XLV. OXIDISING ENZYMES. III. THE OXIDISING ENZYMES OF SOME COMMON FRUITS.

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IN carrying out a general survey of the oxidising enzymes of fruits, certain additional observations have been recorded.

The first point to be emphasised is the fact that divergent results may be obtained in testing for oxidising enzymes, according to whether tests are made on the fresh tissue, or on extracts prepared in various ways from the tissues. Some workers have obtained their results by placing the tissues in the testing reagents. Others have used tissue extracts only. It appears desirable to examine the materials in both ways.

The maximum "oxidising capacity" of the tissue is usually shown when the tissue is placed directly in the reagent. In the case of extracts, the reaction may be modified by the following conditions. First, the enzyme itself may not be extracted; secondly, the relative concentration of enzyme, sugars, organic acids and tannins occurring in the extracts are different from those in the cells, and the "oxidising capacity" of the tissue is not always shown until special steps have been taken to remove some of the products present in solution. In order to understand, however, the nature of the reactions *in situ*, extraction of the various components of the system, its inhibitors, etc., is essential.

Further, it may happen that certain substances, such as organic acids, tannins, etc., prevent even the tissues from giving the reactions, and these must be removed by solvents, *e.g.* alcohol, leaving the enzymes in the tissue residue. Moreover, sometimes the enzymes may be adsorbed by the tissue residue and not readily extracted. In this case, the reactions will only occur when the fragments of the residue are placed in the reagents, the enzyme and reacting substances having been adsorbed on to these surfaces.

It has been stated by various observers that tannins have an inhibiting effect on oxidising enzymes, and the present results confirm this observation. It is further shown that malic acid to the extent in which it occurs in many fruits, even in the form of neutral salts, may also have an inhibiting effect.

Observations have been made on the fruits according to the following procedures. (a) *Reactions of tissues.* Pieces of tissue are placed in the reagents,

viz. alcoholic solution of guaiacum (with or without hydrogen peroxide): 1 % solution of benzidine in 50 % alcohol with hydrogen peroxide; 1 % solution of α -naphthol in 50 % alcohol with hydrogen peroxide. (b) *Reactions of water extract.* A water extract of the pounded tissues is tested with the above reagents. If acid, it is neutralised to litmus. (c) *Reactions with extract of enzymes.* The tissues are pounded rapidly with cold 96 % alcohol, sucked dry on a filter funnel, and the process repeated several times. The residue ("tissue residue") is extracted with water, and in this way a crude extract ("enzyme extract") of the enzymes practically free from sugars, tannins and organic acids is obtained. The enzyme extract is tested for peroxidase with guaiacum and hydrogen peroxide, and for oxygenase with catechol and guaiacum¹. (d) *Extraction of aromatic compounds and their reaction with the enzyme extract.* The tissues are extracted with hot alcohol and the alcohol removed by distilling *in vacuo*. The residual extract is diluted with a little water and precipitated with powdered lead acetate. The precipitate so formed chiefly consists of salts of aromatic substances (including tannins) and organic acids. It is decomposed with the minimal amount of sulphuric acid and filtered from lead sulphate. The filtrate is neutralised to litmus, and tested for the catechol reaction with a few drops of ferric chloride followed by a few drops of a 1 % solution of sodium carbonate. A green colour becoming blue, purple or red on addition of the carbonate denotes the presence of a substance with the catechol grouping. To another portion some enzyme extract [see (c) above] is then added. If a catechol reaction has been given and an oxygenase has been detected, a brown colour may be produced in the mixture followed by a blue colour on adding guaiacum; this denotes that the whole oxidase system is present. In a few cases such a result is only obtained by using the tissue residue itself. If both oxygenase and catechol substance have been detected and yet no oxidase system can be synthesised in the above way, the absence of the reaction is usually due to the presence of organic acids or tannins.

If considerable quantities of malic acid are present, this is removed by adding calcium chloride solution, and then excess of alcohol, to the neutralised filtrate obtained by treating the lead salts with dilute sulphuric acid. The calcium malate is insoluble in alcohol and can be filtered off. The alcohol is removed by distilling *in vacuo*, and the residue tested with the enzyme extract.

If tannin is present, this is removed by adding a little concentrated gelatin solution. After filtering, the filtrate is tested with the enzyme extract.

Employing the above methods, the following fruits have been investigated. The results may vary in detail, according to the variety or ripeness of the fruit used.

