ABSTRACT

BRAIN SEROTONIN LEVELS IN NORMAL AND ENDOTOXIN-POISONED MICE WITH AND WITHOUT A TRYPTOPHAN LOAD

By

Elaine Suzanne Tremblay

The relationship between serotonin levels and increased sensitivity of endotoxin-poisoned mice to tryptophan was investigated. Normal and endotoxin-poisoned mice were injected with a 20 milligram load of tryptophan. The animals were killed by cervical dislocation and the brain tissue was immediately removed. The serotonin was extracted from the tissue directly into butanol and then assayed by the o-phthalaldehyde method. The relative intensity of fluorescence of the brain tissue extracts was compared to the values obtained from normal and endotoxin-poisoned mice without a tryptophan load. An increase in the intensity of fluorescence was obtained in animals given a tryptophan load. This increase is greater and more prolonged in endotoxin-poisoned mice. The entire increase was initially attributed to increased serotonin levels. However, analysis of the extract by thin layer chromatography and autoradiography indicated that tryptophan and/or other tryptophan metabolites may be interfering in this procedure.

# BRAIN SEROTONIN LEVELS IN NORMAL AND ENDOTOXIN-POISONED MICE WITH AND WITHOUT A TRYPTOPHAN LOAD

By

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# INTRODUCTION

It is well established that tryptophan oxygenase activity is depressed in endotoxin-poisoned animals (1,9,10, 42). If tryptophan is administered to endotoxin-poisoned mice at a time when tryptophan oxygenase activity is depressed death frequently occurs within 8 hours (42). This time of death is 24-36 hours sooner than would be expected among mice given endotoxin alone. It has been suggested that this response reflects the funneling of tryptophan in excess into serotonin synthesis. Cyproheptadine, an anti-serotonin drug (54), significantly reduces the sensitivity of endotoxin-treated mice to tryptophan thereby supporting the suggestion that excess serotonin may be a contributing factor in the early deaths (41,42). To date, no quantitative measurements showing increased serotonin levels are available to confirm this hypothesis.

The primary objective of this project is to determine serotonin concentrations in the brain of normal and endotoxin-poisoned mice given a tryptophan load. The brain tissue was chosen for 2 reasons: first, the central nervous system has been previously implicated as a primary target for the biological actions of endotoxin (9) and second, changes in

serotonin levels have been associated with certain disorders involving the central nervous system (18,45).

The extraction and assay procedure reported by Maickel et al. (34) is one of the more sensitive methods available for the measurement of serotonin in the brain. The assay is based on the fluorescence of serotonin after reaction with O-phthalaldehyde (OPT) (35). The fluorescence of tryptophan after reaction with OPT is reported to be negligible compared to the intense fluorescence obtained with serotonin (35). Since it was not anticipated that the presence of tryptophan in the brain after tryptophan loading would interfere with the assay procedure, serotonin determinations were performed in normal and endotoxin-poisoned animals at various times after tryptophan load. Results obtained during the course of these studies did not eliminate the possibility that high concentrations of tryptophan may be responsible for the considerable increase in fluorescence observed in tryptophan loading studies. Hence a number of the experiments in this project deal with the applicability of the procedures developed by Maickel and co-workers to tryptophan loading techniques.

# LITERATURE REVIEW

Serotonin (5-hydroxytryptamine) is synthesized from L-tryptophan (57) by 2 enzymatic reactions. Tryptophan is first hydroxylated in the five position to form 5-hydroxytryptophan (25,32). The latter is decarboxylated to give 5-hydroxytryptamine (33). Following synthesis serotonin can be stored in tissue, presumably bound to protein or lipoprotein components (3,4). If it remains free in the cytoplasm it is subject to immediate destruction by monoamine oxidase which converts 5-hydroxytryptamine to 5-hydroxytryptophal. This compound is subsequently converted to 5-hydroxyindoleacetic acid, the major oxidative product of serotonin (44,57), by aldehyde dehydrogenase.

Initially, the intracerebral conversion of tryptophan to serotonin was questioned. The low activity of tryptophan hydroxylase in the brain tissue and the lack of an adequate enzyme assay procedure made its presence undetectable (48). In vitro conversion of L-tryptophan to 5-hydroxytryptophan with brain stem homogenates was the first evidence of brain hydroxylase activity (25). Weber and Horita (60) found that eviscerated animals given intraperitoneal injections of L-tryptophan showed an increase in brain serotonin levels

equivalent to the increase obtained in normal rats given a tryptophan load. Infusion of the brain with L-tryptophan also resulted in increase brain serotonin. The work of Gal et al. (22) demonstrating the appearance of ($^{14}$C)-5HT in brain of pigeons and rats after intracerebral injection of ($^{14}$C)-L-tryptophan supported the in vivo hydroxylation of tryptophan by brain tissue. These experiments indicate that the central nervous tissue need not depend on the peripheral organs for the first step in the synthesis of serotonin.

The metabolism of serotonin in the rat brain has been extensively investigated by Eccleston and co-workers (6,20,39). Loading doses of tryptophan precursor were administered to increase the concentration of intermediate metabolites. Intraperitoneal injections of tryptophan resulted in an increase in brain serotonin levels which was maintained for a few hours followed by a gradual return to normal (6). Similar results have been reported by Koe (30). 5-Hydroxyindoleacetic acid also increased reaching its maximum level later than serotonin (6). The greater the tryptophan load the higher the tryptophan concentration in the brain. Varying doses of tryptophan failed to alter the maximum concentration of 5-hydroxytryptamine and 5-hydroxyindoleacetic acid but only prolonged the maintenance of the increased levels in the brain (20). No 5-hydroxytryptophan can be detected in the brain at any time after tryptophan loading (6). These observations led to the conclusion that the

hydroxylation of tryptophan is the rate-limiting step in the formation of serotonin. It has been proposed that the regulating factor is either saturation of the enzyme by substrate or a feedback mechanism on the enzyme (6). Studies by Lin and co-workers suggest that brain 5-hydroxytryptamine synthesis is not controlled by feedback inhibition (31).

Similar studies were performed using 5-hydroxytryptophan as the precursor load (39). This compound can be transported across the blood-brain barrier (58). No increase in serotonin could be detected in the rat brain when small doses (10 mg/kg) were injected. A 20 mg/kg dose caused only a moderate increase in serotonin levels. Both doses resulted in a marked increase in 5-hydroxyindoleacetic acid indicating that 5-hydroxytryptophan can enter the brain and be metabolized to 5-hydroxyindoleacetic acid without greatly affecting serotonin concentration. Moir and Eccleston have suggested that 5-hydroxytryptophan derived from the extracerebral source is metabolized in the cytoplasm of the nerve cells rather than the subcellular sites reserved for tryptophan metabolism. Consequently the majority of the serotonin formed from 5-hydroxytryptophan has no access to the storage sites for serotonin and is readily converted to 5-hydroxyindoleacetic acid (39).

Serotonin levels greater than the maximum obtained in normal rats given a tryptophan load can be found in animals

treated with a monoamine oxidase inhibitor before the tryptophan load (20).

Variable results have been presented on the passage of serotonin through the blood-brain barrier (8,14,15). The variation in the dose of 5-hydroxytryptamine injected, the route of injection and the time of serotonin measurements may account for the conflicting reports. The studies by Bulat and Supek (14,15) indicate that serotonin will penetrate the brain and cause an increase in brain serotonin only if a high enough concentration of free serotonin is present in the blood stream.

