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Brain Reserpine Levels Following Large and Small Doses of Reserpine-H^3. (23856)

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Previous studies on fate and distribution of reserpine have consistently demonstrated lack of correlation between pharmacological effects and tissue concentrations(1,2,3). Samples of radioactive reserpine used in several studies unfortunately had C^{14} introduced into the trimethoxybenzoic acid portion of the molecule. Since it was recognized that a somewhat different pattern might be obtained if the methyl reserpate portion of the drug were labeled, attention was drawn to the method of Rowland and Wolfgang(4) for randomly labeling organic compounds with tritium. Application of this method to the labeling of reserpine proved successful and the results of some studies utilizing this material in guinea pigs are recorded below. Parts of this work have appeared in abstract form(5,6).

Methods. Preparation of tritium-labeled reserpine: 9.9 g of reserpine were mixed with 0.30 g of lithium carbonate and irradiated with neutrons for 10 days in the reactor at Brookhaven National Laboratory. The material was extracted with benzene and chromatographed on alumina(1). A yield of 500 mg of tritiated reserpine (reserpine-H^3) was obtained and shown to be pure by paper chromatography(7).* It had a specific activity of 7.8×10^5 dpm/mg with an isotope distribution in the trimethoxybenzoic acid

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and methyl reserpate portions of 35 and 65% respectively.

Exp. I: Twelve male guinea pigs weighing approximately 350 g were injected intravenously with 2 mg of tritiated reserpine and sacrificed in pairs after ⅓, 1, 2, 4, 8, and 24 hours. The blood was heparinized on collection and quickly separated into plasma and cells. Urine was collected when possible. The brains and livers were removed and stored in vials embedded in dry ice until analyzed. The tissues were treated as described previously (2) so as to yield fractions B and C. After the removal of fractions B and C the aqueous residue was brought to pH 8 with alkali and extracted with chloroform to yield fraction MR. Fraction B contained all of the unmetabolized reserpine, fraction C all of the trimethoxybenzoic acid (TMBA) and fraction MR all of the methyl reserpate. *Exp. II*: Twenty-eight male guinea pigs weighing approximately 350 g each were injected subcutaneously with 35 μg of reserpine-H^3 and sacrificed in groups of four after ⅓, 1, 2, 8, 24 and 48 hours. A record was kept of the observable pharmacological responses such as ptosis, myosis and shivering. Brains and livers were removed and analyzed as described above. *Exp. III*: Six male guinea pigs were treated as in Exp. II except that reserpine was labeled with C^{14} in the carboxy carbon of the TMBA moiety. Four of these animals were sacrificed by exsanguination after 48 hours and the remaining two after 72 hours. Livers and brains were removed and extracted as described earlier except that 100 μg of unlabeled reserpine were added as carrier at the time of homogenization of the tissue. The final fraction B was chromatographed on paper(7) and the fluorescent reserpine spot eluted and counted. *Determination of Radioactivity*: Samples obtained from experiments in which animals received 2 mg of reserpine-H^3 intravenously as well as those receiving 35 μg of reserpine-C^{14} were counted in a Tricarb liquid scintillation spectrometer. The remaining samples were combusted(8) and counted in a low-level gas counting system described by Broecker(9) and Giletti(10). This heavily shielded anti-coincidence counter can readily detect 10^{-7} microcuries of H^3 in a sample.

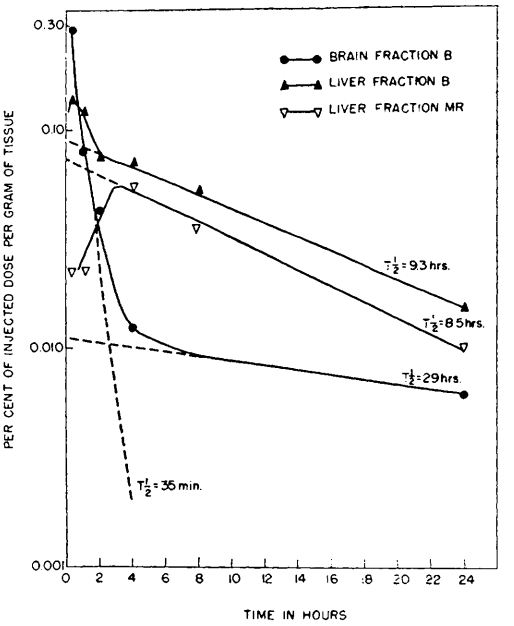

FIG. 1. Percent of inj. tritium recovered in fractions of brain and liver following the intrav. inj. of 2 mg of reserpine-H^3 into guinea pigs.

