II. THE ACTIVATION PHENOMENA OF PAPAIN AND CATHEPSIN.

By ARNULF PURR.

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

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THE discovery [Purr, 1933] of the activating effect of the vitamin C—Fe⁺⁺ complex on arginase and cathepsin demonstrates that a product of plant cellular metabolism may, like sulphhydryl compounds (*e.g.* glutathione), exercise a regulatory action on animal protein metabolism. Later investigations by Maschmann and Helmert [1934], as well as the present work, show that this activating effect of vitamin C—Fe⁺⁺ is not limited to animal proteases, but extends also to papain. The latter is the protease of many plants and appears to be the complete counterpart of cathepsin, the protease of most animal organs and leucocytes. According to our present knowledge, a close relationship exists between the proteolytic enzyme systems of the plant and animal worlds.

The vitamin C—Fe⁺⁺ complex is a physiological product which resembles HCN in its activating effect on papain and cathepsin. The discovery of this new activator complex has cast considerable doubt on the widely held idea that sulphhydryl compounds function as specific activators of these proteinases. Purr and Weil [1934] have likewise found evidence that sulphhydryl may not be a specific activator of arginase. The clarification of these questions is of considerable physiological significance because of the fact that the newly-found activator is a vitamin.

The present investigation deals with the activation phenomena of papain and cathepsin. It is a continuation of a series of studies which has as its goal the explanation of the relation between intracellular enzymes and vitamins. A further aim is to clarify our knowledge of the mechanism of enzyme activation, so that differences between normal and cancerous enzymic processes may more readily be detected. It appears quite probable that the proteinases of specific organs (*e.g.* liver) will differ in some essential points (perhaps in activation mechanism) from those of pathological neoplasms, which have been designated by Waldschmidt-Leitz *et al.* [1933] as unspecific organs with respect to their enzymic equipment.

Activation of papain.

For comparative activation studies, a papain preparation was used which had been reversibly inactivated with H₂O₂ according to a procedure described by Purr [1934]. The use of such an inactivated preparation makes the results obtained independent of the variations in initial activity which are characteristic of natural papain. 1 g. of inactive papain was extracted by standing overnight in the ice-box with 50 ml. of water. After filtration and addition of a drop of toluene the extract may be preserved for a long time in the ice-box.

The initial activity of this extract was determined as follows. 0.20 ml. of papain extract was treated with 20 mg. of FeSO₄, 7H₂O in 1 ml. of H₂O, 5 ml.

of 8 % aqueous gelatin, 2 ml. of acetate buffer of p_H 5 and sufficient water to bring the total volume to 25 ml. 10 ml. were removed immediately and 10 ml. after an incubation period of 20 hours at 30° for analysis. The increase in free amino-groups was estimated by the Van Slyke method, the results being expressed in ml. of 0.05 *N* KOH.

By this procedure, the following activation experiments were carried out. (a) 0.20 ml. of papain extract was incubated for 15 minutes at p_H 7 with 20 mg. cysteine hydrochloride in 2 ml. of water and 20 mg. of $FeSO_4 \cdot 7H_2O$ in 1 ml. of water; (b) 0.20 ml. of papain extract was treated for 1 hour at 30° with 5 ml. of $M/5$ KCN at p_H 7; (c) 0.20 ml. of papain extract was incubated for 1 hour at 30° and p_H 7 with 7 mg. vitamin C in 2 ml. H_2O and 20 mg. $FeSO_4 \cdot 7H_2O$ in 1 ml. of H_2O ; (d) same as (c) except that 20 mg. of pyruvic acid were substituted for the vitamin; (e) same as (c) except that 20 mg. of succinic acid were substituted for the vitamin.

The reaction mixtures were then carried through the same procedure as described for initial activity. The entire series was repeated (except for the HCN experiment) in the absence of iron salt. The results are shown in Table I.