The discovery by Koe and co-workers of the ability of p-chlorophenylalanine (PCPA) to inhibit serotonin synthesis in normal animals (30) has served recently as a valuable tool for the study of serotonin metabolism in a variety of normal and pathological states (17,52). Koe and Weissman found p-chlorophenylalanine to be a selective and potent depletor of brain serotonin in mice, rats and dogs (30). A 75 percent decrease in brain serotonin in mice treated with repeated injections of this drug has been reported. Peripheral stores of serotonin are also depleted with PCPA. The decrease of 5-hydroxytryptamine in mice, however, is considerably less than the decrease obtained in the peripheral stores of 5-hydroxytryptamine in the dog or rat. No inhibition of monoamine oxidase or 5-hydroxytryptophan decarboxylase activity <u>in vivo</u> or <u>in vitro</u> could be detected. The <u>in vitro</u>

inhibition of liver tryptophan hydroxylase by PCPA and the decrease in the hydroxylating ability of liver tissue from PCPA-treated animals suggested inhibition of tryptophan hydroxylase as the *in vivo* mechanism by which p-chlorophenylalanine depletes brain serotonin (30).

The development of a sensitive radioassay for tryptophan hydroxylating activity (32) enabled Lovenberg, Jéquier, and Sjordsma to verify the suggestion that PCPA did lower brain serotonin levels by impairing the biosynthesis of serotonin at the rate limiting tryptophan hydroxylase step. Using soluble enzyme preparations from brain tissue these workers showed that p-chlorophenylalanine is a competitive inhibitor of tryptophan hydroxylase *in vitro* (28). The kinetics of the *in vitro* inhibition indicate that PCPA is competitive with the substrate for the binding sites on the enzyme. The $K_m$ of both tryptophan and p-chlorophenylalanine was $10^{-4}$ with their enzyme preparations. PCPA also caused significant loss in tryptophan hydroxylase activity *in vivo* (28). Rats injected with PCPA (300 mg/kg, ip) showed a rapid decrease in brain stem levels of tryptophan hydroxylase and serotonin. The minimal level was reached after 24 hours and remained at this level for approximately 4 days. Both tryptophan hydroxylase activity and serotonin levels then gradually increased reaching normal levels by the 11th day. Immediately after injection, p-chlorophenylalanine was markedly increased in the brain but had decreased to

undetectable levels by the 6th day. The inhibition of the enzyme in the absence of the inhibitor suggested that the *in vivo* effect of p-chlorophenylalanine on the enzyme was irreversible inactivation rather than competitive inhibition.

An attempt was made by Koe to determine which component of the hydroxylating system is affected by PCPA (29). The observation that the activity of the inhibited enzyme could be restored to normal levels with the incorporation of the hydroxylase suggested that the inhibiting action of PCPA *in vivo* may be due to direct inactivation of the hydroxylase.

The selectivity of p-chlorophenylalanine as a serotonin depletor has been questioned. Though only slight changes in whole brain catecholamine levels in the rat have been reported by Koe (30), a significant change in norepinephrine does occur in some areas of the rat brain (38).

PCPA's effectiveness in inhibiting serotonin synthesis is diminished when large doses of tryptophan are injected. Three consecutive daily doses of 100 mg/kg of PCPA followed 24 hours later by a 500 mg/kg load of tryptophan prevented the increase in serotonin normally obtained in rats with a tryptophan load (30). This inhibition, however, was maintained for approximately one hour only and was followed by a gradual increase in serotonin levels. By the 4th hour after tryptophan, the increase was equal to the increase found in normal rats given a tryptophan load. Serotonin concentrations returned to baseline values by the 8th hour.

It has been suggested that the distribution of tryptophan among the normal *in vivo* pathway of tryptophan metabolism is altered in endotoxin-poisoned mice (40,41,42). Tryptophan can be metabolized extracerebrally through 2 major pathways: the tryptophan oxygenase pathway leading ultimately to the synthesis of nicotinamide adenine dinucleotide or the serotonin pathway. Moon and Berry have studied the effect of endotoxin on the adaptive liver enzyme, tryptophan oxygenase. These authors and others have shown that tryptophan oxygenase activity is depressed soon after endotoxin poisoning (1,9,10,11,40,42). Treatment of endotoxin-poisoned mice with large doses of L-tryptophan at the time of maximum enzyme depression resulted in earlier deaths than would be expected among mice given endotoxin alone. Such deaths are frequently convulsive in nature. Equivalent doses of L-tryptophan had no toxic effect in normal animals. It has been suggested by these workers that the tryptophan load cannot be completely metabolized through the depressed tryptophan oxygenase pathway in endotoxin-poisoned mice and consequently the amino acid is funneled in excess into the serotonin pathway (41,42). An excess production of serotonin, a potent vasoactive compound, has been implicated in the increased sensitivity of endotoxin-poisoned mice to tryptophan. Pretreatment with cyproheptadine, an antiserotonin drug (54), protected the endotoxin-treated animals against the increased lethality of the delayed injection of tryptophan (41,42).

Several methods have been developed to measure the serotonin concentration in various tissues. Spectrofluorometry, due to its sensitivity and specificity, is the present method of choice. An extraction and/or chromatographic procedure is usually used in conjunction with the fluorometric technique.

The most widely used method for the determination of serotonin is the Bogdanski method (13). The extraction is based on the partitioning of serotonin between an aqueous and organic phase. Addition of 3N HCl to the final aqueous phase gives specificity to the fluorometric assay by shifting the fluorescence maximum of the hydroxyindoles (56) thereby distinguishing them from other metabolites. Various modifications of this method have been reported. The extraction procedure of Shore and Olin (51) for the determination of norepinephrine in the brain has been adapted by Mead and Finger (37) for the measurement of serotonin. Serotonin is extracted into n-butanol from a salt-saturated acid phase rather than the alkaline phase used by Bogdanski.

A sensitive technique for the assay of 5-hydroxyindole metabolites in the brain after tryptophan loading was developed by Ashcroft et al. (6). The method involves preparation of brain extract, separation of the metabolites by paper chromatography, elution of various sections of the chromatogram and fluorometric analyses of these segments.

A column chromatographic procedure was recommended by Bertler (12) for analysis of serotonin in brain tissue. Awapara (7) suggested the use of Amberlite CG-50 for separation of amines from precursor amino acids. An efficient separation of tryptophan, tryptamine, serotonin, 5-hydroxytryptophan and 5-hydroxyindoleacetic acid has been recently reported by Ichiyama et al. (27) using a series of columns (Dowex 1-formate, Amberlite CG-50 and Sephadex G-25).

Other methods have been developed to increase the sensitivity of the 5-hydroxyindoles. The techniques are based on the formation of highly fluorescent derivatives. Snyder (53) has developed an assay procedure based on Vanable's observation that serotonin is highly fluorescent when heated in the presence of ninhydrin (59). This reaction is eight times more intense than the native fluorescence of serotonin in mineral acid.

Maickel and Miller have reported a sensitive method for the determination of serotonin. The assay is based on the fluorescence of serotonin after reaction with o-phthalaldehyde (OPT) (34). The reaction of OPT with indole alkylamines is not very specific. Indole does not fluoresce. The intensity of the fluorescence of the substituted indoles after reaction with OPT is dependent on the chemical structure of the compound (35). Addition of side chains such as $[-CH_2CH_2NH_2]^-$ or $[-CH_2COOH]^-$ in the 3 position or substitution of an $[-OH]^-$ or an $[-OCH_3]^-$ group in the 5 position yields only slight

fluorescence. A combination of the 2 to 3 side chains in the 3 position and the hydroxy or methoxy substitution in the 5 position does enhance the fluorescence (Appendix A). O-phthalaldehyde is an aromatic 1,2-dialdehyde. Compounds lacking this structure will not yield any fluorescence when reacted with the substituted indoles.

An extraction procedure has also been devised by these workers (34,36) to allow for the determination of serotonin in small brain samples. Extraction of 1 microgram samples of the substituted indoles indicate that only 5-hydroxytryptophan and 5-methoxytryptamine could be interfering substances in the procedure. The normal brain level of these 2 compounds is negligible and therefore should not cause spuriously high values (36,58).

# MATERIALS AND METHODS

## Removal and Freezing of Brain Tissue

Mice were killed by cervical dislocation. The skin covering the skull was removed and the skull was split vertically with a razor blade. The bone covering the brain was pulled away and the whole brain was removed. It was rinsed in ice cold physiological saline, blotted gently, and weighed to the nearest milligram. If the sample was to be frozen it was immediately dropped into an acetone and dry ice solution. After approximately 15 seconds the frozen brain was removed, blotted, wrapped in saran wrap and stored at -70°C until assayed.

