J. Pharm. Pharmac., 1968, **20**, 41–47 Received June 29, 1967

Subcellular distribution of [^{3}H]ε-aminocaproic acid and its effects on amine storage mechanisms

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ε-Aminocaproic acid (EACA) is an amino-acid reported to cause almost complete depletion of cardiac noradrenaline stores. The present report indicates that this compound can be found in both the particulate and supernatant fractions derived from heart homogenates. The [^{3}H]EACA in the supernatant probably represents a mixture of intra- and extraneuronally located drug. Protriptyline pretreatment decreases the uptake of [^{3}H]EACA suggesting that the amino-acid probably utilizes the amine membrane transport system. EACA, like reserpine, can both impair the retention of exogenously administered amines by adrenergic storage particles and cause the release of amines previously stored in such particles.

THE synthetic amino-acid ε-aminocaproic acid (EACA) is a potent inhibitor of plasminogen activation and has been demonstrated to be an effective therapeutic agent for the control of disordered fibrinolytic states in man (Alkjaersig, Fletcher & Sherry, 1959; Ablondi, Hagan & others, 1959; Nilsson, Sjoerdsma & Waldenström, 1960; Nilsson, Andersson & Björkman, 1966).

Recently, EACA has been reported to inhibit the dual amine uptake-concentration mechanisms of the adrenergic neurons (Obianwu, 1967) and to cause almost complete depletion of cardiac noradrenaline stores (Lippmann, Wishnick & Buyske, 1965; Andén, Henning & Obianwu, in preparation). The EACA-induced depletion of tissue noradrenaline is accompanied by a loss of adrenergic nerve function (Andén, Henning & Obianwu, in preparation). In an attempt to elucidate the mechanisms by which EACA exerts some of its amine-depleting effects, we have examined both the subcellular distribution of [^{3}H]EACA and its effect on amine storage mechanisms.

Experimental

METHODS

In vivo *experiments*. Mice, divided at random into groups of six, were given [^{3}H]α-methylnoradrenaline, 100 μg/kg (30 mc/mM); [^{3}H]metaraminol, 40 μg/kg (100 mc/mM) or [^{3}H]EACA, 200 μg/kg (60 mc/mg) intravenously. The animals were either pretreated or subsequently given various drugs and were killed at appropriate time intervals. A more detailed presentation of the injections schedules can be found in the Results section.

All animals were killed by decapitation and hearts removed and homogenized in the cold. A coarse fraction was obtained by centrifugation of the homogenate in the cold at 2000 *g* for 10 min. The supernatant obtained was then centrifuged at 100,000 *g* for 60 min in a Spinco Model L Ultracentrifuge, providing two more fractions, particulate (sediment) and high speed supernatant. Details of the subcellular fractionation and isolation of [^{3}H]α-methylnoradrenaline and [^{3}H]metaraminol have been

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described previously (Carlsson & Waldeck, 1963; Lundborg & Stitzel, 1967; Stitzel & Lundborg, 1967).

[^{3}H]EACA was isolated by ion-exchange chromatography. Tissue extracts were neutralized to pH 3·5 with 5N potassium carbonate solution and placed on a column of Dowex 50-X4 resin (50 × 4·2 mm). The column was washed with 40 ml of redistilled water and [^{3}H]EACA eluted with 18 ml of 0·4N hydrochloric acid. The first 3 ml of the eluate was discarded. Eluates were freeze-dried and the radioactivity was estimated by liquid scintillation counting. The identity of the isolated [^{3}H]EACA was established by subjecting the freeze-dried eluates, dissolved in a few drops of distilled water, to paper chromatography (butanol–acetic acid–water; 4:1:5, 18–20 hr). All the radioactivity was localized in an area with an Rf value (0·56–0·60) which was similar to that of authentic EACA. The recovery of known amounts of [^{3}H]EACA added to tissue homogenates or extracts ranged from 80–90%. No corrections have been made for recovery.

In vitro *granule experiment.* Bovine adrenal medullae were homogenized in 0·3M sucrose. Unbroken cells and nuclei were removed by centrifugation at 800 *g* for 5 min. The supernatant thus obtained was decanted and centrifuged at 26,000 *g* for 20 min. The granules were then suspended in 0·3M sucrose. The preparation and incubation of the granules were essentially the same as described by Hillarp (1958) and Carlsson, Hillarp & Waldeck (1963). Incubations were made without shaking at 0° and 31° for 30 min.

