Phytochemistry, 1965, Vol. 4, pp. 677 to 685. Pergamon Press Ltd. Printed in England

THE INHIBITION OF THE PHENOLASE FROM APPLE PEEL BY POLYVINYLPYRROLIDONE

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(*Received* 4 *March* 1965)

Abstract—The mechanism of the inhibition of phenolase from apple peel by PVP has been studied and shown to be competitive in nature. Dissociating agents such as certain anionic detergents reversed inhibition by PVP and further increased phenolase activity.

INTRODUCTION

EARLIER work in this laboratory has shown that highly active mitochondria could be isolated from apple peel (Jones and Hulme,[1] Hulme *et al.*[2]) or from flower petals (Hulme *et al.*[3]) when 1% polyvinylpyrrolidone(PVP) was incorporated in the extraction medium. More recently Jones *et al.*[4] have investigated the various factors involved in the use of PVP and other polymers in the isolation of enzymes from apple fruits and have discussed theories concerning the probable mode of action of PVP in protecting mitochondria. In the course of this work it was observed also that the phenolase (*o*-diphenol: O_2 oxidoreductase, E.C. No: 1.10.3.1.) activity of these preparations was considerably lower than that recorded when PVP was omitted from the extracting medium. It was concluded that the phenolase was inhibited by PVP. However, the possibility that partially oxidized (polymerized) phenolic compounds also combined with PVP was not entirely eliminated since evidence that such combinations with PVP can occur has been presented by Gustavson.[5] This paper presents the results of further investigations on the inhibition of phenolase by PVP and the reversal of this PVP inhibition by suitable treatments.

RESULTS

In view of a recent report by Harel *et al.*[6] that their preparation of phenolase from apples showed two pH optima, at pH 5·1 and 7·3 respectively the change of phenolase activity with pH was checked for both the mitochondrial and "purified" enzyme, chlorogenic acid and 4-methylcatechol being used as test substrates. From Fig. 1 it will be seen that maximum activity occurred between pH 4·8 and 5·0 with mitochondria: similar results were obtained with the purified preparation and no secondary pH optimum occurred with either preparation. This agrees with earlier New Zealand work on the phenolase of apples (Walker[7]).

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[1] J. D. JONES and A. C. HULME, *Nature* **191**, 370 (1961).
[2] A. C. HULME, J. D. JONES and L. S. C. WOOLTORTON, *Phytochem.* **3**, 173 (1964).
[3] A. C. HULME, J. D. JONES and L. S. C. WOOLTORTON, *Nature* **201**, 795 (1964).
[4] J. D. JONES, A. C. HULME and L. S. C. WOOLTORTON, *Phytochem.* **4**, 659 (1965).
[5] K. H. GUSTAVSON, *Svensk Kem. Tidskr.* **66**, 359 (1954).
[6] E. HAREL, A. M. MAYER and Y. SHAIN, *Physiol. Plant.* **17**, 921 (1964).
[7] J. R. L. WALKER, *Aust. J. Biol. Sci.* **17**, 360 (1964).

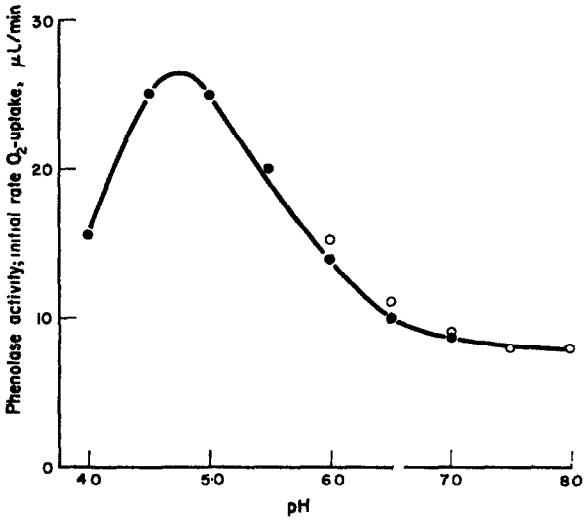

FIG. 1. VARIATION OF PHENOLASE ACTIVITY WITH pH USING MITOCHONDRIA AND 10 µmoles CHLOROGENIC ACID AS SUBSTRATE. SIMILAR RESULTS WERE OBTAINED WHEN 4-METHYLCATECHOL OR "PURIFIED" ENZYME WAS USED.

Phosphate-citrate buffer (●), phosphate buffer (○).

