MULTIPLE FORMS OF THAUMATIN: STRUCTURE AND BIOSYNTHESIS.

A thesis submitted in accordance with
the requirements of the University of London for
the Degree of Doctor of Philosophy.

by

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FEBRUARY 1981

DEPARTMENT OF BIOCHEMISTRY

ROYAL HOLLOWAY COLLEGE

A C K N O W L E D G E M E N T S

The author acknowledges with gratitude the guidance, encouragement and assistance given by his supervisors, Professor J. B. Pridham and Dr. A. Mackenzie and is indebted to other members of the Department of Biochemistry who have advised and helped him during the course of this study, especially to Dr G. Douglas and Dr. D. Settachan. Many thanks are also due to Dr. J. D. Higginbotham and to other staff at the Philip Lyle Memorial Laboratories, Reading for their guidance and for the supplies of fruit without which these studies would not have been possible.

The author is grateful for the financial support of the Science Research Council and of Tate and Lyle Limited.

A B B R E V I A T I O N S

In general the abbreviations and symbols employed in this thesis are those described in the Instructions to Authors for the Biochemical Journal (Biochem. J. (1978) 169, 1-27).

Hepes	N-2-hydroxyethylpiperazine - N'2-ethane-sulphonic acid.
PPO	2,5 - Diphenyloxazole.
BNPS	2-(2-nitrophenylsulphenyl)-3 methyl-3'-bromoindolenine.
ATP	adenosine 5'-triphosphate.
DNA	deoxyribonucleic acid.
DTT	dithiothreitol.
GTP	guanosine 5'-triphosphate.
hnRNA	heterogenous nuclear ribonucleic acid.
mRNA	messenger ribonucleic acid.
MAK	methylated albumin on Kieselguhr.
PBS	phosphate-buffered saline (0·8% NaCl, 0·02% KH_2 PO_4, 0·02% KCl, 0·12% Na_2H PO_4)
P.A.G.E.	polyacrylamide gel electrophoresis.
oligo (dT)	polythymidylic acid.
poly (U)	polyuridylic acid.
RNA	ribonucleic acid.
Na DOC	sodium deoxycholate.
SDS	sodium dodecyl sulphate.
TCA	trichloroacetic acid.
EDTA	ethylenediaminetetraacetic acid.
EGTA	ethyleneglycol-bis-(β-aminoethyl ether) N,N'-tetraacetic acid
TEMED	N,N,N',N'-tetramethylethylenediamine.

N.Z.W.	New Zealand White.
MWt	molecular weight.
2-ME	2-mercaptoethanol.
bisacrylamide	N,N'-Methylene-bis-acrylamide.
Tris	Tris(hydroxymethyl)aminomethane.

ABSTRACT

The aim of the investigation was to determine the structural and biosynthetic relationships between the various forms of the sweet protein, thaumatin, which are found in the tropical plant, Thaumatococcus daniellii.

Three thaumatins, TO, TIand TII were isolated from the aril tissue of Thaumatococcus fruits in, apparently, homogenous forms and the structural relationships between them were investigated using various protein analytical techniques. Antibodies to thaumatin II were raised and used to measure the immunological relatedness of the three proteins by quantitative precipitin and micro-complement fixation methods. The evidence suggests that the thaumatins have almost identical amino acid sequences and conformations. The observed differences in isoelectric points are consistant with the view that the thaumatins represent forms of the same protein which differ only in their relative amide contents.

Studies on the content of Thaumatococcus fruits at different stages of maturation showed that the amount of thaumatin increased during development and that in the ripe fruit up to 60% of the protein in the aril tissue was thaumatin. Interestingly fruits from the Ashanti region of Ghana have roughly similar TI and TII contents together with much lower amounts of TO whilst Kadjebe region material does not contain any TII but has correspondingly larger amounts of TI and TO.

The hypothesis that the thaumatin forms arise by interconversion of a common precursor was tested. No interconversion was observed under conditions of high salt concentration or high pH or in the presence of aril protein extracts.

Polysomes and RNA preparations isolated from aril tissue directed protein synthesis in wheat germ and nuclease-treated reticulocyte lysate systems. SDS P.A.G.E. analysis of the radiolabelled product precipitated by anti-TII showed that only a small proportion (0.05%) of the total protein synthesised was thaumatin. The proportion of thaumatin synthesised was similar in the case of poly(A)^{plus} RNA preparations from ripe and unripe Ashanti and ripe Kadjebe region fruits. Poly(A)^{minus} RNA preparations directed synthesis of a similar proportion of thaumatin to total protein as poly(A)^{plus} RNA.

Various methods were used to determine whether the immunoprecipitable product was a single or a mixture of thaumatins but this problem remained unresolved.

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VII. GENERAL CONCLUSIONS

I N T R O D U C T I O N

I. SWEETENERS.

A. THE SWEET TASTE DETERMINANTS.

Sweetness is a quality of food that is greatly preferred by most humans, this preference is probably inborn since it seems to appear as early as the fourth foetal month.(1)

Unfortunately, sucrose can have serious deleterious effects on human metabolism if taken in excess. One problem is that the metabolism of sucrose yields a relatively large amount of energy. It therefore can contribute to a significant problem in over-nourished individuals where excess energy is stored, principally in the form of fat. Consequently it is desirable that sweeteners with good taste properties and which have no appreciable effect on metabolism should be developed.(2)

The basis of sweetness is the ability of a molecule to interact specifically with certain taste receptors. Man possesses up to half a million taste cells in the mouth which are clustered into groups of 40-60 to form taste buds. Each taste bud has projecting microvilli ($0{\cdot}2\mu$ x $2{\cdot}0\mu$) which are in contact with saliva in the mouth. It is most likely that the receptor sites for sweet molecules are associated with the membranes of the microvilli on the tongue.

Many different molecules can bind reversibly to the taste bud membrane and produce taste effects. The principal tastes are salt, sour, bitter and sweet. Salt tastes are generally caused by alkali and alkaline earth cations whilst hydrogen ions play the dominant role in producing the sensation of sourness.(3) Sweet and bitter molecules are much more complex and therefore can display much higher specificities in their interactions with the taste cell membranes. Not all sweet binding sites on the tongue are identical since some react better with fructose than glucose while others do

the opposite. Thus it seems that the microvilli have many varied receptor sites capable of binding taste molecules which produce conformational changes in the membrane which in turn lead to eventual taste responses. The conformation of the sweet tasting molecule is also important and this may be altered by the binding reaction.[4]

Sweet substances have been examined extensively in attempts to find their common determinants. Shallenberger and Acree[5,6] point out that most sweet molecules have an electronegative atom B and a polarised system A-H with a distance between them of about 3Å. However the D-amino acids (A-H = NH_3^+; B = CO) histidine, leucine, phenylalanine and tryptophan are sweet whilst their L-enantiomorphs are not. This suggested that the receptor also possessed stereospecificity which required a third binding site(X). In 1972 Kier[7] postulated that this third site was hydrophobic in nature and from studies of the sweet amino acids that it was positioned 3·5 Å from the A-H site and 5·5 Å from the B site (Fig.1). This valuable picture of the structure-taste relationship in sweet molecules is still not finalised. It is not possible, for example, to predict from molecular structure the intensity of sweetness of a molecule or even whether it will be sweet at all. Furthermore the model cannot account for the sweetness of dianhydrofurano trehalose[8] which does not contain an A-H, B system with the usual interatomic (A→B) distance of 2·8 - 2·9 Å (Fig. 2).

To date it has not been possible to purify the sweet receptor site from tongue membranes[9,10]. However, it has been found that antibodies to the sweet protein, thaumatin, react with other sweet molecules[11,12] so that it is possible that anti-thaumatin may in the future provide a model system for

Figure 1 The sweet site: atomic pattern.

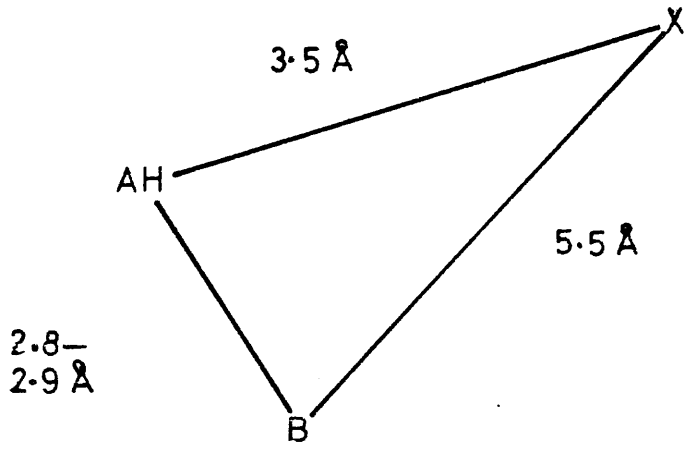
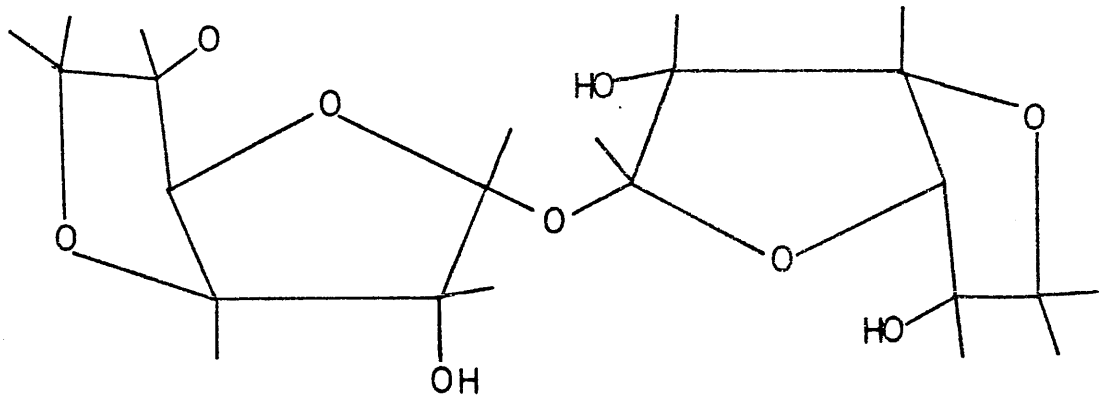


FIGURE 2 The molecular structure of 3,6,3',6'-dianhydro-(furano)-α,α-trehalose.



studying the sweet taste receptor.

B. SWEET TASTING MOLECULES.

The major sweeteners used in the food and drink industry at the present time are sucrose and saccharin. However, industry has a great interest in alternative sweeteners for four major reasons. Firstly, the consumption of sucrose in large quantities can be a major cause of obesity, with attendant diseases, and of dental caries. Secondly, diabetics must have a diet which is strictly controlled with respect to sucrose consumption. Thirdly, since sucrose is used in large quantities considerable commercial savings can be made by reducing its use whilst maintaining the level of sweetness in a given product. Finally, saccharin has a poor taste quality on its own and its safety as a food additive is suspect.

The cost problem can be partially solved by use of corn syrups which are produced by enzymic hydrolysis of corn starch[13]. Unfortunately the major constituent of corn syrups is glucose which has only about half the sweetness of sucrose and so twice as much must be used in foodstuffs and, hence, there is a disadvantage in using corn syrups with respect to caloric content. Glucose can be converted to fructose, which is nearly twice as sweet as sucrose, by enzymic isomerization[14]. High-fructose corn syrups are now finding wide spread applications especially in the soft drinks market[15]. Unfortunately fructose is suspect as a causative agent in atherosclerosis and hyperuricaemia. (198,199)

Other naturally occuring mono and di-saccharides which occur in the diet and which taste sweet are lactose, maltose, isomaltose and α- D -mannose. However, none of these are as sweet as glucose and they would, therefore, have to be used in relatively large quantities which might in some instances prove harmful. (Table 1. shows the relative sweetness of some naturally occuring dietary sugars).

Table 1 The relative sweetness of naturally occuring dietary sugars

Sugar	Sweetness relative to sucrose
Sucrose	1.0
α-D-Glucose	0.5
β-D-Fructose	1.7
Lactose	0.2
Maltose	0.3
α-D-Mannose	-0.4
Isomaltose	-0.3

In contrast to the carbohydrate sweetening agents the artificial sweeteners are all intensely sweet. Of the known materials, none of which bears any obvious structural resemblance to the sugars, only saccharin and cyclamate have been permitted for food use in the United Kingdom. Cyclamate has, however, been banned since 1970 because of evidence that it can induce bladder tumours in animals when given in very high dosage together with saccharin (200). Besides being intensely sweet the artificial sweeteners also differ from the carbohydrate sweeteners in that they are non-nutritive. This is an advantage in dietary control. Since it is calorie-free and relatively cheap due to its extreme sweetness, saccharin is a great boon to the food manufacturers. (Table 2 shows the relative sweetness of some artificial sweeteners).

A synthetic dipeptide L-aspartyl-L-phenylalanine methyl ester (aspartame) which is 250 times sweeter than sucrose, was under development by the Searle Chemical Company and appeared to be a potential sweetener for future mass marketing. Unfortunately it proved to be too toxic for human consumption.

The naturally occuring non-carbohydrate sweeteners have attracted a great deal of industrial attention during the last fifteen years. These materials which are natural dietary constituents are obviously less suspect regarding toxicity than synthetic compounds.

Stevioside, for example, is a glycoside with a steroid aglycone which is obtained from the small shrub, Stevia rebaudiana which is used by the natives of Paraguay as a sweetener. Unfortunately the steroid aglycone is responsible for potent biological side effects and so stevioside has been rejected by food manufacturers in the Western world.

Table 2 The relative sweetness of the artificial sweeteners.

Sweetener	Sweetness relative to sucrose (sucrose=1)	Type of molecule
p-anisyl urea	15	aromatic
cyclamate	30	cyclic
chlorcform	40	acyclic
p-ethoxyphenyl urea (Dulcin)	200	aromatic
saccharin	300	aromatic
1-bromo-5-nitroaniline	700	aromatic
5-nitro-2-r-propoxyaniline	4000	aromatic

Glycyrrhizin, another natural glycoside, is obtained from licorice root. The use of this compound is also limited because of its harmful physiological activity and, in addition, it has a rather undesirable taste.

Naringin dihydrochalcone and neohesperidin dihydrochalcone can be obtained by alkaline hydrogenation of the parent glycosides present in orange peel. Both of the dihydrochalcones possess a Rhamnose - Glucose disaccharide residue which appears to be essential for their sweetness. The isolated disaccharide is not sweet. The dihydrochalcones appear to have no unpleasant physiological side effects but in terms of sweetness they are more expensive to produce than saccharin.

Finally, in the last decade, the intensely sweet tasting proteins thaumatin and monellin and the taste modifying glycoprotein miraculin have been discovered. These materials will be more fully discussed in section I C (Table 3 shows the relative sweetness of the natural sweetening agents).

C. SWEET TASTING PROTEINS.

General. Until recently no macromolecule was known to act as a specific taste stimulus in man, and proteins were considered to be tasteless. However, three taste active proteins, all obtained from plants of West African origin, are now known. Two of the proteins, monellin and thaumatin can be described as chemostimulatory and the third, miraculin (a glycoprotein), as taste-modifying. Monellin and thaumatin are intensely sweet whilst pre-treatment of the tongue with miraculin changes the normal taste sensation of acids from sour to sweet.

It seems likely from antibody binding studies that thaumatin and monellin have similar sweet taste determinants[11,12]. Miraculin probably exerts its effect by a different mechanism.

Table 3. The relative sweetness of natural sweetening agents.

Sweetener	Sweetness relative to sucrose (sucrose=1)
Stevioside	300
Glycyrrhizin	100
Monellin	≐2,000
Thaumatin	≐3,000
Neohesperidin dihydrochalcone	2,000

1. Miraculin

In 1852 W. F. Daniell[16] reported the astonishing properties of the 'miraculous berry of Western Africa' but it was not until 1965 that it was 're-discovered' by Inglett et al[17]. The shrub which bears miracle fruit, Richardella dulcifica (Schum and Thonn), family Sapotaceae, is indigenous to tropical West Africa from Ghana eastwards to the Congo[18]. When grown from seedlings it takes 3 - 4 years before fruiting occurs[19], growing slowly and eventually reaching 6 - 15 feet in height. The branches bear single fruits which are typically 2 cm long, bright red and ellipsoidal. The taste-modifying substance, miraculin, is found in a thin layer of pulp sandwiched between the skin and the large olive-shaped seed. Miraculin, when taken orally, has the property of sweetening sour - or acid-tasting food or drink. The effect is persistent, lasting for several hours and can also modify the overall flavour perception, for example, changing vinegar into a port-like drink.

Miraculin is present only in low concentrations in the fruit and it is, therefore, fairly difficult to purify. It has now been established that miraculin is a glycoprotein of molecular weight $42,000 \pm 3,000$ comprising about 370 amino acid residues[20]. It has a relatively high isoelectric point of about 9. The carbohydrate portion of miraculin has not been extensively studied but probably constitutes about 6% of the molecule[20,21]. Miraculin is inactivated below pH3 and above pH12 and it is sensitive to temperature changes.

Various modes of action have been proposed for miraculin. Kurihara and Beidler[22] proposed that an acid enviroment changed the shape of the molecule sufficiently to allow the carbohydrate moiety to interact with the sweet taste receptor. Dzendolet[23]

suggested that miraculin blocked the sour receptor sites allowing a sweet taste to be generated by the anionic groups of acid molecules.

Attempts have been made to market a miraculin-containing product in the United States but unfortunately miraculin was eventually denied food additive status.

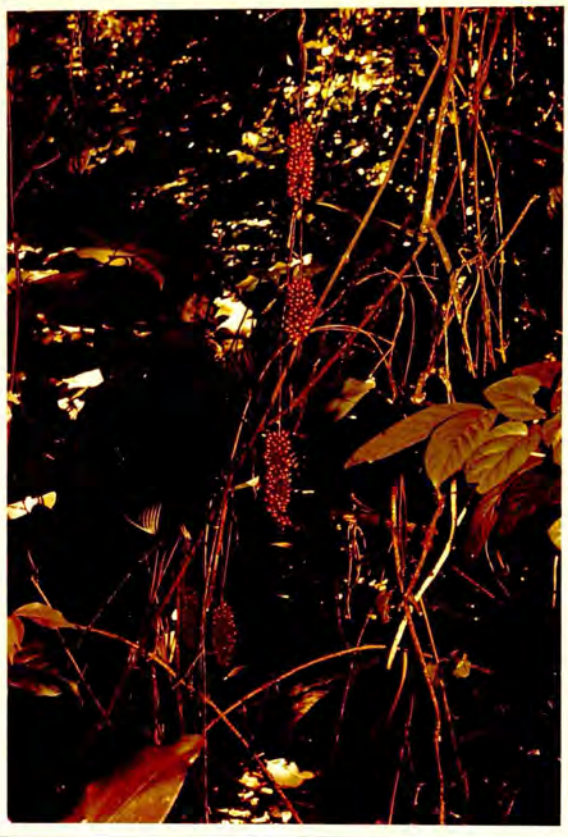
2. Monellin.

The berries of the West African plant Dioscoreophyllum cumminsii were first reported to be intensely sweet in 1895[24]. Irvine[18] made a more detailed description of the plant in 1961 and this was followed by its inclusion in a list of tropical plants with unusual taste properties by Inglett and May in 1968[25]. They named the fruit the 'serendipity berry'.

Dioscoreophyllum cumminsii (family Menispermaceae) is a herbaceous perennial which bears male and female flowers on separate plants. Only fruits from plants found in tropical West Africa, the Sudan and equatorial Africa have been reported to be sweet tasting. However plants bearing non-sweet fruits are found in Rhodesia, Mozambique and Kenya[26]. The plants have heart-shaped leaves which are supported by long, vine-like stems and they grow amongst humid, understorey vegetation in closed forests. The stems grow from tubers at the onset of the rainy season but the aerial vegetation dies down during the dry season. Female plants produce grape-like clusters of small, red berries (up to 100 in each bunch) which are mainly found in deep shade under the bushes from July to October (Fig. 4). Each berry has a thick, fleshy skin which covers a thorny seed surrounded by a whitish mucilage, which tastes extremely sweet. The plant is not cultivated nor is it generally eaten, except by the people of Zaire who also eat the tubers which resemble small yams[26]. Plants of Dioscoreophyllum cumminsii can be reproduced

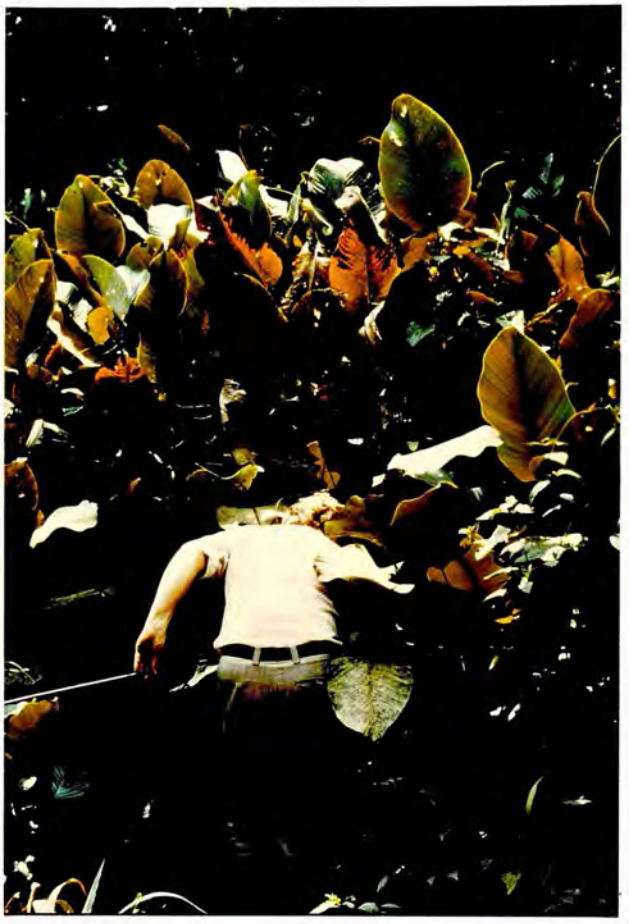
Figure 4 Examples of Dioscoreophyllum cumminsii and Thaumatococcus daniellii plants.

A



A Fruits of Dioscoreophyllum cumminsii in situ (serendipity berries).

B



B A dense patch of Thaumatococcus daniellii plants.

C

C Young fruits, flowers and rhizomes of Thaumatococcus danielli.

D

D Ripe fruit of Thaumatococcus danielli. (Kadjebe region type , centre; Ashanti region type, left and right)

E

E Thaumatococcus danielli fruits at different developmental stages. (Kadjebe type) Unripe, left (stage A); intermediate, centre (stage B); ripe, right (stage D).

from seed, tubers or stem cuttings.

Inglett and May[27] reported initially that the sweet essence of 'serendipity berries' was a high molecular weight carbohydrate. The sweetness was said to be about 1,600 times greater than that of sucrose which aroused worldwide interest in the material as a possible commercial sweetener. In 1972[28,29] two groups working independently characterised the sweet substance as a protein and it was named 'monellin'.

Initial characterisation of monellin was carried out by Morris and Cagan and Van der Wel and Loeve[28,29,30,31].

The discovery that proteins could taste sweet made elucidation of the primary structure of monellin an interesting objective since it was expected that this would shed light on the structural character of the sweet taste determinant. Three amino acid sequence studies have been published[32,33,34]. Monellin has two non-covalently bound subunits, A and B, of 44 and 50 amino acid residues, giving a molecular weight of approx. 11,000. The primary sequence is shown in Figure 3. The isoelectric point of monellin was found to be 9·03.

Heating the molecule above 55-65°C caused loss of the sweet taste[31] and enzymic hydrolysis yielded no sweet peptides[35].

Some sweetness is retained after limited proteolysis with carboxypeptidase[36]. Denaturation of monellin with 8M Urea or with SDS results in irreversible loss of sweetness but 6M guanidine hydrochloride treatment allows full recovery of sweetness[37]. When separated neither the A or B chains of monellin taste sweet[32] even at high concentrations. However the two chains, on remixing, slowly generated a sweet taste which never reached full initial sweetness intensity. Twenty to forty percent of the lysine groups of monellin can be methylated without loss of sweetness but further

Figure 3 The primary sequences of chains I and II of monellin.

Subunit I

Arg-Glu- Ile-Lys-Gly-Tyr-Glu-Tyr-Gln-Leu-Tyr-Val-Tyr-Ala-Ser-Asp-Lys-Leu-Phe-Arg-Ala-Asn-Ile-Ser-Gln-Asn-Tyr-Lys-Thr- ? -Gly- ? -Arg-Leu-Leu-Arg-Phe- ? - ? - ?

Subunit II

Gly-Glu-Trp-Glu-Ile-Ile-Asp-Ile-Gly-Pro-Phe-Thr-Gln-Asn-Leu-Gly-Lys-Phe-Ala-Val-Asp-Glu-Glu-Asn-Lys-Ile-Gly-Gln-Tyr-Gly-Arg-Leu-Thr-Phe-Asn-Lys-Val-Ile-Arg-Pro-Cys-Met-Lys-Lys-Thr-Ile-Tyr-Glu-Glu-Asn

(FROM BOHAK AND LI REF 32)

methylation results in gradual disappearance of taste[38]. All of these results suggest that the factor(s) responsible for the sweet taste of monellin are not present in a simple peptide sequence alone. It appears that secondary, tertiary and quaternary structural integrity is very important in producing sweetness.

Work with various animals by Hellekant et al[39,40] has shown that monellin is not perceived as sweet by guinea pigs, rats, hamsters, dogs, pigs, rabbits or the New World monkey Saguinas midas tamarin[41]. The evidence was gathered by behavioural observations and by electrophysiological recordings. However, the Old World monkeys Cercopithecus aethiops and Macaca fasicularis. both of which, like man, belong to the infraorder Platyrrhina were found to respond to monellin in such a way as to make it seem that they experience the same taste sensations as man. All of the animals tested responded to sucrose, suggesting that monellin has a separate receptor site at which it elicits its sweet taste and which is absent in some species.

Monellin has not been developed further as a commercial sweetening agent since it has serious disadvantages which make it less attractive than alternatives. Firstly, it is unstable under normal storage conditions. Secondly, it has inferior taste qualities and thirdly, the yield of monellin from the fruit is low.

3. Thaumatin

(i) Occurrence. The fruit of Thaumatococcus danielli has long been known for its intensely sweet tasting aril. The plant was first described by Daniell in 1855[42], who reported that the sweet property of the fruit was then already well known and utilised for sweetening breads and wines in West Africa .

The plant grows well without special attention but it is encouraged, not for its fruits but for its large tough leaves which

are used for wrapping foodstuffs. It is common throughout the West African rain-forest zone from Sierra Leone to Zaire and has been reported to be present in the Princes Islands, the Central African Republic and Angola[43]. The photographs in figure 4 show examples of the plant, its fruit and flowers.

Thaumatococcus danielli is a perennial tropical herb which forms part of the ground flora of forests. It belongs to the monocot family Marantaceae. The plants, which are hermaphrodite, bear spike inflorescences at the junction of the swollen petiole base with the rhizome. Up to twelve pairs of purplish-pink flowers form on each inflorescence but usually only 2 to 3 form mature fruits. The majority of fruits have a triangular shape formed by three fleshy pericarp segments which turn from green to red during ripening. Within each fruit there are up to three seeds (generally three) which also change colour during ripening from white to black. At the point of attachment of each seed to the fruit is a yellowy/white fleshy aril. The seeds are surrounded by a gel layer. Sweet protein is found only in the aril tissue.

The sweet property of the arils was 'rediscovered' in 1968 by Inglett and May[25].

(ii) Molecular and Physiological Properties The characterisation of the sweet principle from Thaumatococcus danielli began with the report that it could be extracted from the aril with water[25].

In 1972 Van der Wel and Loeve[44] reported purification and characterisation of the sweet material which they found was composed of two proteins which were designated thaumatin I (TI) and thaumatin II (T II).

T I and T II were purified from an aqueous aril extract by ultrafiltration, gel filtration and ion-exchange chromatography.

Sweetness evaluation of T I and T II by a taste panel gave

identical values for both proteins. The procedure adopted was to determine the concentration of thaumatin required for iso-sweetness with a 7% sucrose solution. On a weight basis thaumatin was 1,600 times sweeter than sucrose and on a molar basis it was 10^5 times sweeter.

T I and T II were both shown to be proteinaceous by their characteristic ultraviolet absorption spectra, the presence of almost 100% polypeptide material (as determined by the biuret method, the 100% yield of amino acids on acid hydrolysis, by their positive reaction with amido black and by the disappearance of their sweet taste after trypsin digestion).

T I and T II were reported to have pI values of almost 12 as judged from starch gel electrophoresis at different pH values and because they were precipitated at pH 12. This was an unexpected finding since the amino acid analysis of the thaumatin proteins showed a normal number of basic and acidic amino acids apart from the absence of histidine. It is more usual to find that proteins with very high pI values like the histones or protamine, for example, have a high proportion of basic amino acids.

Analysis of both T I and T II showed that alanine was N-terminal. Molecular weight determination by ultracentrifugation gave similar molecular weights of 21,000 $\pm$ 600 and 20,400 $\pm$ 600 for T I and T II, respectively. Estimation of molecular weights by gel filtration on Sephadex G - 50 gave 19,500 $\pm$ 1,900 (T I) and 18,000 $\pm$ 1,800 (T II). The procedure of Ellman[45] was used to show that both thaumatins contained no free sulphyryl groups. Since both thaumatins contain 16 cysteine residues they must be present as cystine which presumably gives rise to a highly rigid tertiary structure.

Circular dichroism and proton magnetic resonance measurements indicate that reversible conformational changes occur with both

proteins as the temperature is raised but that at certain temperatures, which vary with pH, irreversible heat denaturation occurs[46]. The irreversible heat-induced transition seems to be responsible for the loss of sweetness of the proteins. The spectrophotometric evidence suggests that tyrosine residues and at least one disulphide chromophore are involved in the conformational change.

The primary structure of T I has now been determined by Iyengar *et al*[47]. The protein consists of a single polypeptide chain of 207 residues. In order to arrive at the complete sequence it was found to be necessary to sequence, automatically, fragments produced by the following treatments: cyanogen bromide cleavage, BNPS - skatole cleavage and trypsin, *Staphylococcus* V8 protease and chymotrypsin digestions. Figure 5 gives the primary sequence of thaumatin I.

Using the method of Chou and Fasman[48], Iyengar *et al*[47] found that it was possible from the known amino acid sequence of thaumatin I to predict that the protein had a low α-helix content which agreed well with the published spectroscopic data[46]. The β-sheet and β-bends contents were predicted to be high.

Two groups of workers have raised antibodies to T I in rabbits. Hough and Edwardson[11] were in the process of developing a sensitive assay for thaumatin in dilute solution using radioimmunoassay when they discovered that saccharin (present as part of a soft drink formulation) was capable of interfering with the thaumatin-anti-thaumatin reaction. They extended this work to other sweet materials, including monellin, and found that they all reacted with anti-thaumatin. The degree of reaction showed a close relationship to the relative sweetness of the molecule.

Table 4 shows the relationship between cross-reactivity with

FIGURE 5 The primary structure of thaumatin I

NH_2-ALA-THR-PHE-GLU-ILE-VAL-ASN-ARG-CYS-SER-TYR-THR-VAL-TRP-ALA-ALA-ALA-SER-LYS-GLY-ASP-ALA-
ALA-LEU-ASP-ALA-GLY-GLY-ARG-GLN-LEU-ASN-SER-GLY-GLU-SER-TRP-THR-ILE-ASN-VAL-GLU-PRO-GLY-
THR-ASN-GLY-GLY-LYS-ILE-TRP-ALA-ARG-THR-ASP-CYS-TYR-PHE-ASP-ASP-SER-GLY-SER-GLY-ILE-CYS-
LYS-THR-GLY-ASP-CYS-GLY-GLY-LEU-LEU-ARG-CYS-LYS-ARG-PHE-GLY-ARG-PRO-PRO-THR-THR-LEU-ALA-
GLU-PHE-SER-LEU-ASN-GLN-TYR-GLY-LYS-ASP-TYR-ILE-ASP-ILE-SER-ASN-ILE-LYS-GLY-PHE-ASN-VAL-PRO-
MET-ASN-PHE-SER-PRO-THR-THR-ARG-GLY-CYS-ARG-GLY-VAL-ARG-CYS-ALA-ALA-ASP-ILE-VAL-GLY-GLN-CYS-
PRO-ALA-LYS-LEU-LYS-ALA-PRO-GLY-GLY-GLY-CYS-ASN-ASP-ALA-CYS-THR-VAL-PHE-GLN-THR-SER-GLU-TYR-
CYS-CYS-THR-THR-GLY-LYS-CYS-GLY-PRO-THR-GLU-TYR-SER-ARG-PHE-PHE-LYS-ARG-LEU-CYS-PRO-ASP-ALA-
PHE-SER-TYR-VAL-LEU-ASP-LYS-PRO-THR-THR-VAL-THR-CYS-PRO-GLY-SER-SER-ASN-TYR-ARG-VAL-THR-PHE-
CYS-PRO-THR-ALA-COOH

anti-thaumatin and sweetness intensity.

Van der Wel and Bel[12] have looked at the cross-reactivity between anti-thaumatin I and monellin by Ouchterlony double diffusion. Their results showed a reaction of identity between T I and monellin but there was no cross-reactivity between iodinated monellin and anti-T I (iodination of monellin splits the two monellin polypeptide chains). They concluded that thaumatin and monellin share an identical conformational determinant which is probably the site responsible for the sweet-taste sensation. The structural feature of thaumatin which is capable of interaction with the sweet-taste receptor and which is also a major antigenic determinant, appears to be similar in all intensely sweet materials. Hough and Edwardson have therefore suggested that use of the anti-T I radioimmunoassay may provide a valuable method for the assay of sweetness *in vitro*.

Thaumatin has been shown to elicit a significant electrophysiological response in the chorda tympani of anaesthetised *Cercopithecus aethiops* (Old World monkey species) but not in the nerve from *Saguinas midas tamarin* (New World monkey species), pig, rabbit, hamster, rat or dog[39,40,41]. Behavioural techniques have shown that this electrophysiological response probably correlates with the ability to perceive sweetness of thaumatin. It would appear, therefore, that not all animals recognise thaumatin as sweet-tasting. Glaser *et al*[49] have recently surveyed 34 species of primate for their taste response to thaumatin. Only the Old World monkeys of the *Cercopithecidae*, *Hylobatidae* and *Pongidae* respond to the protein. This suggests that the capacity to taste thaumatin evolved 38 million years ago. Whether the change in taste perception which occured was a subtle change to the existing sweet-taste receptor or the appearance of a whole new class of receptors is as yet unclear. Comparison of T I with monellin shows that besides both tasting sweet both

Table 4. Immunological cross reactivity with anti-thaumatin and sweetness intensity.

Sweetener	Relative sweetness		Immuno-reactivity
	relative to 10x sucrose	relative to thaumatin (molar)	
thaumatin	3,000	1	1
monellin	2,000	1/3	2.4×10^{2}
neohesperidin dihydrochalcone	1,000	1/100	9.0×10^{5}
4,1,6,6'-tetrachlorosucrose	200	1/720	3.0×10^{6}
L-aspartyl-L-phenylalanyl methyl ester	200	1/970	4.0×10^{6}
1',6,6-trichlorosucrose	100	1/1,500	3.0×10^{7}
saccharin	150	1/1,600	2.0×10^{8}
cyclamate	50	1/6,000	1.0×10^{8}

Taken from reference 11

have relatively high pI values[28,29,44], similar α-helix/β-sheet contents[47] and both react with antibodies raised against thaumatin I [11,12]. However, comparison of the primary structures does not reveal any similar sequences beyond what could be expected by chance.

There are five tripeptide sequences which are present in both sweet-tasting proteins. It is possible that one of these is in a favourable position to interact with the taste receptor.

II. MICROHETEROGENEITY OF PROTEINS

A. GENERAL.

The main features of protein biosynthesis are now well established. The information required for the assembly of amino acids in their correct sequences to form polypeptides is supplied by the nucleotide sequence of the genes. The process is called translation and it involves the matching (via tRNA) of specific nucleotide triplets with their respective amino acids. If the process proceeds accurately the polypeptides which result from a given nucleotide sequence will be identical. However, it has been found that within populations of certain proteins and polypeptides, sub-populations exist which can be distinguished either chemically or physically. Two explanations of this phenomenon are possible. Firstly, differing copies of a gene may exist within the genetic material of an organism giving rise to polypeptide chains which differ slightly. Secondly, post-translational modification of proteins may occur. The latter can give rise to heterogeneity if not all of the synthesised protein is modified identically, or if the rate of modification is slow relative to the half-life of the protein.

B. HETEROGENEITY OF PROTEIN FORMS ARISING FROM DIFFERENT GENE COPIES.

Several gene copies can code for a single protein molecule. This arises, firstly, because the somatic cells of eukaryotic organisms are usually diploid and contain twice the number of chromosomes found in the germ cells. Each gene in a eukaryote, therefore, occurs as two alleles. Secondly, structural genes in genetic material may be multiplied in a single haploid set of chromosomes. Each of the gene copies has the possibility of separate change with time due to mutation and so the proteins which

result may have different amino acid sequences.

In plants, the cereal storage proteins have been examined in order to determine the cause of their heterogeneity. For example the oat prolamin, avenin, consists of two fractions designated $\gamma 1$ and $\gamma 4$ which can be separated by ion-exchange chromatography. $\gamma 1$ and $\gamma 4$ have the same N-terminal amino acid and almost equal molecular weights with a calculated number of residues per molecule of 191 ± 3. Slight differences in their amino acid compositions were found. It was suggested that the two molecules represented translations of closely related structural genes and possibly that they were alloproteins[50]. Zein, the storage protein from maize, has also been studied with respect to its heterogeneity. Righetti et al[51] suggested that the heterogeneity was due to differences in charge and amino acid composition resulting from a combination of post-translational deamidation and the presence of multiple genes. However Viotti et al[52] have now demonstrated that a different mRNA species exists for each zein species. It is therefore likely that more than one copy of the zein gene must exist in each haploid genome.

C. HETEROGENEITY ARISING FROM POST-TRANSLATIONAL MODIFICATIONS.

Post-translational modifications of proteins which result in heterogeneity are usually detected as differences in charges. Deamidation of glutaminyl and asparaginyl residues in a wide variety of proteins has been described. A classical example of this is the modification of cytochrome C. The heterogeneity of this protein was first discovered in 1957, by Paleus and Theorell[53] who separated a number of forms by electrophoresis. In 1964 Flatmark[54] showed that in rat, horse and beef hearts four forms of cytochrome C were present and it was suggested that they were produced in vivo and not as artifacts of the isolation procedure. Later in vitro

conversion of cytochrome C into singly and doubly deamidated forms was demonstrated and the process was shown to be dependent on pH, ionic strength and temperature[55]. In experiments to determine the isoelectric points of unmodified (CyI), singly deamidated (Cy II) and doubly deamidated (Cy III) cytochrome C[56], it was found that Cy II was made up of at least two different components and it was suggested that the two forms contained the same numbers of amide groups but that the positioning of one of the groups was responsible for the differences in pI. The biological activities of Cy II and of Cy III were only 59% and 19%, respectively, of the activity of Cy I[57]. The different deamidated forms also possess different visible absorption and circular dichroism spectra and oxidation-reduction potentials and the rates of reduction by ascorbate and oxidation by O_2 are also dissimilar.

