Biochemical Pharmacology, 1966, Vol. 15, pp. 817–824. Pergamon Press Ltd., Printed in Great Britain.

ACTION OF TRICYCLIC ANTI-DEPRESSANT DRUGS ON CENTRAL PROCESSES INVOLVING ACETYLCHOLINE

A. K. S. Ho, Shirley E. Freeman, W. P. Freeman and Helen J. Lloyd

Department of Pharmacology, University of Melbourne, Parkville, Australia

(*Received* 22 *October* 1965, *accepted* 9 *February* 1966)

Abstract—Five tricyclic antidepressant drugs have been shown to be weak to moderate inhibitors of rat brain cholinesterase.

Amitriptyline has been found to increase the ability of rat cerebral cortex slices to synthesize ACh, both *in vitro* and after chronic treatment with the drug, Rats injected with amitriptylamine did not show an increased synthetic ability until after 8 days treatment.

An attempt has been made to correlate the rate of ACh synthesis with cell types in the cortical slices used. No clear cut effect was found.

The tricycle antidepressants do not affect either the basal or the K^+ stimulated respiration of the cortex.

In view of the known atropine-like activity of these compounds it is suggested that they may inhibit central muscarinic actions of ACh, whilst they stimulate nicotinic actions.

In spite of extensive investigation, the mode of action of the tricyclic group of anti-depressant drugs remains obscure. Studies on their peripheral pharmacology suggest that they affect both divisions of the autonomic nervous system. Thus imipramine has been shown to potentiate the peripheral effects of noradrenaline, and to antagonize the muscarinic actions of acetyl choline.[1, 2] Amitriptyline also possesses a strong atropine-like action, and potentiates the pressor response to exogenous noradrenaline. However, it has been shown[3] to reverse the pressor response to exogenous adrenaline.

It was of interest therefore, to examine the effects of this group of drugs on cholinergic mechanisms in the mammalian brain. An investigation has been made of the anti-cholinesterase activity of the group; their effect on acetyl choline synthesis has been studied, and a brief study has been carried out of their effect on oxidative processes in the rat brain. An attempt has been made to correlate the data obtained with the histological distribution of cell types in the rat cortical layers.

Since it has been demonstrated that the desmethyl derivitives of amitriptyline and imipramine are metabolites of the parent compounds, these derivatives, and also protriptyline have been included in the study.

METHODS

Cholinesterase activity

The activity of rat brain cholinesterase was measured by the ampermetric technique described previously.[4] A uniform, frozen-dried sample of rat brain powder was used as the source of cholinesterase; the amount of crude enzyme was kept constant throughout the series of experiments at 6 mg dry brain per ml solution. The time of incubation

of enzyme–substrate–inhibitor mixtures was 15 min. The drugs were checked initially for any possible interference with the assay. None of them was found to have a current–voltage plateau at −0·7 V. The incubation mixture contained the following substances:

Enzyme extract	1 ml
Acetylthiocholine	1 ml (4×10^{-3} M/l. final concentration)
Drug solution	1 ml
Tris buffer (pH 8·0)	2 ml

The determination of I_{50}, the concentration of inhibitor necessary to produce 50 per cent inhibition, and K_i values were based on the method described by Cohen and Oosterbaan.[5]

Acetylcholine synthesis

Acetylcholine synthesis was studied in cortical slices of rat brain, using methods similar to those of McLennan and Elliott.[6, 7] Sprague-Dawley rats weighing between 150 and 200 g were used. After decapitation, the brain was removed from the skull, and cortical slices were cut according to the method of McIlwain and Rodnight.[8] The slices were weighed on a torsion balance, and immediately transferred to double side-arm Warburg vessels. These vessels contained 4 ml of a carbogenated incubation medium of the following composition:

NaCl	100 mM/l.
KCl	27 mM/l.
$NaHCO_3$	23·5 mM/l.
KH_2PO_4	0·6 mM/l.
$CaCl_2$	1·4 mM/l.
Glucose	9·1 mM/l.

Physostigmine sulphate 0·1 mM/l. was placed in one side arm of the Warburg vessel, and the drug under investigation in the other. After aeration with carbogen, physostigmine and the drug (when present) were tipped into the main compartment of the vessel, which was incubated in the Warburg apparatus for 90 or 120 min at 37·5°. The pH of the incubation medium was checked before and after incubation.

