The very low solidifying point of this C_{20} saturated branched-chain acid has precluded melting-point determinations, and in the absence of these criteria of homogeneity, freedom from isomers or closely related substances cannot be assured. When larger quantities of the substance become available, however, derivatives will be prepared and further resolution attempted.

SUMMARY

A C_{20} saturated acid fraction containing at least three and possibly four methyl groups has been isolated from butterfat.

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The Oxidation of Amines by Extracts of Pea Seedlings

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While a number of enzymes catalysing reactions which lead *in vitro* to hydrogen peroxide formation have been shown to be present in animal tissues little is known about the distribution of such enzymes in higher plants. These enzymes may be of importance not only because of the oxidations they catalyse, but also because the hydrogen peroxide formed can be used by peroxidase (Thurlow, 1925) or catalase (Keilin & Hartree, 1936, 1945) for coupled oxidations. In this connexion Kenten & Mann (1949, 1950) showed that peroxidase systems can oxidize manganese and suggested that hydrogen peroxide produced by flavoprotein enzymes could be used *in vivo* for this oxidation. In the present work colorimetric evidence has been obtained suggesting the presence of enzyme systems producing hydrogen peroxide in extracts of many plants. In an investigation of such enzyme systems in extracts of pea seedlings it has been found that not only diamines (Werle & Pechmann, 1949), but also certain monoamines are oxidized by these extracts with formation of hydrogen peroxide. The oxidation of the monoamines is apparently not due to the plant monoamine oxidase described by Werle & Roewer (1950).

MATERIAL AND METHODS

Plant material. The majority of the plants examined were glasshouse grown in a John Innes potting compost. The rest of the plants were from gardens in the neighbourhood of Rothamsted. The peas and beans were soaked for 24 hr. in water prior to sowing. In some experiments with peas when

seedlings only a few days old were required the peas were germinated in moist washed sand. Pea seedlings were also grown aseptically in boiling tubes on a mineral salts agar medium. Before sowing on this medium the seed coats were sterilized by treatment first with ethanol and then with 0.2% $HgCl_2$ washed off with several changes of sterile water (Thornton, 1930).

Enzyme preparations. Water extracts of the plant material and a dry preparation obtained by precipitation of such extracts with $(NH_4)_2SO_4$ and acetone were used.

(a) **Plant extracts.** The plant material was washed free from soil and ground in a mortar with sand and water, squeezed by hand through madapolam and finally made up to approximately double the wet weight.

(b) **Dry preparation.** Pea seedlings (6–18 days old) were washed free from soil and any obviously diseased material removed. With the older seedlings it was frequently necessary to remove a number of the cotyledons on this account. About 100 g. of seedlings were extracted as above; the extract was then fractionally precipitated with $(NH_4)_2SO_4$ and the precipitate obtained with 30–66% saturation was dialysed overnight against 2 l. of distilled water. The dialysed suspension was centrifuged and the supernatant cooled to 0° and treated with 2 vol. of acetone cooled to –10°. The precipitate was centrifuged off and dried *in vacuo*. The yield was 400–600 mg. The activity of the various fractions was estimated manometrically by measuring the increase in O_2 uptake produced by 0.01 M- β -phenylethylamine in 0.067 M-orthophosphate at pH 8.5. The supernatant obtained after dialysis of the $(NH_4)_2SO_4$ precipitate contained 60–70%, and the acetone precipitate 30–40%, of the activity of the original plant extract. The dry preparation was stored *in vacuo* at room temperature, and under these conditions about 30% of the activity towards amines was lost in a fortnight.

Manometric measurements were carried out in air in the Warburg apparatus at 25°. The volume of the reaction mixture was 3 ml. and KOH was present in the centre cup unless otherwise stated.

Catalase was prepared from horse liver by a method based on that of Agner (1938). The activity of the preparation was estimated by the method of Sumner & Somers (1943). The rate of decomposition of 0.01N-H₂O₂ in 0.0067M-orthophosphate at pH 7 at 0°, by a suitable amount of catalase, was followed by iodine titration of samples taken at 0, 3, 6, 9 and 12 min. From the equation

$$K = \frac{1}{t} \log_{10} \frac{a}{a-x},$$

where a is the initial H₂O₂ concentration and $a-x$ is the concentration at time t in minutes, K values for the time intervals were calculated and the K value at zero time obtained by extrapolation. Using this value for K the Katalasefähigkeit (Kat.f. = $K/g.$ enzyme) was found to be 6000.

Amines were used as the hydrochlorides.

Estimation of aldehydes was done with the bisulphite method of Clift & Cook (1932).

Estimation of ammonia. A preliminary distillation was carried out in the Markham (1942) still from borate buffer pH 9.5. The NH₃ in the distillate was absorbed on to yellow HgO according to the method of Pugh & Quastel (1937). The washed HgO-NH₃ complex was then transferred to the still and decomposed by the addition of 2.5 ml. of 5N-NaOH. The distillate was collected in boric acid and titrated with N/140 HCl.

