Table 4. *Recovery of tyrosine*

Colour was developed in the standard way in a solution containing 1·6 mg. of casein hydrolysate/ml. The extinction was measured and the solution was then diluted to exactly twice the original volume. Tyrosine concentration of the dilute solution was estimated from the standard curve, and sufficient tyrosine was added to it to double its estimated concentration. Colour was developed in the new solution, and its extinction was measured. Figures are the means of six replicates for each solution, with standard deviations.

	Extinction	Tyrosine concn. (μg./ml.)
(*a*) Original solution	$0{\cdot}378 \pm 0{\cdot}028$	—
(*b*) Diluted solution	$0{\cdot}198 \pm 0{\cdot}022$	12·1
(*c*) Diluted solution with 12·1 μg. of added tyrosine/ml.	$0{\cdot}372 \pm 0{\cdot}028$	—

t for (*a*) – (*c*) = 0·46. Not significant.

the optical density produced by the same concentration of tyrosine in different concentrations of protein hydrolysate (Table 4).

Applications

This reaction has been investigated particularly because of its convenience in the study of tyrosine metabolism. As it measures only tyrosine and *p*-hydroxyphenylpyruvic acid, it gives very good agreement between tyrosine disappearance and oxygen consumption in the rat-liver tyrosine oxidase system described by Knox & LeMay-Knox (1951). The method can be easily applied to the estimation of tyrosine in protein hydrolysates, if precautions are taken against interference by tryptophan. Similar precautions must be taken in the estimation of tyrosine in urine, since urine contains sufficient tryptophan derivatives to inhibit colour development seriously. If the urine is diluted 10- to 20-fold, and internal standards are used in addition, the estimation can be performed without difficulty. An estimation performed on a 24 hr. sample of normal urine by this method gave a figure of 72 mg. of excreted tyrosine/day. This is just within the range of 46–72 mg./day for total (free + conjugated) tyrosine excretion reported by Ulrich, Schropp & Martin (1954), who used a microbiological method of estimation. The use of the present method for the determination of tyrosine in blood appears feasible. Hier & Bergeim (1946) report a value of 15 μg./ml. for free tyrosine in human plasma. Estimations of tyrosine in deproteinized human plasma, trichloroacetic acid being used as suggested by Udenfriend & Cooper (1952), give reasonable values of about 15–20 μg. of tyrosine/ml. of plasma. In one instance a plasma ultrafiltrate was prepared and found to contain the same concentration of tyrosine as the same plasma when deproteinized by trichloroacetic acid.

SUMMARY

1. A simple and reliable method of estimating tyrosine has been developed, based on earlier work, which may be used for the determination of 10–250 μg. of tyrosine in 2–4 ml. samples.

2. The effects of a number of interfering substances have been investigated, and precautions are described for the determination of tyrosine in various biological fluids.

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

2. ACTIVATION BY ANIONIC WETTING AGENTS*

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Kenten (1957) has shown that water extracts of broad-bean leaves contain much latent phenolase. Active phenolase was released by brief exposure of the extracts to acidic (pH 3–3·5) or alkaline (pOH 2·5–3) conditions, or by incubating in the presence of ammonium sulphate at about pH 5. The nature of the latent phenolase was not elucidated but it was suggested that either a phenolase precursor or a phenolase–protein-inhibitor complex was present in the leaf extracts. The present work describes some further properties of the latent phenolase and shows that activation follows treatment with certain anionic wetting agents.

* Part 1: Kenten (1957).

MATERIALS AND METHODS

Estimation of phenolase activity. Phenolase activity was estimated either colorimetrically or manometrically. The manometric method is preferable when crude extracts are used since these have low transparency and are liable to contain reducing materials which prevent the accumulation of the phenolic oxidation product. The manometric method is also more suitable when the oxidation of monophenols is studied, first because the initial step in their oxidation involves *ortho*hydroxylation and so does not immediately give rise to coloured oxidation products, and secondly because the oxidation is preceded by a variable and sometimes prolonged lag period.

