CCLXXV. STUDIES ON PANCREATIC PROTEINASE.

II. THE EFFECTS OF VARIOUS COMPOUNDS ON THE ACTIVITY OF THE ENZYME.

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In the foregoing paper [Farber and Wynne, 1935] methods were described for the study of the activity of pancreatic proteinase. These methods have been used in a study of the effects on the activity of the enzyme of chemical substances of various types, including carbohydrates, glycerol, amino-acids, triglycerides, bile and bile salts, indicators and dyes, salts of heavy metals and other salts.

As substrate Kahlbaum's "casein (Hammarsten)" was used throughout, the concentration in the digestion mixtures being 0·80 % in the glycerol experiments and 0·90 % in all others. The digests were buffered at p_H 8·7 with $M/5$ phosphate-borax buffer; solutions of the various compounds were, whenever necessary and practicable, adjusted to p_H 8·7. The exceptions were those salts which formed precipitates at this alkalinity. The relative rates of hydrolysis listed in the tables which follow have been calculated from initial rates which, in turn, were based on determinations spaced, usually, at intervals of 20 sec. The total period of digestion, as a rule, was not more than 60 sec.; changes in p_H during such short time intervals were negligible. Accurate estimations of initial velocity of hydrolysis were readily made with the aid of the procedures described in the previous paper.

Experimental results.

Carbohydrates. Stock buffer solutions of several carbohydrates having the following concentrations were prepared; sugars, molar; dextrin and soluble starch, 18 %; gum acacia, 9 %. Portions of the solutions were added to the digestion mixtures to give the concentrations listed in Tables I and II. Two enzyme preparations were used: the first, a phosphate-borax buffer extract of powdered pancreas prepared as described in the previous paper and used in the experiments with sucrose, maltose and lactose; the second, a 1 % Na_2CO_3 extract

Table I. *The effects of sugars on the activity of the proteinase.*

The figures in columns 2 to 6 represent the initial velocity as % of the initial velocity of hydrolysis in the control digest.

	Concentration M					% inhibition
Sugar	0·02	0·04	0·10	0·20	0·50	at 0·5 M
Glucose	—	90	—	82	58	42
Fructose	—	71	—	63	55	45
Galactose	—	78	—	73	60	40
Sucrose	97	94	89	81	57	43
Maltose	—	88	—	81	61	39
Lactose	95	88	81	78	66	34
Melizitose	—	100	99	83	76	24

Table II. *Effect of polysaccharides on proteinase activity.*

The figures represent initial velocity as % of that in control digests.

	Concentration %					
Substance	0·36	0·72	1·8	3·6	4·5	9·0
Dextrin	—	95	90	88	—	85
Soluble starch	—	100	86	78	—	75
Gum acacia	95	—	96	—	104	—

used with the other carbohydrates. Procedure 1, previously described (p. 2315), was used for the preparation of the digests and for the determination of residual protein. The results are summarised in Tables I and II.

Glycerol. For these experiments the enzyme solution was prepared by precipitating an aqueous extract of pancreas powder by adding *N* acetic acid, concentrating the filtrate at low temperature to one-tenth the volume and neutralising the concentrate with NaOH. Procedure 1 was used throughout; the total volumes of the digestion mixtures were identical (5 ml.), the relative proportions of glycerol and water having been varied to give the glycerol concentrations listed in Table III.

Table III. *The effect of glycerol on proteinase activity.*

Concentration		
M	%	% inhibition
0·22	2	11
0·44	4	19
0·88	8	40
1·30	12	63
1·74	16	73

The results in Tables I, II and III show that both sugars and glycerol, present in moderately high concentrations, retard the action of the enzyme. The individual sugars exhibit differences in their effects at the lower concentrations; at 0·5 *M* concentration, however, the differences are less marked except in the case of lactose and melizitose. Stock solutions of these sugars and of maltose yielded, on standing at 8°, small deposits of crystals increasing in amount in the order, maltose, lactose, melizitose, but slight warming was sufficient to bring the sugars into solution again. The inhibitory powers of the three sugars at 0·5 *M* concentration decreased in the same order as their solubilities. Although the various sugars in the lower concentrations exhibit differences in their effectiveness as inhibitors, the general similarity of their effects (excluding those of lactose and melizitose) in the higher range of concentration leads to the conclusion that in this range the retardation of the activity of the enzyme is very largely a function of the molar concentration of the compounds; the nature of the sugar is of secondary importance. This view is supported by the data in Tables I and II, and in Table IV where the effects of mixtures of sugars are shown.

