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Anti-inflammatory effect of antiplasmin agents,ϵ -aminocaproic acid and trans-4-aminomethyl-cyclohexane carboxylic acid, in rats

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Abstract

1. Both EACA and AMCHA clearly showed an anti-inflammatory effect, by intravenous, intramuscular, or oral route, against inflammatory edema produced in rats by intracutaneous injection of rabbit's anti-rat serum, carrageenin, histamine, serotonin, or bradykinin, as tested by the punch method. 2. The two compounds also showed inhibitory action against the cotton pellet granuloma when used in a larger dose. 3. There was virtually no difference between the two compounds in their anti-inflammatory activity, in spite of the fact that antiplasmin activity of AMCHA is evidently greater than that of EACA. In addition, there was no increase in fibrinolysis at the site of antiserum inflammation in rats. Therefore, it would be difficult to presume that the anti-inflammatory action of these compounds is due to their antiplasmin activity. 4. Salicylates, pyrazolidine derivatives, and non-steroidal antiinflammatory agents like flufenamic acid inhibited degranulation of isolated rat mast cells induced by compound 48/80 and also inhibited ATP-32Pi exchange reaction in rat liver mitochondria, but such actions were not observed in EACA or AMCHA. 5. Anti-inflammatory effect of EACA and AMCHA did not decrease after adrenalectomy but did become weak in hypophysectomized rats. EACA did not increase blood sugar level in normal rats so that its antiinflammatory action is not due to hyperglycemia, and the effect of hypophysectomy may not be correlated to carbohydrate metabolism. 6. Anti- inflammatory effect of EACA and AMCHA appeared more rapidly after intramuscular or oral administration than by intravenous injection but the effect was not fortified after their in vitro incubation with tissues of stomach, intestine, or liver.

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ANTI-INFLAMMATORY EFFECT OF ANTIPLASMIN AGENTS, ε-AMINOCAPROIC ACID AND *TRANS*-4-AMINOMETHYL-CYCLOHEXANE CARBOXYLIC ACID, IN RATS*

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It has been shown that salicylates, pyrazolone derivatives, and other antipyretics inhibit certain experimental allergy and histamine release (1-3). These compounds are also known to inhibit plasma protease, considered to be plasmin, *in vitro*, in a concentration attainable in circulating blood after administration of a therapeutic dose (4, 5). Since activation of proteases, including plasmin, may occur during antigen-antibody reaction and in inflammation (6), and since the activated plasmin can form phlogogenic kinins from kininogens (α_2-globulin) in the plasma, either indirectly or directly (7-15), it is possible to presume anti-allergic or anti-inflammatory action in substances that inhibit the activation or action of plasmin.

Fragmentary reports have already appeared on the anti-allergic effect of an antiplasmin agent, ε-aminocaproic acid (EACA). *trans*-(4-Aminomethyl)-cyclohexanecarboxylic acid (AMCHA), newly introduced by OKAMOTO and OKAMOTO (16) is said to possess far more potent antiplasmin activity than EACA as determined by a fibrinolytic test (16-18). Reports have also been made on the absorption, excretion, and blood level of these two compounds (19-21).

The present report demonstrates that both EACA and AMCHA show a fairly marked inhibitory effect on experimental inflammation in rats, as tested by the punch method, and also gives result of a few experiments carried out for attempted elucidation of the mechanism of such action.

MATERIALS AND METHODS

Anti-inflammatory assay. Evaluation was made by the punch method devised by UNGAR *et al.* (22), in which anti-inflammatory effect of drugs is based on the inhibition of the cutaneous edema provoked by antiserum. Male Wistar rats (150 ±5 g) received intradermally a total of 8 injections in the previously shaved skin

* The main part of this study has been published in Japanese (*Folia pharmac. japon.*, **63**, 560, 1967).

of the back, 4 sites on each side of the midline. One side received the lyophilized anti-rat rabbit serum, 5 mg per site in 0.05 ml physiological saline, while the opposite side was injected with saline of the same quantity, through a 27-gauge needle. Two hours after injection of the antiserum, when the edema reached the maximum, the rats were stunned and bled to death. The excised piece of skin, with the fur-side down, was pinned on a wooden board and injected sites were cut out with a steel punch (diam. 12 mm, area 112 mm^2). Each piece of skin was immediately weighed on a torsion balance. Intensity of inflammation is expressed as $I_c=(W_t-W_s)100/W_s$, where W_t is the weight of inflammatory site and W_s is the weight of the saline-injected site. The I_c value taken from 16 animals, 64 test and 64 control sites, gave a mean of 89 ± 0.68 (S.E.). Anti-inflammatory activity is expressed in terms of per cent inhibition of inflammation, i. e., $(I_c-I_t)\ 100/I_c$, where I_c is the intensity of inflammation in the non-treated control animals and I_t is the same in the animals treated with the drug under test. In each test for a drug, I_t was usually expressed as the mean of 8—12 values obtained from 2—3 animals. Drugs were administered by either intravenous, intramuscular, or oral route, and volume of the solution was 1 ml/150 g body weight. When observation was made on the time course of the activity, the intervals between administration of the drug and injection of phlogogenic substance were varied from 30 min to 24 hr. Intravenous injection was made into the tail vein, and intramuscular injection was divided into both sides of femoral muscle.