In vivo four forms of cytochrome C (Cy I, Cy II, Cy III and Cy IV) are present. Flatmark and Sletten[58] suggested that the turnover of cytochrome C may be passively attributed to three successive deamidations which would give rise to a half-life in vivo of cytochrome C of 80 days or more. However it is possible that the deamidated cytochromes are preferentially degraded[59]. This is supported by the finding that the half-life of cytochrome C in rat kidney mitochondria is only 16 days. This increased susceptibility to degradation may be caused by a change in cytochrome C structure and/or location.

Calf lens α-crystallin consists of basic and acidic polypeptide chains[60] and on high pH urea-polyacrylamide gel electrophoresis four fractions, A1, A2, B1 and B2, separate[61]. In 1968 Schoenmakers and Bloemendal[62] were able to show that in embryonic calf lenses the A1 chain was absent and that it was gradually produced with increasing age of the embryo. Palmer

and Papaconstantinou suggested that the A1 chain was not formed by de novo synthesis but by chemical or enzymic modification of A2. Both chains, A1 and A2, have identical amino acid contents and peptide mapping after chymotryptic digestion revealed the occurence of one'difference-peptide' of similar amino acid content[63]. Strong evidence now exists that a glutamine to glutamic acid conversion is responsible for the difference between A1 and A2[64]. Messenger RNA species from calf lens were translated in two different cell-free systems and in one in vivo system. It was found that no detectable mRNA species coding for A1 was present in calf lens. The possibility that a repression mechanism blocked the translation of the A1 messenger could not be completely ruled out but the presence of A1 in the adult lens was thought to arise by deamidation of one glutamine in the A2 chain[64]. In α-crystallin from both calf and mature bovine lens nuclei extra subunits have been observed, these have been designated AA1, AA2, AA3, N1 and N2. AA1, AA2 and AA3 are products of intracellular degradation of A1 and A2[65,66]. N2 is degraded B2 whilst N1 is deamidated B1. Electrophoretically homogenous preparations of the A-chains actually contain two types of chains, normal and those lacking 5 residues at their C-termini [67]. All of the degraded α-crystallins AA1, AA2, AA3, and N2 have peptides of varying lengths missing from the C-termini . This situation with the α-crystallins is now fairly well understood; the deamidation process is apparently not related to ageing since in calf lens nucleus tissue no deamidation changes occur with age. However limited proteolysis of the subunits increases with age of the tissue. It remains to be seen whether this view of protein ageing derived from these studies with α-crystallin will have any relevance for other proteins. Table 3 shows the origin of the

Table 5. The origin of the various subunits of α-crystallin

Chain	Origin	Content
A_2	primary gene product	$A_2$1-173
A'_2	degraded A_2	$A_2$1-169 or 1-166
A_1	deamidated A_2	$A_1$1-173
A'_1	degraded A_1	$A_1$1-168
AA_1	degraded A_1	$A_1$1-151
AA_2	degraded A	A 1-101
AA_3	degraded A_2	$A_2$1-151
B_2	primary gene product	B_2
B_1	deamidated B_1	B_1
N_0	degraded B_1	$B_1$1-170
N_1	deamidated B_1	B_0
N_2	degraded B_2	$B_2$1-170

subunits of α-crystallin.

Very many other cases of deamidation of proteins and peptides have been reported: in some cases the process may be enzyme-catalysed as in the case of the peptide hormone thyroliberin[68]. However, deamidation more usually appears to proceed spontaneously as with rabbit muscle aldolase[69] and lysozyme[70].

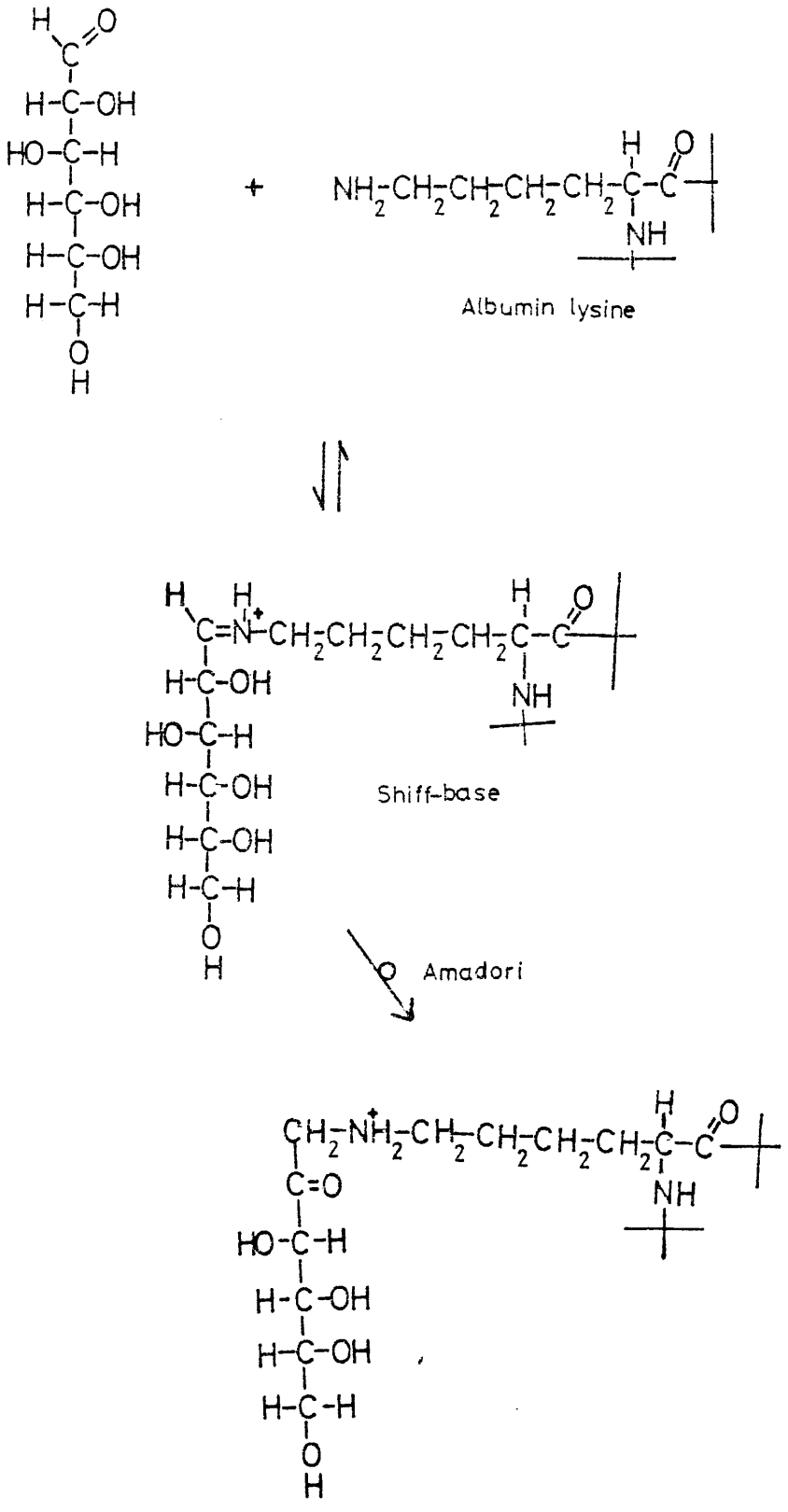
Glycosylation is also a major cause of protein heterogeneity. Variations in glycosylation patterns are responsible for the heterogeneity of very many glycoproteins, for example, ovalbumin[71], human anti-thrombin III[72] and CB - 7 + 8 a component of bovine milk[73].

It has also been reported that human albumin becomes glucosylated non-enzymically *in vitro* and *in vivo*[74] giving rise to Schiff's base derivatives of the lysine residues which make the protein less basic (fig. 6). Lens crystallins can also become glucosylated when exposed to high concentrations of glucose *in vivo* or, *in vitro*, in the presence of high concentrations of G-6-P or glucose[75]. The process of glucosylation of lens crystallins was thought to contribute to the formation of cataracts in diabetics but recent results suggest that it is not a primary factor in humans[76].

Acetylation of proteins occurs *in vivo* and results in heterogeneity in a number of instances. N-terminal acetylation occurs in the acidic proteins A1 and A2 from 50-S ribosomes of *E. coli*[77] and in dogfish M4 lactic dehydrogenase[78]. Alternatively, lysyl residues can be N^{ε}-acetylated and this occurs at specific sites in histones[79], possibly with some effect upon their regulatory role.

Other modifications resulting in heterogeneity include:- γ-carboxylation of N-terminal glutamic acid[80], amidation of C-terminal groups[81], formation of tyrosine sulphate esters[82], and phosphorylation[83]. Removal of acidic or basic terminal

FIGURE 6. The glucosylation of albumin



amino acids[83] and even binding of poly(A) sequences[84] have also been found to be causes of protein heterogeneity.

III RIPENING OF FRUIT

A. PHYSICAL CHANGES OF FRUITS DURING RIPENING

Various physical signals usually accompany the ripening of fruits, these can include changes in colour, either by loss of chlorophyll which unmasks underlying pigmentation or by synthesis of new pigments, alteration of flavour by changes in acidity, bitterness and sweetness, changes in texture, usually softening, abscission and increased wax development on the skin. Ripening does not begin until the fruit has developed considerably from the tissues present at anthesis. It is difficult to stimulate fruits to ripen until they have reached the appropriate point in development, conversly once the process has begun it is not possible to reverse it although it can be slowed[85].

B. HORMONAL REGULATION OF RIPENING

True ripening of fruits must involve significant changes in a range of enzyme activities within the fruit tissue. The control of these changes may be at a variety of levels i.e. transcription, translation or inhibition of enzymes. It is likely that the process is ultimately under the control of phytohormones. Auxins, cytokinins and gibberellins can all act to retard the ageing process in plants. In fruits auxin is able to counteract the stimulatory effects of ethylene or abscisic acid on ripening[86,87].

Ethylene has long been known to be involved in fruit ripening. At one time it was thought that it was the fruit ripening 'trigger' hormone[88,89,90] and this may be true in some cases but in the grape, for example, a consistant, low production of ethylene persists during the beginning of the ripening process. It can be stimulated to produce larger amounts of ethylene but there is no correlation between these levels and ripening[91]. Even in the

classical example of ethylene-induced ripening of the tomato it has been found that the rise in the levels of ethylene does not begin until after ripening has begun[92]. It may be that ethylene is responsible for just part of the ripening process. In the peach the rise in ethylene concentration was found to coincide with the accumulation of dry matter but not with expansion of cells both of which are involved in ripening[93,94].

Abscisic acid has been proposed as a 'trigger' for ripening in some fruits[95,96,97,98,99]. In grapes the evidence is that abscisic acid concentration falls steadily during the first rapid growth phase reaching a low value seven to ten days before ripening becomes evident. Its level then increases and continues to rise until ripening is well established when the level begins to fall. Treatments which delayed or hastened ripening were found to have parallel effects upon abscisic acid levels[95]. Treatment of grapes with abscisic acid brought on ripening but only when the endogenous abscisic acid level was relatively low.

Storage of tomatoes at low air pressure results in a delay in ripening. This is not due to a low concentration of ethylene but to a low partial pressure of oxygen[100]. In the same way restriction of the oxygen supply to avocado slices, by washing, also results in a delay in ripening[101]. The ripening of whole pears can be speeded up either by treatment with oxygen or with auxin oxidation products. Although both the treatments stimulate ethylene production it seems most likely that auxin oxidation products stimulate the ripening process and ethylene synthesis in the case of pear[102].

C. CLIMACTERIC RESPIRATION

Kidd and West[103] were first to note a relatively sudden rise in the level of respiration which occured in apples either

on or off the tree at the beginning of ripening. It was soon established that a similar pattern of respiration (a climacteric) was associated with the ripening of many other fruits. In fact the majority of fruits show the climacteric pattern of respiratory changes associated with ripening. The increase in respiration over the base level varies; in apples it is 60-100%, in banana 200% and in avocado 300-400%. In tomato, as in apple the climacteric rise occurs in detached fruit or in fruit on the plant[104]. On the other hand the avocado only shows a climacteric rise after detachment from the tree[105]. In many fruits (e.g. avocado, banana, mango) the stage of eating ripeness corresponds to the climacteric peak. In others, like the apple and tomato[106], eating ripeness is not reached until some time after the climacteric peak. In 1960 Biale[107] classified all fruits into climacteric and non-climacteric groups: the non-climacteric type of fruit were those which showed a steady decline in rate of respiration after picking. Since the original classification was made more fruits of the non-climacteric category have been shown to, in fact, have a climacteric under the appropriate physiological conditions. The difference between the two groups is either that they have distinct mechanisms of ripening or that the events which occur quickly in the climacteric fruits take place slowly over a long period in the non-climacteric types. It seems more likely that the second hypothesis is correct.

D. NUCLEIC ACIDS AND RIPENING

Two main hypotheses have been proposed to explain the ripening of fruits. The first assumes that in the early stages there is a loss of structural integrity leading to the loss of cellular membrane control of substrate-enzyme associations[108]. The second

is that the process is under complex biochemical control so that anabolic activity occurs which is essential in the subsequent ripening of the fruit. What is known about nucleic acid and protein metabolism, particularly at the beginning of the ripening process, seems to support the second hypothesis.

It is clear that there can be considerable variation between the ripening of different fruits with respect to the synthesis of nucleic acids. Looney and Patterson[109] studied the changes in total RNA content during the climacteric phase in yellow transparent apples. This increased by approximately 50% as respiration increased. The change was attributed largely to an increase in the amount of ribosomal RNA present. Hulme et al[110] have investigated the effect of ethylene on RNA synthesis in preclimacteric apples. Exogenous ethylene firstly stimulates the endogenous production of ethylene which signals the climacteric. Secondly, increased incorporation of $[^{14}C]$-uridine into RNA occurs and after reaching maximum levels this is followed by a surge in the rate of incorporation of $[^{14}C]$-valine into protein. In avocado Richmond and Biale[111] found that $[^{32}P]$ incorporation into rRNA and mRNA was high in the early phase of the climacteric but that this declined to zero by the time the climacteric peak was reached. In late climacteric fruit an upsurge in the synthesis of low molecular weight species of RNA occured. It was thought possible that this might represent synthesis of a specific tRNA species. Methylated albumin on Keiselguhr column separations of the RNA species present during the climacteric rise showed no significant changes in the RNA pattern with development and also no significant changes in total RNA content.

Ku and Romani[112] have studied the ribosomes of pear fruit. The distribution of ribosomes between monosome and polysome forms

remained relatively constant until the climacteric peak after which the polysomes decline. During the climacteric rise changes occured in the rate of turnover of ribosomes; an increase in the rate at the beginning of the climacteric was followed by a decline until at the climacteric peak, no incorporation of radioactive bases into ribosomes could be detected. Chromatography on M A K was used to demonstrate that the RNA newly synthesised at the climacteric rise, was ribosomal. Rattanopanone et al[113] have translated mRNA from tomato fruits in a wheat germ cell-free system. Comparison of the protein products from green and ripe tomatoes gave evidence of changes in the amounts of mRNA coding for specific proteins. The available evidence certainly appears to suggest that RNA synthesis plays an important role in fruit ripening. As a measure of this importance it has been found that actinomycin D is capable of inhibiting the ripening of pears[114].

E. PROTEINS AND RIPENING

Work on the protein contents of ripening fruits has shown that in certain cases, such as apple[115,116], there is a net increase during ripening. In others such as banana[117,118,127,128] the protein content stays relatively constant and in avocado it falls[119].

Polyacrylamide gel electrophoresis has been employed to examine the protein profiles of various fruits[117,120,121] at different stages of ripening. It has been found that changes in specific proteins occur which implies a redirection of protein synthesis.

A large number of investigations of changes in enzyme levels during ripening have been made. The centre of attention has been the enzymes which have the capacity for catalyzing degenerative aspects of ripening[122]. Most of these enzymes increase markedly in activity at some time during fruit growth and during ripening.

In the avocado, for example, fruit softening shows a clear correlation with the activities of the pectic enzymes, polygalacturonase and pectin methylesterase[123,124]. In the same fruit, cellulase activity can also be directly correlated with ripening and the enzyme can be induced by ethylene[125]. Increases in the levels of other enzyme have been noted during maturation for example chlorophyllase[126,116], lipase[116], lipoxidase[129] and phenylalanine ammonia-lyase[130].

Changes in the rate of protein synthesis have been examined by the use of radiolabelled amino acids. Brady et al[117] investigated protein synthesis in ripening banana slices. During the early part of the ethylene induced climacteric rise, both the rate of uptake of labelled lysine into the cells and the rate of incorporation into protein increased. The rate of incorporation was, however, only increased at 13 and 24 hours; at 40 hours which corresponds to the middle of the climacteric rise the rate declined sharply. Richmond and Biale[131] have measured the rate of incorporation of [^{14}C]-labelled leucine and valine into protein in avocado tissue slices. Incorporation was relatively high at the beginning of the climacteric but declined and was virtually zero by the climacteric peak.

Frenkel et al[132] have shown that if intact preclimacteric Bartlett pears are treated with cycloheximide ripening does not occur. Cycloheximide is unable to inhibit ripening if it is administered during the later stages of the climacteric. The climacteric rise was not blocked by cycloheximide. Studies on the rate of incorporation of labelled phenylalanine into protein during ripening showed that a peak occured during the early part of the climacteric rise. These results suggest that proteins necessary for ripening are synthesised early in the climacteric. In banana

slices treatment with cycloheximide or p-fluorophenylalanine blocked the ripening effect of ethylene. Administration of cycloheximide during the climacteric prevented any further rise in respiration but had no immediate effect on the respiration rate of unripe tissue[117].

IV. MESSENGER RNA IN PLANTS

A. GENERAL

In eukaryotic cells genetic information 'flows' from the nucleus to the cytoplasm as RNA transcripts of DNA. These transcripts, known as pre-mRNAs are combined with proteins and undergo processing and modifications to become translatable mRNAs. The process of transcription of the DNA, that is, the formation of RNA complementary to a strand of the chromosomal DNA is under complex control in eukaryotes.

The formation of mature mRNA species from their respective pre-mRNAs can involve several post-transcriptional modifications. Many eukaryote mRNA molecules are converted to mature mRNAs by cleavage and possibly splicing of the mRNA strand. Most eukaryotic mRNAs contain at their 3'-ends a polyadenylated (Poly (A)) tail of 50-200 residues[133], which is added after transcription before and after processing of hnRNA in the nucleus and cytoplasm[134]. This poly (A) sequence does not, however, appear to be an essential requirement for mRNA function since several mRNA species lacking a poly (A) sequence are known; for example the histone mRNAs[135,136]. However, a role for poly (A) in mRNA may have been demonstrated by Soreq *et al*[137] and Williamson *et al*[138]. Globin mRNA from which the poly (A) was enzymically removed with polynucleotide phosphorylase directed globin synthesis in the Kreb's ascites cell-free system at the same initial rate as poly (A)-containing mRNA. However, the rate with the deadenylated mRNA, relatively speaking, became progressively slower with increasing incubation times. This effect may be best attributed to the greater stability of polyadenylated mRNA. Alternative functions of the poly (A) sequence have been suggested including a role in the cleavage of hnRNA and in

transport of mRNA to the cytoplasm[139,140].

At the 5'-termini of eukaryotic mRNAs a capping structure of the form $m^7G^{5'}ppp^{5'}XmYm$ is generally found. The 7 methyl guanosine is linked by a 5',5'-pyrophosphate bridge to the adjacent methylated nucleotide[141]. The function of the 5'-terminal m^7G was first explained by Both et al[142] and Muthukrishnan et al[143]. Messenger RNAs lacking 5'-m^7G were prepared and translated in the wheat germ system. The efficiency of translation was much lower than with the equivalent capped messengers. However, eucaryote mRNAs without a m^7G cap have been found[144,145]. These can be efficiently translated in eucaryotic cell-free systems. The cap structure is less important in the case of mRNAs where the initiator AUG is far from the 5' terminus or in the case of certain ribosomal systems where there is high affinity for the mRNA-binding sequence. The cap also protects the mRNA from 5'-exonucleolytic degradation resulting in increased stability of the messenger in the various cell-free protein synthesising systems and in the Xenopus oocyte in vivo system[146]. Demethylated $G^{5'}ppp^{5'}X$-terminated RNA was as stable as the methylated form but was not as efficiently translated. Enzymic removal of mRNA caps may be a factor in translational control. Methylation of internal adenosine in RNA has also been found to occur but the function of these residues has not been established.

B. PLANT mRNAs

In comparison with animals and micro-organisms little has been done to elucidate the detailed mechanisms of protein synthesis in plants. However, a rather close similarity between plant and animal mRNAs is suggested by studies of mRNA translation. For example, messengers from animal cells can be translated in the wheat-germ cell-free system. Conversely RNA from parsley cell suspension cultures can direct synthesis of phenylalanine ammonia-lyase (PAL),

a plant specific enzyme[147,148,149], in the rabbit reticulocyte lysate system or in Xenopus oocytes[150]. It seems likely that the main structural features of mRNA species in both animal and plant kingdoms are identical.

Higgins et al[151] were first to show that poly (A) occurred in plants and that these sequences were associated with the polysomes in the rapidly labelled fraction of high specific activity, which was thought to represent mRNA.

A number of studies have shown that plant mRNA species can be separated from total cellular or polysomal RNA preparations by affinity chromatography on oligo (dT), poly (U) or cellulose columns all of which act by binding poly (A) sequences[148,149,150,152,153,154,155,156,157].

Gray and Cashmore[158] isolated total polysomal RNA from pea leaves and found that only approximately 50% of the mRNA was capable of binding to poly (U) sepharose. It was found that the mRNAs which did not bind to poly (U) columns (poly $(A)^{minus}$) and those which did (poly $(A)^{plus}$) both coded for the same major polypeptides when they were translated in the wheat germ system. The poly $(A)^{minus}$ RNA fraction was found to be essentially poly(A) free. The possibility that poly (A) in the poly $(A)^{minus}$ species was lost by the action of a ribonuclease during isolation was eliminated. It therefore seems most likely that at least in pea seedlings two classes of each messenger species exist, one with and one without a poly (A) sequence.

Sagher et al[159] have estimated that mRNA species from several plants contain poly (A) sequences 150-250 nucleotides long. Covey and Grierson[160] have shown that the average size of sycamore poly (A) sequences released from polydisperse RNA by nuclease digestion is 160 nucleotides with molecules ranging in size from 50-250

nucleotides.

Wheeler and Hartley[161] have shown that the major mRNA species of spinach chloroplasts do not contain poly (A). This may prove to be another respect in which the chloroplasts resemble prokaryotes which also do not usually carry poly (A).

As stated above, poly $(A)^{minus}$ and poly $(A)^{plus}$ RNA preparations from plants have been successfully translated in various cell-free systems and in one case in the Xenopus oocyte in vivo system. The system of choice has usually been that derived from wheat germ[162] since this has the obvious advantage of being of plant origin; it also has a low background activity and it is relatively easy to prepare and to handle.

C. RECOGNITION OF PLANT mRNA PRODUCTS

Recognition of plant mRNA products synthesised in cell-free protein synthesising systems has, to date, relied on either SDS gel electrophoresis alone or on a combination of this technique and precipitation with specific antibodies. Examples of specific products recognised by the latter technique include phenylalanine ammonia lyase[150], cellulase[163], leghaemoglobin[152], α-amylase[155] and isocitrate lyase[154].

As an alternative to the preparation of isolated RNA fractions for the study of in vitro protein synthesis some workers have used isolated polysomes, for example Verma et al[152] have shown that leghaemoglobin is synthesised in a cell-free system containing wheat-germ post-ribosomal supernatant and soybean root-nodule polysomes. Larkins et al[164] have separated maize membrane-bound and soluble polysomes and added them to a wheat germ system containing tRNA from maize kernels. The membrane bound rather than the soluble polysomes were found to be the principal sites of synthesis in vitro of the maize protein, zein. Zein was recognised by its

solubility in hot ethanol.

M A T E R I A L S A N D M E T H O D S

V MATERIALS.

Radiochemicals (L-[4,5-^{3}H]-isoleucine, 113Ci/mmol; L-[4,5-^{3}H]-leucine, 52Ci/mmol; L-[3,5-^{3}H]-tyrosine, 40Ci/mmol; L-[4,5-^{3}H]-lysine monohydrochloride, 75Ci/mmol; L-[^{35}S]-methionine, 500Ci/mmol; Na[^{125}I], 1200Ci/mmol) were obtained from the Radiochemical Centre, Amersham, Bucks.

Acrylamide, N,N'-methylene-bis-acrylamide, urea and other Analar grade reagents were obtained from BDH Chemicals Ltd, Poole, Dorset. Coomassie Brilliant blue R and G 250, 2-mercaptoethanol, N,N,N',N'-Tetramethylethylenediamine (TEMED), ethylenediamine, DTT, N-ethylmorpholine, Triton X-100, Triton X-405, agar, phenylhydrazine, haemin, human fibrinogen, hydroxyquinolone, sucrose(RNAse free), EGTA, creatine phosphate kinase, ATP, GTP, creatine phosphate, spermine, isoamyl alcohol, sodium deoxycholate and heparin were from Sigma (London) Chemical Company Ltd, Poole, Dorset. NCS tissue solubilizer (Amersham-Searle) was obtained from Hopkins and Williams Ltd, Romford, Essex. PPO was supplied by Koch-Light Laboratories Ltd, Colnbrook, Bucks. Pharmalyte (ampholines) were from Pharmacia (GB) Ltd, Hounslow, Middlesex. CM-cellulose (CM52) was provided by Whatman Lab Sales Ltd, Maidstone, Kent. Dowex resins and hydroxylapatite were from Bio-Rad Laboratories, Watford, Herts. Freund's adjuvant was supplied by Calbiochem Ltd, Bishops Stortford, Herts. Sheep red blood cells and guinea pig complement were from Flow Laboratories, Irvine, Ayrshire. Rabbit Haemolytic serum was from the Wellcome Foundation Ltd, Dartford, Kent. Staphylococcus aureus nuclease was supplied by The Boehringer Corporation (London) Ltd, Lewes, Sussex. Staphylococcus aureus V8 protease was from Miles Laboratories Ltd, Stoke Poges,

Slough. Rabbit anti-human fibrinogen was from Dako immuno-globulins, Mercia Brocades Ltd, Weybridge, Surrey. Iodo-gen was the product of Pierce Chemical Company, Rockford, Illinois. Sagatal was supplied by May and Baker Ltd, Dagenham, Essex. Oligo-(dT) cellulose (Type 2) was obtained from Uniscience Ltd, Cambridge.

Rabbits (lops and N.Z.W.) were obtained from Hylyne Rabbits Ltd, Lymm, Cheshire. Pea seeds (var. Pioneer) were from Suttons Seeds Ltd.

Fruits of Thaumatococcus danielli were provided by Tate and Lyle Ltd, and were divided into four categories A,B,C and D using the following criteria.

A: unripe	pericarp:- green or green and orange.
	seeds:- white.
	arils:- white.
B: intermediate ripeness	pericarp:- orange or orange with some red patches.
	seeds:- purple.
	arils:- white.
C: early ripe	pericarp:- light red.
	seeds:- very dark purple.
	arils:- very pale yellow.
D: ripe	pericarp:- dark red.
	seeds:- black.
	arils:- pale yellow.

METHODS.

A. GENERAL METHODS

1. Protein determination:

i) Protein was measured by the method of Lowry et al[165]. To avoid non-specific colour reactions the protein was initially precipitated with a 7% solution of T C A. ii) Alternatively estimates were made by measurement of absorbance at 280 nm. ($E^{1\%}_{1cm}$ for all forms of thaumatin was 11·05).

2. Measurement of incorporation of labelled amino acids into T C A insoluble material:

Radioactivity in TCA insoluble material was measured by the method of Mans and Novelli[166]. Samples were spotted onto 2·1cm discs of Whatman 3MM paper and dried in an oven at 60°C. The discs were then placed in a 10% TCA solution containing the appropriate unlabelled amino acid to facilitate the removal of unincorporated label. After 5 min the discs were washed, successively, (in minimum volumes of 2ml/disc) in 5% TCA at 90°C (30 min), 5% TCA (5 min), ethanol/ether 3:1 (v/v) (30 min), ethanol/ether 3:1 (v/v) (5 min) and finally ether (5 min). The papers were again dried, placed in scintillation vials and then wetted with water (100µl) before addition of NCS (500µl). Solubilization was allowed to proceed either overnight at 37°C or for 2 hours at 55°C and then the vials were cooled on ice. To the cold vials glacial acetic acid (17µl) was added, followed by 5g/litre toluene/PPO (10ml). The radioactivity in the vials was measured with a Packard Tri-Carb scintillation counter.

3. Polyacrylamide gel electrophoresis (PAGE)

i) Procedure of Gabriel[167]. Protein samples were dissolved at 1mg/ml in electrophoresis buffer (β-alanine, acetic acid 30mM

pH4·5). 0·1% methyl green (5µl) and glycerol (5µl) were added to each sample (5-100µl). The separating gel (6cm) was 15% acrylamide, 0·1% bisacrylamide at pH4·3 and the stacking gel (5mm) was 2·5% acrylamide, 0·625% bisacrylamide at pH6·7. Samples were applied in the positive compartment and a constant current of 1·5mA/tube was applied (4°C) until the marker dye reached the ends of the gels. Gels were removed from their tubes by rimming and then fixed and stained for 1h in Coomassie brilliant blue R250 solution (1·25g in methanol (227ml), glacial acetic acid (46ml), water (227ml)) then destained in destaining solution (methanol (50ml), glacial acetic acid (75ml), water (875ml)).

ii) SDS PAGE by the procedure of Weber et al [168]. Samples were dissolved at 1mg/ml in sample buffer (0·01M sodium phosphate, pH7·0, 1% SDS, 1% 2-mercaptoethanol). The solution was heated in a boiling water bath (2 min) then cooled to room temperature and 0·05% bromophenol blue in 0·02M sodium phosphate buffer, pH7·0, (5µl), glycerol, (5µl) and 2-mercaptoethanol (5µl) were added. The separating gel (6cm) was 10% acrylamide, 0·27% bisacrylamide. Samples were applied in the negative compartment and a constant current of 6mA/tube was applied at room temperature until the marker dye reached the end of the gels. Gels were processed as in section V A 3 i).

iii) Polyacrylamide gel isoelectric focusing by the method of Wrigley[169]. Gels were prepared and electrophoresed basically according to the method of Wrigley[169]. Modifications were made according to van Kleef and Hoenders[170] and to the specific instructions on the use of Pharmalyte (the source of ampholines). The gel formula was as follows:- 30µl TEMED, 11ml acrylamide/bisacrylamide solution (30% acrylamide, 0·8% bisacrylamide (w/v)), 3ml Pharmalyte (pH range 8 - 10·5), 31ml urea solution (50% w/v),

3 ml ammonium persulphate (1% w/v). The gels were cast in tubes 8cm long with an internal diameter of 0·5cm. A space (2cm long) was left at the top of each tube for the application of samples. The anode buffer (top) was 0·01M Hepes and the cathode buffer was 0·01M ethylenediamine. Ampholyte overlay solution was layered onto the top of the gels to a depth of 1·5-2·0cm. (Ampholyte overlay solution: 6M urea, 5% sucrose, 0·75% Pharmalyte, 0·04% DTT). A pH gradient was established by pre-electrophoresis at 350V for 45 min. Protein samples were dissolved in 8M urea, 10% sucrose, 0·04% DTT solution to give a final volume of up to 150µl. The sample was layered between the gel and the ampholyte overlay (to facilitate the application of the sample it was possible to add 0·1% bromophenol blue to the sample). Gels were electrophoresed at a constant voltage of 350V for 4h and finally stained in 0·04% (w/v) Coomassie brilliant blue G250 in 3·5% (v/v) perchloric acid prepared according to Reisner et al[171]. This stain was used because it was not affected by the ampholine. It has the added advantage that it requires no destaining but unfortunately it is not as sensitive as methods using Coomassie brilliant blue R250. The pH gradient was measured either with a surface pH electrode or by slicing the gel, placing the slice into a small volume of water, allowing 1h for it to equilibrate and then measuring the pH with a microelectrode.

B. METHODS

1. Purification of TO, TI and TII

All procedures were carried out at 4°C. Ripe fruit (stage D) were dissected and the arils were removed. The aril tissue was homogenised in 4 volumes (w/v) of 0·01M sodium phosphate buffer pH7·2 in a pestle and mortar until all of the tissue was pulped. The homogenate was centrifuged for 5 min at 2,100g in an MSE minor

bench centrifuge. The supernatant was applied to a column of CM52 (Na^+form) (30 x 1cm) which had been equilibrated with the homogenisation buffer. The thaumatins were eluted with a small volume of homogenisation buffer followed by a linear gradient of 0-0·2M NaCl in the same buffer. The sweet tasting protein peaks were collected, dialysed against deionised water and finally lyophilised.

2. Amino acid analysis

Amino acid analyses were performed according to the procedure of Moore and Stein[172]. Protein samples were hydrolysed in evacuated, sealed ampoules in 6M HCL at 110°C for either 24 or 48h. The hydrolysates were dried on a rotary evaporator at 40°C and dissolved in water. The solution was brought to pH6·5 by addition of an equal volume of 0·2M sodium phosphate buffer pH6·5 The solutions were allowed to stand for 4h to permit oxidation of cysteine to cystine and then brought to pH2 by addition of 1M HCL. The solutions were diluted as necessary with 0·01M HCL containing 5ml/litre of thiodiglycol and aliquots of approximately 1ml of the solutions were used for each chromatogram; this ideally represented 0·2mg of the original sample. Analyses were performed using an amino acid analyser (JLC-6AH/Jeol Ltd).

Tryptophan was determined as described by Fraenkel-Conrat[173]. Dry, weighed protein samples were dissolved in 0·1M NaOH. The absorbance of the solutions was measured at 294·4nm, 280nm, 320nm, and 360nm. The molar concentration of tryptophan and tyrosine could be determined according to the formulae.

molarity tyrosine = (0·592 x absorbance 294·4nm - 0·262 x absorbance 280nm) x 10^{-3}

molarity tryptophan = (0·263 x absorbance 280nm - 0·170 x absorbance 294·4nm) x 10^{-3}

The absorbance at 320nm and at 360nm was used to extrapolate the base line to 280 and 294·4nm.

3. N-terminal analysis

The cyanate method as described by Stark[174] was used in conjunction with automatic amino acid analysis. Protein samples were dissolved in 8M urea solution at a concentration of 20mg/ml. To this was added an equal volume of N-ethylmorpholine buffer (50% v/v N-ethylmorpholine, 8M urea, brought to pH8 by addition of glacial acetic acid) and potassium cyanate 5mg/mg of protein.

Carbamylation was achieved by incubating the mixture overnight at 50°C in a stoppered tube. After incubation glacial acetic acid (1ml) was added to the protein (10mg) with thorough stirring and then the mixture was saturated with acetone. The precipitated protein was pelleted by centrifugation (5 min, 2,100g) in the MSE minor then washed with distilled water saturated with acetone. The carbamylated protein was dried under nitrogen and samples were cyclized by heating at 100°C (1h) in a solution made up of 1 volume 50% (v/v) glacial acetic acid and 1 volume concentrated HCl in sealed evacuated tubes. The cyclized material was dried on a rotary evaporator at 40°C and transferred onto a column of Dowex 50-X2-400 (10 x 1 cm) with 3 x 1ml rinses of distilled water. The column was developed with water at a flow rate of 50ml/h. The first 30ml of eluant was collected and evaporated to dryness on a rotary evaporator at 50°C. The dried material was dissolved in 3M HCl (5ml) and heated at 100°C (30 min). The acid was removed on a rotary evaporator and the residue was dissolved in water (2ml) and transferred to a column of Dowex 50-X2-400 (5 x 0·4cm) with an additional 1ml of water. This column was developed with 6ml of water and the effluent was dried. The residue was dissolved in 0·2M NaOH (2ml) and transferred to a sealed hydrolysis tube and

heated at 100°C for 36h. The hydrolysate was brought to acidic pH with 1M HCl (1ml) and evaporated to dryness. The final residue was dissolved in 0·01M HCl (2ml) and this was applied to the JEOL amino acid analyser. The traces attained were compared with a standard amino acid mixture to facilitate the identification of the N-terminal amino acid.

4. Peptide mapping

Peptide mapping was performed using the procedure of Cleveland et al[175]. SDS PAGE was carried out as described in section V A 3ii) except that 15% acrylamide was employed (see reference 168). Protein samples were dissolved in sample buffer (1mg/ml), (Sample buffer contained: 0·1M NH_4acetate pH 4·0 or 0·1M sodium phosphate pH 6·8, 10% glycerol and 1 x 10^{-4}% bromophenol blue), and heated at 100°C (2 mins). Various concentrations of protease were added to equal volumes of protein solution. The mixtures were incubated at 37°C for different times. The partial protein digests obtained were applied to the SDS polyacrylamide gels. Electrophoresis and staining were as described in section V A 3ii).

5. Preparation of antibodies to thaumatin II

Thaumatin II purified as described in section V B 1 was dissolved in 0·9% NaCl under sterile conditions. Equal volumes of T II solution and Freund's adjuvant were mixed and sonicated until a stable emulsion had formed. The emulsion was stored until required at -20°C in sterile syringes. Two male lop rabbits weighing 2kg were injected either subcutaneously (in the back) or intramuscularly (into the thigh muscle), alternately. The first two injections given contained Freund's complete adjuvant, subsequent injections contained incomplete adjuvant. At each injection 50µg T II were administered for each kilogramme of body weight. The total injection volume was 200µl. Injections were given weekly for four months after which time the

antibody titre was static. After two months rest weekly injections were resumed until the titre had reached a satisfactory level which was maintained with fortnightly booster injections.

Blood samples were obtained via an ear vein. Serum was prepared by allowing the blood to clot at room temperature for 5-6 hours then spinning down the clot. The serum was made 0·1% in sodium azide by addition of a 1% solution and stored at 4°C.

Antibody titre was determined by the precipitin method. A solution of T II (1mg/ml) was placed in increasing quantities into a series of tubes. Aliquots of serum (usually 200µl) were added to the tubes which were incubated for 1h at 37°C and then centrifuged to pack the precipitate. The tube containing the largest pellet indicated the optimum concentrations of antibody and antigen and the antibody titre was calculated accordingly.

