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On the Lachrymatory Factor in Onion (*Allium cepa*) Vapours and Its Precursor

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The isolation of the precursor of the lachrymatory factor in onion is described in detail. The compound was characterized as (+)-S-(prop-1-enyl)-L-cysteine sulphoxide. An enzyme preparation of onion splits it into the lachrymatory factor, pyruvic acid, and ammonia. The lachrymatory factor is very unstable. In mass-spectral studies of the enzymic cleavage of the lachrymatory precursor no higher mass number than 90 could be detected. These studies were in accordance with the idea that the lachrymatory factor is propenylsulphenic acid. It is spontaneously degradated to propionaldehyde from which some 2-methyl-2-pentenal is formed. In mild alkaline solution the precursor of the lachrymatory factor is cyclized to cycloalliin.

In the course of the studies in this laboratory on the sulphur-containing amino acids and their enzymic decomposition products in onion and garlic [1–9] we became interested in the lachrymatory factor (LF) in onion, which was generally assumed to be sulphur-containing. Little work has previously been done on this interesting compound. Kohmann [10] states, however, on somewhat feeble grounds, that the LF is thiopropionaldehyde and that the lachrymatory effect is not due to volatility, but depends on the formation of tiny hovering droplets which squirt out when the onion is cut. Niegisch and Stahl [11] have analyzed the volatile components in onion vapours by mass-spectrometry and deny the identity of the LF with thiopropionaldehyde. They give a variety of eight compounds as tentative lachrymators, the most probable — in their opinion — being 3-hydroxythiopropionaldehyde. Carson and Wong [12] have isolated and identified a great many constituents of onion flavour, none of which has any pronounced lachrymatory effects, however.

After some preliminary experiments it became evident that the LF would be extremely difficult to isolate and characterize as such owing to its instability, probable reactivity and to the fact that it was present in onion vapours in minor amounts only together with all the other volatile constituents. Therefore it was assumed as a working hypothesis that the LF was enzymically formed from a non-volatile and essentially stable precursor when onion was cut or crushed. Only when this compound were isolated in pure form, would

it seem possible to understand the enzymic formation of the LF and characterize it. Work was thus concentrated on the isolation and identification of the precursor. The results of this investigation have already been presented in a short communication [13].

In order to follow the isolation of the precursor, a test system for proving its presence was needed. As the only known property of the precursor was its ability to form the LF, the test system had to be based on this fact. An enzymic preparation which causes the formation of the LF was thus first needed. A crude enzymic preparation was made in the following way. Some onions were homogenized with distilled water and the resulting sludge left standing for two hours with occasional stirring in order to let the enzymic reaction proceed "to the end". In fact, even after about half an hour no lachrymatory effect could be perceived any longer. The sludge was then lyophilized, giving a precursor-free, enzyme-containing powder which was used in the subsequent tests.

To the test system also belonged the determination of the lachrymatory effect. This was performed in the following way. 4—6 drops of the aqueous solution to be tested was placed in a small weighing vial and the pH was adjusted to 6—7. A small amount of the enzyme powder — usually about 5 mg — was added to the solution, the suspension was stirred for 10 sec and the vial held tightly, but without pressure to the eye. Depending on the concentration, the lachrymating effect was perceived after 15—45 sec, usually becoming unendurable after an additional 5 sec. If the lag time exceeded 1 min, the test was described as negative, because the effect that sometimes appeared after that time was always so weak that it could easily be confused with ordinary tiring of the eye.

That the enzyme preparation really contains active enzyme and that the LF is enzymically formed from a precursor was conclusively proved by the following experiments. When water is added to lyophilized powder from relatively undamaged onion peelings, a strong lachrymatory effect is produced, showing that this powder contains both the precursor and the enzyme. If, however, this powder is treated with methanol at 60°C for 1 h to inactivate the enzyme and subsequently taken to complete dryness, it gives no reaction upon addition of water. When, on the other hand, this inactivated powder is mixed with the active enzyme preparation described above, and the mixture is then moistened, a strongly positive reaction ensues. In our opinion the appearance of the LF under the conditions described also disproves the "squirting theory" of Kohmann mentioned earlier.

