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EEFECTS OF REPEATED LITHIUM ADMINISTRATION ON THE SUBCELLULAR DISTRIBUTION OF 5-HYDROXYTRYPTAMINE IN RAT BRAIN

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1 The content and distribution of 5-hydroxytryptamine (5-HT) between subcellular fractions from rat whole brain (excluding cerebellum) were examined following repeated lithium administration.

2 Lithium chloride (3 mEq/kg body wt. s.c.) administered twice daily for 3 days produced no change in the 5-HT content of primary subcellular fractions (Pl—nuclear; P2—crude synaptosomal; SNT—soluble) measured on the 4th day.

3 Similarly, repeated lithium treatment alone did not appear to produce increases in the 5-HT content of either cytoplasmic (S) or vesicular (M2) fractions derived from hypo-osmotically disrupted synaptosomes (P2) when compared to control rats receiving NaCl only.

4 One hour after monoamine oxidase inhibition with tranylcypromine, there was a further selective 25% increase in 5-HT accumulation in the lithium-treated rats over control values in the soluble cytoplasmic fraction (S) and in fractions containing occluded cytoplasm only. This increase did not occur in the synaptic vesicle fraction (M2). This corresponds to an increase in 5-HT turnover rate due to the lithium treatment of approx. 50%.

5 These findings lend additional support to the hypothesis that lithium treatment alters the intraneuronal storage or compartmentation of 5-HT between vesicles and cytoplasm.

Introduction

Lithium has been used effectively for the treatment of mania and depression both alone and in conjunction with a monoamine oxidase inhibitor (Gershon & Yuwiler, 1960; Coppen, Noguera, Baitey, Burns, Swarin, Hare, Gardner & Maggs, 1971; Himmelhoch, Detre, Kupfer, Swartzburg & Byck, 1972). The possible central neurochemical changes underlying the therapeutic effect have been the subject of a number of investigations. For example gross neurochemical alterations which have been noted following chronic lithium treatment include those in various 5-hydroxytryptamine (5-HT) parameters (Perez-Cruet, Tagliamonte, Tagliamonte & Gessa, 1971; Grahame-Smith & Green, 1974; Knapp & Mandell, 1975), γ-aminobutyric acid (GABA) receptor binding (Maggi & Enna, 1980) and dopamine release (Maggi & Enna, 1980). Furthermore some of these changes (e.g. 5-HT and GABA system) appear to be brain region specific (see Ho, Loh, Graves, Hitzemann & Gershon, 1970; Maggi & Enna, 1980). The noradrenergic system is also affected and it has been demonstrated that prolonged treatment increases noradrenaline uptake in synaptosomes (Colburn, Goodwin, Bunney & Davis, 1967; Baldessarini & Yorke, 1970). The molecular basis of its neuropharmacological effect still remains unclear but an effect on neuronal membrane properties is highly apparent. For example specific actions on (Na^+–K^+) ATPase in certain brain areas, monoamine storage/binding mechanisms and dopamine sensitivity have all been reported (see Colburn *et al.*, 1967; Tobin, Akera, Ham & Brody, 1974; Komiskey & Buckner, 1974; Pert, Rosenblatt, Sivit, Pert & Bunney, 1978; Swann, Marini Sheard & Maas, 1980).

There has been particular interest in the role of the brain 5-HT system in the antipsychotic effects of lithium. Knapp & Mandell (1975) have proposed a complex series of events at the 5-

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hydroxytryptaminergic synapse after lithium administration. Short term treatment appears to potentiate tryptophan uptake and 5-HT synthesis whereas long-term treatment has an additional inhibitory effect on tryptophan hydroxylase activity thus restoring synthesis rates to control levels. It has also been shown that repeated lithium administration followed by monoamine oxidase inhibition causes a characteristic 5-HT hyperactivity syndrome in rats with concomitant increases in 5-HT synthesis (Grahame-Smith & Green, 1974). This did not appear to be due to increased brain tryptophan as suggested by Perez-Cruet *et al.* (1971) nor was there any increased postsynaptic 5-HT sensitivity as shown with 5-methoxy-N,N-dimethyltryptamine (5-MeODMT, Grahame-Smith & Green, 1974) suggesting that lithium must be acting presynaptically on some aspect of 5-HT function other than synthesis. However, two independent oberservations suggest that lithium may alter the intraneuronal compartmentation of 5-HT in rat brain resulting in more free cytoplasmic 5-HT, less bound to storage vesicles, more intraneuronal metabolism and decreased feedback inhibition on synthesis (Grahame-Smith & Green, 1974; Collard & Roberts, 1977; Collard, 1978).

