CHARACTERIZATION OF TWO NATURALLY OCCURRING GLYCOPROTEIN-PIGMENT COMPLEXES FROM COTTONSEED PIGMENT GLANDS

By

CHUNG-MIN CHANG

Bachelor of Agriculture

National Northwestern Agricultural College

Shansi, China

1946

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PIGMENT GLANDS

Thesis Approved:

George V. Odell
Thesis Adviser

James E. Webster

D. D. Durham
Dean of the Graduate College

658389

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CHAPTER I

INTRODUCTION

Cottonseed pigments are unique not only in their chemical nature but also in their manner of occurrence in the seed. Most of the pigment of cottonseed are contained in a distinct morphological structure which has been designated the "gossypol gland".

It has been shown (28) that the pigment glands are mechanically very strong and are resistant to the action of a considerable number of organic solvents such as the hydrocarbons, chlorinated hydrocarbons and glycerides. The density of these glands is less than that of the other tissues of this seed. On the basis of these properties a method which employs mechanical desintegration and flotation was devised as a means of separating pigment glands from other tissue and oil (28).

It has generally been assumed that all the pigments exist as two types, free pigment and pigment-protein complexes in the gland, but no accurate determination has heretofore been made of the extent of which this occurs. Consequently, the present investigation was undertaken with the objective of ascertaining the types of the pigment-protein complexes in the pigment glands. Further information has also been sought concerning the carbohydrate content of these pigment-protein complexes.

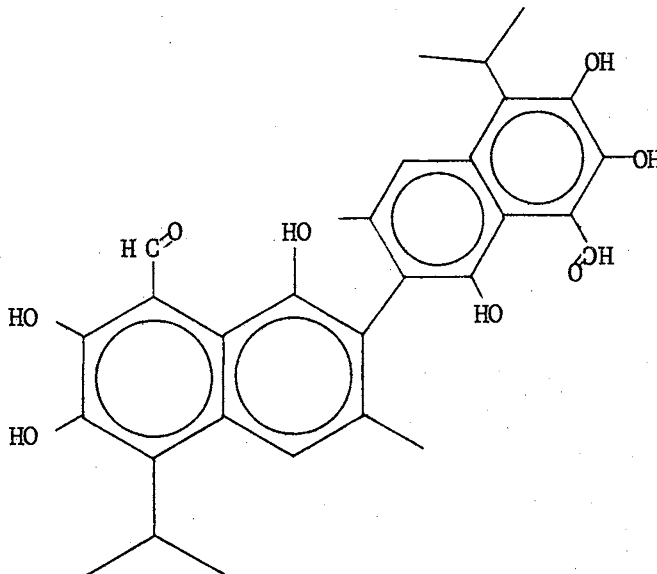
Gossypol, a toxic polyphenolic yellow-pigment is inactivated in cottonseed meal by a cooking process. In this process pigment-protein

complexes are formed. Many of these synthetic complexes have been studied. The data reported in this thesis covers the first isolation, purification and characterization of naturally occurring pigment-glycoprotein complexes from cottonseed pigment glands.

CHAPTER II

REVIEW OF LITERATURE

The first scientific study of the pigments of cottonseed was conducted by Kuhlman in 1861 (1). Gossypol, the principal pigment of cottonseed, was first isolated by Logmore in 1886 and partially characterized by Marchilewski in 1899 (2,3). It has a molecular weight of 518 and the empirical formula is $C_{30}H_{30}O$ (4). The structure was determined by Adams and co-workers in 1958 (5,6); one tautomeric form is shown below. O'Connor and co-workers studied the infrared spectra of



GOSSYPOL

gossypol (7) while Shirley and co-workers (8) have contributed to the study of the chemical reactions of gossypol.

"Bound" gossypol was first proposed by Clark (9). He pointed out that the "bound" gossypol was gossypol that was combined with the protein of the cottonseed. This hypothesis was not confirmed by experimental evidence for many years. Lyman (10) used solid protein reacting with a methanol or ethanol solution of gossypol to prepare gossypol-protein complexes. Lyman and co-workers found that the reactivity towards gossypol was not the same for all the amino groups in protein. There were two types of complexes between gossypol and protein, (a) a bridge formation with one molecule of gossypol between two molecules of protein or (b) the protein was connected to one end of the gossypol and the other reactive end of the gossypol molecule was still free. Both complexes described here involve a Shiff base condensation of formyl groups of gossypol with ϵ amino groups of lysine.

