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THE SUBCELLULAR LOCALIZATION OF 5-HYDROXYTRYPTAMINE IN GUINEA PIG BRAIN

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Abstract—The subcellular distribution of 5-HT in subfractions from guinea pig brain homogenates was assayed by fluorimetric techniques and agrees with that of earlier studies employing the rat fundus strip bioassay method. The present study reaffirms the finding that 5-HT is principally found in a particulate fraction free of myelin and mitochondria and identifiable as consisting largely of pinched-off nerve endings (NEPs). The forces binding 5-HT to the storage sites are quite labile relative to those which bind acetylcholine. Iproniazid raises the level of 5-HT in brain tissue but does not alter the subcellular distribution. Density gradient experiments were carried out in order to separate the 5-HT containing particles from those containing acetylcholine. Evidence is presented suggesting that acetylcholine is preferentially associated with the lighter, smaller nerve ending particles and that 5-HT is associated with the larger, denser NEPs; however, complete overlap occurs.

5-HYDROXYTRYPTAMINE (5-HT) in rat brain is distributed between the particulate and soluble fractions of the cell, and 60 per cent of the total brain 5-HT sediments with "mitochondria".[1] Furthermore, in homogenates of rat brain tissue the nuclei and microsomes are relatively free of 5-HT whereas the cytoplasmic "unbound" 5-HT accounts for 24 per cent of the total 5-HT of the brain.[2] Using the rat fundus strip assay method,[3] Whittaker[4, 5] was able to demonstrate that bound 5-HT in guinea-pig brain is mainly recovered from a fraction of subcellular particles distinct from nuclei, mitochondria and microsomes which is rich in acetylcholine, and which consists largely of particles derived from nerve endings by a "pinching-off" process (nerve ending particles, NEPs).[6, 7] The density and morphology of this fraction is in marked contrast to that which contains pressor amines in other tissues and suggests that 5-HT in brain is located in nerve endings. The assay procedure was found, however, to be slow and tedious when large numbers of fractions had to be assayed, and to be less reproducible than the frog rectus method for acetylcholine. It was decided, therefore, to repeat and extend the earlier observations using a fluorimetric method for assaying 5-HT.[8] A preliminary account of this work has already been given.[9]

METHODS

Preparation of fractions

Preparation of homogenates. Guinea pigs (350–500 g) were killed by decapitation and the brain tissue rostral to the quadrigemina was removed and immediately placed

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in cold (4°C) 0·32 M sucrose. After removal of blood and superficial blood vessels the tissues were blotted on filter paper and weighed. A 10% homogenate in 0·32 M sucrose was prepared using a glass and Perspex homogenizer.[10] The time taken to homogenize the tissue was 1–2 min.

Differential centrifugation. The first step in the fractionation procedure was to separate the homogenate into a number of primary fractions by differential centrifugation. The first particulate fraction (nuclear P_1 fraction) was obtained in a Servall bench centrifuge by centrifuging the original homogenate (*H*) for 11 min at 1000 × *g*. The "crude mitochondrial" fraction (P_2) was prepared from the supernatant of the P_1 preparation by centrifuging at 17,000 × *g* for 1 hr in a Servall Refrigerated Automatic centrifuge. The microsomal fraction (P_3) was separated by centrifuging the supernatant from P_2 at 100,000 × *g* for 1 hr in a Spinco Model L preparative ultracentrifuge. The final supernatant (S_3) was taken to represent soluble cytoplasmic material diluted with suspension medium.

Density gradient centrifugation. Subfractionation of P_2 by equilibrium density gradient centrifugation was carried out essentially as described by Gray and Whittaker.[7] The P_2 pellet was resuspended in 0·32 M sucrose so that 1 ml of the suspension corresponded to 500 mg of fresh tissue. The suspension (2 ml/tube) was then layered on top of a discontinuous density gradient prepared 1–2 hr before use, consisting of 10 ml 0·8 M sucrose/tube layered over an equal volume of 1·2 M sucrose, and centrifuged at 53,500 × *g* for 2 hr in the SW-25 swing-out bucket head of the Spinco Model L preparative ultracentrifuge. Separation into three well-defined sub-fractions took place. The first (*A*) consists of particles less dense than 0·8 M sucrose, the second (*B*) of particles intermediate in density between 0·8 and 1·2 M sucrose, and the third (*C*) of particles denser than 1·2 M sucrose. Electron microscopic examination[1–7] of the *A*, *B* and *C* subfractions of the P_2 fraction has shown them to consist mainly of myelin, NEPs and mitochondria, respectively.

