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A STUDY OF 5-HYDROXYTRYPTAMINE FORMATION FROM L-TRYPTOPHAN IN THE BRAIN AND OTHER TISSUES*

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Abstract—Three methods have been developed to study, in the rat, the synthesis of 5-hydroxytryptamine (5-HT) from the amino acid precursor L-tryptophan (Try): the first involves perfusion of the small intestine *in situ* with a Tyrode's solution containing Try; the second involves i.p. injections of the amino acid into unoperated and into partially or totally eviscerated rats; the third consists in infusing Try into the brain via the internal carotid artery. All these procedures showed synthesis of 5-HT in the brain as measured by fluorometric techniques. The results showed that the brain formed 5-HT from Try and that the brain did not depend on the stomach, intestine, spleen, kidneys, or liver for 5-hydroxytryptophan.

SYNTHESIS of 5-hydroxytryptamine (5-HT) from L-tryptophan (Try) is accomplished in mammalian tissue by two enzymatic steps. First, an aromatic hydroxylation forming 5-hydroxytryptophan (5-HTP), second a decarboxylation of 5-HTP resulting in 5-HT. The enzyme for decarboxylation of 5-HTP has been partially purified and is generally found in all parenchymatous tissue. The mechanism for the oxidation of Try to 5-HTP has not been well studied; enzyme(s) involved in this hydroxylation are unknown, and the localization of this process has not been elucidated.

A particulate, ascorbic acid-dependent enzyme in intestine and kidney, capable of hydroxylating Try, has been reported.[1, 2] An enzyme in the liver of rats is also capable of hydroxylating Try.[3, 4] Renson *et al.*[5] demonstrated that this liver enzyme is phenylalanine hydroxylase and concluded its activity on Try has little physiological importance.

Avian brain is able to hydroxylate Try,[6] but only very recently has such a process been demonstrated in homogenates of mammalian brain.[7] Brain transport systems for 5-HTP do exist,[8] and it is possible that peripherally formed 5-HTP can be transported to the brain.

This communication describes several *in vivo* methods whereby 5-HT synthesis from Try can be shown and demonstrates that rat brain can form 5-HT from Try. Tryptamine assays were done as a control measure because it has been postulated that tryptamine could cause some of the overt behavioral effects seen after administration of a monoamine oxidase inhibitor plus Try[9] and because Weissbach *et al.*[10] have demonstrated Try decarboxylase in kidney *in vitro*.

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METHODS

To demonstrate synthesis of 5-HT from Try in brain and other tissue, three different types of experiments were performed: perfusion of the small intestine, eviscerations, and infusion of brain. Male Sprague–Dawley rats, aged 80 to 90 days corresponding to a weight of 275–325 g, were used in all experiments, this group having reached a maximum in activity of phenylalanine hydroxylase.[11]

Perfusion of the small intestine

Rats were pretreated 16 hr before perfusion with a monoamine oxidase inhibitor, 2-phenylcyclopropylamine (PCP), 5 mg/kg s.c. The rats were provided with water but no food during this period. During ether anesthesia, about 25 cm of small intestine was cannulated at each end and the area between perfused *in situ*. After recovery from anesthesia the perfusion was done with Tyrode's solution, with and without Try, at a rate of 5–7 ml/min, siphoned through the intestinal lumen from a temperature-controlled reservoir 75 cm above the rat. Because the animals required sedation to allow an even perfusion flow, chlorpromazine (2·5 mg/kg) was given s.c. at the beginning of the perfusion. Chlorpromazine at this dose gave ample sedation to calm the rat. Pentobarbital was first used for sedation and anesthesia but was abandoned because of a high mortality in the perfusion experiments. At the end of the perfusion period (2 hr), the rat was sacrificed, by crushing the spinal column in the neck, and exsanguinated. The perfused area of small intestine, as well as the brain, colon, and skin were removed. They were blotted, minced, diluted with 2 parts of 0·01 N HCl, homogenized, and placed in ice until assayed.

