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Received September 21, 1955. P.S.E.B.M., 1955, v90.

Metabolism of Reserpine-C^{14}. II. Species Differences as Studied *in vitro*. (22058)

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The crystalline alkaloid, reserpine, which is currently finding use in the treatment of hypertension(1,2,3) and psychiatric disorders (4,5,6), was identified in extracts from the roots of *Rauwolfia serpentina* Benth by Müller, Schlittler and Bein(7). In a report on the pharmacology of reserpine, Plummer *et al.*(8) mention the wide range in doses necessary to elicit the characteristic tranquilizing response in the various laboratory animals. Since Sheppard, Lucas and Tsien(9) showed that the rat is capable of oxidizing the 4-methoxy carbon of the 3,4,5-trimethoxybenzoic acid (TMBA) moiety to carbon dioxide as well as hydrolyzing reserpine to yield TMBA, it was thought that the ability to degrade reserpine might be related to dose requirements. To test this hypothesis, liver slices of several animal species were tested for their ability to metabolize reserpine. Part of this work was presented at a meeting of the American Chemical Society(10).

Experimental. In vitro: The livers of mature rats, mice, pigeons and guinea pigs were obtained immediately after death from animals sacrificed for the purpose; while those from dogs and rabbits were obtained by biopsy. The livers were sliced freehand and incubated with 200 μg of reserpine-C^{14}. The preparation of fractions as illustrated in Fig. 1, and the methods of counting and synthesis of reserpine labeled with C^{14} in the 4-methoxy carbon of the 3, 4, 5-TMBA moiety are the same as those previously described(9). Subcellular particles of guinea pig liver were prepared according to the method of Schneider (11). Each fraction was incubated with 100 μg of reserpine-C^{14} for 3 hours at 37°C in Krebs bicarbonate buffer. The reaction was stopped with acid and the tissue extracted as previously described(9). *In vivo*: Four male guinea pigs weighing about 400 g were injected with reserpine-C^{14} in vehicle C(9). One animal received 1.0 mg/kg intravenously while the others received 0.5 mg/kg. $C^{14}O_2$ collections were made and counted as previously described(9).

Paper chromatography and radioautography. Aliquots of fraction C and C_B were mounted on Whatman No. 1 filter paper. 40 μg of TMBA were then added to each spot and the sheets chromatographed by descending method with a solvent composed of 5 normal ammonium hydroxide: butanol: isoproponal (3:2:1). Under these conditions TMBA gives an R_F of about 0.61 and fluoresces a bluish purple when exposed to ultraviolet light. To locate the radioactive spots radioautographs were made using Kodak No-Screen X-ray Safety Film.

Results. The results of the *in vitro* studies with liver slices are summarized in Table I. The rat possesses the greatest ability for the oxidation of the 4-methoxy carbon of the 3, 4, 5-trimethoxybenzoic acid moiety to carbon dioxide. Only the pigeon and rabbit seemed to be devoid of this activity. That most of this process depends on oxygen is obvious from the drop in $C^{14}O_2$ recoveries that occurred when incubation was carried out under nitrogen. The rat liver slices, however, show little ability for splitting off TMBA. Surprisingly, the guinea pig liver slices show a very marked ability in this respect, hydrolyzing about 80 per cent of the reserpine in three hours. The mouse and rabbit show fair hy-

