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PLANT PHENOLIC COMPOUNDS AND THE ISOLATION OF PLANT ENZYMES*

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Abstract—The presence of phenolic compounds makes it impossible to isolate active enzymes by conventional techniques from many plant tissues. Phenols combine with proteins reversibly by hydrogen bonding, and irreversibly by oxidation followed by covalent condensations. The chemistry of these reactions is reviewed, and their importance in the isolation of enzymes from plants is discussed. Phenols are effectively removed from H-bonded complexes with protein by adding large amounts of substances which contain groups similar to the peptide bond. A technique is described in which insoluble polyvinylpyrrolidone is used to adsorb phenols and thus obtain active soluble enzymes. Using this technique, mevalonic kinase, phosphomevalonic kinase, glutamyl transferase and alkaline phosphatase have been demonstrated in extracts from peppermint leaves.

INTRODUCTION

THE isolation of enzymes and other cell components from any organism generally involves disruption of the tissues, with inevitable mixing of substances which in the living organism were rigidly compartmentalized. This may result in inactivation of enzymes, or in the isolation of an enzyme which, though active, is more or less modified from its native form. Plant tissues present all of the usual difficulties due to interactions of proteins with other protoplasmic constituents, such as nucleic acids and lipids, as well as to the inherent instability of proteins. In addition they present special problems.

Stahmann[1] and Pirie[2] in recent reviews cite a number of factors which make plant proteins particularly unstable and difficult to work with. Included are vacuole acids, carbohydrates, hydrolytic and oxidative enzymes, phytic acid, and tannins. Adsorption of soluble proteins by cell wall fragments has been discussed in a recent review by Newcomb.[3] Cell walls and vacuoles make up the bulk of most plant tissues, and both contain a variety of materials which can react with proteins, and which cause special problems not encountered with other organisms. Our purpose here is to consider problems caused by the plant phenolic compounds, or tannins.† In living cells these compounds are frequently associated with vacuoles,[4] but information on their localization is limited. There is a tendency to assume that tannins cause difficulty only in a few species of plants, and only because they precipitate proteins.

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† The term "tannin" has no precise chemical meaning. It is used loosely to describe plant phenolic compounds which precipitate proteins, or in a more restricted sense to designate only those plant phenolics which are capable of converting raw hide into leather. In this paper the word will be used primarily in discussing the findings of other investigators and will be used in each case in the same sense that they have used it.

[1] M. A. STAHMANN, *Ann. Rev. Plant Physiol.* **14**, 137 (1963).
[2] N. W. PIRIE, *Ann. Rev. Plant Physiol.* **10**, 33 (1959).
[3] E. H. NEWCOMB, *Ann. Rev. Plant Physiol.* **14**, 43 (1963).
[4] K. ESAU, *Plant Anatomy* (2nd Ed.). Wiley, New York (1965).

In fact, problems due to reactions between plant proteins and plant phenolic compounds are much more prevalent and much more complex than is generally recognized. Animal tissues contain few phenolic compounds other than the amino acid tyrosine, and seldom in high concentration,[5] but in plants, phenolic compounds (in addition to tyrosine) are widespread, and frequently occur in very high concentrations.[5-8]

All phenols, unless sterically hindered, take part in hydrogen bonding,[9] and the bond formed between phenols and N-substituted amides is one of the strongest types of hydrogen bond.[10] Phenol itself has been shown to form complexes with proteins,[11] nylon[12] and polyvinylpyrrolidone (PVP).*[13] In addition, many phenols are readily oxidized to quinones, which are highly reactive compounds.[9, 14, 15] Oxidation of phenols may occur non-enzymatically, or it may be catalyzed by phenol oxidases or peroxidases, enzymes which are widely distributed in plants. Quinones are oxidizing agents and may oxidize essential groups of proteins. More important, they polymerize rapidly, and in the presence of protein they also react rapidly to form covalent bonds to the protein.

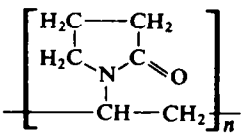
The complex reactions of phenols and quinones with proteins and related compounds have been studied in connection with several seemingly unrelated problems. An understanding of these reactions is important to any one working with plant proteins, but the literature is widely scattered, and much of it is in specialized publications which may not be readily accessible to plant biochemists. We would like to review this literature briefly and also describe an improved technique which we have developed for isolating soluble enzymes from plants which contain phenolic compounds. We hope that the review will not only explain our own results but also provide a basic understanding of the processes involved, thus aiding in the further development of methods for working with plant proteins.

Chemically the plant phenolic compounds are extremely heterogeneous, ranging from simple monomers to very large polymers. In many cases the structures are still unknown. However, most of them belong to one of two biochemical groups:[16] the flavonoid compounds (including the condensed tannins), or the group of C_6–C_3 and C_6–C_1 compounds and their derivatives (such as caffeic acid and gallic acid, and including the hydrolyzable tannins as well as tyrosine and lignin). Compounds of the first type generally contain only phenolic hydroxyl groups as reactive centers. Compounds of the second type commonly contain also carboxyl groups, either free or esterified. In both types of compounds it is usual for some of the hydroxyl groups to be substituted.

* *Abbreviations used*: PVP, polyvinylpyrrolidone; ADP, adenosine diphosphate; ATP, adenosine triphosphate.

[5] J. B. Harborne and N. W. Simmonds, In *Biochemistry of Phenolic Compounds* (Edited by J. B. Harborne), p. 77. Academic Press, London (1964).
[6] J. B. Harborne, In *Biochemistry of Phenolic Compounds* (Edited by J. B. Harborne), p. 129. Academic Press, London (1964).
[7] R. E. Alston and B. L. Turner, *Biochemical Systematics*. Prentice-Hall, Englewood Cliffs, N.J. (1963).
[8] E. C. Bate-Smith, In *Chemical Plant Taxonomy* (Edited by T. Swain), p. 127. Academic Press, London (1963).
[9] R. H. Thomson, In *Biochemistry of Phenolic Compounds* (Edited by J. B. Harborne), p. 1. Academic Press, London (1964).
[10] M. St. C. Flett, *J. Soc. Dyers Colourists* **68**, 59 (1952).
[11] K. H. Gustavson, *The Chemistry and Reactivity of Collagen*. Academic Press, New York (1956).
[12] H. Endres and H. Hörmann, *Angew. Chem.* **75**, 288 (1963).
[13] D. Guttmann and T. Higuchi, *J. Am. Pharm. Assoc., Sci. Edit.*, **45**, 659 (1956).
[14] H. S. Mason, *Advan. Enzymol.* **16**, 105 (1955).
[15] H. S. Mason, In *Pigment Cell Biology* (Edited by M. Gordon), p. 563. Academic Press, New York (1959).
[16] T. Robinson, *The Organic Constituents of Higher Plants*. Burgess, Minneapolis (1963).