Experiments with Mitochondria

When mitochondria, prepared without PVP, were incubated with chlorogenic acid in the presence of various amounts of PVP it was found that the degree of inhibition of oxygen uptake increased regularly until a PVP concentration of 1 per cent was reached but thereafter extra PVP caused little further inhibition. This finding correlates with the observations of Jones *et al.*[4] who found, using mitochondria from similar apples to these used here, that 1% PVP in the extraction medium yielded mitochondria with maximum dehydrogenase activity; they attributed inhibition of mitochondrial activity in the absence of PVP to the action of oxidized phenolics formed during the preparation of the enzyme. Thus we have the significant fact that 1% PVP brings about maximum inhibition of mitochondrial phenolase and gives maximum activity to the mitochondrial dehydrogenases.

Using the procedure suggested by Dixon[8] a plot of the reciprocal of the initial rate of O_2-absorbtion ($1/v$) against inhibitor concentration (i) for two different levels of substrate was found to be linear for PVP concentrations up to 1·0%. The position of the intersection of the two graphs (see Fig. 2*a*) suggested competitive inhibition and gave a value for the inhibitor constant (K_i) of 0·27 per cent or $0{\cdot}97 \times 10^{-4}$ M, assuming an average mol. wt. of 28,000 for the Kollidon-25 grade PVP used in these experiments. A duplicate experiment with 4-methylcatechol as the substrate gave closely similar results (K_i: 0·25 per cent).

The competitive nature of the inhibition of phenolase by PVP was also demonstrated by the experiment shown in Fig. 3 where it may be seen that the addition of extra substrate to the Warburg flask (D) containing 1% PVP and 8µmoles substrate increased the rate of O_2-uptake almost to that observed for 1% PVP and 16 µmoles substrate (flask C).

In view of the evidence presented by Gustavson[5] that PVP can form complexes with vegetable tannins, analogous to tannin-protein complexes, and these complexes could be

[8] M. DIXON, *Biochem. J.* **55**, 170 (1953).

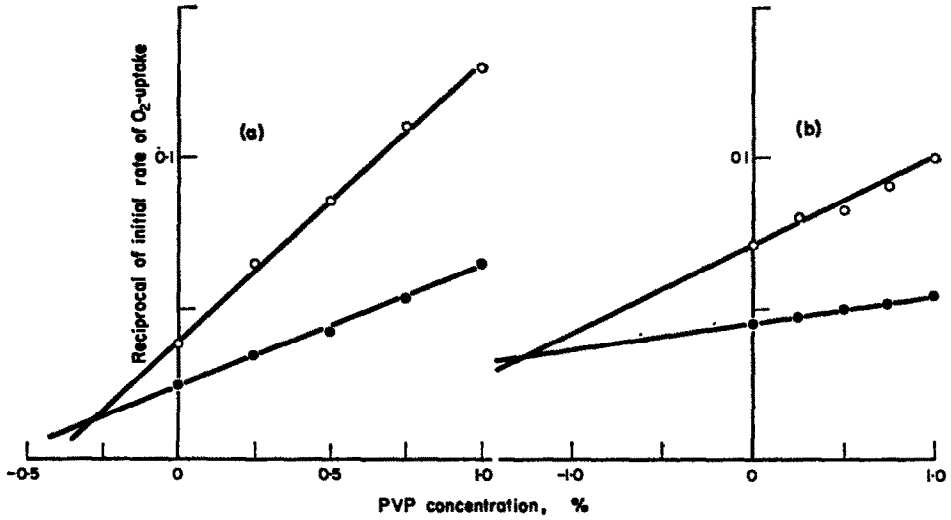

FIG. 2. INHIBITION OF MITOCHONDRIAL (A) AND "PURIFIED" (B) PHENOLASE BY PVP (K-25 GRADE); GRAPHICAL DETERMINATION OF INHIBITOR CONSTANT BY THE METHOD OF DIXON.[8]

Open circles (○) denote 16 μmoles substrate, filled in circles (●) denote 8 μmoles substrate.

