## Degradation of ribonuclease by subtilisin

The study of the kinetics of protein degradation by enzymes is greatly facilitated if, at different times during the proteolysis, the concentration of totally unchanged substrate protein is known[1]. Using a substrate like ribonuclease (RNase) and measuring the change in its activity during degradation would give valuable information concerning this point, provided ribonuclease activity is strictly a property of the intact protein and is uninfluenced by degradation products formed. We have therefore subjected beef ribonuclease to the proteolytic action of subtilisin[2,3] measuring both the extent of hydrolysis in the JACOBSEN-LÉONIS pH-stat[4] and the loss in activity according to KUNITZ[5]. Fig. 1 shows a typical kinetic experiment in 0.1 *N* KCl (substrate conc. 1 %, enzyme concentration 0.004 %, pH 8, 30°). It will be seen that subtilisin will hydrolyze a considerable number of peptide bonds per molecule, *viz.* 22 in the course of 350 min and that the subsequent reaction is very slow. The average chain length of the peptides formed after 350 min is about 6. The degradation of ribonuclease by subtilisin is therefore more intensive than that by pepsin (ANFINSEN[6]) but has features in common with the breakdown of insulin by subtilisin (E. AND N. HAUGAARD (in press)). Fig. 2 shows the relationship between activity and hydrolysis in two cases. Several ribonuclease preparations were used and although they all have approximately the same specific activity they show widely different behavior. In the case of preparation I (made at the Carlsberg Laboratory) it would appear that a considerable number of bonds could be split without serious effect upon the activity while the curve for preparation II (from Nordisk Insulin Laboratory) is much more linear and might just indicate the trivial fact that the breakdown proceeds practically without formation of intermediary products[7,1]. The shape of curve I may be explained in different ways:

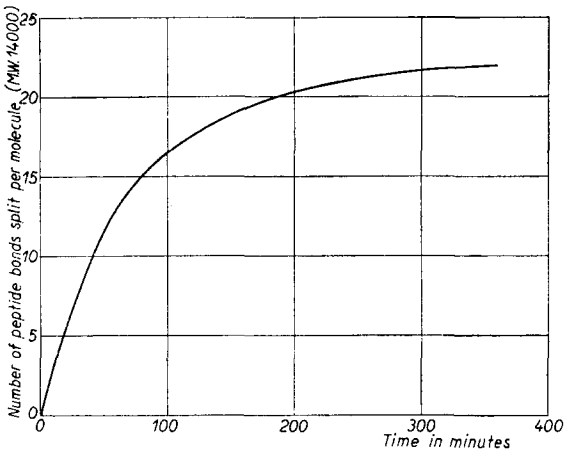


Fig. 1

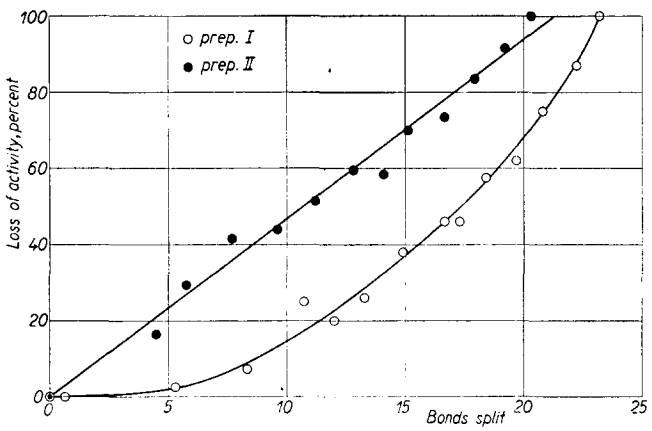


Fig. 2

a. Active intermediary products are formed.

b. The RNase preparations are mixtures of active components with widely different susceptibility to subtilisin.

c. An activator for RNase is formed during the digestion.

Similarly curve II may be interpreted as follows:

e. No active (or inactive) intermediary products are formed.

f. Active intermediary products are formed but

$f_1$: An inhibitor for RNase is formed during the digestion.

