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THE USE OF POLYVINYLPYRROLIDONE IN THE ISOLATION OF ENZYMES FROM APPLE FRUITS

J. D. JONES, A. C. HULME and L. S. C. WOOLTORTON

Agricultural Research Council, Ditton Laboratory, Larkfield, Maidstone, Kent

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Abstract—A comparison was made of the activity of a mitochondrial fraction from apple peel prepared with the addition of various polymers to the isolation medium. The most satisfactory polymer appeared to be polyvinylpyrrolidone (PVP) of approximate mol. wt. 28,000. A study of the effect of PVP concentration in the extraction medium on the activity of various enzymes of the mitochondria, malic enzyme, pyruvic carboxylase and phenolase subsequently isolated from the tissue, showed that the optimal concentration was not identical for all the enzymes. For mature fruit, a concentration of 1% PVP was the best compromise, giving near maximum activity for all the enzymes other than phenolase. Phenolase was directly inhibited by PVP. Darkening of the preparations was prevented by PVP and it appears that, in plant material rich in phenolase and its substrates, one of the ways in which PVP prevents the inactivation of enzymes is by combining with phenolase.

INTRODUCTION

DURING the development of a method[1] to overcome the inhibition of enzyme activity in preparations from plant material containing relatively large amounts of phenolic compounds, several "phenolic-binding" compounds were investigated. It appeared that polymerized phenolics were the main cause of inhibition so that a permanent sequestration into a non-inhibitory phenolic complex would be preferable to an attempt to prevent polymerization, presumably oxidative, by the use of reducing conditions. The prevalence of oxidases in the material under immediate study—apple fruit tissue, especially peel—would bring about a rapid oxidation of reducing agents such as ascorbic acid and cysteine, commonly used in biochemistry, and so obliterate their action during the isolation procedures.

The classic compounds used to combine with phenolics ("tannins") namely proteins (hide powder) were inappropriate since they would merely compete with the proteins being isolated and would also lead to bulky precipitates containing much inactive material. Several new compounds have become available which might simulate in one way or another the reaction of phenolics and proteins. Such compounds are, various forms of powdered nylon, dextran, polyethylene glycol and polyvinylpyrrolidone[2]. Grassmann and his school[3,4] have successfully used various preparations of nylon ("Perlon", "Ultramid") and polyvinylpyrrolidone to combine with phenolic compounds in a manner similar to that of hide powder. Polyvinylpyrrolidone (PVP) is available in various degrees of polymerization and has the advantage, at the lower molecular weights, of being soluble in water. Other workers have used PVP (e.g. Novikoff[5]) to improve the quality of mitochondrial preparations from animal tissue.

Nylon powder was rejected early in our work as being of only limited value and difficult to

[1] J. D. JONES and A. C. HULME, *Nature* **191**, 370 (1961).
[2] K. H. GUSTAVSON, *Svensk Kem. Tidskr.* **66**, 3959 (1954).
[3] W. GRASSMANN, H. HÖRMANN and A. HARTLE, *Makromol. Chem.* **21**, 37 (1956).
[4] K. H. GUSTAVSON, *Das Leder* **14**, 27 (1963).
[5] A. B. NOVIKOFF, *Symposia Soc. Exp. Biol.* **10**, 92 (1957).

separate from the final fruit enzyme preparations. The present communication describes experiments with the use of dextran and various grades of PVP to prevent the inhibition of the activity of mitochondrial preparations and certain soluble enzymes during their isolation.

The *manner* in which PVP reacts with the phenolic compounds in the tissue is important. If it combines with the unpolymerized (soluble) phenolics before they can be attacked by the phenolase present in the tissue, then soluble PVP-phenolics complexes may be formed which would not be "co-precipitated" with, for example, mitochondrial preparations. If, on the other hand, oxidative polymerization to give "tannins" first occurs then PVP will form bulky insoluble complexes with these compounds (Gustavson, 1954), and these complexes are likely subsequently to "contaminate" mitochondrial and other enzyme preparations.

Gustavson[2,4] has shown that hydrogen bonding is the main reaction between PVP and the OH-groups of tannins. Many enzymes combine with their substrates through hydrogen bonding with —OH and —SH groups. PVP might, therefore, inhibit certain enzyme reactions in a competitive manner. If just sufficient PVP were present during the enzyme isolation procedures, then the phenolics may be sequestered preferentially in solution without interfering with enzyme reactions. If, however, excess PVP is present it might also provide the basis for subsequent (competitive or non-competitive) inhibition of enzyme systems especially the complex mitochondrial system. Since we are also interested in the phenolase present in apples there is also the possibility that excess PVP would inhibit phenolase activity through combination with the phenolase—substrate complex as tentatively suggested by Hulme *et al.*,[6] or directly with the enzyme as suggested by Harel *et al.*[7]

The phenolics content of apples, especially peel tissue, falls considerably during the development of the fruit (Hulme, 1957). It was necessary, therefore, to investigate the effect of adding various concentrations of PVP during the preparation of mitochondria and other enzyme systems from fruit at various stages of development, i.e. at various levels of phenolics.

RESULTS

As a first assessment of the value of a compound in overcoming the inhibitory effect of phenolic compounds, the colour of the tissue homogenate and the mitochondrial fraction prepared from it (see Experimental section) is a useful guide. Further evidence may be provided by the activity of the mitochondrial fraction (hereafter called "mitochondria") in the Warburg respirometer. We have found it advisable to measure both O_2-uptake and CO_2-output with the two substrates succinate and malate, since these four parameters may vary relative to one another under different preparative conditions and when the tissue is in different physiological states.

The results of such an assessment of the value of dextran and various proprietary preparations of PVP at 2% concentrations are given in Table 1. Details of the various additions are given in the Experimental section. Kollidon 25 and Kollidon 17 and the more highly purified Plasdones gave similar results and good mitochondrial pellets. Insoluble PVP (Polyclar AT) gave good coloured but very small pellets of low activity. Experiments showed that Polyclar added to active preparations of mitochondria did not, however, inhibit activity and it was concluded that the Polyclar formed a loose attachment to the particles and caused a proportion of them to be brought down in the first low-speed centrifugation. Dextran gave very bulky pellets similar to those obtained without additives. Clearly it did not prevent

[6] A. C. HULME, J. D. JONES and L. S. C. WOOLTORTON, *Nature* **201**, 795 (1964).
[7] E. HAREL, A. M. MAYER and Y. SHAIN, *Physiol. Plantarum* **17**, 921 (1964).

