MOLECULAR PHARMACOLOGY, 11, 558–565

Potency of Inhibitors for γ-Aminobutyric Acid Uptake by Mouse Brain Subcellular Particles at 0°

RICHARD W. OLSEN, J. DOUGLAS BAYLESS, AND MATT BAN

Department of Biochemistry, University of California, Riverside, California 92502

(Received December 6, 1974)

SUMMARY

OLSEN, RICHARD W., BAYLESS, J. DOUGLAS & BAN, MATT (1975) Potency of inhibitors for γ-aminobutyric acid uptake by mouse brain subcellular particles at 0°. *Mol. Pharmacol.*, 11, 585–565.

Binding of γ-amino[^{14}C]butyric acid by sucellular particles of mouse brain homogenates at 0° is apparently due to action of the specific transport system for GABA. Although not energy-requiring, this uptake process showed a saturable dependence of GABA concentration, with an apparent K_m of 28 ± 3 μM. The process was strictly sodium-dependent and sensitive to osmotic shock, freezing and thawing, and treatment with mild detergents. None of the GABA sequestering had the properties expected of neurotransmitter receptor sites, which probably were present in concentrations too low to be detectable by equilibrium dialysis of filtration assays of ligand binding. The potency of inhibition of GABA uptake at 0° was determined for 13 structural analogues of GABA known to inhibit both the transport of GABA and GABA synapses. No inhibition of GABA uptake occurred with 0.3 mM picrotoxin, but high concentrations (0.3 mM) of bicuculline-like compounds did inhibit the process, indicating that inhibition of the "binding" of radioactive GABA to tissue homogenates by bicuculline is not a sufficient criterion by itself to define such binding sites as receptors.

INTRODUCTION

γ-Aminobutyric acid is an inhibitory neurotransmitter substance in the vertebrate central nervous system (1–3) and in invertebrate central and peripheral nervous systems (3–5). Binding studies with radioactive GABA[1] have recently been described (6–11) in attempts to measure GABA receptors, by methods similar to those used for acetylcholine and other receptors (12–15). As with other receptors (15), the doubtful specificity of binding is a serious problem in attempting to identify the receptor sites *in vitro*, since neurotransmitter molecules could bind to other proteins in the tissue, such as enzymes and transport systems. In particular, tissue containing receptors for GABA will also be likely to contain the specific high-affinity transport system for GABA, thought to participate in terminating transmitter action at the synapse (3, 16). Therefore, in radioactive ligand binding assays designed to measure receptor sites in homogenized tissue, one might also detect binding to membrane transport recognition sites or even accumulation by the transport system.

This work was supported by funds from the University of California Agricultural Experiment Station and by Grant GB-42032 from the National Science Foundation.

[1] The abbreviation used is: GABA, γ-aminobutyric acid.

This study presents evidence that the sequestering of radioactive GABA at 0° by crude mitochondrial or synaptosome fractions, described by Roberts and collaborators (17–20), is virtually completely associated with the GABA transport system described for mammalian brain at 37° (21–24). Since GABA receptor sites are apparently not detected at the limits of sensitivity of these binding assays, the upper limit to the quantity of receptor sites in brain can be estimated as approximately 100 pmoles/g, wet weight. K_I values for inhibitors of GABA uptake at 10° have been determined under conditions employed in binding assays for receptors, so that one would know what properties *not* to expect of the receptor sites. These results have been presented in abstract form (25).

EXPERIMENTAL PROCEDURE

Chemicals. All chemicals were purchased from Sigma Chemical Company, with the following exceptions: bicuculline was purchased from Pierce Chemicals; homohypotaurine was a kind gift from Dr. B. Jollès-Bergeret; 4-amino-*trans*-crotonic acid, 4-amino-pentanoic acid, (−)-nipecotic acid, *cis*- and *trans*-3-aminocyclopentane-1-carboxylic acid, and 3-amino-propanesulfonic acid (homotaurine) were gifts from Dr. G. A. R. Johnston; imipramine was the gift of Ciba-Geigy; and chlorpromazine was a gift from Smith Kline & French. Triton X-100 was purchased from Calbiochem; sodium cholate, from Schuchardt, Munich; toluene, from Mallinckrodt; and β-mercaptoethanol, from Eastman. Bicucine methyl ester and bicuculline methiodide were synthesized according to Johnston *et al.* (26). Radioactive γ-aminobutyric acid was obtained from Schwarz/Mann ([4-^{14}C], 10.8 mCi/mmole) and the Radiochemical Center, Amersham ([*U*-^{14}C], 228 mCi/mmole).

