Biochemical Pharmacology, 1964, Vol. 13, pp. 1639–1653. Pergamon Press Ltd., Printed in Great Britain.

HYDROXYLATION OF TRYPTOPHAN BY BRAIN TISSUE *IN VIVO* AND RELATED ASPECTS OF 5-HYDROXYTRYPTAMINE METABOLISM

E. M. GAL, M. MORGAN, S. K. CHATTERJEE and F. D. MARSHALL, JR.

Neurochemical Research Division, Department of Psychiatry, College of Medicine, State University of Iowa, Iowa City, Iowa, U.S.A.

(*Received* 18 *May* 1964; *accepted* 6 *July* 1964)

Abstract—As an evidence for the hydroxylation of L-tryptophan (Try) to 5-hydroxytryptophan (5-HTP) in the brain, it was demonstrated that intracerebrally injected (^{14}C)-L-Try led to the appearance of (^{14}C)-5-hydroxytryptamine (5-HT) in the brains of pigeons and rats. Under identical conditions, the injection of the D-isomer did not give labeled 5-HT, but it decreased the 5-HT level in the brain. Intraperitoneal injection of (^{14}C)-Try in amounts equal to or 17 times larger than those injected i.c. did not lead to the appearance of labeled 5-HT in the central nervous system. Identification of label in 5-HT was proved by column, paper chromatography, enzymatic conversion to 5-hydroxyindoleacetic acid, and by recovery of (^{14}C)-5-HT from its picrate salt. Results of the studies of the efflux and turnover of Try, 5-HTP, and 5-HT are also presented.

STUDIES *in vivo*[1] and *in vitro* have yielded sufficient evidence to show that tryptophan (Try) is the precursor of 5-hydroxyindole compounds and that the most extensively investigated of them, 5-hydroxytryptamine (5-HT) is derived from this dietary amino acid in a two-step process. *In-vitro* hydroxylation of Try to 5-hydroxytryptophan (5-HTP) has been demonstrated recently.[2,3] In the liver, the enzyme responsible for this reaction has been shown to be phenylalanine hydroxylase.[4] However, the authors of this report, supported by corollary evidence, questioned that this enzyme is the exclusive regulatory and contributory site of 5-HTP synthesis. They noted that the administration of amethopterin, an inhibitor of phenylalanine hydroxylation, did not alter the 5-HT levels of the brain. Further observations[5–7] strongly support an extrahepatic hydroxylation of Try to 5-HTP.

These facts, suggestive as they are of hydroxylation of Try to 5-HTP by other tissues, do not constitute direct proof of it. The *in vitro* experiments have failed to show any hydroxylation of Try by brain tissue,[4] implying the absence of a hydroxylating mechanism in the central nervous system. Two preliminary reports[8,9] recently described the *in vivo* hydroxylation of Try in the brain tissue.

This paper is a detailed report of the conditions affecting the hydroxylation of Try by the central nervous system and the relation of this hydroxylation to some aspects of 5-HT metabolism.

EXPERIMENTAL

One hundred and fifty pigeons (400 g) and 100 Sprague-Dawley rats (300 g) were kept on standard diet with water *ad libitum*. Animals were of both sexes. In general,

the experimental and control rats were litter mates. Twelve hours before the animals were injected they were deprived of food and divided into groups according to the experimental schedule. The chemicals were administered either intraperitoneally or intracerebrally. Prior to the i.c. injection the head of each animal was shaved, and the scalp was cleansed with 70% alcohol.

Pigeons. After exposing the cranium of the pigeon by an 18-mm longitudinal incision of the scalp, a fine hole was opened with a dental drill (No. $\frac{1}{2}$ bur) at an approximate intersection of the sagittal and coronal sutures. The solution containing the radioactive substance was injected at about 0·5 μl/sec from a 10 μl Hamilton syringe inserted 9 mm at a 45° angle. To prevent the escape of the radioactive solution because of intracerebral pressure the needle was carefully withdrawn; the hole in the scalp was sealed with collodion and the animal was replaced in its cage.

Rats. The rat was kept under light ether anesthesia ,with the head stereotaxically placed, while the hole was drilled in the cranium; the animal was left in the instrument until it came out of anesthesia. The radioactive compound was then injected by the same procedure used for the pigeon.

(^{14}C) *Compounds.* Several batches of radioactive Try were used and their specific activities (s.a.) were: (3-^{14}C)-DL*-Try, s.a. 4·67 mc/mmole, and (2-^{14}C)-DL-Try, s.a. 0·623 mc/mmole, from Tracerlab, Inc. (Boston, Mass.); (3-^{14}C)-DL-Try, 8·95 mc/mmole, from New England Nuclear Corp. (Boston, Mass.); (3-^{14}C)-5-HT, creatine sulfate, 32 mc/mmole; (3-^{14}C)-L-Try, 12·6 mc/mmole; (3-^{14}C)-D-Try, 12·6 mc/mmole; (2-^{14}C)-tryptamine·bisuccinate, 1·3 mc/mmole, from Nuclear-Chicago (Des Plaines, Ill.); (3-^{14}C)-DL-5-HTP, 2·4 mc/mmole, from California Corp. for Biochemical Research (Los Angeles, Calif.).

Chemicals. Serotonin (5-hydroxytryptamine creatinine sulfate H_2O), 5-HTP, 5-hydroxyindoleacetic acid (5-HIAA), indoleacetic acid (IAA), tryptamine (TA), and N-acetyl-L-Try, 5-methoxytryptamine (5-MHT), were purchased from Sigma Chemical Co. (St. Louis, Mo.).

Drugs. β-Phenylisopropylhydrazine.HCl (PIH) from Lakeside Lab. (Milwaukee, Wis.).

Preparation of tissue extracts. Blood was withdrawn from the heart of each animal with a heparinized needle. The animal was then killed at a predetermined time by decapitation. Tissues were immediately removed, weighed, and processed as described in the various analytic procedures below.

