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Stimulation of the Production of Unesterified Fatty Acids in Nerve Endings of Guinea-Pig Brain *in vitro* by Noradrenaline and 5-Hydroxytryptamine

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1. Noradrenaline (1 mM) and 5-hydroxytryptamine (1 mM) stimulated the production of unesterified palmitate, oleate, stearate and arachidonate in nerve endings (synaptosomes) isolated from combined guinea-pig cerebral cortex and cerebellum. 2. Iproniazid phosphate (0.36 mM) increased the concentrations of the same acids in osmotically ruptured synaptosomes. Further addition of 1 mM-noradrenaline or 1 mM-5-hydroxytryptamine reversed this increase. 3. Noradrenaline (0.01 mM) stimulated the production of unesterified fatty acids in isolated synaptic membranes. 5-Hydroxytryptamine (0.01 mM) stimulated the production of unesterified fatty acids in synaptic membranes and synaptic vesicles.

Noradrenaline and 5-hydroxytryptamine are putative transmitter substances in the central nervous system (Curtis, 1969). Like acetylcholine, they are concentrated in the nerve endings (Michaelson & Whittaker, 1963; Michaelson *et al.*, 1963; Zieher & De Robertis, 1963; Levi & Maynert, 1964) and are bound to vesicles (Michaelson *et al.*, 1963; Maynert *et al.*, 1964). The present theory of synaptic transmission postulates the release of transmitter substances into the synaptic cleft. In the sympathetic nervous system noradrenaline is actively reabsorbed into the nerve endings (Iversen, 1967). A consequence of the release and reabsorption mechanisms is that both the presynaptic and postsynaptic membranes must be exposed to relatively high local concentrations of noradrenaline.

When eserine and carbamoylcholine were injected intraventricularly into rat brain there was a large increase in the concentration of unesterified fatty acids in the cerebral hemispheres (Lunt & Rowe, 1971). The implication of these observations is that an increase in the concentration of unbound acetylcholine stimulates the release of unesterified fatty acids. This raises the question as to whether noradrenaline and 5-hydroxytryptamine when present in unbound forms also stimulate the production of unesterified fatty acids. The present paper reports increases in the concentrations of unesterified fatty acids in nerve endings and in synaptic membranes when these were exposed to noradrenaline. Increases in unesterified fatty acid concentrations were also obtained in nerve endings, synaptic membranes and synaptic vesicles when these fractions were treated with 5-hydroxytryptamine. Preliminary communications of this work have been given (Price & Rowe, 1971; Price *et al.*, 1971).

Experimental

Materials

Male guinea pigs of the Dunkin–Hartley strain of age greater than 3 months were used throughout. L-Noradrenaline bitartrate, L-noradrenaline hydrochloride, L-adrenaline bitartrate, 5-hydroxytryptamine creatinine sulphate and iproniazid (*N*-isonicotinoyl-*N'*-isopropylhydrazine) phosphate were supplied by Sigma Chemical Co. Ltd., London S.W.6, U.K. Except where stated otherwise the hydrochloride of noradrenaline was used. [*Me*-^{3}H]-Methanol was supplied by The Radiochemical Centre, Amersham, Bucks., U.K. Bovine serum albumin (fraction V powder, fatty acid free) was obtained from Pentex Inc., Kankakee, Ill., U.S.A. G.l.c. was performed by using a Pye argon chromatograph. All solvents were redistilled before use.

Methods

Preparation of synaptosomes. Synaptosomes and other subcellular fractions were prepared from combined cerebral cortex and cerebellum of guinea-pig brain by the method of Eichberg *et al.* (1964).

Enzyme assays. Succinate dehydrogenase (EC 1.3.99.1) was determined by the method of Pennington (1961). Lactate dehydrogenase (L-lactate–NAD oxidoreductase; EC 1.1.1.27) was determined by the procedure of Wroblewski &

LaDue (1955). Acetylcholinesterase (acetylcholine acetylhydrolase; EC 3.1.1.7) was determined by the method of Ellman *et al.* (1961).

Incubation of intact synaptosomes. The synaptosomal fraction was collected by aspiration from the discontinuous sucrose gradient (Eichberg *et al.*, 1964) and diluted with Krebs–Ringer bicarbonate medium (Krebs & Henseleit, 1932) containing 11 mM-glucose, to give a final sucrose concentration of 0.45 M (see Bradford, 1969). The diluted suspension was centrifuged at 17000*g* for 30min at 4°C. The sedimented synaptosomes were resuspended in Krebs–Ringer bicarbonate–glucose medium. To a suspension (0.90ml) containing 3–8mg of protein a solution of noradrenaline or 5-hydroxytryptamine (0.10ml; 10mM) in the same buffer was added and immediately mixed. The mixture was incubated in a water bath at 37°C with shaking for 90min.

Incubation of osmotically ruptured synaptosomes. The synaptosomal pellet that had been separated from sucrose as described above was resuspended in water at 0°C (2ml/g of original tissue). The mixture was left at 0°C for 30min (Whittaker *et al.*, 1964). The resulting suspension (0.90ml) containing 3–8mg of protein was mixed with 0.10ml of 0.5M-tris–HCl buffer, pH7.4, containing 3.60mM-iproniazid phosphate. The mixture was incubated at 37°C in a water bath with shaking. In other experiments suspension (0.80ml) was mixed with 0.10ml of buffered iproniazid solution and 0.10ml of an aqueous solution of noradrenaline hydrochloride or 5-hydroxytryptamine creatinine sulphate.