Table IV. *Effect of sugar mixtures on proteinase activity.*

Substance	% inhibition
0·25 *M* glucose + 0·25 *M* fructose	47
0·25 *M* glucose + 0·25 *M* galactose	48

Inhibition by sucrose was directly proportional to the concentration in the range 0·04 to 0·5 *M*. In the case of lactose a similar relationship was observed to hold from 0·1 to 0·5 *M*; with the lower concentrations such proportionality did not exist, owing perhaps to the relatively greater influence in the low concentrations of the reducing group of the sugar. It is proposed in further studies to compare the effects of low concentrations of various sugars, reducing and non-reducing, in order to determine more exactly the relation between the reducing powers of the sugars and their inhibitory properties. The degree of inhibition by glycerol was, within experimental error, directly proportional to the concentration between the limits 2 and 12 %.

It may be suggested that the inhibition by glycerol and the sugars is closely related to the well known fact that these compounds become hydrated in solution, possibly through coordinate linkages of the atoms of the hydroxyl groups with water molecules in the manner indicated by Jordan-Lloyd and Phillips [1933] in the case of proteins. The degree of hydration of sucrose has been investigated by several workers. McBain and Kistler [1929], who review the earlier literature, obtained evidence from direct measurements by ultrafiltration methods that sucrose in dilute solution is hydrated with not less than 4 molecules of water per molecule of sugar. Scatchard [1921] concluded from an analysis of his own and other data that the formation of a penta- or hexa-hydrate best explained the experimental results, though the concentration affected in some measure the degree of hydration. Total removal of water by hydration increases, of course, with the concentration of the sugar. One may assume also that caseinogen in solution becomes hydrated, possibly by coordinate linkage of water molecules with the atoms of the hydroxyl, carboxyl, amide, amino- and imino-groups, but in the case of proteins accurate assessment of the degree of hydration of the various groups is much more difficult. In any case it is probable that the sugars and glycerol, when added to enzyme-protein solutions, reduce in greater or less degree the effective water concentration by competition with both enzyme and its substrate for water.

Polysaccharides. Dextrin and soluble starch as inhibitors were less effective than the sugars; gum acacia was without significant effect (Table II).

Amino-acids. The effects of several amino-acids and of asparagine on the activity of the enzyme were examined at p_H 8·7 according to Procedure 1, the enzyme preparation being a 1 % Na_2CO_3 extract of pancreas powder. The results are shown in Tables V and VI.

Table V. *The effects of amino-acids on proteinase activity.*

The figures represent initial velocity as % of that in control digests.

	Concentration *M*															
Substance	0·0020	0·0024	0·0026	0·0028	0·0030	0·013	0·020	0·024	0·026	0·028	0·030	0·050	0·060	0·065	0·070	0·075
Histidine	94	—	—	—	—	—	92	—	—	—	—	97	—	—	—	—
Phenylalanine	—	97	—	—	—	—	—	94	—	—	—	—	97	—	—	—
Cysteine	—	—	82	—	—	100	—	—	110	—	—	—	—	97	—	—
			85						109							
Leucine	—	—	—	—	99	—	—	—	—	—	99	—	—	—	—	98
Aspartic acid	—	—	—	—	106	—	—	—	—	—	117	—	—	—	—	121
Glutamic acid	—	—	—	96	—	—	—	—	—	105	—	—	—	118	—	—
Asparagine	—	—	—	—	98	—	—	—	—	—	—	—	—	—	—	125

Of the amino-acids examined histidine, phenylalanine and leucine had no very significant effects in the concentrations used. Cysteine (0·026 *M*) exercised a slight accelerating effect in two experiments; with aspartic and glutamic acids

Table VI. *Effect of amino-acid mixtures on proteinase activity.*

The figures represent initial velocity as % of that in control digests.

Mixture	Concentration %			
	0·06	0·30	0·60	1·50
Monoamino-acid mixture prepared from caseinogen	111	115	119	—
Arlington aminoids	97	—	105	107

and asparagine the activation was somewhat more marked. Activation by the amino-acid mixture (Table VI) was similarly quite definite but the identity of the activator is obscure.

