Biochemical Pharmacology, Vol. 19, pp. 2727–2736. Pergamon Press. 1970. Printed in Great Britain

MOLECULAR MECHANISM OF UNCONSCIOUS STATE INDUCED BY BUTYRATE*

A. A. RIZZOLI and L. GALZIGNA

Department of Biochemistry, The University of Padova, Via Marzolo, 3, 35100, Italy

(*Received* 29 *December* 1969; *accepted* 14 *April* 1970)

Abstract—The role of butyrate in the induction of the unconscious state depends on an interaction of this compound with the lecithin of the neural membrane which is followed by the formation of molecular complexes between butyrate and the central chemical transmitters serotonin and dopamine.

SHORT chain aliphatic fatty acids (butyrate, propionate, valerate) and parent compounds (like 4-hydroxybutyrate (= GHB) and 1·4 butanediol)[1] induce a sleep- or anaesthetic-like state when injected into animals. There is however a disagreement about the definition of such a state: Matzusaki,[2–6] who devoted much physiological work to this subject, reported that butyrate, in physiological acute preparations, mimics pretty closely the onset and the characters of normal sleep with the two stages of SWS and REM sleep. On the other hand, Winters *et al.*[7–15] prefer to consider the true action of all fatty acids as that of an epileptogenic agent which could act by disconnecting cortical from subcortical relais.[16]

A previous research carried out by one of us[17] showed that butyrate crossed the blood–brain barrier very slowly with no appreciable regional differences among various parts of the brain and that, *in vitro*, it had no action upon brain AChE and MAO activities. In contrast to earlier reports,[18] Sacks[19] has shown in human experiments that butyrate is metabolized by the brain. The closely related compound GHB affected creatine phosphate, ATP and various metabolites of the Krebs cycle in a way similar to phenobarbital,[20] increased the concentration of aspartic acid[21] and had a biphasic effect on the dendritic response.[22] The critical concentration of this drug in brain tissue for the induction of anaesthesia was found to be 8×10^{-4}M.[23]

In this work we attempted to study the butyrate sleep phenomenon in the light of the present knowledge on the actions and properties of catechol and indole amines in the genesis of sleep. Our search was directed towards two aspects of the phenomenon e.g. the binding of butyrate with model receptors and the possibility of interaction with chemical transmitters suspects.

*A preliminary report of this investigation was given at the 2nd International Meeting of Neurochemistry Society, Milan, 3 September 1969.

Bibliographic assistance was received from UCLA Brain Information Service, a Member of the NINDS Neurological Information Network.

Abbreviations used: AChE, acetylcholinesterase; MAO, monoamine oxidase; GHB, 4-hydroxybutyrate; ATP, adenosine triphosphate; SWS, slow wave sleep; REM, rapid eye movement (paradoxical sleep).

MATERIALS AND METHODS

Young adult Wistar rats (150–200 g body weight) of either sex were used in this study.

Injection of butyrate-1-^{14}C, glucose-U-^{14}C was made intracerebrally through a small hole which was made at 4 mm lateral to midline and 6 mm anterior to the parieto-occipital suture with a 5 mm stoppered no. 14 hypodermic needle.[24] Fractionation of cerebral tissue into subcellular components was carried out according to Potter and Axelrod.[25] Difference spectra were obtained on a Cary 15 recording spectrophotometer by one of us.[26]

Thin-layer chromatography (= TLC) was run on a Silica Gel G plates with chloroform–methanol–water (65:25:4) or diisobutylketone–formic acid–water (40:15:2) as solvents. Plates were subsequently exposed to iodine vapors.