Determination of unesterified fatty acids. Incubation mixture (1.0ml) containing tissue was extracted by the method of Dole (1956) except that the extraction medium contained 0.25M-H_2SO_4. The unesterified fatty acids were then determined as methyl esters by one of the following procedures.

Method 1. The final extract in heptane was concentrated to dryness and methylated with diazomethane (Rowe, 1964). Methyl esters of palmitate, oleate, stearate and arachidonate were determined by g.l.c. on a column of 7% (w/w) Apiezon L at 200°C with methyl heptadecanoate as an internal standard (Lunt & Rowe, 1968). Identities of esters were confirmed by g.l.c. on 5% (w/w) polyethyleneglycol adipate at 175°C. Areas of peaks were measured by triangulation.

Method 2. The extract, containing unesterified fatty acids in heptane, was concentrated to dryness in a pointed tube by warming in a water bath and blowing N_2 on to the surface of the liquid. The residue, dissolved in chloroform–methanol (2:1, v/v; 0.1ml), was transferred to a glass plate coated with 0.25mm Kieselgel G, which had been divided into lanes 1cm wide. The tube was washed five times with chloroform–methanol and five times with diethyl ether. The washings were put on the plate in the same position as the original solution. When dry, the plate was chromatographed in light petroleum (b.p. 40–60°C) – diethyl ether – acetic acid (90:53:9, by vol.). The developed plate was dried. Spots of known volumes (20–100μl) of a solution of palmitic acid in benzene (1.00mg/ml) were put on the developed chromatogram alongside chromatographed fatty acid from tissue. These areas were treated in an identical manner to those containing fatty acid from tissue and were used to determine a calibration curve. The whole plate was sprayed with 0.2% (w/v) 2′,7′-dichlorofluorescein in ethanol–water (24:1, v/v) to detect fatty acids. Areas of the chromatogram corresponding to fatty acid were transferred to a thick-walled ampoule. A solution (0.25ml) of boron trifluoride (9.0–10.4%, w/v) in methanol–benzene (4:1, v/v) containing 50–150μCi of [*Me*-^{3}H]methanol was added. The ampoule was cooled by partial immersion in acetone–solid CO_2 mixture and sealed. It was then heated in a water bath at 100°C for 1h. After cooling in acetone–solid CO_2 mixture, the ampoule was opened and 1ml of methanol–benzene (4:1, v/v) containing 0.25–0.50mg of methyl palmitate was added to its contents. The mixture was transferred to a centrifuge tube. The ampoule was washed successively with 1ml of methanol–benzene containing 0.25–0.50mg of methyl palmitate, and 1.65ml of 0.27M-HCl. Washings were added to the main solution and mixed. The mixture was extracted three times with 2ml of hexane to collect the methyl palmitate. The combined extracts were concentrated to dryness. The residue, dissolved in 0.2ml of diethyl ether, was transferred to a t.l.c. plate coated with Kieselgel G (0.25mm thick). The tube that had contained the extract was washed nine times with 0.2ml of diethyl ether and the washings were added to the plate. When dry the plate was chromatographed in light petroleum (b.p. 40–60°C)–diethyl ether (9:1, v/v). Methyl palmitate was chromatographed as a marker on the same plate and detected by spraying with aq. Rhodamine-6G (1mg/100ml). Unsprayed areas of the chromatogram corresponding to [^{3}H]methyl palmitate were transferred to vials. Phosphor [10ml of xylene containing 0.6% 2,5-diphenyloxazole and 0.015% 1,4-bis-(5-phenyloxazol-2-yl)benzene] was added to each vial and the radioactivity was determined by liquid-scintillation counting in three channels by using a Nuclear-Chicago liquid-scintillation counter. Radioactivity was corrected to 100% efficiency by the channels-ratio method (Bruno & Christian, 1961).

Determination of proteins. After removal of the heptane layer for the determination of fatty acids the specific gravity of the remaining layer was lowered by the addition of an equal volume of methanol. Protein was collected as a pellet by centrifugation. The supernatant was removed and the protein was resuspended in 15ml of chloroform–methanol (2:1,

v/v) to remove lipid and any remaining H_2SO_4. After 2h the protein was collected by centrifugation, digested in 1M-NaOH, and determined by the biuret reaction (Weichselbaum, 1946). This procedure was used unless otherwise stated.

When only small quantities of protein were available the washing with chloroform–methanol was omitted and protein was determined by the method of Lowry *et al.* (1951). Turbidity was removed before measurement of the developed colour by extraction with an equal volume of chloroform.

Both procedures were standardized with bovine serum albumin.

Statistical analysis. Results were expressed as mean±S.D. of values in independent incubations, with the number of incubations given in parentheses. Results were subjected to Student's '*t*' test for significance. A value of $P>0.05$ was considered to be not significant. The upper limit of P is quoted in the tables.