There are many indications that more than one type of bound gossypol exist. The reaction between gossypol and the epsilon amino group of lysine in protein, has been postulated for many years (9). This reaction has been demonstrated by the use of Sager's dinitrofluorobenzene method (11) for end group determination and Carpenter's (12) method for measuring the free epsilon amino lysine content of protein.

A number of gossypol derivatives have been described by Martin (10) including those of ureas, guanidine and amino acids. Ureas will react quantitatively with gossypol in a number ratio of 2:1 at a temperature of 100°C; gossypol-urea products are readily converted to dianilino gossypol by reaction with aniline and ethanol. The guanidine salts generally reacted less quantitatively with gossypol. Gossypol-

amino acid reaction products approach a composition of two moles of amino acid per mole of gossypol. A hydrogen bonded gossypol has not been demonstrated as a "bound" form, but it may exist since phenolic-OH groups are present. Salt and ester derivatives are also possible.

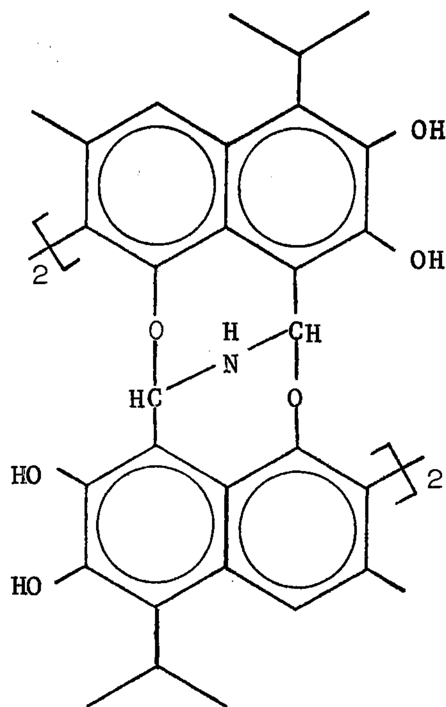
The hydrogen bonding of phenolic compounds to proteins has recently been demonstrated by Loomis (44) in the isolation of plant enzymes. This researcher further proposes that oxidation of hydrogen bonded phenols would by oxidation form quinones and permanent covalent linkages to proteins.

Free gossypol is toxic to many species of animals (4,13,14,15,16), but the "bound" gossypol is non-toxic (9) and this term has been used to denote gossypol which has reacted during milling processes in such a way as to be converted into a non-toxic molecule (3,4,17,18,19,20,21). Eagle (22) found the toxicity of cottonseed pigment gland material could not be attributed to gossypol alone. This observation led to further examination of the composition of the pigment glands.

Gossypurpurin, a naturally occurring purple colored pigment of cottonseed, was first isolated by Boatner (23) from the red crystals (so-called red gossypol) obtained from chloroform extracts of cottonseed kernels (21,24,25). By treating an ethereal extract of cottonseed kernels with dilute ammonium hydroxide (16,26) gossypurpurin has also been obtained. Gossypurpurin was isolated from cottonseed pigment gland material and prepared from diamino-gossypol by Pominski _et al_. (27). Pominski has also proposed $C_{30}H_{12}O_7N$ as a molecular formula of gossypurpurin. Later work (45) indicates gossypurpurin is about twice the size of gossypol and its empirical formula would be $C_{60}H_{64}N_2O_{14}$. Absorbancy maxima at 530-532 mμ and 565-568 mμ were observed. Gossypurpurin

was found to be very stable in the dry state but it was rapidly decomposed by the action of light (24). Even in the absence of light, solutions of gossypurpurin in chloroform, ethyl acetate, diethyl ether, and dioxane turn yellow in a few hours.