In some of the experiments, carrageenin, histamine, serotonin, bradykinin, and formaldehyde solutions were also used as the phlogogenic substance. Table 1

TABLE 1. STANDARD DOSES OF PHLOGOGENIC SUBSTANCES AND THEIR EFFECTS

Phlogogenic substance	Dose	Weight increase of skin (%)	Time of determination after injection (hr)
Anti-rat serum	5 mg	89 ± 0.68 (64)[a]	2
Histamine[b]	20 μg (base)	56 ± 0.80 (16)	1
Serotonin	20 μg (base)	53 ± 0.67 (16)	2
Bradykinin	7.5 μg	50 ± 0.64 (24)	1
Carrageenin	0.5 mg	52 ± 1.32 (24)	3
Formaldehyde	5 mg	93 ± 1.48 (12)	3

a) Number of test sites.

b) In guinea pigs, the others in rats.

gives the doses of phlogogenic substances injected, the time of determination of inflammation after injection, and the intensity of inflammation, I_c, at the time of determination, i. e., the per cent weight increase of skin. Guinea pigs (male, 230 ± 10 g) were also used but only for histamine inflammation and drugs to be tested were given by cardiac puncture. Anti-rat serum was obtained from rabbits which had received 9 intravenous injections of 1 ml of rat serum every other day and blood was drawn 10 days after the last injection, and the serum was lyophilized.

Assay of inhibitory effect on granuloma. The cotton pellet method of ROBINSON

and ROBSON (23) was slightly modified; details have already been reported (24).

Hypophysectomy and adrenalectomy. Rat hypophysis was extirpated by TANAKA's auditory canal method (25). Only the animals in healthy state one week after the operation were used for the experiments. Bilateral adrenalectomy was performed from both sides of the back under ether anesthesia, and the animals were used for the experiment 3 days after the operation, during which period drinking water contained 0.9% saline. Absence of accessory adrenals was confirmed after the experiment.

Measurement of blood glucose. The glucose oxidase-peroxidase method of HUGGETT and NIXON (26) was used. Blood was drawn from the carotid artery.

Test for inhibition of degranulation of rat mast cells. The method was the same as that of YAMASAKI and SAEKI (27) using rat mast cells isolated from peritoneal fluid. The effect was represented as the drug concentration to effect 50% inhibition of mast-cell degranulation produced by 0.5 μg/ml of compound 48/80 in a buffered physiological solution.

ATP-^{32}Pi exchange reaction. Mitochondria were isolated by centrifugation from the liver of Wistar rats (male or female) weighing 150—200 g, by the method of HOGEBOOM (28). For the preparation of homogenate, 0.25 M sucrose solution containing 40 μM of EDTA-Na_2 and 1 mM of Tris-HCl buffer (pH 7.4) was used. The isolated mitochondria were suspended in 0.25 M sucrose solution containing 1 mM of Tris-HCl buffer (pH 7.4). All these procedures were carried out at 0—4°C. The protein contents were measured by the method of LOWRY *et al.* (29).

The medium for ATP-^{32}Pi exchange reaction in mitochondria contained the following in a final volume of 2.0 ml: 0.1 M sucrose, 20 mM KCl, 10 mM Tris-HCl buffer (pH 7.4), 40 μM EDTA-Na_2, 3 mM potassium phosphate buffer (pH 7.4) containing 200,000 cpm of ^{32}P, 3 mM ATP, and the drug under test, which was omitted in the control experiment. After addition of mitochondria corresponding to 2.9 mg of protein to this medium, the reaction was allowed to proceed in test tubes at 25°C. Incorporation of ^{32}P into organic matter was measured by the method of HAGIHARA and LARDY (30). After incubation for 10 min, 0.5 ml of the reaction mixture was added to 1 ml of HAGIHARA's Reagent A in test tubes which were kept in an ice bath. To the mixtures, 1.5 ml of Reagent B was added, and, after left standing for a few min, this mixture was applied to a reverse-phase siliconized Celite column. In a planchet 1 ml of the effluent was placed, dried, and radioactivity was counted by a GM counter.

Measurement of fibrinolytic activity. Standard and heated (60—85°C, 30 min) fibrin plates were used. In a dish of 4.5 cm diameter, 1 ml of fibrinogen solution and 2 ml of buffer solution were placed, one drop of thrombin solution was added while gently moving the dish, and the dish was allowed to stand until the mixture formed a white jelly-like mixture. This was allowed to stand in an incubator of 37°C for 30 min to dry the surface. Fibrinogen solution was prepared from 100 ml of bovine plasma according to the method of ASTRUP and MÜLLERTZ (31), fractionally precipitated with ammonium sulfate, washed with cold distilled water, and dissolved in 40 ml of buffer solution (662 ml of 0.1 M barbital sodium,

338 ml of 0.1 M HCl, and 320 ml of distilled water). Bovine thrombin was dissolved to contain 100 units in 1 ml of physiological saline solution.

