Biochemical Pharmacology, Vol. 17, pp. 1401–1409. Pergamon Press. 1968. Printed in Great Britain

ADDITIONAL EVIDENCE FOR THE EXISTENCE OF SEVERAL FORMS OF MITOCHONDRIAL MONOAMINE OXIDASE IN THE MOUSE

RICHARD F. SQUIRES

Research Laboratory, A/S Ferrosan, Copenhagen, Denmark

(*Received* 10 *November* 1967; *accepted* 31 *January* 1968)

Abstract—Evidence has been presented that there are at least three forms of mitochondrial MAO in the mouse capable of deaminating kynuramine. These forms can be divided into two groups: one of these groups is inhibited by harmine but is relatively resistant to pargyline, while the other is resistant to harmine but is inhibited by low concentrations of pargyline. The harmine resistant group consists of two major forms with relatively short half-lives of 50°. The harmine sensitive fraction is apparently homogeneous with respect to thermal stability and has a significantly longer half-life than the harmine resistant forms. The relative proportions of these three forms vary greatly from one organ to another. It is suggested that some of the pharmacological effects of the MAO inhibitors may depend on specific inhibition of single forms.

THE EXISTENCE of multiple forms of MAO has been suspected for a number of years, and evidence supporting this suspicion continues to accumulate. This evidence may be summarized as follows:

1. The properties of a given preparation of MAO vary considerably according to the substrate used to measure its activity.[1–7, 20, 21] These properties include sensitivity to various inhibitors[1, 3–8, 20, 21] pH optimum,[2, 8] and effect of temperature on chloride ion inhibition.[21]

2. Some inhibitors appear to selectively inhibit a limited fraction of the total MAO activity in some preparations.[3, 8–10, 12, 20]. This indicates that certain substrates may be deaminated by more than one form of MAO present in the same preparation. Substrates which may be deaminated by more than one form of MAO include tyramine,[3, 9, 20] kynuramine,[8, 10, 12] benzylamine,[8] tryptamine[10] and serotonin.[20]

3. MAO preparations from different organs of the same animal differ with respect to substrate preference[13, 15] and apparent affinity constant for oxygen.[14] Similarly, different regions of the dog brain are inhibited to different degrees by certain inhibitors.[22]

Taken together, the above observations suggest that (1) many MAO preparations contain several forms of the enzyme which differ with respect to substrate preference, sensitivity to certain inhibitors, and other properties; (2) a single substrate may be deaminated by several of these forms, and (3) the relative proportions of the different forms varies from one organ to another (and probably from one region of the brain to another).

In the present communication it will be shown that mitochondria prepared from various organs of the mouse contain several forms of MAO capable of deaminating

kynuramine. These forms differ with respect to sensitivity to the inhibitors harmine and pargyline, as well as with respect to thermal stability. The relative proportions of the different forms vary from one organ to another.

MATERIALS AND METHODS

Mice of the NMRI strain were used throughout. Kynuramine di-hydrobromide was purchased from Mann or Sigma. Harmine HCl and harmaline HCl were purchased from Fluka. Pargyline HCl and alpha ethyl tryptamine acetate were generously provided by Abbott and Upjohn, respectively. Mitochondria were prepared by differential centrifugation in ice cold sucrose (250 mM containing 1 mM EDTA). The mitochondria were washed three times in sucrose, and were finally taken up in 100 mM Tris sulfate pH 8·5, containing 1 mM EDTA and stored frozen.

Mitochondria prepared from brain, kidneys, intestine, liver, heart, and lung were taken up in 5, 3, 6, 10, 3, and 3 ml Tris sulfate, respectively. Organs from different animals were not pooled, except in the heat inactivation experiments using mitochondria from heart, lung and kidney, where organs from 5 animals were pooled.

MAO was determined by the fluorometric method of Kraml,[16] slightly modified, using kynuramine as substrate. The mitochondrial preparations described above were usually diluted 10, 20, 80, 240, 5, and 5 times, respectively, for kidney, brain, intestine, liver, lung, and heart, in Tris sulfate. After dilution it was found necessary to homogenize the mitochondrial suspensions in a glass homogenizer to ensure full dispersion.