In some experiments, separations were carried out in more elaborate density gradients, built up out of a series of sucrose solutions (each 3 ml/tube) differing in concentration by 0·1 M in the range 0·8–1·2 M. Layers of 1·4 M (3 ml/tube) and 1·6 M (1·5 ml/tube) sucrose prevented the densest particles from forming a pellet at the bottom of the tube. The layers (total volume 19·5 ml/tube) were allowed to diffuse for 24 hr so as to even out discontinuities. The P_2 fraction (6·5 ml/tube) was pipetted on to the density gradient and after centrifuging at 53,500 × *g* for 2 hr, the gradient was separated into several fractions by puncturing the bottoms of the tubes.[11] Samples were withdrawn for acetylcholine and 5-HT assay, nitrogen estimations and for electron microscopy.

5-Hydroxytryptamine assays

Method. The 5-HT of the various tissue fractions prepared by either differential or density gradient centrifugation was extracted by the alkaline-butanol method.[8] Fluorimetric analyses of the extracted 5-HT were done on the Locarte Fluorimeter Model LFN/4[12] using the following filters: primary side, Locarte LF/2 (254–400 mμ) in combination with a liquid filter, Locarte LFM/H4, containing $NiSO_4$ solution (437·5 g/l.) with a light path of 2 cm; secondary side, Locarte LF/7 (510 mμ cut off).

Specificity of the method. Examination of the activation and fluorescence spectra (Aminco–Bowman Spectrophotofluorometer) within the wavelengths transmitted by

the Locarte filters revealed no significant fluorescent material other than that having the activation and fluorescence characteristics of 5-HT (Fig. 1).

Units of 5-hydroxytryptamine concentration. The 5-HT concentration of the tissue fractions is expressed as mμg free base/g. fresh brain tissue. The percentage distribution of 5-HT among the various subfractions are percentages of the total activities recovered. The percentage recoveries are indicated in each table. The pooled results of a number of similar experiments are expressed as the mean value and the standard deviation from the mean followed by the number of experiments in brackets.

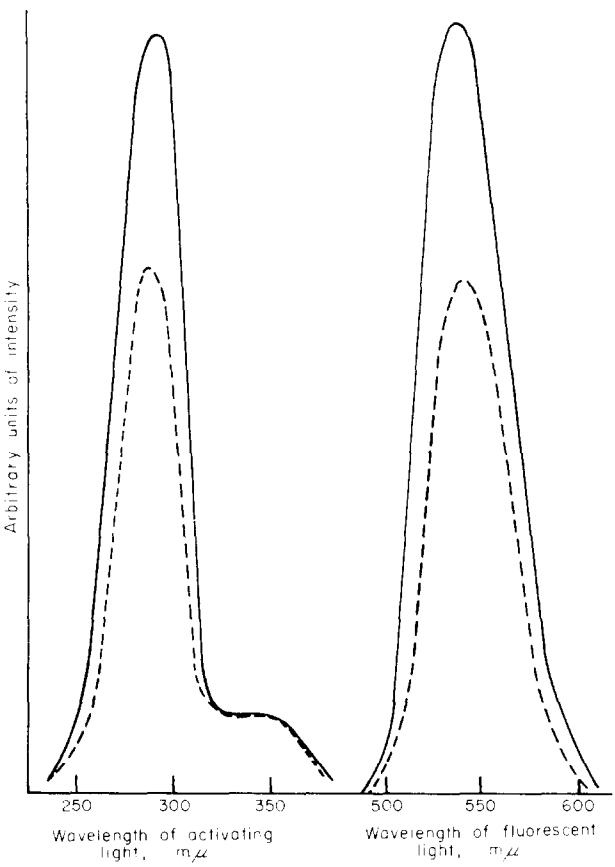

FIG. 1. Activation (left) and fluorescenece (right) spectra of authentic 5-HT (————) and guinea pig brain extract (– – – –). To obtain the activation spectrum, the fluorescence monochromator of the spectrofluorometer was set at 550 mμ and the spectrum from the activating monochromator was scanned. To obtain the fluorescence spectrum, the activating monochromator was set at 300 mμ and the spectrum from the fluorescence monochromator was scanned.

Iproniazid and reserpine treatment. 1-*iso*-Nicotinoyl-2-*iso*propyl-hydrazine (iproniazid, Roche Products Ltd.) was injected subcutaneously in 0·9 % w/v NaCl (100 mg/kg) 12–24 hr before each experiment. Reserpine (Ciba Ltd.) (5 mg/kg) was injected intravenously at various times before each experiment.

