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CHANGES IN THE 5-HYDROXYTRYPTAMINE CONTENT OF RAT, RABBIT AND HUMAN BRAIN AFTER DEATH

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The fall in 5-hydroxytryptamine concentration which occurs in brain tissues in the first 48 hr after death varies in different areas of the brain and is of the same order of magnitude in those areas of the rat and rabbit in which it was estimated, namely, hypothalamus, hippocampal gyrus and frontal cortex, and also in human frontal cortex. The loss of 5-hydroxytryptamine is greater in the hypothalamus than in the cerebral cortex. The extent of this change depends on the temperature and conditions of storage of tissues. It is least in tissues which remain within the skull after death before chemical extraction and in tissues stored at −17° C. Comparison of cerebral 5-hydroxytryptamine content in control animals and animals pretreated with iproniazid suggests that the differences are for the greater part due to the action of mono-amine oxidase.

The hypothesis that the 5-hydroxytryptamine present in mammalian brain has a fundamental role in the central nervous system (Gaddum, 1954; Woolley & Shaw, 1954) has stimulated investigations in man in the hope of finding alterations of the metabolism of 5-hydroxytryptamine in neurological or psychological disturbances (Haverback, Sjoerdsma & Terry, 1956; Feldstein, Hoagland & Freeman, 1959).

The levels of 5-hydroxytryptamine in circulating blood and the urinary excretion of its metabolite, 5-hydroxyindole acetic acid, are poor indicators of 5-hydroxytryptamine metabolism in the central nervous system because 5-hydroxytryptamine is present in much greater quantities in the gut and the blood than in the brain (Dalgliesh, Toh & Work, 1953; Humphrey & Toh, 1954; Amin, Crawford & Gaddum, 1954) and because the excretion of 5-hydroxyindole acetic acid can be greatly altered by certain factors in the diet (Anderson, Ziegler & Doeden, 1958). Thus it would be possible for small but possibly important disturbances of 5-hydroxytryptamine metabolism occurring only within the central nervous system not to show in analysis of 5-hydroxytryptamine in peripheral blood or of metabolites in the urine.

The alternative approach, the estimation of 5-hydroxytryptamine directly in discrete areas of the brain (Costa & Aprison, 1958), requires that the normal range of 5-hydroxytryptamine concentrations be established so that subsequent estimates of 5-hydroxytryptamine in changing conditions may be referred to this range. But biopsy material is available in very small amounts and then only from certain brain

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areas and is presumably most often abnormal, because it is removed with the aim of helping diagnosis. Autopsy specimens, on the other hand, can only be examined several hours after death: during this time, synthesis and oxidation of 5-hydroxytryptamine may proceed at rates differing from those in the living tissue. The present studies were designed to estimate the size of the changes in the 5-hydroxytryptamine content of central nervous tissue after death and to test the validity of estimates of 5-hydroxytryptamine in post-mortem material as an index of concentrations in the living tissue.

METHODS

Material and extraction. The rats were albino females (bred from Glaxo stock). They weighed between 150 and 250 g except in experiments in which the animals were killed by being dropped into liquid nitrogen: these weighed 20 to 50 g. Otherwise they were killed by a blow on the neck and their throats were cut. The rabbits were of mixed breed and sex, weighing between 2.0 and 2.5 kg. They were killed by injecting air into the heart.

Human frontal lobe tissue was obtained directly from the operating theatre. The surgical operations were performed under nitrous oxide (75% in oxygen) and after the administration to the patient of atropine sulphate (0.6 to 0.8 mg subcutaneously), sodium thiopentone (0 to 500 mg intravenously), pethidine hydrochloride (50 to 100 mg intravenously), and succinylcholine chloride (10 to 40 mg intravenously). Hypothermia was induced before operation by cooling to 31° C (rectal temperature).

5-Hydroxytryptamine from brain tissues was extracted with 4 vol. of acetone (Correale, 1956). Specific procedures are described under the appropriate section of the results.