The assay was carried out in a final volume of 400 μl: 200 μl of mitochondria diluted in Tris sulfate, 100 μl inhibitor solution or water, and 100 μl of kynuramine dihydrobromide (100 γ/ml). In the inhibition experiments mitochondria and inhibitor were pre-incubated together for 30 min at 37° before adding kynuramine. After addition of kynuramine the samples were incubated for 20 min at 37°, and the reaction was stopped by adding 1·0 ml of 1N NaOH. The fluorescence was then measured directly in an Aminco–Bowman fluorometer (A = 318 mμ, F = 382 mμ). Preliminary experiments showed that, at the concentrations used, the mitochondrial suspensions contribute to the small blank fluorescence but do not significantly quench the fluorescence of 4-hydroxyquinoline. MAO activity is essentially a linear function of both time and mitochondrial concentration under the conditions described above.

In the thermal inactivation experiments a small amount of concentrated mitochondria was rapidly introduced into a suitable amount of Tris sulfate, pH 8·5, 100 mM, containing 1 mM EDTA, previously heated to 50° in a thermostated water bath. One-ml aliquots were withdrawn at precisely timed intervals and quickly pippetted into chilled glass tubes and kept on ice until the last aliquot had been withdrawn. The MAO activity was then determined as described above.

RESULTS

1. *Partial inhibition by harmine and pargyline*

Preliminary experiments with a few well known MAO inhibitors showed that several of these produced an incomplete or step-wise inhibition when mouse brain mitochondria were used as the source of MAO.

These effects were most pronounced with harmine, harmaline, alpha ethyl tryptamine, and pargyline. The investigation was then extended to mitochondria prepared from several other organs of the mouse. When graded concentrations of harmine HCl

were tested on mitochondria prepared from six different organs of the mouse, in no case did inhibition reach 100% (Fig. 1). Maximum inhibition was always obtained with a concentration of 100 ng/ml: a 10-fold increase in harmine concentration did not significantly increase inhibition. It can also be seen that the maximum inhibition by harmine varies strikingly from one organ to another: liver MAO is uninhibited by harmine up to 1 γ/ml, while MAO in mouse kidney and small intestine is inhibited a maximum of 70 per cent. The values for maximum inhibition of MAO in the other organs lie scattered between those for liver and intestine.

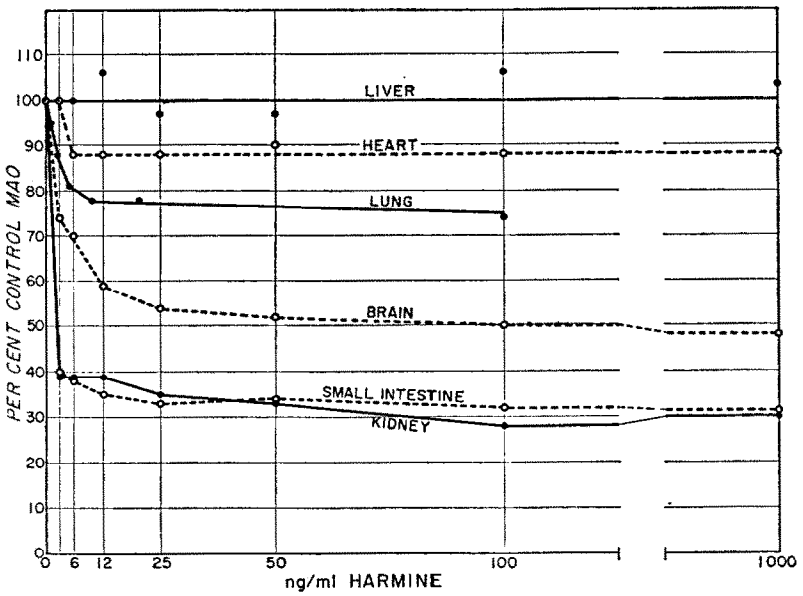

FIG. 1. Inhibition by harmine of MAO in mitochondria from six organs of the mouse. Harmine concentrations are given as the hydrochloride.

