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REVIEW ARTICLE

EXTRACTION OF ENZYMES AND SUBCELLULAR ORGANELLES FROM PLANT TISSUES

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Abstract—During extraction of plant tissues, endogenous phenolics are rapidly oxidized to form quinones, condensed tannins and brown pigments. The oxidation is catalysed by phenoloxidases but only occurs to any appreciable extent when the tissue is homogenized because the phenolics are spatially separated from the phenoloxidases in intact tissue. The products of the phenoloxidase reaction powerfully inhibit plant enzymes and subcellular organelles. Loss of activity of enzymes and subcellular organelles during extraction from tissues containing phenolics (but not tannins) is prevented by thiols and other reducing agents. The effectiveness, usefulness and limitations of thiols and other reducing agents for preventing inactivation of enzymes and subcellular organelles during extraction are reviewed. Various polymers also prevent inactivation and the applications of polymers and reducing agents are compared.

INTRODUCTION

THE MEDIUM for extracting enzymes and subcellular organelles from plant tissues requires careful formulation in order to recover maximum activity in cell-free extracts. For the purpose of this review, the proportion of the total activity of an enzyme or subcellular organelle in the tissue recovered in a cell-free extract will be referred to as the efficiency of extraction. Thus, taking extreme cases, Slack[1] found that extracts of sugar cane internode tissue contain no sucrose synthetase activity unless either cysteine or diethyldithiocarbamate (DIECA) is included in the extracting medium. Similarly, potato tuber mitochondria are completely uncoupled unless either cysteine[2] or metabisulphite[3] is included in the extracting medium. These examples show that modification of the extracting medium can lead to measurement of activity of enzymes and subcellular organelles which cannot otherwise be detected, but there are in addition, countless examples of increased activity as a result of modifying the extracting medium.

Efficiency of extraction is of paramount importance in physiological investigations where the activity of enzymes and subcellular organelles are compared between tissues of different physiological status. Not only is high efficiency of extraction required in such studies, but it is important that the efficiency of extraction is very nearly identical for the tissues compared, especially in experiments comparing the enzyme activity of tissues after treatment with growth regulation substances where the differences in activity are often quite small. It is now

[1] C. R. SLACK, *Phytochem.* **5**, 397 (1966).
[2] J. D. VERLEUR, *Plant Physiol.* **40**, 1003 (1965).
[3] D. M. STOKES, J. W. ANDERSON and K. S. ROWAN, *Phytochem.* **7**, 1509 (1968).

well established that the observed large increase in aldolase activity in ripening banana fruit[4] is an artifact due to increased efficiency of extraction of aldolase as the banana ripens.[5–7]

Many instances of improved efficiency of extraction reported in the literature are attributable to removal of endogenous tannins* and/or preventing formation of oxidation products of endogenous phenolics.† Most plant tissues contain a wide range of phenolic compounds which are oxidized by copper-containing enzymes, broadly classified as phenol oxidases.[9] The most active of these enzymes in plant tissues is *o*-diphenol: O_2 oxidoreductase (E.C. 1.10.3.1, trivial name *o*-diphenoloxidase) which oxidizes *o*-diphenols to the corresponding quinones. Quinones and the brown pigments formed from them by non-enzymic polymerization reactions inhibit many enzymes and the activity of subcellular organelles[1–3, 10–14] including *o*-diphenoloxidase itself.[15, 16] Condensed tannins, which have a lower MW than brown pigments, also form quinones[8] and inhibit enzymes.[10] Inhibition of enzymes and subcellular organelles by tannins and phenolics was reviewed 2 years ago by Loomis and Battaile[10] and they discussed at length the use of various polymers for preventing inactivation of enzymes and mitochondria during extraction by tannins and *o*-diphenoloxidase products.

It has long been known that various reducing agents inhibit browning and improve the efficiency of extraction of enzymes from some plant tissues. In the previous review on extraction, Loomis and Battaile[10] mentioned that reducing agents are of some value for extracting enzymes from plant tissues but concluded that "the use of reducing agents is only an imperfect substitute for actual inhibition of phenol oxidation." Since then, interest in the use of reducing agents has been revived and recent papers by Slack,[1] Verleur,[2] Anderson and Rowan,[11, 17, 18] and Stokes *et al.*[3] call for a reassessment of the incorporation of thiols and other reducing agents in extracting media for improving efficiency of extraction, especially since the polymers have not proved as efficient for extracting plant enzymes and mitochondria as originally believed. This paper extends the review of Loomis and Battaile[10] to include the reducing agents and several other compounds which have been used to prevent the formation of *o*-diphenoloxidase products during extraction. The control of pH, tonicity, ionic strength, addition of detergents and other variables will not be considered. Similarly, the increase in enzyme activity obtained by adding reducing agents to the incubation mixture during assay

* A tannin is defined by Swain[8] as a compound of MW 3500-000 with 1–2 phenolic hydroxy groups per 100 MW.

† A phenolic is defined (for the purpose of this review) as a compound which is a substrate for any of the phenol oxidases Some tannins are included in this definition.

[4] J. M. TAGER and J. B. BIALE, *Physiol. Plantarum* **10**, 79 (1957).
[5] J. B. BIALE, *Advan. Food Res.* **10**, 293 (1960).
[6] J. B. BIALE and R. E. YOUNG, *Proc.* 5th *Inter. Congr. Biochem*, *Moscow*, 1961, Vol. 9, p. 370, Macmillan (Pergamon), New York (1963).
[7] R. E. YOUNG, *Arch. Biochem. Biophys.* **111**, 174 (1965).
[8] T. SWAIN, in *Plant Biochemistry* (edited by J. BONNER and J. E. VARNER), p. 552, Academic Press, New York (1965).
[9] W. D. BONNER, *Ann. Rev. Plant Physiol.* **8**, 427 (1957).
[10] W. D. LOOMIS and J. BATTAILE, *Phytochem.* **5**, 423 (1966).
[11] J. W. ANDERSON and K. S. ROWAN, *Phytochem.* **6**, 1047 (1967).
[12] J M. QUASTEL, *Biochem. J.* **27**, 1116 (1933).
[13] H. S. MASON, *Advan. Enzymol.* **16**, 105 (1955).
[14] M. LIEBERMAN and J. B. BIALE, *Plant Physiol.* **31**, 420 (1956).
[15] J R. L. WALKER, *Australian J Biol. Sci.* **17**, 360 (1964).
[16] J. M. NELSON and C. R. DAWSON, *Advan. Enzymol.* **4**, 99 (1944).
[17] J W. ANDERSON and K. S. ROWAN, *Biochem. J.* **97**, 741 (1965).
[18] J. W. ANDERSON and K. S. ROWAN, *Biochem. J* **101**, 9 (1966).

of an enzyme previously extracted in the absence of a reducing agent has no place in this review and should not be confused with the requirement for a reducing agent in the extracting medium since this requirement cannot be made good by adding the reducing agent after extraction.

