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THE EFFECT OF A TETRACYCLIC ANTIDEPRESSANT COMPOUND, ORG GB94*, ON THE TURNOVER OF BIOGENIC AMINES IN RAT BRAIN

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Abstract—The acute administration of Org GB94 resulted in an increased incorporation of [^{3}H]tyrosine into [^{3}H]noradrenaline in the cerebral cortex, brain stem and mid-brain of the rat. The incorporation of labelled tyrosine into dopamine was slightly increased in the cerebellum. Following the chronic administration of Org GB94, the incorporation of tritiated tyrosine into noradrenaline was increased, whereas the incorporation of this amino acid into dopamine was unaffected. The incorporation of [^{3}H]tryptophan into [^{3}H]serotonin was unaffected after either the acute or chronic administration of the compound. It is concluded that Org GB94 increases the turnover of noradrenaline in the rat brain and in this respect differs qualitatively in its action from the tricyclic antidepressants. When rats were injected intraperitoneally with 3 mg Org GB94 daily for 14 days, the plasma levels decreased from approximately 170 ng/ml at 1 hr after the last dose to approximately 2.5 ng/ml after 24 hr. The effect of Org GB94 on the turnover of noradrenaline was most marked 24 hr after the chronic dose. The brain concentration of Org GB94 decreased from 3400 ng total brain at 1hr after the last dosing to 95 ng/total brain at 24 hr after dosing. There does not appear to be a correlation between the increase in brain noradrenaline turnover and the concentration of the drug in the brain. No significant differences were found between the concentration of Org GB94 after a single dose and the last dose of chronic treatment. There is clinical evidence that Org GB 94 (1,2,3,4,10,14b-hexahydro-2-methyl-dibenzo(c,f,) pyrazino-(1,2-a) azepine monohydrochloride) has antidepressant properties. It is widely accepted that endogenous depression results from a deficiency of noradrenaline and/or serotonin at post synaptic receptor sites within the brain. It is generally thought that the efficacy of the tricyclic antidepressants of the imipramine type is due to their ability to reduce the re-uptake of these biogenic amines from the synaptic cleft into the pre-synaptic neurone thereby increasing the effective concentration of the amines at the receptors. Neurochemical evidence suggests that Org GB94 has effects on brain monoamine metabolism which differs from those of the tricyclic antidepressants.

The antagonism by Org GB94 of the head twitch response in mice to the administration of 5-hydroxytryptophan suggests that the drug acts on tryptaminergic receptors in the rodent brain; studies of its effects on the central nervous system show that it has properties which bear some resemblance to those of minor tranquilizers [1, 2].

The present study was undertaken to extend the scope of the previous studies and to investigate the effects of Org GB94 on the turnover of noradrenaline, dopamine and serotonin. An attempt was also made to correlate changes in brain amine turnover with plasma and brain concentrations of the drug.

The finding that Org GB94 increases the turnover of brain noradrenaline, whereas tricyclic antidepressants of the imipramine type reduce noradrenaline and/or serotonin turnover in the brain [7, 8], suggests that it is unnecessary for a drug to have an effect on amine metabolism similar to tricyclic antidepressants before it is considered to be a potential antidepressant drug.

METHODS

In all experiments, male Wistar rats were used. They were housed under standard animal house conditions, until the commencement of the experiment when they were randomly assigned to cages in groups of five.

Acute experiments. In the first acute experiment, groups of male rats (80–90 g) were treated with Org GB94 (10 or 20 mg/kg i.p.) for 100 min. These doses were approximately 1/6th of the acute LD_{50} [2]; the period of pretreatment had previously been found to coincide with that for the peak effect of the compound on brain amine metabolism [9]. The control group was injected with saline.

Exactly 60 min after the administration of Org GB94, all rats were injected intraperitoneally with 50 μCi of [^{3}H]tyrosine and 50 μCi of [^{3}H]tryptophan (sp. act. 32 mCi/m-mole and 1.5 mCi/m-mole respectively) in a total volume of 0.5 ml. The rats were decapitated exactly 40 min after the injection of the precursor amino acids, the brains rapidly placed on a cooled Petri dish and any adhering blood clots removed. The brains were homogenized in 0.4 N perchloric acid and, after centrifugation, the concentration and radioactivity of tyrosine, tryptophan, noradrenaline, dopamine and serotonin determined by the method of Neff *et al.* [10].