The colorimetric method has proved to be the most convenient one with clarified and dialysed leaf extracts. Here the rate of formation of the red initial oxidation product of dihydroxy-DL-phenylalanine (DOPA) is measured with an EEL (Evans Electroselenium Ltd.) colorimeter with a Bright-Spectrum Blue-Green Filter no. 623, maximum transmission at 495 mμ. The reaction mixtures consisted of 3 mg. of DOPA, with or without wetting agent, and dialysed water extract of broad-bean leaf in a total volume of 6 ml., containing 25 mM-citrate at pH 6. The dialysed water extract of broad-bean leaf was added 10–15 sec. before zero time and it was thoroughly mixed in at zero time. The first reading was taken at 20 sec., and readings thereafter at 30 sec. intervals. The increase in light absorption over the period 20–200 sec. was taken as a measure of the phenolase activity. With this method there is a linear relationship between the amount of liberated phenolase and the rate of formation of the oxidation product of DOPA over a wide range of enzyme concentration (Fig. 1).

Manometric reaction mixtures in a total volume of 3 ml. at 25° consisted of 3 mg. of DOPA or 3 mg. of *p*-cresol in 50 mM-citrate at pH 6, with or without wetting agent, in the main vessel. After equilibration, dialysed water extract of broad-bean leaf was added from the side arm. Potassium hydroxide was present in the centre well, the gas phase was air and the temperature 25°.

Plant material and extraction procedure. The broad-bean leaves used were taken from young plants grown in John Innes Compost in a heated greenhouse. The leaves were 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, the extract being collected in a cylinder surrounded by ice and water. The extract was centrifuged in an MSE (Measuring and Scientific Equipment Ltd.) angle-head centrifuge at about 8000 *g* for 15 min. in a cold room at 0–4°. The supernatant was dialysed for 24 hr. at 0–4° against large volumes of buffer, pH 6–6·5, 4 mM with respect to both phosphate and citrate. The dialysed extract was then frozen and thawed twice, clarified by centrifuging at 8000 *g* for 15 min. and stored frozen at − 18°. These dialysed extracts had only a trace of phenolase activity and could be stored frozen for at least a month without appreciable change either in phenolase activity or phenolase activity released by treatment with anionic wetting agents (latent phenolase). Table 1 shows the activities of phenolase and latent phenolase of some of the preparations used in the present work. Under the conditions of estimation of latent phenolase used in Table 1, the amount of liberated phenolase activity is not directly proportional to the O_2 uptake in the first 10 min. of the reaction (Fig. 1). However, the departure from linearity

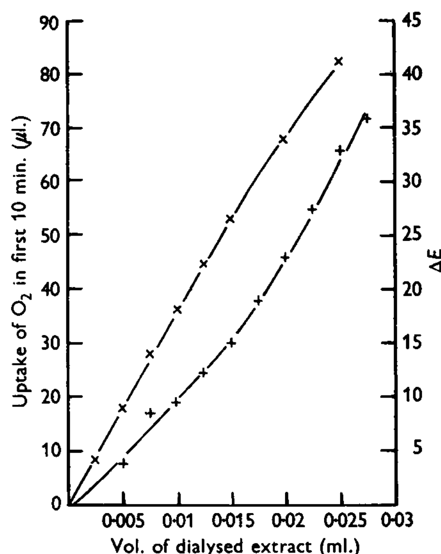

Fig. 1. Effect of varying the amount of dialysed water extract of broad-bean leaf on the oxidation of DOPA in the presence of 4 mM-Aerosol OT at pH 6. +, Manometric measurements of the O_2 uptake; ×, colorimetric measurements of the rate of formation of the oxidation product of DOPA at 21°.

Table 1. *Phenolase and latent phenolase in dialysed water extracts of broad-bean leaf*

Phenolase activity was estimated manometrically with DOPA as substrate in the absence and in the presence of 4 mM-Aerosol OT. The values are corrected for the blank uptakes with heated extract (5 min. at 100°). These blanks were less than 3 µl. of O_2/10 min.

		O_2 uptake in first 10 min. (µl.)		
Dialysed water extract preparation	Total N (mg./ml.)	Phenolase (0·6 ml. of extract in the absence of Aerosol)	Latent phenolase (0·02 ml. of extract in the presence of Aerosol)	Latent phenolase (µl. of O_2/mg. of N/hr. in the presence of Aerosol)
A	0·54	8	30·5	17 100
B	0·71	0	27·0	10 400
C	1·29	0	48·5	11 200
D	0·84	9	58·5	20 900

is not serious and it is considered preferable to express these results in quantitative terms of one of the reactants rather than in the relative colorimetric units. The relationship between the rate of oxidation and amount of enzyme obtained in the manometric experiments of Fig. 1 is similar to that obtained by Adams & Nelson (1938) in manometric studies of mushroom phenolase. Their evidence suggests that this type of relationship may result from a protective action of the protein contained in the preparation on the activity of the phenolase.