1. *Determination of free tryptophan.* The excised tissues were homogenized in 5 ml boiling water/g tissue. After the homogenates were cooled to about 5°, 0·15 ml of 40% trichloroacetic acid (TCA) per ml of homogenate was added. After centrifuging, the free tryptophan content of the supernatants was determined according to the method of Hess and Udenfriend.[10]

2. *Recovery of 5-HT.* Brains were quickly removed, weighed, and homogenized in ice-cold 0·1 N HCl when the *n*-butanol extraction method of Bogdanski *et al.*[11] (method A below) was employed or with ice-cold 0·4 N perchloric acid as required by the method (B, below) of Bertler.[12]

Method A. Each gram of brain was homogenized in 3 ml of 0·1 N HCl. The acid precipitate of each brain was resuspended in 3 ml of acid and recentrifuged. The final

* Prior to July 1963, (^{14}C)-L-tryptophan was not available to the authors; hence the radioactive racemate in the earlier experiments was necessarily used.

supernatants were pooled, and a known volume of each was taken for determination of radioactivity; the remainder was submitted to butanol extraction for the isolation of 5-HT. The blank and standard [in several instances (^{14}C)-5-HT was used as standard] were submitted to the same procedure. The butanol extracts obtained by method A were processed as described elsewhere.[8] In several experiments where the recovery of all the Try metabolites was of paramount importance, the extraction method of Shore and Olin[13] was preferred.

Method B. The brain samples were homogenized with 0·4 N $HClO_4$. The precipitates and supernatants were treated as under method A up to the point of butanol extraction. A measured volume of the ice-cold supernatant (about 6 ml), adjusted to pH 6·5, was applied to a 6 × 30-mm column of Amberlite XE-64 ion-exchange resin converted to Na^+ form as recommended.[12] After the supernatant had passed through the column, it (fraction E) was collected. A known aliquot was taken for radioactive assay; the remainder of the extract was analyzed for Try, 5-HTP, and 5-HIAA content. The column was successively washed with 10 ml of phosphate buffer–Versene solution, followed by 10 ml of double-distilled water (redistilled over Versene). Both washes were routinely checked for radioactivity and contained negligible counts at most. The 5-HT was eluted by 3 ml of 1·2 N HCl followed by two 3 ml acid washes. One ml of the 5-HT eluate was mixed with 0·3 ml of concentrated HCl and was read on the spectrophotofluorometer for its 5-HT content. The remaining 2 ml of the 5-HT eluate was lyophilized and the residue taken up in 0·5 ml of water. Of this solution 0·1 ml was counted for radioactivity, and 0·3 ml was used for further identification.

Recovery of 5-HIAA and IAA. The 5-HIAA content of the brain was determined from column fraction E by the method of Roos.[14] Determination of 5-HIAA and IAA obtained after enzymatic oxidation[15] of 5-HT and TA was done as described in the text. In the experiments with (^{14}C)-DL-5-HTP, the 5-HTP content was calculated from the difference in the counts from fraction E before and after extraction of 5-HIAA.

Chromatography. The chromatographic separation and identification of the components in the acetone extracts obtained by method A were as described earlier.[8] The indoleacetic acids were identified after developing the chromatograms by *n*-butanol:acetic acid:water (v/v 40:10:50). The concentrated solutions of eluates from the ion-exchange columns were run by thin-layer chromatography on silica-G in methyl acetate:isopropanol:25% NH_4OH (v/v 45:35:20) according to the technique of Stahl and Kaldewey.[16]

Measurement of radioactivity. All samples were counted in a liquid scintillation counter (Nuclear-Chicago). The counting efficiency of unquenched (^{14}C)-samples was 78%. In all instances quench corrections were established. For routine counting, 0·1 ml of samples of known volume was added to the counting vials containing 2 ml of absolute alcohol and 13 ml of fluor mixture (POP and POPOP) in toluene. Tissues and protein samples were dissolved in Hyamine solution and occasionally the dry tissue protein was dispersed with Cabosil. In some experiments the radioactive spots from paper chromatograms were cut out and counted directly, according to the technique of Takahashi *et al.*[17] In other instances the radioactive spots were eluted with 0·01 N HCl. After the acid eluates were treated with 2,2-dimethoxypropane to remove water,[18] they were counted directly. After thin-layer chromatography the radioactive substances were extracted from the plates with 1·5 ml of 0·1 N HCl and separated from

silica G by filtration. The filtrates were counted as described for the eluates from paper strips.

Isolation of protein. In the studies with bound Try, the protein was isolated according to Norberg and Greenberg.[19] The protein nitrogen was determined by the Kjeldahl method.

Isolation of 5-HT picrate was done according to Udenfriend and Weissbach.[20]

Enzyme assays. In the enzymatic conversion of the indoleamines to the corresponding indoleacetic acids, a system combining guinea pig liver mitochondrial monoamine oxidase and guinea pig kidney aldehyde dehydrogenase was used according to the assay of Lovenberg *et al.*[15] The mitochondria was prepared according to the technique of Hogeboom and Schneider.[21] The activity of the aldehyde dehydrogenase was verified by the method of Racker.[22]

RESULTS AND DISCUSSION

Estimated losses of indole compounds during isolation from brain tissue

In several experiments the brains of untreated animals were removed and homogenized in the presence of a known amount of labeled 5-HT. The recovery of 5-HT was estimated at each step of the two procedures. The results, which are presented in Table 1, indicate considerable loss of 5-HT by either method of isolation. The

TABLE 1. RECOVERY OF 5-HT FROM BRAIN TISSUE SUBJECTED TO VARIOUS PROCEDURES

Method	Fraction	(^{14}C-5-HT (dpm $\times 10^4$)	Overall recovery (%)
A. *n*-Butanol extraction	Homogenate	9·98*	100
	Butanol/acid extract	4·55	46
	Lyophilization	4·10	41
	Paper chromatogram	2·28	23
	Eluate of chromatogram	1·47	15
B. Ion-exchange column	Homogenate	20·93*	100
	Supernatant and wash	10·50	50
	1·2 N HCl eluate	8·02	38
	Lyophilization	7·34	35
	Eluate from thin-layer chromatography	2·51	11

* (^{14}C)-5-HT was added to brain homogenates.

repeated washings of the precipitates by 0·4 N perchloric acid did not appreciably decrease radioactivity in the precipitates. The recovery of radioactivity in the precipitates was somewhat higher than that found by Giarman and Schanberg.[23] These authors had shown that particles of brain homogenized in sucrose would adsorb significant amounts of 5-HT added exogenously. An analytical assessment of the percentage recovery of 5-HT by the various methods permitted a correction of 5-HT counts and amounts found for the overall losses inherent in the procedures.