Eviscerations

This series of experiments was designed to determine the possible role of the visceral organs in supplying the brain with 5-HT. The rats were starved overnight but did not receive PCP until after surgery. All animals in this series of experiments were injected i.p. with a solution of 0·9% saline containing 5% dextrose (20 ml/kg). In certain experimental rats, Try was added to this solution. All animals were anesthetized with ether for the same period of time, 20 to 25 min.

Partial evisceration refers to removal of stomach, intestines, and spleen. Total evisceration refers to removal of the liver, kidneys, stomach, intestines, and spleen. After closing the abdominal incision, the animals were injected with PCP (5 mg/kg) s.c. and 15 min later injected i.p. with the saline–dextrose solution. Two hr after the last injection, the animals were sacrificed as described above.

These experiments are divided into three groups: (1) not surgically treated, (2) partially eviscerated, and (3) totally eviscerated rats. In each group, there are four divisions: (1) a control receiving only saline–dextrose solution; (2) PCP-treated animals receiving the saline–dextrose solution 15 min later; (3) PCP-treated rats receiving 15 min later a saline–dextrose solution containing Try, 10 mg/ml (this corresponds to a dose of 200 mg/kg); and (4) animals which received the saline–dextrose solution with Try.

Infusion of rat brain

The left hemisphere of male Sprague–Dawley rats was infused with an isotonic saline solution with or without Try (10 mg/ml). These rats were anesthetized with

pentobarbital (40 mg/kg) i.p., and a tracheotomy performed. A polyethylene tube (PE-10) was placed in the left carotid artery, and the external carotid was isolated and ligated. The brain was infused by means of an infusion withdrawal pump (model 600–900, Harvard Apparatus Co.) at a rate of 0·0136 ml/min, which corresponds in the Try-infused animals to 0·136 mg Try/min. PCP (5 mg/kg) was injected s.c. 15 min before infusion. The brain was infused for 1 hr, which represents a dose of 8·16 mg Try.

Spectrophotofluorometric assay of 5-HT was performed as described by Udenfriend *et al.*[12] Biological assay of 5-HT, described by Dalgliesh *et al.*[13] was carried out in several samples to check the fluorometric technique. The method for measurement of tryptamine was that developed by Hess and Udenfriend.[14]

RESULTS

Perfusion of small intestine

The rat small intestine perfused *in situ* for 2 hr with Tyrode's solution (37°) containing Try (3×10^{-3} M) had 2·68 μg 5-HT/g tissue as compared to the perfused control which had 2·05 μg/g (Fig. 1). This increase of 0·61 μg 5-HT is significant as

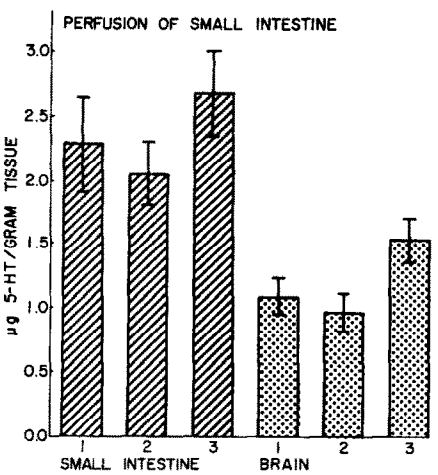

FIG. 1. The small intestine of male rats was perfused for 2 hr at 37°. All animals were treated with chlorpromazine s.c. (2·5 mg/kg) and pretreated 16 to 18 hr before with 2-phenylcyclopropylamine s.c. (5 mg/kg). Bars labeled are: (1) nonperfused controls, (2) perfused controls, and (3) L-tryptophan-perfused animals. Each bar represents results from 6 animals and indicates the 5-hydroxytryptamine (5-HT)/g tissue. The vertical line on each bar is 1 standard deviation. Student *t* analysis was performed between the groups represented on bars 2 and 3 for small intestine and brain and found to have significant values of $P < 0{\cdot}01$ and $P < 0{\cdot}001$ respectively.