Other Assays

Acetylcholine assay was carried out on the frog rectus abdominis muscle. To avoid the disturbing effects of hypertonic sucrose the following procedure was used. Samples (2 ml) of fractions from sucrose density gradients were diluted to 11·5 ml with 0·32 M sucrose and centrifuged at 100,000 × *g* for 30 min. The supernatant was decanted and the pellets resuspended in 2 ml frog Ringer solution containing eserine (10 μM), previously acidified to pH 4. The suspension was heated at 100 °C for 10 min to release bound acetylcholine, cooled, neutralized and assayed.

Succinate dehydrogenase. Succinate dehydrogenase (SDH) was estimated manometrically at 37 °C.[5] One unit (u.) of activity is defined at 1000 μl CO_2/hr liberated by enzyme activity from the bicarbonate buffer of the medium.

Nitrogen was determined by the micro-Kjeldahl method.

Electron microscopy

Morphological examination of fractions was carried out in the Siemens Elmiskop I electron microscope on ultra-thin sections of particulate material embedded in Araldite after fixing and staining the particles in suspension at 0 °C with 1% osmium tetroxide in iso-osmotic veronal acetate buffer (0·2 ml suspension mixed with 1 ml 1% fixative), centrifuging and then dehydrating and staining the ensuing pellet with alcoholic phosphotungstic acid as described by Gray and Whittaker.[7] The electron micrographs were evaluated by classifying the particles in each micrograph into membrane fragments, NEPs and mitochondria. The membrane fragments included myelin fragments and all of the many empty vesicular profiles of greatly varying size, some of which might have been derived from nerve endings or mitochondria and others, small myelin fragments or microsomes. Very small structureless membrane fragments were disregarded. The mitochondria did not include the small mitochondria found in NEPs.

RESULTS

Bound and unbound 5-hydroxytryptamine

In a differential centrifugation of guinea pig brain homogenates into P_1, P_2 and S_2

TABLE 1. PERCENTAGE DISTRIBUTION OF 5-HT IN TOTAL PARTICULATE AND HIGH-SPEED SUPERNATANT FRACTIONS

H (mμg/g)	% Recovered activity *P*	*S*	Recovery (% of *H*)
489 ± 240 (15)	74 ± 9 (5)	26 ± 9 (5)	87 ± 9 (5)

fractions, Whittaker[5] reported that 45 per cent of the brain 5-HT was unbound. Using rat brain tissue Giarman and Schanberg[2] stated that the cytoplasmic unbound 5-HT accounted for 24 per cent of the total 5-HT in the brain.

In order to make a more accurate estimate of the proportion of total brain 5-HT in the free and particle bound forms, homogenates were immediately centrifuged at 100,000 × *g* for 1 hr in the no. 40 head of the Spinco preparative ultracentrifuge.

The results obtained are summarized in Table 1, and are in agreement with those of Giarman and Schanberg.[2]

Distribution of 5-HT in primary fractions

An analysis of the 5-HT content of the nuclear, "crude mitochondrial," microsomal and cytoplasmic fractions is given in Table 2.

TABLE 2. PERCENTAGE DISTRIBUTION OF 5-HT IN PRIMARY FRACTIONS

H (mμg/g)	% Recovered activity P_1	P_2	P_3	S_3	Recovery (% of H)
505 ± 92 (6)	5 ± 1 (6)	41 ± 8 (6)	0 (6)	54 ± 12 (6)	113 ± 19 (6)

Subfractionation of mitochondrial fraction

The distribution of 5-HT and succinate dehydrogenase (SDH) in the myelin, NEPs and mitochondria obtained by density gradient separation of the P_2 fraction is given in Table 3.

TABLE 3. PERCENTAGE DISTRIBUTION OF 5-HT AND SDH AMONG P_2 SUBFRACTIONS

5-HT content of P_2/g original tissue = 253 ± 68 (5) mμg.
SDH activity of P_2/g original tissue = 7·2 ± 1 (3) u.

