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The inhibition of tryptic digestion by certain drugs *

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The increasing therapeutical uses found for proteolytic enzymes like trypsin, and the important role attributed to these biocatalysts in the mechanism of production of certain pathological states (inflammation, rheumatism, arthritis, etc.), induced us to study the inhibitions exerted by several drugs upon the tryptic digestion of natural substrates, endeavouring to establish the possible relationships, if any, existing between molecular structure and inhibitory power.

The influence of many substances on the hydrolytic actions of several proteases has been, at different times, investigated by several authors. RONA found that sodium bisulphite, potassium cyanide, hydroxylamine and phenylhydrazine (among carbonilic reagents) had no action upon peptic and tryptic digestions; JOSEPHSON and EULER observed that sodium bisulphite, potassium cyanide and phenylhydrazine inhibited erepsin, and SCHALES found that they also exerted an inhibitory action on tryptic and papain hydrolisis of egg white. SCHALES and BORROUGHS, and SCHALES, also observed miscellaneous effects upon cathepsins I, II, III, and IV and positive inhibitions of trypsin and chimotrypsin; in SCHALES opinion, these inhibitors modify the structures of the substrate surfaces, thus making them more resistant to attack.

The actions of some oxydizing and reducing agents were investigated by GRASSMAN, DICKERHOF and SCHOENEBECK, who found that whereas cyanide and sulphide ions and cystein in-

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hibited crude trypsin preparations, cystein and gluthation activated the hydrolytic actions of pepsin and cathepsin, a fact which induced BERSIN and HELLERMANN to sugest that the reduced form (—SH) of proteases is the only active one.

On the other hand, GROB found that when working with pure crystalline trypsin, certain reducing agents such as cyanide, sulphide, thioglycolate, and others, inhibited the tryptic digestion of casein; these experimental facts are in disagreement with BERSIN and HELLERMANN'S suggestion, which induced the papers published by PETERS and WAKELIN, WEBB and HYMINGEN, FRAENKEL-CONRAT and others, TAYAU, VISWANATHA and others, with miscellaneous results and often contradictory.

NEUMANN, JOBLING and PETERSEN, and also PECK, discovered the inhibitory action exerted by several soaps upon the tryptic digestion of several substrates.

Most of the results to be found in the literature have, however, been obtained by using analytical methods of restricted applicability and not free from criticism, while others are the consequence of the use of crude enzymatic preparations. These misleading circumstances and the desire to obtain reliable results increased our interest in carrying out the present investigation on the inhibition of the tryptic digestion induced by certain drugs.

Experimental

ANALYTICAL METHOD CHOOSEN.

Owing to interferences of several types produced by the substances tested, the following known techniques could not be used: acidimetric methods with indicators, colorimetric methods like biuret's, Folin's, ninhydrin's and the techniques based on the use of died or azocopulated substrates. The spectrophotometric method of measuring the optical density (280 mμ) of the digestion samples had also to be rejected, as many of the drugs investigated showed a definite absortion capacity, not precipitable by trichloracetic acid, which interferred with the results.

We have used, as a method of choice, Levy's technique of potentiometric determination of amino groups in the presence of formaldehyde (10 %), using 0.1 N NaOH as a reagent, and *p*H 9.0 as the end point of titration.

ENZYME.

Recrystallized trypsin 6.10^{-3}[T.U.]$^{cas}_{v}$, established by KUNITZ'S spectrophotometric method. A stock solution was pre-

parared by dissolving 50-100 mg of the pure enzyme in 0.0025 M HCl and making up to 100 c. c. ; the required amount of this solution was taken and conveniently diluted for each test. The stock solution could be kept several days in the ice chest.

SUBSTRATES.

Solutions containing 1 % casein, 1 % fibrin or 5 % gelatin, in M/15 buffer phosphate *p*H 7.6, were employed in each case.

The 1% casein solution was prepared by heating 15 minutes on the water bath, a suspensión of the pure protein in buffer, and finally adjusting the *p*H to 7.6 by adding 0.1 H NaOH.

