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Inhibition of Aminotripeptidase.* (19755)

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An aminoexotripeptidase which hydrolyzes one peptide bond of a tripeptide such as l-leucylglycylglycine and diglycylglycine (GGG) has been demonstrated in a number of tissues by Ellis and Fruton(1). This enzyme, referred to by these authors as tripeptidase, was inhibited by a number of metal ions and by relatively large concentrations of cysteine, but not appreciably by cyanide, sulfide or citrate ions. Although lymphoid cells are said to be especially rich in tripeptidase(2), it is distributed in other cell types (3), and has been found in several microorganisms(4). Stern and co-workers(5), have shown that human white blood cells have a high tripeptidase content, and that the activity of leucocytes was about 40 times greater than that of erythrocytes. The tripeptidase activity of serum is increased in certain pathological conditions such as bone fracture(6), burns(7) and a number of diseases(8). It has been observed in this laboratory that a tripeptidase is present in synovial effusions of a number of arthritic diseases, and is especially rich in the synovial fluid of patients with rheumatoid arthritis(9).

We have observed, in the present investigation, that the activity of tripeptidase from calf thymus, rabbit skeletal muscle and human synovial fluid, with respect to the hydrolysis of GGG, is inhibited by a group of compounds possessing anti-histamine, local anesthetic and anti-cholinesterase activity in concentrations as low as 0.0001 M.

Methods. Peptidase activity was determined using the titrimetric method of Grassman and Heyde(10). The reaction mixture contained 0.5 or 1.0 ml of the enzyme solution, 0.5 ml of 0.25 M GGG, previously neutralized to pH 7.8, 0.1 ml of inhibitor solution neutralized to pH 7.8 just prior to use, and sufficient 0.02 M veronal buffer at pH 7.8 to bring the total volume to 2.5 ml. The reaction was carried out in stoppered flasks and in a shaking water bath at 37.5°C. In general, reaction was allowed to proceed to 60% hydrolysis of one peptide bond, and 4 or 5 analyses in duplicate were obtained by titration of 0.2 ml samples, removed from the incubation mixture, with 0.015 N KOH in 90% ethanol. The hydrolysis of GGG was zero order to the extent of approximately 60% splitting of one peptide bond. Results are expressed as per cent hydrolysis per hour.

Calf thymus peptidase extracts were prepared according to Fruton(2), and rabbit skeletal muscle extracts according to Smith (11). The extracts obtained were frozen and thawed twice to remove extraneous protein without diminution in total activity. When inhibitor compounds were added, the reaction was followed for a variable time given below, dependent on the extent of inhibition. In the case of benadryl and procaine, the hydrolysis curves were not linear because of decomposi-

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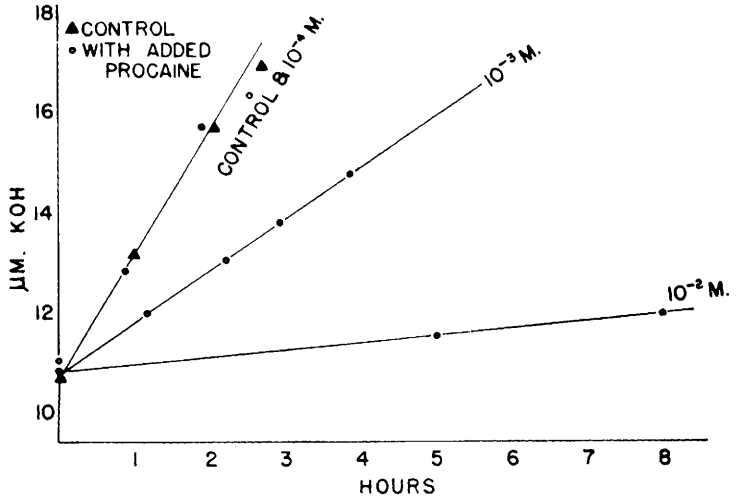

FIG. 1. Effect of procaine on hydrolysis of GGG by calf thymus extract (N = .0714 mg/ml); μM GGG = 1250; pH = 7.8, veronal buffer. Curve at 10^{-2} M procaine concentration is corrected for titration of procaine which represented 2.1 μM, as determined on solutions at zero time.

tion of these substances in the muscle extract during the incubation(12,13). As an approximation, in the case of these two substances, the rate of hydrolysis was obtained by drawing a smooth curve and calculating the rate from the initial 60-minute portion of the curve.