Assay of acetylcholine

Acetylcholine was assayed by the method of Chang and Gaddum.[9] The rectus abdominus of the toad, *Bufo marinus*, was used, either with or without acetone sensitization.[10]

Oxygen uptake

The oxygen uptake of cortical slices was measured in the Warburg apparatus under similar conditions to those obtaining in the experiments concerned with acetylcholine synthesis. However, air rather than carbogen was present as the gaseous phase, and the K^+ concentration of the medium was either 6·5 or 48 mM, depending upon whether the basal or stimulated oxygen uptake was studied.

Histological procedures

Slices of the parietal cortex of the brain were cut according to the method of McIlwain and Rodnight[8] and placed immediately in cold 10% formol saline and allowed to fix for 48 hr, then processed through graded alcohol and cedar wood oil to paraffin wax. Sections were cut at 10 μ, at which thickness compression due to sectioning was negligible. The sections were brought to water through xylol and alcohol and stained for 2 min in 0·1 % aqueous Cresyl Fast Violet.

Measurements were carried out by means of an eye piece grid placed in the eye piece of the microscope and calibrated against a stage micrometer (1 mm divided into 100 parts).

RESULTS

Anticholinesterase activity of the tricyclic antidepressants

All the antidepressants tested were found to have some anticholinesterase potency. Lineweaver-Burke[11] plots of the inhibitory effects of both amitriptyline and imipramine were drawn (Figs. 1 and 2). It is clear that amitriptyline inhibits cholinesterase

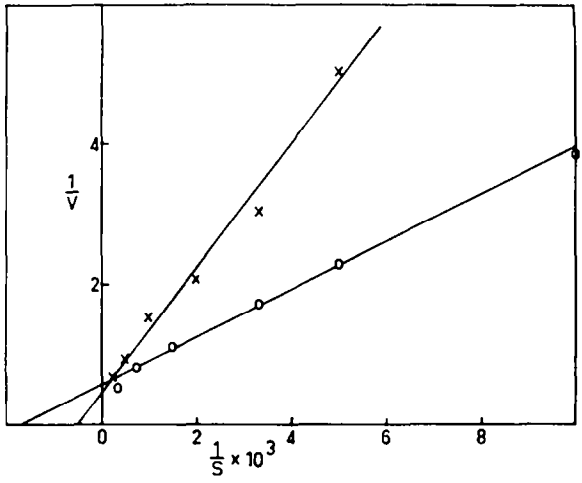


FIG. 1. Lineweaver-Burke plot of the inhibition of brain cholinesterase by amitriptyline. The concentration of amitriptyline was 2×10^{-4} M/1.

competitively; with imipramine the line of best fit for the inhibited enzyme did not cut the vertical axis at the same point as the plot for the uninhibited enzyme. The divergence, however, was not great enough to suggest that the inhibition due to imipramine is non-competitive.

It has been assumed that protriptyline and the desmethyl derivatives would also be competitive inhibitors, because of their structural similarity.

The concentration of antidepressant necessary to bring about 50 per cent inhibition

of rat brain cholinesterase was determined from the relation:

$$\frac{V'}{V} = 1 + \frac{(I)}{K_i(1 + (S)/K_m)}$$

where V' = velocity of the uninhibited reaction;
V = velocity of the inhibited reaction;
(I) = concentration of inhibitor (M/l.);
(S) = concentration of substrate (M/l.);
K_m = Michaelis-Menten constant for the enzyme substrate complex, and
K_i = Enzyme inhibitor constant.

Values for K_i and I_{50} for the five compounds are shown in Table 1. It may be seen that amitriptyline and imipramine are of equivalent inhibitory potency, whilst the

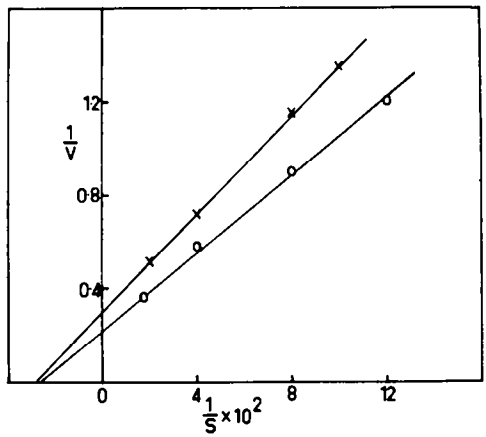


Fig. 2. Lineweaver-Burke plot of the inhibition of brain cholinesterase by i mipramine. The concen tration of imipramine was $2{\cdot}4 \times 10^{-4}$ M/l.