Determination of 5-hydroxytryptamine. Synaptosomes (3–6mg of protein) were extracted with $HClO_4$ containing 2% (w/v) ascorbic acid and 10% (w/v) EDTA as described by Andén & Magnusson (1967). 5-Hydroxytryptamine was isolated by ion exchange on Amberlite CG50 (Andén & Magnusson, 1967) and determined by fluorescence of the complex with *o*-phthalaldehyde in the presence of L-cysteine as described by Curzon & Green (1970).

In an experiment in which synaptosomes were incubated with 1.0mM-5-hydroxytryptamine the synaptosomes were isolated by centrifugation at 0°C at approx. 3000*g* for 10min. The sides of the tube and surface of the pellet were rinsed rapidly with 1ml of Krebs–Ringer bicarbonate–glucose medium at 0°C. The synaptosomes and the combined supernatant and washings were extracted as described above.

Respiration of synaptosomes. Respiration was measured by using a Clark oxygen electrode (see Chappell, 1964).

Intraventricular injection of rats. Female rats, 2 months old, were injected intraventricularly (Noble *et al.*, 1967) under light anaethesia with diethyl ether. Control animals were injected with iproniazid phosphate (0.75μmol) dissolved in 20μl of 0.154M-NaCl. Experimental animals were injected with iproniazid phosphate (0.75μmol) plus noradrenaline hydrochloride (0.15μmol) dissolved in 20μl of 0.154M-NaCl. The rats, which recovered after 1–2min, were killed by decapitation 20min after injection. Cerebral cortices were homogenized in 2ml of distilled water and extracted immediately with Dole (1956) extraction medium (5ml) containing 0.75M-H_2SO_4.

Results and Discussion

Recovery of unesterified fatty acid

The recovery of [1-^{14}C]palmitate (0.23μg; 0.051 μCi) dissolved in 1μl of benzene and added to extracts of a 90min incubation of synaptosomes (5–8mg of protein) was 100.6±6.2% (12). The S.D. of the combined pipetting and counting errors for 1μl of solution of [1-^{14}C]palmitate was 5.59% (42). After methylation of an extract to which [1-^{14}C]-palmitate had been added 96.4±0.9% (4) of the radioactivity was reisolated as methyl ester by t.l.c. with 500μg of added palmitate as carrier for any unesterified [1-^{14}C]palmitate.

The recovery of added [1-^{14}C]palmitate by using method 2 was 76.7±6.4% (6).

Table 1. *Effect of noradrenaline on the concentration of unesterified fatty acids in subcellular fractions of guinea-pig brain*

Isolated fractions prepared from 10.1g of tissue were incubated (in 1.0ml of Krebs–Ringer bicarbonate buffer containing 11mM-glucose) for 90min at 37°C with shaking. Values are given as means±S.D. for four incubations. $P<0.01$ for the increase in unesterified fatty acids in synaptosomes with noradrenaline compared with the control.

Fraction	Content	Protein (mg)	Unesterified fatty acid (mg/g of protein)	
			Control	Noradrenaline bitartrate (1.0mM) present
P1	Nuclei, large myelin, cellular fragments	3.4±0.9	4.9± 0.9	4.7± 1.9
P2A	Myelin	2.1±0.3	8.6± 1.1	7.4± 2.3
P3	Microsomal material	1.9±0.8	7.5± 1.9	7.3± 1.7
S3	Supernatant	1.0±0.01	3.1± 1.0	3.4± 1.7
P2B	Synaptosomes	8.2±1.0	4.5± 0.9	6.7± 0.7
P2C	Mitochondria	2.1±0.1	32.7±17.4	18.3±10.1

Table 2. *Effect of noradrenaline on the concentration of unesterified fatty acid in subcellular fractions and synaptic fractions*

Subcellular fractions and synaptic membrane fractions were prepared from 6.0g of cerebral cortex by the procedure of Rodriguez de Lores Arnaiz *et al.* (1967). Mic 20 and Mic 100 fractions were isolated as described by Kataoka & De Robertis (1967). Synaptic vesicles were isolated as described by Lapetina *et al.* (1967). Isolated fractions were incubated in 1.0ml of 50mM-tris–HCl buffer, pH7.4, with 0.01mM-noradrenaline, or in the absence of noradrenaline (control), for 30min. Fatty acids were determined as the [^{3}H]methyl esters (method 2 in text). Protein was determined by the method of Lowry *et al.* (1951).

Fraction	Content	Protein (mg)	Unesterified fatty acid (mg/g of protein) (*a*) Control	(*b*) Noradrenaline present	$\frac{(b)-(a)}{(a)}$ (%)	Significance (*P*)
P1	Nuclei, myelin, cell fragments	5.85±0.37 (8)	12.9± 3.3 (4)	12.9± 2.6 (4)	—	—
Mic 20	Microsomal material, small synaptosomes	1.22±0.02 (7)	8.3± 1.3 (3)	13.6± 3.0 (4)	72	0.05
Mic 100	Microsomal material	0.80±0.03 (7)	21.9± 6.2 (3)	21.5± 3.2 (4)	—	—
M1 (0.8)	Myelin	0.92±0.01 (6)	24.0± 4.5 (4)	21.1± 2.7 (2)	—	—
M1 (0.9)	Synaptic membrane, myelin	0.30±0.02 (6)	10.2± 1.7 (3)	23.7± 4.1 (3)	133	0.01
M1 (1.0)	Synaptic membrane	0.28±0.03 (8)	44.2± 5.2 (4)	72.7± 2.4 (4)	65	0.0005
M1 (1.2)	Synaptic membrane	0.53±0.02 (8)	11.5± 3.5 (4)	25.6± 4.2 (4)	123	0.01
M1 (p)	Mitochondria	2.79±0.41 (8)	16.3±10.9 (4)	8.8± 3.0 (4)	−86	Not significant
M2A	Synaptic vesicles	0.22±0.02 (7)	12.8± 4.0 (3)	34.6±17.8 (4)	171	Not significant