The isolation of the lachrymatory precursor (LP). Using the lachrymating test described, the LP has been isolated in pure, crystalline form. On the basis of elementary analysis, enzymic cleavage, hydrolysis, and IR-spectroscopy (Fig. 1) it was deduced to be S-(prop-1-enyl)-cysteine sulphoxide. One of the steps in the isolation consists of absorption on cation exchanger and subsequent elution with ammonia. Although the ammonia (generally a mild alkaline reaction) brings about some conversion of the lachrymatory precursor into cycloalliin, a compound isolated from onion and characterized by Virtanen and Matikkala [4, 14], the purification in this step is so considerable that it is retained in the isolation scheme. This conversion into cycloalliin, which alliin is not

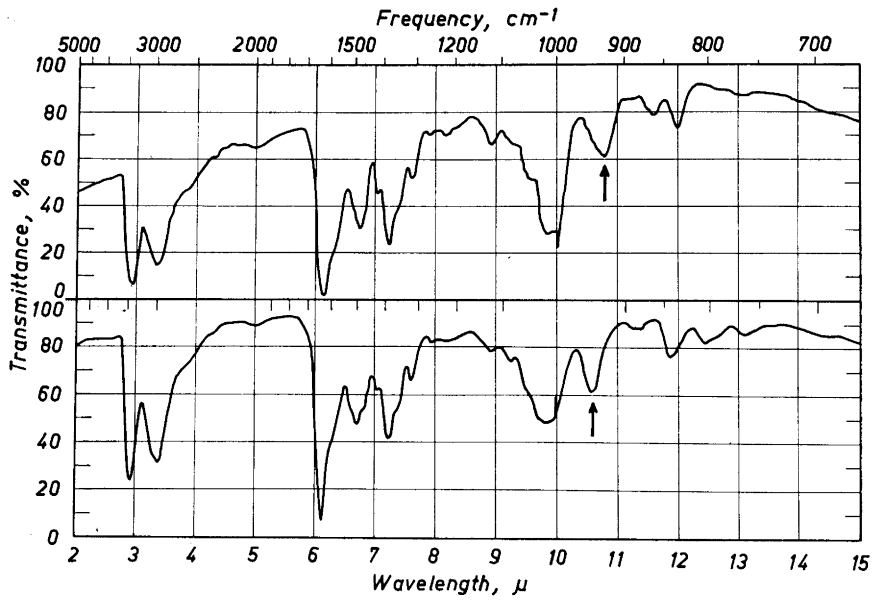


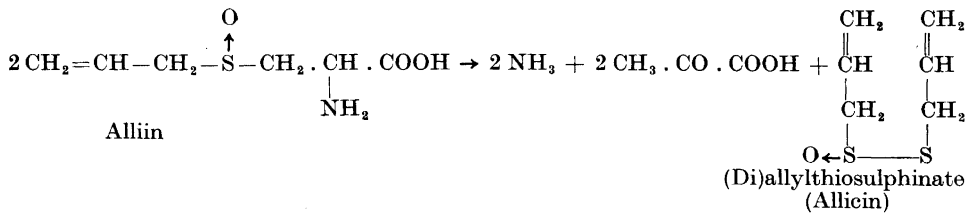
Fig. 1. Above: IR-spectrum of alliin. Below: IR-spectrum of the LP. Note the difference due to the position of the double bond in the molecules (marked by arrows).

subjected to, is also additional proof of the structure. The cyclisation of the LP is slight if the elution with ammonia and the evaporation is performed rapidly at lower temperature. Without these precautions, most of the LP is converted into cycloalliin.

The LP is stable, however, in neutral and 2 N acetic acid solution. Acid hydrolysis splits it into cysteine, propionaldehyde, and probably elementary sulphur. On paper chromatograms developed in butanol-acetic acid the LP travels as alliin and gives the same brownish colour with ninhydrin. Alliin gives, however, no LF with the enzyme preparation, nor does it change into cycloalliin. The optical rotation of the crystalline LP was $[\alpha]_D^{25}$ = about + 74° (because of the slight turbidity of the solution, the exact estimation of the rotation was difficult). The LP is thus (+)-S-(prop-1-enyl)-L-cysteine-S-oxide. IR-spectra of alliin and the LP are presented in Fig. 1.