## Serotonin Extraction and Assay

The methods used for both the extraction of serotonin and the assay of serotonin utilizing OPT reagent were patterned after those of Maickel *et al.* (34) with only slight modification in our laboratory. The addition of cysteine as suggested by Curzon (19) was also incorporated into our procedure.

Reagents: Glass distilled water was used in the preparation of all reagents unless otherwise specified. Reagent grade n-butanol (Mallinckrodt Chemical Works, St. Louis, Mo.) and

practical grade heptane (Matheson Coleman and Bell, Norwood, Ohio) were purified by shaking with equal volumes of 0.1N NaOH, then by shaking twice with equal volumes of distilled water, followed by an equal volume of 0.1N HCl and finally twice with equal volumes of distilled water. The n-butanol was acidified by adding 0.85 ml of concentrated HCl per liter of n-butanol (16). The OPT reagent (4 mg/100 ml of 10N HCl) and L-cysteine (1 mg/ml of 0.1N HCl) were prepared immediately before use. The o-phthalaldehyde and L-cysteine were purchased from the Sigma Chemical Co., St. Louis, Mo.

Standard curve: Solutions of serotonin creatinine sulfate ranging from 0.024-400 μg/ml were prepared in 0.1N HCl containing 0.1% cysteine. Aliquots (0.2 ml) were transfered to 12 x 75 mm test tubes and 1.2 ml of freshly prepared OPT reagent was added to each sample. The tubes were mixed with a vibrating mixer, placed in a boiling water bath for 15 minutes then cooled in tap water. Fluorescence was measured in an Aminco SPF-125 spectrofluorometer (American Instrument Co., Silver Spring, Maryland). Activation and emission wavelengths were set at 360 and 470 mμ (both uncorrected) respectively. Both activation and emission slit widths were set at 2.0 mm.

Assay of brain tissue: Whole mouse brains were homogenized in 3 ml of cold acidified butanol. After centrifugation for 5 minutes at 1000 x g, 2.5 ml of supernatant were pipetted

into a 13 ml glass stoppered centrifuge tube and shaken for 5 minutes with 5 ml of heptane and 0.4 ml of 0.1N HCl containing 0.1% cysteine. The phases were separated by centrifugation and the organic phase discarded. A 0.2 ml aliquot of the acid phase was mixed with 1.2 ml of the OPT reagent and boiled for 15 minutes. Fluorescence was read as previously indicated. The Rank Order test (62) was used to determine significance between groups.

Internal standard: Whole brains from 30 or more mice were homogenized in butanol and centrifuged at 1000 x g. The supernatant fractions were pooled, mixed, and divided into two fractions. One μg of serotonin creatinine sulfate per ml of supernatant was added to one fraction. An equal volume of glass distilled water was added to the second fraction. This latter fraction served as the recovery blank.

## Chromatography and Autoradiography

Chromatographic and autoradiographic procedures used have been previously described (43). The thin layer chromatography procedure was slightly modified by using a 2-dimensional chromatography system with butanol, acetic acid and water (12:5:2) as the second solvent.

## Endotoxin

Heat killed cells of Salmonella typhimurium, strain SR-11, suspended in isotonic non-pyrogenic saline (Baxter Laboratories, Morton, Illinois) served as the source of

endotoxin. Cultures of Salmonella typhimurium were grown for 18 hours at 37°C in Brain Heart Infusion broth to a final concentration of approximately $10^9$ cells per ml. The cells were collected by continuous flow centrifugation (Sorvall RC2-B, KSB Continuous Flow System) at 10,000 x g. The concentrated cells were washed 3 times with isotonic non-pyrogenic saline and resuspended in saline to 10 times their original concentration. Plate counts of the saline suspension were used to determine the cell number. Cells were heat-killed by autoclaving at 6 pounds pressure (115°C for 6 minutes). Lack of growth on subculture in Brain Heart Infusion broth confirmed sterility of the endotoxin preparation. The $LD_{50}$ was determined according to the method of Reed and Muench (47).

## Chemicals

L-tryptophan, serotonin creatinine sulfate, 5-hydroxyindoleacetic acid and 5-hydroxytryptophan were purchased from the Nutritional Biochemical Co., Cleveland, Ohio. Tryptamine and DL-p-chlorophenylalanine were purchased from Sigma Chemical Co., St. Louis, Mo.

## Mice

Female CF-1 mice (Carworth Farms, Portage, Michigan) weighing 18-20 grams were used in all experiments. They were housed five or six per cage with wood shavings serving as litter. Food (Purina Laboratory Chow, Ralston Purina Co., St. Louis, Mo.) and water were available ad libitum.

# RESULTS

## Standard curve: Serotonin concentrations vs. relative intensity of fluorescence

Using a full logarithmic scale, a linear relationship between the relative intensity of fluorescence and the concentration of serotonin was obtained for concentrations of serotonin creatinine sulfate ranging from 0.18-50 μg/ml (Figure 1). This is equivalent to 0.078-21.7 μg of serotonin per ml. At higher concentrations (greater than 100 μg/ml) the solution develops a yellow coloration. This interference causes a decrease in the relative intensity of fluorescence. The lowest serotonin concentration which can be accurately measured gives a fluorescence of approximately 4 times the blank reading. All serotonin concentrations reported in the tables are corrected to free base values.

## Internal standard: Variability in recovery depending upon the point at which the internal standard is added to the extraction procedure

Recovery of exogenous serotonin varied with the method used. When serotonin creatinine sulfate was added to the supernatant fraction of the tissue homogenate the percent recovery ranged from 102-108 percent. If serotonin was added to the butanol and homogenized simultaneously with the brain

Figure 1. Standard curve: Linear relationship between the relative intensity of fluorescence and the concentration of serotonin for concentrations of serotonin creatinine sulfate ranging from 0.18-50.0 μg/ml.