Pargyline also appears to be a selective inhibitor at low concentrations. However, this inhibitor differs from harmine in that by using sufficiently high concentrations pargyline inhibits MAO from all organs almost completely: at 1 γ/ml inhibition is more than 90 per cent in all cases (Fig. 2). Thus, inhibition of MAO by pargyline in most of the organs investigated appeared to take place in two distinct steps. The first step appears to be essentially complete at a pargyline HCl concentration of 50–75 ng/ml, while the second step approaches completion at about 1 γ/ml. The two steps have ID_{50}'s of, roughly 2–3 ng/ml, and 200–300 ng/ml, respectively. Here, first step inhibition by pargyline HCl will be arbitrarily defined as the inhibition produced by a final concentration of 75 ng/ml, corresponding to 100 ng/ml during pre-incubation.

Fig. 2 shows that first step inhibition by pargyline also varies from one organ to another. If the organs are listed in order of increasing first step inhibition by pargyline, it will be seen (Table 1) that this is exactly the reverse of the corresponding list made for harmine. Thus, while harmine is without effect on liver MAO, pargyline inhibits about 95 per cent at 50 ng/ml. First step inhibition for intestine and kidney is near 40 per cent.

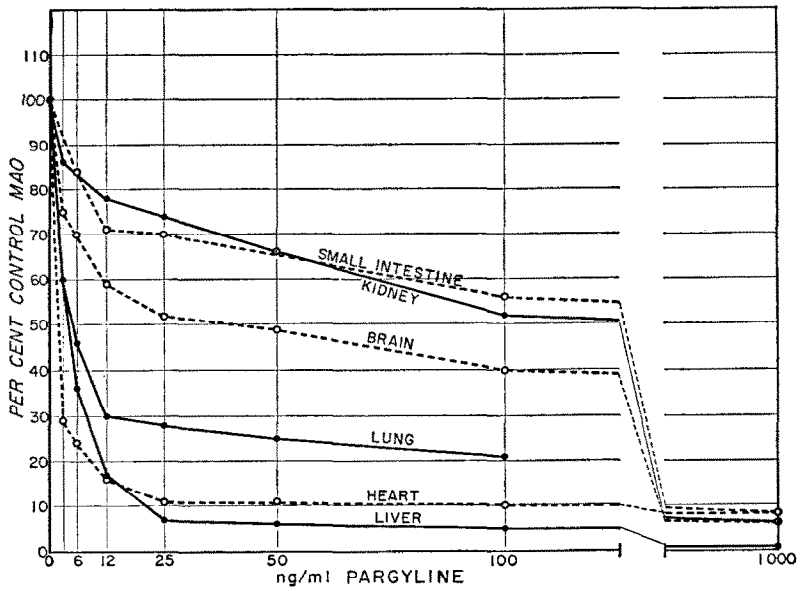

FIG. 2. Inhibition by pargyline of MAO in mitochondria from six organs of the mouse. Pargyline concentrations are given as the hydrochloride.

TABLE 1. MAXIMUM INHIBITION BY HARMINE AND FIRST STEP INHIBITION BY PARGYLINE OF MITOCHONDRIAL MAO FROM VARIOUS ORGANS OF THE MOUSE

Organ	Average maximum inhibition by harmine	Average first step inhibition by pargyline	Total inhibition (harmine + pargyline)
Liver	3 (11)	96 (9)	99
Heart	11 (1)	90 (1)	101
Lung	27 (2)	77 (1)	104
Brain	42 (10)	61 (9)	103
Kidney	66 (9)	43 (6)	109
Intestine	72 (11)	34 (8)	106

The number of separate experiments is given in parentheses. First step inhibition by pargyline is arbitrarily defined as the inhibition produced by preincubating mitochondria with 100 mg/ml pargyline HCl (corresponding to a final concentration of 75 ng/ml) as described under Materials and Methods.

