A Colorimetric Method for the Estimation of Tyrosine

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A considerable need exists for a method of estimating tyrosine. The method first described by Gerngross, Voss & Herfeld (1933), which depends on the reaction between tyrosine and 1-nitroso-2-naphthol in the presence of an oxidizing agent (usually nitric acid), appears to meet the requirements. The red colour which is the result of the reaction is, however, destroyed by light, and neither the original method nor a later adaptation by Thomas (1944) overcomes this disadvantage. The colour fades sufficiently rapidly for the reaction to be unsuitable as a routine method. Maciag & Schoental (1938) were able to stabilize the colour by adding iron alum to the solution, but this reduced the sensitivity of the method considerably; the conditions which they describe are also not the best for accurate work. Udenfriend & Cooper (1952) allowed the red-coloured compound to be further oxidized by nitric acid to a stable yellow pigment, excess of nitrosonaphthol then being extracted with a water-immiscible solvent. A considerable part of the reaction product from tyrosine is extracted by the solvent, and the pigment formed from compounds not containing an amino group, such as *p*-hydroxyphenylpyruvic acid, is completely soluble in the solvent and hence cannot be estimated. This method is more complicated than that to be described.

In view of the simplicity and sensitivity of the Gerngross reaction, factors controlling the intensity of colour development, and the stability of the colour, have been investigated. As a result a method for the estimation of tyrosine has been developed, which can be used for the analysis of solutions containing as little as 1 μg./ml. A preliminary report of the method has been made (Ottaway, 1957).

Ceriotti & Spandrio (1957), who have very courteously allowed me to see their manuscript after the present paper had been submitted for publication, have also re-examined the nitrosonaphthol reaction. Apart from the method of stabilizing the colour, the conclusions reached by Ceriotti & Spandrio with regard to reagent concentrations and general conditions were very similar to those described here.

MATERIALS AND METHOD

L-Tyrosine was obtained from Roche Products Ltd. (Welwyn Garden City, Herts.). 1-Nitroso-2-naphthol was AnalaR grade; it was not recrystallized before use, but solutions in ethanol or acetone were filtered before making up to volume. Protein hydrolysates were Hydrolysed Casein, Allen and Hanbury Ltd., and Casein Hydrolysate, B.D.H. Ltd.

For general work an EEL colorimeter (Evans Electroselenium Ltd., Harlow, Essex) was used. The absorption spectrum of the reaction product was determined with a Unicam SP. 600 spectrophotometer (Cambridge Instrument Co.).

The method of colour production used in the experiments described in this paper was as follows. The nitrosonaphthol reagent was added to 4 ml. of the solution containing tyrosine in a test tube (6 in. $\times \frac{5}{8}$ in.). Conc. HNO_3 was added from a dropping pipette so that the drops fell through the solution and did not touch the walls of the tube. The solution was then placed in a water bath at a selected temperature, care being taken not to agitate the tube and so disturb the HNO_3 at the bottom. At the end of a selected time, the solution, which now showed a red ring at the bottom, was swirled vigorously and returned to the bath for a further time, usually the same as the first, to complete colour development. This procedure was found to give very satisfactory results. The directions of Maciag & Schoental (1938), which are described under 'Recommended procedure', are simpler and give equally good results.

RESULTS

Factors controlling development of colour

Temperature. In Fig. 1 are shown the effects on colour intensity of varying the temperature to which the solution was heated, and also the total length of time for which it was incubated. The colour is destroyed by heating with HNO_3, particularly at 100°. Thus although colour development was very quick when the tubes were heated in a boiling-water bath, full colour was never obtained, nor was it possible to get reproducible results. At temperatures lower than 60°, colour development was very slow; at 40° it required more than 1 hr. After incubating at 100° and 80°, more of the colour was preserved if the tubes were cooled quickly in cold water (see Fig. 1), but at 60° the difference between air-cooling and water-cooling was very slight.

Concentration of acid. Colour intensity increases with nitric acid concentration up to a maximum value. For example, with 100 μg. of tyrosine per tube, optical density rose from 0·015 with 1 drop of conc. HNO_3 to 0·435 with 5 drops; 6 drops per tube produced an optical density of 0·410. Since a large excess of acid destroyed the colour quite rapidly, the minimum volume required for full colour development was chosen. This was about 1 drop (0·022 ml.) of conc. HNO_3/ml. of tyrosine solution, which means that the solution after mixing was approx. 0·35 M with respect to HNO_3.

The effect of acidifying the solution with acids other than nitric, before colour development, was tested. Mineral acids were found to be without effect up to M-concentrations (cf. Ceriotti & Spandrio, 1957). Some complex acids, in high concentrations, inhibited colour development, and these are discussed in the section on 'Interfering substances'.