TABLE 1. EFFECT OF VARIOUS ADDITIVES (AT 2% W/V.) ON THE COLOUR AND ACTIVITY OF MITOCHONDRIAL PREPARATIONS FROM THE PEEL OF COX'S ORANGE PIPPIN APPLES

Additive	pH of super-natant	N-content of mito-chondria	Colour		Activity of mitochondria in Warburg							
					Substrate-succinate				Substrate-malate			
					Per 10 g tissue		Per mg N		Per 10 g tissue		Per mg N	
			Supernatant	Mitochondria	O_2	CO_2	O_2	CO_2	O_2	CO_2	O_2	CO_2
1. PVP—Kollidon 25	7·52	0·608	emerald green	green	740	195	1215	319	348	310	572	510
2. PVP—Kollidon 17	7·50	0·776	dark green	green	755	296	960	382	384	344	495	443
PVP—Kollidon 17	7·46	0·672			672	266	998	396	321	332	478	494
3. PVP—K-30	7·52	0·408	dark green	green	480	100	1176	245	240	260	588	637
4. PVP—Rhone-Poulenc	7·50	0·480	dark green	green	492	88	1025	184	251	248	523	507
5. PVP—Plasdone	7·50	0·520	dark green	green	513	160	986	308	264	300	507	577
6. PVP—Plasdone C	7·57	0·600	light green	green	566	158	951	301	292	272	488	453
7. PVP—Plasdone "special"	7·49	0·600	light green	green	600	139	1000	234	272	240	453	390
8. PVP (insoluble)—Polyclar AT	7·55	0·784	green	brown-green	20	11	4	8	26	14	5	11
9. Dextran	7·52	1·69	brown	brown-green	194	73	115	43	61	120	36	71
10. Nil	7·56	1·29	brown	brown	176	48	135	37	48	40	37	31

Table 2. Details of the source of the tissue used and the colour of the mitochondrial and supernatant preparations obtained

Series	Date (1962)	Av. wt. g/apple	Tissue	PVP concn. %	Cysteine (M)	Colour*	
						Supernatant	Mitochondria
	July						
I	4	10·7	Whole fruit	0	—	B	B
	6	9·9		0·25	—	becoming B	BG
	6	9·9		0·75	—	becoming B	BG
	5	10·5		1·25	—	becoming B	G
	5	10·5		2·5	—	GB	G
	4	10·7		5·0	—	GB	G
	9	11·7		5·0	0·01	G	G
	July						
II	24	30·0	Whole fruit	0	—	B	B
	25	30·0		0·25	—	becoming B	GB
	26	30·6		0·75	—	becoming B	BG
	25	30·0		1·0	—	G	G
	27	30·5		1·25	—	G	G
	24	30·0		2·5	—	G	G
	27	30·5		4·0	—	G	G
	20	23·5		4·0	0·01	G	G
	26	30·6		5·0	—	G	G
	Aug.						
III	16	63·3	Peel	0	—	becoming B	GB
	16	63·3		0	0·03	becoming B	BG
	21	65·5		0·1	—	becoming B	GB
	21	65·5		0·1	0·03	BG	BG
	19	64·0		0·25	—	becoming B	GB
	19	64·0		0·25	0·03	BG	G
	22	66·3		0·5	—	BG	G
	22	66·3		0·5	0·03	G	G
	14	63·1		0·75	—	BG	G
	14	63·1		0·75	0·03	G	G
	17	65·3		1·25	—	G	G
	17	65·3		1·25	0·03	G	G
	20	64·0		2·5	—	G	G
	20	64·0		2·5	0·03	G	G
	15	65·3		5·0	—	G	G
	15	65·3		5·0	0·03	G	G
	Sept.–Oct.†						
IV	26	109·3	Peel	0	—	becoming B	dull G
	26	109·3		0	0·03	becoming B	dull G
	4†	117·0		0·1	—	becoming B	dull G
	4†	117·0		0·1	0·03	BG	G
	28	114·0		0·25	—	BG	G
	28	114·0		0·25	0·03	BG	G
	5†	117·0		0·5	—	G	G
	5†	117·0		0·5	0·03	G	G
	24	105·0		0·75	—	G	G
	24	105·0		0·75	0·03	G	G
	27	112·2		1·25	—	G	G
	27	112·2		1·25	0·03	G	G
	3†	111·0		2·50	—	G	G
	3†	111·0		2·50	0·03	G	G
	25	105·7		5·0	—	G	G
	25	105·0		5·0	0·03	G	G
	1963						
V	5–19 Feb.	Fully mature	Peel–fruit ex 2·8° store	0–4·0	—	G throughout	G throughout

* B = brown, G = green.

polyphenolic browning but brought down extensive amounts of non-mitochondrial nitrogen. Since Kollidon 25 was more readily available than the lower mol. wt. Kollidon 17, it was chosen for further study.

Fruit at five stages of maturity were used. With the first two series, the fruit was too small to obtain good separation of peel and pulp; the whole fruits (minus strigs and seeds) were used in the preparation of mitochondria, etc. With more mature fruit—series III and IV—peel tissue only was used to give a maximum phenolics content. In the final series also, peel was used; this fruit had been in cold storage (2·8°) from 1 October up to the date of analysis. To minimize the effect of any differences in maturity of the fruit within a series, the dates of the experiments throughout the range of PVP concentrations were randomized. Relevant details for each series of fruit are given in Table 2. Colour changes during preparation of mitochondria etc. for the first two series progressed from brown to green with increased concentrations of PVP. In the last three series the tissue and extract remained green throughout the extraction procedure at concentrations of PVP above 0·25% whether or not cysteine was used as an additive during the preparation. Although the particles were a brighter green when cysteine was used, as will be seen later, their phenolic and nitrogen content were little affected by the presence of cysteine.

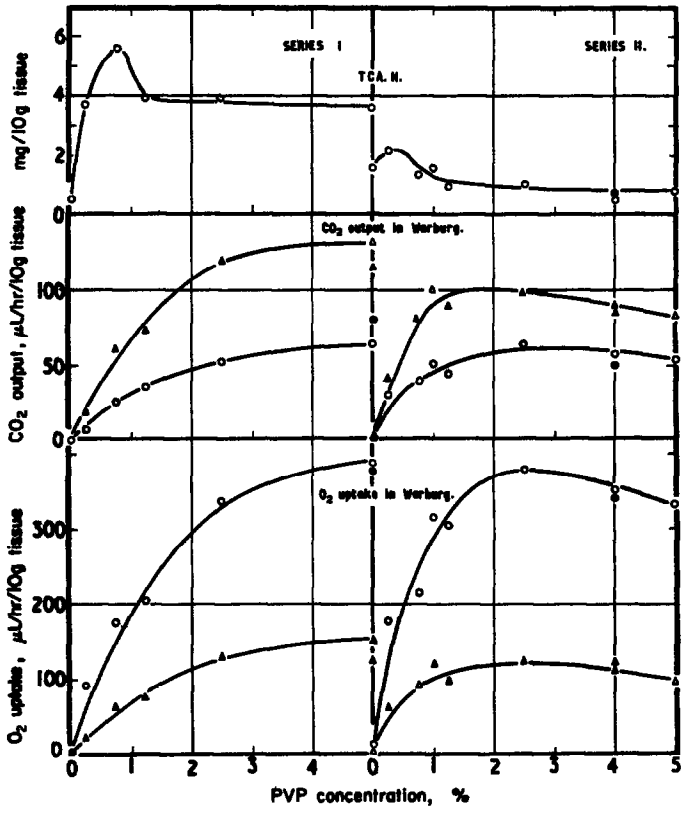

FIG. 1. TCA INSOLUBLE NITROGEN (TCA.N) CONTENT AND WARBURG RESPIROMETER RESULTS FOR THE MITOCHONDRIA OF FRUIT OF SERIES I AND II IN RELATION TO PVP CONCENTRATION.