From fresh, washed, bovine fibrin, a 1 % solution was prepared by mashing 2 g. protein powder with a small quantity of 3N NaOH, suspending the whole in about 100 c. c. of buffer phosphate, heating on the water bath for 15 minutes, diluting with buffer up to 200 c. c., and finally adjusting to *p*H 7.6 by cautious addition of 0.1 N HCl

A 5 % gelatin solution was made by dissolving the protein in buffer phosphate.

INHIBITORS.

Fresh solutions of pure specimens of the substances investigated, the concentrations varying generally from 0.05 to 0.25 M.

APPARATUS.

A microburette calibrated in hundreths of c. c.

A *Meteor* *p*H meter with a sensivility of about ± 0.02, using a Beckmann glass electrode.

DIGESTION TESTS.

Every digestion sample should contain 15 c. c. of the protein solution used as the substrate, and to each blank 9 c. c. of the M/15 buffer solution and 6 c. c. of distilled water are added ; to the rest of the samples 6 c. c. of buffer, from 0 to 6 c. c. of the inhibitor solution, 3 c. c. of the enzyme solution and distilled water up to 30 c. c. are added. The corresponding, routine, control samples are also prepared.

The digestions are carried out in 50 c. c. test tubes, mantained at 37° C in a thermostate, and in each of them, every component is introduced but the enzyme solution, which is only added once the thermal equilibrium of the liquids has been attained. The digestion time chosen is 30 minutes and the digestions are interrupted by pipetting 20 c. c. of each of the

samples into 5 c. c. of a 40 % solution of formaldehyde contained in several 100 c. c. vessels. Each sample is then titrated with 0.1 N NaOH up to *p*H 9.0, with the help of the *p*H meter.

The results are expressed in percentages of inhibition referred to the normal digestion values obtained when a given quantity of trypsin acts upon a certain substrate, under, the established conditions, in the absence of inhibitor.

THE INHIBITORY ACTIONS OF SEVERAL SUBSTANCES UPON TRYPTIC DIGESTION

CARBONYL GROUP REAGENTS

The influences exerted by several carbonyl group reagents on the tryptic digestion of egg white had, previously, been considered by SHALES, RONA, CALANDRA and others, although with contradictory results. Using casein, gelatin and fibrin as substrates, we have obtained the results collected in Table I.

TABLE I

Inhibitions exerted by carbonyl reagents upon tryptic digestion.

Trypsin concentration: 5 γ/c.c.

Inhibitor	Concentration	Percentages of Inhibition		
		Casein	Gelatin	Fibrin
Hydrazine (sulphate)	0.1 M	45 %	37 %	65 %
Phenylhydrazine	0.1 M	42	32	72
	0.05 M	18		
Phenylsemicarbazide	0.1 M	30	37	53
Hydroxylamine (HCl)	0.1 M	34	20	63
Potassium cyanide	0.1 M	33	50	66
	0.02 M	8		
Sodium sulphite	0.1 M	28	26	42

From Table I it is clear that fairly high concentrations (0.1 M) of the carbonyl group reagents are necessary to obtain pronounced inhibitions, and that no fundamental differences can be observed whether casein, gelatin or fibrin, are used as substrates.

The higher molarity of the solutions investigated required

TABLE II

Inhibitions induced by various phenols upon tryptic digestion.
Trypsin concentration: 5 γ/c.c. with casein and gelatin,
10 γ/c.c. with fibrin

Inhibitor	Concentration	Percentages of inhibition		
		Casein	Gelatin	Fibrin
Phenol	0,05 M	42 %		52 %
	0.1	53	73	
o-Cresol	0,05	100		80
p-Cresol	0.1		39	
Pyrocatechol	0.025	65	81	80
Resorcin	0.05	74	63	
	0.025			100
Hydroquinone	0.025			49
	0.05		23	
	0.1	75	40	
Pyrogallol	0.05	57	41	
	0.025			100
Phloroglucin	0.025	32		48
	0.05		50	
o-Nitrophenol	0.05	No		No
	0.1	No	No	
m-Nitrophenol	0.05	76	92	100
p-Nitrophenol	0.05	27	42	42
	0.1	59		
2,4-Dinitrophenol	0.025	No		
	0.05	15	10	
	0.1	63		
Picrid acid	0.02	No		
	0.04	31	44	
p-Chorphenol	0.05	71		90
	0.1	100		
o-Aminophenol	0.05	22	21	
m-Aminophenol	0.05	25	20	
p-Aminophenol	0.05	No	No	

