the introductory phase of glycolysis, is later reconstituted (a) through the transfer of the phosphate group from phosphoglyceric acid to adenylic acid and (b) by way of a reaction between inorganic phosphate and adenylic acid, which forms part of the coenzyme-catalyzed 'phosphorylative oxidation-reduction'.

5. Evidence is provided in support of the view that ATP is the substance through which a link is established between the activity of spermatozoa on the one hand and glycolysis on the other.

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Note added in proof: 27 November 1945. Since this paper was sent to press, the sugar present in seminal plasma has been purified and identified as *d*-fructose by means of optical rotation, reduction value and by the formation of crystalline methylphenyl-fructosazone. Washed spermatozoa can metabolize glucose and fructose alike. Yet in whole semen the natural substrate for the formation of lactic acid, as it is provided by the seminal plasma, is mainly fructose. (Mann (1945), *Nature, Lond.*, in the Press.)

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Euphorbain, a Protease Occurring in the Latex of the Weed *Euphorbia lathyris*

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Euphorbia lathyris, commonly known as Caper Spurge, is a biennial plant which grows to a height of about 3 ft. A survey of its distribution in Australia has shown that it is widespread in Victoria and New South Wales, but less plentiful in South Australia, and only rarely found in Queensland and Western Australia. When the stem or a branch of the living plant is broken near the tip, or when the stem is lanced, milky latex drips from the wound. Collection of the latex is best made in the spring, for then it flows freely.

The pH value of the latex samples examined ranges between 5.1 and 5.3, increasing by 0.5 pH units during dialysis. On addition of alkali either to the latex or acetone powder extracts (described later), a bluish purple colour appears at pH 6.5 reaching maximum intensity at pH 7.5. The colour

changes to deep red as the pH approaches 9.0 and yellow at pH 11.5.

Rod-shaped crystals are visible in the latex under the microscope but they disappear when the latex is dialyzed through cellophan. Both the undialyzed latex and the dialysate give reactions characteristic of an aromatic hydroxy acid.

As reported by Ellis & Lennox (1942), the latex possesses a highly active proteinase for which the name 'euphorbain I'* was suggested. Vines in 1903, however, referred to the work of Buscalioni & Fermi (1898), who demonstrated qualitatively the existence of a gelatinase in *E. lathyris* by placing portions

* 'Euphorbain I' is used to distinguish this protease from that of *Euphorbia cerifera* reported by Castañeda, Gavarron & Balcazar (1942).

of the plant on a layer of gelatin and observing liquefaction.

The present paper describes some of the more important characteristics of the enzyme.

METHODS

Collection and treatment of latex. Latex was collected from a laboratory plot of *E. lathyris* by lancing the stems and allowing the latex to drip into a 10 ml. graduated cylinder. Daily collections of 2 ml. were made from 40 plants over a period of several weeks.

Before measuring its activity, the latex was diluted, unless otherwise stated, with 11.5 times its own volume of water. For dialysis, 1 vol. of latex was diluted with 4 vol. water, and dialyzed through cellophan until KMnO_4 was no longer reduced by the dialysate. This treatment, occupying several hours, hastened the flocculation of the latex particles which could then be removed easily by filtering through cotton-wool, before diluting the dialyzed latex to 12.5 times the volume of the original sample. Gas bubbled through the solution also favoured clotting.

In the field, 1 vol. latex was collected in 3 vol. of acetone, and the precipitate which formed was filtered, washed with acetone, dried *in vacuo* and ground finely to yield an 'acetone powder'. '0.4% acetone powder extract' refers to an extract prepared by incubating 0.4 parts by weight of the acetone powder with 100 parts by weight of water for 1 hr. at 40°, and filtering.

Gelatinase activity (10 min.). This term is defined as the ratio of the reduction in viscosity which occurs during 10 min. incubation at $40^\circ \pm 0.2^\circ$ of a 6.67% (w/v) solution of gelatin containing the enzyme, to the initial viscosity. The digestion flask viscometer of Koch, Orthmann & Degenfelder (1939) was used and the procedure described by Lennox (1943) was followed.