The object of the present study, therefore, was to investigate further this possibility in lithium-treated rats by means of subcellular fractionation techniques. The method of Zieher & De Robertis (1963) was employed to study the subcellular distribution of 5-HT in crude synaptic vesicular and soluble fractions following repeated lithium administration. A preliminary account of this work has already appeared (8th, ISN, Nottingham, Sept. 1981).

Methods

Animal treatment

Male Wistar rats 150–200 g (Anglia Laboratories, Alconbury, Huntingdon) were used in all experiments. LiCl (3 mEq/kg) or NaCl (3 mEq/kg) (Sigma Ltd) were administered subcutaneously twice daily (early a.m. and late p.m.) for three days. Additional drinking water was made available to prevent dehydration. On the fourth day (late a.m.) rats were killed and whole brains immediately removed on to a cooled surface. In other experiments monoamine oxidase inhibition was effected by tranylcypromine (Tcp 20 mg/kg, i.p.) and the brains removed 1 h later.

Subcellular fractionation of brain tissue

Cerebella were removed from the brains and discarded, and pooled samples of 3 brains each were fractionated by a slight modification of the method of De Robertis *et al.* (see Zieher & De Robertis, 1963; De Robertis, de Iraldi, de Lores Arnaiz & Zieher, 1965). The tissue was homogenized in 0.32 M sucrose containing Tcp (1 mM) and disodium edetate (EDTA, 2 mM) and centrifuged at 1000 $g \times 10$ min (0–4°C). The P1 pellet was washed and the pooled supernatants centrifuged at 17,000 $g \times 30$ min to obtain the crude mitochondrial fraction (P2). The P2 fraction was then hypo-osmotically shocked by resuspending in 2 ml/g original wet wt tissue of ice-cold distilled water (containing 1 mM Tcp) and allowed to stand on ice for 15–30 min. The resultant suspension was subfractionated in two different ways. Firstly, by centrifugation at 11,500 $g \times 30$ min a pellet designated M1 was obtained which contained synaptic ghosts and other heavy components (see Results section). The supernatant was then centrifuged 100,000 $g \times 30$ min to give the M2 pellet containing synaptic vesicles. Alternatively a crude particulate fraction designated Wp and a soluble fraction (S) were obtained from the shocked P2 fraction by centrifugation at 100,000 $g \times 30$ min (Wp = M1 + M2). Biochemical assays were carried out on these four fractions.

5-Hydroxytryptamine determination

5-HT content of the subcellular fractions was measured by a modification of the fluorimetric procedure of Curzon & Green (1970). Particulate fractions (M1, M2, Wp) were sonicated in 10 ml ice-cold acid-butanol (Chang, 1964) until totally dispersed and then shaken with 2.0 ml of 0.2 M borate buffer (pH 10) containing 3.3 g NaCl to remove impurities (e.g. sucrose). The butanol extract was then taken through the fluorimetric procedure. The soluble fraction (S) (4 ml) was adjusted to pH 10.0 with a few drops of 1 N NaOH and 2.0 ml of 0.2 M borate buffer (pH 10.0; containing 3.3 g NaCl) plus 8.0 ml of butanol added. Following extraction, 5.0 ml of the butanol layer was taken through the fluorimetric procedure. Total recoveries through the two extraction procedures were: pellets (Wp, M1 & M2) = 50–60%; soluble fraction (S) = 55–65%. Protein was measured by the method of Lowry, Rosenbrough, Farr & Randall (1951).

