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STUDIES ON THE ACUTE TOXICITY OF 5-HYDROXYTRYPTAMINE IN THE RATS PRE-EXPOSED TO OZONE

BY

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ABSTRACT

The present study was conducted, from the view point of the effects of ozone upon rat, to investigate the toxic effects of 5-hydroxytryptamine (5-HT), the changes in the activity of the lung and serum monoamine oxidase (MAO), which metabolizes 5-HT, and the changes in the 5-HT content of various organs.

In the rats exposed previously to ozone, a number of animals died by the intraperitoneal administration of 5-HT, though the control rats without previous exposure to ozone didn't receive any lethal damage by the administration of the same dose of 5-HT. The mortality of the rats exposed previously to ozone increased as the dose of 5-HT was increased and the susceptibility to 5-HT increased as the ozone concentration became higher, but it decreased as the interval between the end of the exposure and 5-HT administration became longer and the increased susceptibility was detectable for as long as 12½ hours.

In the rats exposed to ozone, the activity of the lung and serum MAO was significantly inhibited as compared with the control, and these changes in the activity generally paralleled each other with time. In these rats, the 5-HT content of the lung and spleen was significantly increased, that of the brain was significantly decreased but no significant change in the 5-HT content was noted in the liver and kidney.

INTRODUCTION

The biological and toxic effects of the air pollutants on the human being and animals have been attracting public attention. Only a few studies, however, have been made on the effects of ozone from the immunological point of view, especially in relation to the allergic diseases such as bronchial asthma. Schoettlin and Landau[1] in an epidemiological study on asthmatic patients in Pasadena, California, reported that a low positive correlation was found between the chemical measurements of air pollution and the number of persons having attacks. On the other hand, it was reported that a significantly larger number of persons had asthmatic attacks when the oxidant value was high enough (0.25 ppm) to cause eye irritation. In the laboratory animals exposed to ozone, changes in the content of the chemical mediator of allergic reaction were reported: the lung histamine content of the guinea pig was decreased[2] and the lung 5-hydroxytryptamine (5-HT) content of the rats was increased[3]. Moreover, it was reported that histamine and acetylcholine, causing a syndrome similar to that by anaphylaxis, increased the mortality of animals pre-exposed to ozone[4,5].

5-HT is relatively abundant in the lungs as compared with the other organs and,

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similar to histamine, it causes physiological actions, such as extremely increased sensitivity in *pertussis*-inoculated animals[6-8], capillary permeability[9] and bronchoconstriction[10,11], etc. Therefore, 5-HT has been thought to be related to allergy and anaphylaxis as a mediating substance of antigen-antibody reaction. Humphery and Jaques[12,13] reported that 5-HT is liberated from the platelets *in vitro* by the antigen-antibody reaction. Herxheimer[10] studied in detail the bronchial actions of 5-HT administered by inhalation in guinea pigs. Guinea pigs exposed to a 1% aerosol of 5-HT developed severe dyspnea, followed by convulsions. 5-HT aerosol caused a shock syndrome in the guinea pig similar to that caused by anaphylaxis, histamine and acetylcholine. During anaphylaxis, there is a transient increase in the plasma concentration of both 5-HT and histamine[14].

The present study was planned in an attempt to investigate the toxic effects of 5-HT, the changes in the activity of the enzyme which metabolizes 5-HT and the changes in the 5-HT content of various organs of animals pre-exposed to ozone, as the first step to reveal the relationship between the air pollutants and allergy.

