1957, v96, 728.

5. Sawyer, C. H., *Anat. Rec.*, 1957, v127, 362.

6. Tuchmann-Duplessis, H., Mercier-Parot, L., *C. R. Soc. Biol.*, Paris, 1957, v151, 656.

7. Desclin, L., *ibid.*, 1957, v151, 1774.

8. Benson, G. K., PROC. SOC. EXP. BIOL. AND MED., 1958, v99, 550.

9. Sulman, F. G., Winnik, H. Z., *Lancet*, 1956, v1, 161.

10. Marshall, W. K., Lieberman, D. M., *ibid.*, 1956, v1, 162.

11. Plummer, A. J., Barrett, W. E., Maxwell, R. A., Finocchio, D., Lucas, R. A., Earl, A. E., *Arch. Inter. Pharmacol. et Therap.* (Ghent), 1959, v119, 245.

12. Shore, P. A., Silver, S. L., Brodie, B. B., *Science*, 1955, v122, 284.

13. Brodie, B. B., Pletscher, A. A., Shore, P. A., *ibid.*, 1955, v122, 968.

14. Waalkes, T. P., Weissbach, H., PROC. SOC. EXP. BIOL. AND MED., 1956, v93, 394.

15. McKinlay, H., *J. Anim. Tech. Assn.*, 1951, v2, 2.

16. Benson, G. K., *J. Endo.*, 1958, v17, xvii.

17. ———, *ibid.*, in press.

18. Sulman, F. G., Winnik, H. B., *Nature*, Lond., 1956, v178, 365.

19. Cowie, A. T., Tindal, J. S., *Endocrinology*, 1955, v56, 612.

20. Gaunt, R., Renzi, A. A., Antonchak, N., Miller, G. J., Gilman, M., *Ann. N. Y. Acad. Sci.*, 1954, v59, 22.

21. Tindal, J. S., *J. Endo.*, 1959, v18, xxxvi.

22. Harwood, C. T., Mason, J. W., *Endocrinology*, 1957, v60, 239.

23. Folley, S. J., In *Marshall's Physiology of Reproduction*, 3rd Ed. Chap. 6, p568. Ed. A. S. Parkes, London, Longmans, Green & Co.

24. Johnson, R. M., Meites, J., *Endocrinology*, 1958, v63, 290.

25. Gaunt, R., Gross, F., Renzi, A. A., Chart, J. J., in *First Hahnemann Symposium on Hypertensive Disease*, Ed. J. Moyer, 1959, p219, W. B. Saunders Co., Philadelphia.

26. Meites, J., Nicoll, C. S., Talwalker, P. K., PROC. SOC. EXP. BIOL. AND MED., 1959, v101, 563.

27. Benson, G. K., Cowie, A. T., Folley, S. J., Tindal, J. S., In *Endocrinology of Reproduction*, p457, Ed. C. W. Lloyd, Academic Press.

Received September 28, 1959. P.S.E.B.M., 1960, v103.

Indirect Evidence of Synthesis of Norepinephrine from 3-Hydroxy-Tyramine-1-C^{14} *in vivo*.* (25437)

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Considerable evidence is available that 3-hydroxytyramine is, *in vitro*, a precursor of norepinephrine(1,2,3). However, little evidence is available about the formation of norepinephrine from 3-hydroxytyramine in the intact animal. The low rate at which 3-hydroxytyramine is utilized for conversion into norepinephrine, as well as the high rate of metabolism of 3-hydroxytyramine and norepinephrine *in vivo* makes it difficult to demonstrate this reaction directly in the intact animal. Since Axelrod(4) has shown that the main metabolite of norepinephrine is 3-methoxy-norepinephrine, it occurred to us that conversion of 3-hydroxytyramine to norepinephrine could be demonstrated *in vivo* indirectly by isolation of the end product of biosynthesis, namely, 3-methoxynorepinephrine. Also, since various kinds of stresses have been reported to increase excretion of norepinephrine, it seemed likely that excretion of 3-methoxynorepinephrine would be affected under these conditions.

Materials. Iproniazid phosphate was kindly supplied by Hoffmann-La Roche; 3-hydroxytyramine-1-C^{14} was obtained from New England Nuclear Corp.; 3-methoxytyramine-1-C^{14} was isolated from urine of rats treated with iproniazid; 3-methoxynorepinephrine, 3-methoxyepinephrine and 3-methoxytyramine were kindly supplied by Sterling-Winthrop

* This investigation supported by grant from Nat. Inst. Health, and presented in part to Fed. Am. Soc. for Exp. Biol., Atlantic City, April, 1959.

