanolone was purchased from Sigma Chemical Company (St. Louis), [1,2-^{3}H]pregnanolone from New England Nuclear Corp. (Boston), and other steroids from Schwarz/Mann (Orangeburg, N.Y.). All steroids were recrystallized prior to use.

Distribution studies. Tissues were excised or the entire carcass was taken, homogenized in H_2O, and an aliquot taken for counting in a dioxane-based scintillation solution. To correct for quenching, [^{3}H]pregnanolone was added as an internal standard. For calculation of total body content, the values used for 100 g of rat were: plasma, 3; muscle, 46; and fat, 13·2 g.[7–9]

Induction. The animals were pretreated with single daily doses of phenobarbital sodium, 50 mg/kg s.c., 3-methylcholanthrene, 60 mg/kg i.p., or pregnanolone, 50 mg/kg i.p., for 4 days. Controls received daily injections of the appropriate solvents, i.e. 0·9% NaCl for phenobarbital and pregnanolone, and sesame oil for methylcholanthrene.

Partial hepatectomy. Approximately 65 per cent of the liver was excised and the animals were studied 24 hr after surgery.

Studies in vitro. Rats were killed by decapitation, the liver was rapidly excised, rinsed in ice-cold 1·15% KCl, blotted dry and weighed. The liver was minced with scissors and a 20 per cent homogenate was prepared in buffered sucrose solution (0·2 M sucrose in 0·1 M potassium phosphate buffer, pH 7·4). The homogenate was centrifuged at 9000 *g* for 30 min and the supernatant was used for assay. Pregnanolone (1·6 mg) and [^{3}H]pregnanolone were dissolved in methanol, added to a 125-ml Erlenmeyer flask, and the methanol was allowed to evaporate. The incubation mixture consisted of: an NADPH-generating system containing NADP (0·2 mM) and glucose 6-phosphate (2 mM) in 20 ml of phosphate buffer (pH 7·4) with $MgCl_2$ (1 mM) and nicotinamide (2 mM); 20 ml of 9000 *g* supernatant and 10 ml H_2O. The incubation was carried out at 37° for 30 min in air. The flasks were then placed in an ice bath and 50 ml of cold diethyl ether was added. After shaking, the ether was removed and the extraction repeated three times. The ether extracts were dried under a stream of nitrogen. The residue was resuspended in 100 μl of absolute ethanol and aliquots were taken for liquid scintillation counting and thin-layer chromatography.

The same procedure, only using a 10 ml final volume incubation mixture, was used in studies of pregnanolone metabolism by 9000 *g* supernatants (39 mg protein/flask) from two phenobarbital-pretreated and two control rats, with each performed in duplicate. The reaction was terminated by addition of 5 ml of 1 N HCl and extraction with ether was performed as above. Recovery of ^{3}H from the eight incubated and two non-incubated flasks was 87–100 per cent and did not differ between groups. An aliquot was chromatographed with solvent system A (see below), and 0·5-cm sections were scraped, placed in vials, and counted in a model 3320 Tri-Carb spectrometer equipped with a model 544 Absolute Activity Analyzer (Packard Instrument Company, Downers Grove, Ill.). Total disintegrations per minute recovered accounted for 77–87 per cent of the radioactivity as determined from the extract.

Thin-layer chromatography. Glass plates (20 × 20 cm) were coated with an aqueous slurry of Silica gel GF-254 (E. Merck, Darmstadt, Germany) at a thickness of 250 μ. They were activated by heating at 110° overnight. The solvent systems employed were: (A) chloroform–methanol, 90 : 10; (B) chloroform–methanol, 95 : 5; and (C) ethyl acetate–hexane–acetic acid–ethanol, 72 : 13·5 : 10 : 4·5.

Gas–liquid chromatography. Metabolites from the studies *in vivo* were analyzed as previously described.[6] In the subsequent studies *in vitro*, we employed a model 7411 dual-coiled column gas chromatograph (Packard Instrument Company, Inc., Downers Grove, Ill.), equipped with dual hydrogen-flame detectors and 6 × 2 ft mm (i.d.) glass columns packed with 3% OV-1 or 3% OV-17 on Chromosorb W (Varian Aerograph, Walnut Creek, Calif.). Samples were dissolved in chloroform or carbon disulfide and analyzed with a nitrogen carrier gas flow rate of 25 ml/min at a column temperature of 240°, flash heater 250°, and flame detector 250°. Trimethylsilyl derivatives were formed by dissolving the residue in *N,O*-bis-(trimethylsilyl)-acetamide (BSA); (Pierce Chemical Company, Rockford, Ill.) and allowing the reaction to proceed overnight at room temperature.

Hydrolysis of conjugates. After thin-layer chromatography, the area of non-migrating material was removed from the plate and eluted with ethanol. An aliquot was subjected to solvolysis[10] to hydrolyze sulfate esters and another was treated with β-glucuronidase as previously described.[6] The free steroids were extracted and aliquots taken for thin-layer chromatography, and trimethylsilyl derivatives were prepared for gas–liquid chromatography.