SUBCELLULAR DISTRIBUTION OF *o*-DIPHENOLS AND *o*-DIPHENOLOXIDASE IN THE INTACT CELL AND THE EFFECT OF DISRUPTION OF THE CELL

The subcellular location of *o*-diphenoloxidase is not clearly understood. Whilst it is generally believed to be a soluble enzyme,[9] there are numerous reports of *o*-diphenoloxidase activity associated with mitochondria[19,20] and chloroplasts.[21–22] However, both the *o*-diphenoloxidases of mitochondria and chloroplasts are inhibited by polyvinylpyrrolidone (PVP) of MW 20,000[19] which suggests that *o*-diphenoloxidase is attached to the chloroplast and mitochondrion rather than contained within these particles. More recently Walker and Hulme[23] prepared mitochondria from apple peel with soluble PVP (a compound which lessens the contamination of mitochondria by co-precipitated material[24]) and found that both the mitochondrial and soluble fractions contain two *o*-diphenoloxidases (possibly isoenzymes) which show identical properties on electrophoresis and on chromatography on DEAE-cellulose though Harel *et al.*[19] reported that some properties of the particulate and cytoplasmic *o*-diphenoloxidases are different. However, Harel *et al.* did not include PVP in their extracting medium. Walker and Hulme[23] suggest that the distribution of *o*-diphenoloxidase that they observed could be an artifact, possibly caused by adsorption of cytoplasmic *o*-diphenoloxidase onto the surface of mitochondria. However, soluble enzymes are believed to be synthesized in the cytoplasm by way of the general protein-synthesizing machinery and subsequently pass into the developing mitochondrion, whereas the membrane-bound enzymes of the organelle (e.g. cytochrome oxidase) are synthesized by the mitochondrial system.[25] Thus the results of Walker and Hulme[23] are consistent with localization of *o*-diphenoloxidase in the soluble phase of the mitochondrion. Perhaps the recent discovery of a particle of slightly greater density than a mitochondrion containing high peroxidase activity could shed some light on this problem.[26] Obviously the whole problem of intracellular localization of the mitochondrial *o*-diphenoloxidases needs careful reinvestigation. No attempt has yet been made critically to examine the *o*-diphenoloxidase of chloroplasts in relation to the cytoplasmic *o*-diphenoloxidases; liberation of the enzyme by treatment of isolated chloroplasts with proteolytic enzymes[21] does not rule out the possibility of adsorption from the cytoplasm onto the chloroplast during isolation. The results of Sanderson[27] suggest that tea leaf *o*-diphenoloxidase, previously thought to be located in the chloroplast,[28] is contained in the soluble fraction but no attempt was made to demonstrate that the medium used to extract *o*-diphenoloxidase did not disrupt chloroplasts.

[19] E. HAREL, A. M. MAYER and Y. SHAIN, *Physiol. Plantarum* **17**, 921 (1964).
[20] J. R. L. WALKER and A. C. HULME, *Phytochem.* **4**, 677 (1965).
[21] A. M. MAYER, *Phytochem.* **5**, 1297 (1966).
[22] M. SATO, *Phytochem.* **6**, 1363 (1967).
[23] J. R. L. WALKER and A. C. HULME, *Phytochem.* **5**, 259 (1966).
[24] A. C. HULME and J. D. JONES, in *Enzyme Chemistry of Phenolic Compounds* (edited by J. B. PRIDHAM), p. 97, Pergamon Press, Oxford (1963).
[25] D. B. RODYN and D. WILKIE, *The Biogenesis of Mitochondria*, Methuen, London (1968).
[26] M. PLESNICAR, W. D. BONNER and B. T. STOREY, *Plant. Physiol.* **42**, 366 (1967).
[27] G. W. SANDERSON, *Biochim. Biophys. Acta* **92**, 622 (1964).
[28] L. P. LI and J. BONNER, *Biochem. J.* **41**, 105 (1945).

Many tissues contain high concentrations of *o*-diphenols; e.g. independent authors have reported that tobacco leaf contains 16–22 μmoles,[29] 3–5 μmoles[30] and 3–9 μmoles[31] chlorogenic acid per g fresh weight. Most reviews on the subcellular location of tannins and phenolics state that these compounds occur in the vacuoles of intact plant cells.[8, 32–34] Whilst this is probably true, Swain[8] points out that some of the cytochemical tests used to determine the subcellular location of tannins are equivocal. Concrete evidence demonstrating subcellular location of tannins and phenolics in vacuoles is scant. Thus little oxidation of phenolics occurs in intact plant cells because the phenolics are spatially separated from *o*-diphenoloxidases by the tonoplast. However, in ageing cells, breakdown of the tonoplast occurs[35] and oxidation of the vacuolar phenolics by the cytoplasmic enzymes ensues; it may be significant that this is concomittant with the death of the cell.[36]

The K_m value of chlorogenic acid substrate for *o*-diphenoloxidase prepared from tobacco leaf, apple fruit and potato tuber falls in the range 0·1 to 2·5 mM,[15, 37–41] but most values fall between 0·1 and 0·2 mM and therefore a K_m value of 0·15 mM has been used as the average value. The concentration of oxygen required for half maximum activity of *o*-diphenoloxidase is high (223 μM,[37] 150 μM[38]) in comparison with the respiratory chain (0·4 μM for high affinity system of potato tubers[42]). Thus not only are the phenolics spatially separated from *o*-diphenoloxidase, but the oxidase competes poorly with the respiratory chain at low partial pressures of oxygen. Thus if the tonoplast (but not the plasmalemma) is ruptured, as in ageing cells, oxidation products accumulate at a slow rate. On the other hand, complete disruption of a cell by sonication etc. causes saturation of the cellular debris with oxygen as well as mixing of *o*-diphenoloxidase with the phenolics. Consequently *o*-diphenoloxidase competes with the high affinity system[13] and oxidation of phenolics occurs readily. Thus, assuming that the concentration of oxygen is not limiting and using the average K_m value for *o*-diphenoloxidase and the average concentration of chlorogenic acid in tobacco leaf tissue, then *o*-diphenoloxidase would be operating at approximately 95 per cent of maximum activity in macerates of tobacco leaf tissue. Consequently quinones, condensed tannins and brown pigment accumulate during extraction and inactivation of susceptible enzymes ensues.[1, 10, 11] The degree of inactivation must vary with activity of *o*-diphenoloxidase and the concentration of endogenous phenolics which in turn vary with the physiological state of the tissue.[31, 43–46]

[29] M. SHIROYA, T. SHIROYA and S. HATTORI, *Physiol. Plantarum* **8**, 594 (1955).
[30] M. ZUCKER and J. F. AHRENS, *Plant Physiol.* **33**, 246 (1958).
[31] R. F. DAWSON and E. WADA, *Tobacco Sci.* **1**, 47 (1957).
[32] P. J. KRAMER, in *Encyclopedia of Plant Physiology* (edited by W. RHULAND), Vol. 1, p. 649, Springer-Verlag, Berlin (1955).
[33] O. HARTEL, *Protoplasma* **40**, 338 (1951).
[34] W. G. C. FORSYTH, *Ann. Rev. Plant Physiol.* **15**, 443 (1964).
[35] M. SHAW and M. S. MANOCHA, *Can J. Botany* **43**, 747 (1965).
[36] M. SPENCER, in *Plant Biochemistry* (edited by J. BONNER and J. E. VARNER), p. 793, Academic Press, New York (1965).
[37] D. A. ABUKHARMA and H. W. WOOLHOUSE, *New Phytol.* **65**, 477 (1966).
[38] F. A. M. ALBERGHNINA, *Phytochem.* **3**, 65 (1964).
[39] A. VAN KAMMEN and D. BROUWER, *Virology* **22**, 9 (1964).
[40] E. C. SISLER and H. J. EVANS, *Plant Physiol.* **33**, 255 (1958).
[41] R. A. CLAYTON, *Arch. Biochem. Biophys.* **81**, 404 (1959).
[42] L. W. MAPSON and W. G. BURTON, *Biochem. J.* **82**, 19 (1962).
[43] M. ZUCKER, C. NITSCH and J P. NITSCH, *Am. J. Botany* **52**, 271 (1965).
[44] A. O. TAYLOR and M. ZUCKER, *Plant Physiol.* **41**, 1350 (1966).
[45] H. HYODO and I. URITANI, *Plant Cell Physiol.* **7**, 137 (1966).
[46] H. RUCKENBROD, *Planta* **46**, 19 (1955).

