Pectinesterase in Normal and Abnormal Tomato Fruit

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Although the pectin content of tomatoes is relatively low in comparison with other fruits (Money & Christian, 1950), the activity of pectinesterase (pectin pectyl-hydrolase, EC 3.1.1.11) is particularly high (Kertesz, 1938). Softening of the fruit takes place relatively rapidly during ripening (Hobson, 1959), and the classical mechanism by which the effect is usually explained assumes the solubilization of pectin and de-esterification with pectinesterase followed by its degradation with polygalacturonase (Demain & Phaff, 1957; Deuel & Stutz, 1958). An explanation of the solubilization of pectin involving a movement of calcium in the cell walls was offered by Doesburg (1957), and Ginzburg (1961) gave evidence in favour of a proteinaceous intercellular binding material in addition to the pectic substances. It seems clear, however, that the solubilization of pectin, and its de-esterification by pectinesterase, must precede any appreciable degradation by polygalacturonase. Hence the rate of reaction of polygalacturonase increases with the degree of de-esterification of the pectin substrate. The transformations thus brought about are considered to contribute to a softening of the tissue during ripening (Woodmansee, McClendon & Somers, 1959; McCready & McComb, 1954).

A physiological disorder often known as 'blotchy' ripening shows as harder green, yellow or translucent areas of an otherwise typically soft red tomato fruit (Bewley, Read & Orchard, 1934). Histological studies have shown that the thin-walled parenchymatous cells immediately surrounding the vascular bundles in the affected tissue are reduced by this condition to a brown thickened mass of dead corky cells which must limit conduction of nutrients to the rest of the affected tissue (Gigante, 1954; Seaton & Gray, 1936). The abnormality appears to various extents in all varieties of the tomato, *Lycopersicon esculentum* L., and manurial treatments and environmental conditions also affect the proportion of fruit ripening non-uniformly (Winsor, 1959).

As pectinesterase was thought to be closely connected with the softening of tissue it was considered that the inability of parts of tomato fruit to ripen normally might be due to a lack of this determining enzyme. The present paper therefore records typical values for pectinesterase activity in normal tomato fruit of two varieties at five colour stages of ripening, and contrasts these figures with the diminished activity of the enzyme in areas of the fruit where the expected ripening changes have not taken place. The solubilization of the pectic substances in both normal and abnormal fruit, probably closely connected with firmness, has also been investigated quantitatively.

EXPERIMENTAL

Tomato varieties. The fruits of two contrasting varieties of tomato were studied. 'Potentate' is a widely grown, particularly firm, variety; the seeds were obtained from the Market Growers' Seed Association Ltd., Mill Nursery, London Road, Hassocks, Sussex. 'Immuna' has fruit that are quicker to ripen, at which stage they are much softer than the fruit of 'Potentate'. It is an F_1 hybrid and the seeds were a gift from W. Weibull AB., Landskrona, Sweden.

Tissue samples. Fruit from plants grown in a heated glasshouse were picked immediately before analysis, which was carried out at intervals from May to August. Descriptions of the appearance of fruit at the five chosen ripening stages have been given (Hobson, 1959); 'green' in the tables implies a mature, almost full-size, fruit with no trace of yellow pigment. Tissue for the assay of pectic substances and of enzyme consisted either of radial sections cut through the axis of the fruit, which gave results representing whole tomato fruit, or of the outer walls of the locules of fruit only, i.e. the pericarp, without the internal dividing walls. Determinations of pectic substance and of dry matter were carried out at once; tissue for enzyme studies was stored in sealed jars at $-20°$ and analysed within 4 months. Calibration experiments with normal fruit showed that the activity of pectinesterase diminished at this temperature at a rate of not more than 1·5% per month.

Enzyme preparation. The extraction method of Pithawala, Savur & Sreenivasan (1948) was used, except that the phosphate buffer solution they recommended obscured the end point of the subsequent electrometric titration and was replaced by 0·5M-sodium acetate (Hills & Mottern, 1947). This proved to be a more efficient extractant than NaCl. The pH of the extraction was about 8·0.