Assay. The 5-hydroxytryptamine extract was assayed on the rat stomach (Vane, 1957) in Tyrode solution at 37° C to which hyoscine (10^{-7}) was always added. Extracts were assayed at least twice on each of two rat stomach preparations by bracketing with higher and lower doses of standard 5-hydroxytryptamine solution shown by preliminary and final examination in each experiment to lie on the rectilinear portions of the dose-response curve. The concentration in each extract was thus estimated at least four times.

Blood content of the brain. Blood trapped in the tissues was estimated by a method based on that of King & Gilchrist (1947). Brain tissue (1 g) was ground with 7.5 ml. 0.1 N hydrochloric acid and well mixed. 10 min were allowed for the transformation of haemoglobin into acid haematin. Two ml. of 5% sodium cyanide was added and distilled water to make up the volume to 10 ml. The solution was mixed and the intensity of colour of the cyanhaematin measured by comparison with 5 standard dilutions containing between 0.005 and 0.1 ml. blood in saline similarly treated. The colours developed were read on an SP 300 spectrophotometer using an Ilford T.C. green light filter with maximum transmission between 530 and 550 mμ.

RESULTS

Identification of the substance assayed. The active substance extracted was identified in two ways: by using the ability of reserpine to deplete tissues of 5-hydroxytryptamine (Pletscher, Shore & Brodie, 1956), and by the response of the rat stomach to the test solution in the presence of bromlysergic acid diethylamide.

When reserpine (3 mg/kg) was administered intraperitoneally to rats 16 hr before they were killed, the concentration of the active substance in the hypothalamus, the hippocampal gyrus and in the frontal cerebral cortex was decreased, but was unchanged in the cerebellum (Table 1). This was taken to indicate that most of the substance extracted from the first three tissues and active upon the rat stomach

TABLE 1

EFFECT OF RESERPINE ADMINISTRATION UPON THE ACTIVE SUBSTANCE ASSAYED

Extracts were assayed on the isolated rat stomach and results are expressed as 5-hydroxytryptamine in ng/g wet weight rat brain ± s.e. of mean. The number of animals used is shown in parenthesis

	Area			
Treatment	Hypothalamus	Frontal cortex	Hippocampal gyrus	Cerebellum
None	315·5±19·4 (15)	162·3±13·7 (15)	318·8±45·3 (6)	29·0±2·0 (2)
Reserpine (3 mg/kg 16 hr) (before death)	37·0±22·5 (2)	62·0±16·5 (2)	80·0±11·0 (2)	28·5±5·5 (2)
Reserpine-treated groups as % of controls	11·7	38·0	25·0	96·5

was 5-hydroxytryptamine and that none was present in cerebellar extract. The response of the rat stomach to brain extract in presence of bromlysergic acid diethylamide (10^{-7}) was compared with that to standard 5-hydroxytryptamine solution and that to barium chloride solution. The responses to the extract and to standard 5-hydroxytryptamine were equally diminished though not abolished by the presence of bromlysergic acid diethylamide, whilst those to 5 μg barium chloride—which was found to give a contraction approximately equivalent to that produced by 4 ng 5-hydroxytryptamine—were unchanged.

Contribution of blood 5-hydroxytryptamine. The amount of blood present in rat brain was found to be equal to or less than 0.01 ml./g fresh brain. This was the case whether the animals had been killed by a blow on the head followed by bleeding or by immersion into liquid nitrogen.

This figure is lower than that of 0.024 ml. derived by Nair, Palm & Roth (1960) from the data of Weil-Malherbe, Axelrod & Tomchick (1959), yet was obtained from those rat brains which appeared to be most congested with blood. Humphrey & Jaques (1954) give values of the order of 5-hydroxytryptamine 500 ng/ml. blood for the rat and 5-hydroxytryptamine 200 ng/ml. blood for man. Thus, at the most, only 5-hydroxytryptamine 5 ng/g fresh brain would normally be due to the presence of blood.