Color reactions. Thin-layer chromatograms were sprayed with vanillin-sulfuric acid (3 g vanillin dissolved in 100 ml absolute ethanol and 0·5 ml concentrated sulfuric acid) and heated at 120° for 10 and 20 min.

Crystallization to constant specific activity. After thin-layer chromatography, 1 to 2-cm areas were scraped and placed in Pasteur pipettes and the steroids were eluted with methanol. The methanol was evaporated under nitrogen, purified reference standard (2–4 mg) was added, and dissolved in a minimum volume of methanol with gentle heating. Water was added dropwise with cooling to room temperature until crystals formed. The crystals were pelleted by centrifugation and the supernatant (~1 ml mother liquor) retained. The crystals were suspended in 1 ml water and recentrifuged. An aliquot of crystals was placed on a preweighed pan and dried in an oven at 150° to a constant weight. The weighing pan was then placed in a scintillation vial and radioactivity determined. The remaining crystals were redissolved in methanol and the procedure was repeated through three or more cycles.

RESULTS

Distribution. The time course of changes in distribution in liver, kidney, muscle, fat and brain was studied by sacrificing rats at 3, 10, 30, 90, 180 and 360 min after intravenous injection of a hypnotic dose of pregnanolone (10 mg/kg) plus tritiated tracer. Mean duration of loss of righting reflex was 38 min with a range of 21–45 min. At 3 min, the concentration (counts per minute per gram) was highest in the kidney, followed by muscle, brain, liver and fat respectively (Fig. 1). At all other times (except fat at 180 min), the liver had the highest concentration of radioactivity. After 3 min, concentration in all organs except liver and fat declined rapidly and linearly until 3 hr. Between 3 and 6 hr, little further decline occurred; in fact, there was an increase in counts per minute per gram of liver. Concentrations of radioactivity in brain and fat decreased most rapidly and fell to low levels (less than 1000 counts/min/g) at 6 hr. At this time, the liver had by far the highest value (about 16,600 counts/min/g), compared to the kidney (8700) and muscle (3700).

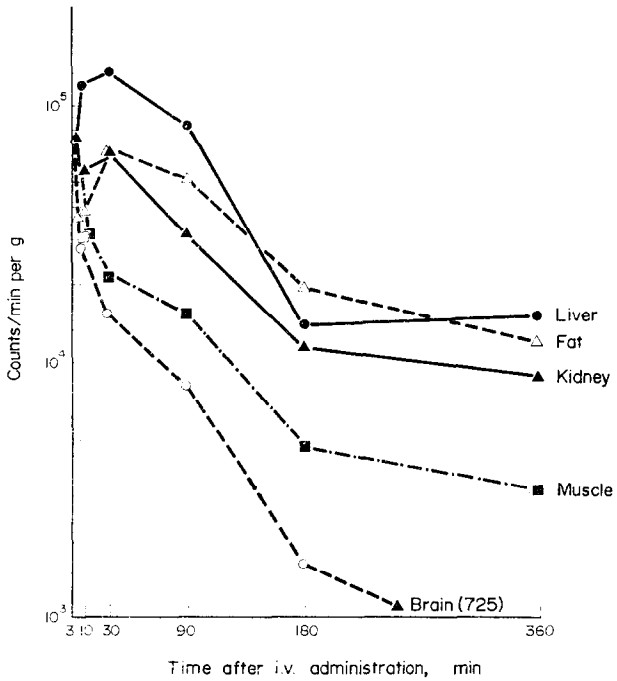

FIG. 1. Change in concentration of radioactivity in various organs after i.v. administration of pregnanolone plus ^{3}H-tracer.

Selective tissue concentration. Two rats were injected intravenously with pregnanolone (10 mg/kg) and sacrificed 30 min later upon awakening. Tissues were removed from one, while the second rat was homogenized intact to serve as a control for calculation of recovery. The liver accounted for 35 per cent of the total radioactivity found in the whole rat (Table 1). Muscle, fat and intestine (first 10 in. of small bowel) accounted for an additional 34 per cent.

Intestinal excretion. Since the liver and intestine concentrated the radioactivity, as shown by tissue : whole body ratios of greater than one (Table 1), the distribution of radioactivity in four sections of small intestine of three rats sacrificed 10 min, 3 hr and 6 hr after injection was determined. In contrast to other organs (Fig. 1), total radioactivity in the intestine increased with time, being highest at 6 hr (Table 2). At 3 and 6 hr, the disintegrations per minute were highest in the fourth section, suggesting secretion into the terminal ileum. The large amount of labeled material present in the gut probably accounts for a major portion of the 24 per cent not located in the first experiment. Only 10 in. (approximately one-fourth of the intestine) was included in that study, whereas a large percentage of radioactivity would have been present in more distal segments by 30 min post-injection, as indicated by the data in Table 2.

