of glucose greater than 100 %. Dische (1947) found that serum proteins in a concentration of 0.1 % produced a 20 % decrease in the intensity of the colour given by glucuronolactone. The failure to observe any decrease in the readings obtained with glucuronolactone in the present work may have been due to the use of a test blank, and of 0.2 % (instead of 0.1 %) ethanolic carbazole.

The similarity of the absorption spectra of the colour complexes of glucose and of hydroxymethylfurfural suggests that the colour from glucose is produced by reaction of carbazole with hydroxymethylfurfural formed from glucose by the action of sulphuric acid. Similarly, the colour from xylose is probably the result of a reaction between furfural and carbazole. Since the absorption spectra of the complexes from glucuronolactone at 2.5 and 24 hr. are both different from the above, and since neither furfural nor hydroxymethylfurfural gives as intense a colour at 2.5 hr. as glucuronolactone, it seems that neither of these compounds is principally responsible for the colour obtained with glucuronolactone and carbazole.

SUMMARY

1. A modification of the carbazole method for uronic acids has been adapted for the estimation of glucuronolactone, glucose and xylose in solutions containing any or all of these compounds in the concentration range 10–100 $\mu\text{g./ml.}$ The results were reproducible to within $\pm 4 \mu\text{g.}$ except for some mixtures containing xylose.

2. The method gave good recoveries of glucuronolactone and glucose added to urine. These recoveries were not affected by the addition of albumin, ketone bodies, urea or arabinose in limited quantities.

3. The method gave figures for the concentrations of glucuronic acid in chondroitinsulphate, hyaluronate and heparin preparations, and in

urine, which were of the same order as previously published figures obtained by other methods.

4. Hydroxymethylfurfural is probably responsible for the colour given by glucose, and furfural for that given by xylose. The material responsible for the colour given by glucuronic acid does not appear to be either of these compounds.

I am indebted to Mr G. M. Ching for the creatinine determinations.

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Latent Phenolase in Extracts of Broad-Bean (*Vicia faba* L.) Leaves

1. ACTIVATION BY ACID AND ALKALI

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(Received 8 March 1957)

Latent phenolase activity has been found in extracts of eggs of the grasshopper *Melanoplus differentialis* (Bodine & Allen, 1938*a, b*), mealworm *Tenebrio molitor* (Bodine & Allen, 1941), *Drosophila* spp. (Ohnishi, 1954; Horowitz & Fling, 1955) and crayfish serum (Bodine & Allen, 1941). The extensive investigations of Bodine and co-workers (Bodine &

Boell, 1935; Bodine, Allen & Boell, 1937; Allen, Ray & Bodine, 1938; Allen & Bodine, 1941; Allen, Otis & Bodine, 1942; Bodine & Tahmisian, 1943; Bodine, Tahmisian & Hill, 1944) with extracts and preparations from grasshopper eggs have shown that active phenolase is released by treatment with certain organic solvents (e.g. acetone, chloroform),

certain detergents, by heating to 60–70°, or by brief exposure to weak alkali (about pOH 4). They have obtained evidence that these effects are due to the presence in the extracts of egg of a copper-protein which is converted into an active phenolase by the various treatments.

Kenten (1955) has reported that much of the phenolase of extracts of broad-bean leaves is present in a masked form and that release of active enzyme follows treatment with certain proteolytic enzymes or anionic wetting agents. The nature of the masked phenolase is not yet clear, but the present work describes some treatments which bring about activation of the phenolase.

MATERIALS AND METHODS

Plant material. Most of the broad-bean leaves used were taken from plants grown in a heated greenhouse in John Innes Compost, but occasionally leaves from garden plants were used. Other plant material was found growing wild or in gardens.

Plant extracts. The plant material was ground with sand and an equal weight of water previously cooled to 0° in a chilled mortar. The slurry was squeezed by hand through strong cotton cloth and the water extract collected in a cylinder surrounded by ice and water.

Precipitation and centrifuging. Unless otherwise stated, precipitations were made at 0–4° with $(\text{NH}_4)_2\text{SO}_4$. Ethanol and acetone were cooled to –10° and the temperature was maintained below 4° during the precipitation. Centrifuging was carried out in an MSE angle-head centrifuge (Measuring and Scientific Equipment Ltd.) at 0–4°.

