I. STUDIES ON THE REVERSIBLE INACTIVATION OF PAPAIN AND CATHEPSIN.

By ARNULF PURR.

*From the Cancer Research Laboratories, Graduate School of Medicine,
University of Pennsylvania, Philadelphia, Pa.*

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THE natural activation of cathepsin and papain by sulphydryl compounds acquired increased physiological interest when it became known [Purr, 1933] that the combination, vitamin C-Fe⁺⁺, can activate not only arginase but also cathepsin. Later, Maschmann and Helmert [1934] found a similar activation of papain by the vitamin C-Fe⁺⁺ complex.

The activation of these two proteinases by such widely dissimilar types of compounds made it of interest to investigate further the mechanism of these activations. The experiments here reported show much promise, contributing not only to our knowledge of the nature of intracellular proteolysis, but also to our understanding of the part played by vitamin C in the regulation of cellular metabolic processes.

Since the initial activities of papain and cathepsin vary considerably for different preparations, it was necessary to devise methods for obtaining entirely inactive enzyme preparations. For comparative studies in enzyme activation, it was essential that these inactive preparations should retain the property of being completely reactivated upon addition of the proper activator.

Reversible inactivators of papain.

I. *Alloxan inactivation and reactivation.* Papain extract was prepared from a commercial Merck papain powder by 6 hours' extraction with 10 parts of water. After centrifuging, 4 ml. of the clear fluid were allowed to stand for 30 minutes at 30° and p_H 7 with 40 mg. of cysteine hydrochloride dissolved in 4 ml. of water. To the thus fully activated enzyme solution were then added 80 mg. of alloxan in 4 ml. of water, and the reaction mixture was incubated for 1 hour at 30° and p_H 7. The volume was brought to 20 ml., and the papain activity determined on 10 ml.; H₂S was bubbled through the remaining 10 ml. for 1 hour at p_H 7, and the degree of reactivation determined. (If cysteine be used for reactivation, it is necessary to use a large excess, 50 mg., because of the presence of excess alloxan.) The measurement of enzymic activity was carried out by adding 5 ml. of 8 % gelatin, 2 ml. of *M* acetate buffer of p_H 5 and sufficient water to bring the volume up to 25 ml. 10 ml. of this were analysed at once for free amino-groups, and a second 10 ml. after 1 hour's incubation at 30°. The increase of free amino-groups, determined by the Van Slyke method, constitutes a measure of the enzymic activity, the results being expressed in ml. of 0.05 *N* KOH.

For the determination of the initial activity before inactivation, 2 ml. of the papain extract, prepared as described above, were mixed with 5 ml. of 8 % gelatin, 2 ml. of *M* acetate buffer of p_H 5 and sufficient water to bring the total volume up to 25 ml. Amino-group determinations were carried out immediately and after 1 hour's incubation at 30° on 10 ml. portions of the mixture, exactly as previously described. For the determination of full activity before inactivation, 2 ml. of the original papain extract were fully activated by passing a stream of H_2S for 1 hour at 30°. The rest of the treatment was identical with that just described.

II. *Methylglyoxal inactivation and reactivation.* The experimental details of this experiment were identical with those described for alloxan, except that for inactivation 48 mg. of methylglyoxal in 5 ml. of water were used in place of the alloxan. The methylglyoxal was prepared by distillation of dihydroxyacetone with dilute sulphuric acid according to the method of Neuberg *et al.* [1917]. Since the same papain extract was used, the initial and full activities before inactivation are the same as those in the case of alloxan.

Table I. *Reversible inactivation of papain by alloxan and methylglyoxal.*

Reversible inactivator	Papain activity before inactivation		Papain activity after inactivation	Reactivation	
	Initial activity	Full activity		Cysteine	H_2S
Alloxan	0.80	3.54	—	—	—
Methylglyoxal	—	—	0.0	3.50	3.65
				—	3.60

The results given in Table I (as NH_2 increase in ml. 0.05 *N* KOH) show not only that alloxan and methylglyoxal completely remove the slight initial activity present in the papain preparation, but also that the enzyme, after inactivation by these compounds, may be completely reactivated, showing that the enzyme itself has not been damaged. In separate experiments with the nitroprusside test for SH groups, it was found that the inactivation is connected with a disappearance of the SH reaction. This finding demonstrates the dependence of papain activity on the presence of SH groups.