O, Succinate substrate; △, malate substrate. Solid symbols refer to mitochondria prepared in presence of 0·03 M cysteine.

Effect of PVP—Concentration on Nitrogen and Phenolic Content and Enzyme Activity of the Mitochondria

Fruit of series I and II. The lower PVP concentrations—especially 0·1%—gave very bulky pellets containing more nitrogen (top curves, Fig. 1). That this extra nitrogen did not lead to a higher enzyme activity is seen from Fig. 1 where, on the basis of O_2-uptake and CO_2-output in the Warburg respirometer, there is a general increase in activity up to an asymptotic value at 4% PVP. The few preparations in which cysteine was used in addition to PVP (filled-in symbols) indicate little effect of this reducing agent although the supernatant frac-

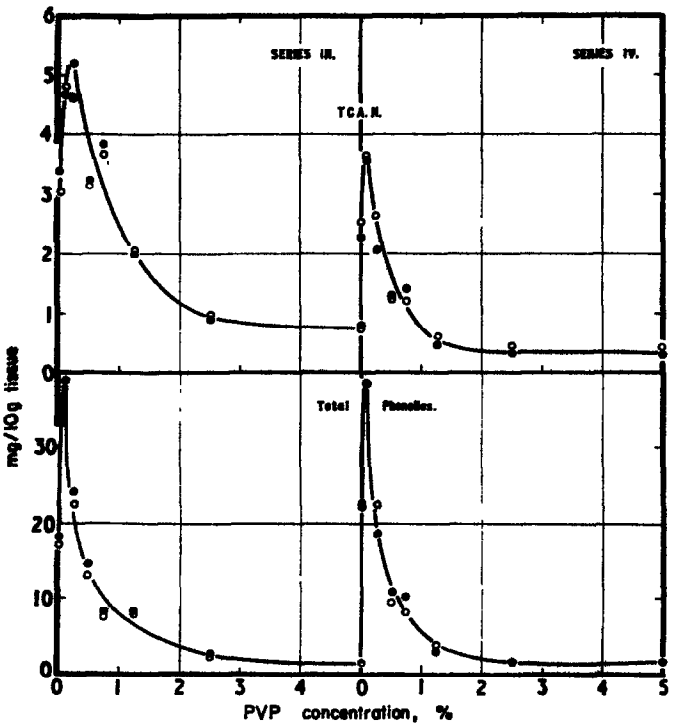

FIG. 2. CHANGES IN TCA INSOLUBLE NITROGEN (TCA.N) AND TOTAL PHENOLICS CONTENT OF MITOCHONDRIA OF SERIES III AND IV IN RELATION TO PVP CONCENTRATION.

Open symbols refer to preparations made in the absence, and solid symbols refer to preparations made in the presence of cysteine.

tions were somewhat greener. No determinations were made of the phenolics content of the preparations.

Fruit of series III and IV. Only peel tissue was used here so that, although the fruit was more mature, the phenolic content (and probably the phenolase content) would be considerably higher.

(a) Nitrogen content and total phenolics, leucoanthocyanidin and flavan content of the mitochondria. The trichloroacetic acid insoluble nitrogen content (TCA.N) and total phenolics content are shown in Fig. 2. The leucoanthocyanidin and flavan contents are given later for convenience in Fig. 8 which includes similar results for series V. The fact that in some cases leucoanthocyanidin content is greater than total phenolic content is due to the empirical

methods used in the determinations; these methods employed calibration tables (Swain, private communication) prepared with pure compounds ((+)-catechin and cacao leucocyanidin) not necessarily the same as those present in apple tissue.

An examination of these results will be deferred until the Discussion section.

(*b*) *Oxidation and decarboxylation of succinate.* The Warburg respirometer results for mitochondria prepared with and without cysteine in the extraction medium are given in Fig. 3. The O_2-uptake increase rapidly with PVP concentration to an asymptotic value reached at between 1 and 2%; the effect of cysteine is marginal but tends to "reinforce" the effect of PVP resulting in a more rapid increase in activity at the lower concentration of PVP. CO_2-output

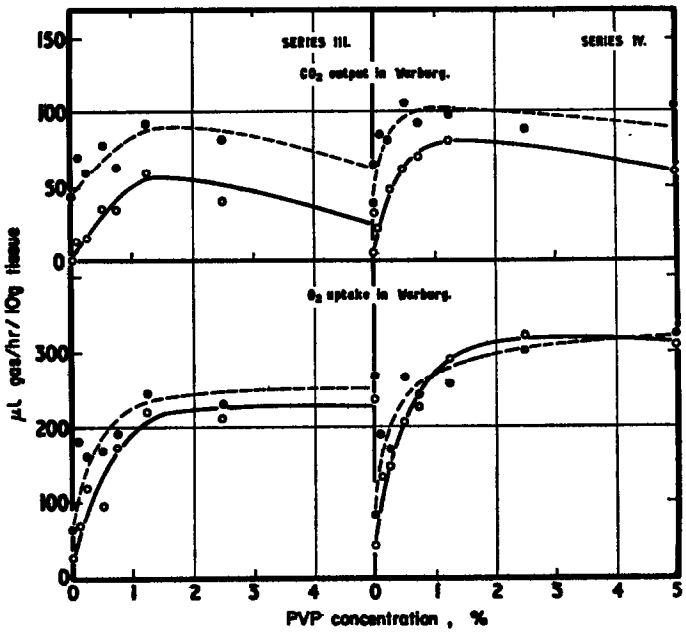

FIG. 3. O_2-UPTAKE AND CO_2-OUTPUT WITH SUCCINATE SUBSTRATE OF MITOCHONDRIA PREPARED FROM FRUIT OF SERIES III AND IV IN RELATION TO PVP CONCENTRATION.