Phenolase activity. This was followed manometrically by measuring the rate of O_2 uptake at 25° when the preparations acted on DL- β -3,4-dihydroxyphenylalanine (DOPA), catechol, adrenaline or *p*-cresol. The volume of the reaction mixtures was 3 ml., the gas phase was air and KOH was present in the centre cup. Phenolase activity was also estimated by a method essentially similar to that of Horowitz & Shen (1952). The rate of formation of the red initial oxidation product of DOPA was followed with an EEL colorimeter (Evans Electroselenium Ltd.) with a Bright Spectrum blue-green filter no. 623, maximum transmission at 495 m μ . Reaction mixtures consisted of 3 mg. of DOPA and enzyme in a total volume of 6 ml., 0.025M with respect to both phosphate and citrate at pH 6. Under these conditions there was no gross departure from a linear rate of oxidation over the first 3.5 min. of the reaction and the rate of oxidation was proportional to the amount of enzyme used, provided that the change on the EEL scale was less than 30 units. For routine determinations 0.05–0.5 ml. of enzyme was added at zero time to the other reagents and, after thoroughly mixing, readings were taken at 20 and 200 sec., and the increase in light absorption over this period was taken as a measure of the enzyme activity. Blank determinations with enzyme previously heated for 5 min. at 100° always gave very small values, rarely exceeding 1 EEL unit in 200 sec.; where the amount of protein was large, a small increase in transparency was observed owing to the settling out of coagulated protein unless the solution was frequently stirred. On no occasion was a red coloration seen in the blank determinations.

Estimation of β -3,4-dihydroxyphenylalanine. This compound was estimated by the colorimetric procedure of Kendal (1949) with an EEL colorimeter and filter no. 623.

Buffers. Phosphate buffers were prepared from solutions of KH_2PO_4 and KOH, citrate and acetate buffers from solutions of the acids and NaOH.

Ammonium sulphate. For most of the work British Drug Houses Ltd. Laboratory Reagent grade $(\text{NH}_4)_2\text{SO}_4$ was used.

pH measurements. These were made with a Cambridge or Beckman 'General Purpose' glass electrode. The Beckman electrode was used exclusively at pH values above 9 since it has a low Na^+ ion error and a negligible potassium error at the highest pH values measured.

EXPERIMENTAL AND RESULTS

It is well known that when broad-bean leaf sap is left to stand it soon begins to darken, first at the surface and then through the whole fluid, which finally resembles ink. It is believed that the production of melanin by the oxidation of tyrosine or DOPA is responsible for the blackening of the sap. Guggenheim (1913) isolated L-DOPA from broad-bean pods (2.5 g./kg. of fresh pod), and in the present work the DOPA content of a number of different samples of broad-bean leaves was found to vary between 3.5 and 9.4 g./kg. fresh wt. There is therefore ample DOPA present to account for the observed blackening of leaf sap. All the water extracts of broad-bean leaves prepared in the present work turned black on standing, although a considerable variation in the rate at which different batches darkened was observed. During attempts to prepare an oxidase free from DOPA from such water extracts it was often found that repeated precipitation with $(\text{NH}_4)_2\text{SO}_4$ gave a preparation with much greater activity towards DOPA or catechol at pH 6 than did a single precipitation.

Effect of repeated precipitation with ammonium sulphate. A water extract of broad-bean leaves was centrifuged at 8000 g for 20 min. and the pellet discarded. All subsequent steps were carried out at room temperature. A portion of the supernatant was set aside and the rest saturated with $(\text{NH}_4)_2\text{SO}_4$. The precipitate was centrifuged off and suspended with water to a volume equal to that of the supernatant taken for precipitation. A sample of this suspension [first $(\text{NH}_4)_2\text{SO}_4$ ppt.] was taken and the rest reprecipitated by saturating with $(\text{NH}_4)_2\text{SO}_4$. The precipitate from centrifuging was suspended in water to a volume equal to that of the suspension taken for precipitation. After sampling this second $(\text{NH}_4)_2\text{SO}_4$ ppt. the cycle was repeated until all the fractions had been obtained. The rate of oxidation of added DOPA or catechol by the fractions at pH 6 was then estimated (Table 1).

The results show that a large increase in the phenolase activity is brought about by repeated precipitation with $(\text{NH}_4)_2\text{SO}_4$. The activity of all of the fractions was virtually completely destroyed by

heating for 5 min. at 100° before testing. The magnitude of the effect brought about by repeated precipitation was extremely variable, often being much less than that shown in Table 1.