Research Inst.; and hog kidney acylase was obtained from Nutritional Biochemicals Corp.

Methods. Groups of 6 rats were treated with 100 mg/kg of iproniazid and 100 μg of 3-hydroxytyramine-1-C^{14}, injected daily. Urine was collected for 24 hours, and samples were hydrolyzed at pH 2 by refluxing them under nitrogen for 30 minutes. After cooling to room temperature, the urine was extracted 4 times with ethyl acetate. The organic layer contained all of the acid metabolites of 3-hydroxytyramine, which were further separated by paper chromatography(5). The aqueous layer was adjusted to pH 4, and the amines were acetylated as described previously(6). The acetylated amines were extracted into methylene chloride, and aliquots of the concentrated extracts were applied for chromatographic separation in the "C" solvent system of Bush(7). Dry chromatograms were then scanned in a calibrated counting chamber, so as to locate all radioactive zones. Material in the radioactive zones was eluted with methyl alcohol and concentrated under nitrogen. Aliquots of concentrated eluates were dried on planchets, and radioactivity measurements were made with Tracerlab SC-16 windowless flow counter on duplicate samples for a time to assure an accuracy of $\pm$ 5%. *Deacetylation.* Dry samples of the acetylated catecholamines or methoxycatecholamines were dissolved in 1 to 2 ml water, and 50 to 100 mg of hog kidney acylase was added. The mixture was incubated 3 hours at 37°C, with air as the gas phase. At end of incubation, *O*-acetyl groups were removed and *N*-acetyl derivatives were extracted from the aqueous phase into ethyl acetate. The combined ethyl acetate extract was concentrated *in vacuo*, and aliquots were chromatographed in the "C" system of Bush for 24 hours, then in amyl alcohol-water for 12 hours, or in a chloroform-formamide solvent system overnight. *Metabolism of 3-methoxytyramine-1-C^{14}.* A group of 6 rats was treated with 100 mg of iproniazid/kg, and 25 μg of 3-methoxytyramine-1-C^{14}, were injected daily. Urine samples were collected for 24-hour periods during 6 days. Urine was treated as described above and radioactive 3-methoxytyramine and 3-methoxy-4-hydroxyphenylacetic acid were isolated. Labeled 3-methoxynorepinephrine could not be detected. *Shock stress of animals.* Rats were stressed in a specially constructed "activity wheel," 15 inches diameter. The wheel was so arranged to permit a shock of moderate intensity (1.5 milliamperes) to be delivered to the feet of rats. When the shock was applied (for 10 seconds at intervals of 30 seconds) the normal response of the animals was to run at high speed to minimize the intensity of the painful stimulus. The procedure was continued for 2 to 3 minutes for each rat.

TABLE I. Mobilities of Catecholamine Triacetate Derivatives and Methoxycatecholamine Diacetate Derivatives in Bush "C" Chromatography System* at 37°C.

Acetate derivate of	Distance from starting line, cm	Mobility in cm/hr
norepinephrine	7- 9	2
methoxynorepinephrine	11-13	3
epinephrine	18-20	4.75
methoxyepinephrine	20-22	5.25
3-hydroxytyramine	23-25	6
3-methoxytyramine	27-29	7

* Descending technic with Whatman No. 1 filter paper prewashed with benzene:methylalcohol (1:1) was used. Catecholamines were developed by passing a narrow strip of paper through solution of 1% $FeCl_3$ and 2% potassiumferricyanide which was then blotted and passed through a solution of 10% KOH in methylalcohol:water (1:1). Methoxycatecholamines were developed by passing a narrow strip through solution of 0.1% dichloroquinone chlorimide in alcohol, blotting and then passing it through a solution of 0.1 m borate buffer pH 10, blotting again and exposing the strip to ammonia vapors.

Results. Mobilities of acetylated catecholamines and methoxy-catecholamines in the "C" solvent system of Bush are listed in Table I. The methoxy derivative moves somewhat faster than its corresponding catecholamine.

A radiochromatogram of the acetylated urine fraction from rats which had been treated for only one day with iproniazid and hydroxytyramine-1-C^{14} shows 2 radioactive peaks with the same mobility as 3-hydroxytyramine triacetate and 3-methoxytyramine diacetate (Fig. 1). However, after the same treatment was prolonged for 3 days, an additional radioactive peak was detected (Fig. 2) which had the same mobility as 3-methoxynorepinephrine diacetate.