Experiments on the tanning of modified collagen[17] and of synthetic polymers[17, 18-22] have established the importance of the peptide or amide linkage in the formation of H-bonded complexes between tannins and protein. Grassmann and coworkers demonstrated in 1937 (see Gustavson[17]) that water-soluble urea-formaldehyde condensation products, containing —CO—NH— as the only reactive group, precipitate tannins from solution. More recently Batzer and Weissenberger[18, 19] and Gustavson and Holm[20, 21] showed that hydrated nylon precipitates tannins. Further investigations of Gustavson established that both soluble[22] and insoluble[23] polyvinylpyrrolidone form stable insoluble complexes with tannins. Hide



Polyvinylpyrrolidone

powder, hydrated nylon and insoluble PVP all have nearly the same capacity for binding tannins. Under the conditions of Gustavson's experiments[23] the amounts of several types of tannins bound ranged from 31 to 44 per cent of the dry weight of the substrate polymers. Tannins are partially removed from all of these complexes by 6–8 M urea. Dilute alkali, or aqueous organic solvents which are capable of H-bonding, such as alcohols or acetone, also de-tan,[17, 23] but in certain cases they dissolve the substrate as well, so that general comparisons cannot be made as they can with urea. From the tanning experiments it is clear that only the —CO—N< group is required for the formation of complexes with vegetable tannins. It was concluded that tannins form H-bonds with the peptide linkages, probably through the peptide oxygen, and with the tannins furnishing the hydrogen. The two types of tannins show very different pH responses in these experiments.[17, 23] Condensed tannins are bound almost independently of pH below 7–8, except for an indirect effect due to the swelling of collagen at pH 3 or below. The binding decreases rapidly above pH 8. Hydrolyzable tannins on the other hand are bound very strongly at pH 3–4 (80 per cent of the weight of substrate), but the binding decreases above pH 5. At pH 6 the amount bound is approximately 25 per cent of that bound at pH 3, and at pH 7·5, only 8–10 per cent.

In the case of condensed tannins the pH effects indicate that the binding involves un-ionized phenolic hydroxyl groups. It seems clear that these groups react to form H-bonds with the substituted amide groupings of the protein or synthetic polymer. In the case of hydrolyzable tannins the pH effects suggest that strong H-bonds are formed by un-ionized carboxyl groups of the tannins, and weaker H-bonds by un-ionized phenolic hydroxyl groups. Hydrolyzable tannins are predominantly derivatives of pyrogallol (1,2,3-trihydroxybenzene) and catechol (*o*-dihydroxybenzene). Chromatography experiments have shown that such phenols, as a result of internal H-bonding, have relatively low affinity for polyamide.

Investigations of the chromatography of phenols and related compounds on polyamide powder (principally nylon 6) have yielded valuable information about the types of interactions

[17] K. H. Gustavson, *The Chemistry of Tanning Processes*. Academic Press, New York (1956).
[18] H. Batzer and G. Weissenberger, *Makromol. Chem.* **7**, 320 (1952).
[19] H. Batzer, *Makromol. Chem.* **8**, 183 (1952).
[20] K. H. Gustavson and B. Holm, *J. Am. Leather Chemists' Assoc.* **47**, 700 (1952).
[21] K. H. Gustavson, *J. Polymer Sci.* **12**, 317 (1954).
[22] K. H. Gustavson, *Svensk Kem. Tidskr.* **66**, 359 (1954).
[23] K. H. Gustavson, *Leder* **14**, 27 (1963).

involved. This work has been reviewed recently by Endres and Hörmann[12] and by Egger.[24] Phenolic compounds in general are adsorbed on polyamides by hydrogen bonding. The affinity increases (R_f decreases) with an increase in the number of phenolic hydroxyl groups on the molecule, except when the hydroxyl groups are ortho to each other or otherwise favorably arranged to allow internal H-bonding. In this case, additional hydroxyl groups decrease the affinity for polyamide. For example, phloroglucinol (1,3,5-trihydroxybenzene) has an R_f of 0·07 with water as the developing solvent, while pyrogallol (1,2,3-trihydroxybenzene) has an R_f of 0·36 and phenol has an R_f of 0·21.[12] The eluting strength of solvents increases in the order: water, ethanol, methanol, acetone, dilute NaOH, formamide, dimethylformamide. Surprisingly, tyrosine migrates with the solvent front, having very little affinity for the polyamide. This is not the only instance of anomalous behaviour on the part of tyrosine. Tyrosine is much less soluble in water than are other amino acids, including phenylalanine.[25, 26] The phenolic hydroxy group seemingly reduces the polarity of tyrosine, rather than increasing it as one would expect. The water solubility of tyrosine is greatly increased in the presence of urea.[26, 27] In addition, the sublimation temperature of tyrosine is considerably higher than that of phenylalanine and most other amino acids.[25] These observations apparently have not been explained, but they suggest unique interactions between the phenol portion and the alanine portion of tyrosine.

Brown and Wright[28] have investigated interactions between milk proteins and tea polyphenols by means of electrophoresis. They found that tea infusion drastically altered the electrophoretic mobilities of milk proteins. In the presence of 7 M urea, tea infusion had no influence on protein mobility. The investigators concluded that protein–polyphenol complexes are formed and that the interactions are due, at least initially, to hydrogen bonding. Further evidence of complexing was obtained during attempts to separate the colored tea polyphenols by gel filtration. The colored material of tea extracts would not move with water on a Sephadex column and was only eluted on addition of 0·1 N NaOH. When casein was added to the tea infusion before putting it on the column the brown color passed straight through the column with the protein.

Although large amounts of phenolic substances can be bound to protein by hydrogen bonding there have been many indications that other, more stable, bonds are also formed, and that they are formed very rapidly.[17] It now appears that these stable bonds result from oxidation of phenols to quinones, and copolymerization of the quinones with protein. The enzymatic oxidation of phenols, and the reactions of quinones with proteins, have been reviewed by Mason,[14, 15, 29] by Yasunobu,[30] and by Bouchilloux.[31] Gustavson[17] has also reviewed quinone–protein reactions. Mason's 1955 review[14] is especially comprehensive. Evidence is cited for the covalent bonding of quinones to proteins by 1,4-addition of sulfhydryl groups and the imino group of proline, in addition to free amino groups. Sulfhydryl groups and terminal α-amino groups react much more readily than do the ϵ-amino groups of lysine. N-terminal proline also reacts rapidly. The initial product is a catechol- or quinol–protein

[24] K. Egger, *Planta Med.* **12**, 265 (1964).
[25] J. P. Greenstein and M. Winitz, *Chemistry of the Amino Acids*, Vol. 1, p. 565. Wiley, New York (1961).
[26] Y. Nozaki and C. Tanford, *J. Biol. Chem.* **238**, 4074 (1963).
[27] P. L. Whitney and C. Tanford, *J. Biol. Chem.* **237**, PC 1735 (1962).
[28] P. J. Brown and W. B. Wright, *J. Chromatog.* **11**, 504 (1963).
[29] H. S. Mason, *Nature* **175**, 771 (1955).
[30] K. T. Yasunobu, In *Pigment Cell Biology* (Edited by M. Gordon), p. 583. Academic Press, New York (1959).
[31] S. Bouchilloux, In *Plant Phenolics and their Industrial Significance.* (Edited by V. C. Runeckles), p. 1 1962 Symposium Plant Phenolics Group of North Americia (1963).

compound, which is readily oxidized by free quinone to the corresponding quinone–protein. Quinones with a second reactive ring position available can react with a second reactive group of protein, resulting in cross-linking.