Effects of noradrenaline and 5-hydroxytryptamine

When subcellular fractions isolated by the procedure of Eichberg *et al.* (1964) were incubated with 1mM-noradrenaline for 90min the yield of unesterified fatty acids from the synaptosomes was significantly increased (by 49%, Table 1). The distribution of acetylcholinesterase, lactate dehydrogenase and succinate dehydrogenase in tissue fractions prepared by this procedure were similar to those reported for guinea pig brain (Whittaker, 1965). Electron microscopy indicated that the P2B fraction consisted predominantly of synaptosomes. Noradrenaline did not increase the concentration of unesterified fatty acids in the crude nuclear (P1), microsomal (P3) and supernatant (S3) fractions. The yield from myelin (P2A) incubated with noradrenaline was not significantly different from that of the control.

Similar results were obtained when subcellular fractions, isolated by the procedure of Rodriguez de Lores Arnaiz *et al.* (1967) were incubated with 0.01mM-noradrenaline for 30min (Table 2). The crude nuclear fraction (P1), myelin [M1(0.8)], and microsomal (Mic 100) fractions were unaffected. Noradrenaline increased the concentration of unesterified fatty acids in the Mic 20 fraction by 72%. This fraction was shown by Kataoka & De Robertis (1967) to contain small synaptosomes which themselves contained histamine. The distribution of acetylcholinesterase in subcellular fractions, including submitochondrial fractions, of guinea-pig brain (Table 3) was similar to that reported for the corresponding fractions of rat brain (De Robertis *et al.*, 1966; Rodriguez de Lores Arnaiz *et al.*, 1967). The mitochondrial marker, succinate dehydrogenase, however, was concentrated to a greater extent in fractions M1 (1.2) and M1 (p) than was monoamine oxidase in rat brain (De Robertis *et al.*, 1966; Rodriguez de Lores Arnaiz *et al.*, 1967). The concentrations of succinate dehydrogenase and lactate dehydrogenase in fraction M1 (1.2) indicated that this fraction containing occluded mitochondria and cytoplasm. This is consistent with the view that fraction M1 (1.2) contained intact synaptosomes.

The results with mitochondria were ambiguous with both fractionation procedures. The means of the yields of unesterified fatty acids were decreased by noradrenaline, but these decreases were not statistically significant. With mitochondria the situation was

Table 3. *Distribution of protein, lactate dehydrogenase, succinate dehydrogenase and acetylcholinesterase between subcellular fractions and synaptic fractions*

Fractions were prepared from 4.7g of cerebral cortex as described for Table 2. Proteins were determined by the method of Lowry *et al.* (1951). RSA, relative specific activity.

		Lactate dehydrogenase			Succinate dehydrogenase			Acetylcholinesterase		
		Activity			Activity			Activity		
Primary fractions	% of recovered protein	% of recovered activity	μmol/min per g of protein	RSA	% of recovered activity	μmol/min per g of protein	RSA	% of recovered activity	μmol/min per g of protein	RSA
Homogenate	—	—	451	1.00	—	16.3	1.00	—	30.1	1.00
Nuclear	9.5	4.3	203	0.45	8.4	13.9	0.85	5.4	15.7	0.52
Crude mitochondria	42.2	26.7	286	0.63	88.4	32.8	2.01	53.6	35.3	1.17
Mic 20	8.4	5.4	293	0.65	2.8	5.3	0.33	17.5	58.1	1.93
Mic 100	5.9	1.2	88	0.20	0.4	0.9	0.06	14.5	68.0	2.26
Supernatant	34.1	62.4	828	1.84	0.0	0.0	0.00	9.0	7.3	0.24
Total Recovery (%)	92.4	92.6	—	—	88.7	—	—	85.3	—	—
Submitochondrial fractions*										
M1 Total membranes	—	—	54.8	1.00	—	40.4	1.00	—	35.4	1.00
M1 (0.8) Myelin	25.2	26.9	75.4	1.38	2.3	3.7	0.09	40.1	66.4	1.87
M1 (0.9) Synaptic membranes	7.1	2.6	25.5	0.47	0.4	2.3	0.05	19.9	117.9	3.33
M1 (1.0) Synaptic membranes	8.2	1.3	11.0	0.20	1.5	7.2	0.18	20.0	102.0	2.88
M1 (1.2) Synaptic membranes	21.5	24.4	79.9	1.46	22.5	41.3	1.02	9.8	19.4	0.55
M1 (p) Mitochondria	38.1	44.9	83.2	0.87	73.3	76.0	1.88	10.2	11.0	0.31
M2A Vesicles and membranes	—	—	55.0	—	—	0.0	—	—	21.2	—

* Fraction M1 (0.32) was not analysed.

probably complicated by the release of fatty acids by phospholipase A, which is active during incubation *in vitro* (see Chefurka, 1966), and by oxidation of the added noradrenaline by mitochondrial monoamine oxidase.