When rats were stressed after one day of

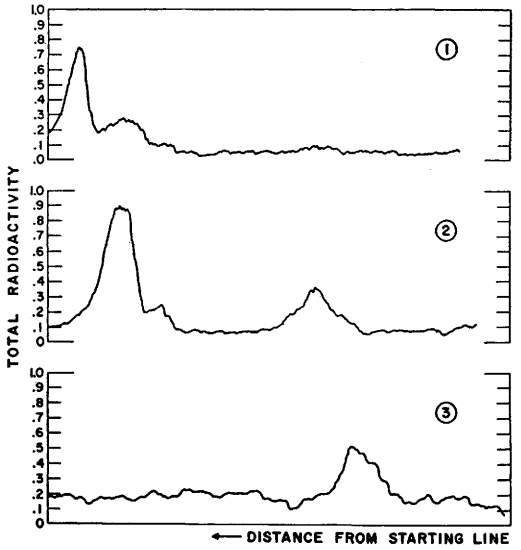

FIG. 1. Paper chromatography separation of acetylated amine fraction in urine of rats treated one day with 100 mg/kg of iproniazid and 100 μg 3-hydroxytyramine-1-C^{14}.

FIG. 2. Paper chromatography separation of acetylated amine fraction in urine of rats treated 3 days with 100 mg/kg of iproniazid and 100 μg 3-hydroxytyramine-1-C^{14}.

FIG. 3. Paper chromatography separation of acetylated amine fraction in urine of stressed rats treated for first 3 days with 100 mg/kg of iproniazid and 100 μg 3-hydroxytyramine-1-C^{14}, and for the last 2 days with iproniazid only.

A 24 hr urine specimen was collected at end of treatment period.

treatment with iproniazid and 3-hydroxytyramine-1-C^{14}, the amine fraction yielded the same 2 radioactive peaks as in the unstressed rats. However, a relatively higher excretion of labeled 3-methoxytyramine was found in stressed rats. When rats were stressed 2 days after last injection of 3-hydroxytyramine-1-C^{14}, all radioactivity of the amine fraction excreted in the next 24 hours was associated with 3-methoxynorepinephrine diacetate (Fig. 3). Table II shows radioactivity of 3-methoxynorepinephrine under various experimental conditions. It is evident that 3-methoxynorepinephrine is still excreted 8 days after last injection of 3-hydroxytyramine.

The radiochemical purity of 3-methoxynorepinephrine was established by rechromatography and deacetylation with hog kidney acylase to *N*-acetyl 3-methoxynorepinephrine. After chromatography in the "C" system of Bush for 24 hours, radioactivity was associated with *N*-acetyl 3-methoxynorepinephrine. The same sample was chromatographed in amyl alcohol water overnight, and then in chloroform formamide solvent, and radioactivity was still found to be associated with *N*-acetyl methoxynorepinephrine. The lower recovery of radioactivity after deacetylation and rechromatography (Table II) partially reflects a loss from the procedure, but is also a result of increased purity, since rechromatography of the *N*-acetyl compounds results in a better separation of norepinephrine from 3-methoxynorepinephrine.

Another radioactive sample of acetylated 3-methoxynorepinephrine diacetate was diluted with 30 mg of carrier of nonradioactive 3-methoxynorepinephrine diacetate (m.p. 123°-125°) and after 2 recrystallizations from ethyl acetate constant specific activity was obtained.

Discussion. Formation of 3-methoxynorepinephrine *in vivo* may proceed by 2 possible pathways, (a) hydroxylation of 3-hydroxytyramine to norepinephrine followed by *O*-methylation to 3-methoxynorepinephrine, or (b) *O*-methylation followed by hydroxylation. Since 3-methoxy-tyramine-1-C^{14} could not be converted into 3-methoxynorepinephrine, it must be concluded that formation of 3-methoxynorepinephrine proceeds through the first pathway, and 3-methoxynorepinephrine

TABLE II. Radioactivity of 3-methoxynorepinephrine Derivatives Isolated from 24 Hour Rat Urine by Paper Chromatography.*

Days after completion of treatment‡	Radioactivity, cpm†		
	3-methoxynorepinephrine diacetate		N-acetyl 3-methoxynorepinephrine stressed
	Unstressed	Stressed	
0	1950§	4600§	2450
2	1200	3100	1900
4	1050	2600	1100
6	850	2000	750
8	620	1300	550

* Rats were treated with 100 mg/kg of iproniazid and 100 μg of 3-hydroxytyramine-1-C^{14} daily for 3 days. Iproniazid alone was then continued until urine was collected.

