is completely hydrolyzed *in vivo** (Bray *et al.* 1946), but only to an extent of 10 % by liver extracts. This apparent discrepancy may not be as great as it appears at first, since under the dynamic conditions prevailing in the living animal products of a reaction of this type are continuously removed, so that it can proceed further towards completion than under static conditions such as those used in *in vitro* experiments. Thus it is possible that amides which appear to be hydrolyzed with difficulty *in vitro* could be completely hydrolyzed *in vivo*. Phenylacetamide is excreted (as phenaceturic acid) much more slowly than benzamide or benzoic acid (as hippuric acid). Since, however, phenylacetic acid is eliminated as slowly as phenylacetamide, it would seem that it is the conjugation with glycine which is delayed rather than the hydrolysis of the amide.

The fact that the liver extracts used in this study were active in hydrolyzing asparagine and glutamine suggests that it might be profitable to investigate the possible identity of the liver enzyme(s) with asparaginase or glutaminase.

* These findings have been confirmed during the present investigation.

SUMMARY

1. The hydrolysis of 18 amides by rabbit-liver extracts has been studied and the results compared with some obtained on the rabbit *in vivo*.

2. A suitable pH for the reaction was 7·4.

3. Rabbit-kidney extracts behave in a qualitatively similar manner, but are less active.

4. The action of papain, pepsin and trypsin upon some of the amides is recorded.

5. There is considerable difference between the stability of the amides to hydrolysis by HCl or NaOH and by liver extracts.

We are indebted to the Royal Society for a Government Grant which defrayed part of the cost of this work. The results for the percentage hydrolysis of amides by acid and alkali were obtained by Mr P. B. Wood, whom we wish to thank.

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Leaf Protease of Tobacco and other Plants

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The work described in this paper was undertaken to obtain information on the properties and activities of protein- and peptide-splitting enzymes of the green leaves of tobacco and other plants. These enzymes will be referred to as proteases.

Greenberg & Winnick (1945) list eleven plant proteases that have been obtained in a relatively high state of purity. All, with the exception of bromelin, pinguinain and solanain, are obtained from latex. These three are obtained from fruits, although bromelin also occurs in the green leaves of the pine-apple. Other plant proteases which have been studied in detail are those of seeds, both dormant and germinating. Reports on proteases in the green leaves of plants that do not produce latex are few, and frequently amount to little more than an assertion of their presence, based in much of the early work on evidence that seems open to question.

The presence of proteases in the green leaf of tobacco has been noted by Loew (1900) (action on egg albumin; none detected on casein, fibrin or gelatin); Oosthuizen & Shedd (1913) (possibly present in cured leaves); Fodor & Reifenberg (1925, 1926) (present in green and dried leaves, peptone substrate); Silberschmidt (1934) (stem sap of *Nicotiana-Datura* grafts, peptone substrate); and by Nito & Kitamura (1936) ('trypsin' and 'erepsin' in fresh leaves, 'trypsin' lost on drying). Much of the diffi-

culty in working on the protease of green leaves is due to its low concentration—the activity/g. protein N of tobacco sap is seldom as high as 1% of that of a commercial papain preparation.

MATERIALS AND METHODS

Leaves of *Nicotiana tabacum* var. White Burley from glasshouse plants were used for most of the work, but leaves of other plants listed later were also used. The leaves were minced twice in a domestic meat mincer and the sap squeezed by hand through madapollam. The crude sap was freed from much cell debris and chloroplast material by centrifugation for 15 min. at 3000 r.p.m. (*c.* 1500 *g*); the supernatant fluid is subsequently referred to as 'sap'. The fibrous residue remaining after the expression of the crude sap was washed twice with a volume of distilled water approximately equal to that of the crude sap, and squeezed dry by hand. The residue is referred to as 'fibre'.

Measurement of protease activity

Changes in the viscosity of gelatin due to the action of protease were first used, but were abandoned as the low activity necessitated long periods of measurement during which spontaneous precipitation occurred leading to inaccuracy. The method finally adopted was that of measuring the increase of amino N produced by the incubation at 40° of protease-containing material with 0·8% (w/v) gelatin at pH 5. Michaelis buffer was used because it has a wide useful range and, unlike citrate, does not interfere with the subsequent estimation of amino N. Amino-acid N was determined by a modification of the method of Pope & Stevens (1939) which allows the use of small quantities of test material and eliminates interference by reducing materials.