Tissue. White male Wistar mice were killed by decapitation under ether anesthesia. Whole brains or dissected regions were homogenized in a glass tube by a motor-driven Teflon pestle (five passes at 600 rpm) in 10 volumes of ice-cold buffer (1 mM sodium phosphate, pH 7.1, in 0.32 M sucrose). Homogenates were centrifuged at 1000 × *g* for 10 min in a Sorvall SS-34 rotor; the pellet was resuspended in 10 volumes of the phosphate-sucrose buffer and centrifuged at the same speed to give pellet P_1. The combined supernatants were then centrifuged at 15,000 × *g* for 20 min in the same rotor. This crude mitochondrial pellet, P_2, was used in most of the experiments, resuspended by homogenization in 0.1 M NaCl–0.01 M Tris-HCl–1 mM β-mercaptoethanol, pH 7.8. The buffer was found to give results comparable to experiments performed in a mouse Ringer's solution. The supernatant from the centrifugation at 15,000 × *g* was centrifuged for 1 hr at 100,000 × *g* in Beckman Spinco rotor 65 to give the high-speed microsomal pellet P_3. In some cases fraction P_2 was further fractionated by sucrose gradient centrifugation (27). The pellet was resuspended in 5 original volumes of 0.32 M sucrose containing 1 mM sodium phosphate buffer, pH 7.1, and 5 ml were layered on top of 16-ml layers of 1.2 M and 0.8 M sucrose. After centrifugation for 2 hr at 25,000 rpm in a Spinco SW-27 rotor, the turbid material banding at the two interfaces was (separately) removed with a syringe, diluted 2-fold with 0.1 M NaCl–10 mM Tris-HCl, pH 7.8, and pelleted by centrifugation for 30 min at 100,000 × *g* in a Spinco 60 Ti rotor. The pellets were resuspended by rehomogenizing them as before in volumes of buffer to yield protein concentrations suitable for assays (see below). Protein was determined by the method of Lowry *et al.* (28).

Binding assays. All operations were carried out at 0–4°, and 0.1 mM aminooxyacetic acid was included to prevent GABA metabolism (29).[2] For filter assays, tissue was resuspended in 60 original volumes of buffer, generally 0.1 M NaCl in 10 mM Tris-HCl, pH 7.8, to yield 0.2–0.4 mg of protein/per milliliter. Radioactive GABA was added, the time was noted, and 2.0-ml aliquots were quickly added to tubes containing 0.2 ml of the buffer with or without nonradioactive GABA and/or inhibitors. After a 30-min incubation, 1.0-ml aliquots

[2] T. Claudio and R. W. Olsen, unpublished experiments.

(duplicates) were filtered by suction through soaked Millipore filters (GSWPO2500). The filters were then washed with 3 ml of buffer at 0° and dried, and radioactivity was counted in 5 ml of a solution composed of 2 volumes of toluene, 1 volume of Triton X-100, and 3 g of 2,5-diphenyloxazole per liter, using a Beckman CPM-100 counter. Samples were prepared in the absence of fluorescent light so that no fluorescence would contribute to the measured radioactivity. The blank in filter experiments was taken as the amount of GABA retained on the filter in the presence of tissue and 2 mM nonradioactive GABA. This was generally 200–300 cpm, or 5–10% of the experimental values. The amount taken up by tissue at 30 min represented less that 5% of the total radioactive GABA present in the sample, so that the initial concentration was virtually unchanged.

For equilibrium dialysis assays, tissue was resuspended in 5 original volumes of Tris-sodium buffer to give 3–4 mg of protein per milliliter. Aliquots of 0.3 ml (triplicates) were incubated in dialysis sacs for various times in gently rotating Erlenmeyer flasks containing 100 volumes of buffer with the radioactive GABA, additional GABA if desired, or other drugs. Nonspecific ionic binding and Donnan effects were unlikely at the ionic strengths used, i.e., over 0.1. After suitable times (6–16 hr, during which uptake was stable) the sacs were removed and 0.1 ml of the contents of both the sac and the buffer were counted. Nonradioactive GABA at 2 mM completely prevented uptake in dialysis experiments.