Enzyme assay. The method used was mainly that of Kertesz (1951*b*). 0·02M-Sodium oxalate solution was mixed with a 1% (w/v) solution of apple pectin from British Drug Houses Ltd., Poole, Dorset (240, later 250 grade, having an average analysis of 8·55% of moisture, 1·76% of ash and 4·75% of OMe by the method of Heron, Reed, Stagg & Watson, 1954), diluted and brought to pH 7·5 with 3N-NaOH. On the addition of 0·1 vol. of

enzyme in 0·5 M-sodium acetate, the final concentrations became: pectin, 0·4%; sodium acetate, 0·05 M; sodium oxalate, 2 mM. The reaction, carried out at 27°, was stopped after 15 min. by the addition of 1 ml. of Teepol (Shell Chemical Co. Ltd., London) which had been previously filtered and brought to pH 7·35 with 25% (v/v) HCl. In this assay method the acid groups liberated by the esterase were being titrated, but a stoicheiometric liberation of methanol is inferred from the results of other workers and of experiments described below. Hence a unit of activity is defined as the quantity of enzyme which would liberate 1 m-mole of methanol from pectin in 1 min. at 27° in 0·05 M-sodium acetate at pH 7·5.

Methanol estimation. In some of the experiments, after an enzyme reaction had been stopped with Teepol, the mixture was adjusted to pH 3·0–3·2 with a measured quantity of 25% (v/v) HCl and 25 ml. was pipetted into the distillation head of a Hoskins micro-Kjeldahl distillation apparatus together with a speck of Silicone M.S. antifoam 'A' (Hopkin and Williams Ltd., London). About 15 ml. of the distillate was collected in a receiver surrounded by crushed ice. A calibration curve was constructed by distilling similar quantities of the reaction mixture used as a blank during the enzyme assay to which had been added known amounts of AnalaR methanol. Finally, the methanol in the distillate was determined, in earlier experiments by the method of Reid & Truelove (1952) and later by the method of Pollard & Kieser (1951).

Determination of dry matter. Representative samples of about 100 g. of fresh tomato-fruit tissue were dried in a forced-draught oven at 80° for 48 hr.

Determination of total and insoluble pectic substances. A method by Potter & McComb (1957) for separating, extracting and degrading the pectic substances in potatoes was adapted for use with tomato tissue. Only small quantities of starch occur in tomato fruit, even when green (Saywell & Robertson, 1932), and these did not interfere with the determinations. All the conditions suggested by McCready & McComb (1954) as necessary for a quantitative extraction of the pectic substances were fulfilled by the following procedure.

About 50 g. of fresh fruit tissue was thoroughly disintegrated in a macerator (Measuring and Scientific Equipment Co. Ltd., London) with an equal weight of water. A portion (40 g.) of this mixture was weighed into a tared beaker and used for the determination of the total pectic substances. The remaining slurry was thoroughly ground in a large pestle and mortar and a second 40 g. portion was weighed into a 250 ml. centrifuge bottle for the determination of the insoluble fraction. Degradation of the pectin by autolysis was effectively prevented by the addition of 2 drops of Teepol to each sample at this stage. About 200 ml. of water was added to the second tissue sample and the whole placed on a rotary shaker for 30 min. The tissue was then centrifuged at 2000*g* for 15 min. and the supernatant, containing most of the soluble pectic substances, carefully poured off. The extraction was repeated with a further quantity of water and the insoluble material again centrifuged down. Finally about 200 ml. of 95% (v/v) ethanol was slowly added to both tissue samples. After 1 hr., with occasional stirring, both precipitates were filtered off (Whatman no. 5 paper) and washed twice with about 50 ml. of 75% (v/v) ethanol. All ethanolic filtrates were kept for further analysis. The precipitated substances were pressed and drained well and transferred to beakers. The procedure for the enzymic degradation of the pectin and subsequent determination of the galacturonic acid produced with a carbazole–sulphuric acid reagent followed that of McCready & McComb (1952), as used by Potter & McComb (1957), except that Pectinase (L. Light and Co. Ltd., Colnbrook, Bucks.) was employed instead of Pectinol 100 D. Fresh D-galacturonic acid standards were prepared every 2 days and were stored at 0°. Reproducible results were only obtained if both the concentrated sulphuric acid and all solutions containing galacturonic acid were brought to this temperature before being mixed. It was necessary to add the galacturonic acid solutions slowly with constant shaking, with the tubes kept in an ice bath. Glass-stoppered tubes were used to keep the uptake of moisture by the acid to a minimum, and the time allowed for development of the colour was standardized at exactly 25 min. The absorption of the solution was measured on a Hilger Spekker photoelectric absorptiometer with an Ilford 604 spectrum filter (absorption max. 517 mμ).