analyzed by the Student *t* test ($P < 0{\cdot}01$). The control animal, nonperfused, had 2·28 μg 5-HT/g small intestine, which was higher than the perfused control and lower than the experimental animals, but in neither case was there a statistically significant change. Each bar on the graph represents the results from 6 rats. Also on this graph are the 5-HT content of the brains. The Try-perfused rats had 1·52 μg 5-HT/g brain, whereas the perfused controls had 0·96 μg/g, a significant change ($P < 0{\cdot}001$). The

rats perfused with Try showed signs of excitation during the last half hour of the 2-hr perfusion, manifested by piloerection, exophthalmia, incessant struggling in their cages, and gnawing at the cage. The nonperfused and perfused control rats were calm and sedated throughout the experiment.

Animals not pretreated with monoamine oxidase inhibitor did not show a significant change in 5-HT content after perfusion. The brains from the Try-perfused rats had 0·74 μg 5-HT/g, which was a significant increase ($P < 0{\cdot}01$) over the nonperfused and perfused control rats which had 0·50 μg and 0·40 μg/g respectively. None of these animals showed excitation in the 2-hr perfusion period. Because chlorpromazine causes hypothermia, three perfusions were performed on rats pretreated with chlorpromazine and monoamine oxidase inhibitors, with the temperature of the Tyrode's solution reduced to 21°. The intestines and the brains of these animals had the same 5-HT levels as the rats perfused at 37°.

Synthesis of 5-HT occurred at the same rate in perfused animals without chlorpromazine as in the chlorpromazine treated, but perfusion was very difficult. In two experiments, rats anesthetized with pentobarbital also synthesized 5-HT from Try. In preliminary experiments, reserpinized animals formed 5-HT from Try, but the amount of increase in the tissue of these rats was not as great.

Data obtained from the fluorometric assay were confirmed by results of a bioassay for 5-HT in rat colon, as described by Dalgliesh *et al.*[13] In three preliminary experiments with ^{14}C-labeled Try, the synthesis of 5-HT was shown. ^{14}C-5-HT was chromatographically separated from other Try and Try metabolites and assayed for activity.

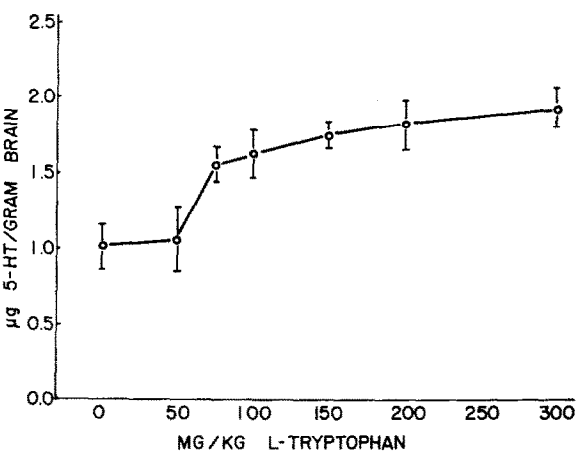

FIG. 2. 5-Hydroxytryptamine (5-HT) in the brain of rats after various doses of L-tryptophan. These animals were all pretreated 15 min before injection of L-tryptophan with 2-phenylcyclopropylamine s.c. (5 mg/kg). The L-tryptophan was injected in a saline–dextrose solution (20 ml/kg). Two hr after the i.p. injection of L-tryptophan the animals were sacrificed and 5-hydroxytryptamine assayed.

Partial and total eviscerations

These experiments determined: (1) the amount of 5-HT formed in the brain after i.p. injections of Try and (2) the influence that visceral organs had on the synthesis of 5-HT from Try in the brain. Dose–response relationships between the i.p. injections of Try and changes in total brain 5-HT indicated that little increase of 5-HT was seen until a dose of 75 mg Try/kg was reached, as shown in Fig. 2. From this dose upward

to 300 mg/kg, there was a steady but slow rate of increase. Only animals receiving 75 mg Try/kg or more showed signs of excitation; all rats received PCP (5 mg/kg). A dose of 200 mg Try/kg was chosen as optimal; larger doses tended to be toxic. Figure 3 shows the 5-HT levels in the rat brain at various time intervals after the administration of Try i.p. (200 mg/kg). This dose of Try was given 15 min after a subcutaneous dose