MATERIALS

[^{3}H]α-methylnoradrenaline and [^{3}H]metaraminol were prepared by the research laboratory of Hässle Ltd. in co-operation with this department. [^{3}H]EACA was kindly donated by Dr. K.-F. Benitz of Lederle Laboratories, Pearl River, New York. [^{14}C]Adrenaline was obtained from New England Nuclear Corp.

Results

Subcellular distribution of [^{3}H]EACA *in the mouse heart.* The subcellular distribution of [^{3}H]EACA ½, 1, 2 and 4 hr after its intravenous administration (400 μg/kg) is shown in Table 1. After ½ hr only about 6% of the [^{3}H]EACA retained by the heart was localized in the particulate fraction, while after 4 hr 28% of the remaining tritiated compound was associated

TABLE 1. SUBCELLULAR DISTRIBUTION OF [^{3}H]EACA IN THE MOUSE HEART

Time (hr)	[^{3}H]EACA — ng/g ± s.e.m.*		$\frac{p}{p+s} \times 100$
	Particulate	Supernatant	
0·5	1·91 ± 0·25	32·83 ± 4·42	6·1 ± 1·6
1·0	2·05 ± 0·23	29·54 ± 0·72	6·5 ± 0·7
2·0	4·56 ± 1·10	19·09 ± 1·76	18·3 ± 2·9
4·0	4·39 ± 0·22	11·68 ± 0·93	27·5 ± 1·5

[^{3}H]EACA (400 ng/kg, i.v.) was given and the animals killed at various time intervals thereafter. Each experiment used six pooled hearts.
* Mean of four experiments.

with this fraction. During the 4 hr following its injection, there was an increase in the amount of EACA found in the particulate fraction with a concomitant decrease in the supernatant content.

Effects of blockade of the neuronal membrane pump on the uptake and subcellular distribution of [^{3}H]EACA. Substances capable of releasing tissue amines have been separated into two groups depending on their ability to utilize the amine transport mechanism located in or on the adrenergic axonal membrane. Protriptyline, a compound which markedly inhibits this uptake mechanism, was found to decrease the accumulation of [^{3}H]EACA in both subcellular fractions by about 32% (Table 2).

TABLE 2. EFFECT OF PROTRIPTYLINE ON THE UPTAKE AND SUBCELLULAR DISTRIBUTION OF [^{3}H]EACA

Treatment	No. of experiments	[^{3}H]EACA — ng/g ± s.e.m.		$\frac{P}{P+S} \times 100$
		Particulate	Supernatant	
Control	6	1·86 ± 0·19	28·13 ± 1·20	6·2 ± 0·5
Protriptyline	3	1·23 ± 0·18	19·15 ± 0·33	6·0 ± 0·7

Protriptyline-treated animals were given the drug (10 mg/kg, i.p.) 1 hr before the administration of [^{3}H]EACA (400 ng/kg, i.v.). Each experiment used six pooled hearts.

Effect of EACA *on the total uptake of* [^{3}H]α*-methylnoradrenaline and its metabolic conversion to* [^{3}H]α*-methylnormetanephrine.* EACA exerted a moderate inhibitory effect on the total accumulation of [^{3}H]α-methylnoradrenaline (Fig. 1). The impairment of [^{3}H]α-methylnoradrenaline uptake was most pronounced $\frac{1}{2}$ hr after an intraperitoneal injection of EACA (1 g/kg) and then gradually declined over the next 12 hr. The early stages of uptake blockade were accompanied by a slight increase in the amount of *O*-methylated metabolite, [^{3}H]α-methylnormetanephrine, which could be recovered from the heart (Table 3). A decrease in amine uptake impairment was accompanied by a gradual decrease in the amount of [^{3}H]α-methylnormetanephrine formed.