INHIBITION OF ACTIVITY OF ENZYMES AND SUBCELLULAR ORGANELLES BY PHENOLICS AND PRODUCTS OF THE *o*-DIPHENOLOXIDASE REACTION

Several explanations have been offered for the low activity of enzymes and subcellular organelles in cell-free extracts containing products of the *o*-diphenoloxidase reaction. Quinones condense readily with free sulphydryl groups and terminal α-amino and imino groups of proteins and less readily with the ϵ-amino group of lysine.[10, 13] It follows that quinones readily react with proteins and inhibit enzymes. Hulme *et al.*[47, 48] suggested that loss of soluble protein (and presumably soluble enzymes) occurs by co-precipitation of protein with polymerized forms of oxidized phenolics. This hypothesis is supported by the results of Sanderson[27] who found that the amount of protein remaining in the soluble fraction of extracts of tea leaves was inversely related to the concentration of phenolics in the extract. In a later communication, Sanderson[49] reported that the effect of tea leaf phenolics on the activity of *o*-diphenoloxidase of tea leaf was to cause precipitation of the enzyme; preincubation of a mixture of tea leaf phenolics and *o*-diphenoloxidase, either aerobically or anaerobically, had no effect on enzyme activity, though preincubation under oxygen slightly increased the loss of activity by precipitation.

If precautions are not taken to prevent co-precipitation, soluble enzymes are recovered in the particulate fractions; e.g. recovery of *o*-diphenoloxidase of tea leaves in the mitochondrial fraction.[27] This effect can be demonstrated *in vitro* with synthetic complexes of soluble enzymes and crystalline tannic acid which sediment at 7500 *g* min.[50] Similarly, Hulme *et al.*[47] suggested that mitochondria are co-precipitated with protein-phenolic complexes, yielding "inactive agglomerates".

Plant enzymes and mitochondria are reported to be inhibited by some plant phenolics *per se*[10, 24, 51] though this is thought to be less important than loss of activity caused by the oxidized and polymerized forms.[14, 47] However, it is noteworthy that enzyme and mitochondrial preparations reported to be inhibited by phenolics have not been shown to be free of *o*-diphenoloxidase. Thus Lieberman and Biale[14] reported that chlorogenic acid inactivated sweet potato mitochondria but since DIECA and ascorbate (inhibitors of *o*-diphenoloxidase) prevented inactivation of the mitochondria, they concluded that their mitochondrial preparations contained *o*-diphenoloxidase and that the quinone of chlorogenic acid, produced by the action of *o*-diphenoloxidase, was the inhibitory form. Using jack bean, Quastel[12] demonstrated inhibition of urease by the addition of phenolics but this too was attributed to the formation of quinones. On the other hand Hulme *et al.*[24] found that if contamination of apple mitochondria with *o*-diphenoloxidase and *o*-diphenols was prevented by extracting the tissue with PVP, then no inhibition by chlorogenic acid was observed.

COMPOUNDS USED IN EXTRACTING MEDIA TO PREVENT FORMATION OF THE PRODUCTS OF *o*-DIPHENOLOXIDASE ACTIVITY

The formation of oxidation products of *o*-diphenoloxidase activity can be prevented in a variety of ways:

1. Removal of phenolic substrates: e.g. adsorption.
2. Inhibition of *o*-diphenoloxidase activity.

[47] A. C. HULME, J. D. JONES and L. S. C. WOOLTORTON, *Phytochem.* **3**, 173 (1964).
[48] J. D. JONES, A. C. HULME and L. S. C. WOOLTORTON, *Phytochem.* **4**, 659 (1965).
[49] G. W. SANDERSON, *Biochem. J.* **95**, 24p (1965).
[50] J. L. GOLDSTEIN and T. SWAIN, *Phytochem.* **4**, 185 (1965).
[51] A. C. HULME, J. D. JONES and L. S. C. WOOLTORTON, *Nature* **201**, 795 (1964).

3. Removal of quinones (formed as a result of *o*-diphenoloxidase activity) by reduction of the quinone back to the *o*-diphenol. Alternatively, quinone may be removed by condensation with another compound to yield a product which will not undergo further oxidation and which is non-inhibitory.

No single class of compounds appears to be specific to any one of these groups in its type of action and in many cases the type of action varies with the concentration.

Polymers

Soluble PVP was used by Hulme *et al.*[24, 47] for the preparation of mitochondria from the peel of apple fruit, free from contamination by phenolics; details of this work have been reviewed by Loomis and Battaile.[10] The same technique, using insoluble forms of PVP, has been extended to the extraction of soluble enzymes from various tissues,[10, 48, 52] and other polymers have also been successfully used, e.g. polyethylene glycol,[7, 53] and polycaprolaktam.[27] (Polyethylene glycol has also been used at very high concentration for the preparation of chloroplasts,[54, 55] but as this appears to be mainly an osmotic effect it will not be discussed here.) Mitochondria prepared with PVP exhibit higher rates of oxygen uptake on addition of TCA cycle intermediates than mitochondria prepared without PVP in the extracting medium.[47] Various factors appear to contribute to the increased efficiency of extraction obtained with PVP. PVP inhibits purified preparations of *o*-diphenoloxidase from apple fruit,[19, 20] potato tuber[37] and tobacco leaf[56] though the mechanism of the inhibition is uncertain.[19, 20, 48] The degree of inhibition of *o*-diphenoloxidase by a given concentration of PVP varies greatly but is rarely greater than 75 per cent using chlorogenic acid as substrate.[20, 37, 56] PVP also binds phenolics and tannins (see Loomis and Battaile[10]) thereby removing endogenous substrates of *o*-diphenoloxidase though there is evidence that PVP itself is bound by phenolics associated with mitochondria.[20] PVP also binds phenolics reported to inhibit enzymes and mitochondria. However, Walker and Hulme[20] reported that a relative excess of PVP did not bind chlorogenic acid; one explanation could be that PVP only binds phenolics of molecular weight greater than chlorogenic acid. More recently, Andersen and Sowers[57] investigated the absorption of various phenolics by insoluble PVP. Their results show that the amount of phenolic removed is dependent on the amount of PVP added; e.g. using a solution of 0·03 mM chlorogenic acid, 2·5 per cent (w/v) PVP absorbed 79 per cent and 5·0 per cent (w/v) PVP absorbed 93 per cent. Presumably higher concentrations of chlorogenic acid require still higher concentrations of PVP to effect the same absorption. Since tobacco leaf tissue contains approximately 100 times the concentration of chlorogenic acid used by Andersen and Sowers (see Refs.[29–31]), then extremely high concentrations of PVP would have to be used to quantitatively absorb the chlorogenic acid from this tissue. Alternatively, the phenolics could be absorbed by using a vast excess of extracting medium containing PVP, but dilution of the extract is clearly undesirable. Furthermore, the optimum pH for absorption of phenolics by PVP is 3·5. Therefore PVP would seem to be of little value for increasing the efficiency of extraction of enzymes and subcellular organelles from tissues rich in chlorogenic acid and possibly other low molecular weight phenolics. This