Table I. *Reactivation of inactive papain.*

Reactivator	Activity (ml. 0.05 <i>N</i> KOH)		Time of incubation (hours)
	Without Fe^{++}	With Fe^{++}	
None	0.00	0.00	20
Cysteine	4.85	5.00	20
HCN	3.10	—	20
Vitamin C	0.00	0.05	20
"	—	0.80	70
Pyruvic acid	0.00	0.05	20
Succinic acid	0.00	0.00	20

It is evident that neither vitamin C nor the vitamin C— Fe^{++} complex is able to reactivate the purified, reversibly inactivated papain used. A possible explanation for this finding, which is contrary to the results obtained by Maschmann and Helmert [1934], is suggested by the appreciable activation obtained on longer incubation (70 hours). It appeared likely that certain contaminating substances, which were removed by the purification and inactivation process, might be responsible for the variations in the results.

The experiment was therefore repeated using a 1:10 aqueous extract (6 hours' extraction) of commercial unpurified papain (Merck). The experimental details were similar to those described for Table I, except that 2 ml. of the papain extract were used. The results are shown in Table II.

Table II. *Activation of unpurified commercial papain.*

Activator	Activity in ml. 0.05 <i>N</i> KOH	
	Without Fe^{++}	With Fe^{++}
None	0.70	0.71
Cysteine	5.10	5.20
HCN	5.65	—
Vitamin C	0.80	2.41
Pyruvic acid	2.10	2.20
Succinic acid	0.65	0.75

In agreement with Maschmann and Helmert's [1934] results, it was found that the vitamin C— Fe^{++} complex activates unpurified commercial papain. The

pyruvic acid—Fe" complex likewise produces a similar degree of activation. In the absence of iron salts, vitamin C produces no activation, while under the same circumstances pyruvic acid produces nearly as high an activation as with iron.

The failure of the vitamin C—Fe", and the pyruvic acid—Fe" complexes to activate purified inactive papain led to the conclusion that these combinations are able to activate papain only indirectly. Observations made during a search for a possible contaminating substance, which might play a part in the activations produced by the above-mentioned complexes, led to a study of proteins containing fixed SH groups, first described by Hopkins and Dixon [1922]. Proteins of this type, which occur in all plant and animal tissues, contain SH groups which are resistant to mild oxidation. Furthermore, the SH groups of these water-insoluble proteins cannot be removed by washing with water or dialysis through animal membranes, which indicates that these groups are an essential part of the structure of the protein molecule. Aqueous solutions of such proteins cannot be obtained by direct extraction. However, if the organs are freed from blood, minced and thoroughly washed with water, most of the SH proteins may then be extracted with excess secondary phosphate. It was found that these dissolved proteins are not attacked enzymically by activated papain. The extracts thus prepared give a positive nitroprusside test, which is intensified by addition of KCN, indicating the presence of fixed, reducible SS groups in addition to SH. The same conclusion was reached by Hopkins and Dixon [1922].

The following series of experiments was carried out to determine to what extent the addition of solutions of SH- and SS-proteins influences the activation of purified papain by vitamin C—Fe", and whether these proteins may not be among the contaminating substances in commercial papain which cause the latter to respond differently to vitamin C—Fe".

Preparation of fixed SH- and SS-proteins.

The organ, or preferably muscular tissue, of horse, ox, rabbit *etc.*, is freed from fat and nerve tissue and finely ground. 100 g. of the ground tissue are repeatedly kneaded with small portions of 0.25 % NaCl solution until the mass becomes greyish white. Most of the NaCl is removed by kneading with 2 volumes of distilled water. The mass is filtered with suction and the residue thoroughly kneaded with a small amount of $M/10$ Na_2HPO_4 . More phosphate solution, up to a total volume of 150 ml., is then added. The mixture is shaken 2 or 3 hours and centrifuged. The supernatant fluid, which contains the desired proteins, may be preserved with toluene for several days in the ice-box.