The hardening of cuticle and related structures of invertebrates has been shown in several cases to be due to quinone tanning of proteins, the quinones arising by enzymatic oxidation of phenols. Much of the available fundamental knowledge of quinone–protein reactions is due to investigations of these tanning processes.[14, 17, 32, 33]

Recently the importance of quinones in vegetable tanning has been emphasized.[23, 34] Endres[34] concludes that the essential reaction of vegetable tanning is 1,4-addition of free amino groups of the protein to bifunctional low molecular weight quinones formed by oxidation of phenols, resulting in covalent cross-linkage. Quinones are bound irreversibly on polyamide (nylon 6).[12, 35] Analyses showed slightly less than one mole of quinone bound per two moles of free amino groups of the polyamide, and blocking the amino groups completely removed the affinity for quinones. This was taken as evidence for successive 1,4-additions of two terminal amino groups of the polyamide to each quinone molecule.

Wood and Ingraham[36] have shown that when phenol oxidase oxidizes ^{14}C-phenol, in the presence of oxygen and ascorbate, radioactive material is bound irreversibly to the enzyme. Quinone addition reactions are thought to be responsible.

When beer ages, or is exposed to oxidizing conditions, a precipitate frequently appears which is referred to as "haze". Chemical analyses have shown that beer hazes contain proteins, "tannins", and in some cases heavy metals.[37, 38]

The beer haze problem has been investigated intensively in the past few years, with particular emphasis on a search for agents which would specifically remove phenolic compounds from beer. Soluble PVP,[38] nylon 66 and nylon 6,[39] and keratin[40] were all used with considerable success by different investigators. McFarlane and Bayne[41] tested several types of cross-linked insoluble PVP, and found that one of these, originally designated "Agent AT-496" was particularly promising. Compared with nylon, the new polymer removed more of the haze precursors and fewer other substances. It had the obvious advantage over soluble PVP that it could be used in excess without leaving any soluble residue in the beer. Agent AT-496 was later referred to simply as "Agent AT", and it is now available commercially as "Polyclar AT". In subsequent investigations McFarlane and coworkers have studied the binding of various flavonoid phenolic compounds by Polyclar AT and have used it for analysis of polyphenols,[42–45] as well as for stabilizing beer. For colorimetric analysis, beer polyphenols are adsorbed on Polyclar AT and then eluted with *N*-methyl-2-pyrrolidone.[42]

[32] D. GILMOUR, *The Biochemistry of Insects*. Academic Press, New York (1961).
[33] R. H. HACKMAN, In *The Physiology of Insecta* (Edited by M. ROCKSTEIN), Vol. 3, p. 471. Academic Press, New York (1964).
[34] H. ENDRES, *Leder* **12**, 294 (1961). From *Chem. Abstr.* **56**, 13062e (1962).
[35] H. ENDRES, *Z. Anal. Chem.* **181**, 331 (1961).
[36] B. J. B. WOOD and L. L. INGRAHAM, *Nature* **205**, 291 (1965).
[37] W. I. BENGOUGH and G. HARRIS, *J. Inst. Brewing* **61**, 134 (1955).
[38] W. D. MCFARLANE, E. WYE and H. L. GRANT, *European Brewery Conv., Proc. Congr., 5th Baden-Baden*, p. 298 (1955).
[39] G. HARRIS and R. W. RICKETTS, *European Brewery Conv., Proc. Congr., 7th Rome*, p. 290 (1959).
[40] R. VAN CRAENENBROECK and R. LONTIE, *European Brewery Conv., Proc. Congr., 9th Brussels*, p. 513 (1963).
[41] W. D. MCFARLANE and P. D. BAYNE, *European Brewery Conv., Proc. Congr., 8th Vienna*, p. 278 (1961).
[42] W. D. MCFARLANE, *J. Inst. Brewing* **67**, 502 (1961).
[43] W. D. MCFARLANE and M. J. VADER, *J. Inst. Brewing* **68**, 254 (1962).
[44] W. D. MCFARLANE and P. T. SWORD, *J. Inst. Brewing* **68**, 344 (1962).
[45] W. D. MCFARLANE, P. T. SWORD and G. BLINOFF, *European Brewery Conv., Proc. Congr., 9th Brussels*, p. 174 (1963).

Chapon *et al.*[46-48] have studied the formation of beer hazes, and changes in their solubility, and suggest that their findings apply generally to work with plant proteins.[46] Freshly formed beer haze dissolved rapidly and almost completely on the addition of PVP of molecular weight 40,000, while if the haze was allowed to age, its solubility in PVP decreased greatly. PVP 40,000 (non-dialyzable) had the additional effect of converting dialyzable phenolic substances of beer into soluble non-dialyzable complexes. It was shown[48] that a number of purified proteins and polyphenols can act as precursors of precipitates which are essentially the same as natural beer hazes. Among the factors which influence haze formation, ionic strength was found to be important. Haze formation was greatly accelerated at ionic strengths of less than 0·08.[46]

Interference by phenolic compounds in the isolation of plant enzymes has been noted by several investigators, and in some cases methods have been devised for overcoming the inhibition.

Quastel[49] in 1932 found that certain polyhydric phenols inhibited urease very strongly, and that thiols could prevent the inhibition. Evidence was obtained which indicated that the actual inhibitors were quinones and that even traces of quinones were extremely inhibitory. Difficulty in assaying enzymes of *Kalanchoë blossfeldiana* led Ehrenberg[50] to investigate the inhibition of phosphatases by tannins extracted from *Kalanchoë*. Phosphatase from *Phaseolus multiflorus* was strongly inhibited by the *Kalanchoë* tannins. This inhibition was reversed by the addition of hide powder, about 10 mg of hide powder per mg of tannin being required for complete reversal. The phosphatase activity of suspensions of leaf powder of *Kalanchoë* was increased as much as 150 per cent by the addition of lightly chromed hide powder. (Chrome tanning of hide protein increases its capacity to take up vegetable tannins.[17]) Addition of soluble proteins was said to reverse the inhibition of the *Phaseolus* phosphatase but to have no effect on the activity of the *Kalanchoë* enzyme.

Friedrich[51] showed that β-glucosidase from bitter almonds was strongly inhibited by addition of tannic acid, and that the inhibition could be reversed by addition of lightly chromed hide powder.

Forsyth and coworkers[52] measured the solubility and activity of several enzymes during the curing of cacao beans. They prepared acetone powders of the beans and extracted the powders several times with aqueous acetone, a procedure which should remove phenolic material that is not covalently bonded. As the curing progressed the acetone powders obtained became browner and contained increasing amounts of phenolic materials. At the same time the enzymes (and all other proteins) became insoluble in water. The enzymes retained a small amount of activity even after they had become completely insoluble.

