Biochemical Pharmacology, Vol. 41, No. 12, pp. 1863–1869, 1991
Printed in Great Britain.

0006-2952/91 $3.00 + 0.00

ENZYMIC OXIDATION OF CAPSAICIN

ANGELIKA BOERSCH, BRIAN A. CALLINGHAM, FRED LEMBECK* and DENNIS F. SHARMAN†

Department of Pharmacology, University of Cambridge, Tennis Court Road, Cambridge CB2 1QJ, U.K.; and *Department of Experimental and Clinical Pharmacology, University of Graz, Universitaetsplatz 4, A-8010 Graz, Austria

(*Received* 28 *August* 1990; *accepted* 18 *January* 1991)

Abstract—The oxidation of capsaicin (8-methyl-*N*-vanillyl-6-nonenamide) has been investigated by means of electrochemical, enzymic and chemical procedures. Capsaicin appears to form a fluorescent dimer comparable with those known to be formed from some other compounds bearing the vanillyl(4-hydroxy-3-methoxybenzyl-) group. If such a dimer of capsaicin were to be formed in tissues, it would bind tightly to lipid structures and its formation would prove difficult to follow. Tests on other substances bearing the vanillyl group that might be used to investigate the dimerization reaction in tissues and tissue extracts showed that 4-hydroxy-3-methoxyphenylacetic acid is a poor second substrate for peroxidase reactions. It was found that 2-methoxy-4-methylphenol (creosol) was more suitable. These results support the suggestion that the oxidation of capsaicin may be involved in some of its biological actions.

Capsaicin (8-methyl-*N*-vanillyl-6-nonenamide), the pungent chemical found in *Capsicum* fruits, exerts its major pharmacological effects on C-fibre afferent sensory neurones. Its action results in a series of responses which range from stimulation of the sensory fibres to irreversible destruction of the nerves. The pungent action depends largely on the presence of the 4-hydroxy-3-methoxybenzyl (vanillyl) group at the end of a long lipophilic side chain [1].

The vanillyl group also occurs in the major metabolic products of catecholamines in the body [2, 3] and its formation is part of the inactivation mechanism for catecholamines. It was, thus, unexpected that the vanillyl moiety should impart such biological activity to a molecule such as capsaicin. The possibility that metabolic changes to the vanillyl group might take place when capsaicin is present in the membranes or internal structures of the sensory neurones was the stimulus for the present study.

The oxidative coupling of phenols to form dimers has been studied extensively [4] and the formation of a fluorescent dimer (5,5'-dehydro-di-4-hydroxy-3-methoxyphenylacetic acid) from 4-hydroxy-3-methoxyphenylacetic acid (homovanillic acid, HVA) has been used to determine the concentration of this metabolite of 3,4-dihydroxyphenylethylamine (dopamine) in nervous tissues and body fluids [6, 7]. The same reaction has been employed to measure the activity of some oxidative enzymes where hydrogen peroxide is one of the products of the enzyme activity. In such assays, HVA acts as the hydrogen donor in the peroxidase reaction and the formation of a fluorescent dimer can be measured as an index of the rate at which the oxidative enzyme reaction takes place [8–11].

There are examples from plant biochemistry which suggest that the oxidative dimerization of vanillyl groups is important in cross-linking between cellular and subcellular components [12, 13].

The present work was undertaken to determine if a dimer could be formed from capsaicin using enzymic and chemical methods. Because capsaicin and, presumably, any dimer of capsaicin, is lipophilic and binds to tissue membranes, other compounds possessing the vanillyl group were also investigated to try to find an analogue with similar oxidation properties but which was more soluble in aqueous solutions, that might be used in studies with tissues to detect whether or not dimerisation could take place.

MATERIALS AND METHODS

Electrochemistry. Oxidation–reduction (redox) potentials (E_0') were estimated from single sweep voltammograms obtained with the cyclic voltammetry apparatus described by McCreery *et al.* [14]. The carbon paste (3.3 g carbon powder [Ultracarbon UCP-IM] well mixed with 2.0 g Dow-Corning silicone high vacuum grease) electrode was prepared in a glass capillary tube (i.d. 2 mm). An anodic potential, which increased at a rate of 2 V/min, was applied to the electrode, starting at 0 V with respect to a Ag/AgCl reference electrode. (All voltages used for electrochemical studies in these experiments are with reference to Ag/AgCl). Measurements were made at room temperature in a volume of 1 mL of 0.2 M potassium phosphate buffer of the required pH containing the substance under investigation at a concentration of 3×10^{-4} M. The solution was stirred continuously by means of a magnetic stirrer. E_0' was estimated by extrapolation of the linear portion of the voltammogram to the zero current axis. This does not provide a precise determination of the redox potentials of the substances under study but enables a reasonable comparison of the oxidation characteristics of the different compounds to be made.

† To whom correspondence should be addressed.