Buffers. Phosphate buffers were prepared from solutions of KH_2PO_4 and KOH, citrate buffers from solutions of citric acid and sodium citrate, pyrophosphate buffers from solutions of $K_4P_2O_7$ and $Na_2H_2P_2O_7$.

Measurements of pH. These were made with a glass electrode.

Wetting agents. The commercial wetting agents used were Triton X 100 (an alkyl aryl polyether alcohol, Rohm and Haas Co.) and Lissapol N (an ethylene oxide condensate with substituted phenol or fatty acid, Imperial Chemical Industries Ltd.), both non-ionic, and Aerosol OT 100% (sodium dioctylsulphosuccinate, Hardman and Holden Ltd.), which is anionic. For convenience in plotting on a molar basis and comparison with the other wetting agents, it has been assumed that the last-named compound was pure sodium dioctylsulphosuccinate.

RESULTS

Activation of latent phenolase by treatment with anionic wetting agents

Effect of the nature and concentration of wetting agent. The effect of the presence of different wetting agents on the phenolase activity of dialysed water extracts of broad-bean leaf has been studied manometrically and colorimetrically.

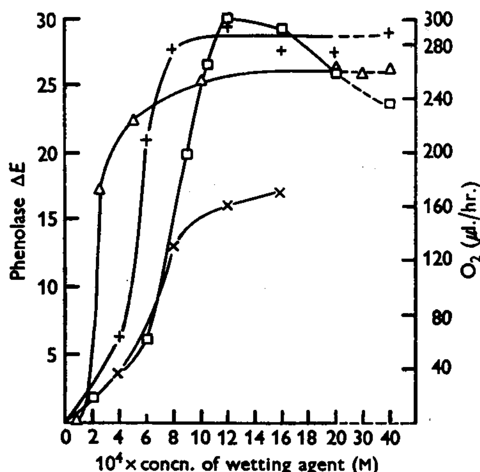

Fig. 2. Effect of different anionic wetting agents on the phenolase activity of dialysed water extract of broad-bean leaf at pH 6. △, Aerosol OT, *p*-cresol as substrate, 0·1 ml. of dialysed extract, activity measured manometrically; +, Aerosol OT; □, sodium dodecyl sulphate; ×, sodium oleate, activity measured colorimetrically with DOPA as substrate at 19–20° with 0·014 ml. of dialysed extract.

The manometric experiments were made with *p*-cresol as substrate and varying amounts of Aerosol OT. With *p*-cresol the O_2 uptake began after a lag period which was variable and sometimes as long as 30 min. In the second hour the rate of O_2 uptake remained practically constant, and it is from measurements in this period that the activities in Fig. 2 have been calculated. The autocatalytic nature of the reaction with monophenol but not with *o*-dihydric phenol is typical of the behaviour of other plant phenolases (e.g. Dawson & Tarpley, 1951).

The results obtained with some of the anionic agents (Fig. 2) show that the phenolase activity increases as their concentration is increased until a maximum is reached; further increase in wetting agent is then either without effect (Aerosol OT) or reduces the activation (sodium dodecyl sulphate). Whereas the shapes of the curves given in Fig. 2 were reproducible, the position relative to the abscissa varied somewhat in different experiments. The maximum activity obtained with sodium dodecyl sulphate (SDS) was about the same as that obtained with Aerosol OT. In the experiments with sodium oleate some oleic acid came out of solution at pH 6 and decreased the transparency of the solution. For this reason it was not possible to study the effects of high concentrations of sodium oleate by the colorimetric method.

A number of other wetting agents were tested under the conditions of Fig. 2 at 0·5, 3 and 20 mM, where the molecular weight was known, and at about 0·2, 1·3 and 8 g./l. where this was unknown. No activation was observed either with the cationic wetting agents cetyltrimethylammonium bromide (CTAB) and cetylpyridinium bromide, or with the nonionic agents saponin, Triton X 100 and Lissapol N.

When the dialysed extract was heated for 3 min. at 100° before testing, no phenolase activity was detected in the presence of the anionic wetting agents.