Materials and Methods

a. Exposure of animals

Rats were used as experimental animals because they have a high lung concentration of 5-HT as compared with several other species[15] and show a high susceptibility to 5-HT: rat smooth muscles such as of the bronchiole[15,16], colon[17] and uterus[18], etc. are found to be very responsive to it. Six to seven-week old male Sprague-Dowley rats weighing 245±23 grams were used. They were housed in an air-conditioned laboratory, fed with a standard diet and exposed to clean air or ozone for 3 hours in a stainless steel and glass whole-body-exposure chamber (about 1×1×1 m). Ozone was obtained from clean air by a dielectric type ozone generator and the ozone concentration was continuously measured with a monitor by the chemiluminescence method. Chamber ventilation was done by changing about half of the air every minute. The temperature range and humidity range were maintained at 25± 2°C and 40 to 60%, respectively.

b. Effects of 5-HT upon rats pre-exposed to air or ozone

Three experiments were conducted for the mortality studies: (I) Animals were injected with the various doses of 5-HT from 15 to 20 minutes after exposure to air or 5 ppm of ozone, (II) animals were exposed to various concentrations of ozone and after 15 to 20 minutes a dose of 30 mg/kg body weight of 5-HT was injected and (III) animals were injected with a dose of 30 mg/kg of 5-HT at various intervals after exposure to 5 ppm of ozone. 5-HT was administered intraperitoneally as a saline (0.9% sodium chloride) solution and then, regardless of the dose, the injected volume was kept below one milliliter for each animal.

c. Enzymatic activity of the lung and serum MAO of rats exposed to ozone

Animals exposed to air or 5 ppm of ozone were sacrificed by withdrawing blood by heart puncture within one hour after exposure without anesthetization, as the enzymatic activity may be affected by the anesthetic agent. The serum was immediately obtained by centrifugation. The lungs were homogenized in the Potter-Elvehjem type homogenizer using an ice-cold saline (0.9% sodium chloride) solution. Serum and lung homogenate was stored in liquid nitrogen

till the assay of enzymatic activity was performed.

The MAO activity levels were determined by the method of Nakano and Ito[19]. The activity of MAO was measured by a colorimeter using 1-[(4-aminomethylphenyl) azo]-2-naphthol as the substrate. As the lungs might be edematous by exposure to ozone, the unit of lung MAO activity was expressed as micromoles of 1-[(4-formylphenyl) azo]-2-naphthol, formed by the enzyme reaction, per hour per total lung per 100 grams of body weight at 37°C. The unit of serum MAO activity was expressed as nanomoles per hour per milliliter of serum at 37°C.

d. Measurement of the water content and protein content of the lung and the 5-HT content of various organs

Anamals exposed to air or 5 ppm of ozone for 3 hours were immediately sacrificed under pentobarbital sodium anesthetization. Various organs were taken out and the blood on the surface was removed gently with a filter paper. After weighing, the right lung was dried at 110±1°C for 3 days and weighed again. The difference in the weight between the fresh and dried lung was assumed to indicate the water content of the fresh lung, and the dried lung weight was assumed to indicate the lung parenchyma weight. A part of the fresh lung was homogenized in an ice-cold saline solution and the protein content of the homogenate was determined by the method of Gornall *et al.*[20]. Various organs were homogenized in 0.1 N hydrochloric acid and the 5-HT content of the homogenate was determined by the fluorometric method described by Bogdanski *et al.*[21].

Table 1. 5-HT toxicity in rats pre-exposed to air or ozone

Exposure time (3 hours)	5-HT* (mg/kg)	Mortality No. died/No. tested	(%)
Clean air	30	0 / 6	0
	40	0 / 6	0
	50	0 / 6	0
Ozone 5±0.2 ppm	10	3 / 16	19
	20	3 / 12	25
	30	7 / 12	58
	40	5 / 6	83

* All animals were intraperitoneally injected 15 to 20 minutes after the end of exposure.

Results

a. Mortality studies

Table 1 shows that animals exposed to ozone (5±0.2 ppm, 3 hours) were considerably more susceptible to the action of 5-HT. A few minutes after the 5-HT injection, every rat presented symptoms such as hyperpnea, leading to extremely labored breathing, tremble and cyanosis, which were assumed to be partly due to 5-HT poisoning. Signs of cyanosis were observed by the color of the snout, tail, ear and sole. No rat of the control died, but a large number of the rats exposed to ozone died with convulsions in 10 minutes to 3 hours after injection, and the other rats, which did not die within 3 hours, survived for at least two weeks till they were sacrificed for autopsy. It was reported that the lethal dose 50 (LD_{50}) of 5-HT in the rat is 30 mg/kg body weight by the intravenous route and approximately 117 mg/kg by the subcutaneous route[22]. Therefore, the LD_{50} by the intraperitoneal route is considered to be between these two values. Table 1 shows that a dose of 50 mg/kg of 5-HT produced no death in the control groups, but a dose of 10 mg/kg produced a 19% mortality and a dose of 30 mg/kg a 58% mortality in the groups exposed to ozone. Therefore, the