The concentration of unesterified fatty acid in synaptosomes was increased by noradrenaline, 5-hydroxytryptamine (Table 4) and adrenaline (Table 5). During the period of incubation the synaptosomes respired, and the respiration was unaffected by 1.0mM noradrenaline bitartrate, 5-hydroxytryptamine creatinine sulphate, adrenaline hydrochloride or methylamine hydrochloride. No increases were obtained with acetylcholine in the presence of eserine, γ-aminobutyrate, dibutyryladenosine cyclic monophosphate, reserpine, or methylamine hydrochloride (Table 4). With acetylcholine and dibutyryladenosine cyclic monophosphate the reservation must be made that in the experiments reported the concentrations of unesterified fatty acid in the controls were high. Thus the fatty acid-releasing potential of these preparations may have been fully realized before the addition of the experimental reagents. The negative result with methylamine indicated that the increases

Table 4. *Effect of amines and other reagents on the concentration of unesterified fatty acids in synaptosomes*

Reagents were present in concentrations of 1.0mM unless otherwise indicated.

Reagent	Unesterified fatty acid (mg/g of protein) (a) Control	(b) Reagent present	(b)−(a)/(a) (%)	Significance (P)
Noradrenaline bitartrate	1.2±0.1 (6)	3.6±0.6 (6)	195	0.001
Noradrenaline hydrochloride	1.4±0.2 (6)	3.9±1.1 (6)	176	0.001
5-Hydroxytryptamine creatinine sulphate	4.4±0.4 (4)	6.7±0.8 (4)	52	0.001
γ-Aminobutyrate	2.9±0.2 (4)	3.4±1.1 (4)	17	Not significant
Acetylcholine chloride+eserine (0.10mM)	9.7±1.9 (4)	10.8±1.9 (4)	11	Not significant
Reserpine (0.30mM)	2.9±0.2 (4)	2.6±0.3 (4)	−10	Not significant
N^6,O^2-Dibutyryladenosine 3′:5′-cyclic monophosphate (0.10mM)	9.7±0.4 (4)	10.4±1.1 (4)	7	Not significant
Methylamine hydrochloride*	6.4±1.1 (6)	5.5±0.9 (4)	−14	Not significant

* Results from the independent determinations of fatty acids in reisolated synaptosomes and in the incubation fluid were used to determine these values.

Table 5. *Comparison of the effects of noradrenaline and adrenaline on the concentrations of unesterified fatty acids in incubated synaptosomes and in the incubation medium, in the presence of bovine serum albumin*

Synaptosomes were incubated in Krebs–Ringer bicarbonate–glucose buffer containing 3% (w/v) bovine serum albumin. Values are means±S.D. for four incubations.

Addition	Unesterified fatty acid (mg/g of protein) Synaptosomes	Incubation fluid	Increase in incubation medium (% of control)	Significance (P)
None (control)	2.2±0.5	6.1±0.8	—	—
Noradrenaline hydrochloride	2.1±0.1	8.7±1.2	41	0.02
Adrenaline bitartrate	1.9±0.3	7.6±0.4	24	0.02

with noradrenaline, adrenaline and 5-hydroxytryptamine were not non-specific effects caused by amine groups. Albumin was necessary to obtain an effect with adrenaline, but not with noradrenaline. Adrenaline and noradrenaline are compared by using the same tissue preparation in Table 5. With both amines the increases in unesterified fatty acid were in the incubation fluid and not in the synaptosomes. Adrenaline, although not present in detectable quantities in the brains of mammals, has been reported to be present in the brains of lizard and frog (Brodie & Bogdanski, 1964).

Noradrenaline and 5-hydroxytryptamine increased the concentrations of unesterified fatty acid in synaptosomes isolated from guinea-pig cerebral cortex, from cerebellum and from the remainder of the brain, excluding the medulla oblongata (Table 6). The degree of stimulation with noradrenaline was of the same order as the reported distribution of noradrenaline in different regions of rat brain (Miller *et al.*, 1970).

The experiments described above indicated that noradrenaline and 5-hydroxytryptamine increased the concentration of unesterified fatty acid in isolated synaptosomes. The relative contributions of endogenous and added amines are unknown. The concentrations of amines inside the synaptosomes are also unknown. When isolated synaptosomes are incubated with low concentrations of noradrenaline or 5-hydroxytryptamine there is an active uptake into the synaptosomes (Bogdanski *et al.*, 1970; Kuriyama & Speken, 1970).