TABLE 3. EFFECT OF EACA PRETREATMENT ON THE FORMATION AND RETENTION OF [^{3}H]α-METHYLNORMETANEPHRINE ([^{3}H]α-MeNM)

	Time after EACA administration	No. of experiments	[^{3}H]α-MeNM ng/g
Control ..	0	6	19·85 ± 1·10
EACA	($\frac{1}{2}$ hr)	4	26·04 ± 1·65
EACA	(1 hr)	4	27·76 ± 2·23
EACA	(2 hr)	4	20·34 ± 1·86
EACA	(4 hr)	4	19·26 ± 0·74

At varying intervals after the administration of EACA (1 g/kg i.p.) [^{3}H]α-methylnoradrenaline was given (100 μg/kg i.v.) and the [^{3}H]α-methylnormetanephrine content determined 15 min later. Values are means ± s.e.m. and each experiment used six pooled hearts.

Effect of EACA *pretreatment on the uptake of* [^{3}H]α*-methylnoradrenaline into subcellular fractions of the mouse heart.* EACA pretreatment (1 g/kg) greatly impaired the retention of [^{3}H]α-methylnoradrenaline in the particulate fraction derived from heart homogenates (Table 4). This impairment was accompanied by a moderate increase in the amount of tritiated compound found in the supernatant fraction. The inhibitory

effect of EACA on amine uptake appeared to be maximal 1–2 hr after its administration while 12 hr after EACA administration there was a significant recovery of the granular storage function.

TABLE 4. EFFECT OF EACA PRETREATMENT ON THE UPTAKE OF [^{3}H]α-METHYLNORADRENALINE ([^{3}H]α-MeNA) INTO SUBCELLULAR FRACTIONS OF THE MOUSE HEART

Treatment	Time (hr)	No. of experiments	[^{3}H]α-MeNA – ng/g ± s.e.m.		$\frac{P}{P+S} \times 100$
			Particulate	Supernatant	
Control ..	0	6	23·44 ± 2·12	32·55 ± 2·31	41·9
EACA	0·5	4	10·59 ± 2·32	37·72 ± 4·20	21·9
EACA	1·0	4	9·69 ± 1·11	45·56 ± 1·91	17·5
EACA	2·0	4	8·87 ± 0·91	40·53 ± 1·53	21·8
EACA	4·0	4	11·69 ± 1·20	35·24 ± 2·07	24·9
EACA	12·0	4	19·30 ± 1·38	27·95 ± 2·79	40·8

Animals were pretreated with EACA (1 g/kg, i.p.) and at various intervals thereafter were given [^{3}H]α-methylnoradrenaline (100 μg/kg, i.v.) and killed 15 min later. Each experiment used six pooled hearts.

Effect of EACA *on the release of* [^{3}H]*metaraminol from subcellular fractions of the mouse heart.* Mice were pretreated with [^{3}H]metaraminol (40 μg/kg) intravenously either 15 min or 23¼ hr before EACA (1 g/kg, i.p.). All animals were killed 45 min after EACA administration. EACA caused a loss of [^{3}H]metaraminol from both the particulate and the supernatant fractions (Table 5) at both the shorter and longer time intervals after [^{3}H]metaraminol administration. However, the loss appeared to be greater from the particulate than from the supernatant fractions as evidenced by the decline in the percentage of labelled amine recovered in the particulate fraction (relative to the P + S fractions) (Table 5). EACA appeared to cause a more pronounced loss from the particulate fraction of animals which had been given [^{3}H]metaraminol 23¼ hr previously than those given the amine 45 min before.

TABLE 5. EFFECT OF EACA ON THE RELEASE OF [^{3}H]METARAMINOL ([^{3}H]MA) FROM SUBCELLULAR FRACTIONS OF THE MOUSE HEART

Treatment	No. of experiments	[^{3}H]MA – ng/g ± s.e.m.		$\frac{P}{P+S} \times 100$
		Particulate	Superantant	
[^{3}H]MA (1 hr)	4	6·40 ± 0·86	29·76 ± 3·46	18·8 ± 1·19
[^{3}H]MA (1 hr) + EACA ..	3	3·81 ± 0·46	22·04 ± 1·96	14·7 ± 0·69
[^{3}H]MA (24 hr) ..	4	6·77 ± 0·40	20·06 ± 1·12	25·2 ± 0·54
[^{3}H]MA (24 hr) + EACA	6	1·17 ± 0·09	12·12 ± 0·92	9·9 ± 0·30

[^{3}H]MA (40 μg/kg, i.v.) was given either 15 min or 23¼ hr before the injection of EACA (1 g/kg, i.p.) Animals were killed 45 min after EACA administration. The figures in parenthesis indicate the time elapsed between [^{3}H]MA injection and decapitation. Each experiment used six pooled hearts.