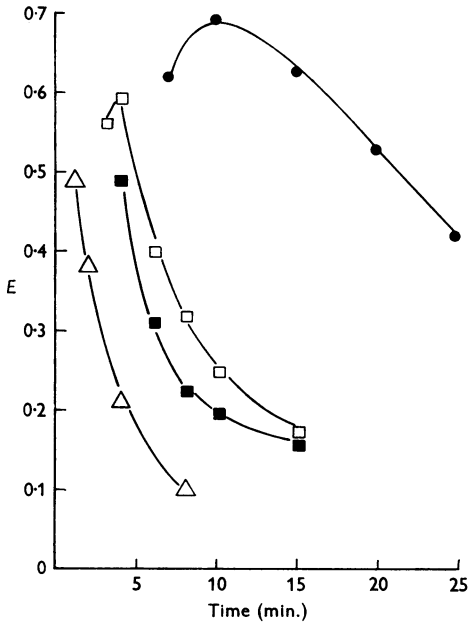

Fig. 1. Effect of temperature and heating time on colour formation and extinction. ●, 60°; □, 80°, water-cooled; ■, 80°, air-cooled; △, 100°.

Ethanol. Ethanol was found to reduce the colour intensity considerably. It may inhibit colour development, but it is probable that it also causes the colour to fade more rapidly, as the following experiment shows. Colour was developed in tubes containing 100 μg. of tyrosine by the addition of 0·03 ml. of 1 % nitrosonaphthol in ethanol, and to the cooled solutions were added varied volumes of ethanol, with sufficient water to make the total volume up to 8 ml. The rate of colour fading ranged from 21 %/hr. with no added ethanol, to 100 % with 2 ml. of added ethanol (see also Fig. 3).

Ethanol and other alcohols were judged to be unsuitable solvents for the nitrosonaphthol reagent. Acetone impedes colour development to a much less extent than ethanol and, if added after development was complete, was found not to increase the rate of fading at all.

Concentration of nitrosonaphthol. The effect on colour intensity of varying the amount of nitrosonaphthol reagent added to 4 ml. of tyrosine solution is shown in Table 1. The optimum amount of reagent varied according to the amount of tyrosine to be estimated; with 250 μg. of tyrosine, maximum colour development required the addition of 1000 μg. of nitrosonaphthol. Even with this amount of reagent, Beer's Law was never obeyed as Table 1 shows, and a great disadvantage was that a white haze formed in the solutions on cooling. This was evidently some insoluble oxidation product of the reagent (since it was most intense in the blank), although its colour showed that it was not nitronaphthol. It did not form if less than 250 μg. of nitrosonaphthol was added to 4 ml. of tyrosine sample. This was hardly enough to ensure adequate sensitivity, so the quantity finally decided on was 300 μg./sample.

The haze which formed when this quantity of reagent was used was a very finely divided precipitate and not easy to centrifuge down. It was found more convenient to remove it by adding a suitable solvent, such as acetone, while diluting the solutions to a convenient volume for the colorimeter.

Table 1. *Effect of nitrosonaphthol concentration on colour intensity*

Volume of solution in each tube was 4 ml. The nitrosonaphthol added was dissolved in 0·1 ml. of acetone. After the addition of 4 drops of conc. HNO_3 to each tube, colour development was carried out for 12 min. at 60°. Each tube was read in the colorimeter against a blank containing the same amount of nitrosonaphthol. Figures represent optical densities.

	Optical density					
Nitrosonaphthol added (mg.) ...	0·1	0·2	0·3	0·4	0·7	1·0
Tyrosine/tube (μg.)						
100	0·390	0·428	0·496	0·498	0·502	0·492
250	0·568	0·744	0·790	—	0·880	0·900

Table 2. *Effect of inorganic salts on colour stability*

Each tube contained 100 μg. of tyrosine and colour development was carried out for 12 min. at 60°. The salts were introduced either before or after colour development, by the addition of varying volumes of M-salt solution. The total volume was made up to 4 ml. before development, or 8 ml. after. Figures represent the optical densities when the samples were read against the appropriate blanks, and the values in the first half of the Table are the means of six observations.