Open symbols refer to preparations made in the absence, and solid symbols refer to preparations made in the presence of cysteine.

reaches a maximum at approximately 1·5% PVP for series III and at 1% for the more mature fruit. Cysteine brings about an increased CO_2-output even in absence of PVP and the activities at the highest concentration of PVP are considerably higher in its presence, although, especially series III, activity tends to fall off generally at the higher concentrations of PVP. No experiments were done with malate as substrate in these two series of fruit.

(*c*) *Activity of succinic and malic dehydrogenases, diaphorase ($NADH_2$) and $NADH_2$-cytochrome-c-reductase.* The effect of PVP concentration, in presence and absence of cysteine, on the dehydrogenases is shown in Fig. 4. We have always found[8] succinic dehydrogenase activity to be low by the method of determination used. At the lower concentrations of PVP, the bulky pellets appeared to retain small amounts of cysteine (though not enough to effect the nitrogen content of the preparations—Fig. 2) which reduced the dye used in the succinic

[8] A. C. HULME, J. D. JONES and L. S. C. WOOLTORTON, *Phytochem.* 3, 173 (1964).

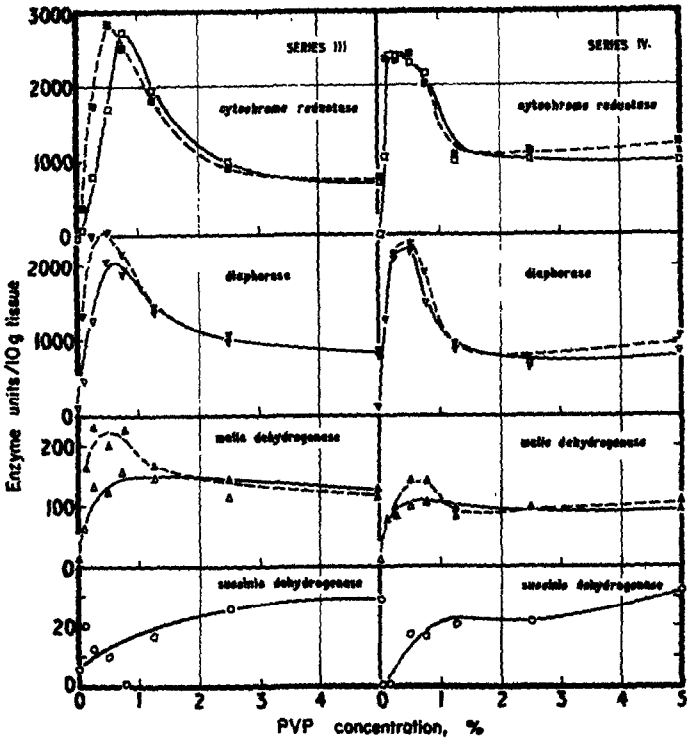

FIG. 4. ACTIVITY OF THE DEHYDROGENASES OF THE MITOCHONDRIA OF THE FRUIT OF SERIES III AND IV IN RELATION TO PVP CONCENTRATION.

The open symbols refer to preparations made in the absence, and solid symbols refer to preparations made in the presence of cysteine.

dehydrogenase determinations and made the results unreliable at such low activities. Although the "blank" readings compensated for this reduction there are indications in Fig. 4 that the results even for malic dehydrogenase are effected by the presence of residual cysteine in the

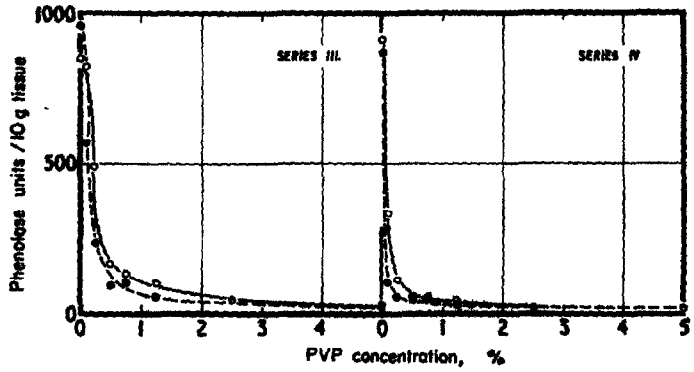

FIG. 5. PHENOLASE ACTIVITY OF MITOCHONDRIA PREPARED FROM FRUIT OF SERIES III AND IV IN RELATION TO PVP CONCENTRATION.

Open symbols refer to preparations made in the absence, and solid symbols refer to preparations made in the presence of cysteine.

pellets although these had been washed before use. The results for the dehydrogenases in general indicate most strikingly that maximum activity is obtained at between 0·5 and 1% concentrations of PVP and that high concentrations begin quite rapidly to have an inhibitory effect. Once again cysteine in the extracting medium is seen to reinforce the action of PVP in reducing inhibition at the lower levels of PVP concentration. As might be expected from the lower phenolics content of the more mature fruit (series IV) maximum activities are attained at lower concentrations of PVP.

(*d*) *Phenolase activity.* The activity of this enzyme in the mitochondrial preparations is shown in Fig. 5. With both series of fruit this falls very rapidly as the PVP concentration is increased. The presence of cysteine in the extraction medium reduces the concentration of PVP needed to bring about a rapid inhibition of phenolase activity.

Effect of Concentration of PVP on Activity, Nitrogen, and Phenolics Content of Mitochondrial and Supernatant Fractions from the Same Tissue

Fruit tissue of series V. With this fruit, taken from the cold store, a more extensive examination was made of the effect of PVP concentration on various enzymes etc. of the peel tissue. The addition of cysteine to the extracting media was discontinued since it appeared to add no new principle to the action of PVP and its reducing action might be an embarrassment to a study of the soluble enzymes.

In addition to the determinations made on the mitochondria of the earlier series, phenolase, malic enzymes and carboxylase activity were measured in the supernatant liquid remaining after removal by centrifugation of the mitochondria.[9] The nitrogen and phenolics content of the polyethylene glycol (PEG) precipitates containing these enzymes were also determined. The derivation of the various fractions to be discussed will be seen from the Experimental section.

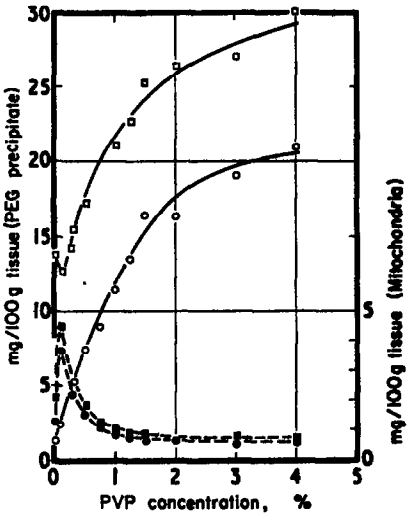

FIG. 6. TOTAL NITROGEN (■), TCA-INSOLUBLE NITROGEN (●) OF THE MITOCHONDRIA, AND TOTAL NITROGEN (□), TCA-INSOLUBLE NITROGEN (○) OF THE PEG PRECIPITATES, IN RELATION TO PVP CONCENTRATION, PREPARED FROM FRUIT OF SERIES V.