Table 1. Anticholinesterase activity of tricyclic antidepressants on rat brain acetyl cholinesterase

	K_i	I_{50}
Amitriptyline HCl	$1{\cdot}26 \times 10^{-4}$	$1{\cdot}20 \times 10^{-3}$ M/l
Imipramine HCl	$1{\cdot}23 \times 10^{-4}$	$1{\cdot}25 \times 10^{-3}$ M/l
Desmethyl amitriptyline HCl	$2{\cdot}59 \times 10^{-4}$	$2{\cdot}05 \times 10^{-3}$ M/l
Desmethyl imipramine HCl	$2{\cdot}52 \times 10^{-4}$	$2{\cdot}51 \times 10^{-3}$ M/l
Protriptyline HCl	$2{\cdot}14 \times 10^{-4}$	$2{\cdot}37 \times 10^{-3}$ M/l

desmethyl derivatives and protriptyline approximately one half as potent. None of the compounds however, can be considered to be potent anticholinesterases.

The effect of the tricyclic antidepressants on acetyl choline synthesis

Preliminary experiments were carried out to test the effect of amitriptyline on the *in vitro* synthesis of acetylcholine (ACh) in rat brain cortex slices. Concentrations of

amitriptyline at 10^{-4}, 10^{-5} and 10^{-6} M/l. were tested. A difficulty was encountered because of the marked atropine-like action of amitriptyline at the highest concentration used. This was sufficient to affect the sensitivity of the test preparation. Consequently the finding of a slight depression of ACh synthesis at 10^{-4} M amitriptyline must be viewed with some caution. However, at 10^{-5} M amitryptyline there was a slight but significant ($P \leqslant 0{\cdot}05$) increase in ACh synthesis in the presence of the anti-depressant (Table 2). Amitriptyline at 10^{-6} was without effect.

TABLE 2. THE EFFECT OF AMITRIPTYLINE (10^{-5} M) ON THE *in vitro* SYNTHESIS OF ACh BY RAT CORTEX SLICES

	Rate of synthesis of ACh in μg/g/hr			
Control	30·2	37·5	31·3	26·5
Test	34·2	51·2	38·0	34·0

Because of the atropine-like action of amitriptyline we decided to study the effects of chronic administration on the ability of the brain to synthesize ACh. Female rats, weighing from 160–180 g were injected with the drug for periods of from 2–14 days. An initial dose of 5 mg/kg was given on the first day, followed by 3·5 mg/kg on each succeeding day. Control animals were injected with sterile saline. At the end of the period of treatment the animals were killed in the usual way; and two slices from one hemisphere were incubated in the high K^+ medium as described above.

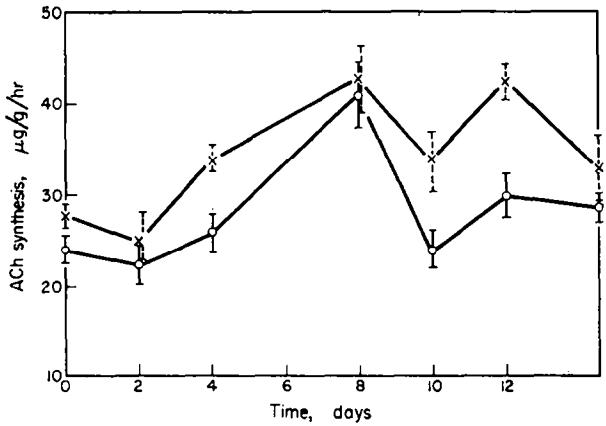


FIG. 3. The effect of chronic administration of amitriptyline to rats. Acetyl choline synthesis was measured in brain slices taken from such rats, and may be seen to rise after day 4. The lower curve O——O represents ACh synthesis in the first slice taken from the cortex; the upper curve ×——× represents ACh synthesis in the second slice.