The inactivation of papain by alloxan and methylglyoxal differs sharply from that brought about by iodoacetic acid. The inhibition produced by the latter, which is irreversible, will be discussed later in more detail.

III. *The system "aerobic dehydrogenase-peroxidase."* The application of this system to the reversible inactivation of papain is suggested by the illuminating work of Thurlow [1925], who found that the system aerobic dehydrogenase-peroxidase catalyses the formation of H_2O_2 from molecular oxygen. An aerobic dehydrogenase, such as xanthine oxidase, is able, in the presence of hypoxanthine, to make molecular oxygen directly available as a hydrogen acceptor, and, in the presence of excess oxygen, to produce H_2O_2 . As Thurlow found, the quantity of H_2O_2 formed is very small at the beginning of the experiment, but as soon as the greater part of the hypoxanthine is converted into xanthine, the quantity increases remarkably. Bach and Chodat [1903] have defined as an oxygenase a system which is able to convert molecular oxygen directly into H_2O_2 . Thurlow showed that the system xanthine dehydrogenase-hypoxanthine falls within the classification of oxygenases.

In contrast to this are the anaerobic dehydrogenases, such as lactic dehydrogenase, which do not possess the ability to react directly with molecular oxygen

Only the addition of a small quantity of methylene blue enables the system to react with molecular oxygen. The methylene blue acts as an intermediate carrier (hydrogen acceptor) between oxygen and the dehydrogenase substrate.

Peroxidase is an enzyme which activates the oxygen in the H_2O_2 molecule. This active condition of the oxygen enables it to transfer its oxidation potential very easily to other substances, which themselves are oxidisable.

If such a system (aerobic dehydrogenase + substrate + peroxidase) is added to a papain preparation, and care is taken that the oxygen of the air comes into intimate contact with the solution, a complete inactivation of the papain is obtained. This inactivation occurs only when all the components mentioned are present, the separate components being without effect on the enzymic activity.

The procedure for the inactivation was as follows: 30 mg. of milk xanthine oxidase (prepared by the method of Dixon and Thurlow [1924]), 1 mg. of hypoxanthine and 10 mg. of milk peroxidase [Elliott, 1932] were dissolved in 2 ml. of water and added to 2 ml. of the papain extract previously described. 1 ml. of $M/10$ phosphate buffer of p_{H} 7 was added. Air was then bubbled through the solution for 3 hours, after which 5 ml. of 8 % gelatin, 2 ml. of M acetate buffer of p_{H} 5 and sufficient water to bring the total volume up to 25 ml. were added. Amino-determinations were carried out immediately, and after 1 hour's incubation at 30° , on 10 ml. portions. The results are shown in Table II as NH_2 increase in ml. of $0.05N$ KOH. A similar inactivated reaction mixture was treated with H_2S for 1 hour at p_{H} 7 and the degree of reactivation obtained measured as previously described. The determination of initial and full activity of the papain before inactivation was carried out as described under Table I.

The following control experiments were carried out: (a) 2 ml. of papain extract were diluted with 2 ml. of water and 1 ml. of $M/10$ phosphate buffer of p_{H} 7; air was then bubbled through for 3 hours and the papain activity determined as described above; (b) same as (a) except that 1 mg. of hypoxanthine was added; (c) same as (a) except that 1 mg. of hypoxanthine and 30 mg. of milk xanthine oxidase were added; (d) same as (a) except that 30 mg. of milk xanthine oxidase and 10 mg. of milk peroxidase were added.

Table II. *Reversible inactivation of papain by respiration ferments.*

Reversible inactivator	Papain activity before inactivation		Papain activity after inactivation	Reactivation	
	Initial activity	Full activity		Cysteine	H_2S
	0.90	3.50	—	—	—
D + Hy. + Per. + O_2	—	—	0.05	3.50	3.40
O_2 (3 hours)	—	—	0.90	—	—
Hy. + O_2	—	—	0.90	—	—
Hy. + D + O_2	—	—	0.85	—	—
D + Per. + O_2	—	—	0.90	—	—

D=aerobic dehydrogenase; Hy.=hypoxanthine; Per.=peroxidase.