In the second acute experiment groups of 20 male rats (80–90 g) were treated with Org GB94 (20 mg/kg i.p.) for 100 min. Tritiated tyrosine and tryptophan were injected as described above before killing the animals by decapitation. The brains were dissected into the brain stem region (pons, medulla), mid-brain (thalamus, hypothalamus, hippocampus, striatum, tegmentum, colliculus and "amygdaloid cortex"), cere-

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bellum and cortex as described elsewhere [9]. The reliability and reproducibility of this dissection method has been checked histologically. In this experiment brain areas from two rats were pooled for all determinations. The concentrations and radioactivities of the amines, tyrosine and tryptophan were determined by the method of Neff *et al.* [10].

Chronic experiments. In the chronic experiment, groups of 10 rats, initially weighing 50–60 g, were injected i.p. with Org GB94 twice daily for a period of 3 weeks; the dose given was 30 mg/kg/day. The body weight, food and fluid intake were determined thrice weekly. The control groups were injected with physiological saline. After 3 weeks treatment, one group of the drug treated rats was killed 2 hr after the last injection of Org GB94; the tritiated amino acids were injected 40 min before decapitation. A second experimental group was killed 22 hr after the first group. The radioactivity and concentration of the amino acids and amines was determined in extracts of the whole brain (after removal of the cerebellum) by the method of Neff *et al.* [10].

The results of these experiments are expressed as the specific activity of the amino acids and amines. The incorporation of the labelled precursor amino acids into the amines is expressed as the conversion index (ratio of the specific activity of the amine to that of its precursor) essentially as described by Costa and and coworkers [10, 11]. This method of expressing the results fails to correct for the efflux of the amines from their storage "pools". However, providing the estimations are made shortly after injection of the labelled precursors it can be used for comparative studies for the incorporation rates of labelled amino acids into the amines.

All results are expressed as the mean values together with the 95% confidence limits instead of the 90% confidence limits for the percentage change as described by van Riezen and Delver [13].

Plasma and brain concentration of Org GB94 after acute and chronic administration. Rats (body wt approx. 100 g) were dosed intraperitoneally with 3 mg of Org GB94 per a day during 14 days. On day 14 the rats were killed 1, 2, 3 and 24 hr after administration of the compound. Blood was collected in heparinized conical flasks and the total brain was dissected and homogenized in 4 ml of perchloric acid solution. Rats, receiving a single i.p. dose of 3 mg Org GB94 were killed at 1, 2 and 4 hr after dosing, blood and brain samples were collected at these times. Plasma was obtained by centrifugation of the blood samples at 3000 rpm for 15 min.

Org GB94 was isolated from the plasma and brain homogenates by a procedure which can be summarized as follows: to samples of 1 ml of plasma or 1 g of brain homogenate, 100 μl of concentrated ammonia was added together with an amount of the internal standard (10, 10d_2-Org GB94) which was approximately equal to the expected Org GB94 concentration. These mixtures were extracted twice with 5 ml *n*-hexane and combined hexane extracts were evaporated to dryness under nitrogen. The *plasma extracts* were purified from interfering materials by high pressure liquid chromatography using a micro-Porasil column 30-cm, lay by 4-mm i.d. and the elution system hexane: isopropanol = 9 : 1 (v/v) containing 4% ethanol and 0·1% ammonia.

The acidified brain extracts were purified by washing with three 5-ml portions of hexane followed by a back extraction with two 5-ml portions of hexane at pH = 10. After purification the extracts were evaporated to dryness and the residues were redissolved in 10 μl methanol. For quantification, 2 μl of this methanolic solution were injected into a gas chromatograph–mass spectrometer system (GC–MS) consisting of

(i) a Varian Aerograph type 2740 Gaschromatograph equipped with a 2-m long by 2-mm i.d. glass column filled with 1% OV-1 on gaschrom Q, operating at 230° column temperature;

(ii) a Varian MAT CH7 mass spectrometer equipped with a peak matching device and operating at 70 eV electron energy and approx. 135° ion source temperature;

(ii) a Varian MAT Spectro System 100 MS.

The peak heights of *m/e* 264 and *m/e* 266, the molecular ions of Org GB94 and of 10,10d_2-Org GB94, respectively, were continuously monitored. From these data the concentration of Org GB94 per ml of plasma or in total brain was calculated using a BASIC computer program. After single-dose-only single determinations were performed whereas after chronic treatment the samples were measured in triplicate experiments. Tissue samples estimated at the same time as the determination of the turnover rate were estimated in quadruplicate.