Urinary excretion. Only 1·6 per cent of an intravenous dose was excreted in the urine during the first 1·5 hr, 1·3 per cent during the next 2 hr and 5 per cent during the next 2·5 hr. Thus, only 8 per cent of the total administered dose was excreted by the kidney in 6 hr. At 6 hr, the rats were sacrificed and homogenized. By differential solvent

TABLE 1. DISTRIBUTION OF RADIOACTIVITY IN TISSUES OR IN THE ENTIRE BODY OF RATS KILLED 30 min AFTER INTRAVENOUS INJECTION OF 10 mg/kg OF PREGNANOLONE SUSPENSION CONTAINING 100 μc/kg [1,2-^{3}H]PREGNANOLONE

Tissue or organ	Radioactivity (dis./min × 10^3)	Per cent of whole rat (dis./min)	Radioactivity (dis./min × 10^3/g wet wt.)	Ratio tissue : whole body distribution
Liver	9662·0	35·0	1170·0	5·9
Muscle	5007·0	18·0	77·8	0·4
Fat	3081·0	11·0	169·0	0·9
Small intestine (first 10 in.)	1459·0	5·3	917·0	4·7
Serum	1060·0	3·8	151·0	0·8
Kidneys	321·0	1·2	260·0	1·3
Lungs	234·0	0·8	190·0	1·0
Brain	103·0	0·4	82·0	0·4
Heart	72·8	0·3	130·0	0·7
Uterus	32·2	0·1	179·0	0·9
Thyroid	25·8	<0·1	60·1	0·3
Spleen	22·7	<0·1	52·8	0·3
Ovaries	8·34	<0·1	208·0	1·1
Adrenals	3·09	<0·1	155·0	0·8
Total	21,091·00	~76·0		
Whole rat	27,689·00	100·0	199·0	

extraction (chloroform–methanol, 2 : 1, vs. ethyl acetate and benzene), it was found that 94 per cent of the remaining labeled steroids were transformed to highly polar or conjugated forms or to both. Because these are usually rapidly cleared by the kidney, it was concluded that they were stored in tissues and present in very low concentrations in the blood.

Liver metabolites. Analysis of a chloroform–methanol extract of liver from rats killed 30 min after intraperitoneal administration of pregnanolone (40 mg/kg) revealed that only 10 per cent of the radioactivity was unchanged pregnanolone. Thin-layer chromatography revealed five migrated peaks; the majority of counts remained at the origin (Fig. 2, top). The second most rapidly migrating peak (Fig. 2, peak B) corresponded to

TABLE 2. RADIOACTIVITY IN SECTIONS OF SMALL INTESTINE AFTER INJECTION OF PREGNANOLONE WITH TRITIATED TRACER*

Section	Time after injection: 10 min (% total dis./min)	3 hr (% total dis./min)	6 hr (% total dis./min)
1	45·2	10·1	14·6
2	30·4	21·2	14·2
3	15·6	16·4	24·5
4	8·8	52·2	46·7
Total dis./min × 10^3	1246·0	1879·0	2730·0

* Dose as in Table 1. The small bowel was excised, divided into four segments of equal length (approximately 8 in.), and each was extracted exhaustively with chloroform–methanol; the extract was dried and an aliquot counted in a toluene-based scintillation solution.

authentic pregnanolone. By studies of the migration of 5β-pregnanes of known structure (Table 3), we postulated the presence of zero to three or four hydroxyl groups in compounds occurring in peaks A through E respectively. After solvolysis of material eluted from the area of nonmigration and repeat chromatography, two peaks were found (Fig. 2, middle). The major one proved to be pregnanolone. The second coincided with the most rapidly migrating peak (peak A) seen in the initial chromatogram. No migrating steroids were found after treatment of the eluent from the origin with β-glucuronidase (Fig. 2, bottom).

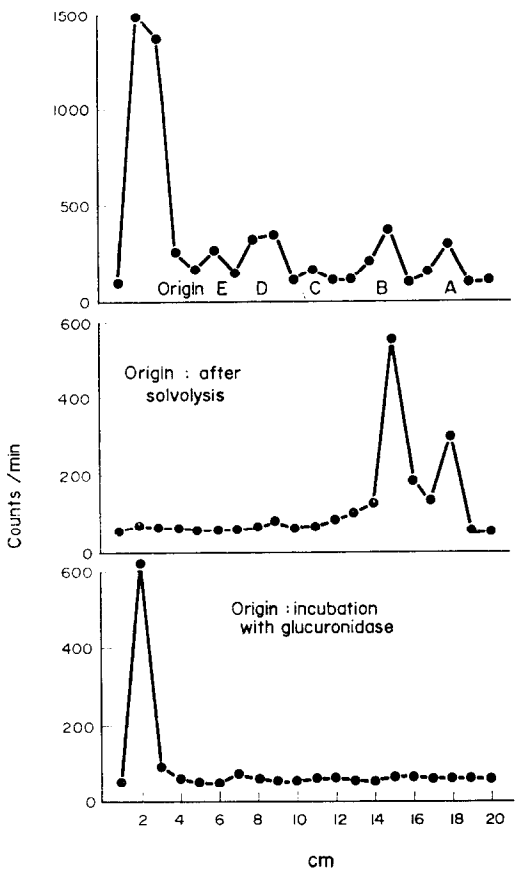

FIG. 2. Top: Thin-layer chromatography of chloroform–methanol extract of liver from rats killed 30 min after intraperitoneal administration of pregnanolone (40 mg/kg) with added [1,2-^{3}H]pregnanolone (100 μc/kg). The migration of peak B corresponds to that of authentic pregnanolone. Middle: Repeat chromatography after solvolysis to hydrolyze sulfate esters. Bottom: Repeat chromatography after β-glucuronidase treatment.