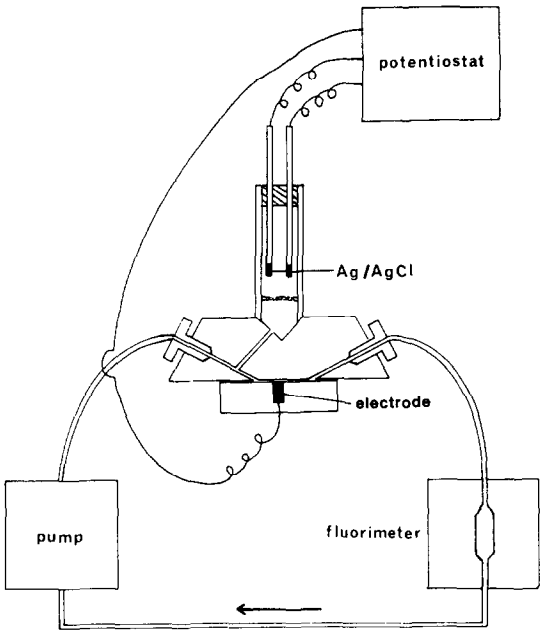

Fig. 1. Diagram of apparatus to measure fluorescence of compounds produced by oxidation at an electrode.

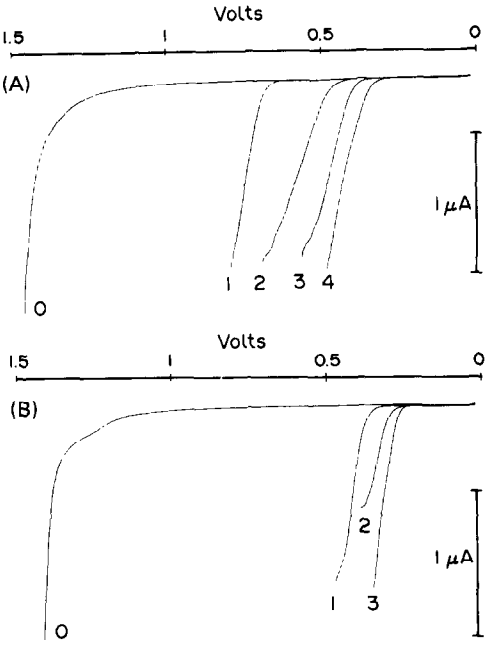

Fig. 2. Partial single sweep voltammograms obtained with phenolic compounds at a carbon paste electrode. (A) 0: potassium phosphate buffer, 0.2 M, pH 7.4; 1: 4-hydroxybenzoic acid; 2: 4-hydroxy-3-methoxybenzoic acid (vanillic acid); 3: 4-hydroxy-3-methoxyphenylacetic acid (HVA); 4: capsaicin (8-methyl-*N*-vanillyl-6-nonenamide). (B) 0: potassium phosphate buffer, 0.2 M, pH 7.4; 1: 2-methoxyphenol (guaiacol); 2: 4-hydroxy-3-methoxycinnamic acid (ferulic acid); 3: 2-methoxy-4-methylphenol (creosol). All of the compounds investigated were present in the electrochemical cell at a concentration of 3×10^{-4} M in potassium phosphate buffer, 0.2 M, pH 7.4.

Enzyme assays. Peroxidase (donor: hydrogen peroxide oxidoreductase; EC 1.11.1.7) assays were carried out in conical polystyrene tubes (Sarstedt No. 57.477). The total volume incubated was 1.2 mL and consisted of 5–50 μL of enzyme solution and 20 μL of a solution of the compound to be studied as the second substrate for the enzyme reaction, made up to a volume of 200 μL with 0.2 M potassium phosphate buffer of the appropriate pH. One millilitre of an aqueous solution of hydrogen peroxide was then added and the mixture incubated at 37° up to 20 min. After incubation, 2 mL of an aqueous solution containing 10 g/L Tris, 200 mg/L cysteine and 100 mg/L catechol were added to stop the reaction. The fluorescence of this mixture was measured in a Locarte filter fluorimeter with an activation wavelength of 313 nm and a fluorescence wavelength of 430 nm (interference filters). The instrument was standardized against the fluorescence of a block of Araldite CY212 resin. The activity of the enzymes, when given in mUnits, was calculated from the activity quoted by the supplier. The activation and fluorescence spectra of the compounds formed from capsaicin and creosol (2-methoxy-4-methylphenol) were determined by means of a Perkin-Elmer luminescence spectrometer LS 50.

The rate of disappearance of the second substrate in the peroxidase reaction was measured by recording the oxidizing current at a carbon electrode similar to that used for the redox potential measurements. The electrode potential was held at +0.5 V. Polarographic measurements were used by Doskocil [15] to study peroxidase reactions.