Attempts to synthesize the LP have so far been unsuccessful. The coupling of 1-Br-prop-1-ene to cysteine and the subsequent sulphoxidation seems an obvious route. Although the coupling has been tried in a variety of solvents and temperatures, it could not be realized, probably depending on the low reactivity of a bromine atom located on a doubly bonded carbon atom. The coupling of 1,1-dibromo-propane to cysteine and the subsequent formation of the double bond in the product by removal of hydrobromic acid was another possibility tried in vain.

The enzymic cleavage of the LP. According to Stoll and Seebeck [15] the enzymic splitting of alliin proceeds in the following way:



These authors believed that allylsulfenic acid was formed as the primary product in the reaction and that allylthiosulfinate (allicin) was formed spontaneously from two molecules of allylsulfenic acid. Because of the striking similarity in structure between alliin and the LP it was thought possible that the enzymic cleavage proceeds analogously. In an experiment on the enzymic degradation of the LP (see below), the recovery of ammonia was theoretical and that of pyruvic acid almost so. Among the reaction products there also was some propionaldehyde (or a compound giving the same dinitrophenylhydrazone) but no amino acids or other ninhydrin-positive compounds. On the basis of these results it seemed clear that the enzymic cleavage of the alanine part of the LP leads to pyruvic acid and ammonia. The fate of the sulphur-containing part of the molecule seemed to differ from alliin, however. In fact, the data indicate that in the case of the LP no allicin-like "double molecule" is formed (see below).

Mass-spectral proofs of the constitution of the LF. Because of the instability of the LF, demonstrated in preliminary experiments, no attempts to trap and isolate substantial amounts of it could be made. The proof of the structure of the LF is therefore largely mass-spectrometric, even if the enzymic splitting of the LP with the subsequent spontaneous conversion of the LF into propionaldehyde already led to a probable formula [17]. Using pure LP and an enzyme preparation which through gelfiltration on Sephadex and lyophilisation was

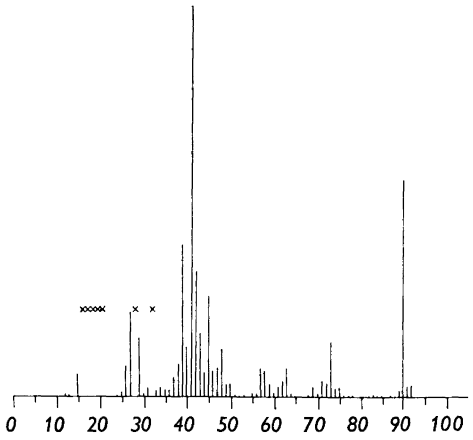


Fig. 2. Mass-spectrum of the enzymic splitting of (+)-S-(prop-1-enyl)-L-cysteine sulphoxide in H_2O. Reaction time 1 min.

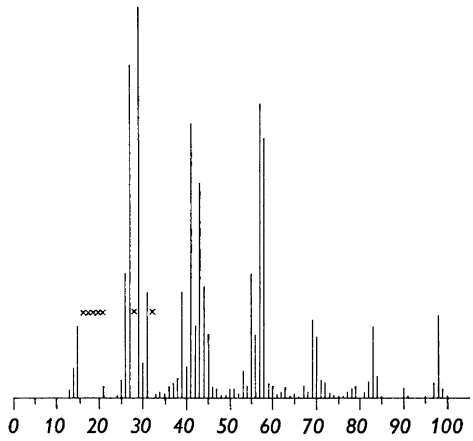


Fig. 3. Mass-spectrum of the enzymic splitting of (+)-S-(prop-1-enyl)-L-cysteine sulphoxide in H_2O. Reaction time 2 h.

purified from lowmolecular substances and accordingly was mass-spectrometrically "empty", mass-spectral studies were performed [16]. The mass-spectra of the volatile substances formed when the LP was mixed with purified enzyme and water or deuterium oxide are shown in Figs. 2, 3, and 4. In these figures the background is subtracted and those peaks omitted which could not be measured due to the jamming effect of the considerable excess of water always present in these experiments.