[52] G. W. SANDERSON, *Biochem. J.* **98**, 248 (1966).
[53] A. M. BADRAN and D. E. JONES, *Nature* **206**, 622 (1965).
[54] J. H. MCCLENDON and L. R. BLINKS, *Nature* **170**, 577 (1952).
[55] J. H. MCCLENDON, *Plant Physiol.* **29**, 448 (1954).
[56] W. S. PIERPOINT, *Biochem. J.* **98**, 567 (1966).
[57] R. A. ANDERSEN and J. A. SOWERS, *Phytochem.* **7**, 293 (1968).

would explain why PVP is not effective for preparing mitochondria from potato tuber[24] nor for preventing inactivation of cucumber mosaic virus during extraction from tobacco leaves,[57] since both these tissues contain high concentrations of chlorogenic acid. Other more efficient inhibitors of *o*-diphenoloxidase prevent inactivation of cucumber mosaic virus.[58, 59]

Rowan,[60] in reviewing the work of Jones *et al.*[61] on the oxidative phosphorylation of mitochondria during the climacteric rise in apple fruits, points out that mitochondria prepared with PVP by the method of Hulme *et al.*[47] appear uncoupled. The more recent results of Hulme *et al.*[62] show that mitochondria from apple, prepared with PVP, exhibit poor respiratory control (respiratory control ratios 1·2–1·6). Thus whilst PVP is a suitable reagent for preventing inactivation of soluble enzymes, the use of PVP for extracting mitochondria for studies of oxidative phosphorylation seems less satisfactory.

Walker and Hulme[20] reported that approximately 50 per cent of the *o*-diphenoloxidase of apple peel was found in the mitochondrial fraction when the tissue was extracted without PVP in the extracting medium. One of the difficulties of determining the subcellular distribution of *o*-diphenoloxidase in tissues extracted with media containing PVP lies in the fact that PVP itself inhibits *o*-diphenoloxidase activity. This can be overcome by treating the extracts with anionic detergents which reverse the inhibition.[20] Using this technique, Walker and Hulme[20] showed that the activity of *o*-diphenoloxidase in the mitochondrial fraction was reduced by approximately 50 per cent when PVP was included in the extracting medium but approximately 25 per cent of the total activity in the tissue was still recovered in the mitochondrial fraction. The observation that mitochondria prepared with PVP contain the same two *o*-diphenoloxidases as the soluble fraction,[23] suggests that mitochondria prepared with PVP could be contaminated with cytoplasmic *o*-diphenoloxidases. Walker and Hulme[20] reported that PVP was a competitive inhibitor of *o*-diphenoloxidase (a point disputed by Harel *et al.*[19]) and combines with the *o*-diphenol-substrate complex. They provided evidence that mitochondria could bind PVP and they therefore suggested that mitochondria could bind to the PVP-*o*-diphenol-substrate complex, thus explaining the particulate occurrence of *o*-diphenoloxidase when mitochondria are prepared with PVP in the extracting medium.

Copper Chelating Agents

DIECA is a most efficient inhibitor of *o*-diphenoloxidase [9, 15, 37, 56] because it chelates with copper which forms the active centre of this enzyme. Furthermore DIECA combines with the quinone product, formed at concentrations of DIECA insufficient to completely inhibit *o*-diphenoloxidase, provided that the molar ratio of DIECA to *o*-diphenol exceeds 1·3.[56] Ethyl xanthate shows a similar effect to DIECA, though DIECA inhibits *o*-diphenoloxidase more powerfully.[56] DIECA has been successfully used for the extraction of sucrose synthetase from sugar cane[1] and both DIECA and ethyl xanthate have been used for the extraction of various viruses from plant tissues; e.g. extraction of cucumber mosaic virus from tobacco leaf.[58, 59] (See Harrison and Pierpoint[58] for other examples.) DIECA has not been used to inhibit *o*-diphenoloxidase during extraction of mitochondria from plants, presumably because DIECA might be expected to inhibit the respiratory chain. However, cytochrome oxidase of beef heart mitochondria which contains both iron and copper[63] is not inhibited either by

[58] B. D. Harrison and W. S. Pierpoint, *J. Gen. Microbiol.* **32**, 417 (1963).
[59] W. S. Pierpoint and B. D. Harrison, *J. Gen. Microbiol.* **32**, 429 (1963).
[60] K. S. Rowan, *Int. Rev. Cytol.* **19**, 301 (1966).
[61] J. D. Jones, A. C. Hulme and L. S. C. Wooltorton, *Phytochem.* **3**, 201 (1964).
[62] A. C. Hulme, M. J. C. Rhodes and L. S. C. Wooltorton, *Phytochem.* **6**, 1343 (1967).
[63] L. Ernster and C. Lee, *Ann. Rev. Biochem.* **33**, 729 (1964).

ethylenediaminetetra acetate or by 8-hydroxyquinoline;[64] DIECA caused a small inhibition of this cytochrome oxidase but was thought to be the result of an artifact.[64] Consequently DIECA and ethyl xanthate might prove useful for preventing damage to mitochondria by *o*-diphenoloxidase products during extraction.

The property of DIECA and ethyl xanthate to combine with quinones (which form in small amounts even at high concentrations of these compounds) seems to be of critical importance in the role these compounds play in preventing accumulation of *o*-diphenoloxidase products during extraction. Cyanide is also a powerful inhibitor of *o*-diphenoloxidase but does not afford protection of tobacco leaf peptidase during extraction at pH 5·2,[17] presumably because cyanide does not combine with the small quantities of quinone produced. In addition, the cyanide ion is probably the active species in inhibiting *o*-diphenol oxidase. Since approximately 10^{-4} of the cyanide is dissociated at pH 5·2, then the concentration of CN^- may not have been high enough to inhibit *o*-diphenoloxidase effectively.

Mercaptobenzothiazole is a powerful inhibitor of *o*-diphenoloxidase.[56, 65] The mechanism of inhibition was formerly thought to be similar to many of the thiols discussed later. However, Palmer and Roberts[66] have recently reported that very low concentrations of mercaptobenzothiazole inhibit *o*-diphenoloxidase and that the inhibition is reversed by equally low concentrations of cupric ion. Palmer and Roberts demonstrated that 2 moles mercaptobenzothiazole complex with 1 mole cupric ion. They suggested that mercaptobenzothiazole combines with copper at the active centre of *o*-diphenoloxidase, thus making an inactive complex.

Mercaptobenzothiazole, like ethyl xanthate and DIECA, is thought to be removed by traces of quinone according to the mechanisms discussed under thiols.