The formation of fluorescent oxidation products from phenolic compounds was also investigated by combining electrochemical oxidation with fluorescence measurements (Fig. 1). A carbon paste, a glassy carbon and a platinum electrode were used in these studies. The anodic potential on the electrode was increased in steps of 0.1 V starting at 0 V and kept constant for 2–5 min to enable a clear rate of increase in fluorescence to be established. The rates of increase were normalized with respect to the maximum rate of increase observed and are expressed as percentages of this maximum.

Chemical oxidation of 4-hydroxy-3-methoxyphenyl compounds. The compounds studied were oxidized with potassium hexacyanoferrate III in aqueous solutions containing ammonia, sodium acetate or Tris. The fluorescent product was stabilized by the addition of cysteine [6]. The concentrations of the chemicals are given in the Results section.

Chemicals and reagents. The following phenolic compounds were used: capsaicin (8-methyl-*N*-vanillyl-6-nonenamide), 4-hydroxy-3-methoxyphenylacetic acid (homovanillic acid; HVA), 4-hydroxy-3-methoxybenzoic acid (vanillic acid; VA), 4-hydroxybenzoic acid, DL-4-hydroxy-3-methoxymandelic acid (VMA) and 4-hydroxy-3-methoxycinnamic acid (ferulic acid), all from the Sigma Chemical Co. (Poole, U.K.); 4-hydroxy-3-methoxybenzylamine HCl and 4-methylphenol (*p*-cresol) from the Aldrich Chemical Co. (Gillingham, U.K.); 2-methoxyphenol (guaiacol) from Koch-Light

Table 1. Estimated redox potentials (E_0') of capsaicin and some phenolic compounds

Compound	Estimated redox potential (E_0') (V) pH 7.0	pH 7.4	pH 7.8
p-OH BA	0.70 ± 0.002	0.69 ± 0.004	0.64 ± 0.002
VA	0.47 ± 0.003	0.47 ± 0.010	0.43 ± 0.002
HVA	0.39 ± 0.002	0.37 ± 0.010	0.35 ± 0.002
Capsaicin	0.34 ± 0.004	0.33 ± 0.005	0.29 ± 0.003
Creosol		0.30 ± 0.005	
Guaiacol		0.38 ± 0.002	
Ferulic acid		0.30 ± 0.004	

The values are given as mean ± SE calculated from 4–10 observations. *p*-OH BA: 4-hydroxybenzoic acid; VA: 4-hydroxy-3-methoxybenzoic acid (vanillic acid); HVA: 4-hydroxy-3-methoxyphenylacetic acid (homovanillic acid). The redox potentials were measured at a concentration of each compound of 3×10^{-4} M dissolved in potassium phosphate buffer, 0.2 M, of the appropriate pH. Voltages are with reference to an Ag/AgCl electrode.

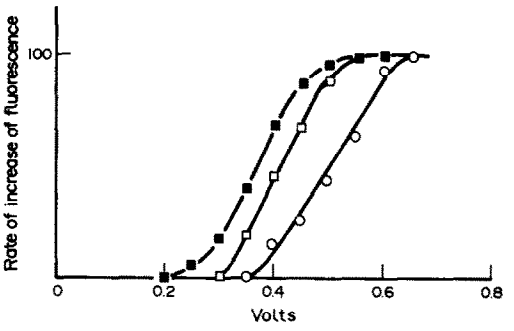

Fig. 3. Fluorescence developed from phenolic compounds oxidized electrochemically. Solutions (100 μg/mL) of each compound in potassium phosphate buffer (0.1 M, pH 7.5) were passed through an electrochemical cell containing a carbon paste electrode. The applied anodic voltage was increased stepwise and the rate of increase in fluorescence recorded. The rate of increase of fluorescence observed at each potential was normalised with respect to the maximum rate of increase in fluorescence observed for each compound. Values are means of three observations. (■) 2-Methoxy-4-methylphenol (creosol); (□) 4-hydroxy-3-methoxyphenylacetic acid (HVA); (○) 4-methylphenol (*p*-cresol).

(Haverhill, U.K.); 2-methoxy-4-methylphenol (creosol) prepared by the method described by Schwarz and Hering [16].

Cysteine and catechol were obtained from Hopkin and Williams Ltd (Chadwell Heath, U.K.) and recrystallized from ethanol and water, respectively. Tris was obtained from Sigma and hydrogen peroxide from Fisons Scientific Apparatus (Loughborough, U.K.). All other chemicals were of analytical reagent grade. Carbon powder (Ultracarbon UCP-IM) was obtained from Johnson, Matthey plc, (Royston, U.K.) and Dow-Corning silicone high vacuum grease from Hopkin and Williams. Single distilled water was used throughout. The enzymes, horseradish peroxidase, type II and bovine lactoperoxidase were obtained from Sigma.