Interactions of phenolic compounds with proteins are important in the curing of several plant products. Forsyth[53] has reviewed the physiological aspects of the curing of tea, cacao and tobacco, with special emphasis on the role of phenols and quinones, and their reactions with proteins.

Hathway and Seakins[54] showed that pectinase was inhibited by the addition of tannins

[46] L. Chapon, B. Chollot and E. Urion, *Bull. Soc. Chim. Biol.* **43**, 429 (1961).
[47] L. Chapon, B. Chollot and E. Urion, *European Brewery Conv., Proc. Congr., 8th Vienna*, p. 319 (1961).
[48] B. Chollot, L. Chapon, and E. Urion, *European Brewery Conv., Proc. Congr., 8th Vienna*, p. 334 (1961).
[49] J. H. Quastel, *Biochem. J.* **27**, 1116 (1933).
[50] M. Ehrenberg, *Biochem. Z.* **325**, 102 (1954).
[51] H. Friedrich, *Arch. Pharm.* **288**, 583 (1955).
[52] W. G. C. Forsyth, V. C. Quesnel and J. B. Roberts, *J. Sci. Food Agri.* **9**, 181 (1958).
[53] W. G. C. Forsyth, *Ann. Rev. Plant Physiol.* **15**, 443 (1964).
[54] D. E. Hathway and J. W. T. Seakins, *Biochem. J.* **70**, 158 (1958).

and that the enzyme could be partially recovered from the enzyme–tannin complex by precipitation with 80 % acetone. Porter and Schwartz[55] isolated a water soluble substance from grape leaves which had been shown to inhibit pectinase and cellulase and found it to be a tannin. The inhibitor could be removed from extracts by hide powder, gelatine, caffeine, or nicotine sulfate. The same group[56] screened extracts from leaves of sixty-one plant species for ability to inhibit enzymes. Extracts from twenty-nine species inhibited pectinase, and extracts from fourteen inhibited cellulase.

Schwimmer[57] found that ethanolic extracts of potato tubers contain substances, apparently phenolic, which inhibit potato phosphorylase. Boser[58, 59] studied the inhibition of malic dehydrogenase and glucose-6-phosphate dehydrogenase by known phenolic compounds, especially flavonoids. Several of these compounds were potent inhibitors at a concentration of 3×10^{-4} M, and in some cases the inhibition was partly or completely reversed by addition of serum proteins. Of the compounds tested, anthocyanins were among the most effective enzyme inhibitors. Catechin and epicatechin did not in themselves inhibit, but they yielded "acid condensates" which were very inhibitory.

Goldstein and Swain[60] have studied the inhibition of several soluble enzymes by a purified hydrolyzable tannin and by a condensed tannin extract. The tannins precipitated all of the enzymes, but most of the precipitated enzymes retained some activity. Soluble enzyme activity could be obtained from the precipitates with varying degrees of success by addition of borate, caffeine, polyethylene glycol, polyvinyl alcohol, methyl cellulose, non-ionic or cationic detergents, or soluble PVP. In almost all cases PVP was the most effective.

Phenol oxidase of tea leaves has been regarded as a particulate enzyme and has proven difficult to isolate in soluble form. However, Sanderson[61] showed recently that it is completely soluble if a large quantity of powdered nylon is added during the extraction of the tissue.

The isolation of active mitochondria from plant tissues has been hampered in several cases by the presence of phenolic compounds. Tager[62] was unable to obtain mitochondria or soluble protein from banana pulp by conventional techniques. He suggested that tannins were responsible. When tripotassium phosphate was added to the isolation medium, mitochondria and soluble proteins could be extracted, but the mitochondria were extensively damaged by the high pH. Addition of egg albumen made it possible to isolate active mitochondria. Recently Badran and Jones[63] have obtained soluble phenol oxidase from bananas by precipitating tannins with polyethylene glycol.

Lieberman[64] isolated active mitochondria from apple fruit by removing the peel (which is especially rich in phenolic compounds) and homogenizing the pulp at a pH of 8·4 to 9·9 with ascorbic acid added. Particles isolated at lower pH values were not active.

Jones and Hulme[65] obtained highly active mitochondria from the peel of apples by adding soluble PVP to the extraction medium to bind phenolic compounds. Subsequently Hulme, Jones and Wooltorton have used this technique very successfully in further investiga-

[55] W. L. Porter and J. H. Schwartz, *J. Food Sci.* **27**, 416 (1962).
[56] T. A. Bell, J. L. Etchells, C. F. Williams and W. L. Porter, *Botan. Gaz.* **123**, 220 (1962).
[57] S. Schwimmer, *J. Biol. Chem.* **232**, 715 (1958).
[58] H. Boser, *Hoppe-Seyler's Z. Physiol. Chem.* **316**, 284 (1959).
[59] H. Boser, *Planta Med.* **9**, 456 (1961).
[60] J. L. Goldstein and T. Swain, *Phytochem.* **4**, 185 (1965).
[61] G. W. Sanderson, *Biochem. Biophys. Acta* **92**, 622 (1964).
[62] J. M. Tager, *South African J. Sci.* **54**, 324 (1958).
[63] A. N. Badran and D. E. Jones, *Nature* **206**, 622 (1965).
[64] M. Lieberman, *Plant Physiol.* **35**, 796 (1960).
[65] J. D. Jones and A. C. Hulme, *Nature* **191**, 370 (1961).

tions of mitochondria from apples[66-70] and rose petals.[71] They also found[67] that use of soluble PVP greatly increased the activity of soluble malic enzyme and pyruvic carboxylase in extracts obtained from apple fruit. References 68 and 69 include detailed discussions of their findings. They found that ordinary PVP contained impurities which made it ineffective; pharmaceutical grade PVP was required. Best results were obtained when the pH of the homogenate was 7 to 7·5. They found also that merely preventing the oxidation of phenolic compounds did little good; it was necessary to remove them.

Barber and Hassid[71a] found that addition of soluble PVP made it possible to isolate active cellulose-synthesizing particles from the cotton boll.

Phenolic compounds also make it difficult to isolate viruses from many species of plants. Thresh[72] was able to restore infectivity in virus-phenolic mixtures by dilution, or by addition of pH 8 buffer, nicotine sulfate, or alumina. Cadman[73] has reported similar findings. Brunt and Kenten[74] found that infective virus could be extracted from cocoa leaves only if excess protein was added to the medium. Either soluble protein or hide powder was effective, but the authors preferred hide powder because of the ease of separating it from the extract. Their data suggest that the amount of protein relative to the leaf weight is more important than the concentration of protein in the extracting fluid.

Precipitation with tannins has been reported as a technique for purifying or concentrating enzymes and other proteins. Acetone[75] or caffeine[76] has been used to recover the protein from the tannin complex.