There were several qualitative tests conducted with gossypurpurin in order to determine its relationship to gossypol (22). Fehling's test, Tollen's test, hydroxylamine hydrochloride and phenylhydrazine hydrochloride tests were found to give positive reactions with both gossypurpurin and gossypol thus indicating the presence of one or more carbonyl groups. Both pigments also gave positive results with ferric chloride, pyroboroacetate, and stannic chloride, thus indicating the presence of two orthophenolic hydroxyls and a hydroxyl, para or ortho to a carbonyl (27). Concentrated sulfuric acid reacted with gossypurpurin to produce a yellow-green color which turned to orange after ten minutes at room temperature but a dark red color developed immediately with gossypol. Boatner (27,28) observed that gossypurpurin reacted with glacial acetic acid to produce a green precipitate, whereas gossypol gave a yellow precipitate. In the visible region there were two absorbancy maxima: one at 530 mμ and one at 565-566 mμ (for gossypurpurin in chloroform solution). There was no peak for gossypol in this region. In the ultraviolet region, the absorbancy maxima of gossypurpurin at 326-327 mμ and 370-371 mμ indicated that gossypurpurin was structurally related to gossypol which has peaks at 288-289 mμ and at 363.5mμ. The ready conversion of gossypurpurin to gossypol upon contact with acid seems to indicate that their basic structures are similar (27). The following figure shows the proposed structure of gossypurpurin (29).



GOSSYPURPURIN

Cottonseed pigments are unique in their manner of occurrence in the seed. Most of cottonseed pigments are contained in distinct morphological structures which are relatively large ovid or spherical bodies 100 to 400 microns on the long axis (29). Brefeld _et al._ (30) noted the occurrence of pigment glands in cottonseed in 1887 and other investigators reported the gland membrane was composed of two layers (22,31). The internal structure of the cottonseed pigment gland, in which discrete particles ranging in size from one micro to less than 0.2 microns in diameter are held within a membranous mesh-like network, has been demonstrated with the electronmicroscope. The gland is very sensitive to water. The outside walls of the gland appear to consist of platelets which exist as flattened compartments, each of which has a complex

internal structure (32). Stanford (21) suggested that gossypurpurin may be concentrated in the outer wall of the gland and the gossypol deposited in the interior.

The presence of pigment glands can be detected in the 15-day embryo as well as in the mature seed (33,34). The formation of the water-sensitive gland wall in the moist surroundings of the developing seed might logically be attributed to the protective action of the encircling layer of cells, which always form before the pigment gland develops (35). The pigments of moist seed undergo relatively large changes during storage (36,37,38), whereas dry seeds show less change than the moist seed (39). Many of the transformations occurring during the storage of moist seeds can be attributed to the inadequate protective action of the sheath of cells surrounding the pigment glands. Further changes may be attributed to the more active participation of the gland pigments in the respiration of the seed (37,39,40) and their conversion into deeply colored phenol and quinone oxidation products.

It has been shown (41) that the pigment glands owe their mechanical strength to a thick encompassing wall, composed of cellulose impregnated with a pectinaceous material and exteriorly coated with a thick layer of cutin. This wall is readily ruptured by contact with water and a few water-miscible alcohols, ketones and ethers of low molecular weight.

Eagle and Davis (10) found that cottonseed pigment glands were slightly less toxic when administered in oil, as compared to the administration in water. When gossypol is administered in oil, it is more toxic than when administered in water.

As stated in the introduction it has generally been assumed that all the pigments exist as two types free pigment and pigment-protein

complexes in the gland, but no accurate determination has heretofore been made of the extent to which this occurs. Consequently, the present investigation was undertaken with the objective of ascertaining the types of the pigment-protein complexes in the pigment glands. Further information has also been sought concerning the carbohydrate content of these pigment-protein complexes. Gossypol, a toxic polyphenolic yellow-pigment is inactivated in cottonseed meal by a cooking process. In this process pigment-protein complexes are formed. Many of these synthetic complexes have been studied. The data reported in this thesis covers the first isolation, purification and characterization of naturally occurring pigment-glycoprotein complexes from cottonseed pigment glands.

CHAPTER III

EXPERIMENTAL PROCEDURE

Isolation of Pigment Glands from Cottonseed

Decorticated cottonseeds, Gossypium hirsutum, were crushed by a roller and expelled through a screw press. The cottonseed meal obtained from the crude cake was extraced 3 times with cold n-hexane. Isolation of the pigment glands from this cottonseed meal was effected by the method of semi-pilot plant scale as reported by Spadaro et al. (42). The meal was passed through a "Wiley" mill with a one mm screen.

In the isolation of cottonseed pigment gland material, about 10 pounds of the hexane-extracted cottonseed meal was used in the following manner. A dispersing solvent mixture of commercial hexane and tetrachloroethylene of specific gravity 1.378 was added to the meal. About 10 gallons of solvent mixture was required for 10 pounds of cottonseed meal. The dispersed mixture of cottonseed meal was allowed to settle for several hours and the pigment glands were collected by skimming from the top of the solvent mixture container. The glands were placed on a Buchner funnel and air dried to remove the excess solvent.

