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ELECTROPHORETIC INVESTIGATION OF ENZYMES FROM DEVELOPING *LYCOPERSICON ESCULENTUM* FRUIT

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Key Word Index—*Lycopersicon esculentum*; Solanaceae; tomato fruit ripening; plant proteins; electrophoresis, isoenzymes.

Abstract—Acetone powders were prepared from tomato fruit tissue sampled during development and the proteins were separated by polyacrylamide disc gel electrophoresis The gels were stained to show up general proteins, lipoproteins, glycoproteins and certain enzymes Minor changes in protein and glycoprotein patterns accompanied development Most enzymes exhibited more than one active band, with maximum diversity and specific activities usually appearing in extracts from mature green tissue and least with over-ripe tissue. The results support the view that enzyme synthesis accompanies the climacteric respiration rise at the expense of non-metabolic protein.

INTRODUCTION

VARIATIONS in the activities of many enzymes during the development of fruits have been extensively investigated with attention mainly being focused on the changes with ripening.[1–3] The key factors governing the initiation of the ripening phenomenon remain obscure but there is considerable evidence to show that the main biochemical changes during ripening result from a coordinated synthesis of selected proteins, probably involving degradation of other proteins, as in the tomato the total amount present on a fresh weight basis falls during development. Within this limit, a change in protein composition is probable, leading to the production of what has been called "the enzymes of ripening".[2]

Polyacrylamide disc gel electrophoresis is a useful techique for the study of fruit proteins as it is relatively simple, versatile and reproducible. The small amount of protein in tomato locule walls, together with the presence of a range of deleterious substances, necessitates preliminary concentration as an acetone powder and subsequent extraction with salt solutions.

The proteins from tomato fruit at closely defined stages of development were separated into their main components which were located by general protein stains. Further gels were specifically tested for the presence of certain terminal oxidases, dehydrogenases, esterases and hydrolases selected for examination because of their potential importance in the onset and furtherance of ripening. During ripening a number of the enzymes appeared to change in specific activity, often accompanied by characteristic changes in the isoenzyme patterns.

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RESULTS

Extraction procedures and general protein patterns

Acetone powders were prepared from tomato fruit at four stages of development, viz. small green (SG), mature green (MG—close to the beginning of the climacteric respiration rise), red (RED) and over-ripe (OR—post-climacteric), and the proteins extracted by an alkaline–salt solution containing sucrose. Although the amount of protein extracted per gram acetone powder did not alter significantly from stage to stage during development (Table 1), (which allowed direct comparison of enzyme activities in gels from different stages, on the basis of the fresh weight of tissue) there were significant differences between each successive stage. The proteins separated electrophoretically in acrylamide gels were subsequently stained to show the general distribution of components (Fig. 1). Common bands between the various stages of development were established by coelectrophoresis, and each stage gave a characteristic staining pattern containing constituents of widely differing charge and size.

TABLE 1. ACETONE-INSOLUBLE NITROGEN AND PROTEIN CONTENT OF TOMATO FRUIT OUTER LOCULE WALLS DURING DEVELOPMENT

	Stage of development				Significance level	Least significant difference at $P = 0.05$
	Small green	Mature green	Red	Over-ripe		
N content of acetone powder (mg per g)	7.8	6.6	7.2	7.1	$P < 0.05$	1.0
Protein extracted* from acetone powder (mg per g)	28.7	22.3	26.1	22.0	-	
N content per unit fresh weight (mg per 100 g)	48.4	22.4	18.3	15.3	$P < 0.001$	3.0
Protein extracted* per unit fresh weight (mg per 100 g)	178.1	75.7	66.0	47.7	$P < 0.001$	19.4

* For extraction details see Experimental section.

Most of the bands also showed up as glycoproteins although again the differences between the stages of development were small. Further gels were examined for lipoproteins and the result with MG extracts is given in Fig. 2(A). All eight bands also showed up with RED extracts, but OR fruit gave only four (*e–h*), and SG two (*b* and *e*). The positions of most of the active zones were confirmed by the use of fluorescein, but a number of additional regions also became coloured and this stain is, perhaps, less specific for lipoproteins than is Sudan black.