The fact that no peak of greater mass than 90 — the low peaks 91 and 92 represent the same molecule with heavier isotopes — has been detected and that this group with a constant ratio between peaks 90, 91, and 92 is the last

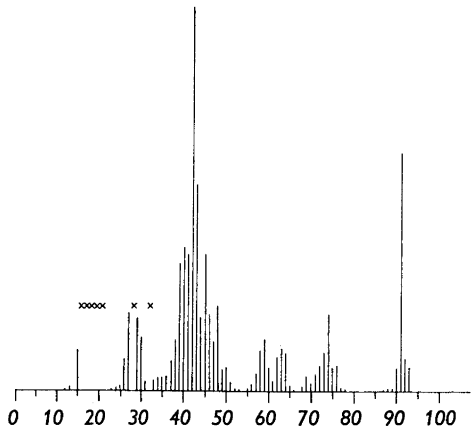


Fig. 4. Mass-spectrum of the enzymic splitting of (+)-S-(prop-1-enyl)-L-cysteine sulphoxide in D_2O. Reaction time 1 min.

to disappear when the ionisation energy is decreased, indicates a molecular weight of 90 for the compound. Niegisch and Stahl [11] also found this peak in their studies on flavour substances in onion. From the proportions between the peaks of the 90 group it is also concluded that the molecule contains one sulphur atom. The fact that the molecular weight is an even number indicates that no nitrogen is present. Several of the peaks would be difficult to explain if the molecule did not contain oxygen. Thus the formula C_3H_6OS was the most probable for the LF [16], in agreement with the formula $CH_3-CH=CH-SHO$ which is also suggested by what is "left over" from the enzymic cleavage [17].

The mass-spectra also give additional proofs for this structure. The peak 15 indicates the presence of a methyl group. The peak 73 (= 90 minus a hydroxyl group) increases to 74 in deuterium oxide, indicating that in both instances the fragment is OH and not OD. This shift is particularly evident when low ionisation energies are used. From this it can be concluded that no preformed OH exists in the molecule, because a real hydroxyl group would exchange its hydrogen for deuterium in deuterium oxide.

The formation of the fragment of mass 73 is rather to be compared with the formation of the analog fragment of mass 61 — molecular weight 78 minus 17 — occurring in the mass-spectra of dimethyl sulphoxide. The shift of the molecular peak to 91 indicates the presence of one exchangeable hydrogen atom, thus corroborating the suggested structure.

On the basis of the enzymic cleavage and especially of the mass-spectral findings, the LF is probably propenylsulphenic acid. No other aliphatic sulphenic acid has as yet been known.

The fate of the LF. As mentioned earlier, the enzymic cleavage also leads to the formation of some propionaldehyde. This is also evident from the mass-spectra where after longer reaction times peaks attributable to propionaldehyde emerge while the peaks of propenylsulphenic acid decrease. This phenomenon indicates that the LF or propenylsulphenic acid with the loss of sulphur spontaneously changes into propionaldehyde. The following mechanism is tentatively suggested for the reaction:

$$CH_3-CH=CH-\overset{\uparrow O}{S}H + H_2O \rightarrow CH_3-CH=CHOH + [H\overset{\uparrow O}{S}H]$$

$$CH_3-CH=CHOH \xrightarrow{\text{spont.}} CH_3 \cdot CH_2 \cdot CHO$$

$$[H\overset{\uparrow O}{S}H] \xrightarrow{\text{spont.}} H_2O + S$$

As hydrogen sulphoxide has never been isolated, its existence is of course only hypothetical. Propenyl alcohol rearranges spontaneously into propionaldehyde. Part of the propionaldehyde is further condensed into 2-methyl-2-pentenal, a compound shown in this laboratory to be present in onion vapours [18]. The slow emerging of a peak with mass number 98 in the mass-spectra of the volatiles from the enzymic cleavage after considerable reaction times supports this concept.