On the back skin of a rat 5 mg of rabbit's anti-rat serum was given by intracutaneous injection to produce antiserum inflammation. The injected skin was punched as described above after various time intervals (immediately after, and 3, 5, 10, 30, 60, and 120 min) and the skin was placed gently on the fibrin plate, with the subcutaneous tissue touching the plate. The dish was covered, placed in an incubator (37°C), and dissolution of fibrin was tested at various time intervals during the next 30 min to 24 hr. The control experiment was made on the skin injected with saline solution not containing antiserum.

Chemicals and reagents. EACA and AMCHA used were crystalline products supplied by the Daiichi Pharmaceutical Co., Tokyo. Phlogogenic substances used were carrageenin (Genu Carrageenan, Pectin Factory, Copenhagen), dextran (Meiji Seito Co., av. mol. wt., 54,000), histamine hydrochloride (Wako Pure Chemical Co., Osaka), serotonin creatinine sulfate (Sigma Chemical Co., U. S. A.), bradykinin (Protein Research Institute, Osaka University), and formaldehyde (Formalin, J. P.). Compound 48/80 was kindly donated by the Burroughs Wellcome Laboratories, New York, and disodium salt of ATP was a product of Sigma Chemical. Bovine thrombin was purchased from Mochida Pharmaceutical Co. All other reagents used were the purest commercial grade.

RESULTS

A. Anti-inflammatory effect

1. Inhibition of antiserum edema. After intravenous injection of 100 mg/kg of EACA or AMCHA, the rats were divided into seven groups of 2—3 each and antiserum was injected intracutaneously on their back 0.5, 1, 2, 3, 5, 8, and 24 hr later. Edema intensity, i. e., weight increase of skin, was compared with that of non-treated control, and percentage of inhibition of edema was calculated as mentioned before. The same experiments were carried out with intramuscular and oral administration of these drugs. The results are shown in Fig. 1.

In the case of intravenous injection, edema inhibition was the most remarkable when antiserum was injected 2 hr after the administration of the drug, both EACA and AMCHA. In other words, effect of the drugs bacomes maximum 2 hr after their administration. In the oral administration, the maximum inhibition appeared after 30 min while that appeared 1 hr after intramuscular administration. It is of interest that the effect of intravenous administration reached the maximum later than that of oral or intramuscular administration. The effect decreased with longer interval between administration of drug and antiserum injection but some effect remained even 24 hr after the drug administration. There was no dif-

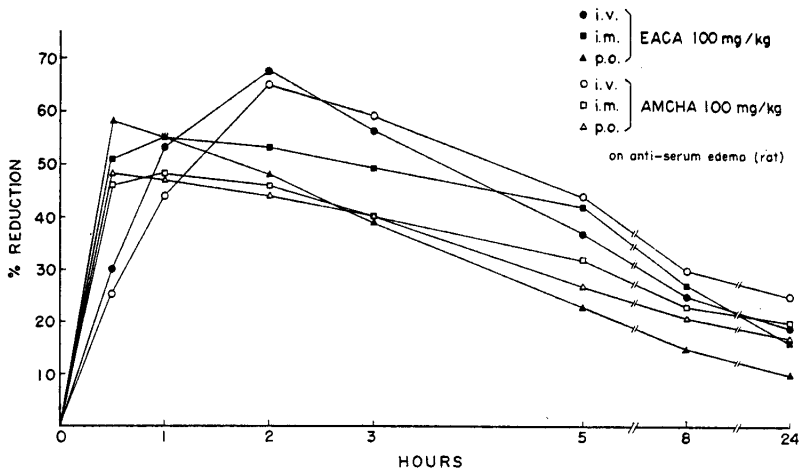

Fig. 1. Time course of the anti-inflammatory effects of EACA and AMCHA on antiserum-induced cutaneous edema in rats. Ordinate: % reduction in weight increase of skin produced by antiserum 5 mg per site; abscissa: time intervals at which antiserum was injected after administration of the drugs. Each point represents the mean of 8—12 test and 8—12 control sites from 2—3 rats.