Results. The percentage of injected tritium found/g of tissue in brain and liver fraction B and liver fraction MR after the intravenous injection of 2 mg of reserpine-H^3 can be found in Fig. 1. No MR could be found in the brain. Maximum tritium concentrations were found within 20 to 30 minutes in brain and liver fraction B, which then decreased rapidly reaching in 2 to 4 hours more stable levels showing biological half-lives of 29 and 9.3 hours respectively. The tritium in liver MR fraction, however, reached its maximum only after 3 to 4 hours but then decreased with a biological half-life of 8.5 hours which was comparable to that for liver fraction B.

Urine and blood tritium levels can be found in Table I. It is observed that the tritium content of plasma fraction B + MR remained relatively constant over the entire experimental period. This was true also for the same fraction of the blood cells but at a very much lower level. The tritium content of fraction C of the plasma was very much lower than that observed for fraction B + MR. No tritium was detectable in fraction C of the blood cells. In urine, however, most of the tritium could be accounted for in fraction C

TABLE I. Blood and Urine Tritium Content following Intravenous Injection of 2 mg of Reserpine-H^3 into Male Guinea Pigs.

Time (hr)	% of inj. dose of 2 mg of reserpine-H^3 found in: Urine fraction B + MR*	Urine fraction C†	Blood plasma fraction B + MR	Blood plasma fraction C	Blood cell fraction B + MR	Blood cell fraction C
⅓			.66	.12	.13	ND
			1.43	.20	.20	"
1			1.00	.08	.14	"
			.80	.21	.17	"
2	.066	2.0	.60	.17	.07	"
			.93	.14	.10	"
4	.38	2.0	1.10	.03	.14	"
	1.26	2.6	1.33	.14	.07	"
8	.45	5.4	.63	.10	.07	"
	.66	5.2	.58	.07	.07	"
24	1.08	4.2	.66	ND	.07	"
0– 5	.22	4.6				
5–24	.76	1.1				

* Fraction B + MR would include tritium derived from reserpine and methyl reserpine.

† Fraction C would include all of the trimethoxybenzoic acid.

ND means none detected.

rather than in fraction B + MR.

The results of Exp. II in Table II show that the tritium content reached a maximum in the brain in 20 minutes and in the liver in 60 minutes after injection and was still detectable at lower levels in fraction B of brain and liver after 48 hours. The rate of decrease in tritium concentration was very much slower compared to that of the experiment utilizing the intravenous route and this was probably the result of the slow absorption of reserpine from the injection site. In spite of the low levels of reserpine administered and the small amounts which entered the tissues, the curves were still biphasic in nature. Evaluation of the pharmacological effects of the single subcutaneous injection of 35 micrograms showed that observable ptosis came on after about 2 hours, increased in intensity and became accompanied by myosis, and shivering, after about 8 hours. The intensity of effect fell off somewhat after 24 hours and was practically gone after 48 hours. Thus, during the period when all observable effects had appeared, reached a maximum and disappeared, the tritium concentration of the brain remained unchanged. It was necessary to be certain that the low levels of tritium being measured were from reserpine and not from stray tritium atoms which had been stripped off of the reserpine by exchange reactions. Another group of guinea pigs were injected with reserpine-C^{14} and sacrificed after 48 and 72 hours. The results of experiment III in Table III showed that approximately 70% of the C^{14} recovered could be accounted for in the spot on the paper chromatogram occupied by the unlabeled reserpine which had been added as carrier at the time of extraction. This was so after 72 hours, when no effect was evident, as well as after 48 hours, when approximately one-half

TABLE II. Concentration of Tritium in Tissue Fraction B and Graded Responses of the Animals following Subcutaneous Injection of 35 μg (Approx. 0.1 mg/kg) of Reserpine-H^3.

Time, hr	Brain % I.D./tissue	Brain μg/g*	Liver % I.D./tissue	Liver μg/g	Inj. site, % I.D./tissue	Graded response†
⅓	.18	.023			30.65	
	.12	.013	.83	.019		
1	.13	.014	1.11	.022	4.77	
	.13	.014				
2	.11	.012	.15	.004		+
	.10	.011				
8	.080	.009				+++
	.080	.009			1.14	
24	.11	.012			.35	++
	.10	.011				
48	.08	.009	.08	.002	.18	±
	.09	.009				

* This column is calculated on the assumption that all of the tritium is contained in reserpine.

† Mild ptosis, +; ptosis plus myosis, ++; marked ptosis, myosis and shivering, +++.

TABLE III. C^{14} Content of Guinea Pig Brains 48 and 72 Hours after Subcutaneous Injection of 35 μg of Reserpine-C^{14} and Its Recovery as Reserpine on a Paper Chromatogram.