EXPERIMENTAL AND RESULTS

COLORIMETRIC EVIDENCE OF

HYDROGEN PEROXIDE PRODUCTION

The blue coloration obtained when benzidine and H₂O₂ are added to most plant and animal tissues is known to be due to the oxidation of benzidine by peroxidase or to the peroxidase-like action of haem or haematin derivatives. Therefore, a blue coloration obtained with tissue and benzidine in the absence of added H₂O₂ suggests the formation of H₂O₂ by the tissue. Goldacre (1949) found that extracts of pea epicotyls gave a blue coloration with benzidine in absence of added H₂O₂. This observation has been confirmed and positive tests have been obtained with pea roots and cotyledons as well as epicotyls, but under the conditions used the blue colour was frequently faint and faded rapidly. Owing to the catalytic activity of plant extracts the H₂O₂ formed is unlikely to accumulate. If the extracts were incubated with added Mn⁺⁺ and pyrophosphate at pH 7 the intensity of the subsequent benzidine test was much increased. In pyrophosphate solution the H₂O₂ formed by the plant extracts can be used by peroxidase systems to oxidize added Mn⁺⁺ to Mn⁺⁺⁺, which may accumulate as manganipyrophosphate. Since manganipyrophosphate oxidizes benzidine such an accumulation would explain the great increase in intensity of the benzidine test.

The colorimetric test was negative with extracts which had been heated for 5 min. in a boiling-water bath, and its intensity was considerably reduced by the previous addition of catalase to the extracts.

The tests were made in 25 ml. beakers with reaction mixtures consisting of 0.5-1 ml. plant extract, 0.5 ml. 0.2M-pyrophosphate (pH 7), with and without 0.2 ml. 0.01M-MnSO₄, and water to 3 ml. The reaction mixtures were allowed to stand for 15-30 min. at room temperature. Benzidine acetate (4 drops of 0.2% (w/v) in 10% (v/v) acetic acid) was then added and the intensity of the blue coloration noted. Where catalase was used 1 mg. of Kat.f. 6000 was added.

A number of extracts of different plants were tested in this way. The strongest tests were given by extracts of pea (*Pisum sativum* L.) and lupin (*Lupinus* sp.) seedlings in the presence of MnSO₄. Extracts of other plants gave negative tests in absence of MnSO₄, but in presence of MnSO₄ strongly positive tests were obtained with freshly prepared extracts of the leaves of barley (*Hordeum vulgare* L.), and spinach (*Beta vulgaris* L.), weaker tests were given by parsley (*Petroselinum hortense*, Hoffm.), tobacco (*Nicotiana tabacum* L.), lettuce (*Lactuca sativa* L.) and carrot seedlings (*Daucus carota* L.).

These results suggest that enzyme systems producing H₂O₂ were present in the plant extracts.

COLORIMETRIC EVIDENCE OF AMINE OXIDATION

A thermolabile fraction of the H₂O₂ producing system was isolated by precipitating pea extracts with (NH₄)₂SO₄, and suspending the precipitates in water. These suspensions were inactive in the benzidine tests, but could be reactivated by adding boiled extract. It was found by means of benzidine tests that the thermostable fraction passed into CHCl₃ from alkaline, but not from acid, extracts of pea seedlings. This suggested that the thermostable fraction was basic, and that the H₂O₂-producing enzyme might be the diamine oxidase described by Werle & Pechmann (1949) in extracts of various leguminous plants and of lavender leaves. In agreement with this it was found that the addition of putrescine reactivated the precipitate obtained with (NH₄)₂SO₄ in the benzidine tests and markedly increased the strength of the tests given by the pea extract. When the tests were made with the pea and lupin extracts with added putrescine in the presence of MnSO₄ the accumulation of manganipyrophosphate was so great as to impart a pink coloration to the reaction mixtures. But similar results were obtained when β-phenylethylamine was added in place of putrescine which suggested that the extracts oxidized mono- as well as di-amines. This was further investigated in manometric experiments. The benzidine tests given by the other plant extracts

in the presence of MnSO_4 were not markedly increased by adding β -phenylethylamine or putrescine. This suggests that H_2O_2 is produced by these extracts by the oxidation of substrates other than amines. Preliminary experiments indicate that one such substrate may be lactic acid.

MANOMETRIC STUDIES OF THE OXIDATION OF AMINES BY EXTRACTS OF PEA SEEDLINGS

The effect of various amines on the O_2 uptake. The increased O_2 uptake of extracts of pea seedlings in presence of several different amines suggested that an amine oxidase was present in the extracts (Table 1). The amines most readily oxidized were the diamines putrescine, cadaverine, agmatine, 1:6-diaminohexane and histamine, and the phenylalkylamines β -phenylethylamine, di- β -phenylethylamine, tyramine, and adrenaline, and tryptamine. The oxidation of tyramine and adrenaline was not due to tyrosinase or polyphenol oxidase since neither tyrosine nor catechol were oxidized under the same conditions. The oxidation of β -phenylethylamine and di- β -phenylethylamine differs from that of the diamines in having a short lag period. This is shown in Fig. 1, where the increased O_2 uptake due to the addition of amines to pea-seedling extract is plotted against time. The aliphatic monoamines were not readily attacked, but some increase in O_2 uptake was obtained with colamine, ethylamine, allylamine and *n*-heptylamine. Of the aminoacids tested L-lysine gave a small but definite increase in the rate of O_2 uptake. These results were

obtained in a number of experiments using different extracts, and therefore the increased O_2 uptake observed only provides an approximate estimate of