The radioactivity measurements were carried out with a Packard Tri-Carb liquid scintillation counting apparatus. Sodium *n*-butyrate-1-^{14}C, glucose-U-^{14}C, 5-hydroxytryptamine-3-^{14}C-creatinine sulphate were obtained from the Radiochemical Center, Amersham, England. 5-hydroxytryptamine creatinine sulphate was purchased from

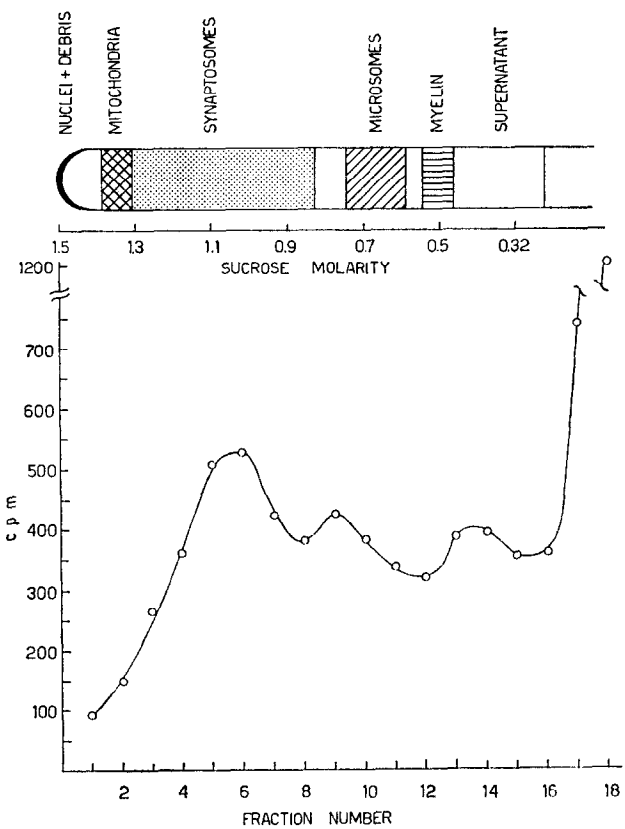

FIG. 1. Subcellular distribution of butyrate-^{14}C in a homogenate of cerebral hemispheres 120 min after intracerebral injection in the rat. Values are given as counts per minute. The histogram at the top shows the corresponding "fractions" according to Potter and Axelrod.[25]

SIGMA, St. Louis, Mo., and β-γ-dipalmitoyl-L-α-lecithin from Fluka AG, Buchs, Switzerland. Serotonin oxalate, DL-norepinephrine, acetylcholine chloride and dopamine HCl were Calbiochem.

All other reagents were Merck pure grade.

EXPERIMENTAL

First we checked butyrate distribution among subcellular particles. Figures 1 and 2 show the distribution of ^{14}C 120 min after the intracerebral injection of butyrate ^{14}C

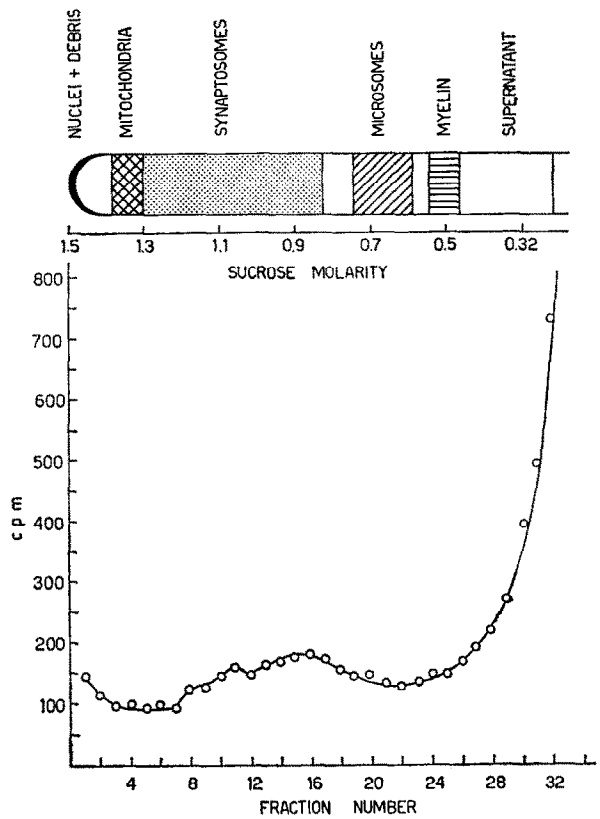

FIG. 2. Subcellular distribution of glucose-U-^{14}C in an homogenate of cerebral hemispheres 120 min after intracerebral injection in the rat. Values are given as counts per minute. The histogram at the top shows the corresponding "fractions" according to Potter and Axelrod.[25]

or glucose ^{14}C utilized as reference compound. Animals had been lightly anaesthetized by ether; at the time of their sacrifice they had completely recovered and showed no apparent neurological damage.