It was found that from day 4 onwards there was a tendency for a greater synthesis of ACh to occur in the treated rat brains. This tendency reached a maximum at 8 days (see Fig. 3) when both the brain slices produced a significantly increased amount of ACh ($P < 0{\cdot}01$). This increase was less obvious at 10 days, when only the second

slice showed a significantly increased ACh production. At 12 and 14 days both slices showed significant increases ($P \leqslant 0{\cdot}05$).

It will be noted from Fig. 3 that the second cortical slice showed a consistent tendency to produce a greater amount of ACh than the first, or top-most slice. The difference was only significant in some circumstances (see in particular, day 12).

In general the results show some scatter, We found, in common with other workers in the field, that different animals show considerably different rates of ACh synthesis. Our range in values of ACh synthesized in untreated animals is from 15–42 μg/g/hr, whilst McLennan and Elliott[6, 7] found a range of from 18–52 μg/g/hr. It is clear that small changes in the ACh synthesizing power of the brain, due to the drug treatment, will not be easy to detect.

Histological examination of the tissue

The reason for the apparent difference in ACh synthesis between the first and second slices of the cortex was pursued further. It is known that the number of neuronal cells present in the cortex varies with depth. A histological investigation was carried out to determine whether the cortical slices used in our experiments showed any clear-cut difference in cell distribution.

It was noted that cortical slices obtained in the usual way, and then subjected to histological processing, varied considerably in thickness. Variation occurred from one part of a slice to another, and also occurred between slices. The average thickness of the first cortical slice (allowing for 10 per cent shrinkage due to processing) was 0·26 mm, but slices varied from 0·13 to 0·38 mm in thickness. The average thickness of the second slice was 0·21 mm; the same variation in thickness was found.

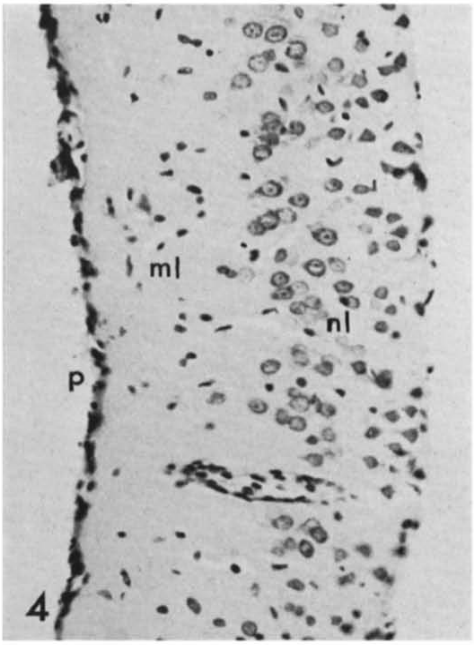
As may be seen from Fig. 4; the first slices were composed of pia mater, and approximately equal amounts of molecular layer and neuronal layer. Some of the thinner slices were composed almost entirely of molecular layer (Fig. 6). The second slices (Fig. 5) were composed of neuronal layer with an annulus of molecular layer, which in some cases had a volume approaching that of the neuronal layer. The annulus of molecular layer is an inevitable consequence of the geometry of the brain, and the method of slicing it.

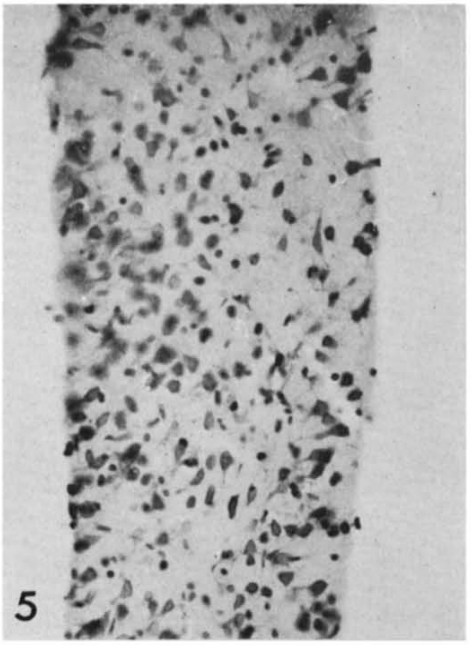
It is clear that, whilst it is probable that the second layer cut from the cortex contained a slightly larger proportion of neuronal cells than the first, many instances must have occurred when the neuronal cells were equally distributed in the two slices.