The values in Table 1 represent averages of three different samples. It is apparent from these values that about 40%–50% of 5-HT was lost during paper chromatography, with further losses encountered during the elution of the chromatograms. This yielded about a 38% recovery of the 5-HT originally applied to the paper. Similar

losses of 5-HT by paper chromatography have been reported by Correale.[24] Recovery of 5-HT after thin-layer chromatography seldom exceeded 30%. No significant losses, however, were observed during the chromatography of Try or 5-HTP. After the radioautography of the butanol extracts, it was noted that about 10% of the radioactive Try originally injected into the brain remained a major contaminant of the 5-HT extract. Repeated washings with borate buffer of the butanol extracts did not eliminate traces of radioactive Try but further decreased the recovery of 5-HT. The ion-exchange method[11] permitted an effective separation of 5-HT from Try, 5-HTP, and 5-HIAA. Under properly controlled conditions, method B was found to be most suitable for the isolation of radioactive 5-HT. Nevertheless, the 5-HT fractions were consistently checked by thin-layer chromatography for the presence of radioactive Try.

Rate of conversion and efflux of intracerebrally administered (^{14}C)-DL-5-*HTP and* (^{14}C)-5-*HT*

In order to establish some relationship between the hydroxylation rate of Try and the appearance of 5-HT, the conversion of 5-HTP to 5-HT and 5-HT to 5-HIAA was followed at varying times after intracerebral injection of (^{14}C)-DL-5-HTP or (^{14}C)-5-HT. This permitted the comparative measurement of the efflux of these compounds and served to give some idea of the relative rates of conversion. After intracerebral injection, radioactivity rapidly spread throughout the central nervous system. The results of others[25] had indicated a slight but insignificant conversion of the D-isomer to 5-HT and 5-HIAA in man. It was therefore assumed that only the L-isomer contributed to 5-HT synthesis. The 9·2 μg of (^{14}C)-DL-5-HTP injected i.c. was equivalent to 2·5 or 3 times the amounts of 5-HT found in the average pigeon brain. Table 2 gives the results of these experiments. The values under total radioactivity represent the sum of the radioactivity found in the supernatants and precipitates without reference to the nature of the radioactive components. On the other hand, the values under (a), (b) and (c) represent 5-HTP, 5-HT, and 5-HIAA from the supernatants which have passed through the ion-exchange column. The obvious discrepancy between the sums of the values under (a), (b) and (c) and the values under total radioactivity recovered can be ascribed to the loss of radioactive material to the acid precipitates. The radioactive substances adsorbed by the precipitates were not recoverable. From the data given under (a) and (b) for 5-HTP and 5-HT, the efflux rates expressed as the time required for these substances to decrease to their halves averaged 30 and 23 min respectively. There was a noticeable variation of the efflux rate of 5-HTP from one time interval to another. Such variations have been reported previously for several other intracerebrally administered amino acids.[26] A closer examination of the data in Table 2 indicates that at any time interval the ratio, which is the sum of radioactivity in 5-HT and 5-HIAA to 5-HTP, is fairly constant. Calculated from the sum of (b) and (c) the percentage distribution of label averaged 62·1% for 5-HT and 37·9% for 5-HIAA for the various periods.

The relatively high values under (a) indicate that the *in vivo* decarboxylation of exogenous 5-HTP was slower than would have been expected in light of the *in vitro* results of others.[27] Decarboxylase assays of fraction E (from the ion-exchange column) would be expected to yield a high conversion of 5-HTP to 5-HT if the L-isomer were

TABLE 2. RECOVERY OF RADIOACTIVITY IN PIGEON BRAIN AFTER INTRACEREBRAL INJECTION OF (^{14}C)-5-HTP AND (^{14}C)-5-HT

Compound	Injected	Time	Total radioactivity recovered		5-HTP		5-Hydroxytryptamine			5-HIAA		Total	Ratio
	(μg)	(min)	(dpm $\times 10^5$)	(%)	(a) (dpm $\times 10^4$)	(%)	(b) (dpm $\times 10^3$)	(%)	($m\mu$moles)	(c) (dpm $\times 10^3$)	(%)	(a, b, c) (%)	
(^{14}C)-DL-5-HTP	9·20	0	2·07	100	20·70	100						100·0	$\frac{b+c}{a}$
		30	1·44	69	7·80	38	7·9	3·8	1·59	5·0	2·4	44·2	0·17
		60	1·11	53	4·60	22	6·1	3·0	1·25	2·4	1·1	26·1	0·18
		120	0·48	23	2·80	13	3·8	1·7	0·71	0·9	0·5	15·2	0·18
		240	0·24	12	0·67	3	1·3	0·6	0·25	0·4	0·2	3·8	0·25
(^{14}C)-5-HT	0·35	0	1·39	100			139·1	100·0	1·99			100·0	$\frac{c}{b}$
		30	0·54	39			22·5	16·0	0·32	16·1	11·5	27·5	0·71
		60	0·31	22			18·5	13·0	0·26	10·6	7·6	20·6	0·57
		120	0·14	10			9·0	6·0	0·12	4·9	3·5	9·5	0·54
		240	0·03	2			2·2	1·6	0·03	1·3	0·9	2·5	0·59

present in large quantities. Experimental evidence of such assays indicates no appreciable recovery of (^{14}C)-5-HT. It is therefore suggested that the presence of the D-isomer with its possible slow efflux accounts for the high radioactivity.

No accurate estimate of the conversion rate *in vivo* of the intracerebrally injected 5-HTP to 5-HT could be made because it was not technically feasible to recover without considerable losses 5-HTP, 5-HT, and 5-HIAA which had been bound to the protein. However, from the experiments with (^{14}C)-5-HT one can get an approximate idea of the losses of 5-HT to the protein precipitate. These losses were about 10% for the first 30 min, and 1%–2% for the next 4 hr. This indicates that in the experiments with (^{14}C)-DL-5-HTP the bulk of radioactivity in the protein precipitates was represented by the amino acid. The decrease of radioactivity following the intracerebral administration of labeled 5-HT presumably might be due to the rapid efflux of 5-HIAA rather than of 5-HT *per se*. Over 60% of the injected 5-HT disappeared within the first half hour. This was followed by a slower and more gradual decrease over a period of several hours. However, even 4 hr after injection, detectable amounts of labeled 5-HT remained in the central nervous system. It is likely that the 1–4 hr values of 5-HT represent "bound" 5-HT. This binding of exogenously supplied (^{14}C)-5-HT might have occurred through an exchange with unlabeled 5-HT at the storage sites. This would imply that "bound" 5-HT need not derive exclusively from an "on-the-site" decarboxylation of its precursor. Germane to this is a recent report[28] of prolonged storage in mouse brain of intravenously administered (^{14}C)-5-HT.