Changes occurring in tissues after death

Immediate changes. In order to detect changes in brain 5-hydroxytryptamine occurring immediately after death, the procedure of immersing small animals in liquid gas (Richter & Crossland, 1949) was adopted. The rats were dropped into liquid nitrogen for 30 sec and the frozen brains were removed from the skull and extracted at once with ice-cold acetone. The concentration of 5-hydroxytryptamine in the brain of normal rats killed in this way was not significantly different from that of a group of normal rats of the same weight killed by a blow on the head and bled by cutting the throat (Table 2).

Table 3 shows the concentrations of brain 5-hydroxytryptamine in rats killed by the two methods after being treated with one of four drugs which may be expected to cause such changes. These are: iproniazid, reserpine, allobarbitone and leptazol.

TABLE 2

EFFECT OF DEATH BY STUNNING AND FREEZING IN LIQUID NITROGEN ON THE 5-HYDROXYTRYPTAMINE CONTENT OF RAT BRAIN

Mean levels are expressed as a % ±s.e. of the levels in rats killed by stunning and bleeding. The number of animals used is shown in parenthesis. Probability of difference between means= $0{\cdot}3>P>0{\cdot}2$

Expt.	Bled	Liquid N_2
A	100±6·90 (3)	92·7±15·13 (3)
B	100±1·97 (2)	88·1±6·60 (4)

TABLE 3

COMPARISON OF DEATH BY BLEEDING AND DEATH BY INSTANTANEOUS FREEZING ON BRAIN 5-HYDROXYTRYPTAMINE CONTENT

Rats treated with various drugs intraperitoneally and killed either by bleeding or by immersion in liquid nitrogen. 5-Hydroxytryptamine concentration expressed as ng/g wet weight. The mixture of allobarbitone and urethane contained diallylbarbituric acid 0.1 g/ml. and urethane 0.4 g/ml.

Drug	No. of animals in each group	Dose/kg	Interval between drug administration and death	Mean 5-hydroxytryptamine concentration	
				Bled	Liquid N_2
No drug	3	—	—	397	386
Iproniazid	2	100 mg	5 hr	484	512·5
Reserpine	2	3 mg	5 hr	85	51·5
Reserpine	3	3 mg	16 hr	146	146
Allobarbitone and urethane	2	1 ml.	15 min	337	304
Leptazol	2	80 mg	30 sec	380	395

There were no significant differences ($P>0.1$) in brain 5-hydroxytryptamine levels between the animals stunned and bled and those dropped into liquid nitrogen (that is, when enzymatic action was prevented after death). It was concluded that no striking changes in brain 5-hydroxytryptamine occurred in the first few min after death and that valid estimates of 5-hydroxytryptamine in tissues extracted at this time could be made by means of the simpler method of killing.

Long-term changes. All the animals in these experiments were killed by a light blow on the head. Rat brains were then treated in one of three ways: they were either removed from the skull and extracted at once with acetone ("zero time" controls); or removed from the skull, weighed and placed in air-tight containers at different temperatures (−17° C, +4° C, +16° C, +28° C) and left for the time required (0 to 48 hr) ("separated"); or left *in situ* in the skull at the temperature and for the times required ("unseparated"): after the time required had elapsed the brain parts were removed from the skull, weighed and extracted with acetone. Rabbit brain parts were removed from the skull immediately after death and were either extracted at once ("zero time" controls) or stored for a period of time in air-tight containers ("separated"), after which they were extracted with acetone.

Pieces of human frontal lobes were received from the operating theatre and extracted with acetone within 30 min of removal from the body ("zero time") or stored in air-tight containers for 48 hr ("separated") and at this time extracted with acetone.

TABLE 4

DEPENDENCE OF LOSS OF ACTIVITY IN "SEPARATED TISSUES" AT ROOM TEMPERATURE UPON THE AREA OF THE BRAIN EXAMINED

The figures represent mean 5-hydroxytryptamine concentrations (ng/g wet weight)±s.e. The figures in parentheses indicate the number of estimations