Intracerebral injection was necessary in view of preliminary findings showing that to achieve a good ^{14}C concentration in brain subcellular particles it was necessary to inject i.v. or i.p. too large quantities of the labelled compound. Butyrate, in fact, crosses the blood–brain barrier slowly and in small amounts.[17]

It is evident that butyrate (or its catabolites) was bound to membrane-rich fractions i.e. synaptosomes (which sediment between 0·8 and 1·3 M sucrose) and microsomes (which sediment between 0·6 and 0·8 M sucrose). On the other hand, metabolites of glucose-^{14}C (mostly glutamic and aspartic acid)[27] were recovered mainly in the supernatant fraction.

In experiments *in vitro* butyrate-^{14}C was added to brain homogenate in 0·32 M sucrose. The homogenate was incubated at 37° for 15 min in a Dubnoff metabolic incubator under O_2 stream. Extraction by chloroform–methanol (2:1) or by 0·1 N HCl showed that the recovered radioactivity was six times higher in lipid than in protein fraction.

The attempt of detecting the lipid specifically involved in the binding was unsuccessful since the TLC methods used were unable to differentiate the bound from the free fatty acid.

We tried therefore to simplify experimental conditions, looking at the possibility of interaction of butyrate with different species of lipids *in vitro*.

In a test tube we added different amounts of pure synthetic (dipalmitoyl-lecithin) or brain extracted lipids and of butyrate-^{14}C. The lipids were brought into solution by chloroform while butyrate-^{14}C was dissolved in bidistilled water. After 3 min of

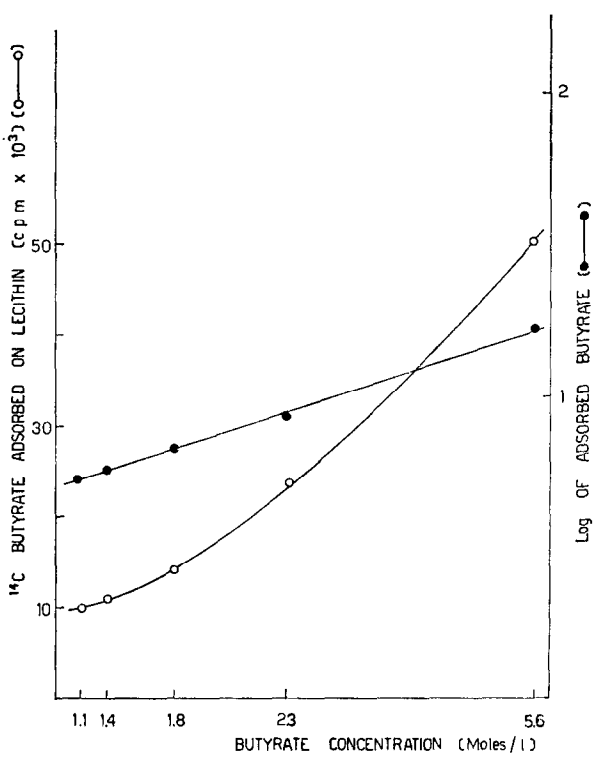

FIG. 3. A solution of lecithin in chloroform (mg/ml) was partitioned with an equal volume of a 5·6 M solution of butyrate-^{14}C in water at 4° and left overnight. The residual radioactivity was measured in both phases. The different concentrations of butyrate-^{14}C are put on the x axis while on the y axis is put radioactivity found in the chloroform phase.

energic shaking, tubes were left overnight in cold room at 4° to allow the separation of phases.