to obtain a partial inhibition of the digestion (specially if compared with the very small concentration of the enzyme solution used) seems to suggest that the action of the inhibitors is not exerted upon trypsin but on the substrates employed (casein, gelatin and fibrin) by probably inducing a modification of some sort of the protein surface, which partially protects it against hydrolytic attack.

PHENOLS.

The important pharmacological actions specific of many phenols provides a special interest to the investigation of their

inhibitory actions on the tryptic digestion, which has previously been considered by KALIPADA BASU, and by DIEMAIR and BOECKHOFF, with miscellaneous results.

The results stated in Table II clearly show that phenol and cresols are very active inhibitors of the tryptic digestions of casein, gelatin and fibrin. Among diphenols, resorcin is more potent than o- and p-derivatives, and pyrogallol and phloroglucin are both also active. m-Nitrophenol is a good inhibitor but the o-isomer is inactive, whereas the p-nitroderivative shows a weaker activity. 2-4-Dinitrophenol and picric acid are weak inhibitors while p-chlorphenol is fairly potent. o- And m-aminophenols possess some weak action whereas the p-derivative shows to be inactive. The preceeding results seem to show that the more dissociated the phenolic group is, the less is the activity developed by the substance as an inhibitor of the digestions.

AROMATIC ACIDS.

Although many of the following acids possess definite pharmacological and therapeutical properties, none of them exert any inhibitory influence upon tryptic digestion : benzoic, salycilic, m- and p-hydroxybenzoic, p-aminobenzoic, p-nitrobenzoic, 2-4-dichlorophenoxyacetic, phenylacetic, phenylpropionic, cinnamic, amygdalic, gentisic, phthalic, dichlorophthalic, sulphanilic, sulphosalycilic, nitrophenylacetic and hippuric acids. Benzamide, phthalilglutamide, sulphanilamide and cumarine are neither inhibitors. It seems that the complete dissociation

TABLE III

Inhibitions of the tryptic digestion produced by certain acids.

Trypsin concentration: 5 γ/c.c.

Inhibitor	Concentration	Percentages of inhibition		
		Casein	Gelatin	Fibrin
p-Hydroxyphenylacetic	0.1	61	71	
	0.05	32		
p-Nitrophenylacetic	0.1	No		No
2,4-Dinitrophenylacetic	0.1	33		32
o-Nitrophenylpropionic	0.1	35		
p-Nitrophenylpropionic	0.1	33		36
Naphthalenacetic	0.06	40		

of the sodium salts of the foregoing substances is also here responsible for their inactivity (in spite of the fact that many of the acids contain, in addition, phenolic groups).

In the following Table III are included some acids which, however, exert a certain inhibition of the tryptic digestion

AROMATIC AMINES.

The scarce solubility of many aromatic amines at the *p*H 7.6 of the experiments has limited our investigations to only a few substances : aniline, o-toluidine, p-nitroaniline and 2-5-dichloraniline prove themselves to be inactive, o- and m-phenylene-diamine possess a weak activity, whereas p-phenylene-diamine and alpha-aminopyridine are definite inhibitors at 0.1 M concentrations. (Table, IV).

TABLE IV

Inhibitions exerted by some aromatic amines.

Trypsin concentration: 5 γ/c.c.

Inhibitor	Concentration	Percentages of inhibition	
		Casein	Gelatin
Aniline	0.1	No	No
o-Toluidine	0.1	No	No
p-Nitroaniline	Saturated	No	No
2,5-Dichloraniline	Saturated	No	No
o-Phenylenediamine	0.1	12	10
m-Phenylenediamine	0.1	15	11
p-Phenylenediamine	0.1	35	39
alpha-Aminopyridine	0.1	37	43

PYRIDINE, DERIVATIVES AND RELATED COMPOUNDS.