Specificity of the method of Pope & Stevens (1939)

The method depends on the decomposition of copper phosphate at a controlled pH by amino-acids with the formation of amino-acid-copper complexes. Peptides also behave in the same way and their N is included in the amino N figures obtained. Since the method is here used for the estimation of extent of protein splitting, this lack of specificity is immaterial. Pope & Stevens found that about 80% of the amino N of completely hydrolyzed gelatin was estimated by this method. They used solutions containing from 0·028 to 0·4 mg. amino N/ml. after final dilution with buffered copper phosphate, and found that low results were obtained in the lower part of this range. In this work, final concentrations were only of the order of 0·004–0·024 mg. amino N/ml. and it was found that in this range about 80% of amino N was estimated when added as glycine which is all estimated at higher concentrations. Nevertheless the method is useful at these low concentrations and gives reproducible results.

A number of substances present in plant sap and fibre may be estimated as amino N, but would not interfere in the measurement of protease activity if their concentration remained unchanged during incubation, e.g. sugars, pectin and pectic acid, and organic acids. These are considered below.

Sugars. Sucrose and reducing sugars may account for up to 10% of the leaf dry weight and non-cellulosic polysaccharides (mainly starch) for a further 20–30% (Ward, 1942). Glucose and sucrose were found not to interfere in the concentrations likely to be encountered; further confirmation was obtained by adding salivary amylase to sap containing starch and soluble sugars, and incubating in the usual way with gelatin. At the end of incubation the amylase was still active in degrading added starch, but had produced no apparent increase in the protease activity measured.

Pectic acid. Methanol is present in tobacco sap (Holden, 1945) and its concentration (0·05 mg./ml.) indicates the possibility of the presence of about 0·5 mg./ml. of pectic acid. It was thought that the many free carboxyl groups of pectic acid might interfere. Its formation during incubation would be likely to interfere only with sap protease estimations, since when formed in fibre it appears to remain attached (Holden, 1945). It was found that pectic acid at a conc. of 1 mg./ml. did not increase the blank values.

Organic acids. Of the organic acids present in the leaf that are likely to interfere, oxalic may be excluded since it is not soluble at the pH values used (Vickery & Pucher, 1931). Malic acid does not interfere at the concentrations likely to be encountered, but citric acid does. Since malic acid may be converted to citric acid in water-cultured leaves in the dark (Vickery, Pucher, Wakeman & Leavenworth, 1937) interference from this cause must be considered. Most of the protease estimations were carried out in the presence of a reducing agent which lessens the likelihood of the change from malic to citric acid. It was found that the addition of 1 mg. of malic acid/ml. of sap did not affect the protease value found, and that sap dialyzed against 0·02 M-thioglycollic acid at 5° had lost no activity in spite of the loss of 75% of its initial 'amino N' content, and hence presumably of a major portion of its organic acids.

Amino-acid oxidase. If this were present low results would be obtained owing to destruction of amino-acids. Estimations of ammonia were made on samples incubated in the usual way and for twice the usual time and the difference between initial and final values was no more than 0·01 mg./ml.

Method of estimation

The enzyme solution (0·5 ml.) was pipetted into a 15 ml. tube and 0·4 ml. of distilled water added, followed by 0·1 ml. of a M-solution of activator or inhibitor. A 1% (w/v) gelatin solution (4 ml.) in Michaelis buffer (pH 5·0) at about 30° was then added and the contents of the tube mixed. A 2 ml. sample was then withdrawn into a small beaker, two drops of starch indicator added followed by iodine until the development of a permanent blue colour. The iodine stops the action of the enzyme and removes any reducing substances that would interfere in the subsequent titrations. After 30 min. excess iodine was removed quantitatively with 0·005 N-thiosulphate. From this point the procedure of Pope & Stevens (1939) was followed except that 15 ml. of copper phosphate suspension were used and the samples brought to a final volume of 25 ml. before filtration. Titration was carried out using 0·005 N-thiosulphate and a 1 ml. burette delivering below the surface of the fluid; 5 ml. samples of the filtrate were used with the addition of 0·25 ml. of glacial acetic acid and 0·5 ml. of

saturated KI. With starch indicator, the end point was not very sharp and increased accuracy could be obtained by the electrometric titration of Foulk & Bawden (1926) as modified by Bonner (1946). Gelatin and water blanks were run with each set of estimations, and for absolute values determinations were made on glycine solutions.