DISCUSSION

According to the results presented above, there is a considerable difference between sulphur-containing compounds formed during the enzymic cleavage of alliin and of the LP. The former gives rise to a double molecule, diallylthiosulphinate (allicin), but no peak of mass number 90 has been detected in mass-spectra of crushed garlic, nor of alliin plus alliinase, indicating that the supposed intermediary "single molecule", if at all existing, must be very short-lived. The question of how allylthiosulphinate is formed is therefore open. If, after all, allylsulphenic acid should be the first splitting product, it must immediately be converted into a compound which is less volatile and therefore not detectable in mass-spectral studies in water solution. The suggestion [18] that it would proportionate to allylsulphinic acid and allylmercaptan from which by elimination of water diallylthiosulphinate might be formed, is possible but not demonstrated.

The LP on the other hand gives rise to propenylsulphenic acid, the LF. No peak with higher mass number than 90 could be detected in the mass-spectra (with the exception of mass number 98 which appears only after longer reaction times and which can be accounted for as a condensation product of

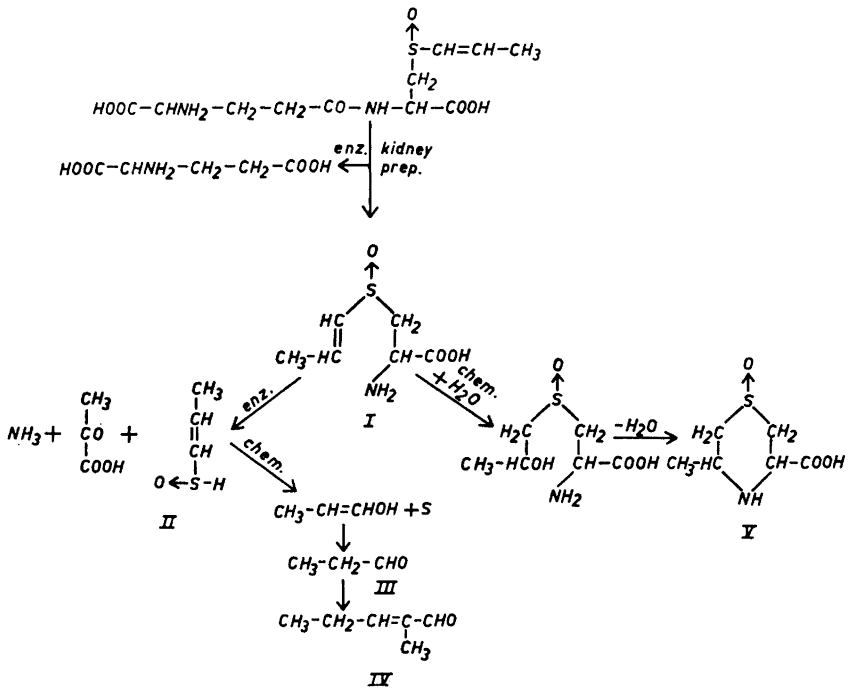


Fig. 5. Enzymic splitting of γ-L-glutamyl-S-(prop-1-enyl)-L-cysteine sulphoxide and the compounds formed enzymically and chemically from (+)-S-(prop-1-enyl)-L-cysteine sulphoxide I. II propenylsulphenic acid, III propionaldehyde, IV 2-methyl-2-pentenal, V cycloalliin.

propionaldehyde, 2-methyl-2-pentenal). This is of course only to be expected as the volatility of the possible allicin-analogon (mass-number 162) surely would be too low to give a peak in the presence of considerable amounts of water. For this reason the enzymic cleavage of the LP was repeated in the presence of hydroquinone and of ascorbic acid, which would be expected to reduce the thiosulphinic acid to the corresponding disulphide, the volatility of which should be sufficient for the mass-spectral measurements. In neither instance, however, could a peak above mass number 90 be detected. From these facts it was concluded that no dipropenylthiosulphinic acid is formed in the enzymic cleavage of the LP.

The various enzymic and nonenzymic reactions discussed above are summarized in Fig. 5. The formation of the LP by enzymic hydrolysis of γ-glutamyl-S-(prop-1-enyl)-cysteine sulphoxide, isolated from onions by Virtanen and Matikkala [14, 19, 20] is also included in the figure.