Table 2. Effect of various concentrations of ozone on susceptibility of rats to 5-HT poisoning

Exposure time (3 hours)		Mortality No. died/No. tested	(%)
Clean air		0 / 6	0
Ozone	1 ppm	0 / 12	0
	3	2 / 12*	17
	4	3 / 12	25
	5	7 / 12	58
	6	3 / 4**	75

All animals were intraperitoneally injected with a dose of 30 mg/kg of 5-HT 15 to 20 minutes after the end of exposure.

* Two animals died 2 and 5 days after the 5-HT injection.

** Two of six animals exposed to ozone died at the end of exposure.

Table 3. Effect of length of intervals between the end of exposure and 5-HT injection on susceptibility of rats to 5-HT poisoning

Exposure time (3 hours)	Test interval	Mortality No. died/No. tested	(%)
Clean air	15 minutes	0 / 6	0
Ozone 5±0.2 ppm	15 minutes	7 / 12	58
	3.5 hours	5 / 10	50
	12.5 hours	3 / 9	33
	24 hours	1 / 9*	11

All animals were intraperitoneally injected with a dose of 30 mg/kg of 5-HT.

* One animal died 2 days after the 5-HT injection.

dose of 30 mg/kg of 5-HT was selected as the standard test dose for further studies because this dose produced no death in the control groups but showed a high mortality in the groups exposed to ozone.

Table 2 shows the degree of the lethal effect of 5-HT when the ozone concentration was changed. In the case of the administration of 30 mg/kg of 5-HT 15 to 20 minutes after the end of exposure, the mortality increased as the ozone concentration increased. Animals pre-exposed to 1 ppm of ozone did not die and the mortality of the animals pre-exposed to 3 ppm of ozone was 17%, two of the twelve animals dying in 2 and 5 days, as compared to death coming generally within 3 hours after the injection.

The lethal concentration 50 (LC_{50}) of ozone in the rats exposed to ozone for four hours was reported to be in the region of 10 to 12 ppm by Diggle and Gage[23] and 3.6 to 6.4 ppm by Stokinger[24]. Moreover, Mittler *et al.*[25] reported that the LC_{50} in in the rats by a single three-hour exposure to ozone was 21.8 ppm. In this experiment, a study was made wether a single three-hour ozone (5 ppm) exposure caused the rats to die, because the LC_{50} values reported by several investigators were not entirely in agreement. But, all of six animals exposed to ozone survived for at least two weeks till they were sacrificed for autopsy. Even if taking into consideration the differences in the age, sex and strain of the rats, it is probable that the minimal lethal concentration of ozone in the rats by the single three-hour exposure to ozone is approximately 6 ppm, because two of the six animals pre-exposed to 6 ppm of ozone died at the end of the exposure as shown in Table 2. Therefore, a concentration of 5 ppm of ozone was selected as the standard concentration for further studies because the rats did not die from a single exposure at this concentration whereas the combined ozone-5-HT treatment, as shown in Table 1, produced a high mortality at this concentration.

The results of the experiments in which 30 mg/kg of 5-HT were administered at various intervals from the end of the exposure to ozone (5±0.2 ppm, 3 hours) are shown in Table 3. The mortality decreased as the interval was increased and the increased susceptibility to 5-HT was detect-

able for as long as 12½ hours after the end of exposure.

