Biochemical Pharmacology, 1964, Vol. 13, pp. 1457–1474. Pergamon Press Ltd., Printed in Great Britain.

RETENTION OF 5-HYDROXYTRYPTAMINE BY SUBCELLULAR FRACTIONS OF RAT BRAIN HOMOGENATES*

C. N. GILLIS, N. J. GIARMAN and D. X. FREEDMAN†

Laboratory of Psychopharmacology, Departments of Psychiatry and Pharmacology, Yale University School of Medicine, New Haven, Conn., U.S.A.

(*Received* 15 *July* 1964; *accepted* 28 *July* 1964)

Abstract—After their incubation with ^{14}C-labeled amine, microsomal fractions of rat brain homogenates *in vitro* retained up to 12 times the concentration of 5-HT-^{14}C (on the basis of their protein content) found in the corresponding mitochondrial fractions. This difference was maintained over the range of concentrations, 100–4,000 mμg of 5-HT-^{14}C per ml of medium. No clear evidence of saturation was seen even when the highest concentration of amine was used. While lowering the temperature of a 30-min incubation to 4° significantly reduced the retention when compared with that at 37°, similar treatment, when the duration of incubation was 10 min, failed to alter the magnitude of retention. Retention was increased (at 37°) when the duration of incubation was 30 or 60 min; however, significant retention took place even when the time of incubation was zero (i.e. when the microsomal fractions were mixed briefly with the labeled amine and immediately subjected to analysis). Microsomal fractions retained up to 12·7 times the concentration of 5-HT-^{14}C in the medium; the corresponding figure for 5-HTP-^{14}C was 3·3. It was found that microsomal as well as mitochondrial fractions of both rat and guinea pig brains contained significant amounts of endogenous 5-HT; in the case of guinea pig brains, the concentration of amine (in terms of the wet weight of fractions) in the microsomal was always higher than in the mitochondrial fractions. The physiological significance of retention of 5-HT by microsomal particles is not clear but may represent, *in vitro*, the process of binding of 5-HT synthesized in the brain, without the limitations on the process normally set *in vivo* by the presence of the cell membrane.

ENDOGENOUS 5-hydroxytryptamine (5-HT) is found mainly in association with subcellular particulate structures in the brain of the rat,[1] guinea pig,[2] and rabbit.[3] Michaelson and Whittaker[2] observed that 5-HT was found principally in a subcellular fraction, which consisted largely of "pinched-off nerve endings." This fraction was prepared by equilibrium density gradient centrifugation of a crude mitochondral preparation. Inouye *et al.*,[3] however, reported that the microsomal as well as mitochondrial fractions of rabbit brain were rich in 5-HT.

Subcellular structures of brain are known to concentrate norepinephrine after their incubation with this amine.[4,5] It has been suggested that similar structures also concentrate exogenous 5-HT.[6, 7] The present study was undertaken to examine in detail the ability of brain organelles *in vitro* to take up 5-HT and its precursor 5-hydroxytryptophan (5-HTP) from incubation media; in addition, the influence of

* Aided by Grants MHO3363-04 and H-7249 from the U.S. Public Health Service.

† And with the technical assistance of S. PACHL.

factors that may alter such retention of exogenous amine was examined. It was also of interest to compare the ability of mitochondria to concentrate exogenous 5-HT *in vitro* with that of microsomal fractions, since only the former have been shown[2] to contain endogenous 5-HT.

METHODS

Two freshly excised whole brains from decapitated male rats (Sprague-Dawley, 150–200 g) were homogenized in nine volumes of chilled 0·3 M sucrose containing 60 μg of the *p*-tolyl ether of choline (TEC) per ml, by means of ten passes of a Teflon pestle in a smooth glass homogenizing tube*. Subsequent centrifugation was carried out at 4° in a Spinco preparative ultracentrifuge. Nuclei and cell debris were separated by centrifugation at 1,000 *g* for 10 min. A mitochondrial fraction was isolated from the cloudy supernatant by centrifuging at 17,000 *g* for 60 min (cf. Michaelson and Whittaker[2]) and the remaining supernatant fluid centrifuged at 105,000 *g* for 30 min to give a microsomal pellet. Both mitochondrial and microsomal pellets were resuspended, by gentle hand-homogenization, in 10 ml of the medium containing varying amounts of radioactive 5-HT or 5-HTP. Unless otherwise indicated, incubations were carried out in the sucrose/TEC medium described above. The suspensions were incubated at either 4° or 37°, for varying periods of time, after which complete separation of particulate and supernatant fractions was achieved by centrifugation at 132,000 *g* for 30 min with a no. 40 Spinco rotor. In some experiments 5-HT-3-^{14}C was added to the suspensions, which were shaken briefly and then subjected to the above separation procedure. These experiments are described subsequently as having been carried out with a zero time of incubation.