Time after completion of colour development (min.)	Optical density 10	20	40	60	120	Percentage drop in intensity, 20–60 min.
Addition before or after development						
None	0·420	0·396	0·372	0·344	—	13
1 ml. of NaCl; after	0·414	0·402	0·400	0·392	0·368	2
1 ml. of NaCl, 1 drop of $FeCl_3$; after	0·422	0·414	0·414	0·406	0·390	2
1 ml. of NaCl; before	0·470	0·450	0·450	0·440	—	2
2 ml. of NaCl; before	0·440	0·430	0·424	0·410	—	4·5
3 ml. of NaCl; before	0·380	0·390	0·362	0·348	—	4·5
0·5 ml. of Na_2SO_4; before	0·494	0·460	0·440	0·420	—	8·5
1 ml. of Na_2SO_4; before	0·340	0·320	0·312	0·300	—	6

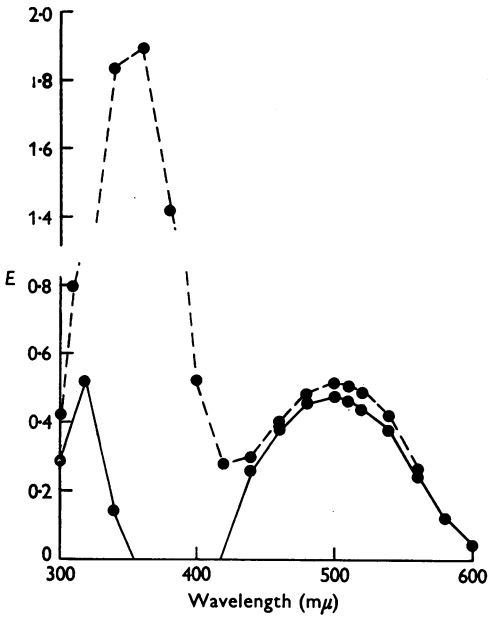

Fig. 2. Absorption spectrum of pigment, developed from 100 μg. of tyrosine and 300 μg. of 1-nitroso-2-naphthol in a final volume of 8 ml. The blank contained nitroso-naphthol: stabilized with acetone and NaCl (continuous line); stabilized with acetone, NaCl and $FeCl_3$ (broken line).

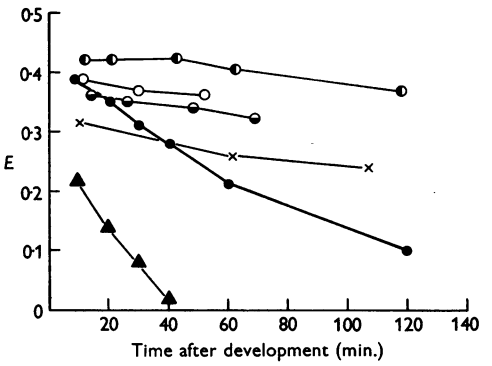

Fig. 3. Effect of various substances on the stability of the red colour developed by 100 μg. of tyrosine. Each addition was made after colour development was complete. ●, Acetone; ○, acetone, tubes kept in dark; ◒, acetone, 0·125M-NaCl; ◐, acetone, 0·125M-NaCl, 2 drops of $FeCl_3$ soln.; ×, acetone, 1% potassium ferricyanide; ▲, 25% ethanol.

Characteristics and stability of the colour

The red pigment obtained during the reaction has the absorption spectrum shown in Fig. 2 (full line). There is a sharp peak with a maximum at 320 mμ and a broader peak with a maximum at 500 mμ. The latter band was used for the estimation of tyrosine. Beer's Law was obeyed only in the range 0–80 μg. of tyrosine, even with monochromatic light (see also Maciag & Schoental, 1938).

The pigment is destroyed by light at a rate sufficiently fast to make quantitative application of the method difficult (Table 2 and Fig. 3). In the conditions used here the loss of intensity is about 20%/hr. In the dark the colour is quite stable (Fig. 3). Gerngross *et al.* (1933) and Thomas (1944) describe procedures which make allowance for the fading colour, but neither is very suitable for routine use. It seemed likely that destruction of the pigment was due to light absorbed in the band centred at 320 mμ, and various compounds with a strong absorption in this region were tried as stabilizers. The addition, after colour development, of an equal volume of 1% potassium ferricyanide was found to be effective, but it absorbed enough light at 500 mμ to reduce the sensitivity of the method considerably. The same objection applies to the addition of an equal volume of saturated

ferric ammonium sulphate, which was recommended by Maciag & Schoental.