In order to obtain a higher concentration of pigment glands, the first preparation of dried pigment glands was subjected to a second flotation process using the same type solvent mixture and the same conditions as before. Air dried pigment glands obtained from the second flotation process were seived using a U. S. standard sieve series:

sieve no. 40, 60, 100 with openings of 420, 250, 140 micons, respectively. The fine, light, yellowish-green pigment glands were obtained after they had passed through the 100 mesh sieve. This material was placed in glass containers with aluminum foil for protection from light and then stored at 4°C.

Isolation of Gossypol-free Pigment Glands Residue from Cottonseed Pigment Glands

Pigment glands were extracted for five minutes with five percent water in acetone in a Waring blendor. The mixture was filtered through a Buchner funnel, with a water aspirator suction, on Whatman filter paper No. 1. The residue of gland walls was re-extracted with five percent water in acetone, filtered and then washed with acetone. This procedure was repeated three times. Gossypol-free pigment gland residue was obtained.

Isolation of Pigment-Protein Complexes from Gossypol-free Pigment Gland Residue

Twenty grams of the gossypol-free pigment gland residue were extracted with 200 ml of distilled water. The mixture of glands and water was agitated for 10 minutes and centrifuged in an International Chemical CL Centrifuge at 2000 rpm for 10 minutes, then filtered through a Buchner funnel with a water aspirator suction onto Whatman filter paper No. 1. The residue was re-extracted twice with the same amount of water. The filtrates were combined and the crude purple-protein complex was obtained by lyophilization.

Purification of the Crude Purple-Protein Complex by Gel Filtration

The crude purple-protein complex was dissolved in a minimum amount of deionized water. This solution was dialyzed at 4°C against several changes of a large volume of pre-cooled water during a 48 hour period. The solution was then placed in a 500 ml round bottom flask and freeze-dried. The purple material was stored in a refrigerator for further purification.

Preparation of Sephadex Gel Columns

Each column was packed in a vertical glass tube, 1.2 cm internal diameter, with glass wool placed on the bottom of the tube to support the gel. A layer of sand, 0.5 cm depth, prevented blockage of the glass wool by gel particles.

Sephadex gel-filtration media G-25, lot no. 4649 and G-75 lot no. 423M were suspended in distilled water and allowed to swell for 2-3 days. The smallest particles were removed by decantation. Each column was prepared by pouring a dilute slurry of gel particles in water into the vertical tube already partly filled with water, while allowing an excess of liquid to percolate through the growing gel bed. The addition of gel was continued until a desired bed height was obtained. A water reservior was then connected to the top of the column and a flow of water maintained at a rate of approximately 30 ml/hr. for 2 days. After the bed settled to a constant height, it was adjusted to the desired height by addition or removal of gel, or by the addition of fresh gel previously swelled with water.

Crude purple-protein was dissolved in distilled water (2 ml) and the solution applied to the top of a column. The column effluent was collected in 3 ml fractions with an automatic fraction collector. Proteins were estimated spectrophotometrically by reading each fraction in a Beckman DU Spectrophotometer, 1 cm light path, at 280 mμ. The Klett-Summerson colorimeter was also used to assay for colored substances. The wavelengths were selected according to the assay being performed on the effluent fraction; ninhydrin assay for amino acids and biuret assay for proteins were observed at 570 mμ and 550 mμ, respectively. The gossypurpurin was estimated at 540 mμ.

Purification of Complexes by Paper Electrophoresis

About 0.05 gram of Sephadex-purified material of purple-protein complex (from Sephadex G-75) was dissolved in 0.1 ml of phosphate buffer (pH 8.4) and placed on the center of the paper strips of the Spinco Model-R low voltage electrophoresis cell, (Durrum ridge pole apparatus). After 20 hours of operation at a current of 50 ma, at 4°C. The pigment complex was eluted from the paper. Some lighter purple-protein complexes migrated toward the positive pole in the paper strips for a distance of 1-2 cm from the origin, but the largest amount of the purple-protein remained at the origin. This material appeared as a heavy band and was removed by cutting out from the paper strips and eluting with distilled water. The eluate was filtered through Whatman filter paper No. 1. The filtrate was freeze-dried on a lyophilizer and stored in solid form at 4°C.