6. Immunoelectrophoresis(176)

Immunoelectrophesis was carried out on glass slides (8·5 x 9·5cm) coated with 1% agar (to a depth of 1·5mm) buffered with 0·2M barbital buffer pH 8·6. The antigen samples (10µl) were placed in the wells cut at intervals across the centre of the plate and electrophesis was carried out on a cooled platter at 4V/cm (in an LKB multiphor apparatus). The electrophoretic buffer was 0·1M barbital buffer pH 8·6. Electrophoresis was stopped when the bromophenol blue marker (placed in a side well) had migrated to the end of the plate. After electrophoresis troughs were cut between the wells and the gel was removed. Antiserum was placed into the troughs and diffusion was allowed to proceed at 4°C for 48h in a damp box. After the gels had been pressed, washed and dried they were stained for 10 minutes in ethanol: acetic acid: water, 4·5: 1: 4·5 containing 0·5% Coomassie Brilliant Blue R-250 and then destained in a fresh solution without the dye, until the background colour was faint. Finally the

plates were rinsed with water and dried.

7. Ouchterlony double diffusion[196]

Agar coated glass slides were prepared as described in section V B 6. Wells were cut in a hexagonal pattern around a central well. Serum (20µl) was placed in the central well and antigen (10µl) was placed in the outside wells. The plates were stored in the cold (4°C) for 48h to allow precipitin lines to form, then they were stained as described in section V B 6.

8. Quantitative precipitin analysis of T O, T I and T II reaction with anti-T II

Quantitative precipitin analysis was carried out according to Prager and Wilson[177]. 0·9% NaCl (200µl) containing various quantities of thaumatins O, I and II was added to antiserum (200µl) having a titre of 35µg/ml in small polypropylene tubes. The tubes were incubated at 20°C overnight and the precipitate formed was pelleted by centrifugation (2,100g for 20 min). The precipitate was washed twice with cold 0·9% NaCl (400µl) and the washed pellet was dissolved in 0·5M NaOH (400µl). The absorbance (280nm) of this solution was measured.

9. Quantitative micro-complement fixation analysis

Quantitative micro-complement fixation was performed using the method of Wasserman and Levine[178]. The diluent was that described by Arnheim and Wilson[179]. Seven hundred microliter reaction volumes were used containing 300µl diluent (0·14M NaCl, 0·01M Tris/HCl pH 7·45, 0·5 mM $MgSO_4$, 0·15mM $CaCl_2$), 100µl antiserum (titre 400 ng/ml), 100µl complement (34·5 CH_{50}/ml), 100µl antigen solution (1-200 ng thaumatin) and 100µl sensitized sheep erythrocytes (5 x 10^7 cells/ml).

All components of the assay mixture except sheep erythrocytes were incubated for 18h (4°C). The sheep erythrocytes were added

and the final mixture was incubated for a further 1h at 37°C. The reaction was stopped by cooling on ice. The unlysed erythrocytes and cellular debris were removed by centrifugation for 4 min in a Unipan micro-centrifuge. The absorbance of the supernatant was measured at 413nm.

Sheep red blood cells were supplied as 50% whole blood in Alsever's solution. An aliquot (1ml) was washed in 5ml diluent (3 times). The cells were collected between washes by centrifugation for 1 min at 500g. The washed erythrocytes were suspended in diluent (4·5ml) to make 6% erythrocytes. (the percentage was determined by mixing 1ml erythrocytes with 1M NH_4 OH (25ml) to lyse the cells and reading the absorbance at 541nm; 6% erythrocytes should give an absorbance of 0·48 - 0·50). Rabbit haemolytic serum (titre 1 in 2000 or 1 in 3000) was diluted to 1 in 150 and added to the suspended cells (30µl added to 4·5ml of 6% erythrocytes); this mixture was incubated at 37°C for 15 min. The sensitized cells were stored at 4°C and used within 48 hours. Immediately before use the cells were adjusted to a concentration of 5 x 10^7 cells/ml (dilution factor 1 in 20). The concentration of complement used was calculated for maximum sensitivity by measuring the haemolysis of sheep red blood cells in the standard complement fixation assay of 0·5 - 10 CH_{50} of complement in the absence of antiserum and antigen.

10. Development of a procedure for quantitative extraction of aril protein

i) Effect of pH, salt and multiple extractions on protein recovery. All procedures were carried out at 0-4°C. Arils were excised from fruit and minced with scissors. The minced arils were weighed and homogenised in 20 volumes of buffer, either buffer A (pH 6·0, 0·01M sodium phosphate) or buffer B (pH 7·7, 0·01M sodium phosphate). The samples were firstly homogenised by hand with a

pestle and mortar until all of the tissue had been pulped and then sonicated for 30 s, at full power, setting 5 on an MSE sonicator.

The homogenate was centrifuged to remove insoluble material at 100,000g for 90 min. The supernatant (1st extract) was removed and the pellet was resuspended in the original buffer (either A or B) and recentrifuged. The supernatant (2nd extract) was removed and the pellet was either used directly for estimation of the remaining protein content or resuspended in the original buffer (either A or B) containing 0·3M NaCl and incubated at 4°C overnight, then stirred and recentrifuged. The supernatant (3rd extract) was removed and the pellet was resuspended in the original buffer (either A or B) containing 0·3M NaCl and finally recentrifuged, the supernatant being removed (4th extract). Aliquots of the supernatants were used for protein estimation as described in section V A 1 i). Pellets were treated with 1M NaOH for 1-2h at room temperature and the solutions were used directly for protein estimation without TCA precipitation.

ii) Test of a procedure for sequential extraction of soluble and high pH, salt-soluble protein. Arils were excised and then chopped and weighed. Homogenisation was in buffer A as in section i). The homogenate was centrifuged at 100,000g for 90 min and the supernatant was saved (1st extract). The pellet was resuspended in buffer A and then recentrifuged (supernatant was 2nd extract). The pellet was either taken for protein estimation or resuspended in buffer B containing 0·3M NaCl and incubated at 4°C overnight. After incubation the mixture was shaken and then recentrifuged (supernatant was 3rd extract). The pellet was resuspended in buffer B containing 0·3M NaCl and finally recentrifuged (supernatant was 4th extract). Aliquots of the supernatants were used for protein estimation as described in section V A 1 i). Pellets were treated

with 0·1M NaOH for 1-2h at room temperature and the solutions were used directly for protein estimation without TCA precipitation.

11. Measurement of quantities of T O, T I and T II in different developmental stages of fruit.

Fruits of different maturities, unripe, intermediate, early ripe and ripe (stages A-D) were used. Arils were excised, counted and weighed. The proteins were extracted from the arils as described in section V B 10 ii). The high salt extracts were dialysed against buffer A for 24h. The extracts were applied to ion-exchange chromatography columns (CM 52 (Na^+)) which were developed as described in section V B 1. Sweet protein was estimated by measurement of absorbance at 280nm of each fraction containing T O, T I or T II. Total protein was estimated as described in section V A 1 i).

12. The effect of high pH, temperature and salt concentrations on the thaumatin forms.

Method 1: Purified sweet proteins T O, T I and T II were dissolved (1mg/ml) in 0·01M sodium phosphate buffer pH 7·7 containing 0·3M NaCl and filtered through sterile millipore filters into sterile ampoules which were sealed. The ampoules were incubated at 37°C for 77h or 240h. After incubation the solutions were stored frozen until analysis. Samples were analysed by P.A.G.E. as described in section V A 3 i).

Method 2: Samples of T I and T II were dissolved in 0·01M sodium phosphate buffer pH 7·7 (1mg/ml) and dialysed against a solution of ammonium sulphate in the same buffer so that the final ammonium sulphate concentration of the thaumatin solution reached 45%. The solutions were incubated at room temperature for 144h and then dialysed against water at 4°C for 24h.

After this time the solutions were removed from their dialysis bags and frozen until analysis. Samples were analysed by P.A.G.E.

as described in section V A 3 i).

13. Radioiodination of thaumatin using Iodo-gen

Iodination was achieved using the method of Markwell and Fox[180]. Iodo-gen (2mg) was dissolved in chloroform (100µl) and placed into a scintillation vial. The chloroform was removed under a stream of nitrogen gas at room temperature. The dry vessel was rinsed with P B S to remove any loose flakes of iodo-gen. Thaumatin (20mg) was dissolved in P B S (1ml) and this was added to the reaction vessel. Na $[^{125}I]$ (5µCi, specific activity 1,200Ci/mmol) was added and mixed. The reaction was allowed to proceed for 10 min at room temperature during which time the vessel was agitated periodically to aid diffusion. The sample was removed from the reaction vessel, made 0·25M in sodium iodide and dialysed exhaustively against distilled water. Finally the sample was applied to an ion-exchange column (CM52 Na^+) and eluted as described in section V B 1. The protein content of the fractions was determined by measurement of the absorbance at 280nm. The $[^{125}I]$ content was measured by precipitation of an aliquot (50µl) with 10% TCA and bovine serum albumin (1mg/ml) as carrier. The precipitate was collected and washed with acetone: ether (1:1) v/v (3 times, 20 volumes) and finally dried. The dry material was dissolved in 1M NaOH (0·5ml), neutralized with glacial acetic acid and then added to a scintillation vial with 10ml of triton scintillant. [Toluene: triton: PPO (2 parts toluene containing PPO 5g/1 to 1 part triton X - 100)]. Disintegrations per minute were estimated in the tritium channel of a Packard tri-carb scintillation counter.

14. Exposure of $[^{125}I]$ thaumatin II to an aril homogenate

Arils of stage D Kadjebe region friut which had previously been frozen in liquid N2 and stored at -70°C were thawed and homogenised in an equal volume of 0·01M sodium phosphate buffer pH 7·2. $[^{125}I]$

labelled thaumatin was added to a sample of homogenate (1ml) and incubated at 26°C for 15h. Controls were frozen without incubation. After incubation the samples were extracted with 0·01M sodium phosphate pH 7·7, 0·3M NaCl (2 x 5ml) and diluted with 0·01M sodium phosphate pH 7·2 (2 volumes). This extract was applied to an ion-exchange column (CM52 Na^+) which was eluted as described in section V B 1. Fractions were assayed for protein content and $[^{125}I]$ as described in section V B 13.

15. Protein synthesis in intact arils

Arils were removed from early ripe fruit tissue and injected (5µl) with L-(4,5-^{3}H) -leucine (0·5µCi). Control arils were treated in the same way except that cycloheximide (2·5mg/ml) was included in the injection. The injected arils were incubated in a petri dish with a piece of moist tissue, in a dark cupboard, at 26°C for 16h. After this time protein was extracted by homogenisation in 0·01M sodium phosphate pH 7·7 containing 0·3M NaCl (2 x 5ml) and an aliquot was taken to determine incorporation into TCA insoluble material (section V A 2). The extract was dialysed overnight against deionized water and then applied to an ion-exchange chromatography column (CM52 Na^+) and eluted as described in section V B 1. Each fraction was treated with a final concentration of 10% TCA to precipitate protein (1mg bovine serum albumin added as carrier) and the precipitates were washed twice with 5% TCA (20 volumes) and once in ethanol (this dissolved albumin and thaumatin). The protein was finally reprecipitated by adding 10 volumes of ether, dried and dissolved in 0·5M NaOH (1ml). This solution was neutralised with glacial acetic acid and used for scintillation counting with triton: toluene: PPO scintillant (section V B 13).

16. Preparation of rabbit reticulocyte lysate

Rabbit reticulocyte lysate was prepared by the method of Hunt and Jackson[181]. The lysate was made messenger dependent as described by Pelham and Jackson[182]. A young New Zealand White rabbit weighing 2kg was injected (subcutaneously) on four successive days with 1·25% phenylhydrazine (1·4ml). On the ninth day after the first injection the rabbit was anaesthetised with 'Sagatal' and bled by cutting the jugular vein. The blood was drained into 1 litre of ice cold saline buffer containing 300 units of heparin. (Saline buffer contained: 0·13M NaCl, 5mM KCl and 7·5mM $MgCl_2$ brought to pH 7·4 by addition of NaOH). The cells were collected by centrifugation at 2°C (2,500g,10 min) and the loosely packed cells were resuspended and washed in saline buffer (3 times). The washed cells were lysed by addition of an equal volume of deionized water with gentle swirling for 1-2 min. The lysate was centrifuged at 22,000g for 10 minutes and the supernatant was stored as small aliquots under liquid nitrogen.

Lysate was made messenger dependent as follows. An aliquot was removed from storage and warmed gently. As it began to thaw 1mM haemin solution was added to make a final concentration of 20μM. When completely thawed 0·1M $CaCl_2$ (8μl) and 10μg nuclease (15,000 units/mg from Staph aureus) were added and digestion was allowed to occur for 15 minutes at 20°C. After this time 0·1M EGTA (16·5μl) was added to inactivate the nuclease and the treated lysate was stored either under liquid nitrogen or for up to 30 minutes on ice.

17. The rabbit reticulocyte lysate cell free system

The standard assay mixture contained in a final volume of 50μl: lysate (25μl), 10mM Tris-HCl pH 7·6, creatine Ⓟ kinase (2·5μg), 1mM ATP, 200μM GTP, 10mM creatine phosphate, 0·15mM alanine, 0·025mM arginine, 0·025mM asparagine, 0·1mM aspartic acid, 0·025mM cysteine, 0·025mM glutamine, 0·1mM glutamic acid, 0·1mM glycine, 0·1mM histidine,

0·025mM isoleucine, 0·15mM leucine, 0·1mM lysine, 0·025mM methionine, 0·075mM phenylalanine, 0·05mM proline, 0·1mM serine, 0·075mM threonine, 0·025mM tryptophan, 0·025mM tyrosine, 0·15mM valine, 100mM KAc, 1mM MgAc and 12½ µl containing RNA and labelled amino acid (the appropriate cold amino acid was omitted).

18. Preparation of the wheat germ S-30 cell free extract

Wheat germ extract was prepared in a cold room at 4°C. Wheat germ (2g) was ground with an equal weight of sand in a mortar for 1 min. The homogenate was scraped into a centrifuge tube and mixed with 2 volumes (w/v) of 20mM Hepes buffer pH 7·6 containing 100mM KCl, 1mM MgAc, 2mM $CaCl_2$ and 1mM DTT. After mixing this material was centrifuged at 30,000g for 90 s. The upper fatty layer and pellet were discarded and the supernatant was layered onto a column of Sephadex G-25 (25 x 1cm) which had been equilibrated with 20mM Hepes buffer pH 7·6 containing 100mM KCl, 5mM MgAc and 1mM DTT. The column was eluted with the equilibration buffer at a flow rate of 3ml/ min and fractions of 0·5ml were collected. Fractions with absorbance at 260nm of greater than 90 OD's/ml were pooled and centrifuged at 30,000g for 20 min. The supernatant was stored at -70°C in small aliquots.

The standard incubation mixture contained in a final volume of 50µl: wheat germ S-30 fraction (20µl), 12mM Hepes-KOH pH 7·6, 2mM DTT, 8mM creatine phosphate, creatine Ⓟ Kinase (2µg), 1mM ATP, 20µM GTP, 95·2 µM spermine and amino acids at 30µM except the amino acid to be added as label which was added cold at a concentration of 6µM (the 20 amino acids used were those listed in section V B 17).

19. Preparation of RNA from rabbit reticulocytes[183]

A rabbit was made anaemic as described in section V B 16 and was bled on the sixth day into 100ml of Richs' Saline containing heparin (300 units). Richs' Saline contained 0·14M NaCl, 50mM KCl,

5mM $MgCl_2$. The cells were collected by centrifugation at 4°C (5,000g, 10 min) and washed twice with 3 volumes of Richs' Saline. The cells were lysed by addition of 4 volumes of lysing solution containing 2mM $MgCl_2$, 1mM DTT and 0·1mM EDTA (brought to pH 7·0 with NaOH). The lysate was diluted with 1 volume of a solution containing 1% SDS, 0·4M NaCl and to this solution an equal volume of phenol: metacresol: hydroxyquinolone: water (1000: 140: 1: 160 v/v/w/v) was added. This mixture was shaken vigorously for 10 minutes at room temperature and then the phases were separated by centrifugation (6,000g, 5 min). The aqueous layer was removed and the phenol layer and interphase were re-extracted with an equal volume of 0·1M Tris-HCl pH 9·0. The aqueous phases were pooled and re-extracted twice with an equal volume of phenol solution. The RNA was precipitated by addition of ammonium acetate to make a 0·24M solution and 2 volumes of cold (-20°C) ethanol[184]. After precipitation overnight at -20°C the RNA was collected at 100,000g (1h) and washed in ethanol to remove the phenol. The washed pellet was dissolved in glass distilled deionized water and the absorbtion spectrum was measured in the region 230-320nm to determine the RNA concentration (20 OD units were taken to be equivalent to 1mg RNA) and also to ascertain that the preparation was free of contaminating phenol.

20. Extraction and purification of polysomes

i) Magnesium precipitation of polysomes[185 - 186].

Polysomes were prepared from rat liver by the method of Palmiter[185]. Tissue was homogenised in 5 volumes (w/v) of buffer A (25mM Tris-HCl pH 7·5, 25mM NaCl, 5mM $MgCl_2$, 2% Triton X-100 and 1mg/ml heparin) with 15 strokes of a loose fitting glass-teflon homogeniser. The homogenate was centrifuged (27,000g, 5 min) and the supernatant was decanted into an equal volume of buffer B (20mM Tris-HCl pH 7·5,

20mM NaCl, 204mM $MgCl_2$, 1·6% Triton X-100). This mixture was incubated at 0°C. After 1h, aliquots (≏ 25ml) were layered over 10ml pads of buffer C (25mM Tris-HCl pH 7·5, 25mM NaCl, 5mM $MgCl_2$, 0·2M sucrose) in 50ml centrifuge tubes and centrifuged (27,000g. 10 mins). The supernatants were removed by aspiration and the pads were partly removed. The sides of the tubes were washed with distilled water and the remaining supernatant was removed by aspiration. The sides of the tubes were wiped dry and the pellets were dissolved in buffer for application to a sucrose density gradient or stored at -70°C.

A variation of this technique was employed for isolation of polysomes from arils and pea sub-apical segments as described by Akalehiywot[186]. The tissue was homogenised in 8 volumes (w/v) of buffer A (25mM Tris-HCl pH 7·5, 25mM NaCl, 5mM $MgCl_2$, 1% Triton X-100, 5mM 2-ME). The incubation time was increased to 1·5h. Buffer C contained 25mM Tris-HCl pH 7·5, 25mM NaCl, 5mM $MgCl_2$ and 1M sucrose.

ii) Preparation of polysomes by high speed centrifugation through a sucrose pad. Polysomes were prepared from pea sub-apical tissue segments as described by Verma et al[163]. Frozen tissue was ground under liquid nitrogen in a pestle and mortar until a fine powder was obtained. The powder was then scraped into another pestle and mortar (to avoid freezing the buffer) with 6 volumes (w/v) of extraction medium (150mM Tris-actate pH 8·5, 200mM sucrose (RNAse free), 50mM KCl, 20mM $MgA\bar{c}$, 4mM 2-ME and 0·4% Nonidet P-40 containing heparin (1mg/ml)) and mixed thoroughly. The slurry was centrifuged (23,000g, 10 min). Aliquots of the supernatant were layered over pad buffer (3ml containing 50mM Tris-acetate pH 8·5, 1·5M sucrose, 20mM KCl, 10mM $MgA\bar{c}$) in 10ml centrifuge tubes and these were centrifuged (105,000g, 90 min). The clear pellets obtained were resuspended gently with a small glass rod in resuspension buffer (50mM Tris-acetate pH 8·5, 20mM KCl, 10mM $MgA\bar{c}$). This solution was centrifuged

(10,000g, 5 min) to remove any insoluble material. Aliquots of the polysome solution were applied to sucrose density gradients.

Arils were homogenised in 5 volumes (w/v) extraction medium. The polysomes were collected through a 10ml sucrose pad in a 50ml centrifuge tube. In some experiments heparin was omitted from the extraction medium.

iii) Sucrose density gradient fractionation of polysomes. Aliquots of polysome solutions in up to 0·5ml of various buffers were layered over 15ml linear sucrose gradients of 125-500mg/ml formed from sucrose solutions in buffer containing 50mM Tris-acetate pH 8·5, 20mM KCl and 10mM MgAc. Sucrose gradients were centrifuged in a swing-out rotor (6 x 16·5ml) (91,000g, 100 min). The gradients were analysed by pumping through a continuous flow cell in a Pye Unicam SP 1800 spectrophotometer and absorbance at 260nm was measured. Smoothness of this procedure was ensured by using an LKB peristaltic pump to layer 50% sucrose solution into the bottom of the gradient tube.

iv) Washing of polysomes on hydroxylapatite[187]. Polysomes were suspended in buffer E (1ml) (50mM Tris-HCl pH7·4, 25mM KCl, 5mM $MgCl_2$). Dry, hydroxylapatite crystals were suspended in 1mM NaH_2PO_4, the suspension was allowed to settle for 10-15 min and the settled crystals (1ml) were resuspended in buffer F (0·15 M KH_2PO_4, 60mM Tris, 10mM $MgCl_2$ pH6·55). The resuspended crystals were poured onto a moistened filter paper disc held on a sintered glass support over a Buchner flask so that the effective diameter was 1cm. This apparatus was kept on ice. After pouring a gentle vacuum was applied until the settled crystals were about to go dry and then they were washed with 10 volumes of buffer F. At this point the crystal thickness was about 3-4mm. Polysomes were added to the crystal surface and washed with a further 10-15 volumes of buffer F.

As the crystal surface began to dry buffer G (0·3M KH_2PO_4, 0·152M Tris, 20mM $MgCl_2$ pH 6·5) was added and the vacuum disconnected. The flask was rinsed in buffer G and the vacuum line reconnected. Polysomes were eluted with a further 5-10 volumes of buffer G and recovered by centrifugation (160,000g,1h).

21. Extraction and purification of poly $(A)^{plus}$ RNA

RNA was isolated by the method of Verma et al[163]. Aril tissue was dissected out and frozen in liquid nitrogen (the frozen tissue was usually stored at -70°C until required). The frozen tissue was powdered in a pestle and mortar under liquid nitrogen. The powdered material was transferred to a flask and shaken with 3-4 volumes of buffer H (0·1M Tris-acetate, 0·1M NaCl, 2mM Na_2 EDTA, 1% SDS, pH 9·0 containing 500μg/ml heparin) for 2 minutes at 20°C. An equal volume of phenol: chloroform: isoamyl alcohol (50:50:1v/v/v) was added and shaken vigorously for 10 min. The phases were separated by centrifugation (6,500g, 5 min) and the phenol phase was re-extracted with an equal volume of buffer H. The combined aqueous phases were re-extracted twice with the original volume of phenol: chloroform: isoamyl alcohol and made 0·24M in ammonium acetate (by addition of a 2·4M solution) and 66% in ethanol (by addition of 2 volumes of cold ethanol (-20°C)). Precipitation was allowed to occur overnight at -20°C and the precipitate collected by centrifugation (30,000g, 10 min). The RNA was washed three times in cold ethanol (20 volumes), dried and dissolved in buffer I (0·4M NaCl, 10mM Tris-acetate, 0·5% SDS, pH 7·6). Insoluble material was removed by centrifugation (30,000g, 5 min) and the supernatant was applied to a column of oligo (dT)-cellulose (2ml in a 2ml syringe) at a flow rate of 2ml/min. The absorbance (260nm) of the eluant was monitored with a Uvicord (LKB Ltd). Non-absorbed RNA (poly $(A)^{minus}$) was collected and precipitated with ethanol. The column was eluted with buffer J

(buffer I minus 0·5% SDS). When the absorbance of the eluant had returned to the base line the poly $(A)^{plus}$ RNA was eluted with 10mM Tris-acetate pH 7·6. The poly $(A)^{plus}$ RNA was collected and precipitated with ethanol.

RNA samples were subjected to P.A.G.E. as described by Loening (188). Electrophoresis was at 15 volts for 20 min followed by 90 min at 40 volts. At the end of this period the gels were removed from their tubes and washed in water (15 min) and fixed in 30% ethanol overnight. After a water wash (1h) the gels were stained in 0·2% Toluidine blue (1h) and destained in water.

22. Assay for mRNA activity

Standard incubation mixtures containing either the wheat germ cell free extract or the rabbit reticulocyte lysate (section V B 17,18) were incubated at 26 or 37°C (37°C: lysate only). At the end of the incubation period (60 min) aliquots were withdrawn and spotted onto paper discs for measurement of TCA insoluble label as described in section V A 2.

23. Measurement of thaumatin synthesis in cell-free systems

Incubation mixtures containing nuclease treated reticulocyte lysate or wheat germ system were prepared as described in sections V B 17 and V B 18 except that larger volumes were used. The mixtures were supplemented with the appropriate RNA fractions. Thaumatin synthesised under these conditions was identified by immunoprecipitation and SDS P.A.G E. as described by Cashman and Pitot(189). After incubation the reaction was stopped by addition of $\frac{1}{3}$ volume of a solution containing 5% Triton X-100, 5% NaDOC and 10mM appropriate cold amino acids. Aliquots (10µl) were removed and spotted onto paper discs for determination of the total TCA insoluble label as described in section V A 2. Polysomes were removed from the mixture by centrifugation (160,000g, 1h) and further aliquots (10µl)

were removed for determination of incorporation of label into released proteins. A pre-immunoprecipitation was performed using human fibrinogen (10μg) and rabbit anti-fibrinogen serum (40μl, titre 300μg/ml). The precipitate was removed after 1h at 37°C. Thaumatin was precipitated by addition of excess anti-thaumatin II serum (section V B 5) together with thaumatin II (10μg) which was added as carrier. The precipitate was collected by centrifugation after a 1h incubation at 37°C and washed (three times) in P B S containing 1% Triton X-405 (v/v) and 10mM of the appropriate cold amino acids (500μl). The final immunoprecipitate was dissolved in buffer containing 10mM sodium phosphate pH 7·2, 2% SDS, 2% DTT and 25% glycerol by heating in a boiling water bath (5 min). Bromophenol blue (5μl) was added and the sample was subjected to SDS P.A.G.E. as described in section V A 3_{ii}). The gels were removed from their tubes and immediately frozen in cardice/methanol. The frozen gels were sliced into 2mm segments which were placed into scintillation vials and treated with 90% NCS in water (0·6ml) for 2h at 55°C. Scintillation counting was performed as described in section V A 2.

24. Dissociation and isoelectric focusing of anti-thaumatin II - thaumatin complex.

Immunoprecipitates were collected and washed in 1% Triton X-405 as described in section V B 23. The final precipitate was suspended in a solution (50μl) containing 1% SDS, 0·01M 2-ME and dissolved by heating in a boiling water bath at 100°C for 2 min. The solution was cooled and made 6M in urea by addition of a solution containing 0·01M 2-ME and 9M urea (100μl) and incubated for 30 min at 20°C. SDS was removed from this solution by a modification of the method of Weber and Kuter[215]. Dowex AG-X2 (200-400 mesh) was extensively washed on a Buchner funnel with the following solvents: 2M NaOH, distilled water, 1M acetic acid, distilled water and finally 0·05M

Tris-acetate buffer, pH 7·8. If not required immediately the resin was stored in this form. Prior to use a small amount of resin was packed into a 1ml syringe to give a ½ml column of resin and equilibrated with a solution containing 6M urea, 0·01M 2-ME. The immunoprecipitate solution was passed through this column which was developed with the equilibration solution. Protein-containing fractions of the effluent were pooled and made 10% in sucrose and 8M in urea by addition of solid sucrose and urea. The final protein solution was subjected to isoelectric focusing as described in section V A 3 iii).

RESULTS AND DISCUSSION

VI. RESULTS AND DISCUSSION

A. PURIFICATION OF T O, T I AND T II

The purification of T I and T II was first reported by Van der Wel and Loeve in 1972[44]. A similar procedure was adopted for the preparation of the thaumatin forms in this project, however the ultrafiltration and gel filtration steps were omitted since it was found that pure products could be obtained by using only an ion-exchange procedure. The purification was greatly facilitated by the fact that in mature tissue thaumatin represents a large proportion of the total protein content and that all three sweet proteins are relatively basic polymers. The purification of T O, T I and T II by chromatography on a CM-cellulose ion-exchange column is illustrated in Figure 7.

With some aril extracts from Ashanti fruit the final non-sweet fraction was relatively smaller but generally the pattern was consistent. The degree of purity of the thaumatin products was assessed by P.A.G.E. Three methods were employed; P.A.G.E. at acid pH, P.A.G.E. in the presence of SDS and P.A.G. isoelectric focusing in the pH range 8-10·5. SDS P.A.G.E. would be expected to give the most reliable indication of the purity of the thaumatins since all contaminating proteins would enter the gel. With the other two methods only those proteins with specific characteristics would migrate into the gel. Typical gels are shown in figure 8. Single protein bands were obtained with the various pure thaumatin samples with all three electrophoretic methods.

These results showed that the preparations were free from major contaminants. The single band obtained in the case of SDS gels suggested that the sweet proteins all possessed one polypeptide chain or alternatively that if they had subunit structures all of

Figure 7. Chromatography of Thaumatococcus aril extract on a CM-cellulose column.

In a typical experiment aril tissue (2g) yielded 207mg of total protein which was fractionated into three components: TO (2.8mg), TI (43.8mg) and TII (42.2mg) all of which were sweet tasting. The column was eluted with buffer (0.01M sodium phosphate pH 7.2; 56ml) and then a linear salt gradient of 0-0.2M NaCl in buffer (200ml). The flow rate was 0.2ml/min. Fractions of 4ml were collected.

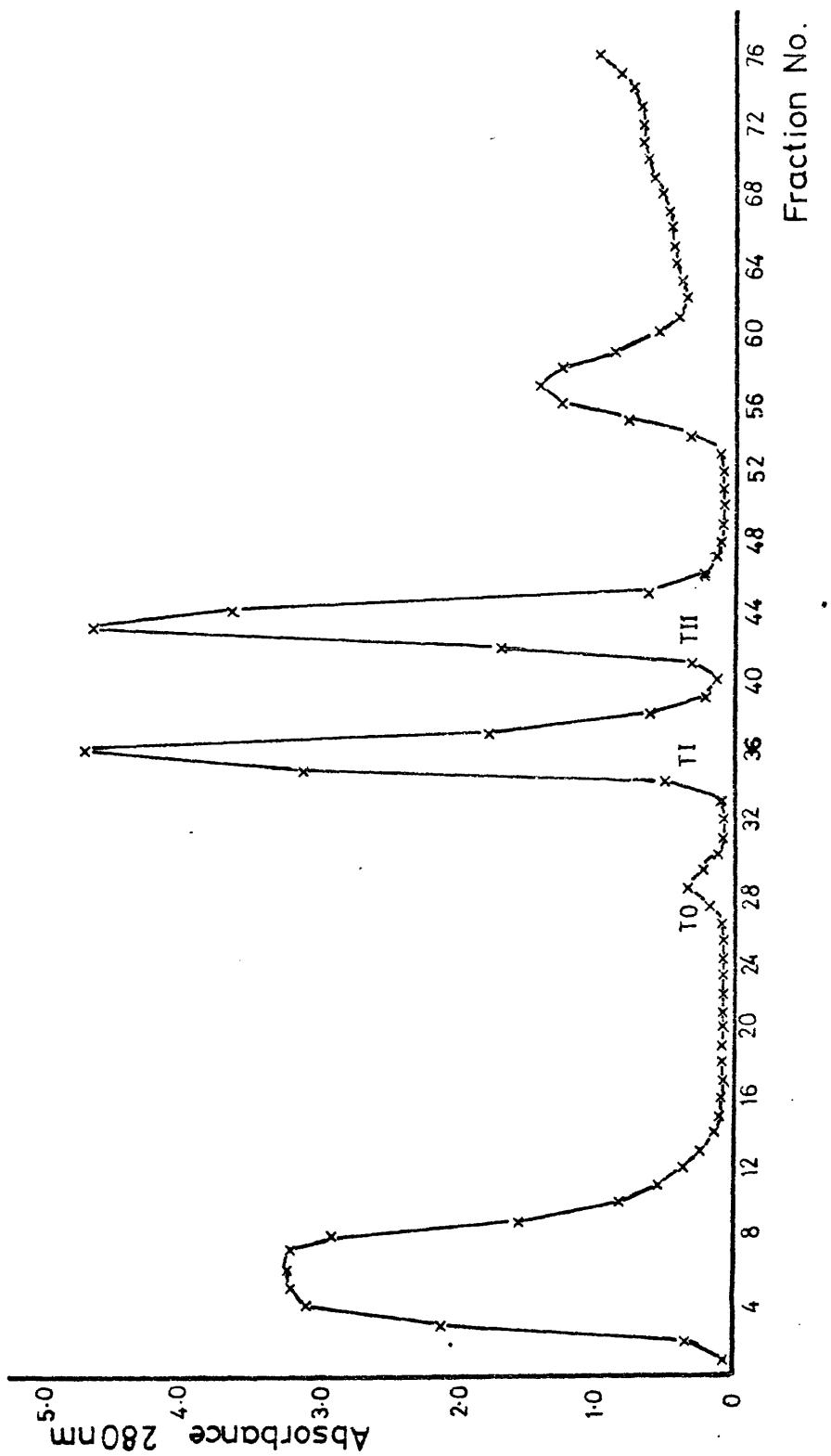
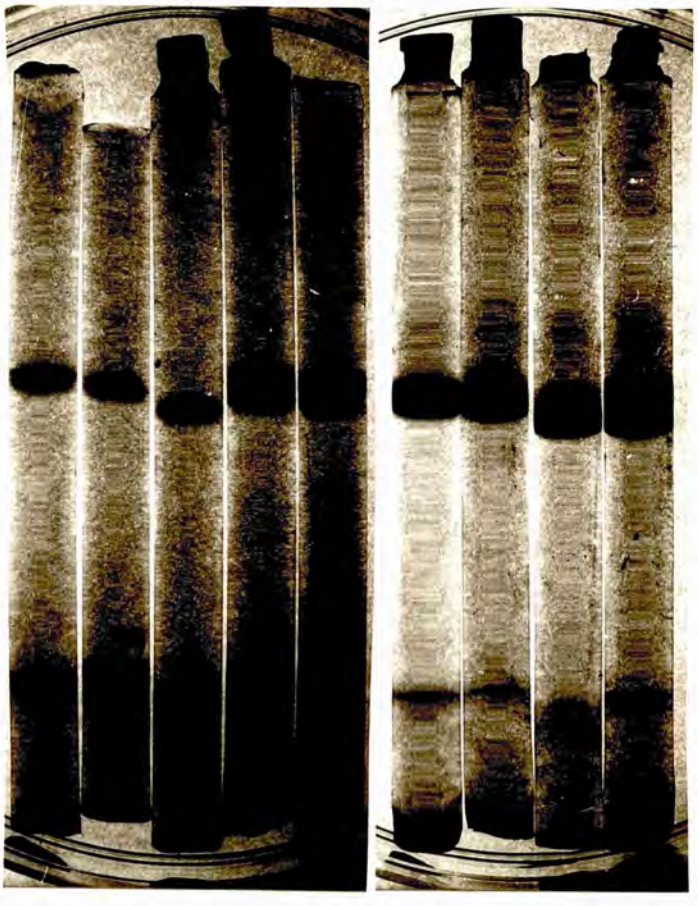
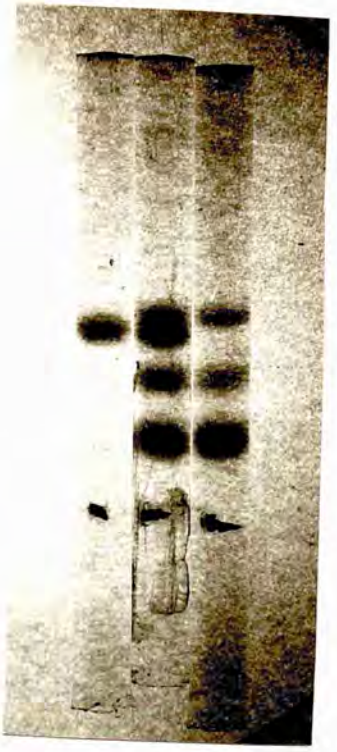

Absorbance 280nm
5·0
4·0
3·0
2·0
1·0
0
4
8
12
16
20
24
28
32
36
40
44
48
52
56
60
64
68
72
76
Fraction No.
TO
TI
TII

Figure 8 Polyacrylamide gel electrophoresis of purified thaumatins.



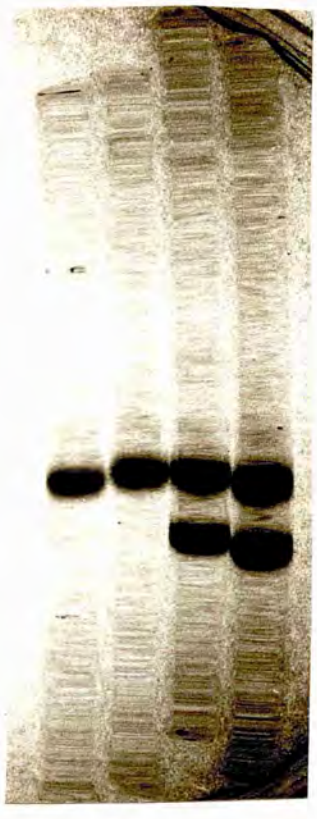
a b c d e f g h i

P.A.G.E. at acid pH was as described in section V A 3(i) a) TO (5µg), b) TI (5µg), c) TII (5µg), d) TI (5µg)+ TII (5µg), e) TO (5µg)+ TII (5µg), f) TO (100µg), g) TI (100µg), h) TII (100µg), i) TI (100µg)+ TII (100µg). The band present near the end of each gel was at the same position as the methyl green dye front (the band was also present in gels to which no protein was applied).



a b c

P.A.G.E. in the presence of SDS was as described in section V A 3(ii) a) TO (10µg)+ TI (10µg)+ TII (10µg), b) TI (30µg)+ trypsinogen (20µg)+ lysozyme (20µg)+ β-lactoglobulin (20µg), c) trypsinogen (20µg)+ lysozyme (20µg) + β-lactoglobulin (20µg). The bromophenol blue dye front was marked with indian ink.



a b c d

Polyacrylamide gel isoelectric focusing was performed as described in section V A 3(iii). a) TI (40µg), b) TI (40µg), c) TI (40µg) + TII (40µg), d) TI (40µg) + TII (40µg).

the component chains were of identical molecular weight.

B. FRUIT DEVELOPMENT AND THAUMATIN CONTENT

1. Development of a procedure for quantitative extraction of Thaumatococcus aril protein.

In order to measure the amounts of T O, T I and T II present in fruits at different stages of ripening it was first necessary to be sure that all of the sweet protein could be extracted. Initially arils from stage C Kadjebe fruit were extracted under different conditions of pH and ionic strength to test the effect of these parameters on protein extractability. The results in Tables 6a and 6b show clearly that total aril proteins were extracted more effectively at pH 7·7 than at pH 6·0 and at high rather than low salt concentration: the tissue which had been extracted with a high salt/high pH buffer retained only approximately 10% of total protein material. It was, therefore, decided to adopt these conditions for extraction of total aril protein. Another possibility would have been to have solubilised the proteins using a non-ionic detergent, Triton X-100 for example, but this would have been difficult to remove from the extract. The extraction procedure that was eventually adopted fractionated the aril proteins into two groups. The easily extracted protein (extracted at pH 6·0 and low ionic strength) and the more tightly bound protein (extracted at pH 7·7 and high ionic strength). The tissue samples were extracted twice with each buffer. The results (Tables 7 and 8) show that the extraction of protein is almost complete after the full extraction procedure. About 30% of the protein is extracted at pH 6·0 with low ionic strength and the remaining 70% by the second treatment. It is likely that the protein extracted at pH 6·0 corresponds to that protein which is normally unbound *in vivo* since the pH of aril juice is approximately 6 and presumably it has a low salt content.