Immediately after the initial samples had been taken a drop of chloroform was added to each tube, the tubes stoppered and incubated at 40° for 24 hr. At the end of this time, a second 2 ml. sample was taken and treated in the same manner as the first. From the difference in the values obtained by titration of initial and final samples the number of units/ml. was calculated. If the activity of fibre is to be estimated 1 g. is weighed into the tube and double amounts of water, activators or inhibitors and gelatin solution are added. Samples of 2 ml. are taken and treated like the samples from estimations in liquids.

The unit of activity is defined as that activity which leads to the production of 1 mg. of amino N, as estimated by the method used, on incubation at pH 5 for 24 hr. at 40°. The protease unit is the amount of protease which produces 1 mg. of amino N, as estimated by the method used, on incubation at pH 5 with 0·8% (w/v) gelatin for 24 hr. at 40°.

RESULTS

Preliminary observations on the protease

Protease activity is shown both by sap and fibre of tobacco and other leaves, about 60% of the total activity of healthy tobacco leaves being present in the sap. The activity of both sap and fibre was increased by reducing agents such as sodium thioglycollate, the reagent most frequently used in routine experiments. Activators and inhibitors were used at a final concentration of 0·02M. Sodium sulphide, sodium hyposulphite, potassium cyanide, ascorbic acid, cysteine and glutathione were also effective as activators. Activity was unaffected by manganese, was reduced to about 25% of its thioglycollate value by iodoacetic acid and was abolished by copper. The pH optimum of the protease was found to be *c.* 5 with gelatin as substrate. The enzyme has thus many properties in common with other plant enzymes of the papain type.

Method of partial purification

If tobacco sap is heated to 55° for a few minutes much of the normal plant protein is denatured and can be removed by centrifugation (Bawden & Pirie, 1938). Such heating leads to some loss of total activity, but may increase the purity (i.e. activity/g. protein N) by as much as tenfold. The addition of one twentieth volume of ethanol to heated sap adjusted to pH 4·2 gives a further increase in purity. Table 1 shows the progress of a typical purification. The purification at each step may vary widely, but the overall results are usually similar to the example given. Precipitation of the enzyme has been attempted under many conditions, but does not seem to give as satisfactory results as methods based on the precipitation of impurities. Attempts have been made to prepare fractions of sap protein with enhanced protease content by using $(NH_4)_2SO_4$, ethanol and acetone. These have not been successful, all the fractions prepared having much the same activity. Protease present on fibre is not removed by water, 5% NaCl or glycerol solutions and has not been purified.

Properties of the enzyme

Fig. 1 shows the variation of the activity of the protease with the pH of the medium. Determinations were made by adding buffered 1% gelatin to samples of sap, adding activator and water and determining the pH after the initial and final samples were taken. Michaelis buffer was used.

Variation of activity with substrate concentration was measured in the usual way. Difficulty was encountered in handling samples containing more

Table 1. *Partial purification of tobacco-sap protease*

	Protease (units/ml.)	Protein N (mg./ml.)	Protease (units/g. protein N)	Total protease (units)	Protease units as % of initial value
(*A*) Crude sap	0·39	2·22	176	35·1	100
(*B*) *A* spun*	0·40	1·82	247	34·8	99
(*C*) *B* heated and spun*	0·28	0·82	342	21·8	62
(*D*) *C* spun at pH 4·2*	0·25	0·34	735	18·2	52
(*E*) *D*+5% (w/v) ethanol, spun*	0·21	0·23	910	15·3	44
(*F*) *E* concentrated by ultrafiltration and dialyzed	—	—	—	—	—
(*G*) Fraction of *F* insoluble in Michaelis buffer, pH 5	0·09	0·175	515	2·1	6
(*H*) Fraction of *F* soluble in Michaelis buffer, pH 5	0·33	0·11	3000	7·65	22

* Refers only to the supernatant liquid.