Radioactive GABA was either [4-^{14}C]GABA, 10.8 mCi/mmole, or [*U*-^{14}C]GABA, 228 mCi/mmole. Filter assays contained 1–2 × 10^5 cpm/assay in 1 ml; dialysis assays contained 3000–4000 cpm/aliquot counted (0.10 ml). Millipore filtration assays allowed the use of more radioactivity but less tissue; they were faster to perform but could result in loss of bound radioactivity during washing. The efficiency of radioactivity measurement, using an internal standard of [^{14}C]toluene, was determined as 82%.

Inhibitory constants were determined by the method of Dixon and Webb (30), using three to five concentrations of both GABA and inhibitor.

RESULTS

Both filter assays and equilibrium dialysis assays detected considerable binding of [^{14}C]GABA by mouse brain homogenate fractions at 0°, as described by Roberts and co-workers (17–20). This uptake of radioactive GABA was linear with protein concentration from 1 to 10 mg/ml and was virtually unaffected by varying the pH values from 7.0–7.8 (not shown).

The amount of GABA taken up by mouse brain crude mitochondrial fraction (P_2) is plotted in Fig. 1 as a function of free GABA concentration. Saturation was observed at a maximum value of 5.5 ± 1.0 nmoles of GABA per milligram of protein, or 100 nmoles/g of wet tissue (average of 12 experiments, filter and dialysis, SE 20%, extreme variation 60%). This quantity is clearly too large to represent simple binding to protein sites and is consistent with the occurrence of carrier-mediated uptake into membrane-bound space. Metabolic poisons (Table 1) did not inhibit the process.

A reciprocal plot of the uptake data is also shown in Fig. 1 and suggests that a single uptake system was involved under these conditions. The apparent K_m for GABA was 28 ± 3 μM (extreme variation). Similar K_m values were obtained for other tissue fractions, although the quantity of GABA taken up was generally lower than for P_2 (Table 1). This K_m value agrees well with the value obtained from initial velocity measurements of GABA transport in brain slices at 37° (21, 22).

The following experiments made it unlikely that significant contributions to the measured GABA uptake were due to systems other than the high-affinity, specific, sodium-dependent transport system. When brain tissue was frozen and thawed either once or twice on the same day and then assayed, approximately 50% of the GABA uptake was lost; i.e., fraction P_2 accumulated about 3 nmoles of GABA per milligram of protein. Tissue frozen for

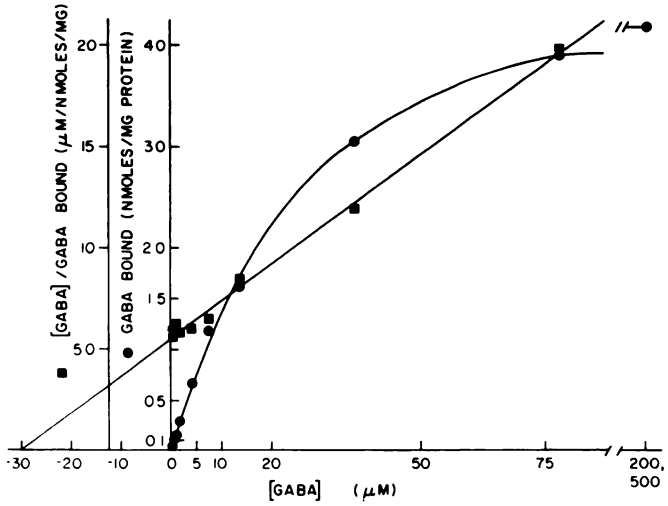

FIG. 1. *GABA sequestering at 0° by mouse brain fraction P_2.*

Tissue samples of 0.3 mg of protein per milliliter were incubated for 30 min with varying concentrations of GABA, including 2×10^5 cpm/ml of [^{14}C]GABA, and then filtered, washed, and counted for radioactivity. Radioactivity retained on the filter in the presence of tissue and 2 mM nonradioactive GABA was subtracted from each assay. Each point represents duplicate determinations; variation was less than 2%.

