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DISPOSITION OF ^{3}H-EPSILON AMINO CAPROIC ACID AND ITS INTERACTION WITH ADRENERGIC NEURONES

BY

HOPÉ O. OBIANWU

From the Department of Pharmacology, University of Göteborg, Sweden

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Epsilon amino caproic acid (EACA), a potent inhibitor of plasminogen activation, has been demonstrated to be an effective therapeutic agent for the control of disordered fibrinolytic states in man (Alkjaersig, Fletcher & Sherry, 1959; Ablondi, Hagan, Philips & Renzo, 1959).

In a recent review (Nilsson, Andersson & Björkman, 1966), the efficacy of EACA in the treatment of a variety of haemorrhagic states in man was discussed. Apart from its antifibrinolytic action, EACA appears to possess other important pharmacological actions. For example, it causes about 90% depletion of tissue noradrenaline (Lippmann, Wishnik & Buyske, 1965; Lippmann & Wishnick, 1965; Andén, Henning & Obianwu, unpublished). The depletion of tissue noradrenaline is accompanied by loss of adrenergic nerve function (Andén, Henning & Obianwu, unpublished). EACA has also been reported to inhibit the dual uptake-concentration mechanism of the adrenergic neurones (Obianwu, 1967). Despite the potential usefulness of this compound in therapeutics and pharmacology, little is known about its physiological disposition.

The present report describes the uptake and retention of ^{3}H-EACA by various tissues of the rat. Since some of the pharmacological actions of EACA are antagonized by procedures which inhibit the storage and release of noradrenaline, the effect of these procedures on the disposition of ^{3}H-EACA is also reported.

METHODS

Male Sprague-Dawley rats 200–250 g were used. The left jugular veins were cannulated under light ether anaesthesia as described by Popovic & Popovic (1960) and the rats were used 2–3 days later. In some the right superior cervical ganglia were excised and the rats were used 8–10 days after the operation.

Reserpine (Serpasil), 5 mg/kg, desipramine, 10 mg/kg and protriptyline, 10 mg/kg, were administered intraperitoneally; α-amphetamine, 0.5 mg/kg, and ^{3}H-EACA, 0.2 mg/kg, were administered intravenously via the jugular cannula. ^{3}H-EACA, specific activity 60 μc/mg, was kindly donated by Dr. K-F. Benitz of Lederle Laboratories, Pearl River, New York.

The rats were killed by dislocation of the neck at various intervals after administration of ^{3}H-EACA.

Tissues were immediately removed and homogenized in 8 ml. ice-cold, 0.4 N perchloric acid (3 ml./g tissue in case of the liver), the homogenizer (Ultra-Turrax) being rinsed with a further 2 ml. 0.4 N perchloric acid. The homogenate was centrifuged in the cold at 2,000 *g* for 10 min.

³H-EACA was isolated by ion exchange chromatography. Tissue extracts were neutralized to pH 3.5 with 5 N potassium carbonate solution and applied to the column of Dowex 50-X4 (50×4.2 mm), resin 200–400 mesh. The column was washed with 40 ml. redistilled water and ³H-EACA eluted with 18 ml. 0.4 N hydrochloric acid, the first 3 ml. being discarded. Eluates were freeze-dried and ³H-EACA was estimated by liquid scintillation counting as previously described by Carlsson & Waldeck (1965).

The identity of the isolated ³H-EACA was established by subjecting the freeze-dried eluates (dissolved in a few drops of distilled water) to paper chromatography (butanol, acetic, water, 4:1:5, 18–20 hr). All the radio activity was localized in an area with an Rf value (0.56–0.6), similar to that of authentic ³H-EACA.

Recoveries

No corrections were made for recoveries. Where known amounts of ³H-EACA (0.1–0.8 μg) were added to tissue homogenates, recoveries ranged from 80–90% (mean, 85%).