Extraction and measurement of radioactive substances

The method used was based on that previously employed in these laboratories.[8] Mitochondrial and microsomal pellets were homogenized in 70% (v/v) ethanol and were allowed to stand in ice for 1 hr. After centrifugation at low speed for 15 min the supernatant was decanted and the radioactivity of a 0·1-ml aliquot determined in a liquid scintillation counter, with the phosphor described by Bray.[9] A second extraction was carried out by suspension of the pellets in 5·0 ml of trichloracetic acid (5%). After centrifugation and aspiration of the supernatant material, the radioactivity of a 0·1-ml aliquot was measured by means of the same phosphor. Results (expressed as counts per minute/milligram protein) are uncorrected for quenching, which was uniformly less than 5% (^{14}C internal standards). The results presented are also uncorrected for recovery of either 5-HT or 5-HTP, which in both cases was greater than 90%. When 5-HT-^{14}C was used, the radioactivity measured reflected mainly unchanged 5-HT. Paper chromatography (butanol:acetic acid:water, 12:3:5), followed by radiochromatogram scanning, revealed that over 90% of the label in ethanol extracts was associated with material having the same R_f value as authentic 5-HT.

The method of Bogdanski *et al.*[10] was used to measure the endogenous 5-HT of frations prepared as described above.

The protein content of both mitochondrial and microsomal pellets was estimated by the method of Lowry *et al.*[11] with serum albumin as a standard.

* The clearance of the pestle (new) was 0·013 to 0·018 cm with a tolerance of ± 0·001 cm.

*Electron microscopy**

Microsomal and mitochondrial fractions, isolated in the manner described above, were fixed in buffered osmium tetroxide,[12] embedded in Epon, and sectioned on an ultramicrotome (LKB) at 300–500Å thickness. Electron micrographs were examined in an RCA (EMU-3F) electron microscope.

MATERIALS

DL-5-Hydroxytryptophan-1-^{14}C (2·85 mc/mmole) and serotonin, purchased as 5-hydroxytryptamine-3-^{14}C creatinine sulfate (11·4 mc/mmole), were obtained from Calbiochem and the Nuclear-Chicago Corp. respectively. The appropriate volume of isotope solution was added to incubation media with a microliter syringe. Throughout this paper, the concentrations of these substances mentioned refer to the amount of base per milliliter of incubation medium.

In some instances (see Results) the weight of the pellets was calculated by subtraction of the weight of the empty Spinco tube from the weight of the tube containing the pellet. Where the significance of data was evaluated statistically, Student's "t" test was the method used.[13]

RESULTS

Influence of amine concentration on the retention of 5-hydroxytryptamine-^{14}C by subcellular particles

When incubation was carried out for 30 min at 37° in 100 to 4,000 mμg 5-HT-^{14}C/ml incubation medium (0·3 M sucrose), the concentration of labeled amine in the microsomal fraction was 9–12 times greater than that in the mitochondrial fractions (Fig. 1). Retention of 5-HT by microsomal fractions† was not significantly changed by increasing the amine concentration from 100 to 1,000 mμg/ml of incubation medium. Between 1,000 and 2,000 mμg/ml, retention of 5-HT was approximately doubled. However, when 4,000 mμg/ml was used, retention was only slightly (and not significantly) increased in magnitude.

Stability of 5-hydroxytryptamine-^{14}C bound to microsomal pellets

In several experiments, microsomal pellets isolated after incubation for 30 min at 37° with 250 mμg, 2,000 mμg or 4,000 mμg of 5-HT-^{14}C/ml were rehomogenized gently in fresh 0·3 M sucrose. After centrifugation of the suspensions at 132,000 *g* for 30 min, the resulting microsomal pellets were found to have retained averages of 52%, 58%, and 70%, respectively, of their original 5-HT-^{14}C content.