Table III shows the effect of various activators on inactive papain in the presence and absence of fixed SH- and SS-proteins. The experimental details were identical with those described for Table I, except that in the cases indicated 7.0 ml. of the above-described fixed SH- and SS-protein solutions were added with the activator. The enzyme solution must not contain cathepsin. The time of incubation in each case was 20 hours.

It is evident from these results that purified inactive papain, which is not activated by vitamin C, pyruvic acid or succinic acid (or by their iron complexes), is nevertheless activated by these complexes in the presence of fixed SH- and SS-proteins. The magnitude of the activation is of the same order as that obtained with cysteine—Fe". These findings demonstrate not only that the observed papain activation effects are dependent on the presence of accompanying substances, but also that these substances are to be sought for in the

Table III. *The effect of fixed SH- and SS-proteins on the activation phenomena of papain.*

Activator	Activity in ml. 0.05 N KOH	
	Without Fe"	With Fe"
None	0.00	0.00
Cysteine	4.80	5.00
Fixed SH- and SS-proteins	3.10	2.90
HCN	3.10	—
HCN + fixed SH- and SS-proteins	5.40	—
Vitamin C	0.00	0.05
Vitamin + fixed SH- and SS-proteins	3.10	4.90
Pyruvic acid	0.00	0.05
Pyruvic acid + fixed SH- and SS-proteins	3.10	4.20
Succinic acid	0.00	0.00
Succinic acid + fixed SH- and SS-proteins	3.10	4.70
Fixed SH- and SS-proteins + cysteine + papain (without gelatin)	0.08	0.05

class of proteins which contain fixed SS groups. The activator systems (vitamin C—Fe", pyruvic acid—Fe" and succinic acid—Fe"), in the presence of the papain complex, convert these SS-proteins into the SH-form, which is then able to activate the enzyme. It is also evident from Table III that this conversion of SS- into SH-protein (as a step in the activation process) requires the presence of bivalent iron. This reduction of SS to SH can also be followed by means of the nitroprusside reaction, as, for example, in the system HCN + fixed SS-protein. The part played by the bivalent iron in this process is not yet clear. Possibly the relationship is to be sought in an activation, by the iron, of dehydrogenases which are always found in the fixed SH- and SS-protein solutions prepared as described. The activation of papain by the system succinic acid—fixed SH- and SS-protein—Fe" supports this view. It will be of interest to attempt to establish a connection between papain activation and the flavins (vitamin B₂). Our knowledge of this field has been greatly extended by the recent work of György *et al.* [1934] and Wagner-Jauregg *et al.* [1934].

It frequently happens that the protein solutions prepared as previously described contain mostly fixed SH-protein and very little of the SS-form. This can be seen from the high activation of inactive papain which they produce. In these cases also the nitroprusside reaction is not intensified by HCN or other activators. This latter finding in particular is evidence supporting the assumption that the conversion of fixed SS-protein into the SH-form by the various activators (vitamin C, pyruvic acid and succinic acid in presence of bivalent iron) is responsible for the activating effect of the latter. Further evidence for this is presented in the next section of this paper, in which a series of papain activation experiments was carried out with a solution of fixed SS-protein prepared by treatment of the tissue extract with alloxan.

Conversion of fixed SH-protein into the SS-form by means of alloxan.

It has been pointed out [Purr, 1935] that alloxan converts SH groups into SS groups without appreciably affecting the structure of the protein molecule. On the basis of this finding the following method was evolved for converting fixed SH-proteins into the SS-form.

100 ml. of a muscle extract prepared as previously described were treated for 5 hours at 37° with 0.7 g. of alloxan. A trace of toluene was then added and the excess alloxan removed by dialysis through an animal membrane (fish bladder), the desired protein being non-dialysable. At the end of the dialysis

the protein solution was divided into 2 parts, one of which was used immediately in the activation experiments. The other part was centrifuged to remove a precipitate which had formed during the dialysis, and the liquid was dried in a vacuum-desiccator over P_2O_5 . The fine powder thus obtained contains mostly fixed SS-proteins (little SH) and was used directly.