TABLE 1

Uptake of GABA by mouse brain particles at 0°

Uptake was determined by filter assays and equilibrium dialysis assays. See EXPERIMENTAL PROCEDURE for details.

Condition or fraction[a]	Percentage of P_2
	%
P_2	100
P_1	40 ± 10
P_3	40 ± 10
Synaptosomes	35 ± 5
Myelin	35 ± 5
"Fresh" frozen P_2	50 ± 10
Frozen 1 month	18 ± 5
Frozen 3 months	9 ± 3
Sodium azide	100 ± 7
2,4-Dinitrophenol	95 ± 7
Osmotic shock	10 ± 10
Triton X-100, 0.1%, w/v	0
Sodium cholate, 0.5%, w/v	0
Na^+-free solution	0
3 mM Na^+	20 ± 20
100 mM Na^+	100 ± 10

[a] Fresh P_2 unless otherwise labeled.

longer periods of time lost more of its capacity to take up GABA (Table 1). Uptake was drastically reduced by osmotic shock (rehomogenizing tissue in distilled water and assaying in the usual buffer) and completely inhibited by the mild detergents Triton X-100 and sodium cholate. Detergents were studied in dialysis assays only, with detergent present both in the samples and in the buffer.

Furthermore, no uptake of GABA at 0° occurred when all homogenizations and assays were performed in sodium-free buffer (100 mM NaCl replaced by choline chloride). This was true of fraction P_2, synaptosomes, and synaptosome membranes (high-speed pellet of osmotically shocked fraction P_2) from total brain, cerebral cortex, and cerebellar cortex. No binding was observed in the absence of sodium. If even a little sodium was present, some uptake occurred: at 3 mM Na^+, 20% of the uptake observed at 100 mM Na^+ was found (Table 1) [100 mM Na^+ is necessary for maximal GABA transport at 37° (16–24)].

Thus osmotic fragility, sodium dependence, the quantity taken up, and the K_m value for GABA suggest that the GABA transport system carries out an energy-independent exchange of GABA across membranes, even at 0°. Under our conditions, in the presence of Na^+, this results in some net accumulation by the tissue of labeled exogenous GABA. No binding to

receptor sites was detected in these experiments. Our assays would have detected such sites if the quantity exceeded 10 pmoles/g of wet tissue, assuming a K_D of 0.1 μM, or 1000 pmoles/g of tissue, assuming a K_D of 10 μM for the transmitter binding to receptor. It is not unreasonable that receptor sites might be present in concentrations lower than that (12–15).

The uptake of GABA is also known to be blocked by chlorpromazine and related compounds (17, 21). In our experiments chlorpromazine and imipramine inhibited uptake noncompetitively at 0° by as much as 100% (at 0.5 mM inhibitor). The I_{50} values are given in Table 2.

K_I values for inhibition of GABA uptake by 13 structural analogues are shown in Table 2, as determined by Dixon plots (30), e.g., Fig. 2. GABA uptake was measured

TABLE 2

Inhibitors of GABA uptake by mouse brain particles at 0°

K_I values were obtained graphically. See EXPERIMENTAL PROCEDURE for details.

Compound	K_I (competitive) or I_{50} (noncompetitive)
	μM
Competitive	
GABA	28 ± 5[a]
DL-2,4-Diaminobutyric acid	26 ± 4
DL-β-Hydroxy GABA	45 ± 10
(−)-Nipecotic acid	60 ± 10
trans-4-Aminocrotonic acid	60 ± 20
cis-3-Aminocyclopentane-1-carboxylic acid	65 ± 10
trans-3-Aminocyclopentane-1-carboxylic acid	120 ± 10
β-Guanidinopropionic acid	135 ± 20
Homohypotaurine	150 ± 30
4-Aminopentanoic acid	200 ± 50
Homotaurine	500 ± 100
β-Alanine	500 ± 100
Imidazoleacetic acid	600 ± 100
Noncompetitive	
Picrotoxin	>1 mM
Imipramine	120 ± 40
Chlorpromazine	90 ± 20
Bicuculline	500 ± 50
Bicucine	>500
Bicucine methyl ester	100 ± 30
Bicuculline methiodide	>500

[a] Apparent K_m.