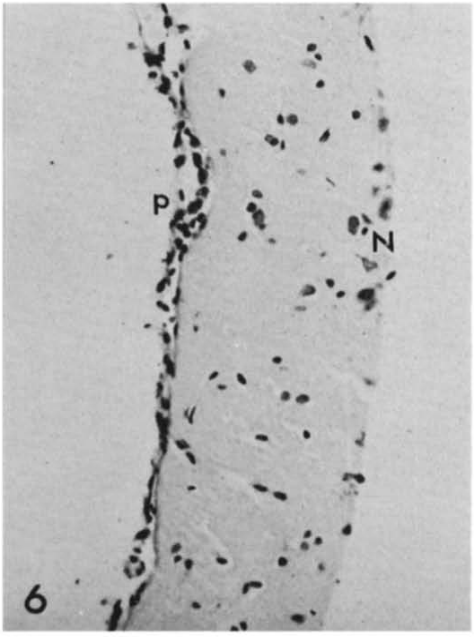
This means that one cannot state with any confidence that ACh synthesis is greater in the neuronal layer than in the molecular layer, although the evidence seems to point in this direction.

The effect of tricyclic antidepressants on brain slice respiration

The increased ability of the cortex to synthesize ACh after chronic treatment with amitriptyline could represent a stimulation of metabolic processes, leading to an increased level of acetyl co-enzyme A available for the acetylation of choline. On the other hand, it could represent a more specific effect on the enzyme choline acetylase. It has been shown previously in this laboratory[12] that amitriptyline has a stimulatory effect on the conversion of heart glycogen phosphorylase *b* to phosphorylase *a*. Should this represent an increase in the cellular level of cyclic 3: 5 AMP then other rate limiting steps in glycolysis (for example phosphofructokinase) could also be relieved.







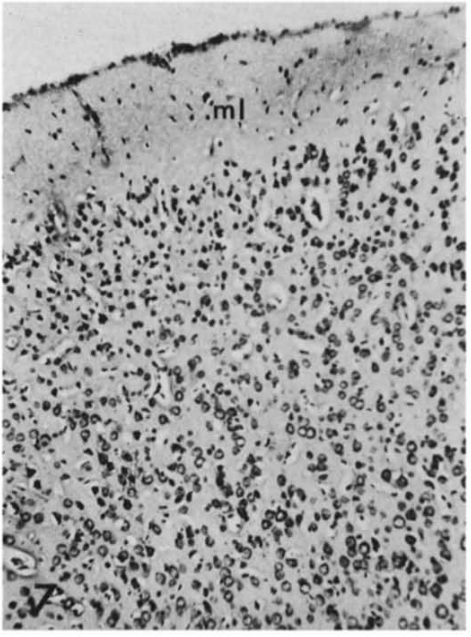


FIG. 4. Section of a typical first slice from the parietal cortex, showing pia mater (p), molecular layer (*ml*) and neuronal layer (nl). Scattered microglia are present in the molecular layer. Magnification $\times 320$.

FIG. 5. A typical second slice from the parietal cortex, showing a larger population of neurones than the first slice. Magnification $\times$ 320.

FIG. 6. A thin slice from the parietal cortex, showing that in some instances slices taken consisted almost entirely of pia mater (p) and molecular layer. A few neuronal cells are present at N. Magnification $\times$ 320.

FIG. 7. A coronal section through fixed whole brain, showing the molecular layer and the distribution of neuronal cells. Magnification $\times$ 100.

facing page 822

The effect of amitriptyline and imipramine on the oxygen uptake of cortex slices was investigated, to determine whether these drugs have a stimulatory effect on oxidative metabolism. The concentrations of amitriptyline were 2×10^{-4}, 1×10^{-4} and 1×10^{-5} M; imipramine was used at 1×10^{-4} M.

Neither of the antidepressants was found to produce any change in the oxygen consumption of the cortical slices. The drugs were tested both on the basal oxygen uptake of the tissue, and after stimulation with 48 mM K^+.

It is appreciated that these drugs do not exert their therapeutic effect until after appriximately 8 days treatment. It may consequently be unwise to look for changes in oxidative metabolism in an acute experiment. The difficulty, however, of picking up what could be expected to be a small change in respiration in chronically treated animals would seem to be overwhelming.