The therapeutical qualities of nicotinic acid and nicotinamide, of the hydrazides of isonicotinic and cyanacetic acids, and of piperazine, directed our attention upon these drugs.

Pyridine, nicotinic acid and nicotinamide appear to be strong inhibitors of the tryptic digestion, whereas the o- and p-carboxylic acids, i.e. picolinic and isonicotinic acids, exhibit no action whatever, at 0.1 M concentrations. Among antitubercular drugs, while isonicotinic hydrazide is inactive, cyanacetic hydrazide shows a powerful inhibitory action. Maloiic hydrazide is weakly active, but malonamic hydrazide is inactive (neither of these hydrazides have tuberculoustatic properties). Piperidine and piperazine are fairly good inhibitors. (Table V).

TABLE V

Inhibitions exerted by pyridine, derivatives and related compounds

Trypsin concentration: 5 γ/c.c.

Inhibitor	Concentration	Percentages of inhibition		
		Casein	Gelatin	Fibrin
Pyridine	0.1 M	35		80
Nicotinic ac.	0.1	28	15	
Isonicotinic ac.	0.1	No	No	
Picolinic ac.	0.1	No	No	No
Piperidine	0.1	100	64	
Nicotinamide	0.1	13	19	
Isonicotinhidrazide	0.1	No	No	
Cyanacetylhydrazide	0.1	100	32	
Malonamyl-hydrazide	0.1	No	No	
Malonyl-dihydrazide	0.05	10	24	
8-Oxyquinolein	0.1	No	No	
Piperazine	0.1	20	21	

INDOLE AND DERIVATIVES.

The biological importance of the indole derivatives does not need to be emphasized to justify their study as inhibitors of the tryptic digestion.

It can be seen in the following Table VI, that isatin and indolyl-3-acetic acid are strong inhibitors, whereas indol, tryptophane, histamine and histidine, have no influence on the tryptic digestion of casein and gelatin, at the concentrations investigated.

TABLE VI

Inhibitions induced by indole and derivatives

Trypsin concentration: 5 γ/c.c.

Inhibitor	Concentration	Percentages of inhibition	
		Casein	Gelatin
Indole	Saturation	No	No
Isatine	Saturation	20	11
Indolyl-3-acetic ac.	0.1 M	65	33
Tryptophane	0.01	No	No
Histamine	0.006	No	No
Histidine	0.006	No	No

AMINOACIDS.

Among the aminoacids investigated only glutamic and aspartic acids showed positive inhibitions, but asparaguine, cysteine, cystine and tyrosine exerted no inhibitory action on the tryptic digestion of casein and gelatin; these results are in agreement with the observations published by GROB, FARBER and WYNE, although VISWANATHA states that cystein is not an inhibitor. Aminoacids being the products of the hydrolisis of proteins were postulated by BAYLISS to be inhibitors of the proteic hydrolisis.

TABLE VII

Inhibitions induced by some aminoacids.
Trypsin concentration: 5 γ/c.c.

Inhibitor	Concentration	Percentages of inhibition	
		Casein	Gelatin
Glutâmic ac.	O, 1 M	59	15
Aspartic ac.	0.0075	15	
	0.015		23
Asparaguine	0.05	No	
Cysteine	Saturation	No	No
Cystine	Saturation	No	No
Tyrosine	0.02	No	No

TABLE VIII

Inhibitions induced by certain sodium soaps.

Inhibitor	Concentration	Enzyme Concentration γ/c c.	Percentages of inhibition		
			Casein	Gelatin	Fibrin
Stearate	0.02 M	5	63	37	
	0.01	10			100
	0.005	10			100
Palmitate	0.02	5	55	52	
	0.01	10			100
Oleate	0.02	5	79	70	
	0.005	10			100
	0.0025	10			100
	0.0012	10			100
Elaidinate	0.02	5	70	41	
	0.01	10			100
	0.002	10			65
Linoleate	0.02	5	69	75	
	0.01	5	69		
	0.0025	10			100
	0.0012	10			100
Undecilenate	0.025	5		34	
	0.025	10	77		
	0.012	10	77		
	0.006	10			100

FATTY ACIDS.