Triglycerides. Emulsions (20 % by volume) of triacetin, tributyrin and triolein in 3 % gum acacia were prepared. The first two emulsions were reasonably stable but triolein in gum acacia solution gave a very unstable emulsion which separated, immediately after shaking, into two layers. A very stable emulsion of triolein was obtained with the aid of 0·2 % sodium oleate solution. Portions of the emulsions were transferred to the digestion mixtures to give the concentrations listed in Table VII. Strictly speaking, these cannot be regarded as truly molecular concentrations since the substances, at least in the higher concentrations, were not in true solution.

Table VII. *Effect of triglycerides on proteinase activity.*

The figures represent initial hydrolysis as % of that in control digests.

Substance	Concentration *M*									
	0·0081	0·020	0·041	0·066	0·081	0·092	0·132	0·184	0·264	0·368
Triacetin	—	—	—	—	—	56	—	41	—	29
Tributyrin	—	—	—	80	—	—	63	—	40	—
Triolein (gum acacia)	95	—	96	—	102	—	—	—	—	—
Triolein (sodium oleate)	104	91	74	—	—	—	—	—	—	—

The three triglycerides, when properly emulsified, had marked inhibitory effects. In the case of triolein emulsified in gum acacia solution the degree of inhibition was relatively very slight but triolein in sodium oleate was definitely inhibitory in a concentration corresponding to 0·04 *M*. Control experiments indicated that part of this effect was due to the sodium oleate. Inhibition by the well-emulsified triglycerides was probably due to the formation of protective films around the micelles of the reacting substances. The opposite effect of emulsions was observed by Wasteneys and Borsook [1928] in experiments on protein synthesis. In this case the emulsion apparently provided means for the extension of the surface area of enzyme or substrate or both.

Bile and bile salts. Sodium taurocholate and sodium glycocholate (Eastman) and fresh ox bile were used. The enzyme was a 33 % glycerol extract of pancreas powder. The results are summarised in Tables VIII and IX. They confirm the observations of Ringer [1921; 1922], of Vonk *et al.* [1933] and of others, that in alkaline solution bile and bile salts diminish the rate of breakdown of protein by trypsin. The results with sodium glycocholate are, however, contrary to those of Willstätter and Persiel [1925] who observed that this salt, in 0·002 *M* concentration, had no effect on tryptic activity determined by the increase in acid titratable in alcoholic solution.

Table VIII. *Effect of ox bile on proteinase activity.*

% concentration	% inhibition
0·002	3
1·0	0
10·0	33
20·0	77
40·0	88

Table IX. *Effect of bile salts on proteinase activity.*

The figures represent % inhibition.

	Concentration *M*		
	0·001	0·01	0·04
Sodium glycocholate	11	22	62
Sodium taurocholate	32	51	77

Indicators and dyes. In carrying out these experiments the solution of the indicator was first mixed with freshly prepared aqueous enzyme extract and the mixture was allowed to stand before addition to the reaction mixture. 2 ml. portions of enzyme solution were pipetted into several small test-tubes; varying amounts of *M*/5 phosphate-borax buffer solution (p_H 8·7) and of the indicator solution were then added to give the desired concentrations of indicator in a total volume of 3 ml. Control tubes contained 2 ml. enzyme solution and 1 ml. buffer solution. After standing for 20 min. at 25°, 1 ml. indicator-enzyme solution was added to a solution containing 1·5 ml. 3 % caseinogen and 2·5 ml. buffer solution; the reaction was followed as in Procedure 1. In experiments in which Procedure 2 was used the reaction mixture contained twice the above amounts of all consti-

Table X. *Effect of indicators on proteinase activity.*

The figures represent initial velocity as % of that in control digests.

	Concentration *M* × 10^{-4}														
Substance	0·42	0·59	0·65	0·72	0·84	1·2	1·3	1·4	4·8	8·4	9·6	12	13	17	96
Phenol red	—	—	108	—	—	—	94	—	—	—	—	—	89	—	—
Bromophenol blue	—	104	—	—	—	106	—	—	—	—	—	96	—	—	—
Thymol blue	—	—	—	107	—	—	—	100	—	—	—	—	—	—	—
Bromothymol blue	97	—	—	—	95	—	—	—	—	94	—	—	—	—	—
Phenolphthalein*	—	—	—	—	—	—	—	—	95	—	85†	—	—	—	74‡
Methyl orange	—	—	—	—	95	—	—	—	—	—	—	—	—	94	—

* This was dissolved in 30 % aqueous alcohol solution. The alcohol introduced into the reaction mixture with the indicator was shown by a control experiment to have no effect on the rate of hydrolysis.