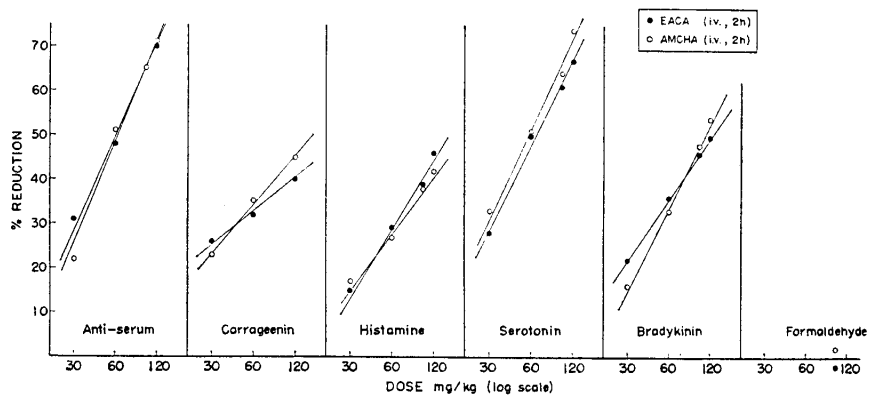

Fig. 2. Anti-inflammatory effects of EACA and AMCHA on the cutaneous edema produced by various phlogogenic substances. Ordinate: % reduction of weight increase; abscissa: dose (log scale). Standard inflammations were produced by intradermal injection of phlogogenic substances in doses per site listed in Table 1. EACA and AMCHA were injected intravenously 2 hr before the injection of phlogogenic substances. Histamine inflammation was produced in guinea pig, the others in rat. Each point represents the mean of 8—12 test and 8—12 control sites from 2—3 animals.

ference in such effect between EACA and AMCHA, and the degree of the effect was hardly distinguishable between these two drugs. Intravenous route gave slightly better effect than oral or intramuscular route in either

drug. In the dose used (100 mg/kg), half-life of the effect lies between 5 and 8 hr by any route of administration.

Dose-effect relationship in the inhibitory effect of EACA and AMCHA, administered by intravenous route 2 hr before antiserum, is shown by a straight line based on log dose scale, as indicated on the far left in Fig. 2. There is no difference between the two drugs in the effect, and their dose for 50 % inhibition of edema is around 60 mg/kg.

Since some effect of both drugs was seen even after 24 hr, possible cumulative effect was examined by intravenous injection of 100 mg/kg of either drug during the forenoon every day for 1—5 days. Three animals each were taken after each administration, and antiserum injection was given 20 hr after the last administration of the drug. As shown in Fig. 3,

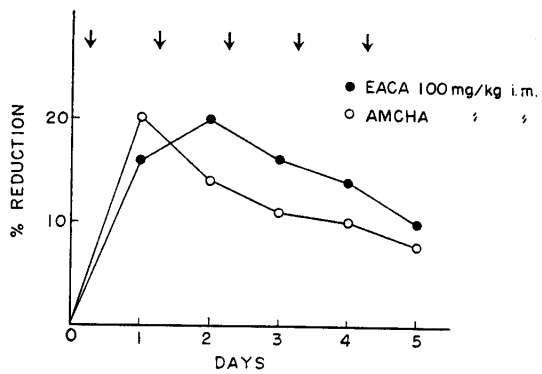

Fig. 3. Anti-inflammatory effects of successive daily injection of EACA and AMCHA. Ordinate: % reduction of weight increase; abscissa: days of determination of the effect. Arrows indicate an intramuscular injection of drugs (100 mg/kg), 20 hr prior to every anti-inflammatory test. Each point represents the mean of 8 test and 8 control sites from 2 rats.

increase in the effect, suggesting accumulation of the action, was seen until second administration of EACA but the residual effect rather tended to decrease 20 hr later after the third injection. This decrease in the effect already appeared after second injection of AMCHA. These results indicate that repeated injection of 100 mg/kg dose every 24 hr does not produce accumulation of the effect.

2. *Inhibition of edema produced by carrageenin, histamine, serotonin, bradykinin, and formaldehyde.* Anti-inflammatory dose-effect curves were obtained for intravenously injected EACA and AMCHA against inflammatory edema produced by the title substances (guinea pigs for histamine, rats for others). Experiments were carried out exactly as in the foregoing

case using antiserum edema, and the results are shown in Fig. 2. Both drugs were ineffective on formaldehyde inflammation but showed inhibitory effect on inflammation by other substances. There was no difference between the two drugs in the intensity of their effect. The dose for 50 % edema inhibition was 85—120 mg/kg for carrageenin, 100—115 mg/kg for histamine, 60 mg/kg for serotonin, and 110—120 mg/kg for bradykinin inflammation.

3. Inhibition of granuloma. After implantation of cotton pellets, the drug was given by intramuscular injection every day for six days and the pellets were taken out 24 hr after the last injection. The result shown in Table 2 indicates that 200 mg/kg/day of EACA gives a slight inhibition

Table 2. Effect of EACA, AMCHA and hydrocortisone acetate on cotton pellet granuloma in rats

Treatment	Daily dose (mg/kg)	Pellet weight (mg)	Granulation tissue (mg±S. E.)	Effect (%)	*P*
Controls	—	8.2	13.3±0.53		
EACA	100	8.2	13.0±0.49		
Controls	—	7.2	12.1±0.51		
EACA	200	7.2	10.3±0.34	15.1	< 0.05
EACA	500	7.2	9.2±0.39	24.1	< 0.001
Controls	—	7.4	10.8±0.35		
AMCHA	100	7.4	10.4±0.88		
AMCHA	200	7.4	10.1±0.36	5.5	< 0.1
AMCHA	500	7.4	8.5±0.27	21.3	< 0.001
Controls	—	10.5	17.2±0.48		
Hydrocortisone	10	10.5	12.6±0.25	26.3	< 0.001
Controls	—	7.2	12.1±0.51		
Hydrocortisone	40	7.2	7.4±0.24	38.8	< 0.001

4—6 animals per group.

but the reliable inhibitory effect required a large dose of 500 mg/kg/day of both drugs. In this dose, the effect of these drugs was close to that of 10 mg/kg/day of hydrocortisone.