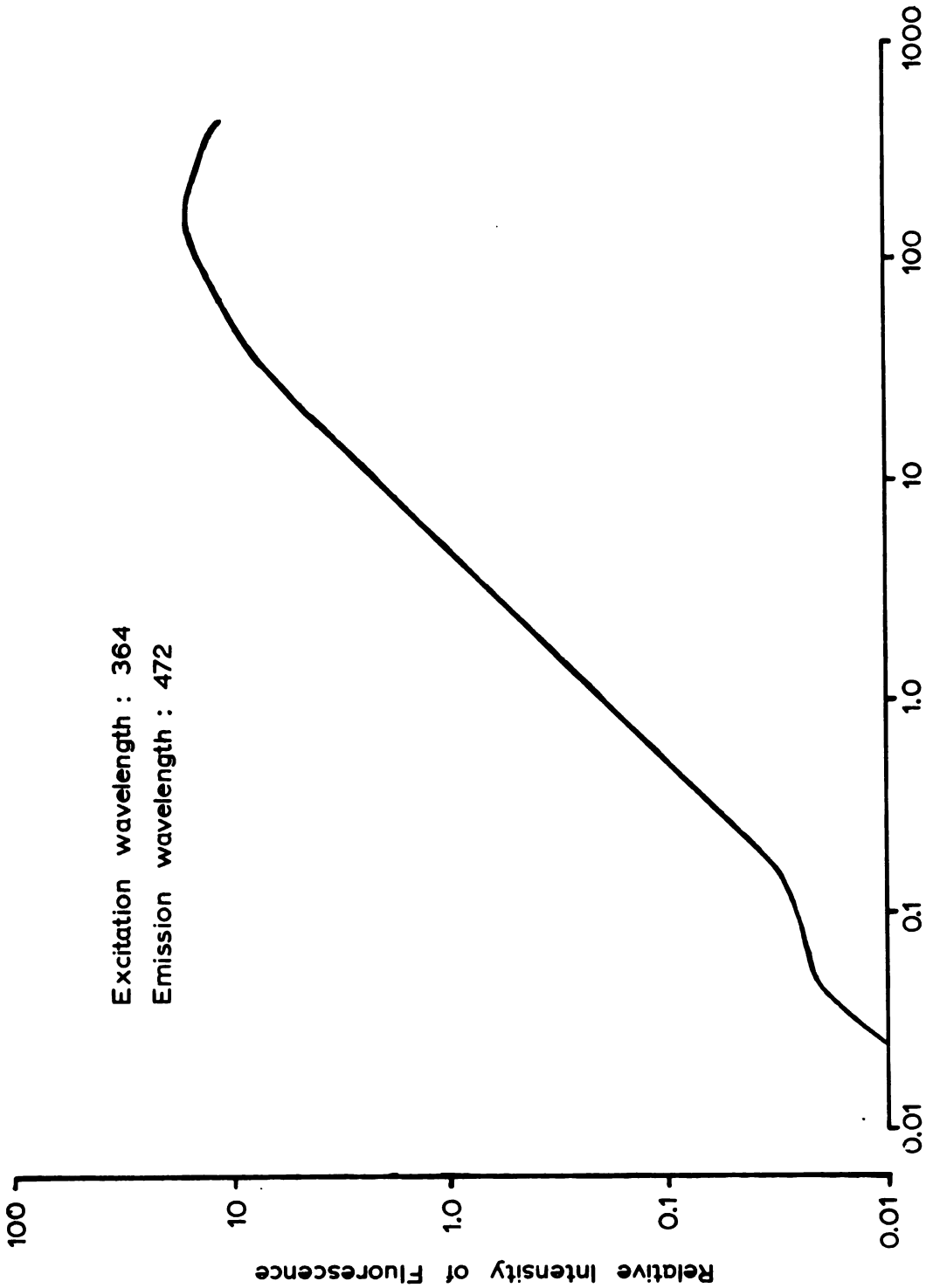
Excitation wavelength : 364
Emission wavelength : 472
Relative Intensity of Fluorescence
100
10
1.0
0.1
0.01
0.01
0.1
1.0
10
100
1000
µg of Serotonin creatinine sulfate / ml

Figure 1

tissue recovery of exogenous serotonin was variable and ranged from 57-76 percent (Table 1). Preliminary studies using radioactive serotonin indicated that a considerable amount of the exogenous serotonin remained in the precipitated fraction of the tissue homogenate. The mechanism or specificity of the binding was not further investigated.

Serotonin concentrations in brain tissue of normal mice at different times of the day

Considerable variation in the serotonin levels was observed in normal mice within a 12 hour period. Higher values appeared in the morning and lower ones in the evening (Table 2). A statistically significant difference in 5-hydroxytryptamine concentrations is observed when comparing the 9 A.M. value to 12, 3 and 9 P.M. values. Since determinations were made at irregular time intervals within a 12 hour period no exact diurnal pattern could be established.

Effect of freezing on serotonin concentrations in mouse brain tissue

Eighteen mice were separated into 3 groups. The brain tissue of the first group was removed and assayed immediately for serotonin. The whole brains from the other 2 groups were frozen in a dry ice and acetone solution for 15 seconds, wrapped in saran wrap and placed in a freezer at $-70^{o}C$. One group was assayed for serotonin concentration at 1 day after freezing, the second group at 6 days after freezing. No significant change in serotonin concentration was observed even after 6 days of freezing (Table 3). In this experiment

TABLE 1

Internal Standard: variability in percent recovery depending on whether serotonin creatinine sulfate is added prior to homogenization or added to the supernatant of tissue homogenate

| Conditions | Percent recovery of serotonin | Range of percent recovery |
|---|---|---|
| 10 μg serotonin creatinine sulfate added prior to homogenization | 67.3 ± 2.6 (6)* | 57-76 |
| 2.5 μg serotonin creatinine sulfate added to supernatant fraction of tissue homogenate | 105.5 ± 1.5 (4) | 102-108 |

*mean ± standard error (no. of samples)

TABLE 2

Serotonin concentrations (μg/g wet weight of tissue) in brain tissue of normal mice assayed at different times of the day

| Time | Serotonin concentration (μg/g wet weight) |
|---|---|
| 9:00 A.M. | 0.68 ± 0.02 (16) *a |
| 10:00 A.M. | 0.64 ± 0.02 (6) b |
| 12:00 P.M. | 0.61 ± 0.04 (8) c |
| 3:00 P.M. | 0.58 ± 0.02 (8) d |
| 9:00 P.M. | 0.58 ± 0.03 (5) e |

*mean ± standard error (no. of samples)

| | | | |
|---|---|---|---|
| a vs e | $P < 0.01$ | b vs c,d,e | N.S.S. |
| a vs c | $P < 0.05$ | c vs d,e | N.S.S. |
| a vs d | $P < 0.05$ | d vs e | N.S.S. |
| a vs b | N.S.S. | | |

N.S.S. = not statistically significant

TABLE 3

Serotonin concentrations (μg/g wet weight of tissue) in brain tissue of normal mice assayed without freezing and after 1 and 6 days of freezing at -70°C

| Duration of Freezing (-70°C) | Serotonin concentration (μg/g wet weight) |
|---|---|
| 0 day | 0.69 ± 0.03 (6)* |
| 1 day | 0.68 ± 0.03 (6) |
| 6 days | 0.69 ± 0.03 (5) |

*mean ± standard error (no. of samples)

all mice were killed at the same time of day to avoid changes due to the circadian periodicity of brain 5-hydroxytryptamine.

Depression of brain serotonin concentrations with repeated injections of p-chlorophenylalanine

All serotonin concentrations obtained thus far have been relatively close values. To assure that the method is actually measuring serotonin and not merely a sample artifact, p-chlorophenylalanine, a known depletor of brain serotonin in mice, was injected and serotonin concentrations were determined. Mice treated with a 6 milligram dose of PCPA daily for 3 consecutive days showed significant decrease in brain serotonin 24 hours after the last PCPA injection (Table 4). This dose and treatment schedule reduced serotonin concentrations to approximately 55 percent its normal value. These results indicate that serotonin is present in the extract and changes in serotonin levels can be detected with this assay.

Effect of endotoxin on brain serotonin

Endotoxin-poisoned CF-1 mice will not show sensitivity to tryptophan until 10 hours after injection of the bacterial poison (40). Hence the serotonin levels of endotoxin-poisoned mice subsequent to the 10 hour period become crucial in studies on the role of serotonin in sensitivity of CF-1 mice to tryptophan. Table 5 shows the concentration of brain serotonin in CF-1 mice at 10, 11, 13, 16 and 22 hours after

TABLE 4

Depression of brain serotonin levels in mice given 6 mg of p-chlorophenylalanine daily for 3 consecutive days

| Treatment | Dose | Serotonin Concentration (μg/g wet weight) | Percent recovery of serotonin |
|---|---|---|---|
| Saline | ---- | 0.58 ± 0.01 (7) *a | ---- |
| PCPA | 6 mg PCPA daily for 3 consecutive days# | 0.26 ± 0.01 (8) b | 54.4 |

*mean ± standard error (no. of samples)

#animals were killed 24 hours after last PCPA injection

a vs b $P < 0.001$

TABLE 5

Brain serotonin concentrations (μg/g wet weight of tissue) in CF-1 mice at 10, 11, 13, 16, and 22 hours after endotoxin-poisoning

| Time (hours) | | Serotonin concentration (μg/g wet weight) | |
|---|---|---|---|
| Hours after endotoxin# | Time of killing | Endotoxin-treated[a] | Normal[b] |
| 10 | 9:00 A.M. | 0.73 ± 0.03 (6)* | 0.68 ± 0.02 (16) |
| 11 | 10:00 A.M. | 0.67 ± 0.03 (9) | 0.64 ± 0.02 (6) |
| 13 | 12:00 P.M. | 0.63 ± 0.03 (9) | 0.61 ± 0.04 (8) |
| 16 | 3:00 P.M. | 0.65 ± 0.03 (9) | 0.58 ± 0.02 (8) |
| 22 | 9:00 P.M. | 0.68 ± 0.03 (5) | 0.58 ± 0.03 (5) |

#all endotoxin injections were given at the same time of day

*mean ± standard error (no. of samples)

a vs b N.S.S.

endotoxin-poisoning. There is no statistically significant difference between these values and the values found in normal mice at the same time periods.