Electron microscopy

Particulate fractions were fixed in suspension after washing in isotonic saline (0.9% w/v NaCl solution) and then fixed in 3% glutaraldehyde in Sorensens phosphate buffer (pH 7.3) for 1 h. After post-fixation in 1% osmium tetroxide for 1 h the pellets were dehydrated through a series of alcohols and embedded. Sections were cut on a Porter-Blum MT-2 ultramicrotome and stained with methanolic uranyl acetate and aqueous lead citrate. Grids were pre-

pared and examined on an AE1 801 electron microscope.

Results

Serum lithium concentration

Rats were given 3 mEq/kg LiCl or NaCl subcutaneously, twice daily, for 3 days at approximately 10 h 00 min and 17 h 00 min. On the fourth day approximately 19 h after the last injection, serum ion concentrations were found to be 0.83 ± 0.07 mEq/l (LiCl-treated rats, mean ± s.e.mean, $n = 15$ animals) and 0.08 ± 0.03 mEq/l (NaCl-treated rats, $n = 11$ animals) by atomic absorption spectrophotometry. These serum lithium concentrations at the time of subcellular fractionation studies were very near the optimum human therapeutic range (0.9–1.4 mEq/l E; Prien, Caffey & Klett, 1972). Grahame-Smith & Green (1974) obtained concentrations of 1.1 mEq/l using a similar treatment protocol.

Effect of lithium treatment on 5-hydroxytryptamine in primary subcellular fractions

Fractionation of the homogenate into P1, P2 and SNT fractions 17 h following 3 days' lithium treatment showed no difference in either 5-HT concentration or percentage fractional distribution between drug-treated and control groups (Table 1). Approximately 45% of the total recovered 5-HT was localized in the crude mitochondrial P2 fraction and 25% in the SNT. This is a similar distribution to that found in an early study by Zieher & De Robertis (1963); however, they found a lower proportion in the SNT fraction.

Effect of lithium treatment on 5-hydroxytryptamine content of P2 subfractions; effect of tranylcypromine pretreatment

Although there was no apparent change in the P2 content of 5-HT after lithium treatment, very small changes in the P2 subfractions (following hypo-osmotic shock) were sought. The recovery of 5-HT in Wp + S over the total original P2 fraction was 78%, and the recovery in M1 + M2 over the total original Wp fraction = 80.4% (mean 4–6 determinations). Results show (Table 2) that the percentage distribution of 5-HT between the P2-derived subfractions was again very similar to that shown by Zieher & De Robertis (1963). Around 60% was found in the M1 fraction (mitochondria, debris, synaptosome ghosts), 12% in M2 (synaptic vesicles) and 25% in S (intrasynaptosomal cytoplasm). Treatment with lithium for 3 days did not alter this distribution of neurotransmitter.

The large proportion of 5-HT in M1 could be taken to indicate a low sensitivity of 5-HT nerve endings to osmotic shock (see EM section) or that the 5-HT of nerve endings is not primarily in synaptic vesicles. However, on a protein basis, fraction M2 contained a roughly equal 5-HT concentration to M1. In contrast Zieher & De Robertis showed that approx. 50% of choline acetylase and acetylcholine are found in the M2 fraction.

In terms of the 5-HT concentration in fractions M1 and M2 per g original wet wt of brain tissue or protein content of fractions (Table 2), lithium treatment did not cause a significant alteration. There was similarly no change in Wp or S fractions. When monoamine oxidase was inhibited (Tcp administered 20 mg/kg i.p.) for 1 h before brain removal, changes in 5-HT were seen; when comparing control groups

Table 1 Primary subcellular distribution of 5-hydroxytryptamine (5-HT) between fractions derived from Li^+-treated and control rats

Drug treatment	*Unit expressed*	*Primary subcellular fraction* P1	P2	SNT
NaCl (× 3 days)	[5-HT]	0.188 ± 0.01	0.334 ± 0.02	0.193 ± 0.01
	%	26.3	46.7	27
LiCl (× 3 days)	[5-HT]	0.235 ± 0.03	0.354 ± 0.01	0.199 ± 0.01
	%	29.8	44.9	25.3

Values are mean ± s.e.mean ($n = 6$).
[5-HT] = 5-HT concentration μg/g wet wt original brain tissue; % = % distribution between fractions (% fraction over P1 + P2 + SNT).
LiCl-treated not significantly different from controls (NaCl) – Student's t test.