	A (myelin)	*B* (NEPs)	*C* (mitochondria)	Recovery (% of P_2)
5-HT	12 ± 6 (5)	64 ± 13 (5)	24 ± 16 (5)	106 ± 36 (5)
SDH	0 (3)	29 ± 10 (3)	71 ± 15 (3)	110 ± 27 (3)

Effect of iproniazid on 5-HT

Iproniazid inhibits the catabolism of 5-HT and the levels of amine in the brain rise under the influence of this drug.[13] The effect of 100 mg/kg iproniazid on the concentration and distribution of 5-HT in guinea pig brain and subfractions of the brain is presented in Table 4. The absence of 5-HT in the P_3 fraction (Table 2) indicated that

TABLE 4. PERCENTAGE DISTRIBUTION OF 5-HT IN SUBFRACTIONS OF BRAIN FROM IPRONIAZID-TREATED GUINEA PIGS

H (mμg/g)	Total P and S *P*	*S*	Recovery (% of *H*)	Primary fractions P_2*	S_2	Recovery (% of *H*)
1360 ± 180 (5)	74 (2)	26 (2)	100 (2)	51 ± 8 (3)	49 ± 8 (3)	98 ± 8 (3)

P_2 (mμg/g)	Subfractions of P_2 *A*	*B*	*C*	Recovery (% of P_2)
500	18 (2)	79 (2)	3 (2)	96 (2)

* Subfraction P_1 contains 5 per cent of particulate bound 5 HT (Table 2) and was not routinely assayed.

the supernatant (S_2) from the "crude mitochondrial" preparation could represent the compartment for "unbound" amine. Accordingly, this fraction was used in this series of experiments.

Effect of reserpine on iproniazid-treated guinea pigs

Brodie[14] showed that within 1 hr after a single injection of reserpine the brain is depleted of 75 per cent of its normal levels of 5-HT. Within 3 hr there is a 90 per cent depletion which persists for 24 hr. In view of the finding that iproniazid raises the absolute levels of 5-HT in both the total particulate and soluble fractions (Table 4), although the percentage distribution remains unaltered, it was of interest to study the effect of reserpine on 5-HT distribution in iproniazid treated guinea pigs. Reserpine was injected intravenously (5 mg/kg) to iproniazid pretreated animals at 45 min, 5 hr and 10 hr before killing. The results obtained are summarized in Table 5.

TABLE 5. PERCENTAGE DISTRIBUTION OF 5-HT IN BRAIN OF IPRONIAZID AND RESERPINE PRETREATED GUINEA PIGS

Time after reserpine (hr)	H (mμg/g)	P_2* (% of H)	S_2
0·75	1417	54	45
5	1205	51	49
10	1049	57	43

* P_1 discarded: see footnote to Table 4.

Attempted separation of 5-hydroxytryptamine containing particles from those containing acetylcholine

In more elaborate density gradient experiments an attempt was made to separate the 5-HT containing particles of the P_2 fraction from those containing acetylcholine.

Figure 2 shows the results of one such experiment with a P_2 fraction prepared from animals pretreated with iproniazid (100 mg/kg) subcutaneously 12 hr before the experiment, to raise 5-HT levels to a value which would enable the 5-HT distribution to be measured in a larger number of subfractions. After centrifuging the density gradient was split into six fractions. The first of these corresponded to a bulky white layer rich in myelin but containing relatively few NEPs and almost no mitochondria. The next fractions (2–5) corresponded to a cloudy zone of suspended material stretching from 0·8 to 1·2 M sucrose. Electron microscopy showed that this consisted mainly of NEPs, small and with a relatively clear cytoplasm in the upper fractions, large, dense and shrunken in the lower fractions. The last fraction contained a tan coloured layer floating on 1·6 M sucrose and consisted mainly of mitochondria.

The upper diagram shows the relative specific activity (i.e. the percentage of the total recovered activity found in a fraction divided by the percentage of the total recovered nitrogen found in that fraction) of the various fractions with respect to acetylcholine and 5-HT.

Values above 1·0 indicate a relative concentration of activity compared with the parent fraction (i.e. the elimination of inactive material). In the lower diagram the blocks represent the nitrogen contents (as a percentage of total recovered nitrogen) of each fraction. Each block has been subdivided into three zones corresponding to the

three classes of particles seen in the electron microscope (membrane fragments, mitochondria and NEPs); the relative heights of the three zones are the proportions of these three classes in each fraction. This method of plotting the results has been adopted to give some indication of the *absolute* as well as the relative composition of the various fractions. Thus the number of NEPs in the bulky white layer corresponding to fraction 1 though a relatively small proportion of the total number of particles present may be absolutely larger than the proportional figure might suggest because of the large bulk of this fraction. However, the zones do not represent the percentage of nitrogen associated with the various particle types except on the unwarranted assumption that all particles contained equal amounts of nitrogen; in fact there is no