Fig. 1. The initial course of oxidation of mono- and diamines by pea-seedling extract. Amine concentration, 0.01 M.; orthophosphate, 0.067 M. +—+, cadaverine, 0.5 ml. extract, pH 6; ○—○, putrescine, 0.5 ml. extract, pH 7.5; ●—●, di- β -phenylethylamine, 1 ml. extract, pH 8.5; □—□, β -phenylethylamine, 1 ml. extract, pH 8.5.

the relative rates of oxidation at pH 7. Furthermore, the optimum pH for oxidation varies with the different amines. It is clear, however, that the

Table 1. *Effect of various amines on the O_2 uptake of extracts of pea seedlings*

(Reaction mixtures consisted of 1 ml. tissue extract (5- to 6-day old pea seedlings) in 0.067 M-orthophosphate at pH 7. The substrates, 0.3 ml. 0.1 M were added from the side arm as solutions of hydrochlorides except where otherwise stated. Uptake by extract in absence of added substrate 20–30 μl . O_2 /hr.)

Substrate added	Increased O_2 uptake (μl /hr.)	Substrate added	Increased O_2 uptake (μl /hr.)
Methylamine	5	Ephedrine	<5
Ethylamine	27	Colamine	24
<i>n</i> -Butylamine	6	Choline	<5
<i>n</i> -Amylamine	10	Ethylenediamine	15
<i>iso</i> Amylamine	5	1:3-Diaminopropane	<5
<i>n</i> -Heptylamine	39	Putrescine	342
Allylamine	31	Cadaverine	378
<i>cyclo</i> Hexylamine	<5	1:6-Diaminohexane	150
Aniline*	<5	Agmatine†	190
Benzylamine	30	Spermine	29
β -Phenylethylamine	134	Histamine	101
β -Phenyl- β -hydroxyethylamine	9	DL-Ornithine‡	<5
Di- β -phenylethylamine	133	L-Lysine	24
Dimethylamine	<5	L-Alanine	<5
Trimethylamine	10	L-Tyrosine§	<5
Hordenine†	<5	L-Tryptophan	<5
Tyramine	54	Guanine§	<5
Tryptamine	49	Adenine§	<5
Adrenaline	54	D-Glucosamine	5

* Added as suspension of free base.

† Added as sulphate.

‡ Added as hydrobromide.

§ Added as suspension.

oxidation of the monoamines is not due to the plant monoamine oxidase described by Werle & Roewer (1950) in *Colchicum bormulleri* Freyn., *Salvia uliginosa* Benth., *Cannabis sativa* L. var. indica Lam. and *Momordica balsamina* L., which oxidizes butylamine and methylamine more readily than tyramine and tryptamine. With the diamines, in contrast to the present results, Werle & Pechmann (1949) found that, at pH 7, putrescine was oxidized much more readily than cadaverine by extracts of lavender leaves.

Effect of pH. The variation of activity with pH was tested in 0.067M-orthophosphate, using 0.01M- β -phenylethylamine, 1:3-diaminopropane, putrescine, cadaverine and 1:6-diaminohexane. Extracts of 7- to 13-day old pea seedlings were added from the side arm after equilibration. The initial pH was determined using separate reaction mixtures and the final pH with the experimental reaction mixtures. In no case was any appreciable change of pH observed.

Fig. 2. Effect of pH of the medium on the rate of oxidation of amines by pea-seedling extract. Amine concentration, 0.01M; orthophosphate, 0.067M. ●—●, cadaverine; □—□, putrescine; +—+, 1:6-diaminohexane; ○—○, β -phenylethylamine.

In Fig. 2 the O_2 uptake in 20 min. is plotted against pH. The results are plotted in this way rather than as velocities, since with β -phenylethylamine there is an initial lag in the O_2 uptake (Fig. 1).

With the series of diamines the pH optima and the activity were dependent on the length of the methylene chain. Activity was negligible with 1:3-diaminopropane, optimal with putrescine (1:4-diaminopentane), decreased slightly with cadaverine (1:5-diaminopentane) and markedly with 1:6-diaminohexane.

The optimum pH was about 7.5 for putrescine, 6.0 for cadaverine, and 8 for 1:6-diaminohexane; thus the optimum varied, but not in a regular manner, with increase in methylene chain. The

possibility that the differences in pH optimum were related to differences in ionization of the amines was investigated. Titration curves of the three amines up to pH 9.7 were very similar and showed that below about pH 8 the amines were completely ionized. Bredig (1894) found for putrescine $K = 5.1 \times 10^{-4}$, and for cadaverine 7.3×10^{-4} at 25°. Alles & Heegard (1943) with the monoamine oxidase of animal tissues, using two series of monoamines, found varying pH optima which could not be related to the ionization constants of the amines. It is of interest that the pH optimum for the action of animal amine oxidase on β -phenylethylamine is pH 6 (Alles & Heegard, 1943), whereas with pea amine oxidase it is about pH 8.5. Under optimum conditions of pH this monoamine is attacked much less readily than putrescine and cadaverine by pea amine oxidase.