† Recovery as 3-methoxynorepinephrine averaged 70-90% of the total radioactivity excreted in the urine with the exception of (§) whose values were .37 and .85% respectively.

‡ On day designated, electric shock was given to stressed rats and a 24 hr urine sample was collected from stressed and unstressed rats.

is the last step in biosynthesis of norepinephrine. The finding that 3-hydroxytyramine is a precursor of norepinephrine is in agreement with many studies *in vitro*. However, these findings are the first evidence of synthesis of norepinephrine from 3-hydroxytyramine *in vivo*. It is known that certain stresses increase excretion of norepinephrine(8), and in our stress experiments a higher excretion of 3-methoxynorepinephrine was also observed.

Of special interest may be the discrepancy in the biological half-life of 3-hydroxytyramine and norepinephrine. It was found that labeled 3-hydroxytyramine was no longer excreted 48 hours after last injection of 3-hydroxytyramine-1-C^{14}. However, radioactive 3-methoxynorepinephrine was still being excreted 8 days after last injection of 3-hydroxytyramine. Although a more quantitative study is necessary to permit exact calculation of turnover rates of these 2 catecholamines, it is evident that their half-life is different.

The fact that norepinephrine-C^{14} is stored for at least 8 days, while 3-hydroxytyramine-1-C^{14} appears to be completely replaced by synthesis of endogenous hydroxytyramine in 48 hours, suggests that the turnover rate of dihydroxyphenylalanine to hydroxytyramine is faster than the turnover of 3-hydroxytyramine to norepinephrine. This may mean that conversion of hydroxytyramine to norepinephrine is the rate-limiting step in the synthesis.

Summary. 1. Formation *in vivo* of norepinephrine from 3-hydroxytyramine-1-C^{14}, can be demonstrated indirectly by isolating the end product of biosynthesis, namely, 3-methoxynorepinephrine. 2. Labeled 3-methoxynorepinephrine was isolated as a diacetate derivative from urine of unstressed and stressed rats treated with iproniazid and 3-hydroxytyramine-1-C^{14}. Radiochemical purity of the diacetate and the *N*-acetyl derivative was established by paper chromatography, and by recrystallization of the diacetate derivative to constant specific activity. 3. Radioactivity of 3-methoxynorepinephrine isolated from urine of rats was higher in stressed rats. 4. Labeled 3-methoxynorepinephrine continued to be excreted for 8 days after treatment with 3-hydroxytyramine-1-C^{14} in contrast to radioactive 3-hydroxytyramine and 3-methoxytyramine which disappeared from urine after 48 hours.

The authors thank Dr. S. Bernard Wortis for encouragement of this research, Stephen L. Chorover and Dr. Hans L. Teuber for help in stress experiments, and Linda E. Law for technical assistance.

1. Hagen, P., *J. Pharmacol.*, 1956, v116, 26.
2. Neri, R., Hayano, M., Stone, D., Dorfman, R. I., Elmadjan, F., *Arch. Biochem.*, 1956, v60, 297.
3. Creveling, C. R., Udenfriend, S., *Fed. Proc.*, 1959, v18, 1497.
4. Axelrod, J., Senoh, S., Witkop, B., *J. Biol. Chem.*, 1958, v233, 697.
5. Goldstein, M., Friedhoff, A. J., Simmons, C., *Biochim. et Biophys. Acta,* in press.
6. ———, *Experientia,* 1959, v15, 80.
7. Bush, I. E., *Biochem. J.*, 1951, v50, 370.
8. Euler, U. S.v., *Noradrenaline,* 1956. Charles C Thomas.

Received October 1, 1959. P.S.E.B.M., 1960, v103.

Rabies Virus Isolated from Brown Fat of Naturally Infected Bats. (25438)

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The physiological nature of brown fat is obscure, but is now receiving increased attention primarily because of numerous isolations of several kinds of viruses from brown fat of various animals(1) and an inference that it may play a special role in host-virus relationship(2). Sulkin *et al.*(1) adduced evidence from bats experimentally infected with rabies virus that this virus may be present in brown fat when absent from other organs, and that it may propagate in this tissue. We here present evidence of the presence of rabies