Disc electrophoresis was used to establish the purity of complexes obtained from paper electrophoresis. A synthetic polyacrylamide gel

system marketed by Conalco was utilized for these tests according to the directions furnished with the system. A summary of the isolation and purification system utilized in this study can be seen in Figure 1.

Determination of the Molecular Weight and Purity

About 5 mg of purified purple-protein complex from paper electrophoresis were dissolved in 2 ml of distilled water and centrifuged in a Beckman Model E Analytical Ultracentrifuge at 7928 rpm for 18 minutes. Photographs were taken at 2 minute intervals after the speed of 7928 had been reached. A continuous band was obtained and an average S value was calculated from the average distance of the band. Urease (S = 19, M. W. = 500,000) was used as a reference to estimate the molecular weight of the purple-protein complex. The molecular weight was approximated at 60,000,000 by this technique.

Ten mg of the yellow-protein complex from Sephadex G-75 were dissolved in 2 ml of water and centrifuged in the same manner as above at 59,780 rpm. Photographs were taken at 30, 40, 50, and 60 minutes after reaching maximum speed. The average S value was calculated as about 6.00 The molecular weight of the yellow-protein complex was near 100,000 with urease used as a reference.

Separation of the Pigment from the Purified Protein-Pigment Complex

Ten mg of purified purple-protein complex were treated with 10 ml of 3% water in 1,4-dioxane. The complex was completely dissociated from the pigment within 6 hours. The mixture was filtered through Whatman filter paper No. 1. The protein residue was washed twice with 5 ml