By such a procedure we were able to demonstrate (Figs. 3 and 4) that different concentrations of butyrate were subtracted from the water phase when a fixed amount of lecithin was added. Other species of lipids were ineffective. If butyrate concentrations are plotted against the logarithm of the subtracted quantity we obtain a linear function. On the other hand a linear relationship was also obtained when a fixed amount of butyrate was added to different concentrations of lecithin. This means that Freundlich's equation ($Y = \beta C^{\alpha}$) can describe the phenomenon and thus the interaction lecithin–butyrate can be considered as a true adsorption process. In the experiments of

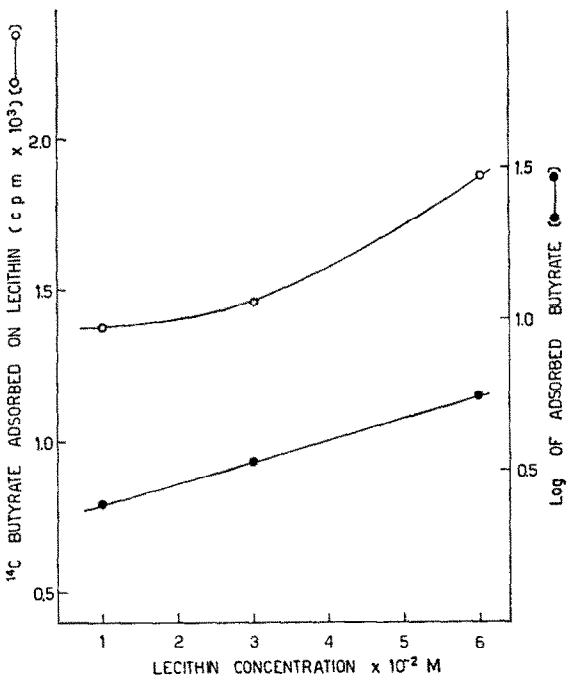

FIG. 4. A solution of lecithin in chloroform was partitioned with 0·01 M butyrate-^{14}C in water at 4° and left overnight. The residual radioactivity was measured in both phases.
The different concentrations of lecithin are put on the x axis while on the y axis is put radioactivity found in the chloroform phase.

partition either choline or palmitate were ineffective in altering butyrate-^{14}C distribution in a manner similar to that of lecithin.

We were also interested to ascertain if some of the neurochemical transmitter suspects was capable of interacting with butyrate by forming molecular complexes. We carried out a differential spectrophotometric analysis of mixtures of butyrate with central chemical transmitters (acetylcholine, serotonin, noradrenaline and dopamine). The difference spectra showed a highly significant interaction of the neurotransmitter with butyrate in the case of serotonin and dopamine yielding a spectral effect which has already been described as due to a molecular interaction (Fig. 5).[26]

The serotonin–butyrate interaction is not limited to the excited state of molecules and was also demonstrated at the ground state. In fact TLC of serotonin, butyrate and of the complex serotonin–butyrate (carried out with acetic acid 2%–water as solvent) showed that this last one had an R_f value of 0·698 while pure serotonin had a value of 0·774 and butyrate a value of 0·819 thus proving a net change in the polar state of the molecules due probably to their electrostatic interaction.