RESULTS

Preliminary Experiments

In connection with investigations of monoterpene biosynthesis we attempted to obtain active enzymes from leaves of peppermint and other monoterpene-producing plants. Extracts prepared from peppermint leaves by conventional techniques browned rapidly, and no active enzyme, other than phenol oxidase, could be found in them. It was clear that the plant tissues contained enzyme inhibitors, but the nature of these inhibitors was not known. It seemed likely that they were phenols, quinones formed by phenol oxidase activity, or organic acids. Many extraction and purification procedures were tested, most of them designed to remove or neutralize these compounds and to prevent oxidation. Techniques tested included the use of large amounts of buffer; addition of reducing agents, cyanide, and metal chelating agents; decolorizing with charcoal; dialysis and gel filtration to remove compounds of low molecular weight; addition of polyethylene glycol, soluble PVP, or albumen. These techniques were

[66] A. C. HULME and L. S. C. WOOLTORTON, *Nature* **196**, 388 (1962).
[67] A. C. HULME, J. D. JONES and L. S. C. WOOLTORTON, *Proc. Roy. Soc. (London) Ser. B* **158**, 514 (1963).
[68] A. C. HULME and J. D. JONES, In *Enzyme Chemistry of Phenolic Compounds* (Edited by J. B. PRIDHAM), p. 97. Pergamon Press, Oxford (1963).
[69] A. C. HULME, J. D. JONES and L. S. C. WOOLTORTON, *Phytochem.* **3**, 173 (1964).
[70] J. D. JONES, A. C. HULME and L. S. C. WOOLTORTON, *Phytochem.* **3**, 201 (1964).
[71] A. C. HULME, J. D. JONES and L. S. C. WOOLTORTON, *Nature* **201**, 795 (1964).
[71a] G. A. BARBER and W. Z. HASSID, *Nature* **207**, 295 (1965).
[72] J. M. THRESH, *Ann. Appl. Biol.* **44**, 608 (1956).
[73] C. H. CADMAN, *J. Gen. Microbiol.* **20**, 113 (1959).
[74] A. A. BRUNT and R. H. KENTEN, *Virology* **19**, 388 (1963).
[75] R. BENTLEY, In *Methods in Enzymology* (Edited by S. P. COLOWICK and N. O. KAPLAN), Vol. 1, p. 340. Academic Press, New York (1955).
[76] W. MEJBAUM-KATZENELLENBOGEN, W. DOBRYSZYCKA, J. BOGUSLAWSKA-JAWORSKA and B. MORAWIECKA, *Nature* **184**, 1799 (1959).

tested both on fresh tissues and on acetone-dried tissues, using glutamyl transferase[77] and mevalonic kinase[78] as test systems in most cases. Some of the procedures were helpful with other species, but none were effective with peppermint. It was possible to prevent peppermint extracts from browning, but none of the extracts had any convincing amount of enzyme activity. Extracts prepared with albumen became considerably browner than other extracts, suggesting that the albumen formed soluble complexes with the browning products.

It was found that addition of insoluble proteins (collagen or hide powder), plus buffer and sodium ascorbate, during the homogenization of peppermint leaves, yielded extracts which did not brown rapidly, and in which several active enzymes could be detected. An extract prepared with 0·2 g of collagen per g of fresh tissue was shown to have mevalonic kinase activity, and the same extract after gel filtration on Sephadex G-50 showed weak glutamyl transferase activity. An extract prepared with 0·2 g of "purified" hide powder per g of tissue, and fractionated by gel filtration on Sephadex G-50, was shown to contain glucose-6-phosphate dehydrogenase as well as weak glutamyl transferase activity. The solid residue from each of these extraction procedures turned deep brown on standing in air. The extracts themselves browned slowly.

It was evident that the insoluble proteins had removed substances which were strongly inhibitory to the peppermint enzymes; it seemed likely that the inhibitors were the phenolic compounds which are the substrates of the browning reaction. It was also clear that the substances did not need to be oxidized in order to be inhibitory. Since the use of foreign protein, even such presumably inert proteins as collagen and hide powder, introduces a possibility of contaminating the extract, a synthetic polymer seemed preferable. Insoluble PVP (Polyclar AT) was tested and found to be very effective.

Experiments with Insoluble PVP

Preliminary experiments showed that Polyclar AT adsorbed both browning substrates and browning products from peppermint extracts. To determine the amount of Polyclar required, peppermint leaves were extracted with water in the presence of 0·5, 1·0, 1·25 and 1·5 g of the polymer per g of fresh tissue. With 0·5 g the extract was somewhat brown; with 1·0 g the extract was initially clear but there seemed to be very slight browning after 16 hr at room temperature. With 1·25 or 1·5 g, the extract was clear and remained clear.

The extraction procedure which was then devised consisted of washing the leaves with distilled water and grinding them in a mortar with liquid nitrogen, adding 1·5 g of Polyclar for each g of fresh tissue, and mixing thoroughly by continued grinding. A solution of buffer and sodium ascorbate was added slowly so that it froze, and was mixed by further grinding. The mixture was then thawed, squeezed through bolting silk, and centrifuged. The extract was further purified by gel filtration on Sephadex G-50. This extract contained mevalonic kinase and also phosphomevalonic kinase. From 62 mμmoles of (±) mevalonic acid, 15 mμmoles of phosphomevalonate and 1 mμmole pyrophosphomevalonate were formed in 165 min. at 37°. Thirty per cent of the mevalonic acid, or 60 per cent of the physiologically active (+) isomer, was phosphorylated.

On further investigation we found that variable amounts of soluble PVP are formed from Polyclar AT either by grinding in a mortar or by homogenizing in a blendor type of homogenizer. The amounts formed represent a very small fraction of the Polyclar (on the order of 0·02 per cent) but nevertheless are a substantial contaminant to the small amounts of protein

[77] W. D. Loomis, *Plant Physiol.* **34**, 541 (1959).
[78] W. D. Loomis and J. Battaile, *Biochim. Biophys. Acta* **67**, 54 (1963).

extracted from the plant tissues. The soluble PVP formed in this way was precipitated, at least in part, by trichloroacetic acid, but the precipitate frequently redissolved. To avoid contamination of the extracts by PVP, the procedure was modified. Polyclar, buffer and sodium ascorbate are now mixed to form a thin paste, and chilled in an ice bath. Preferably this is allowed to stand for several hours so that the Polyclar is fully hydrated. The washed plant tissue is then ground with liquid nitrogen in a mortar, and the frozen powder is added to the Polyclar–buffer–ascorbate slurry and stirred in gently. The liquid is expressed by squeezing through bolting silk, and the residue is extracted again with ice-cold, glass-distilled water. Most of the solid material is removed by the bolting silk; what remains is precipitated by centrifuging.