Effect of substrate concentration. The effect of substrate concentration on the initial reaction

Fig. 3. Effect of substrate concentration on the rate of oxidation. ○—○, β -Phenylethylamine, 0.067M-orthophosphate pH 8.5, 1 ml. pea-seedling extract; ●—●, putrescine, 0.067M-orthophosphate pH 7.5, 0.1 ml. pea-seedling extract.

velocity was tested with putrescine and β -phenylethylamine in 0.067M-orthophosphate buffer at the optimum pH for the amines used (Fig. 3). Over the range 0.01–0.001M-substrate there was little change in initial velocity with the diamine used, but with β -phenylethylamine the initial velocity increased with substrate concentration, maximum velocity being reached at about 0.01M.

Effect of inhibitors. The effect of a number of inhibitors on the oxidation of β -phenylethylamine and putrescine by pea-seedling extract is shown in Table 2. At a concentration of 0.01M-cyanide, the oxidation of both mono- and di-amine was inhibited strongly; at 0.001M-cyanide oxidation of the diamine was only slightly inhibited, whereas a 60% inhibition of the monoamine was produced. Azide

at the same concentration was a much less effective inhibitor than cyanide, but semicarbazide was more effective causing 86–98 % inhibition at a concentration of 0.001M. The results with azide and semicarbazide on the oxidation of the diamine are in agreement with those obtained by Werle & Pechmann (1949). The comparative ineffectiveness of azide suggests that the inhibition observed with cyanide is not due to its property of complex formation with heavy metals, and the sensitivity towards

mono- and di-amines with the age of the pea seedlings has been investigated.

Peas sown in compost were harvested at various stages of growth, and the activity of extracts towards cadaverine and β -phenylethylamine determined. A typical set of results is given in Fig. 4 where the change in Q_{O_2} (μ l. O_2 /mg. dry wt./hr.) of the extracts with the age of the seedling is plotted. The seeds after soaking, but before germination, showed no activity towards either amine. A measurable activity first appeared 3–4 days after sowing.

Table 2. *Effect of inhibitors*

(Reaction mixtures consisted of 1 ml. extract of 7- to 15-day old pea seedlings in 0.067M-orthophosphate at pH 7, 0.3 ml. of 0.1M-amine hydrochloride was added from the side arm. KOH was omitted from the centre cup in the experiment on KCN inhibition.)

Inhibitor	Concentration of inhibitor (M)	Percentage inhibition	
		β -Phenylethylamine	Putrescine
KCN	0.01	90	99
	0.001	60	15
NaN ₃	0.01	37	27
	0.001	9	10
Semicarbazide	0.01	100	98
	0.001	86	98
Ephedrine	0.1	15	19
isoAmylamine	0.1	83	62
1:3-Diaminopropane	0.1	92	97
<i>p</i> -Benzoquinone*	0.006	52	—
Iodoacetate	0.01	8	0

* Added as suspension 2 mg./vessel.

semicarbazide suggests that the activity of the enzyme may depend on the presence of a carbonyl group. This explanation for the inhibiting effect of semicarbazide on the oxidation of diamines by extracts of pea seedlings was also put forward by Werle & Pechmann (1949). The inhibiting effect of cyanide could then be due to the formation of cyanhydrin, as suggested by Zeller (1942) for animal diamine oxidase. *p*-Benzoquinone inhibited the oxidation of the monoamine, but with the diamine it caused an increase in the O_2 uptake probably due to coupled oxidation of the benzoquinone by peroxidase and the H_2O_2 formed during the oxidation of the amine. Iodoacetate did not inhibit even when incubated for 3 hr. with the extract before addition of the amines (Friedenwald & Herrmann, 1942).

Variation in activity with age of pea seedling. Werle & Pechmann (1949) showed that extracts of the resting seeds of leguminous plants were unable to oxidize diamines. Activity appeared on germination reaching a maximum in red clover seedlings after about 5 days of germination. In the present work the correlation of the activities towards both

Fig. 4. Variation in rate of amine oxidation with age of pea seedling. Amine concentration 0.01M, orthophosphate concentration 0.067M. ●—●, Cadaverine, pH 6; ○—○, β -phenylethylamine, pH 8.5. The Q_{O_2} was based on the increased O_2 uptake over the first 20 min.

The activity towards both amines increased rapidly over the next few days, was maximal over the period 7–18 days, and then decreased. This decrease in activity paralleled the breakdown of the cotyledons. At later stages of growth (24–45 days) it was not possible to use cotyledon material for the extract. A slight, but measurable, activity was still present in the compost-grown seedlings at 30–45 days and also in extracts of leaves of garden pea plants at the cropping stage. The extracts from the seedlings always attacked the diamines more readily than the monoamines. This difference in the rate of attack appeared to be greatest in the first few days of activity. Although only slight activity was found in the leaves of adult pea plants, extracts of root nodules from these plants sometimes showed activity comparable with cotyledons.