TABLE 3. DISTRIBUTION OF RADIOACTIVITY AFTER ADMINISTRATION OF (3 ^{14}C)-L-TRYPTOPHAN TO PIGEONS

Tryptophan injected (dpm × 10^5)	Time (min)	Brain	Plasma*	Kidney	Liver	Small intestine
		(total disintegration × 10^3/min)				
2·65†	10	108·8	0·7	1·1	8·1	3·5
	30	81·1	0·8	4·1	14·6	9·2
	60	70·1	0·9	8·7	10·1	11·8
	120	60·4	0·7	3·0	14·8	14·1
	240	27·4	0·4	3·4	8·7	7·0
45·20‡	30	7·1		26·3	177·8	397·5
	60	14·2		48·4	293·7	407·5
	120	14·5		69·2	304·7	240·5

Each value represents the average of four animals.
* Expressed as dpm × 10^3/ml.
† Intracerebral.
‡ Intraperitoneal.

Distribution of intraperitoneally and intracerebrally administered (^{14}C)-L-*tryptophan*

In a series of experiments (^{14}C)-L-Try was intraperitoneally injected into pigeons to investigate its penetration and to test the eventual contribution of this amino acid to the 5-HT pool in the central nervous system. The radioactivity in the cerebral 5-HT was followed to see whether intracerebrally injected radioactive Try, after its efflux and its hydroxylation in the liver or in the intestine, was not returned to the central nervous system as labeled 5-HTP in sufficient amounts to account for the (^{14}C)-5-HT recovery from the brain. The results in Table 3 reveal that, with i.p. injection of

amounts of (^{14}C)-L-Try 17 times larger than those administered i.c., only 0·1 %–0·2 % of the total activity appeared in the brain after 30 min. In 2 hr the cerebral radioactivity seemed to level off. Concomitantly there was an abrupt drop in the activity of the small intestine and a continued increase of activity in the kidneys and liver. The values reported for the organs listed in Table 3 represent 15 %–17 % recovery of the total radioactivity administered. Over 50 % of the radioactivity appeared in the carcass. After intracerebral injection of (^{14}C)-L-Try, the recovery of radioactivity from the same organs varied between 10 % and 50 % of the total. These values imply a fairly rapid efflux of L-Try from the brain.

The earlier studies[26] of the degree of penetration of various intracerebrally injected amino acids had already revealed that maximal penetration varied between 29 % and 73 %. Therefore, mindful of the possibility of incomplete penetration of intracerebrally injected L-Try, several animals were killed at 1 and 10 min after administration. The results showed about 60 % penetration of L-Try. Similar values were obtained from a count vs. time curve by extrapolating to zero time.

In a report[9] of the hydroxylation of DL-Try, the effect of D-Try on the hydroxylation was unclear. Thus, the earlier values reported for the specific activity of 5-HT were based on calculations which tentatively assumed a contribution from both isomers. The experiments with (^{14}C)-L-Try, however, clearly demonstrated a much higher incorporation of label in 5-HT, which implied that the presence of the D-isomer might have been inhibitory to the hydroxylation of the L-isomer. Furthermore, during the first 30 min, intracerebrally administered D-Try reduced the 5-HT content of the pigeon brain by about 50 % in both normal and PIH-treated animals. This observation was accepted as a further corollary to the inhibitory nature of D-Try to the synthesis of 5-HTP. These results, along with additional information on the relative efflux rates of the isomers and of the racemate, are given in Table 4. The differences in the efflux rates

TABLE 4. EFFLUX OF DL-, L-, AND D-(^{14}C)-TRYPTOPHAN FROM PIGEON BRAIN AND THEIR EFFECT ON CEREBRAL 5-HT LEVELS

Time (min)	Administered tryptophan present			5-Hydroxytryptamine content		
	DL	L	D	DL	L	D
	(%)			(μg/g)		
30	48	31	84	0·63	0·82	0·30
60	37	26	63	0·58	0·76	0·17
120	30	22	47	0·65	0·68	0·14
240		10			0·72	
Estimated avg. $T_{1/2}$	59	44	105			

Amounts of the amino acids injected were: 17·45 μg DL-Try (8·86 × 10^5 dpm); 4·08 μg L-Try (5·3 × 10^5 dpm); 4·10 μg D-Try (5·56 × 10^5 dpm).

between D- and L-Try are in agreement with the observations of Lajtha and Toth,[26] who found that leucine, phenylalanine, and lysine showed faster efflux when administered as the L-isomer. In experiments with DL-Try, the efflux rates were found to lie between the rates for the two isomers, and the 5-HT levels were not significantly lower than the average of 0·70 μg/g brain.

Unlike the efflux of the D-isomer, that of L-Try was rather rapid during the first 30 min. The time–activity curves obtained from observations at various intervals beyond 30 min indicated a slower rate of decrease in the specific activity of L-Try.

Hydroxylation of tryptophan

Until recently very little information was available on the hydroxylation of Try in the brain and the few studies related to this problem only indirectly supported the ability of the brain to perform enzymatic hydroxylation of Try to 5-HT. The lack of direct evidence, because of difficult experimental conditions and unsatisfactory methods, greatly contributed to the continued and persistent belief in the inability of the brain to synthesize 5-HTP.