Eluents from the peaks after thin-layer chromatography were subjected to gas–liquid chromatography (Table 4). In the most rapidly migrating peak (Fig. 2, peak A), a single major peak with a retention time of 11·2 min was found. Peak B had several peaks on gas–liquid chromatography but, the quantity of pregnanolone accounted for

TABLE 3. MIGRATION OF PREGNANOLONE AND POSSIBLE 5β-PREGNANE METABOLITES AFTER ONE-DIMENSIONAL THIN-LAYER CHROMATOGRAPHY ON SILICA GEL G WITH VARIOUS SOLVENT SYSTEMS

Number of hydroxyl groups	Compound (5β-pregnane derivatives)	R_f in solvent system *		
		A	B	C
0	3,20-dione(pregnanedione)	0·80	0·93	0·80
1	3α-ol-20-one(pregnanolone)	0·71	0·80	0·72
	17α-ol-3,20-dione	0·67		
	3α-ol-11,20-dione	0·55		
2	3α,17α-diol-20-one	0·60	0·63	0·70
	3α,20β-diol	0·54	0·54	
	3α,20α-diol(pregnanediol)	0·50	0·51	0·68
	3α,6α-diol-20-one	0·35	0·18	0·41
3	3α-17α,20β-triol	0·44	0·18	0·54
	3α,11β,21-triol-20-one	0·36		
	3α,17α,20α-triol(pregnanetriol)	0·30	0·12	0·60
	3α,17α,21-triol-11,20-dione (tetrahydrocortisone)	0·28		
4	3α,11β,17α,21-tetrol-20-one (tetrahydrocortisol)	0·14		

* A = chloroform–methanol, 90 : 10; B = chloroform–methanol, 95 : 5; C = ethyl acetate–hexane–acetic acid–ethanol, 72 : 13·5 : 10 : 4·5.

the total radioactivity present. Peak C had two major and one minor components which based on their migration on thin-layer chromatography and their gas–liquid chromatography retention times, were assumed to be 3α-ol-11, 20-dione and 3α,20α- and 3α,20β-diols. Peak D had a single major component whose R_f value and retention time were

TABLE 4. TENTATIVE IDENTIFICATION OF PREGNANOLONE METABOLITES FROM THE LIVER OF AN 18-day-old RAT KILLED 30 min AFTER ADMINISTRATION OF PREGNANOLONE

Thin-layer chromatography		Gas–liquid chromatography (OV-1)	Tentative identity
Peak*	R_f	Retention time (min)	
A	0·87	11·2	? (Not pregnanedione)
B	0·72	7·5	Pregnanolone
C	0·55	10·0	3α-ol-11,20-dione, pregnanediol(s)
D	0·43	10·3	6α-hydroxy-pregnanolone
E	0·30	5 Small peaks	Triols
Origin			Sulfate esters
After solvolysis			
A	0·87	11·2	Presumably has a 3-OH group because formed an ester
B	0·75	7·5	Pregnanolone

* Peaks correspond with those of the chromatogram shown in Fig. 2.

consistent with those of a hydroxylated pregnanolone, possibly the 6α-hydroxy derivative. The slowest migrating peak (peak E) had five small components with relatively long retention times consistent with triol or tetrol (or both) configurations.

Intestine metabolites. The pattern of metabolites, as displayed after one-dimensional thin-layer chromatography, differed between sections of the intestine and changed with time after injection. At 10 min, all sections had a large (about 50 per cent the total counts per minute) free pregnanolone peak ($R_f \sim 0{\cdot}7$) with three smaller distinct peaks ($R_f \sim 0{\cdot}9$, 0·6, 0·4). This pattern was similar to that found in the liver.

In extracts from the first section of small bowel, and to a lesser extent from the second portion, there was a significant peak of labeled material which remained at the origin. This was composed chiefly of glucuronide esters (95 per cent), about 75 per cent of these being pregnanolone, and another 10 per cent being from the peak migrating at R_f 0·6. The remaining 5 per cent of the originally nonmigrating material proved to be the sulfate ester of pregnanolone. This ratio of glucuronide : sulfate esters was the reverse of that seen in liver extracts.