Galacturonic acid and ethanol-soluble oligouronides. The ethanolic filtrates from both the insoluble and total pectic fractions were concentrated under partial vacuum until almost all the organic solvent had been removed. Excess of 10% (w/v) $CaCl_2$ was added, and the resulting precipitate centrifuged down after standing overnight. This was then dissolved in the minimum quantity of 50% (v/v) HCl and analysed qualitatively for D-galacturonic acid by descending paper chromatography, on Whatman no. 1 papers (30 cm. × 50 cm.), with the top layer from a mixture of ethyl acetate–acetic acid–water (2:1:2, by vol.) as solvent. After overnight development the paper was sprayed with 2% (w/v) *p*-anisidine hydrochloride in butan-1-ol (Hough, Jones & Wadman, 1950). Quantitative analyses of the same solutions were made by the method of Potter & McComb (1957).

Determination of protein. Protein was precipitated overnight from about 180 ml. of the enzyme solution by the addition of 10% of its weight of trichloroacetic acid. The precipitate was washed twice with about 40 ml. of 5% (w/v) trichloroacetic acid and its nitrogen content determined by the Kjeldahl procedure.

Enzyme inhibitors. In studying their possible inhibition of the enzyme, the polyphenols were extracted from normal locule-wall tissue and whole-fruit samples at both the green and red ripening stages. Their action on the enzyme was compared with substances similarly obtained from green and red tissue from the two differently coloured areas of 'blotchy' ripened fruit. Fresh or deep-frozen samples (100 g.) were dropped into hot methanol, boiled for 1 min., thoroughly macerated and further extracted three times for 1 hr. with sufficient 80% (v/v) methanol to cover the tissue. The combined methanolic filtrates were evaporated below 40° under reduced pressure to about 50 ml., adjusted to pH 8·3 with 1 N-NaOH and excess of saturated lead acetate was added. After 1 hr. at 0° the precipitate was centrifuged off, washed once and resuspended in 50 ml. of water. The pH was brought to 3·0 with 2 N-H_2SO_4 and the precipitate filtered off. The filtrate was exhaustively extracted with 10 ml. portions of ethyl acetate and the organic solvent evaporated below 40° under reduced pressure. The residue was made up to 10 ml. with water and the effect of 4 ml. portions on pectinesterase activity, under standard conditions, was investigated.

Qualitative chromatographic analyses of the major polyphenolic acids present in normal and 'blotchy' ripened tissue were carried out on Whatman no. 1 paper (15 cm. × 50 cm.) with benzene–acetic acid–water (125:72:3, by vol.), 2% (v/v) acetic acid, or butan-1-ol–acetic acid–water (63:10:27, by vol.) as solvent. Ultraviolet light, both before and after exposure to ammonia vapour, a ferric chloride–potassium ferricyanide mixture and diazotized *p*-nitroaniline were used for detecting the results of chromatography (Smith, 1960). Caffeic acid, chlorogenic acid and ferulic acid were tentatively identified by comparison with authentic samples (L. Light and Co. Ltd.).

The effect on pectinesterase activity of the addition of quantities of each of these polyphenolic acids was also investigated. Solutions containing up to 10 mg. of caffeic acid, chlorogenic acid and ferulic acid [the last in 4% (v/v) ethanol] were added to standard reaction mixtures for measuring the activity of the enzyme.