As shown in Table II, the reversible inactivating effect on papain is obtained only by the simultaneous action of xanthine oxidase (aerobic dehydrogenase), hypoxanthine (substrate for the dehydrogenase), peroxidase and excess oxygen. From these findings, which for the first time demonstrate the dependence of

intracellular proteolysis on the respiration ferments, it may be concluded that the following intermediary steps are responsible for the papain inactivation:

IV. *Hydrogen peroxide*. Preliminary experiments on the reversible inactivation of papain with hydrogen peroxide gave satisfactory results. A method was therefore developed which permits the preparation of large quantities of inactive papain in a stable, pulverisable form.

To an aqueous solution of 5 g. of papain (Merck) in 100 ml. are added 3 ml. of 30 % H_2O_2 , and the mixture is allowed to stand 1 hour at 30° . The enzyme is then precipitated by addition of 600 ml. of 95 % alcohol, centrifuged, and the residue washed twice with 95 % alcohol. After the final centrifuging, the residue is redissolved in 100 ml. of water (any insoluble matter is removed by centrifuging) and treated again with H_2O_2 and alcohol as described above. This procedure is repeated five times. The final precipitate is dried *in vacuo* over P_2O_5 and powdered.

For reactivation experiments, the inactive powder was extracted with 40 parts of water and centrifuged. 1 ml. of the liquid (comparable in activity with 2 ml. of crude papain extract before inactivation) was brought up to 5 ml. with water, mixed with 20 mg. of cysteine HCl in 2 ml. and allowed to activate for 30 minutes at p_{H} 7. The measurement of enzymic activity, as well as the determination of initial and full activity before inactivation, were carried out as previously described.

Table III. *Reversible inactivation of papain by hydrogen peroxide.*

Reversible inactivator	Papain activity before inactivation		Papain activity after inactivation	Reactivation	
	Initial activity	Full activity		Cysteine	H_2S
H_2O_2	0.80	3.30	0.0	3.35	3.40

Table III shows that papain inactivated by hydrogen peroxide can be fully reactivated, just as is the case with the other inactivators described in this paper. The method described for the inactivation of papain by hydrogen peroxide possesses the advantage of being easily performed. The technique is therefore peculiarly adapted to the preparation of large amounts of papain, so that comparative activation studies on this enzyme are made much simpler.

Reversible inactivation of cathepsin.

I. *Alloxan*. Rat liver was used as the source of cathepsin. Liver from well-bled rats was finely ground in a micro-mincing machine (Leitz). The "brei" thus obtained was suspended in 10 parts of 90 % glycerol and used directly for the enzyme studies.

To obtain an estimate of the initial activity, 5 ml. of this suspension were treated with 5 ml. of 8 % gelatin (Kahlbaum), 1 ml. of *N* acetic acid and sufficient water to bring the volume up to 25 ml. (final $p_H=4$). By means of the Van Slyke method, amino-nitrogen was estimated immediately, and again after 24 hours' incubation at 30° , on 10 ml. portions of the reaction mixture. As before, the increase in amino-nitrogen, expressed in ml. of 0.05*N* KOH, indicates the cathepsin activity. A second portion of the glycerol suspension was fully activated by allowing 5 ml. to stand for 15 minutes at 30° and p_H 7 with 20 mg. of cysteine hydrochloride and 30 mg. of $FeSO_4 \cdot 7H_2O$ in 3 ml. of water. The determination of the full activity then followed exactly as described for the initial activity.

To demonstrate the inactivating effect of alloxan, 5 ml. of the glycerol suspension were fully activated by cysteine, then allowed to stand for 1 hour at 30° and p_H 7 with 30 mg. of alloxan, after which activity determinations were carried out as before. A second portion of the glycerol suspension was fully activated by cysteine, inactivated by alloxan and finally reactivated by passing H_2S for 1 hour at p_H 7. The results obtained are shown in Table IV.

II. *Methylglyoxal*. The experimental procedure was the same as that described for alloxan, except that 30 mg. of methylglyoxal were used in place of the alloxan. The results are shown in Table IV.