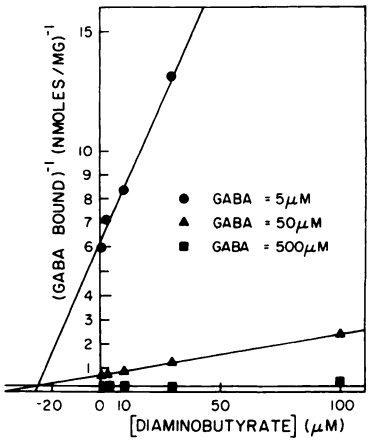

FIG. 2. *Inhibition of GABA uptake at 0° by* DL-*2,4-diaminobutyric acid at three different concentrations of GABA*. Experimental procedure was the same as in Fig. 1.

at varying concentrations of inhibitor and three to four concentrations of GABA. The following compounds, listed in order of decreasing potency, were competitive inhibitors: DL-2,4-diaminobutyric acid, DL-β-hydroxy-GABA, (−)-nipecotic acid, 4-amino-*trans*-crotonic acid, *cis*- and *trans*-3-aminocyclopentane-1-carboxylic acid, β-guanidinopropionic acid, homohypotaurine, 4-aminopentanoic acid, homotaurine, β-alanine, and imidazoleacetic acid. K_I values of over 600 μM were found for taurine, glycine, DL-α-aminobutyric acid, L-glutamine, DL-glutamic acid, and *cis*-4-aminocyclohexane-1-carboxylic acid.

Picrotoxin, an antagonist of GABA synapses (1, 2, 31–33), was only a very weak inhibitor of GABA uptake, causing 10–20% inhibition at 0.4 mM and no inhibition at 0.3 mM.

Some other antagonists of GABA synapses, the bicuculline-like compounds (1, 2, 32, 34, 35), when present at high concentrations (above 0.1 mM), significantly inhibited GABA uptake at 0°. The inhibition by these compounds was also apparently noncompetitive (this study and ref. 25).[3] Bicuculline did not inhibit GABA uptake by more than 50% (at 0.4–0.5 mM); however, bicuculline is not soluble at higher concentrations than this (34).[3] Bicucine

[3] R. W. Olsen, M. Ban, and T. Miller, manuscript submitted for publication.

methyl ester (0.3 mM) inhibited uptake almost completely; this compound also inhibited GABA transport at 37°, apparently because it is more stable chemically than bicuculline (25)[3]. These weak effects of bicuculline on GABA uptake probably are not responsible for the biological activity of bicuculline.

DISCUSSION

Homogenized brain particulate fractions containing intact or lysed synaptosomes, with postsynaptic membranes attached (27, 36), are likely to contain receptor sites for neurotransmitters. Various laboratories have attempted to measure the receptors for GABA in such preparations by binding assays (6, 9, 11). The identification of the binding sites detected is complicated by the possibility of extraneous binding of GABA to other proteins, especially those involved in membrane transport.

It is known that homogenized membranes reseal and perform carrier-mediated exchange of small molecules; in the presence of sodium brain homogenates can accumulate GABA at 0° (17). This raises the question whether any binding to receptor sites in brain homogenates at 0° can be detected in the presence of this uptake system. Could it be possible, under conditions that break membranes, or in Na^+-free buffer, or in purified brain regions rich in GABA, to measure GABA binding to receptor sites? Could it be possible to inhibit selectively either receptor sites or transport sites and thus distinguish between the two?

The answer to the first question is apparently negative. These studies show that brain fraction P_2 or synaptosomes take up considerable amounts of GABA at 0° by a mechanism that is completely eliminated by sodium-free conditions, by mild detergents, and by other treatments that damage membranes. We did not see any Na^+-independent binding at the limits of sensitivity of our assays. This result differs from that of Peck *et al.* (6), who observed high levels of binding in Na^+-free buffer. Unless receptor sites are sensitive to these treatments, they must be present in amounts too low to be detectable in our filtration or equilibrium dialysis binding assays, i.e., less than 100 pmoles/g of wet tissue, assuming (35) a dissociation constant (K_D) of the putative receptor for GABA of roughly 1 μM. Detection of such sites will require more sensitive assays or an enrichment in the number of sites by suitable tissue fractionation.