RESULTS

Pectin content of normally ripening fruit. A 'mature' green tomato goes through the colour stages to become fully ripe in about 7 days. During this time, as shown in Table 1, the total pectic content decreased by about one-third and the proportion that was insoluble fell markedly at each stage. The observation that fruit of the variety 'Immuna' was less firm than that of 'Potentate' at the stages of ripeness beyond 'mature' green (Hobson, 1959) may well be partly due to a lower proportion of insoluble pectin in the former variety.

A number of fruits have been shown to contain free galacturonic acid (McClendon, Woodmansee & Somers, 1959). It was possible that this acid, together with ethanol-soluble oligouronides, could form a significant fraction of the total uronides present (Foda, 1957; Borenstein, Stier & Ball, 1955) which would be lost in the filtrate when the longer-chain uronides were precipitated. The results in Table 2 demonstrate, however, that the loss of these ethanol-soluble acids is comparatively insignificant, and the concentration found was approximately that reported by McClendon *et al.* (1959) in American varieties of tomato fruit.

Pectin content of abnormally ripened fruit. The abnormal fruit used for analysis were selected so that they showed approximately equal areas of apparently normal and of 'blotchy' tissue in order to facilitate direct comparison. The results given in Table 3 show that the red and the green areas from 'blotchy' fruit contained similar amounts of pectin; this is in direct contrast with the decrease in pectic substances encountered as evenly coloured

Table 1. *Pectic substances in ripening tomato fruit*

The analyses were carried out as described in the text. Each value is the mean of three determinations, and the pectic content is expressed as % (w/w) of anhydrouronic acid in fresh tissue from whole fruit.

	Stage of ripeness					Significance of the F test	Least significant difference between means ($P = 0.05$)
	Green	Green–orange	Orange–green	Orange	Red		
Variety 'Potentate'							
Total pectic substances	0·27	0·22	0·19	0·19	0·16	$P < 0.050$	0·07
Insoluble pectic substances	0·27	0·18	0·11	0·08	0·03	$P < 0.001$	0·06
Insoluble pectic substances (% of total)	100·0	81·8	57·9	42·1	18·8	—	—
Dry matter in fruit (%)	6·16	5·62	5·64	5·66	5·38	—	—
Variety 'Immuna'							
Total pectic substances	0·30	0·26	0·24	0·23	0·19	$P < 0.001$	0·04
Insoluble pectic substances	0·23	0·16	0·11	0·06	0·03	$P < 0.001$	0·06
Insoluble pectic substances (% of total)	76·7	61·5	45·8	26·1	15·8	—	—
Dry matter in fruit (%)	6·62	6·30	6·02	5·94	6·09	—	—

Table 2. *Content in ripening tomato fruit of galacturonic acid and oligouronides soluble in aqueous ethanol*

The analyses were carried out as described in the text.

	Variety 'Potentate'		Variety 'Immuna'	
Stage of ripeness	Galacturonic acid [% (w/w) of fresh tissue]	Dry matter (%)	Galacturonic acid [% (w/w) of fresh tissue]	Dry matter (%)
Green	0·0030	5·95	0·0025	6·85
Green–orange	0·0029	5·80	0·0024	6·70
Orange–green	0·0034	5·70	0·0023	6·32
Orange	0·0035	5·55	0·0030	6·24
Red	0·0050	5·37	0·0060	6·66

fruit matured. It is also clear that 'blotchy' fruit contains twice the amount of pectic material found in typical, uniformly ripening, red fruit (Table 1). The red areas of 'blotchy' fruit contain less insoluble pectin than do the green areas. Moreover, the red areas contained considerably more insoluble pectin, and the green regions less, than were shown by uniformly-ripening red and typical green fruit respectively.

Pectinesterase activity in normal and abnormal fruit. Average figures are given in Table 4 for the activity of pectinesterase in samples of whole fruit of the two varieties 'Potentate' and 'Immuna'. Both showed increasing activity as the fruit matured, with a maximum rise as the orange colour began to appear. At all the colour stages 'Immuna' gave a greater activity than did 'Potentate'.

The investigation was then extended to include samples drawn from 'blotchy' and adjacent, apparently normal, ripe tissue. In the first series of experiments, tissue from the outer walls of the locule was analysed for enzyme activity. For the second series, tissue samples were taken by cutting radial segments from the appropriate sides of the fruit. As shown in Table 5, for both of the sampling methods employed, pectinesterase activity was significantly higher in the normal areas than in those showing symptoms of 'blotchy' ripening.