III. *Iodoacetic acid*. 5 ml. of glycerol suspension were fully activated by keeping with 26 mg. of cysteine hydrochloride in 2 ml. of water for 30 minutes at 30° and p_H 7. A second 5 ml. portion was first activated, then inactivated by allowing it to stand for 30 minutes with 15 mg. of neutralised iodoacetic acid. Attempts were made to reactivate similarly inactivated reaction mixtures by treatment with 3-fold quantities of cysteine hydrochloride (30 minutes at 30°) and with H_2S (1 hour). Activity determinations were made on all reaction mixtures by the usual procedure. The results are shown in Table IV.

Table IV. *Effect of alloxan, methylglyoxal and iodoacetic acid on cathepsin activity.*

Inactivator	Initial activity	Full activity	SH test* before inactivation	$\frac{1}{2}$ hour inactivation		1 hour inactivation		Reactivation	
				Enzymic activity	SH test	Enzymic activity	SH test	H_2S	Cysteine
None	0.50	1.50	++	—	—	—	—	—	—
Alloxan	—	—	++	0.8	+	0.0	—	1.50	1.20
Methylglyoxal	—	—	++	—	—	0.0	—	1.60	—
Iodoacetic acid	—	—	++	0.0	++	0.0	+	0.0	0.0

* Nitroprusside colour reaction.

As shown in Table IV, alloxan and methylglyoxal differ from monoiodoacetic acid in their mode of action on cathepsin. Cathepsin inactivated by the latter cannot be reactivated by direct addition of cysteine or H_2S , in contrast to alloxan- or methylglyoxal-inactivated cathepsin. The same difference is shown by a study of the SH colour reaction (nitroprusside test). It was found that a parallelism exists between the disappearance of sulphhydryl from the cathepsin (and also papain) complex and the reversible inactivation by alloxan, methylglyoxal, H_2O_2 , or by the system aerobic dehydrogenase-substrate-peroxidase. In the case of iodoacetic acid inactivation, however, no relation exists between loss of catheptic activity and loss of SH. Even after the enzyme has been com-

pletely inactivated, a strong SH test is obtained, which, however, eventually disappears. This irreversible inactivation of papain and cathepsin by iodoacetic acid does not involve a destruction of the enzyme complex. If iodoacetic acid-inactivated cathepsin or papain is treated with 80 % alcohol, according to the procedure of Maschmann and Helmert [1933], the enzyme may be reactivated practically to its original activity with cysteine.

A series of experiments was carried out on the inactivation of cathepsin by iodoacetic acid, to determine the effect of varying the order of adding the various reagents. (a) 6 ml. of 1:10 glycerol extract of acetone-ether-dried pig liver were activated with 25 mg. of cysteine hydrochloride in 2 ml. of water by keeping for 30 minutes at 30° and p_H 7. The mixture was then treated with 25 mg. of iodoacetic acid in 2 ml. of water and again allowed to stand for 1 hour. (b) The same quantities of cysteine hydrochloride and iodoacetic acid were added simultaneously to 6 ml. of glycerol extract and kept for 1 hour at 30° and p_H 7. (c) The same quantities of cysteine hydrochloride and iodoacetic acid were mixed and kept for 1 hour at 30° and p_H 7. 6 ml. of fully activated glycerol extract were then added. (d) To 6 ml. of fully activated glycerol extract were added 5 ml. of 8 % gelatin and then 25 mg. of iodoacetic acid in 2 ml. of water; 1 ml. of *N* acetic acid and sufficient water were then added to bring the volume to 25 ml. (p_H 4). The mixture was incubated for 24 hours at 30°. The various reaction mixtures were then treated with H_2S or cysteine hydrochloride (6×26 mg.) for 1 hour and, after addition of the appropriate reagents, tested for possible enzyme reactivation.

It was found that the order of adding the various reagents is immaterial. In each case the enzyme was found to be completely inactivated, and no reactivation was obtained with either H_2S or cysteine. The same results were obtained with papain. It should be mentioned that the inhibition with iodoacetic acid appears not to be a time reaction, but is effective immediately, even when excess SH groups are present.