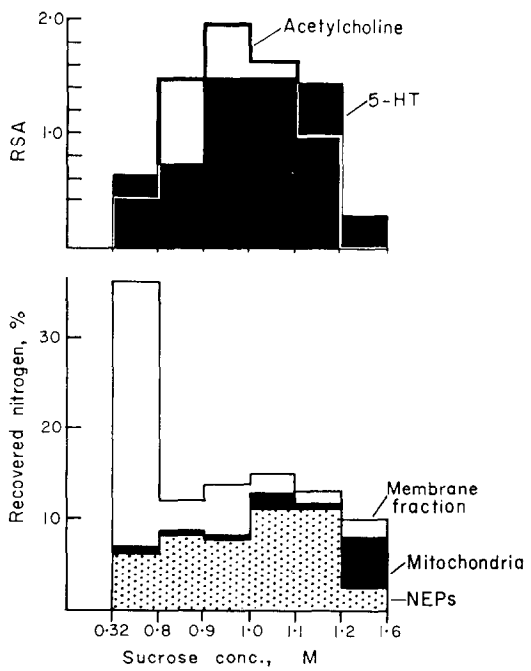

FIG. 2. Distribution of particle types (membrane fragments, mitochondria, NEPS) (lower diagram) and pharmacologically active substances (acetylcholine, 5-HT) (upper diagram) in a continuous sucrose density gradient. The envelope of the lower diagram represents the nitrogen content of each fraction as a percentage of the total recovered nitrogen (recovery 107 per cent; nitrogen content of starting material, 4·33 mg/vol. equivalent to 1 g brain tissue). Each block is divided in proportion to the morphological composition (for details, see text) of the fraction. The upper diagram represents the relative specific activity (percentage of total recovered activity occurring in fraction divided by the percentage of total recovered nitrogen occurring in fraction) of acetylcholine and 5-HT in each fraction. Recoveries: acetylcholine, 88 per cent; 5-HT, 100 per cent. Composition of starting material/vol. equivalent to 1 g brain tissue: acetylcholine, 2·05 mμmole; 5-HT, 639 mμg.

way of telling at present what proportion of the total nitrogen is associated with any one particle type.

It will be seen that the fractions having the highest relative specific activity with respect to acetylcholine and 5-HT (fractions 2–5) are also those consisting mainly of NEPs. There is some evidence that acetylcholine is preferentially associated with the

fraction containing the lighter, smaller NEPs and that 5-HT is associated with the fractions containing the larger, denser NEPs. It must be emphasized, however, that overlap is complete and the density gradient separation has not resulted in the sharp separation of particles into chemically and morphologically distinct types.[15]

DISCUSSION

The results obtained by the alkaline-butanol extraction of 5-HT from guinea pig brain subfractions and its assay by fluorimetric techniques are in very close agreement with earlier studies[5] employing the rat fundus strip bioassay method. Whereas Whittaker[5] reported the percentage distribution for 5-HT in the particulate and soluble fractions as 60–75 and 25–40 per cent, respectively, it is now shown (Table 1) to be 74 and 26 per cent. Giarman and Schanberg[2] report essentially the same total particulate to soluble ratio. Similarly, Whittaker's[5] estimates of the percentage distribution among the primary fractions as: P_1, 13 per cent; P_2, 47 per cent and S_2 40 per cent need only minor correction to: P_1, 5 per cent; P_2, 41 per cent and S_2, 54 per cent. Both the earlier[5] and the present study show that 5-HT is principally found in the "crude mitochondrial" fraction. The results also confirm Giarman and Shanberg's[2] contention that there is release of bound 5-HT during fractionation.

The sucrose density gradient separation of the "crude mitochondrial" fraction (P_2) into relatively pure subfractions of myelin (A), NEPs (B) and mitochondria (C) has been illustrated by Gray and Whittaker.[6] The 29 per cent SDH activity observed in the subfraction B might not be due to contamination with extracellular mitochondria less dense than 1·2 M sucrose but rather with the intracellular mitochondria seen to be enclosed within the NEPs.

It was shown by Whittaker[5] that of the recovered 5-HT from the P_2 fraction, 20 per cent was in the myelin (A layer), 54 per cent in the pinched-off nerve endings (B layer) and 26 per cent in the mitochondrial pellet (C layer). The present results, 12 per cent (A layer), 64 per cent (B layer) and 24 per cent (C layer), again confirm the earlier findings.