$f_2$: An activator for RNase is removed during the digestion.

Since our small stock of preparation I was exhausted in preliminary experiments (Fig. 2) we concentrated on Prep. II of which we had ample quantities. It was tested for purity according to HIRS, MOORE AND STEIN[8] and found to be satisfactory (Fig. 3). The breakdown by subtilisin was studied as described above, except that we determined both the activity towards RNA (KUNITZ) and, following a suggestion by W. ANDERSEN, the activity towards the cyclic phosphate of uridylic acid (kindly presented to us by Professor A. TODD) thus separating the influence of subtilisin upon the two functions of RNase: the diesterase activity and the action upon cyclic phosphate (DEase-

and CPase-activity respectively) (see [9, 10] and [11]). The method for the measurement of CPase-activity will be described in detail in a later publication, but the principle was that of determining the shift in absorption spectrum occurring when the ring is opened. Fig. 4 shows the results. Although the experimental errors (as in Fig. 2) are rather large, it is evident that the DEase- and CPase-activity do not disappear simultaneously. This observation may be understood by assuming that

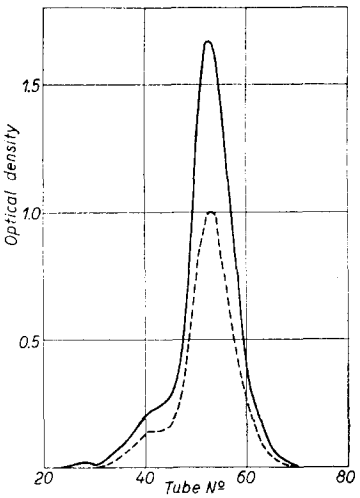


Fig. 3. RNase on XE-64, pH = 6.5. —— = $OD_{280}$ blank 0.010; – – – = $OD_{570}$ (ninhydrin) blank 0.100 corrected for blank readings.

$e_1$: A selective activator for CPase is liberated in the digestion.

f. Active products are formed, and

$f_3$: They show a higher ratio CPase/DEase than the original RNase, indicating different sites for the two functions in RNase.

$f_4$: They show the same ratio CPase/DEase but a selective inhibitor of DEase is formed.

Experiments to be reported later by one of us (RICHARDS) has made it likely that $f_4$ actually represents the facts. It has been shown that an active intermediary product (AIP) *is* formed and may be separated in the digest from intact RNase by chromatography on IRC-50 columns[8]. The detailed description of its properties will be given elsewhere but it should be mentioned here that it is not able to pass cellophane membranes (*cf.*[12]) and especially that its ratio CPase/DEase is the same as that of intact RNase. The presence in the digest of a selective DEase inhibitor which is removed in the column is suggested by the fact that the sum of the DEase activities of the separated peaks of RNase and AIP is greater than that of the original digest.

The phenomena described here are reminiscent of observations by PORTER[13] on the breakdown of ribonuclease by pepsin and carboxypeptidase, but the interpretation given by this author is different from ours.

Our results emphasize very strongly the error that may arise if enzyme activity is used as a measure of the amount of intact substrate in kinetic experiments.

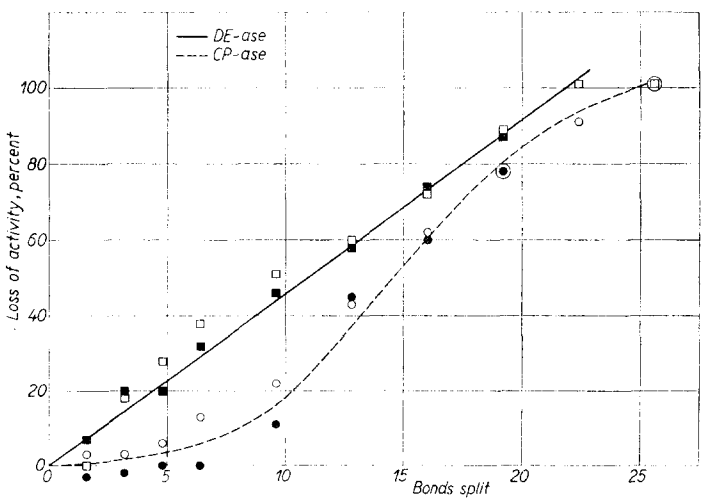


Fig. 4

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## The nature of the reaction between diisopropylfluorophosphate and chymotrypsin