A series of activation studies was carried out on reversibly inactivated papain using the dialysate and the dry powder obtained from alloxan-treated muscle extract, with and without HCN, vitamin C, pyruvic acid and succinic acid. Duplicate experiments were run with the addition of 20 mg. of $FeSO_4 \cdot 7H_2O$.

(a) *Before alloxan treatment.* Control experiments were run with a solution of fixed SH- and SS-proteins before alloxan treatment. These experiments were identical with those recorded in Table III.

(b) *Dialysate after alloxan treatment.* These experiments were identical with those described in (a) except that 10 ml. portions of the alloxan-treated dialysate were used instead of the untreated solution of fixed SH and SS groups.

(c) *Dry powder from alloxan-treated dialysate.* These experiments were identical with those described in (a) except that 60 mg. of the dry powder obtained from the alloxan-treated dialysate were used instead of the untreated solution of fixed SH- and SS-proteins.

Table IV. *Effect of fixed SS-proteins on the activation phenomena of papain.*

Activator	Activity before alloxan treatment		Activity after alloxan treatment			
			Dialysate		Dry powder	
	Without Fe^{++}	With Fe^{++}	Without Fe^{++}	With Fe^{++}	Without Fe^{++}	With Fe^{++}
None	0.00	0.00	0.00	0.00	0.00	0.00
Cysteine	4.90	5.00	4.80	5.00	4.90	5.00
Fixed SH- and SS-proteins	3.80	3.80	2.50	2.50	0.70	0.80
HCN	3.10	—	3.10	—	3.10	—
HCN + fixed SH- and SS-proteins	5.30	—	4.70	—	—	—
Vitamin C	0.00	0.05	0.00	0.00	0.00	0.00
Vitamin C + fixed SH- and SS-proteins	3.80	4.30	2.40	4.80	0.70	2.90
Pyruvic acid	0.00	0.05	0.00	0.00	—	—
Pyruvic acid + fixed SH- and SS-proteins	3.70	4.20	2.50	2.50	0.70	0.70
Succinic acid	0.00	0.00	—	—	—	—
Succinic acid + fixed SH- and SS-proteins	3.65	4.10	2.40	2.30	0.65	0.50

The results, shown in Table IV, indicate that it is possible to convert fixed SH-protein into the SS-form. It is also clear that the activation of inactive papain, obtained with fixed SS-proteins in the presence of the vitamin C— Fe^{++} complex, is an indirect effect resulting from the formation of fixed SH groups. The vitamin C— Fe^{++} complex is able, in the presence of the papain complex, to convert fixed SS-protein into the corresponding SH-form. This follows from the fact that addition of vitamin C— Fe^{++} scarcely affects the papain activity obtained with a fresh, untreated solution of fixed SH- and SS-protein (containing much SH-protein and very little of the SS-form). The observed activation is due merely to SH groups present in the protein solution, since approximately the same degree of activation results in the absence of vitamin C— Fe^{++} . When however a mixture of fixed SH- and SS-proteins (alloxan treatment) is used, the partial papain activation can be increased to full activation by addition of vitamin C— Fe^{++} . This is true also when the latter is replaced by HCN. Full papain activation is obtained also with pyruvic acid— Fe^{++} and succinic acid— Fe^{++} , provided the fresh (untreated) SH- and SS-protein solution is also added. However,

for unknown reasons these two complexes lose their activating effects when alloxan-treated preparations of SH- and SS-proteins are used.

Distribution of fixed SH- and SS-proteins.