[9] A. C. HULME, J. D. JONES and L. S. C. WOOLTORTON, *Proc. Roy. Soc.* B 158, 514 (1963).

(*a*) ***Nitrogen and phenolic content of the mitochondrial fraction and the PEG precipitates (soluble enzymes).*** The total and TCA insoluble nitrogen (TCA.N) of the mitochondria and the PEG precipitates, as the PVP concentration in the extracting medium is increased, are shown in Fig. 6. It should be mentioned here that PEG does not precipitate PVP so that the nitrogen content of the PEG precipitates *should* not include any nitrogen directly due to PVP even when no TCA treatment of the precipitates has been given (see Discussion). The total phenolics content of the mitochondria and the PEG precipitates is given in the top section of Fig. 7 and the leucoanthocyanidin and flavan components are shown in Fig. 8 which includes similar results for series III and IV fruit. Comments on these results will be reserved for the Discussion.

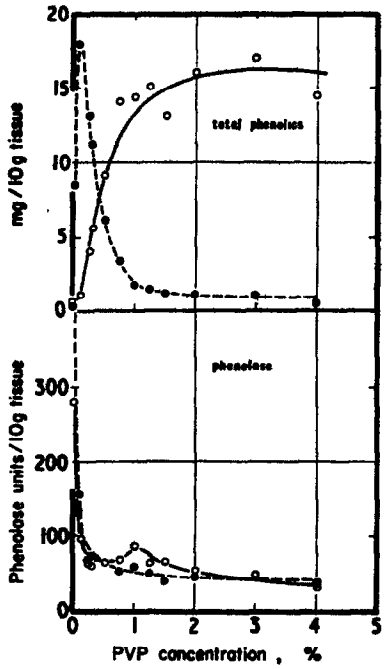

FIG. 7. TOTAL PHENOLICS CONTENT (UPPER CURVES) AND PHENOLASE ACTIVITY (LOWER CURVES) OF MITOCHONDRIA (●) AND PEG PRECIPITATES (○), IN RELATION TO PVP CONTENT, PREPARED FROM FRUIT OF SERIES V.

(*b*) ***Oxidation and decarboxylation of succinate and malate by the mitochondria.*** The results are given in the left-hand section of Fig. 9. The general trend of O_2-uptake and CO_2-output are reminiscent of the trend of activity of the mitochondria from fruit of series III and IV (Fig. 4) with optima at a PVP concentration of 0·75% (w/v).

(*c*) ***Activity of the dehydrogenases of the mitochondria.*** The activities of succinic and malic dehydrogenases, diaporase and cytochrome-c-reductase are given in the right-hand section of Fig. 9. The trend of activity as the PVP concentration is increased are similar to those of the preparations of series III and IV.

(*d*) ***Activity of malic enzyme (M.E.), carboxylase and phenolase.*** Phenolase activity of both mitochondria and PEG precipitates are shown in the lower section of Fig. 7. As with the fruit of series III and IV the activity in the mitochondria decreases rapidly as PVP concentration is increased. Surprisingly, so does the activity in the PEG precipitates: the "hump"

at approximately 1% PVP concentration may or may not be significant. Where then has the phenolase gone? This point will be considered in the Discussion.

Malic enzyme (M.E.) and carboxylase in the PEG precipitates (Fig. 10) increase as the PVP concentration is increased to an asymptotic value at 1% PVP concentration for M.E. and 2% PVP concentration for carboxylase. The small amount of these enzymes in the mitochondrial fraction is augmented at very low concentrations of PVP probably because they are

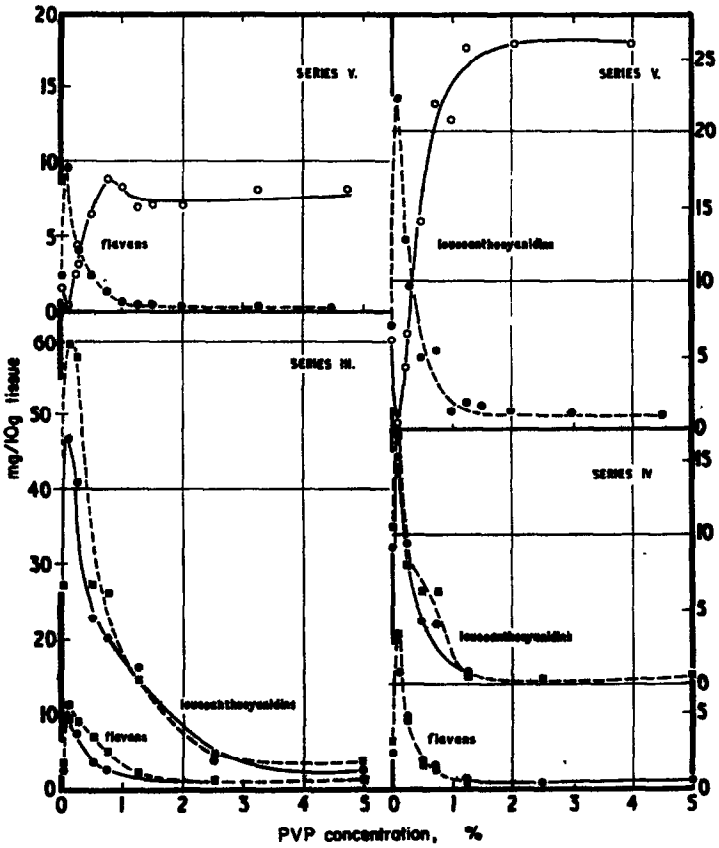

FIG. 8. LEUCOANTHOCYANIDIN AND FLAVAN CONTENTS OF MITOCHONDRIA OF FRUIT OF SERIES III AND IV AND OF MITOCHONDRIA AND PEG PRECIPITATES OF FRUIT OF SERIES V IN RELATION TO PVP CONCENTRATION.

Solid symbols refer to mitochondria (● prepared in absence, ■ prepared in presence of cysteine—series III and IV only). Open symbols refer to PEG precipitates.

mechanically difficult to wash from the bulky mitochondrial pellets obtained at these low concentrations of PVP.

DISCUSSION

In early July Cox's Orange Pippin apples contain as much as 6 per cent of the dry wt. of the tissue, and the peel tissue of the same fruit at maturity as much as 3 per cent of the dry wt. in the form of "total phenolics". It is not surprising, therefore, that the activity of mitochondria and other enzymes isolated from such tissue is very low, since oxidative polymerization of phenolic compounds occurs rapidly as soon as the tissue is macerated.