RESULTS

Effect of Org GB94 on amine turnover. In both acute and chronic experiments the gross behaviour of the rats appeared to be unaffected by Org GB94. There was no evidence of sedation or hypothermia. In the first acute experiment the higher dose of Org GB94 reduced the specific activity of tyrosine and increased that of noradrenaline (Table 1). The conversion index for noradrenaline was raised as was that for serotonin although the rise did not reach the 95% level of statistical significance.

The concentrations of tyrosine and noradrenaline were reduced following administration of Org GB94; this effect was more marked with the 20 mg/kg dose. In the second acute experiment, the specific activity of noradrenaline in the cortex, midbrain and brainstem was raised following the administration of Org GB94; the conversion index for this amine was also significantly increased in these brain regions (Table 2). Org GB94 treatment did not change the specific activity of serotonin in the cortex, cerebellum or brain stem, but it did increase the specific activity of this amine in the midbrain; there was no significant change in the conversion index for serotonin in any of the brain regions. The specific activity of dopamine was raised in the brain stem and its conversion index increased in the mid-brain. Apart from a slight decrease in the endogenous concentration of tyrosine in the midbrain, the steady-state levels of the precursors and amines were not significantly affected by Org GB94 treatment.

In the chronic experiment the experimental rats had a slightly lower body weight (approximately 10 per cent) compared with the control animals. However, there were no signs of gross behavioural abnor-

Table 1. Effect of the acute administration of different doses of Org GB94 on the turnover of biogenic amines in the whole rat brain

Drug and dose (mg/kg)	Tyrosine	Dopamine	Noradrenaline	Tryptophan	Serotonin
		Concentration (nmoles/g)			
Control	84·10	4.09	3.45	13·30	3·89
Org GB94 (10)	69.4* (−32.0)	3.73 (−38, +34)	3.01 (−32, +11)	14.80 (−35, +89)	3.49 (−47, +51)
Org GB94 (20)	62.8* (−39, −9)	3.72 (−38, +34)	2.09* (−53, −23)	11.50 (−49, +47)	3.80 (−42, +64)
		Specific activity (dpm/g wet wt)			
Control	60.7	37.9	22.1	66.3	23.4
Org GB94 (10)	47.0* (−37, −6)	36.9 (−39, +55)	28.8 (−7, +73)	69.2 (−26, +48)	28.1 (−36, +120)
Org GB94 (20)	52.5 (−29, +5)	40.9 (−32, +72)	43.2*(+49, +91)	64.1 (−32, +37)	31.1 (−29, +150)
		Conversion index (sp. act. amine/sp. act. precursor)			
Control	—	1.25	0.72	—	0.43
Org GB94 (10)	—	1.57 (−21, +102)	1.23* (+21, +104)	—	0.48 (−40, +116)
Org GB94 (20)	—	1.56 (−22, +98)	1.65* (+62, +120)	—	0.57 (−29, +115)

Rats treated with Org GB94 (10 or 20 mg/kg i.p.) for 100 min; control animals were injected with physiological saline.

All rats were given a pulse injection of triated tryptophan and tyrosine 40 min before decapitation.

* Difference between control and experimental group significant at $P < 0.05$. Results given as mean values; 95% confidence limits for the percentage change are shown in parenthesis.

malities and the animals were healthy for the complete period of treatment. It was essential to administer the drug parenterally throughout the chronic treatment because it was found in initial experiments that the local anaesthetic properties of the compound made drinking too unpalatable for the rats; consequently they drastically reduced their fluid intake.

In the chronic experiment, the specific activity and the conversion index was increased in the groups of rats which were treated with Org GB94 and killed either 2 or 24 hr after the last dose (Table 3). Although there was no significant change in the concentration of the catecholamines following the chronic treatment, brain tryptophan levels were reduced as was the specific activity of serotonin. There was no significant change in the conversion index for serotonin.

Changes in the plasma and brain concentrations of Org GB94 after acute and chronic administration. Mean values of Org GB94 plasma levels and brain levels at various times after chronic or single i.p. doses are shown in Figs. 1 and 2. The data are compiled in Tables 4 and 5.