When maximum inhibition due to harmine is added to the first step inhibition produced by pargyline, the sum in all six cases lies between 98 and 109 per cent. This finding strongly suggests that in the mouse there are two or more forms of MAO which are capable of deaminating kynuramine, and that these forms can be divided into two groups, one which is inhibited by harmine but not by low concentrations of pargyline, and a second which is inhibited by low concentrations of pargyline but not by harmine.

Under the present conditions harmine and harmaline appear to behave almost identically: both substances give the same maximum inhibition in the organs tested, and both give half maximum inhibition at about 3 ng/ml. Alpha ethyl tryptamine

produces the same maximum inhibition as harmine and harmaline but a higher concentration (about 600 ng/ml) is required to produce half-maximum inhibition. So far, no other inhibitors have been found which produce an inhibition pattern similar to that of pargyline.

2. *Heat inactivation*

Another, independent, line of evidence was sought to confirm the existence of several forms of MAO. A series of experiments was therefore performed to determine whether the forms might differ with respect to thermal stability. Youdim and Sourkes[12] have reported a non-homogeneous thermal inactivation of MAO in rat liver mitochondria, using kynuramine as substrate. This finding suggested the presence, in rat liver mitochondria, of two or more forms of MAO with different thermal stabilities.

When mouse liver mitochondria were heated at 50° in 100 mM Tris sulfate, pH 8·5, containing 1 mM EDTA, a non-homogeneous inactivation curve was obtained which indicated the presence of at least two forms of MAO having half-lives of approximately 2 and 10 min, and constituting, respectively 84 per cent and 11 per cent of the total MAO activity (Fig. 3(a)). A small fraction of MAO activity (about 5%) not shown in Fig. 3(a), appeared to have a half-life of about 46 min.

It was also of interest to investigate the possible heterogeneity with respect to thermal stability within the harmine sensitive, and the harmine resistant groups of MAO in the mitochondria prepared from the other organs. This was done by carrying out an additional assay on the heated samples in which harmine, at a final concentration of 1 γ/ml, was added together with kynuramine. Thus, two thermal inactivation curves were obtained: the first derived from an assay without added inhibitor, and a second from an assay carried out in the presence of 1 γ/ml of harmine. In all of the organs. examined, with the possible exception of intestine, the harmine resistant fraction of MAO appeared to be heterogeneous with respect to thermal stability. In all these cases the harmine resistant fraction appeared to consist of two forms having half-lives of 1–2 min, and of 6–12 min, respectively (see Table 2). In the case of intestine, several heating experiments suggested the presence of a small amount of a 9-min component, but this was not evident in the experiment shown in Fig. 3(b).

The harmine sensitive fraction, in all the cases where this could be accurately measured, appeared to be homogeneous with respect to thermal stability. The apparent half-life of this fraction ranged from 29 to 65 min at 50° (see Table 2).

At 55°C also (with all other conditions the same as at 50°) the harmine sensitive fraction appeared to be homogeneous and had a measured half-life ranging from 15 to 24 min (see Table 2).

It must be emphasized that the magnitude of the half-lives of the different forms of MAO are very sensitive to changes in the experimental conditions, including washing of the mitochondria, pH, and type of buffer used (sodium borate buffer, for example, gives results different from those obtained with tris sulfate). In addition there may be other critical variables such as mitochondrial swelling[1] which were not controlled in the present work.

Taken together, however, the thermal inactivation experiments clearly indicate the existence of three forms of MAO in the mouse which can deaminate kynuramine: two of these are totally resistant to inhibition by harmine and have relatively short half-