In order to test the extraction procedure, five extracts were made, with and without Polyclar and with and without reducing agents. A large amount of peppermint leaf tissue was ground in liquid nitrogen, and the frozen powder was divided into equal samples by measuring with a 5 ml beaker. The samples were extracted with various combinations of buffer, Polyclar AT, ascorbate and 2-mercaptoethanol, as indicated in Table 1. A portion of each extract was fractionated on Bio-Gel P-10 polyacrylamide gel to remove substances of low molecular weight. Ten fractions were thus obtained: five original extracts and the high-molecular-weight fraction from gel-filtration of each extract. These fractions were analyzed for protein and for the enzymes mevalonic kinase, alkaline phosphatase, and glutamyl transferase. The results, except for glutamyl transferase activities (which were consistently very low are shown in Table 1. Due to differences in the volumes of the extracts the amount of fresh

Table 1. Protein content and enzyme activities of extracts from peppermint leaves

Extract	Extraction additions*	Protein in extract (mg/g fresh tissue)	Mevalonic kinase activity†	Alkaline phosphatase activity‡
crude				
1	B, A	4·8	0·9	0
2	B, P	5·6	0·2	40
3	B, A, P	7·0	3·2	40
4	B, M, P	7·5	0	0
5	B, M	2·6	0·5	0
Gel filtered				
1G	B, A	1·8	0·2	35
2G	B, P	6·2	0	55
3G	B, A, P	4·9	2·7	75
4G	B, M, P	3·8	0	47
5G	B, M	1·9	0·4	11

* B = buffer; P = Polyclar AT; A = sodium ascorbate; M = mercaptoethanol.

† mμmoles of mevalonate phosphorylated per 10 mg fresh tissue in 180 min. The reaction mixture contained: buffer, Tris plus maleate, 16 μmoles of each, KOH to give pH 6·2; $MnSO_4$, 0·5 μmole; ATP, 2 μmoles; mevalonic acid, 30 mμmoles (100 mμc); enzyme extract, 0·1 ml for crude extracts, 0·15 ml for gel-filtered extracts. Total volume, 0·16 ml. for crude extracts, 0·21 ml for gel-filtered extracts. Temperature 37°.

‡ μmoles of *p*-nitrophenyl phosphate hydrolyzed per 10 mg fresh tissue in 50 min. The reaction mixture contained: buffer, Tris, 3·0 m-moles, acetic acid to give pH 8·0; *p*-nitrophenyl phosphate, 3 μmoles; enzyme extract 0·1 ml in all cases. Total volume, 3·1 ml. Temperature, 25°. Readings at 10 min intervals showed the reaction rates were constant.

tissue represented in each assay ranged from 7·9 to 12·9 mg. For ease of comparison, all results are expressed in terms of 10 mg of fresh tissue.

It is not clear whether the consistently low glutamyl transferase activity of peppermint extracts is due to an actual low level of the enzyme, or to inadequacy of the extraction and assay techniques. However, the extraction procedures definitely removed substances which would interfere with the colorimetric determination of hydroxamic acid in the transferase assay. Extracts 1 and 5 turned pink on addition of trichloroacetic acid, before the addition of ferric chloride. These same extracts were intensely yellow at pH 8. None of the other extracts behaved in this way, indicating that the substances responsible for the colours could be removed either by gel filtration or by adsorption on Polyclar AT.

The alkaline phosphatase assay was repeated later, after the extracts had been stored for 3 months in the freezer, with occasional thawing. The results agreed generally with the initial assay, except for a striking decrease of activity in extract number 3. It would appear that the 0·25 M ascorbate damaged proteins when left over such a long period of time. The same concentration of mercaptoethanol was apparently harmful even in the hour or so required for preparing the extracts. This may be due to exchange reactions which would disrupt disulfide bridges of the protein.

Preliminary evidence indicates that peppermint extracts prepared with Polyclar AT contain an enzyme or enzymes which act on geraniol. When ^{14}C-geraniol is incubated with the extract plus ATP a labeled water, soluble product is formed. From the ATP requirement, and from R_f values in paper chromatography,[79] it appears that the new compound may be geranyl pyrophosphate. Extracts which have been heated do not form the compound.

TABLE 2. EFFECT OF POLYCLAR AT AND *N*-METHYLPYRROLIDONE ON GLUTAMYL TRANSFERASE ACTIVITY OF A PUMPKIN SEEDLING ACETONE POWDER

Additives		Hydroxamate formed (μmoles)
Polyclar (mg)	Methyl-pyrrolidone (mg)	
0	0	4·5
0	103 (0·1 ml)	4·3
1	0	4·0
2	0	3·7
5	0	4·0
10	0	3·6
20	0	3·6
50	0	3·4
100	0	3·8

The reaction mixture contained: buffer, potassium maleate, pH 6·4, 100 μmoles; glutamine, 17 μmoles; hydroxylamine hydrochloride (neutralized with $NAHCO_3$), 10 μmoles; $MnSO_4$, 1 μmole; Na_2HAsO_4, 20 μmoles; ADP, 0·2 μmoles; Polyclar AT and methylpyrrolidone as indicated. Total volume, before adding Polyclar or methylpyrrolidone, 2·0 ml. Enzyme, 10 mg of a pumpkin seedling acetone powder (equivalent to 130 mg fresh tissue). Reaction time, 180 min; temperature, 35°.

[79] F. CRAMER, W. RITTERSDORF and W. BÖHM, *Ann. Chem.* **654**, 180 (1962).

As a general test for possible enzyme inhibition, pumpkin seedling glutamyl transferase was assayed in the presence of varying amounts of Polyclar AT, and also of *N*-methylpyrrolidone. The results are shown in Table 2. It is clear that neither reagent has any great inhibitory action on this enzyme, even when added in large amounts. The apparent reduction of about 10 per cent caused by Polyclar AT is probably due to removal of phenolic compounds which form interferring colored complexes with ferric ion. Polyclar treatment of pumpkin extracts causes a decrease in light absorption in the 270 mμ region, as well as a slight reduction in the visible color formed when $FeCl_3$ is added.

In preliminary experiments we have found that addition of Polyclar AT makes it possible to extract soluble proteins from apple fruit and from Canada thistle leaves (*Cirsium arvense*). Without Polyclar no protein could be extracted from apples, and very little from thistle leaves.

DISCUSSION

Phenolic compounds combine with proteins reversibly by hydrogen bonding, and irreversibly by oxidation followed by covalent condensations. Techniques for isolating enzymes from plants which contain phenolic compounds should then specifically separate the phenols from the proteins, and at the same time prevent oxidation of the phenols.

Those phenols which form H-bonded complexes with protein are effectively removed by adding large amounts of substances which contain groups similar to the peptide linkage. To date, the most satisfactory agents have been various grades of PVP, soluble PVP for mitochondria, and insoluble PVP for soluble enzymes. Proteins and synthetic polyamides have also been used successfully.