Table 2 5-Hydroxytryptamine (5-HT(concentrations and percentage distributions in subcellular fractions from rats treated with LiCl alone or LiCl plus tranylcypromine (20 Mg/kg × 60 min)

Drug treatment		*WP* [5-HT]	*WP* %	*S* [5-HT]	*S* %	*M1* [5-HT]	*M1* %	*M2* [5-HT]	*M2* %
× 3 days No tranylcypromine	NaCl (Control)	0.202	77.7	0.058	22.3	0.126 (2.9)	65	0.024 (3.1)	12.1
		(NS)		(NS)		(NS)		(NS)	
	LiCl	0.208	76.7	0.064	23.3	0.142 (3.3)	63.1	0.026 (3.5)	11.5
× 3 days plus tranylcypromine (20 mg/kg; 60 min)	NaCl (Control)	0.337	75.2	0.111	24.8	0.225 (5.2)	60.5	0.055 (7.2)	15.0
		($P = 0.05-1$)		(P 0.05)		(P 0.005)		(NS)	
	LiCl	0.411	74.7	0.139	25.3	0.288 (6.6)	62.8	0.055 (7.4)	11.1

Mean (no s.e.mean, $n = 6-8$ experiments). P value from Student's t test.
Figures in () parentheses = ng 5-HT/mg protein.
[5-HT] = 5-HT concentration μg/g wet wt original tissue; % = % distribution of 5-HT between subcellular fractions.

with and without Tcp pretreatment there was an increase in the 5-HT content of all fractions: Wp/M1 = 70% increase, S = 90% increase and M2 = 130% increase. Furthermore, following Tcp treatment subcellular fractions containing soluble material (S primarily, also Wp and M1) in the lithium-treated group accumulated more 5-HT than the control group (NaCl plus Tcp) by around 25% (Table 3). This futher increase did not occur in the vesicular fraction (M2). Similarly, calculation of 'synthesis' or turnover rates by the method of Neff & Tozer (1968) in the NaCl- and LiCl-treated rat fractions (by subtraction of basal 5-HT levels before Tcp administration) showed increases in turnover of around 50% in fractions containing soluble material only (S, Wp/M1) but not in M2 (Table 3). In whole brain it has been shown that a similar lithium-treatment protocol produces a 70% increase in 5-HT turnover rate (Grahame-Smith & Green, 1974).

Electron microscopical examination of subcellular fractions

In order to determine the morphological composition of the particulate fractions (M1 and M2) containing 5-HT, electron microscopy was performed. Firstly the original P2 fraction (starting material, Figure 1) can be seen to comprise many intact synaptosomes 0.5–1 μM diameter, large and small intact mitochondria, myelin fragments etc. Following hyposmotic disruption of P2, the Wp fraction was not examined but the similar 'heavy' M1 fraction (crude particu-

Table 3 Summary of 5-hydroxytryptamine (5-HT) changes in subcellular fractions after various treatments (extrapolated from Table 2)

Treatment comparison	*Unit expressed*	*WP*	*S*	*M1*	*M2*
(a) 5-HT concentration LiCl *vs* NaCl (No Tcp)	% ↑ over base	3 (NS)	10 (NS)	13 (NS)	8 (NS)
(b) 5-HT concentration LiCl *vs* NaCl (+ Tcp)	" "	22 ($P = 0.05$)	25 ($P = 0.05$)	28 ($P = 0.005$)	0 (NS)
(c) 5-HT concentration NaCl (+ Tcp) *vs* NaCl (No Tcp)	" "	67	91	78	129
(d) Increase in 5-HT 'turnover rate due to LiCl treatment	% ↑ turnover rate	50.4	42	48	0