RESULTS

Disposition of ³H-EACA in rat tissues

Figure 1 shows that ³H-EACA was taken up and retained in all the tissues examined (liver, kidney, salivary glands and heart). 0.5 hr after its administration, the highest concentration was found in the liver and the lowest in the heart. However, the levels

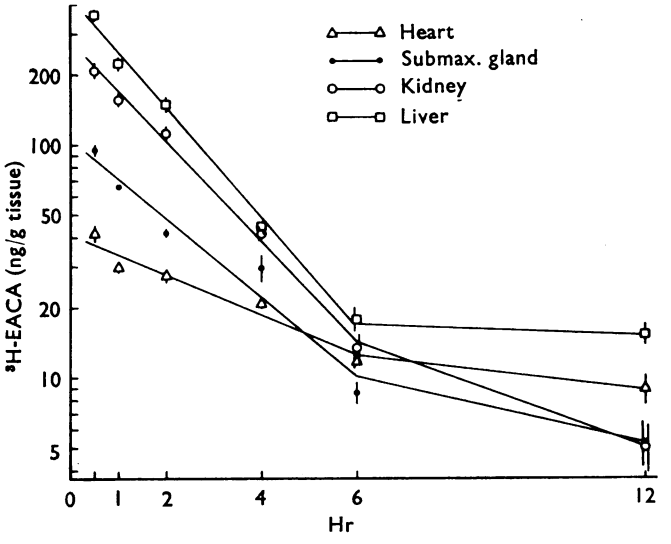

Fig. 1. Disposition of ³H-EACA in rat tissues. Animals were killed at various intervals after intravenous administration of ³H-EACA, 0.2 mg/kg. Each point is a mean of six determinations ±S.E. of the mean.

declined more rapidly in the liver and the kidney than in the heart and salivary glands. For example, after 4 hr, the amount of ³H-EACA retained in the heart was 50% of that retained after 0.5 hr compared with only 12% retained in the liver. This may indicate that EACA is more rapidly eliminated from tissues with poor adrenergic innervation—that is, liver and kidney—than those rich in adrenergic innervation—that is, heart and salivary gland.

The elimination of ^{3}H-EACA appears to be biphasic with an initial rapid phase, which lasted up to 6 hr, followed by a much slower phase. Due to the very low binding of ^{3}H-EACA in the heart and salivary glands, the levels could not be followed with adequate precision after 12 hr.

Effect of sympathetic denervation on the uptake and retention of ^{3}H-EACA

If the amount of ^{3}H-EACA taken up and bound is related to the degree of adrenergic innervation of organs, degeneration of the nerves should inhibit its uptake and retention.

Eight to ten days after removal of the right superior cervical ganglion, the amounts of ^{3}H-EACA retained at various intervals in the right (denervated) and the left (innervated) submaxillary glands are as shown in Table 1. Preliminary observation showed that in unoperated control animals no significant difference existed between the abilities of the right and left submaxillary glands to retain ^{3}H-EACA or ^{3}H-α-methylnoradrenaline.

0.5 hr after the administration of ^{3}H-EACA, the amount found in the denervated gland was about 35% less than that found in the innervated gland.

TABLE 1

EFFECT OF DENERVATION ON THE UPTAKE AND RETENTION OF ^{3}H-EACA BY THE RAT SUBMAXILLARY GLANDS

The right superior cervical ganglia were removed under hexobarbitone anaesthesia and the animals were used 8–10 days after the operation. They were given ^{3}H-EACA, 0.2 mg/kg (intravenously), at zero time and killed after the intervals shown below. Glands from two animals were pooled for each determination and each value is a mean of two determinations±S.E. of the means

Time (hr)	^{3}H-EACA (ng/g innervated gland)	^{3}H-EACA (ng/g denervated gland)	
0·5	121·0±2·6	75·7±6·3	$P<0·025$
1·0	62·5±0·5	51·1±3·3	$P>0·05$
3·0	52·3±1·4	26·9±0·9	$P<0·005$
6·0	14·4±1·2	6·1±0·9	$P<0·05$

Influence of reserpine treatment on the retention of ^{3}H-EACA

Reserpine impairs the storage mechanism of the amine storage granules. If ^{3}H-EACA is retained at sites similar to those at which noradrenaline is bound, reserpine treatment should inhibit its retention in organs rich in adrenergic innervation but not in those poor in adrenergic innervation. Table 2 shows that reserpine treatment (5 mg/kg intraperitoneally) significantly inhibited the ability of the heart to retain ^{3}H-EACA but not that of the liver. The retention of ^{3}H-EACA in the submaxillary gland was not statistically different from that of the controls (see Discussion).