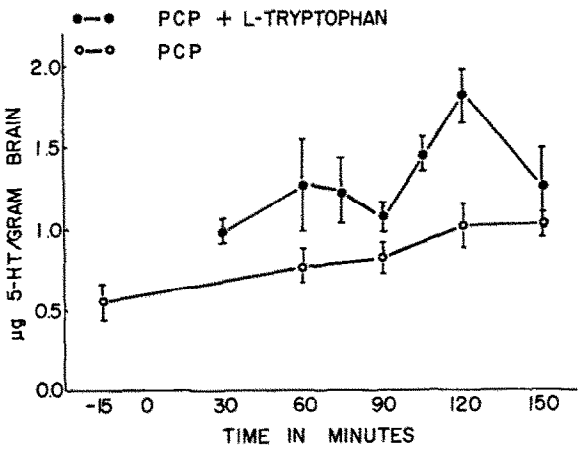

FIG. 3. The levels of 5-hydroxytryptamine (5-HT) in the brain of rats at various time intervals after (1) s.c. injection of 2-phenylcyclopropylamine (PCP) and (2) after a pretreatment with PCP followed 15 min later with i.p. injection of L-tryptophan (200 mg/kg).

of PCP (5 mg/kg). There was a rather irregular rise, reaching a peak of 5-HT in 120 min. This graph also demonstrates the change in 5-HT levels after the administration of the monoamine oxidase inhibitor alone. There was a steady increase of 5-HT in the rat brain for 135 min, followed by an apparent leveling. The level of 5-HT 165 min after PCP was nearly the same as in 8 animals pretreated for 20 hr; that is, 1·00 μg 5-HT/g brain and 1·09 μg/g respectively.

Table 1 is a synopsis of all the experiments from the unoperated and the partially and totally eviscerated rats. In the uneviscerated animals, the brain levels of 5-HT for each group were: (1) 0·56 μg/g, (2) 1·01 μg/g, (3) 1·81 μg/g, and (4) 0·82 μg/g. The statistical significance between the group with PCP and the PCP + Try as determined by Student's t test was $P < 0{\cdot}001$. In comparing the control group with the Try group, a significant difference was found ($P < 0{\cdot}001$). Behaviorally, these animals had a comparable response to the rats in the perfusion experiments in that only the PCP + Try-treated rats became agitated, first obvious between 70 and 80 min after the injection of Try. None of the rats in this series of experiments received chlorpromazine.

The 5-HT levels in the brains of partially eviscerated rats were: control, 0·62 μg/g; PCP, 1·05 μg/g; PCP + Try, 1·78 μg/g; and Try, 0·84 μg/g. The P values between the groups, PCP vs. PCP + Try and control vs. Try are both $< 0{\cdot}01$. Behavioral changes occurred only in the group receiving PCP + Try.

In totally eviscerated rats, the 5-HT levels changed in the same manner as in the uneviscerated and partially eviscerated animals. The control, PCP, PCP + Try, and

Try groups had 0·52 μg/g 1·06 μg/g, 1·83 μg/g, and 0·72 μg 5-HT/g brain, respectively, with behavioral changes in the form of agitation and excitation only in the PCP + Try group. Statistically, the difference between groups PCP and PCP + Try was very significant ($P < 0·001$). The difference between control and Try groups was significant ($P < 0·02$).

TABLE 1. A SYNOPSIS OF ALL THE DATA FROM THE EVISCERATION EXPERIMENTS

One standard deviation.