Synovial fluid was obtained by aspiration of knee joints of patients with rheumatoid arthritis using ethyl chloride anaesthesia. The fluid was promptly centrifuged at 3000 rpm for 20 minutes. When stored in the refrigerator, tripeptidase activity of synovial fluid did not decrease over a period of two months. The inhibiting effect of the following substances was studied: Procaine hydrochloride, pronestyl (procaine amide hydrochloride), benadryl (β-dimethylaminoethyl benzhydryl ether hydrochloride), pyribenzamine (N'-pyridyl-N'-benzyl-N-dimethylethylenediamine hydrochloride), cocaine hydrochloride, eserine salicylate, sodium salicylate, diisopropylfluorophosphate (DFP), and tetraethylpyrophosphate (TEPP).

Results. The hydrolysis of GGG by calf thymus extract in the presence of procaine is shown in Fig. 1. Marked inhibition was observed in the presence of 0.001 M procaine. Fig. 2 presents the results of a similar experiment using the synovial fluid obtained from a patient with rheumatoid arthritis. It is noted that in contrast to the constant rates observed with the thymus enzyme extract, increasing rates of hydrolysis were found with

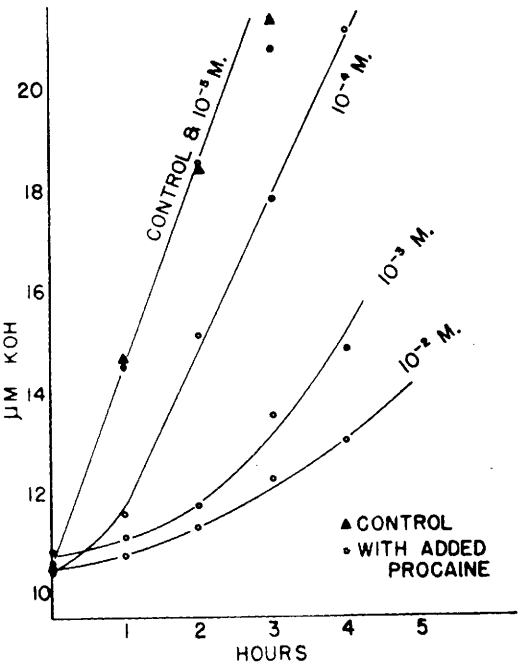

FIG. 2. Effect of procaine on hydrolysis of GGG by synovial fluid from a patient with rheumatoid arthritis (N = 42 mg/ml). Curve at 10^{-2} M procaine concentration is corrected for titration of procaine which represented 2 μM as determined on solutions at zero time.

TABLE I. Inhibition of Rabbit Skeletal Muscle Tripeptidase. Concentration of GGG in incubation mixture .05 M; nitrogen of tripeptidase test solution 2.79 mg/ml; final concentration of all substances listed below was .001 M.

Added substance	% of original tripeptidase activity
None	100
Pyribenzamine	8.7
Benadryl	25.8*
Procaine	30.4*
Eserine	31
Cocaine	41
Pronestyl	57.7
Sodium salicylate	100
Diisopropylfluorophosphate	100
Tetraethylpyrophosphate	100

* Calculated from initial 60 min period.

synovial fluid, suggesting the simultaneous hydrolysis of procaine in synovial fluid, probably by an independent esterase reaction(14).

Pyribenzamine, benadryl, procaine, eserine, cocaine, and pronestyl were found to be effective inhibitors of rabbit muscle tripeptidase as shown in Table I. DFP and TEPP, powerful inhibitors of cholinesterase, did not inhibit tripeptidase when present in relatively high concentration.

Discussion. Ellis and Fruton(1) have prepared highly purified tripeptidase from calf thymus. This enzyme demonstrated marked specificity towards optically active tripeptides, and showed little or no activity with respect to dipeptides, tetrapeptides, tripeptides containing β-amino acids, and substrates split by endopeptidases. In view of this, they concluded that calf thymus tripeptidase is specifically adapted to act on peptide chains of a particular chain length characteristic of tripeptides composed of α-amino acid residues.