TABLE 2

EFFECT OF RESERPINE TREATMENT ON THE BINDING OF ^{3}H-EACA

Rats were injected with reserpine, 5 mg/kg intraperitoneally, and 6 hr later with ^{3}H-EACA, 0·2 mg/kg intravenously. The animals were killed 2 hr after ^{3}H-EACA. The control animals did not receive reserpine. Each value is a mean of four determinations±S.E. of the means

	^{3}H-EACA (mg/g tissue)		
	Heart	Salivary gland	Liver
Control	27·2±1·8	41·7±1·6	148·1±11·7
Reserpine (5 mg/kg I.P.)	22·3±1·4 $P<0·05$	39·3±2·1 $P>0·25$	180·0±20·5 $P>0·10$

The result may indicate that part of the ^{3}H-EACA taken up by the heart, which is associated with the adrenergic nerves, is retained extragranularly—that is, not particle-bound.

Influence of desipramine and protriptyline on the uptake of ^{3}H-EACA

Two different uptake-concentration mechanisms of the adrenergic neurones, the amine transport across the nerve cell membrane and the subsequent incorporation into the amine storage granules have been demonstrated (Carlsson, Hillarp & Waldeck, 1963; Hillarp & Malmfors, 1964; Lindmar & Muscholl, 1964; Carlsson & Waldeck, 1965; Malmfors, 1965). The former mechanism can be selectively blocked by agents such as desipramine or protriptyline, while the latter mechanism can be selectively blocked by such agents as reserpine or prenylamine (Segontin).

It has been shown that desipramine pretreatment completely prevented EACA from depleting tissues of noradrenaline and blocking adrenergic nerves (Andén, Henning & Obianwu, unpublished). It was therefore considered of interest to see if such treatment would affect the uptake of ^{3}H-EACA by the tissues. Table 3 shows that desipramine treatment significantly reduced the amounts of ^{3}H-EACA retained by the heart and salivary glands, while those retained by the liver and the kidney were greatly increased. The ^{3}H-EACA content of the skeletal muscle was not significantly changed. Protriptyline gave results similar to desipramine.

TABLE 3

EFFECT OF DESIPRAMINE (DMI) AND PROTRIPTYLINE (PTP) ON THE UPTAKE AND BINDING OF ^{3}H-EACA

Values of ^{3}H-EACA are in ng/g tissue. Rats were injected with DMI, 10 mg/kg intraperitoneally, or PTP, 10 mg/kg intraperitoneally, and 0·5 hr later with ^{3}H-EACA, 0·2 mg/kg intravenously. Control animals received only ^{3}H-EACA. The animals were killed 1 hr after ^{3}H-EACA. Each value is a mean of six to seven determinations ± S.E. of the mean

	Heart	Salivary gland	Kidney	Liver	Femoral muscle
Control	38·4±3·6	75·2±7·9	191·1±20·1	325·8±22·7	117·0±8·7
DMI	26·4±1·3 $P<0·025$	49·9±3·4 $P<0·025$	355·4±44·1 $P<0·005$	608·3±48·0 $P<0·001$	131·8±16·0 $P>0·25$
PTP	25·3±1·0 $P<0·01$	50·0±2·9 $P<0·025$	—	628·8±40·4 $P<0·001$	—

Influence of amphetamine on the retention of ^{3}H-EACA

The adrenergic nerve blockade induced by EACA can be prevented or terminated by ⊕-amphetamine if amphetamine is given before or after EACA, respectively (Andén, Henning & Obiawu, unpublished). In order to determine to what extent a direct interaction between amphetamine and EACA is involved in this antagonism, the following experiments were performed. Rats were given ⊕-amphetamine, 0.5 mg/kg (intravenously), 0.5 hr before ^{3}H-EACA and killed 1 hr later. In another series, rats were given amphetamine, 0.5 mg/kg intravenously, 1 hr after ^{3}H-EACA and killed after another hr.

TABLE 4

EFFECT OF AMPHETAMINE TREATMENT ON UPTAKE AND BINDING OF ^{3}H-EACA

Rats were treated with amphetamine, 0·5 mg/kg (intravenously) 0·5 hr before (A) or 1 hr after (B) ^{3}H-EACA, 0·2 mg/kg intravenously. The animals were killed 1 hr (A) or 2 hr (B) after ^{3}H-EACA. Each value is a mean of four determinations±S.E. of the mean

	Heart ^{3}H-EACA (ng/g)	
	A	B
Control	35·6±5·1	27·2±1·7
Amphetamine 0·5 mg/kg I.V.	31·8±3·2 $P>0·25$	28·6±0·7 $P>0·20$

The results presented in Table 4 show that amphetamine did not significantly influence the amount of ^{3}H-EACA retained by the heart. This may indicate that antagonism of the EACA-induced adrenergic nerve blockade by amphetamine is not a direct interaction between the two substances, such as competition for common binding sites.