Male Wistar rats (A. J. Tuck and Son, Rayleigh, U.K.) were killed by stunning and decapitation and the parotid salivary glands were removed and freed from adhering tissue. They were then homogenized in 10 volumes (w/v) of 1 mM potassium phosphate buffer, pH 7.4 in a glass homogenizer. The homogenate was centrifuged at 11,000 *g* for 5 min. The supernatant was used for the enzyme studies both fresh and after storage at −20° for up to 10 days. Human saliva was diluted 1:1 with 0.2 M potassium phosphate buffer, pH 7.4.

RESULTS

Electrochemical oxidation

Figure 2A and B shows partial, single sweep voltammograms for seven of the phenolic compounds studied. Table 1 gives estimates of the redox potentials (E_0') obtained for these compounds. The results show that creosol and ferulic acid have similar redox potentials at pH 7.4 and that capsaicin has a redox potential about 0.03 V higher. All of the other compounds showed redox potentials that were higher than that of capsaicin. When the pH of the buffer was reduced to 7.0, this had little effect on the redox potentials of 4-hydroxybenzoic acid, VA, HVA or capsaicin. At pH 7.8, there was a small reduction in E_0' for these compounds.

It was found that when capsaicin was investigated, only one voltammogram sweep could be performed. The electrode was then found to be poisoned and would not give a satisfactory second response with capsaicin or any other phenolic compound. Poisoning of the electrode was not significant with the other compounds, apart from ferulic acid, until about six sweeps had been made. In the above experiments, not more than four phenolic compounds were tested on the same electrode and, except for capsaicin and ferulic acid, were tested in different sequences. Capsaicin or ferulic acid were always tested last and then a fresh electrode prepared.

The electrochemical formation of fluorescent oxidation products from capsaicin, HVA, creosol and *p*-cresol was also investigated. The relative rates of formation of fluorescent derivatives with respect to electrode potentials are illustrated in Fig. 3. This shows that fluorescent oxidation products, presumably dimers, are formed from creosol, HVA and *p*-cresol. The voltages at which fluorescence is obtained from creosol and HVA correspond with the single sweep voltammogram described above. The oxidation of capsaicin, in similar experiments, was investigated with a carbon paste, a glassy carbon or a platinum electrode in the electrochemical oxidation-fluorescence apparatus. Only on one occasion did a small increase in fluorescence occur. It was again found that, after a short exposure to capsaicin, the electrodes were severely poisoned and no longer oxidized HVA.

The formation of fluorescent oxidation products of 4-hydroxy-3-methoxyphenyl compounds by peroxidase

HVA has been employed successfully in several assays of oxidative enzymes to measure the rate of

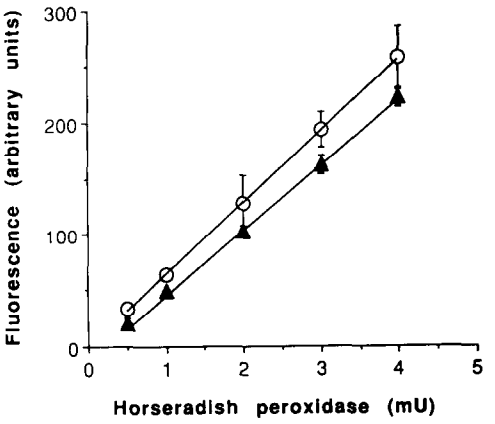

Fig. 4. Assay of horseradish peroxidase activity with 2-methoxy-4-methylphenol (creosol, ○) or capsaicin (▲) as indicating second substrates. Incubations were made with $[H_2O_2] = 8 \times 10^{-5}$ M and [second substrate] = 5.5×10^{-5} M. Incubation time: 5 min at 37°. Results are mean ± SE (N = 3).

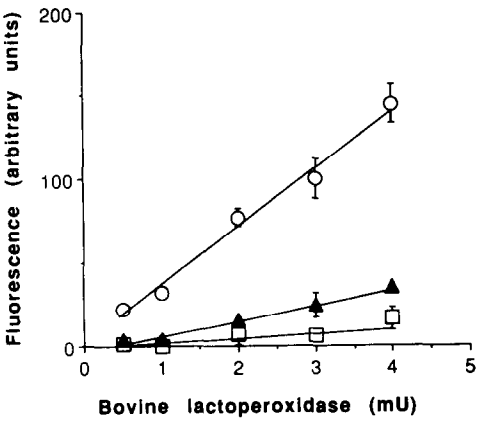

Fig. 5. Assay of bovine lactoperoxidase activity with 2-methoxy-4-methylphenol (creosol, ○), capsaicin (▲) and 4-hydroxy-3-methoxyphenylacetic acid (HVA, □) as indicating second substrates. Incubations were made with $[H_2O_2] = 8 \times 10^{-5}$ M and [second substrate] = 5.5×10^{-5} M. Incubation time: 5 min at 37°. Results are given as mean ± SE (N = 4).

formation of hydrogen peroxide [8–11] by means of the peroxidase reaction. In these assays, a relatively large amount of peroxidase is used. It was decided to investigate if HVA could be used to measure peroxidase activity in an attempt to find an alternative second substrate that could be of help in determining whether, or not, capsaicin might be oxidized in tissues. It was found that HVA was unsuitable as an indicating second substrate in the horseradish peroxidase reaction. Fluorescence could be measured when 25 or 50 mUnits of enzyme were present in the incubation mixture together with HVA (1.1×10^{-3} M) but no significant fluorescence could be detected when 10 mUnits were tested even when the incubation time was 20 min. Further tests showed that the system was very sensitive to the concentration of H_2O_2 and that a concentration of 10^{-4} M gave an optimal yield of fluorescence.