† A small precipitate was formed.

‡ A heavy precipitate developed.

Table XI. *Effect of indicators and dyestuffs on proteinase activity.*

The figures represent initial velocity as % of that in control digests.

	Concentration *M* × 10^{-4}							
Substance	0·084	0·17	0·34	0·84	1·7	3·4	17·0	34·0
Congo red	107	100	—	—	88	—	—	—
Safranine	—	—	—	97	113	—	109	—
Trypan red	—	—	—	110	110	—	106	—
Methylene blue	—	94	93	—	—	92	—	—
Rosolic acid	—	91	99	—	—	101	—	—
Bismarck brown	—	—	—	—	101	100	—	110

tuents. The caseinogen concentration in the digests was therefore 0·9%; the concentrations of the indicators shown in Tables X and XI refer to those in the indicator-enzyme solutions before addition to the substrate. The concentrations in the reaction mixtures were, therefore, one-fifth of these values.

Quastel [1931; 1932] has shown that certain indicators and dyestuffs have marked inhibitory effects on the oxidation of glucose, lactate, succinate and formate by *Bact. coli* and on the action of fumarase and urease. Both basic and acidic dyes were toxic toward fumarase, whereas only basic dyes inhibited oxidations by *Bact. coli*. Acidic dyes were entirely inert toward urease although most basic dyes were toxic. These and other observations led Quastel to the conclusion that the groupings responsible for the attachment of urease to its substrate consist of the primary amino-group and the secondary imino-group. Investigations of this kind would appear to offer a very promising approach to the study of the nature of the active groups of enzymes in general. Our own efforts in this direction were concerned with a survey of the effects of representative dyestuffs on proteinase activity with the hope that a subsequent study of the possible protective effects of known chemical compounds in the case of toxic dyes might lead to some understanding of the nature of the chemical groups responsible for the union of the proteinase with its substrate. It has been found however that, even though used in concentrations considerably higher than those which Quastel found to be decidedly toxic in many cases towards the enzymes enumerated above, the dyestuffs examined were quite inert toward the proteinase. Furthermore, the acidic or basic nature of the dye had little or no relation to its effect on the enzyme. The results which are recorded have, therefore, little positive interest. They do suggest that the active group of the proteinase differs from those of the enzymes studied by Quastel. But the question of the degree of purity of the enzyme is one which introduces difficulties of interpretation. It is quite possible that impurities present in the enzyme preparations protected the enzyme from the toxic influence of added dyestuff, though enzyme and dyestuff were in preliminary association for 20 min. Quastel's urease preparation was a partially purified aqueous extract; the fumarase solutions were cell-free preparations made from bacteria, erythrocytes and brain; for the oxidation experiments suspensions of bacteria and of minced muscle were used. Some of these preparations, like the enzyme solutions used in the present work, must have contained large amounts of non-enzymic material. Further work on the influence of dyestuffs on the activity of purified proteinase preparations is now in progress.

Salts. In Tables XII and XIII the effects of various salts are recorded. In all experiments the enzyme-salt mixtures were allowed to stand for 20 min. at 25° before addition to the substrate-buffer solution. An aqueous solution of the enzyme was used and the method of preparing the digests was similar to that employed in the case of the indicators. The enzyme solutions containing calcium chloride and lead acetate developed precipitates on standing, the amounts varying with the concentration of the salt. Sodium nitroprusside caused the development of a purple colour in the enzyme solution; the other salts caused no visible effect. The concentrations of the salts given in the tables are those in the enzyme-salt solutions; in the digests the salt concentrations were one-fifth of the recorded values.

Unlike many enzymes the proteinase was apparently unaffected by the presence of salts of the heavy metals in concentrations as high as 3×10^{-3} *M*.

Each of the compounds calcium chloride, sodium cyanide, potassium ferrocyanide and potassium ferricyanide exercised an activating effect on the pro-

Table XII. *Effect of salts of heavy metals on proteinase activity.*

The figures represent initial velocity as % of that in control digests.