A comparison of the activity of pectinesterase in different areas of 'blotchy' ripened fruit with the mean values for normally ripening tomatoes demonstrates that, though the red areas of 'blotchy' specimens show generally characteristic values, the enzyme in green areas from the same fruit has significantly less activity. In fact the activity of the enzyme in the abnormal regions of 'Potentate' tomatoes is only at a level usually found in fruit just beginning to change colour. For 'Immuna' the value is even lower than that encountered in normal green fruit. Similarly, there is a consistent drop, from normal to abnormal tissue extractions, for the specific activity of pectinesterase.

High concentrations of potassium in fertilizers reduce the proportion of fruit showing uneven ripening (Winsor, 1959). It was thought possible that one of the pathways by which potassium could exert this effect might be through alterations of the pectinesterase activity. Fruit from plants grown with and without the addition of fertilizers supplying potassium were accordingly analysed for pectinesterase (Table 6). A significant decrease of over 40% was shown in the enzyme activity of fruit grown under conditions of potassium deficiency. The specific activity of the enzyme in the protein extract also fell sharply but this trend just failed to be statistically significant. A similar effect of nutrients on the enzyme in tobacco leaves was shown by Holden & Tracey (1948).

After some enzyme assays the medium was also analysed for its methanol content. From the amount of alkali consumed during the period of the enzyme action, the amount of methanol that should have been produced was calculated. A highly significant relation between the calculated and the experimental values was found, the correlation coefficients being 0·958 and 0·883 for the methods of Reid & Truelove (1952) and Pollard & Kieser (1951) respectively. Applying Student's t test to these series of paired results, there was no significant difference between them at a P value of 0·05. This was confirmatory evidence that pectinesterase action was being followed.

Although there have been a number of reports showing an inhibition of pectic enzymes (especially polygalacturonase) by plant phenolic substances (Pollard, Kieser & Sissons, 1958; Ramaswamy & Lamb, 1958), none of these has referred to the tomato fruit. No inhibition of pectinesterase activity by the phenolic components extracted from any of the colour stages of normal fruit or from

Table 3. *Content of pectic substances in normal and 'blotchy' areas of the same tomato fruits*

Analyses were carried out as described in the text. Each value is the mean of two determinations. The pectin content is expressed as % (w/w) of anhydrouronic acid in fresh tissue from whole tomato fruit.

Tissue analysed	Condition	Total pectic substances	Insoluble pectic substances	Insoluble pectic substances (%)	Dry matter (%)
Variety 'Potentate'					
Radial segments of whole fruit	Normal	0·35	0·14	40·0	5·71
	'Blotchy'	0·36	0·20	55·5	5·66
Outer walls of the locules	Normal	0·39	0·18	46·2	5·45
	'Blotchy'	0·42	0·29	69·0	5·17
Variety 'Immuna'					
Radial segments of whole fruit	Normal	0·32	0·12	37·5	5·89
	'Blotchy'	0·33	0·20	60·6	5·68
Outer walls of the locules	Normal	0·41	0·27	66·1	5·38
	'Blotchy'	0·38	0·31	82·0	5·04

Table 4. *Pectinesterase activity in two varieties of tomato fruit during ripening*

The method of assay is described in the text. The upper line of values (*a*) applies to whole fruit of the variety 'Potentate' and the lower line (*b*) to whole fruit of the variety 'Immuna'.