In another experiment, two groups of rats were intraperitoneally administered with 30 mg/kg of 5-HT before exposure to ozone. The first group (eight animals) and the second group (ten animals) were exposed to ozone (5±0.2 ppm) for 3 hours, 3½ hours and 15 minutes after the injection, respectively. Only one death occurred five days after the exposure in the first group, although, in the previous experiment, the mortality was more than 50% in the groups which were given an injection of 5-HT 15 minutes or 3½ hours after the exposure to ozone as shown in Table 3. Thus, the increased mortality from a combined ozone-5-HT treatment was detected only when exposure to ozone preceded the 5-HT injection.

b. Enzymatic activity of the lung and serum MAO

Not only the 5-HT itself but also the enzymes which produce 5-HT and destroy 5-HT were found to be present in the lungs[26]. It is commonly known that 5-HT is oxidatively deamidized and inactivated by MAO. From Table 3 which shows that the mortality decreased as the interval between the end of exposure and 5-HT injection became longer, it is probably considered that HAO inhibited by ozone recovered with time. As shown in Fig. 1, the activity level of the lung MAO was significantly reduced to 63% ($P<0.01$) 1.5 hours after the exposure began, then slightly recovered to 75% ($P<0.05$) 1.5 hours after that and thereafter this level was maintained for 12.5 hours in the animals exposed to ozone. The activity level of serum MAO tended to change in parallel with that of the lung MAO (Fig. 2).

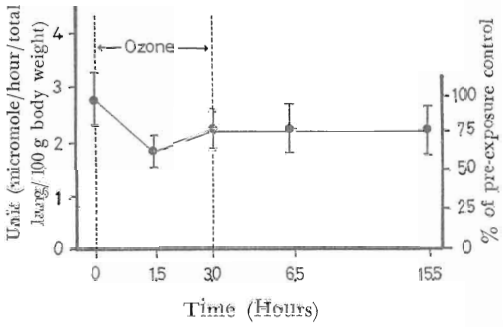

Fig. 1. Change of activity of lung MAO with time

Ten rats in each group were exposed to ozone (5±0.2 ppm) for 3 hours. Figure shows the mean value ± standard deviation and % of pre-exposure control.

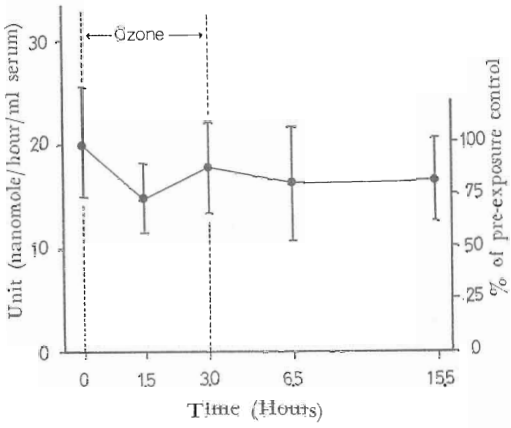

Fig. 2. Change of activity of serum MAO with time

c. Water content and protein content to the lungs

As shown in Table 4, in the animals exposed to ozone, as compared with the control animals, the average of the lung weight was about two-fold ($P<0.001$) and the average of the lung water content and lung protein content was higher ($P<0.001$), respectively. On autopsy, the lungs were edematous with hemorrhage. The increase of these in the animals exposed to ozone is attributed to the leakage of the plasma protein and blood cells in the alveoli.

Table 4. Water content and protein content of lungs in rats pre-exposed to air or ozone

Exposure time (3 hours)	Weight (g/100g body weight)	Water content (%)	Protein content (mg/100g body weight)
Clean air (n=15)	0.43±0.03	78.8±0.4	73±6
Ozone 5±0.2 ppm (n=14)	0.83±0.10*	85.6±1.2*	99±7*

Table shows the mean value ± standard deviation.
* $P<0.001$

Table 5. 5-HT content of various organs of rats after exposure to air or ozone for 3 hours