Effect of altered incubation time on the retention of 5-HT

Figure 2 illustrates that retention of 5-HT increased in microsomal fractions when the duration of incubation time was lengthened. Retention after 60 min of incubation was significantly greater ($P < 0{\cdot}05$) than that after 10 min. However, significant uptake of 5-HT took place even when the incubation time was zero (Fig. 2; see methods).

* We are very grateful to Dr. R. G. Barnett for preparing and examining the electron micrographs.

† In this study the subcellular elements with which 5HT-^{14}C is associated are defined only on the basis of their common sedimentation characteristics; accordingly the term "microsomal fraction" is used to describe these and all other components that can be isolated by centrifugation between 17,000 *g* (for 60 min) and 105,000 *g* (for 30 min).

Effect of altered temperature of incubation on the retention of 5-HT

Increasing the temperature of a 10 min incubation from 4° to 37° did not increase the amount of 5-HT bound to microsomal particles (Table 1). With a 30-min period of incubation, however, retention was significantly ($P < 0.05$) decreased by lowering the temperature of the medium from 37° to 4°.

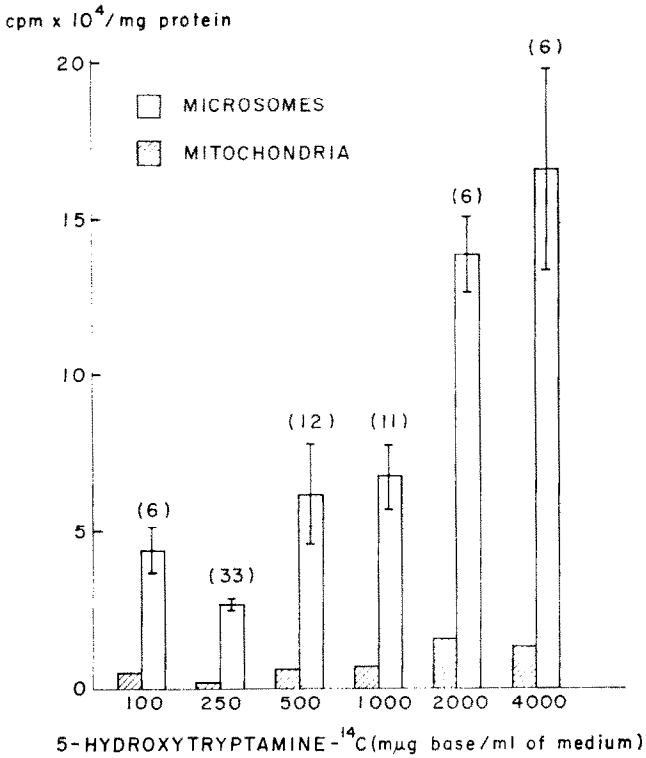

FIG. 1. The influence of amine concentration on the retention of 5-HT-^{14}C by microsomal and mitochondrial fractions of rat brain. The figures in parentheses refer to the number of individual observations. Vertical bars indicate the standard error of the mean values. In all cases, incubation was carried on for 30 min at 37°.

Influence of pH on the retention of 5-hydroxytryptamine

The pH of unbuffered suspensions of particles measured both before and after incubation was always within the range 7·0–7·4. Attempts were made to examine the effect of altering pH by carrying out incubation in 0·3 M sucrose, containing 0·067 M Sorensen's phosphate buffer at pH 6·6, 7·2, and 7·8. It was found (Table 2) that mean uptake by microsomal particles was maximal at pH 7·2. The use of buffered sucrose, however, invariably led to the production of a smaller volume of particulate sediment and to considerably greater variability in uptake than was seen when unbuffered sucrose was used. The reason for this finding is unclear, but it is similar to that previously reported for subcellular fractions of brain[14] and heart.[15]