Experiments were performed in which osmotically ruptured synaptosomes were incubated with iproniazid phosphate. Osmotically ruptured synaptosomes would be expected to contain both bound and unbound endogenous noradrenaline (Michaelson *et al.*, 1963; Maynert *et al.*, 1964) and 5-hydroxytryptamine (Maynert *et al.*, 1964). Iproniazid phosphate is an inhibitor of monoamine oxidase in brain mitochondria (Zeller & Barsky, 1952) and inhibits the oxidation of 5-hydroxytryptamine and adrenaline by ox liver mitochondria (Zeller *et al.*, 1955). In seven experiments in which synaptosomes from one batch of guinea pigs were incubated for periods varying from 5 to 90min, iproniazid phosphate (0.36mM) increased the concentration of unesterified fatty acid by 17–101% (upper limits of $P = 0.05$–0.001). In one of these experiments in which synaptosomes were incubated for 20min iproniazid phosphate increased the concentration of 5-hydroxytryptamine from 0.10±0.01 (4) nmol/g of protein to 0.13±0.01 (4) nmol/g of protein ($P<0.01$) and the concentration of unesterified fatty acid was increased from 4.8±0.5 (4) mg/g of protein to 8.4±0.5mg/g of protein ($P<0.001$). The concentration of unesterified fatty acid from the same preparation, which had been incubated with iproniazid phosphate plus 1.0mM of added 5-hydroxytryptamine was 5.0±0.2 (4) mg/g of protein. In another experiment, in which synaptosomes were prepared from a different batch of guinea pigs, iproniazid phosphate decreased the concentration of fatty acid from 4.8±0.3 (4) mg/g of protein to 3.3±0.4 (4) mg/g of protein (4) ($P<0.01$). Iproniazid phosphate plus added 1.0mM-noradrenaline decreased the concentration of unesterified fatty acid further to 2.5±0.5 (4) mg/g of protein. Although these experiments, performed with a complex system, are inconclusive they are in accord with the view that small changes of endogenous noradrenaline and 5-hydroxytryptamine can regulate the concentration of unesterified fatty acid. Further, high concentrations of these amines at the appropriate site decrease the concentration of unesterified fatty acid.

Isolated synaptic membranes released unesterified

Table 6. *Effects of noradrenaline and 5-hydroxytryptamine on the concentrations of unesterified fatty acids in synaptosomes from different regions of the brain*

Values are means±S.D. from four incubations. Amine was present at a concentration of 1.0mM. 'Remainder' means whole brain minus cerebral hemispheres, cerebellum and medulla oblongata.

			Unesterified fatty acid (mg/g of protein)			
			(*a*)	(*b*)	$\frac{(b)-(a)}{(a)}$	
Expt. no.	Brain region	Amine	Control	Amine present	(%)	Significance (P)
1.	Cerebral cortex	Noradrenaline	2.7±0.3	5.2±1.7	93	0.05
	Cerebellum	Noradrenaline	3.0±0.7	4.7±0.4	56	0.01
	'Remainder'	Noradrenaline	2.1±1.8	5.6±0.7	169	0.01
2.	Cortex	5-Hydroxytryptamine	5.5±1.3	12.2±1.7 (2)	121	0.01
	Cerebellum	5-Hydroxytryptamine	4.7±1.4	9.9±1.2	109	0.01
	'Remainder'	5-Hydroxytryptamine	2.4±0.3	4.5±1.0	87	0.02

fatty acids when incubated with 0.01 mM-noradrenaline for 30 min (Table 2). An increased amount of unesterified fatty acid was obtained with fractions M1 (0.9), M1 (1.0) and M1 (1.2). The mean yield of unesterified fatty acid from synaptic vesicles was higher than that of the control but there was a large scatter of results and the increase was not statistically significant. The distribution of acetylcholinesterase in the synaptic membranes from this fractionation is shown in Table 3 and was similar to that obtained from whole rat brain by Rodriguez de Lores Arnaiz *et al.* (1967). The M1 (0.9) and M1 (1.0) fractions were high in acetylcholinesterase activity and were almost devoid of lactate dehydrogenase and succinate dehydrogenase activities. The M1 (1.2) fraction, however, was low in acetylcholinesterase activity and contained both lactate dehydrogenase and succinate dehydrogenase activities, in accord with this fraction containing intact synaptosomes. 5-Hydroxytryptamine stimulated the release of unesterified fatty acids in both synaptic membranes and in synaptic vesicles as shown in Table 7. In contrast with noradrenaline, 5-hydroxytryptamine did not stimulate the release of unesterified fatty acid from the Mic 20 fraction.

When the synaptic membrane fraction M1 (1.0), containing 0.4–0.7 mg of protein, was incubated for 30 min with iproniazid phosphate (0.36 mM) in tris buffer, the concentration of unesterified fatty acids [19.0±5.3 (4) mg/g of protein] was not significantly different from the concentration [21.1±5.4 (4) mg/g of protein] in the same membrane fraction incubated without iproniazid phosphate. Noradrenaline (0.1 mM), on the other hand, increased the concentration to 27.6±2.0 mg/g of protein ($P<0.05$). These observations support the view that the increases in concentration of the unesterified fatty acids in osmotically ruptured synaptosomes caused by the

Table 7. *Effect of 5-hydroxytryptamine on the concentration of unesterified fatty acids in synaptic membranes and synaptic vesicles*

Fractions, prepared as described for Table 2, were incubated with 5-hydroxytryptamine (0.01 mM), or in the absence of 5-hydroxytryptamine (control) in 1.0 ml of 50 mM-tris–HCl buffer, pH 7.4, for 30 min. Fatty acids were determined as their [^{3}H]methyl esters (method 2 in text). Proteins were determined by the method of Lowry *et al.* (1951).