Distribution of activity. In the early stages of growth (8-day old seedlings) the activity was mainly confined to the cotyledon and shoot, the root showing only a weak activity (Table 3). At a later stage (16-day old seedlings) when sufficient leaves had developed to enable differentiation between stem and leaf, activity was found in leaf, stem and root, though this was low in comparison with that of the cotyledon. In view of the fact that the cotyledons were so much more active than the other parts of the seedlings a few experiments were made with pea seedlings grown aseptically. These

Table 3. *Distribution of amine oxidase in pea seedlings*

(The activity of extracts of different parts of pea seedlings was tested manometrically. Reaction mixtures consisted of 0.2–1.5 ml. tissue extracts in 0.067 M-orthophosphate at pH 6 (cadaverine) or 8.5 (β -phenylethylamine) and 0.3 ml. 0.1 M-amine hydrochloride added from the side arm. The Q_{O_2} and activity per ml. extract was based on the increase in O_2 uptake over the first 20 min.)

Part	8-day seedlings					16-day seedlings				
	β -Phenylethylamine			Cadaverine		β -Phenylethylamine			Cadaverine	
	Average wet wt. (g./seedling)	Increased O_2 uptake (μ l./ml. extract/hr.)		Q_{O_2}	Q_{O_2}	Average wet wt. (g./seedling)	Increased O_2 uptake (μ l./ml. extract/hr.)		Q_{O_2}	Q_{O_2}
		extract/hr.	Q_{O_2}				extract/hr.	Q_{O_2}		
Root	0.16	33	2	91	5	0.58	15	3	21	4
Shoot	0.12	184	11	264	14	—	—	—	—	—
Cotyledon	0.64	870	16	1680	32	0.64	1086	46	2331	97
Leaf	—	—	—	—	—	0.44	81	2	151	4
Stem	—	—	—	—	—	0.45	42	2	84	5

seedlings proved to be as active towards amines as those grown in compost and it is therefore clear that the high activity of the cotyledons was not due to infection.

RESULTS WITH OTHER PLANTS

Extracts of leaves of lavender (*Lavendula spica* Cav.) and of red clover (*Trifolium pratense* L.) and of seedlings of garden lupins (*Lupinus hybridus* Hort.) and of tree lupins (*L. arboreus* L.), shown by Werle & Pechmann (1949) to oxidize diamines, were tested manometrically with putrescine and β -phenylethylamine. In every case oxidation of both mono- and di-amine was found, the diamine always being oxidized more readily than the monoamine. Neither the mono- nor the di-amine were oxidized by extracts of spinach (*Beta vulgaris* L.), marrow (*Cucurbita pepo* L.), lettuce (*Lactuca sativa* L.), barley (*Hordeum vulgare* L.), parsley (*Petroselinum hortense* Hoffm.), and tobacco (*Nicotiana tabacum* L.).

THE COURSE OF THE OXIDATION

A quantitative study has been made of the total O_2 uptake during the oxidation of the amines and of the products of oxidation. The results obtained depended on whether the extract or the dry preparation of enzyme was used to catalyse the reaction. In these experiments β -phenylethylamine and putrescine were used as representatives of the mono- and di-amines attacked. The results suggest that the primary attack on the amines is an oxidative deamination according to the equation:

If the peroxide formed is decomposed by catalase the net reaction is

Hydrogen peroxide formation

Some evidence that H_2O_2 is formed during the oxidation of diamines by extracts of pea seedlings was obtained by Werle & Pechmann (1949). Using the cerous hydroxide method they were able to demonstrate 20% of the H_2O_2 required by Eqn. 1. In the present work the formation of H_2O_2 during the oxidation of the amines was shown, with the dry preparation, by the effect of catalase on the total O_2 uptake, and, with the extract, by the coupled oxidation of the phenols, quinol and *p*-cresol, through peroxidase present in the extract (Thurlow, 1925; Stephenson, 1943), and also of ethanol in the presence of added catalase (Keilin & Hartree, 1936, 1945; Philpot, 1937; Kohn, 1937).

Dry preparation. Using the dry preparation in the presence of added catalase with putrescine as substrate the total O_2 uptake corresponded to 1 atom O_2 /mol. amine, in accordance with Eqn. 2. In absence of added catalase the total uptake approached 1 mol. O_2 /mol. amine (Eqn. 1) and the rapid uptake was succeeded by a slow output. This increased uptake in absence of added catalase and the subsequent slow output were due to the accumulation and slow decomposition of H_2O_2 since, on addition of catalase from the side arm of the manometer vessel, a rapid output of gas took place causing a drop in the overall uptake from 1 mol. to 1 atom O_2 /mol. amine (Fig. 5a). Tabor (1951) has demonstrated H_2O_2 formation by animal diamine oxidase in the same way. The results obtained with β -phenylethylamine as substrate were similar except that the total O_2 uptake in presence of added catalase was greater than 1 atom O_2 /mol. amine, and the difference in uptake in presence and absence of added catalase was therefore not as great as with putrescine as substrate (Fig. 5b).