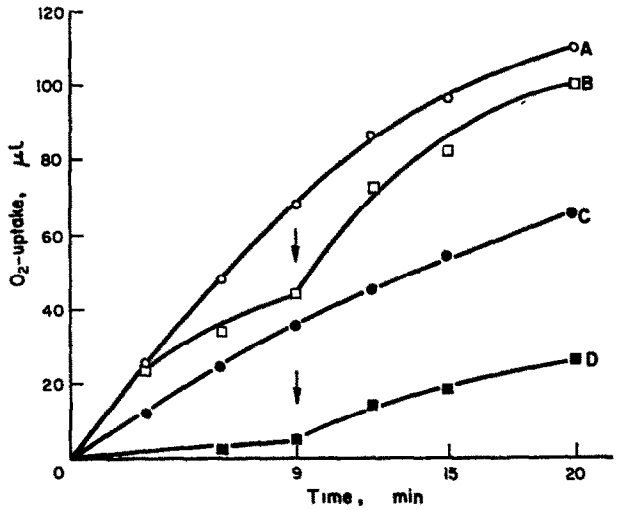

FIG. 3. REVERSAL OF PVP INHIBITION OF MITOCHONDRIAL PHENOLASE BY ADDITION OF EXTRA SUBSTRATE.

Double side-arm flasks contained 16 μmoles chlorogenic acid (A), 8 μmoles chlorogenic acid (B), 16 μmoles chlorogenic acid + 1% PVP (C), 8 μmoles chlorogenic acid + 1% PVP (D). Extra substrate (8 μmoles) added from second side-arm to flasks B and D at point indicated by arrow.

split by 6–8 M urea solutions the action of dissociating agents such as urea and synthetic detergents on PVP-inhibited mitochondria was investigated. The results of these experiments are recorded in Table 1 where it will be seen that the anionic detergents sodium dodecyl sulphate and Manoxol OT (sodium dioctyl sulphosuccinate) not only reversed the inhibition of phenolase activity caused by PVP but also brought about the further activation of both preparations up to a similar limiting value. High concentrations (5 M) of urea, which are known to affect H-bonding, also partially reactivated the PVP-inhibited mitochondria.

Table 1. Reactivation of phenolase in mitochondria prepared from equal amounts of apple peel with or without the addition of 1% PVP in the extraction medium

Dissociating agent	Concentration mM	Phenolase activity (initial rate of O_2-uptake, μl/min) Without PVP	With PVP
None (control)	—	4·9	0·2
Sodium dodecyl sulphate	0·5	6·5	2·0
	1	7·6	4·2
	5	16·4	15·0
	10	17·5	16·4
Manoxol OT (Sodium dioctyl sulphosuccinate	0·5	11·8	7·0
	1	12·5	9·7
	5	13·9	13·2
	10	18·0	13·2
Manoxol IB (Sodium di-*iso*-butyl sulphosuccinate)	1	4·8	0·1
Urea	500	4·8	0·2
	5×10^3	3·9	2·1
Lissapol N	1	4·8	0·2
Cetyl pyridinium bromide	1	4·1	3·0
Cetyl tri-methyl ammonium bromide	1	3·4	2·0

These PVP-inhibition and detergent-activation effects were further demonstrated by incubating an aliquot of the mitochondrial fraction, prepared in the absence of PVP, with 1% PVP for 60 min, after which time it was centrifuged, washed with buffer and finally resuspended in buffer solution. An equal volume of the same mitochondrial preparation was similarly treated, but without the PVP, to serve as a control. The reults shown in Table 2 indicate that PVP binds onto the mitochondria thereby inhibiting phenolase activity. This inhibition was not removed by washing but was overcome by treatment with Manoxol OT suggesting that the detergent removes PVP from the mitochondrial complex. Attempts to estimate the PVP released by detergent activation using the iodine titration method of Dwyer

Table 2. Reactivation of phenolase activity of mitochondrial fraction (prepared in the absence of PVP) after treatment with 1% PVP. (Warburg flasks contained 0·5 ml enzyme, 0·1 M phosphate-citrate buffer, pH 5·0, 10 μmoles chlorogenic acid and additions as listed)

Additions to Warburg flask	Mitochondria prepared without PVP. Initial rate O_2-uptake μl/min	Mitochondria treated with 1% PVP. Initial rate O_2-uptake μl/min
None	26*	17
1% PVP	12·5	—
1% PVP + 0·01 M Manoxol OT	35	—
0·01 M Manoxol OT	35	32

* Identical with original, i.e. no loss of activity by washing with buffer.

and Lewandowski[9] were inconclusive owing to the small quantities involved and the non-specificity of the method.