Gelatinase activity (2 min.). In order to measure proteolytic activity on small volumes of liquid, a micro-viscometer was constructed which required only 3.0 ml. of the incubation mixture, containing 0.8 ml. of the enzyme solution. The enzyme and gelatin were used in the same ratio as when measuring gelatinase activity (10 min.), but a digestion period of 2 min. was adopted. Since the time of flow of the enzyme-gelatin mixture was usually between 20 and 30 sec., it was possible to make frequent measurements in the one viscometer.

As shown in Fig. 1, the apparatus comprises a viscometer bulb A of 0.8 ml. capacity with a constriction of 1 mm. diameter both above and below the bulb (B_1 and B_2 respectively), attached through a three-way tap C of 2 mm. bore

to a reservoir D, 2 cm. diameter and 5 cm. long, with its outlet slightly above the level of the viscometer bulb. The lower connexion from the tap leads to a 0.7 mm. bore capillary tube E, 10 cm. long, which is attached through a rubber stopper to a 100 ml. Erlenmeyer flask F. The tube G, passing through the stopper, ensures that the air inside the flask remains at atmospheric pressure. One arm H of the tap C is ground at the tip so that it passes under the brass stop J attached to the stem of the viscometer. When H is adjacent to J, the three tubes from the tap are interconnected. By turning the tap anti-clockwise through 90° the reservoir is connected to the bulb, and by turning it anti-clockwise through an additional 90° the long arm I of the tap is brought to rest against the stop and the viscometer bulb is connected to the capillary tube. The stop enables the position of the tap to be exactly reproduced.

To measure the gelatinase activity of an enzyme, the solutions required were warmed to $40^\circ \pm 0.2^\circ$ in a thermostat bath, 1 vol. 10% (w/v) gelatin solution was adjusted to the required pH value (usually 7.0) with 0.1 vol. NaOH solution of appropriate concentration, and 0.8 ml. enzyme solution was added to 2.2 ml. of the adjusted gelatin in a test-tube at 40°. After 80 sec. the mixture was transferred to the reservoir of the viscometer, the tap being open to the bulb so that the liquid filled the bulb and rose to a level slightly above the upper constriction. The level of water in the 40° thermostat bath was kept above the level of the liquid in the viscometer. At 120 sec. after mixing the enzyme and gelatin, the viscometer tap was turned to connect the bulb of the viscometer with the capillary tube beneath and the time was taken for the surface of the liquid to pass from the upper constriction B_1 to the lower constriction B_2 . The tap was then turned to allow liquid from the viscometer and the reservoir to drain into the flask before making the next measurement.

A calibration curve for the viscometer relating time of flow with viscosity in millipoises was constructed from data obtained with 40% sucrose tested at 25 and 40°, as suggested by Koch *et al.* (1939). Gelatinase activity (2 min.) was calculated as the ratio of the reduction in viscosity of the solution containing the enzyme and 6.67% (w/v) gelatin during 2 min. incubation at 40° to the initial viscosity.

Rennase activity. This was measured by the method of Balls & Hoover (1937) and expressed as the reciprocal of the time of clotting in minutes at 40°. The time for the formation of flecks of clot on the walls of the test-tube was taken as the time of clotting, since this interval was more sharply defined than that required for detectable thickening of the bulk of the milk. When using euphorbain there was a longer interval between these two stages than with papain.

Although the milk-clotting method is simple, rapid and requires only a small sample, the clotting time is affected by pH changes, and some activators and inhibitors could not be used in high concentration owing to their effect on the pH value. Milk powder reconstituted with 0.5 and 1.0 M buffer solutions instead of 0.1 M buffer, to increase the buffering capacity, clotted spontaneously on storage because, no doubt, of the proximity of the pH values of the final preparations to the isoelectric point of casein (4.7). The rennase activity of euphorbain preparations was found to be less reproducible than the gelatinase activity. This was primarily because the interval required for the clotting of milk could not be measured as accurately as the time of

Fig. 1. Micro-viscometer used to measure gelatinase activity of small volumes of diluted latex.

flow of gelatin solution through a viscometer, and moreover, the pH value at which the rennase activity is measured is several units lower than the optimum value for the gelatinase activity of euphorbain, and therefore slight variations in pH may be more important.