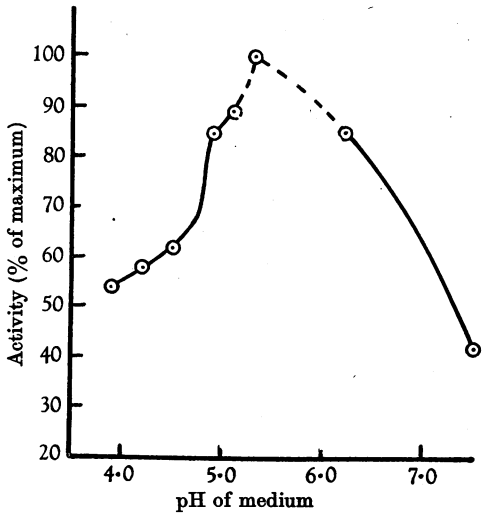

Fig. 1.

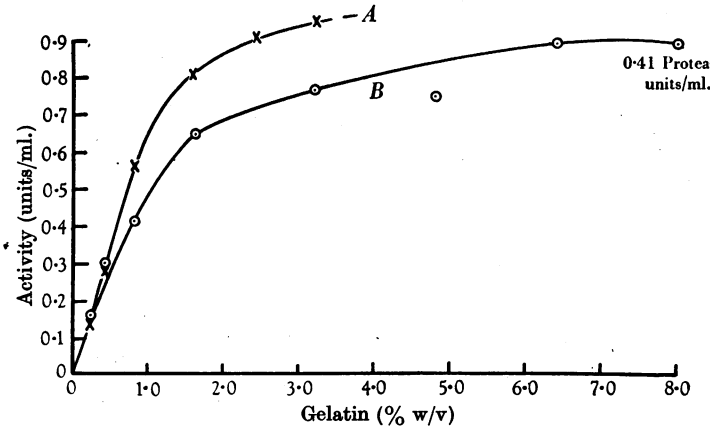

Fig. 2.

Fig. 1. Protease activity at various pH values expressed as percentage of the maximum activity found. Portions of the same sample of partially purified tobacco sap were used for all determinations.

Fig. 2. Variation of activity with substrate concentration in partially purified samples of tobacco-sap protease containing (*A*) 0·56 and (*B*) 0·41 protease units/ml.

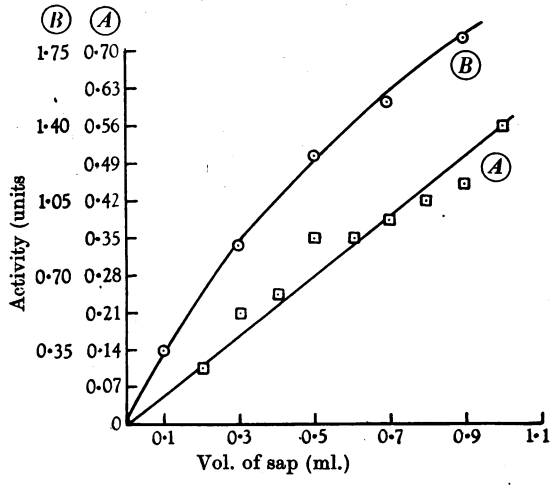

Fig. 3.

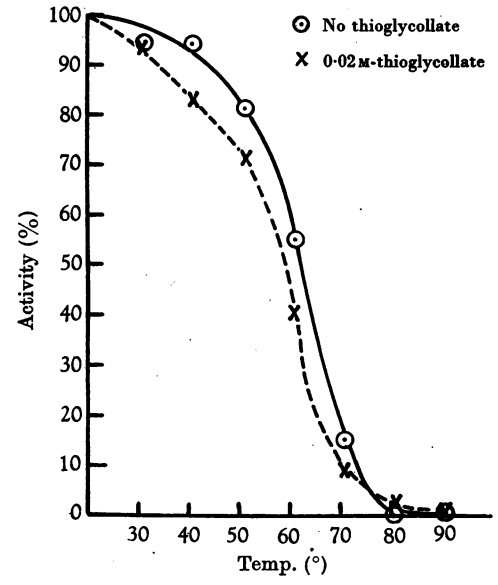

Fig. 4.