When creosol or capsaicin (final concentration 5.5×10^{-5} M) was used as indicating second substrates in the horseradish peroxidase reaction a more intense fluorescence was obtained whereas HVA, under similar conditions, did not yield a significant fluorescence. A measurable fluorescence could be obtained within 5 min with 0.5 mUnits of enzyme with both capsaicin and creosol. Both of these compounds gave a linear response to the amount of enzyme present (up to 4 mUnits) in the incubation mixture and yielded a similar intensity of fluorescence (Fig. 4).

Analysis of the characteristics of the fluorescence derived from capsaicin and creosol showed that the wavelengths for maximum activation were 318.5 nm (capsaicin) and 319 nm (creosol). The wavelengths for maximum fluorescence were 424 nm (capsaicin) and 425.5 nm (creosol). These can be compared with the results reported in the literature [5, 7–10, 17] for the fluorescence of the HVA dimer which report that the wavelength of maximum activation is 305–315 nm and the wavelength of maximum fluorescence is 425–430 nm. Neither 4-hydroxy-3-methoxybenzylamine nor ferulic acid produced a fluorescence in the horseradish peroxidase assay. Ferulic acid does not give rise to a fluorescent product when oxidized chemically [18].

Capsaicin, creosol and HVA were also tested as indicating second substrates for bovine lactoperoxidase activity. Figure 5 shows that capsaicin appears not to be as good a second substrate for this enzyme as creosol. Again, HVA is very poor in this respect. It was found also with lactoperoxidase, that concentrations of H_2O_2 above 10^{-4} M gave rise to much lower yields of fluorescence when creosol was used as the second substrate.

The standard assay for peroxidase with guaiacol as indicating second substrate as described by Maehly and Chance [19] was used to examine the effect of increasing the concentration of H_2O_2. It was found that maximum apparent horseradish peroxidase activity occurred with a concentration of H_2O_2 of about 5×10^{-3} M. This is more than 10-fold greater than the maximal concentration of H_2O_2 when creosol or capsaicin is used.

Peroxidase activity in human saliva could be detected with either creosol or capsaicin as the second substrate. Capsaicin yielded approximately 70% of the fluorescence given by creosol. Once again, HVA gave rise to very little fluorescence.

Similar results were obtained with an extract of rat parotid salivary glands. It was also found that the development of fluorescence derived from creosol was subject to a delay of about 6 min. Treatment of the extract of salivary glands with MnO_2 [20] to oxidize substances with a redox potential lower than that of creosol reduced the delay to about 2.5 min but did not abolish it completely.

Because of these unexpected results with HVA, the rate of disappearance of the second substrate, while the peroxidase reaction was taking place, was