In a second group of experiments mice were given endotoxin at various times of day and brain serotonin concentrations were measured at 2, 4, and 6 hours after endotoxin. In this study the time of injection was varied. All mice were killed at the same time of day and still, as can be seen in Table 6, no significant alterations in serotonin concentrations could be observed.

Changes in the relative intensity of fluorescence in brain extracts prepared from normal and endotoxin-poisoned mice given a 20 mg load of L-tryptophan

Both normal and endotoxin-poisoned mice were injected subcutaneously with a 20 mg load of L-tryptophan. Endotoxin-poisoned mice were injected with tryptophan 10 hours after 1 $LD_{50}$ dose of endotoxin and brain tissue was assayed for serotonin at 1, 3, 6, and 12 hours after tryptophan. Mice given tryptophan alone were assayed at these same time periods and served as controls. A statistically significant increase in the relative intensity of fluorescence was observed among both groups of mice (Figure 2). Values were significantly higher in the endotoxin-poisoned mice at 1 and 6 hours when compared to mice given tryptophan alone. In normal mice given a tryptophan load the increase in the relative intensity of fluorescence was maintained for approximately 3 hours after

TABLE 6

Brain serotonin concentrations (μg/g wet weight of tissue) in CF-1 mice at 2, 4, and 6 hours after endotoxin-poisoning

| Experiment treatment# | Duration (hours) | Serotonin concentration (μg/g wet weight) |
|---|---|---|
| Control[a] | ---- | 0.56 ± 0.02 (6)* |
| Endotoxin[b] | 2 | 0.55 ± 0.02 (6) |
| | 4 | 0.54 ± 0.01 (6) |
| | 6 | 0.55 ± 0.02 (6) |

#Time of endotoxin injection varied. All animals were killed at the same time of day.

*mean ± standard error (no. of samples)

a vs b N.S.S.

Figure 2. Relative intensity of fluorescence of brain extracts from normal and endotoxin-treated mice given a 20 mg load of L-tryptophan at time 0. Endotoxin was injected 10 hours prior to the tryptophan load. Serotonin determinations were made at 1, 3, 6, and 12 hours after tryptophan. Values are compared to fluorescence of extracts from normal and endotoxin-poisoned mice without a tryptophan load. Zero, 1, 3, 6, and 12 hours correspond to 9 A.M., 10 A.M., 12 P.M., 3 P.M. and 9 P.M. respectively.

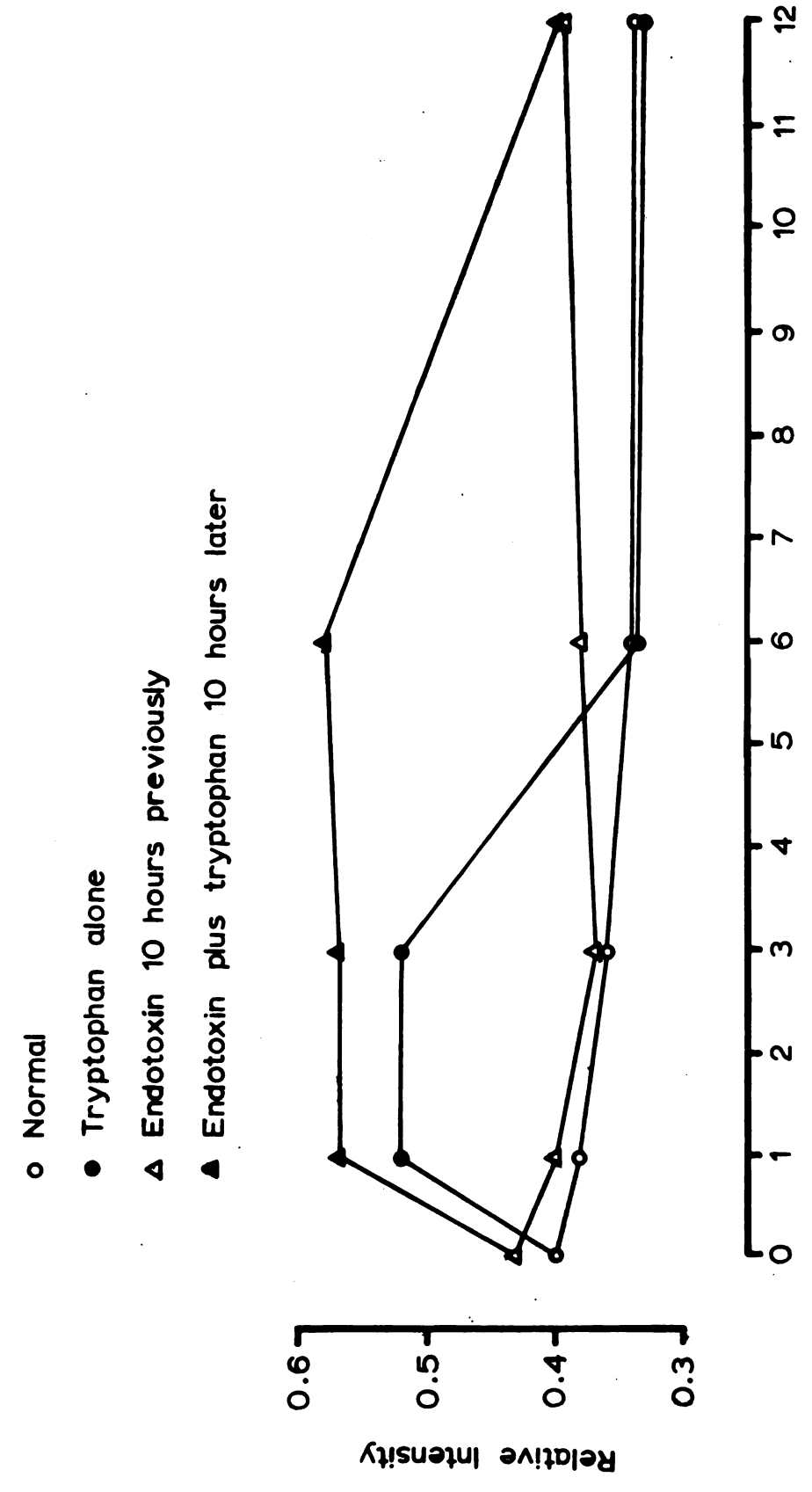
o Normal
● Tryptophan alone
Δ Endotoxin 10 hours previously
▲ Endotoxin plus tryptophan 10 hours later
Relative Intensity
0.6
0.5
0.4
0.3
0
1
2
3
4
5
6
7
8
9
10
11
12
Time (Hours)

Figure 2

tryptophan. This was followed by a gradual decrease which returned to baseline levels by 12 hours. In endotoxin-poisoned mice the increase in relative intensity of fluorescence was maintained for at least 6 hours after tryptophan and returned to control endotoxin levels by 12 hours.