It should be noted that because of our limited experience in analyses of brain tissue and also due to the fact that the brain and plasma samples were stored for a long period, the accuracy (deviation from the "true" value) of the analyses is expected to be

Table 2. Effect of the acute administration of Org GB94 on the turnover of biogenic amines in rat brain areas

	Tyrosine		Dopamine		Noradrenaline		Tryptophan		Serotonin	
	Cortex	Cerebellum	Cortex	Cerebellum	Cortex	Cerebellum	Cortex	Cerebellum	Cortex	Cerebellum
Concentration (nmoles/g)	73.62 65.84 (−29, +12)	71.35 57.76 (−39, +8)	3.67 3.70 (−33, +51)	.1.03 0.81 (−43, +9)	2.16 2.02 (−33, 31)	3.28 2.82 (−37, +17)	19.12 18.89 (−44, +77)	17.37 18.24 (−31, +60)	2.95 3.28 (−12, +41)	3.27 3.63 (−13, +42)
Sp. act. (dpm/g)	51.34 50.49 (−18, +19)	59.30 61.88 (−15, +28)	20.31 27.26 (−13, +107)	18.08 21.47 (−80, +66)	17.33 27.00* (+3, +136)	5.31 8.08 (−42, +98)	51.98 56.57 (−36, +85)	89.33 97.14 (−15, +38)	22.88 30.53 (−11, +99)	11.51 16.10 (−12, +123)
Conversion index (sp. act. amine: sp. act. amino acid)	—	—	0.79 1.08 (−16, +119)	0.70 0.71 (−99, +131)	0.67 1.07* (+2, +145)	0.18 0.35 (145, +101)	—	—	0.53 0.65 (−7, +60)	0.15 0.20 (−10, +82)
	Brain stem	Mid-brain	Brain stem	Mid-brain	Brain stem	Mid-brain	Brain stem	Mid-brain	Brain stem	Mid-brain
Concentration	98.42 81.25 (−52, +43)	83.01 71.18* (−20, −8)	0.40 0.60 (−20, +108)	2.96 2.98 (−21, +28)	3.43 3.26 (−14, +5)	1.51 1.46 (−26, +25)	17.43 17.39 (−22, +27)	9.22 10.93 (−3, +45)	3.62 3.68 (−35, +59)	2.34 2.41 (−19, +30)
Sp. Act.	24.68 34.27 (−23, +150)	22.42 30.90 (−12, +116)	25.28 49.38* (+1, +180)	43.18 51.72 (−10, +59)	13.22 24.69* (+12, +212)	28.54 50.09* (+18, +161)	37.93 56.48 (−2, +127)	77.80 81.84 (−16, +32)	41.38 65.15 (−40, +119)	28.92 36.82* (0, +62)
Conversion index	—	—	2.04 2.75 (−36, +181)	1.71 2.36* (+6, +79)	1.07 1.79* (+2, +182)	1.19 2.29* (+24, +196)	—	—	1.30 1.38 (−43, +96)	0.44 0.54 (−11, +65)

Rats treated with Org GB94 (20 mg/kg i.p.) for 100 min; control animals were injected with physiological saline. All rats were given a pulse injection of tritiated tryptophan and tyrosine 40 min before decapitation.

* Difference between control and experimental animals significant at $P < 0.05$. Results given as mean values; 95% confidence limits for the percentage change are shown in parenthesis.

Table 3. Effect of the chronic administration of Org GB94 on the turnover of biogenic amines in the rat brain

	Tyrosine		Dopamine		Noradrenaline		Tryptophan		Serotonin	
	2 hr	24 hr	2 hr	24 hr	2 hr	24 hr	2 hr	24 hr	2 hr	24 hr
					Concentration (nmoles/g)					
Control	113.3	98.0	4.95	4.36	0.59	0.67	11.25	12.35	3.38	1.80
Org GB94	96.4	60.7	4.37	5.16	0.56	0.76	7*.65	8*.27	3.33	2.10
	(−47, +37)	(−18, +103)	(−39, +29)	(−2, +43)	(−46, +64)	(−19, +60)	(−53, 0)	(−52, −6)	(−28, +35)	(−35, +112)
					Specific activity (dpm/g wet wt)					
Control	100.5	92.3	49.7	45.8	230.6	169.8	116.4	99.6	164.1	103.1
Org GB94	59.3	64.7	34.5	35.1	661*.2	325*.9	64.2	117.2	77*.5	77.9
	(−99, +62)	(−81, +90)	(−99, +37)	(−85, +190)	(+13, +149)	(+46, +98)	(−76, +29)	(−85, +84)	(−100,0)	(−76, +134)
					Conversion index (sp. act. amine/sp. act. precursor)					
Control			0.99	0.87	6.77	3.20			1.41	1.05
Org GB94			1.09	1.16	12.00	6*.65			0.79	1.45
			(−36, +87)	(−84, +99)	(−63, +75)	(+12, +109)			(−100, +77)	(.19, +78)

Rats treated with Org GB94 for 2 weeks (30 mg/kg i.p. daily) and killed either 2hr or 24hr after the last dose of the drug. Control animals were injected daily with phyrinogical saline. All rats were given a pulse injection of tritiated tryptophan and tyrosine 40 min. before decapitation.