Detection of specific proteins as enzymes

Other gels were examined for indications of change in activity during the development of fruit for representative enzymes from various pathways thought to play a part in ripening. Tests were carried out for two terminal oxidases, tyrosinase and peroxidase, using catechol and benzidine, respectively, as substrates. Active zones on the gels for MG, RED and OR extracts coincided with the lipoprotein bands *e–h* in Fig. 2(A). Extracts from SG fruit showed only one zone at a similar position to band *e*. The substitution of guaiacol

for benzidine in tests for peroxidase revealed a further zone of activity at an R_{BPB} (relative mobility compared with bromophenol blue) of 0·12–0·15. Thus all the sites of tyrosinase activity and most of the peroxidase bands occurred on the gels at points coincident with those staining for lipoproteins.

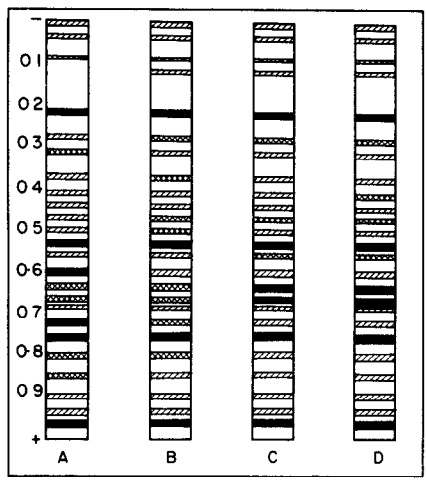

FIG 1 DISCONTINUOUS GEL ELECTROPHORESIS OF PROTEINS FROM TOMATO FRUIT DURING DEVELOPMENT
Amido black or Coosmassie blue was used as a stain after the separation of alkaline-salt extracts of proteins from, A, small green fruit, B, mature green, C, red and D, over-ripe fruit In this and the following Figures, the areas that are blocked-in represent heavily staining bands at these points on the gels, double hatching represents moderate staining, and single hatching represents light staining The proteins were separated anodally, and the numbers on the left of the Figure indicate the distance bands moved relative to bromophenol blue

The esterase pattern revealed that there were 13 centres of activity in MG fruit (Fig. 2(B), but band *d* was absent in SG and *c* and *f–h* absent in RED and OR fruit extracts. Acid phosphatase distribution was somewhat less complex and that for SG fruit is shown

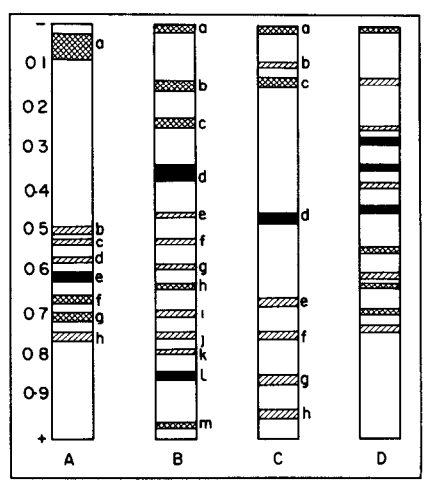

FIG 2 ELECTROPHORETIC SEPARATION OF TOMATO FRUIT PROTEINS
The gels were stained for, A, lipoproteins from mature green fruit, B, esterase from mature green fruit, C, phosphatase from small green fruit, and D, $NADH_2$-diaphorase from mature green fruit

in Fig. 2(C). A high level of activity at this stage was spread between eight bands and all of these except *f* and *g* were maintained in MG fruit Further reductions in activity later in development left only three regions (*a*, *c* and *d*) which showed up with the staining mixture.

A zone close to the mid-point of the gels with SG extracts responded to both β-glycerophosphatase and ATPase staining mixtures, and with both enzymes reaction was less intense later in development. Other weakly staining bands were detected but with glycerophosphatase these were exclusively in the lower half of the gels and with ATPase in the upper half

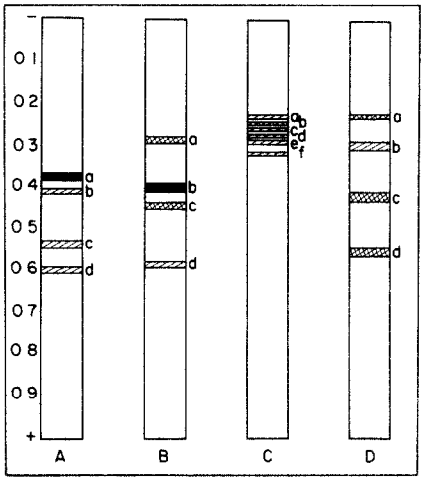

FIG 3 ELECTROPHORETIC SEPARATION OF TOMATO FRUIT PROTEINS
The gels were stained for A, fumarase, B, $NADP^+$-malic enzyme, C glutamic dehydrogenase, and D, phosphofructokinase All extracts were from mature green fruit tissue except C which was from small green fruit