When active phenolase was obtained from water extracts by repeated precipitation with $(\text{NH}_4)_2\text{SO}_4$ it could be precipitated with only slight loss of activity by the addition of 2 vol. of acetone or ethanol. However, repeated precipitation of centrifuged water extracts with 2 vol. of acetone or ethanol failed to bring about an increase in phenolase activity at pH 6. The pH of the water extracts was in the neighbourhood of pH 6, and when repeated precipitation with $(\text{NH}_4)_2\text{SO}_4$ was carried out in the presence of phosphate buffer at pH 6 the increases in activity obtained were negligible compared with those found with extracts without added buffer. With unbuffered solutions, as each cycle of precipitation is carried out the pH falls owing to the acidity of the $(\text{NH}_4)_2\text{SO}_4$. The results of the following experiments show that exposure of the bean-leaf extracts to suitable acidities does in fact increase the phenolase activity at pH 6 and that exposure to alkaline conditions has a similar effect.

Effect of acid and alkali. For these experiments a preparation free from the naturally occurring polyphenols was made from a centrifuged water extract of broad-bean leaf by dialysis for 24 hr. at 0–4° against large volumes of buffer, pH 6, 0.004M with respect to phosphate and citrate. Portions of the dialysed extract (40–50 ml.) were held below 10° and $\text{N-H}_2\text{SO}_4$ or N-KOH was added dropwise with brisk stirring. Samples (2 ml.) were withdrawn at various pH values into cold (0–5°) 0.2M-phosphate-citrate (1 ml.), of pH 6 when the samples were alkaline and pH 6.5 when the samples were acid. The pH of these mixtures was 6–6.5 except when the pH of the dialysed extract was outside the range 2–12, when further adjustment with acid or alkali was necessary. The whole operation was carried out rapidly and the addition of acid or alkali and sampling was complete within 10–15 min. Within 1 hr. of preparation the phenolase activity of the samples was estimated colorimetrically at room temperature (18.5–20°). The results of one such experiment are given in Fig. 1. The values of phenolase activity are the increase in light absorption, in EEL-scale units, which would be brought about by the action of 1 ml. of the samples of dialysed water extract (corrected for dilution by the addition of KOH or H_2SO_4 and the phosphate-citrate buffer) under the standard conditions. All the values given in Fig. 1 were obtained with a single extract. Not all of the samples were tested for thermolability but the most active samples (those exposed to a pH or pOH of 2–4) were completely inactivated by heating for 2–3 min. at pH 6–6.5. Furthermore, if the dialysed extract was heated

before exposing it to acid and alkaline pH no phenolase activity was found. The results (Fig. 1) show that within limits, exposure of the water extracts to acidity or alkalinity outside the range pH 6–7 increases the phenolase activity towards DOPA at pH 6. With different batches of extract the results varied somewhat in the magnitude of the effects and the ratio of the maximum activities reached at acid and alkaline pH values. In general, the maximum activity released by exposure to acid pH was greater than that obtained by exposure to alkaline pH. The maximum activities were obtained on the acid side in the range pH 2.8–3.8 and on the alkaline side pOH 2.2–3.2; with increase in acidity or alkalinity outside this range the activity decreased, suggesting that it was destroyed by too

Table 1. *Effect of repeated precipitation with ammonium sulphate on the phenolase activity of water extract of broad-bean leaves*

Reaction mixtures consisted of 3 mg. of DOPA or 5 mg. of catechol in 0.1M-phosphate at pH 6 in the main vessel. After equilibration 0.5 ml. of water extract or $(\text{NH}_4)_2\text{SO}_4$ precipitate was added from the side arm. Water extracts contained about 3 g. of DOPA/l. and had O_2 uptakes in the absence of added phenol of 7–8 $\mu\text{l.}$ of O_2 /0.5 ml. in the first 30 min. Uptakes with the $(\text{NH}_4)_2\text{SO}_4$ precipitates in the absence of added phenol varied between 4.5 and 6.5 $\mu\text{l.}$ of O_2 in 30 min. Blank determinations with heated extract or precipitate were less than 4 $\mu\text{l.}$ of O_2 in 30 min.