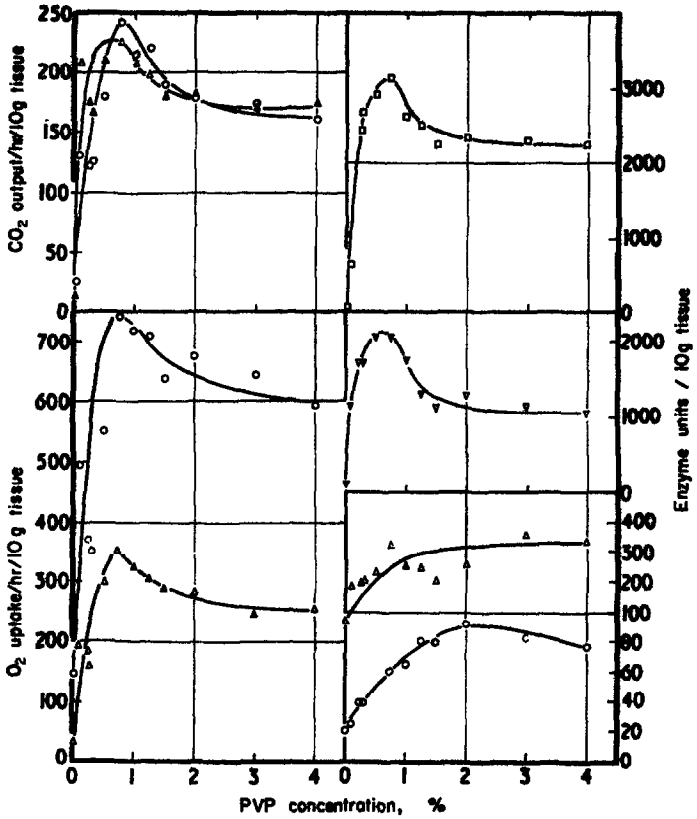

FIG. 9. LEFT-HAND CURVES: O_2-UPTAKE AND CO_2-OUTPUT OF MITOCHONDRIA WITH SUCCINATE (○) AND WITH MALATE (△) SUBSTRATES IN RELATION TO PVP CONCENTRATION. Right-hand curves: dehydrogenase activity of mitochondria in relation to PVP concentration. ○, succinic dehydrogenase; △, malic dehydrogenase; ▽, diaphorase; □, cytochrome-c-reductase. All the results are for fruit of series V.

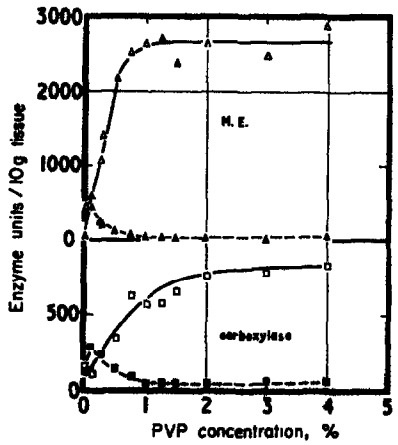

FIG. 10. MALIC ENZYME (M.E.) AND PYRUVIC CARBOXYLASE ACTIVITY OF MITOCHONDRIA (SOLID SYMBOLS) AND PEG PRECIPITATES (OPEN SYMBOLS) IN RELATION TO PVP CONCENTRATION; SERIES V FRUIT.

Addition of PVP to the medium used to extract the enzymes from the tissue removes the inhibition progressively as the concentration of PVP is increased. Concomitant reduction in the browning of the extracts as isolation of the mitochondria proceeds (Table 2) suggests that the PVP is combining either with the phenolase or with its substrates in the tissue to prevent the interaction of the two. An indication of the complexity of the problem is given by the fact that the maximum activity of the mitochondrial preparations does not coincide with their maximum nitrogen contents (Fig. 1). If the sole action of polymerized phenolics (tannins) was to precipitate protein then (in spite of the differential centrifugation procedures used in preparing the mitochondria) it might be expected that the "protein" nitrogen in the mitochondrial pellets would decrease with increasing concentrations of PVP. Instead of this a maximum nitrogen content is found at, in this young whole-fruit tissue, 0·75% PVP.

With peel tissue of more mature fruit, containing at least as much phenolic material, the oxidative decarboxylation effected by the mitochondrial preparations increases from a very low value if PVP is omitted from the extracting medium to an approximately steady value at PVP concentrations between 1·5 and 5·0%. Cysteine alone brings about some reversal of the inhibition and when used with PVP gives some overall increase in the rates of decarboxylation (Fig. 3).

When we turn to some of the individual dehydrogenases of the mitochondria the picture is different. Activity increases up to a PVP concentration of between 0·5 and 0·75 PVP. Discounting the effect of cysteine, preparations from the least mature fruit requires most PVP to give maximum activity (Fig. 4, series III). This is in agreement with the fact that the fruit presumably contains the highest amount of phenolics. Diaphorase and cytochrome-c-reductase activity both decrease quite rapidly in series III and IV (Fig. 4) at concentrations of PVP between 1 and 2%.

This effect of PVP appears to be quite different from its "removal" of tannin inhibition of the enzymes. The nitrogen and phenolics content of the preparations have fallen to a low value (Fig. 2) by the time the dehydrogenase activity begins to fall so that actual physical loss of enzyme cannot be the important factor. It could be significant that the two dehydrogenases most affected (diaphorase and cytochrome-c-reductase, Fig. 4) are flavin enzymes; Goldstein and Swain[10] have shown that two non-flavin enzymes, alcohol and lactic dehydrogenases, are not inhibited by PVP. They, however, used 1% PVP to remove tannin inhibition of these enzymes and their conditions could correspond with our "non-inhibitory" PVP concentrations.

The inactivation of diaphorase and cytochrome-c-reductase by higher concentrations of PVP is also seen in the results with the peel of senescing fruit (series V) (Fig. 9). Here the inhibition has passed right along the electron-transport chain and is clearly evident in the reduced O_2-uptake and CO_2-output of the mitochondrial preparations with succinate and malate substrates. With this material, the phenolics and phenolase content of the tissue will be lower so that more free PVP should be available for any dehydrogenase inactivation for a given original concentration of PVP.

An examination of the effect of PVP on "soluble" non-mitochondrial enzymes brings out the following points (Fig. 10). Without the use of PVP the PEG precipitates from the supernatant solutions have no malic enzyme of carboxylase activity. As the PVP concentration is increased activity rises to an asymptotic value at ~ 1% PVP concentration; there is thus no apparent inactivation of these enzymes at higher concentrations of PVP. The small

[10] J. L. Goldstein and T. Swain, *Phytochem.* 4, 185 (1965).

amount of activity in the mitochondrial preparations at very low concentrations of PVP is probably due to mechanical contamination in the bulky pellets obtained under these conditions.