	Frontal cortex		Hippocampal gyrus		Hypothalamus	
Time after death in hr:	0	48	0	48	0	48
Rabbit	55·0±2·0 (2)	43·0±8·0 (2)	328·5±70·5 (2)	193·0±27·0 (2)	429·0±123·0 (2)	153·5±0·5 (2)
48 hr as % of 0 hr		78·2		57·5		35·8
Rat	162·3±13·7 (15)	117·9±15·1 (9)			315·5±19·4 (15)	111·2±13·6 (9)
48 hr as % of 0 hr		73·0				35·0
Man	82·0±11·5 (4)	67·0±9·0 (4)				
48 hr as % of 0 hr		81·5				

Loss of 5-hydroxytryptamine from different areas of the brain. When " separated " brain tissues were left for 48 hr at room temperature, there was a decrease in the 5-hydroxytryptamine content of all three areas examined (cerebral cortex, hippocampal gyrus and hypothalamus). This decrease was highly significant ($P<0.001$) for the rat hypothalamus and just significant ($P=0.05$) for the cerebral cortex (Table 4). The extent of the decrease was of the same order in the rat and rabbit. The extent of the fall in hippocampal 5-hydroxytryptamine determined in the rabbit lay between that of the cerebral cortex and of the hypothalamus. The value found for the loss of 5-hydroxytryptamine from human cerebral cortex was of the same order as that of this area in the rat.

Effect of duration of storage before extraction with acetone. The rate of loss of brain 5-hydroxytryptamine was determined at 0, 6, 12, 24 and 48 hr of storage at room temperature after rat hypothalami and cerebral cortices removed from the whole brains were left for the stated periods (Fig. 1). Three animals were used for each time and tissue. The rate of loss of 5-hydroxytryptamine was similar in the two areas and was not significantly different in the first 12 hr from that in the subsequent 36 hr.

Effect of temperature and of conditions of storage. 5-Hydroxytryptamine concentrations were estimated in whole " unseparated " rat brains from animals left for 48 hr at +28° C, +16° C, −17° C. In rat brains which remained inside the

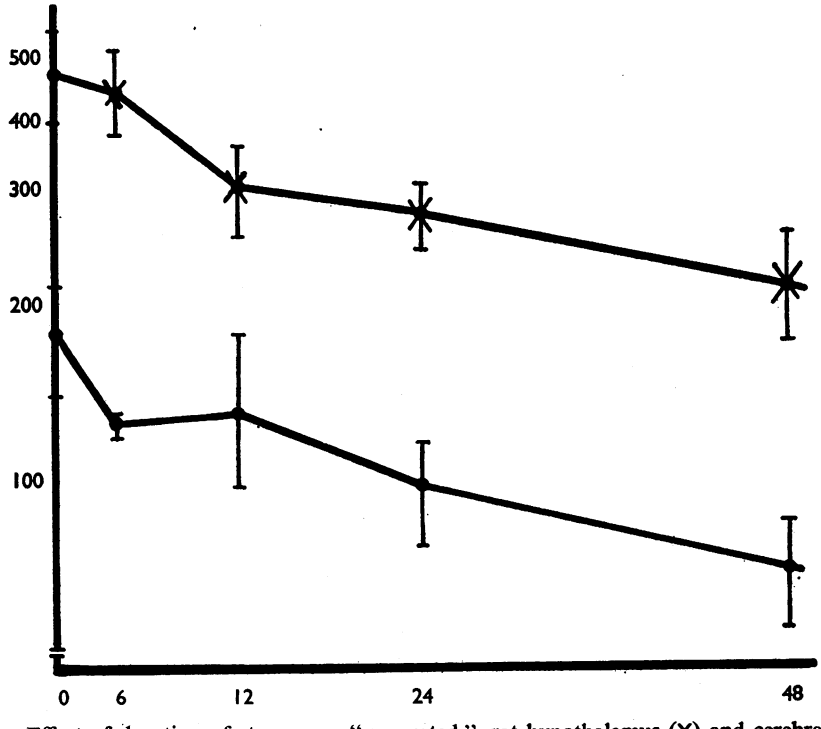

Fig. 1. Effect of duration of storage on " separated " rat hypothalamus (X) and cerebral cortex (●). Abscissa: storage time (hr). Ordinate: mean 5-hydroxytryptamine concentration (ng/g wet weight). Range marks are standard errors.