Thiols

Many authors have reported that the activity of enzymes and mitochondria in extracts prepared from plant tissues is greatly enhanced by including thiols in the extracting medium (Table 1). Enzymes extracted from leaf tissue show a larger increase in efficiency of extraction when thiols are included in the extracting medium than enzymes from other tissues.[69] Many authors routinely include thiols in the medium for extracting enzymes[72–74] and subcellular organelles,[75–77] but apart from those references cited in Table 1 (though this list cannot claim to be exhaustive), the effect of these thiols on the efficiency of extraction of the enzyme has not been reported. With the exception of the work of Clayton,[41] all thiols so far tested inhibit *o*-diphenoloxidase activity[11, 15, 56, 65, 66] but the greater enzyme activity of extracts prepared with thiols has been correlated with preventing accumulation of *o*-diphenoloxidase products in only a few cases.[1, 11] However, some enzymes are not inhibited by the products of the *o*-diphenoloxidase reaction and therefore do not respond to including thiols in the extracting medium (e.g. invertase from sugar cane tissue[1]); whilst some tissues do not appear to contain *o*-diphenoloxidase and/or *o*-diphenols and similarly show no response to including

[64] D. E. GRIFFITHS and D. C. WHARTON, *J. Biol. Chem.* **236**, 1850 (1961).
[65] J. K PALMER, in *Phenolics in Normal and Diseased Fruits and Vegetables* (edited by V. C RUNECKLES), p. 7, Plant Phenolics Group of North America, Montreal (1965).
[66] J. K. PALMER and J. B. ROBERTS, *Science* **157**, 200 (1967).
[72] G. V. JOHNSON, H. J. EVANS and TE MAY CHING, *Plant Physiol.* **41**, 1330 (1966).
[73] A. E. CLARKE and B. A. STONE, *Phytochem.* **1**, 175 (1962).
[74] P K. POTTINGER and I. T. OLIVER, *Biochim. Biophys. Acta* **58**, 303 (1962).
[75] W. D. BONNER and D. O. VOSS, *Nature* **191**, 682 (1961).
[76] D. SPENCER and S. G. WILDMAN, *Biochem.* **3**, 954 (1964).
[77] R. I. B. FRANCKI, N. K. BOARDMAN and S. G. WILDMAN, *Biochem.* **4**, 865 (1965).

TABLE 1. ENZYMES AND SUBCELLULAR ORGANELLES REQUIRING THIOLS IN THE EXTRACTING MEDIUM FOR MAXIMUM EFFICIENCY OF EXTRACTION

Enzyme or organelle	Species	Tissue	Thiol	Thiol dependent increase in activity (fold)	Reference
Carbonic anhydrase	*Lactuca sativa* *Tropaeolum majus* *Solanum tuberosum*	Leaf	Cysteine	Inactive without	67
Triose phosphate dehydrogenase	*Zea mays* (and other cereals)	Leaf	{Thioglycollate Cysteine Glutathione}	Inactive without	68
Nitrate reductase	*Lycopersicum esculentum* and many other spp.	Leaf	Cysteine	30	69
Nitrite reductase	*Lycopersicum esculentum*	Leaf	Cysteine	5	70
Mitochondrial oxidative phosphorylation	*Solanum tuberosum*	Tuber	Cysteine	Uncoupled without	2
Peptidase	*Nicotiana tabacum*	Leaf	{Thioglycollate Cysteine}	4–6	11, 17
Sucrose synthetase	*Saccharum officinarum*	Elongating internode	{Cysteine DIECA}	Inactive without	1
Aminoacyl-s-RNA synthetases	*Nicotiana tabacum* *Phaseolus vulgaris*	Leaf Plumule Radicle	Thioglycollate Thioglycollate Thioglycollate	3–4 2–3 2–3	18 this paper (Table 2)
[UDPG-dependent glucosylation of *o*-coumaric acid]	*Melilotus alba*	Not specified	{Mercaptoethanol Cysteine}	Inactive without	71

[67] J. R. G. BRADFIELD, *Nature* **159**, 467 (1947).
[68] R. H. HAGEMAN and E. R. WAYGOOD, *Plant Physiol.* **34**, 396 (1959).
[69] G. W. SANDERSON and E. C. COCKING, *Plant Physiol.* **39**, 416 (1964).
[70] G. W. SANDERSON and E. C. COCKING, *Plant Physiol.* **39**, 423 (1964).
[71] A. KLEINHOFS, F. A. HASKINS and H. J. GORZ, *Phytochem.* **6**, 1313 (1967).

a thiol in the extracting medium. Thus the activity of aminoacyl-s-RNA synthetases in the plumule and the radicle of french bean seedlings show a marked requirement for thioglycollate in the extracting medium whilst the synthetases extracted from the cotyledon do not (Table 2). Similarly, Palmer[78] has recently reported that coupled mitochondria with good respiratory control can be prepared from Jerusalem artichoke tuber without the addition of a thiol to the extracting medium.

The concentration of thiol causing 50 per cent inhibition of a given amount of *o*-diphenol-oxidase varies widely, e.g. 1 mM-thioglycollate, 10 mM-cysteine[56] (inhibitor constants cannot be applied since the nature of the inhibition is not known), but even at high concentrations of the most powerful inhibitor (thioglycollate), small amounts of quinone form. Therefore the crucial part of the effectiveness of thiols in preventing accumulation of *o*-diphenol-oxidase products (and hence the effect of thiols on the efficiency of extraction) depends on removal of any quinone as it is formed. The thiols can be arbitrarily grouped into two classes,

TABLE 2. EFFECT OF INCLUDING POTASSIUM THIOGLYCOLLATE DURING EXTRACTION AND INCUBATION UPON ACTIVITY OF AMINOACYL-S-RNA SYNTHETASES FROM VARIOUS TISSUES OF FRENCH BEAN SEEDLINGS

Tissue	Concentration of thioglycollate (mM)		Enzyme protein (mg/ml)	Specific activity (units/mg protein)
	Extracting medium	Incubating medium		
Cotyledon	0	0	0·171	9·1
	0	17·5	0·171	10·4
	25	17·5	0·180	8·4
Radicle	0	0	0·0149	33·2
	0	17·5	0·0149	33·2
	25	17·5	0·0143	58·2
Plumule	0	0	0·0870	22·3
	0	17·5	0·0870	20·6
	25	17·5	0·0812	50·7

depending on the type of reaction between thiol and quinone. (1) Thiols which reduce quinones (as they form) back to the *o*-diphenol; such thiols (thioglycollate[56] and mercaptobenzothiazole[56, 65, 66]) tend to be powerful inhibitors of *o*-diphenoloxidase. (2) Thiols which combine with quinones to form a product which is not further oxidized and does not inhibit enzymes; such thiols (cysteine,[15, 56, 79] DIECA,[56] and ethyl xanthate[56]) tend to be less powerful inhibitors of *o*-diphenoloxidase than those of class (1). Glutathione[56, 79, 80] and thiourea[79] also combine with quinones. However, glutathione does not increase the efficiency of extraction of the cell-free system from sweet clover catalysing the UDPG-dependent glucosylation of *o*-coumaric acid, though other thiols (cysteine and mercaptoethanol) are most effective.[71] Thiourea has not been examined in relation to the efficiency of extraction. The reactions of thiols with quinones, formed by *o*-diphenoloxidase, are complicated by the removal of thiol both by autoxidation, catalysed by metal ions,[81] present in plant extracts,

[78] J. M. PALMER, *Nature* **216**, 1208 (1967).
[79] R. E. HENZE, *Science* **123**, 1174 (1956).
[80] A. C. HULME, *Advan. Food. Res.* **8**, 297 (1958).
[81] A. E. MARTELL and M. CALVIN, *Chemistry of the Metal Chelate Compounds*, p. 341, Prentice-Hall, Englewood Cliffs, N.J. (1952).

and by enzymic oxidation;[82] e.g. Hackett *et al.*[83] found very rapid oxidation of cysteine by preparations of mitochondria from potato tuber. Furthermore, thiols in class (2) only combine with quinones when the molar ratio of thiol to *o*-diphenol exceeds a critical value[15, 56] (e.g. 1·5 for cysteine/chlorogenic acid[56]).