The soaps of the higher fatty acids, whether saturated or not, are strong inhibitors of fibrin, casein and gelatin digestions. Lower fatty acids, from heptilic down to formic, do not inhibit tryptic digestion, and the same can be said of monochloracetic, oxalic, succinic, tartaric and citric acids, as previously observed HANSON and SMITH, but in disagreement with several other authors.

TENSOACTIVE SUBSTANCES.

Several commercial detergents of miscellaneous compositions, including sulphonated lauric and oleocetílic alcohols,

TABLE IX

Inhibitions induced by some commercial detergents.

Trypsin concentration: γ/c.c.

Commercial name	Composition	Conc %	Percentage of inhibition		
			Casein	Gelatin	Fibrin
Gardinol paste, Sixley 1/k 21	80 % sodium cetilate and 20 % stearate.	0.5	52	41	83
D. O. 8, Sixley III/B	Na oxiethylated and sulphonated laurate.	0.5	67	21	44
Gardinol WA Sixley 1K/24	Na oleocetil-sulphonate.	0.5	64	50	100
Setal MB Sixley 1/F 5	Na salts of C_{16-18} fatty acids.	0.5	45	45	74
Gardinol WA Sixley 1K/24	Na salt of sulphonated oleocetilic alcohol.	0.5	68	44	100
Gardinol J Sixley 1K/24	Na salt of sulphonated stearic alc.	0.5	66	39	100
Gardinol CA Sixley 1K/21	Na salt of mixt. cetilic and stearic acids.	0.5	45	44	
Carboxymethyl-celulose		0.5	23	12	62
Na Laurylsulphonate		0.5	66		100
Na Tetrapropilenebenzosulphonate		0.5	50		100

behave as very potent inhibitors of the tryptic digestion of casein, gelatin and fibrin. The commercial names specified in Table IX are taken form Sixley's Modern Detergent Index. Vol. II.

MISCELLANEOUS DRUGS.

Ethyl alcohol, glycol, glycerol, poliethyleneglycol, thiouracil, thiourea, guanidine, creatinine, uric acid, chloramphenicol, nitrofurazone and nitrofurazolidine are inactive, whereas phenergan (N-dimethylamin-methyl-ethyl-dibenzo-parathiazine), glucosamine and ascorbic acid behave as definite inhibitors of the digestion of casein and gelatin.

TABLE X

Inhibitions induced by miscellaneous drugs.

Inhibitor	Concentration	Trypsin conc.	Percentage of inhibition	
			Casein	Gelatin
Phenergan	0.25 %	5γ/ c. c.	100	100
Glocosamine	0.1 M	5	21	60
Ascorbic ac.	0.1 M	5	70	33

INORGANIC IONS.

Several negative ions of reducing character have been studied, but only cyanide and sulphide showed a marked inhibitory action upon the tryptic digestions of casein and gelatin; ferrocyanide, thyocyanide, nitrite and hydrochloride ions are inactive. Among positive ions Cu^{--}, Hg^{--}, Zn^{--}, Cd^{--} and Sn^{--} exert very definite actions on the digestion of both substrates. Some of these ions had previously been investigated by CREWTHER, GREEN, GLADNER, NEURATH and others.

TABLE XI

Inhibitions exerted by some inorganic ions.