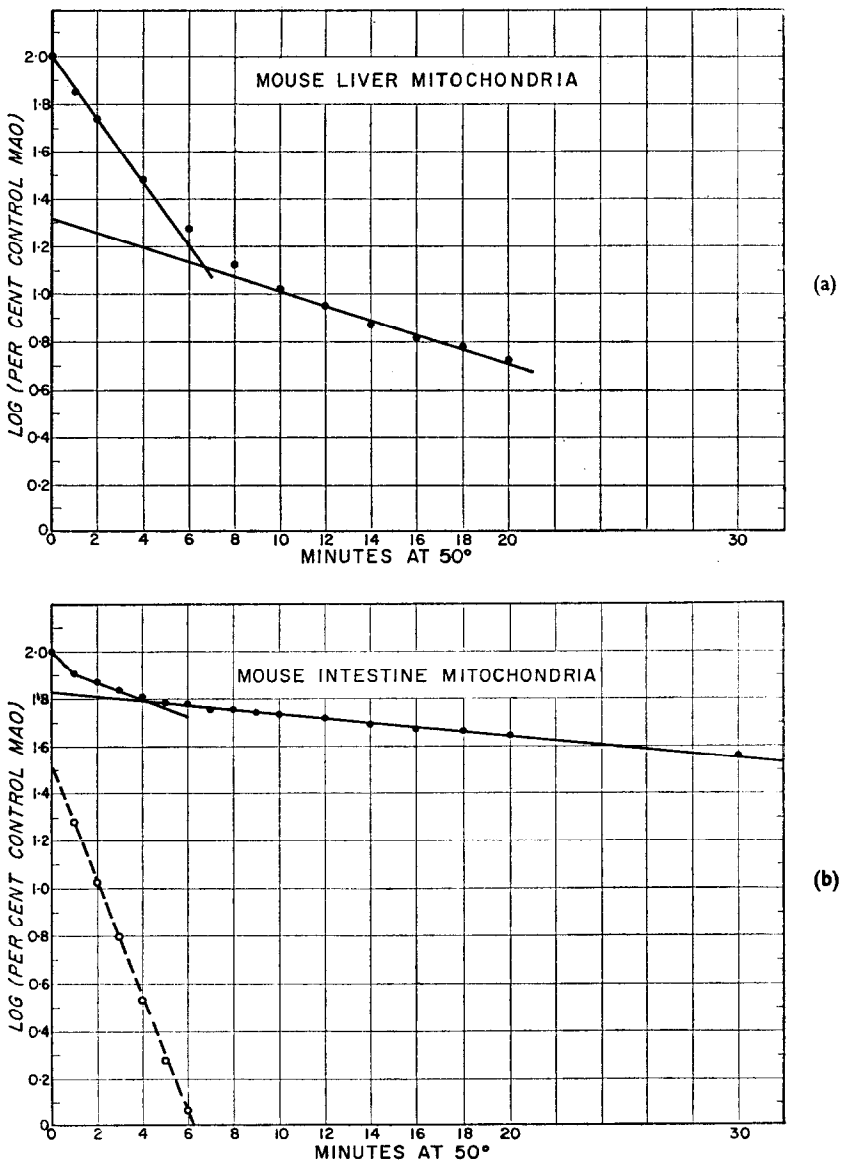

FIG. 3. Thermal inactivation of MAO in mitochondria from mouse liver and intestine. The curves (○) derived from assays carried out without additions, while the curve (●) derived from an assay carried out in the presence of harmine HCl (1γ/ml), added together with kynuramine. The liver and intestine mitochondria were isolated from the organs of a single mouse.

lives, while the third form, which is inhibited by harmine, has a significantly longer half-life.

DISCUSSION

The results presented in this communication, taken together with the results of other workers, appear to support the following model: (1) there are several forms of

MAO present in a single animal, which differ with respect to a number of physicochemical properties including substrate preference, sensitivity to inhibitors, pH optimum, and thermal stability. (2) Some substrates may be deaminated by two or more forms. (3) The relative proportions of these forms vary from one organ to another. (4) In the mouse and the rat (R. Squires, unpublished) the several forms

TABLE 2. THE HALF-LIVES OF THE THREE FORMS OF MAO IN THE MOUSE CAPABLE OF DEAMINATING KYNURAMINE

	Half-lives (min)			
Organ	A at 50°	B at 50°	C at 50°	C at 55°
Liver	1·6	10	46	15
Heart	2·0	10	–	ND
Lung	1·7	6·8	65	ND
Brain	1·8	8·0	29	15
Kidney	2·2	–	45	ND
Intestine	1·6	–	29	18

A and B are the harmine resistant forms. C is the harmine sensitive form.
–indicates that there was insufficient data for a reliable determination.
ND = not determined.

deaminating kynuramine can apparently be divided into a harmine resistant and a harmine sensitive group. The harmine resistant group is inhibited by low concentrations of pargyline while the harmine sensitive group is not.