In these experiments the reaction mixtures consisted of 0.0025 M-putrescine or β -phenylethylamine

in 0.067M-orthophosphate at pH 7.5 and 8.5 respectively, 2.5 mg. dry preparation and 0.1 mg. catalase (Kat.f. 6000, where present). With the dry preparation with β -phenylethylamine as substrate some coupled oxidation of ethanol was obtained in presence of added catalase, though the increase in O_2 uptake was not as great as with putrescine as substrate.

oxidation either of ethanol in the presence of added catalase or of quinol and *p*-cresol (Tables 4 and 5). With β -phenylethylamine as substrate the formation of H_2O_2 could not be demonstrated by either of these methods. Since β -phenylethylamine is attacked much less rapidly than putrescine, much larger amounts of extract are required to produce a suitable rate of oxidation. Quantitative studies on

Fig. 5. Formation of hydrogen peroxide during the oxidation of putrescine and β -phenylethylamine by the amine oxidase preparation. Amine concentration, 0.0025M; orthophosphate, 0.067M. Catalase 0.1 mg. Kat.f. 6000 (where present). (a) Putrescine; (b) β -phenylethylamine. $\square$ — $\square$, No catalase; $\circ$ — $\circ$, catalase added at $\downarrow$; $\bullet$ — $\bullet$, catalase added at zero time. The dotted lines show the uptake for 1 atom and 1 molecule of oxygen/molecule amine.

Table 4. Coupled oxidation of phenols during the oxidation of putrescine by extracts of pea seedlings

(Reaction mixtures consisted of 0.2 ml. extract of 6-day old pea seedlings in 0.067M-orthophosphate at pH 6.5, 0.2 ml. of 0.02M-quinol or *p*-cresol, and 0.2 ml. of 0.025M-putrescine added from the side arm.)

Reaction system	Total O_2 uptake ($\mu l.$)
Extract	4
Extract + quinol or <i>p</i> -cresol	2
Extract + putrescine	66
Extract + putrescine + quinol	114
Extract + putrescine + <i>p</i> -cresol	117

Extract. When the extract is used to catalyse the oxidation of the amines, H_2O_2 does not accumulate. With putrescine as substrate, however, the formation of H_2O_2 can be demonstrated by the coupled

Table 5. Coupled oxidation of ethanol during the oxidation of putrescine by extracts of pea seedlings

(Reaction mixtures consisted of 10 mg. catalase (Kat.f. 6000), 5 mg. ethanol, 0.4 ml. of 0.025M-putrescine in 0.067M-orthophosphate at pH 7.5. 0.2 ml. extract of 7-day old pea seedlings was added from the side arm.)

Reaction system	Total O_2 uptake ($\mu l.$)
Extract	10
Extract + putrescine	126
Extract + putrescine + catalase	145
Extract + putrescine + ethanol	126
Extract + putrescine + ethanol + catalase	231

the O_2 uptake suggest that under these conditions the H_2O_2 formed is used for the coupled oxidation of substrates present in the extract. Quinol and *p*-cresol inhibited the oxidation of β -phenyl-

Table 6. *Oxygen uptake and aldehyde and ammonia formation during the oxidation of β -phenylethylamine and putrescine*

(Reaction mixtures consisted of extract of 8- to 12-day old pea seedlings, or dry preparation, in 0.067 M-orthophosphate at pH 7.5 (putrescine) or 8.5 (β -phenylethylamine). Catalase 0.1 mg. (Kat.f. 6000) was added where the dry preparation was used. Amine hydrochloride was added from the side arm.)

Enzyme	Amine added (μ mol.)	Total O ₂ uptake (μ mol.)	Aldehyde formed (μ mol.)	Ammonia formed (μ mol.)
<i>β-Phenylethylamine</i>				
Extract 1.5 ml.	5	11.3	1.1	5.8
	7.5	16.4	3.0	7.4
	10	20.5	4.5	10.5
Dry preparation 10 mg.	5	3.2	3.8	5.4
	7.5	5.2	5.6	8.3
	10	7.2	7.8	9.9
<i>Putrescine</i>				
Extract 0.2 ml.	5	3.1	4.6	5.9
	7.5	4.2	6.8	8.8
	10	5.4	8.5	11.8
Dry preparation 5 mg.	5	2.5	4.8	5.4
	7.5	3.7	6.8	7.5
	10	5.1	8.6	11.2

ethylamine by the extract, possibly owing to the formation of quinones by coupled oxidation. As shown earlier (Table 2), *p*-benzoquinone inhibits the oxidation of β -phenylethylamine.

Total oxygen uptake, aldehyde and ammonia formation

The total O₂ uptakes, and the amounts of aldehyde and NH₃ formed during the oxidation of putrescine and β -phenylethylamine by pea amine oxidase are shown in Table 6.