Much of the nitrogen (constant quantity of approximately 10 mg) in the PEG precipitates is removed by extraction with TCA (Fig. 6). It cannot represent PVP since with no PVP the value is the same. This is most likely to be the nitrogen of the tris buffer occluded in the gelatinous PEG precipitate, since PEG does not precipitate PVP and the precipitates were not washed initially. This is supported by the fact that this constant quantity of N is removed from the precipitates even when no PVP has been used. The phosphorus determination on the TCA treated and untreated PEG precipitates show a similar pattern suggesting a similar occlusion of phosphate from the extraction medium.

The leucoanthocyanidins and the flavans in the mitochondrial preparations (Fig. 8) follow the general pattern of the total phenolics with the former tending to be removed more readily at the lower concentrations of PVP. The additional presence of cysteine (series III and IV) appear to be of little particular interest. For the peel of mature apples (series V), the pattern of change in total phenolics, leucoanthocyanidins and flavans in the PEG precipitates with increase in PVP concentration is the inverse of the pattern in the mitochondrial preparations. Thus, in the present context, there seems to be no special interest in the individual groups of phenolics and in the subsequent argument "total phenolics" only will be considered

At this point we have two views as to the action on PVP on phenolase and phenolics. Our own tentative suggestion, based largely on work with flower petals,[6] that "the protective action (on non-phenolase enzymes) of PVP is due to its combination with a phenolase-substrate complex probably through attachment to the (phenolic) substrate moiety", and the view of Harel *et al.*[7] that this action is a result of direct combination of PVP with the phenolase.

Let us examine how the present results bear on these two hypotheses. The similarity in the pattern of total phenolics and nitrogen as the PVP concentration is increased does, at first sight, lend some support for the view that phenolics and PVP combined, attach to and inactivate the phenolase either via the phenolics "end" or via the PVP "end" of such a complex. The very rapid decrease in phenolase activity which occurs between 0 and 0·1% PVP, at which latter concentration both N and phenolics content are at a maximum, agrees with this suggestion. Here, perhaps, some collateral evidence in the form of the effect of TCA treatment on the mitochondrial content of phenolics and N might be considered. The effect of TCA treatment of mitochondria is shown in Table 3. Similar results for the PEG precipitates cannot be entered in evidence because of the likelihood, already mentioned, that some of the N (that fraction likely to be removed by the TCA treatment) in these precipitates comes from the tris buffer used. The results shown in Table 3 can easily be reconciled with the assumption that both PVP and phenolic compounds can combine individually with the phenolase. They could hardly be reconciled with our earlier postulate that the two combine as one unit with the phenolase.

The high values for the phenolics and nitrogen content of the mitochondria at 0·1 and 0·25% PVP, taken in conjunction with the bulkiness of the precipitates and the peak activity (small though it is to the total activity in the tissue) of M.E. and carboxylase, suggest a general agglomeration of enzyme and "tannin" material co-precipitating with the mitochondria at these lower concentrations of PVP.

As PVP concentration is increased, more of the potential tannin remains unprecipitated with the mitochondria and appears later in the PEG precipitates. These precipitates remain light in colour and, presumably, the phenolics have gone through to these precipitates in the

TABLE 3. THE EFFECT ON THE NITROGEN AND PHENOLICS CONTENT OF TREATING MITOCHONDRIA WITH 7·5% TCA

PVP conc. %	Decrease brought about by TCA extraction (mg/10 g original tissue)	
	Total phenolics	Nitrogen
0	7·4	0·79
0·10	14·5	0·83
0·25	8·4	0·45
0·30	5·4	0·30
0·50	3·2	0·29
0·75	—	0·29
1·0	1·3	0·26
1·25	0·6	0·31
1·5	0·8	0·20
2·0	0·9	0·61
3·0	0·5	0·37
4·0	0·6	0·11

more or less unoxidized and unpolymerized state. We had stated earlier[6] that unpolymerized phenolics may inhibit mitochondrial and other enzymes but this suggestion was based on rose petal preparations which, although virtually free of phenolase, contained considerable quantities of ellagic acid which has itself, without any polymerization, protein "tanning" action. It also, without oxidation, inactivates mitochondrial succinoxidase.[11]

Finally, there is the fact that when PVP is entirely absent, phenolase activity is maximal in the mitochondria, although a considerable amount of phenolic material is already present (Fig. 7). Incidentally there is also considerable phenolase activity in the supernatant fraction (PEG precipitates) at this point which agrees with the findings of Harel *et al.*[7] that "soluble" phenolase increase in mature stored apples; our series V fruits were in this state.

All these factors suggest strongly that the PVP itself prevents phenolase activity directly rather than combining with the phenolic substrates to prevent their attachment to the phenolase. It is, however, not completely ruled out that, in addition, partially oxidized (polymerized) phenolics also combine with PVP so that no further oxidation by phenolase can occur. This would explain why the PEG precipitates obtained in the presence of increasing amounts of PVP have high M.E. and carboxylase activity (Fig. 10) in spite of containing large amounts of phenolics (presumably, as already stated, in the unoxidized, unpolymerized state). This view is in agreement with the recent results of Harel *et al.*[7]

Where, however, has the phenolase activity gone? It is absent from both mitochondria and PEG precipitates (soluble fraction) when PVP is present. We have found it also to be absent from the tissue debris centrifuged off in the early stages of the isolation technique. Unlike the soluble dehydrogenases of Goldstein and Swain[10] it is not released by PEG; both Goldstein and Swain and ourselves used PEG 4000 and the relatively small differences in concentration of PEG (25% as against 40%) should not be a critical factor. Recent work in this laboratory by Dr. J. R. L. Walker (on leave from the Cawthron Institute, Nelson, New Zealand) shows that the phenolase is still present in both mitochondria and PEG precipitates and its activity can be released by suitable treatments. Preliminary results also suggest that,

[11] A. C. HULME and J. D. JONES, *Enzyme Chemistry of Phenolic Compounds* (Edited by J. B. PRIDHAM), p. 97, Pergamon Press, Oxford (1963).

unlike the work of Harel *et al.*,[7] PVP (Kollidon 25) is a competitive reversible inhibitor of our apple phenolase. Possibly the different molecular weights and preparations of PVP used by Harel *et al.* and by ourselves is the source of these conflicting views. We feel that the resolution of this problem awaits a study of purified apple phenolase; this we are now undertaking.