Fig. 3. Variation of activity with protease concentration, (*A*) 0·28 protease unit/ml., (*B*) 1·22 protease units/ml.

Fig. 4. The effect of heat on tobacco-sap protease. Portions of partially purified protease were heated for 5 min. at the temperatures indicated, in the absence of added substrate. Activities subsequently measured are expressed as percentages of that of the unheated sap.

than 4% (w/v) gelatin owing to gelling during sampling. Typical results are shown in Fig. 2. The concentration of gelatin which gives half the maximum velocity of hydrolysis is about 0·8%.

An approximately linear relationship was found between the amount of enzyme present and the extent of hydrolysis over the enzyme concentration range usually employed (Fig. 3). The effect of heat

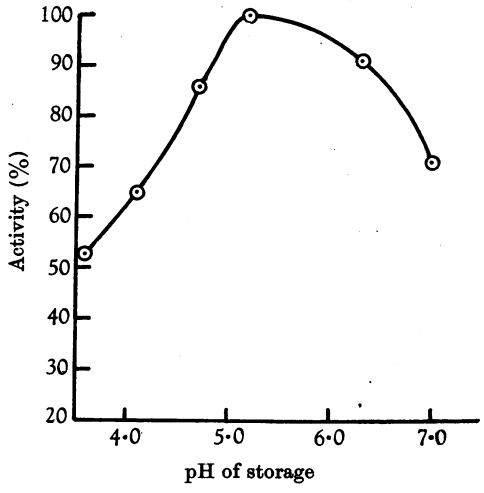

Fig. 5. The effect of pH on the stability of tomato-sap protease kept at 5° for 20 hr. The activities are expressed as percentages of the maximum observed activity in this experiment.

on enzyme activity was determined by heating samples of sap at pH 5 in the absence of added substrate to a desired temperature, maintaining it at that temperature for 5 min. and cooling rapidly. The activity of the heated samples was then measured in the usual manner. Fig. 4 shows that some inactivation occurs even at 40° and that the presence of reducing agent has little effect. The influence of the temperature of incubation on the activity measured was measured at four temperatures. Enzyme action at 25° was 57%; at 20°, 25%; at 5°, 14% of the activity at 40° using gelatin as substrate. The pH of maximum stability on storage at 5° was measured on samples of tomato sap. Values of pH were measured 1 hr. after adjustment and at the end of 20 hr. at 5° and were found not to have changed, except for the sample initially at 6·5 which had changed to 6·3 and the sample at 7·4 which had changed to 7·0. Final pH values have been used in Fig. 5. The pH of maximum stability seems to be in the region of the optimum for protease activity, about pH 5.

The course of protease activity in the presence of added substrate was followed for both sap and fibre for 7 days at 40°. In Fig. 6 the values for the sap have been multiplied by 3·3 in order to show an equivalent activity.

A number of substrates have been tested with tobacco-leaf protease. The protein present in both crude and spun sap is split to some extent, but the amount of amino N released is small compared with that which occurs in the presence of added gelatin.

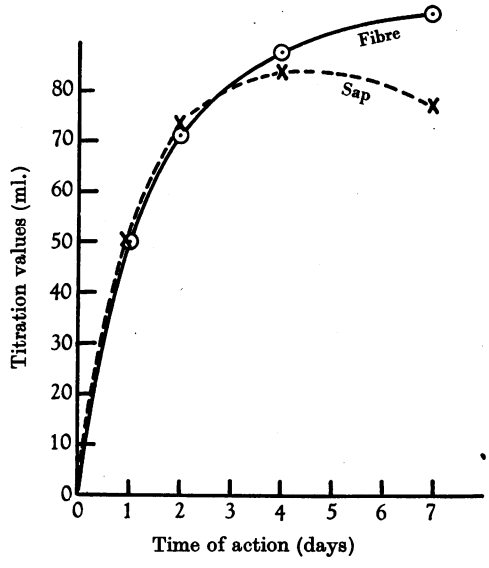

Fig. 6. Progress curve for the hydrolysis of gelatin (0·8%, w/v) by tobacco protease over an extended period. Titration values for equal samples withdrawn at intervals are plotted against time. The values for sap have been multiplied by 10/3.