Experiments with PEG-precipitate

The supernatant liquid from the mitochondrial preparations was treated with polyethylene glycol (PEG) to obtain the PEG-precipitate (see Experimental section). From the results recorded in Table 3 it will be seen that the phenolase activity of the PEG-precipitate, prepared from PVP-containing medium, was markedly increased in the presence of certain anionic detergents and, to a lesser extent, 5 M urea. In the case of the alkyl sulphosuccinate (Manoxol) esters the size of the alkyl group also determined the extent of the reactivation, the dioctyl ester being the most effective.

TABLE 3. REACTIVATION OF PHENOLASE IN PEG-PRECIPITATES BY DISSOCIATING AGENTS

Dissociating agent	Type	Concentration mM	Phenolase activity. (Initial rate O_2-uptake μl/min)
None (control)	—	—	2·6
Sodium dodecyl sulphate	Anionic detergent	1	15·0
		5	20·0
		10	25·0
Manoxol IB (sodium di-*iso*-butyl sulphosuccinate)	Anionic detergent	1	2·6
		10	2·6
Manoxol MA (Sodium di-(methyl-amyl) sulphosuccinate)	Anionic detergent	1	3·4
		5	6·7
		10	14·7
Manoxol OT (sodium dioctyl sulphosuccinate)	Anionic detergent	0·5	16·0
		1	24·4
		5	28·0
		10	28·0
Manoxol N (sodium di-nonyl sulphosuccinate)	Anionic detergent	0·5	8·7
		1	18·7
		5	20·0
		10	20·0
Manoxol TR (sodium di-tridecyl sulphosuccinate)	Anionic detergent	1	19·4
		5	19·4
		10	23·4
Lissapol N (*p*-Isoöctylphenyl ether of polyethyleneglycol)	Non-ionic detergent	1	2·6
Cetyl-trimethyl ammonium bromide	Cationic detergent	1	4·5
Urea		5×10^3	9·7

The new high level of phenolase activity brought about by treatment of the PEG-precipitate with anionic detergents still remained after their removal as was demonstrated by the following experiment: 3 ml of PEG-precipitate was treated with 1 ml of 0·03 M Manoxol OT dissolved in pH 5·0 phosphate-citrate buffer and a second 3 ml of PEG-precipitate was treated with buffer only to serve as a control. After standing at 20° for 20 min both tubes were centrifuged at 80,000 *g* for 20 min, the precipitates washed once with buffer and finally resuspended in buffer solution. When tested against 10 μmoles of chlorogenic acid initial rates of

[9] R. F. DWYER and R. J. LEWANDOWSKI, *Anal. Biochem.* 9, 133 (1964).

O_2-uptake of 33·0 μl/min and 8·0 μl/min (control) were recorded. It was found that identical amounts of phenolic and nitrogenous material were removed from these preparations by both treatments. This eliminates the possibility that the detergent activation is brought about by removal of phenolic material. The phenolase activity of the PEG-precipitate could also be restored by treatment with acetone: 10 ml of PEG-precipitate was treated with 30 ml of cold (−20°) acetone and the precipitate, after centrifugation and resuspension in 10 ml buffer solution, showed an increase in the rate of O_2-uptake from 8·0 μl/min to 30·0 μl/min.

The ability of certain anionic detergents to activate phenolase was used to study the distribution of the enzyme between the mitochondria and PEG-precipitates prepared from equal amounts (25 g) of apple peel, both with and without PVP in the extraction medium. The results given in Table 4 show that the total phenolase activity, as released by sodium dodecyl sulphate or Manoxol OT, was similar for both preparations. The distribution of activity between the mitochondria and the PEG precipitates was different depending on the presence or absence of PVP from the extracting medium.

Table 4. Distribution of phenolase activity in preparations made from equal amounts of apple peel, with or without the addition of 1% PVP to the extraction medium

	Phenolase activity (initial rate O_2-uptake: μl/min/g peel)					
	Minus PVP preparation			Plus PVP preparation		
Detergent added	Mito-chondria	PEG-ppt.	Total activity	Mito-chondria	PEG-ppt.	Total activity
None	8·7	0·7	9·4	0·6	0·7	1·3
0·01 M Sodium dodecyl sulphate	16·8	15·0	31·8	9·2	30·7	39·9
0·01 M Manoxol OT	17·6	20·8	38·4	9·6	37·2	46·8

Experiments with "Purified" Phenolase

Since the original mitochondrial preparations were known to contain appreciable amounts of phenolic material[4, 6] and these might play a part in the binding of PVP, further experiments on the inhibition of phenolase by PVP were performed using a "purified" form of the enzyme from which much of the phenolic material had been removed. Details of the method of preparation of this enzyme from mitochondria, which involved a fractionation on a Sephadex column in the final stages, are given in the Experimental section. During this purification procedure the phenolics/TCA-insoluble N ratio fell from 2·55 to 0·32.