Table 6 The effect of pH and salt conditions on extraction of aril protein.

6A Arils from stage C fruit (mg of protein extracted/g of aril tissue.)

ARIL SAMPLE	EXTRACTION BUFFER	FIRST EXTRACTION	SECOND EXTRACTION	THIRD EXTRACTION	FOURTH EXTRACTION	RESIDUE	TOTAL EXTRACTED
(i)	A	16.1	2.0	30.7	4.3	8.0	61.1
(ii)	A	16.4	2.1	-	-	40.0	58.5
(iii)	B	24.9	3.0	25.1	3.4	6.3	62.7
(iv)	B	24.7	4.1	-	-	36.0	64.8

Arils (16) from Nadjebe region fruit were chopped and four 0.7g samples were taken. The 3rd and 4th extractions were made with buffers containing 0.3M NaCl. (Buffer A: 0.01M sodium phosphate pH 6.0 and buffer B: 0.01M sodium phosphate pH 7.2)

Arils from
(2) stage B fruit(mg of protein extracted/g of aril tissue.)

ARIL SAMPLE	EXTRACTION BUFFER	FIRST EXTRACTION	SECOND EXTRACTION	THIRD EXTRACTION	FOURTH EXTRACTION	RESIDUE	TOTAL EXTRACTED
(i)	A	9.7	1.8	24.8	3.7	5.4	45.4
(ii)	A	9.9	4.1	-	-	29.8	43.8
(iii)	B	12.7	3.6	20.7	3.2	3.7	43.9
(iv)	B	13.7	3.1	-	-	25.8	42.6

Arils (22) from Kadjebe region fruit were chopped and four 1.0g samples were taken. Extractions were made as for table 6A.

Table 7 The differential extraction of aril protein.

EXTRACTION BUFFER	pH 6.0 LOW SALT		pH 7.7 HIGH SALT			
ARIL SAMPLE	FIRST EXTRACTION	SECOND EXTRACTION	THIRD EXTRACTION	FOURTH EXTRACTION	RESIDUE	TOTAL EXTRACTED
(i)	13.4	0.4	35.8	2.4	2.0	54.0
(ii)	15.2	1.2	34.0	1.2	2.2	53.8
(iii)	15.0	0.4	-	-	34.8	50.2

(mg protein extracted/g of aril tissue)

Arils from Kadjebe region fruits stage B were chopped and three 0.5g samples were taken. The first and second extractions were made with 0.01M sodium phosphate pH 6.0. The third and fourth extractions were with 0.01M sodium phosphate pH 7.7 containing 0.3M sodium chloride. Protein remaining in the residue was removed for estimation with 1M sodium hydroxide.

Table 8 The differential extraction of aril protein.

EXTRACTION BUFFER	pH 6.0 low SALT		pH 7.7 HIGH SALT			
ARIL SAMPLE	FIRST EXTRACTION	SECOND EXTRACTION	THIRD EXTRACTION	FOURTH EXTRACTION	RESIDUE	TOTAL EXTRACTED
(i)	14.2	0.3	33.2	2.7	3.4	53.8
(ii)	11.5	0.2	31.4	2.6	3.1	47.8
(iii)	16.2	0.3	-	-	32.1	48.6
(iv)	-	-	-	-	51.2	51.2

(mg protein extracted/g of aril tissue)

Arils from Kadjebe region fruits stage 3 were chopped and three 0.9g samples were taken. The first and second extractions were made with 0.01 M. sodium phosphate pH 6.0. The third and fourth extractions were with 0.01M. sodium phosphate pH 7.7 containing 0.3M. sodium chloride. Protein remaining in the residue was removed for estimation by extraction into 1M. sodium hydroxide.

2. Distribution of T 0, T I and T II in different development stages of fruit.

A study of the quantitative distribution of the thaumatin forms during fruit development was then made using the procedure developed in section VI B 1.

It is clear from the data in Tables 9-12 that a dramatic rise in sweet protein levels occurs during the ripening of Thaumatococcus fruits and this seems to account almost entirely for net protein synthesis during this period. However, the true rates of protein synthesis are probably masked by different rates of protein degradation. It is not possible to relate the rise in sweet protein levels to fruit age in a precise way since the exact age of the fruits involved in this study were unknown. However typical data for the ripening times of Thaumatococcus fruits have been gathered by Tate and Lyle Ltd working in Ghana. The total time which elapses between fruit set and the ripe fruit stage (D) is approximately 110 days. The 'unripe' stage (A) is reached after 12 days, the intermediate (B) after 23 days, the nearly ripe (C) after 70 days and the ripe (D) after 110 days.

There does not appear to be any definite correlation between the ratios of the different thaumatin forms and fruit maturation stages. In fact the ratio of T I to T II was very variable ranging from 0·4 to 2·0. It is possible that the samples taken were not truely representative because of the small number of individual fruits used but a large number of fruits of each ripening stage from a single fruit batch was not available. The amount of T 0 was always much smaller than the amounts of T I and T II.

The low salt extractions at pH 6 released a higher proportion of sweet protein to non-sweet protein and also of T I to T II than the high salt extraction at pH 7·7. It seems likely that the

Table 2 Content of TO, TI and TII in Ashanti region fruits at different developmental stages.

DEVELOPMENTAL STAGE	SOLUBLE					PARTICULATE				
	TOTAL PROTEIN	TO	TI	TII	TOTAL SWEET	TOTAL PROTEIN	TO	TI	TII	TOTAL SWEET
A	6.4	0.1	0.6	0.6	1.3	24.3	-	0.4	1.0	1.4
B	19.3	-	1.7	2.1	3.8	27.6	-	0.8	2.0	2.8
D+C	22.9	0.8	8.0	8.0	16.8	56.3	0.5	8.6	16.2	25.3

	SOLUBLE + PARTICULATE						
	TOTAL PROTEIN	TO	TI	TII	TOTAL SWEET	% SWEET	RATIO $\frac{TI}{TII}$
A	30.7	0.1	1.0	1.6	2.7	8.7	0.6
B	46.9	-	2.5	4.1	6.6	14.2	0.6
D+C	79.2	1.3	16.6	24.1	42.0	53.4	0.7

Fruit of the Ashanti region type collected in Bunso were divided according to their maturity. The thaumatin content of each division was estimated by the procedure described in section V E 11. The sample sizes were A (unripe) 9 fruits, 20 arils. B (intermediate) 20 fruits, 47 arils. C and D (early ripe and ripe) 8 fruits, 15 arils. The values given are as mg of protein/g of aril tissue.

Table 10 Content of TC, TI and TII in Ashanti region fruits at different developmental stages.

DEVELOPMENTAL STAGE	SOLUBLE					PARTICULATE				
	TOTAL PROTEIN	TC	TI	TII	TOTAL SWEET	TOTAL PROTEIN	TC	TI	TII	TOTAL SWEET
A	23.9	0.1	2.8	1.9	4.8	17.0	0.0	0.8	0.4	1.2
B	46.1	0.3	8.8	4.4	13.5	19.9	0.1	1.3	0.9	2.3
D+C	56.2	0.4	22.0	13.7	36.1	44.8	0.2	10.0	7.3	17.5

	SOLUBLE + PARTICULATE						
	TOTAL PROTEIN	TC	TI	TII	TOTAL SWEET	% SWEET	RATIO $\frac{TI}{TII}$
A	40.9	0.1	3.6	2.3	6.0	14.6	1.5
B	66.0	0.4	10.6	5.3	16.3	24.3	2.0
D+C	101.0	0.6	32.0	21.0	53.6	53.1	1.5

Fruit of the Ashanti region type were divided according to their maturity. The thaumatin content of each division was estimated by the procedure described in section V B 11. The sample sizes were A (unripe) 6 fruits, 16 arils. B (intermediate) 6 fruits, 14 arils. C and D (early ripe and ripe) 6 fruits, 17 arils. The values given are as mg of protein/g of aril tissue.

Table 11 Content of To, TI and TII in Ashanti region fruits at different developmental stages.

DEVELOPMENTAL STAGE	SOLUBLE					PARTICULATE				
	TOTAL PROTEIN	To	TI	TII	TOTAL SWEET	TOTAL PROTEIN	To	TI	TII	TOTAL SWEET
A	2.4	0.0	0.1	0.1	0.2	9.6	0.1	0.3	0.2	0.6
C	31.0	0.5	8.3	7.1	15.9	41.6	0.3	5.9	6.1	12.3
D	28.6	0.3	5.5	11.8	17.6	38.1	0.2	3.9	11.1	14.5

	SOLUBLE + PARTICULATE						
	TOTAL PROTEIN	To	TI	TII	TOTAL SWEET	% SWEET	RATIO $\frac{TI}{TII}$
A	12.0	0.1	0.4	0.3	0.8	6.3	1.6
C	72.6	0.8	14.2	13.2	28.2	39.1	1.1
D	66.7	0.5	9.0	22.9	32.4	48.7	0.4

Fruit of the Ashanti region type collected in Jamasi were divided according to their maturity. The thaumatin content of each division was estimated by the procedure described in section V B 11. The sample sizes were A (unripe) 6 fruits, 16 arils. C (early ripe) 6 fruits, 14 arils. D (ripe) 6 fruits, 17 arils. The values given are as mg of protein/g of aril tissue.

Table 12 Content of TO and TI in Kadjebe region fruits at different developmental stages.

DEVELOPMENTAL STAGE	SOLUBLE				PARTICULATE			
	TOTAL PROTEIN	TO	TI	TOTAL SWEET	TOTAL PROTEIN	TO	TI	TOTAL SWEET
A	12.9	0.3	5.9	6.2	21.4	0.1	4.5	4.6
B	23.1	0.2	13.3	13.5	24.0	0.1	5.9	6.0
D+C	48.5	1.2	34.9	36.1	38.7	0.7	16.9	17.6

	SOLUBLE + PARTICULATE				
	TOTAL PROTEIN	TO	TI	TOTAL SWEET	SWEET
A	34.3	0.4	10.4	10.8	31.3
B	47.1	0.3	19.2	19.5	41.5
D+C	87.2	1.9	53.8	55.7	63.8

Fruit of the Kadjebe region type collected in Alavaijo were divided according to their maturity. The thaumatin content of each division was estimated by the procedure described in section V B 11. The sample sizes were A (unripe) 6 fruits, 17 arils. B (intermediate) 6 fruits, 18 arils. C and D (early ripe and ripe) 6 fruits, 18 arils. No thaumatin II was detected in any of the samples. The values given are as mg of protein/g of aril tissue.

thaumatins are generally held to insoluble cellular material by a non-specific ionic type interaction. It should be noted for example that T II which is more positively charged is most strongly held.

The results show strikingly that fruit from the Kadjebe region do not contain T II whilst the thaumatin content is made up for by a greater content of T I. (This is a result which has been obtained many times using ripe Kadjebe-type fruits when these have been used as a source of T O and T I. Unfortunately only one batch of Kadjebe region fruit which contained mature and immature material was available). This raises the interesting subject of taxonomic differences between Thaumatococcus plants from Kadjebe and Ashanti and whether fruits from the former area lack a specific gene for T II production. The two most likely hypotheses to account for the regional protein difference are: (i) the sweet proteins are different gene products and the gene for T II is absent or non-functional in Kadjebe fruit; (ii) the sweet proteins are the same gene product and either T II is derived from T I by an enzyme absent in Kadjebe fruit or T II is broken down as fast as it is made in Kadjebe but not in Ashanti fruit.

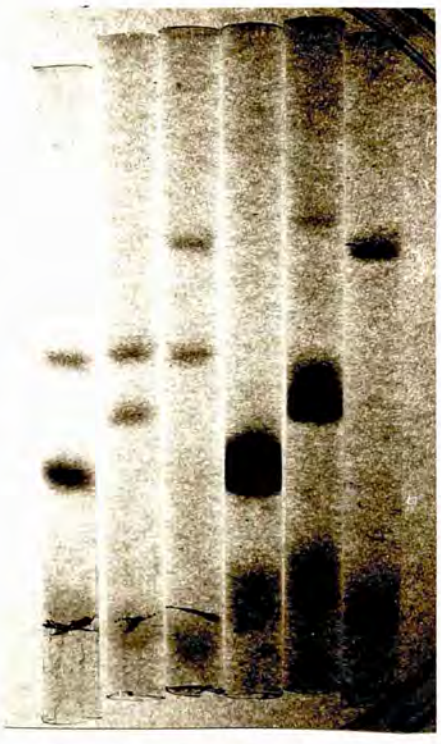
If the thaumatins are different gene products then they are likely to have fundamental structural differences. Attempts were therefore made to discover differences between the three forms.

C. STRUCTURAL ANALYSIS OF T O, T I AND T II

1. Molecular weight determination of T O, T I and T II

Small differences between the molecular weights of T I and T II were reported by Van der Wel and Loeve[44] who used both ultracentrifugation and gel filtration methods. In this present study this was reinvestigated by SDS P.A.G.E. Various proteins of known molecular weight were used to calibrate the gel system. All the sweet proteins co-chromatographed on SDS polyacrylamide gels (Fig.9) which suggests strongly that they have identical molecular weights of

Figure 9 SDS P.A.G.E. of thaumatin and molecular weight standards.



a b c d e f

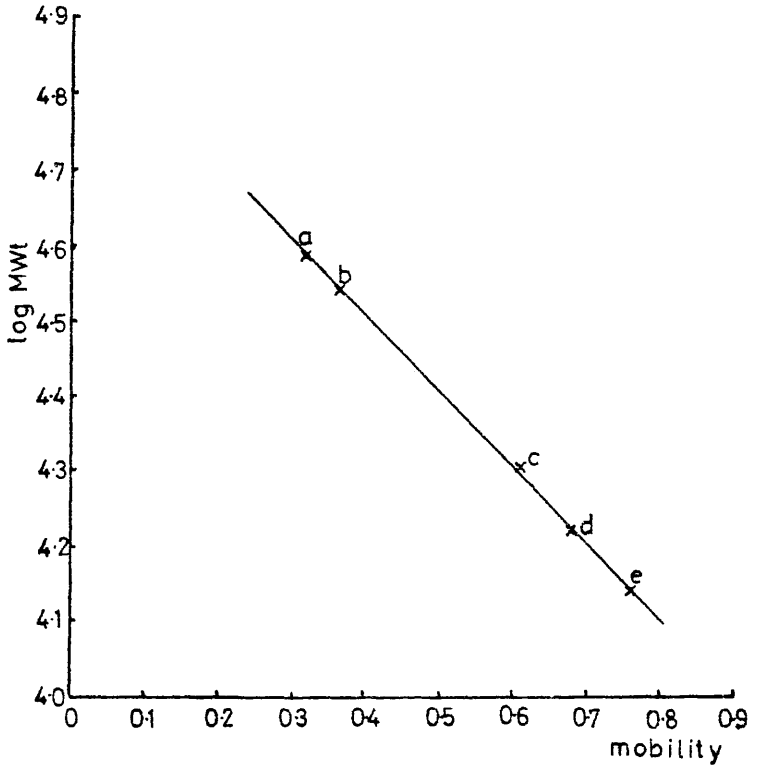
Electrophoresis was as described in section V A 3(ii). a) lysozyme (10µg) + TI (5µg), b) myoglobin (10µg) + TI (5µg), c) pepsin (10µg) + TI (5µg), d) lysozyme (50µg), e) myoglobin (50µg), f) pepsin (50µg). The bromophenol blue dye front was marked with indian ink.

Figure 10 SDS P.A.G.E.: mobility against molecular weight of standard proteins.

Various protein molecular weight standards were subjected to SDS P.A.G.E. on 10% polyacrylamide gels (5mA/gel for 7.5h).

a) RNA polymerase b) pepsin c) soybean trypsin inhibitor d) myoglobin e) lysozyme.

A thaumatin mixture (TO, TI and TII) was applied to five gels and the molecular weight was calculated.



20,200 ± 250 (from 5 gels) (Fig.10). This can be compared with the results of Van der Wel and Loeve[44] who quoted molecular weights of 21,000 ± 600 and 20,400 ± 600 for T I and T II respectively obtained by ultracentrifugation and 19,500 ± 1,900 and 18,000 ± 1,800 calculated from gel filtration on Sephadex G-50. The molecular weight of T I calculated from the amino acid composition is 22,415[47].

2. Amino acid analysis of T 0, T I and T II

Amino acid analysis of the thaumatins was performed in order to discover whether measurable differences in composition occurred between the three forms. Obviously proteins with similar molecular weights but differing amino acid compositions are not likely to be derived from a single precursor.

The results of the amino acid analyses are shown in Table 13 which indicates that the three sweet proteins are almost identical. The small differences which exist could be accounted for entirely by experimental error. The results for T I and T II agree well with those given by Van der Wel and Loeve[44]. It is interesting to note that the thaumatins do not contain a large proportion of the basic amino acids, histidine being absent. A high proportion of basic amino acids might have been predicted from the reported pI of thaumatin.

3. N-terminal analysis of the thaumatins

Initially N-terminal analysis of the three forms by the dansylation method was used[190] but unfortunately the results obtained were difficult to interpret because of the production of dansylated fractions which could not be identified together with dansylated alanine. The cyanate method was therefore employed. This procedure yields as a final product the free N-terminal amino acid which can readily be characterised with an amino acid analyser. The results obtained with T 0, T I and T II were identical indicating the presence of N-terminal alanine (see Fig.11). With T 0, however,

Table 13 Amino acid composition of thaumatins 0,I and II.

Values given in moles/mole of protein

Amino acid	TO	TI	TII	known sequence TI	figures taken from ref 44	
					TI	TII
lysine	11	11	11	11	11	11
histidine	0	0	0	0	0	0
arginine	20.7	17.4	17.4	14	-	-
cystine	11.9	11.7	11.2	12	12.4	13.2
aspartic acid	23.5	22.9	21.0	22	21.6	19.9
threonine	17.5	19.0	19.4	20	19.1	17.4
serine	13.4	15.1	15.3	14	11.8	13.2
glutamic acid	11.1	10.0	10.0	10	10.3	10.4
proline	12.2	12.2	12.1	12	12.8	12.3
glycine	24.0	22.9	21.5	24	21.3	19.8
alanine	16.9	16.1	15.3	16	14.2	13.7

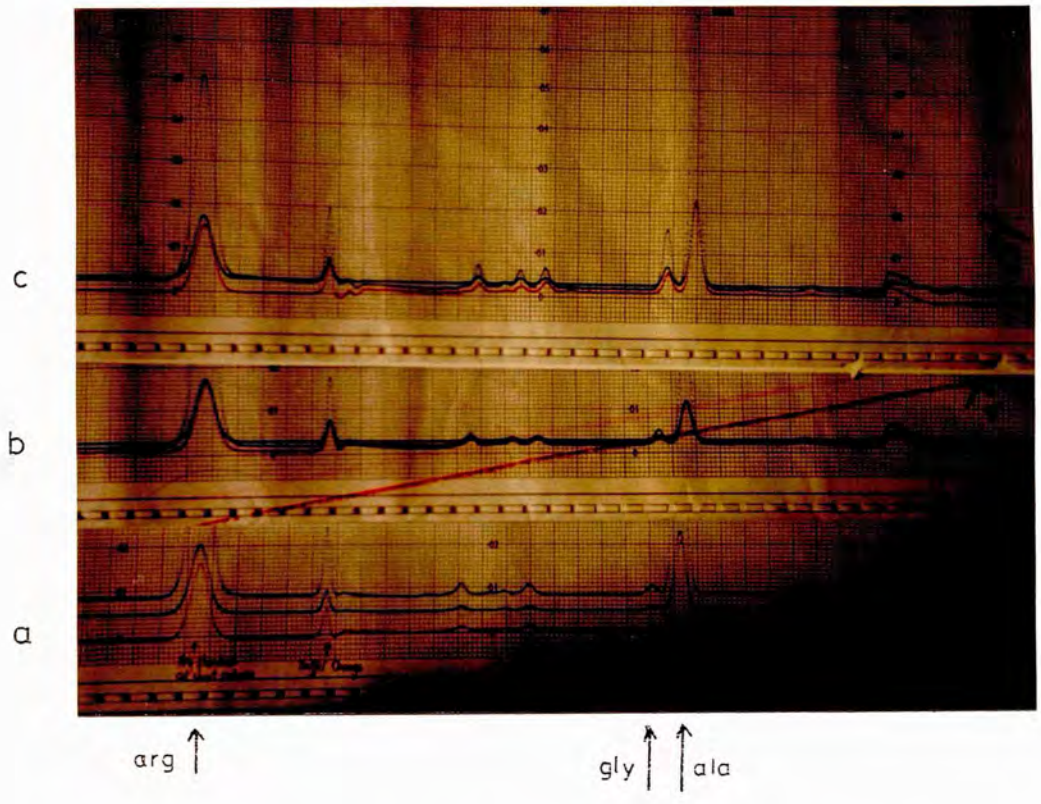
Amino acid	T.	TI	III	known sequence II	figures taken from ref 44	
					II	III
cysteine	14.9	14.7	14.1	16	14.4	13.3
valine	9.4	9.5	9.7	10	9.0	8.6
methionine	2.0	2.0	2.0	1	1.0	1.3
isoleucine	7.6	7.6	7.2	8	7.3	7.3
leucine	10.0	9.3	8.7	9	9.6	9.5
tyrosine	8.3	8.6	7.2	8	7.6	8.3
phenylalanine	10.9	10.6	10.1	11	11.1	10.6
	measured spectrophotometrically					
tryptophan	2.3	2.4	2.9	3	3.9	3.7
tyrosine	4.6	5.9	6.1	-	-	-

Thaumatins I and II were prepared from Ashanti type fruit as described in section (V 2 1). Samples were hydrolysed for 24 and 48h and in duplicate. The sample sizes were as follows:- 24h: T; 1.5mg, 1.8mg; II; 6.0mg, 5.3mg; III; 5.5mg, 4.6mg; 48h: I; 1.2mg, 2.1mg; II; 6.7mg, 5.0mg; III; 4.1mg, 7.1mg. Approximately 0.2mg of each protein sample was used for each chromatogram. Peak area was calculated from the formula:-

area = peak height x peak width at half peak height

Peak area was used to calculate the amino acid concentration by comparison with a standard having a known amino acid content.
The lysine value was set to 11 and the other values were adjusted accordingly.

Figure 11 N-terminal analysis of TO, TI and TII.



Samples of TII, TI and TO were used for N-terminal analysis by the cyanate method as described in section V B 3. The N-terminal amino acid was identified by amino acid analysis. A sample of arginine was included on the short column of the analyser as a reference. c) TO, b) TI and a) TII.

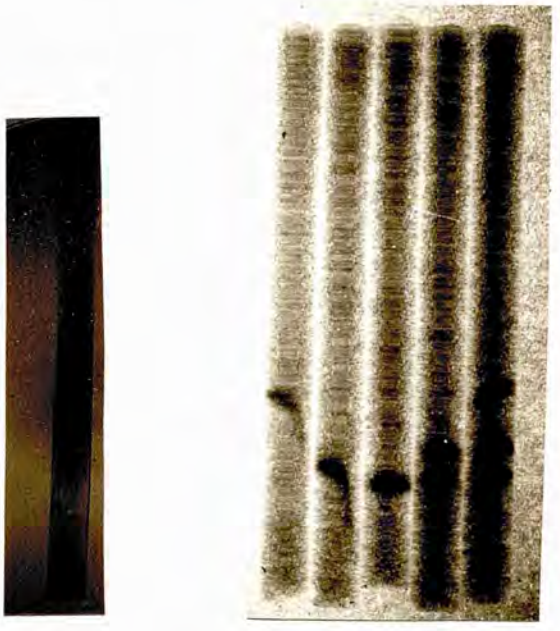
a small amount of glycine, together with the alanine, was detected (Fig.11(c)). This was considered to be a minor contaminant. The result with T I and T II confirms earlier published data[44] obtained by the dinitrofluorbenzene method and also in the case of T I from sequence data[47].

4. Determination of the pIs of T 0, T I and T II

The fact that the three thaumatins could be separated by ion-exchange chromatography (see section VI A) suggested that the pI values were different and so these were investigated. Initially isoelectric focusing was thought to be unsuitable for these measurements because Van der Wel and Loeve[44] had reported values of about 12 for T I and T II and ampholines giving pH values greater than 11 were not available. However, it was noted that the sweet proteins did not migrate towards the cathode when examined on polyacrylamide gels at pH 8·6 containing urea. This suggested, therefore, that their pI values were significantly lower than 12 and that isoelectric focusing could be used. Hence samples were focused in the presence and absence of urea (Fig. 12 and 13).

In the presence of urea the pI of the proteins can be seen to be in the range 8·0-8·3 (T 0, 8·04; T I, 8·18; T II 8·24). In the absence of urea they have slightly higher pI values as judged by their increased mobilities on the gels. Salaman and Williamson[191] have measured the pI values of various proteins in the presence and absence of urea. They found that of the ten proteins they examined only in one case, bovine serum albumin, was the pI changed by more than one pH unit in the presence of urea. Thaumatin, therefore, appears to resemble the majority of the proteins used in the above study in which only minimal differences in pI were noted under the two different electrophoretic conditions. The difference in the pI values of T 0, T I and T II was of the order to be

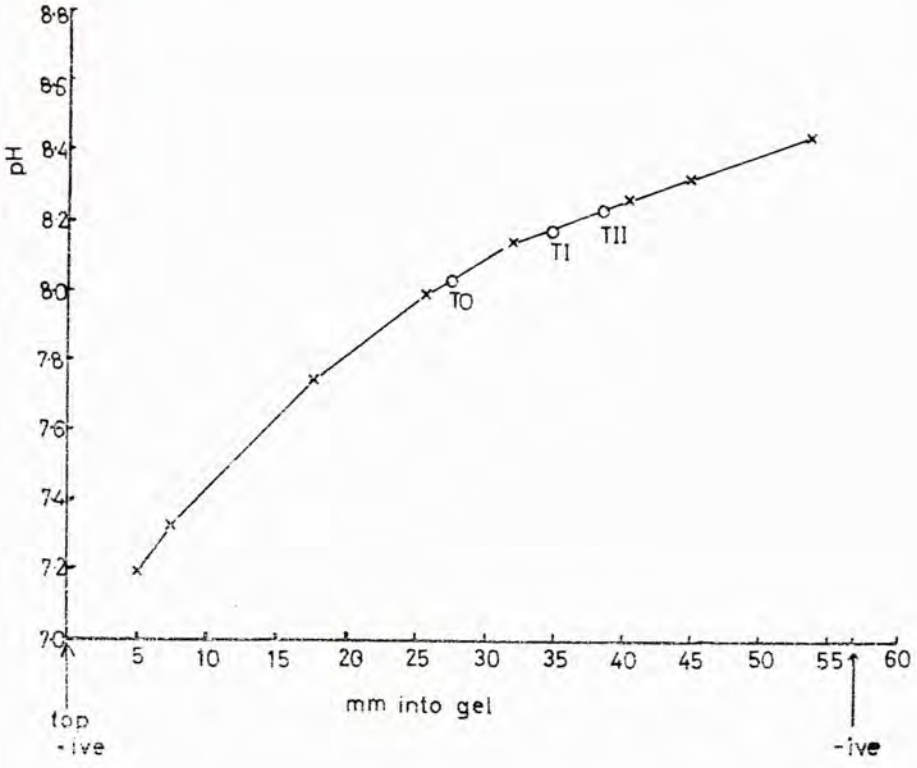
Figure 12 P.A.C.I.F. of TO, TI and TII in the presence and absence of urea.



a b c d e f

Polyacrylamide gel isoelectric focusing was performed as described in section V B 3(iii) except that urea was omitted from the sample and gel in the case of gels b-f. a) TO (20µg)+ TI (20µg)+TII (20µg), b) TO (10µg), c) TI (10µg), d) TII (10µg), e) TI (10µg)+ TII (10µg), f) TO (10µg)+ TI (10µg)+ TII (10µg).

Figure 13 P.A.G.I.F. a plot of the pH gradient.



TO, TI and TII were subjected to P.A.G.I.F.. After focusing the gels were cut longitudinally. One half was stained whilst the other was used for measurement of the pH at various points using a surface pH electrode. The gel increased in length during staining; this was taken into consideration in plotting the positions of TO, TI and TII on the graph.

expected if the structures differed by a single amide group as could occur by *in vivo* deamidation[192] as follows: T II → T I → T 0.

5. Fingerprinting of T C, T I and T II by SDS P.A.G.E. after limited proteolysis

Fingerprints of the thaumatin forms were obtained in order to be able to assess possible differences in amino acid sequences. Initially tryptic digests were chromatographed on paper or thin layer chromatograms in two dimensions. The fingerprints obtained for the different thaumatin forms were observed to be essentially identical but there were a small number of 'difference peptides'. Unfortunately difficulties were encountered with reproducibility using these techniques and it was not possible to determine the exact location or numbers of the 'difference peptides'. Column chromatography was, therefore, investigated as a method for the separation of the thaumatin tryptic peptides. This method should have allowed easy recovery and analysis of the 'difference peptides'. However, here again great difficulty was encountered in obtaining reproducible results.

Finally, the method of Cleveland *et al*[175] was employed for fingerprint analysis. This method has many advantages over the previous techniques used. Very small quantities of protein are required which in the case of small samples of protein being available still allows various conditions of proteolysis to be examined. Furthermore the peptides can be quickly analysed by SDS P.A.G.E. Samples of the three thaumatins were treated with papain, chymotrypsin and the protease from *Staphylococcus aureus*. The *Staph. aureus* digestion was repeated in the presence of both phosphate and acetate buffers. In phosphate buffer this enzyme cleaves peptide bonds on the carboxyl-terminal side of either aspartate or glutamate residues whilst in acetate buffer only glutamoyl bonds are cleaved[193]. It was hoped that if the thaumatin proteins differed only in their

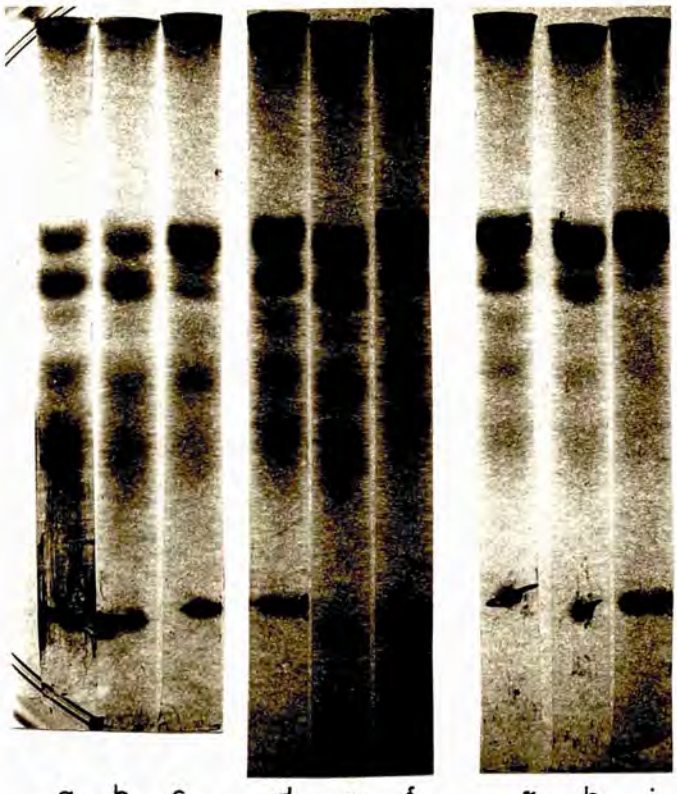
relative degrees of amidation then the fingerprints obtained for papain and chymotrypsin would be identical since these enzymes cleave at sites other than glutamine/glutamic acid and asparagine/aspartic acid. However if the patterns obtained with Staph. aureus protease were different then it would have shown that different amidation patterns existed which could have related to differences of pI between the thaumatins. Furthermore it would have been possible to tell whether amidation differences were due to glutamine/glutamic acid or asparagine/aspartic acid because of the buffer dependent properties of the Staph. aureus protease.

Fig.14 shows that the thaumatins gave identical fingerprints under all of the digestion conditions employed. This is strong evidence that they have very similar amino acid sequences. Evidently if T C, T I and T II differ in their relative degrees of amidation then the difference groups are not among those accessible to Staph. aureus protease perhaps for conformational reasons. The results also suggest that the conformations of the thaumatins are similar since any gross differences would have resulted in the accessibility of a different set of sites to the protease.

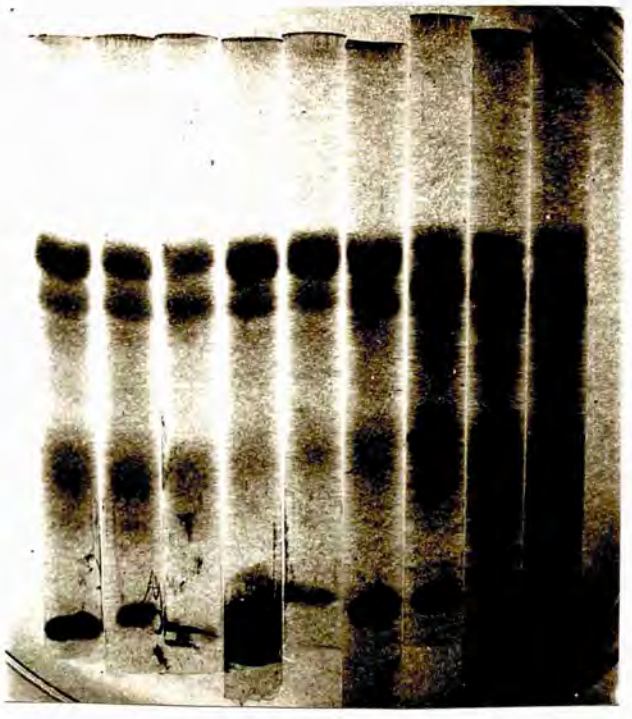
Overall the evidence indicates that the thaumatins have almost identical structures. This conclusion is based on the similarity between their molecular weights, amino acid compositions, N-terminal amino acid residues, isoelectric points and fingerprint patterns. The differences between them could perhaps best be explained by differences in the numbers of amide groups present. Against this must be set the fact that identical peptide patterns resulted from digestion of the thaumatins with Staph. aureus. protease.

The pI differences between T C, T I and T II are of the magnitudes expected if the thaumatins differ only in their amide group contents. Analysis of the total amide contents was attempted

Figure 14 Peptide maps of TO, TI and TII.

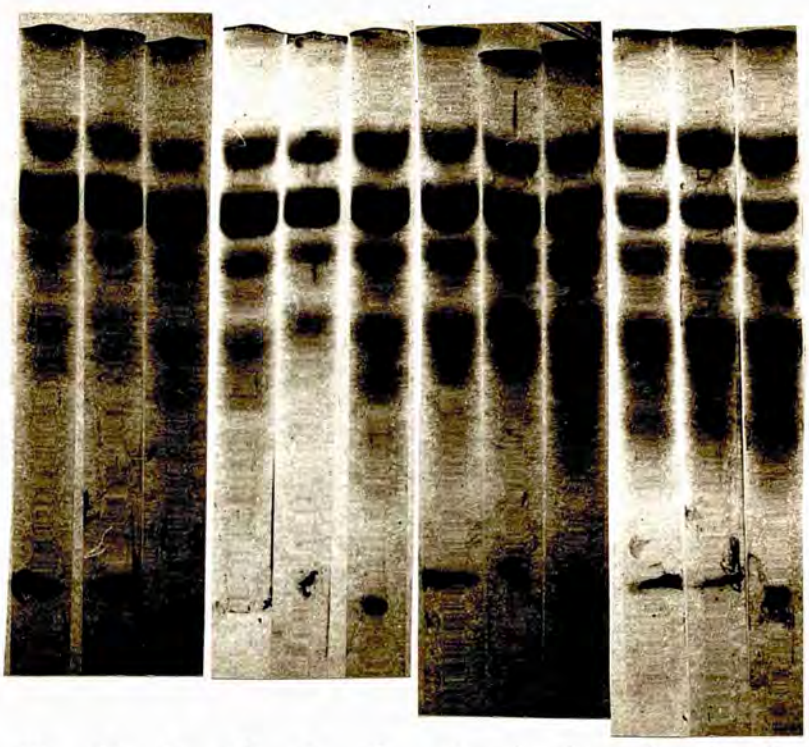


Peptide maps were made as described in section V B 4. Each digest was of 20µg of TO, TI or TII with various amounts of papain in a final volume of 40µl of phosphate buffer for 1h. a) TI, 1µg papain; b) TII, 1µg papain; c) TO, 1µg papain; d) TI, 0.4µg papain; e) TII, 0.4µg papain; f) TO, 0.4µg papain; g) TI, 0.13µg papain; h) TII, 0.13µg papain; i) TO, 0.13µg papain.



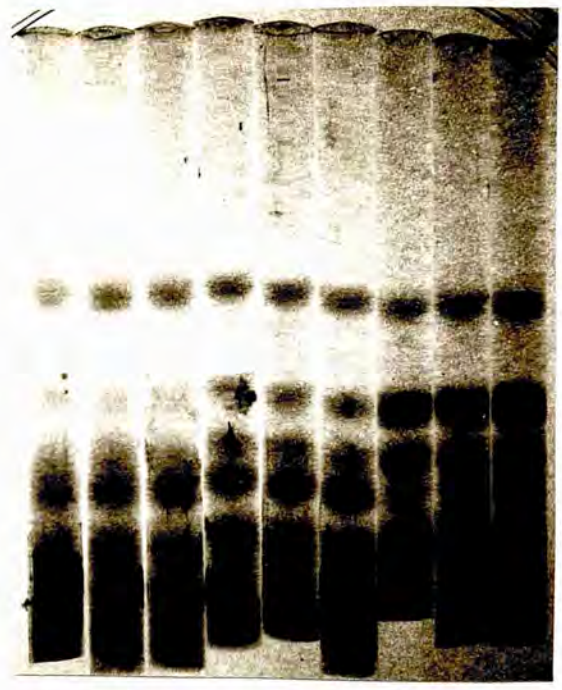
a b c d e f g h i

Peptide maps were made as described in section V B 4. Each digest was of 20µg of TO, TI or TII with various amounts of α-chymotrypsin in a final volume of 40µl of phosphate buffer. Incubation times were as stated. a) TI, 5µg α-chymotrypsin, 30min; b) TII, 5µg α-chymotrypsin, 30min; c) TO, 5µg α-chymotrypsin, 30min; d) TI, 2.5µg α-chymotrypsin, 60min; e) TII, 2.5µg α-chymotrypsin, 60min; f) TO, 2.5µg α-chymotrypsin, 60min; g) TI, 5µg α-chymotrypsin, 60min; h) TII, 5µg α-chymotrypsin, 60min; i) TO, 5µg α-chymotrypsin, 60min.



a b c d e f g h i j k l

Peptide maps were made as described in section V B 4. Each digest was of 20µg of TO, TI or TII with various amounts of Staphylococcus aureus V8 protease in a final volume of 40µl phosphate buffer. Incubation times were as stated. a) TI, 1.7µg protease, 30min; b) TII, 1.7µg protease, 30min; c) TO, 1.7µg protease, 30min; d) TI, 5µg protease, 30min; e) TII, 5µg protease, 30min; f) TO, 5µg protease, 30min; g) TI, 5µg protease, 60min; h) TII, 5µg protease, 60min; i) TO, 5µg protease, 60min; j) TI, 10µg protease, 60min; k) TII, 10µg protease, 60min, l) TO, 10µg protease, 60min.



a b c d e f g h i

Peptide maps were made as described in section V B 4, except that 10% acrylamide gels were used. Each digest was of 15µg of TO, TI or TII with 15µg Staphylococcus aureus V8 protease in a final volume of 30µl of acetate buffer. Incubation times were as stated. a) TO, 60min; b) TI, 60 min; c) TII, 60min; d) TO, 45min; e) TI, 45min; f) TII, 45 min; g) TO, 30min; h) TI, 30min; i) TII, 30min.

by treatment of the proteins with concentrated HCl at 37°C for 10 days. The free ammonia generated was collected by the Conway microdiffusion method[194] and determined with ninhydrin[195]. Initial experiments showed that all forms of the protein possessed a very similar amide content (T 0, 0·372 μmol/mg; T I, 0·503 μmol/mg; T II, 0·440 μmol/mg) but the procedure was not accurate enough to show small differences between the thaumatin forms.