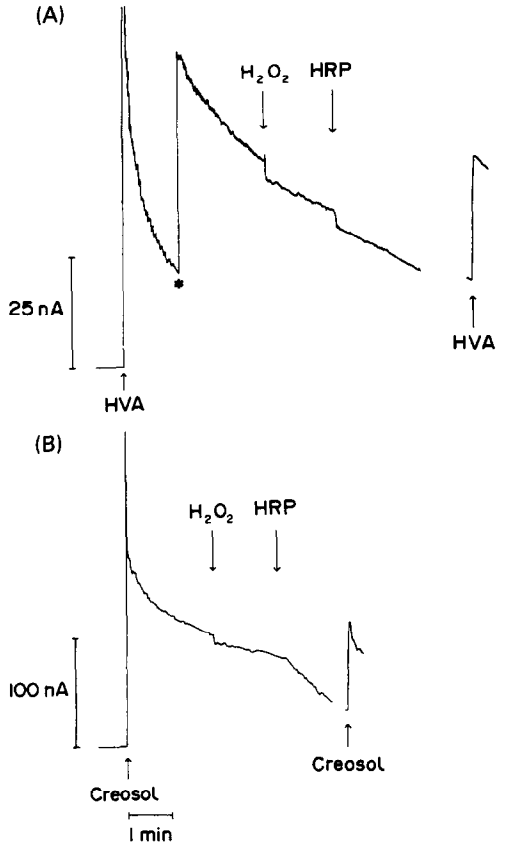

Fig. 6. Electrochemical oxidation current-time tracings showing the utilization of the second substrate in the horseradish peroxidase reaction. The tracing shows the effect of addition of the second substrate (A) 4-hydroxy-3-methoxyphenylacetic acid (HVA) or (B) 2-methoxy-4-methylphenol (creosol) followed by H_2O_2 and horseradish peroxidase (HRP) on the current flowing at a carbon paste electrode with an anodic potential maintained at +0.5 V (versus Ag/AgCl) in a stirred solution. The tracing for HVA was recorded at an amplification four times greater than that for creosol. Concentration of H_2O_2: 10^{-4} M; amount of enzyme: 300 mUnits; concentration of creosol or HVA: 3×10^{-5} M in potassium phosphate buffer, 0.2 M, pH 7.4. The addition of creosol or HVA at the end of the tracing to give an increase in concentration of 3×10^{-5} M enabled an estimate of the rate of utilization of the second substrate to be made. *Pen adjusted.

determined electrochemically. Figure 6 shows that there was no change in the slope of the current-line tracing when the peroxidase reaction should have been taking place if HVA was present as the second substrate, whereas there was an increase in the slope when creosol was present. Estimates of the rate of utilization of different phenolic compounds in the horseradish peroxidase reaction by this method were variable but indicated that creosol (5 ± 2 nmol/min; mean ± SE), guaiacol (10 ± 3 nmol/min) and capsaicin (18 ± 6 nmol/min) were readily oxidized whereas no utilization of HVA or VMA cold be detected.

Chemical oxidation

A fluorescent dimer can be prepared from HVA by oxidation with $FeCl_3$ [7] or potassium hexacyanoferrate III [6]. The latter method was used to oxidize capsaicin, creosol and HVA. When the oxidation was carried out in the presence of 2.5 M ammonium hydroxide and oxidation allowed to proceed for 4 min, the fluorescence yield from capsaicin was approximately 10% of that from HVA. Shorter oxidation times lessened the difference but the yield of fluorescence was always lower. The yield of fluorescence was increased by over 3-fold when the oxidation of capsaicin was carried out in 0.05 M Tris (pH 10.4). A similar intensity of fluorescence was obtained from creosol under these experimental conditions and, with both compounds, the conversion was very rapid, a maximal fluorescence being obtained within 1 min.

Preliminary experiments to obtain a sample of the fluorescent oxidation product of capsaicin have indicated the formation of at least two compounds when the oxidation with potassium hexacyanoferrate III (in amounts equimolar with capsaicin) was carried out in Tris (10 g/L), water or sodium acetate (10 g/L) and sodium acetate (10 g/L) to which a few crystals of Tris were added. One of these compounds was intensely fluorescent and was soluble in diethylether or ethanol but was only slightly soluble in water. The second compound was not soluble in these solvents. Further experiments will be carried out in order to obtain sufficient material to characterise the fluorescent compound.

DISCUSSION

Capsaicin, the pungent constituent of *Capsicum* fruits, has a variety of pharmacological actions on sensory neurones [21, 22]. Initially, the neurones are stimulated to release neurotransmitter substances including substance P. There is rapid desensitization to this effect. It can be followed by neuronal block, by long-lasting impairment of function and, finally, irreversible neurodegeneration can take place. The property of pungency, with capsaicin and related compounds, appears to be associated with the 4-hydroxy-3-methoxyphenyl (vanillyl) group [1, 23] and desensitization has been attributed to the acylamine group in the side chain [24]. The action of capsaicin involves an increase in intracellular calcium [25].

The vanillyl group occurs in the normal metabolites of catecholamines in the body and its formation is important in their inactivation [2, 3]. The vanillyl group, by itself, therefore, might be considered as being comparatively innocuous. The importance of this group in the action of capsaicin suggested that a metabolic change might be involved. The vanillyl group of capsaicin can be hydroxylated *in vitro* by liver microsomes [26].

Two observations in plant biochemistry indicated that oxidation of the vanillyl moiety should be considered in regard to the actions of capsaicin. The gelling of the water soluble pentosans in wheat flour, a process important in dough making and bakery, depends on cross-links formed by the oxidative dimerization of ferulic acid [13, 27]. Cross-linking, through dimerization of ferulic acid, is also thought to be important in the structure of the cell wall in

graminaceous plants [12, 28, 29]. The possibility that a similar reaction leading to cross-linking between molecules of capsaicin could take place was, therefore, investigated.

HVA has been used as an indicating second substrate in enzyme reaction systems incorporating horseradish peroxidase. Thus, the possibility that a similar dimer of capsaicin (the (*N*,*N*')-di-8-methyl-6-nonenoic acid derivative of 6,6'-dehydro-di-2-methoxy-4-aminomethylphenol) might be formed in peroxidatic reactions was studied. It was found that significant amounts of the fluorescent dimer could be formed from HVA only when there was a large amount of horseradish peroxidase, and a small amount of H_2O_2, present in the incubation mixture. Similar results were obtained with peroxidase enzymes from animal sources, bovine lactoperoxidase and salivary peroxidase. This means that HVA should not be used as an indicating second substrate to determine the activity of peroxidatic enzymes in tissues whereas it is satisfactory for measuring the formation of small amounts of H_2O_2 in the presence of an excess of horseradish peroxidase.