B. Anti-inflammatory effect in adrenalectomized and in hypophysectomized rats

Oral and intravenous dose-effect curves of EACA and AMCHA on anti-rat serum edema were examined with adrenalectomized and hypophysectomized rats. Results are shown in Fig. 4 in comparison with that of normal rats. Edema was stronger in the adrenalectomized than normal rats, but weaker in hypophysectomized rats. Due to such a difference,

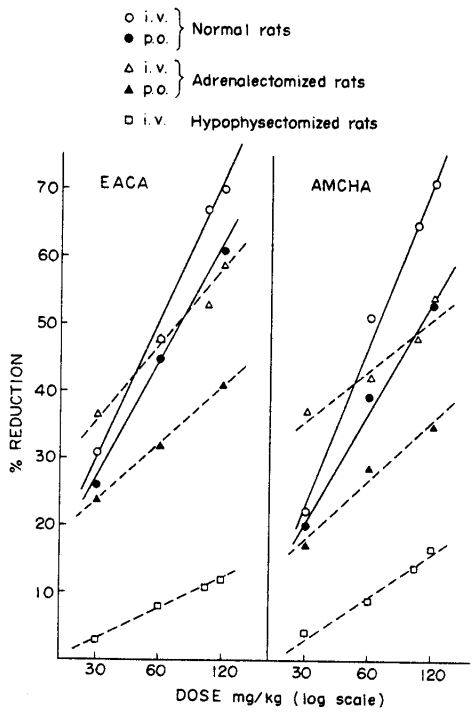

Fig. 4. Anti-inflammatory effects of EACA and AMCHA in adrenalectomized and in hypophysectomized rats. Ordinate: % reduction of weight increase; abscissa: dose (log scale). *See* also Table 3.

edema intensity is shown as the observed values of per cent weight increase of skin in Table 3.

TABLE 3. ANTI-INFLAMMATORY EFFECTS OF EACA AND AMCHA IN ADRENALECTOMIZED AND IN HYPOHYSECTOMIZED RATS ON ANTISERUM-INDUCED CUTANEOUS EDEMA.

Figures denote percentage of weight increase of skin with S. E., and number of test sites in parentheses. Dose of antiserum, 5 mg in 0.05 ml per site.

	Control			Dose (mg/kg) 30	60	100	120
Normal rats	89±0.74 (20)	EACA	i.v.	62±0.9 (8)	46±0.6 (8)	30±1.0 (8)	28±1.0 (8)
			p.o.	66±2.1 (4)	50±0.7 (4)		35±1.8 (4)
		AMCHA	i.v.	70±1.8 (8)	44±1.2 (8)	31±1.0 (8)	26±1.1 (8)
			p.o.	74±0.8 (4)	55±1.9 (4)		42±1.9 (4)
Adrenalectomized rats[a]	107±1.17 (16)	EACA	i.v.	56±1.1 (8)	47±0.9 (8)	42±1.0 (8)	37±1.0 (8)
			p.o.	83±2.1 (4)	73±2.0 (4)		64±1.1 (4)
		AMCHA	i.v.	56±1.1 (8)	49±1.6 (8)	44±1.5 (8)	39±1.8 (8)
			p.o.	86±1.8 (4)	77±1.4 (4)		70±1.3 (4)
Hypophysectomized rats[b]	77±1.17 (12)	EACA	i.v.	74±1.4 (8)	70±0.9 (8)	67±1.6 (8)	
		AMCHA	i.v.	76±1.3 (8)	71±0.9 (8)	67±1.0 (8)	

a) Experiment performed 3 days after the operation. Drinking water contained 0.9% NaCl.
b) Experiment performed 7 days after the operation.

Slope of the dose-effect curves of two drugs differs in the adrenalectomized and hypophysectomized rats. Considering the data in Fig. 4 and Table 3, the effect of two drugs cannot be said to decrease by adrenalectomy although hypophysectomy clearly decreases their effect.