Organs removed	n	Control	n	PCP	n	PCP + L-Try	n	L-Try
		5-Hydroxytryptamine per gram brain (μg)						
None	9	0·56 ±0·09	8	1·01 ±0·14	7	1·81 ±0·17	7	0·82 ±0·11
Intestines Stomach Spleen	9	0·62 ±0·12	8	1·05 ±0·12	9	1·78 ±0·26	10	0·82 ±0·17
Intestines Stomach Spleen Kidneys Liver	8	0·52 ±0·10	8	1·06 ±0·10	7	1·83 ±0·25	5	0·72 ±0·11
		Tryptamine per gram brain (μg)						
None	5	0·04 ±0·05	5	0·11 ±0·07	5	0·61 ±0·37	5	0·59 ±0·28
Intestines Stomach Spleen	4	0·01 ±0·01	5	0·06 ±0·06	7	0·58 ±0·15	9	0·61 ±0·24
Intestines Stomach Spleen Kidneys Liver	4	0·08 ±0·03	5	0·15 ±0·01	5	0·64 ±0·14	4	0·67 ±0·21

TABLE 2. 5-HYDROXYTRYPTAMINE (5-HT) CONTENT OF TISSUES FROM ANIMALS WHOSE BRAINS WERE INFUSED WITH L-TRYPTOPHAN PLUS SALINE (L-TRY) AND WITH SALINE (CONTROL).

One standard deviation.

	Brain infusions		Number		
	Control	L-Try	C	L-Try	P
Brain	0·72 ±0·06	1·14 ±0·13	7	12	⩽0·001
Spleen	4·29 ±0·71	4·69 ±0·87	7	9	N.S.
Liver	0·54 ±0·20	0·39 ±0·18	7	8	N.S.
Small intestine	1·07 ±0·16	1·20 ±0·21	3	8	N.S.
Colon	5·33 ±0·41	5·64 ±0·53	7	9	N.S.

The results of the tryptamine determinations are also presented in Table 1. In the controls and PCP-treated animals, regardless of organs present or removed, there was essentially no tryptamine or less than 0·1 μg/g, which would be below assay sensitivity. In the PCP + Try and in the Try animals there was an equal increase, and this increase was seen in the unoperated and in the partially and totally eviscerated rats.

Infusion of rat brain

Try infusion increased the 5-HT content of the brain to 1·14 μg/g, which was 0·42 μg/g more than was found in the saline-infused control (Table 2). The statistical significance is very high ($P \ll 0{\cdot}001$). Tryptamine levels after infusion were also measured. The saline-infused control had essentially no tryptamine, whereas the Try-infused brains had 0·20 μg tryptamine/g brain, a significant change ($P < 0{\cdot}001$).

Table 2 gives the values for changes in 5-HT content of the organs in the control and the Try-infused rats. The 5-HT content in μg/g tissue of these organs in control and treatment groups were respectively: spleen 4·29 vs. 4·69; liver 0·54 vs. 0·39; small intestine 1·07 vs. 1·20; and colon 5·33 vs. 5·64. These differences were not statistically significant. Tryptamine was also assayed in these peripheral organs, and in no instance was a significant amount found within the limits of the sensitivity of the assay procedure.

DISCUSSION

These experiments show several methods by which the hydroxylation and decarboxylation of Try can be accomplished. They do not, however, demonstrate hydroxylation of Try alone but always in conjunction with the decarboxylation. The results indicate clearly that the central nervous tissue is capable of hydroxylating and decarboxylating Try, and that the first step need not be carried out in a peripheral organ.

In the experiments related to the perfusion of the small intestine *in situ*, it is apparent that the small intestine was capable of hydroxylating Try. Concurrent increases in the brain did not exclude the possibility that the increases of 5-HT in the central nervous system were from decarboxylation of 5-HTP formed in peripheral organs and transported to the brain.

Not only did the brain levels of 5-HT increase during perfusion of the small intestine but several peripheral tissues such as the colon and the spleen also demonstrated increases. It was notable in some of the preliminary studies that the skin did not show increases during the time interval of perfusion. This would tend to verify the findings of Schindler *et al.*,[15] who found that the mast cells formed 5-HT very slowly.

In the perfusion controls there was a tendency toward some depletion of 5-HT as a result of the perfusion itself, although this was not statistically significant. The apparent depletion of 5-HT in this experiment may be explained by an elevation of intraluminal pressure or increased motility[16, 17] releasing 5-HT into the intestinal lumen.