Tests for $NADH_2$-diaphorases revealed a wide distribution of reactive sites and that for MG fruit is shown in Fig. 2(D). There were minor but characteristic reductions in both the number and intensity of the bands with extracts from all the other stages of ripeness More specific dehydrogenases were then examined starting with those from the tricarboxylic acid cycle. Fumarase separated as four zones of activity in MG extracts (see Fig 3(A)). but only bands *a* and *c* persisted in RED and OR fruit SG fruit extracts showed only one weakly staining band at position *a* Malic dehydrogenase showed little change in activity with development, there always being two reactive sites, a more intense one with an R_{BPB} of about 0·44 and a further weaker one composed of three bands between 0·49 and 0·55. The $NADP^+$-activated "malic" enzyme by which pyruvate is formed directly from malate was distributed between four centres of activity with MG fruit extracts, and this is illustrated in Fig. 3(B). Of these only band *c* gave a reaction for malic dehydrogenase as well. Band *c* was not visible with SG extracts and neither *c* nor *d* with RED or OR extracts. A single active zone for *iso*citric dehydrogenase in SG fruit (R_{BPB} 0·54) was augmented by another band (R_{BPB} 0·40) at the MG and subsequent stages of development The total activity progressively decreased towards over-ripeness

Glutamic dehydrogenase is often present in plant tissues in closely related isoenzymic forms[4] Figure 3(C) indicates that SG fruit extracts contained six zones of activity with

[4] YUE, S B (1969) *Plant Physiol* **44**, 453

band *b* staining the most heavily. A similar pattern emerged with MG fruit extracts but the total activity was considerably greater. RED tissue gave only weak responses confined to bands *a* and *b*, and with OR tissue activity persisted only in band *a*.

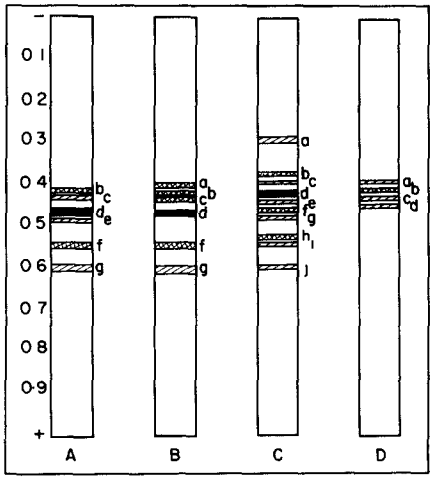

FIG 4 ELECTROPHORETIC SEPARATION OF TOMATO FRUIT PROTEINS
The gels were stained for, A, 6-phosphogluconate dehydrogenase, B, phosphoglucomutase, C, phosphohexose isomerase, and D, glucose-6-phosphate dehydrogenase All extracts were from mature green fruit tissue

Phosphofructokinase probably controls the rate of glycolysis by the Embden–Meyerhof scheme through activation by phosphate.[5] Four active zones were detected in MG extracts (*a–d* in Fig. 3(D)) but band *a* did not show up when RED or OR extracts were examined. SG fruit showed reaction only in zones *b* and *d*. No marked change in overall activity during development was apparent Control experiments were made to eliminate the possibility of interference from fructose-1,6-diphosphatase, fructokinase and adenylate kinase.

Four enzymes concerned with the metabolism of glucose were examined, namely 6-phosphogluconate dehydrogenase, phosphoglucomutase, phosphohexose isomerase and glucose-6-phosphate dehydrogenase. Comparative examples of the activities shown by MG fruit extracts are given in Fig. 4 It will be seen that on the basis of activity distribution the enzymes fall into two pairs, A with B and C with D. With the first pair, activity was maximal at the MG stage and the other three stages of development showed far fewer reactive zones. The second pair, on the other hand, were about as active at the SG stage as at the MG, activity only falling away towards over-ripeness The number of isoenzymes was also much more uniform throughout development.

Glutamate–oxaloacetate transaminase activity was found to be concentrated into four zones following electrophoresis and each of these was represented at the four stages of development. The total activity decreased throughout development. Leucine aminopeptidase also separated into four bands but their positions were not related to those of the previous enzyme. The combined activity of the peptidase reached a peak at the RED stage of development.