As for possible specific blocking agents, picrotoxin does not inhibit GABA uptake at 0°, so that it might be a specific GABA receptor antagonist. Some evidence, however, suggests that it is more likely an ionophore-blocking agent (33). Bicuculline-like compounds at high concentrations (above 0.1 mM) block GABA uptake at 0°, and thus inhibition of GABA binding by high concentrations of bicuculline cannot be taken as proof, by itself, that binding sites are receptors. There are other reasons to question the specificity of bicuculline,[3] and some evidence suggests that it too may be an ionophore-blocking agent (32). Others (6, 9, 34) have suggested that bicuculline at low concentrations is a specific competitive antagonist of GABA.

Chemical analogues might produce a specific blockage of GABA receptors or transport sites. Therefore accurate K_I values are needed for all possible compounds which might be used to distinguish between the two sites. With regard to the transport system, we present here K_I values (Table 2) for inhibition by several GABA analogues of GABA uptake at 0°, measured under conditions generally used in binding assays designed to measure receptor sites. The structure-activity relationships made evident by these inhibitor studies are in general accord with those proposed by Iversen and Johnston (21–23, 37) from data on GABA transport at 37° by brain slices. The data suggest a somewhat but not fully extended configuration for the GABA molecule in its recognition by the transport system. The greater activity of nipecotic acid and *cis*-3-aminocyclopentane-1-carboxylic acid, compared to *trans*-3-aminocyclopentane-1-carboxylic acid, suggest that compounds active in the transport system have a separation between the two charge centers of about 4.6–5.0 A, preferably 4.6 A. Receptors sites appear to have a similar but not identical specificity, requiring a quite extended configuration

and a charge separation of 5.0–5.4 A (37). Our values for inhibition of GABA uptake should be useful in determining what chemical specificity to expect or not to expect from real receptor sites. A few of the compounds have been suggested as specific GABA uptake or receptor blockers in some tissues. For example, (−)-nipecotic acid or 2,4-diaminobutyrate may inhibit GABA transport more strongly than they inhibit GABA receptors (37), while 3-aminopropanesulfonic acid (homotaurine) may bind to GABA receptors more strongly than to transport sites (1, 9). These compounds have not been carefully investigated by neurophysiologists, however. More accurate estimates of affinities for postsynaptic receptors in suitable preparations of central nervous system are needed before binding to receptors can be convincingly distinguished from binding and/or uptake by transport mechanisms on the basis of the effects of selective inhibition.

We suggest that, at present, no GABA blocking agent is known that shows clear specificity for receptor sites vs. transport sites, so that the two are very difficult to distinguish. Therefore a clear-cut assay *in vitro* for GABA receptors is not yet established. Snyder and collaborators (9) have described a suitably low concentration of GABA binding sites in brain membrane fractions which are detectable in Na^+-free buffer by a centrifugation assay. Binding was inhibited by bicuculline and GABA analogues, but more work is needed to determine whether these are receptor sites.

ACKNOWLEDGMENTS

We thank Drs. Michael F. Dunn, Clement E. Furlong, Randall C. Willis, Thomas Miller, and Ning G. Pon for helpful discussions. The gift of several compounds from Dr. Graham Johnston is gratefully acknowledged.