JANSEN *et al.*[1,2] were the first to study the inhibition of chymotrypsin using $^{32}P$ labeled diisopropylfluorophosphate ($DF^{32}P$). They showed that one mole $DF^{32}P$ was bound per mole of active enzyme, that a fluorine ion was released during the reaction and that the reaction product consisted of the original protein with a diisopropylphosphate (DIP) rest[3]. Moreover it was demonstrated by SCHAFFER *et al.*[4,5] that this reaction product yielded O-serinephosphate on acid hydrolysis.

In the course of our study of the pharmacological action of organophosphates we became interested in the character of the bond resulting from the reaction of DFP on mammalian cholinesterase[6]. In view of the limited supply of purified cholinesterase available at the time, we decided first to obtain more basic information about the inhibition of crystalline chymotrypsin by $DF^{32}P$. It was assumed that this inhibition could serve as a satisfactory model for the corresponding reaction on cholinesterase. This hypothesis was supported by the fact that other esterases could be demonstrated to produce serinephosphate on acid hydrolysis. This was shown by SCHAFFER *et al.*[7] for electric eel cholinesterase, and by us for purified bovine cholinesterase, serum pseudo-cholinesterase and stroma ali-esterase[8]. SCHAFFER *et al.*[5] have repeatedly stressed that these findings do not necessarily mean that the primary point of attack of DFP on the chymotrypsin molecule would involve the hydroxyl group of a serine molecule. The isolated serinephosphate might be produced during and as a result of the acid hydrolysis. It appeared to us that breakdown of chymotrypsin-DFP by enzymic methods, avoiding the acid hydrolysis, might yield a product which on analysis would provide information about the primary side of reaction of DFP.

For the breakdown of the chymotrypsin-DIP a polyvalent commercial pancreatic enzyme preparation was used*. This preparation was able to split a number of proteins, including chymotrypsin, into amino acids. It seems reasonable to assume on the basis of the specificity of proteolytic enzymes, that the presence of an unusual group in the protein molecule (like the DIP group in the chymotrypsin-DIP) might interfere with the normal breakdown of such a protein. Thus a peptide might result, containing this unusual group (in the present case the $^{32}P$ labeled DIP) in addition to a number of amino acids. Hydrolysis of chymotrypsin-DIP by cotazym indeed produced a single peptide which contained 50 to 60% of the original DIP group. The isolation of this peptide from the hydrolysate could be effected by distribution between water and phenol, followed by paper chromatography in butanol-acetic acid-water and butanol-water, or better still by zone electrophoresis on a starch column[9] at pH 4.6, followed by paper chromatography in butanol-acetic acid-water.

The isolated peptide had the following properties:

1. It was stable against hydrolysis by cotazym.

2. It moved rapidly towards the anode on the starch column, suggesting a high acidity (oddly it hardly moved on paper electrophoresis).

3. On alkaline treatment (pH 11.0) and heating at 100° C for 5 minutes all labeled phosphorus was split off as DIP, indicating that the labeling group was still present as DIP.

4. On acid treatment (pH 2.0) at 100° C the peptide did not produce DIP and yielded serinephosphate on continued acid hydrolysis with 2 *N* HCl (15 h 100° C).

5. Per DIP group the original peptide was shown to consist of the following amino acids: proline (1), leucine (1), aspartic acid (1), serine (1), and glycine (2 or 3).

The sequence phosphoseryl-glycine, demonstrated by SCHAFFER *et al.*[10], could obviously occur in the present peptide. We do not know yet where the DIP group is situated, but in view of the mild treatment we applied, it seems reasonable to assume that this localisation will not be different