TABLE 5

EFFECT OF STORAGE TEMPERATURE ON 24 HR LOSS OF 5-HYDROXYTRYPTAMINE FROM "SEPARATED" AND "UNSEPARATED" HYPOTHALAMI

The 5-hydroxytryptamine levels are expressed as a % of the level of control tissues extracted immediately after death. The number of animals is shown in parenthesis

	20° C	4° C	−17° C
"Separated"	57·4 (5)	54·3 (7)	86·4 (6)
"Unseparated"	116·2 (5)	97·4 (11)	95·3 (12)

skull after death, the loss of 5-hydroxytryptamine was least in those brains stored at the highest temperature and greatest in those stored at the lowest temperature (Fig. 2). However, when rat hypothalami were stored under the "separated" or the "unseparated" condition for 24 hr at the three temperatures 20° C, 4° C, − 17° C, little 5-hydroxytryptamine was lost in 24 hr from "unseparated" hypothalami stored *in situ* at 4° C or − 17° C and there was apparently a small gain at 20° C, whereas, on the contrary, the loss of 5-hydroxytryptamine in "separated" hypothalami was significant ($P<0.05$) at 20° C and 4° C but small and insignificant at − 17° C (Table 5).

Storage under nitrogen. The possibility that the difference observed between 5-hydroxytryptamine loss in "separated" and "unseparated" hypothalami was related to a difference in access by atmospheric oxygen was tested by storing hypothalami under nitrogen.

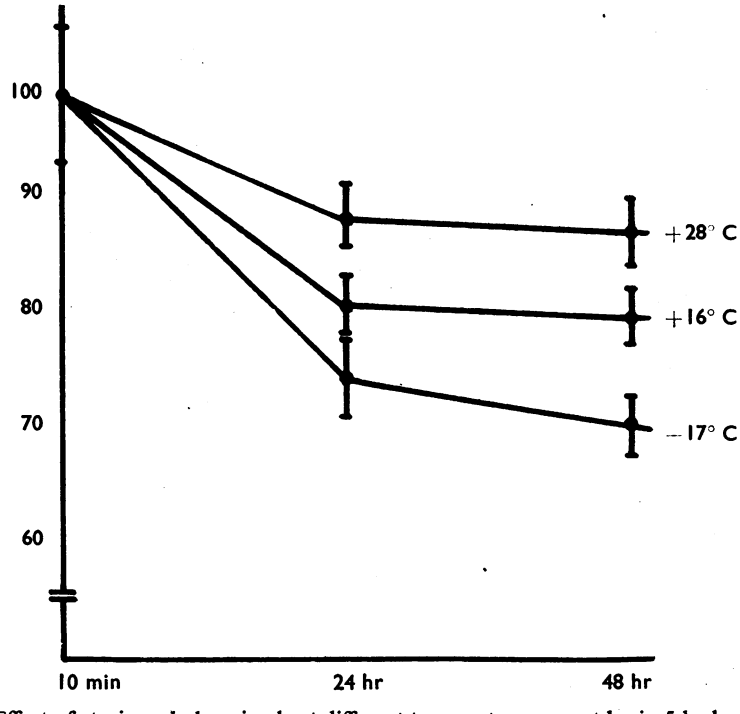

Fig. 2. Effect of storing whole animals at different temperatures on rat brain 5-hydroxytryptamine content. Abscissa: time (hr). Ordinate: brain 5-hydroxytryptamine concentration expressed as % of the concentration at zero time (10 min). Range marks are standard errors.

Whereas the 5-hydroxytryptamine content of hypothalami stored in atmospheric air fell to 35% in 48 hr, that of hypothalami stored under nitrogen apparently fell only to 70%. Bromlysergic acid diethylamide, however, only prevented half this activity. The greater activity in the hypothalami under nitrogen was therefore due to the presence of another substance which was not identified.

Effect of previous administration of iproniazid on the loss of 5-hydroxytryptamine in "separated" tissues. The 5-hydroxytryptamine concentration in "separated" hypothalami and cerebral cortex in untreated control animals was compared with that in animals given iproniazid (100 mg/kg injected intraperitoneally) 5 hr before killing.