Fraction	Increased O_2 uptake ($\mu\text{l.}$ /0.5 ml. in the first 30 min.)	
	Expt. 1. DOPA as substrate	Expt. 2. Catechol as substrate
Centrifuged water extract	2	4
1st $(\text{NH}_4)_2\text{SO}_4$ ppt.	5.5	20.5
2nd $(\text{NH}_4)_2\text{SO}_4$ ppt.	18.5	36
3rd $(\text{NH}_4)_2\text{SO}_4$ ppt.	91.5	56.5
4th $(\text{NH}_4)_2\text{SO}_4$ ppt.	226	—
5th $(\text{NH}_4)_2\text{SO}_4$ ppt.	176.5	—

Fig. 1. Effect on the phenolase activity at pH 6 of brief exposure of dialysed water extracts of broad-bean leaf to different pH values at 5–10°.

extreme conditions. In alkaline solutions the pH decreases as the temperature increases, owing to the variation of pK_w with temperature, whereas the pOH, which is the important factor, remains unaltered. It is for this reason that the values given in Fig. 1 for pH have been calculated to the pH at 25° with the values for K_w given by Harned & Owen (1939). At 25°, $pK_w = 14$ and the $pOH = 14 - pH$. The effect of temperature on the pH of the acidified dialysed water extracts was small and differences between 5° and 25° were less than 0.2.

Effect of incubating dialysed water extracts of broad-bean leaves in the absence and presence of ammonium sulphate on the phenolase activity

It was found that when the precipitates, obtained from water extracts of broad-bean leaves, by saturation with $(NH_4)_2SO_4$, were suspended in water and allowed to stand at room temperature their phenolase activity at pH 6 often increased. The rate of increase in activity was found to be dependent on temperature, being more rapid at 37° than at 0°. Little or no increase was observed when the suspensions were stored frozen at -18° for 14 days, but subsequent incubation at 37° brought about a large increase in activity. A typical set of results obtained with a second $(NH_4)_2SO_4$ ppt. is shown in Table 2. No increase in activity was obtained when the preparation was heated for 5 min. at 100° before incubating for 15 hr. at 37°. Heating the fractions stored at the various temperatures for 5 min. at 100° before testing with DOPA destroyed 95-100% of the activity.

Experiments with dialysed water extracts of broad-bean leaves showed that the increase in phenolase activity obtained on incubation at 37° depended on the pH and the presence of $(NH_4)_2SO_4$ (Table 3).

Mixtures were made containing 6 ml. of dialysed water extract with and without the addition of 0.75 g. of $(NH_4)_2SO_4$ in a total volume of 10 ml., 0.03 M with respect to both phosphate and citrate at pH 3, 4, 5 or 6. A few drops of toluene were added to inhibit the growth of micro-organisms. (No evidence that either toluene or chloroform has any activating effect was obtained.) Samples were taken as soon as the mixtures were prepared and the activity towards DOPA at pH 6 was measured manometrically. It was found that adjustment of the pH before measuring the activity was not necessary, as the pH of the manometric reaction mixtures was not affected by the addition of the 0.1 ml. samples tested. The dialysed-extract mixtures were then incubated at 37° and samples were taken at different times, and the activity towards DOPA at pH 6 was measured. The results show that initially the highest activity is present in those samples at pH 4. On incubation, however, the

largest activity is reached in the sample containing $(NH_4)_2SO_4$ at pH 5. In the absence of $(NH_4)_2SO_4$ an increase in activity is observed only with the mixture at pH 5 and the effect is small compared with that obtained in the presence of $(NH_4)_2SO_4$ at the same pH. At pH 3 the activity is markedly unstable and in other experiments where phenolase was estimated colorimetrically with DOPA it was found that 0.5-1 hr. at 37° and pH 3 destroyed 95-100% of the activity. Prolonged incubation at any of the experimental pH values leads finally to complete loss of phenolase activity regardless of the presence of $(NH_4)_2SO_4$. If the samples incubated at pH 6 for 72 hr. are then acidified to pH 4 little or

Table 2. *Effect of ageing at different temperatures on the activity of an ammonium sulphate precipitate towards DOPA at pH 6*

A second $(NH_4)_2SO_4$ ppt. from a water extract of broad-bean leaves was stored at different temperatures and the activity towards DOPA at pH 6 was measured manometrically under the conditions of Table 1.

Conditions of storage	Increased O_2 uptake in first 30 min. (μ l./0.4 ml. of fraction)
4 hr. at 0°	12
15 hr. at 0°	54
15 hr. at 37°	235
15 hr. at 37°, then 7 days at 0°	443
8 days at 0°	237
8 days at -18°	13

Table 3. *Phenolase activity at pH 6 of dialysed water extracts of broad-bean leaves after incubation at acid pH in the absence and in the presence of ammonium sulphate*