	Concentration $M \times 10^{-4}$			
Substance	0·67	3·3	9·9	33
Mercuric chloride	106	102	101	—
Silver nitrate	101	98	101	—
Cupric chloride	106	95	101	112
Lead acetate	—	108	—	99

Table XIII. *Effect of various salts on the activity of pancreatic proteinase.*

The figures represent initial velocity as % of that in control digests.

	Concentration $M \times 10^{-4}$						
Substance	0·67	1·65	3·3	6·7	9·9	16·5	33
Magnesium sulphate	—	100	—	98	—	—	97
Sodium fluoride	—	108	—	—	104	—	98
Sodium nitroprusside	100	—	97	—	—	96	—
Calcium chloride	121	—	141	—	110	—	106
			135				
Sodium cyanide	119	—	140	172	107	—	99
			134				
			136				
Potassium ferrocyanide	100	—	129	—	—	102	—
Potassium ferricyanide	102	—	127	—	—	118	—

teinase (Table XIII). Some years ago it was generally accepted that calcium salts activate trypsin, but experiments of Waldschmidt-Leitz [1924] caused a revision of this opinion. In the present experiments the activity of the proteinase was definitely stimulated by calcium. It is possible that this activation was due to the removal of concomitant inhibitors present in the rather impure enzyme preparation employed. It is proposed to test this possibility by using more highly purified preparations.

The activation by cyanide was quite definite. Willstätter *et al.* [1926] obtained results which led them to conclude that cyanide inhibits the activity of the protease of the pumpkin. However, Ambros and Harteneck [1929], on examining the problem, showed that the effect was due to the inhibitory influence of cyanide on the peptidases present in the enzyme preparation. The result was a marked reduction in the total acid titratable in alcoholic solution. When the effect on the peptidases was taken into account, the proteinase was shown to be activated by cyanide, a finding with which the present results with pancreatic proteinase are in agreement. Activation by cyanide has been well established in the case of other proteolytic enzymes such as papain and bromelin.

The mechanism of the acceleration of enzyme action by cyanide has, in recent years, been a subject of considerable interest. Krebs [1930] suggested that cyanide activation is due to the removal of toxic heavy metals by the formation of cyanide-metal complexes. In the present work, however, salts of heavy metals in moderately high concentration had no apparent inhibitory or toxic effects even after preliminary association with the enzyme for 20 min. Activation of the enzyme by cyanide cannot, therefore, be explained on the basis of such a detoxication process. Cyanide appears to exercise a positive activating influence; this is true, also, of potassium ferrocyanide and potassium ferricyanide. The explanation in each case must await further experiments.

SUMMARY.

1. The influence of various chemical compounds on the initial rate of hydrolysis of caseinogen at p_H 8·7 by pancreatic proteinase was investigated with the aid of micro-methods for the determination of residual proteins. The substances investigated included carbohydrates, glycerol, amino-acids, asparagine, triglycerides, bile and bile salts, indicators and dyes, salts of heavy metals and other salts.

2. Mono- and di-saccharides in moderately high concentrations (*e.g.* 0·5 *M*) exercised marked inhibitory effects upon the activity of the proteinase. These effects are believed to be largely independent of the configurations of the sugars and to be related to a disturbance of the effective water concentration of the digestion mixture.

3. Dextrin and soluble starch were less effective inhibitors than the sugars. Gum acacia was without significant effect in moderately high concentration.

4. The degree of inhibition by glycerol was in direct proportion to the concentration. The effect observed is believed to be related to a disturbance of the effective water concentration of the enzyme digest.

5. Of the amino-acids investigated, aspartic and glutamic acids exercised definite accelerating effects; acceleration by cysteine was less marked; the others were relatively ineffective.

6. Asparagine caused definite activation.

7. Triacetin, tributyrin and triolein, when properly emulsified, were inhibitory. Unstable emulsions of triolein in gum acacia had no appreciable effect.

8. Fresh ox bile and bile salts were inhibitory.

9. Indicators and dyes, used in concentrations which Quastel found to be inhibitory towards fumarase and urease, had no significant effect on proteinase activity.

10. Salts of heavy metals in concentrations as high as 3×10^{-3} *M* were without effect.

11. Calcium chloride, sodium cyanide, potassium ferrocyanide and potassium ferricyanide stimulated the activity of the enzyme.

12. Activation of the proteinase by cyanide was apparently not due to removal of toxic heavy metals.

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