It is shown in Fig. 3 that the structures of the substances found to be inhibitors approximate the dimensions of the tripeptide substrate. All are tertiary amines and, like GGG, have a positively charged onium group at the pH of the reaction mixture, 7.8. The dense lines trace the longest chain length from the onium nitrogen atom, which for all substances is either 8 or 9 carbon atoms. It would appear,

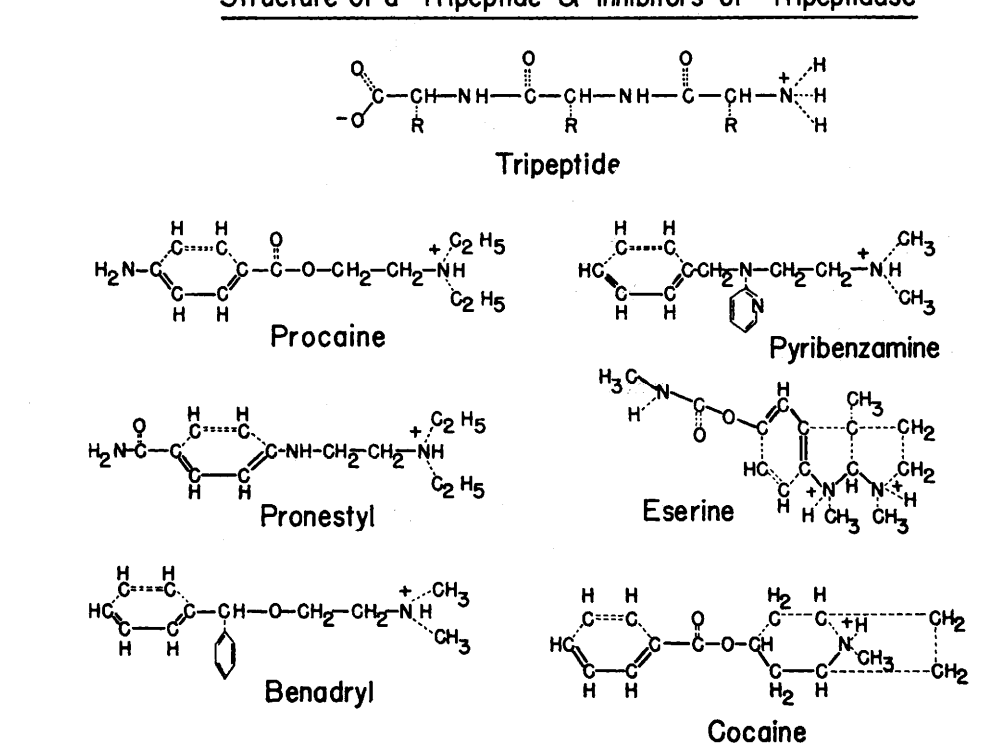

FIG. 3. Structures of a tripeptide and inhibitors of tripeptidase to demonstrate similarity in chain length and presence of onium groups.

therefore, that the inhibition observed is related to similarity of inhibitor to substrate, both as to structure and charge.

It has been shown that both true and pseudocholinesterase are inhibited by DFP (15). Jansen and Balls(16) suggest that the mode of inhibition by DFP of all esterases may be similar to the observed inhibition by this agent of both the esterolytic and proteolytic activity of β and γ chymotrypsin and of trypsin. Since DFP did not inhibit the tripeptidase of rabbit skeletal muscle (Table I) and in view of the apparent inability of the purified calf thymus tripeptidase to hydrolyze procaine, as indicated by the constant slope of the hydrolysis rate of GGG in the presence of procaine, it would appear that tripeptidase does not have esterolytic properties.

The observation that the group of compounds studied are inhibitors of aminoexotripeptidase, which is widely distributed in a variety of tissues, raises the question of the relationship between peptidase inhibition and the pharmacological action of these substances. Pyribenzamine, benadryl, procaine, cocaine, and eserine are local anaesthetic agents, and block nerve conduction(17). DFP, which did not inhibit tripeptidase in the present investigation, does not block nerve conduction in concentrations which completely inactivate nerve cholinesterase(18). The possibility arises, therefore, that peptidase inhibition may play a role in local anaesthetic activity. Whether the local effects of anihistaminic substances such as pyribenzamine and benadryl in inflammatory skin disease, as contact dermatitis and eczema(19), are related to peptidase inhibition cannot at present be determined.

Summary. A group of compounds known to possess antihistamine, local anaesthetic, and anti-cholinesterase activities were observed to inhibit aminoexotripeptidase from several sources in concentrations as low as 0.0001 M. The inhibiting substances were pyribenzamine, benadryl, procaine, pronestyl, cocaine and eserine; diisopropylfluorophosphate and tetraethylpyrophosphate did not inhibit. The mechanism of the observed inhibition and its possible physiological significance is discussed.

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