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DISCUSSION

The method adopted for the concentration of the protein fraction from tomato wall tissue provided a starting material that was essentially free from acetone-soluble carbohydrates, polyphenols, organic acids and most pigments thus minimizing protein denaturation. Calculated from the *N* content of the acetone powders, wall tissue contained only about 0.3% protein on a fresh weight basis for SG fruit and about half this figure for fruit later in development. The acetone powders themselves contained about 4% protein on the same basis, about half of which was brought into solution by the extraction medium. On balance, past reports[6] are in favour of a loss of protein during the ripening period. Davies and Cocking[7,8] examined the changes in protein levels in locule tissue on a "per cell" basis and concluded that there were two main synthetic periods, the second coincident with the climacteric respiration rise. In view of the apparent continuing increases in cell size, comparisons of these results with others is difficult.

Visual inspection of the gels allowed comparative classification of the depths of staining shown by the active zones. In a proportion of cases the depth of stain was confirmed by densitometry, or, in the case of acid phosphatase by eluting the coloured product and measuring its absorbance.[9] However, data on comparative enzyme activities from the gels is at best semi-quantitative, although the present results are supported by published activity figures for peroxidase,[10,11] acid phosphatase,[12] malic dehydrogenase[13] and phenolase[14] during tomato fruit ripening.

Terminal oxidases such as tyrosinase and peroxidase increased in activity considerably during maturation, the extra activity being in part associated with isoenzymes of lower molecular weight. Extensive purification of peroxidase by Ku *et al*.[11] prior to electrophoresis allowed the detection of several further bands of activity additional to those reported here.

The isoenzyme patterns of esterase and phosphatase have often been used as biochemical markers in genetic studies.[15,16] The specificity with the commonly used substrates is often not very high but this approach can often give meaningful results. The phosphatase band near the middle of the gel (band *d* in Fig. 2(C)) also showed activity during tests for β-glycerophosphatase and ATPase, whereas it is possible that all three enzymes separate at the same point on the gel, a broad specificity by a single enzyme species is more likely.

Tests for specific enzymes need to be accompanied by control experiments, especially where one of the products of a reaction forms the substrate for a second enzyme. The multiple specificity of certain bands was demonstrated by cutting the gel in half lengthways after protein separation and subjecting each part to the appropriate stain.

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The relative importance of the Embden–Meyerhof and the hexose monophosphate pathways for glycolysis has been studied by radiochemical tracer techniques.[17] The latter scheme, it was concluded, assumed increasing importance with ripening. From the present work the first enzymes in the hexose monophosphate pathway do appear to be particularly active at the MG stage, but impressions gained merely from results with the two enzymes investigated may be misleading.

The proliferation of isoenzymes during development might be caused by sub-unit formation brought about by electrophoresis. However, not all the additional bands were of lower molecular weight than the original zone, and separations carried out under non-dissociating conditions, i.e. in the presence of 10 mM 2-mercaptoethanol,[18] in no case affected the number of bands shown up.

It is clear that the onset of the climacteric respiration is brought about by the release of some constraint which controls the respiration prior to this point. In the tomato there is some evidence to support the concept[19] that at a certain point in development the production of ethylene rises. This promotes metabolic changes leading to the climacteric rise in respiration and concomitant synthesis of new enzymic protein. These new enzymes of ripening[2] apparently come from storage of structural protein as a fall in total protein accompanies ripening. Studies on the enzyme patterns during the development of fruits,[20–22] experiments using protein inhibitors[20,23] and evidence on the synthesis of specific enzymes[24,25] provide a basis for this interpretation.

A significant number of the enzymes examined in this study show a maximum diversity of components in extracts from MG tissue, tissue that was taken from fruit which contained, on average, sufficient internal ethylene to allow the first signs of colour change to occur within 48 hr. Further enzymes gave indications of maximum specific activity at the MG stage within a constant number of components. Tissue taken from fruit about 10 days prior to the MG stage, and referred to as large green (LG), showed none of the physical signs of ripening and would almost certainly have contained a very low ethylene concentration. Tests with this LG tissue indicated that whereas some enzymes already showed patterns typical of MG fruit (e.g. esterase and 6-phosphogluconate dehydrogenase), others (e.g. glutamate dehydrogenase and peroxidase) were still characteristic of SG fruit. These observations lead to the suggestion that during development isoenzymes are formed specifically to carry out the changes associated with the final stages of the process. As the full complement of isoenzymes is accumulated, perhaps mediated by ethylene, the tissue then moves into the ripening period with its attendant respiration rise. By the time the full red colour is attained by the fruit many of these isoenzymes have disappeared perhaps because their function is over.