			Unesterified fatty acid (mg/g of protein)			
Fraction	Content	Protein (mg)	(*a*) Control	(*b*) 5-Hydroxytryptamine present	$\frac{(b)-(a)}{(a)}$ (%)	Significance (P)
M1 (1.0)	Synaptic membranes	0.25±0.02 (8)	57±1 (4)	90±13 (4)	58	0.02
M2A	Synaptic vesicles	0.11±0.01 (10)	62±9 (5)	127±23 (5)	107	0.001
Mic 20	Microsomal material, small synaptosomes	0.58±0.02 (6)	39±2 (3)	38±2 (3)	—	—

Table 8. *Effect of noradrenaline on the molecular composition of unesterified fatty acid in subcellular fractions of guinea-pig brain*

Values are means±S.D. for four determinations. Results are from the experiment described in Table 1.

	Fatty acid composition (% w/w as methyl ester)							
	Palmitate		Oleate		Stearate		Arachidonate	
Fraction	Control	Noradrenaline	Control	Noradrenaline	Control	Noradrenaline	Control	Noradrenaline
P1	36.3± 7.3	33.0± 9.0	13.5±3.4	17.0±4.3	28.0± 1.4	27.5± 3.9	22.5±3.7	22.5± 1.7
P2A	30.5± 8.4	35.3± 7.2	19.5±4.8	13.5±4.8	26.3± 1.9	26.2± 1.5	23.8±4.4	24.2± 3.4
P2B	26.0± 6.1	26.3±12.8	15.5±1.7	11.8±3.8	34.0± 4.7	33.3± 6.9	25.0±8.5	28.5±15.9
P2C	23.5± 7.1	22.0± 5.6	31.5±3.1	34.5±3.4	25.8± 4.1	19.8± 3.4	19.3±3.3	23.8± 6.2
S3	46.0±11.9	48.2± 8.6	24.8±7.7	20.8±8.7	22.5±10.4	23.0±10.3	0.00	0.00

addition of iproniazid phosphate were due to increased concentrations of unbound amine rather than iproniazid phosphate itself.

Molecular composition of unesterified fatty acids

The unesterified fatty acids detected in synaptosomes after incubation were palmitate, oleate, stearate and arachidonate. These accounted for more than 90% (w/w) of the C_{12}–C_{20} fatty acids. The molecular distributions obtained from synaptosomes and other subcellular fractions are shown in Table 8. The mitochondrial fraction (P2C) could be distinguished by a high proportion of oleate and the supernatant fraction by an absence of arachidonate. The absence of arachidonate in the supernatant fraction has been reported for rat brain (Lunt & Rowe, 1968).

In incubated synaptosomes the molecular composition of the unesterified fatty acids depended on the region of the brain from which the synaptosomes had been isolated (Table 9). Thus the proportion of palmitate in synaptosomes from the cortex was less than that in synaptosomes from the cerebellum ($P < 0.01$ and 0.001 in Expts. 1 and 2 respectively) and the 'remainder' ($P < 0.001$ and 0.01).

In experiments where noradrenaline stimulated a high increase in the concentration of unesterified fatty acids in intact synaptosomes there was selective stimulation of the yield of palmitate, compared with stearate and oleate (Table 10). In the experiment where the concentration of unesterified fatty acid in osmotically ruptured synaptosomes was depressed by iproniazid phosphate plus 1.0mM-noradrenaline, the concentration of palmitate was selectively decreased (Table 10). Thus the concentration of palmitate was most affected by noradrenaline.

The present investigations indicate that noradrenaline, 5-hydroxytryptamine and iproniazid phosphate, when administered *in vitro*, increase the concentration of unesterified fatty acids in synaptosomes. With noradrenaline the effect was located in the synaptic membranes and with 5-hydroxytryptamine in the synaptic membranes and synaptic vesicles. The source of the fatty acids is unknown. Adenylate cyclase is concentrated in synaptosomes and synaptic membranes of rat brain (De Robertis *et al.*, 1967). The distribution of this enzyme parallels the distribution of the effect of amines on the concentrations of unesterified fatty acids reported here. Thus hydrolysis of esters, mediated by cyclic AMP (adenosine 3′:5′-cyclic monophosphate) is a possible source of fatty acids and this could be controlled by the concentrations of amine. Synaptosomes from combined guinea-pig cortex and cerebellum contain triglyceride lipase (Price *et al.*, 1971) and this lipase is stimulated by noradrenaline (Price *et al.*, 1971). In whole rat brain the concentration of triglyceride is approx. 0.2 μmol/g of tissue (Rowe, 1969). This concentration, in guinea-pig synaptosomes, would be insufficient to account for the stimulated release without a concomitant synthesis of triglyceride. Phospholipases A_1 and A_2 (Cooper & Webster, 1970)

Table 9. *Molecular composition of unesterified fatty acid in synaptosomes isolated from different regions of guinea-pig brain*

Values are derived from the experiment shown in Table 6. 'Remainder' is whole brain minus cerebral hemispheres, cerebellum and medulla oblongata.