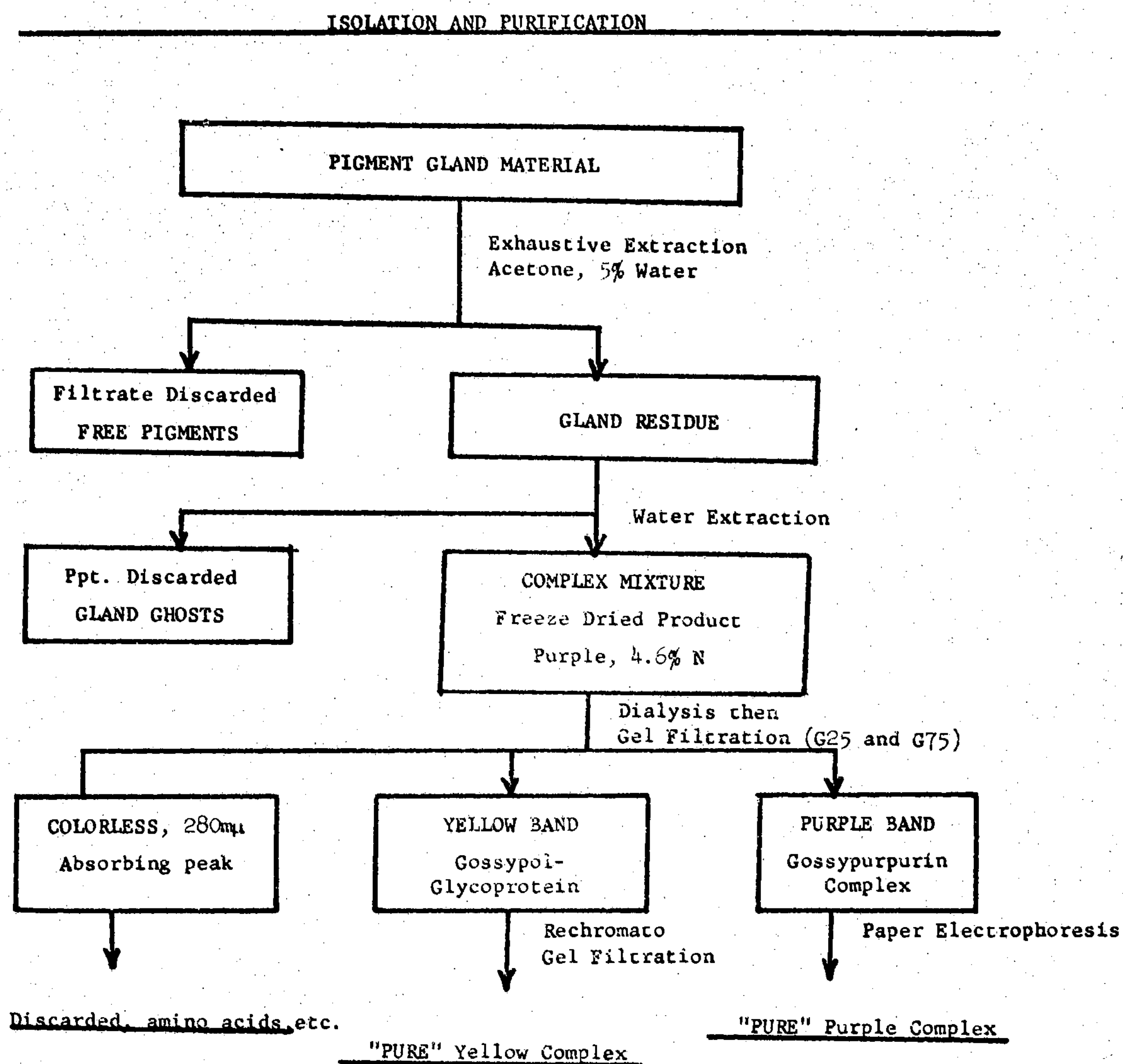


Figure 1. Isolation and Purification of Pigment-protein Complexes from Cottonseed Pigment Gland Material

portions of dioxane. The total pigment solution was condensed on a rotary evaporator to 5 ml, and then placed on a silicic acid column. Chloroform was used as an elution solvent to separate pure purple pigment. The chloroform solution of pigment was also reacted with freshly distilled aniline and the resulting reddish brown solution was heated on a steam bath for 20 minutes. The absorption spectra was then determined for all solutions on the Cary-14 recording spectrophotometer.

Amino Acid Analysis of Pigment-Protein Complexes

Three mg of purified-protein complexes were dissolved in 1 ml of 6 N HCl and transferred to a tube having an internal diameter of 1.2 cm. The tube was sealed by drawing out in a microburner and placed in an oven at 105°C for 20 hours. After cooling the tube was scratched with a sharp file and the contents transferred to a centrifuge tube. The sample was centrifuged and filtered through Whatman No. 1 paper. The filtrate was evaporated on a rotary evaporator, then transferred with 2 ml of water to a small flask and finally dried by lyophilization. The quantitative analysis of amino acids in the hydrolysate of the purple-protein complex was carried out with a Beckman Spinco amino acid analyzer. The yellow-protein complex was hydrolyzed and by the same procedure as for the purple-protein complex.

Identification of Carbohydrates

Three mg of the purple-protein complex were hydrolyzed with 2 N hydrochloric acid at 105°C for 16 hours. The hydrolysate from the mild acid hydrolysis was freeze-dried. Hydrolysates were first examined by descending paper chromatography on Whatman No. 2 paper using the

solvents (a) pyridine : butanol : water (3:2:1) and (b) butanol : acetic acid : water (4:1:5). Reducing substances on the chromatograms were detected with aniline acid oxalate spray reagent.

Two ml of N, N-Dimethylformamide were added to the dry sample, and the container was heated in a water bath at 50°C for one hour. This solution was used to prepare the trimethylsilyl sugar derivatives for gas chromatographic identification. 0.5 ml Pyridine was added with 0.4 ml hexamethyl disilizane and 0.2 ml trimethyl silichloride while swirling the flask. Then a 5 μl aliquot of this mixture was used for gas chromatography in a Perkin-Elmer 801 Gas chromatograph. The glass column (6 ft x 1/8 inch) was packed with SE-52 on Chomosorb Q. Nitrogen was the carrier gas with a flow rate of 60 ml per minute. The column temperature was programmed at a rate of 4° per minute from the initial temperature of 120°C to the final temperature of 250°C.

CHAPTER IV

RESULTS AND DISCUSSION

Isolation and Purification of the Pigment-Protein Complexes

After the free pigments were extracted from cottonseed pigment gland material with acetone-5% water, cold water was used for extracting the water soluble intraglandular material from gossypol-free pigment glands. The pigment-protein complexes were completely dissolved in water within 10 minutes with agitation. It can be deduced that water ruptured the pigment gland wall and the inner layer of mucilage material rapidly dissolved in water.