A dialysed water extract of broad-bean leaves was incubated at 37° under the conditions described in the text, and the activity towards DOPA was measured at the stated times. Manometric reaction mixtures consisted of 3 mg. of DL-DOPA in buffer pH 6, 0.1 M with respect to phosphate and citrate. At zero time 0.1 ml. samples of the dialysed extract mixtures were added from the side-arm. The values given are corrected for the blank O_2 uptakes when DOPA was omitted or when the samples were heated (5 min. at 100°) before testing. These uptakes were less than 4 μ l. of O_2 in 10 min.

pH of incubation	...	3	4	5	6				
(NH ₄) ₂ SO ₄	...	-	+	-	+				
Time of incubation (hr.)		Uptake of O ₂ (μl. in 10 min.)							
0		17	12	59	53	9	21	5	1
6		1	1	49	63	15	67	2	4
24		-1	-1	11	24	15	79	5	12
48		0	2	0	-2	14	62	-1	6
72		0	2	0	2	-1	10	2	1

no increase in activity is observed, which suggests that the material from which activity is derived has been altered by the incubation.

Effect of ammonium sulphate on the stability of the phenolase activity released by acidifying dialysed water extract of broad-bean leaves

The phenolase activity released by brief exposure of dialysed water extracts of broad-bean leaves to acid pH is unstable in phosphate-citrate buffer at pH 6.5 even at 0–5°. However, the presence of $(\text{NH}_4)_2\text{SO}_4$ markedly reduces the rate of loss of the activity (Table 4).

Buffer solutions at pH 6.5, 0.1M with respect to phosphate and citrate and containing varying amounts of $(\text{NH}_4)_2\text{SO}_4$, were made up. A sample of dialysed water extract of broad-bean leaves was then acidified to pH 3.3 at 0–5° by the gradual addition of 0.2M- H_2SO_4 , with brisk stirring. Samples (2 ml.) of the acidified extract were immediately added to samples (4 ml.) of the buffer solutions which had been cooled previously to 0–5°. The activity of these mixtures towards DOPA was then determined colorimetrically at pH 6. The activity was again estimated after the mixtures had been stored at 0–5° for 1, 3 and 22.5 hr. The final pH of these mixtures was in the range 6.4–6.5. Control experiments in which samples of the dialysed extract before acidifying were added to the phosphate-citrate buffer mixtures had a barely measurable phenolase activity (about 50 times less than that of the acidified-extract mixtures) and showed no significant change on storing at 0–5° for 22.5 hr.

The results (Table 4) show that at pH 6.5 the activity is more stable in the presence of $(\text{NH}_4)_2\text{SO}_4$, and that the stability increases with increase in $(\text{NH}_4)_2\text{SO}_4$ concentration. This effect of $(\text{NH}_4)_2\text{SO}_4$ explains why incubation of dialysed extracts at pH 5 in the presence, but not in the absence, of $(\text{NH}_4)_2\text{SO}_4$ brings about a large increase in the phenolase activity. It is probable that at pH 5 two processes, formation of active phenolase and destruction of active phenolase, are taking place. Assuming that $(\text{NH}_4)_2\text{SO}_4$ has a similar protective action at pH 5 as at pH 6.5, then the presence of $(\text{NH}_4)_2\text{SO}_4$ would reduce the rate of destruction of the active phenolase and so favour its accumulation. The increase in activity observed when suspensions of $(\text{NH}_4)_2\text{SO}_4$ precipitates from water extracts of broad-bean leaves are incubated is probably dependent on $(\text{NH}_4)_2\text{SO}_4$ retained by the precipitates.

Effect of pH on reaction velocity with various substrates and after various treatments of enzyme

From centrifuged water extracts of broad-bean leaves, second $(\text{NH}_4)_2\text{SO}_4$ precipitates were prepared as described previously except that the preparation

was held below 5° throughout. When DOPA and adrenaline were used as substrates part of the precipitate was stored frozen at –18° while the rest was incubated in the presence of toluene at 37°. The rate of oxidation of the two substrates at different pH values was then tested manometrically. With *p*-cresol the preparation was tested as soon as it was prepared and again after incubation.

Table 4. *Effect of ammonium sulphate on the stability of the phenolase activity released by acidification of dialysed water extract of broad-bean leaf*

Mixtures containing acidified dialysed water extract of broad-bean leaf, $(\text{NH}_4)_2\text{SO}_4$ and phosphate-citrate buffer at pH 6.5 were prepared as described in the text and stored at 0–5°. The activity of these mixtures towards DOPA at pH 6 was measured colorimetrically at 18–19.5° at the stated times.