The following results are submitted in support of the existence of an extrahepatic synthesis of 5-HTP. In a series of experiments in which (^{14}C)-L-Try was injected into the brain, labeled 5-HT was recovered from that organ. The results are shown in Table 5. It was found that during the first hour a maximum of 0·37% of the total

TABLE 5. RECOVERY OF (^{14}C)-5-HT FROM PIGEON AND RAT BRAIN AFTER INTRACEREBRAL INJECTION OF (^{14}C)-L-TRYPTOPHAN

Expt. no.	Animal	Time (min)	5-HT (dpm)	5-HT (μg)	Radioactivity in 5-HT* (%)	5-Hydroxytryptamine dpm × 10^7/mmole)
5-R	Rat	10	1,463	1·00	0·28	25·7
214	Pigeon	30	1,950	1·42	0·36	24·3
215		30	1,980	1·39	0·37	25·0
222		120	375	1·20	0·05	5·5

In all experiments 4·08 μg of (^{14}C)-L-tryptophan ($5·3 \times 10^5$ dpm) was injected. 5-HT was recovered by the method of Bertler.[12] All values represent (^{14}C)-5-HT found in the acid eluate of the supernatants and are uncorrected for the recovery given in Table 1.

* Based on the amount of L-tryptophan administered.

administered radioactivity would appear in 5-HT. If these values are corrected for the 38% recovery given in Table 1, then the actual conversion of Try to 5-HTP as reflected by the recovered 5-HT will increase to 0·95%–0·97%. The disappearance of labeled 5-HT was slower than that expected from the turnover values reported by Udenfried and Weissbach[20] but faster than that obtained from the turnover studies reported elsewhere in this paper. From these experiments, the $T_{1/2}$ of 5-HT in pigeon brain was 42 min, with a calculated rate of 0·5 μg/g/hr of 5-HT synthesis. This value agrees very closely with that computed from the rate of disappearance of intracerebrally injected (^{14}C)-5-HT. It was also noticed that, after i.c. administration of (^{14}C)-DL-Try, the radioactivity in 5-HT never exceeded 0·17%, and that (^{14}C)-5-HT could not be recovered from the brain after 20 min unless the animals were pretreated with PIH.

In the experiments with L-Try, the acid eluates containing 5-HT were worked up by method B above. The appearance of radioactivity in a single spot corresponding to the R_F of the 5-HT marker confirmed that all the radioactivity in the 1·2 N HCl eluate of the brain extract was in the 5-HT. The results in Fig. 1 demonstrate the effectiveness of thin-layer chromatography as a tool for a clear-cut separation of 5-HT from labeled Try. This is further evidence of the presence of labeled 5-HT in

the brain after i.c. injection of (^{14}C)-L-Try. Significantly, i.c. injection of 4·1 μg of (^{14}C)-D-Try yielded no labeled 5-HT by either method B or its combination with thin-layer chromatography. The radio autography in Fig. 2 is presented as further proof for the appearance of labeled 5-HT in the brain extracts obtained by method A. Finally, i.p. injection of (^{14}C)-Try in amounts equal to or 10–20 times larger than those injected i.c. did not lead to the appearance of labeled 5-HT in the brain.

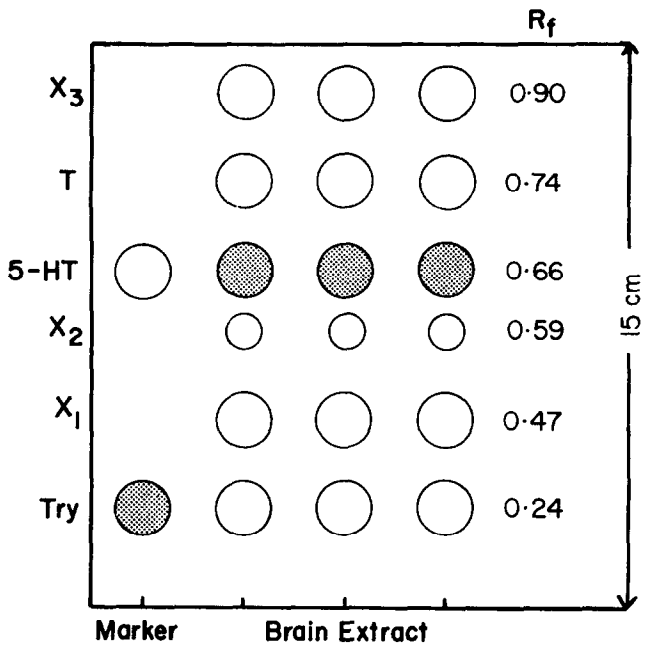

FIG. 1. Thin-layer chromatogram of pooled 5-HT eluted (expts. 202, 203, 204) after separation in a column of cation-exchange resin. Animals were given i.p. injection of 18·65 mg PIH·HCl/kg 2 hr before i.c. injection of (^{14}C)-L-tryptophan. They were killed 20 min later. Plate thickness about 1 mm Solvent system: methylacetate:isopropanol:NH_4OH 25% (45:35:20). Marker contained a mixture (^{14}C)-L-tryptophan and unlabeled 5-HT creatine sulfate. Components: tryptophan, 5-HT, tryptamine, and x_1, x_2, x_3 (unknown compounds with fluorescence). Stippled circles indicate presence of radioactivity.

In the brain extracts of animals injected intracerebrally with labeled L-Try, the various chromatographic methods revealed the presence of a metabolite with 0·1 %–1 % of the original radioactivity and the same R_F as 5-HT. However, it was decided that further characterization of this metabolite as well as others would be necessary for an unequivocal proof of identity. In several experiments, therefore, the chromatograms of brain extracts were cut into strips around the radioactive spots of R_F 0·69–0·73 (5-HT) and R_F 0·83–0·87 (TA) as revealed by radioautography. The adsorbed substances were eluted from the strips with 2 ml of 0·01 N HCl. A 0·1-ml aliquot of each eluate was counted, and the remainder was submitted to the following methods of identification:

(*a*) *Enzymatic conversion of R-NH_2 to R-COOH.* The eluates were concentrated to 0·2 ml at 4°. The concentrates corresponding either to R_F 0·70 or R_F 0·83 were mixed with 0·2 ml carrier solution of unlabeled 5-HT or TA containing 0·175 μmole of either