C. Effect on degranulation of isolated rat mast cells induced by compound 48/80

Degranulation of mast cells by compound 48/80 or antiserum is considered to be dependent on energy generating capacity of these cells since this reaction is inhibited by uncouplers of oxidative phosphorylation or respiratory inhibitors in a glucose-free medium (32, 33). The uncoupling properties of salicylates, pyrazolone derivatives, and some non-steroidal anti-inflammatory agents have been shown by ADAMS and COBB (34), BRODY (35), and by WHITEHOUSE (36). Recently, YAMASAKI and SAEKI (27) reported that these non-steroidal anti-inflammatory agents inhibit degranulation of mast cells by compound 48/80 in a medium not containing glucose and that this activity roughly parallels their anti-inflammatory effect against antiserum inflammation examined by the punch method. Table 4 shows the effect of EACA and AMCHA on mast-cell degranulation by compound 48/80 together with the effect of some non-steroidal anti-inflammatory agents and some known uncouplers (2, 4-dinitrophenol,

TABLE 4. EFFECTS OF EACA, AMCHA, AND OTHER DRUGS ON MAST-CELL DEGRANULATION BY COMPOUND 48/80 AND ON INFLAMMATORY EDEMA BY ANTISERUM

Drug	Conc. of 50% inhibition of M. C. degranulation	Anti-inflammatory effect	
		Dose	Reduction of weight increase
	mM	mg/kg	%
EACA	25	100[a]	67±1.2
AMCHA	65	100[a]	65±1.0
Sodium salicylate	1.5	250	43±0.5
Acetylsalicylic acid	1.1	250	33±1.0
Phenylbutazone	0.03	30	48±1.1
Oxyphenbutazone	0.006	30[b]	52±0.5
Flufenamic acid	0.018	30	40±2.0
Mefenamic acid	0.062	30	49±1.3
Indomethacin	0.05	30[b]	57±1.3
2, 4-Dinitrophenol	0.02	30[c]	38±1.1
Dicumarol	0.02	30[c]	32±1.1
Warfarin sodium	0.16	30[c]	38±0.5
Amytal sodium	0.5	30	30±1.1

[a] Injected i. v. 2 hr before, [b] i. m. 3 hr before, and [c] s. c. 30 min before injection of antiserum; while all the others were injected i. m. 2 hr before.

dicumarol, warfarin sodium), and also their inhibitory effect on antiserum inflammation. While non-steroidal anti-inflammatory agents showed a marked inhibitory action on degranulation like known uncouplers, EACA and AMCHA did not show any apparent activity despite their similar anti-inflammatory effect.

D. Effect on ATP-^{32}Pi exchange reaction in mitochondria

As shown in Table 5, ATP-^{32}Pi exchange reaction in rat liver mitochondria is clearly inhibited by 0.01—0.05 mM of phenylbutazone, oxyphenbutazone, and flufenamic acid, effect of the last named being more marked than that of dinitrophenol. However, such an effect was not observed in either EACA or AMCHA.

TABLE 5. EFFECT OF EACA AND AMCHA ON THE ATP-^{32}Pi EXCHANGE REACTION IN RAT LIVER MITOCHONDRIA

	Concentration (mM)	c. p. m.	Inhibition (%)
None		2,383	
EACA	1	2,660	(−13)
	5	2,619	(−10)
AMCHA	1	2,313	3
	5	2,269	5
Flufenamic acid	0.01	0	100
Phenylbutazone	0.05	650	67
	0.1	235	91
Oxyphenbutazone	0.01	1,597	33
	0.05	658	68
2,4-Dinitrophenol	0.01	136	94

Mitochodria, 2.9 mg protein/2 ml, 10 min incubation at 25°C.

E. Anti-inflammatory effect and blood sugar

It is known that anaphylactoid edema in rats produced by dextran or egg white is inhibited in alloxan-diabetes and increased in insulin-induced hypoglycemia (37, 38). Similar phenomena have been observed in the edema by antiserum or other phlogogenic substances, as tested by the punch method (39). Fig. 5 shows the relationship between blood sugar in rats after administration of alloxan, glucose, or insulin, and intensity of anti-rat serum edema, with the results obtained after intravenous injection of EACA. As will be clear from these data, EACA hardly gave any effect on blood sugar in a dose that showed anti-inflammatory effect, indicating that its anti-inflammatory mechanism is not related to hyperglycemia.

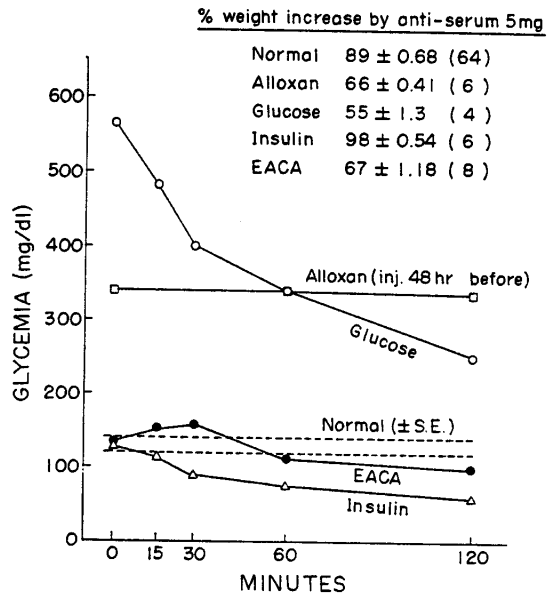

Fig. 5. Glycemia after treatment with EACA, alloxan, glucose or insulin. EACA 100 mg/kg (i. v.), glucose 10 g/kg (i. v.) or insulin 20 i. u./kg (s. c.) was injected just before zero min, and alloxan monohydrate 30 mg/kg (i. v.) 48 hr before. Accompanying table indicates intensities of inflammatory edema produced by antiserum injected intracutaneously at the point between 0 and 15 min in abscissa. Figures in parentheses denote number of test and control sites.