D. IMMUNOLOGICAL COMPARISON OF T 0, T I AND T II

1. Production of antiserum to thaumatin II

Hough and Edwardson[11] and Van der Wel and Bel[12] have raised antibodies to thaumatin I in rabbits. These workers did not, however, report on the cross-reactivity between the antibodies and the other thaumatin forms. In the present investigation thaumatin II was chosen as antigen for two reasons, firstly, because it seemed most likely that, at least in fruit from the Ashanti region of Ghana, T II was the primary product of the thaumatin gene whilst the other forms were derived by post-translational modifications.

Secondly, it was thought desirable to raise antibodies to thaumatin II rather than to T I since anti-T I had already been studied[11,12].

After the primary course of injection (using two animals) the antiserum titre reached a plateau at approximately 20-25 μg (1ml serum at equivalence with 20-25μg thaumatin II).

After the secondary injections and subsequent booster injections the antibody titre slowly rose to approximately 70 μg. This level of antibody was adequate for the purposes of the project, however, the low value suggests that thaumatin II is rather a 'poor' antigen.

2. Test of antibody specificity by immunoelectrophoresis

Immunoelectrophoresis was used to assess the specificity of the antiserum for T II. Samples of aril extract and purified

thaumatins were subjected to electrophoresis and then allowed to diffuse against control and anti-thaumatin II sera. The final plates obtained are shown in figure 15.

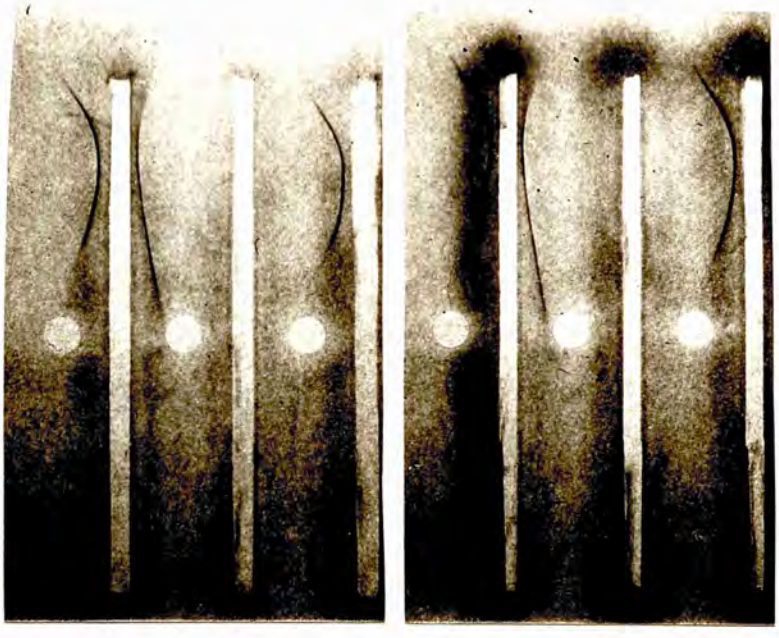
Purified T II, a mixture of purified T O, T I and T II and an aril extract all gave rise to single lines of precipitation. No precipitin arcs other than those associated with the thaumatins were observable in the case of immunoelectrophoresis of the aril extract. Therefore, it must be presumed that the antibody preparation was specific for thaumatin . As expected the mixture of thaumatins and the aril extract gave extended precipitin arcs because of the degree of separation of T O, T I, and T II on the agar gel. All three thaumatins reacted with anti-T II demonstrating that T O, T I and T II share at least some antigenic determinants. Ouchterlony double diffusion, quantitative precipitin and micro-complement fixation techniques were next used to examine the immunological relationship between the sweet proteins.

3. Determination of the degree of cross-reactivity between anti T II and the three thaumatin forms.

When using the Ouchterlony method[196] to compare different antigens wells are cut in agar to form a hexagonal pattern around a central well. The antibody is placed in the central well and the various antigens in the other wells. Identical antigens give a single curved precipitin line whilst completely different antigens give straight lines. If one antigen, A, has some of the antigenic determinants of a second antigen, B, to which antibody in the central well has been raised then a curved precipitin band forms between A, B and anti-B with a spur coming off at a tangent in the direction of A (see Fig.16).

The plates obtained by this method (Fig.17) show that single curved precipitin lines were obtained in the case of each thaumatin form (the reaction of identity). The absence of any spurs is

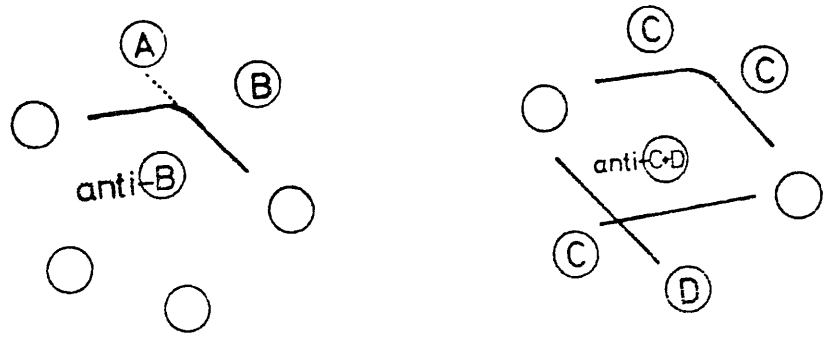
Figure 15 Immunoelectrophoresis of thaumatin.



a A b B c C d D e E f F

Immunoelectrophoresis was performed as described in section V B 6. Troughs A and C contained 50µl and troughs D and F, 100µl of anti-TII antiserum (titre 70µg/ml, mixture of sera from two rabbits). Troughs B and E contained 50 and 100µl of pre-immune serum respectively. Wells a and c contained TII (2µg) and wells d and f, TII (4µg). Well b contained 1µg each of TO, TI and TII. Well e contained aril extract (20µg prepared as described in section V B 1).

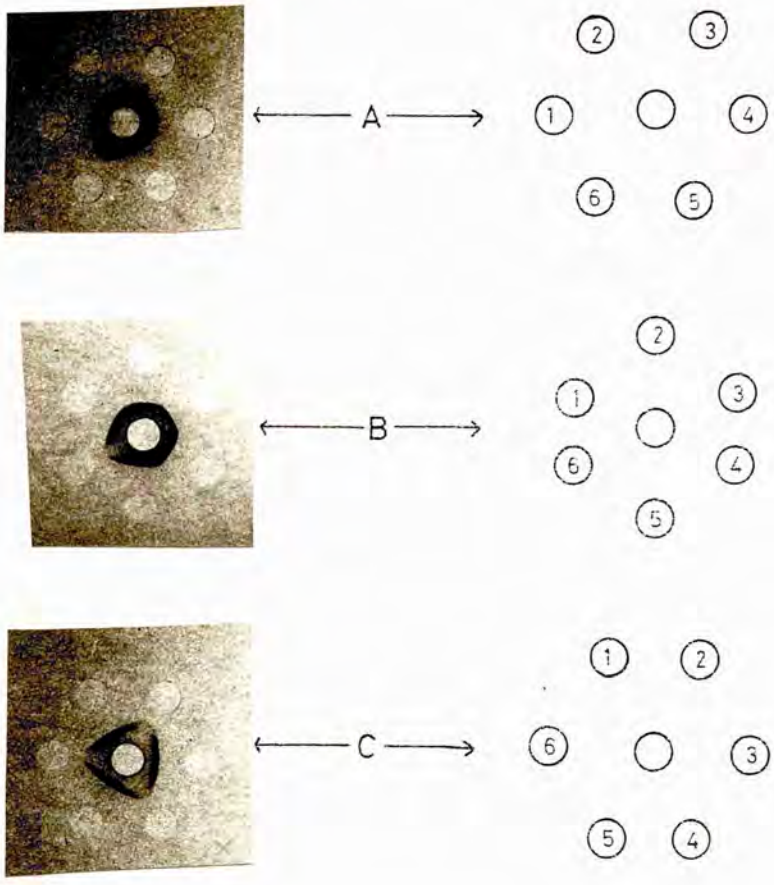
Figure 16 Ouchterlony double diffusion.



On the left ; antigen A has some of the antigenic determinants of antigen B.

On the right ; the central well contains antibodies to antigens C and D which do not share antigenic determinants.

Figure 17 Ouchterlony double diffusion of thaumatin.



Ouchterlony double diffusion was carried out as described in section V B 7. Anti-TII antiserum was placed in the central well, thaumatin samples were dissolved in 0.1M barbital buffer pH 8.6 and placed in the outside wells. A; the central well contained anti-TII antiserum (20µl, titre 70µg/ml), the outside wells were filled as follows 1) TII, 40µg; 2) TI, 40µg; 3) TO, 40µg; 4) TII, 40µg; 5) TI, 40µg; 6) buffer. B; the central well contained anti-TII antiserum (10µl, titre 70µg/ml), outside wells contained 1) TII, 20µg; 2) TI, 20µg; 3) TO, 20µg; 4) TII, 20µg; 5) TI, 20µg; 6) buffer.

C; the central well contained anti-TII antiserum (10µl, titre 70µg/ml), outside wells contained 1) TII, 2.0µg; 2) buffer; 3) TI, 2.0µg; 4) buffer; 5) TO, 2.0µg; 6) buffer.

evidence of a close similarity between T O, T I and TII. Ouchterlony double diffusion is not as sensitive to small antigenic differences between proteins as the quantitative precipitin or micro-complement fixation techniques. According to Prager and Wilson[197] (working with bird lysozymes) the results of the latter two methods can be related graphically by plotting percent cross-reaction in the quantitative precipitin method against the index of dissimilarity in the micro-complement fixation method. (Index of dissimilarity is the factor by which the antiserum concentration must be raised in order for a heterologous antigen to produce a complement fixation curve with a peak height equal to that produced by the homologous antigen. Cross-reactivity is calculated from the relative absorbance at the points of maximum protein precipitation in the quantitative precipitin method). This plot gives a straight line which can be expressed by a simple equation:

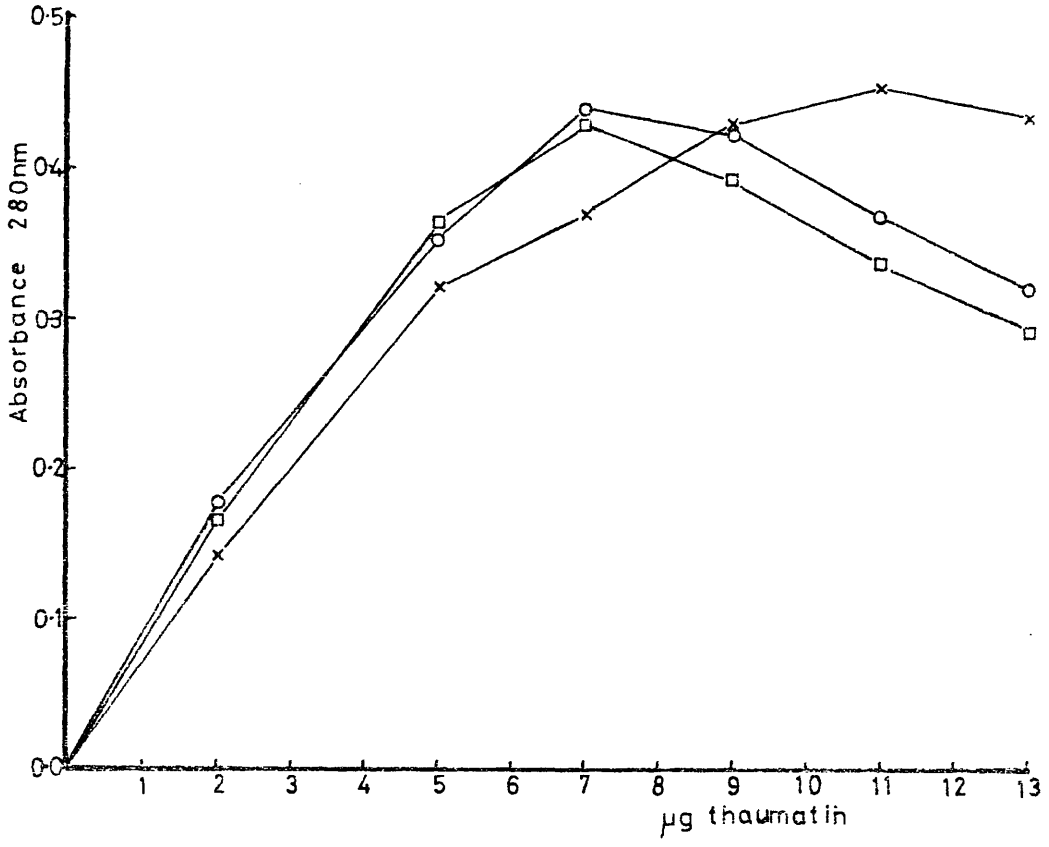
$$\log y = \frac{2{\cdot}24\ (100 - Z)}{100}$$

where y = index of dissimilarity and z = percent cross-reactivity.

The threshold for the formation of spurs, as opposed to lines of identity, in the lysozyme system was at about 80% cross-reactivity which corresponded to an index of dissimilarity of 3. When percent cross-reaction was plotted against the known percent sequence differences in the same lysozyme system and for other proteins a general relationship was seen. It was calculated that if this was applied to the thaumatins a cross-reactivity of >80% would imply a range of 0-30 amino acid sequence differences between forms.

Hence, quantitative precipitin and micro-complement fixation were employed in order to determine the percent cross-reactivity between the thaumatin forms and the results are shown in figures 18 and 19.

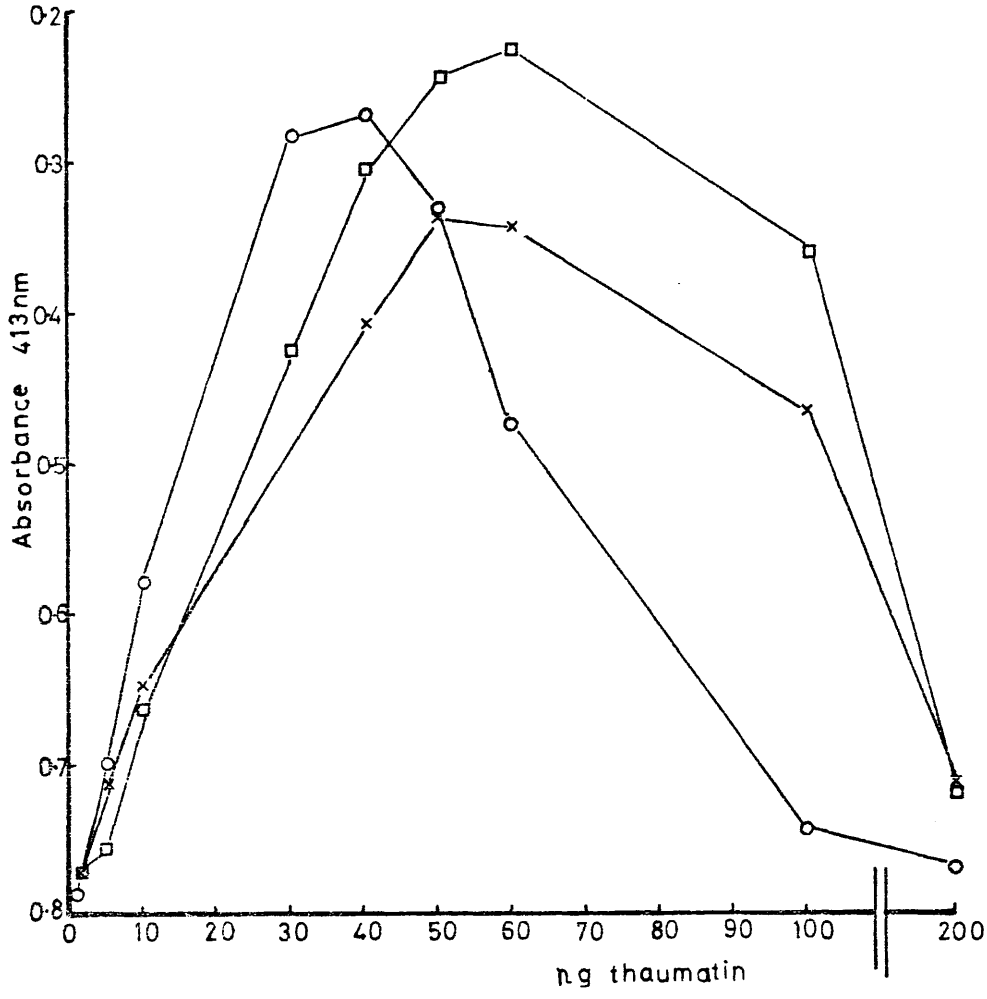
Figure 1: Quantitative precipitin analysis of the thaumatin forms.



TO —×—, TI—o—, TII —□—,

Quantitative precipitin analysis was performed as described in section II B 9 using a range of 0-13µg of thaumatin and a fixed volume of antiserum (200µl titre 35µg/ml). The precipitates were dissolved in 0.5 M NaOH (400µl) and the absorbance of this solution was measured at 280nm.

Figure 19 Micro-complement fixation analysis of the thaumatin forms.



TO—×—, TI—o—, TII—□—.

Micro-complement fixation analysis was performed as described in section V B 9. Each assay mixture contained a fixed quantity of anti-TII antiserum (100 µl:400 ng/ml), diluent (300µl), complement (100µl:50 CH_{50}/ml), thaumatin (100µl:1-200mµg), sensitized sheep erythrocytes (100µl:5×10^{7} cells/ml). After incubation cell lysis was estimated by measurement of absorbance (413nm) of the cell free supernatant.

Quantitative precipitin analysis gave a value of 100% for the cross-reactivity of the forms and micro-complement fixation showed a very close similarity between the three thaumatins with an estimated index of dissimilarity of approximately 1·25 between T O and T II and of approximately 1·09 between T I and T II. These values give cross-reactivities of 96% and 98% respectively. It is likely that these cross-reactivities resulted from a percent sequence difference of from 0-4% for T I/T II and from 0-6% for T O/T II.

In conclusion, the immunological evidence again shows that strong conformational and sequence similarities exist between the thaumatin proteins. The recorded differences are of the order expected if the proteins differ only in their amide contents or possibly other substituent groups on the polypeptide chain.

E. STUDIES ON THE BIOSYNTHETIC RELATIONSHIP BETWEEN T O, T I AND T II

1. Attempted interconversion of multiple forms of thaumatin

If the thaumatin forms differ only in the ratio of asparagine and glutamine to aspartate and glutamate then it is possible that *in vivo* post-translational deamidation produces the multiple forms. Attempts were therefore made to imitate these changes *in vitro*.

i) Effect of pH, temperature and salt concentration on the thaumatins. A number of proteins which exist in various forms differing only in their amide contents have been described (see section II C) [192]. Cytochrome C is a typical example. The sequential deamidation and conversion of CyI → Cy II → Cy III was shown to occur *in vitro*. The process was dependent upon temperature, ionic strength and pH. In this present investigation conditions (see section V B 12) which would have resulted in extensive deamidation of cytochrome C were chosen and applied to the purified thaumatins. It was found that no interconversion of the thaumatin forms occured (as judged by P.A.C.E.) under the various incubation conditions

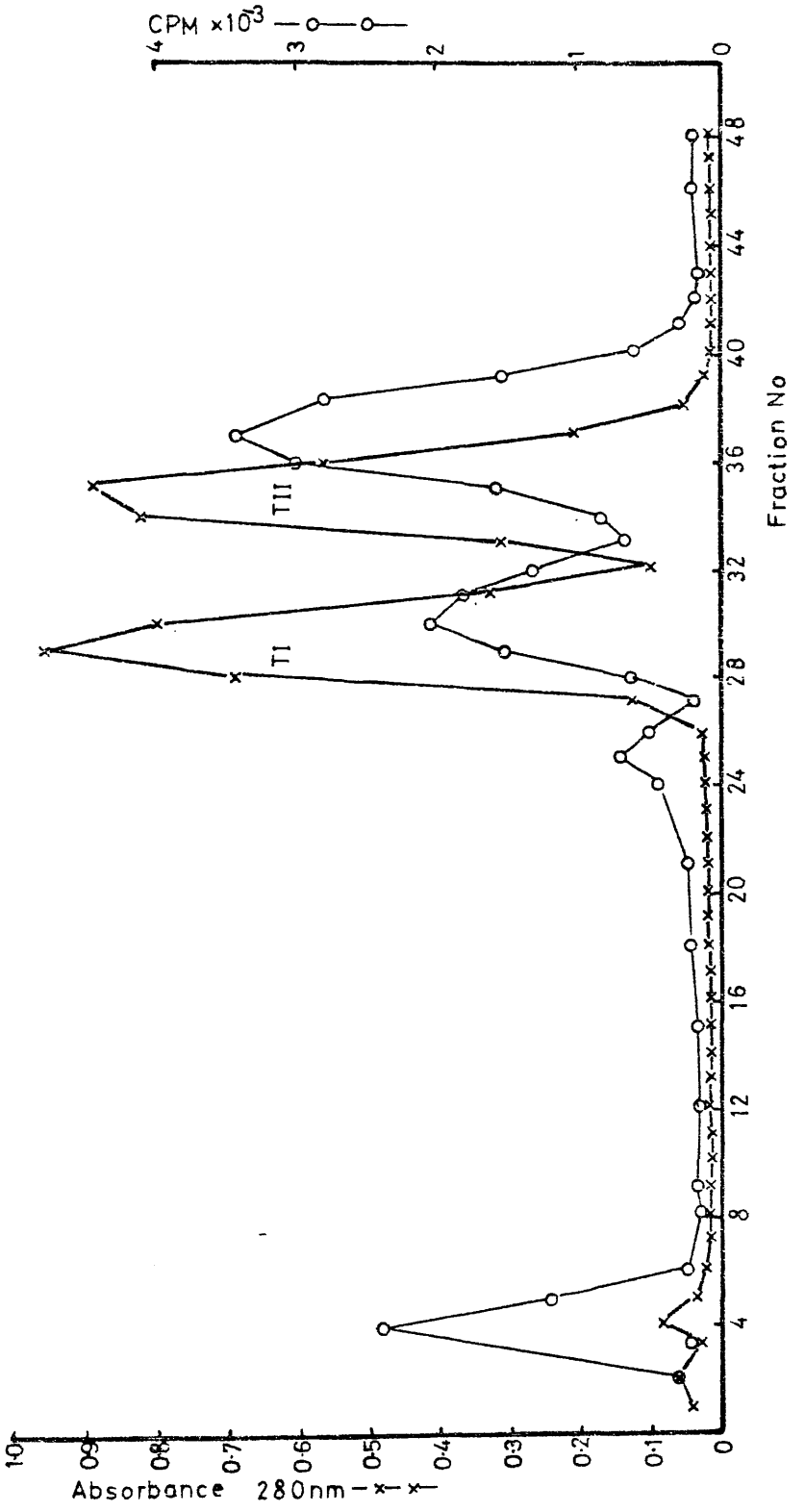
employed. In addition no electrophoretic mobility changes were observed and hence, the amide groups present in all of the thaumatin forms must be relatively stable. Therefore if deamidation does result in formation of T I and T 0 from T II *in vivo* then either it occurs spontaneously over a long period or it is enzyme catalysed.

ii) Attempted conversion of radioiodinated T II to T I and T 0 in the presence of aril extracts. If the thaumatins T I and T 0 are derived enzymically from T II then it was considered that T II might be quickly converted to the other forms in the presence of an extract of fruit obtained from the Kadjebe region: these fruits do not contain measurable quantities of T II. In order to test this hypothesis labelled thaumatins were prepared using the iodinating reagent 1, 3, 4, 6 tetrachloro - 3α, 6α - diphenylglycoluril (Iodo-gen). This is a simple procedure and has advantages over other iodination methods in that it is a mild reaction and no addition of extraneous protein or reducing agent is necessary. The radio-iodinated proteins obtained (T I and T II) were re-chromatographed on an ion-exchange column in order to determine whether they had retained their basic characters and were still identifiable.

Figure 20 shows a typical ion-exchange elution profile for the iodinated thaumatins. The radioactive peaks were slightly off-set from the protein (A_{280}) peaks. A change in the ionization of tyrosine phenolic hydroxyl groups produced by iodination may be responsible for this shift. The observation that the protein peaks did not correspond with the radioactivity shows that most of the thaumatin was not iodinated. The low yield may result from the inaccessibility of tyrosine residues due to the folding of the thaumatin molecules (thaumatin apparently contains 8 cystine residues and so it is probably highly folded), or the choice of unfavourable conditions for the reaction.

Figure 20. CM-cellulose chromatography of iodinated thaumatins.

[^{125}I]-labelled thaumatins I and II (10mg I, 10mg II) prepared as described in section V B 13 were examined by chromatography on CM-cellulose. The column was eluted with buffer (0.01M sodium phosphate pH 7.2; 20ml) and then a linear salt gradient of 0-0.2M NaCl in buffer (200ml). The flow rate was 0.2ml/min. Fractions of 4ml were collected (the final 20 fractions were discarded). The protein and [^{125}I] contents were measured as described in section V B 13.


CPM ×10³
Absorbance 280nm
Fraction No
TI
TII

The final specific activities of the thaumatins were 500DPM/μg and 250DPM/μg for T I and T II respectively.

[^{125}I] -T II was incubated with an homogenate of aril tissue from fruits of the Kadjebe region and the mixture was then examined by ion-exchange chromatography (Fig.21). No conversion of the labelled material to less basic forms was detected. The recovery of radioiodinated thaumatin from the incubation mixture was low. It is possible that this loss was caused by the release of proteases by homogenisation of the tissue. However the procedure was sufficiently sensitive to have allowed a 2% conversion of T II to either T I or T 0 to have been observed. The failure to detect enzymic conversion of T II to other thaumatin forms in vitro does not necessarily mean that this does not occur in vivo. It may be a slow process or iodination of T II may have produced an unsuitable substrate for the enzyme(s) involved or the enzyme(s) may have been rapidly inactivated during and after preparation of the extract. A further possibility is that the fruit which had been picked 4-6 days prior to freezing and storage may have lost the necessary activity.

It is conceivable that multiple forms of thaumatin are produced by synthesis rather than degradation i.e. amidation of T 0 to T I and T I to T II. However it was considered impracticable to test this hypothesis by adding labelled T 0 or T I to crude extracts of fruit because of the high levels of native T 0 and T I present which would have reduced the specific activity of added labelled tracer. For a similar reason the examination of the effect of aril extracts from Ashanti fruit on T II were considered not to be feasible.

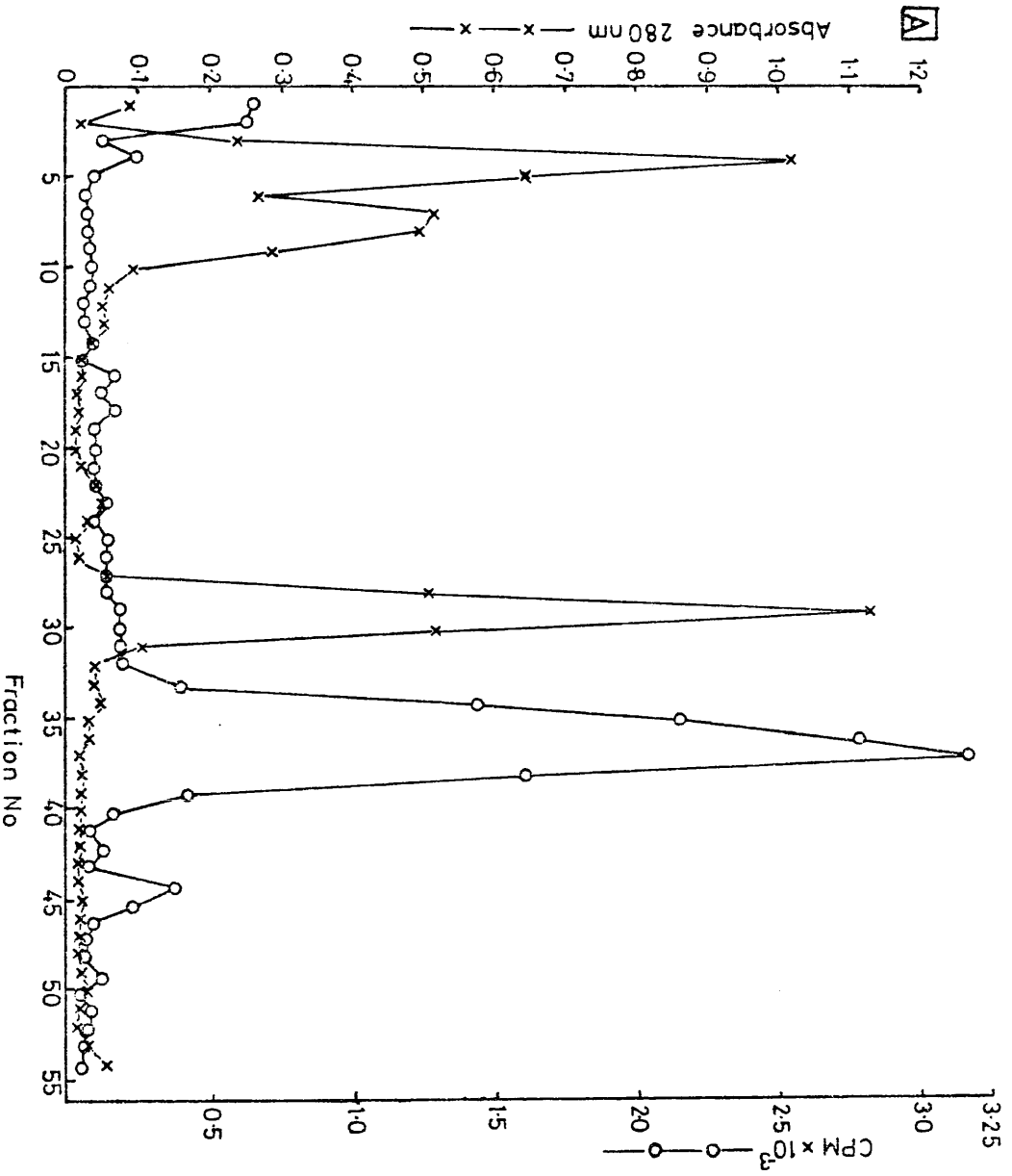
F. STUDIES ON THE BIOSYNTHESIS OF THAUMATIN

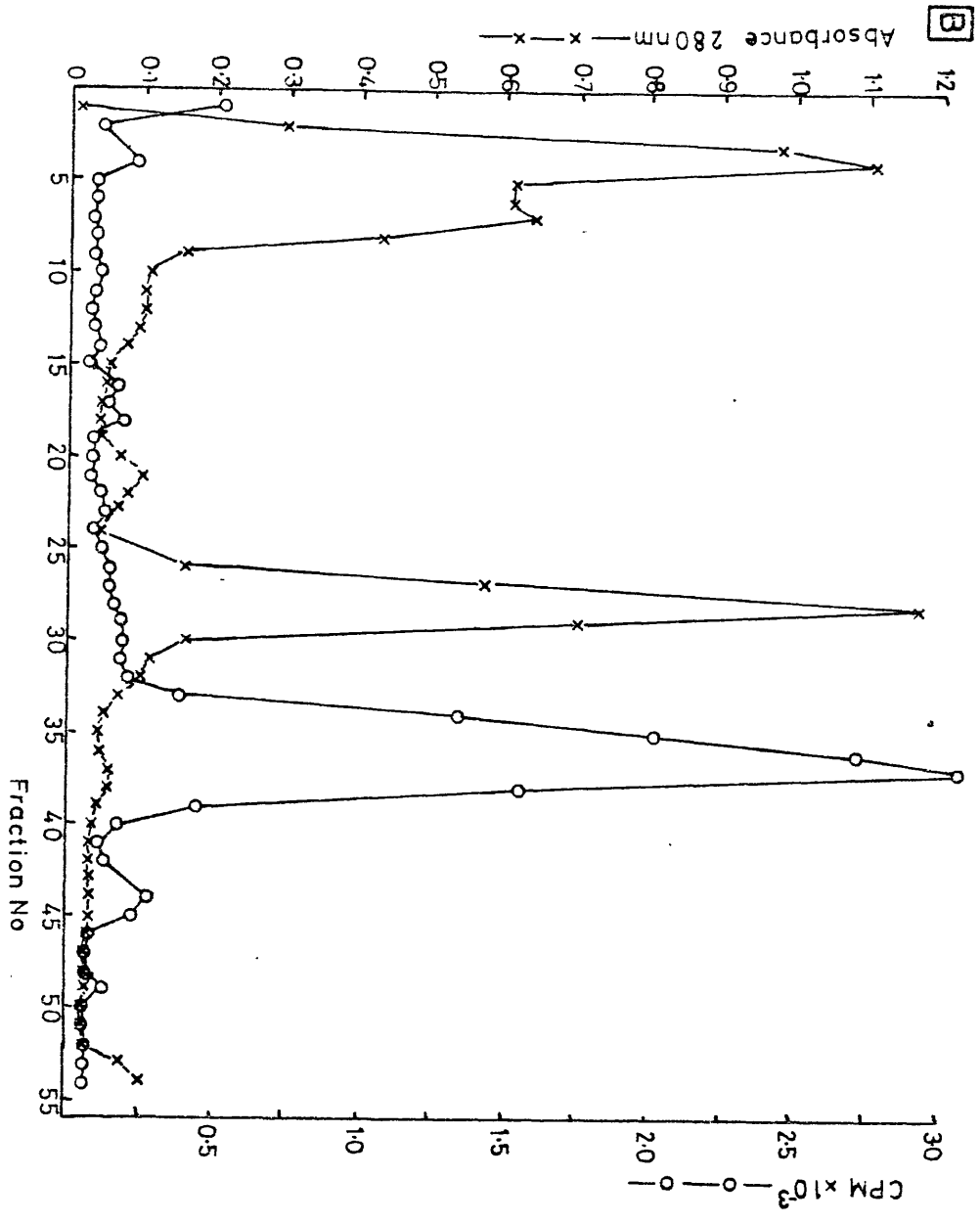
Following the attempted interconversion of the sweet proteins it was decided to investigate the biosynthesis of thaumatin in order to determine the relationship between the three forms.

Figure 21. CM-cellulose chromatography of iodinated TII after incubation with a Kadjebe fruit extract.

A [^{125}I]-labelled thaumatin II (100,000 D.P.M., 0.4mg) was added to a homogenate of Kadjebe region fruit (stage D) and the mixture was immediately frozen. After storage for at least 15h at -20°C the thaumatin was extracted with 0.01M sodium phosphate pH 7.7, 0.3M NaCl buffer and subjected to CM-cellulose chromatography. The column was eluted with buffer (0.01M sodium phosphate pH 7.2 , 30ml) and then a linear salt gradient of 0-0.2M NaCl in buffer (200ml). The flow rate was 0.2 ml/min. Fractions of 4ml were collected (the final 10 fractions were discarded). The protein and [^{125}I] contents were measured as described in section V B 14.

B As in A except that the mixture of homogenate and labelled thaumatin was incubated at 26°C for 15h before freezing.


A
Absorbance 280 nm
Fraction No
CPM x 10³


B
Absorbance 280nm — x — x —
CPM x10³ — o — o —
Fraction No

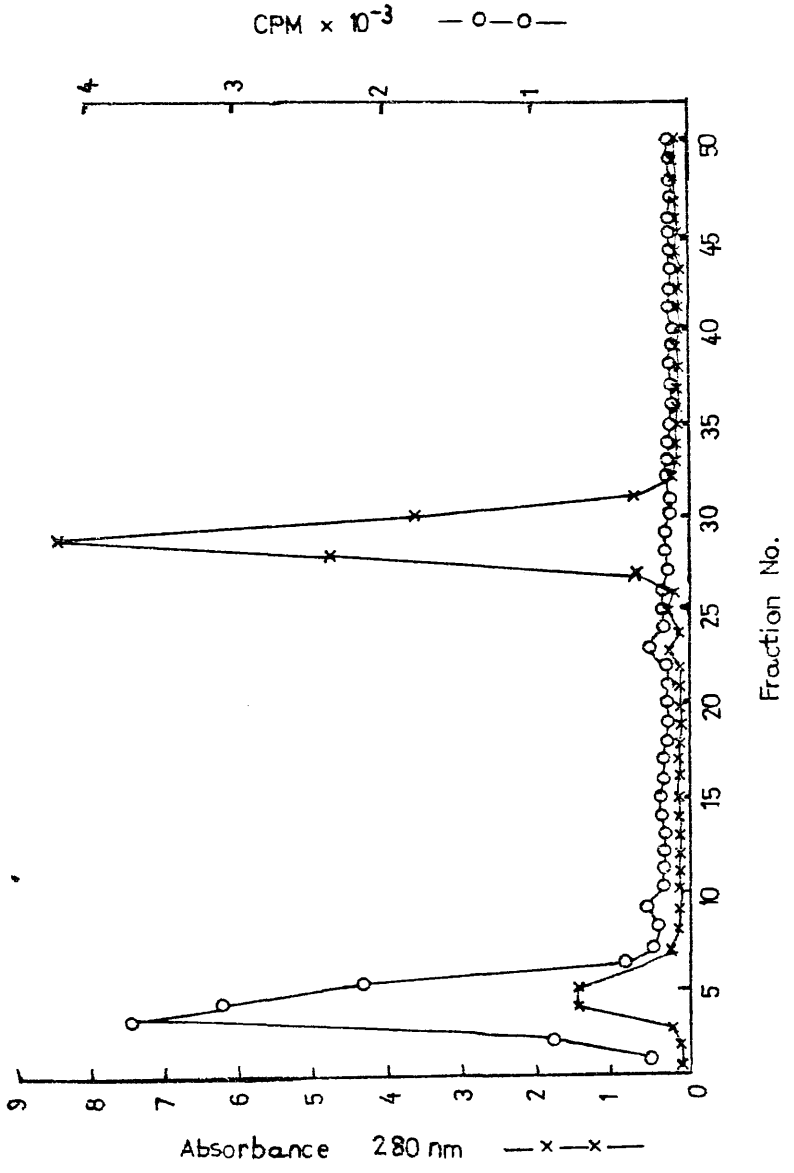
1. Synthesis of Thaumatococcus aril proteins in intact arils

Under ideal conditions it would have been possible to have introduced a labelled amino acid into intact Thaumatococcus fruit on the growing plant and then after various times to have extracted the thaumatins and measured the radioactivity of the three forms. The information gained would then have revealed whether the thaumatin forms were products of a single gene and which thaumatin form was the primary product. If in the short term T 0, T I and T II had all been produced it would have been strong evidence that they were translated from different mRNA species. Unfortunately, fruiting plants were unavailable because of the difficulties of growing them under greenhouse conditions in the U.K.. Therefore, as an alternative, isolated, developing arils from fresh fruits (stage C) were instead injected with L-[4,5 - ^{3}H] -leucine and the products were analysed by ion-exchange chromatography. The results (Fig.22) show that the arils actively synthesised proteins as judged by the incorporation of labelled amino acid into TCA precipitable material which could be inhibited by cycloheximide. No radioactive peaks corresponding to the sweet proteins were observable although under the conditions of the experiment as little as 1-2% incorporation of leucine into the thaumatins would have been detectable. It is possible that in vivo the synthesis of thaumatin does proceed slowly over a long period of time with negligible turnover. The developmental study presented in section B supports this idea. On the other hand, it is possible that the physiological state of the aril tissue had changed during the time which elapsed between picking the fruit and the experiment. Such changes may have resulted from water stress, lack of nutrients, chilling or, simply, detachment of the fruit from the plant. A climacteric rise in respiration may occur after picking the fruit which could possibly result in

Figure 22 Protein synthesis in intact Thaumatococcus arils

Each sample (in both experiments) consisted of 10 arils from Kadjebe fruits (stage C) which were treated by injection of L-[4,5-^{3}H]-leucine ± cycloheximide. After incubation a protein extract was prepared and an estimate was made of the incorporation of leucine into protein as described in section V B 15 (see table below). The profile (opposite) shows the result of fractionation of sample B on CM-cellulose. The column was eluted with buffer (0.01M sodium phosphate pH 7.2, 30ml) and then a linear salt gradient of 0-0.2 M NaCl in buffer (200ml). The flow rate was 0.2ml/min. Fractions of 4ml were collected (the final 15 fractions were discarded). The protein and TCA insoluble [^{3}H] contents were measured as described in section V B 15.