EXPERIMENTAL

Polyacryamide gel electrophoresis Analytical electrophoresis was carried out in 10 × 0·5 cm precision-bore glass tubes in conjunction with a Quickfit & Quartz Ltd P A G E apparatus The separation gel (1·4 ml in each

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tube) was based on system no 1 described by Maurer[26] except that additional HCl was added to the first component mixture to bring the PH to 8 3, and the proportion of H_2O increased to 1 6 parts to give 7% acrylamide concentration Immediately prior to use 0 3 ml of a spacer-gel based on system no 19[26] but substituting 3 5 parts of 50% sucrose for the H_2O[27] to give a 2 67% acrylamide concentration was polymerized on top Anode and cathode buffer solns consisted of 0 05 M Tris–0 4 M glycine pH 8 3 diluted with 2 vol H_2O before use, and 0 1 ml of a 0 01% solution of bromophenol blue was added to the cathode buffer as a tracking dye, allowing relative mobility to be calculated for the various zones of activity

Extraction and separation The general principles recommended by Clements for banana tissue[28] were followed for the preparation of acetone powders from the outer locule walls of tomatoes (*Lycopersicon esculentum* Mill, cv Amberley Cross) Definitions of the various stages of development have already been given[1,29] Acetone powders were stored under vacuum at −20° to delay denaturation The proteins were extracted overnight by adding 10× the wt of 0 3 M K phosphate, 0 05 mM K_2EDTA and 0 1 M KCl pH 8 The tissue was squeezed in a nylon bag and the resulting liquid centrifuged at 0°, mixed with 0 125 vol of 80% sucrose sol and 0 225 ml placed on top of the spacer gel Electrophoretic separation was carried out anodally for about 4 hr at 3 mA per tube at 2°, with coolant at 0° circulating through a coil in the anode compartment

Staining and associated techniques Naphthalene black 12B (0 5% in 7% HOAc) and Coomassie brilliant blue R-250[30] (differentiating in 7% HOAc MeOH, 1 1) were used as general protein stains Lipoproteins were detected with a 1% soln in Sudan black B in acetone H_2O (3 1) used overnight alternatively a 0 1% soln of 2 7-dichlorofluorescein in EtOH–H_2O (1 1) for 1 hr followed by differentiation in 3% HOAc Glycoproteins were stained by the method of Felgenhauer,[31] leaving the gels in Schiff's reagent for at least 3 days Tyrosinase was detected by prolonged exposure to 1 5 mM catechol and L-proline in 80% EtOH, and peroxidase by guaiacol[32] or benzidine[33] (carcinogenic) Esterases were located using Fast blue[34] and confirmed with Fast red[35] Acid phosphatase distribution was detected using α-naphthyl phosphate as substrate[35] after soaking the gels in NaOAc buffer pH 5 5 at 0° for 2 hr, glycerophosphatase according to Burstone[36] using mixed isomers as substrate, and ATPase according to Hall[37] Standard methods were used for $NADH_2$-diaphorase[38] malic dehydrogenase[39] (except that 0 45 M Tris–HCl pH 7 1 was used as buffer), glutamic dehydrogenase,[33] glutamate-oxaloacetate transaminase,[33] phosphoglucomutase[33] and phosphohexose isomerase[33] For fumarase,[33] 6-phosphogluconate dehydrogenase,[33] $NADP^+$-dependent "malic" enzyme,[40] leucine aminopeptidase[36] aldolase[41] (confirmed by method A[42]) glucose-6-phosphate dehydrogenase[33] and phosphofructokinase, the gels were soaked in NaOAc buffer prior to development To retain activity with these last two enzymes 10 mM 2-mercaptoethanol was added to the extractant soln and for phosphofructokinase to the cathode buffer as well The staining mixture for phosphofructokinase[43] was incorporated in an agar overlay[33] Densitometer tracings were made using a Vitatron TLD instrument; total nitrogen by the method of Biale *et al*[44] and protein nitrogen by the method of Lowry *et al*[45] using bovine serum albumin as standard

Acknowledgement—The skilful technical assistance of Mrs J Finney in this work is greatly appreciated

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