The biological assays for 5-HT from the intestine of perfused rats confirm the fluorometric analysis. In addition to the above techniques, preliminary experiments utilized ^{14}C-labeled DL-Try and it was found after chromatographic separation that there was radioactivity in both Try and 5-HT isolated from the perfused intestine. Attempts were made to isolate labeled 5-HTP but were unsuccessful, perhaps because of low specific activity of the labeled precursor.

The chlorpromazine used as a sedative in these animals had no effect on the increases of 5-HT at the dose level used, although changes have been reported at ten times this dose.[18] Results were more reliable when animals were treated with a low dose of this major tranquilizer. Because chlorpromazine and anesthesia tend to cause hypothermia, the temperature of the perfusion medium was reduced to 21°. This temperature did not alter the synthesis of 5-HT.

Monoamine oxidase inhibition was used in these experiments to accentuate the increase of 5-HT formation by preventing its oxidation. Perfusion experiments performed in an attempt to show increases of 5-HT from Try without monoamine oxidase inhibition failed to show a statistically significant increase. The 5-HT levels in the perfused small intestine were much more variable than they were in the presence of the monoamine oxidase inhibitor. It is possible that the perfusion-induced release is somewhat blocked by the monoamine oxidase inhibitor, as in the case of release of 5-HT by reserpine in the stomach of rat.[19] In animals not treated with PCP, the central nervous tissue in the Try-perfused control showed a significant increase of 5-HT, compared to the perfused control.

The dose–response curve indicates that at doses of less than 50 mg/kg little of Try injected is used for the formation of 5-HT. Higher doses, 75 mg/kg or more, however, show synthesis of the amine. A dose of 200 mg Try/kg was chosen because it produced a substantial increase of 5-HT and was not toxic. Larger doses of Try in the presence of PCP often were lethal in 2 hr. A time–response curve for the formation of 5-HT from Try (200 mg/kg) indicated that the maximal formation occurred at 2 hr and then decreased toward the level of the monoamine oxidase-inhibited control. The latter phenomenon would seem to indicate that much of this newly formed 5-HT was not tightly bound.

With apparently optimal conditions for time and dose (2 hr and 200 mg Try/kg), the effect that the visceral organs might play in supplying the brain with hydroxylated Try was studied. The effect of removal of intestine and liver in subacute experiments has been studied.[20–23] In control animals with the visceral organs intact, i.p. injections of Try in monoamine oxidase-inhibited animals resulted in significant increases of 5-HT in the brain. Partial eviscerations were then performed because the organs involved are known to contain large amounts of 5-HT and because Cooper and Melcer[1] have demonstrated some Try hydroxylation at these sites. The results indicated clearly that none of these organs had any effect on the amount of 5-HT formed in the brain from i.p. injection of Try.

Freedland *et al.*[3] demonstrated that liver phenyalanine hydroxylase also hydroxylated Try. Cooper and Melcer[1] demonstrated that the kidneys can hydroxylate Try *in vitro*. In order to elminate the effects of these organs, total eviscerations were performed. Try was then injected, and the brain levels of 5-HT still increased to that of the controls. The evidence from these experiments suggests that the brain can synthesize 5-HT from Try and that 5-HTP is not first supplied by a visceral organ.

In all experiments with animals which had received a monoamine oxidase inhibitor and then Try, the rats became excited about 1 hr later, manifested by piloerection, general hyperactivity, and increased sensitivity. This has been studied by Hess and Doepener,[9] and they concluded that it was impossible to determine the cause of the excitation, since 5-HTP would cause as much excitation as Try after monoamine oxidase inhibition.

In an attempt to correlate the behavioral effects with an increase of tryptamine or 5-HT, tryptamine assays were done in parallel with the 5-HT assays in the evisceration experiments. Within the sensitivity of the assay procedure, essentially no tryptamine was present in the control and PCP-treated animals. In the PCP + Try and in the Try-treated animals an equivalent increase of tryptamine occurred regardless of the degree of evisceration. These results indicate that the excitation was not due to

tryptamine alone, since both the PCP + Try and Try groups should have shown the same excitation. The Try-treated animals showed no agitation beyond that of the controls. Erspamer *et al.*[24] noted that tryptamine was a better substrate *in vitro* for monoamine oxidase than was 5-HT. It was therefore surprising that monoamine oxidase inhibition had no effect on the tryptamine level.