Experiment 1	Aril sample	cycloheximide	TCA insoluble CPM/25µl
	A	-	285
	B	-	2099
	C	+	157
Experiment 2	D	-	1277
	E	-	1672
	F	+	566


CPM x 10^{-3} —o—o—
4
3
2
1
0
1
2
3
4
5
6
7
8
9
Absorbance 280 nm —x—x—
5
10
15
20
25
30
35
40
45
50
Fraction No.

the synthesis of a spectra of proteins different from those found when fruits are still attached to the plant. Alternatively the fruit used in these experiments (stage C) may have been at a stage of development in which very little thaumatin synthesis was taking place.

It would have been possible to have reinvestigated thaumatin synthesis by scaling up the experiment and using a larger amount of labelled amino acid with a higher specific activity. However, if thaumatin synthesis had occurred under such conditions it would have been difficult to have detected sweet protein synthesis in view of the accompanying synthesis of proteins other than thaumatins which would have also occurred. It would not have been possible to have used the antibody to T II to detect the product(s) because of the large amounts of the thaumatins present in the tissue. Attention was next turned to the possibility of using an in vitro system.

2. Synthesis of Thaumatococcus aril proteins in a cell-free system

It was considered likely that the failure to detect synthesis of thaumatin in intact arils injected with L-[4, 5 - ^{3}H]-leucine could be overcome by using a system in which newly synthesised thaumatin was not swamped by the presence of endogenous sweet protein. The aim of these investigations was to develop a heterologous cell-free system capable of synthesising thaumatin and, hence, to discover the nature of the primary thaumatin gene product. A heterologous protein synthesising system would be unlikely to possess the means to bring about specific post-translational modifications of the primary product, which would be advantageous if, for example, deamidation reactions were responsible for the production of multiple forms of thaumatin from a single form. However, it was recognised that the absence of specific post-translational modification enzymes in the in vitro system might result in the synthesis of a sweet

protein precursor or precursors differing from the native thaumatins. For example, the initial product of protein synthesis may retain its N-terminal methionine group (the initiating amino acid) as does the precursor of egg white lysozyme[201]: such a molecule would not differ significantly in its properties from native thaumatin and would be easily recognisable. This may not be the case if the thaumatin precursor were to be significantly larger than the native protein as occurs with a majority of secreted proteins[202].

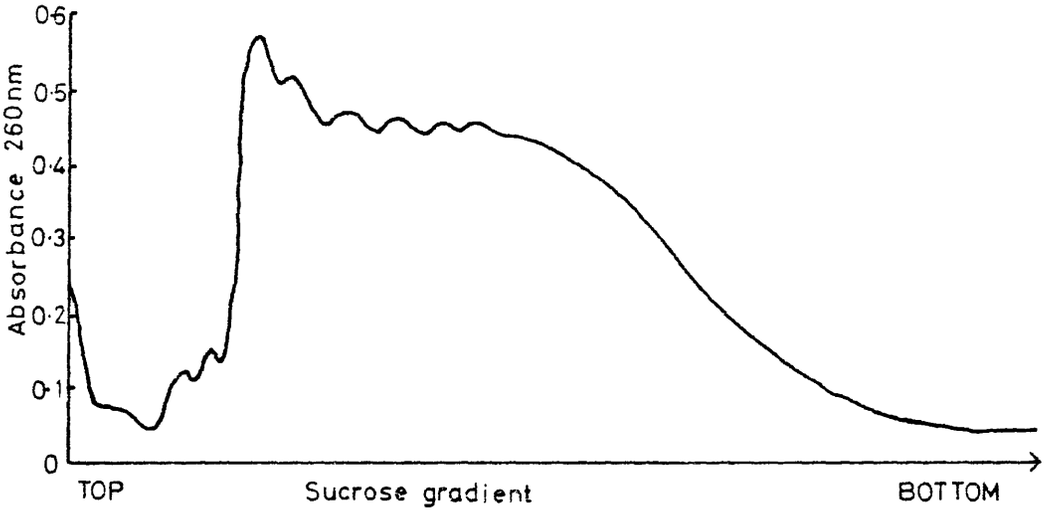
For the study of thaumatin synthesis two potential sources of active thaumatin mRNA were examined, firstly, polysomes and secondly isolated RNA fractions.

i) Preparation of polysomes. Initially the procedure for assessing the quality of polysomes (sucrose density fractionation) was tested using polysomes prepared from rat liver by the magnesium precipitation method of Palmiter[185]. The rat liver polysome profile shown in Figure 23 demonstrates that the yield and quality of this preparation was good. The sucrose density gradient fractionation procedure used successfully separated the polysomes into the different size classes present.

The magnesium precipitation method as developed by Palmiter[185] has been adapted by Akalehiywot et al[186] for the isolation of polysomes from plant sources. This technique was, therefore, used in a first attempt to prepare polysomes from aril tissue. However, no precipitation occured, probably because this technique is not applicable in situations in which the concentration of polysomes is low[185].

A second method of preparation was used[163] which involved extraction of the polysomes at pH 8·5 with a solution of high ionic strength followed by high speed centrifugation through a 'sucrose pad'. The high pH and relatively concentrated salt solutions have

Figure 23 Polysomes from rat liver.



Fresh rat liver (8g) from a starved female rat gave a yield of 605 OD units (260nm) of polysomes extracted by the method of Palmiter (185). The absorbance ratio 260/280nm was 1.87. A polysome sample (20 OD units (260 nm)) resuspended in buffer (25mM Tris-HCl pH7.5, 25mM NaCl, 5mM $MgCl_2$, 200µl) was fractionated on a linear sucrose gradient.

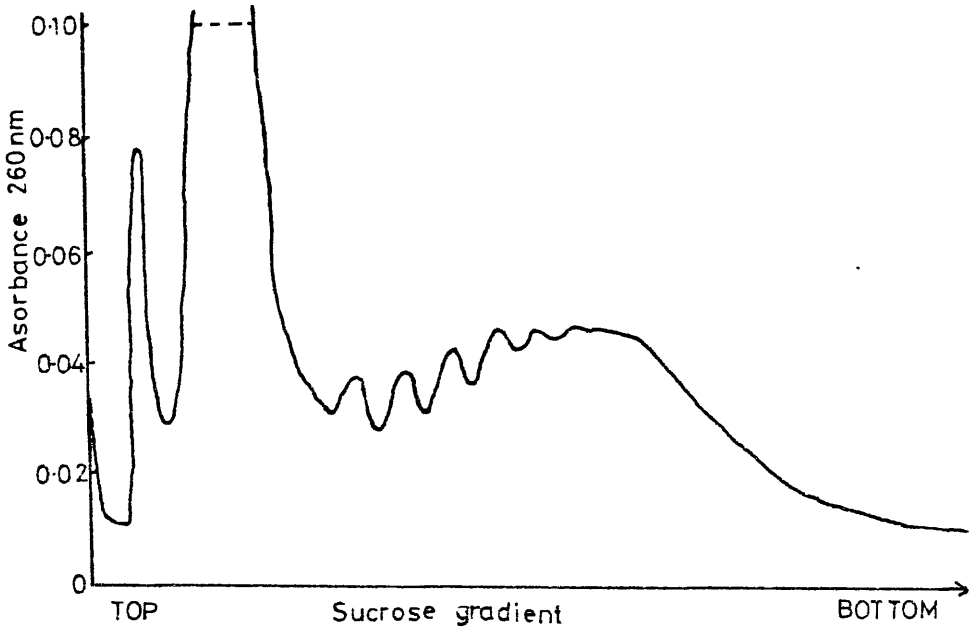
been found to protect polysomes from ribonuclease action. High pH was shown to reduce the activity of acid ribonuclease and to help to prevent the association of ribonuclease with the ribosomes[203]. High salt conditions probably help to remove ribonuclease from the ribosomes[204]. This method was first tested by preparing polysomes from pea tissue. A typical pea polysome profile is shown in Figure 24 which shows that the organelles obtained were of reasonable quality and compared favourably with those published[163]. The high monosome to polysome ratio may indicate that some degradation had occured but nevertheless considerable quantities of larger polysomes were obtained.

This second method was then applied to the isolation of polysomes from Thaumatococcus aril tissue in the presence and absence of heparin. The polysome profiles (Figs.25 and 26) show that in contrast to the polysomes obtained from pea those from arils appeared to have a higher proportion of large polysomes indicating that the associated mRNA was intact. The inclusion of heparin as an inhibitor of ribonuclease (Fig.25) increased the proportion of large polysomes and for this reason it is unlikely that the polysomes were merely ribosomal aggregates produced as artifacts of the isolation procedure. The yield of polysomes corresponded to approximately 100 unit (260nm)/g of aril tissue. This value is low compared to those quoted for animal and plant tissues[185,186] and explains the failure of the magnesium precipitation method to yield polysomes.

Aril polysome preparations were assayed for their ability to direct the synthesis of thaumatin in cell-free protein synthesising systems.

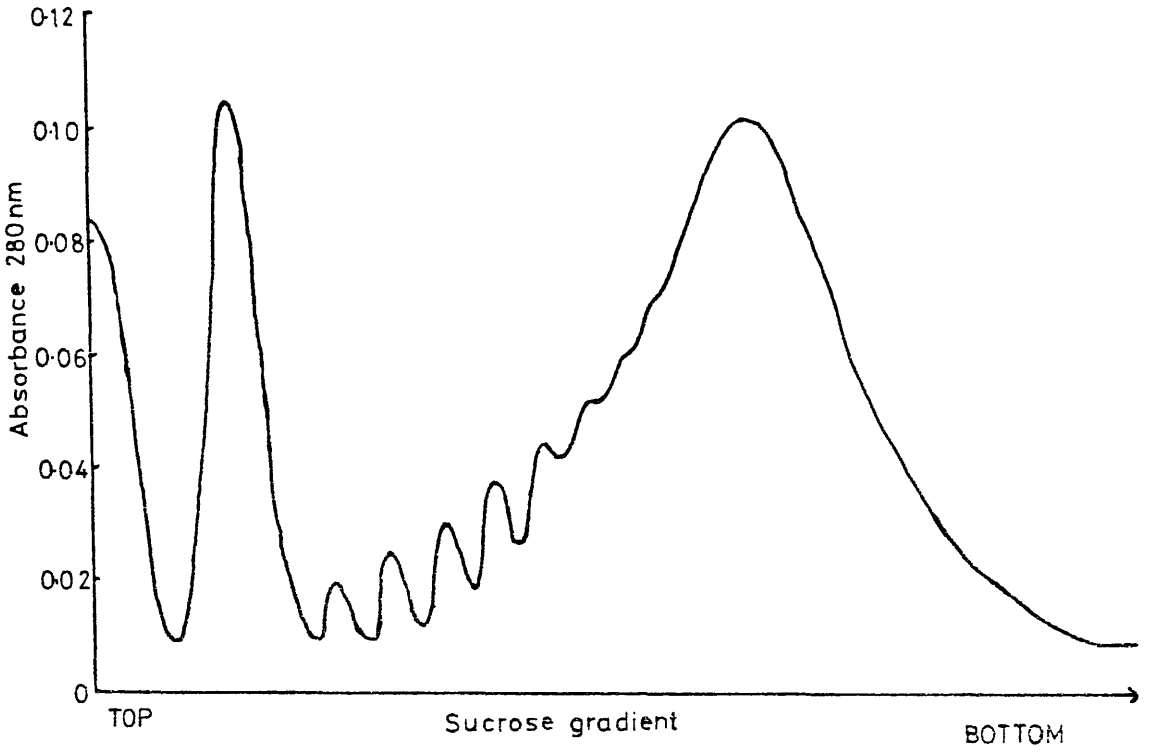
ii) Isolation of RNA fractions from Thaumatococcus arils. In view of the low concentration of polysomes per gram of aril tissue, rather than using polysomes as the starting material for

Figure 24 Polysomes from pea.



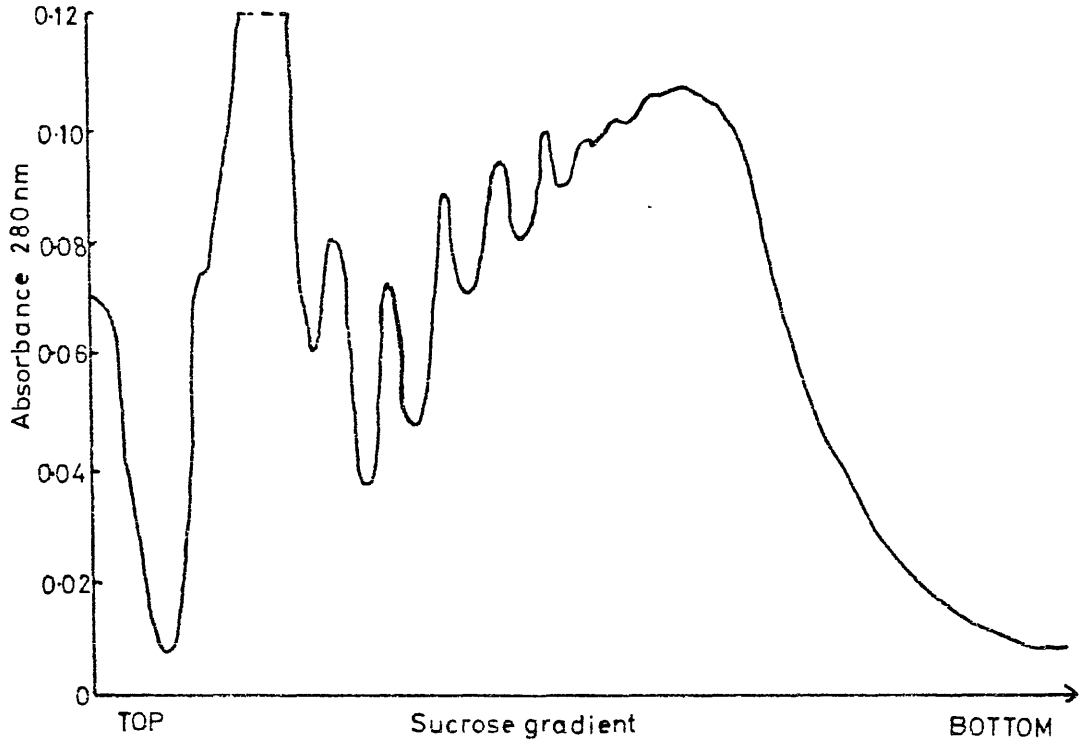
Ten day pea (variety Pioneer) sub-apical segments (6g) gave a yield of 9 OD units (260nm) of polysomes extracted by the method of Verma (163). The absorbance ratio 260/280nm was 1.87. A polysome sample (4.5 OD units 260nm)) suspended in buffer (100µl) containing 50mM Tris-acetate pH 8.5, 20mM KCl, 10mM MgAc) was fractionated on a linear sucrose gradient.

Figure 25 Polysomes from Thaumatococcus arils.



Frozen aril tissue (30g) from Ashanti type fruit (stage D) gave a yield of 22 OD units (260nm) of polysomes extracted by the method of Verma (163). The absorbance ratio 260/280nm was 1.70. A polysome sample (4.4 OD units (260nm)) suspended in buffer (100µl) containing 50mM Tris-acetate pH8.5, 20mM KCl, 10mM MgAc, was fractionated on a linear sucrose gradient.

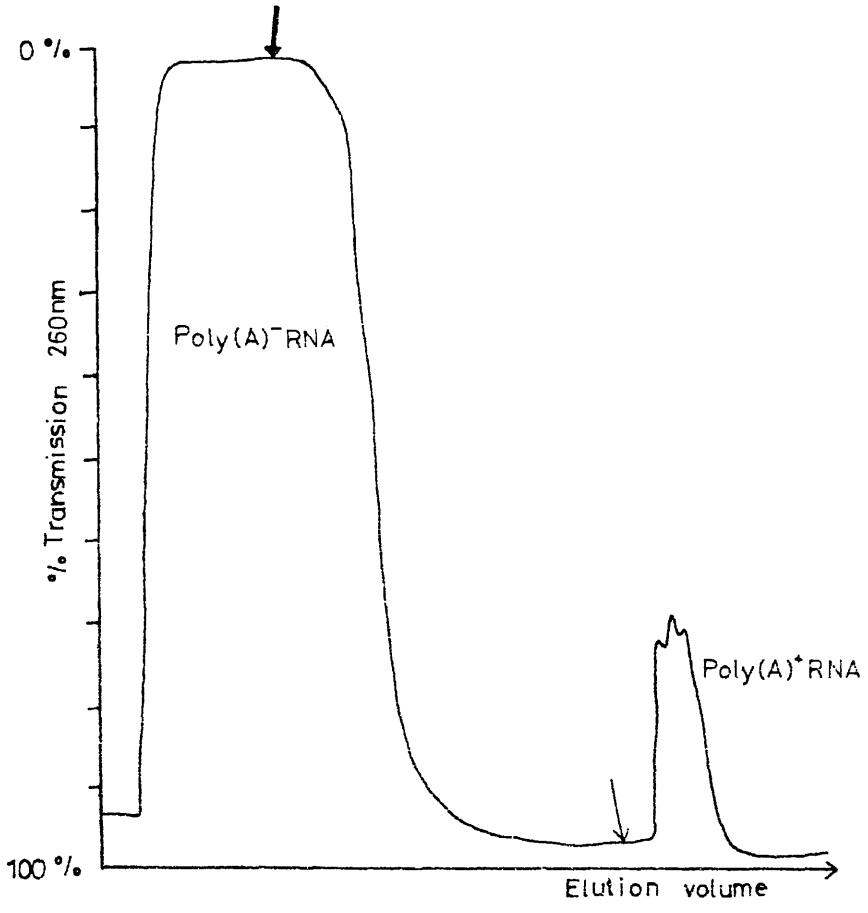
Figure 26 Polysomes from Thaumatococcus arils prepared in the absence of heparin.



Frozen aril tissue (25g) from Ashanti type fruit (stage D) gave a yield of 26 OD units (260nm) of polysomes extracted by the method of Verma (163) except that heparin was omitted from the extraction buffer. The absorbance ratio 260/280nm was 1.71. A polysome sample (6.5 OD units (260nm)) suspended in buffer (100µl) containing 50mM Tris-acetate pH8.5, 20mM KCl, 10mM $MgAc_2$, was fractionated on a linear sucrose gradient.

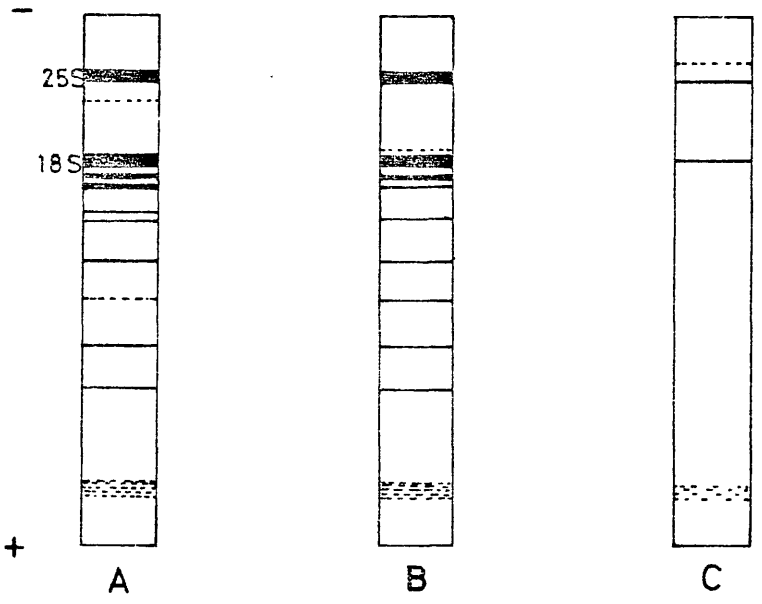
isolating mRNA, the alternative procedure of isolating mRNA from total RNA was attempted. This involved the preparation of total RNA directly from the aril tissue, using phenol: chloroform extraction according to the method of Verma et al[163] followed by the isolation of poly $(A)^{plus}$ RNA using oligo (dT)-cellulose affinity chromatography. It was hoped that this method would be more efficient with respect to yields of mRNA per gram of aril tissue. The phenol extraction method typically yielded about 4 OD units (260nm) of RNA per gram of aril tissue as compared to 1 OD unit of polysomes by the previous method. Figure 27 shows an elution profile obtained by fractionation of total RNA on an oligo (dT)-cellulose column. Generally about 0·4% of the total RNA samples was retained on the columns as poly $(A)^{plus}$ RNA (equivalent to 1 μg poly $(A)^{plus}$ RNA per gram aril tissue). As a rough estimate of the degree of degradation samples of the total and fractionated RNAs were subjected to gel electrophoretic analysis as described by Lœning[188]. In the cases of the total and poly $(A)^{minus}$ RNA fractions (Fig.28) the presence of two major bands at the top of the gel representing the 25S and 18S ribosomal RNAs suggests that much of the RNA remained undegraded although the occurrence of additional, multiple, faint bands may indicate that at least some degradation had taken place. The gel containing the poly $(A)^{plus}$ RNA fraction is more difficult to interpret in these terms since the expected pattern of RNAs is not known. Further, only small quantities of this RNA were applied to the gels because of the extremely low yields and requirement for in vitro mRNA assays. The 25S and 18S RNAs were still the major species, showing that the poly $(A)^{plus}$ fraction was contaminated by ribosomal RNAs. Some workers recycle the partially purified poly $(A)^{plus}$ RNA through the affinity column in order to free it from contaminating ribosomal RNAs[205,206]. However, it was felt that

Figure 27 Oligo(dT)-cellulose chromatography of Thaumatococcus aril RNA.



RNA (270 OD units (260nm)) isolated from stage D, Kadjebe type arils (100g) was dissolved in buffer containing 10mM Tris-acetate pH7.6, 0.4M NaCl, 0.5% SDS (20ml) and applied to an oligo(dT)-cellulose column at a flow rate of 2ml/min. The column was eluted with buffer containing 10mM Tris-acetate pH7.6, 0.4M NaCl (25ml) until the absorbance of the eluant was stable and finally with buffer (10mM Tris-acetate, pH7.6). The poly(A)^{minus} RNA and poly(A)^{plus} RNA (yield 0.5 OD units (260nm)) fractions were collected.

Figure 28 SDS P.A.G.E. of Thaumatococcus aril RNA



RNA samples were subjected to SDS P.A.G.E. in 2.6% acrylamide, 0.13% bisacrylamide gels as described by Loening (188). A: total RNA (0.6 OD units (260nm)). B: minus poly(A) RNA (0.6 OD units (260nm)). C: plus poly(A) RNA (0.3 OD units (260nm)).

some ribosomal RNA contamination would be beneficial serving as a carrier for the very small amount of poly $(A)^{plus}$ RNA present in the final recovery step involving ethanol precipitation,

The polysome and RNA preparations were then tested for their ability to direct the synthesis of aril proteins in both wheat germ and rabbit reticulocyte in vitro protein synthesising systems.

iii) Assays for mRNA activity. The possibility existed that mRNA from the arils of Thaumatococcus fruit would not be translated efficiently in a cell-free system derived from an animal e.g. the reticulocyte lysate. For this reason a wheat germ cell-free extract which is commonly used for translation of plant mRNAs was first examined. This system has low endogenous protein synthesising activity and it is easy to prepare. The wheat germ system has been reported not to synthesise high molecular weight proteins efficiently but this was considered to be unimportant in view of the low molecular weights of the thaumatins[207,208]. When aril polysomes were introduced into the wheat germ incubation mixture there was a marked inhibition of the endogenous mRNA activity (Table 14). In addition when rabbit reticulocyte RNA was added to the system together with the polysome preparation its ability to direct protein synthesis was blocked. It was considered possible that heparin contamination (see section V B 20ii)) of the polysomes was responsible for these inhibitory effects as this material is known to be an inhibitor of protein synthesis[185]. Hoffman and Ilan[187] had reported that polysomes could be purified by adsorption chromatography on hydroxylapatite. The procedure had been designed to separate liver polysomes from contaminating glycogen. This method was, therefore, used in an attempt to remove heparin, or possibly other extraneous materials, from the polysome fraction and hydroxylapatite-treated polysomes were shown to stimulate protein synthesis in the wheat germ system (Table 15).

Table 14 Wheat germ system: addition of polysomes.

Addition to standard incubation	C.F.I.
none	712
2.5μg reticulocyte RNA	1059
2.5μg reticulocyte RNA + 1.5μg polysomes	2478
3.1μg polysomes	2?1
1.55μg polysomes	35?
0.77μg polysomes	555
0.38μg polysomes	677

Polysomes prepared from Ashanti type fruit collected in Bunso (25g of arils from stage D fruit gave a yield of 24.6 OD units (260nm)) were suspended in deionised water at a concentration of 24.6 OD units/ml and added at various concentrations to the standard wheat germ system containing 1-[4,5-]^{3}H -leucine (1μCi) as the labelled amino acid. At the end of the incubation period (1h) 2x20μl aliquots were withdrawn from the mixture (total volume 50μl) and spotted onto paper discs for determination of the C.F.I. figure (TCA insoluble material).

Table 15 Wheat germ system: addition of purified polysomes.

Addition to standard incubation	C.P.M.
none	333
5.0μg reticulocyte RNA	2095
6.3μg polysomes	640
4.45μg polysomes	647
3.15μg polysomes	525
1.57μg polysomes	394

Polysomes prepared from Hadjebe type fruit (30g of arils from stage D fruit gave a yield of 23.8 OD units (260nm). After washing on hydroxylapatite the final yield was 4.0 OD units (260nm).) were suspended in deionised water at a concentration of 50.2 OD units/ml and added at various concentrations to the standard wheat germ system containing L-[4,5-^{3}H]-isoleucine (1μCi) as the labelled amino acid. At the end of the incubation period (1h) 2x20μl aliquots were withdrawn from the mixture (total volume 50μl) and spotted onto paper discs for determination of the C.P.M. figure (TCA insoluble material).

It would appear, therefore, that heparin or possibly other molecules (such as polyphenols[209]) were removed by the hydroxylapatite treatment and that these were at least partly responsible for the observed inhibition of protein synthesis.

In view of the low incorporation of label into total protein which was obtained using the wheat-germ system it was decided to examine aril RNA preparations for their ability to direct protein synthesis in an alternative cell-free system.

The most efficient available eukaryotic cell-free system is the unfractionated reticulocyte lysate. One problem with the system is that exogenous mRNA has to compete with the endogenous messenger species (mainly globin mRNA) in order to be translated. Because of the endogenous components the translation products of the exogenous mRNA cannot be detected by simply measuring incorporation of radioactive amino acids in the system and so a method for product recognition is required. However, nuclease treatment of the reticulocyte lysate system largely destroys the endogenous mRNA without seriously harming the ability of the lysate to translate exogenous mRNA (the Ca^{++} dependent nuclease is first rendered inactive by the addition of EGTA)[182]. Under these conditions the translation of the exogenous mRNA can exceed that of residual endogenous mRNA by many times and messenger RNA activity can therefore be measured without a method for specific product recognition being available.

In the present study nuclease-treated reticulocyte lysate was used for the translation of mRNA preparations from Thaumatococcus because of the advantages described above. Initially the effects of hæmin (an agent known to inhibit the formation of the repressor of initiation in the reticulocyte lysate[210,211]), temperature and time of incubation upon incorporation of labelled amino acid were determined. Lysates which had low activities under all conditions

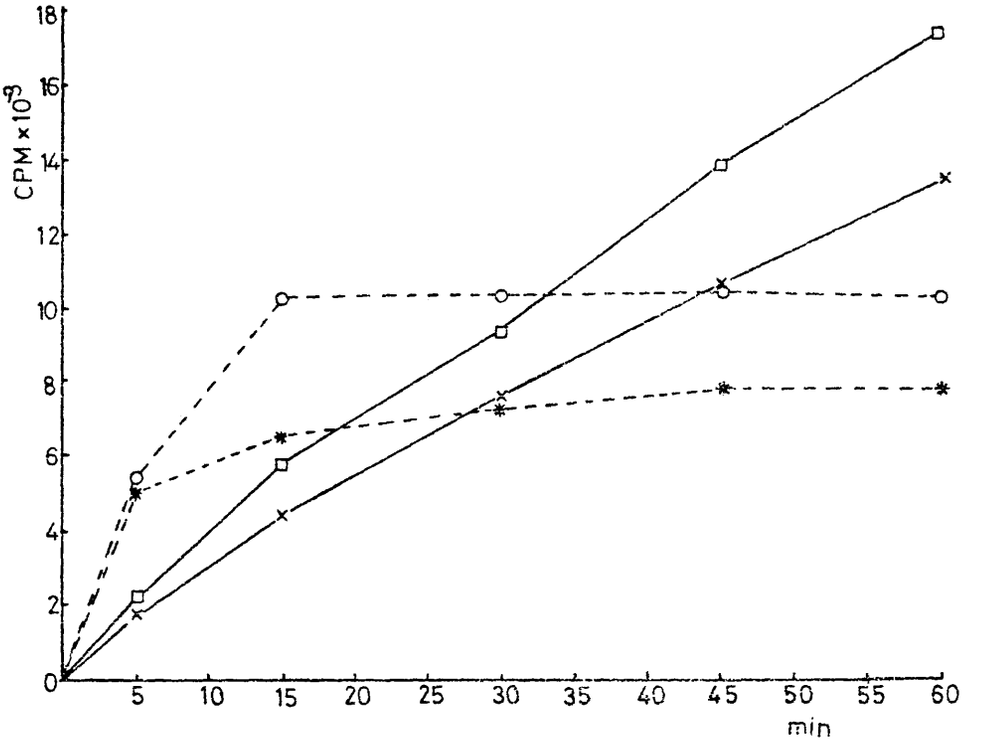
were discarded. The time course of leucine incorporation into protein under the direction of endogenous mRNA in the untreated reticulocyte lysate is shown in figure 29. At 26°C this system continued to synthesise protein at a linear rate for 1h, whilst at 37°C synthesis only continued at a significant rate for 15 min. The net synthesis of protein was greater after 1h at 26°C than at 37°C. Haemin stimulated protein synthesis at 37°C but inhibited it at 26°C. These properties of the reticulocyte lysate are similar to those described by Woodward et al[212] who examined a large number of lysates.

In the nuclease-treated system (Fig.30) supplemented with rat liver poly $(A)^{plus}$ RNA a higher net synthesis of protein occured at 37°C in the presence of hæmin than under any other conditions examined. Hæmin had the same temperature-related effect as was observed in the untreated system. The net synthesis of protein reached a maximum after 30 min of incubation. It is unclear why the nuclease-treated system should have stopped synthesising protein earlier than the non-treated system.

Preparations of aril polysomal material and isolated RNA fractions were then assayed for messenger RNA activity using the nuclease-treated reticulocyte lysate system by first measuring the incorporation of labelled amino acid into TCA precipitable material. It was assumed that only a small proportion of the protein synthesised would be thaumatin (see section VI F 1). Accordingly attempts were made to maximise the amount of label incorporated into total protein so that labelled thaumatin could be more easily detected.

The results of assaying the mRNA activity of the polysome preparations using the nuclease-treated reticulocyte lysate are shown in Table 16. As in the case of the wheat germ system the polysomes which had not been purified on hydroxylapatite inhibited

Figure 29 Time course of leucine incorporation in the reticulocyte lysate.

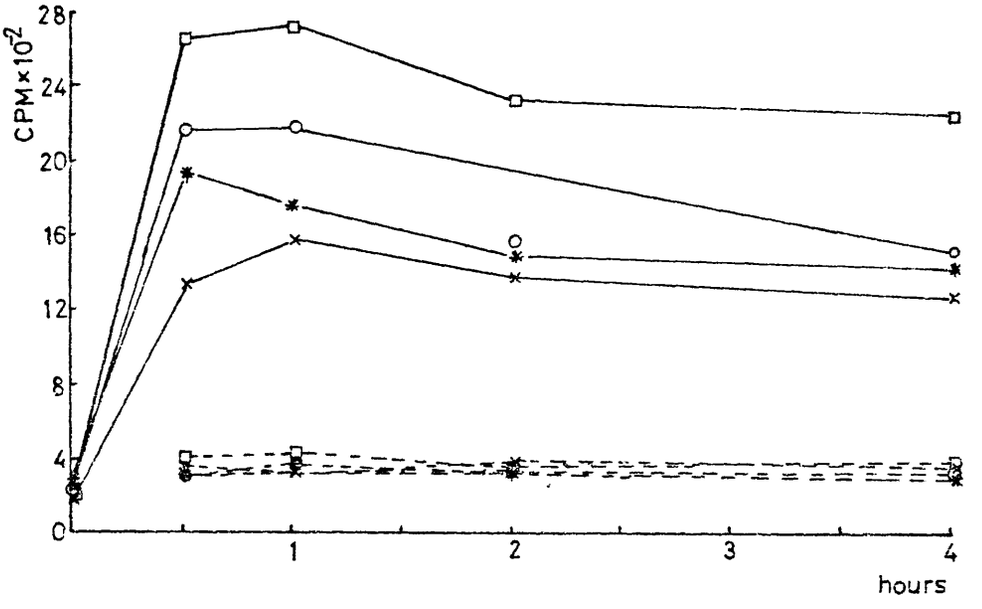


Incubations of the rabbit reticulocyte lysate cell-free system were as described in section V B 17 except that the volumes were increased (200µl). The labelled amino acid L-[4,5-^{3}H]-leucine (2µCi) was not omitted from the mixture of cold amino acids. The lysate was not nuclease treated.

Solid lines: incubation at 26 °C -□-□- haemin not added to the lysate, -x-x- haemin added.

Dotted lines: incubation at 37 °C -*-*- haemin not added to the lysate, -o-o- haemin added.

Figure 30 Time course of leucine incorporation in the nuclease-treated reticulocyte lysate.



Incubations of the rabbit reticulocyte lysate cell-free system were as described in section V B 17 except that the volumes were increased (200µl). The labelled amino acid was L-[4,5-^{3}H]-isoleucine (2µCi). The lysate was nuclease treated. Aliquots of the incubation mixture (15µl) were withdrawn at various time intervals and the CPM in TCA precipitable material was determined by the paper disc method.

Solid lines: supplemented with rat liver poly(A)$^{+}$ RNA (6.2µg/incubation). Dotted lines: unsupplemented. -□-□- haemin added, incubation at 37°C; -×-×- haemin added, incubation at 26°C; -*-*- no haemin, incubation at 37°C; -o-o- no haemin, incubation at 26°C.

Table 16 Nuclease-treated reticulocyte lysate system: addition of polysomes.

Addition to standard incubation	C.P.M.
none	1690
1.5µg polysomes	1478
0.8µg polysomes	1783
0.4µg polysomes	1788

Polysomes prepared from Ashanti type fruit collected in Sunso (30g of arils from stage D fruit gave a yield of 22.0 OD units (260nm)) were suspended in deionised water at a concentration of 5.6 OD units/ml and added at various concentrations to the nuclease treated reticulocyte lysate system containing L-[4,5-^{3}H]-leucine (2µCi) as the labelled amino acid. At the end of the incubation period (1h) at 26°C, 2x20µl aliquots were withdrawn from the mixture (total volume 50µl) and spotted onto paper discs for determination of the TCA insoluble label (C.P.M.).

the endogenous level of protein synthesis in the nuclease-treated reticulocyte lysate. The use of polysomes prepared in the absence of heparin did not reduce the level of inhibition (Table 17). It was, therefore, assumed that some material, other than heparin, present in the polysome preparation was responsible for the observed inhibition of protein synthesis. As in the case of the wheat germ system polysomes purified by adsorption chromatography on hydroxylapatite stimulated protein synthesis in the nuclease-treated reticulocyte lysate (Table 18). Despite removal or at least partial removal of the inhibitor the level of stimulation of protein synthesis was still low (2-3x above background). The increased level of incorporation probably represents the translation of polysomal mRNA although other interpretations are feasible such as stimulation of the residual endogenous activity. A further possibility would be that the polysomes were contaminated by residual inhibitor which was not completely removed by the hydroxylapatite treatment. No further attempts were made to purify the Thaumatococcus polysomes.