It was observed by chance that the colour faded much less rapidly if the solutions to be analysed contained NaCl. Further investigation showed that the addition to the solution, after colour development, of an equal volume of 0·5 M-NaCl was as effective in stabilizing the pigment as ferricyanide. No reason was found why this should be so. It was observed that the salt was slightly more effective if added before colour development, although concentrations above M diminished the colour, and that the effect was temporary, as slow fading began about an hour after adding the salt solution. Sodium chloride was found to be very much more effective than an equimolar concentration of Na_2SO_4. This last observation appeared to rule out the possibility that stabilization was a result of reducing the concentration of dissolved oxygen. It is noteworthy that Ceriotti & Spandrio (1957) were able to produce a stable pigment by carrying out the reaction in approx. 2 N-HCl. It is possible that this is due to the presence of Cl^- ions, rather than to pH.

The addition, after colour development, of a few drops of 0·5 M-$FeCl_3$ appeared to give stabilization for a rather longer period than NaCl alone. Ferric chloride by itself was ineffective at this concentration. The intense absorption of what is presumably the undissociated Fe_2Cl_6 molecule in the region of 300 mμ is shown in Fig. 2 (broken line).

Recommended procedure

In the light of the observations described, the following procedure is suggested for the estimation of tyrosine in solutions which do not contain interfering substances (for a discussion of these, see below).

Reagents. 0·3% 1-Nitroso-2-naphthol in acetone: the solution is filtered before making up to volume. It is stable indefinitely, but must be protected from evaporation.

Concentrated nitric acid.

Stabilizing reagent: the precise composition of this solution may be varied, according to the final volume of sample which is required. For the volumes described below, the solution was made up as follows. To 50 ml. of M-NaCl was added 75 ml. of acetone, and the mixture was made up to 300 ml.; 5 ml. of 0·5 M-$FeCl_3$ was added immediately before use. (A precipitate, probably of the double salt of $FeCl_3$ and NaCl, develops if the complete solution is allowed to stand overnight.)

Standard tyrosine solution: 25 mg. of tyrosine is dissolved in a few millilitres of boiling water, and this solution is quickly diluted to 100 ml. It is stable for several weeks if kept in the refrigerator.

Procedure. To 2 ml. of solution, containing 10–200 μg. of tyrosine, is added 0·1 ml. of nitrosonaphthol reagent. The solutions are mixed and placed in a water bath at 60° for 5 min., or until temperature equilibrium has been reached. The solution is then removed from the bath and 4 drops of conc. HNO_3 are added from a dropping pipette. Since, as shown above, the amount of nitric acid added affects the amount of colour developed, it is as well always to use the same pipette. Colour development starts almost immediately. The solution is returned to the water bath for 1 min. It is then removed, mixed by swirling, and allowed to cool in air for about 5 min., after which the stabilizing reagent is added. A volume (6 ml.) of the stabilizing reagent described above was usually added to the cooled solution, but the composition of this reagent and the volume to be added may be altered as convenient, so long as the final solution contains 0·12 M-NaCl and 25% of acetone. Colour intensity is best read within 10–45 min. after adding the stabilizing reagent, with an OB2 (blue) filter (Evans Electroselenium Ltd.). A reagent blank and suitable standards, prepared by diluting the stock tyrosine solution, are run at the same time.

Interfering substances

Fe^{2+}, Co^{2+} and Ni^{2+} ions interfere by forming coloured complexes with nitrosonaphthol. Fe^{3+} ions do not seriously interfere unless they are present in high concentrations, when their effect may be lessened by acidifying the solution with dilute HCl before adding the colour reagent. Cu^{2+} ions in low concentrations (below 0·01 M) do not interfere.

Of the protein-precipitating reagents, tungstic and molybdic acids interfere seriously, particularly if phosphate is also present. The effect of ethanol has been described above. Trichloroacetic acid is without effect on the reaction.

Strong reducing agents, such as cysteine or ascorbic acid, inhibit colour development. They are best removed by warming the solution to 60° with a drop of conc. HNO_3 before adding the colour reagent. Glucose does not interfere in concentrations up to 0·02 mg./ml., if colour development is

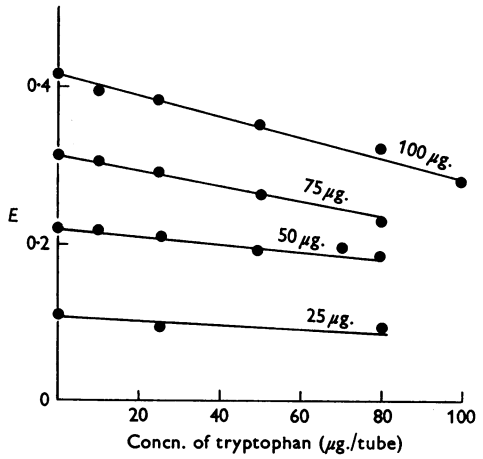

Fig. 4. Inhibition of colour production by tryptophan. Tyrosine concentration was 25, 50, 75 or 100 μg./tube.