Final concn. of $(\text{NH}_4)_2\text{SO}_4$ (M) ...	0	0.17	0.5	1.0	2.3
Time of storage (hr.)	Loss of activity (%)				
1	20	23	14	11	0
3	40	36	27	15	0
22.5	70	61	40	19	0

Fig. 2. Effect of pH on the oxidation of phenols by second $(\text{NH}_4)_2\text{SO}_4$ precipitates from water extracts of broad-bean leaves. DOPA as substrate, enzyme preparation A: Δ , frozen for 45 hr.; $\blacktriangle$, incubated at 37° for 45 hr. Adrenaline as substrate, enzyme preparation B: $\circ$, frozen for 30 hr.; $\bullet$, incubated at 37° for 40 hr. *p*-Cresol as substrate, enzyme preparation C: $\square$, freshly prepared; $\blacksquare$, incubated at 37° for 40 hr. Experiments in phosphate (0.08M)-citrate (0.08M) buffer at a phenol concentration of 1 g./l. With DOPA and adrenaline as substrates the O_2 uptake in the first 15 min. with 0.1 ml. and 0.2 ml. of preparation is plotted against the final pH. With *p*-cresol the O_2 uptake in the first hr. with 0.1 ml. of preparation is plotted.

The results (Fig. 2) show that with frozen or freshly prepared precipitates appreciable activity is found only below pH 5, the optimum with all three substrates being in the neighbourhood of pH 4. After incubation the shape of the pH curve changes and the highest activities are found at pH 5–6 with adrenaline and at pH values above 4 with DOPA and *p*-cresol. With *p*-cresol, at all of the pH values tested there was a lag period of about 10–15 min. before the O₂ uptake started. The acid pH optimum found with the frozen or freshly prepared precipitates is probably a consequence of the activation of the latent phenolase brought about by the acid conditions.

The variation of activity with pH for all three substrates was studied under the same experimental conditions with a preparation from potato tuber (*Solanum tuberosum* L.) made in the same way as the broad-bean leaf second (NH₄)₂SO₄ precipitate. With this potato phenolase preparation there was little or no activity at pH values below 4 and with all three substrates the rate of oxidation increased with increase in pH values from 4 to 6. There was a 10 min. lag period with *p*-cresol as substrate before the O₂ uptake commenced. Similar experiments with a second (NH₄)₂SO₄ precipitate from a water extract of red-clover leaves [*Trifolium pratense* var. *sativum* (Crone) Schreb.] and with a crude chloroplast preparation (Arnon, 1949) from spinach-beet leaves (*Beta vulgaris* L.) with adrenaline as substrate showed that the activity increased with increase in pH value from 3.5 to 6. Phenolase has been reported absent from pea seedlings (Kenten & Mann, 1952) and a second (NH₄)₂SO₄ precipitate from pea leaves made in the same way as that from broad-bean leaves showed no activity with adrenaline over the range pH 3–6, either before or after incubation at 37° for 18 hr. in the presence of toluene.

These results suggest that no latent phenolase was present in the potato, clover, spinach-beet and pea preparations.

Tests for the presence of a phenolase inhibitor in the water extracts of broad-bean leaves

The experiments so far have shown that activation of an enzyme occurs; this might occur by the removal of an inhibitor. Accordingly, water extracts were tested together with ultrafiltrate from the extract to find out if an inhibitor of small molecular size was involved, and also with boiled extract, since boiling might liberate an inhibitor from an enzyme-inhibitor complex present in the extract.

Portions of the supernatant prepared from a water extract of broad-bean leaves centrifuged at 8000 g for 20 min. were treated in one of the following ways: (a) heated for 5 min. at 100°; (b) filtered

Table 5. *Oxidation of DOPA present in fractions of a water extract of broad-bean leaf by broad-bean-leaf phenolase*

Reaction mixtures comprised: in the main vessel 3 mg. of DOPA or amounts of extract, boiled extract or ultrafiltrate containing 3 mg. of DOPA, in 0.1M-phosphate at pH 6. After equilibration 0.3 ml. of incubated (NH₄)₂SO₄ precipitate [fraction (c), see text] was added from the side arm. After 1 hr. an equal volume of 4% (w/v) metaphosphoric acid was added to the reaction mixtures and the DOPA content estimated. The values for DOPA oxidized were obtained by difference between the DOPA content of the complete reaction mixtures and those from which the (NH₄)₂SO₄ precipitate had been omitted. Correction has been made for the blank O₂ uptake of extract (14 μl.), boiled extract (7 μl.), ultrafiltrate (6 μl.), DOPA (2 μl.) and the (NH₄)₂SO₄ precipitate (5 μl.).