Iproniazid raised the 5-hydroxytryptamine content of both cerebral cortex and hypothalamus extracted at the time of death (Table 6). The loss of 5-hydroxytryptamine which occurred in storage at room temperature for 48 hr was less in

TABLE 6

EFFECT OF IPRONIAZID ADMINISTRATION ON THE LOSS OF 5-HYDROXYTRYPTAMINE AFTER DEATH

Figures represent mean 5-hydroxytryptamine concentrations (ng/g wet weight)±s.e.

	Frontal cortex		Hypothalamus	
	0 hr	48 hr	0 hr	48 hr
Iproniazid	223·5±22·8 (8)	205·8±32·9 (8)	628·0±38·3 (8)	478·4±53·5 (8)
48 hr as % of 0 hr		92·0		76·0
Controls, untreated	162·3±13·7 (15)	117·9±15·1 (9)	315·5±19·4 (15)	111·2±13·6 (9)
48 hr as % of 0 hr		73·0		35·0

the animals treated with iproniazid than in the controls: for the cerebral cortex it was 8% in the treated animals compared with 27% in the untreated. The respective figures for the hypothalamus were 24% and 65%.

DISCUSSION

Although several pharmacologically active substances can theoretically be present in a brain extract, it is most likely that the active substance assayed in these experiments was 5-hydroxytryptamine, since its concentration was diminished by previous administration of reserpine (Pletscher *et al.*, 1956) and increased by previous administration of iproniazid (Bogdanski, Weissbach & Udenfriend, 1956), and its action on the rat stomach was blocked by bromlysergic acid diethylamide (Vane, 1957). It is unlikely that the substance assayed was acetylcholine, since hyoscine was present during the bioassay; or substance P, since its concentration is unchanged by reserpine administration (Paasonen & Vogt, 1956); or γ-aminobutyric acid, since the amount of this substance might be expected to increase rather than decrease after death (Elliott & Jasper, 1959), and a similar argument can be applied to such other substances as cadaverine and putresceine.

Adrenaline and noradrenaline cause a relaxation of the rat stomach and if present in a brain extract could abolish or diminish the contraction due to 5-hydroxytryptamine. But the administration of iproniazid would be expected to increase their concentration in the brain as well as that of 5-hydroxytryptamine. In any case these amines appear to be destroyed during the extraction procedure (Joyce, 1961).

The amount of 5-hydroxytryptamine in a brain extract contributed by blood 5-hydroxytryptamine was relatively small. This view agrees with that of Amin *et al.* (1954), who found little difference in the 5-hydroxytryptamine content of brain areas in dogs which had or had not been perfused with warm saline through the carotid artery until the effluent from the jugular vein was bloodless.

The results of experiments on early changes in 5-hydroxytryptamine levels after death (Table 2) showed a mean brain 5-hydroxytryptamine content for rats killed in liquid nitrogen which was about 10% lower than that of unfrozen rats, but this difference was not statistically significant. Crossland (1951), on the contrary, had found a 13% increase in brain acetylcholine if the tissue was frozen with liquid air before chemical extraction. The idea that there is no acute loss of 5-hydroxytryptamine in brain tissue in the first 4 min after death can only be reconciled with the high turnover rate which has been reported for brain 5-hydroxytryptamine (Brodie, Spector, Kuntzman & Shore, 1958) if it is assumed that even after arrest of the circulation the activities of the synthetic and the destructive enzymes proceed at approximately the same rate, although the figures given by Bogdanski, Weissbach & Udenfriend (1957) show that in both hypothalamus and cerebral cortex *in vitro* activity of the mono-amine oxidase is greater than that of 5-hydroxytryptophan decarboxylase. Studies of enzymes in the cerebellum of the rabbit (Smith, Robins, Eydt & Daesch, 1957) have in fact shown that many enzymes are stable until at least 6 hr after death.