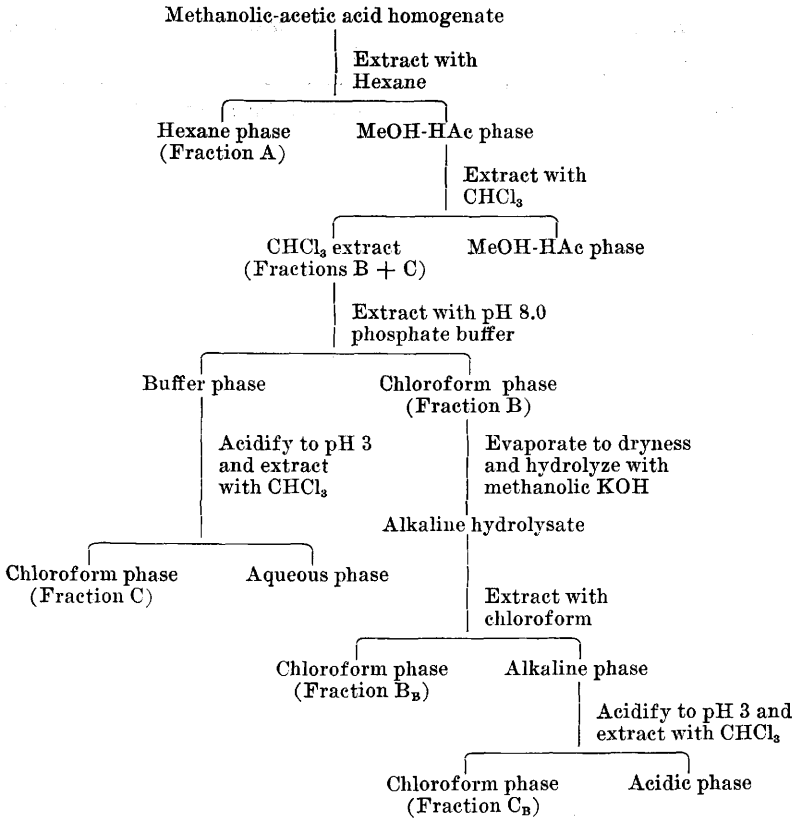

FIG. 1. Flow sheet for fractionation of liver homogenate.

drolytic activity while the dog and pigeon show practically none. The subcellular particles which possess most of the esteratic activity are the microsomes, as can be seen in Table II. The esteratic activity observed in the other fractions is less, and a portion is probably due to contamination with microsomes.

Radioautographs of paper chromatograms of Fractions C and C_B showed that all of the radioactivity coincided with the bluish purple fluorescence of TMBA.

The urinary excretion of C^{14} by the guinea pig is summarized in Table III. As can be seen, approximately 22% of the injected dose is excreted in the first 24 hours with some C^{14} still being excreted up to 96 hours. As confirmation of the *in vitro* studies indicating a slight ability to oxidize the 4-methoxy carbon of TMBA, some respiratory $C^{14}O_2$ could be detected.

Discussion. Among the animals used in these experiments, the dog is recognized as requiring the smallest and the rat the largest dose of reserpine to produce its typical tranquilizing response. It is apparent from the results presented that the dose requirement of reserpine is not directly related to the ability of liver slices to hydrolyze reserpine nor to demethylate the 4-methoxy group of TMBA or both. If it were, the pigeon would require the smallest and the guinea pig the largest dose of reserpine. The metabolic activity of other tissues must necessarily be considered when studying the response of the whole animal.

Because of the strong esteratic activity present in the guinea pig liver, it was supposed that the guinea pig would rapidly hydrolyze reserpine *in vivo* and excrete the TMBA via the urine. Therefore, it might be possible to demonstrate that pharmacological activity was lost when practically all of the TMBA was excreted, or, if some product of reserpine

TABLE I. *In Vitro* Metabolism of Reserpine-C^{14} by Liver Slices of Different Animals. 500 mg of liver slices incubated with reserpine-C^{14} in Krebs bicarbonate buffer at 37°C for 3 hr. All samples in duplicate.

Species	No. of animals	Atmosphere	% of added C^{14} recovered in fractions: CO_2	A*	B†	C‡	Total recovery
Dog	5	O_2	8.51	0	83.4	1.21	93.1
	1	N_2	0	0	96.2	.34	96.5
Pigeon	5	O_2	0	0	89.1	1.25	90.4
	1	N_2	0	0	86.0	1.28	87.3
Rabbit	5	O_2	0	0	80.1	15.7	95.8
	1	N_2	0	0	93.8	10.3	104.1
Mouse	5	O_2	5.6	.15	65.0	31.0	101.7
	1	N_2	—	—	—	—	—
Rat	5	O_2	19.7	.10	58.1	1.37	79.27
	1	N_2	1.3	.06	92.8	2.09	96.25
Guinea pig	5	O_2	2.67	1.51	3.19	80.6	87.97
	1	N_2	3.08	.62	2.25	91.8	97.75

* Fraction A—Contains fat-like substances.
† " B— " reserpine-like substances.
‡ " C— " trimethoxybenzoic acid-like substances.

were responsible for the pharmacological activity, such activity would continue beyond the time when all the TMBA were excreted. The results presented here not only do not support the latter supposition but also demonstrate that hydrolysis does not occur nearly as rapidly *in vivo* as *in vitro*. The reason for this great difference is probably that the reserpine is taken up by tissues which are not very active in metabolizing it(9).