Effect of chain length on the activating capacity of sodium n-*alkyl sulphates.* The activating effect of a number of sodium *n*-alkyl sulphates has been studied with the results shown in Fig. 3. The experiments were made by the colorimetric method with DOPA as substrate; a single batch of dialysed water extract of broad-bean leaf was used throughout. No activation was found when the dialysed extract was heated for 5 min. at 100° before testing. When sodium butyl sulphate was tested under the conditions of Fig. 2 a slight activating effect was found at about M concentration. Tests showed that the presence of high concentrations of sodium butyl sulphate inhibited the phenolase activity

towards DOPA; at 2M inhibition was complete, at M about 70% whereas at 0·1M there was no appreciable effect. These tests were made with a dialysed water extract, previously activated by treatment with Aerosol OT, as enzyme. Accordingly, the curve given in Fig. 3 was obtained by mixing the enzyme extract with sodium butyl sulphate before testing for phenolase activity. To 0·8 ml. of solution containing varying amounts of sodium butyl sulphate in 40 mM-citrate buffer, pH 6, 0·1 ml. of dialysed extract was added and immediately after mixing a 0·1 ml. sample was withdrawn and the phenolase activity estimated colorimetrically, as described previously, in a mixture containing 3 mg. of DOPA in a total volume of 6 ml., and containing 25 mM-citrate, pH 6. In this way, the final concentration of sodium butyl sulphate in the colorimetric-reaction mixtures was kept below 0·05M. When SDS was tested in this way by mixing with the dialysed extract immediately before estimating phenolase a plot of the phenolase activity against wetting-agent concentration gave a curve closely resembling that obtained when the wetting agent was present in the colorimetric-reaction mixtures, but its position was shifted to the right so that the maximum activity was reached at a wetting-agent concentration some four times higher. It will be shown later (Table 2) that this effect is probably due to the fact that the ratio of the volume of extract to that of wetting agent is much higher when these are mixed before testing than when the wetting agent is present in the colorimetric-reaction mixtures. Since 0·1M-sodium butyl sulphate does not significantly inhibit the phenolase activity and tests with this concentration in the colorimetric reaction mixtures failed to activate, it must be concluded that the activation curve of sodium butyl sulphate comparable with those of the other alkyl sulphates lies above 0·1M but at a somewhat lower region than that shown in Fig. 3.

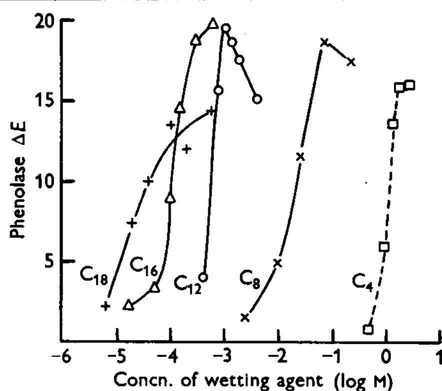

Fig. 3. Effect of sodium *n*-alkyl sulphates on the phenolase activity of dialysed water extract of broad-bean leaf at pH 6. +, Octadecyl; △, cetyl; ○, dodecyl; ×, octyl; activity measured colorimetrically with 0·011 ml. of extract under the conditions of Fig. 2. □, Butyl, activity measured as described in the text. DOPA as substrate. Temp., 18–19·5°.

The results suggest that over the range of chain length of 4–18 carbon atoms the concentration of sodium *n*-alkyl sulphate required to produce a given amount of activation of the latent phenolase decreases with increase in chain length. With the largest amount of sodium cetyl sulphate and the larger amount of sodium octadecyl sulphate all of the wetting agent was not in solution. The low solubility of sodium octadecyl sulphate is probably responsible for the anomalous shape of its activation curve.

Effect of time of exposure of the dialysed extract to wetting agent. Whereas the release of active phenolase from the dialysed extracts by the anionic wetting agents is a rapid process at relatively high concentrations of wetting agent, as the concentration decreases the rate of liberation of phenolase decreases.