Chromatographic purification of the pigment-protein complexes on a Sephadex G-25 column is shown in Figure 2. The pigment-protein can not be separated from free amino acids on this column. The effluent volume from 40 ml to 80 ml, a mixture of pigment-glycoprotein was applied to a Sephadex G-75 column and two components, a purple-complex and a yellow-complex were obtained as is shown in Figure 3. Gel filtration with Sephadex G-50, Figure 4, also separates amino acids from the complexes but will not separate the purple and yellow complex.

The purple-complex was further purified by paper electrophoresis. Figure 5 shows acrylamide gel electrophoresis A and B for checking the purity of the purple-complex after paper electrophoresis. "A" was applied for detection of pigment-protein complex after paper electrophoresis and there were at least six different proteins in the impure

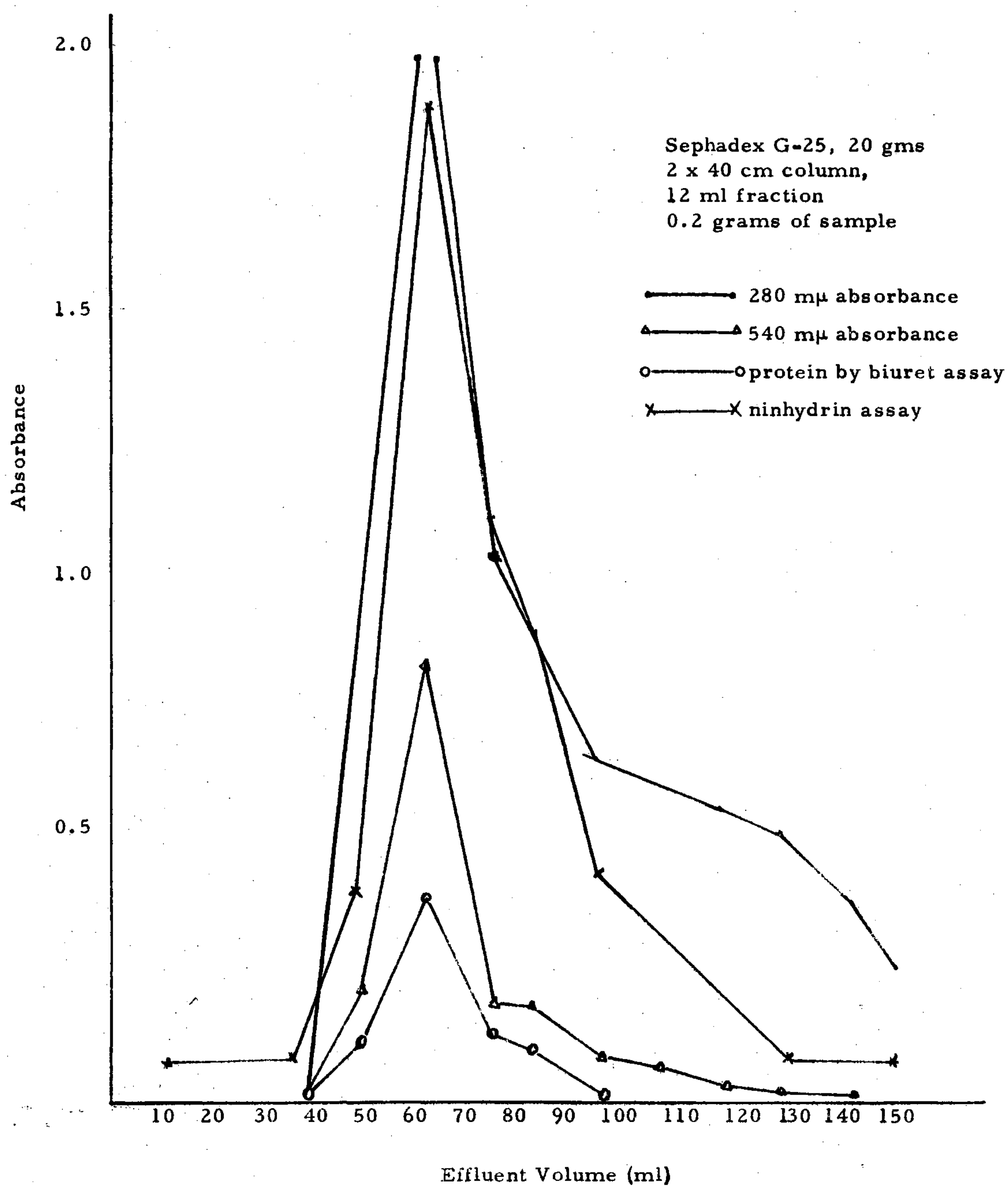


Figure 2. Gel Filtration Chromatography of Gossypurpurin-Glycoprotein Complex (crude isolate)