That paragyline is a selective inhibitor of certain forms of MAO is supported by observations from other laboratories:

(1) Fähse has shown that when inhibition of MAO in mouse brain (using tyramine as substrate) is plotted as a function of inhibitor dose administered *in vivo*, the slope of the curve obtained with pargyline was strikingly less than the slopes obtained with 6 other known MAO inhibitors.[23]

(2) Parmar *et al.*[22] have shown that a single dose of pargyline (25 mg/kg) given to dogs results in different degrees of MAO inhibition in different regions of the brain: MAO in the hypothalamus was inhibited more than 90 per cent while MAO in the cerebellum was inhibited only 44 per cent. Three other well known MAO inhibitors gave distinctly smaller regional variations in MAO inhibition. Tyramine was used as substrate in these experiments.

(3) Maitre has also presented evidence which suggests stepwise inhibition of MAO in rat brain, liver and heart by pargyline and another acetylenic MAO inhibitor, SU 11739.[20]

(4) Partially heat inactivated MAO in rat liver mitochondria has two pH optima, using kynuramine as substrate. Activity at the pH 6·5 optimum was inhibited 48 per cent by pargyline ($6{\cdot}2 \times 10^{-8}$M) while activity at the pH 7·4 optimum was inhibited only 11 per cent.[12]

The existence of multiple forms of MAO complicates the problem of relating inhibition of the enzyme(s) to the pharmacological effects of the MAO inhibitors. It appears not unlikely that a given pharmacological effect is specifically related to inhibition of a single form. Poor correlations between pharmacological effects and

MAO inhibition have been reported by several investigators.[10, 17–19] While some of these anomalies may be due to effects of the MAO inhibitors unrelated to inhibition of the enzyme, others may be explained by assuming that the pharmacological effect is specifically related to inhibition of a single form, while the assay procedure measured mainly inhibition of other forms.

The genetic and structural basis of MAO multiplicity is unknown. Youdim and Sourkes[12] have suggested that rat liver mitochondria contain two distinct types of MAO only one of which is dependent on a riboflavin-containing coenzyme. The other type may contain a metal cofactor, but this does not appear to be copper. The physical separation and characterization of the various forms of MAO has been severely impeded by the fact that they are firmly bound to the mitochondrial outer membrane[28] from which they have proved to be difficult to remove without loss of activity. However, in a recent preliminary communication Youdim and Sandler[29] report the separation, by polyacrylamide gel electrophoresis, of three forms of MAO solubilized from rat liver mitochondria. Similarly solubilized MAO from human placental mitochondria could be separated into two bands by gel electrophoresis.

There does not seem to be a single genetic or structural basis for all isoenzyme systems. Systems have been described in which (1) the various forms are distinct, nonallelic protein monomers, as with the human carbonic anhydrases B and C,[24] (2) the active isoenzymes are tetramers consisting of two types of nonallelic protein subunits occuring in all possible combinations (the lactic dehydrogenases),[27] (3) the different forms differ mainly with respect to state of aggregation and conformation (mushroom tyrosinase),[25] (4) the different forms consist of a single protein species bound to varying amounts of sialic acid (human kidney alkaline phosphatase).[26]

The experiments of Parmar *et al.*[22] strongly suggest that the relative proportions of the different forms of MAO in dog brain vary from one anatomical area to another. This raises the possibility that some of the forms may be compartmentalized in different types of neurones. For example, neurones containing serotonin, noradrenaline, and dopamine, respectively, may contain different forms of MAO which are more or less specific for the deamination of these amines.