RESULTS

Activity of material from various sources. The gelatinase activity (10 min.) of 0.4% acetone powder extracts and of 0.4% ethanol powder extracts, prepared from latex collected during two seasons in various parts of Victoria and New South Wales, ranged between 0.30 and 0.48. Ethanol powder extracts were prepared as described for acetone powder extracts except that the latex was precipitated with 3 vol. ethanol instead of acetone. The gelatinase activity (10 min.) of a 0.4% extract of a medicinal grade of papain powder was 0.40. The yields of powder varied between 9 and 12 g./100 ml. *Euphorbia* latex.

Stability during storage. Acetone powder preparations retained their original activity during 12 months' storage in the laboratory. The rennase activity of a 1 in 20 dilution of the latex, on the other hand, fell from 0.26 to 0.12 during 14 days at 7°, and the rennase activity of a 0.8% acetone powder extract fell from 0.17 to 0.05 during 12 days at 7°. The original activity was not restored by saturation with H₂S.

Optimum pH value. The effect of pH value on the gelatinase activity (10 min.) was studied by adding

Fig. 2. pH-gelatinase activity curve for euphorbain.

the requisite amount of 0.1M-NaOH or HCl to a portion of an acetone powder extract, and making similar adjustments to 50 ml. portions of 10% (w/v) gelatin by reference to the titration curve for the particular grade of gelatin employed (Lennox, 1943). After mixing 25 ml. enzyme extract with 50 ml. gelatin solution and taking 50 ml. for the estimation of the gelatinase activity, the remainder was held at 40° and its pH value was measured with the glass electrode 10 min. after mixing, that is, at the same stage as the viscosity was measured for

calculation of the gelatinase activity. The results in Fig. 2 show that the enzyme was most active at about pH 8, although variation in pH value between 7 and 9 had little effect.

Effect of temperature on activity. The activity of an acetone powder extract measured after storage for 17 hr. at temperatures between 7 and 55° (Table 1) shows that complete inactivation occurred at 55°.

Table 1. Temperature inactivation of *Euphorbain*

Gelatinase activity (10 min.) after 17 hr. at			
7°	22°	40°	55°
0.35	0.33	0.30	Nil

In the experiment reported in Fig. 3, the viscosity of the enzyme-gelatin mixture was measured at 4 min. intervals at each of the temperatures indicated. The 0.4% acetone powder extract reduced

Fig. 3. Gelatin viscosity reduction curves for euphorbain at various temperatures.

the viscosity of the gelatin at all the temperatures tested, but temperature inactivation is evidenced by a flattening of the 70° curve so that, after 30 min., it failed to reach as low a viscosity as the 60° curve.

Inhibitors. As shown in Fig. 4, reducing agents did not increase the gelatinase activity of euphorbain. On the contrary, all except H₂S reduced it slightly during 20 min. incubation at 40°. Of the two oxidizing agents reported in Fig. 4, only I₂ lowered the activity appreciably, such lowering being more pronounced after dialysis than before. The loss during dialysis of material which partially protects the enzyme against inactivation is also evident from Fig. 5.

Similar results were obtained on measuring rennase activity of the latex during incubation with the same series of compounds. I_2 again produced the greatest inactivation, reducing the rennase activity from 0.40 to 0.04 within 120 sec. at 40° in the undialyzed latex, and from 0.40 to nil in the dialyzed latex under the same conditions.

Fig. 4. Action of oxidizing and reducing agents on the gelatinase activity during incubation.

Fig. 5. Effect of I_2 concentration on gelatinase activity, $\circ$ — $\circ$ undialyzed latex, $\bullet$ — $\bullet$ dialyzed latex.