* Difference between control and experimental group significant at $P < 0.05$. Results given as mean values: The 95% confidence limits for the percentage change are shown in parenthesis.

not greater than 20 per cent. The values thus indicate more the order of magnitude rather than being an accurate estimation of the tissue concentrations of Org GB94.

No significant differences were observed in plasma or brain levels of Org GB94 between the chronic administration of the drug and one single dose.

DISCUSSION

The results of this investigation show that Org GB94 increases the rate of incorporation of tritiated tyrosine into noradrenaline which suggests that the drug increases the turnover of the amine. Following the "pulse" injection of the labelled amino acids, a proportion of the precursors mix with the endogenous tyrosine and tryptophan. The accumulation of [^{3}H]serotonin and [^{3}H]noradrenaline during any interval is dependent on the amine synthesis rate, the main specific activity of the precursor amino acid during the interval and the degree of retention of the newly formed amine by the endogenous stores. During the relatively short time interval of the present studies, most of the labelled amines formed are still

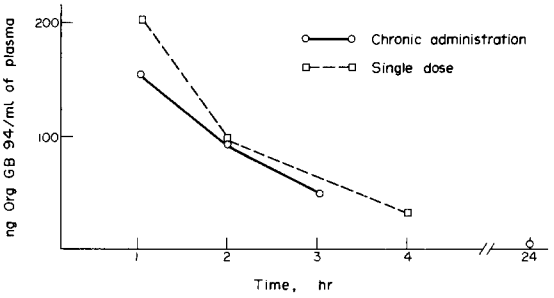

Fig. 1. Mean values of Org GB94 expressed as ng/ml of plasma, at various times after the (last) i.p. dose (30 mg/kg) to rats; single dose or after chronic treatment (1 dose/day).

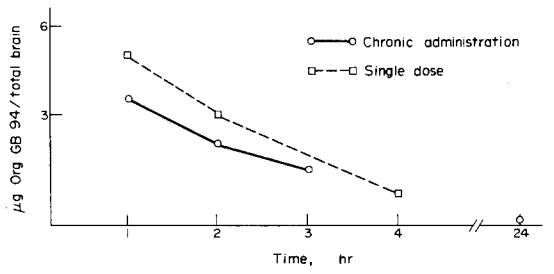

Fig. 2. Mean values of Org GB94, expressed as μg/total brain at various times after the (last) i.p. dose (30 mg/kg) to rats; single dose or after chronic treatment (1 dose/day).

Table 4. Concentrations of Org GB94 circulating in the plasma and localized in the brain of individual rats at 2 and 24 hr after a chronic i.p. dose of the compound

Number of the rat in the expt	Days of treatment	Time after dosing (hr)	ng Org GB94 per ml plasma	ng Org GB94 per total brain
1	14	2	65	1300
2	14	2	65	1700
3	14	2	167	3400
4	14	2	103	2200
	mean ± S.D.		100 ± 48	2150 ± 911
5	14	24	2.8	90
6	14	24	1.8	35
7	14	24	2.4	115
8	14	24	2.7	140
	mean ± S.D.		2.4 ± 0.5	95 ± 45

Table 5. Concentrations of Org GB94 circulating in the plasma and localized in the brain of individual rats, after both, chronic and a single i.p. dose of the compound

Number of the rat in the expt	Days of treatment	Time after dosing (hr)	ng Org GB94 per ml plasma	ng Org GB94 per total brain
9	13	1	188	5700
10	14	1	63	1000
11	14	1	174	3900
	mean ± S.D.		142 ± 68	3400 ± 2400
12	13	3	28	600
13	13	3	73	1700
14	14	3	68	1400
	mean ± S.D.		56 ± 25	1200 ± 600
15	single dose	1	203	4800
16	single dose	2	95	3000
17	single dose	4	28	700

retained at the time of the death of the animals so that the [^{3}H]catecholamine and [^{3}H]serotonin content will reflect the rate of synthesis of these amines [14]. Under the conditions described for our investigations it has been found that the rate of incorporation of tritiated amino acids into the catecholamines and serotonin is linear for approximately 60 min following injection. It is not unreasonable to assume that the turnover rate of the brain amines is equal to their rate of synthesis, which implies that a steady state exists in which the synthesis and transport of the amines into a metabolic compartment equals their breakdown and exit [15]. This seems to be a valid assumption as the blood brain barrier largely prevents the influx of catecholamines or serotonin which may be formed at the periphery [16].