The inhibitory effect of PVP upon the "purified" enzyme was investigated in a manner similar to that used for the mitochondria and, as can be seen from Fig. 2*b* and Table 5, the "purified" enzyme was found to be far less sensitive to PVP than the original mitochondrial-bound enzyme. Moreover the inhibitory effect of PVP could be removed, and the original phenolase activity recovered, by washing the purified particulate enzyme preparation with buffer solution alone. As clearly shown (see Table 2) PVP could not be washed off from PVP inhibited mitochondria. Similarly there was far less stimulation by Manoxol-OT and other anionic detergents (see Table 5). A comparison of the inhibitory effects of different molecular weight polymers of PVP was also made using this purified enzyme (see Table 6) and it was

TABLE 5. COMPARISON OF THE EFFECT OF PVP AND MANOXOL-OT ON THE MITOCHONDRIAL FRACTION (PREPARED WITHOUT PVP) AND "PURIFIED" PHENOLASE EXTRACTED FROM IT.

Additions to flask	Mitochondrial fraction		Purified enzyme	
	Activity initial rate O_2-uptake μl/min	Relative activity %	Activity initial rate O_2-uptake μl/min	Relative activity %
Experiment I				
None	15·3	100	14·0	100
0·5% PVP	8·4	55	12·0	86
1·0% PVP	5·2	34	10·0	72
Experiment II				
None	4·9	100	12·0	100
0·001 M Manoxol-OT	12·5	255	19·0	158
0·01 M Manoxol-OT	18·0	368	19·0	158

N.B. Relative activities should be compared since different amounts of enzyme were used in each experiment.

TABLE 6. VARIATION OF INHIBITOR CONSTANT (K_I) WITH MOLECULAR WEIGHT OF PVP USING "PURIFIED" ENZYME PREPARATION

Grade of PVP	Average molecular weight	K_I (%)	K_I (10^{-4} M)
K-17	11,000	1·36	12·4
K-25	28,000	1·25	4·7
K-30	40,000	0·84	2·1

found that the inhibitor constant (K_I) decreased more or less regularly with increasing molecular weight.

Spectrophotometric Experiments

The possibility of PVP exerting its protective action by combination with unoxidized phenolic compounds was also investigated spectrophotometrically. This was done by recording the u.v. absorption spectra of dilute solutions (0·0001 M) of chlorogenic acid or (+)-catechin, both common phenolics in apples, in pH 5·0 phosphate-citrate buffer before and after the addition of a relative excess (0·004%) of PVP. The characteristic spectra of these compounds remained unaltered by the presence of PVP, a finding in agreement with those of Harel *et al.*[6]

As an alternative approach to the problem of the mechanism of PVP binding, the i.r. spectra of mitochondria prepared with and without PVP were compared using the KBr disc technique and a Hilger and Watts H800 i.r. spectrophotometer. PVP shows a strong "amide III" absorption band at 1280 cm^{-1}, characteristic of the pyrrolidone ring, but unfortunately both mitochondrial spectra showed similar high absorptions in this region so that the presence of PVP could not be established.

DISCUSSION

The results reported in this paper indicate that PVP behaves as a competitive inhibitor of phenolase if purified enzyme preparations are used but acts in a more complex manner when the enzyme is intimately bound up with mitochondrial or other particulate components of the plant cell. Our results differ in certain respects from those obtained by Harel *et al.*[6] who found pH optima at 5·1 and 7·3 for the phenolase of "Grand Alexander" apples. They also claimed on the basis of experiments carried out with chloroplasts and the monomer, N-vinyl pyrrolidone, at pH 7·3, that the inhibition of phenolase by PVP was irreversible.

According to Dixon[8] and Dixon and Webb[10] the present results suggest that the inhibition of phenolase by PVP is most probably of a partially competitive nature (Type Ib) where the substrate and inhibitor occupy adjacent sites on the enzyme. Although this inhibition by PVP was easily removed by simply washing the "purified" enzyme this was not the case for the mitochondrial enzyme which appears to be intimately associated with the polyphenols, lipids and other complex constituent materials of the mitochondria. It is therefore suggested that, in the case of the mitochondrial-bound enzyme, the PVP may also form attachments to these other extraneous materials thereby giving a more permanent inhibition of the phenolase attached to the mitochondria.