Fixed SH- and SS-proteins, which, on the basis of the above results, play a part in the activation of papain by vitamin C—Fe²⁺, are widely distributed in plants and animal organs. As examples of animal tissue, ox muscle, rabbit muscle, rabbit liver and rat sarcoma (Philadelphia #1) were extracted by the previously described method for obtaining solutions of these proteins. The activating effects of these solutions (7 ml.) on inactive papain were then measured in the presence of iron or vitamin C—Fe²⁺, the experimental details being identical with those previously described. As examples of the plant kingdom, barley meal and jack bean meal were studied. The quantities used were 100 mg. of meal, 0.2 ml. of inactive papain, 20 mg. of FeSO₄·7H₂O (in 1 ml. of water) and 7 mg. of vitamin C (in 1 ml. of water). The total volume was brought to 7 ml., and the mixture was then incubated for 30 minutes at 37° and *p*_H 7. The measurement of papain activity followed as usual. Similar experiments were carried out without the addition of vitamin.

Table V. *Distribution of fixed SH- and SS-proteins in plant and animal cells, and their relation to the vitamin C activation of papain.*

Activator	Activity without vitamin C (Fe ²⁺ present)	Activity with vitamin C (Fe ²⁺ present)	Time of incubation (hours)
None	0.00	0.00	20
Cysteine	5.00	—	20
Ox muscle	3.70	5.0	20
Rabbit muscle	3.10	4.80	20
Rabbit liver	3.80	4.90	20
Rat sarcoma	3.50	5.0	20
Barley meal	0.70	2.50	20
Barley meal and cysteine	5.00	—	20
Jack bean meal	1.00	2.10	20

Table V shows that fixed SH- and SS-proteins probably occur in most plant and animal cells. This is evident from the papain activations obtained in the presence or absence of vitamin C. As previously stated, the activation obtained with the protein solutions in the absence of vitamin is due only to the fixed SH-protein present. The increased activation which results on addition of vitamin C—Fe²⁺ is due to the conversion of the SS-proteins into the SH-form by the vitamin C—Fe²⁺ in the presence of the papain complex. HCN has a similar effect.

Experiments with SS-glutathione.

Hopkins and Dixon [1922] and Bernheim and Dixon [1928] found that fixed SH-proteins (bound to the cell residue) would reduce oxidised glutathione to the SH-form. It was of interest, therefore, to investigate the effects of SS-glutathione, fixed SH- and SS-protein suspension and vitamin C on papain activation.

A solution of oxidised glutathione was prepared by passing oxygen through a solution of 100 mg. of reduced glutathione in 10 ml. of water, in the presence of a trace of FeSO₄·7H₂O. Twelve hours' oxygenation were usually sufficient to complete the reaction, the course of which was followed by means of the nitroprusside test, and by the iodimetric procedure of Woodward and Fry [1932].

To 10 mg. of oxidised glutathione in 1 ml. water were added 20 mg. of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (in 1 ml. of H_2O) and 6 ml. of a 1:5 aqueous suspension of ox muscle, which had previously been minced and thoroughly washed with water. The mixture was incubated at p_{H} 7 and 37° for 1 hour, after which 0.2 ml. of inactive papain extract was added. The incubation was continued for an additional hour. The papain activity was then measured as previously described. Control experiments were carried out in a similar manner on oxidised glutathione alone and muscle suspension alone. In a second series, 10 mg. of oxidised glutathione were treated with 20 mg. of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 7 mg. of vitamin C and 2 ml. of inactive papain, in a total volume of 6 ml. After 1 hour's incubation at p_{H} 7 and 37° , the papain activity was measured in the usual manner. A control was run without oxidised glutathione. The results are shown in Table VI. Time of incubation in all cases was 20 hours.

Table VI. *Relation of fixed SH-proteins and the vitamin C—Fe" complex to glutathione metabolism.*

Activator	Activity (ml. 0.05N KOH)	
	Without Fe"	With Fe"
None	0.00	0.00
Cysteine	4.9	4.30
Oxidised glutathione	0.50	0.30
Ox muscle	3.5	3.40
Oxidised glutathione and ox muscle	4.6	4.90
Vitamin C	0.00	0.05
Vitamin C and oxidised glutathione	0.60	1.50