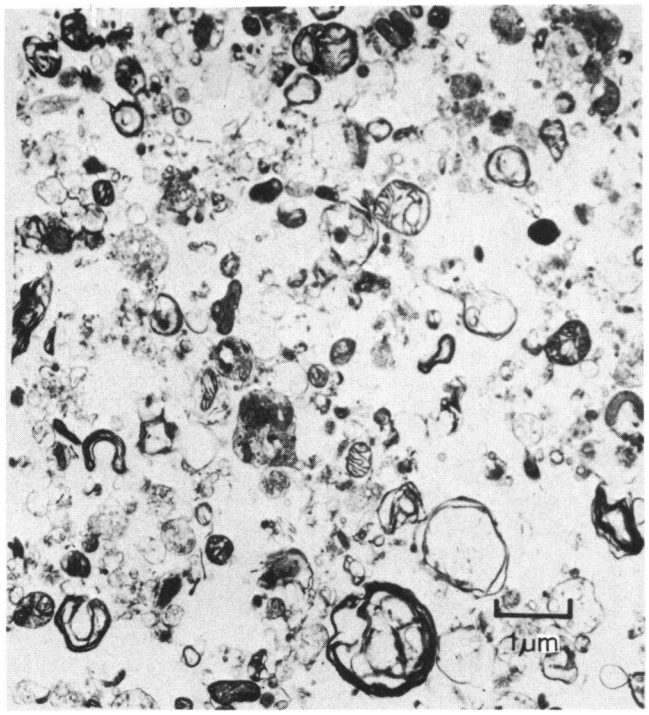

Figure 1 P2 fraction (starting material) including intact synaptosomes, large and small nutochondria and myelin fragments. Magnification × 17,250.

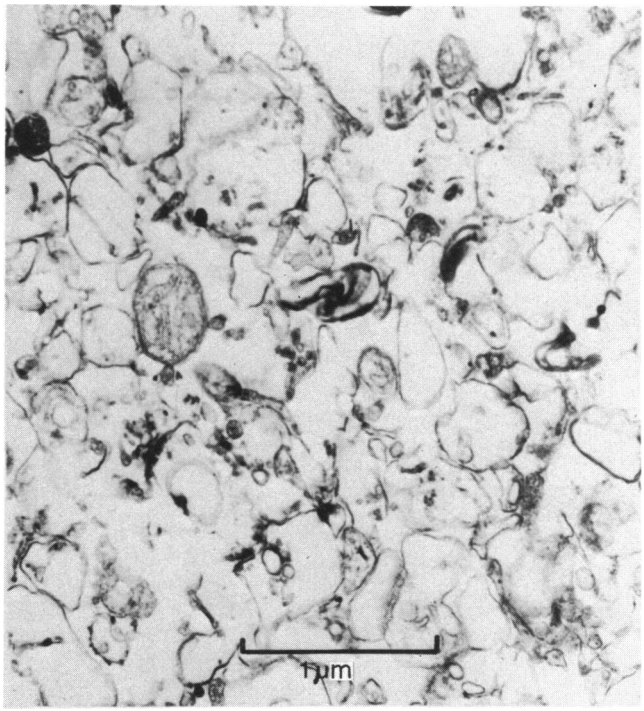

Figure 2 M1 fraction (crude particulate) containing empty synaptic ghosts, 'torn' synaptosomes, myelin fragments and mitochondria. Magnification × 49,000.

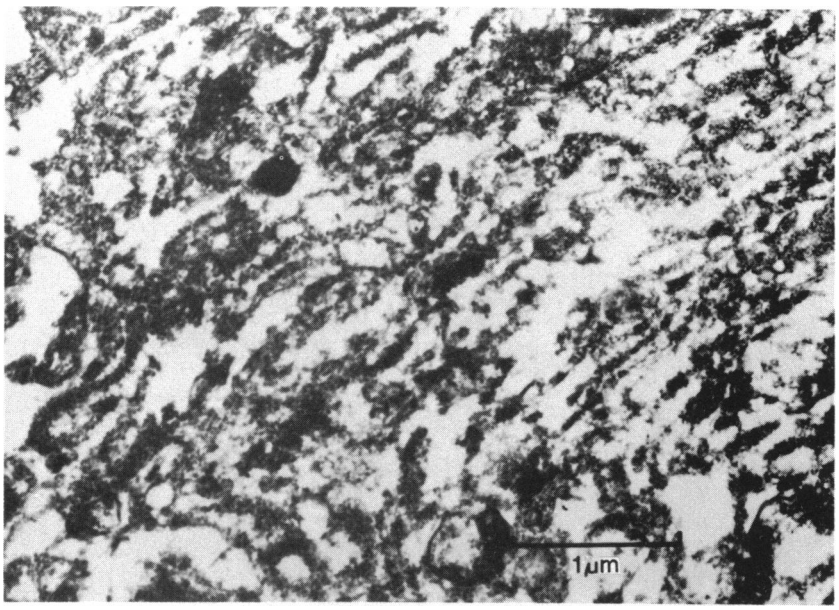

Figure 3 M2 fraction (sonicated) containing largely amorphous membranous material. Magnification × 48,000.