This hypothesis is supported by the reactivation effects of anionic detergents and other dissociating agents such as urea and acetone since these phenomona were much more marked with the mitochondrial and PEG-precipitate fractions than with the less complex purified enzyme. Furthermore, PVP bound to these fractions could not be washed off by buffer alone whereas this was possible with the purified enzyme preparation (Table 2).

In a recent publication Goldstein and Swain[11] have reported studies of the inhibition of certain enzymes by tannins and the reversal of this inhibition by various cationic and non-ionic detergents, anionic detergents having been found to be ineffective. Phenolase was not however included among the enzymes studied. Their findings, in so far as they are applicable, were in marked contrast to the present results which are more in keeping with those obtained by Kenten[12] who demonstrated that the latent tyrosinase of broad bean leaf (*Vicia faba*, L.) was strongly activated by anionic but not by non-ionic and cationic detergents. Robb *et al.*[13] have suggested that the activation of latent broad bean tyrosinase is brought about by changes in the tertiary structure of the enzyme protein and by modification of the hydrogen and hydrophobic bonding. On the basis of the present results it is suggested that a similar mechanism operates with our preparations and that the various dissociating agents act not only by weakening the links binding PVP to the particle-bound phenolase but also by bringing about activation along the lines suggested by Robb *et al.*[13]

EXPERIMENTAL

All the apples used in these experiments were of the cultivar Cox's Orange Pippin picked when mature, just prior to the onset of the respiration climacteric, and stored in air at 3°. Mitochondrial fractions (hereafter referred to as "mitochondria") were prepared from the peel of these apples as previously described (Hulme *et al.*[14]) except that 0·01 M-cysteine HCl was added to the extraction media to prevent the browning that otherwise occurred when

[10] M. DIXON and E. C. WEBB, *Enzymes*, Longmans, Green and Co., London (1958).
[11] J. L. GOLDSTEIN and T. SWAIN, *Phytochem.* **4**, 185 (1965).
[12] R. H. KENTEN, *Biochem. J.* **68**, 244 (1958).
[13] D. A. ROBB, L. W. MAPSON and T. SWAIN, *Nature* **207**, 503 (1964).
[14] A. C. HULME, J. D. JONES and L. S. C. WOOLTORTON, *Proc. Roy. Soc.* (*London*) **B158**, 514 (1963).

PVP was omitted. The supernatant liquid from the mitochondria prepared with PVP was subsequently treated with 40% (w/v) polyethylene glycol, mol. wt. 4000, to yield a second enzymically active preparation, hereafter referred to as the PEG-precipitate (see Hulme *et al.*[14]).

A "purified" phenolase, low in phenolic impurities, was required for certain experiments and this was obtained from mitochondria, prepared without PVP, using the procedure described by Walker.[7] In brief this involved treatment of the mitochondria with cold (−20°) acetone, extraction with Na_2CO_3 (0·01 M)–cysteine (0·005 M) solution, further acetone precipitation steps and final fractionation on a column of Sephadex G-75. This preparation was soluble in the carbonate-cysteine solution but insoluble at pH 5·0; it was therefore used as a fine suspension in phosphate-citrate buffer.

Phenolase activity was estimated manometrically at 30° by conventional Warburg techniques and expressed as initial rates of O_2-uptake, averaged over the first 3 min. The Warburg flasks contained 0·1 M phosphate-citrate buffer at pH 5·0 together with enzyme and other additions, with either chlorogenic acid or 4-methylcatechol as substrated in a final volume of 3 ml. The former compound was the preferred substrate because it is probably one of the substrates for phenolase in the apple fruit.

The nitrogen and phenolics content of the various enzyme preparations were measured by the methods described by Hulme *et al.*[2] whilst the sources of chemicals were the same as those listed by Jones *et al.*[4] Manoxol esters were supplied by Hardman and Holden Ltd. (Manchester) and Lissapol N was obtained from I.C.I. Ltd.

Acknowledgements—We wish to thank Hardman and Holden Ltd. for gifts of Manoxol esters and I.C.I. Ltd. for a gift of Lissapol N, L. S. C. Wooltorton for preparing some of the enzyme preparations and Dr. D. F. Meigh for assistance with the i.r. measurements. One of us (J. R. L. W.) is grateful to the Agricultural Research Council for financial assistance.