Poly $(A)^{plus}$ RNA was next assayed for mRNA activity in the nuclease-treated reticulocyte lysate (Table 19). Cycloheximide (5μg/ml), an inhibitor of protein synthesis at the level of initiation and elongation, was added to some incubations so that it would be possible to determine which proportion of the L-[4,5-^{3}H]-leucine incorporated into TCA precipitable material was assimilated by 'true' protein synthesis. In the case of incubations containing no exogenous RNA there was only a small reduction in the quantity of radio-labelled TCA-insoluble material produced in the presence of cycloheximide. This result is interesting since it indicates that only a small proportion of the endogenous incorporation of amino acids into TCA precipitable material can be regarded as 'true' protein synthesis. It is at present unclear what is responsible for this effect though

Table 17 Nuclease-treated reticulocyte lysate system: addition of polysomes. (heparin omitted)

Addition to standard incubation	C.P.M.
none	2555
10.5µg reticulocyte RNA	47473
6.4µg polysomes	745
3.2µg polysomes	848
1.6µg polysomes	805
0.8µg polysomes	913

Polysomes prepared from Ashanti type fruit collected in Bunso (25g of arils from stage D fruit gave a yield of 26.0 OD units (260nm). Heparin was not used in the extraction buffer.) were suspended in deionised water at a concentration of 24.6 OD units/ml and added at various concentrations to the nuclease treated reticulocyte lysate system containing L-[4,5-^{3}H]-leucine (2µCi) as the labelled amino acid. At the end of the incubation period (1h) at 26°C, 2x20µl aliquots were withdrawn from the mixture (total volume 50µl) and spotted onto paper discs for determination of the TCA insoluble label (C.P.M.).

Table 18 Nuclease treated reticulocyte lysate system: addition of purified polysomes.

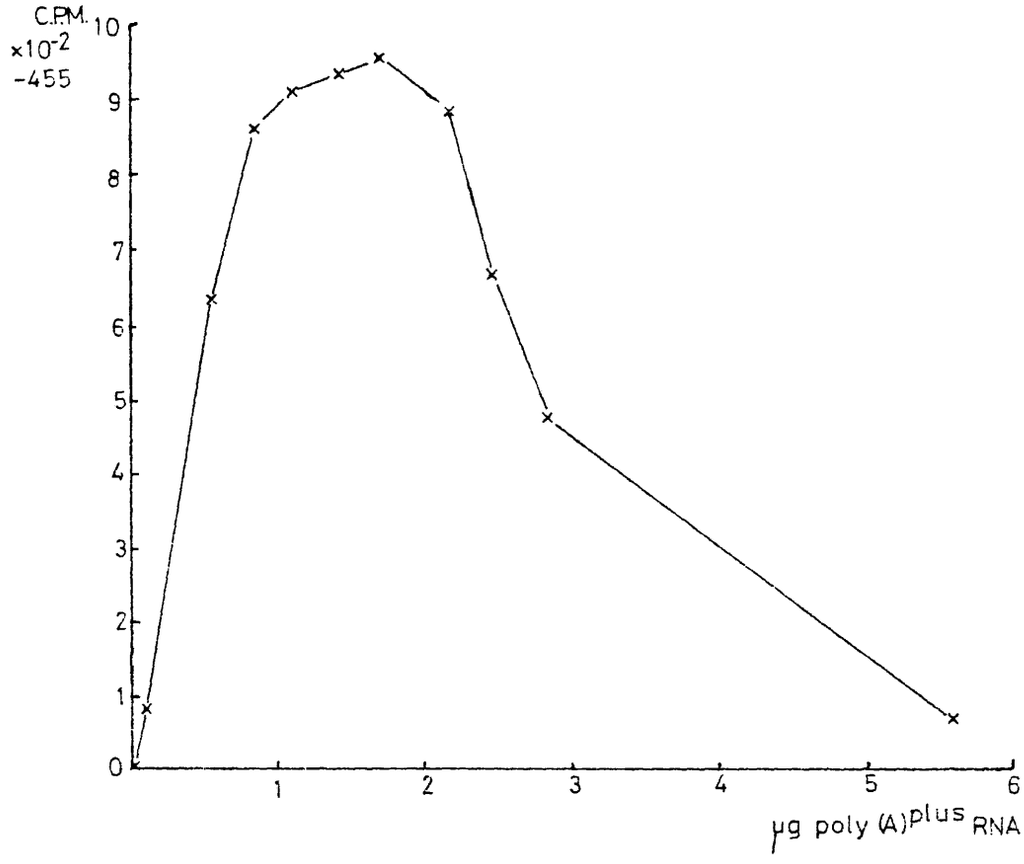
Addition to standard incubation	C.P.M.
none	862
12.6µg polysomes	1615
8.9µg polysomes	2483
6.3µg polysomes	1814

Polysomes prepared from Hadjebe type fruit (30g of arils from stage 5 fruit gave a yield of 23.5 OD units (260nm) . After washing on hydroxylapatite the final yield was 6.0 OD units (260nm).) were suspended in deionised water at a concentration of 50.2 OD units/ml and added at various concentrations to the standard nuclease treated reticulocyte lysate system containing L-[4,5-^{3}H]-isoleucine (1µCi) as the labelled amino acid. At the end of the incubation period (1h) 2x20µl aliquots were withdrawn from the mixture (total volume 50µl) and spotted onto paper discs for determination of the C.P.M. figure (TCA insoluble material).

Table 13 Nuclease treated reticulocyte lysate system plus addition of poly(A) mRNA.

Addition to standard incubation	C.I.U.
none	455
5μg/ml cycloheximide	391
10.5μg reticulocyte mRNA	5237
10.5μg reticulocyte mRNA 5μg/ml cycloheximide	611
5.6μg poly(A) mRNA	519
2.8μg poly(A) mRNA	933
2.2μg poly(A) mRNA	1338
1.7μg poly(A) mRNA	1412
1.1μg poly(A) mRNA	1365
0.84μg poly(A) mRNA	1316
0.55μg poly(A) mRNA	1057
0.28μg poly(A) mRNA	695
0.11μg poly(A) mRNA	531
1.1μg poly(A) mRNA 5μg/ml cycloheximide	407

Table 19



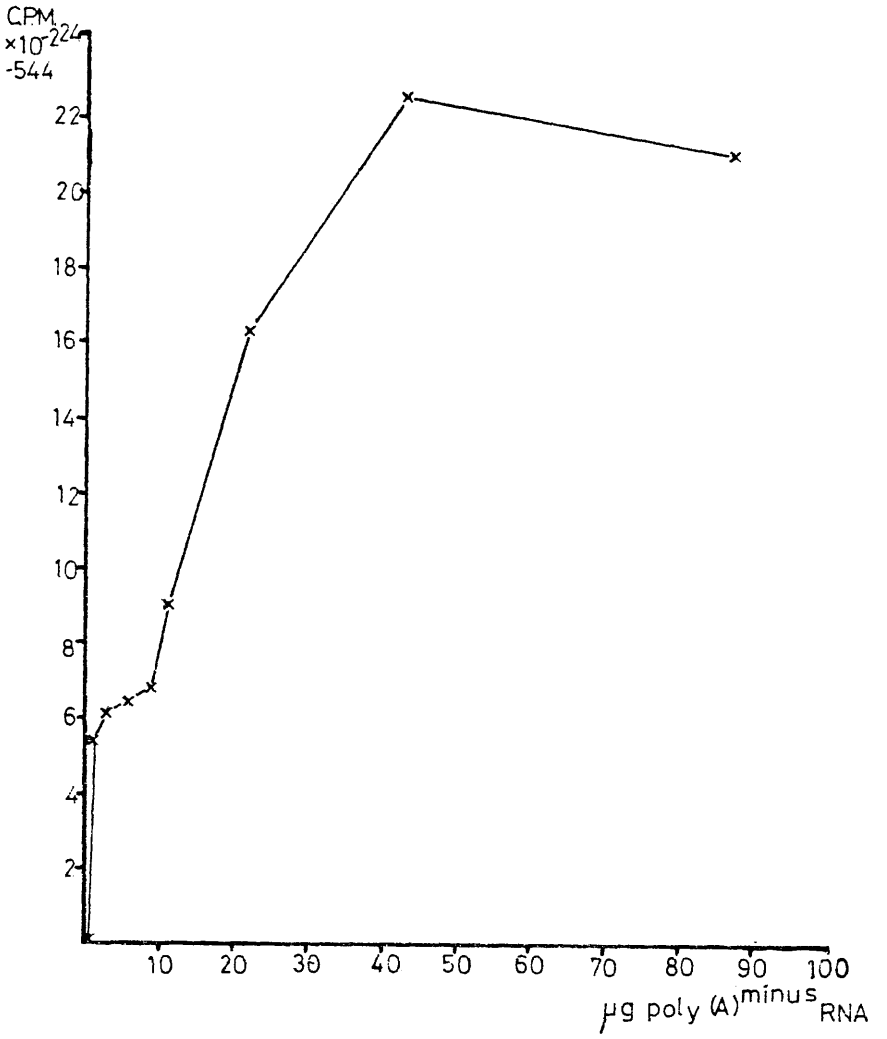
Poly (A)RNA prepared from Nadjebe type fruit (100g of arils from stage I fruit gave a yield of 396 OD units (260nm) RNA. After oligo (dT)-cellulose chromatography of this material 1.24 OD units of poly (A) plus RNA were obtained.) was added at various concentrations to the standard incubation mixture containing L-[4,5-^{3}H]-isoleucine (1µCi) as the labelled amino acid. At the end of the incubation period (1h) 2×20µl aliquots were withdrawn from the mixture (total volume 50µl) and spotted onto paper discs for determination of the C.P.M. figure.(TCA insoluble label)

it is possible that it is caused by a specific interaction between the labelled amino acid(s) (or labelled impurities) and the paper disc (see section V A 2), alternatively a synthetic mechanism in the lysate could be involved. The mRNA -dependent synthesis of protein in the system on the other hand was almost completely inhibited by cycloheximide. If only cycloheximide-sensitive incorporation is considered then a 15-fold stimulation of protein synthesis was achieved under the experimental conditions used. This level of stimulation of the system could only be attributed to the presence of active mRNA in the poly $(A)^{plus}$ RNA preparation. Poly $(A)^{minus}$ RNA fractions were assayed and these also stimulated protein synthesis (Table 20). The presence of mRNA in the poly $(A)^{minus}$ RNA fraction indicates either that some non-polyadenylated mRNA was present or alternatively that some poly (A) sequences were not of sufficient length to stick to the oligo (dT)-cellulose affinity column.

The response of the reticulocyte lysate to different concentrations of both the poly $(A)^{plus}$ and poly $(A)^{minus}$ RNA preparations was not linear. In the case of poly $(A)^{plus}$ RNA the greatest stimulation of protein synthesis occured at between 20 and 40 μg RNA/ml in the incubation mixture, higher concentrations inhibited protein synthesis. Using poly $(A)^{minus}$ RNA the optimum RNA concentration was about 20 times greater. These results suggest that the inhibition caused by the higher RNA concentrations is closely associated with the mRNA content which would be highest in the poly $(A)^{plus}$ RNA preparations. It is possible that damaged mRNA molecules in the preparations which cannot be translated properly but which are still able to compete for ribosomes and initiation factors in the system are the inhibitory agents. The possibility that an inhibitor of protein synthesis is purified in parallel with the poly $(A)^{plus}$ RNA cannot, however, be ruled out.

Table [illegible] Nuclease treated reticulocyte lysate [illegible] system: addition of poly(A) [illegible] RNA.

Addition to standard incubation	[illegible]
none	544
86.4 µg RNA	2[illegible]5[illegible]
43.2 µg RNA	2[illegible]17
21.6 µg RNA	21[illegible]4
10.8 µg RNA	144[illegible]
8.64 µg RNA	122[illegible]
5.76 µg RNA	1192
2.88 µg RNA	1165
1.44 µg RNA	10[illegible]5



Minus

Poly (A) RNA prepared from Ashanti type fruit (40g of arils from stages A and B fruit gave a yield of 517 OD units (260nm) of RNA. Poly (A) minus RNA was removed by chromatography on oligo (dT)-cellulose.) was added at various concentrations to the standard incubation mixture containing L-[4,5-^{3}H]-isoleucine (0.5µCi) as the labelled amino acid. At the end of the incubation period (1h) at 37°C, 2×20µl aliquots were withdrawn from the mixture (total volume 50µl) and spotted onto paper discs for determination of the C.P.M. figure. (TCA insoluble label)

It was decided to use the nuclease-treated reticulocyte lysate rather than the less active wheat-germ system for assaying for specific thaumatin mRNAs. Isolated RNA preparations were preferred as sources of messenger because they stimulated the reticulocyte lysate system more than the polysome preparations.

iv) Analysis of total protein synthesised *in vitro* under the direction of aril RNA. Labelled products resulting from the incubation of poly $(A)^{plus}$ or poly $(A)^{minus}$ RNAs in the nuclease-treated reticulocyte lysate system were analysed by polyacrylamide gel electrophoresis in the presence of SDS. In view of the results of the investigation into the synthesis of proteins in intact arils (see section VII F 1) translation of mRNA in an *in vitro* system was not expected to give a high yield of thaumatin. However it was hoped that it would be possible to show that proteins of a similar size to thaumatin were synthesised and therefore that any thaumatin mRNA present would be likely to be successfully translated.

In the case of both poly $(A)^{plus}$ and poly $(A)^{minus}$ RNA, labelled proteins with a wide range of molecular weights were synthesised (Figure 31). However the bulk of the protein products were within the molecular weight range 10,000 - 25,000 .Most appeared to have molecular weights of *ca* 14,000. The profiles were broadly similar for both of the RNA preparations. Because of the large number of different protein species present the resolution of the gel system was insufficient for individual protein forms to be seen as separate peaks of radioactivity. As expected there was no peak of radioactivity on the gel at $\triangleq$20,000 MWt which could be interpreted as a large quantity of labelled thaumatin being present. However proteins of a similar molecular weight were synthesised. It was decided therefore to select thaumatin by specific immunoprecipitation using anti-T II.

v) Immunoelectrophoretic detection of thaumatin synthesised

Figure 31 Analysis of Thaumatococcus RNA cell-free products.

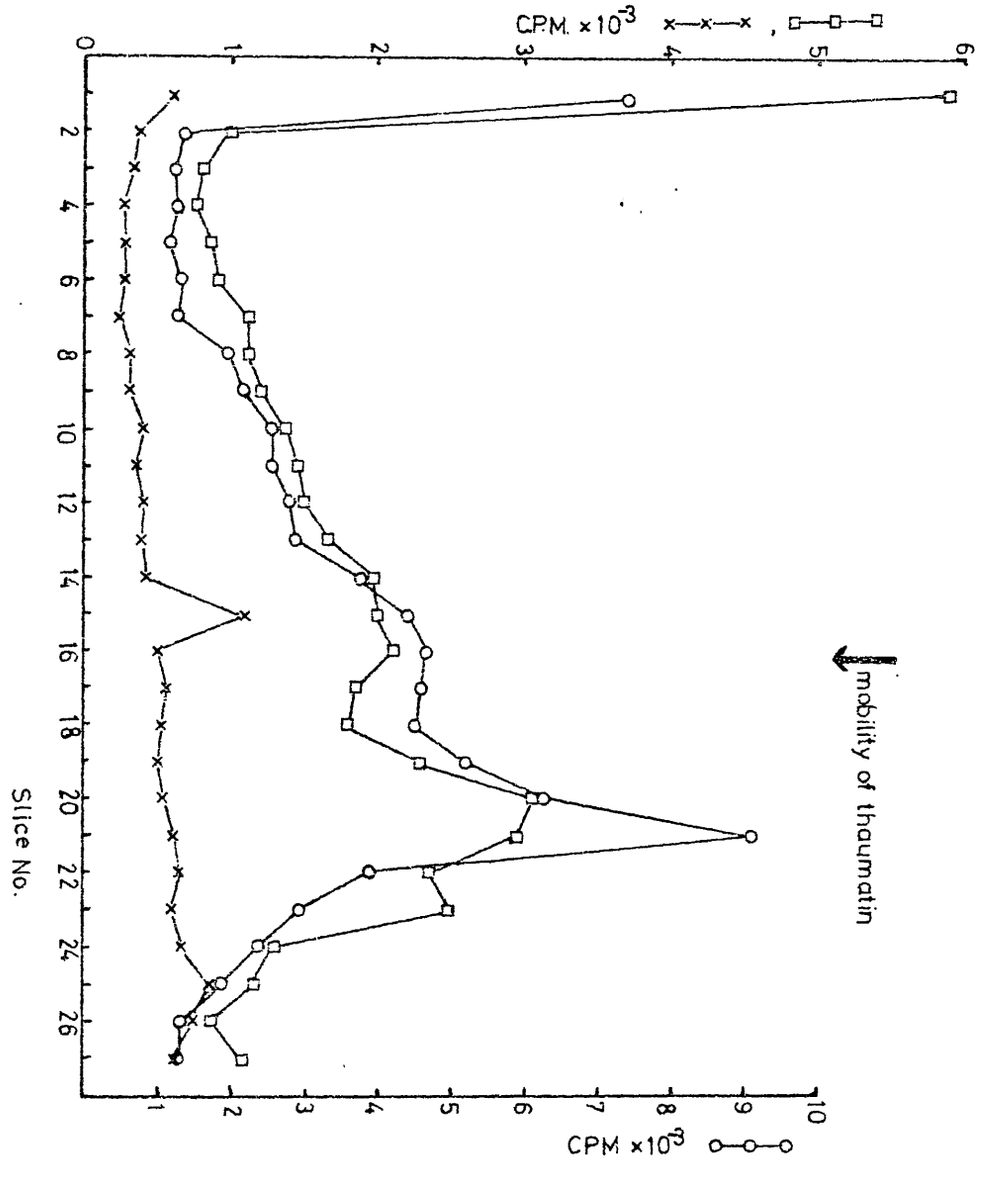
Incubations of the nuclease treated rabbit reticulocyte lysate cell-free system were as described in section V B 19 except that the volumes were increased to 100µl. The labelled amino acids were L-[4,5-^{3}H]-leucine (2µCi), L-[4,5(n)-^{3}H]-isoleucine (2µCi), L-[3,5-^{3}H]-tyrosine (2µCi), L-[4,5-^{3}H]-lysine monohydrochloride (2µCi). Aliquots of the incubation mixtures (5µl) were withdrawn at the end of the incubation period (1h at 37°C) and used to determine the incorporation of label into TCA precipitable material. The remainder of each incubation mixture (90µl) was precipitated with 5 volumes of ethanol. The pellet was washed (3×) with 80% ethanol containing 30mM NaCl. The final pellet was dissolved in sample buffer (200µl) and aliquots (100µl) were subjected to SDS P.A.G.E. on 10% gels. The gels were sliced and subjected to scintillation counting as described in section V B 23.

——o——o— incubation supplemented with 2.7µg poly(A) plus RNA.

——□——□— incubation supplemented with 72µg poly(A) minus RNA.

——×——×— incubation unsupplemented.

Addition to system	CPM in 5ul aliquot
no addition	792
plus 2.7ug poly(A) RNA	9533
minus 72ug poly(A) RNA	5184



in vitro. In view of the likelihood that thaumatin synthesis in the reticulocyte lysate would constitute only a small proportion of the total protein synthesised a sensitive assay was needed to detect the sweet protein product. Immunoprecipitation with anti-T II was, therefore, employed in conjunction with SDS polyacrylamide gel electrophoresis.

Preliminary experiments in which polysomes were used to direct protein synthesis in the nuclease-treated reticulocyte lysate failed to yield sufficient thaumatin for detection by these methods. Later poly $(A)^{plus}$ and poly $(A)^{minus}$ RNA fractions were used and several different preparations were employed to investigate the possibility that there were differences between the Kadjebe and Ashanti region fruit and between ripe and unripe tissues. The radioactivity profiles of the thaumatin immunoprecipitates obtained by SDS P.A.G.E. in each case are shown in figure 32. A radioactive band which migrated at the same rate as thaumatin occurred in the case of all of the RNA supplemented incubations but not in controls in which exogenous RNA was absent. Although the peaks were not large (i.e. only 2-3 times the background level at the peak maximum) it can be assumed that they represented thaumatin synthesis on the basis of their immunoprecipitation with anti-T II and their molecular weights which were calculated to be just over 20,000.

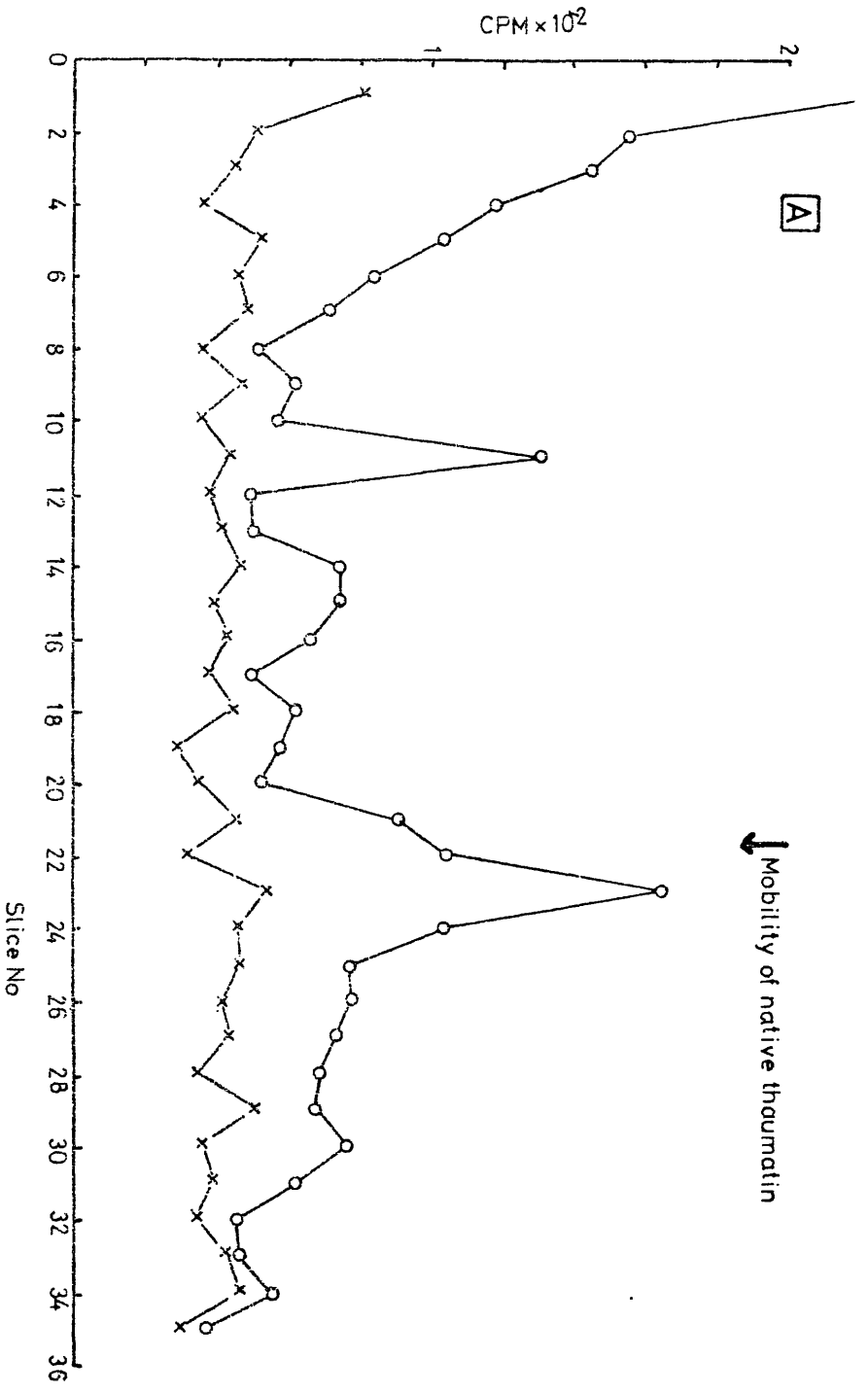
Only a small proportion (0·05-0·1%) of the total protein synthesised in the system was thaumatin. The proportion was the same when mature fruits (stage D) of either the Ashanti or Kadjebe regions were used as the source of the poly $(A)^{plus}$ RNA. If thaumatin synthesis in the intact aril system (section VI F 1) represented a similar percentage of the total protein synthesis then it is clear that the methods used to detect the newly synthesised thaumatin were not of sufficient sensitivity.

Figure 32 Immunoelectrophoretic detection of thaumatin synthesised in vitro

Incubations of the nuclease treated rabbit reticulocyte lysate cell-free system were incubated for 1h at 37°C

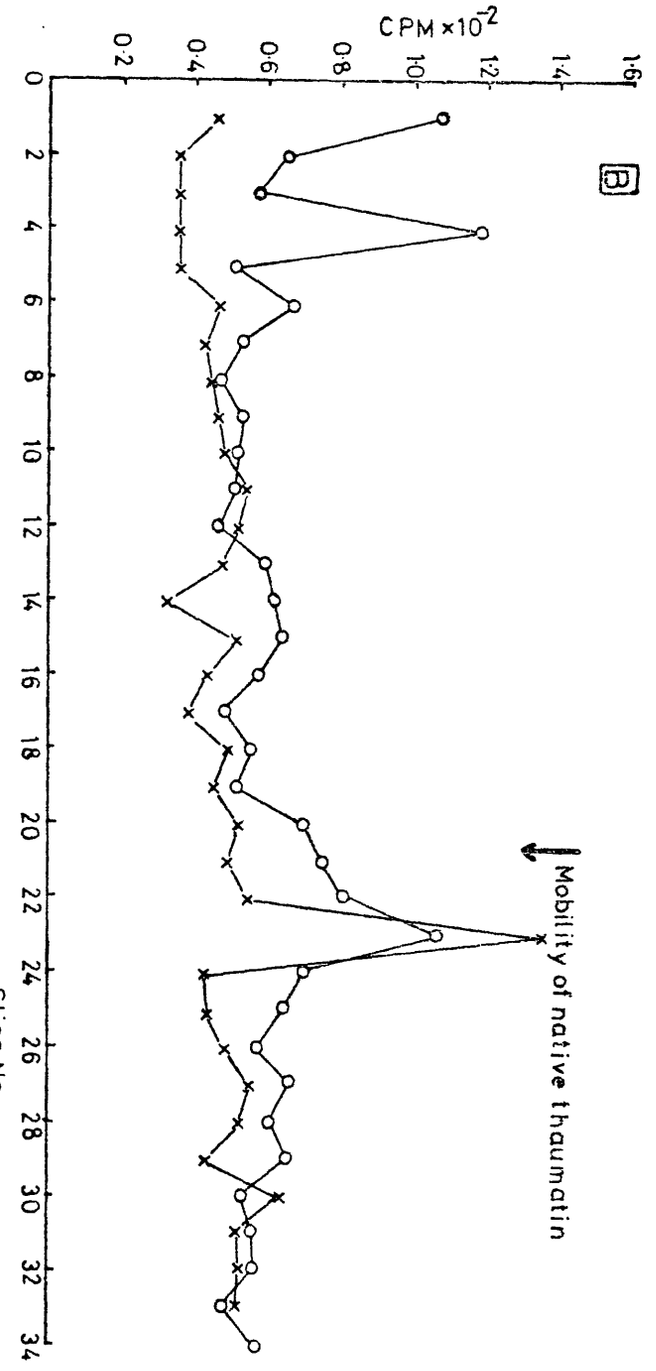
[A] Incubations (350µl) contained the following labelled amino acids:- L-[4,5(n)-^{3}H]-isoleucine (14µCi), L-[4,5-^{3}H]-leucine (21µCi).—x—x— no RNA added;—o—o— plus 5.2µg poly(A) RNA from Ashanti type aril tissue (stage D) added.

addition to system	CPM in 10 µl aliquot	
	total protein	released chains
no addition	2053	1845
plus 5.2ug poly(A) RNA	12137	8167


CPM x 10⁻²
1
2
A
Mobility of native thaumatin
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32
34
36
Slice No

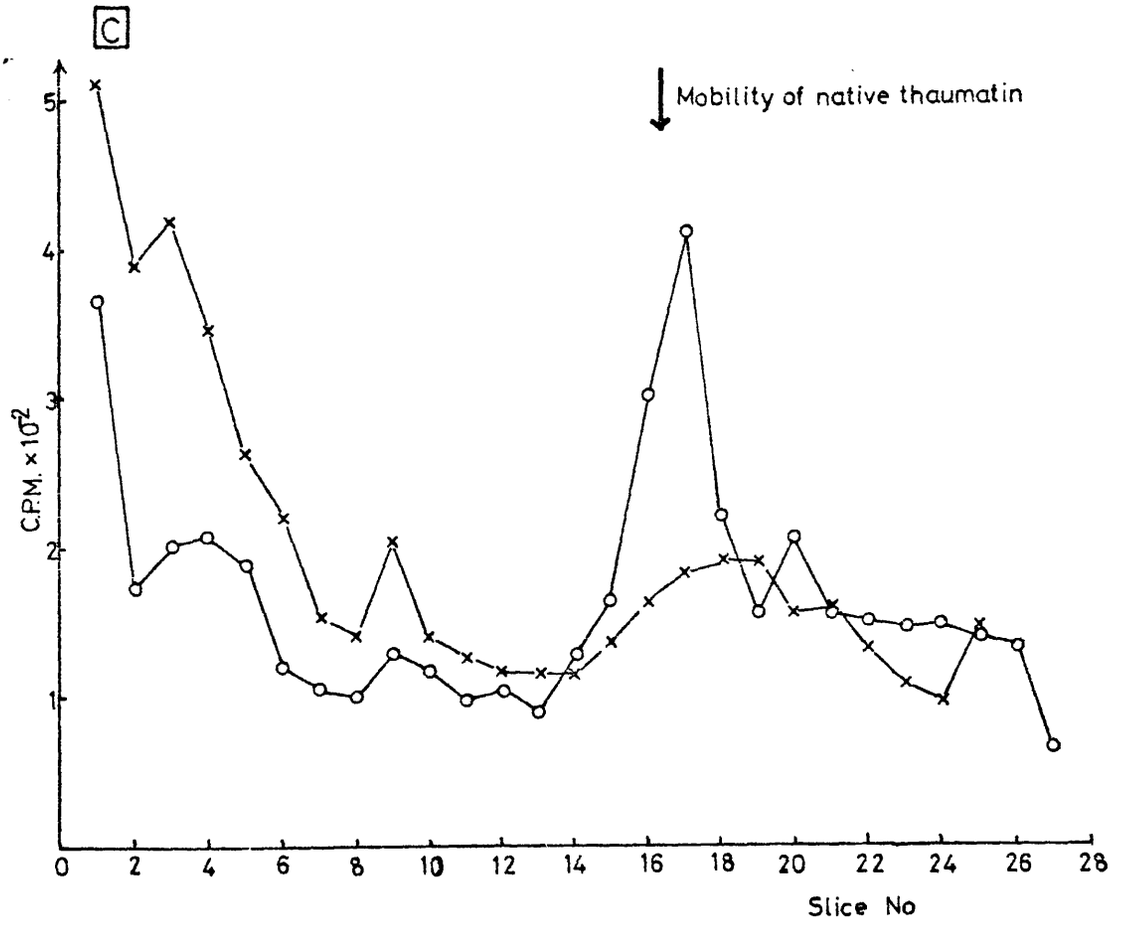
Incubations (500µl) contained the following labelled amino acids:- L-[4,5(n)-^{3}H]-isoleucine (20µCi), L-[4,5-^{3}H]-leucine (30µCi). —x—x— no RNA added; —o—o— plus 8.4µg poly(A) RNA from Kadjebe type aril tissue (stage D) added.

addition to system	CPM. in 10µl aliquot	
	total protein	released chains
no addition	2164	1634
plus 8.4µg poly(A) RNA	5570	4159


B
CPM ×10⁻²
0·2
0·4
0·6
0·8
1·0
1·2
1·4
1·6
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32
34
Slice No
Mobility of native thaumatin

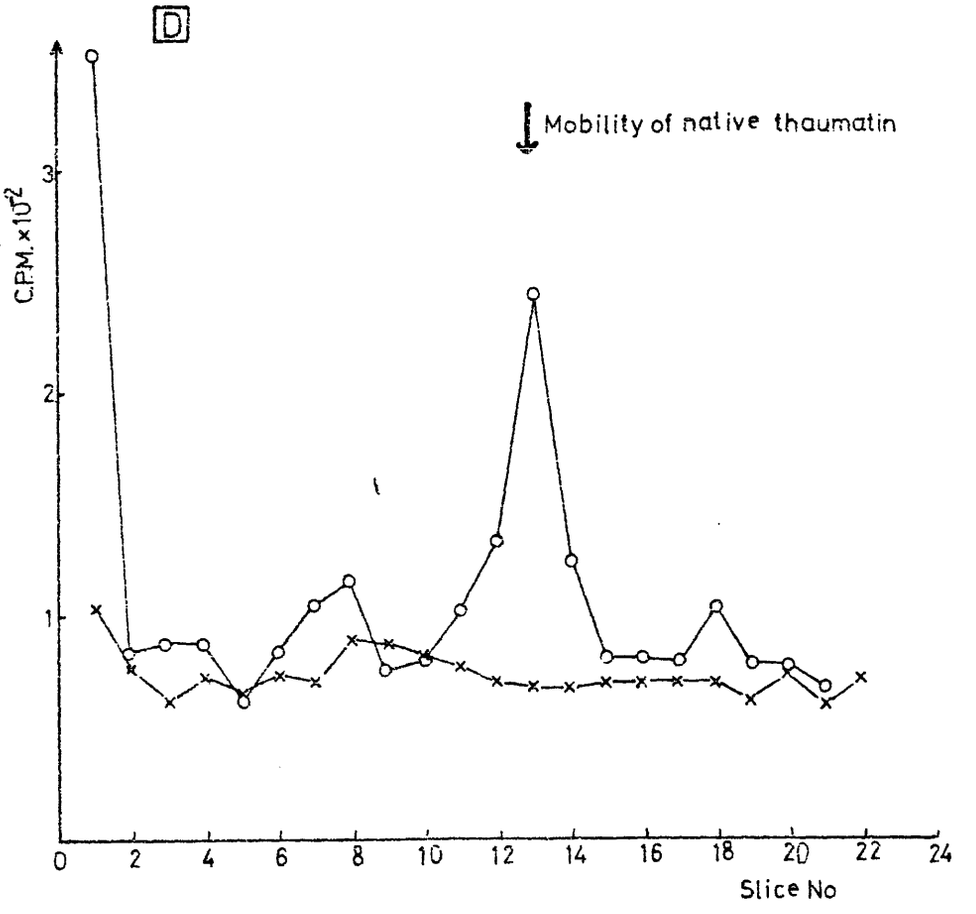
[C] Incubations (500µl) contained the following amino acids:- L-[4,5-^{3}H]-lysine monohydrochloride (20µCi), L-[4,5(n)-^{3}H]-isoleucine (20µCi), L-[4,5-^{3}H]-leucine (20µCi), L-[3,5-^{3}H]-tyrosine (20µCi), L-[^{35}S]-methionine (24µCi).—x—x— no RNA added; —o—o— 13.6µg poly(A) plus RNA from Ashanti type aril tissue (stages A and 3) added.

addition to system	CPM in 10µl aliquot	
	total protein	released chains
no addition	5098	4189
13.6µg poly(A) plus RNA	23371	21814



[D] Incubations (500µl) contained the following amino acids:- L-[4,5-^{3}H]-lysine monohydrochloride (10µCi), L-[4,5(n)-^{3}H]-isoleucine (10µCi), L-[4,5-^{3}H]-leucine (10µCi), L-[3,5-^{3}H]-tyrosine (10µCi). —x—x— no RNA added, —o—o— 432µg poly(A) minus RNA from Ashanti type aril tissue (stages A and B) added.

addition to system	CPM in 10µl aliquot	
	total protein	released chains
no addition	1326	1083
432µg poly(A) minus RNA	6271	5483



The poly $(A)^{plus}$ RNA preparation from immature Ashanti fruits (stages A and B) did not synthesise a higher proportion of thaumatin than RNA from mature tissue (stage D) suggesting that the level of thaumatin synthesis was not very variable over the range of developmental stages of fruit A → D. Finally poly $(A)^{plus}$ and poly $(A)^{minus}$ RNA preparations from immature Ashanti region fruits were both shown to contain thaumatin mRNA, which may therefore occur in both polyadenylated and non-polyadenylated forms. On the other hand a proportion of the thaumatin mRNAs may have short (10-20 bases) or blocked poly (A) sequences which would not bind effectively to the oligo (dT) column[213]. The amount of thaumatin synthesised under the direction of both poly $(A)^{plus}$ and poly $(A)^{minus}$ RNA fractions was approximately 0·05% of the total protein synthesised. Whilst it is possible that thaumatin mRNA was discriminated against by the protein synthetic 'machinery' of the reticulocyte lysate it is more likely that thaumatin mRNA represents a small proportion of the total mRNA occuring <u>in vivo</u>. This would agree with the studies using intact arils (section VI F 1) and indicates that thaumatin is a minor product of protein synthesis in the aril and that the final large accumulation of sweet protein found in mature tissue could only be explained by its very slow rate of turnover. On the other hand the proportion of thaumatin mRNA may be changed significantly as a consequence of picking, transport and storage of the fruit, either by preferential degradation of thaumatin mRNA or by a change in the rates of synthesis of the different mRNAs present <u>in vivo</u>.

vi) Is T I, T II or T 0 the primary <u>in vitro</u> product ? The structural and immunological studies on T I, T II and T 0 demonstrate a very close relationship between the sweet proteins. This would be expected if the thaumatins are derived from a single precursor form (in turn derived from a single gene) by a simple chemical or enzymic

mechanism. Investigation of this possibility failed to uncover any interconversion of forms. As an alternative it was decided to examine the thaumatin mRNA product to see if it was a single form and if so whether it was T 0, T I, T II or a different precursor.

Translation of aril RNA preparations (section VI F 2 v)) in the reticulocyte lysate system was used to show that the primary thaumatin mRNA product (which was identified by its reaction with anti-T II antiserum) had essentially the same relative mobility as thaumatins 0, I and II in 10% polyacrylamide gels containing SDS and that, therefore, it had a similar molecular weight. The messenger product could be detected only after immunoprecipitation with anti-T II which effectively removed the large amounts of non-thaumatin proteins present. Once the immunoprecipitation step was performed the thaumatin could be recovered by heating at 100°C for 2 min to dissolve the immunoprecipitate in buffer containing SDS. Unfortunately the different thaumatin forms treated with SDS in this way could not be identified on the basis of their charge differences due to the binding of negatively charged SDS molecules. An initial attempt to overcome this problem was made in which the complete aril RNA-directed reticulocyte lysate incubation mixture was fractionated by ion-exchange chromatography on CM-cellulose as described in section VI A. The fractions from this column were then treated with anti-T II and the immunoprecipitates analysed by SDS P.A.G.E.. Unfortunately no radioactive proteins of molecular weight ≏20,000 were identified in any of the fractions eluted from the ion-exchange column. One problem was that some of the fractions contained a large quantity of radioactive material which may have masked any labelled thaumatin present. It is also possible that the small quantity of labelled thaumatin under investigation was lost during CM-cellulose chromatography. However, it is unclear by what mechanism this could have occured.