At room temperature, 0·5 ml. of dialysed water extract of broad-bean leaf was added to 4·5 ml. of 15 mM-citrate containing varying amounts of SDS

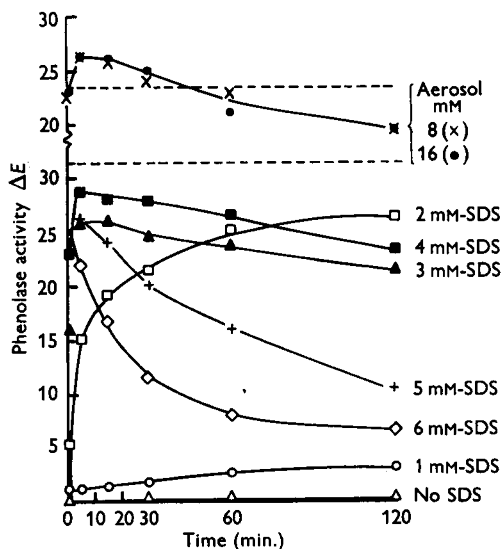

Fig. 4. Effect of time of exposure of dialysed water extract of broad-bean leaf to sodium dodecyl sulphate and Aerosol OT at pH 6 on the phenolase activity. Mixtures containing dialysed extract and wetting agent were maintained at 19–20·5° and the phenolase activity was estimated colorimetrically with DOPA at 19–20·5° at various times. ×, ●, Aerosol OT. △, ○, ◇, +, □, ▲, ■, sodium dodecyl sulphate. The horizontal broken lines show the activities obtained when the dialysed extract was added directly to the colorimetric reaction mixtures containing the wetting agent at 1·5 mM.

or Aerosol OT, pH 6. Immediately after mixing and again at later times, 0·15 ml. portions were taken and the phenolase activity was estimated colorimetrically with DOPA as substrate as described previously. A typical set of results is shown in Fig. 4. They show that the rate of release of active phenolase depends on the concentration of SDS. In the absence of SDS the increase in phenolase activity over a 3 hr. period is barely measurable. With increase in SDS there is an increase in the rate of release of active phenolase. In the experiment of Fig. 4 at a concentration of 4 mM-SDS or greater it is practically complete within 1 min. of mixing. The released phenolase is, however, unstable in these relatively high concentrations of SDS. This can be seen from the rapid loss of the released phenolase activity which takes place in the mixtures containing 5 and 6 mM-SDS. With Aerosol OT the results were similar to those obtained with SDS with the exception that high concentrations of Aerosol did not bring about a rapid loss of the liberated activity. The highest activities obtained by mixing the dialysed extract with SDS before testing were generally a little less than when the dialysed extract was added directly to colorimetric reaction mixtures containing SDS. The reverse was generally found with Aerosol OT (e.g. Fig. 4). The same pattern of response of the dialysed extracts to SDS and Aerosol OT was seen in different experiments but the response to a given concentration of agent varied slightly. This was most likely due to variation in the amounts and composition of the dialysed extracts used. In these experiments, the concentration of wetting agent which gave the maximum phenolase activity was higher than that required when the wetting agent was added directly to the colorimetric reaction mixtures. This may be because under the former conditions the volume of extract relative to that of the agent is much greater. It is shown in Table 2 that increasing the volume of dialysed extract diminishes the effect of both SDS and Aerosol OT.

The results of these experiments suggested that the negative results with the cationic and non-ionic wetting agents which were obtained in the previous experiments could have arisen if these agents are capable of liberating phenolase only at a slow rate. Accordingly CTAB and Triton X 100 were again tested. Mixtures containing CTAB at 0·5, 3 or 20 mM or Triton X100 at 0·2, 1·2 and 8 g./l. final concentration and 0·5 ml. of dialysed water extract in a total volume of 5 ml., containing 20 mM-citrate, pH 6, were made up at room temperature and tested colorimetrically for phenolase activity. They were tested again after standing at room temperature for 10 min., 1 hr. and 3 hr. No measurable phenolase activity was found with any of these mixtures. In control mixtures with Aerosol OT at the same concentration as CTAB and made at the same time, large amounts of phenolase activity were found.

Effect of wetting agents on activated broad-bean phenolase. The failure to demonstrate activation of the latent phenolase in the dialysed water extracts by treatment with the cationic and non-ionic wetting agents could be explained if these agents inhibit or destroy active phenolase. Accordingly, the effect of these agents on an activated broad-bean phenolase preparation has been tested. It has been shown previously (Kenten, 1957) that when the precipitate obtained by saturating a water extract of broad-bean leaves with $(NH_4)_2SO_4$ is suspended in water and stored at 0°, the latent phenolase which it contains becomes active. For the tests described below a preparation corresponding to the second $(NH_4)_2SO_4$ precipitate of Kenten (1957) was aged at 0° in the presence of toluene for 6 weeks.