Putrescine

With putrescine as substrate using 0.2 ml. of extract the O₂ uptake reached completion in about 90 min. and corresponded to rather more than 1 atom O₂/mol. amine. Werle & Pechmann (1949), using extracts of pea or clover seedlings with cadaverine as substrate, found O₂ uptakes of about 1 mol. O₂/mol. amine. In their experiments larger amounts of more concentrated seedling extracts were used and it is probable that the greater O₂ uptakes observed were due to the H₂O₂ formed being used in secondary oxidations. Using 5 mg. of the dry preparation together with added catalase in place of the extract the total O₂ uptake corresponded more closely to 1 atom O₂/mol. amine. In both cases the values found for aldehyde and NH₃ were in the neighbourhood of 1 mol./mol. amine, the values for NH₃ being slightly higher than this, particularly where the extract was used, and those for aldehydes slightly lower. It therefore appears that the reaction proceeds according to Eqn. 2 and as with animal diamine oxidase (Zeller, 1942) only one of the —NH₂ groups of the diamine is attacked. Tabor (1951), with animal diamine oxidase, was unable to

use the bisulphite method since the reaction products (particularly that of cadaverine) reacted with I₂ even in absence of bisulphite. In the present work using putrescine, experiments showed that the reaction products did not react directly with I₂ sufficiently rapidly to affect the results of the bisulphite method.

β -Phenylethylamine

The total O₂ uptake with β -phenylethylamine as substrate using the dry preparation in presence of added catalase varied between 1.2 and 1.5 atoms O₂/mol. amine. The amounts of NH₃ found were in agreement with Eqn. 2, but the aldehyde values were somewhat low. With the extract it was necessary to use at least 1.5 ml. in order that the increased O₂ uptake in presence of β -phenylethylamine should be complete within 3 hr. Under these conditions the O₂ uptake was rather more than 2 mol. O₂/mol. amine. The NH₃ produced corresponded to complete deamination of the amine, but the amounts of aldehyde as estimated by bisulphite titration were low, varying between 22 and 45 % of theory. The bisulphite titration was unsatisfactory with these large amounts of extract as the end point was indefinite. The fact that the amounts of aldehyde found were so low suggested that the extra O₂ uptake was in fact due to further oxidation of the phenylacetaldehyde formed as the primary oxidation product. It was found that large increases in the O₂ uptake were produced on the addition of phenylacetaldehyde to pea-seedling extract or to the dry preparation, presumably owing to oxidation of the aldehyde to phenylacetic acid. The total increase in O₂ uptake was, however, insufficient to account for the oxidation to the corresponding acid

of all the aldehyde added. No increase in O_2 uptake was produced by the addition of acetaldehyde, benzaldehyde and a number of other aldehydes to the extracts or dry preparations. Preliminary tests suggest that the oxidation of phenylacetaldehyde is accompanied with H_2O_2 formation. The oxidation of β -phenylethylamine to the corresponding aldehyde would require 2 atoms of O_2 if the H_2O_2 formed was used in secondary oxidations. Similarly, further oxidation of the aldehyde to phenylacetic acid would require a further 2 atoms of O_2 giving a total uptake of 4 O_2 atoms/mol. amine which is in close agreement with that obtained experimentally with the extract. According to the values obtained by bisulphite titration, however, the aldehyde is not completely oxidized. With the dry preparation the O_2 uptake in excess of 1 mol. O_2 /mol. amine can be accounted for by further oxidation of aldehyde as shown by the bisulphite estimations. It is not yet clear, however, why the oxidation, particularly with the enzyme preparation, stops when large amounts of aldehyde are still present.

Isolation of phenylacetaldehyde 2:4-dinitrophenylhydrazone. Using the dry preparation it has been proved that phenylacetaldehyde is a primary product of the oxidation of β -phenylethylamine by its isolation as the 2:4-dinitrophenylhydrazone.

A reaction mixture containing 10 ml. 0.1M- β -phenylethylamine, 500 mg. dry preparation, 10 mg. catalase (Kat.f. 6000) and 100 ml. 0.2M-orthophosphate, pH 8.5, in a total volume of 300 ml. was shaken in air at room temperature for 4 hr. The oxidation was followed by estimating the aldehyde present in 2 ml. samples taken at half-hourly intervals. Aldehyde was rapidly formed during the first hour at the end of which the amount present was 50% of the theoretical. Thereafter the increase was slow, and had virtually ceased at 4 hr., when the amount present was 75% of the theoretical. The residual solution (284 ml.) which had the characteristic hyacinth odour of phenylacetaldehyde, was brought to pH 2 with 3N-HCl and centrifuged. To the centrifugate 80 ml. of a saturated solution of 2:4-dinitrophenylhydrazine in 2N-HCl was added, and the mixture was allowed to stand overnight at room temperature. The yellow precipitate was then centrifuged off and extracted several times with boiling ethanol. The combined filtered ethanol extract was concentrated and left in the refrigerator overnight. The yellow crystals were filtered off and dried *in vacuo*. Wt. 96 mg. Yield: 35% theory based on amine added, 46.5% based on the bisulphite titration. The m.p. (uncorr.) was 111.5–113.5°. After recrystallization from ethanol the m.p. (uncorr.) was 116.5–117.5°, unchanged on mixture with an authentic sample (m.p. (uncorr.) 118–119°) prepared according to Campbell (1936).

DISCUSSION

The results of the colorimetric tests described in the present work suggest that hydrogen peroxide is produced by enzyme systems present in extracts of many plants, and that this hydrogen peroxide can be used by the extracts for the oxidation of added

Mn^{++} . Since under certain conditions the oxidized manganese accumulates and gives a blue coloration with benzidine, it is frequently possible by addition of Mn^{++} to demonstrate hydrogen peroxide formation in extracts which give negative tests in absence of added Mn^{++} .