On the one hand serotonin itself dissolved in 0·01 M KH_2PO_4–0·15 M KCl, pH 8·0

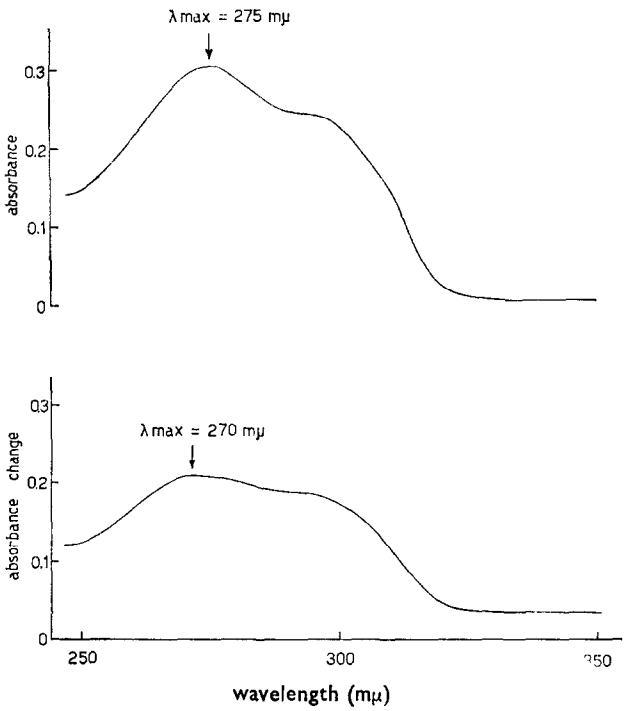

FIG. 5. One ml of 0·1 M butyrate in phosphate buffer 0·1 M pH 6·8 is mixed with 1 ml of 0·1 M serotonin oxalate (Calbiochem) and the mixture is diluted 1:1000 with buffer before the spectral analysis. The difference spectrum is obtained with a Cary 15 spectrophotometer by placing the serotonin-butyrate mixture in the reference chamber and two separate solutions of butyrate and serotonin in the sample chamber.

In this way a positive peak is obtained as a result of an hypochromic effect.

was bound by an acetone powder of brain phospholipids extracts in a way proportional to the concentration of lipids (Fig. 6). On the other hand addition of serotonin in equimolar amount to a test tube in which butyrate-^{14}C was adsorbed on lecithin caused a net "discharge" of butyrate from the chloroform to the water phase so that the partition coefficient, that initially was about 3, was reduced to 0·89 (Table 1). In a similar system in which serotonin-^{14}C was bound to phospholipids, butyrate, at various concentrations, was proved to be ineffective in altering ^{14}C partition (Table 2). Also these experiments were performed in cold room at 4°. A chemical interaction *in vitro*

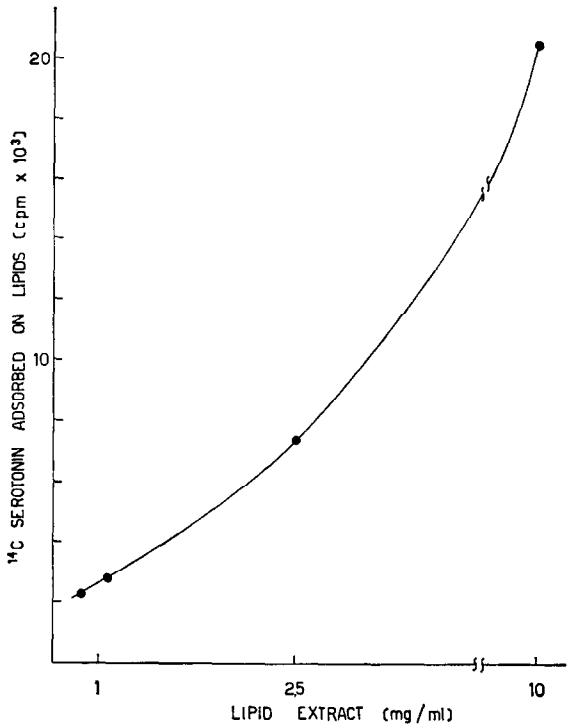

FIG. 6. Brain extracted phospholipids (10 mg/ml) in chloroform were partitioned with fixed amounts of 0·01 M 5-hydroxy-3-^{14}C-tryptamine in 0·01 M KH_2PO_4–0·15 M KCl pH 8 buffer at 4° and left overnight. The residual radioactivity was measured in both phases. The different concentrations of lipids are put on the x axis while on the y axis is put radioactivity found in the chloroform phase.

TABLE 1. EFFECT OF SEROTONIN ON THE PARTITION COEFFICIENT OF BUTYRATE IN A WATER–CHLOROFORM SYSTEM

	Aqueous phase (1 ml) (cpm ± SE)	Organic phase (1 ml) (cpm ± SE)	Partition coefficient
0·01 M Lecithin + 0·01 M Butyrate-^{14}C	92,000 ± 1500	273,000 ± 5500	2·88
0·01 M Lecithin + 0·01 M Butyrate-^{14}C + 0·01 M Serotonin oxalate	175,000 ± 6000	155,000 ± 7300	0·89

Mean of four experiments.

resembling the one shown with butyrate and serotonin was also proved for the butyrate–dopamine system. It is then clear that the model we will postulate later on for explaining butyrate activity *in vivo* might utilize also dopamine as the key transmitter in agreement with some ideas expressed by Gessa *et al.*[28] It is evident that only more refined neurophysiological experiments can ascertain unequivocally the type of transmitter involved in the phenomenon *in vivo*.