Fraction used as substrate	DOPA oxidized (mg.)	O ₂ uptake (μl./hr.)
DOPA	1.14	203
Centrifuged water extract	0.81	185
Centrifuged water extract, heated at 100°	0.78	180
Ultrafiltrate	0.83	176

through cellophan at 0° in an apparatus of the type described by Paterson, Pirie & Stableforth (1947), the filtrate passing in the first 4–5 hr. being used; (c) precipitated twice by saturation with (NH₄)₂SO₄ as described previously, and incubated at 37° in the presence of toluene for 18 hr. to give an active phenolase preparation. Fractions (a) and (b) and a portion of the original supernatant were stored at 0° until the incubation of the (NH₄)₂SO₄ precipitate was complete. The amount of DOPA present in the various fractions was then estimated. No DOPA was detected in the (NH₄)₂SO₄ precipitate and the amounts present in the extract, boiled extract and ultrafiltrate were found to be 3.1, 3.1 and 3.0 mg./ml. in the experiment given in Table 5. The rate of oxidation of the DOPA present in these fractions by the incubated (NH₄)₂SO₄ precipitate was then followed manometrically by measuring the O₂ uptake and by estimating the disappearance of DOPA colorimetrically. The O₂ uptakes when the incubated (NH₄)₂SO₄ precipitate acts on water extract, boiled water extract or ultrafiltrate are about 90% of that obtained when it acts on added pure DOPA. The disappearance of DOPA from the extract fractions is, however, only about 70% of that found with pure DOPA. This discrepancy is probably due to the presence of substances (e.g. ascorbic acid) in the extract fractions which are capable of reducing the oxidation product, DOPA quinone, to DOPA. Thus a larger O₂ uptake would be obtained for a given loss of DOPA in these crude systems. The true inhibition of phenolase action by the extract fractions is therefore most likely to

be that calculated from the measurements of the O_2 uptake, i.e. about 10 %.

Effect of inhibitors. Phenolases from several sources have been shown to be copper-protein enzymes (e.g. Mallette, 1950) and their activity is inhibited by metal-complexing agents such as diethyldithiocarbamate (Dawson & Tarpley, 1951). The effect of a number of such complexing agents has been tested at pH 6 with the second $(NH_4)_2SO_4$ precipitate from a water extract of broad-bean leaves, activated before testing by incubation in the presence of toluene for 18 hr. The results (Table 6) show that agents which are known to inhibit the action of phenolases (Dawson & Tarpley, 1951) inhibited the broad-bean phenolase preparation. This suggests that, like the known phenolases, broad-bean phenolase is a metalloprotein enzyme.

DISCUSSION

The results of the present work show that the phenolase activity at pH 6 of preparations from broad-bean leaves can be greatly increased by brief exposure to mildly acid (pH 3–3.5) or alkaline (pOH 2.5–3) conditions. In the presence, but to a much less extent in the absence, of ammonium sulphate large increases in the phenolase activity at pH 6 are brought about when dialysed extracts of broad-bean leaves are incubated at 37° at about pH 5. These facts provide an explanation for the increase in activity which is observed when water extracts of broad-bean leaves are repeatedly precipitated with ammonium sulphate. The acidity of the ammonium sulphate causes the pH to fall to the neighbourhood of pH 5, which brings about some increase in activity, and in the time which elapses between the preparation of the fractions and testing a further increase in activity takes place.

Studies of the pH-activity relationships before and after treatment show that before activation preparations have an optimum activity in the

neighbourhood of pH 4, but little activity at pH 5–6. In general, after activation the activity increases with increase in pH up to pH 6 with no definite optimum pH.

There appear to be two possible explanations of the results. On the one hand it is possible that the pH-activity curves obtained before treatment are those of the native phenolase and that the change in the curve is due to modification of the enzyme protein. In agreement with this possibility all of the treatments which lead to an increase in the phenolase activity at pH 6, namely treatment with acid and alkali, and incubation at pH 5 in the presence of ammonium sulphate, are likely to lead to modification of the properties of a labile protein. On the other hand it is possible that the phenolase enzyme in the extracts is present in a masked form from which it is liberated by the various treatments. It has been shown that brief exposure of the broad-bean-leaf preparations to acid pH increases the phenolase activity at pH 6 and that the activity at pH 6 of the preparation increases with decrease in the pH, to which it has been exposed until about pH 3 is reached. From these data and the pH-activity curves of the activated preparations it would be expected that the untreated preparations would show an acid pH optimum in the neighbourhood of pH 4. The pH-activity curves of the untreated preparations would then be the resultant of the extent of liberation of active enzyme and its activity at the particular pH value. Thus these results suggest that the presence of a masked phenolase in the untreated extracts is the more likely possibility.