As an alternative it was decided to use isoelectric focusing in urea gels to identify the thaumatin precursor. This system requires that the proteins to be focused are dissolved in a solution containing 8M urea. It was hoped that this urea concentration would effectively dissolve the immunoprecipitate and break the anti-T II-thaumatin association: Awdeh et al[214] have described the analysis of specific immunoglobulins by this method. When the procedure was tested using [^{125}I]-labelled thaumatin (section V B 13) the recovery of thaumatin from the immunoprecipitates was very low (< 5%) even when the urea concentration was increased to 10M. To overcome this problem it was decided to dissolve the immunoprecipitate in an SDS solution, then to remove the detergent in the presence of urea on an ion-exchange resin (as described by Weber and Kuter[215]) and finally to examine the free thaumatin by polyacrylamide gel isoelectric focusing (pH range 8·0-10·5) in the presence of urea. This procedure was initially tested using an immunoprecipitate of [^{125}I]-T II. The radioactivity profile of the resulting gel is shown in figure 33: the recovery of thaumatin was approximately 40%. A further test was made using another protein (α2U-globulin) which was also available as a labelled immunoprecipitate in order to determine whether the method could be applied more generally. α2U-globulin, the rat urinary protein (synthesised in a short term hepatocyte culture with medium containing tritiated leucine), was immunoprecipitated using specific anti-α2U antibody and the immunoprecipitate was dissociated and analysed by P.A.G.I.F in the pH range 4-6·5. The labelled α2U-globulin was recovered with an efficiency of $\triangleq$50% (Fig 34). This preliminary work, therefore, suggested that this procedure could be a useful and rapid analytical method for examining minute quantities of labelled proteins synthesised in in vitro systems and isolated as immunoprecipitates.

When this method was applied to the identification of the

Figure 33 P.A.G.I.F. of Immunoprecipitated $[^{125}I]$-TII

An immunoprecipitate of $[^{125}I]$-thaumatin II (15µg, 3750 CPM.) was dissociated as described in section V B 24 and subjected to P.A.G.I.F.. The gel was sliced and the slices prepared for scintillation counting as described in section V B 23.

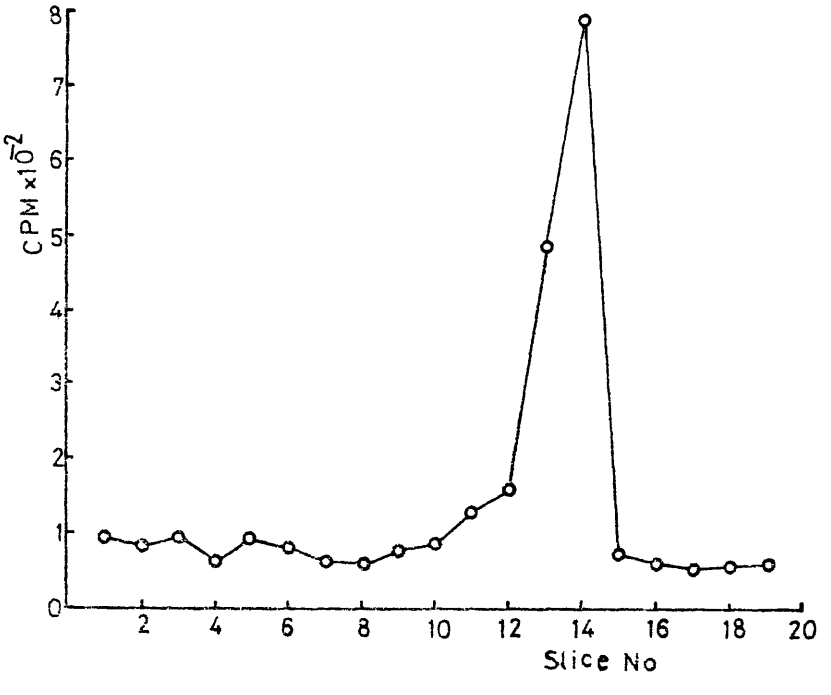
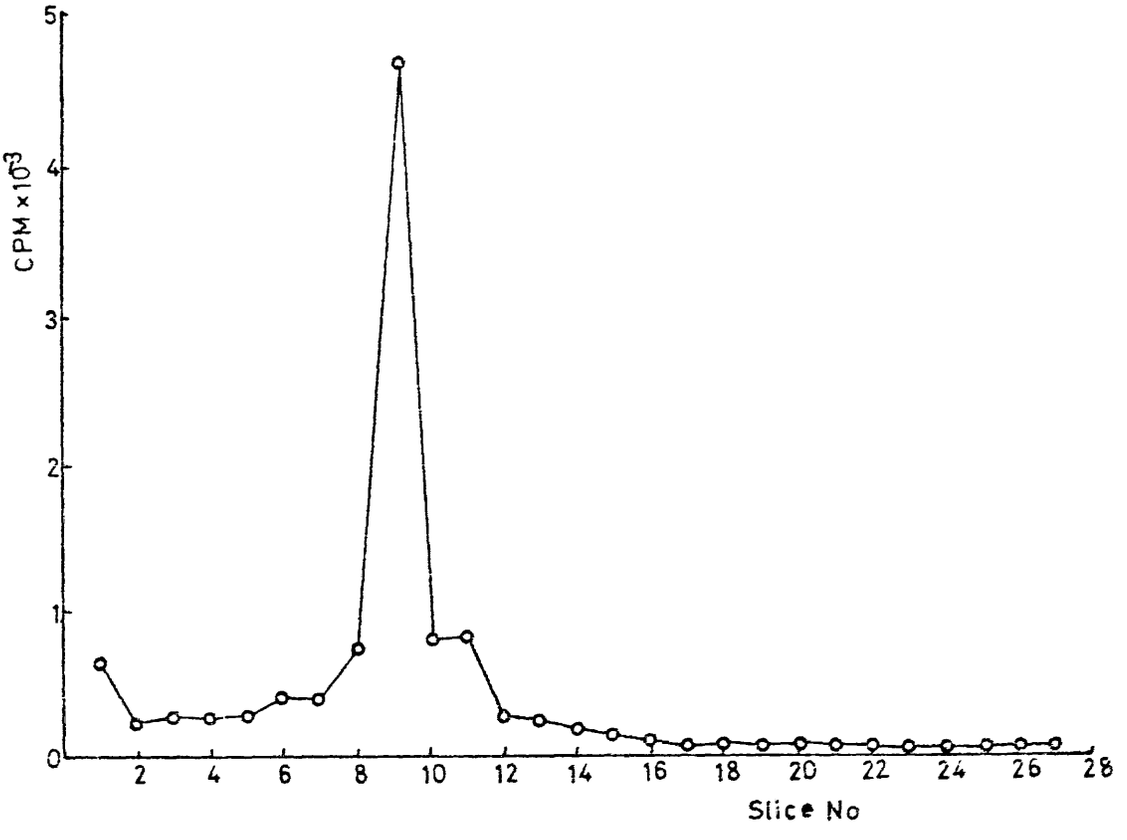


Figure 34 P.A.C.I.F. of immunoprecipitated [^{3}H]-α2U

An immunoprecipitate of [^{3}H]-α2U (20µg, 10,000 C.P.M.) was dissociated as described in section V B 24 and subjected to P.A.C.I.F.. The gel was sliced and the slices prepared for scintillation counting as described in section V B 23.



thaumatin form synthesised in an aril RNA directed nuclease-treated reticulocyte lysate system no radioactive species were detected on the gels (pH range 8-10·5) under conditions which were calculated to yield a measurable amount of labelled thaumatin precursor (it was assumed that thaumatin precursor would represent 0·05% of total protein synthesised (see section VI F 2 γ)). It is possible that the pI of the precursor was not in the pH range of the gel due to differences in conformation or amino acid composition which might exist if thaumatin is synthesised as a pre-form. On the other hand it is more likely that the failure to detect the thaumatin messenger product after immunoprecipitation and P A.G.I.F. was related to the small quantity of thaumatin synthesised in the cell-free system. This difficulty remained unresolved.

The above work leaves unsettled the question of the origin of the thaumatin forms. Further studies directed at solving this problem should involve a re-evaluation of the conditions for the dissociation of the anti-T II-thaumatin complex and the use of larger quantities of labelled amino acids and aril mRNA in order to increase the quantity of labelled thaumatin messenger product available for analysis.

GENERAL CONCLUSIONS

VII. GENERAL CONCLUSIONS

Thaumatin is found in large quantities, up to 50% of total protein, in the arils of the fruits of Thaumatococcus danielli. At present there is no satisfactory explanation available for the presence of such a large quantity of thaumatin in the tissue. The only known biological activity of thaumatin is its ability to stimulate the sweet taste receptors of man and some species of old world monkey.

Net synthesis of thaumatin was found to occur over the ripening period examined, a period of approximately 100 days. It was found that fruits from the Ashanti region of Ghana contain three thaumatin forms T 0, T I and T II whilst those from the Kadjebe region have only T 0 and T I. T I, in the Kadjebe fruits and T I and T II in the Ashanti fruits were always found in much greater quantities than T 0. However in the Ashanti fruits no definite correlation occured between the contents of T I and T II which were present in similar amounts. The interesting difference in thaumatin constituents between the plants from different regions could be explained in a number of ways. If the three thaumatin forms are different gene products then the gene for T II may be absent or non-functional in the Kadjebe region fruit. On the other hand if some post-translational modification is responsible for the change then the mechanism for conversion to T II may be absent or alternatively T II may be quickly degraded to the other thaumatin forms. Attempts were made to discover which of these possibilities accounted for the observed differences between the plants and therefore to determine the route of thaumatin biosynthesis.

Amino acid and N-terminal analyses, molecular weight determination and fingerprinting gave identical results in the case of all three

thaumatin forms suggesting strongly that they share similar primary structures. This was confirmed by immunological examination of the relationship between the forms using antibodies raised against thaumatin II. The thaumatins were not distinguishable by the Ouchterlony double diffusion and quantitative precipitin methods but the more sensitive micro-complement fixation technique showed that minor differences were present. The immunological and fingerprinting studies suggest not only that the sequences but also the conformations of the sweet proteins bear a close resemblance. Small differences were observed between the pI's of the thaumatins. These could be accounted for by differences in the numbers of amide groups present i.e. if T II contained one more amide than T I and two more than T 0. This would also be consistant with the immunological variations observed by micro-complement fixation. On the other hand in fingerprinting experiments using the protease from Staph.aureus which is known to cleave specifically at glutamic acid and aspartic acid residues, there were no differences in the peptide fragments generated. However the technique used involved only partial digestion of thaumatin so that it is possible that the relevant groups were not effected.

The possibility that the observed differences in pI between the thaumatin forms were due to the relative degree of amidation of the molecules was investigated further by subjecting the thaumatin forms to conditions in vitro which had been shown to result in deamidation of other proteins such as cytochrome C, aldolase and lysozyme. [^{125}I] labelled thaumatin II which was considered to be the most highly amidated form was treated with a fruit extract. No conversion was detected under any of the conditions employed. It may be, however, that the deamidation mechanism is very slow.

Another approach to the problem of the origin of the different

thaumatin forms was to study their biosynthesis. Arils were labelled by injection of L-[4,5-^{3}H]-leucine and attempts were made to detect any synthesis of thaumatin. Under the conditions used no labelled thaumatin could be identified. One difficulty was the presence of large quantities of thaumatin in the tissue which made it impracticable to test specifically for the presence of labelled thaumatin by use of the anti-T II antibody.

Purified polysomes and deproteinized RNA preparations from the aril tissue were shown to direct protein synthesis in the wheat-germ and rabbit reticulocyte cell free systems. In the case of the translation of deproteinized RNA (poly $(A)^{plus}$ and poly $(A)^{minus}$) preparations it was possible to identify a small proportion of the polypeptides as thaumatin (i.e. they reacted with anti-T II and had the same relative mobility as thaumatin during SDS P.A.G.E.) The poly $(A)^{plus}$ and poly $(A)^{minus}$ RNA preparations gave roughly the same proportion of thaumatin to total protein so that it can be assumed that at least some thaumatin mRNA has a 3′poly(A) sequence. The presence of thaumatin mRNA in the poly $(A)^{minus}$ fraction indicates that not all of the thaumatin messages are polyadenylated. However it may be that some thaumatin mRNAs have poly (A) sequences which are too short to interact with oligo (dT)-cellulose, alternatively the poly (A) may have been removed from some thaumatin mRNAs by nuclease activity or mechanical damage during the isolation procedure.

Immature and mature fruits from the Ashanti region of Ghana and mature fruits from the Kadjebe region were all found to contain thaumatin mRNA. The proportion of thaumatin to total protein synthesised <u>in vitro</u> was in each case similar and indicates that the low amount of thaumatin mRNA present was not a function of fruit development stage or fruit type.

A number of possible explanations are available to explain why

the mRNA for thaumatin is present in much lower amounts than would be expected from the abundance of thaumatin in the mature fruits. Firstly the rate of thaumatin synthesis may be slow *in vivo*. (In this case it must be assumed that the rate of breakdown of thaumatin is very slow). Secondly it is possible that the physiological state of the tissue is altered, by picking and transport from Ghana, in such a way that the thaumatin message is broken down and therefore occurs only in very small quantities in the available tissue. Thirdly the thaumatin message may be preferentially destroyed during isolation or finally the thaumatin message may be inefficiently translated in the cell-free systems investigated.

The polypeptide material precipitated from the messenger directed system by the anti-T II antiserum had the same mobility on 10% polyacrylamide gels in the presence of SDS as native thaumatin so that it is likely that thaumatin is not synthesised as a precursor of significantly higher molecular weight.

No evidence was obtained about whether the three thaumatin forms arise from a single precursor form or from different mRNA molecules coding for T 0, T I and T II.

The reason why experiments to determine the pI of the cell-free thaumatin product(s) was unsuccessful remains unclear. It is possible that the thaumatin precursor had a significantly different pI from the native forms.

REFERENCES

(1) Bradley R.M. and Mistretta, C.M. (1972) in Oral Physiology (Emmelin, N. and Zotterman, Y. eds) Pergamon, Oxford.

(2) Higginbotham, J.D. (1979) Spectrum (British Science News) 164, 7-9.

(3) Beidler, L.M. (1971) in Handbook of Sensory Physiology. Vol IV. Taste. (Beidler, L.M. ed) Springer Verlag, New York.

(4) Birch, G.G. and Shallenberger, R.S. (1973) in Molecular Structure and Function of Food Carbohydrate (Birch, G.G. and Green, L.P. eds) Applied Science, London.

(5) Shallenberger, R.S. and Acree, T. E. (1967) Nature (London) 216, 480-482.

(6) Shallenberger, R.S. and Acree T.E. (1971) in Handbook of Sensory Physiology. Vol IV. Taste. (Beidler,L.M. ed) Springer Verlag, New York.

(7) Kier, L.B. (1972) J. Pharm. Sci. 61, 1394-1397.

(8) Birch, G.G. and Lee, C.K. (1971) in Sweetness and Sweeteners (Birch, G.G., Green, C.F. and Coulson, C.B. eds) Applied Science, London.

(9) Dastoli, F.R. and Price, S. (1966) Science 154, 905-907.

(10) Dastoli, F.R. (1972) Experientia 28, 387-392.

(11) Hough, C.A.M. and Edwardson, J.A. (1978) Nature 271, 383-384.

(12) Van der Wel, H. and Bel, W.M. (1978) Chem. Senses Flavor 3, 99-104.

(13) Kooi, E.R., Harjes, C.F. and Gilkison, J.S. (1962) U.S. Patent. 3,042,584. (July 3, 1962).

(14) Newton, J.M. and Wardrip, E.K. (1974) in Symposium: Sweeteners (Inglett, G.E. ed) Avi Publishing Co, Inc., Westport, Connecticut.

(15) Brooks, G.A., Warnecke, M.O. and Long, J.E. (1974) in Symposium: Sweeteners. (Inglett, G.E. ed) Avi Publishing Co. Inc., Westport, Connecticut.

(16) Daniell, W.F. (1852) Pharm. J. 11. 445-448.

(17) Inglett, G.E., Dowling, B., Albrecht, J.J. and Hoglan, F.A. (1965) J. Agric Food Chem. 13, 284-290.

(18) Irvine, F.R. (1961) Woody Plants of Ghana Oxford University Press, London.

(19) Adansi, M.A. (1970) Ghana J. Agric. Sci. 3, 207.

(20) Giroux, E.L. and Henkin, R.I. (1974) J. Agric. Food Chem. 22, 595-601.

(21) Kurihara, K. and Beidler, L.M. (1968) Science 161, 1241-1243.

(22) Kurihara, K. and Beidler, L.M. (1969) Nature 222, 1176-1179.

(23) Dzendolet, E. (1969) Percept. Psychophys. 6, 187-188.

(24) Hutchinson, J. and Dalziel, J.M. (1927) Flora of West Tropical Africa 1, Crown Agents, London.

(25) Inglett, G.E. and May, J.F. (1968) Econ. Bot. 22, 326-330.

(26) Orchard, B. (1972) Unpublished report, Tate and Lyle Group R. and D., Reading.

(27) Inglett, G.E. and May, J.F. (1969) J. Food Sci. 34, 408-411.

(28) Morris, J.A. and Cagan, R.H. (1972) Biochim. Biophys. Acta 261, 114-122.

(29) Van der Wel, H. (1972) FEBS Lett. 21, 88-90.

(30) Morris, J.A., Martenson, R., Deibler, G. and Cagan, R.H. (1973) J. Biol. Chem. 248, 534-539.

(31) Van der Wel, H. and Loeve, K. (1973) FEBS Lett. 29, 181-184.

(32) Bohak, Z. and Li, S-L. (1976) Biochim. Biophys. Acta 427, 153-170.

(33) Frank, G. and Zuber, H. (1976) Hoppe-Seylers Z. Physiol. Chem. 357, 585-592.

(34) Hudson, G. and Biemann, K. (1976) Biochem. Biophys. Res. Commun. 71, 212-220.

(35) Morris, J A. and Cagan, R.H. (1976) J. Agric. Food Chem. 24, 1075-1077.

(36) Hough, C.A.M. (1976) Unpublished results, Tate and Lyle Group R. and D., Reading.

(37) Morris, J.A. and Cagan, R.H. (1975) Proc. Soc. Exper. Biol. Med. 150,265.

(38) Morris, J.A., Cagan, R.H., Martenson, R.E. and Deibler, G. (1978) Proc. Soc. Exper. Biol. Med. 157, 194-201.

(39) Brouwer, J.N., Hellekant, G., Kasahara, Y., Van der Wel, H. and Zotterman, Y. (1973) Acta Physiol. Scand. 89, 550-557.

(40) Hellekant, G. (1976) Chem. Senses Flavor 2, 97-104.

(41) Hellekant, G., Glaser, D., Brouwer. J.N. and Van der Wel, H. (1976) Acta Physiol. Scand. 97, 241-250.

(42) Daniell, W.F. (1855) Pharmacol. J. 15, 158-160.

(43) Higginbotham, J.D. (1979) in Developments in Sweeteners I (Hough, C.A.M., Parker, K.J. and Vlitos, A.J. eds) Applied Science Publishers Ltd., Barking, England.

(44) Van der Wel, H. and Loeve, K. (1972) Eur. J. Biochem. 31, 221-225.

(45) Ellman, G.L. (1959) Arch. Biochem. Biophys. 82, 70-77.

(46) Korver, O., Van Gorkom, M. and Van der Wel, H. (1973) Eur. J. Biochem. 35, 554-558.

(47) Iyengar, R.B., Smits, P., Van der Ouderaa, F., Van der Wel, H., Van Brouwershaven, J., Ravestein, P., Richters, G. and Van Wassenaar, P.D. (1979) Eur. J. Biochem. 96, 193-204.

(48) Chou, P.Y. and Fasman, G.D. (1977) Trends Biochem. Sci. 2, 128-131.

(49) Glaser, D., Hellekant, G., Brouwer, J.N. and Van der Wel, H. (1978) Folia Primatol. 29, 53-63.

(50) Kim, S.I.,Charbonnier, L., and Mossé, J. (1978) Biochem. Biophys. Acta 537, 22-30.

(51) Righetti, P.G., Gianazza, E.. Viotti, C. and Soave, C. (1977) Planta 136, 112-124.

(52) Viotti, C., Sala, E., Alberi, P. and Soave, C. (1978) Plant Sci. Letts. 13, 365-376.

(53) Paleus, S. and Theorell, H. (1957) Acta Chem. Scand. 11, 905.

(54) Flatmark, T. (1964) Acta Chem. Scand. 18, 1656-1666.

(55) Flatmark, T. (1966) Acta Chem. Scand. 20, 1487-1496.

(56) Flatmark, T. and Vesterberg, O. (1966) Acta Chem.Scand. 20, 1497-1503.

(57) Flatmark, T. (1967) J. Biol. Chem. 242, 2454-2459.

(58) Flatmark, T. and Sletten, K. (1968) J. Biol. Chem. 243, 1623-1629.

(59) Robinson, A.B. McKerrow, J.H. and Legaz, M. (1974) Int. J. Peptide Protein Res. 6, 31-35.

(60) Bloemendal, H. (1969) Exp. Eye Res. 8, 227-302.

(61) Bloemendal, H. Berns, A.J.M., Zweers, A., Hoenders, H.J. and Benedetti, E.L. (1972) Eur. J. Biochem. 24, 401-406.

(62) Shoenmakers, J.G.G. and Bloemendal, H. (1968) Nature 220, 790-791.

(63) Shoenmakers, J.G.G. Gerding, J.J.T. and Bloemendal, H. (1969) Eur. J. Biochem. 11, 472-481.

(64) Bloemendal, H., Berns, A.J.M,, Van der Ouderaa, F. and De Jong, W.W.W. (1972) Exp. Eye Res. 14, 80-81.

(65) van Kleef, F.S.M., Nijzink-Maas, M.J.C.M. and Hoenders, H.J. (1974) Eur. J. Biochem. 48, 563-570.

(66) van Kleef, F.S.M., Willems-Thijssen, W. and Hoenders, H.J. (1976) Eur. J. Biochem. 66, 477-483.

(67) De Jong, W.W.W., van Kleef, F.S.M. and Bloemendal, H. (1974) Eur. J. Biochem. 48, 271-276.

(68) Taylor, W.L. and Dixon, J.E. (1976) Biochim. Biophys. Acta 444, 428-434.

(69) Midelfort, C.F. and Mehler, A.H. (1972) Proc. Nat. Acad. Sci. USA 69, 1816-1819.

(70) Tallan, H.H. and Stein, W.H. (1953) J. Biol. Chem. 200, 507-514.

(71) Huang, C.-C., Mayer, H.E., Jr. and Montgomery, R. (1970) Carbohydr. Res. 13, 127-137.

(72) Borsodi, A . and Narasimhan, T.R. (1978) Br. J. Haematol. 39, 121-128.

(73) Shimizu, M., Kanno, C. and Yamauchi, K. (1978) Agric. Biol. Chem. 42, 981-988.

(74) Day, J.F.,Thorpe, S.R. and Baynes, J.W. (1979) J. Biol. Chem. 254, 595-597.

(75) Stevens, V.J., Rouzer, C.A., Monnier, V.M. and Cerami, A. (1978) Proc. Nat. Acad. Sci. USA 75, 2918-2922.

(76) Pande, A., Garner, W.H. and Spector, A. (1979) Biochem. Biophys. Res. Communs. 89, 1260-1266.

(77) Terhorst, C., Wittman-Liebold, B. and Möller, W. (1972) Eur. J. Biochem. 25, 13-19.

(78) Allison, W.S., Admiraal, J. and Kaplan, N.O. (1969) J. Biol. Chem. 244, 4743-4749.

(79) Wangh, L., Ruiz-Carrillo, A. and Allfrey, V.G. (1972) Arch. Biochem. Biophys. 150, 44-56.

(80) Nelsestuen, G.L., Zylkovicz, T.H. and Howard, J.B. (1974) J. Biol. Chem. 249, 6347-6350.

(81) Lowry, P.J. and Chadwick, A. (1970) Nature 226, 219-222.

(82) Gregory, R.A., Tracy, H.J. and Grossman, M.I. (1966) Nature 209, 583.

(83) Martenson, R.E., Kramer, A.J. and Deibler, G.E. (1976) J. Neurochem. 27, 1529-1531.

(84) Barbenhenin, E.K. and Klee, C.B. (1978) J. Biol. Chem. 253, 2446-2454.

(85) Kidd, F. and West, C. (1945) Plant Physiol. 20, 467-504.

(86) Venrell, M. (1969) Aust. J. Biol. Sci. 22, 601-610.

(87) Frenkel. C. and Dyck, R. (1973) Plant Physiol. 51, 6-9.

(88) Marei, N. and Crane, J.C. (1971) Plant Physiol. 48, 249-254.

(89) McGlasson, W.B. (1970) in The Biochemistry of Fruits and their Products 1,(Hulme, A.C. ed) Academic Press, London and New York.

(90) Pratt, H.K. and Goeschl, J.D. (1969) Ann. Rev. Plant Physiol. 20, 544-584.

(91) Coombe, B.G. and Hale, C.R. (1973) Plant Physiol. 51, 629-634

(92) McGlasson, W.B., Dostal H.C. and Tigchelaar, E.C. (1975) Plant Physiol. 55, 218-222.

(93) Chalmers, D.J. and Van den Ende, B. (1975) Aust. J. Plant Physiol. 2, 623-634.

(94) Jene, P.H. and Chalmers, D.J. (1976) Aust. J. Plant Physiol. 3, 429-434.

(95) Coombe, B.C. and Hale, C.R. (1973) Plant Physiol. 51, 629-634.

(96) Hale, C.R. and Coombe, B.G. (1974) R. Soc. N.Z. Bull. 12, 831-836.

(97) Looney, N.E., McGlasson, W.B. and Coombe, B.G. (1974) Aust. J. Plant Physiol. 1, 77-86.

(98) Milborrow, B.V. (1974) Ann. Rev. Plant Physiol. 25, 259-307.

(99) Milborrow, B.V. and Robinson, D.R. (1973) J. Exp. Bot. 24, 537-548.

(100) Stenvers, N. and Bruinsma, J. (1975) Nature 253, 532-533.

(101) Tingwa, P.O. and Young, R.E. (1974) Plant Physiol. 54, 907-910.

(102) Frenkel, C. (1975) Plant Physiol. 55, 480-484.

(103) Kidd, F. and West, C. (1945) Plant Physiol. 20, 467-504.

(104) Clendenning, K.A. (1941) Can. J. Res. 20, 197-202.

(105) Biale, J.B. (1946) Am. J. Bot. 33, 363-373.

(106) Hulme, A.C. (1958) Adv. Fd. Res. 8, 297-302.

(107) Biale, J.B. (1960) in Handbuch der Pflanzen physiologie 12, (Ruhland, W. ed),536,Springer Verlag, Berlin.

(108) Sacher, J.A. (1966) Plant Physiol. 41, 701-708.

(109) Looney, N.E. and Patterson, M.E. (1967) Phytochemistry 6, 1517-1520.

(110) Hulme, A.C., Rhodes, M.J.C. and Wooltorton, L.B.C. (1971) Phytochemistry 10, 749-756.

(111) Richmond, A. and Biale, J. B. (1967) Biochim, Biophys. Acta 138, 625-627.

(112) Ku, L.L. and Romani, R.J. (1970) Plant Physiol. 45, 401-407.

(113) Rattanapanone, N., Spiers, J. and Grierson, D. (1978) Phytochemistry 17, 1485-1486.

(114) Frenkel, C., Klein, I. and Dilley, D.R. (1968) Plant Physiol. 43, 1146-1153.

(115) Lewis, T.L. and Martin, D. (1965) Aust. J. Biol. Sci. 18, 1093-1101.

(116) Rhodes, M.J.C. and Wooltorton, L.S.C. (1967) Phytochemistry 6, 1-12.

(117) Brady, C.J., Palmer, J.K., O'Connell, P.B.H. and Smillie, R.M. (1970) Phytochemistry 9, 1037-1047.

(118) Sacher J.A. (1966) Plant Physiol. 41. 701-708.

(119) Wong, B. (1967) The relationship between permeability characteristics, active uptake, incorporation of amino acids and the amount of protein and RNA in senescing avocado fruit tissue. MA thesis. Calif. State Univ., Los Angeles.

(120) Clements, R.L. (1970) in The Biochemistry fruits and their Products 1 ,(Hulme, A. C. ed) Academic Press, London and New York.

(121) Frenkel, C., Klein, I. and Dilley, D. R. (1968) Plant Physiol. 43, 1146-1153.

(122) Sacher, J.A. (1973) Ann. Rev. Plant Physiol. 24, 197-224.

(123) Barmore, C.R. and Rouse, A.H. (1976) J. Am. Soc. Hort. Sci. 101, 294-296.

(124) Zauberman, G. and Schieffmann-Nadel, M. (1971) Plant Physiol. 49, 864-865.

(125) Pesis, E., Fuchs, Y. and Zauberman, G. (1978) Plant Physiol. 61, 416-419.

(126) Looney, N.E. (1967) Nature 214, 1245-1246.

(127) Sacher, J.A. (1967) Soc. Exp. Biol. Symp. 21, 260-303.

(128) Wade, N.L., O'Connell, P.B.H. and Brady, C.J. (1972) Phytochemistry 11, 975-979.

(129) Wooltorton, L.S.C., Jones, J.D. and Hulme, A.C. (1965) Nature 207, 999-1000.

(130) Riov, J., Monselise, S.P. and Kahan, R.S. (1969) Plant Physiol. 44, 631-635.

(131) Richmond, A. and Biale, J.B. (1966) Plant Physiol. 41, 1247-1253.

(132) Frenkel, C., Klein, I. and Dilley, D.R. (1968) Plant Physiol. 43, 1146-1153.

(133) Brawerman, G. (1974) Ann. Rev. Biochem. 43, 621-642.

(134) Perry, R.P. (1976) Ann. Rev. Biochem. 45, 605-629.

(135) Adesnik, M. and Darnell, J.E. (1972) J. Mol. Biol. 67, 397-406.

(136) Greenberg, J.R. and Perry, R.P. (1972) J. Mol. Biol. 72, 91-98.

(137) Soreq, H., Nudel, U. Solomon, R., Revel, M. and Littauer, U.Z. (1974) J. Mol. Biol. 88, 233-245.

(138) Williamson, R., Crossley, J. and Humphries, S. (1974) Biochemistry 13, 703-707.

(139) Mendecki, J., Lee, S.Y. and Brawerman, G. (1972) Biochemistry 11, 792-798.

(140) Marbaix, G., Huez, G., Burny, A., Cleuter, Y., Hubert, E., Leclercq, M., Chantrenne, H., Soreq, H., Nudel, U. and Littauer, U.Z. (1975) Proc. Nat. Acad. Sci. USA. 72, 3065-3067.

(141) Shatkin, A.J. (1976) Cell 9, 645-653.

(142) Both, G.W., Bannerjee, A.K. and Shatkin, A.J. (1975) Proc. Nat. Acad. Sci. USA 72, 1189-1193.

(143) Muthukrishnan, S., Filipowicz, W., Sierra, J.M., Both, G.W., Shatkin, A.J. and Ochoa, S. (1975) J. Biol. Chem. 250, 9336-9341

(144) Nomoto, A., Lee, Y.F. and Wimmer, E. (1976) Proc. Nat. Acad. Sci. USA. 73, 375-380.

(145) Frisby, D., Eaton, M. and Fellner, P. (1976) Nucleic Acids Res. 3, 2771-2787.

(146)Furuichi, Y., La Fiandra, A. and Shatkin, A.J. (1977) Nature 266, 235-239.

(147) Ragg, H., Schröder, J. and Hahlbrock, K. (1977) Biochim. Biophys. Acta 474, 226-233.

(148) Schröder, J. (1977) Arch. Biochem. Biophys. 182, 488-496.

(149) Schröder, J., Betz, B. and Hahlbrock, K. (1977) Plant Physiol. 60, 440-445.

(150) Schröder, J., Kreuzaler, F. and Schmock, J. (1977) FEBS Lett. 81, 10-12.

(151) Higgins, T.J.V., Mercer, J.F.B. and Goodwin, P.B. (1973) Nature 246, 68-70.

(152) Verma, D.P.S., Nash, D.T. and Schulman, H.M. (1974) Nature 251, 74-77.

(153) Scragg, A.H. and Thurston, C.F. (1975) J. gen. Microbiol. 89, 155-162.

(154) Scragg, A.H., John, P.C.L. and Thurston, C.F. (1975) Nature 257, 498-501.

(155) Higgins, T.J.V., Zwar, J.A. and Jacobsen, J.V. (1976) Nature 260, 166-169.

(156) Rattanapanone, N., Grierson, D. and Stein, M. (1977) Phytochemistry 16, 629-633.

(157) Hammett, J.R. and Katterman, F.R. (1975) Biochemistry 14, 4375-4379.

(158) Gray, R.E. and Cashmore, A.H. (1976) J. Mol. Biol. 108, 595-608.

(159) Sagher, D., Edelman, M. and Jakob, K.M. (1974) Biochim. Biophys. Acta 349, 32-38.

(160) Covey, S.N. and Grierson, D. (1976) Eur. J. Biochem. 63, 599-606.

(161) Wheeler, A.M. and Hartley, M.R. (1975) Nature 257, 66-67.

(162) Weeks, D.P. and Marcus, A. (1971) Biochim. Biophys. Acta 232, 671-684.

(163) Verma, D.P.S., Maclachlan, G.A., Byrne, H. and Ewings, D. (1975) J. Biol. Chem. 250, 1019-1026.

(164) Larkins, B.A., Bracker, C.E. and Tsai, C.Y. (1976) Plant Physiol. 57, 740-745.

(165) Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.

(166) Mans, R.J. and Novelli, G.D. (1961) Arch. Biochem. Biophys. 94, 48-53.

(167) Gabriel, O. (1971) Methods in Enzymology 22, 565-578.

(168) Weber, K., Pringle, J.R. and Osborn, M. (1972) Methods in Enzymology 26, 3-27.

(169) Wrigley, C.W. (1971) Methods in Enzymology 22, 559-564.

(170) van Kleef, F.S.M. and Hoenders, H.J. (1973) Eur. J. Biochem. 40, 549-554.

(171) Reisner, A.H., Nemes, P. and Bucholtz, C. (1975) Analytical Biochem. 64, 506-516.

(172) Moore, S. and Stein, W.H. (1963) Methods in Enzymology 6, 819-831.

(173) Fraenkel-Conrat, H. (1957) Methods in Enzymology 4, 247-269.

(174) Stark, G.R. (1972) Methods in Enzymology 25, 103-120.

(175) Cleveland, D.W., Fischer, S.G., Kirschner, M.W. and Laemmli, U.K. (1977) J. Biol. Chem. 252, 1102-1106.

(176) Grabar, P. and Williams, C.A. (1953) Biochim. Biophys. Acta 10, 193-194.

(177) Prager, E.M. and Wilson, A.C. (1971) J. Biol. Chem. 246, 7010-7017.

(178) Wasserman, E. and Levine, L. (1961) J. Immunology 87, 290-295.

(179) Arnheim, N. and Wilson, A.C. (1967) J. Biol. Chem. 242, 3951-3956.

(180) Markwell, M.A.K. and Fox, C.F. (1978) Biochemistry 17, 4807-4817.

(181) Hunt, T. and Jackson, R.J. (1974) Hematologie und Bluttransfusion 14, 300-307.

(182) Pelham, H.R.B. and Jackson, R.J. (1976) Eur. J. Biochem. 67, 247-256.

(183) Neinhuis, A.W., Falvey, A.K. and Anderson, W.F. (1974) Methods in Enzymology 30, 621-630.

(184) Osterburg, H.H., Allen, J.K. and Finch, C.E. (1975) Biochem. J. 147, 367-368.

(185) Palmiter, R.D. (1974) Biochemistry 13, 3606-3614.

(186) Akalehiywot, T., Gedamu, L. and Bewley, J.D. (1977) Can. J. Biochem. 55, 901-904.

(187) Hoffman, W.L. and Ilan, J. (1974) Biochim. Biophys. Acta 336, 199-214.

(188) Loening, U.E. (1967) Biochem. J. 102, 251-257.

(189) Cashman, D. and Pitot, H.C. (1971) Analytical Biochem. 44, 559-569.

(190) Narita, K. (1970) in Protein Sequence Determination (Needleman, S.B. ed) 38, Chapman and Hall, Ltd., London.

(191) Salaman, M.R. and Williamson, A.R. (1971) Biochem. J. 122, 93-99.

(192) Robinson, A.B. and Rudd, C.J. (1974) in Current Topics in Cellular Regulation 8, (Horecker, B.L. and Stadtman, E.R. eds) 247-295, Academic Press, London and New York.

(193) Houmard, J. and Drapeau, G.R. (1973) Proc. Nat. Acad. Sci. USA 69, 3506-3509.

(194) Wilcox, P.E. (1967) Methods in Enzymology 11, 63-76.

(195) Spies, J.R. (1956) Methods in Enzymology 3, 468-471.

(196) Ouchterlony, Ö. (1958) in Progress in Allergy 5, (Kallós, P. ed) Karger, Basel and New York.

(197) Prager, E.M. and Wilson, A.C. (1971) J. Biol. Chem. 246, 7010-7017.

(198) Yudkin, J. (1967) Am. J. Clin. Nutr. 20, 108-115.

(199) Perheentupa, J. and Raivio, K. (1967) Lancet 2, 528-531.

(200) Price, J.M., Biava, C.G., Oser, B.L., Vogin, E.E., Steinfeld, J. and Ley, H.L. (1970) Science 167, 1131-1132.

(201) Palmiter, R.D., Gagnon, J., Ericsson, L.H. and Walsh, K.A. (1977) J. Biol. Chem. 252, 6386-6393.

(202) Blobel, G. and Dobberstein, B. (1975) J. Cell. Biol. 67, 852-862.

(203) Ramagopal, S. and Hsiao, T.C. (1973) Biochim. Biophys. Acta 299, 460-467.

(204) Breen, M.D., Whitehead, E.I. and Kenefick, D.G. (1971) Plant Physiol. 49, 733-739.

(205) Palmiter, R.D., Gagnon, J. and Walsh, K.A. (1978) Proc. Nat. Acad. Sci. USA 75, 94-98.

(206) Harris, B. and Dure III, L. (1978) Biochemistry 17, 3250-3256.

(207) Cheah, K.S.E., Grant, M.E. and Jackson, D.S. (1979) Biochem. J. 182, 81-93.

(208) Scott, M.P., Storti, R.V., Pardue, M.L. and Rich, A. (1979) Biochemistry 18, 1588-1594.

(209) Goldstein, J.L. and Swain, T. (1965) Phytochemistry 4, 185-192.

(210) Legon, S., Brayley, A., Hunt, T. and Jackson, R.J. (1974) Biochem. Biophys. Res. Commun. 56, 745-752.

(211) Balkow, K., Hunt, T. and Jackson, R.J. (1975) Biochem. Biophys. Res. Commun. 67, 366-375.

(212) Woodward, W.R., Ivey, J.L. and Herbert, E.(1974) Methods in Enzymology 30, 724-731.

(213) Taylor, J.M. (1979) Ann. Revs. Biochem. 48, 681-717.

(214) Awdeh, Z.L., Williamson, A.R. and Askonas, B.A. (1968) Nature 219, 66-67.

(215) Weber, K. and Kuter, D.J. (1971) J. Biol. Chem. 246, 4504-4509.