Compounds of the second class should in theory provide permanent protection of enzymes in extracts of plant tissues provided that all the endogenous *o*-diphenol is converted to the condensation product before the supply of thiol is depleted by enzymic and non-enzymic oxidation to less than 1·5 times the original amount of *o*-diphenol. Compounds of the first class only provide temporary protection of enzymes since the endogenous *o*-diphenol concentration virtually remains constant until the supply of thiol is exhausted. In practice, both classes of thiols provide the same degree of protection of enzymes during extraction; e.g. the efficiency of extraction of tobacco leaf peptidase with media containing either thioglycollate or cysteine is similar.[11] The deciding factor in the time of protection of enzymes for both classes of thiols appears to be autoxidation of thiol by traces of metal ions in the extract. The temporary protection of enzymes can be readily demonstrated by using barely adequate concentrations of thiol to inhibit the accumulation of *o*-diphenoloxidase products.[11] Thus high concentrations of thiol are essential to ensure protection of the enzyme for the duration of an experiment or until the enzyme can be separated from the phenolics present in the extract. However, Verleur[2] is of the opinion that cysteine can only be used to prevent the accumulation of *o*-diphenoloxidase products during preparation of potato tuber mitochondria if the concentration of phenolics in the tissue is not too high; tissues containing high concentrations of phenolics require an equally high concentration of cysteine but, at high concentrations, endogenous oxidation of cysteine[83] is equally high.

The time of protection afforded by a given amount of a specified thiol (or alternatively the amount of thiol required to provide protection for a given time) varies greatly, even between samples of a single species. Thus the minimum concentration of thioglycollate necessary to provide protection of tobacco leaf peptidase for 1 hr varied from 0·5 to 10 mM[11] and serves to emphasize the need to use high concentrations of thiols. Such variation is presumably due to variation in activity of *o*-diphenoloxidase, concentration of endogenous *o*-diphenols, and the various other factors regulating the rate of removal of thiol. This might explain why Hulme *et al.*[47] observed only transient inhibition of browning by cysteine (concentration unspecified) during extraction of mitochondria from apples.

Thiols in class (2) (e.g. cysteine) do not combine with quinones at molar ratios of thiol to *o*-diphenol less than approximately 1·5 but rather tend to reduce the quinone back to the phenol. This subject is discussed in detail by Pierpoint.[56]

To the best of the author's knowledge, neither mercaptoethanol nor dithiothreitol have been investigated in relation to their effect on the activity of *o*-diphenoloxidase, though they are both often included in extracting media. Recently, mercaptoethanol has been reported to have a spectacular effect on the efficiency of extraction of a cell-free extract from sweet clover catalysing the UDPG-dependent glucosylation of *o*-coumaric acid.[71] This report is particularly interesting because activity of this system could not be detected in the absence of mercaptoethanol, even though the extracting medium contained 1% (w/v) insoluble PVP ("Polyclar AT").

At the beginning of this review, it was pointed out that adding thiol during assay of an enzyme prepared from plant tissues in the absence of thiol, does not restore activity to the

[82] S. M. Bocks, *Biochem. J.* **98**, 9c (1966).
[83] D. P. Hackett, D. W. Hass, S. K. Griffiths and D. J. Niederpruem, *Plant Physiol.* **35**, 8 (1960).

level obtained if the enzyme is extracted in the presence of thiol. This suggests that the poor activity of extracts prepared without thiols is not due to oxidation of SH groups of the enzyme associated with enzyme activity since this should be reversed by thiols. Nevertheless, it is possible that irreversible oxidation of SH groups associated with enzyme activity might occur during extraction and therefore part of the function of thiols might be to prevent irreversible oxidation.

Ascorbate

Many authors have used ascorbate in attempts to increase the efficiency of extraction of enzymes[11, 37, 71] and subcellular organelles,[21, 47, 84] but no author has reported great success with this approach. Ascorbate readily reduces quinones with the regeneration of the phenol and is widely used for this reason to measure spectrophotometrically *o*-diphenoloxidase activity.[37, 85, 86]

Reports of the effect of ascorbate on the activity of *o*-diphenoloxidase are variable.[41, 56, 65, 87, 88] In a comprehensive investigation of this problem, Pierpoint,[56] using tobacco leaf *o*-diphenoloxidase, found that ascorbate inhibits the initial rate of oxidation of *o*-diphenol in the presence of excess *o*-diphenol. In incubation mixtures containing low concentrations of *o*-diphenol and ascorbate and an excess of enzyme, ascorbate only partially inhibits the rate of oxidation of *o*-diphenol, but the duration of the oxidation is prolonged due to the continuous reduction of quinone as it is formed, by ascorbate. The quinone does not accumulate and inhibition of *o*-diphenoloxidase (by its own reaction products) does not occur until the supply of ascorbate is exhausted. Large amounts of ascorbate incubated with small amounts of *o*-diphenol cause an initial inhibition of *o*-diphenoloxidase, but the inhibition is removed with time due to the oxidation of ascorbate by quinone which continues to form at a slow rate. Thus the mechanism of action of ascorbate is similar to the mechanism of action of thiols in class (1) (thioglycollate and mercaptobenzothiazole). If a vast quantity of ascorbate is included in the medium for extracting plant tissues, transitory inhibition of browning should occur, but for most purposes the amount required is impractical (compare with the high concentration of thiosulphate required to inhibit browning and protect tobacco leaf peptidase[11]). Some idea of the amount of ascorbate that might be required can be judged from the following observation; Pierpoint[56] found that 20 μmoles of ascorbate inhibited the initial rate of oxidation of 8 μmoles of chlorogenic acid by tobacco leaf *o*-diphenoloxidase but, after only 25 min, oxidation proceeded at the same rate as the initial rate of a control containing no ascorbate.