		Percent distribution (w/w as methyl ester)			
		Expt. 1		Expt. 2	
Region	Fatty acid	Synaptosomes	Synaptosomes + noradrenaline	Synaptosomes	Synaptosomes + 5-hydroxytryptamine
Cortex	Palmitate	19.5±3.1	20.8±6.4	25.0±3.6	25.0±1.0
	Oleate	20.3±3.3	14.8±3.4	19.0±0.8	16.0±1.0
	Stearate	32.8±3.3	26.3±4.4	29.5±3.7	32.5±5.5
	Arachidonate	27.0±3.2	40.0±12.9	27.0±4.1	26.0±5.0
Cerebellum	Palmitate	45.8±9.5	41.8±3.0	40.8±3.7	36.5±3.7
	Oleate	14.0±3.7	14.8±2.5	18.3±2.9	17.8±0.9
	Stearate	19.8±2.9	22.0±1.8	25.3±3.3	27.3±3.6
	Arachidonate	20.5±3.7	21.5±6.0	16.0±1.4	18.5±3.1
'Remainder'	Palmitate	34.8±0.9	30.8±4.6	37.8±2.8	41.7±8.0
	Oleate	18.3±2.4	17.5±1.3	19.5±2.5	15.0±2.6
	Stearate	23.3±0.9	24.8±2.2	24.3±3.6	21.7±4.0
	Arachidonate	23.8±3.0	27.3±4.3	18.3±3.6	21.7±1.5

Table 10. *Effect of noradrenaline on the concentration of unesterified acids in synaptosomes*

Experiments 1 and 2 are reported in Table 4. Experiment 4 is reported in the text.

Expt. no.	Conditions	Change in unesterified fatty acid (%)	Change in unesterified fatty acid Significance (*P*)		Yield of unesterified fatty acid (mg/g of protein) Palmitate	Oleate	Stearate	Arachidonate
1	Intact synaptosomes+noradrenaline bitartrate (1.0mM)	+195	0.001	Control (6)*	0.84±0.10	0.16±0.05	0.15±0.06	0.09±0.06
				Expt. (6)	3.02±0.56	0.20±0.05	0.24±0.08	0.19±0.09
				$\frac{\text{Expt.}-\text{Control}}{\text{Control}}$ (%)	+260	+25	+60	+111
				Significance (*P*)	0.001	Not significant	Not significant	Not significant
2	Intact synaptosomes+noradrenaline hydrochloride (1.0mM)	+176	0.001	Control (6)	0.94±0.10	0.16±0.05	0.15±0.06	0.17±0.03
				Expt. (6)	2.98±0.75	0.34±0.13	0.24±0.11	0.36±0.19
				$\frac{\text{Expt.}-\text{Control}}{\text{Control}}$ (%)	+217	+112	+60	+112
				Significance (*P*)	0.001	0.02	Not significant	0.05
3	I Intact synaptosomes+cyclic AMP (2μM)+caffeine (0.5mM)+ theophylline (0.5mM)	+11	Not significant	Control (4)	0.97±0.11	0.11±0.06	0.15±0.04	0.12±0.03
				Expt. I (3)	1.06±0.08	0.10±0.02	0.15±0.03	0.15±0.05
				Expt. II (4)	1.97±0.28	0.15±0.02	0.19±0.06	0.19±0.06
	II, I+noradrenaline hydrochloride (1.0mM)	+85	0.01	$\frac{\text{Expt. II}-\text{Control}}{\text{Control}}$ (%)	+86	+50	+27	+27
				Significance (*P*)	0.001	0.01	Not significant	Not significant
4	Osmotically ruptured synaptosomes+ iproniazid phosphate (0.36mM)+ noradrenaline hydrochloride (1.0mM)	−48	0.001	Control (4)	1.63±0.04	0.73±0.07	1.03±0.20	1.13±0.51
				Expt. (4)	0.57±0.11	0.55±0.21	0.76±0.20	0.60±0.26
				$\frac{\text{Expt.}-\text{Control}}{\text{Control}}$ (%)	−65	−25	−26	−47
				Significance (*P*)	0.001	Not significant	Not significant	Not significant

* Numbers of independent incubations are given in parentheses.

and lysophospholipase (Marples & Thompson, 1960; Cooper & Webster, 1970) have been demonstrated in nervous tissue and these enzymes may account for the release stimulated by amine. There is also the possibility of other bound forms of fatty acid being available for release.

The above observations suggest the possibility that the release of noradrenaline and 5-hydroxytryptamine, during synaptic transmission *in vivo*, regulates the concentration of unesterified fatty acids in membranes. In peripheral nervous tissue there is an active re-uptake of noradrenaline (Axelrod & Kopin, 1969), which would ensure a concentration of amine in the vicinity of the presynaptic membrane. Intraventricular injection *in vivo* of noradrenaline into whole rat brain in the presence of iproniazid phosphate decreased the concentration of unesterified fatty acids in cerebral cortices [0.86 ± 0.09 (5) mg/g of protein] relative to that obtained by the injection of iproniazid phosphate alone [1.18 ± 0.10 (4); $P < 0.01$]. An increase in the concentration of unesterified fatty acids in rat brain after electroconvulsive shock has been reported (Bazan, 1971). Both release and uptake of fatty acid in a membrane might be expected to change the structure of that membrane. Thus the observations reported in the present paper may reflect changes in structure and properties of synaptic membranes during synaptic transmission *in vivo*.

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