Various organs			Clean air (n=15)	Ozone (5 ppm) (n=14)
Lung	μg/g	Fresh tissue	2.39±0.13	3.59±0.15*
	μg/g	Parenchyma	11.3±0.7	25.4±2.6*
	μg/100g	Body weight	1.02±0.10	2.98±0.46*
Brain	μg/g	Fresh tissue	0.65±0.05	0.41±0.07*
Spleen	μg/g	,,	2.70±0.14	3.75±0.25*
Liver	μg/g	,,	1.28±0.15	1.29±0.07
Kidney	μg/g	,,	0.22±0.10	0.24±0.13

* $P<0.001$

In another experiment, a dose of 30 mg/kg of 5-HT was injected to each of the ten animals before the exposure to ozone. The average value of the lung weight and water content of the animals, which were exposed for 3 hours to ozone (5±0.2 ppm) 15 minutes after the 5-HT injection, was 0.54±0.06 gram per 100 grams of body weight and 82.3±2.0 (%), respectively.

d. 5-HT content of various organs

No significant differences were found in the weight of the brain, spleen, kidney, liver and total body in the group exposed to ozone as compared with those of the control group. Table 5 shows that the average value of the 5-HT content of the lung and spleen increased significantly ($P<0.001$) and that of the brain decreased significantly ($P<0.001$), although no significant change was noted in the liver and kidney. Not only the 5-HT content per gram of lung parenchyma but also that per 100 grams of body weight increased more than two-fold ($P<0.001$). The average value of the 5-HT content of the fresh lung, spleen and brain in the control animals was equal to the reported value in the rat (2.30, 2.80 and 0.63 μg/g, respectively)[26-28].

Discussion

Although lung edema might be considered as the cause or etiology of death because the lung weight and its water content are increased and on autopsy the lungs were edematous in the animals exposed to ozone, it is unlikely that edema is the direct cause of death, as the rats did not die for at least two weeks till they were sacrificed for autopsy after a single exposure to

ozone (5 ppm, 3 hours). The organs of the rat contain about 125 μg of 5-HT per kilogram of body weight[29]. As an extremely large dose of 50 mg/kg of 5-HT produced no death in the control animals, as shown in Table 1, the simple additive effect of the endogenous and exogenous 5-HT cannot bring death to the animals. Moreover, as the increase in mortality was detected only when the exposure to ozone preceded the 5-HT injection, it should not be thought that death with convulsion occurred within a short period by the simple additive effect of ozone and 5-HT toxicity. Exposure to ozone, of course, must induce pulmonary lesions and functional changes in the lung, and the toxic effects of 5-HT, which were accelerated by the inhibition of the MAO activity, must affect the various tissues. The accelerated toxic effects of 5-HT might produce a severe constriction of the bronchial muscle. But the direct cause of death cannot be bronchoconstriction, as on autopsy the lungs of the dead animals were not inflated in appearance. Borst *et al.*[30] reported that dogs receiving an intravenous injection of 5-HT showed a marked pumonary vasoconstriction and a marked rise in the pulmonary artery pressure. Therefore, the circulation disturbance of the lungs might be produced by the accelerated toxic effects of 5-HT in the animals pre-exposed to ozone. On the other hand, Sanyal and West[31] reported that anaphylactic shock in the rat is characterized by the progressive circulatory collapse and the target organ is the small intestine. The rat small intestine is found to be very sensitive to 5-HT[32]. Accordingly the accelerated toxic effects of 5-HT, inducing an exteremely increased constriction and peristalsis of the gastrointestinal tract, might cause death after the combined ozone-5-HT treatment. Therefore, the pre-exposure to ozone might bring death to the animals by the enhanced effect, causing a severe disturbance of the pulmonary and extrapulmonary function or a syndrome similar to that by anaphylaxis.

The metabolism of endogenous 5-HT in a rat is very intense: blood 5-HT could be renewed every 3½ hours, intestine 5-HT every 8 to 9 hours and the total 5-HT of the organism every 12 to 14 hours[33]. The results of the author's experiment showed that the mortality decreased as the interval between the end of the exposure and 5-HT injection became longer, as shown in Table 3. It was possible that this decrease in the mortality might be attributed to the metabolization of 5-HT by MAO, though the inhibited MAO activity did not still recover till 12½ hours after the end of exposure, as shown Fig. 1 and 2. Otherwise, it might be dependent on the recovery of the physiological function injured by ozone.