Thus the results of our investigation may be interpreted as evidence that the acute and chronic administration of Org GB94 increases the turnover of noradrenaline. This confirms the previous findings that Org GB94 increases the rate of depletion of noradrenaline, and to a lesser extent of dopamine, which occurs following the administration of the tyrosine hydroxylase inhibitor α-methyl-*p*-tyrosine [9]. However, in the previous study it was found that this compound also decreases the turnover of serotonin in the mouse brain. The reason for the different effects of Org GB94 on the turnover of this amine in the rat and mouse is unclear. From the study of the acute effects of Org GB94, there is evidence that the turnover of noradrenaline is increased in three of the four regions of the rat brain studied; the effect on dopamine and serotonin turnover was less marked. There is evidence from the previous studies of Org GB94 that striatal dopamine metabolism is affected by this drug [9] and it is not without interest that most of the neuroleptics in clinical use have been found to increase the turnover of dopamine in the basal ganglia [17, 18].

The data demonstrate that a large biological variation occurs in the concentration of Org GB94 in plasma and brain 2 and 24 hr after the last chronic dose. From the results shown in Table 3, it is apparent that the major change in the conversion index occurs 24 hr after the last chronic dose when the plasma and brain concentrations of the drug are low. No significant differences are observed between the levels after a single dose and after a dose in a chronic treatment (1 dose per day, Table 5). This conclusively suggests that there is no accumulation of the unchanged drug in brain or plasma after longer treatment. This is also in agreement with the relatively low levels measured 24 hr after the last chronic dose (see Figs. 1 and 2).

The relatively high levels of Org GB94 in the brain (3–5 μg/g brain) 1 hr after dosing as compared with the plasma levels of the unchanged drug (e.g. 160–200 ng/ml plasma 1 hr after dosing) confirms the earlier observed results [28, 29] that Org GB94 is concentrated in the brain after i.p., i.v. or p.o. administration.

The results of these experiments suggest that Org GB94 has an action on brain amine metabolism which appreciably differs from that of the tricyclic antidepressants of the imipramine type. In common with the many investigators who have studied the action of the tricyclic antidepressants on amine turnover in the rat brain, Schubert, Nyback and Sedvall [19], showed that these drugs reduce the turnover of noradrenaline and/or serotonin; their relative effect [24] on the turnover of these amines is dependent on their chemical structure. Leonard and Kafoe [24] have also shown that imipramine, desipramine and amitriptyline reduce the turnover of noradrenaline and serotonin following the chronic administration of these drugs. Presumably the tricyclic antidepressants reduce amine turnover by feedback inhibition of synthesis at the rate limiting step; this is thought to be a consequence of the inhibition of the amine re-uptake mechanism which results in an increased amine concentration at the post synaptic receptor sites [20–23]. There is no evidence from either the present study nor the previous study, in which Org GB94 was found to have no effect on the uptake of [^{14}C]serotonin into rat cerebral cortex slices [9], to suggest that the drug acts in the same way as the tricyclic antidepressants although Goodlet and Sugrue [25] found that this drug reduced the uptake of metaraminol in some brain regions *in vitro* and interpreted the results as suggesting that Org GB94 had a qualitatively similar action to the imipramine type of tricyclic antidepressants in that it inhibited the re-uptake of noradrenaline. From the present study it appears that Org GB94 had a profile qualitatively different from that of the dibenzazepine antidepressants. If it is assumed that endogenous depression is the result of a deficiency of biogenic amines at receptor sites within the brain, then it can be reasoned that Org GB94 exerts its antidepressant action by increasing the availability of noradrenaline at these receptors by increasing the rate of amine synthesis and release. Investigations are now in progress to test the validity of these assumptions. If following the completion of even more extensive trials it is unequivocally established that Org GB94 is a clinically effective antidepressant drug then clearly it will be necessary to reconsider the all too frequent assumption that for a compound to be considered as a potential antidepressant it must reduce amine re-uptake into nerve-endings. A precedent for this view has already been established by the finding that the tricyclic antidepressant iprindol does not affect amine turnover or re-uptake *in vivo* [24–27].

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