It is important to recognize, as Gustavson[20, 21] has emphasized, that the adsorption of phenols from dilute solution by polyamides involves free, or accessible, amide groups. In ordinary nylon most of the amide groups are tied up by internal hydrogen bonding. Polyamides synthesized from mixtures of monomers have irregular structure and, as a result, reduced internal hydrogen bonding. Methylation of the amide nitrogen atom has a similar effect.[80, 81] Batzer and Weissenberger[18, 19] and Gustavson and Holm[20, 21] used a mixed polymer nylon for their tanning experiments. They also hydrated the polymer by dissolving it in hot methanol and pouring it into cold water, thus making the amide groups accessible. Both groups of investigators found that the unmodified polymer as obtained from the manufacturer adsorbed very little tannin.

In PVP there is no possibility of internal H-bonding; the polymer is a strong H-acceptor but cannot act as an H-donor. This undoubtedly has much to do with its capacity for adsorbing phenolic compounds, and with the high water solubility of most grades of PVP. Even in PVP, however, there is apparently considerable possibility for blocking of amide groups. An aqueous "paste" of Polyclar AT is reported to be at least 50 per cent more effective in removing tannins than is the dry powder.[42] Gustavson[23] soaked the dry powder in water for 24 hr before use, in order to hydrate it, and we also have found that thorough wetting of the polymer requires some time.

The same H-bonding characteristics which make PVP effective in adsorbing phenols should make it relatively inert with respect to possible interactions with protein. Singer[82]

[80] C. E. H. Bawn, *The Chemistry of High Polymers*. Interscience, New York (1948).
[81] R. Hill, *J. Soc. Dyers Colourists* **68**, 158 (1952).
[82] S. J. Singer, *Advan. Protein Chem.* **17**, 1 (1962).

has pointed out that proton-accepting solvents have little tendency to disrupt intrapeptide H-bonding, whereas proton-donating solvents tend to cause considerable disruption. The same principles should apply to PVP. The fact that soluble PVP has been used as a plasma substitute in blood transfusions speaks for its inertness. Soluble PVP added during the isolation of amphibian yolk platelets protects the structure of the platelets.[83]

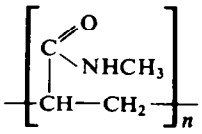
It is common practice to add reducing agents such as ascorbate or thiols during the extraction of enzymes from plant tissues, and these are clearly of some value. However, one should realize that they do not actually prevent the oxidation of phenols; rather, they rapidly remove any quinone that is formed, preventing its accumulation and thus reducing the probability that it will react with protein. Ascorbate reduces the quinones,[84, 85] while thiols also react with them to form thioethers.[15, 29, 31, 86] In one respect reducing agents can be harmful. It has been shown that, in the presence of oxygen, reducing agents activate the ortho-hydroxylation of monophenols by phenol oxidase.[14, 84] The results of Wood and Ingraham[36] cited above are probably an example of this. Among the phenols that can be hydroxylated in this way are tyrosyl residues of protein.[30, 31, 85, 87] This would obviously produce a modified protein; it would also introduce the possibility of "self tanning" of the protein via quinonoid intermediates. Clearly the use of reducing agents is only an imperfect substitute for actual inhibition of phenol oxidation.

The question of the optimum pH for extraction is important and needs further attention. In the experiments described here we used buffers of pH 7·4 or 7·5 because they were satisfactory in the past for extracting plant enzymes; but a somewhat lower pH would probably be better when an agent such as PVP is used, in order to suppress ionization of phenols. The ionized phenols cannot hydrogen bond with either protein or PVP, and at the same time they are more readily oxidized than are the un-ionized forms. We have found active mevalonic kinase in peppermint extracts made with either water or pH 6·4 maleate buffer in the presence of Polyclar AT and ascorbate. Incidental observations from experiments with extracts from pumpkin seedling acetone powders indicate the importance of pH. These extracts, which were pale yellow, were decolorized by Polyclar AT after acidification with trichloroacetic acid, or at the undetermined pH produced by extracting the powder with distilled water. At pH 7·75 Polyclar removed no color. Data of Jones and Hulme[65] showed slightly greater succinic oxidase activity in apple peel mitochondria prepared at pH 7·15 with PVP than in mitochondria prepared in the same way at pH 7·5.

It is probable that some phenolic compounds which do not form strong H-bonded complexes with proteins or PVP, are readily oxidized to quinones. One would expect such behaviour especially of catechol derivatives, and of free tyrosine. These compounds would not be removed effectively by adsorption on PVP and would remain in the extract as latent inhibitors. Gel filtration or dialysis should remove such materials, provided adequate precautions are taken to prevent oxidation. In this connection, it would be especially interesting to test poly-*N*-methylacrylamide. This polymer, if appropriately cross-linked, would very likely combine a capacity for gel filtration with a capacity to adsorb phenols. Poly-*N*,*N*-dimethylacrylamide, with a structure analogous to PVP, might also be useful.

Organic solvents have been used to some extent in the isolation of enzymes, and they undoubtedly have much unrealized potential for work with phenol-containing plant tissues.

[83] R. A. WALLACE and S. KARASAKI, *J. Cell Biol.* **18**, 153 (1963).
[84] M. ROLLAND and S. LISSITZKY, *Biochim. Biophys. Acta* **56**, 83 (1962).
[85] S. LISSITZKY and M. ROLLAND, *Biochim. Biophys. Acta* **56**, 95 (1962).
[86] S. ROSTON, *J. Biol. Chem.* **235**, 1002 (1960).
[87] I. W. SIZER, *Advan. Enzymol.* **14**, 129 (1953).



Poly-*N*-methylacrylamide

Several hydrogen-bonding organic solvents, in mixture with water, have been shown to be more or less effective in stripping tannins from leather.[17, 88, 89] Stripping ability of the aqueous solvents increased in the order: methyl, isopropyl, ethyl, *tert.* butyl alcohols, methyl cellosolve, methyl cellosolve acetate, acetone, dioxane.[88] Only a few of the solvents, notably methyl alcohol, were at all effective in the anhydrous state; water alone was ineffective. Bendall and Gregory[90] were able to obtain soluble phenol oxidase from acetone powders of tea leaves, provided the acetone used in preparing the powders contained about 20 per cent water. They suggested removal of tannins as a possible explanation.

Several other areas in the use of organic solvents should be explored. For example, it might be advantageous to add reducing agents, or oxidase inhibitors, when making acetone powders. Use of other solvents, particularly those which act as strong H-acceptors in the formation of hydrogen bonds[82] (e.g. dimethylformamide, dimethylsulfoxide, *N*-methylpyrrolidone) might be of value. Butanol has been used to dissociate lipoprotein complexes,[91] and the techniques developed might be adaptable to protein–phenol complexes. We have found that extraction of plant acetone powders with methanol or methylpyrrolidone removes considerable amounts of pigment. We have not investigated this further, except to note that peppermint acetone powders which had been extracted with methylpyrrolidone still browned rapidly when moistened with water. They clearly contained active phenol oxidase as well as phenol oxidase substrates. A recent review by Singer[82] summarizes interactions of proteins with non-aqueous solvents.

EXPERIMENTAL

Materials

Peppermint plants were the Black Mitcham variety of *Mentha piperita* L., grown in the greenhouse from the same clone we have used previously.[92] Enzyme extracts were made from the tips, consisting principally of young leaves which were still expanding.