DISCUSSION

It would appear from the present investigation that the tricyclic antidepressants have definite, but not marked, effects on cholinergic mechanisms in the rat brain.

Our findings of a mild anti-cholinesterase activity throughout the series is in contrast to that of Osborne and Sigg[13] who reported an I_{50} value for imipramine of $0{\cdot}4 \times 10^{-6}$ M/l. This value is more than a thousand times lower than was found in the present study. The difference would seem too great to ascribe to a different assay technique (Osborne and Sigg used a colorimetric method), the only other possible difference is that those authors used a purified preparation of acetyl cholinesterase, whereas we used a brain extract. Brain cholinesterase is likely to be heterogeneous; some fractions may have a very low affinity for the antidepressants.

The increased ability of cortical slices from chronically treated animals to synthesize ACh would appear to have therapeutic significance, particularly in view of the fact that the effect is only clearly demonstrable after 8 days treatment. This time interval coincides approximately with the period required for the antidepressant effect of the drugs to become manifest.

The atropine-like action of amitriptyline has been commented upon by other authors,[6,14] and may modify the muscarinic actions of ACh in the CNS.

The increased ability of the treated rat cortex to synthesize ACh, and the decreased ability to degrade it, would seem at variance with the atropine-like action of the antidepressants. One may however, speculate that chronic treatment with these drugs may potentiate nicotinic actions of ACh in the CNS, whilst muscarinic actions are blocked.

The difficulty of obtaining cortical slices which can be referred to a particular cell type makes it impossible to say definitely where in the cortex the changes seen are operative. Krnjevic and Phillis[15] have demonstrated ACh sensitive neurones in the cat cerebral cortex but it is not possible to equate these neurones with our "second slice."

The present discussion does not exclude the possibility of an effect on adrenergic mechanism for these drugs. It is possible indeed that the suggested potentiation of a nicotinic mechanism may modulate catecholamine release in the CNS. Such a postulate would resolve the apparent anomaly that CNS depressants such as pentobarbitone and γ-butyrolactone have been shown to increase cortical levels of ACh.[16]

Acknowledgements—This work was carried out with the aid of a grant from the University of Melbourne Medical Research Committee. Mr. Ho and Miss Lloyd were recipients of University of

Melbourne post-graduate scholarships. The authors are indebted to Dr. K. Cairncross for reading the manuscript. The drugs used were a gift from Merck, Sharp and Dohme (Australia) Pty, Ltd.

REFERENCES

1. E. E. Sigg, *Canad. Psychiat. Ass. J.* **4**, 875 (1959).
2. K. D. Cairncross S. Gershon and I. D. Gust, *J. Neuropsych.* **4**, 224 (1963).
3. K. D. Cairncross, *Archs int. Pharmacodyn. Ther.* **154**, 438 (1965).
4. A. K. S. Ho, B. M. Paddle and S. E. Freeman, *Biochem. Pharmac.* **14**, 151 (1965).
5. J. A. Cohen and R. A. Oosterbaan, *Handbuch Exp. Pharm.* **15**, 326 (1963).
6. H. McLennan and K. A. C. Elliott, *Am. J. Physiol.* **163**, 605 (1950).
7. H. McLennan and K. A. C. Elliott, *J. Pharm. exp. Ther.* **103**, 35 (1951).
8. H. McIlwain and R. Rodnight, *Practical Neorochemistry*. Churchill, London (1962).
9. H. C. Chang and J. J. Gaddum, *J. Physiol.* **79**, 255 (1933).
10. H. C. Chang, Y. T. M. Lin and T. Y. Lin, *Proc. Soc. exp. Biol. Med.* **70**, 129 (1949).
11. H. Lineweaver and D. Burke, *J. Am. Chem. Soc.* **56**, 658 (1934).
12. D. G. Satchell, K. D. Cairncross and S. E. Freeman, *Biochem. Pharmac.* **13**, 1683 (1964).
13. M. Osborne and E. B. Sigg, *Archs int. Pharmacodyn. Ther.* **129**, 273 (1960).
14. V. G. Vernier, *Dis. nerv. Syst.* **22**, 7 (1961).
15. K. Krnjevic and J. W. Phillis, *J. Physiol.* **166**, 296 (1963).
16. N. J. Giarman and K. F. Schmidt, *Br. J. Pharmac.* **20**, 563 (1963).