Capsaicin was a better indicating second substrate for the horseradish peroxidase reaction, yielding sufficient intensity of fluorescence, presumably due to the formation of a dimer comparable with that formed from HVA, to enable less than 1 mUnit of peroxidase activity to be measured after 5 min incubation. However, capsaicin binds tightly to lipid components of tissues and would be unsuitable as an indicator of peroxidase activity in tissue homogenates because of the variable amount of binding that might take place. The dimer of capsaicin would also be tightly bound to lipid components of the tissue.

It has been proposed [30] that *p*-cresol be used as an indicating second substrate for peroxidase reactions. However, *p*-cresol has a higher redox potential than compounds possessing a vanillyl group and would be more subject to interference by tissue components than the latter. Creosol, the vanillyl analogue of *p*-cresol, was tested and found to be suitable as an indicating second substrate for the peroxidatic reactions but would be subject to the pitfalls described by Hirsch and Parks [31] if used for continuous rate measurements. Creosol has a redox potential close to that of capsaicin and is less likely to bind tightly to lipid components of cells. It is suggested that creosol be used to indicate whether, or not, capsaicin can be oxidized by tissues to form a dimer.

The experiments made with extracts of rat salivary glands showed that there are tissue components that can interfere with the use of creosol as a quantitative enzyme activity indicator although they also demonstrate that dimerization can take place in such extracts. Pretreatment of the tissue extract with MnO_2 [20] reduced such interference but manganese can modify peroxidase systems [32]. In the experiments reported earlier [20], a large excess of peroxidase was employed and no inhibition or activation of this enzyme due to MnO_2 treatment was observed.

The finding that the two major metabolites of catecholamines HVA and VMA are poor second substrates for the peroxidase enzymes used here might contribute to their lack of biological action in the concentration at which they occur in the body.

The present experiments have shown that capsaicin can be oxidized by electrochemical, enzyme and chemical methods. One of the products appears to be a fluorescent dimer similar to those formed from HVA and other phenolic compounds. The fluorescence does not arise from splitting the capsaicin molecule at the amide group because 4-hydroxy-3-methoxybenzylamine did not give a fluorescent product when tested in the peroxidase reaction.

It has been reported [33] that capsaicin can be oxidized in an electrochemical detector for HPLC but the proposed reaction which involved the demethylation of the methoxy group on the vanillyl ring would require greater energy than is necessary for oxidative dimer formation.

If capsaicin is oxidized to a dimer after it is bound to the lipid membranes of sensory afferent neurones then the cross-linking between capsaicin molecules or, possibly, between capsaicin and another suitable group present on the membrane or intracellular lipid structures, might cause a major disturbance of physiological function. The possibility that cross-linking between densely packed, non-myelinated fibres, comparable with the cross-linking of cellulose components of plant cells, takes place should also be considered.

Acknowledgements—A. Boersch was in receipt of a Scholarship from the Studienstiftung des deutschen Volkes to enable a year of study at Cambridge University.

The authors would like to thank Mr M. Bootman for his help with the measurement of the activation and fluorescence spectra.

REFERENCES

1. Szolcsányi J and Janscó-Gábor A, Sensory effects of capsaicin congeners. Part I. Relation between chemical structure and pain producing potency of pungent agents. *Arzneim-Forsch* **25**: 1877–1881, 1975.
2. Armstrong MD, McMillan A and Shaw KNF, 3-Methoxy-4-hydroxy-*d*-mandelic acid, a urinary metabolite of norepinephrine. *Biochim Biophys Acta* **25**: 422–423, 1957.
3. Axelrod J, Methylation reactions in the formation and metabolism of catecholamines and other biogenic amines. *Pharmacol Rev* **18**: 95–113, 1966.
4. Musso H, Phenol coupling. In: *Oxidative Coupling of Phenols* (Eds. Taylor WI and Battersby AR), pp. 1–94. Edward Arnold Ltd, London and Marcel Dekker, Inc, New York, 1967.
5. Corrodi H and Werdinius B, Oxidative conversion of homovanillic acid to a fluorescent compound. *Acta Chem Scand* **19**: 1854–1858, 1965.
6. Anden N-E, Roos B-E and Werdinius B, On the occurrence of homovanillic acid in brain and cerebrospinal fluid and its determination by a fluorometric method. *Life Sci* **2**: 448–458, 1963.
7. Sharman DF, A fluorimetric method for the estimation of 4-hydroxy-3-methoxyphenylacetic acid (homovanillic acid) and its identification in brain tissue. *Br J Pharmacol* **20**: 204–213, 1963.
8. Guilbault GG, Kramer DN and Hackley E, A new substrate for fluorometric determination of oxidative enzymes. *Anal Chem* **39**: 271, 1967.