Metabisulphite and Dithionite

Browning of freshly cut tissue slices has long been known to be prevented by reducing agents other than thiols. As long ago as 1945, Bourne and co-workers[89, 90] pretreated slices of potato tubers with dithionite before extraction, though no comparison was made with extracts from control slices. Bjork[91] reported that omission of dithionite from the extracting

[84] W. S. PIERPOINT, *Biochem. J.* **71**, 518 (1959).
[85] M. A. EL-BAYOUMI and E. FRIEDEN, *J. Am. Chem. Soc.* **79**, 4854 (1957).
[86] J. K. PALMER, *Plant Physiol.* **38**, 508 (1963).
[87] P. BARUAH and T. SWAIN, *Biochem. J.* **55**, 392 (1953).
[88] G. L. FARKAS, Z. KIRALY and F. SOLYMOSY, *Virology* **12**, 408 (1960).
[89] E. J. BOURNE and S. PEAT, *J. Chem. Soc.* 877 (1945).
[90] S. A. BARKER, E. J. BOURNE and S. PEAT, *J. Chem. Soc.* 1705 (1949).
[91] W. BJORK, *Biochim. Biophys. Acta* **95**, 652 (1965).

medium used to extract endonucleases from potato tuber resulted in the formation of a dark product which absorbed the enzymes and Quastel[12] reported that dithionite prevented the inhibition of urease by various *o*-diphenols. Inhibition of *o*-diphenoloxidase by dithionite[41] is consistent with these observations.

Anderson and Rowan[11] reported that metabisulphite was more efficient than thiols for preventing inactivation of tobacco leaf peptidase by *o*-diphenoloxidase products during extraction from tobacco leaf tissue. Furthermore, the protection of peptidase against inactivation was permanent and the minimum concentration of metabisulphite required to provide maximum protection did not vary with the physiological state of the tissue nor with the amount of tissue extracted per volume of extracting medium. The reason for the superior efficiency of metabisulphate and the lack of variation in the amount required for maximum protection was attributed to complete inhibition of *o*-diphenoloxidase, but no detailed investigation has been made of the nature of the inhibition of *o*-diphenoloxidase by either metabisulphite or dithionite. Palmer[65] has suggested that dithionite is rapidly oxidized, thus depleting the assay mixture of oxygen and inhibiting *o*-diphenoloxidase.

TABLE 3. EFFECT OF INCLUDING POTASSIUM METABISULPHITE DURING EXTRACTION AND INCUBATION UPON ACTIVITY OF AMINOACYL-S-RNA SYNTHETASES FROM VARIOUS TISSUES OF FRENCH BEAN SEEDLINGS

Tissue	Concentration of metabisulphite (mM)		Enzyme protein (mg/ml)	Specific activity (units/mg protein)
	Extracting medium	Incubating medium		
Cotyledon	0	0	0·233	12·9
	0	3·5	0·233	12·8
	5	3·5	0·229	13·6
Plumule	0	0	0·253	20·4
	0	3·5	0·253	22·4
	5	3·5	0·226	38·7

Metabisulphite is as efficient as thiols for extracting nitrate reductase from tomato seedlings (E. C. Cocking, Nottingham, personal communication) and metabisulphite has been used successfully for extracting phosphofructokinase from tomato fruit (D. Chalmers and K. S. Rowan, Melbourne, personal communication). Metabisulphite increases the efficiency of extraction of aminoacyl-s-RNA synthetases from the plumule (but not the cotyledon) of french bean seedlings (Table 3) in much the same manner as thioglycollate (Table 2) and demonstrates that metabisulphite increases the efficiency of extraction of an enzyme from a species outside the family *Solanaceae*.

The use of metabisulphite for protecting plant enzymes from loss of activity during extraction by *o*-diphenoloxidase products has recently been extended to the preparation of highly coupled mitochondria from potato tuber,[3] a tissue rich in phenolics. Since the concentration of metabisulphite required to prevent accumulation of *o*-diphenoloxidase products is independent of *o*-diphenoloxidase activity and concentration of endogenous phenolics, then metabisulphite should prove useful for preparing coupled mitochondria from tissues containing very high concentrations of phenolics. (Verleur[2] has pointed out that

cysteine would probably be ineffective in such tissues.) The respiratory control and oxidative phosphorylation of mitochondria prepared with metabisulphite[3] are comparable to the best reported for plant mitochondria.[2,92–94] B. J. Miflin (Newcastle, personal communication) has found metabisulphite equally as efficient as PVP for the preparation of mitochondria from barley seedlings for studies on mitochondrial nitrate and nitrite reductases.[95] These examples suggest that metabisulphite could be a suitable reagent for extracting mitochondria and soluble enzymes from tissues containing very high concentrations of phenolics. However, collaborative work in the Botany Department, University of Melbourne (unpublished), indicates that the effect of metabisulphite on efficiency of extraction decreases if the pH exceeds 7·5.

Bovine Serum Albumin (BSA)

BSA is routinely included in media for extracting plant mitochondria[2,3,92] because it adsorbs free fatty acids which are otherwise adsorbed by and uncouple plant mitochondria.[96] Weinbach and Garbus[97,98] demonstrated that BSA powerfully binds phenolics and reverses the uncoupling effects of phenols of low MW. Watson and Smith[99] point out that the effectiveness of BSA in improving the properties of mitochondria from *Aspergillus niger* may be due to binding of phenolics in addition to binding of free fatty acids.

Mitochondria from *A. niger* appear to be less delicate than plant mitochondria, since although they are uncoupled when prepared without BSA, addition of BSA after extraction reverses the uncoupling. On the other hand, mitochondria from potato tuber, extracted and assayed with BSA in the medium, are uncoupled unless either cysteine[2] or metabisulphite[3] are included in the extracting medium. At the moment, there is no unequivocal evidence to indicate that BSA has any effect on the efficiency of extraction of mitochondria from plant tissues; the medium for extracting carrot root mitochondria has been reported to require BSA[100,101] but this needs reinvestigation with the oxygen electrode since state (4) was not demonstrated.[60] However, in studies with the oxygen electrode, Wiskich and Bonner[93] reported that mitochondrial preparations from potato tuber were more consistently active when BSA was included in the extracting medium.

Other proteins (e.g. collagen, albumin, gelatin) have been used to extract enzymes and mitochondria free from contamination by phenolics, but with limited success.[10]

Acetone

Acetone powders have been widely used for the extraction of plant enzymes, but the work of Sanderson and Roberts[102] is of special interest because they prepared peptidase from tea leaf, a tissue rich in phenolics, in the form of an acetone powder. The enzyme could not be solubilized and had to be assayed by taking samples of the powder. Sanderson and Roberts suggested that extraction of the tissue with acetone effected separation of peptidase from the

[92] H. IKUMA and W. D. BONNER, *Plant Physiol.* **42**, 67 (1967).
[93] J. T. WISKICH and W. D. BONNER, *Plant Physiol.* **38**, 594 (1963).
[94] J. T. WISKICH, R. E. YOUNG and J. B. BIALE, *Plant Physiol.* **39**, 312 (1964).
[95] B. J. MIFLIN, *Nature* **214**, 1133 (1963).
[96] L. DALGARNO and L. M. BIRT, *Biochem. J.* **87**, 586 (1963).
[97] E. C. WEINBACH and J. GARBUS, *J. Biol. Chem.* **241**, 169 (1966).
[98] E. C. WEINBACH and J. GARBUS, *J. Biol. Chem.* **241**, 3708 (1966).
[99] K. WATSON and J. E. SMITH, *Biochem. J.* **104**, 332 (1967).
[100] L. DALGARNO and L. M. BIRT, *Biochem. J.* **83**, 195 (1962).
[101] L. DALGARNO and L. M. BIRT, *Biochem. J.* **83**, 202 (1962).
[102] G. W. SANDERSON and G. R. ROBERTS, *Biochem J.* **93**, 419 (1964).

phenolics (soluble in acetone) and that acetone irreversibly precipitated peptidase without inactivating it, though Loomis and Battaile[10] noted that acetone powders of peppermint leaves browned rapidly when extracted with aqueous solutions. However, this technique would appear to be of limited use, since as Sanderson and Roberts themselves point out, it prevents an assessment of intracellular distribution of the enzyme; studies on reaction kinetics and mechanisms would not be possible until the enzyme could be solubilized.