Table 2. *Effect of varying the volume of dialysed water extract of broad-bean leaf on the activation of phenolase by sodium dodecyl sulphate and Aerosol OT*

Mixtures containing varying amounts of dialysed water extract of broad-bean leaf and 2 mM-wetting agent in a total volume of 5 ml. of 15 mM-citrate, pH 6, were made up and kept at room temperature (20–21° with SDS, 16–16·5° with Aerosol OT). Samples equivalent to 0·015 ml. of dialysed extract in the experiment with SDS and 0·025 ml. in that with Aerosol were withdrawn at various times and the phenolase activity was estimated colorimetrically at room temperature with DOPA at pH 6. Different batches of dialysed extract were used in the two experiments. The amount of wetting agent carried over into the colorimetric reaction mixtures in the samples was insufficient to bring about appreciable activation of dialysed extract.

	Phenolase activity					
	SDS			Aerosol OT		
Extract (ml.) ...	0·1	0·5	1·5	0·2	0·8	3·2
Time of exposure (min.)						
0·5	19·0	5·0	1·0	25·0	20·0	3·5
5	24·0	11·5	1·0	32·0	26·5	3·5
15	20·5	13·5	1·0	31·5	27·5	4·5
60	12·0	19·0	1·5	21·0	26·0	8·0

The colorimetric method of estimating phenolase activity was used, the conditions being similar to those of Fig. 2. The concentration of wetting agent in the reaction mixtures was either 3 mM or 1·3 g./l. Under these conditions the rate of oxidation of DOPA was inhibited by 55–60 % in the presence of saponin, about 10 % in the presence of SDS and less than 10 % in the presence of Aerosol OT, Lissapol N, CTAB or cetylpyridinium bromide. With the exception of saponin, it is therefore unlikely that the failure to obtain activation with the non-ionic and cationic wetting agents was due to inhibition of active phenolase.

Effect of pH *on the capacity of Aerosol OT and sodium dodecyl sulphate to activate the latent phenolase of dialysed water extract of broad-bean leaf.* It has been shown (Kenten, 1957) that much active phenolase is liberated from extracts of broad-bean leaf by brief exposure to mildly acid or alkaline conditions (pH 3·5 or 11·5). In phosphate–citrate or pyrophosphate buffers over the range pH 5·5–8·5 there is little or no increase in the phenolase activity of a dialysed water extract of broad-bean leaf at about 20° during 2–3 hr. Furthermore, under these conditions there appears to be little loss of latent phenolase. Hence the effect of pH on the capacity of the anionic wetting agents to activate the latent phenolase can be studied over the range pH 5·5–8·5 without appreciable activation taking place in the absence of wetting agent.

In the experiments with Aerosol OT, mixtures containing 6 mM-Aerosol OT and 0·8 ml. of dialysed water extract of broad-bean leaf in a total volume of 5 ml., containing 10 mM-pyrophosphate buffer, were made up at various pH values and kept at room temperature (18–19°). The phenolase activity of 0·1 ml. samples of the mixtures was estimated colorimetrically with DOPA as substrate at different times. After 2 hr. latent phenolase plus active phenolase was estimated by taking 0·1 ml. samples into 4·9 ml. of 30 mM-citrate containing 5 mM-Aerosol OT, pH 6. Time (5 min.) was allowed for activation of the latent phenolase and then 1 ml. of DOPA (3 mg.) was added and the rate at which it was oxidized was followed colorimetrically and taken as a measure of latent plus active phenolase.

With sodium dodecyl sulphate a different batch of dialysed extract was used. At the pyrophosphate and wetting-agent concentrations used in the experiments with Aerosol OT a small crystalline precipitate formed in the mixtures at about pH 8. If the pyrophosphate concentration was decreased to 4 mM and the sodium dodecyl sulphate to 3 mM no precipitate formed at about pH 8, and these conditions were used in the experiments. In a total volume of 5 ml., 1 ml. of dialysed water extract was present and 0·15 ml. samples were withdrawn for phenolase estimations. At the end of the experiment, latent plus active phenolase was estimated in the same way as in the Aerosol OT experiments except that the sodium dodecyl sulphate concentration was 1·7 mM.