The value of the addition of PVP to extracting media as a protection of enzyme activity in plant material containing quantities of phenolics is amply confirmed, It is, however, clear that the optimal concentration of PVP must be determined for each tissue depending on the enzymes under survey. In our own apple-peel tissue the soluble enzymes require somewhat higher concentrations of PVP than the mitochondrial system. It has already been shown[11] for mitochondria from the peel from mature apples that the coupling of oxidation with phosphorylation (as evidenced by the P:O ratios) reaches a maximum when 0·75% PVP is used in the extraction medium and decreases very little up to 4% PVP. We conclude that with peel of *mature fruit* 0·75%–1% is a suitable compromise giving near the maximum activity for the mitochondrial enzymes, malic enzyme and pyruvic carboxylase. For the study of changes in phenolase activity both in mitochondrial preparations and in the supernatant fraction the situation is clearly reversed; the addition of PVP in the extraction medium is designed to inhibit the action of this enzyme. This inhibition may, however, be reversed in both the mitochondrial and supernatant fractions enabling changes in phenolase activity to be studied. Details of this procedure will be described elsewhere (Walker and Hulme.[12]

If, in fact, PVP combines with and inactivates phenolase it will be most valuable in the examination by spectrophotometric methods of plant material rich in phenolics. For example, the oxidation products of phenolic compounds seriously interfere with the determination of proteins and nucleic acids by such methods.

EXPERIMENTAL

Apple Fruits Used

Cox's Orange Pippin apples from 50 trees grown on Malling IX root stocks in a grassed-down orchard were used throughout these experiments.

The various series of fruits were picked on the following dates in 1962: series I, 4–9 July; series II, 24–27 July; series III, 16–22 August; series IV, 24 September–5 October. Series V fruit consisted of apples picked in bulk on 1 October and stored at 2·8° until taken for analysis (5–19 February, 1963). The average wt. of the apples in the various samples are given in Table 2.

Mitochondrial Preparations

The mitochondrial fraction was prepared as previously described.[8,9] The extraction media containing the various concentrations of PVP on a w./v. basis were prepared the night before use and left in a room at 1°. The mitochondrial preparations were washed once with 0·2 M sucrose before use.

With certain samples in series I and II and duplicate samples of all of series III and IV fruit, preparations were made in which 0·01 M or 0·03 M cysteine was incorporated in the extraction medium. Details of these samples are given in Table 2.

"Soluble" Enzyme Preparations

These were prepared from the supernatant extract after removing the mitochondrial fraction as previously described for the preparation of malic enzyme and pyruvic carboxyl-

[12] J. R. L. WALKER, and A. C. HULME, *Phytochem.* 4, 677 (1965).

ase.[9] Essentially, this is a precipitation with polyethylene glycol (PEG). The results given for the enzymes present in these precipitates refer to the total material precipitated by 38–40% (w/v.) PEG, i.e. they represent the combined results for "PEG 1" and "PEG 2" precipitates as described in the previous paper. There was, in fact, little activity in the PEG 1 precipitates (4% PEG).

The resuspended pellets obtained after centrifugation of the precipitated material at 12,000 *g* for 15 min were used for measuring "enzyme activity of PEG precipitates".

Measurement of the Activity of the Mitochondria

Gaseous exchange in the Warburg respirometer, activity of succinic and malic dehydrogenases, diaphorase ($NADH_2$) and $NADH_2$-cytochrome-c-reductase were all determined exactly as described previously.[8] The Warburg results were expressed as μl gas/hr/10 g tissue averaged over a 2 hr run. The dehydrogenases were expressed as units/10 g tissue. One unit was the amount of mitochondrial preparations, which caused a corrected initial rate of change in absorbance of 0·01/min at the appropriate wavelength, the "initial" rate being the rate measured between 30 and 90 sec from the addition of the substrate.

Measurement of the Activity of Malic Enzyme and Pyruvic Carboxylase

These were measured in the PEG precipitates by the methods previously described.[9] Activity of M.E. was expressed in units/10 g tissue, one unit being the amount of enzyme preparation causing an increase in absorptivity of 0·01/min at a wavelength of 340 mμ. One enzyme unit of carboxylase activity was the amount of enzyme preparation required to produce 1 μl CO_2 in 5 min at 25° in the Warburg respirometer.

Measurement of Phenolase Activity

This was measured at 25° in a Warburg respirometer. An equilibration time of 5 min before the manometer taps were closed (zero time) was used; thereafter readings were taken every 5 min for a period of 30 min. The flasks contained sucrose 400 μmoles; KH_2PO_4 adjusted to pH 7·0 with KOH, 37·5 μmoles; $MgSO_4$ 10 μmoles; $MnSO_4$ 0·1 μmole; chlorogenic acid adjusted to pH 7·0, 40 μmoles; 0·5 to 1 ml. mitochondrial preparation or PEG precipitate, and water to make 2 ml. Control experiments with boiled enzyme showed no O_2-uptake. Chlorogenic acid was used as substrate because it is one of the chief phenolase substrates in the apple[13]; experiments showed that with the most active phenolase preparations, substrate was not limiting at 40 μmoles per flask. One unit of phenolase was taken as the amount of enzyme preparation required to catalyse the uptake of 1 μl O_2/5 min measured over 30 min from zero time.

CHEMICAL ESTIMATIONS

The *phenolics content* of the various enzyme preparations was determined by the methods of Swain and Hillis[14] with the minor modifications described by Hulme *et al.*[8]

Nitrogen determinations were made by the method described by Hulme *et al.*[8] which consisted of a combination of Kjeldahl digestion followed by estimation of the ammonia formed with ninhydrin. TCA-insoluble nitrogen was the nitrogen determined after washing the preparations twice with 7·5% TCA at room temperature.

[13] A. C. Hulme, *Biochem. J.* **53**, 337 (1953).
[14] T. Swain and W. E. Hillis, *J. Sci. Food Agr.* **10**, 63 (1959).

All results, whether enzyme activity or chemical constituents, are expressed on the basis of 10 g original tissue.

Chemicals

The sources of most of the chemicals used were as previously described. Chlorogenic acid was used as received from Fluka A.G., Dextran was "Dextran 40" from Pharmacia, Sweden. The various preparations of PVP were as follows: Kollidon 17 (mol. wt. = 11,000) and 25 (mol. wt. = 28,000) were pharmaceutical grade preparations obtained from Badische Anilin-u-Soda-Fabrik A.G.; "Rhone-Poulenc" (France) was a pharmaceutic grade of PVP of mol. wt. approximately 40,000; K-30 (mol. wt. = 40,000) was from the General Aniline and Film Corporation, New York; the various "Plasdone" preparations are a pharmaceutical grade obtained from the General Aniline and Film Corporation, New York, and "Polyclar AT" is a high-molecular-weight insoluble PVP from the same source.

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