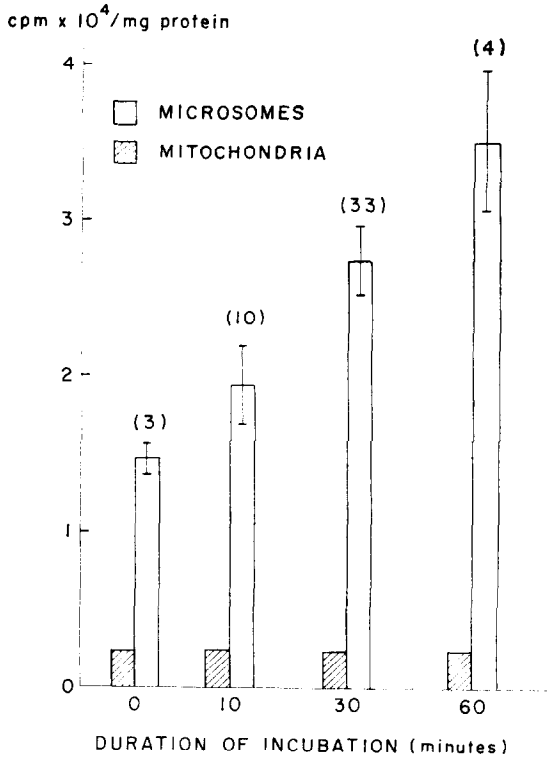

FIG. 2. Effect of altered time of incubation at 37° on the retention of 5-HT-^{14}C by microsomal and mitochondrial fractions of rat brain. In all cases, the concentration of 5-HT-^{14}C employed was 250 mμg/ml. Figures in parentheses refer to the number of individual observations. Vertical bars indicate the standard error of the mean values.

TABLE 1. THE INFLUENCE OF TEMPERATURE OF INCUBATION ON THE RETENTION OF 5-HYDROXYTRYPTAMINE-^{14}C

In all experiments 250 mμg 5-hydroxytryptamine/ml incubation medium were used

Incubation		5-hydroxytryptamine-^{14}C
Temperature	Duration (min)	(cpm × 10^4/mg protein ± S.E.M.)
4°	10	1·65 ± 0·14 (5)*
37°	10	1·94 ± 0·26 (10)
4°	30	1·67 ± 0·13 (6)
37°	30	2·74 ± 0·23 (33)†

* Figures in parentheses refer to the number of values contributing to the mean.

† Significantly ($P < 0·05$) different from mean retention after 30 min of incubation at 4°.

Specificity of uptake by microsomal particles

Slices of the brain of rat[8] and of dog[16] have been shown to concentrate 5-HTP but not 5-HT from the medium in which they were incubated. It was therefore appropriate to investigate the ability of microsomal particles to concentrate 5-HT in contrast to 5-HTP. To this end, equimolar concentrations of both the substances

(equivalent to 250 mμg of 5-HT/ml) were incubated with microsomal fractions for 30 min at 37°. The results of these experiments, expressed as the ratio, cpm × 10^4 per g wet weight/cpm × 10^4 per ml medium, indicated that 5-HTP was concentrated by a factor of 3·3 (mean of 6 determinations), while the microsomal elements retained 12·7 (mean of 3 determinations) times the concentration of 5-HT in the medium.

TABLE 2. THE EFFECT OF pH ON THE RETENTION OF 5-HYDROXYTRYPTAMINE-^{14}C
In all experiments 250 mμg 5-hydroxytryptamine-^{14}C/ml incubation medium were used

Medium	pH	Number of samples	Retention (cpm · 10^4/mg protein ± S.E.M.)
Sucrose + TEC	7·0–7·4	11	3·18 ± 0·49
Sucrose + TEC + phosphate buffer	6·6	4	1·15 (range 0·6–1·5)
Sucrose + TEC + phosphate buffer	7·2	7	1·79 ± 1·25
Sucrose + TEC + phosphate buffer	7·8	3	1·63 (range 0·7–3·0)

TABLE 3. THE ENDOGENOUS 5-HYDROXYTRYPTAMINE OF MICROSOMAL AND MITOCHONDRIAL FRACTIONS OF BRAIN (mμg/g wet weight)

Species	Microsomal fraction	Mitochondrial fraction
Rat*	259 ± 41 (16)†	224 ± 22 (16)
Guinea pig‡	379 (range 310–500, n=3)	188 (range 170–200, n=3)

* Two rat brains contributed an average of 2·67 g in the mitochondrial and 0·18 g in the microsomal fraction

† Figures in parentheses refer to the number of individual values.