By increasing the concentration of I_2 in the enzyme solution and incubating for 2 min. at 40° before mixing with the gelatin, marked inactivation was again demonstrated (Fig. 5). In the dialyzed

latex almost complete inactivation was obtained with $0.01M-I_2$. Attempts to restore the original gelatinase activity by half-saturating or saturating the enzyme solution with H_2S and incubating for 2 min. at 40° after inactivating with $0.01M-I_2$ for 2 min. at 40° were unsuccessful. $K_3Fe(CN)_6$ was a much less effective inactivator than I_2 for, as shown in Fig. 6, only partial inactivation was obtained

Fig. 6. Effect of $K_3Fe(CN)_6$ concentration on gelatinase activity, $\circ$ — $\circ$ undialyzed latex, $\bullet$ — $\bullet$ dialyzed latex.

even at $0.1M$ concentration. The influence of the concentration of I_2 and $K_3Fe(CN)_6$ on the rennase activity of acetone powder extracts is shown in Fig. 7. In these experiments the inactivator-enzyme

Fig. 7. Effect of I_2 concentration and $K_3Fe(CN)_6$ concentration on rennase activity of acetone powder extracts, $\circ$ — $\circ$ I_2 series, $\square$ — $\square$ $K_3Fe(CN)_6$ series.

mixtures were incubated for 30 min. at 40° before measuring the rennase activity, and the inactivation conditions were therefore more severe than those reported in Figs. 5 and 6 for the same concentrations. Slight activation of the euphorbain occurred at I_2 concentrations below $0.001M$. This is analogous to the activation of yeast endotrypsin with low concentrations of CN^- and inhibition with high concentrations, reported by Abderhalden,

Caemmerer & Pincussohn (1909). The failure to activate euphorbain with cysteine (Fig. 4) was not due to the use of too low a concentration, for dialyzing the latex in running water for 20 hr., in order to remove all traces of reducing compounds, and incubating for 2 min. at 40° with 0.002–0.08M-cysteine did not affect the gelatinase activity (2 min.).

The inhibiting effects of ICH_2COOH , AgNO_3 , ZnSO_4 and CuSO_4 are shown in Table 2.

Table 2. *Effect of some inhibitors at 0.02M concentration on gelatinase activity (2 min.)*

Inhibitor	Gelatinase activity (2 min.)	
	Undialyzed	Dialyzed
Nil	0.48	0.48
	0.48	0.47
ICH_2COOH	0.24	0.35
	0.23	0.36
AgNO_3	0.01	Nil
	0.02	0.01
ZnSO_4	0.16	0.11
	0.15	0.10
CuSO_4	0.03	0.05
	0.02	0.04

Evidence that the protease was not inhibited and activated by O_2 and H_2 , respectively, is shown in Table 3. The Pt foil was included to catalyze any such changes, and N_2 gas was bubbled simultaneously through a third sample of latex to ensure that the control received the same agitation as the test samples.

Table 3. *Gelatinase activity after gases are passed through fivefold diluted latex for 20 hr. at room temperature (15°)*

(The combined latex and washings from each test were diluted to 10 times the volume of the undiluted latex before measuring the activity.)

Treatment of latex	Gelatinase activity (2 min.)		
	O_2 passed in presence of Pt foil	H_2 passed in presence of Pt foil	N_2 passed
Undialyzed	0.54	0.50	0.52
	0.52	0.50	0.48
Dialyzed	0.47	0.48	0.49
	0.47	0.46	0.48

Action on nematodes. Attempts to digest nematodes by incubating them for 18 hr. at 40° in a 0.4% acetone powder extract of euphorbain were unsuccessful. An extract of papain powder diluted until it had the same gelatinase activity as the euphorbain extract partially digested

the same species of nematodes when tested under the same conditions.