DISCUSSION

While this manuscript was in preparation, a communication from Dr. Bengt Melander (based on the paper presented by Granstrand, Lindgren, Nybäck & Sedvall (1967) at the XII Scandinavian Congress of Physiology, Åbo, August, 1966) giving support to some of the present findings was received. According to Melander the levels of non-labelled EACA in various organs of rat were determined 0.5 hr after its administration (1,000 mg/kg intraperitoneally). The relative levels of EACA in the heart and submaxillary glands (the only organs included in both studies) are of similar order as the ones reported in the present studies. This may suggest that tracer doses of EACA are essentially disposed in a similar manner as pharmacological doses.

However, after a very large dose (5,000 mg/kg, intravenously) McNicol, Fletcher, Alkjaersig & Sherry (1962) found a somewhat different distribution in the rabbit. Apart from the fact that their data were based on only single determinations (one animal each at two intervals), most of the pharmacological actions had dissipated at the intervals (8 and 18 hr) when determinations were made. The data presented in Fig. 1 indicate that ^{3}H-EACA is eliminated in a biphasic manner, an initial rapid rate (0.5–6 hr) followed by a much slower rate. Data obtained during the slow phase of elimination may not give the entire picture of the disposition of this substance. Although species difference might have contributed to the different results, this is unlikely as this substance appears to be eliminated in a similar manner in different species.

Previous studies show that EACA has no significant effect on the amine levels of the brain (Lippmann & Wishnick, 1965 ; Andén, Henning & Obianwu, unpublished), possibly due to its inability to penetrate the blood brain barrier. Therefore the brain was not included in the present studies. However, the report of Granstrand *et al.* (1967) indicates that about 20% of the amount of EACA found in the heart was retained in the brain, suggesting a limited accessibility of this substance to the brain.

Although EACA may be generally distributed throughout the peripheral tissues, its retention appears to be related to the degree of adrenergic innervation of these organs. For example, 4 hr after its administration the amounts retained in the heart, submaxillary

gland, kidney and the liver were 50%, 31%, 19% and 12% respectively (calculated as percentage of the amounts retained after 0.5 hr).

The relative rates of elimination of ^{3}H-EACA may also in part depend on the plasma level of ^{3}H-EACA. The plasma half-life of EACA in rabbit is about 30 min (McNicol *et al.*, 1962) (cf. results in Fig. 1) and since the liver, for example, has a greater blood supply than the salivary glands, a fall in the plasma level of EACA will cause a greater decline in the tissue level of EACA from the former organ than from the latter. However, significant amounts of EACA could still be recovered from the tissues at a time when the plasma concentrations were hardly detectable (McNicol *et al.*, 1962; Melander, Glinieki, Granstrand & Hanshoff, 1965). Further, cellular uptake of EACA has been demonstrated in the rat diaphragm *in vitro* (McNicol et al., 1962). These observations suggest partial intracellular localization of EACA.

Agents which cause depletion of tissue noradrenaline may be lipid or non-lipid soluble. The former group of substances, such as reserpine and prenylamine (Segontin), do not utilize the amine transport mechanism, the membrane pump, to get into the neurones, while the latter group, such as guanethidine and metaraminol, do utilize it. Effective blockade of the membrane pump completely abolishes the pharmacological actions of the latter group of substances (Carlsson & Waldeck, 1965; Stone, Potter, Stavorski, Ludden & Totaro, 1964). As already noted above (see Results) blockade of the membrane pump with desipramine completely abolished the tissue depletion of noradrenaline and the adrenergic nerve blockade induced by EACA. The present studies show that desipramine and protriptyline, two potent blockers of the membrane pump, inhibited the amounts of ^{3}H-EACA retained in organs rich in adrenergic innervation. The extent of this inhibition is difficult to estimate since such treatments apparently raised the levels of ^{3}H-EACA retained at sites not specific for the storage of noradrenaline (judged by the levels in the liver and kidney).

Further support for the suggestion that the uptake and retention of ^{3}H-EACA are, at least in part, dependent on intact adrenergic nerve function is provided by the finding that denervation of the salivary glands inhibited the uptake of this substance by about 35%. Even if one disregards the possibility that the amount of ^{3}H-EACA retained extracellularly in the heart and the submaxillary glands after desipramine might have been raised, the degree of inhibition of uptake of ^{3}H-EACA by membrane pump blockade is about 33%.