F. Fibrinolytic test for antiserum inflammation

In order to know whether the afore-mentioned anti-inflammatory effect of EACA and AMCHA was due to their antiplasmin activity, it is necessary to examine whether the mechanism of inflammatory edema produced in the rat involves activation of plasmin or not. Rats were given intracutaneous injection of 5 mg of antiserum and the skin at injected sites was punched immediately and 3, 5, 10, 15, 30, 60, and 120 min later. Punched pieces of the skin were embedded in standard and heated plates, and examined periodically up to 24 hr. It was thereby found that there was no fibrinolysis, the same as in the control skin pieces obtained from the site injected with saline solution.

G. Anti-inflammatory effect of EACA and AMCHA incubated with tissues

As stated above, the anti-inflammatory effect of EACA and AMCHA appears much earlier after oral or intramuscular administration than by intravenous injection and is especially rapid after oral administration. However, rise in blood level of these compounds has been reported to be

slower after oral administration than after subcutaneous injection in rabbits (19) and in man (21). This fact suggests the possibility that the drugs administered through the oral route might be transformed into other effective substance(s) after passing through the digestive tissues. Therefore, EACA was incubated with gastric, intestinal, and liver tissue pieces from rats, in Tyrode solution at 37°C for 30 min and its anti-inflammatory effect was examined by intravenous administration. However, the effect of EACA was not increased but rather decreased by the treatment (Table 6).

TABLE 6. ANTI-INFLAMMATORY EFFECT ON ANTISERUM EDEMA OF EACA INCUBATED WITH TISSUES

Tissue	% Reduction of weight increase	
	Time of antiserum injection after drug administration	
	1 hr	2 hr
None	53±1.34 (8)[b]	67±1.18 (8)
Stomach	24±1.84 (8)	34±2.05 (8)
Intestine	18±1.73 (8)	26±1.09 (8)
Liver	15±1.87 (8)	28±1.22 (8)
Liver[a]	18±2.53 (8)	28±4.18 (8)

15 mg of EACA was incubated with 1.1 g tissue slices in 10 ml of 0.9 % saline (pH 7.2, adjusted) or [a]Tyrode solution (pH 8.0) for 30 min at 37°C. Filtrate was injected i.v. in a dose corresponding to 100 mg per kg. [b] Number of test sites.

DISCUSSION

There are many classical literatures suggesting the role of proteolytic enzymes in allergic responses (40-43), and it is now well known that activation of tissue proteolytic enzymes occurs at the site of allergic or inflammatory reaction (6). These enzymes probably initiate a complex chain reaction, which involves the formation of chemical mediators, leading to increased vascular permeability and to other events of inflammatory processes. Such a protease activated at the site of allergy and inflammation is not a unity and can be discriminated by its substrate specificity and pattern of inhibitors. Plasmin (fibrinolysin), one of such proteolytic enzymes, is formed from plasminogen, a precursor present in plasma euglobulin fraction, by its activation through the action of plasminogen activators (fibrinokinase) which are formed in many tissues or in plasma. Because of the difficulty of isolating plasmin in enzymically pure state, its properties have not been clarified strictly and it is still not clear whether it is a single enzyme or not (44, 45). However, its relation to inflamma-

tion is being increasingly regarded important because of the recent observations that plasmin takes part in the kinin forming system, either acting directly on plasmakininogen or indirectly as an activator of pre-enzyme (7-15).

It is known that salicylates and some kinds of anti-inflammatory agent inhibit various enzymes (38, 46-48). UNGAR, DAMGAARD and HUMMEL (4) reported that the inhibitory effect of some anti-inflammatory agents including salicylates and pyrazolone derivatives on Arthus-type arthritis of guinea pigs paralleled their *in vitro* plasmin inhibitory effect.

The present series of experiments also indicate that the antiplasmin agents, EACA and AMCHA, evidently inhibit the reverse Arthus-type inflammatory reaction of the rat skin. These two compounds also inhibited inflammatory edema produced by carrageenin, histamine, serotonin, and bradykinin, although they were ineffective against formaldehyde inflammation. Such an effect was considerably marked in a dose level of 50 mg/kg, which is a little larger than the usual clinical dose of 500 mg/day for 3—4 days. Inhibitory effect on cotton pellet granuloma was also observed with a larger dose (over 200 mg/kg/day) for six days. However, it would be difficult to consider that such an effect of these compounds is due to their antiplasmin properties, whether it be the inhibition of plasminogen activation or of fibrinokinase activation. The main reason for this is, first that there was no clear difference between these two compounds in their anti-inflammatory effect in rats, in spite of the fact that inhibitory effect of AMCHA against plasmin, tested with fibrinolytic or caseinolytic activity, is far stronger than that of EACA, either *in vitro* (16-18) or *in vivo* (16, 20), and second that there was no evidence of increased fibrinolysis in the skin tissue at the site of antiserum inflammation of the rat. The anti-inflammatory action of these two compounds may, therefore, be accredited to a pharmacological action other than antiplasmin activity.