Apart from the classical proteolytic zymogens many examples of such masked activity are now known, and a number of them are cited by Swartz, Kaplan & Frech (1956). These authors have demonstrated that in extracts of a number of micro-organisms two enzymes (a diphosphopyridine-nucleotide pyrophosphatase and a 5'-nucleotidase) are present largely in an inhibited state. They have obtained evidence that the enzymes are present in the extracts in combination with a protein inhibitor. Presumably owing to the difference in the stability of the enzyme and inhibiting protein towards heat and acid, active enzyme can be liberated by brief heating at 100° or by acid treatment (pH 1–2 for 10 min.). Swartz *et al.* (1956) suggest that the enzyme and inhibitor are bound by a salt type of linkage.

It has been reported previously (Kenten, 1955) that incubation with certain proteolytic enzymes or treatment with certain anionic wetting agents brings about a large increase in the phenolase activity at pH 6 of water extracts of broad-bean leaves. These results and the results of the present work can be explained by assuming that in the

Table 6. *Effect of inhibitors on broad-bean-leaf polyphenolase*

Manometric reaction mixtures were similar to those described in Table 3. The inhibition was calculated from the uptakes obtained in the first 10–15 min. of the reaction.

Inhibitor	Concn. (mM)	Inhibition (%)
Diethyldithiocarbamate	1.0	93
	0.1	81
Potassium ethyl xanthate	10.0	90
	1.0	49
Salicylaldehyde	1.0	49
Sodium azide	1.0	47

extracts there is either a phenolase-inhibitor complex, in which the components are bound through interaction of ionic groups, or a phenolase precursor (prophenolase). If the former assumption is correct then H^+ ion, OH^- ion and the anionic wetters, by virtue of their capacity to combine with proteins with accompanying alteration of the net charge, would be capable of dissociating such a complex. If the inhibitor is released in this way it must be unstable, as it has not been possible so far to demonstrate that the release of active enzyme is reversible, which suggests that dissociation must be accompanied by modification of the inhibitor. If prophenolase is present in the extracts then the agents which lead to activation could act by modifying the prophenolase molecule in such a way that the active centre is unmasked.

The presence of a phenolase inhibitor in extracts of broad-bean leaves has been sought, but such an inhibitor was not found to be present in amounts sufficient to account for the results described. Attempts to separate an inhibiting protein fraction from the extracts by fractionation with ammonium sulphate have not been successful.

Whatever the state of the masked phenolase in the extracts the question arises whether it is present as such *in vivo* or whether it is formed during the extraction process. It is not clear how the answer to this question can be found experimentally. The possibility of regulation of metabolism by cellular synthesis of specific enzyme inhibitors has been often suggested (e.g. Swartz *et al.* 1956). In support of this suggestion are the several known examples of species-specific enzyme-inhibitor relationships involving protein inhibitors (e.g. Swartz *et al.* 1956). Recognition of this type of phenomenon is comparatively recent, and both it and the conversion of proenzyme into enzyme may prove to be important mechanisms of cellular regulation.

Preliminary tests with extracts from a limited number of different plants have not so far revealed the presence of a masked phenolase with the properties of that found in broad-bean-leaf extracts. This may be owing to the extreme ease with which active phenolase is liberated from the masked form in those species tested. Masked phenolase has been found in extracts from grasshopper eggs, mealworms, extracts of *Drosophila* and crayfish serum, and possibly in extracts of *Neurospora* (Horowitz & Shen, 1952) since these show an increase in phenolase activity on standing. It seems unlikely, therefore, that the broad bean will prove to be unique among the higher plants in giving extracts with latent phenolase activity.

SUMMARY

1. Water extracts of broad-bean leaves contain much latent phenolase activity.
2. Active phenolase is released by brief exposure of the extracts to acid (pH 3-3.5) or alkaline (pOH 2.5-3) conditions, or by incubating in the presence of ammonium sulphate at about pH 5.
3. Evidence has been obtained that the extracts contain little material capable of inhibiting broad-bean phenolase.
4. The nature of the latent phenolase has not been elucidated but it is suggested that either a phenolase precursor or a phenolase-protein inhibitor complex is present in the leaf extracts.

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