Some work has also been performed in order to purify the LP-splitting enzyme. Fractionation with ammonium sulphate, dialysis, treatment with charcoal, calcium phosphate gel *etc.*, all lead to some purification but gel filtration on Sephadex seems to give the best results. When the effluent from the Sephadex column was collected in fractions and the enzymic activity determined by mass-spectrometry there seemed to be two peaks with similar enzymic activity, indicating the presence of two separate enzymes with considerably differing molecular weights.

Another indication of the presence of two LP-splitting enzymes in onion is the behaviour of the enzyme(s) upon pH fractionation. When the pH of enzyme extracts is brought to 4.5, a precipitate is formed. This precipitate shows enzymic activity, but so does the supernatant too. That an incomplete precipitation cannot be in question is shown by the fact that no more precipitate is formed from the supernatant even when the pH is lowered to 2.5, an acidity in which the enzyme is destroyed.

The question whether there really are two distinct LP-splitting enzymes, is, however, in the light of repeated gelfiltration experiments, rather confused. In some cases only one peak was obtained, and complete separation was never achieved even on very long columns. This inseparability might indicate that there are not two distinct enzymes but rather a more or less continuous system of enzymes with different molecular weights, with maxima around relatively high or low molecular weights, respectively. If this is so, the question arises whether such a complex enzyme system really exists *in vivo*. It is of course entirely possible that the enzyme disintegrates partly during isolation, but that some of the fragments retain activity all the same. Further work elucidating the true nature of the enzyme(s) is in progress.

Part of this work has been published earlier [13, 16, 17, 19].

EXPERIMENTAL

Isolation of the LP. Whole onions were freezed to −80°C with solid carbon dioxide, crushed with a hammer, and homogenized in 70 % ethanol at −80°C in order to inactivate the enzymes. The suspension was filtered through cotton and the extraction repeated once with 70 % ethanol at room temperature. The pooled clear extracts were filtered through an Amberlite IR-120-H+ column (300 ml resin/kg fresh weight). The amino

acids were eluted with 1 N ammonia and the effluent continuously and as rapidly as possible taken to dryness *in vacuo* with the exclusion of oxygen in order to minimize the conversion of the LP to cycloalliin at an alkaline reaction and the oxidation of the LP. The residue was dissolved in 0.5 N acetic acid and chromatographed with the same solvent on a Dowex 1 × 8 column in acetate form. The fractions containing the neutral amino acids were taken to dryness *in vacuo* with the exclusion of oxygen and dissolved in a 0.5 N pyridine buffer adjusted to pH 2.7 with formic acid and fractionated on a Dowex 50 × 8 column, equilibrated and eluted with the same buffer. The fractionation was followed by paper chromatography of the fractions using butanol-acetic acid-water (63:10:27, v/v/v) as solvents. The compounds: LP, cycloalliin, and S-methylcysteine sulphoxide — in the order mentioned — were the first major amino acids to emerge from the column. The LP-containing fractions were taken to dryness as before and separated from contaminants, chiefly cycloalliin, on a cellulose column with the butanol-acetic acid solvent. The proper fractions were taken to dryness and the LP precipitated twice from water-acetone and once from water-alcohol. In this way 129 mg of pure material, in addition to approximately 200 mg of less pure (evaporated motherliquors, *etc.*), was obtained from 5 kg of fresh onions. The pure compound consisted of white crystals, decomposing at 146—148°C. Elementary analysis of a LP preparation isolated in this laboratory by Matikkala [20] gave: C 40.56; H 6.40; N 7.71; S 18.01; O 27.21. Calc. for $C_6H_{11}NSO_3$: C 40.66; H 6.26; N 7.90; S 18.09; O 27.08.

The enzymic cleavage of the LP. (a) Pyruvic acid. 2 mg of the LP, 20 mg of the enzyme, and 10 ml of water were stirred for 2 h and centrifuged. 3 ml of a dinitrophenylhydrazine solution (approx. 50 % excess) was added to 5 ml of the supernatant. The mixture was heated to 80°C and left to cool overnight. In order to remove excess dinitrophenylhydrazine, the mixture was evaporated *in vacuo* and dissolved in 5 ml of water. A paper chromatogram shows that the dinitrophenylhydrazones of pyruvic acid and propionaldehyde are present. The amount of pyruvic acid dinitrophenylhydrazone (roughly estimated from the spot size compared with a series of authentic spots run on the same paper) is somewhat smaller than the theoretical, probably due to losses in handling. Pyruvic acid, however, obviously is a main reaction product.