At 3 and 6 hr, the major peak (greater than 50 per cent of total counts per minute) in all sections of the small intestine was that of the conjugates. The terminal section contained the largest free pregnanolone peak, suggesting that hydrolysis of conjugates occurred during passage along the bowel. At 3 hr, a large peak migrating at $R_f \sim 0{\cdot}9$ was present in extracts from the first section of intestine. The amount of this metabolite gradually declined, none being found in the last section of gut, suggesting that absorption had occurred during passage through the small intestine.

Studies in vitro. Incubation of pregnanolone with a 9000 *g* hepatic supernatant and

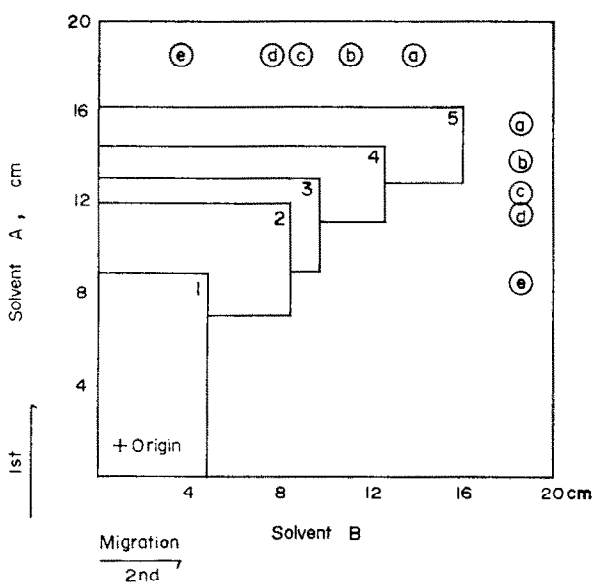

FIG. 3. Two-dimensional thin-layer chromatogram of extract after incubation of pregnanolone plus ^{3}H-tracer with a hepatic 9000 *g* supernatant and an NADPH-generating system. Standards: (a) pregnanedione; (b) pregnanolone; (c) 5β-pregnane-3α,17α-diol-20-one; (d) 5β-pregnane-3α,20β-diol; (e) 5β-pregnane-3α,6α-diol-20-one. Numbers indicate areas scraped and eluted for repeat chromatography (see Fig. 4).

an NADPH-generating system led to the formation of metabolites apparently similar except for the lack of conjugates, to those found in the liver after parenteral administration of pregnanolone.

After a 30-min incubation, about 60 per cent of the substrate was metabolized. For initial identification, two-dimensional thin-layer chromatography was performed and the chromatogram was examined under ultraviolet light, after spraying with vanillin, and by scraping sections of the plate for scintillation counting. These studies allowed the definition of the five principal areas containing pregnanolone and its metabolites (Fig. 3). Using five standards as coordinate landmarks, these areas were scraped, eluted, and aliquots were counted and rechromatographed in various solvent systems (Fig. 4). Adjacent lanes from these chromatograms were either scraped and counted, or eluted for reverse isotope crystallization.

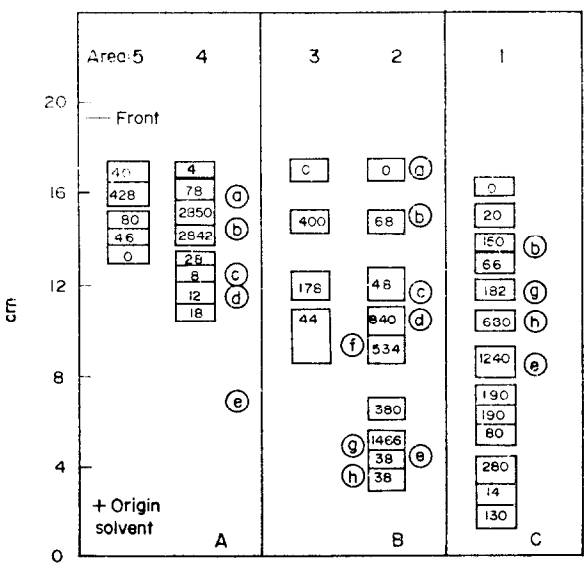

FIG. 4. Chromatography of areas eluted from two-dimensional chromatogram of pregnanolone and metabolites shown in Fig. 3. Squares indicate areas scraped for scintillation counting and numbers within indicate disintegrations per minute. Standards: (a–e) as in Fig. 3, (f) pregnanediol; (g) 5β-pregnane-3α,17α,20β-triol; (h) pregnanetriol. See text for description of solvent systems A, B and C.

Area one, containing the most polar metabolites remaining near the origin had about 20 per cent of the total radioactivity. The presence of two metabolites, 5β-pregnane-3α,6α-diol-20-one and pregnanetriol, was confirmed by crystallization to constant specific activity (Table 5); the 6-hydroxy derivative appeared to be the major metabolite. Another highly polar metabolite, present in trace amounts as indicated by scintillation counting, was not identified.

Area two, containing 25 per cent of total radioactivity, consisted of three steroids, the 20α- and 20β-pregnanediols and 6α-hydroxy pregnanolone (Fig. 4 and Table 5).