I. The Apple (*Pyrus malus*). The fruit turns brown on injury. (a) *Reactions of tissues.* In guaiacum tincture the tissue usually turns blue, though in some varieties the reaction may be slow. It is accelerated by bruising the tissue.

¹ Since peroxidase has always been present with oxygenase, it has not been necessary to supply a peroxidase from another source.

On addition of hydrogen peroxide, the reaction is obtained at once. The benzidine and α -naphthol tests are positive. (b) *Reactions of water extract*. All the tests are positive, though the reaction with guaiacum alone may be delayed or slight. (c) *Enzyme extract*. This gives both the peroxidase and oxygenase reactions. (d) *Extraction of aromatic compounds and their reaction with enzyme extract*. When the solution, obtained by decomposition with sulphuric acid of the lead salts of the aromatic compounds and malic acid, is neutralised, a yellowish precipitate is obtained. On taking up this precipitate in very dilute sulphuric acid, the solution gives a strong catechol reaction and is precipitated by gelatin; the solution also does not react with the enzyme extract. The substance precipitated appears to be a catechol tannin which is soluble in alcohol and the dilute acid of the fruit itself, but is insoluble in water. The filtrate from the tannin precipitate also gives the catechol reaction, though less strongly. When tested with enzyme extract and guaiacum no blueing is obtained. The filtrate is then treated with 2–3 cc. of a concentrated calcium chloride solution and excess of alcohol added. A white flocculent precipitate of calcium malate is formed. This is filtered off and the alcohol removed from the filtrate by distilling *in vacuo*. The residue taken up in water still gives a catechol reaction. With tissue residue and guaiacum it gives a blue colour. With the enzyme extract and guaiacum it may give a positive reaction if the liquid is slightly acid to litmus.

Conclusion. The apple fruit contains an oxidase, that is an oxygenase, a peroxidase, and an aromatic substance with the catechol grouping. The oxygenase will activate the oxidation of the catechol aromatic compounds *in situ* in the cell with the production of a brown colour and of a system which will blue guaiacum: it will also activate the oxidation of catechol supplied artificially with the production, in the presence of the peroxidase, of a similar system. A large part of the aromatic compounds of the fruit appears to be in the form of a catechol tannin, and this cannot be activated by the enzyme *in vitro*. After removing the tannin and malic acid, the residuum of aromatic compounds left is activated by the oxygenase, with difficulty in solution, but more readily when fragments of the tissue residue are suspended in the solution.

II. The Quince (*Pyrus cydonia*). The cut surface of the fruit turns brown less readily than that of the apple, but on pounding in a mortar, considerable browning is produced. (a) *Reactions of tissues*. The tissue gives no blue colour with guaiacum tincture, except on adding hydrogen peroxide. The benzidine and α -naphthol tests are positive. (b) *Reactions of water extract*. This gives none of the above tests even after neutralisation. (c) *Enzyme extract*. This gives the peroxidase but only a slight oxygenase reaction, unless fragments of tissue residue are added, when they become deep blue. (d) *Extraction of aromatic compounds and their reaction with enzyme extract*. The procedure and reactions are very similar to those for the apple. In the same way, though in greater quantity, the fruit appears to contain a catechol tannin which is

soluble in alcohol and dilute acids. On neutralising the filtrate (from the decomposition of the lead salts) this tannin is precipitated. The filtrate from the tannin precipitate still gives the catechol reaction but contains no perceptible amount of tannin. It, however, gives no blue colour with guaiacum and either the enzyme extract or tissue residue. After removing the malic acid as calcium malate and distilling the filtrate *in vacuo*, the residue gave the catechol reaction less strongly, but would again give no blue colour with guaiacum and either the enzyme extract or tissue residue.

Conclusion. The quince fruit contains an oxidase (oxygenase, peroxidase and aromatic substance with the catechol grouping). The oxygenase will activate the oxidation of the catechol aromatic compounds *in situ* in the cell causing browning, but the blueing of guaiacum is largely inhibited owing to the tannin and malic acid present. The oxygenase is not readily extracted from the tissue residue, so that only this residue itself gives a good reaction with catechol solution and guaiacum. No reactions could be obtained between the plant aromatic compounds and enzymes *in vitro* even when the tissue residue was employed. A neutralised water extract of the tissues gives no reactions, probably because of the inhibitory action of the malates.