Hr after inj.	μg of reserpine/g of brain	% of total C^{14} on paper recovered in reserpine spot
48	.015	78
48	.009	67
48	.012	73
48	.018	75
72	.012	78
72	.010	57

of the animals showed only slight ptosis. These results were true for both brain and liver fraction B. No C^{14} was detectable in the total volume of blood obtainable by exsanguination indicating that the reserpine present in these tissues could not be from the contaminating blood. The levels of C^{14} found in these experiments were quite comparable with the levels of tritium found in the experiments utilizing animals which had received tritiated reserpine.

Discussion. In general the results of the experiment utilizing 2 mg of reserpine-H^3 per animal intravenously agree with those found with similar doses of reserpine-C^{14}. In both cases the concentration *vs.* time curves could be resolved into two components one having a short and the other having a long biological half-life. Attempts were made to account for these two components by examination of subcellular fractions. Trial experiments indicated that while most of the reserpine could be found in the particulate matter of the cell no one fraction could be assigned the unique property of accumulating and retaining reserpine. The biological decay curves for tritium in all of the particulate fractions were resolvable into two components as was the case for the complete homogenate. It should be recognized that the distribution of reserpine found in the subcellular fractions may reflect more of a redistribution after homogenization than the actual situation in the intact cell.

Any attempt to compare directly the urinary excretion values obtained in these experiments with those obtained with reserpine-C^{14} will find that the tritium values are low particularly for fraction C. However, when one considers the fact that only $\frac{1}{3}$ of the tritium in the reserpine molecule resides in the TMBA moiety the values become quite comparable.

The tritium content of the plasma B + MR fraction is probably derived mostly from its MR content since in the studies with reserpine-C^{14}(2) and those reported by Hess *et al.*(3) for unlabeled reserpine, blood levels of reserpine quickly drop to very low values. The biological half-life for reserpine in plasma may be calculated from the data of Sheppard *et al.*(2) as being approximately 50 minutes.

It is interesting to note that while the distribution of reserpine between plasma and blood cells is in favor of the plasma, that between plasma and cells of tissues favors the latter. It appears likely that this ability to concentrate reserpine against a concentration gradient is in some way related to its prolonged action.

The presence of what appeared to be two components in the brain and liver fraction B concentration *vs.* time curves suggested the need for studies utilizing much smaller and more pharmacological doses of reserpine. It was felt that the short half-life component might have reflected the entrance and release of a physiological excess of reserpine and therefore might be confusing the picture. The much smaller dose of 35 micrograms/animal was given subcutaneously to allow for a slower onset and development of symptoms. The short half-life component was markedly reduced but its presence was still evident. Though this point is still not fully explained the experiment pointed up the persistent presence of reserpine throughout and beyond the period of observable pharmacological responses. The earlier conclusions that the intensity of the pharmacological effects of reserpine bear no relationship to its concentration in the brain have been strongly reinforced by the experiments reported here. While the paper chromatographic identification of reserpine should not in any way be construed as absolute proof of the nature of the tritiated material present in the brain and liver it is certainly very strong evidence. One may safely state that the material cannot be isoreserpine, reserpine N-oxide, didehydro or

tetradehydro-reserpine since reserpine is satisfactorily separated from these substances. The comparable levels of tritium and C^{14} labeled reserpine found in the livers and brains after 48 hours strongly suggest that practically all of the tritium is biologically stable.

The question as to the site and mechanism of action of reserpine still remains to be answered. The lowering of the levels of serotonin and norepinephrine by reserpine in brain and other tissues observed by a number of workers(11,12,13), while probably offering some hint as to the mechanism of action, fails to explain the time course of events following the administration of this drug. Shore *et al.* (11) have stated that one is not able to correlate the observable effects of reserpine with the concentration of serotonin in the brain of rabbits.

Perhaps it would be of value to look to some tissue other than the brain as the primary site of action of reserpine. One could suggest that initially reserpine acts on some metabolic pathway of a vital organ such as the liver with the result that there occurs a quantitative change in the output of some substance or substances involved in the normal functions of the central nervous system. Such an effect could explain the lag observed in the onset of symptoms of reserpine action. This hypothesis is currently being used as a guide to further work on the mechanism of action of reserpine.

Summary. (1) Reserpine randomly labeled with tritium had a time-concentration relationship in liver and brain similar to that observed for reserpine labeled with C^{14} in the TMBA moiety. (2) Throughout and beyond the entire period of observable pharmacological response, reserpine was found to be present in brain and liver. (3) The concentration of reserpine in the brain varied little during the entire period of observable activity and bore no relationship to the intensity of response. (4) The material present in the brain was indistinguishable from reserpine by paper chromatography.

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