CONCLUDING REMARKS

It is impossible to make any predictions about the suitability of the various reagents available for preventing the accumulation of products of *o*-diphenoloxidase for increasing the efficiency of extraction of individual enzymes from specific tissues. To make such predictions it is necessary to know: (1) the *o*-diphenoloxidase activity of the tissue; (2) the subcellular location of *o*-diphenoloxidase; (3) the nature and the amount of the phenolics and tannins in the tissue; (4) the mechanism of action of *o*-diphenoloxidase(s), and which of the endogenous phenolics is a substrate for the oxidase; (5) the mechanism of action of the various compounds for preventing accumulation of the products of *o*-diphenoloxidase; (6) the susceptibility of the enzyme being extracted to products of the *o*-diphenoloxidase reaction. Our knowledge of any one of these variables throughout the plant kingdom is strictly limited. In the absence of this information, the only definitive way of ascertaining which particular compound or procedure is the most suitable for preventing inactivation of enzymes during extraction from a particular tissue is to experimentally compare all the methods available.

The following recommendations are suggested as a tentative guide until more information becomes available. The reducing agents (cysteine, thioglycollate, metabisulphite, DIECA and dithionite)* have proved particularly effective for increasing efficiency of extraction of enzymes and subcellular organelles from tissues containing *o*-diphenoloxidase substrates of low MW (e.g. chlorogenic acid in tobacco leaf and potato tuber). Verleur[2] has suggested that cysteine (and presumably other thiols) would be ineffective for the preparation of coupled mitochondria from plant tissues containing very high concentrations of endogenous phenolics, but there is reason to believe that metabisulphite should be effective for preparing mitochondria from such tissues.[3] Perhaps a combination of the method of Stokes *et al.*[3] with the rapid preparation of mitochondria recommended by Palmer,[78] could prove useful for the preparation of tightly coupled mitochondria. No information is available regarding the effect of the reducing agents on the efficiency of extraction of enzymes from tissues containing condensed tannins, but since many condensed tannins are inhibitory *per se*, then reducing agents may be of little use in such tissues. Polymers, on the other hand, powerfully bind tannins and oxidation products of *o*-diphenoloxidase activity of high MW and polymers would appear to be of most use in tissues containing such compounds (e.g. unripe banana fruit, peppermint leaf and tea leaf). The ability of polymers to quantitatively bind phenolics of low MW (e.g. chlorogenic acid) at physiological pH is suspect. Polymers are ineffective in tissues containing these phenolics.

Careful application of the range of compounds now available for preventing the accumulation of products of the *o*-diphenoloxidase reaction should assist many aspects of research in

* Some workers,[103, 104] choose to include a mixture of these compounds in their extracting media.

[103] J. S. HAWKER, *Biochem. J.* **105**, 943 (1967).
[104] D. R. DILLEY, *Plant Physiol.* **41**, 214 (1966).

plant biochemistry, particularly the problem of subcellular localization of enzymes. These compounds should prove useful in deciding whether or not there is any biochemical evidence for the existence of lysosomes in plants, for although there is considerable histochemical evidence for the occurrence of lysosomes in plant tissues,[105–108] these reports are lacking biochemical substantiation.[109]

EXPERIMENTAL

The following methods were used to prepare the data reported in Tables 2 and 3:

Plant Material

French bean seedlings (*Phaseolus vulgaris* cultivar. Canadian Wonder) were grown in the dark in aerated water culture at 25° for 6 days.

[^{32}P]*Pyrophosphate* was prepared by the method of Lee Peng[110] and diluted with unlabelled pyrophosphate to adjust the specific activity to 0·25 μc/μmole.

Extraction of Tissues

Tissues were ground in a glass mortar and pestle at 2° for 4 min using 3·3 ml of extracting medium/g fresh weight of tissue. The extracting medium contained 0·1 mM tris-maleic acid-KOH buffer (pH 7·5), 20 mM $MgCl_2$ and potassium thioglycollate or potassium metabisulphite as required. The supernatant fraction was recovered by centrifugation at 25,000 *g* for 15 min and again at 125,000 *g* for 60 min. A sample of the supernatant solution was retained for estimating protein and the remainder was diluted 60-fold in the appropriate extracting medium and used as the source of enzyme.

Assay of Aminoacyl-s-RNA Synthetases

Assays were performed at 40° with incubation mixtures containing 0·7 ml enzyme solution (containing 14 μmoles $MgCl_2$ and 70 μmoles tris-maleic acid–KOH buffer, pH 7·5), 2 μmoles ATP, 2 μmoles [^{32}P]pyrophosphate, and 1 μmole each of the L-amino acids and amides commonly found in protein (except cystine). The final volume was 1·0 ml. After 15 min, 2 ml ice-cold trichloroacetic acid was added to terminate the reaction. (No protein precipitate formed.) Charcoal (50 mg), suspended in 4·5 ml of 0·1 M pyrophosphate (adjusted to pH 8 with H_3PO_4), was added and the charcoal filtered, layered onto a disc of filter paper, and counted as described by Smith and Fowden.[111] The exchange rates were calculated following the method of Davie *et al.*[112] and are expressed as the difference in the rate of exchange in mμmoles/min (aminoacyl-s-RNA synthetase units) between mixtures with and without added amino acids.

Estimation of Protein

Protein was precipitated from undiluted extracts with trichloroacetic acid (final concentration 10%, w/v) and the precipitate washed twice with acetone. The washed precipitate was extracted with 0·1 N NaOH at 50° for 2 hr and an aliquot used for estimating protein by the method of Lowry *et al* [113] using bovine serum albumin as standard.

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[105] P. B. GAHAN, *J. Exp. Botany* **16**, 350 (1965).
[106] F. P. BOUCK and J. CRONSHAW, *J. Cell. Biol.* **25**, Pt. 1, 79 (1965).
[107] A. M. FLINN and D. L. SMITH, *Planta* **75**, 10 (1967).
[108] P. B. GAHAN and A. J. MAPLE, *J. Exp. Botany* **17**, 151 (1966).
[109] M. J. C. RHODES and L. S. C. WOOLTORTON, *Phytochem.* **6**, 1 (1967).
[110] C. H. LEE PENG, *Biochem. Biophys. Acta* **22**, 42 (1956).
[111] I. K SMITH and L. FOWDEN, *Phytochem.* **7**, 1065 (1968).
[112] E. W. DAVIE, V. V. KONINGSBERGER and F. LIPMAN, *Arch. Biochem. Biophys.* **65**, 21 (1956).
[113] O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR and R. J. RANDALL, *J. Biol. Chem.* **193, 265** (1951).