‡ Tissue rostral to the quadrigeminal bodies was used in these determinations.

The fluorescence and activation spectra obtained from extracts of microsomal fractions were identical with those of authentic 5-hydroxytryptamine. It can be seen (Table 3) that in microsomal fractions of both total rat brain and guinea pig brain sectioned rostrally to the quadrigeminal bodies, the concentration of the endogenous 5-HT was greater than in the corresponding mitochondrial fractions. Subcellular fractions of guinea pig brain were prepared by the technique described earlier (see Methods) for rat brain. These results have been calculated and expressed in terms of the wet weight of the appropriate fractions in order to give an indication of the concentration of the amine relative to each subcellular species.

Electron microscopy

Electron micrographs of microsomal fractions revealed the presence of many vesicular structures of different sizes. Also present were strands of endoplasmic reticular membrane and many densely stained particles, which in some instances were enclosed within vesicles. No mitochondria were present in this fraction.

The mitochondrial fraction was quite heterogenous and contained, in addition to mitochondria showing various degrees of integrity, nerve-ending fragments, myelin figures, and all the structures seen in the microsomal pellets.

DISCUSSION

It is apparent from this study that microsomal fractions of rat brain homogenates,

like those of rabbit brain[7] have the ability to retain 5-HT after their incubation in media containing the amine. The absolute amount of 5-HT-^{14}C retained by mitochondrial fractions was always higher than that of microsomal fractions. However, if retention is expressed in terms of the protein content of the fractions, it is seen (Fig. 1) that the capacity of microsomal fractions in this regard is up to 12 times that of mitochondrial fractions prepared from the same homogenate; furthermore, this differential is maintained over the entire range of concentrations used (100–4,000 mμg 5-HT-^{14}C/ml medium). Since electron micrographic examination revealed in the mitochondrial fraction some of the subcellular structures characteristically found in the microsomal fraction, the observation of retention in both fractions is not unexpected. If, as seems very likely, the same subcellular element or elements are responsible for the observed retention in both fractions, the lower concentration (in terms of protein) of the amine in mitochondrial fractions can be explained by the probability that they contain some subcellular particles (e.g. mitochondria), which do not contribute to amine retention but which increase considerably the protein content of such fractions.

Inouye and his colleagues[7] reported a direct relationship between the incorporation of exogenous 5-HT by brain microsomal fractions *in vitro* and its concentration in the medium. While our results (Fig. 1) do not show a clear proportionality between the concentration of 5-HT-^{14}C and its retention, neither is there any convincing evidence of saturation at the highest concentration used (4,000 mμg/ml). These observations, together with the fact that significant retention of 5-HT-^{14}C took place even when the time of incubation was zero (see Methods) or when the temperature of incubation was 4° (Table 1), suggest that the retention observed is nonspecific. However, the process is dependent partially on temperature, since the magnitude of retention at 37° was significantly ($P < 0{\cdot}05$) greater than that at 4°, after 30 min of incubation .It is of interest that these characteristics of 5-HT retention by microsomal fractions are similar to those reported previously for norepinephrine retention by similar fractions.[5]

Despite our observations, which suggest that microsomal elements are largely unable to control the magnitude of amine retention *in vitro*, some evidence of specificity was seen. Thus the concentration of 5-HT-^{14}C in microsomal pellets was over 12 times that in the medium, while the corresponding figure for 5-HTP-^{14}C was 3. In contrast, Schanberg[8] reported that slices of rat brain were able to concentrate 5-HTP, but not 5-HT, *in vitro*, Schanberg suggested that 5-HTP was concentrated by a process of "facilitated transport," since the magnitude of uptake was decreased by dinitrophenol or by lowering the temperature of incubation. It is likely that the cellular disruption produced by homogenization results in a radical change in the behavior of brain structures in their binding of both 5-HT and 5-HTP, such that the microsomal elements *in vitro* can concentrate the amine and, to a much more limited degree, its precursor. The failure to achieve saturation of retention *in vitro* over a wide range of concentration, and the relatively slight effect on retention caused by lower temperatures or shorter duration of incubation, make it probably that discrete control over the concentration and retention of amines (both 5-HT and norepinephrine) requires the integrity of the cell.