The moderate effect of reserpine on the retention of ^{3}H-EACA in the heart and the lack of effect on its retention in the salivary glands probably indicate that the fraction of EACA which is associated with the adrenergic nerves in the two organs is largely not particle-bound.

Recently, Lundborg & Waldeck (1966) and Lundborg & Stitzel (1967) demonstrated the existence of at least two amine storage mechanisms in the adrenergic storage granules, one of which is insensitive to reserpine action. The present finding may also indicate that the fraction of EACA which is associated with the adrenergic nerves is largely bound at sites resistant to reserpine action.

However, it has been recently shown (Stitzel, Lundborg & Obianwu, unpublished) from subcellular distribution studies that ^{3}H-EACA was largely localized in the supernatant, very little ^{3}H-EACA being recovered from the particulate fraction.

The observation that desipramine and protriptyline treatment raised the levels of ^{3}H-EACA in the liver and kidney is surprising. This indicates that these agents delay the elimination of EACA from these tissues. Recently, Sulser, Owens & Dingell (1966) reported that both desipramine and protriptyline prolonged the central actions of amphetamine and substantially raised its level in the brain. They suggested that these agents inhibited the metabolism of amphetamine.

In the present studies no metabolite of EACA was detected, though the possibility that the method used in estimating ^{3}H-EACA may have been unsuitable for detecting any possible metabolites cannot be ruled out. However, previous studies indicate that 60–90% of administered EACA is recovered unchanged in urine (Nilsson, Sjoerdsma & Walderström, 1960 ; Hallesy, Hine & Yuda, 1961 ; Melander *et al.*, 1965) in man, dog, rabbit and rat. These data suggest that EACA is largely eliminated by renal excretion.

The renal clearance rate of EACA in man is about 75% of creatinine clearance (McNicol *et al.*, 1962). This suggests that the kidney handles EACA primarily by filtration and reabsorption. It would appear, then, that desipramine and protriptyline interfere with the renal excretion of EACA, though inhibition of its metabolism cannot be ruled out yet. In any event, the possibility that these agents interfere with renal function does not seem to have been investigated and deserves consideration.

The ability of amphetamine-like substances to antagonize the guanethidine-induced adrenergic nerve blockade is well documented (Day, 1962 ; Day & Rand, 1963 ; Chang, Costa & Brodie, 1965 ; Obianwu, unpublished). The mechanism of this antagonism is not well understood. It has been suggested that amphetamine exerts its antagonistic action by competing with guanethidine for specific binding sites at the adrenergic nerve terminals (Day & Rand, 1963 ; Chang, Costa & Brodie, 1965). It is also suggested that the amphetamine-induced inhibition of uptake of guanethidine is due to the same competition (Chang *et al.*, 1965).

The present finding that amphetamine in doses which antagonize the EACA-induced adrenergic nerve blockade had no effect on the amount of ^{3}H-EACA retained by rat heart is not in accord with the above explanation. However, the possibility that amphetamine might have exerted some antagonistic action on the uptake of ^{3}H-EACA cannot be completely ruled out, in view of the fact that only a small fraction of the ^{3}H-EACA taken up is associated with the adrenergic nerves.

The present studies demonstrate that ^{3}H-EACA is generally distributed in the body and provide evidence indicative that a fraction of the amount retained in organs rich in adrenergic innervation is associated with the adrenergic nerves.

SUMMARY

1. Disposition of ^{3}H-EACA and its interaction with adrenergic neurones have been studied in the rat.

2. ^{3}H-EACA was generally distributed and was eliminated in a biphasic manner.

3. Desipramine and protriptyline reduced the amount of ^{3}H-EACA retained by the heart and submaxillary gland while increasing that retained by the liver and kidney.

4. Denervation of the submaxillary gland reduced the uptake of ^{3}H-EACA by this organ.

5. Reserpine pretreatment significantly inhibited retention of ^{3}H-EACA by the heart but not by the salivary glands or the liver.

6. Amphetamine in doses which readily antagonize the EACA-induced adrenergic nerve blockade failed to influence the retention of ^{3}H-EACA by the heart.

7. It is concluded that about 30% of the amount of ^{3}H-EACA retained by the heart and the submaxillary glands is associated with the adrenergic nerves and that desipramine and protriptyline inhibit elimination of ^{3}H-EACA from the liver and kidney.

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