As pointed out previously(9), the increase in the reserpine-containing fractions of rat liver slices incubated under nitrogen, indicates that demethylation probably occurs while TMBA is still esterified with methyl reserpate rather than after hydrolysis has occurred. This was confirmed again with the rat and also found to occur with dog liver slices.

The preponderance of esteratic activity found in the microsomes agrees with the findings of workers concerned with other esterases (12,13). Whether this esterase is a general or specific one remains to be determined.

The radioautograms demonstrate that the C^{14} content of fractions B and C reside in the TMBA moiety. It must still be demonstrated that the alkaloidal portion of the TMBA ester in fraction B is methyl reserpate. Thus far, paper chromatographic or electrophoretic studies fail to distinguish clearly between such alkaloids as reserpine, deserpidine or syringoyl methyl reserpate, indicating that small changes in the reserpine molecule would be extremely difficult to detect by this method.

TABLE II. Hydrolysis of Reserpine by Subcellular Particles of Guinea Pig Liver.

Fraction	% of added C^{14} present in fractions: B	C	Total
Homogenate	8.1	83.3	91.4
Nuclei	29.1	58.6	87.7
Mitochondria	35.3	52.5	87.8
Microsomes	9.0	91.9	100.9
Supernatant	10.6	75.7	86.3

TABLE III. Excretion of C^{14} in Urine and Feces of Guinea Pigs after Single Intravenous Injection.*

Rat No.	Interval, hr	Urine: B†	Urine: C‡	Feces: B†	Feces: C‡
1	1-24	.15	8.69	.68	.82
	24-96	.0	.35	.0	.0
2	1-24	.16	7.07	.58	.61
	24-96	.13	.43	.0	.0
3	1-24	.95	4.17	1.32	—
	24-96	.0	.83	.0	.0
4	1- 6	.73	21.9	1.62	1.66
	6-24	.13	1.13	.85	.15
	24-48	.07	.30	.26	.0

* Rats No. 1-3 received 0.5 mg/kg while No. 4 received 1.0 mg/kg.
† Fraction B—Contains reserpine and reserpine-like substances.
‡ Fraction C—Contains TMBA and TMBA-like substances.

Summary. 1. Liver slices of rat, mouse, dog, guinea pig, rabbit and pigeon were incubated with reserpine-C^{14}. 2. Demethylation of the 4-methoxy position of trimethoxybenzoic acid moiety of reserpine was observed to occur, with rat liver slices most active in this respect. 3. Hydrolysis of reserpine was observed to occur with liberation of free trimethoxybenzoic acid. Guinea pig liver slices were most active in this respect. 4. Esteratic activity resides chiefly in the microsomes of guinea pig liver. 5. Demethylation or hydrolysis or both by liver slices is not correlated with dose requirement for typical tranquilizing response. 6. Rate of excretion of C^{14} by the guinea pig was studied *in vivo* after intravenous injection of reserpine-C^{14}.

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Received September 27, 1955. P.S.E.B.M., 1955, v90.

Delaying Action of Gonadotrophins on Ovulation in the Hen.* (22059)

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The ovulation-inducing hormone (OIH) in the hen is probably the luteinizing fraction (LH)(1-3), although this action of LH independently of the follicle stimulating hormone (FSH) has not been demonstrated in either birds or mammals(4). As in other spontaneously ovulating animals such as the rat(5) and cow(6), OIH release in the hen is apparently effected over nervous pathways (7-12). The interval from OIH release (or from neural excitation for OIH release) to ovulation is estimated to be not more than about 8 hours(11-15), although it may be greater in some hens(10). A number of pharmacological agents which block LH release in the rat(5,16) were shown recently to suppress ovulation of the first (or C_1) follicle of the hen's cycle following a single injection 38 hours before expected ovulation(17). The agents included anesthetic [diallylbarbituric acid (Dial), pentobarbital Na (Nembutal), and Phenobarbital Na], anticholinergic (atropine sulfate) and antiadrenergic (SKF-501 and Dibenamine) drugs. It seemed highly improbable that some, at least, of these drugs (*e.g.,* atropine or Nembutal) could directly block the OIH release mechanism 30 hours following their injection. Suppression of ovulation was attributed instead to disruption of nervous controls over pituitary gonadotrophin secretions required for maintenance and maturation of the ovarian follicle. The prevalence of follicular atresia in hens injected 38 hours before expected C_1 ovulation and sacrificed shortly after the hour of normally expected

* These results were presented at the 44th Annual Meeting of the Poultry Science Assn., East Lansing, Mich., Aug. 9-12, 1955.