Attempts to identify these systems have so far shown that both mono- and di-amines are oxidized by extracts of pea seedlings with the production of hydrogen peroxide. Of the monoamines tested those most readily attacked are mono- and di- β -phenylethylamine, tyramine and tryptamine, while the aliphatic monoamines such as methylamine and butylamine are only feebly attacked. The plant monoamine oxidase described by Werle & Roewer (1950) oxidizes methylamine and butylamine more readily than tyramine and tryptamine. With the animal monoamine oxidase tyramine, tryptamine and *isoamylamine* are most readily attacked (Blaschko, Richter & Schlossmann, 1937), though the relative rates at which these are attacked differ with different tissues and species (Blaschko, 1941; Alles & Heegard, 1943). The latter two enzymes are not inhibited by 0.001M-cyanide, which causes a 60% inhibition of the oxidation of monoamines by pea-seedling extracts. It appears, therefore, that the pea-seedling enzyme which attacks monoamines differs from the previously described plant and animal monoamine oxidases.

Of the diamines tested no activity is observed with 1:3-diaminopropane; putrescine and cadaverine are rapidly attacked, at about the same rate, and 1:6-diaminohexane is much less readily attacked. With animal diamine oxidase, using the $\alpha\omega$ series of diamines the rate of oxidation increases with the length of the methylene chain up to five methylene groups (Zeller, 1942). The available evidence suggests that the oxidation of both mono- and diamines by the plant extracts used in the present work may be brought about by the same enzyme. The plant extracts which oxidized diamines also oxidized monoamines and the relative rates of attack on the two types of amine were similar. Both activities appear at about the same time in germinating pea and lupin seedlings and are, in general, affected in the same way by inhibitors. As with animal diamine oxidase, and in contrast to animal monoamine oxidase, both cyanide and semicarbazide inhibit the action though the oxidation of the diamines, in particular, is less sensitive to cyanide than is the case with animal diamine oxidase. Amines which are not themselves attacked inhibit the oxidation of both mono- and di-amines to the same extent. Of the amines tested in this way the diamine 1:3-diaminopropane is the strongest inhibitor, while ephedrine, which is well known to inhibit animal monoamine oxidase, is a much less effective inhibitor than *isoamylamine*.

While the above findings suggest that the oxidation of both mono- and di-amines is catalysed by one enzyme the fact that the oxidation of monoamines, but not that of diamines, is inhibited by *p*-benzoquinone suggests the possibility that specific mono- and di-amine oxidases may be present in the plant. Further evidence is necessary to distinguish between these two possibilities. Until such evidence has been obtained it appears preferable to refer to the enzyme as plant amine oxidase rather than diamine oxidase even though the diamines are more readily attacked than the monoamines.

As with the amine oxidases of animal tissues the primary attack on the amines consists of an oxidative deamination according to the equation

The formation of hydrogen peroxide during the oxidation suggests that plant amine oxidase is a flavoprotein. It seems of particular interest that tryptamine is among the amines attacked by the enzyme. According to Skoog (1937) and Gordon & Nieva (1949), tryptamine can function as a precursor of 3-indolylacetic acid in plants. The action of the enzyme on tryptamine presumably leads to the formation of 3-indolylacetaldehyde. It has already been mentioned that an enzyme is present in pea seedling extracts which oxidizes phenylacetaldehyde. Preliminary experiments suggest that this enzyme is widely distributed among higher plants. It is possible that 3-indolylacetaldehyde is one of its substrates and that 3-indolylacetic acid could be formed from tryptamine by the action of amine oxidase followed by that of the aldehyde oxidase. The hypothesis has been advanced that the herbicidal activity of 2-phenoxyethylamine derivatives is due to their conversion to derivatives of 2-phenoxyacetic acid by the living plant (Jones, Metcalfe & Sexton, 1949).

SUMMARY

1. A colorimetric test, based on the oxidation of added Mn^{++} to Mn^{+++} , is described for the detection of hydrogen peroxide in plant extracts. By means of this test evidence has been obtained that hydrogen peroxide is produced by enzyme systems present in extracts of many higher plants.

2. The oxidation of both mono- and di-amines is catalysed by extracts of pea and lupin seedlings and leaves of lavender and red clover. It is not yet known whether this is due to the presence of specific mono- and di-amine oxidases.

3. With the growth conditions used, activity appears in pea seedlings, mainly in the cotyledons, 3–4 days after germination and is maximal over the period 7–18 days. Only slight activity is found in the adult plant.

4. Diamines, in particular putrescine and cadaverine, are more readily attacked than monoamines. Of the monoamines tested β -phenylethylamine and di- β -phenylethylamine are most readily attacked.

5. The oxidative deamination of β -phenylethylamine and putrescine is catalysed according to the equation

As with animal diamine oxidase only one of the terminal amino groups is attacked.

6. The phenylacetaldehyde formed by the action of the plant amine oxidase on β -phenylethylamine was oxidized by an aldehyde oxidase present in the preparation used.

7. It is suggested that 3-indolylacetic acid may be formed by the successive action of the plant amine oxidase and of the aldehyde oxidase on tryptamine.

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