Influence of EACA *on the* in vitro *uptake of* [^{14}C]*adrenaline by bovine adrenal medullary granules.* EACA, in a concentration of $1{\cdot}5 \times 10^{-5}$M, caused a slight inhibition of the uptake of [^{14}C]adrenaline by bovine adrenal granules. Higher concentrations did not markedly increase the inhibitory effect. EACA $2{\cdot}6 \times 10^{-4}$M caused a 35·8 ± 1·42% inhibition of [^{14}C]adrenaline (4 exp.) while at $5{\cdot}2 \times 10^{-4}$M inhibition was 41·8 ± 3·5% (2 exp.). The concentration of adrenaline (labelled and unlabelled) was 3×10^{4}M. The inhibitory effect of EACA could not be attributed to a

lysis of the granules since an estimation of the adrenaline content in the incubation media showed no appreciable difference between the control flasks and those to which EACA had been added.

Discussion

The uptake mechanisms present in adrenergic nerves consist of two major components, transport through the nerve cell membrane and incorporation into an amine storage granule complex (Carlsson & others, 1963). Both of these mechanisms can be selectively blocked by drugs. Reserpine is known to impair the storage mechanism of the adrenergic granules (Green & Sawyer, 1960; Campos & Shideman, 1962), thereby greatly inhibiting their ability to retain a variety of substances such as [^{3}H]noradrenaline (Stitzel & Lundborg, 1967), [^{3}H]α-methylnoradrenaline (Carlsson, Lundborg & others, 1967) and [^{3}H]guanethidine (Chang, Costa & Brodie, 1965). Antidepressant agents such as protriptyline and desmethylimipramine can block the uptake of circulating (Carlsson & Waldeck, 1965) or neurally released (Malmfors, 1964) amines at the level of the cell membrane. The above agents are, therefore, useful tools in assessing the role played by the two uptake mechanisms.

The data presented in Table 1 indicate that intravenously administered [^{3}H]EACA is initially bound to the amine storage particles to only a small extent, most of the drug being recovered from the supernatant fraction. The amount of [^{3}H]EACA found in the supernatant fraction then declined while there was some increase in the particulate content. The amount found in the supernatant fraction probably represents a mixture of intra- and extra-neuronally located drug. The relatively rapid loss of [^{3}H]EACA observed from this fraction may be indicative of a rapid elimination from the blood with a concomitant decrease in extraneuronal concentrations. However, some of the labelled substance is probably associated with adrenergic nerves since it was found in a subcellular fraction usually associated with adrenergic granules and protriptyline pretreatment reduced the amount recovered from subcellular fractions by about 32%. The latter observations indicates that EACA probably utilizes the amine membrane transport system. Thus it is likely that EACA can be taken up into adrenergic nerves by an active process, and once accumulated is bound to some extent within the storage particles.

A quantitative estimation of the inhibition of uptake caused by protriptyline is difficult since EACA is generally distributed through body tissues, and therefore would tend to accumulate extraneuronally after membrane pump blockade. However, since protriptyline can reduce the amount of [^{3}H]EACA taken up, it is probable that at least 32% of that taken up by the mouse heart is associated with adrenergic nerves. This view is further supported by the observation of Obianwu (to be published) that denervation reduces the amount of EACA retained by sympathetically innervated tissues.