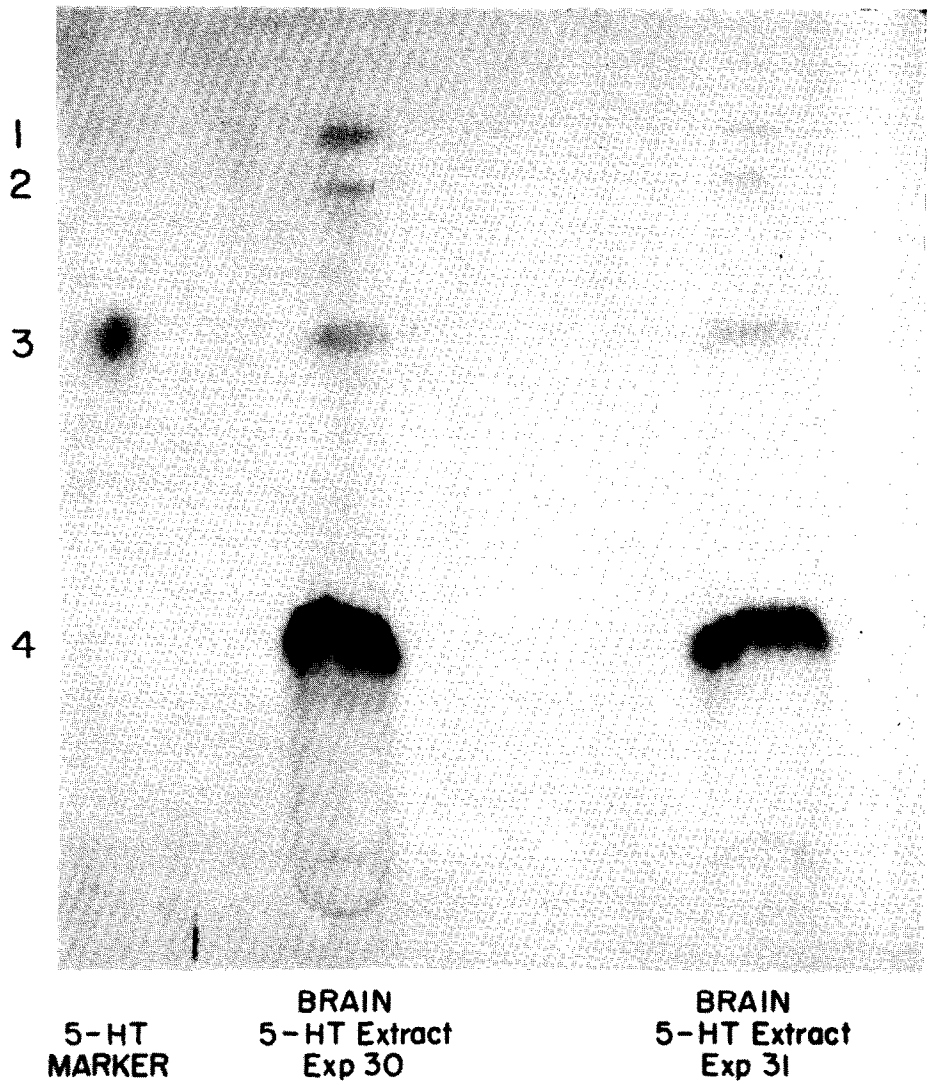

FIG. 2. Radiochromatography of the butanol extract of pigeon brains after i.c. injection of (^{14}C)-DL-tryptophan. Animals were given i.p. injection of 18·65 mg PIH·HCl/kg prior to administration of tryptophan; Expt. 30 was a 10-min animal; Expt. 31 was a 40-min animal. Solvent system A. Components: (1) melatonin; (2) tryptamine; (3) 5-HT; (4) tryptophan.

amine. The labeled markers from the chromatograms were treated identically and served as controls. The amine solutions were pipetted into a mixture containing 60 μmoles nicotinamide (0·3 ml), 14 μmoles NAD (0·4 ml), 250 μmoles phosphate buffer (pH 7·4), 0·5 ml aldehyde dehydrogenase (supernatant of 20% guinea pig kidney homogenate), and 0·5 ml mitochondrial suspension of guinea pig liver sucrose homogenate with the monoamine oxidase activity corresponding to O_2 (N) 70. The total volume was 2·5 ml. The mixture was incubated at 37° for 1 hr. After incubation and cooling, the mixture was acidified with 0·1 ml of concentrated HCl and centrifuged. The indoleacetic acids were extracted with ether as recommended.[14] A known volume of the ether extract (about 1/3) was shaken with 1·5 ml of 0·5 N phosphate buffer, pH 7·0. After the ether layer had been removed, 0·1 ml of the phosphate buffer was counted. The remaining 2/3 of the original ether extract was concentrated to 0·5 ml in vacuum under nitrogen. The concentrate was applied to Whatman 1 paper and developed by descending paper chromatography in solvent B. The radioactivity and the R_F of the unknown samples corresponded to the oxidation product of (^{14}C)-5-HT and (^{14}C)-TA markers, i.e. 5-HIAA and IAA respectively. The results of these experiments are presented in Table 6. It is concluded that the radioactive substances eluted from the paper chromatograms of brain extracts in solvent A, which by their *R_F's* corresponded to 5-HT and TA, were indeed these two amines, the precursors of 5-HIAA and IAA.

(*b*) *Picrate salt.* Two hours before i.c. injection of (^{14}C)-DL-Try, four pigeons were treated with PIH. They were killed 30 min later, and the cerebral 5-HT was extracted according to method B. The four acid extracts were pooled, 10 mg of 5-HT·HCl was added as carrier, and the picrate salt was isolated as recommended.[20] After verifying the melting point of the 5-HT picrate, the picrate was split by 5 ml of 3 N HCl. The picric acid was removed by continuous ether extraction and the solution of 5-HT·HCl was lyophilized. The residue was dissolved in 0·15 ml of water and the solution applied to Whatman 1 paper in two aliquots and run by ascending chromatography in solvent A. After development of the chromatogram, one of the two spots (both of which corresponded to 5-HT) was cut out and directly counted; the other spot was developed by Ehrlich's reagent.[29] Although the radioactivity recovered in 5-HT was only 20% of the original counts in the pooled acid extracts, this carrier method offered additional proof for cerebral hydroxylation of Try.

Turnover of cerebral 5-HT and tryptophan

In a series of experiments, DL-Try (2·28 mg or 100 μc) was given i.p. to rats and pigeons. At various time intervals the animals were killed in the cold room, and their brains were quickly removed, washed free of blood with ice-cold water, blotted dry, weighed, and homogenized in 0·4 N $HClO_4$. The homogenates were worked up according to method B, which permitted separation of free Try, bound Try, and 5-HT. The purity of 5-HT in the eluate was determined by thin-layer chromatography. The free Try content in fraction E (cf. method B) was determined through its conversion to norharman.[10] The bound Try was estimated by counting a Hyamin solution of 5-mg samples of protein which had been obtained after a thorough washing,[19] drying, and weighing of the acid precipitates of the brain homogenates.