Work is in progress to determine the inhibition patterns with harmine and pargyline using substrates other than kynuramine. Preliminary results from this laboratory indicate that, using D,L noradrenaline, dopamine, or serotonin as substrates, harmine inhibits mouse brain MAO a maximum of between 80 and 90 per cent at concentrations below 100 ng/ml.

Acknowledgements—I wish to express my sincere thanks to Dr. J. Buus Lassen for providing the animal material, to Miss Else Sæderup for excellent technical assistance, and to Mr. P. Svendsen, director of research A/S Ferrosan, for encouragement and support during the course of this investigation.

REFERENCES

1. V. Z. GORKIN, *Pharmac. Rev.* **18**, 115, (1966), and references contained therein.
2. G. A. ALLES and E. V. HEEGAARD, *J. biol. Chem.* **147**, 487, (1943).
3. W. HARDEGG and E. HEILBRON, *Biochim biophys. Acta* **51**, 553 (1961).
4. A. BURGER and S. NARA, *J. mednl. Chem.* **8**, 859 (1965).
5. E. A. ZELLER and S. SARKER, *J. biol. Chem.* **237**, 2333 (1962).
6. Z. HUSZTI and J. BORSY, *Biochem. Pharmac.* **13**, 1151, (1964).
7. Z. HUSZTI and J. BORSEY, *Biochem. Pharmac.* **15**, 475 (1966).

8. L. M. BARBATO and A. G. ABOOD, *Biochim. biophys. Acta* **67**, 531 (1963).
9. K. OGAWA *et al.*, *J. Pharmac. exp. Ther.* **155**, 449 (1967).
10. R. SQUIRES and J. BUUS LASSEN, *Biochem. Pharmac.* **17**, 369 (1968).
11. E. WERLE and F. ROEWER, *Biochem. Z.* **322**, 320 (1952).
12. M. B. H. YOUDIM and T. L. SOURKES, *Can. J. Biochem.* **43**, 1305 (1965).
13. D. W. HOPE and A. D. SMITH, *J. Biochem.* **74**, 101 (1960).
14. W. J. NOVICK, *Biochem. Pharmac.* **15**, 1009 (1966).
15. R. E. MCCAMAN, *Fedn. Proc.* **20**, 344 (1961).
16. M. KRAML, *Biochem. Pharmac.* **14**, 1684 (1965).
17. J. H. BIEL, A. HORITA and A. E. DRUCKER, in *Psychopharmacological Agents*. (Ed. M. Gordon), vol. 1, pp. 359–443. Academic Press, New York, 1964.
18. C. L. ZIRKLE and C. KAISER, *ibid.* pp. 445–554.
19. G. R. PSCHEIDT, *Int. Rev. Neurobiol.* **7**, 191 (1964).
20. L. MAITRE, *J. Pharmac. exp. Ther.* **157**, 81 (1967).
21. M. H. VAN WOERT and G. C. COTZIAS, *Biochem. Pharmac.* **15**, 275 (1966).
22. S. S. PARMAR *et al. Biochem. Pharmac.* **16**, 222 (1967).
23. C. FÄHSE, *Pharmak. Forsch. Fortschr.* **5**, 148 (1964).
24. J. MCD. ARMSTRONG, D. V. MYERS, J. A. VERPOORTE and J. T. EDSALL, *J. biol. Chem.* **241**, 5137 (1966).
25. R. L. JOLLEY, and H. S. MASON *J. biol. Chem.* **240**, PC1 489 (1965).
26. P. J. BUTTERWORTH and D. W. MOSS, *Nature, Lond.* **209** 805 (1966).
27. C. R. SHAW and E. BARTO, *Proc. natn. Acad. Sci. U.S.A.* **50**, 211 (1963).
28. G. L. SOTOCASSA, BO KUYLENSTIERNA, L. ERNSTER and A. BERGSTRAND, in *Methods in Enzymology*, vol. X, p. 448. Academic Press, New York (1967).
29. M. B. H. YOUDIM and M. SAUNDLER, *Biochem. J.* **105**, 43P (1967).