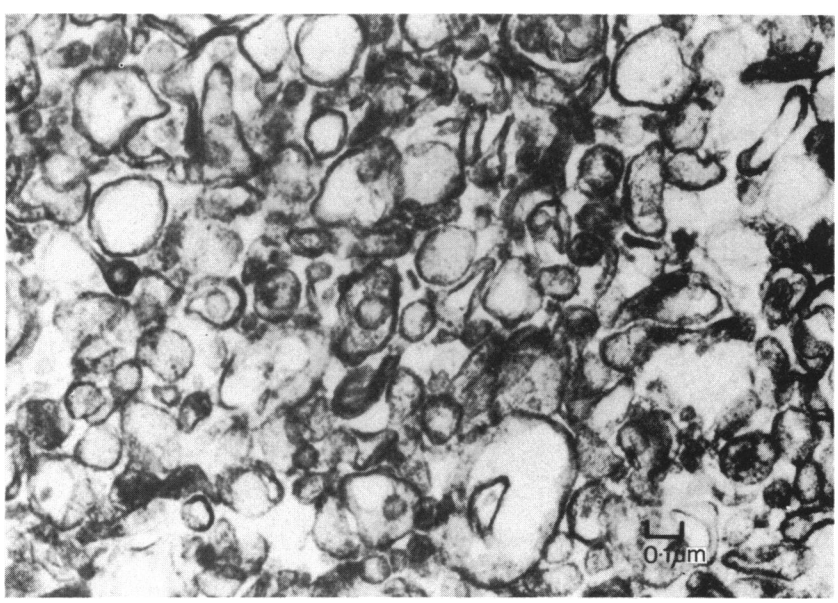

Figure 4 M2 fraction (unsonicated) containing small agranular vesicles together with larger ones and also granulated vesicles. Magnification × 96,000.

late) contained some empty synaptic ghosts, 'torn' synaptosomes (some containing a few synaptic vesicles), myelin fragments and mitochondria (Figure 2). The composition of the M2 'vesicular' fraction (100,000 $g \times 30$ min) depended exclusively on the P2-disruption technique. Ultrasonic disruption as suggested by Maynert, Levi & De Lorenzo (1964) produced a fraction essentially devoid of vesicles and containing largely amorphous membranous material (Figure 3). Hypo-osmotic shock with water gave a polymorphic vesicle fraction as described by De Robertis *et al.* (1965). Typical, small agranular vesicles (approx. 0.1 μM) were intermingled with larger ones which may represent resealed membrane fragments (Figure 4). Also granulated vesicles (containing osmium deposit) were present.

Discussion

The percentage distribution of 5-HT between the P2-derived subcellular subfractions revealed a similar pattern to that obtained by Zieher & De Robertis (1963) with 12% residing in the M2, crude vesicular fraction. Furthermore the actual 5-HT concentration in M2 (around 3 ng/mg protein) is similar to that obtained by Tamir & Gershon (1979), supporting the morphological integrity of this fraction. However, these authors used an improved vesicle-isolation technique, by controlling the hypo-osmotic conditions to which the vesicles are exposed. They also examined 5-HT-binding protein which was highly enriched in the vesicles lending support to the feeling that at least a proportion of 5-HT has a synaptic vesicular localization function. An electron microscopic examination of our fractions showed a similar morphology of particles to De Robertis *et al.* (1965) with the M2 fraction comprising both agranular and granular vesicles of different sizes. The partially disrupted M1 and Wp fractions obviously contain entrapped cytoplasm and therefore must account for the high percentage of transmitter found in these fractions. A comparison of P2-disruption techniques revealed that brief sonification of the P2 fraction, as suggested by Maynert *et al.* (1964), gives a very poor yield of synaptic vesicles and is not recommended for this type of study.