Polyclar AT (insoluble PVP) and *N*-methyl-2-pyrrolidone were obtained from General Aniline and Film Corp. Dyestuff and Chemicals Division, 435 Hudson Street, New York, N.Y. Polyclar AT was purified essentially as described by McFarlane and Vader.[43] It was boiled for 10 min in 10% HCl and washed with glass-distilled water until free of Cl^-. It was then washed with acetone and dried. With one batch of Polyclar this washing removed considerable soluble yellow material. With a later batch it did not appear that anything was washed out. Methylpyrrolidone was purified by adding 5 drops concentrated HCl per liter

[88] H. B. MERRILL, D. H. CAMERON, H. L. ELLISON and C. P. HALL, *J. Am. Leather Chemists' Assoc.* **42**, 536 (1947). From *Chem. Abstr.* **42**, 2460d (1948).

[89] H. B. MERRILL, D. H. CAMERON, H. L. ELLISON and C. H. WRIXTON, *J. Am. Leather Chemists' Assoc.* **44**, 54 (1949). From *Chem. Abstr.* **43**, 4040d (1949).

[90] D. S. BENDALL and R. P. F. GREGORY, In *Enzyme Chemistry of Phenolic Compounds* (Edited by J. B. PRIDHAM), p. 7. Pergamon Press, Oxford (1963).

[91] R. K. MORTON, In *Methods in Enzymology* (Edited by S. P. COLOWICK and N. O. KAPLAN), Vol. 1, p. 25. Academic Press, New York, (1955).

[92] J. BATTAILE and W. D. LOOMIS, *Biochim. Biophys. Acta* **51**, 545 (1961).

and distilling *in vacuo*, discarding the first 10 per cent of the distillate.[42] The colorless redistilled methylpyrrolidone was stored in a refrigerator.

American standard hide powder was obtained from the Marshall Laboratory, Ridgway, Pennsylvania. Before use it was boiled for 40 min in H_2O. The insoluble residue was washed thoroughly with water, acetone, chloroform, dilute base and dilute acid, and finally water, and dried. This drastic "purification" procedure was used in order to minimize the likelihood of contaminating the plant extracts with foreign protein or enzymes.

Collagen, from tendon, was obtained from Sigma Chemical Co., St. Louis, Missouri. Before use it was washed several times with water and dried. Bio-gel P-10, cross-linked polyacrylamide, was obtained from Bio-Rad Laboratories, Richmond, California. Sephadex G-50, cross-linked dextran, was obtained from Pharmacia, Uppsala, Sweden. (±) [2-^{14}C] Mevalonic acid was obtained in the form of the *N,N'*-dibenzylethylenediamine salt from Tracerlab, Inc., Waltham, Massachusetts. It was converted to the sodium salt before use.[78] ^{14}C-Geraniol was isolated and purified from rose geranium cuttings (*Pelargonium graveolens*) which had been exposed to $^{14}CO_2$.[93] ATP and ADP were obtained as sodium salts from Pabst Laboratories, Milwaukee, Wisconsin. Glutamine was obtained from the Pierce Chemical Co., Rockford, Illinois. Tris was Sigma primary standard grade tris(hydroxymethyl)aminomethane. Sodium ascorbate was also obtained from Sigma. *p*-Nitrophenyl phosphate was obtained from Calbiochem, Los Angeles, California.

Analytical Methods

Mevalonic kinase was assayed as described previously.[78] Briefly, ^{14}C-mevalonate was used as the substrate, and the reaction products were separated by paper chromatography. The radioactive compounds were located on the paper by autoradiography with Kodak single coated medical X-ray film, and radioactivity was counted by means of a Vanguard Autoscanner 880 automatic chromatogram scanner. Counts were determined from the recorder trace by cutting out the peaks and weighing them. Due to heavy loading with buffer and other solutes the chromatograms streaked considerably. However, the *n*-butyl alcohol–formic acid–water (77:10:13 by vol.) solvent system gave a clear separation of mevalonic acid from the phosphorylated derivatives in spite of the streaking, and it was therefore used in routine assays. When separation of pyrophosphomevalonate from phosphomevalonate was required, two-dimensional chromatography was used, with *tert.* butyl alcohol–formic acid–water (40:10:16 by vol.) as the second solvent system.

Glutamyl transferase was assayed as described previously.[77] The absorption of the ferric ion–glutamyl hydroxamate complex was measured with a Bausch & Lomb Spectronic 20 spectrophotometer at 540 mμ.

Alkaline phosphatase was measured spectrophotometrically, using *p*-nitrophenylphosphate as the substrate and measuring the formation of *p*-nitrophenol by following absorbancy changes at 410 mμ, with a Bausch & Lomb Spectronic 20 spectrophotometer.[94, 95]

For protein determinations, tissues were extracted with 5% trichloroacetic acid, or trichloroacetic acid was added to extracts to a final concentration of 5 per cent. The precipitate, or the insoluble residue, was analyzed by the micro-Kjeldahl method as described by Steyermark.[96] The weight of nitrogen was multiplied by 6·25 to obtain the weight of protein.

[93] D. J. Baisted and W. D. Loomis, Unpublished data.
[94] A. Garen and C. Levinthal, *Biochim. Biophys. Acta* **38**, 470 (1960).
[95] *Data sheet on* E. coli *alkaline phosphatase*, Worthington Biochemical Corp., Freehold, N.J. (1963).
[96] A. Steyermark, *Quantitative Organic Microanalysis* (2nd Ed.). Academic Press, New York (1961).

Preparation of Peppermint Extracts

All procedures were carried out in the cold, either in an ice bath, or in a cold room or refrigerated centrifuge at about 2°.

The extracts used for the experiment shown in Table 1 were prepared as follows: A large amount of peppermint leaf tissue was ground in a mortar with liquid nitrogen, and a 5 ml beaker was used as a measure to obtain equal samples of the frozen powder. One sample was transferred to a screw-cap vial and weighed (2·3 g). The frozen tissue samples were added to 20 ml of ice-cold solutions containing different combinations of 0·3 M potassium phosphate, pH 7·5; 0·25 M sodium ascorbate; and 0·25 M 2-mercaptoethanol; with or without 2·5 g Polyclar AT. The various combinations are indicated in the Table. The extracts were squeezed through 97 mesh bolting silk, made to 20 ml each, and centrifuged for 30 min at 35,000 ***g***. From each extract, 4·0 ml was fractionated by gel filtration on a 20 ml column of Bio-Gel P-10 polyacrylamide gel, eluting with glass distilled water. Calibration of the columns showed that large molecules started to appear after a forerun of 10 ml. It was arbitrarily assumed that the protein fraction would be diluted 50 per cent, and hence a 6·0 ml fraction was to be taken. However, the columns contracted slightly when the extracts were put through them, and highly refractive solutes began to appear before 6·0 ml had been collected. This fraction was therefore removed when the first drop of refractive material appeared, and varied between 5·3 and 5·8 ml.

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