The reduction of SS-glutathione to the SH-form by fixed SH groups, which was first followed analytically by Hopkins [1926], has now been demonstrated enzymically by means of papain activation. Such a reduction process is probably related to intracellular proteolysis. A similar reduction of oxidised glutathione (or oxidised papain?) is obtained when oxidised glutathione and vitamin C—Fe" are added to inactive papain. Since neither oxidised glutathione—Fe" nor vitamin C—Fe" is able to activate inactive papain, it appears likely, as in the case of fixed SS-proteins, that the vitamin C—Fe" complex and the papain are jointly responsible for the reduction of the SS group. In the absence of papain no reduction can be observed. The activation of papain by vitamin C—Fe" apparently depends on the presence of SS groups, either in the glutathione molecule or in fixed SS-protein.

Activation of cathepsin.

In these experiments, an inactive cathepsin preparation was used, which was made by acetone-ether extraction of minced rabbit liver [Purr, 1934]. The dry powder thus obtained was extracted for 12 hours with 10 parts of 90 % glycerol and then filtered. 5 ml. of the filtrate were kept for 30 minutes at p_{H} 7 and 30° with 6 ml. of a solution of fixed SH- and SS-proteins (prepared from ox muscle as previously described), 20 mg. of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ in 1 ml. of water and 7 mg. of vitamin C in 1 ml. of water. After incubation the enzymic activity was measured essentially as described for papain. 5 ml. of 8 % gelatin and 2 ml. of *M* sodium acetate buffer of p_{H} 4 were added. 10 ml. of the reaction mixture were analysed immediately for amino-nitrogen, and 10 ml. after 24 hours' incubation at 37° by the Van Slyke method, the results being expressed in ml. of 0.05N KOH. Controls were run in the absence of (a) vitamin C, (b) fixed SH- and SS-protein and (c) fixed SH- and SS-protein and vitamin C. A similar reaction was carried out using 20 mg. of pyruvic acid in place of the vitamin C.

Also the full activity was determined by keeping 5 ml. of cathepsin solution for $\frac{1}{2}$ hour at p_H 7 and 30° with 20 mg. $FeSO_4 \cdot 7H_2O$ in 1 ml. of H_2O and 20 mg. of cysteine hydrochloride in 1 ml. of H_2O . The activity of the enzyme was measured in the usual way; time of action 24 hours.

Table VII. *The effect of fixed SH- and SS-proteins on the activation phenomena of cathepsin.*

Activators	Activity (ml. 0.05 N KOH) (with Fe^{++})
None	0.00
Cysteine	1.40
Vitamin C	0.6
Fixed SH- and SS-proteins	0.40
Vitamin C and fixed SH- and SS-proteins	1.50
Pyruvic acid	0.00
Pyruvic acid and fixed SH- and SS-proteins	0.25
Fixed SH- and SS-protein and gelatin (no cathepsin)	0.00
Fixed SH- and SS-protein and cysteine and cathepsin (no gelatin)	0.01

The results shown in Table VII indicate that, as far as they have been studied, the activation phenomena of cathepsin are quite similar to those of papain. Only in the case of pyruvic acid did the results differ. Here it was found that the system fixed SH- and SS-protein—pyruvic acid— Fe^{++} does not increase catheptic activity.

SUMMARY.

1. It has been shown that the activation of papain by the vitamin C— Fe^{++} complex is dependent upon the presence of contaminating substances. Among the latter were found fixed SS-proteins and SS-glutathione.

2. Evidence is produced to show that the activation of papain and cathepsin by the vitamin C— Fe^{++} complex or by HCN is an indirect effect. The real activators are sulphydryl compounds, which are formed from the SS compounds which accompany the enzyme complex (fixed SS-proteins and SS-glutathione), by the action of the activator system mentioned. In contrast to arginase activation, the activation of papain and cathepsin is probably produced only by sulphydryl compounds.

3. It is concluded that papain and cathepsin in the active state are fixed SH-proteins of definite constitution, while the reversibly inactivated forms of these enzymes appear to be SS-proteins.

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