Table 2. *Activity of tobacco protease with various substrates*

	(units/ml.)
Crude sap*	0·18
Spun sap†	0·03
Spun sap + gelatin	0·45
Spun sap + wheat gluten	0·40
Spun sap + egg albumin	0·07
Spun sap + peptone	0·45
	(units/g. dry wt.)
Washed fibre	3·10
Washed fibre + gelatin	4·50

* Final concentration of sap protein *c.* 0·14%.
† Final concentration of sap protein *c.* 0·11%.

Table 2 shows the amount of amino N formed by tobacco protease acting on various substrates and its own proteins at pH 5. Added substrates had a final concentration of 0·8% (w/v); with sap alone the usual volume of 0·5 ml. was used.

Inhibition by iodoacetate

Tobacco-leaf protease is not completely inhibited in the presence of 0·02M or even 0·1M-iodoacetate though no activity can be detected in the presence of 0·02M-copper salts. It is possible that the pro-

tease is a mixture of enzymes, some stable to iodoacetate and some inhibited by it. The ratio of full activity in the presence of thioglycollate to the residual activity shown in the presence of iodoacetate does not change appreciably during the course of preliminary purification, and attempts to demonstrate the existence of different pH optima in the presence of thioglycollate and the presence of iodoacetate have not been successful.

Leaf protease of other plants

The leaves of a number of plants have been examined for the presence of protease. In all those so far examined, the enzyme is activated by thioglycollate and is active at pH 5. Table 3 lists some of the plants tested. In those for which a range of values is given, samples varying in fertilizer treatment were used. The leaves of some plants do not yield sap on mincing (hollyhock, *Althea rosea*; box, *Buxus sempervirens*), while laurel (*Prunus laurocerasus*) gives a sap containing only traces of protein. These leaves were however found to contain protease in amounts similar to those in tobacco.

protease content may be found in plants grown under different cultural conditions (Table 3: tobacco, potato, beet), and that caution must therefore be exercised in drawing conclusions from the examination of plants of unknown antecedents and cultural background. In spite of these variations in protease concentration, none of the plants examined has approached the protease content of the pineapple, which is typical of those plants containing the well characterized plant proteases. The plants examined do, however, appear to form a homogeneous group when protease contents are compared on a basis of units/g. protein N, or units/g. total N. All the values quoted for sap protease/g. protein N (with the exception of pineapple) fall within the range found in tobacco, although the concentrations in sap on a volume basis may differ by a factor of six.

It is probable that the proteases described in this paper have not been examined in any detail before because they occur in such small concentrations. It must be assumed, however, that the protease levels found are adequate for the metabolic needs of the plants; if the total plant protein were equivalent as substrate to gelatin, all the leaf protein could be