		No. of determinations	Stage of ripeness: Green	Green–orange	Orange–green	Orange	Red	Significance of the F test	Least significant difference between means ($P = 0.05$)
Units/100 g. of fresh tissue	(*a*)	7	3·28	3·82	4·30	4·13	4·22	$P < 0.05$	0·60
	(*b*)	8	4·91	6·65	6·86	6·87	6·96	$P < 0.01$	1·06
Protein extracted (mg./100 g. of fresh tissue)	(*a*)	5	96·2	100·7	78·7	85·2	104·5	$P < 0.01$	13·2
	(*b*)	7	82·7	72·2	81·2	94·1	122·5	$P < 0.001$	20·8
Specific activity (units/mg. of protein)	(*a*)	5	0·036	0·038	0·054	0·049	0·039	$P < 0.05$	0·014
	(*b*)	7	0·065	0·102	0·094	0·077	0·061	$P < 0.01$	0·022
Dry matter (%)	(*a*)	7	6·02	5·55	5·41	5·44	5·40	—	—
	(*b*)	8	6·48	6·16	6·09	6·08	6·14	—	—

'blotchy' ripened tissue could be demonstrated outside the limits of experimental error. The addition *in vitro* of amounts ofp olyphenolic acids up to a concentration of 0·008 % resulted in a small but significant inhibition of the enzyme activity by ferulic acid, but caffeic acid and chlorogenic acid were without effect. Thus it seems unlikely that the action of phenolic inhibitors can explain the low activity of the enzyme in 'blotchy' tissue.

DISCUSSION

There is evidence to suggest that the presence of green areas in the walls of 'blotchy' tomatoes cannot be attributed merely to delayed ripening of the tissue (Winsor, Davies & Massey, 1962). It is clear from the present work that the disorder is associated with an unusually high pectin content of the fruit. An abnormality of tomatoes known as 'gray-wall' in North America, in some ways similar to 'blotchy' ripening, also results in an increase of the pectin content of affected fruit (Hall & Dennison, 1956). There are also indications that the high activity of polyphenol oxidase in 'blotchy' tissue exceeds that in any immature fruit except at the very young stage (Kidson, 1960). Moreover, the effects of the disorder are not confined to the outer fruit walls, but are also found in the chemical composition of the locular juices (Winsor & Massey, 1959). These results suggest that the green areas of non-uniformly ripened fruit cannot be regarded as isolated regions of typically unripe tissue, but rather as areas which have followed a divergent maturation pathway.

There are three methods commonly used for the estimation of pectic substances. Pectin may be precipitated quantitatively as calcium pectate under carefully controlled conditions (Kertesz, 1951*a*). Kertesz & McColloch (1950), using this method, recorded a rather lower total of pectic substances in American varieties of tomato than those reported in the present paper. The insoluble portion appeared not to decrease appreciably as the fruit became over-ripe, and their conclusion was that a correlation between ripening processes and changes in the pectic substances was not apparent. A similar pattern of results was found by Woodmansee *et al.* (1959), except that the amount of total pectin was much closer to that found in the present investigation. By the same method of analysis, Money & Christian (1950) showed wide variation in the values for pectin in ripe tomatoes (probably English) but the average approximated to that reported here for the red stage of 'Potentate'. The estimation of pectin may also be carried out by a controlled decarboxylation of D-galacturonic acid on which the polymer is based. Using this method, Foda (1957) recorded concentrations

Table 5. *Pectinesterase activity in normal and abnormal areas of tomato fruit*

The method of assay is described in the text. The upper line of values (*a*) refers to values for tissue from the outer locule walls only and the lower line (*b*) to values for segments of tissue (equivalent to the whole fruit of Table 4) from the appropriate side of the fruit. 'Significance level' is the significance between the two areas of the fruit, obtained by Student's *t* test for the paired results.

		No. of determinations	Normal tissue	Abnormal tissue	Drop in activity from normal to abnormal areas (%)	Significance level	Standard error of the mean difference
Variety 'Potentate'							
Units/100 g. of fresh tissue	(*a*)	6	7·21	5·51	24	$P < 0·05$	±0·534
	(*b*)	4	4·60	3·59	22	$P < 0·05$	±0·258
Protein extracted (mg./100 g. of fresh tissue)	(*a*)	6	85·5	86·9	—	—	—
	(*b*)	4	68·1	66·7	—	—	—
Specific activity (units/mg. of protein)	(*a*)	6	0·097	0·075	—	$P < 0·05$	±0·0058
	(*b*)	4	0·068	0·053	—	$P < 0·05$	±0·0045
Dry matter (%)	(*a*)	6	5·26	5·01	—	—	—
	(*b*)	4	5·34	5·39	—	—	—
Variety 'Immuna'							
Units/100 g. of fresh tissue	(*a*)	4	9·66	6·90	29	$P < 0·05$	±0·693
	(*b*)	5	6·27	4·54	28	$P < 0·01$	±0·239
Protein extracted (mg./100 g. of fresh tissue)	(*a*)	4	89·6	85·6	—	—	—
	(*b*)	5	89·1	92·8	—	—	—
Specific activity (units/mg. of protein)	(*a*)	4	0·108	0·081	—	$P < 0·05$	±0·0082
	(*b*)	5	0·074	0·050	—	$P < 0·01$	±0·0042
Dry matter (%)	(*a*)	4	5·41	4·96	—	—	—
	(*b*)	5	5·84	5·77	—	—	—