Inhibitor	Concentration	Trypsin	Percentage of inhibition	
			Casein	Gelatin
S^{--}	0.1 M	5γ/c. c.	67	77
Cu^{--}	0.1 M	5		82
	0.025	5	75	
Hg^{--}	0.05	5		63
	0.02	5	67	
Zn^{--}	0.02	5	30	
Cd^{--}	0.02	5	43	
Sn^{--}	0.005	5	No	
	0.02	5	45	49

Discussion

Although several authors have pointed out that the rH of the medium exerts a definite influence upon the enzymatic protein hydrolyses, and BERSIN and HELLERMANN stated that proteases are activated by reducing agents, we have not been able to confirm this in the case of trypsin. Our observations show, on the contrary, that certain reducing agents like cyanide, sulphide, sulphite, several phenols and the carbonyl group reagents inhibit the tryptic digestions of casein, gelatin and fibrin, whereas thiocyanides, ferrocyanides, iodides, oxalates, tartrates, citrates, aromatic hydroxyacids and others, do not exert any action in spite of their reducing character. (Sizer has published his observations on the activity of alpha-chimotrypsin, which apparently is not affected by oxidizing or reducing agents in the interval between — 400 and + 500 mV).

Carbonyl group reagents (like hydrazine, phenylhydrazine, hydroxylamine, phenylsemicarbazide and others) are powerful inhibitors of the tryptic activity, when present at fairly high (0.1 M) concentrations, a fact that seems to suggest that their action is exerted upon the substrates involved rather than on the enzyme, by probably inducing a certain protection of the macromolecular surface against hydrolysis.

The capacity of phenols to inhibit the tryptic hydrolyses of the natural substrates tested could be atributed to the steric hindrance promoted by polar bindings established between the inhibitors and the proteic substances. Among diphenols and mononitrophenols it has been found that the m-isomers (resorcine m-nitrophenol) are better inhibitors than the o- and p-isomers ; it is known that m-derivatives show a smaller tendency than the o-isomers, to establish intramolecular hydrogen bridges. On the other hand, Teresi and Murray have found a different capacity to bind proteins, between the three isomeric mono-nitrophenols : 1 mol seroalbumin binds only 6 mols o-nitrophenol, whereas the same protein is capable of binding up to 22 and 25 mols of m- and p-nitrophenol, respectively. It seems that the inhibitory power of the phenols is smaller, the higher the dissociation of the hydroxyl group, which could also explain the strong inhibition exerted by resorcine and m-nitrophenol, against the very weak actions of 2-4-dinitrophenol and picric acid.

Aromatic acids and hydroxyacids are not inhibitors, whereas p-hydroxyphenylacetic, 2-4-dinitrophenylacetic, o- and p-nitrophenylpropionic acids and naphthaleneacetic acid possess definite inhibitory powers on the tryptic hydrolysis of proteins.

Among aromatic amines, o-, m- and p-phenylenediamines show only weak inhibitory actions.

Pyridine is a good inhibitor and while nicotinic acid is weaker, neither picolinic nor isonicotinic acids show any positive activity. Alpha-amynopyridine, piperidine, piperazine, isatine and indol-3-acetic acid are only active at fairly hingh concentrations (0.1 M).

The sodium soaps of higher fatty acids (stearic, palmitic, oleic, elaidinic, linoleic, undecilenic) are very powerful inhibitors of the tryptic digestion of proteins, even at very low concentrations, and so are all the conmercial detergents we have studied (sulphonated fatty alcohols, sulphonated tetrapropylbenzene, etc.) whereas the sodium salts of formic, acetic, monochloracetic, valerianic, heptilic, oxalic, succinic, tartaric and citric acids, show no activity; inhibitory power seems to be associated with detergency.

Ethyl alcohol, glycol, glycerol and butylglycol are no inhibitors.

Summary

1. The inhibition of the tryptic digestion of casein, gelatin and fibrin, by several drugs have been investigated and no fundamental differences have been observed with the use of each of the three substrates.

2. The carbonyl group reagents investigated and several phenols and derivatives prove themselves to be good inhibitors of the digestion; soaps and detergents in particular possess a very strong inhibitory power, even at higher dilutions.

3. The study of the inhibitory action exerted by many aromatic acid and hydroxyacids, aromatic amines, aminoacids, heterocyclic compounds and several other drugs, derivatives, and inorganic ions, has lead to the miscellaneous results stated in the corresponding Tables.

4. No connection between tryptic activity and the presence of oxido-reducing agents (rH) has been detected.

5. Temptative interpretations of the inhibitions observed in certain cases are put forward.

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