(*b*) Amino acids. The rest of the above supernatant was used for paper chromatographic demonstration of amino acids, of which there were none, however.

(*c*) Ammonia. 2 mg of the LP, 20 mg of the enzyme, and 10 ml of water was magnetically stirred in a closed distillation apparatus. The tip of the apparatus extended below the surface in the receiving flask, which contained 30 ml of 0.01 N sulphuric acid. After 1 h, 10 drops of 6 N sodium hydroxide were added and about 5 ml distilled over. The amount of ammonia in the distillate was determined by the Nessler method, giving 0.198 mg. A blank made with the enzyme, but omitting the LP, gave 0.012 mg. The enzymically formed ammonia is thus 0.186 mg. As the theoretical amount is 0.192 mg, it is evident that the LP splits off one molecule of ammonia.

The acid hydrolysis of the LP. 11 mg of the LP was dissolved in 100 ml 4 N hydrochloric acid, and the solution was distilled into a solution of dinitrophenylhydrazone in 2 N hydrochloric acid until about 2/3 had distilled over. The distillate became opaque (elementary sulphur?), and a precipitate formed. The distillate was extracted with chloroform, and the extract was washed with hydrochloric acid and water, and taken to dryness *in vacuo*. The residue was inseparable from authentic propionaldehyde dinitrophenylhydrazone when chromatographed on paper impregnated with dimethylformamide with the solvent cyclohexane saturated with dimethylformamide. The distillation residue was taken to dryness *in vacuo* a few times in order to remove the hydrochloric acid. It contained cysteine, identified through its chromatographic behaviour and through chromatographic inseparability of its oxidation product from authentic cysteic acid.

The instability of the LF. 1 mg of the LP, 10 mg of the enzyme, and 0.5 ml of water was put into each of two identical weighing vials. The first was immediately tightly closed while the second was kept open. After 3 h, the lachrymatory effect could still barely be recognized in the open vial, but after standing overnight it had completely vanished. Then the closed vial was opened, but no lachrymatory effect could be felt. This is taken as a proof of the instability of the LF.

Preparation of the mass-spectra. The mass-spectra were taken with a modified model 21—401 mass-spectrometer of the California Engineering Corporation. The LP (usually 1 mg), the enzyme (usually 4 mg), and prefarably deaerated water (usually 4 drops) were

put into a small "finger" and immediately stoppered and frozen at −80°C. At this temperature the finger was attached to the main vacuum of the mass-spectrometer and vacuum pumped for 3 min. The vacuum valve was then closed and the finger warmed to 30°C for 3 min. Then the valve to the analyzator was opened, with the pressure-guarding device disconnected, so that a relatively large sample of the volatiles entered into the mass-spectrometer.

Gel-filtrations of the enzyme. The enzyme was usually extracted in the cold with pH 7.4 Sörensen buffer, and the extract was cleared from slimy debris with pH 7.4 calcium phosphate gel [21] (1 mg/3 ml soln.). This clear solution was subsequently fractionated on columns of Sephadex G−50 medium, equilibrated and eluted with the same buffer.

pH-Fractionation of the enzyme. An onion was homogenized and extracted three times at 2−4°C with pH 7.4 Sörensen buffer. As all the extracts showed enzymic activity, they were pooled. Calcium phosphate gel was added, the solution was stirred for 1 h and centrifuged. 2 N sulphuric acid was added to the clear solution until the pH reached 4.5, the solution was stirred for 20 min and cold-centrifuged. The precipitate showed enzymic activity when dissolved in pH 7.4 buffer. A sample of the clear supernatant from the centrifugation also showed enzymic activity when the pH was brought back to 7.4. More sulphuric acid was added to the rest of the supernatant until the pH reached 2.5. Although it was stirred for 1 h, no precipitate formed. This solution showed no enzymic activity when the pH was brought back to 7.4.

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