REFERENCES

1. Curtis, D. R. & Johnston, G. A. R. (1973) *Ergeb. Physiol. Biol. Chem. Exp. Pharmakol.*, 69, 97–188.
2. Krnjevićcc, K. (1974) *Physiol. Rev.*, 54, 418–540.
3. Iversen, L. L. (1973) in *Perspectives in Neuropharmacology* (Snyder, S. H., ed.), pp. 75–111, Oxford University Press, New York.
4. Roberts, E. (ed.) (1960) *Inhibition in the Nervous System and Gamma-aminobutyric Acid*, Pergamon Press, New York.
5. Kravitz, E. A. (1967) in *The Neurosciences: A Study Program* (Quarton, G. C., Melnechuk, T. & Schmitt, F. O., eds.) pp. 433–444, Rockefeller University Press, New York.
6. Peck, E. J., Schaeffer, J. M. & Clark, J. H. (1973) *Biochem. Biophys. Res. Commun.*, 52, 394–400.
7. Olsen, R. W., Lee, J. M. & Ban, M. (1975) *Mol. Pharmacol.*, 11, 566–577.
8. Olsen, R. W., Lee, J., Wunderlich, K. & Reynard, M. (1974) *Fed. Proc.*, 33, abstr. 1714, p. 1527.
9. Zukin, S. R., Young, A. B. & Snyder, S. H. (1974) *Proc. Natl. Acad. Sci. U. S. A.*, 71, 4802–4807.
10. De Robertis, E. & Fiszer de Plazas, S. (1974) *J. Neurochem.*, 23, 1121–1125.
11. Fiszer de Plazas, S. & De Robertis, E. (1975) *J. Neurochem.*, in press.
12. Olsen, R., Meunier, J.-C., Weber, M. & Changeux, J.-P. (1973) in *Pharmacology and the Future of Man, Proc. 5th Int. Congr. Pharmacol. (San Francisco)*, Vol. 5. pp. 118–129, Karger, Basel.
13. Hall, Z. W. (1972) *Annu. Rev. Biochem.*, 41, 925–952.
14. Hammes, G. G., Molinoff, P. B. & Bloom, F. E. (1973) *Neurosci. Res. Program Bull.*, 11 (3).
15. Cuatrecasas, P. (1974) *Annu. Rev. Biochem.*, 43, 169–214.
16. Snyder, S. H., Young, A. B., Bennett, J. P. & Mulder, A. H. (1973) *Fed. Proc.*, 32, 2039–2047.
17. Sano, K. & Roberts, E. (1963) *Biochem. Pharmacol.*, 12, 489–502.
18. Weinstein, H., Varon, S., Muhleman, D. R. & Roberts, E. (1965) *Biochem. Pharmacol.*, 14, 273–288.
19. Varon, S., Weinstein, H., Kakefuda, I. & Roberts, E. (1965) *Biochem. Pharmacol.*, 14, 1213–1224.
20. Kuriyama, K., Weinstein, H. & Roberts, E. (1969) *Brain Res.*, 16, 479–492.
21. Iversen, L. L. & Neal, M. J. (1968) *J. Neurochem.*, 15, 1141–1149.
22. Iversen, L. L. & Johnston, G. A. R. (1971) *J. Neurochem.*, 18, 1939–1960.
23. Beart, P. M. & Johnston, G. A. R. (1973) *J. Neurochem.*, 20, 319–324.
24. Simon, J. R. & Martin, D. L. (1973) *Arch. Biochem. Biophys.*, 157, 348–355.
25. Olsen, R. W. (1974) *Abstr. 4th Annu. Meet. Soc. Neurosci.*, abstr. 508, p. 362.
26. Johnston, G. A. R., Beart, P. M., Curtis, D. R., Game, C. J. A., McCulloch, R. M. & Maclach-

lan, R. M. (1972) *Nat. New Biol.*, **240**, 219–220.
27. Whittaker, V. P. & Barker, L. A. (1972) *Methods Neurochem.*, **2**, 1–52.
28. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *Biol. Chem.*, **193**, 265–275.
29. Wallach, D. P. (1961) *Biochem. Pharmacol.*, **5**, 323–331.
30. Dixon, M. & Webb, E. C. (1964) *Enzymes*, Ed. 2, Academic Press, New York.
31. Gerschenfeld, H. M. (1973) *Physiol. Rev.*, **53**, 1–129.
32. Shank, R. P., Pong, S. F., Freeman, A. R. & Graham, L. T. (1974) *Brain Res.*, **72**, 71–78.
33. Takeuchi, A. & Takeuchi, N. (1960) *J. Physiol. (Lond.)*, **205**, 377–391.
34. Curtis, D. R., Duggan, A. W., Felix, D. & Johnston, G. A. R. (1971) *Brain Res.*, **32**, 69–96.
35. Curtis, D. R., Johnston, G. A. R., Game, C. J. A. & McCulloch, R. M. (1974) *J. Neurochem.*, **23**, 605–606.
36. De Robertis, E. (1967) *Science*, **156**, 907–914.
37. Johnston, G. A. R. (1975) in *GABA in Nervous System Function* (Roberts, E., Chase, T. N. & Tower, D., eds.), Raven Press, New York, in press.