III. The Pear (*Pyrus communis*). The fruit turns brown readily on injury. (a) *Reactions of tissues.* The tissue turns blue with guaiacum tincture and gives positive reactions with the benzidine and α -naphthol tests. (b) *Reactions of water extract.* Positive reactions with all three tests. (c) *Enzyme extract.* This gives both the peroxidase and oxygenase reactions. (d) *Extraction of aromatic compounds and their reaction with enzyme extract.* The filtrate, after decomposing the lead salts with sulphuric acid, gives no precipitate on neutralisation, and a very slight, if any, reaction for tannin. It gives a strong catechol reaction. On addition of the enzyme extract, browning occurs and a deep blue colour on subsequent addition of guaiacum.

Conclusion. The pear fruit contains an oxidase (peroxidase, oxygenase and aromatic substance with the catechol grouping). The oxygenase will activate the oxidation of catechol artificially supplied, with the production, in the presence of the peroxidase, of the oxidase system. When the separate extracts of aromatics and enzymes are reunited, the oxidase system is obtained.

A comparison of the oxidase reactions of the three fruits is rendered more comprehensible if the average percentages [Wehmer, 1911] of tannin and free acids are noted.

		Pear	Apple	Quince
Organic acid (chiefly malic)	...	0.1-0.2 %	0.7-1.2 %	0.84-3.5 %
Tannin		0.05	0.1-0.3	

(No reference to the percentage of tannin in the quince could be found, but large quantities were obtained on extraction.) The tannin and organic acids inhibit the oxidising reactions in both the tissues and extracts, though more so in the latter, the effect of the tannin being to precipitate the enzymes. The presence of tannin also renders the extraction of the enzymes more difficult,

even after the bulk of the tannin is removed by alcohol. This is especially so in the case of the oxygenase, *e.g.* in the quince, which appears to be precipitated and adsorbed by the tissue fragments.

IV. The Plum (*Prunus domestica*). Both red and purple varieties were investigated separately. The fruit turns brown on injury. (a) *Reactions of tissues*. The tissues give a positive reaction with guaiacum alone, and also with both benzidine and α -naphthol tests. (b) *Reactions of water extract*. The same results as in (a) were obtained. (c) *Enzyme extract*. This gives both peroxidase and oxygenase reactions. (d) *Extraction of aromatic compounds and their reaction with enzyme extract*. A heavy whitish precipitate (red plums) or purple precipitate (purple plums) is obtained with lead acetate. After decomposition with sulphuric acid and neutralisation, the extract gives a good catechol reaction, but no reaction with the enzyme extract and guaiacum. After removal of the malic acid, the extract still gives the catechol reaction and, on addition of the enzyme extract and guaiacum, a blue colour. The latter reaction is better if the mixture is slightly acid to litmus.

The same results were obtained with greengage and damson fruits.

The percentages of acid and tannin are as follows: acid, chiefly malic (plum, 0.77–1.01 %; greengage, 0.82 %; damson, 0.92 %); tannin (damson, 0.04 %) [Wehmer, 1911].

Conclusion. The plum, greengage and damson all contain an oxidase (peroxidase, oxygenase and aromatic substance with the catechol grouping). Hence the tissues brown on injury and give the oxidase reaction. The oxygenase will activate the oxidation of catechol solution with the production of a peroxide, etc. The extracted aromatic compounds and enzymes from the fruit when recombined will give a blue reaction with guaiacum after the removal of the malic acid.

V. The Banana (*Musa sapientum*). On injury, *e.g.* pounding in a mortar, the skin turns brown but the flesh is less affected. The results recorded below given by the flesh of the fruit varied somewhat in individual fruits and in fruits of different degrees of ripeness. On the whole, however, the reactions were as stated. (a) *Reactions of tissues*. The skin turns blue with guaiacum alone: the flesh only after addition of hydrogen peroxide. Both skin and flesh give positive reactions with the benzidine and α -naphthol tests. (b) *Reactions of water extract*. The extract of the skin gives a positive reaction with guaiacum alone: that of the flesh no reaction until hydrogen peroxide is added. Extracts of both skin and flesh give positive reactions with the benzidine and α -naphthol tests. (c) *Enzyme extract*. From both skin and flesh these give the peroxidase and oxygenase reactions. (d) *Extraction of aromatic compounds and their reaction with enzyme extract*. The solution derived from decomposition with sulphuric acid of the lead salt from the skin gives the catechol reaction. On addition of the enzyme extract from the skin followed by guaiacum, a blue colour is obtained. The reaction takes place more readily after precipitation with gelatin owing to the presence of a certain amount of

tannin. The solution from the decomposition of the lead salt from the flesh gives a doubtful catechol reaction, and no blue colour with the enzyme extract from the flesh followed by guaiacum. After removal of malic acid by the usual method, the residue gives no catechol reaction and again no result after addition of enzyme extract and guaiacum.