Drug treatments revealed a variety of changes which may be relevant to the behavioural changes seen following administration of lithium to rats. Repeated lithium treatment alone did not produce significant changes in either 5-HT content or percentage distribution between primary subcellular fractions and P2-derived subfractions. However, when 5-HT catabolism was prevented by administration of a monoamine oxidase inhibitor (MAOI), brain fractions containing soluble material (M1 and Wp) and the supernatant (S) isolated from the lithium-treated rats accumulated 25% more 5-HT than the fractions from control animals. This was not accompanied by an increase in vesicular 5-HT. Calculations of 'turnover rates' indicated increases of around 50% in fractions containing cytoplasm only, but not in the synaptic vesicles. This percentage increase is quite close to that obtained by Grahame-Smith & Green (1974) in rat whole brain following an identical lithium plus MAOI treatment-protocol (70% increase). They found that repeated lithium plus an MAOI produced a syndrome of behavioural hyperactivity in rats indistinguishable from that produced by L-tryptophan plus an MAOI. The increased 5-HT turnover/synthesis did not appear to be due to higher endogenous brain tryptophan levels as suggested by previous authors (Sheard & Aghajanian, 1970; Perez-Cruet *et al.*, 1971).

Knapp & Mandel (1975) found a biphasic effect of lithium on synaptosomal 5-HT biosynthetic capacity with increased tryptophan uptake following short-term treatment (3–5 days) (with concomitant increases in 5-HT turnover) followed by a compensatory decrease in tryptophan hydroxylase activity up to 21 days' treatment and a return of 5-HT turnover to normal levels. However, 10 days' lithium treatment does increase rat brain 5-HIAA and thus may be derived from an impairment of the intraneuronal 5-HT storage mechanism (Collard & Roberts, 1977). In fact, Grahame-Smith & Green found that one dose of lithium potentiates the hyperactivity produced by L-tryptophan plus an MAOI without altering 5-HT synthesis rate. This suggested that a greater amount of 5-HT is released and active under such conditions, perhaps also due to an altered intraneuronal compartmentation. Support for this hypothesis is provided by the study of Collard (1978) where the decrease in rat forebrain 5-hydroxyindoleacetic acid (5-HIAA) concentration produced by raphe stimulation is abolished by chlorimipramine in control rats but not in lithium-pretreated rats. Thus, the 5-HIAA produced by stimulation may have been derived from metabolism of 5-HT which remained predominantly in the intracellular compartment. It has been suggested that Li^+ interferes with stimulus-release coupling in such a way that 5-HT is released from the vesicular storage sites into the cytoplasm rather than into the synaptic cleft (Collard, 1978). Inhibition of MAO would then result in leakage of 5-HT from the nerve terminal.

In the present study if lithium pretreatment resulted in a change in the intraneuronal compartmentation of 5-HT as hypothesized with an inability of vesicles to bind or store the transmitter and an increased cytoplasmic 5-HT pool, this would not be apparent until after prevention of 5-HT catabolism as demonstrated. Any increase in free cytoplasmic

5-HT would be counteracted by MAO action. Interestingly, Komiskey & Buckner (1974) have shown that chronic lithium treatment increases the uptake and retention of (−)-erythro metaraminol (MA) by synaptosomes, a drug concentrated by the neuronal uptake mechanism, in agreement with other studies (Colburn *et al.*, 1967; Baldessarini & Yorke, 1970). However, (−)-octopamine uptake was unaffected. Since this drug is further concentrated by the vesicular storage mechanism and can be degraded by MAO it was concluded that lithium was acting at the level of the vesicular membrane to impede aromatic amine uptake and binding.

In conclusion, the present results support the contention that repeated lithium treatment can alter 5-HT turnover in the rat brain. One of the contributing factors may be an altered intraneuronal compartmentation of the monoamine between synaptic vesicles and intraneuronal cytoplasm. This action is apparent after inhibition of MAO and may help to explain the behavioural changes seen following such treatment. Further investigation of the 5-HT metabolite, 5-HIAA, level in these subcellular fractions may help to elucidate fully the synaptic mechanism of action of lithium.

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