Table 3. *Reproducibility of the reaction in protein hydrolysate solutions*

Regression coefficients were calculated from the data below for the following solutions: (1) pure tyrosine in water; (2) a 3 mg./ml. solution of a casein hydrolysate containing about 0·1 % of tyrosine, to which were added amounts of tyrosine varying from 5 to 20 μg./ml.; (3) a solution of a casein hydrolysate estimated to contain 1·65 % of tyrosine. Colour development was carried out in the standard way; the total volume in each tube before development was 4 ml. Figures are the means of four replicates at each concentration.

(1) Standard tyrosine solution

Tyrosine concn. (x) (μg./ml.)	5	10	15	20	25
Optical density (y)	0·088	0·180	0·246	0·316	0·396

(2) Low-tyrosine casein hydrolysate

Added tyrosine concn. (x) (μg./ml.)	0	5	10	15	20
Optical density (y)	0·052	0·150	0·236	0·304	0·386

(3) High-tyrosine casein hydrolysate

Estimated tyrosine concn. (x) (μg./ml.)	4·25	8·5	12·75	17	21·25
Optical density (y)	0·062	0·142	0·210	0·282	0·340

Regression coefficients, b, were calculated for the equations $y = a + b(x - \bar{x})$ by the method of least squares.

	Regression coefficient (b)	Variance of b
Solution 1	0·375	0·00064
Solution 2	0·412	0·00014
Solution 3	0·371	0·00099

t for $b_1 - b_2 = 1·32$, t for $b_1 - b_3 = 0·1$ } i.e. neither difference is significantly different from zero

carried out at 60°. Above this concentration there is a slight inhibition, amounting to 6 % at 0·25 mg. of glucose/ml.

Tryptophan. This compound does not itself give a colour in the conditions described here, but it inhibits colour development to an extent depending on its concentration. The percentage inhibition of colour development is independent of tyrosine concentration (Fig. 4) so that it is possible to use internal standards. This precaution is probably not necessary for general work, since the inhibition of colour development is very small when the tryptophan in a solution is less than 5 μg./ml. As tryptophan amounts to less than 2 % of most proteins, the use of internal standards may be neglected when analysing protein hydrolysates containing less than 25 mg. of total amino acids/ml.

Specificity

A list of many *p*-alkylated phenols which had been found to give a colour with 1-nitroso-2-naphthol was given by Gerngross *et al.* in their original paper. These authors also gave a list of related compounds which they had found not to react. These lists have not been generally checked, but a certain number of compounds, mainly of biological interest, have been tested in the present method. Adrenalin, thyroxin, 3:4-dihydroxyphenylalanine, 3:5-dihydroxyphenylpyruvic acid, homogentisic acid and *p*-hydroxybenzoic acid did not react. Tyrosine, *p*-hydroxyphenylpyruvic acid, glycyltyrosine and *p*-cresol gave the characteristic red colour. Glycyltyrosine gave a more intense colour than an equivalent concentration of free tyrosine. Tyrosine in combination in proteins (except alkali-denatured protein) also reacts, but the use of this method for estimating tyrosine in unhydrolysed proteins is limited to those proteins which do not precipitate in dilute HNO_3 solutions. The impression was gained that tyrosine contained in proteins does not give such an intense colour as the free amino-acid. *o*- and *m*-Tyrosine give a brown pigment, which is easily distinguished from that given by *p*-tyrosine. The extinction of the brown pigment is only one-third of that of the red tyrosine pigment when measured at 500 mμ.

Sensitivity and reproducibility

Dilution of the nitrosonaphthol reagent tenfold, in order to minimize the yellow masking colour, enabled the presence of 1 μg. of tyrosine/ml. to be detected in a solution. This limit of sensitivity is not suitable for quantitative use at this concentration, however; the lower limit for quantitative work is about 10 μg./tube. No upper limit for tyrosine concentration was found, but above 200 μg./ml. the curve relating concentration to colour intensity begins to flatten considerably, so that the sensitivity of the method is much less in this range. This is so even if the reagent concentration is increased. The standard deviation of replicate estimations over the range 25–200 μg./tube was 2·6 % of the mean.

The reproducibility of the method in the analysis of more complex solutions was tested by comparing the slope of the line relating optical density to concentration of tyrosine in water and in casein hydrolysate solutions (Table 3); and by comparing

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	0·378±0·028	—
(*b*) Diluted solution	0·198±0·022	12·1
(*c*) Diluted solution with 12·1 μg. of added tyrosine/ml.	0·372±0·028	—

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).