Percent recovery and relative intensity of fluorescence of other indole derivatives

The possibility was considered that the increase in the relative intensity of fluorescence observed in the brain extracts of normal and endotoxin-poisoned mice given a load of tryptophan was not entirely due to an increase in serotonin. In an effort to determine whether other metabolites appearing as a consequence of tryptophan loading could be involved a variety of compounds were carried through the extraction procedure in the absence of tissue and their percent recovery was determined (Table 7). A high percent recovery was obtained with tryptophan, tryptamine, 5-hydroxytryptophan and 5-hydroxytryptamine. Only a low percent of the 5-hydroxyindoleacetic acid could be recovered. The relative intensity of fluorescence of standard solutions of these compounds before extraction was also measured (Table 7). The fluorescence of the hydroxyindoles is very similar. The relative intensity of 5-hydroxyindoleacetic acid is considerably less after extraction due to its low percent recovery. From the data in Table 7 it is readily apparent that concentrations of tryptamine and tryptophan approximately

TABLE 7

Relative intensity of fluorescence before extraction and percent recovery with butanol extraction of 5-hydroxytryptamine, 5-hydroxytryptophan, 5-hydroxyindoleacetic acid, tryptamine and L-tryptophan

| Compound | Relative intensity of fluorescence before extraction (20 μg/ml) | Percent recovery with butanol extraction |
|---|---|---|
| 5-hydroxytryptamine | > 10,000 | 90 |
| 5-hydroxytryptophan | 9,600 | 90 |
| 5-hydroxyindoleacetic acid | 6,800 | 14 |
| Tryptamine | 28 | 85 |
| L-tryptophan | 23 | 75 |

350-400 times greater than those of serotonin and 5-hydroxytryptophan are needed to give an equivalent amount of fluorescence.

Identification of tryptophan as an interfering compound

Normal and endotoxin-poisoned mice were injected with a 20 mg load of L-tryptophan containing 0.5 μc ($5.0 \times 10^{-6}$ mM) benzene ring-$^{14}$C-D,L-tryptophan. Mice were killed 1, 3, 6, and 12 hours after tryptophan and the extraction procedure was performed as usual. Two-dimensional thin layer chromatography of the extract was used to separate the metabolites. Color reactions on the thin layer plates with van Urk's and Prochazca's reagents, autoradiographs of the plates, and radioactive counting of the areas corresponding to the hRf value of tryptophan confirmed the presence of tryptophan in the extracts. No other radioactive or colored compounds were observed including the area where serotonin would have been located. This information suggests that tryptophan is present in the extracts in considerably higher concentration than serotonin. Preliminary attempts to scrap the plates and quantitate counts using liquid scintillation spectrometry confirm the visual data by showing that significant counts above background were found in the area of tryptophan migration and not in the area of serotonin migration (data not shown).

# DISCUSSION

The range of linearity between the relative intensity of fluorescence and the concentration of serotonin obtained in this study (Figure 1) shows a greater sensitivity (0.078 μg of serotonin/ml) than that reported by Maickel and co-workers (0.25 μg of serotonin/ml) (34). This increase in sensitivity may be due to the incorporation of cysteine into the reaction mixture. Curzon has previously observed a four-fold increase over that of Maickel with this modification (19). Contrary to reports by other investigators (55) very low and consistent readings were obtained with the blank. The reason for this unusual consistency is not known.

The percent recovery of exogenous serotonin has been reported to be 95-105 percent (34). However, to obtain this high percent recovery exogenous serotonin must be added to the supernatant fraction of the tissue homogenate. This fraction contains only a limited amount of tissue to which the serotonin may bind. Percent recovery is reduced to 56-75 percent if the serotonin is added prior to the homogenization and carried through the entire extraction procedure (Table 1). Preliminary experiments using tritiated serotonin indicate that considerable binding of serotonin to

the precipitable portion of the homogenate does occur. Similar results have been reported with other extraction procedures (5,24,61). A certain percentage of this loss may also be due to the degradation of serotonin by homogenization or to *in vitro* oxidation of the biogenic amine. To avoid the variability of percent recovery obtained when serotonin is added prior to homogenization, the internal standard in this project was prepared by adding the serotonin to the supernatant fraction. Thus the internal standard served as a valuable tool for day to day standardization of our methods but the actual percent recovery of tissue serotonin may be ranging from 70-100 percent.

The variability of brain 5-hydroxytryptamine concentrations obtained at different times of the day (Table 2) agrees with the circadian periodicity previously reported in mice (2). The rat is essentially the same as the mouse in both phasing and amplitude (49). In both animals serotonin concentration reaches its peak in the morning and its minimum level in the evening. A circadian rhythmicity for tryptophan oxygenase also occurs (46). Tryptophan oxygenase shows lowest activity in the morning and greatest activity during the dark hours. The inverse relationship between brain serotonin and tryptophan oxygenase suggests the interdependence of the 2 pathways. Curzon and co-workers have data confirming the postulated correlation between decreased 5-hydroxytryptamine and tryptophan oxygenase activity (26).

Variation in brain 5-hydroxytryptamine may also be caused by factors other than circadian periodicity such as age (2), sex (2), and living conditions (23) of the mice. Though the number of mice per cage was relatively constant throughout this project, the age of the mice and the amount of time they remained grouped varied with each experiment. This may explain the variability obtained in brain serotonin assayed at the same time of day but in different experiments.

The range in serotonin concentrations found in our experiments (0.51-0.79 μg/g wet weight of tissue) is in close agreement with previously reported values. A mean value of 0.53 μg/g was obtained by Koe (30) in male Charles River Swiss CD strains utilizing the method of Mead and Finger (37). Using an acetone extraction and spectrophotometric assay, Albretch detected values ranging from 0.53-1.05 μg/g in ZBC-Bittner mice (2). Concentrations of 0.74-0.95 μg/g were reported in albino Swiss male mice by Giacolone et al. (23) using the method of Shore and Olin (51) and Shore (50).

In evaluating the results obtained with endotoxin-poisoning two aspects must be considered; first, the difference in values between normal and endotoxin-poisoned mice assayed at the same time of day and second, the change in serotonin levels in endotoxin-poisoned animals at different times after endotoxin. There is no statistically significant difference in serotonin concentrations between normal mice and endotoxin-poisoned mice assayed at the same time of

day (Table 5). Neither is there any significant difference between serotonin concentrations when comparing the 10, 11, 13, 16, and 22 hour time periods in endotoxin-poisoned mice. Normal mice did show a significant change in brain serotonin between the 9 A.M. value and the 12, 3 and 9 P.M. values due to the circadian periodicity (Table 2). The absence of any significant difference in serotonin levels in endotoxin-poisoned mice at any time may be due to an alteration or elimination of the circadian periodicity found in the normal animals.

Koe and Weissman have reported a 75 percent decrease in mouse serotonin concentrations after administration of p-chlorophenylalanine (316 mg/kg) daily for 3 consecutive days (30). Only a 50 percent decrease was observed in CF-1 mice (Table 4). The difference may be due to the preparation of the suspension. To avoid any unnecessary toxic effects to the mice p-chlorophenylalanine was suspended in saline and injected as a neutral solution rather than the strong acid, pH 1.5, recommended by Koe (30).

It is impossible to draw any final conclusions on the data for serotonin concentration in the brain of normal and endotoxin-poisoned mice given a tryptophan load due to the possible interference of tryptophan and/or other indole metabolites. The observation that tryptophan is the only compound giving any color reaction after spraying of the thin layer chromatograms suggests that tryptophan is present in

much higher concentrations than any other metabolite in the extract. The increase in fluorescence found in endotoxin-poisoned mice given a load of tryptophan is greater at 1, 3 and 6 hours after tryptophan than the increase observed in normal mice given the same load (Figure 2). Furthermore, this increase in the relative intensity of fluorescence is maintained at least 3 hours longer in endotoxin-poisoned animals. Radioactive counts of the area on the thin layer plates corresponding to the hRf value for tryptophan were higher in all endotoxin-poisoned mice compared to the counts in normal mice at the same time. Unpublished data in our laboratory show clearly that the $C^{14}$ label of the benzene ring labeled tryptophan remains in the endotoxin-poisoned mice for significantly longer periods of time than in normal mice. While the quantitative distribution of this label in vivo among tryptophan and its various metabolites has not been determined, it is assumed that a significant amount of the label is still associated with intact tryptophan.

The interference of tryptophan in these samples does not exclude the possibility that an increase in 5-hydroxytryptamine may be contributing to the increase in fluorescence. If metabolism of tryptophan in the mouse brain after tryptophan load is similar to the metabolism of tryptophan in the rat brain reported by Eccleston and co-workers (6,20,21,39) it is possible that there is a prolonged increase in serotonin concentrations in the brain of endotoxin-poisoned mice

due to an increase in tryptophan availability over a longer period of time. A more efficient method than the one used in this project for the separation of serotonin from other metabolites in tryptophan loading is necessary before any conclusive results can be obtained.