Table 6. *Effect of two levels of potassium on the pectinesterase activity of ripe tomato fruit*

Samples of ripe tomato fruit (variety 'Potentate'), of the best quality available, were taken from two plots in a factorial nutritional trial in a glasshouse. Both plots received similar manurial treatments except that one was given no potassium and the other had 12 oz./yd.^{2} (407 g./m.^{2}) as K_2SO_4 distributed over it during the growing season. The method of assay is described in the text, and each value is the mean of four determinations. 'Significance level' is the significance of the difference between fruit from the two plots, obtained by Student's *t* test for the paired results.

	Source of the fruit		Significance level	Standard error of the mean difference
	Potassium-treated plot	Potassium-deficient plot		
Units/100 g. of fresh tissue	4·13	2·48	$P < 0·05$	±0·346
Protein extracted (mg./100 g. of fresh tissue)	109·3	120·3	—	—
Specific activity (units/mg. of protein)	0·044	0·022	—	—
Dry matter (%)	6·20	5·74	—	—

1·5–2 times those found in the present work. The third method of analysis involves a preliminary precipitation with ethanol followed by the estimation of pectin by an enzymic degradation of the molecule (McCready & McComb, 1952). This method was employed by Luh, Leonard & Dempsey (1954) and they showed that the amounts of pectic substances in three American varieties of tomato were generally similar to those given in Table 1. Stier, Ball & Maclinn (1956) also found, in ripe fruit of American origin, quantities of pectin comparable with those given in Table 1, with a continuous fall in pectin as the fruit became overripe. However, it is by no means certain that these three methods for the analysis of pectin determine the same chemical entity. This may explain in part some of the large variations in values that occur when results based on differing analytical methods are compared. However, when similar techniques are employed there appears to be quite close agreement in the pectin content of English and American varieties of tomato.

Evidence that the pectic acid chains in a number of plant species may not be exclusive polymers of D-galacturonic acid is accumulating. A number of non-uronide sugars appear to take an intimate part in the molecule as well (Williams & Bevenue, 1954; Aspinall & Fanshawe, 1961). Hence any method of

analysis based on pectic substances' being exclusively composed of galacturonic acid residues must lead inevitably to a low result. Until the composition and structure of the pectic substances in tomato fruit are known more exactly, and a value for the galacturonic acid content of a typical molecule worked out, results like those reported here, which are calculated on the assumption that pectin is exclusively composed of D-galacturonic acid residues, probably give low approximations of the true values.

Only infrequent accounts have appeared on the trend in activity of pectinesterase during the ripening of fruit (Ulrich, 1957). Experiments by Kertesz (1938) showed a twofold increase in activity as green tomato fruit became 'almost ripe' with a similar increase to the 'ripe' stage. However, the extraction of the enzyme was probably not quantitative. Pithawala *et al.* (1948) reported an increase in activity of about one-third in comparing green with ripe tissue, and each ripening series in Table 4 shows about a 40% increase during maturation.

The work of Hamson (1952) showed that firmer varieties of tomato fruit, as measured by a pressure tester, exhibited a greater activity of pectinesterase than softer ones. Since 'Immuna' became considerably less firm in all the later stages of ripeness than 'Potentate' (Hobson, 1959), it might be expected that 'Potentate' would have the greater enzyme activity of the two varieties, but the reverse situation was found to apply. Hall & Dennison (1960) were unable to show any significant correlation between pectinesterase activity and the firmness of tomato fruit, which is consistent with my own results (Hobson, 1962).