The results from the last series of experiments clearly verify that the brain can synthesize 5-HT from Try. Although the infusion was set up so that the left side of the brain should have been receiving all the infused Try, no attempt was made to separate the halves so that an increase on only one side would cause a 50% dilution in the assay. Even so, there was a highly significant increase of 5-HT in the brain. Assays of 5-HT of peripheral tissues were performed in order to determine whether circulating Try had reached peripheral organs and if it had been hydroxylated and subsequently returned to the brain. These assays clearly demonstrate that the Try which did enter the general circulation was not synthesized into 5-HT in significant amounts. This was true in all organs assayed.

In the infusion studies tryptamine was again assayed, and it was found that it increased in the brain but in no other tissue.

REFERENCES

1. J. R. Cooper and I. Melcer, *J. Pharmac. exp. Ther.* **132**, 265 (1961).
2. J. Cooper, *Ann. N.Y. Acad. Sci.* **92**, 208 (1961).
3. R. A. Freedland, I. M. Wadzinski and H. A. Waisman, *Biochem. biophys. Res. Commun.* **5**, 94 (1961).
4. *Ibid.*, **6**, 227 (1961).
5. J. Renson, H. Weissbach and S. Udenfriend, *J. biol. Chem.* **237**, 2261 (1962).
6. E. M. Gal and F. D. Marshall, in *Progress in Brain Research*, H. E. Himwich and W. A. Himwich, Eds., vol. 8, p. 56. Elsevier, Amsterdam (1964).
7. D. G. Grahame-Smith, *Biochem. biophys. Res. Commun.* **16**, 586 (1964).
8. S. Udenfriend, H. Weissbach and D. F. Bogdanski, *J. biol. Chem.* **224**, 803 (1957).
9. S. M. Hess and W. Doepener, *Arch. int. Pharmacodyn.* **134**, 89 (1961).
10. H. Weissbach, W. King, A. Sjoerdsma and S. Udenfriend, *J. biol. Chem.* **234**, 81 (1959).
11. R. A. Freedland, M. C. Krakowski and H. A. Waisman, *Am. J. Physiol.* **202**, 145 (1962).
12. S. Udenfriend, H. Weissbach and B. B. Brodie, in *Methods of Biochemical Analysis*, D. Glick, Ed., vol. 6, p. 96. Interscience, New York (1958).
13. C. E. Dalgliesh, C. C. Toh and T. S. Work, *J. Physiol. (Lond.)* **120**, 298 (1953).
14. S. M. Hess and S. Udenfriend, *J. Pharmac. exp. Ther.* **127**, 175 (1959).
15. R. Schindler, M. Day and G. A. Fischer, *Cancer Res.* **19**, 47 (1959).
16. E. Bülbring and A. Crema, *J. Physiol. (Lond.)* **146**, 18 (1959).
17. *Ibid.*, p. 29.
18. D. X. Freedman and N. J. Giarman, *Ann. N.Y. Acad. Sci.* **96**, 98 (1962).
19. K. S. Kim and P. A. Shore, *J. Pharmac. exp. Ther.* **141**, 321 (1963).
20. G. M. Tyce, G. H. C. Stobie, E. V. Flock and J. L. Bolland, *Fed. Proc.* **21**, 301 (1962).
21. G. M. Tyce, E. V. Flock and C. A. Owen, Jr., *Fed. Proc.* **22**, 632 (1963).
22. G. Bertaccini, *Naturwissenschaften* **45**, 548 (1958).
23. G. Bertaccini, *J. Physiol. (Lond.)* **153**, 239 (1960).
24. V. Erspamer, R. Ferrini and A. Glässer, *J. Pharm. Pharmac.* **12**, 761 (1960).