The physiological significance of retention of 5-HT to microsomal particles is not clear. However, the existence of endogenous amine in those fractions of brain homo-

genates that also have the ability to retain exogenous amine *in vitro*, may point to a role of such particles in the storage of 5-HT synthesized in the brain. Our study reveals many similarities between the retention of amines by microsomal fractions of brain and of heart;[15] it is tempting to speculate therefore that the extensive cellular disruption caused by homogenization not only deprives the cell of its ability to control amine binding but also to some extent decreases the specific identity of tissues, as reflected by studies *in vivo* where a differential ability to limit the concentration of amines is seen.

Michaelson and Whittaker[2] reported that the bulk of the endogenous 5-HT of brain homogenates was associated with "pinched-off" nerve endings that sediment with the mitochondria: these particles are believed to be formed during the homogenization of brain.[17] It is conceivable that such a process may proceed to the point of disruption of the particles themselves, with consequent liberation of their contents. Some of the vesicles so liberated from the nerve particles might be expected to sediment in the microsomal fraction. Since endogenous 5-HT is associated with these particles,[2] our finding (Table 3) of as much (and in some cases more) 5-HT in the microsomal as in the mitochondrial fractions offers some support for this suggestion. However, Michaelson and Whittaker[2] found no 5-HT in the microsomal fraction prepared from guinea pig brain tissue rostral to the quadrigemina. We felt this difference merited closer examination. Using the same portion of brain and conditions of differential centrifugation as employed by these workers (see Methods), we found that in all instances microsomal fractions contained a higher concentration of 5-HT than did the corresponding mitochondrial fractions. Since, in these experiments, the activation and fluorescent spectra of the material measured were identical with those of authentic 5-HT, there can be little doubt that the microsomal fraction of both rat and guinea pig brains contained significant quantities of the amine. At this time no explanation can be offered to reconcile our findings with those of Michaelson and Whittaker.

Acknowledgement—We are grateful to Mr. K. Rozitis for carrying out the protein estimations.

REFERENCES

1. N. J. GIARMAN and S. M. SCHANBERG, *Biochem. Pharmacol.* **1**, 301 (1958).
2. I. A. MICHAELSON and V. P. WHITTAKER, *Biochem. Pharmacol.* **12**, 203 (1963).
3. A. INOUYE, K. KATOAKA and J. SHINAGAWA, *Nature (Lond.)* **194**, 286 (1962).
4. B. L. MIRKIN, N. J. GIARMAN and D. X. FREEDMAN, *Biochem. Pharmacol.* **12**, 214 (1963).
5. B. L. MIRKIN and C. N. GILLIS, *Biochem. Pharmacol.* **12**, 1173 (1963).
6. E. W. MAYNERT, R. LEVI and K. KURIYAMA, *Fed. Proc.* **23**, 350 (1964).
7. A. INOUYE, K. KATOAKA and J. SHINGAWA, *Nature (Lond.)* **198**, 291 (1963).
8. S. M. SCHANBERG. *J. Pharmacol. exp. Ther.* **139**, 191 (1963).
9. S. BRAY, *Analyt. Biochem.* **1**, 279 (1960).
10. D. F. BOGDANSKI, A. PLETCHER, B. B. BRODIE and S. UDENFRIEND, *J. Pharmacol. exp. Ther.* **117**, 82 (1956).
11. O. LOWRY, N. ROSENBROUGH, A. FARR and R. RANDALL, *J. biol. Chem.* **193**, 265 (1951).
12. J. B. CAULFIELD, *J. biophys. biochem. Cytol.* **3**, 827 (1957).
13. L. BERNSTEIN and M. WEATHERALL, *Statistics for Medical and other Biological Students*, Livingstone, London (1952).
14. B. L. MIRKIN, N. J. GIARMAN and D. X. FREEDMAN, *Biochem. Pharmacol.* **13**, 1027 (1964).
15. C. N. GILLIS, J. *Pharmacol. exp. Ther.* In press (1964).
16. S. E. SMITH, *Brit. J. Pharmacol.* **20**, 178 (1963).
17. E. G. GRAY and V. P. WHITTAKER, *J. Anat. (Lond).* **96**, 79 (1962).