In the apparent absence of effective quantities of inhibitors in both normal and abnormal tissue, the relatively small drop in pectinesterase activity in 'blotchy' areas can hardly, it seems, be the exclusive cause of the failure of the hard, necrotic and discoloured regions of the tomato to ripen normally. Pectinesterase is only one of a complex of pectic enzymes present in the fruit, however, and preliminary results with polygalacturonase suggest much wider contrasts in activity between normal and abnormal tissue.

SUMMARY

1. A progressive fall in total and insoluble pectic substances occurred as 'mature' green tomato fruit ripened.

2. A physiological disorder, 'blotchy' ripening, resulted in an approximate doubling of the content of pectic substances in both apparently normal and the abnormal regions of affected fruit as compared with uniformly ripened fruit.

3. A rise in the activity of pectinesterase occurred as 'mature' green fruit became fully red. Areas showing 'blotchy' ripening had activities close to those usually found in the early stages of normal ripening.

4. It is concluded that the failure of areas of fruit to ripen normally is unlikely to be due primarily to the reduced activity of pectinesterase.

5. Less pectinesterase activity was found in the fruit from plants grown in potassium-deficient soil.

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The Metabolism of Progesterone by Animal Tissues *in vitro*

4. CONJUGATE FORMATION DURING THE METABOLISM OF [4-^{14}C]PROGESTERONE BY FEMALE-RAT-LIVER HOMOGENATE*

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It is well established that steroids that have undergone metabolism *in vivo* are excreted in urine and bile almost entirely in the form of water-soluble conjugates. However, of the many investigations that have been made of steroid metabolism *in vitro*, there have been relatively few reports of any extensive degree of conjugate formation. When conjugate formation has been reported to occur, yields were usually low (e.g. Isselbacher & Axelrod, 1955; Dutton, 1956; Fishman & Sie, 1956; Wotiz, Ziskind, Lemon & Gut, 1956). Previous studies of progesterone metabolism by liver *in vitro* have been carried out at low tissue:steroid ratios (1000:1 or less) (Wiswell & Samuels, 1953; Taylor, 1954) because of the low sensitivity of the analytical methods then available. With the introduction of 4-^{14}C-labelled steroids of relatively high specific activity, it has now become possible to work at much more nearly physiological levels. The metabolism of [4-^{14}C]progesterone has therefore been investigated at tissue:steroid ratios of about 56 000:1. In preliminary experiments, not reported in the present paper, it was found that water-soluble metabolites, probably conjugates, were formed when progesterone was incubated with female-rat-liver homogenate. Therefore it was decided to establish the optimum conditions necessary for conjugate formation during progesterone metabolism *in vitro* and to investigate the nature of the conjugates. Standard hydrolytic methods have been carried out after incubation, and the effect of adding co-factors known to be required for the formation of steroid glucuronides and steroid sulphate to preincubated and non-preincubated homogenates has been investigated. A known method of separating glucuronides and sulphates has also been applied to the conjugates before hydrolysis. Preliminary accounts of this work have been published (Cooke & Taylor, 1960, 1962).

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MATERIALS AND METHODS

Nicotinamide and ATP (L. Light and Co. Ltd., Colnbrook, Bucks), NAD$^+$, NADH, NADPH (C. F. Boerhinger und Soehne G.m.b.H., Mannheim, Germany), uridine diphosphate glucose (UDP-glucose), (98–100% pure, sodium salt, pentahydrate) and uridine diphosphate glucuronic acid (UDP-glucuronic acid) (98–100% pure, ammonium salt) (Sigma Chemical Co., St Louis, Mo., U.S.A.) and potassium hydrogen saccharate (laboratory grade) (British Drug Houses Ltd., Poole, Dorset) were all used without further purification.

The methods used for the purification of solvents were as described by Taylor (1954). Assay of radioactivity was carried out as described by Taylor & Scratcherd (1961). Throughout the present paper quantities of radioactivity are expressed as counts/min. actually determined.