Inhibition of the storage mechanism in adrenergic granules leads to a depletion of the endogenous catecholamines (Bertler, Hillarp & Rosengren, 1961; Carlsson, Hillarp & Waldeck, 1962; Kirshner, 1962) and to

depletion of exogenous amines previously administered (Hertting, Axelrod & Whitby, 1961). The released amines appear to be preferentially metabolized by monoamine oxidase (Kopin & Gordon, 1962). However, if this enzyme is inhibited or an amine resistant to its action is administered, such as α-methylnoradrenaline or metaraminol the released amine accumulates in the axoplasm (Stitzel & Lundborg, 1967; Carlsson & others, 1967). The data presented in Table 3 and Fig. 1 indicate that EACA

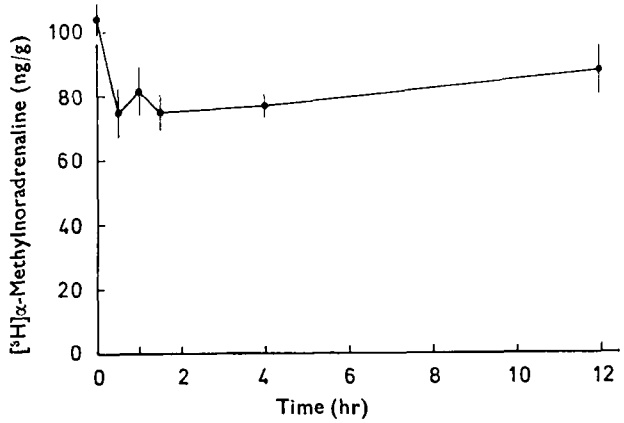

FIG. 1. Effect of EACA on total uptake of [³H]α-methylnoradrenaline in the mouse heart. [³H]α-methylnoradrenaline (100 mg/kg, i.v.) was given at various intervals after EACA (1 g/kg, i.p.) and animals were killed 15 min after injection of the labelled amine. Vertical lines are standard errors of the mean.

greatly inhibits the retention of [³H]α-methylnoradrenaline by the particulate fraction and that this reduction was accompanied by an increase in the level of labelled amine found in the supernatant fraction. Apparently EACA, like reserpine, can impair the retention of exogenously administered amines by adrenergic storage particles. The ability of EACA to impair uptake processes present in adrenergic granules is further supported by the finding that it can cause a moderate inhibition of [¹⁴C]adrenaline uptake into bovine adrenal medullary granules.

After administration of metaraminol this amine, like noradrenaline, accumulates and is retained in sympathetically innervated tissues (Andén, 1964: Shore, Busfield & Alpers, 1964; Gram & Wright, 1966). Previous studies have indicated that there is a gradual transfer of this amine from a labile to a more stable pool with time (Crout, Alpers & others, 1964; Carlsson & Waldeck, 1966) and that reserpine more effectively depletes metaraminol given 24 hr rather than 1 hr previously (Stitzel & Lundborg, 1967). It was of interest, therefore, to see if EACA also could displace metaraminol from subcellular pools. The distribution studies indicate that EACA, like reserpine, was more effective as a depleting agent when given 24 hr after [³H]metaraminol than 1 hr after its administration. The primary site of action of EACA was the particulate fraction since depletion was accompanied by a decrease in the P/(P + S) ratio. The EACA-induced depletion of tissue monoamine (Lippmann & others, 1965;

Lippmann & Wishnick, 1965) probably results from this action of EACA on the granular storage mechanism.

The adrenergic nerve blockade caused by EACA does not set in until severe depletion of tissue noradrenaline has occurred. However, function returns when tissue amine levels are still low (Andén, Henning & Obianwu, in preparation). In both the rat and the cat this occurs 12–18 hr after EACA administration. The present finding that EACA-induced blockade of amine storage mechanisms has partially recovered 12 hr after its administration appears to coincide with recovery of adrenergic nerve function. Reports by Andén, Magnusson & Waldeck (1964), Lundborg (1963) and Andén & Henning (1966) also point to the importance of the recovery of uptake rather than the restoration of tissue amine levels for the return of nerve function. In this respect EACA appears to exert similar actions to reserpine on amine storage and nerve function.

Acknowledgements. The research reported in this manuscript has been supported by the Swedish State Medical Research Council (B 67-14X-155-03 A) and the Faculty of Medicine, University of Göteborg, Sweden. R.E.S. would like to thank West Virginia University for the leave of absence which allowed him to carry out this work and the SSMRC for their financial support. The highly competent technical assistance of Miss Lena Ramstedt is gratefully acknowledged.

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