Conclusion. (The results, as mentioned above, given by extracts of enzymes and aromatic compounds of the flesh vary, but on the whole they may be represented as follows.) Both the skin and flesh of the fruit contain peroxidase and oxygenase. The skin also contains substances with the catechol grouping, but these are practically absent from the flesh. The oxygenase of both skin and flesh catalyses the oxidation of catechol, artificially supplied, with great rapidity followed by blueing on addition of guaiacum. The skin oxygenase also activates the oxidation of its own aromatic compound with the production of a blue colour on addition of guaiacum. No reaction could be obtained between the enzyme and aromatic extracts of the flesh.

VI. The Orange (*Citrus aurantium*). The fruit does not brown on injury. (a) *Reactions of tissues.* Rind: positive result with guaiacum and hydrogen peroxide and with the other two tests. Pulp: slight local positive reaction with benzidine and α -naphthol tests only. (b) *Reactions of water extract.* Rind: positive reaction only with benzidine test. Pulp: after neutralisation to litmus, no reactions with any of the three tests. (c) *Enzyme extract.* Both rind and pulp give some positive reaction with guaiacum and hydrogen peroxide and with the benzidine test. Neither gives the oxygenase reaction. (d) *Extraction of aromatic compounds.* No catechol reaction could be detected.

VII. The Lemon (*Citrus limonum*). The fruit does not brown on injury. (a) *Reactions of tissues.* Rind: positive reaction with guaiacum and hydrogen peroxide and with the other two tests. Pulp: positive reaction with benzidine and α -naphthol tests only. (b) *Reactions of water extract.* Rind: positive reaction with benzidine and α -naphthol tests only. Pulp: after neutralisation to litmus, positive reaction with benzidine test only. (c) *Enzyme extract.* Rind: good positive reactions with guaiacum and hydrogen peroxide and with the other two tests. Pulp: positive reaction only with benzidine test. With the other two tests positive reaction only if tissue residue is used. Neither extract gives the oxygenase reaction. (d) *Extraction of aromatic compounds.* No catechol reaction could be detected.

VIII. The Lime (*Citrus limonum*, var. *acida*). The fruit does not brown on injury. (a) *Reactions of tissues.* Rind: positive reactions with guaiacum and hydrogen peroxide and with the other two tests. Pulp: positive reaction with the benzidine test only. (b) *Reactions of water extract.* Not tried. (c) *Enzyme extract.* Rind: positive reactions with guaiacum and hydrogen peroxide and with the other two tests. Pulp: negative reactions with all three tests but positive reaction with the benzidine test if the tissue residue is used. Neither extract gives the oxygenase reaction. (d) *Extraction of aromatic compounds.* No catechol reaction could be detected.

Conclusion. The fruits of the orange, lemon and lime contain a peroxidase in both the rind and the pulp. No substance giving the catechol reaction could be detected in any of the fruits and there is no oxygenase. In the rind the peroxidase reaction is strongest in the lime, less in the lemon and least in the orange. From the pulp, the peroxidase is most readily extracted in the case of the orange, less in the lemon and least in the lime. These fruits afford an example of the possibility of obtaining a greater oxidation reaction with the crudely separated peroxidase than with the tissue itself.

IX. The Raspberry (*Rubus idaeus*). The fruit does not brown on injury. (a) *Reactions of tissues.* Some positive reaction only with benzidine and α -naphthol tests. (b) *Reactions of water extract.* These cannot be detected because of the anthocyan pigment. (c) *Enzyme extract.* Positive reactions with guaiacum and hydrogen peroxide and with the other two tests. No oxygenase reaction is given. (d) *Extraction of aromatic compounds.* No catechol reaction could be detected.

Conclusion. The raspberry fruit contains a peroxidase but no oxygenase and no substance giving the catechol reaction. This fruit affords another example of greater oxidation powers of crudely prepared peroxidase than of the tissue itself.

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REFERENCE.

Wehmer (1911). *Die Pflanzenstoffe*. Jena.