That the majority of the animals survived when 5-HT was administered before the exposure to ozone remains unexplainable. Matzen[34] reported that a large dose (1 mg) of 5-HT has been found to reduce pulmonary edema in the mouse supposed to be caused by the exposure to ozone. Also in this experiment, rats pretreated with 5-HT showed a slight decrease in the lung water content than in the rats without pretreatment with 5-HT. However, the small decrease of edema alone cannot explain the cause of the survival of the animals given 5-HT before exposure to ozone. Moreover, Gray *et al.*[35] reported that pretreatment with 5-HT (20 mg/kg) increased significantly the survival rate of the rats exposed to the lethal dose of X-ray. Reports of several studies show that ozone may be a radiomimetic substance and this was confirmed in several ways and especially by Fetner, who demonstrated that the chromo-

some breakage in the human cell culture exposed to 8 ppm of ozone for 10 minutes is equivalent to that produced by 200 r.[36-40] Gray *et al.*, assumed that the protective effect of 5-HT may be due to its vasoconstrictive property producing a temporary tissue anoxia.

The increase of the lung 5-HT by exposure to ozone may possibly be due to the inactivation of the lung MAO. Another possible answer may be that the exposure to ozone results in an increased activity of 5-hydroxytryptophan decarboxylase, which is responsible for the conversion of 5-hydroxytryptophan to 5-HT. But, Weissbach *et al.*[26] reported that the decarboxylase activity was relatively weak, less than 15 μg of 5-hydroxytryptophan being converted to 5-HT per gram of lung per hour, and that MAO destroyed 960 μg of 5-HT per gram of lung per hour in the rat. Therefore, though the activity of the lung MAO was reduced to 65% in the animals exposed to ozone, as show in Fig. 1, the increase of 5-HT following exposure to ozone is not enhanced so much by the activation of the decarboxylase, even if the enzyme was activated by the ozone. Moreover, another possible cause may simply be the trapping of the fluid containing 5-HT in the edematous lung because of the increased lung protein content and hemorrhage, which were produced by the exposure to ozone. All of the blood 5-HT are normally found in the platelets and not in the plasma[41]. Then, the retention of the platelets in the edematous lung would be a reasonable explanation. Similary, the possible cause of the increased 5-HT in the spleen may be the destruction of the platelets in the spleen.

Skillen *et al.*[28] reported that a significant decrease in the brain 5-HT of the rats exposed to 6 ppm of ozone for 4 hours was demonstrated. The fate of the released 5-HT remained in question: whether it was destroyed in the brain or transported to the other organs. 5-HT has been attracting attention as a substance playing an important role in the central nervous system, like adrenaline, noradrenaline, etc. As the sedative action of reserpine, which releases 5-HT from many tissues, including the brain, is considered to be related to the liberation of the brain 5-HT[42,43], the decrease of the brain 5-HT following the exposure to ozone might probably be useful in relieving the stress from the exposure to ozone, which causes irritation of the nose and throat and headache, etc.

Nakazawa[44] reported that patients with bronchial asthma were more susceptible than the healthy subjects not only to chemical mediators but also to the irritant gases, such as sulfur dioxide. Therefore, the possibility may be that pre-exposure to air pollutants promotes allerigic reaction. A few studies reported that the animals pre-exposed to air pollutants, such as ozone, nitrogen dioxide and sulfur dioxide, showed anaphylactic attacks by the inhalation of albumin used as a sensitizing antigen[45-47]. The authors described that especially the exposure to ozone brought about a severe dyspneic attack as compared with nitrogen dioxide and sulfur dioxide. Though the mechanism of the enhancement of the susceptibility to 5-HT of the animals pre-exposed to ozone was not definitely clarified in this study, the increased susceptibility to 5-HT remains as an interesting problem in relation to the increased susceptibility to histamine or acetylcholine of the animals pre-exposed to ozone.

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