After the injection of (^{14}C)-Try, a maximum of 0·4%–0·6% radioactivity could be found in 1–24 hr samples of the brain. The results of these experiments are given in

TABLE 6. IDENTIFICATION OF LABELED INDOLE COMPOUNDS FROM PIGEON BRAIN AFTER INTRACEREBRAL INJECTION OF (^{14}C)-TRYPTOPHAN

Expt. no.	Substance	Solvent A	Eluate activity from chromatogram	Activity of acidic extract after enzymatic oxidation		Activity of acidic extract after chromatography		Solvent B	Corresponding component
		(R_F)	(dpm)	(dpm)	(%)	(dpm)	(%)	(R_F)	
51	5-HT	0·72	1,500	1,298	87	429	33	0·79	5-HIAA
80	5-HT	0·70	945	710	75	138	19	0·77	5-HIAA
Marker	5-HT	0·70	4,000	3,575	89	1,200	34	0·77	5-HIAA
17	Tryptamine	0·87	2,902	2,700	93	1,020	38	0·93	IAA
30	Tryptamine	0·84	1,164	930	79	190	20	0·94	IAA
Marker	Tryptamine	0·85	2,000	1,680	84	748	45	0·90	IAA
17	N-Acetyl-Try	0·49	1,902					0·50*	Try
Marker	N-Acetyl-Try	0·46						0·50*	Try

Solvent A: isopropanol:NH_4OH:water (v/v 40:20:40). Solvent B: *n*-butanol-acetic acid-water (v/v 40:10:50).
* After alkaline hydrolysis of N-acetyl-tryptophan.

Fig. 3 and 4. The curves represent the changes in the specific activities of free Try, protein-bound Try, and 5-HT. In Fig. 3 the specific activity of free Try increased during the first 4 hr, then gradually decreased over a period of 30 hr in pigeon brain. The estimated half-lives of protein-bound Try and free Try were 48 and 12 hr respectively. As show in Fig. 4, somewhat different results were obtained from rat brain.

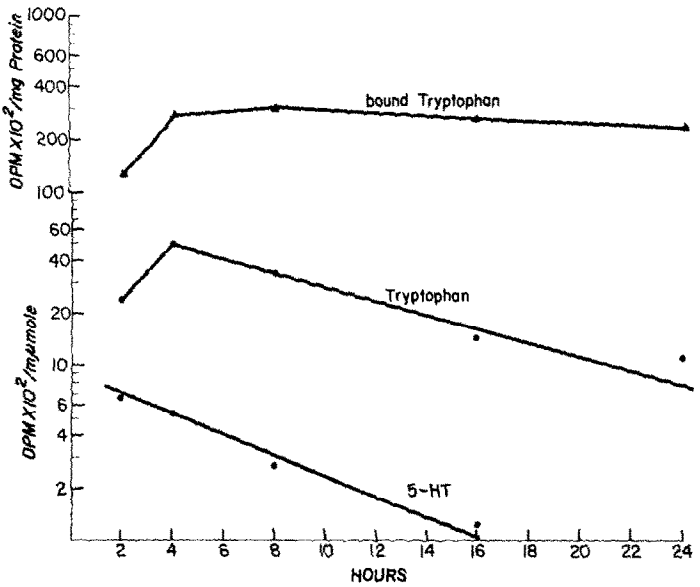

FIG. 3. Changes in the specific activity of 5-HT and free and protein-bound tryptophan after i.p. injection of (^{14}C)-DL-tryptophan (2·28 mg or 100 μc) into pigeons. Each point represents average of two animals.

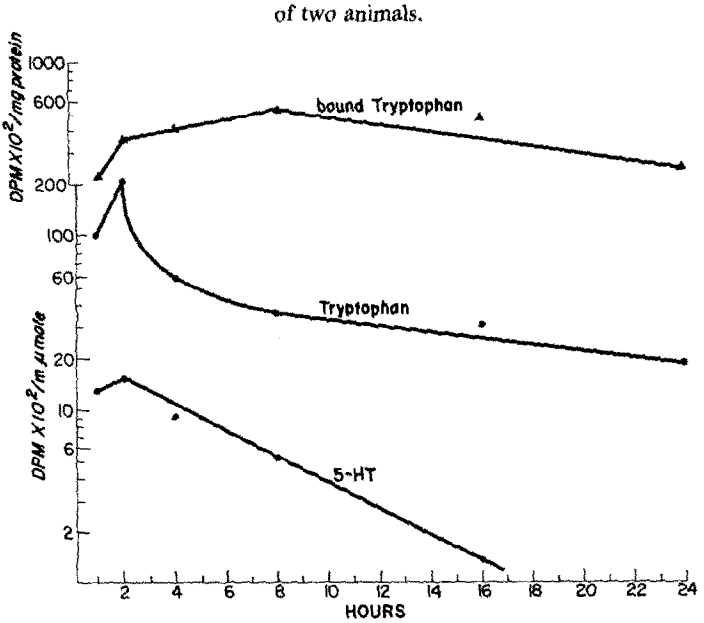

FIG. 4. Changes in the specific activity of 5-HT and free and protein-bound tryptophan after i.p. injection of (^{14}C)-DL-tryptophan (2·28 mg or 100 μc) into rats. Each point represents average of two animals.

The specific activity of free Try increased through an initial 2-hr period whereas that of the protein-bound Try continued increasing for another 6 hr. The specific activity of free Try rapidly decreased during this 2–8 hr period, after which it entered a slow phase of decay parallel to that of the bound Try. This type of decay curve indicates a composite mechanism consisting of efflux and exchange with the Try of the protein pool. From the efflux rate of free Try for the first 8 hr, a half-life of 45 min was calculated; this was obtained from the fast-decaying phase of the curve after its correction from the extrapolated section of the slow-decaying phase. The half-lives of free Try calculated from the slow-decaying phase (8–24 hr) and a half-life of the protein-bound Try are very nearly identical.