Area three, with 4 per cent of the total radioactivity, contained pregnanolone due to trailing, and perhaps a trace quantity of 5β-pregnane-3α,17α-diol-20-one.

Area four, with 45 per cent of the total radioactivity, contained pregnanolone alone.

TABLE 5. REVERSE ISOTOPE DILUTION WITH CRYSTALLIZATION TO CONSTANT SPECIFIC ACTIVITY OF COMPOUNDS ELUTED FROM CHROMATOGRAMS SHOWN IN FIG. 4

Area*	Compound	No. of crystallizations	Radioactivity (dis./min/mg)
1	5β-Pregnane-3α,6α-diol-20-one	1	208
		2	183
		3	167
		4	170
	Pregnanetriol	1	76
		2	58
		3	64
2	5β-Pregnane-3α,20β-diol	1	172
		2	101
		3	75
		4	69
		5	67
		6	68
	Pregnanediol	1	84
		2	79
		3	72
		4	78
		5	76
4	Pregnanolone	1	5950
		2	6950
		3	6200
5	Pregnanedione	1	136
		2	73
		3	95
		4	80

* Areas selected were adjacent to standards as indicated in Fig. 4.

Area five, containing 3–4 per cent of the total radioactivity, consisted mainly of pregnanedione.

Enzyme induction. To stimulate the activity of the mixed-function oxidase system, rats were pretreated with phenobarbital or methylcholanthrene. The duration of loss of the righting reflex was called sleep. Pregnanolone sleep time was decreased about 50 per cent by phenobarbital pretreatment (Table 6). A 2-fold increase in the dose of pregnanolone doubled the sleep time, which then approximated that seen in untreated control animals.

The effect of phenobarbital pretreatment on the duration of hypnosis caused by pentobarbital was more pronounced than its effect on pregnanolone sleep time. Induced rats did not sleep at all, whereas controls slept 54 $\pm$ 2 min. A 2-fold increase in the dose of pentobarbital resulted in a sleep time of 16 $\pm$ 2 min. Thus phenobarbital pretreatment produced a 2-fold decrease in the duration of pregnanolone sleep compared to about a 7-fold decrease in the duration of action of pentobarbital.

Pretreatment with methylcholanthrene or pregnanolone did not affect either pregnanolone or pentobarbital sleep time (Table 6).

TABLE 6. INFLUENCE OF PHENOBARBITAL, 3-METHYLCHOLANTHRENE AND PREGNANOLONE PRETREATMENT ON PREGNANOLONE AND PENTOBARBITAL SLEEP TIME IN RATS*

Pretreatment	Hypnotic agent	Dose (mg/kg i.v.)	Sleep time (min)	Control	P
Phenobarbital (50 mg/kg s.c. × 4 days)	Pregnanolone	10	16·0 ± 4·3	36·4 ± 4·3	<0·05
	Pregnanolone	20	30·7 ± 3·0		
	Pentobarbital	25	0	53·5 ± 1·7	
	Pentobarbital	50	15·7 ± 2·1		
3-Methylcholanthrene (60 mg/kg i.p. × 4 days)	Pregnanolone	10	49·2 ± 7·4	41·6 ± 4·2	NS
Pregnanolone (50 mg/kg i.p. × days 4)	Pregnanolone	10	47 (3)	33 (8)	NS
	Pentobarbital	25	46 (3)	57 (3)	NS
Pregnanolone (50 mg/kg i.p. × 6 days)	Pregnanolone	10	35 (5)		
	Pentobarbital	25	54 (5)	56·6 ± 13·2	

* Values are expressed as means ± S.E. The number of animals, when less than 10, is given in parentheses. NS = not significant.

Metabolism in vitro *after pretreatment.* To evaluate the effect of phenobarbital pretreatment on the hepatic metabolism of pregnanolone, 9000 *g* supernatants were prepared and incubated with pregnanolone and an NADPH-generating system. Extracts were subjected to thin-layer chromatography and radioactivity was determined in the scraped sections. By this means it was determined that 50 per cent of substrate remained unchanged after a 30-min incubation with hepatic supernatants