Mast-cell degranulation by compound 48/80 depends on the ATP-forming capacity of these cells (32, 33) and is inhibited by salicylates, pyrazolidine derivatives and a series of non-steroidal anti-inflammatory agents (27), which are uncouplers of oxidative phosphorylation (34-36). Neither EACA nor AMCHA inhibited this degranulation, and the two compounds did not inhibit the ATP-^{32}Pi exchange reaction in mitochondria, differing from salicylates, pyrazolidine derivatives and flufenamic acid. If the mechanism of the anti-inflammatory effect of many of non-steroidal anti-phlogistics involves the interference of energy metabolism, which may be required for the synthesis of ground substance in connective tissue and for reactivity of vascular endothelium (27, 36), the mechanism of

anti-inflammatory action of EACA and AMCHA must be regarded as different in quality from the action of these other compounds.

Since the anti-inflammatory effect of EACA and AMCHA did not markedly decrease after the removal of adrenals, their action may not be due to stimulation of the pituitary-adrenal axis. However, after hypophysectomy a decrease in the action was observed. This suggests the presence of some relation of the action to hypophyseal secretion other than the above system. The growth hormone of the hypophysis raises blood sugar, decreases glucose resistance in normal animals, and antagonizes the action of insulin (49-51). On the other hand, anaphylactoid inflammation induced by dextran or ovomucoid is inhibited in alloxan diabetic rat while such a reaction is potentiated in insulin hypoglycemia (37, 38). MASADA (39) recently reported that anti-rat serum inflammation produced by the same method as in the present experiments also underwent similar changes as in anaphylactoid inflammation. The decreased inhibitory effect of EACA and AMCHA on antiserum inflammation in hypophysectomized rat suggested that both drugs might have promoted the secretion of growth hormone and raised the blood sugar level, but these two compounds injected intravenously in normal animals failed to show any effect on their blood sugar level; therefore, the presumed mechanism of action must be denied.

A remaining possibility is to attribute the action of these compounds to their metabolites into which they might be transformed. The fact that the anti-inflammatory action of EACA and AMCHA appeared more rapidly after intramuscular or oral administration than by intravenous injection does not agree with the changes in blood level of these compounds (20, 21), and leaves a doubt on the formation of an effective intermediate in the digestive tract or other tissues. It would not be easy to elucidate the actual state of this problem but, at least, increase in the effect of these two compounds could not be proved by incubating them with pieces of gastric, intestinal, or liver tissue.

As described above, EACA and AMACH showed a relatively marked anti-inflammatory action against antiserum allergic inflammation and anaphylactoid inflammation by other phlogogenic substances in rats. The compounds also showed inhibitory effect against granuloma formation. These effects may not be due to the so-called antiplasmin action, and seem to be considerably different in the mechanism from the action of the known non-steroidal anti-inflammatory agents. Further studies are desirable to clarify this problem, and it would also be necessary to examine carefully whether such anti-inflammatory effect observed in rats

would be proved in clinical application.

SUMMARY

1. Both EACA and AMCHA clearly showed an anti-inflammatory effect, by intravenous, intramuscular, or oral route, against inflammatory edema produced in rats by intracutaneous injection of rabbit's anti-rat serum, carrageenin, histamine, serotonin, or bradykinin, as tested by the punch method.

2. The two compounds also showed inhibitory action against the cotton pellet granuloma when used in a larger dose.

3. There was virtually no difference between the two compounds in their anti-inflammatory activity, in spite of the fact that antiplasmin activity of AMCHA is evidently greater than that of EACA. In addition, there was no increase in fibrinolysis at the site of antiserum inflammation in rats. Therefore, it would be difficult to presume that the anti-inflammatory action of these compounds is due to their antiplasmin activity.

4. Salicylates, pyrazolidine derivatives, and non-steroidal anti-inflammatory agents like flufenamic acid inhibited degranulation of isolated rat mast cells induced by compound 48/80 and also inhibited ATP-^{32}Pi exchange reaction in rat liver mitochondria, but such actions were not observed in EACA or AMCHA.

5. Anti-inflammatory effect of EACA and AMCHA did not decrease after adrenalectomy but did become weak in hypophysectomized rats. EACA did not increase blood sugar level in normal rats so that its anti-inflammatory action is not due to hyperglycemia, and the effect of hypophysectomy may not be correlated to carbohydrate metabolism.

6. Anti-inflammatory effect of EACA and AMCHA appeared more rapidly after intramuscular or oral administration than by intravenous injection but the effect was not fortified after their *in vitro* incubation with tissues of stomach, intestine, or liver.

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