The presence of a persistently higher specific activity in Try than in 5-HT precluded an accurate calculation of the turnover rates for 5-HT. In a report[30] of the turnover of some of the catecholamines in guinea pig brain after i.p. administration of (^{14}C)-tyrosine, it was correctly suggested that the half-lives obtained for the catecholamines would be maximal values, owing to continued resynthesis from the high labeled precursor amino acid. The observations presented in this paper support such an explanation. The half-lives given in Table 7 clearly indicate that the values obtained for

TABLE 7. CALCULATED HALF-LIFE ($T_{1/2}$) OF 5-HT IN PIGEON AND RAT BRAIN

Animal	Substance	Condition	Brain wt. (g)	5-Hydroxytryptamine		
				$T_{1/2}$ (min)	(μg/g/hr)	(μg/brain/day)
Rat	DL-Try	i.p.	1·8	240	0·05	2·16
Pigeon	DL-Try	i.p.	2·0	300	0·07	3·36
	L-Try	i.c.	2·0	42	0·50	24·00
	5-HT	i.c.	2·0	35	0·60	28·80

The values are based on average normal cerebral concentration of 5-HT; 0·70 μg/g (pigeon) and 0·40 μg/g (rat).

intraperitoneally administered (^{14}C)-Try are for 5-HT within the same order of magnitude as those obtained[30] for cerebral norepinephrine (0·033 μg/g/hr).

The half-life values of 4 and 5 hr for 5-HT are in sharp contrast to those obtained after i.c. administration of either labeled Try or exogenous (^{14}C)-5-HT. The half-lives of 42 and 35 min seem to be in better agreement with the results obtained by Udenfriend and Weissbach.[20] These values are consistent with the occurrence of a fast turnover of amines in the central nervous system; however, they do not imply a very active on-the-site synthesis. Indeed the experiments described in this paper indicate that the ability of the brain to synthesize 5-HT is rather limited. Sources other than the brain, therefore, through a constant supply of 5-HTP are the major contributors to the 5-HT pool in the brain. From studies *in vivo* and *in vitro* of hepatic hydroxylation, it is estimated that under normal circumstances the liver can adequately take care of the sysnthesis and production of 5-HTP from the available pool of free Try.

In conclusion the present findings indicate that the synthesis of 5-hydroxytryptophan in the brain cannot account for all the 5-hydroxytryptamine in this organ. Consequently, the brain must rely on other tissues for the supply of most of this amino acid.

Acknowledgments—The authors are indebted to Dr. M. Finkelstein of Lakeside Laboratories, Inc. for samples of Catron (PIH). One of us (S. K. C.) gratefully acknowledges a Fulbright travel grant. This work is supported by a grant from the State of Iowa Mental Health Research Fund.

REFERENCES

1. S. Udenfriend, E. Titus, H. Weissbach and R. E. Peterson, *J. biol. Chem.* **219**, 335 (1956).
2. R. A. Freedland, I. M. Wadzinski and H. A. Waisman, *Biochem. biophys. Res Commun.* **5**, 94 (1961).
3. J. R. Cooper and I. Melcer, *J. Pharmacol. exp. Ther.* **132**, 265 (1961).
4. J. Renson, H. Weissbach and S. Udenfriend, *J. biol. Chem.* **237**, 2261 (1962).
5. G. Bertaccini, *J. Physiol. (Lond.)* **153**, 239 (1960).
6. L. J. Weber and A. Horita, *Fed. Proc.* **22**, 625 (1963).
7. G. M. Tyce, E. V. Flock and C. A. Owen, *Fed. Proc.* **22**, 633 (1963).
8. E. M. Gal, M. Poczik and F. D. Marshall, *Biochem. biophys. Res. Commun.* **12**, 39 (1963).
9. E. M. Gal and F. D. Marshøll, in *Progress in Brain Research.* Vol. 8; p. 56. *Brain Biogenic Amines*, H. F. Himwich and W. A. Himwich, Eds. Elsevier, Amsterdam (in press) (1964).
10. S. Hess and S. Udenfriend, *J. Pharmacol. exp. Ther.* **127**, 175 (1959).
11. D. F. Bogdanski, A. Pletscher, B. B. Brodie and S. Udenfriend, *J. Pharmacol. exp. Ther.* **117**, 82 (1956).
12. A. Bertler, *Acta physiol. scand.* **51**, 75 (1961).
13. P. A. Shore and J. S. Olin, *J. Pharmacol. exp. Ther.* **122**, 295 (1958).
14. B.-E. Roos, *Life Sci.* **1**, 25 (1962).
15. W. Lovenberg, R. J. Levine and A. Sjoerdsma, *J. Pharmacol. exp. Ther.* **135**, 7 (1962).
16. E. Stahl and H. Kaldewey, *Z. phys. Chem.* **323**, 182 (1961).
17. H. Takahashi, T. Hattori and B. Maruo, *Analyt. Biochem.* **2**, 447 (1961).
18. N. F. Bousquet, J. E. Christian, A. M. Knevel and J. L. Spahr, *Analyt. Biochem.* **3**, 519 (1962).
19. E. Norberg and D. M. Greenberg, *Cancer* **4**, 383 (1951).
20. S. Udenfriend and H. Weissbach, *Proc. Soc. exp. Biol. (N.Y.)* **97**, 748 (1958).
21. G. H. Hogeboom and W. Schneider, *J. biol. Chem.* **186**, 417 (1950).
22. E. Racker, *J. biol. Chem.* **177**, 883 (1949).
23. N. J. Giarman and S. Schanberg, *Biochem. Pharmacol.* **1**, 301 (1958).
24. P. Correale, *J. Neurochem.* **1**, 22 (1956).
25. J. A. Oates and A. Sjoersdma, *Proc. Soc. exp. Biol. (N.Y.)* **108**, 2 (1961).
26. A. Lajtha and J. Toth, *J. Neurochem.* **9**, 199 (1962).
27. A. Yuwiler, E. Geller and S. Eiduson, *Arch. Biochem.* **89**, 143 (1960).
28. J. Axelrod and T. Inscoe, *J. Pharmacol. exp. Ther.* **141**, 161 (1963).
29. J. B. Jepson, *Lancet* **2**, 1009 (1955).
30. S. Udenfriend and P. Zaltzman-Nirenberg, *Science* **142**, 394 (1963).