The difference between the percentage loss (Table 4) of 5-hydroxytryptamine from hypothalamus and cerebral cortex is likely to be of practical significance where human tissues are extracted with acetone some time after removal from the skull; any correction factor applied to estimates of potency in order to obtain values at the time of death must be appropriate to the area of the brain concerned and to the temperature at which they are kept (Table 5). If the tissues were kept 48 hr, for example, at room temperature in the " separated " state, the concentration of 5-hydroxytryptamine in the hypothalamus would be expected to be about 35% of that present at the time of death, whereas in the cerebral cortex it would be of the order of 80% of that at death.

A striking result of the storage experiments was that a substantial loss of 5-hydroxytryptamine occurred only when the brain had been removed from the skull (" separated "). The experiment in which storage of " separated " tissues took place under nitrogen did not support the idea that this loss was entirely due to the degree of access of atmospheric oxygen. When, however, iproniazid was given before death the observations suggested that mono-amine oxidase might be at least partly responsible for the loss of 5-hydroxytryptamine after death. That this mono-amine oxidase inhibitor does not entirely prevent the loss of 5-hydroxytryptamine may be due to metabolism of the amine by another route (Weissbach, Lovenberg, Redfield & Udenfriend, 1961). An increase in both cortical and hypothalamic 5-hydroxytryptamine occurred after the administration of iproniazid (Table 6). This is not in agreement with the report of Revzin & Costa (1960), who found that after the administration of large doses of mono-amine oxidase inhibitors there was no increase in cerebral cortical 5-hydroxytryptamine whereas concentrations in the

brain stem could increase fourfold. In the experiments reported here cerebral cortical 5-hydroxytryptamine rose to 138% of that of untreated animals and hypothalamic 5-hydroxytryptamine to 200%.

The effect of temperature on the loss of 5-hydroxytryptamine from tissues stored after death is probably a consequence of several different processes: First, an increase in activity of both the synthetic and the destructive enzymes would be expected to occur within this range with an increase in temperature. Second, the rate of autolysis also increases with temperature, and this alters the *p*H of tissues and consequently enzymic activity and also causes the breakdown of cellular barriers between 5-hydroxytryptamine and mono-amine oxidase, thus increasing destruction of the amine. Third, a breakdown of cellular barriers may also occur at low temperatures, since these and the subsequent thawing increase the denaturation of proteins (Meryman, 1960). This factor is probably particularly effective when whole animals are stored at − 17° C. In these cases the time elapsing between removal from the deep-freeze and acetone extraction is lengthened by the fact that it is difficult to remove the brain from the frozen skull of the adult rat.

The results obtained here can be interpreted in the light of these considerations. In the "separated" tissues, less 5-hydroxytryptamine was lost at − 17° C when both autolysis and activity of the destructive enzyme was slowed than at the higher temperature. In the "unseparated" tissues, on the contrary, a small loss of 5-hydroxytryptamine activity occurred at the low temperature of storage, and in the hypothalamus kept at 20° C there was a slight gain.

In considering the best methods of examining 5-hydroxytryptamine content of human nervous tissue, it may be noted that the storage of bodies at 4° C before autopsy, as is the practice in most hospitals, is unlikely to affect hypothalamic 5-hydroxytryptamine greatly, since this condition is comparable to the "unseparated" experiments. As the content of the hypothalamus changes most under the "separated" conditions, the conclusion is probably valid for other brain areas as well. It is often hospital practice to store, subsequently, the brain parts in the refrigerator or in the deep-freeze before 5-hydroxytryptamine extraction is carried out. Here, the results show that storage in the deep-freeze of small pieces of brain is preferable. If 5-hydroxytryptamine is extracted immediately from brain parts on removal from the deep-freeze, losses resulting from permeability changes should be minimal, since most physical destruction following the application of low temperatures to biological tissues occurs during thawing (Meryman, 1960). Thus, if brain parts were placed in the deep-freeze immediately after removal from the skull and stored until acetone extraction could be carried out, a loss of only 10 to 20% of the 5-hydroxytryptamine concentration present at the time of death would be expected in hypothalamic tissues and an even smaller loss from other parts of the brain.

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