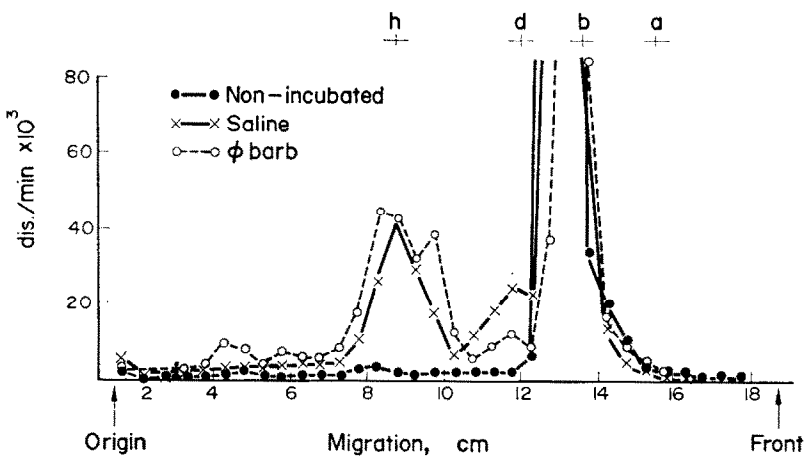

FIG. 5. Radioactivity in 0·5-cm sections of thin-layer chromatograms of extracts from incubations of pregnanolone plus ^{3}H-tracer with 9000 *g* hepatic supernatants from phenobarbital-pretreated (●————●) or saline-treated controls (○- - - - -○) and an NADPH-generating system. Extracts from nonincubated flasks are shown (×————×). Solvent system A. Bars above the radiochromatogram indicate the migration of standards: (a) pregnanedione; (b) pregnanolone; (d) 5β-pregnane-3α,20β-diol; (h) pregnanetriol.

from pretreated rats, as opposed to 71 per cent with controls, and 95 per cent with non-incubated flasks.

The pattern of metabolites differed in that extracts from the pretreated rats exhibited a prominent peak with an R_f of 0·48 and a minor peak at R_f 0·17 (Fig. 5). Peak areas from comparable chromatograms, as determined by a radiochromatogram scanner, were eluted and reverse isotope dilution was performed. Extracts from the controls contained both 20α- and β-diols, 6-hydroxy pregnanolone and 5β-pregnane-3α,17α,20β-triol (Table 7). The same metabolites, with the exception of a relatively larger amount of the 20β-triol accounting for the prominent peak, were identified in extracts from the pretreated samples. Pregnanetriol was not identified in extracts from either group.

Eluent from the major peak was also examined by gas–liquid chromatography.

TABLE 7. REVERSE ISOTOPE DILUTION WITH CRYSTALLIZATION TO CONSTANT SPECIFIC ACTIVITY OF AREAS FROM CHROMATOGRAMS SIMILAR TO THOSE SHOWN IN FIG. 5

Migration* (cm)	Compound	Crystallizations	Control (dis./min/μg)	Phenobarbital pretreated (dis./min/μg)
11–12·5	Pregnanolone	1	4·04	6·19
		2	3·42	4·91
		3	3·09	5·17
		4	3·36	5·55
		5	3·22	4·99
9–10·5	Pregnanediol	1	1·34	0·59
		2	0·59	0·25
		3	0·24	0·10
		4	0·12	0·10
		5	0·09	0·09
		6	0·09	
9–10·5	5β-Pregnane-3α,20β-diol	1	0·46	
		2	0·11	
		3	0·08	
		4	lost	
		5	0·12	
		6	0·07	
6·5–8	5β-Pregnane-3α,6α-diol-20-one	1	8·02	1·05
		2	7·95	0·99
		3	8·26	0·86
		4	8·21	1·05
		5	8·09	0·88
7·5–9	5β-Pregnane-3α,17α,20β-triol	1	3·40	4·34
		2	0·68	1·19
		3	0·54	1·16
		4	0·21	1·04
		5	0·30	1·03
		6	0·23	
6·5–8	Pregnanetriol	1	2·33	0·38
		2	0·73	0·30
		3	0·24	0·23
		4	0·20	0·17
		5	0·08	0·11

* Migration indicates the area eluted.

Pregnanolone and both diols were identified by retention times (relative to 5α-cholestane) of free steroid and trimethylsilyl derivatives which were identical to those of standard compounds on both OV-1 and OV-17 columns. Comparable aliquots of the incubation extract contained approximately 13 μg pregnanolone in the case of controls and 7 μg in the pretreated, confirming the isotopic data indicating more rapid metabolism of pregnanolone by hepatic supernatants from phenobarbital-pretreated rats.

Inhibition. Decreased hepatic activity was produced by partial hepatectomy and by pretreatment with the known drug-metabolizing enzyme inhibitor, SKF-525A (β-diethylaminoethyl diphenylpropyl acetate HCl).[11] Hepatectomy caused an increase in pregnanolone sleep time of 46 per cent over that of sham-operated controls (Table 8). In contrast, progesterone sleep time was increased 450 per cent. The sleep time of pregnanedione, a metabolite of progesterone and a precursor of pregnanolone, was increased to an intermediate degree (216 per cent).

TABLE 8. EFFECT OF PARTIAL HEPATECTOMY (~65%) ON THE DURATION OF HYPNOSIS

Treatment*	Sleeping time (min)	S.D.	% Increase over controls
Progesterone (20 mg/kg)			
Hepatectomy	110	±10	550
Sham operation	36	±19	100
Controls	17	± 8	
Pregnanedione (3·5 mg/kg)			
Hepatectomy	30	±16	330
Sham operation	17	±10	114
Controls	7	± 5	
Pregnanolone (2 mg/kg)			
Hepatectomy	21	± 5	113
Sham operation	13	± 3	67
Controls	9	± 2	

* Drugs in a propylene glycol–ethanol–water (1 : 1 : 1) solution were administered i.v. 24 hr after surgery.