The comparison of the two inactivation effects, the one reversible (alloxan *etc.*) and the other irreversible (iodoacetic acid) gives rise to conjecture as to the active groupings in papain and cathepsin. The reversible inactivation seems to be connected with a destruction of the SH groups in the papain and cathepsin complexes, and is probably related to interference in the reaction of enzyme with activator. The irreversible inactivation, on the other hand, appears to be concerned not only with an action on the SH groups, but also on a second essential grouping, which seems to be even more important to the enzyme than the SH groups. According to Willstätter *et al.* [1922] an enzyme consists of a colloidal carrier and an active group. If this is correct, then it appears probable that the papain and cathepsin molecules are equipped with at least two active groups, one of which may safely be specified as the SH group.

IV. *Reversible inactivation of liver cathepsin by organic solvents.* The following method was adopted for the preparation of large quantities of inactive cathepsin, as required for comparative activation experiments on this enzyme. Fresh pig's liver was ground in a micro-mincer and suspended in 15 parts (by weight) of absolute acetone. After shaking continuously for 1 hour the suspension was filtered by suction. This procedure was repeated three times. The residue was then extracted three times with 15 parts of an acetone-ether mixture (1:1), each extraction requiring 1 hour. Finally the residue was extracted three times with 15 parts of ether. After drying, the residue was finely ground and passed through a sieve and then extracted for 12 hours with 10 parts by weight of 85 % glycerol. After filtration the clear liquid contained the inactive cathepsin.

If, as sometimes happens, the cathepsin is not yet entirely inactive, excess 95 % alcohol may be added to the glycerol extract until no further precipitation occurs. Ordinarily 10–15 volumes of alcohol are required. After 6 hours' standing, the precipitate is filtered or centrifuged off and dried with ether. A glycerol extract is then prepared from the dried powder as previously described. The enzyme is now completely inactive. Table V shows the initial and full activity of the enzyme at the various stages of the procedure just described.

Table V. *Reversible inactivation of cathepsin by organic solvents.*

Treatment	Organ	Activity before inactivation		Activity after inactivation	Reactivation	
		Initial	Full		H ₂ S	Cysteine
Glycerol suspension 1 : 10 (fresh liver)	Pig liver	1.0	2.40	—	—	—
Glycerol extract 1 : 10 from dried liver after treatment 7 × with acetone, 3 × acetone-ether (1 : 1) and 3 × ether	„	—	—	0.6	1.60	1.90
Glycerol extract 1 : 10 after alcohol treatment of the above glycerol extract	„	—	—	0.00	1.10	1.00
Glycerol suspension 1 : 10 from fresh liver	Rabbit liver	0.90	2.20	—	—	—
Glycerol extract 1 : 10 from dried liver after treatment 4 × with acetone, 3 × acetone-ether (1 : 1) and 3 × ether	„	—	—	0.00	1.0	1.00

The final treatment with alcohol appears not to be necessary in the case of rabbit liver, where only a few minutes elapsed between the death of the animal and the first treatment of the liver mince with acetone.

CONCLUSIONS.

I. *Reversible inactivation of papain.*

1. A method is described for the reversible inactivation of papain by means of alloxan, methylglyoxal, hydrogen peroxide and with the system milk xanthine oxidase + hypoxanthine + milk peroxidase. From the observation that the reversible inactivation of papain proceeds parallel with the disappearance of the SH colour reaction with sodium nitroprusside it is concluded that the destruction of the SH groups in the papain complex (the same applies to the cathepsin complex) through the reagents mentioned is responsible for the reversible inactivation.

2. A relation between typical respiration ferments and intracellular proteolysis was shown by the reversible inactivation of papain by the system aerobic dehydrogenase + hypoxanthine + peroxidase.

3. A method is described for the preparation of large quantities of reversibly inactivated papain in stable form.

II. *Reversible inactivation of cathepsin.*

1. Cathepsin is also reversibly inactivated by alloxan or methylglyoxal. The loss of enzymic activity runs parallel with the loss of SH.

2. On the basis of the reversible and irreversible inactivations of papain and cathepsin the possibility is suggested that the two enzyme systems require at least two active groups for their activity, of which groups one can be said with certainty to be an SH group.

3. A method is described for the preparation of large quantities of inactive cathepsin.

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