TABLE 2. EFFECT OF BUTYRATE ON THE PARTITION COEFFICIENT OF SEROTONIN IN A WATER–CHLOROFORM SYSTEM

	Aqueous phase (1ml) (cpm)	Organic phase (1 ml) (cpm)	Partition coefficient
Brain phospholipids extract (10 mg/ml) + 0·01 M Serotonin-3-^{14}C creatinine sulphate	97,540	20,870	0·21
Brain phospholipids extract (10 mg/ml) + 0·01M Serotonin-3-^{14}C creatiuine sulphate + 0·01 M Butyrate	96,162	16,188	0·16

Mean of two experiments.

DISCUSSION

The several papers of Matzusaki[2–6] dealing with the action of butyrate on the middle and rostral portion of the pons do not exclude that the status induced by the administration of this compound could resemble an absence or a catatonic state, sharing some features of degeneralized non-convulsant epilepsy.[8]

In view of the accurate electrophysiological studies of Winters *et al.*[7–15] on the convulsant properties of butyrate molecules, we will try to elucidate the synaptic mechanism of butyrate in a whole sequence of events. For what is more strictly pertaining to neurophysiological aspects we shall restrict ourselves to a quick glance; in fact a comprehensive survey of the complex action of fatty acids molecules on CNS was beyond the aim of the present study.

When butyrate molecules (or butyrate congeners, among which the most effective, probably for permeability reasons, is GHB) arrive at the synaptic junction they fix themselves to lecithin of synaptic structures. This behaviour is similar to that of other anaesthetic agents studied by us.[29]

As soon as the neurochemical transmitter is liberated by synaptic endings, (in view of our *in vitro* findings we will mention here mainly serotonin) on its way to postsynaptic receptors it will pass through the membrane phospholipids which have previously fixed the butyrate molecule. We demonstrated that secondary forces must be involved in the butyrate–lecithin binding as it is an adsorption process. The liberation of serotonin will provide a molecule able to compete with lecithin for butyrate. In fact a complex of electrostatic nature will probably be formed between serotonin and butyrate. As serotonin is bound in the complex its chemical potential will be very different from that of free serotonin and the equilibrium:

$$\text{bound serotonin} \rightleftharpoons \text{free serotonin}$$

will be shifted toward the left hand side.

Since chemical potential of serotonin in the synapsis must be crucial for regulating its supply from presynaptic vesicles, this means that additional molecules of serotonin will be liberated in the synaptic cleft.

At present time we do not know the physiology of serotoninergic pathways; some of them might act as modulatory to the arousal cholinergic system.[30] As a matter of fact GHB in hypnotic doses induces a disorganization of reticular control system resulting in the inability to enforce arousal response, to modulate the amplitude of

sensory input and to modulate the bulbar motor system and, at the same time, this drug does not reduce the sensitivity of the reticular neurons *per se*.[8]

Another possibility is that in a cholinergic synapse—serotonin binding was found to be pre-eminent in "cholinergic" nerve endings[31]—serotonin release and binding with butyrate could interfere with acetylcholine release (or action) through a Ca^{2+} transport dependent mechanism resulting in a significant loss of the incoming impulses.[32]

These hypotheses correlate well with the demonstrated action of butyrate on the middle and rostral portion of the pons,[5] from which it starts an ascending serotoninergic system involved in sleep phenomena.[33]

One of the results of the postulated interference, at whatsoever level it could be, is the production of REM signs. The fact that the action of butyrate and congeners is dose dependent, switching the response of the animal from SWS to a REM sleep, and finally to a convulsive status and death leads us to consider butyrate as a molecule interfering in a multidimensional manner on arousal systems. This is not a mere tautology. An "inhibited" function may be consequent to a change in an exciting system and vice versa, as it was stated first by Sherrington and Hughlings Jackson.[34]

To sum up, the butyrate molecule binds itself to lecithin, by a true adsorption process.

Butyrate is also capable of binding with serotonin and dopamine when it passes through synaptic membranes by forming a molecular interaction complex. The formation of this complex may alter the equilibrium in the synaptic cleft and induce the secretion of additional transmitter or it may interfere with acetylcholine release.