DISCUSSION

On the evidence available the known plant proteases can be divided into two classes. The first class, comprising the papain-like proteases, possess some or all of the following properties: they are activated by reducing agents and inactivated by oxidizing agents; they act optimally, usually at pH 5, although in some instances an optimum pH value of 7 has been reported; they are more resistant to high temperatures than other proteases; and they digest nematodes and some other internal parasites. Examples are: asclepain m (*Asclepias mexicana*) (Greenberg & Winnick, 1940 a, b), bromelin (*Ananas sativus*) (Willstätter, Grassmann & Ambros, 1926 a, b), ficin (*Ficus carica*) (Walti, 1937, 1938), mexicanin (*Pileus mexicanus*) (Castañeda et al. 1942), papain (*Carica papaya*) (Mendel & Blood, 1910–11; Bersin & Logemann, 1933; Balls, 1941; Hellerman, 1937; Maschmann, 1934), pinguinain (*Bropelia pinguin* L.) (Asenjo & de Fernandez, 1942), tabernamontanain (*Tabernamontana grandiflora*) (Jaffé, 1943 b), protease of *Nepenthes* (Stern & Stern, 1932), protease of yeast (Grassmann, v. Schoenebeck & Eibeler, 1931), protease of wheat (Balls & Hale, 1936), and the protease of sprouted wheat (Mounfield, 1938).

Proteases in the second class are not activated by reducing agents, but they are inactivated by heavy metals and some oxidizing agents; they act optimally at pH 8; they have a low temperature tolerance; and they do not digest nematodes. Examples are: hurain (*Hura crepitans*) (Jaffé, 1943 a), solanain (*Solanum elaeagnifolium*) (Greenberg & Winnick, 1940 a, b), and the protease of pumpkin (*Cucurbita pepo*) (Willstätter, Grassmann & Ambros, 1926 a). The properties of euphorbain show that it also belongs to the second class.

Failure to inactivate euphorbain by removal of reducing agents, by dialysis and by bubbling O_2 through the solution emphasizes its difference from ficin, which loses activity if dialyzed aerobically but not if dialyzed in an atmosphere of N_2 (Winnick, Cone & Greenberg, 1944).

Partial inactivation of the enzyme by iodine at a concentration as low as 0.002M suggests that it is readily iodinated. Combination of enzyme with iodine has been demonstrated for pepsin (Herriott, 1936–7).

No evidence was obtained to suggest that the rennase activity of the latex was due to a separate component from that responsible for gelatinase activity. The action of inhibitors, for example, was essentially the same regardless of the method used to measure proteolysis.

SUMMARY

1. Latex collected from *Euphorbia lathyris* contains a protease for which the name 'euphorbain' has been proposed.

2. The activity is assessed by measuring the viscosity reduction effect on gelatin or by the milk-clotting method. The gelatinase activity is measured in the digestion flask viscometer or in a specially designed micro-viscometer.

3. A solid preparation which retains its proteolytic activity for at least 1 year is prepared by adding acetone to the latex.

4. Euphorbain acts optimally at pH 8, is heat-labile, is not activated by reducing agents, but is almost completely inactivated by heavy metals and by I_2 at 0.02M concentration.

5. The activity of the latex is not affected by

dialysis and it remains unaffected when O_2 , H_2 or N_2 are subsequently bubbled through the solution. Dialysis, however, removes protective materials from the latex and renders the enzyme more readily inactivated.

6. Euphorbain does not fall in the papain class of proteases. Its properties resemble those of the plant proteases hurain and solanain.

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Phosphoric Esters of the Pancreas: Choline Glycerophosphate

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Choline glycerophosphate was found by Karrer & Salomon (1926) among the products of hydrolysis of lecithin by lipase. It was stated to be a sticky, oily substance, intensely hygroscopic. We have found a similar material in the 'lead-soluble' fraction of an extract of commercial dried pancreas powder. After hydrolysis of the ester, choline and glycerophosphate were isolated.

The isolation of choline glycerophosphate resulted from an attempt to prepare choline sphingosine phosphate from the lead acetate-soluble fraction of an extract of commercial dried beef pancreas powder, according to a modification of the method previously described for fresh swine pancreas (King & Small, 1939). As a result there was obtained a fraction, of which the analytical figures were closer