The results (Table 3) show that as the pH increases from 5·9 to 8·35 the rate of activation of the latent phenolase by the wetting agent decreases. The effect was more marked with Aerosol OT than with sodium dodecyl sulphate. Under the conditions used at about pH 8 the amount of activation by Aerosol OT is barely measurable in a 2 hr. period, whereas at pH 6 activation is virtually complete in 10 min. Such results could be obtained if the latent phenolase itself or the active phenolase derived from it were unstable at about pH 8. These possibilities are, however, excluded by the results of the estimations of latent plus active phenolase at the end of the experiments. These results show that after the 2 hr. incubation period

Table 3. *Effect of* pH *on the capacity of Aerosol OT and sodium dodecyl sulphate to activate the latent phenolase of dialysed water extract of broad-bean leaf*

Phenolase activity of mixtures containing dialysed water extract and Aerosol OT (6 mM) or SDS (3 mM) in pyrophosphate buffer was followed colorimetrically. Latent + active phenolase was estimated after the 2 hr. incubation period by treatment of samples of the mixtures with wetting agent at pH 6 before measuring their activity towards DOPA. Temperature 18–19°.

	Phenolase activity											
	With Aerosol				Without Aerosol				With SDS		Without SDS	
pH ...	5·9	6·8	7·65	8·35	5·9	6·8	7·65	8·35	5·9	8·25	5·9	8·25
Time of exposure (min.)												
0·5	22·5	2·0	0·5	0·5	0	0	0	0	24·0	1·0	0	0·5
10	30·0	2·0	1·0	1·0	—	—	—	—	26·0	2·0	—	—
30	31·5	5·0	0·5	1·0	—	—	0	0	24·5	2·5	—	—
60	30·0	6·0	1·0	1·0	0	0	0	0	22·5	3·5	—	0
120	29·5	8·0	1·5	1·5	0	0	0	0	16·5	4·5	0	0
Latent + active phenolase at 120 min.	33·5	30·5	29·5	29·5	31·0	30·0	29·5	28·0	12·0	22·0	19·0	22·0

almost as much phenolase can be liberated from the alkaline mixtures by treatment with wetting agent at pH 6 as that initially liberated in the mixtures at pH 6.

DISCUSSION

The results of the present work show that the latent phenolase of dialysed water extracts of broad-bean leaves can be activated by treatment with certain anionic wetting agents at pH 6. With the homologous series of sodium *n*-alkyl sulphates tested the concentration at which activation took place decreased with increase in chain length. Two properties of this series of compounds which appear to be especially relevant to the activation phenomenon are their capacity to form micelles and to combine with proteins. The critical concentration at which transition from simple strong electrolytes to micelles takes place with the sodium *n*-alkyl sulphates has been shown to decrease with increase in chain length from 12 to 18 carbon atoms (Powney & Addison, 1937). The critical concentration of micellar formation for SDS at about 20° is in the neighbourhood of 6 mM. With increase in salt concentration there is a steady decrease in the critical concentration, which at about 0·1 M-Na^+ ion concentration reaches a value of about 1·5 mM (Powney & Addison, 1937; Corrin & Harkins, 1947). The Na^+ ion concentration was less than 0·1 M in the experiments with SDS and in some activation of the latent phenolase was half maximal at 0·8 mM, and measurable activation was found at 0·2 mM, a concentration well below the critical point. Furthermore, the critical concentration of micellar formation for SDS is unaffected by variation in pH over the range 4·5–12 (Powney & Addison, 1937), whereas a change in pH from 6 to 8 greatly reduces the activation of latent phenolase by SDS. Therefore, although it is conceivable that the pronounced solvation properties of micelles could be connected with the activation of the latent phenolase, the results appear to exclude such a mechanism.

Kenten (1957) has discussed the reasons for believing that the known properties of the latent phenolase of extracts of broad-bean leaf are most readily explained by assuming that in the extracts there is either a phenolase–protein-inhibitor complex of the type described by Swartz, Kaplan & Frech (1956), in which the components are bound through interaction of ionic groups, or a phenolase precursor (prophenolase). It is generally thought that the ionic wetting agents produce their effects on proteins by combining with them primarily through electrostatic forces. With the anionic wetting agents there is much evidence that combination takes place at the cationic sites on the protein molecule with accompanying alteration of the net electrical charge (Klotz, 1953; Putnam, 1948). Therefore a phenolase–inhibitor complex held together by salt linkages might be expected to dissociate after combination with an anionic wetting agent. With a prophenolase such combination might permit configurational changes in the molecule which unmask the catalytic centre. In this connexion, it is of interest that the results of studies of the combination of albumin with large anions (Karusch, 1952) or SDS (Neurath & Putnam, 1945) suggest that unfolding or expansion of the protein molecule takes place as the number of bound ions increases.