In any one of these cases there will be an alteration in the arousal system leading to a multimodal response.

REFERENCES

1. G. L. Gessa, P. F. Spano, L. Vargiu, F. Crabai, A. Tagliamonte and L. Mameli, *Life Sci.* **7**, 289 (1968).
2. M. Matzusaki and H. Takagi, *Brain Res.* **4**, 206 (1967).
3. M. Matzusaki and H. Takagi, *Brain Res.* **4**, 223 (1967).
4. M. Matzusaki, *Brain Res.* **11**, 251 (1968).
5. M. Matzusaki, *Brain Res.* **13**, 247 (1969).
6. H. Takagi and M. Matzusaki, *Jap. J. Physiol.* **18**, 380 (1968).
7. R. J. Marcus, W. D. Winters, K. Mori and C. E. Spooner, *Int. J. Neuropharmac.* **6**, 175 (1967).
8. W. D. Winters and C. E. Spooner, *Electroenceph. clin. Neurophys.* **18**, 287 (1965).
9. W. D. Winters and C. E. Spooner, *Int. J. Neuropharmac.* **4**, 197 (1965).
10. W. D. Winters and C. E. Spooner, *Electroenceph. clin. Neurophys.* **20**, 83 (1966).
11. W. D. Winters, K. Mori, C. E. Spooner and R. O. Bauer, *Anesthesiology* **28**, 65 (1967).
12. W. D. Winters, in *Recent Advances in Biological Psychiatry* (Ed J. Wortis), Vol. 9, Plenum Press, New York (1967).
13. W. D. Winters, in *Psychopharmacology: a Review of progress*, 1957–1967 (Ed. D. H. Efron), PHS Publication (1968).
14. W. D. Winters, K. Mori, M. B. Wallach, R. J. Marcus and C. E. Spooner, *Electroenceph. clin. Neurophys.* **27**, 514 (1969).
15. W. D. Winters, in *Drugs and the Brain*, (Ed. P. Black), Johns Hopkins (1969).
16. H. Laborit , G. Laborit and C. Baron, *Ann. Anesth. Franc.* **9**, 163 (1968).
17. A. A. Rizzoli, *Boll. Soc. Ital. biol. Sper.* **45**, 1108 (1969)
18. J. H. Quastel and A. H. M. Wheatley, *Biochem. J.* **27**, 1753 (1933).
19. W. Sacks, *J. appl. Phys.* **12**, 311 (1958).
20. M. C. Fleming and S. La Court. *Biochem. Pharmac.* **14**, 1905 (1965).

21. R. K. MARGOLIS, *Biochem. Pharmac.* **18**, 1243 (1969).
22. T. BAN and M. HOJO, *Jap. J. Pharmac.* **19**, 89 (1969).
23. N. J. GIARMAN and R. H. ROTH, *Science* **145**, 583 (1964).
24. R. BALAZS and W. A. COCKS, *J. Neurochem.* **14**, 1035 (1967).
25. L. T. POTTER and J. AXELROD, *J. Pharmac. exp. Ther.* **142**, 291 (1963).
26. L. GALZIGNA, *Biochem. Pharmac.* **18**, 2485 (1969).
27. R. VRBA, *Nature Lond.* **195**, 663 (1962).
28. G. L. GESSA, F. CRABAI, L. VARGIU and P. F. SPANO, *J. Neurochem.* **15**, 377 (1968).
29. L. GALZIGNA, G. MANANI, E. MANZIN, G. GRITTI and A. A. RIZZOLI, *Acta Anaestesiol.* **XIX**, suppl. 7 (1968).
30. G. C. CELESIA and H. H. JASPER, *Neurology* **16**, 1053 (1966).
31. C. D. WISE and H. W. RUELIUS, *Biochem. Pharmac.* **17**, 617 (1968).
32. C. TORDA, *Psychol. Rep.* **24**, 807 (1969).
33. M. JOUVET, *Science*, **163**, 32 (1969).
34. C. R. B. JOYCE, in *New Horizons in Psychology*, (Ed. B. FOSS), Penguin, London (1966).