Table 3. *Sap and fibre protease content of a number of plants*

		Sap (units/ml.)	Sap (units/g. protein N)	Fibre (units/g. dry wt.)	Fibre (units/g. N)
SOLANACEAE:					
Tobacco, *Nicotiana tabacum*	(1)	0·03–0·63	25–1040	0·8–12·4	20–270
Potato, *Solanum tuberosum*	(2)	0·60–2·18	150–780	8·6–13·8	160–390
Woody nightshade, *Solanum dulcamara*		1·02	1020	—	—
Tomato, *Lycopersicum esculentum*		0·79	370	—	—
CHENOPODIACEAE:					
Sugar beet, *Beta vulgaris*	(3)	0·14–0·67	60–400	1·8–6·7	70–320
CUCURBITACEAE:					
Marrow, *Cucurbita pepo* var. *ovifera*		0·42	244	1·2	51
Bryony, *Bryonia dioica*		0·21	130	1·6	31
CRUCIFERAE:					
Cabbage, *Brassica oleracea*		0·35	263	12·5	330
LEGUMINOSAE:					
Bean, *Phaseolus vulgaris*		0·63	375	1·02	133
GRAMINEAE:					
Wheat, *Triticum vulgare*	(4)	2·7–4·1	350–580	2·5–3·5	120–210
Cocksfoot, *Dactylis glomerata*		0·14	202	0·63	77
BROMELIACEAE:					
Pineapple, green leaves of *Ananas sativus*		6·5	13,000	8·85	—
Papain (British Drug Houses, 0·005 mg. N/ml.)		0·42	84,000	—	—

Results are from single determinations except (1)–(4) which are the ranges with (1) 80 samples, (2) 16 samples, (3) 54 samples, (4) 6 samples of plants with varied fertilizer treatments at different seasons.

DISCUSSION

The methods described in this paper have been used to measure the protease content of plants grown under different fertilizer conditions and infected with different viruses. It is hoped to publish these results shortly. They show that very wide variations in

split by its enzymes in 2–10 days at pH 5 and 40°. Thus it appears that the levels in some plants are abnormally high. It is difficult to see what evolutionary advantage the remarkably high protease activity in latex and fruits has for the plants concerned, since an abnormal resistance to pathogens does not seem to be conferred; pineapple, papaw and fig all suffer from virus diseases.

No evidence has been found to show that the protease is a mixture of enzymes or that the protease of one plant is distinguishable from another. It has been customary to name a protease after the generic name of the plant in which it is found. This custom, both here and in future work, implies an indefinite extension in the number of proteases. It is therefore recommended that the proteases described in this paper shall be considered identical until evidence to the contrary is adduced, and that they be called leaf proteases, adding where necessary the name of the plant.

SUMMARY

1. Protease activity is shown by both sap and fibre of the green leaves of the plants examined.

2. This activity is less than one tenth of that shown by the leaves of pineapple.

3. The activation of the enzyme by reducing agents and the pH optimum suggest that the protease is similar to the papain enzymes.

4. It is suggested that the enzyme be referred to as 'leaf protease' until evidence is obtained of the existence of more than one protease in the leaf or of differences between the leaf proteases of different plants.

5. The method of Pope & Stevens (1939) has been modified to allow smaller amounts of amino nitrogen to be estimated in the presence of reducing agents.

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The Free Amino Groups of Haemoglobins

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Since the original work of Reichert & Brown (1909) on the crystal form of the haemoglobins from a large number of species, many workers have shown them to differ in chemical and physical properties. Among the properties studied were the CO-combining capacity (Douglas, Haldane & Haldane, 1912), oxygen dissociation (Macela & Seliškar, 1925), absorption spectra (Anson, Barcroft, Mirsky & Oinuma, 1925), amino-acid composition (Vickery & White, 1933; Block, 1934), rate of alkali denaturation (von Krüger, 1887) and electrophoretic mobility (Landsteiner, Longsworth & van der Scheer, 1938). Apart from the occasional ability of haemoglobins of closely related species such as the horse and the donkey to form mixed crystals, the property which has brought out marked similarities as well as differences is their immunological behaviour. The most extensive survey was made by Hetkoen & Boor (1931), who showed that, although the haemoglobins were species-specific, cross reactions occurred between the antibodies and antigens of allied species. The extensive work of Landsteiner (1945) and others has suggested a relationship between the antigenic behaviour of a protein and its chemical structure. As an investigation of the free α-amino-groups of a protein brings out some details of the molecular structure (Sanger, 1945), it was of interest to make a comparative study of the terminal residues of several haemoglobins, and to see if any correlation between structure and immunological behaviour could be detected.

EXPERIMENTAL

Preparation of DNP-amino-acids

The method for the preparation of 2:4-dinitrophenylamino-acids (DNP-amino-acids) using 1:2:4-fluorodinitrobenzene (FDNB) has been previously described (Sanger, 1945).