# CONCLUSION

The method of Maickel and co-workers for serotonin determinations in brain tissue was used to measure brain serotonin levels in normal and endotoxin-poisoned mice with and without a tryptophan load. Analyses of the extracts indicate that this method is not applicable to tryptophan loading studies due to the possible interference of tryptophan and/or other tryptophan metabolites. This method is adequate for serotonin determinations in brain tissue of animals not given a tryptophan load. PCPA-treated CF-1 mice showed a marked decrease in serotonin concentrations with repeated injections of p-chlorophenylalanine. A dose of 1 $LD_{50}$ of Salmonella typhimurium endotoxin gave no statistically significant change in serotonin concentrations within a 22 hour period.

# LITERATURE CITED

LITERATURE CITED


1. Agarwal, M. K., W. Hoffman and F. Rosen. 1969. The effect of endotoxin and thorotrast on inducible enzymes in the isolated, perfused rat liver. Biochim. Biophys. Acta 177: 250-258.

2. Albrecht, P., M. B. Visscher, J. J. Bittner and F. Halberg. 1956. Daily changes in 5-hydroxytryptamine concentration in mouse brain. Proc. Soc. Exptl. Biol. Med. 92: 703-706.

3. Alivisatos, S. G., A. D. Papaphilis, F. Ungar and P. K. Seth. 1970. Chemical nature of binding of serotonin in the central nervous system. Nature 226: 455-456.

4. Alivisatos, S. G., F. Ungar, P. K. Seth, L. P. Levitt, A. J. Garoulis and T. S. Meyer. 1971. Receptors: localization and specificity of binding of serotonin in the central nervous system. Science 171: 809-812.

5. Ansell, G. and M. Beeson. 1968. Rapid and sensitive procedure for the combined assay of noradrenaline, dopamine and serotonin in a single brain sample. Anal. Biochem. 23: 196-206.

6. Ashcroft, G. W., D. Eccleston and T. B. B. Crawford. 1965. 5-Hydroxyindole metabolism in rat brain. A study of intermediate metabolism using the technique of tryptophan loading - I. J. Neurochem. 12: 483-492.

7. Awapara, J., V. E. Davis and O. Graham. 1960. Chromatographic methods for the identification of biogenic amines. J. Chromatog. 3: 11-19.

8. Axelrod, J. and J. K. Inscoe. 1963. The uptake and binding of circulating serotonin and the effect of drugs. J. Pharmacol. Exptl. Therap. 141: 161-165.

9. Berry, L. J. 1964. Effect of endotoxin on the level of selected enzymes and metabolites. Bacterial Endotoxins (Landy, M. and Braun, W., eds). Institute of Microbiology, Rutgers, The State University, New Brunswick.

10. Berry, L. J. and D. S. Smythe. 1963. Effects of bacterial endotoxins on metabolism. VI. The role of tryptophan pyrrolase in response of mice to endotoxin. J. Exptl. Med. 118: 587-603.

11. Berry, L. J. and D. S. Smythe. 1964. Effects of bacterial endotoxins on metabolism. VII. Enzyme induction and cortisone protection. J. Exptl. Med. 120: 721-732.

12. Bertler, A. 1961. Effect of reserpine on the storage of catechol amines in brain and other tissues. Acta Physiol. Scand. 51: 75-83.

13. Bogdanski, D. F., A. Pletscher, B. B. Brodie and S. Udenfriend. 1956. Identification and assay of serotonin in brain. J. Pharmacol. Exptl. Therap. 117: 82-88.

14. Bulat, M. and Z. Supek. 1967. The penetration of 5-hydroxytryptamine through the blood-brain barrier. J. Neurochem. 14: 265-271.

15. Bulat, M. and Z. Supek. 1968. Passage of 5-hydroxy-tryptamine through the blood-brain barrier, its metabolism in the brain and elimination of 5-hydroxy-indoleacetic acid from the brain tissue. J. Neuro-chem. 15: 383-389.

16. Chang, C. C. 1964. A sensitive method for spectrofluoro-metric assay of catecholamines. Int. J Neurophar-mac. 3: 643-649.

17. Cremata, V. Y., Jr. and B. K. Koe. 1966. Clinical-pharmacological evaluation of p-chlorophenylalanine: a new serotonin-depleting agent. Clin. Pharmacol. Therap. 7: 768-776.

18. Curzon, G. and A. R. Green. 1968. Effect of hydrocorti-sone on rat brain 5-hydroxytryptamine. Life Sci. 7: 657-663.

19. Curzon, G. and A. R. Green. 1970. Rapid method for the determination of 5-hydroxytryptamine and 5-hydroxy-indoleacetic acid in small regions of rat brain. Brit. J. Pharmacol. 39: 653-655.

20. Eccleston, D., G. W. Ashcroft and T. B. B. Crawford. 1965. 5-Hydroxyindole metabolism in rat brain. A study of intermediate metabolism using the technique of tryptophan loading - II. J. Neurochem. 12: 493-503.

21. Eccleston, D., G. W. Ashcroft, T. B. B. Crawford and R. Loose. 1966. Some observations of the estimation of tryptamine in tissues. J. Neurochem. 13: 93-101.

22. Gal, E. M., M. Morgan, S. K. Chatterjee and F. D. Marshall, Jr. 1964. Hydroxylation of tryptophan by brain tissue in vivo and related aspects of 5-hydroxytryptamine metabolism. Biochem. Pharmacol. 13: 1639-1653.

23. Giacolone, E., M. Tansella, L. Valzelli and G. Garattini. 1968. Brain serotonin metabolism in isolated aggressive mice. Biochem. Pharmacol. 17: 1315-1327.

24. Giacolone, E. and L. Valzelli. 1969. A spectrofluorometric method for the simultaneous determination of 2-(5-hydroxyindol-3-yl)ethylamine (serotonin) and 5-hydroxyindol-3-yl acetic acid in the brain. Pharmacol. 2: 171-175.

25. Grahame-Smith, D. G. 1964. Tryptophan hydroxylation in brain. Biochem. Biophys. Res. Comm. 16: 586-592.

26. Green, A. R. and G. Curzon. 1968. Decrease of 5-hydroxytryptamine in the brain provoked by hydrocortisone and its prevention by allopurinol. Nature. 220: 1095-1097.

27. Ichiyama, A., S. Nakamura, Y. Nishisuku and O. Hayaishi. 1970. Enzymatic studies on the biosynthesis of serotonin in mammalian brain. J. Biol. Chem. 245: 1699-1709.

28. Jéquier, E., W. Lovenberg and A. Sjoerdsma. 1967. Tryptophan hydroxylase inhibition: the mechanism by which p-chlorophenylalanine depletes rat brain serotonin. Mol. Pharmacol. 3: 274-278.

29. Koe, B. K. 1967. Inhibiting action of p-chlorophenylalanine and α-methyl-p-chlorophenylalanine on liver phenylalanine and tryptophan hydroxylase. Med. Pharmacol. exp. 17: 129-138.

30. Koe, B. K. and A. Weissman. 1966. p-Chlorophenylalanine: a specific depletor of brain serotonin. J. Pharmacol. Exptl. Therap. 154: 499-516.

31. Lin, R. C., N. H. Neff, S. H. Ngai and E. Costa. 1969. Turnover rates of serotonin and norepinephrine in brain of normal and pargyline-treated rats. Life Sci. 8: 1077-1084.

32. Lovenberg, W., E. Jéquier and A. Sjoerdsma. 1967. Tryptophan hydroxylation: measurement in pineal gland, brainstem, and carcinoid tumor. Science. 155: 217-219.

33. Lovenberg, W., H. Weissbach and B. Udenfriend. 1962. Aromatic L-amino acid decarboxylase. J. Biol. Chem. 237: 89-93.

34. Maickel, R. P., R. H. Cox, Jr., J. Saillant and F. P. Miller. 1968. A method for the determination of serotonin and norepinephrine in discrete areas of rat brain. J. Neuropharmacol. 7: 275-281.