Pretreatment with SKF-525A had no effect on pregnanolone sleep time, whereas it doubled pentobarbital sleep time (Table 9).

TABLE 9. INFLUENCE OF SKF-525A (7 mg/kg i.p.) ON PREGNANOLONE AND PENTOBARBITAL SLEEP TIME IN RATS

Hypnotic agent	Dose (mg/kg i.v.)	Duration (min)*		Difference	P
		Treated	Control		
Pregnanolone	10	37 ± 6·4	34·4 ± 3·4	+2·6	NS
Pentobarbital	25	109 ± 6·8	54·5 ± 1·6	+54·5	0·01

* Values are expressed as means ± S.E.M.; N = 6.

Hypnotic response in Gunn rats. These animals have a genetically determined defect in glucuronide conjugation leading to chronic unconjugated hyperbilirubinemia.[12,13] It was postulated that if pregnanolone were metabolized by the same mechanism as is bilirubin, then sleeping time would be prolonged in homozygous recessive (affected) rats. This was not found to be the case. Duration of hypnosis after intravenous administration of pregnanolone (10 mg/kg) was 28–46 min (N = 5), equivalent to that seen with Sprague–Dawley rats. Unaccountably, heterozygotes appeared to be unusually susceptible, all four animals dying 15 min after intravenous administration of 10 mg/kg. The LD_{50} for adult Sprague–Dawley rats was 27·5 mg/kg.[2]

DISCUSSION

The results of the initial time-course distribution study indicated that from 30 to 180 min a sharp and approximately parallel fall in steroid concentrations occurred in liver, fat, kidney, muscle and brain. Only 3 per cent of the injected dose was found in the urine during the first 3·5 hr after administration, indicating excretion via the intestine to be the principal route, at least initially. Between 3 and 6 hr, the rate of fall decreased, except in the brain where the concentration of steroid continued to fall sharply, and the liver in which the steroid concentration remained unchanged.

The surprising lack of accumulation in body fat presumably resulted from rapid metabolism to more polar compounds and conjugates. This finding probably explains previous observations that repeated administration of hypnotic doses of pregnanolone did not result in prolonging the duration of hypnosis, in contrast to results with thiopental (L. Gyermek, unpublished observations).

The early appearance of radioactivity in the upper small intestine suggested prompt biliary excretion. Alterations in the pattern of metabolites during passage through the bowel may indicate the presence of an enterophepatic cycle, such as that seen with other steroids.

Pregnanolone was metabolized chiefly by conjugation with glucuronic acid and sulfate, and to a lesser extent by reduction at C-20 and hydroxylation at C-6 and C-17. In the bowel, about 95 per cent of the conjugated steroids (mainly pregnanolone) were glucuronides, in contrast to their virtual absence in liver tissue, where large amounts of sulfated pregnanolone and an unidentified metabolite were found.

The rate of pregnanolone metabolism was relatively resistant to manipulations having marked effects on the metabolism of progesterone and barbiturates. Phenobarbital pretreatment caused only a 2-fold decrease in the duration of pregnanolone-induced hypnosis but a 7-fold decrease in pentobarbital hypnosis. Conney *et al.*[14] found that phenobarbital pretreatment completely abolished the hypnotic effect of doses of progesterone and other steroids which had produced a sleep time of more than 2·5 hr in untreated rats. Similarly, partial hepatectomy increased the duration of progesterone-induced hypnosis to an extent greater than its effect on pregnanolone. Methylcholanthrene and SKF-525A had no effect on pregnanolone-induced duration of sleep, whereas pentobarbital sleep time was significantly prolonged by SKF-525A pretreatment.

Our results with hepatectomy and phenobarbital pretreatment indicate that, although hepatic metabolism plays some part in terminating the hypnotic effect of pregnanolone, this role is much less than in the case of progesterone. The concentration of radioactivity in brain after intravenous administration of pregnanolone fell

sharply between 3 and 10 min, and declined more rapidly than that in other tissues thereafter. Our previous study[6] showed that the percentage of radioactivity present as free pregnanolone decreased from 37 per cent when rats fell asleep to 3 per cent when awakening, thus indicating that both total steroid and pregnanolone concentrations decrease rapidly during the period of hypnosis. Furthermore, our data showed that rats awoke at brain concentrations of pregnanolone equivalent to those present when the rats fell asleep, ruling out acute tolerance as a basis for terminating hypnosis. Since hepatic metabolism is relatively unimportant, we propose that redistribution may be a major factor in terminating its hypnotic effect. A similar mechanism, based on distribution into aqueous compartments and binding to various tissues, has been postulated as responsible for terminating thiopental anesthesia.[15]

Studies *in vitro* with livers from induced animals previously have been shown to cause not only increased rates of disappearance of substrate but changes in ratios of polar to nonpolar metabolites and other alterations in metabolic products.[14] The principal change seen with phenobarbital pretreatment in these studies was an increased formation of the 3α,17α,20β-triol metabolite.

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