The experimental results are in agreement with the suggestion that the anionic wetting agents activate through their capacity to combine with proteins. In the first place, no activation could be demonstrated with the non-ionic wetting agents tested. Secondly, it is known that the affinity of anions for proteins increases with the size of the anion (Steinhardt, Fugitt & Harris, 1942; Boyer, Ballou & Luck, 1947). This has been demonstrated with a variety of proteins and anions and in particular with octyl, decyl and dodecyl sulphates and serum albumin (Karusch & Sonenberg, 1949). Thus it would be expected that with increase in chain length the concentration of alkyl sulphate required to activate the latent phenolase would decrease.

It might be expected that a shift in pH from 6 to 8 would decrease the activation brought about by the anionic wetting agents since the net negative charge on a protein increases with increase in pH value. However, systems that have been tested experimentally do not all conform with this expectation. For example, although Boyer *et al.* (1947) found that the amount of sodium caprylate bound by albumin decreased with increase in pH from 6·8 to 11·8, Klotz, Burkhard & Urquhart (1952) found no pronounced change in the amount of methyl orange or azosulphathiazole anions bound by albumin over the range pH 6–9. If in fact activation of the latent phenolase does depend on combination of the anions at the cationic groups of proteins then the fact that a shift in pH value from 6 to 8 brings about such a large decrease in the amount of activation suggests that the iminazolium groups (p*K* 5·6–7·0; Cohn & Edsall, 1943) and terminal α-ammonium groups (p*K* 7·6–8·4; Cohn & Edsall, 1943) may be the sites at which combination takes place.

There is also diverse behaviour of protein towards the cationic wetting agents. These, through their capacity to combine at the negative sites on protein (e.g. Klotz, 1953), also might be expected to activate the latent phenolase. No evidence that they brought about any activation was obtained. Although Steinhardt & Zaiser (1950) have shown

that both cations and anions of about equal size are bound by wool, the failure of the cationic agents to activate latent phenolase would be consistent with the observation of Klotz, Gelewitz & Urquhart (1952) that organic cations of a wide variety of structures do not form complexes with serum albumin with an affinity even approaching that of complexes with anions of similar structure. Thus while the present results support the suggestion that the anionic wetting agents activate the latent phenolase by their ability to combine with proteins they do not help to decide whether a phenolase–inhibitor complex or a prophenolase is involved.

The latent phenolase of diapause grasshopper eggs is similar to that of extracts of broad-bean leaves in that activation follows treatment of it with anionic wetting agents such as Aerosol OT, sodium oleate and the long-chain alkyl sulphates (Bodine & Allen, 1938; Allen, Otis & Bodine, 1943; Bodine, Tahmisian & Hill, 1944; Bodine & Hill, 1945). The nature of the latent phenolase of grasshopper eggs has not yet been elucidated, but its properties are also consistent with the presence of either a phenolase–inhibitor complex or a prophenolase.

In the present work the maximum phenolase activities released by treatment of the bean-leaf extracts with Aerosol OT and sodium cetyl, dodecyl or octyl sulphates were about the same. It is not known whether these treatments do in fact bring about the activation of all of the latent phenolase present in the extracts. However, the results of preliminary tests suggest that treatment of the extracts with these anionic wetting agents leads to the release of about the same or a greater amount of phenolase than does activation by exposure to acid or alkaline pH or proteolytic enzymes (Kenten, 1957). This suggests that treatment of the extracts with the anionic wetting agents gives the best measure of latent phenolase. Furthermore, Aerosol OT is the preferred activating agent since it does not show the toxic effects at relatively high concentration found with alkyl sulphates.

SUMMARY

1. The latent phenolase of extracts of broad-bean leaf is activated by treatment with anionic wetting agents at pH 6. A number of cationic and non-ionic wetting agents were without effect.

2. With the homologous series of sodium *n*-alkyl sulphates tested, the concentration at which activation of the latent phenolase took place decreased with increase in chain length.

3. At pH 8 the rate of activation with sodium dodecyl sulphate and Aerosol OT was very slow or negligible compared with that at pH 6.

4. It is suggested that the anionic wetting agents activate through their capacity to combine with the cationic groups of protein. Dissociation of a phenolase–protein-inhibitor complex or configurational changes in a prophenolase might follow such combination.

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