Inouye *et al.*[16] have suggested that Whittaker's[5] results were in serious error due to the presence of substance P. Reasons have been given for not accepting their criticisms.[17] The good agreement between the earlier and the present results, which could not be affected by substance P, is a further reason for rejecting the Japanese workers' arguments.

The iproniazid and reserpine effects are similar to those reported by Giarman and Schanberg[2, 18] and the significance of these findings is discussed by these authors. However, two points are raised by the present investigations. The 5-HT storage particle is intimately associated with that subcellular fraction from guinea pig brain homogenates shown to consist mainly of pinched-off nerve endings. The forces binding the 5-HT to the storage site are quite labile relative to those which contain acetylcholine,[5] as evidenced by the redistribution of the particulate 5-HT to the soluble compartment with the manipulations involved in differential centrifugation. The brain 5-HT storage particle is different from the heavy 5-HT granules of dog duodenal mucosa which are more dense than 1·6 M sucrose.[19] However, the 5-HT storage particles from dog brain[20] and those from rat brain [21] show sedimentation characteristics similar to those described here for guinea pig brain. There has been much discussion about the possible role of 5-HT as a central nervous system transmitter. The present findings,

while not proving that 5-HT is a transmitter, are consistent with such a function, and strongly suggest a location of the bound amine in nerve endings. The alternative possibility is that particulate 5-HT is located in storage granules distinct from nerve endings but similar in sedimentation and density characteristics, even in complex gradients. Such a possibility would be hard to disprove with present techniques. Not all particles in the nerve ending fraction can be positively identified as such by electron microscopy. At the low levels of 5-HT present in brain, a relatively small contamination of the nerve-ending fraction with particles containing a high concentration of 5-HT would suffice to account for the observed results and would be difficult or impossible to exclude on morphological grounds. Positive evidence on this point could only come from a fractionation procedure which separated these hypothetical particles from NEPs. Until such a separation can be demonstrated by methods of superior resolving power to those at present available, the simplest hypothesis is to assume that 5-HT is indeed stored in the NEPs themselves.

REFERENCES

1. E. J. Walaszek and L. G. Abood, *Fed. Proc.* **16**, 133 (1957).
2. N. J. Giarman and S. Schanberg, *Biochem. Pharmacol.* **1**, 301 (1959).
3. J. R. Vane, *Brit. J. Pharmacol.* **12**, 344 (1957).
4. V. P. Whittaker, *Biochem. Pharmacol.* **1**, 351 (1959).
5. V. P. Whittaker, *Biochem. J.* **72**, 694 (1959).
6. E. G. Gray and V. P. Whittaker, *J. Physiol.* **153**, 35P (1960).
7. E. G. Gray and V. P. Whittaker, *J. Anat. (Lond.)* **96**, 79 (1962).
8. D. F. Bogdanski, A. Pletscher, B. B. Brodie and S. Udenfriend, *J. Pharmacol.* **117**, 82 (1956).
9. I. A. Michaelson and V. P. Whittaker, *Biochem. Pharmacol.* **11**, 505 (1962).
10. W. N. Aldridge, R. C. Emery and B. W. Street, *Biochem. J.* 77, 326 (1960).
11. R. J. Britten and R. B. Roberts, *Science* **131**, 32 (1960).
12. E. M. Marshall, G. S. Stirling, A. C. Tait and A. Todrick, *Brit. J. Pharmacol.* **15**, 35 (1960).
13. S. Spector, D. Prokop, P. A. Shore and B. B. Brodie, *Science* **127**, 704 (1959).
14. B. B. Brodie, In *5-Hydroxytryptamine* (Edited by G. P. Lewis). p. 64. Pergamon Press, London (1958).
15. F. de Robertis, A. Pellegrino de Iraldi, G. Rodriguez de Lores Arnaiz and L. Salganicoff, *J. Neurochem.* **9**, 23 (1962).
16. A. Inouye, K. Kataoka and J. Shinagawa, *Nature, Lond.* **194**, 286 (1962).
17. V. P. Whittaker, *Nature, Lond.* **195**, 1100 (1962).
18. S. Schanberg and N. J. Giarman, *Biochem. Pharmacol.* **11**, 187 (1962).
19. R. V. Baker, *J. Physiol.* **143**, 80P (1958).
20. R. V. Baker. D. Phil. Thesis, Oxford (1959).
21. L. T. Potter and J. Axelrod, *Nature, Lond.* **194**, 581 (1962).