9. Snyder SH and Hendley ED, A simple and sensitive fluorescence assay for monoamine oxidase and diamine oxidase. *J Pharmacol Exp Ther* **163**: 386–392, 1968.
10. Tipton KF, A sensitive fluorometric assay for monoamine oxidase. *Anal Biochem* **28**: 318–325, 1969.
11. Sharman DF and Cooper TR, A semi-automated fluorimetric method for measuring acetylcholinesterase activity in small volumes of cerebrospinal fluid and tissue extracts using acetylcholine as a substrate. *J Neurosci Methods* **16**: 301–308, 1986.
12. Hartley RD and Jones EC, Diferulic acid as a component of cell walls of *Lolium multiflorum*. *Phytochemistry* **15**: 1157–1160, 1976.
13. Markwalder HU and Neukom H, Diferulic acid as a possible crosslink in hemicelluloses from wheat germ. *Phytochemistry* **15**: 836–837, 1976.
14. McCreery RL, Dreiling R and Adams RN, Voltammetry in brain tissue: quantitative studies of drug interactions. *Brain Res* **73**: 23–33, 1974.
15. Doskocil J, A polarographic study of the kinetics of some peroxidatic reactions. *Bull Int Acad Tchéque Sci* **52**: 153–169, 1951.
16. Schwarz R and Hering H, Creosol. *Organic Syntheses* **33**: 17–19, 1953.
17. Murphy GF, Robinson D and Sharman DF, The effect of tropolone on the formation of 3,4-dihydroxyphenylacetic acid and 4-hydroxy-3-methoxyphenylacetic acid in the brain of the mouse. *Br J Pharmacol* **36**: 107–115, 1969.
18. Gjessing LR, Vellan EJ, Werdinius B and Corrodi H, Oxidative conversion of 3-methoxy-4-hydroxyphenyl (vanyl) compounds to fluorescent substances. *Acta Chem Scand* **21**: 820–821, 1967.
19. Maehly AC and Chance B, The assay of catalases and peroxidases. *Methods Biochem Analysis* **1**: 357–424, 1954.
20. Elliott J, Callingham BA and Sharman DF, Semicarbazide-sensitive amine oxidase (SSAO) of the rat aorta. Interactions with some naturally occurring amines and their structural analogues. *Biochem Pharmacol* **38**: 1507–1515, 1989.
21. Buck SH and Burks TF, The neuropharmacology of capsaicin: review of some recent observations. *Pharmacol Rev* **38**: 179–226, 1986.
22. Maggi CA and Meli A, The sensory-efferent function of capsaicin-sensitive sensory neurons. *Gen Pharmacol* **19**: 1–43, 1988.
23. Szallasi A and Blumberg PM, Resiniferatoxin, a phorbol-related diterpene, acts as an ultrapotent analog of capsaicin, the irritant constituent in red pepper. *Neuroscience* **30**: 515–520, 1989.
24. Szolcsányi J and Jancsó-Gábor A, Sensory effects of capsaicin congeners. Part II. Importance of chemical structure and pungency in desensitizing activity of capsaicin-type compounds. *Arzneim-Forsch* **26**: 33–37, 1976.
25. Wood JN, Winter J, James IF, Rang HP, Yeats J and Bevan S, Capsaicin-induced ion fluxes in dorsal root ganglion cells in culture. *J Neurosci* **8**: 3208–3220, 1988.
26. Miller MS, Brendel K, Burks TF and Sipes IG, Interaction of capsaicinoids with drug-metabolizing systems. *Biochem Pharmacol* **32**: 547–551, 1983.
27. Geissmann T and Neukom H, On the composition of the water soluble wheat flour pentosans and their oxidative gelation. *Lebensm-Wiss u Technol* **6**: 59–62, 1973.
28. Hartley RD, Whatley FR and Harris PJ, 4,4′-Dihydroxytruxillic acid as a component of cell walls of *Lolium multiflorum*. *Phytochemistry* **27**: 349–351, 1988.
29. Shibuya N, Phenolic acids and their carbohydrate esters in rice endosperm cell walls. *Phytochemistry* **23**, 2233–2237, 1984.
30. Roth M, Fluorimetric assay of enzymes. *Methods Biochem Anal* **17**: 189–285, 1969.
31. Hirsch HE and Parks ME, Fluorometric oxidase assays: pitfalls caused by action of ultraviolet light on lipids. *Anal Biochem* **122**: 79–84, 1982.
32. Kenten RH and Mann PGJ, The oxidation of manganese by peroxidase systems. *Biochem J* **46**: 67–73, 1950.
33. Kawada T, Watanabe T, Katsura K, Takami H and Iwai K, Formation and metabolism of pungent principle of *Capsicum* fruits. XV. Microdetermination of capsaicin by high-performance liquid chromatography with electrochemical detection. *J. Chromatogr* **329**: 99–105, 1985.