35. Maickel, R. P. and F. P. Miller. 1966. Fluorescent products formed by reaction of indole derivatives and o-phthalaldehyde. Anal. Chem. 38: 1937-1938.

36. Maickel, R. P. and F. P. Miller. 1968. The fluorometric determination of indolealkylamines in brain and pineal gland. Adv. Pharmacol. 6A: 71-77.

37. Mead, J. and K. Finger. 1961. A single extraction method for the determination of both norepinephrine and serotonin in brain. Biochem. Pharmacol. 6: 52-53.

38. Miller, F. P., R. H. Cox, Jr., W. R. Snodgrass and R. P. Maickel. 1970. Comparative effects of p-chlorophenylalanine, p-chloroamphetamine and p-chloro-N-methyl-amphetamine on rat brain norepinephrine, serotonin and 5-hydroxyindole-3-acetic acid. Biochem. Pharmacol. 19: 435-442.

39. Moir, A. T. B. and D. Eccleston. 1968. The effects of precursor loading in the cerebral metabolism of 5-hydroxyindoles. J. Neurochem. 15: 1093-1108.

40. Moon, R. J 1971. Tryptophan oxygenase and tryptophan metabolism in endotoxin-poisoned and allopurinol-treated mice. Biochim. Biophys. Acta. 230: 342-348.

41. Moon, R. J. and L. J. Berry. 1968. Effect of tryptophan and selected analogues on body temperature of endotoxin-poisoned mice. J. Bacteriol. 95: 764-770.

42. Moon, R. J. and L. J. Berry. 1968. Role of tryptophan pyrrolase in endotoxin poisoning. J. Bacteriol. 95: 1247-1253.

43. Morris, K. M. 1970. Tryptophan metabolites found in urine of normal and endotoxin-poisoned mice. Thesis. Master of Science degree, Dept. of Microbiology and Public Health, Michigan State University.

44. Neff, N. H., T. N. Tozer and B. B. Brodie. 1967. Application of steady-state kinetics to studies of the transfer of 5-hydroxyindoleacetic acid from brain to plasma. J. Pharmacol. Exptl. Therap. 158: 214-218.

45. Page, I. H. 1968. Serotonin. Year Book Medical Publishers, Inc., Chicago.

46. Rapoport, M. I., R. D. Feigin, J. Bruton and W. R. Beisel. 1966. Circadian rhythm for tryptophan pyrrolase activity and its circulating substrate. Science. 153: 1642-1644.

47. Reed, L. and H. Muench. 1938. A simple method of estimating fifty percent and points. Am. J. Hyg. 27: 493-499.

48. Renson, J., H. Weissbach and S. Undenfriend. 1962. Hydroxylation of tryptophan by phenylalanine hydroxylase. J. Biol. Chem. 237: 2261-2264.

49. Scheving, L., W. Harrison, P Gordon and J. Pauly. 1968. Daily fluctuation (circadian and ultradian) in biogenic amines of the rat brain. Am. J. Physiol. 214: 166-173.

50. Shore, P. A. 1959. A simple technique involving solvent extraction for the estimation of norepinephrine and epinephrine in tissues. Pharmacol. Rev. 11: 276-277.

51. Shore, P. A. and J. S. Olin. 1958. Identification and chemical assay of norepinephrine in brain and other tissues. J. Pharmacol. 122: 295-300.

52. Sjoerdsma, A., W. Lovenberg, K. Engelman, W. Carpenter, Jr., R. Wyatt and G. Gessa. 1970. Serotonin now: clinical implications of inhibiting its synthesis with para-chlorophenylalanine. Annals of Internal Medecine. 73: 607-629.

53. Snyder, S. H., J. Axelrod and M. Zweig. 1965. A sensitive and specific fluorescence assay for tissue serotonin. Biochem. Pharmacol. 14: 831-835.

54. Stone, C. A., H. C. Wenger, C. T. Ludden, J. M. Stavorski and C. Ross. 1960. Antiserotonin-antihistaminic properties of cyproheptadine. J. Pharmacol. Exptl. Therap. 131: 73-84.

55. Thompson, J. H., Ch. A. Spezia and M. Angulo. 1970. Fluorometric detection of serotonin using o-phthalaldehyde: an improvement. Experientia. 26: 327-329.

56. Udenfriend, S., D. Bogdanski and H. Weissbach. 1955. Fluorescence characteristics of 5-hydroxytryptamine. Science. 122: 972-973.

57. Udenfriend, S., E. Titus, H. Weissbach and R. Peterson. 1956. Biogenesis and metabolism of 5-hydroxyindole compounds. J. Biol. Chem. 219: 335-344.

58. Udenfriend, S., H. Weissbach and D. F. Bogdanski. 1957. Increase in tissue serotonin following administration of its precursor 5-hydroxytryptophan. J. Biol. Chem. 224: 803-810.

59. Vanable, J. W., Jr. 1963. A ninhydrin reaction giving a sensitive quantitative fluorescence assay for 5-hydroxytryptamine. Anal. Biochem. 6: 393-403.

60. Weber, L. J. and A. Horita. 1965. A study of 5-hydroxytryptamine formation from L-tryptophan in the brain and other tissues. Biochem. Pharmacol. 14: 1141-1149.

61. Welch, A. and B. Welch. 1969. Solvent extraction method for simultaneous determination of norepinephrine, dopamine, serotonin and 5-hydroxyindoleacetic acid in a single mouse brain. Anal. Biochem. 30: 161-179.

62. White, C. 1952. Biometrics. 8: 33.

# APPENDIX

Table 1A. Fluorescence of Substituted Indoles after Reaction with OPT

| Compound | $R_1$ (5 position) | $R_2$ (3 position) | $F_u$ [a] |
|---|---|---|---|
| N-Acetyl-5-methoxytryptamine | $CH_3O-$ | $-CH_2CH_2NHCOOH_3$ | 287 |
| 5-Methoxytryptamine | $CH_3O-$ | $-CH_2CH_2NH_2$ | 275 |
| 5-Methoxyindole-3-acetic acid | $CH_3O-$ | $-CH_2COOH$ | 120 |
| N-Acetyl-5-hydroxytryptamine | HO- | $-CH_2CH_2NHCOCH_3$ | 114 |
| 5-Hydroxytryptophan | HO- | $-CH_2CH(NH_2)COOH$ | 71 |
| 5-Hydroxytryptamine | HO- | $-CH_2CH_2NH_2$ | 50 |
| 5-Hydroxyindole-3-acetic acid | HO- | $-CH_2COOH$ | 47 |
| α-Methyl-5-hydroxytryptophan | HO- | $-CH(CH_3)CH(NH_2)COOH$ | 36 |
| 5-Methoxygramine | $CH_3O-$ | $-CH_2N(CH_3)_2$ | 14 |
| 5-Methyltryptophan | $CH_3$ | $-CH_2CH(NH_2)COOH$ | 1.6 |
| N,N-Dimethyltryptamine | H- | $-CH_2CH_2N(CH_3)_2$ | 1.5 |
| Tryptophan | H- | $-CH_2CH(NH_2)COOH$ | < 1 |
| Tryptamine | H- | $-CH_2CH_2NH_2$ | < 1 |
| 5-Hydroxyindole | HO- | -H | < 1 |
| 5-Methoxyindole | $CH_3O-$ | -H | < 1 |
| N-Acetyltryptophan | H- | $-CH_2CH(NHCOCH_3)COOH$ | < 1 |
| Tryptophol | H- | $-CH_2CH_2OH$ | 0 |
| Indole-3-acetic acid | H- | $-CH_2COOH$ | 0 |
| 5-Bromogramine | Br- | $-CH_2N(CH_3)_2$ | 0 |
| Indole | H- | -H | 0 |

[a] $F_u$ = fluorescence units per $10^{-9}$ mole