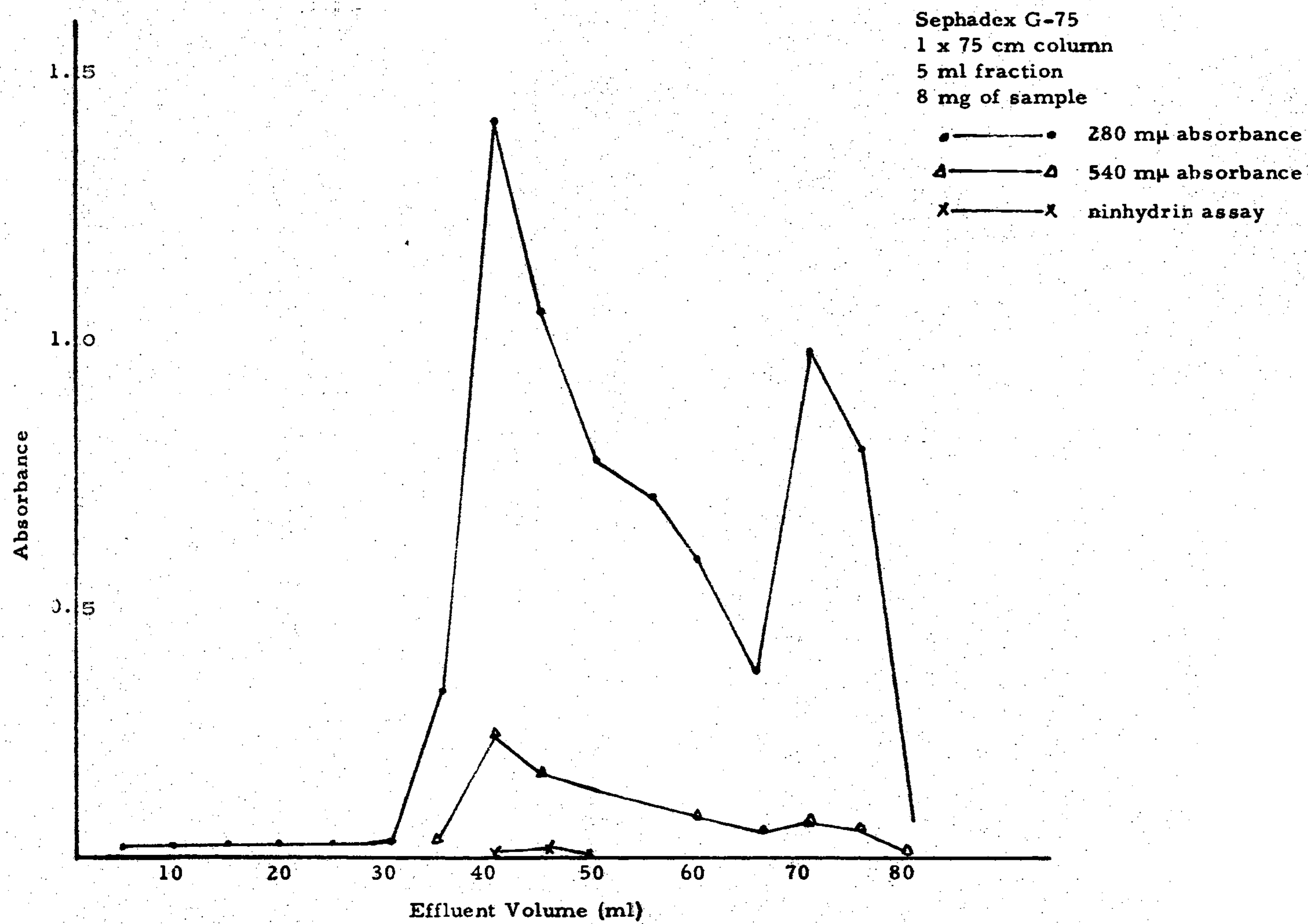


Figure 3. Gel Filtration Chromatography of Gossypurpurin-Glycoprotein Complex

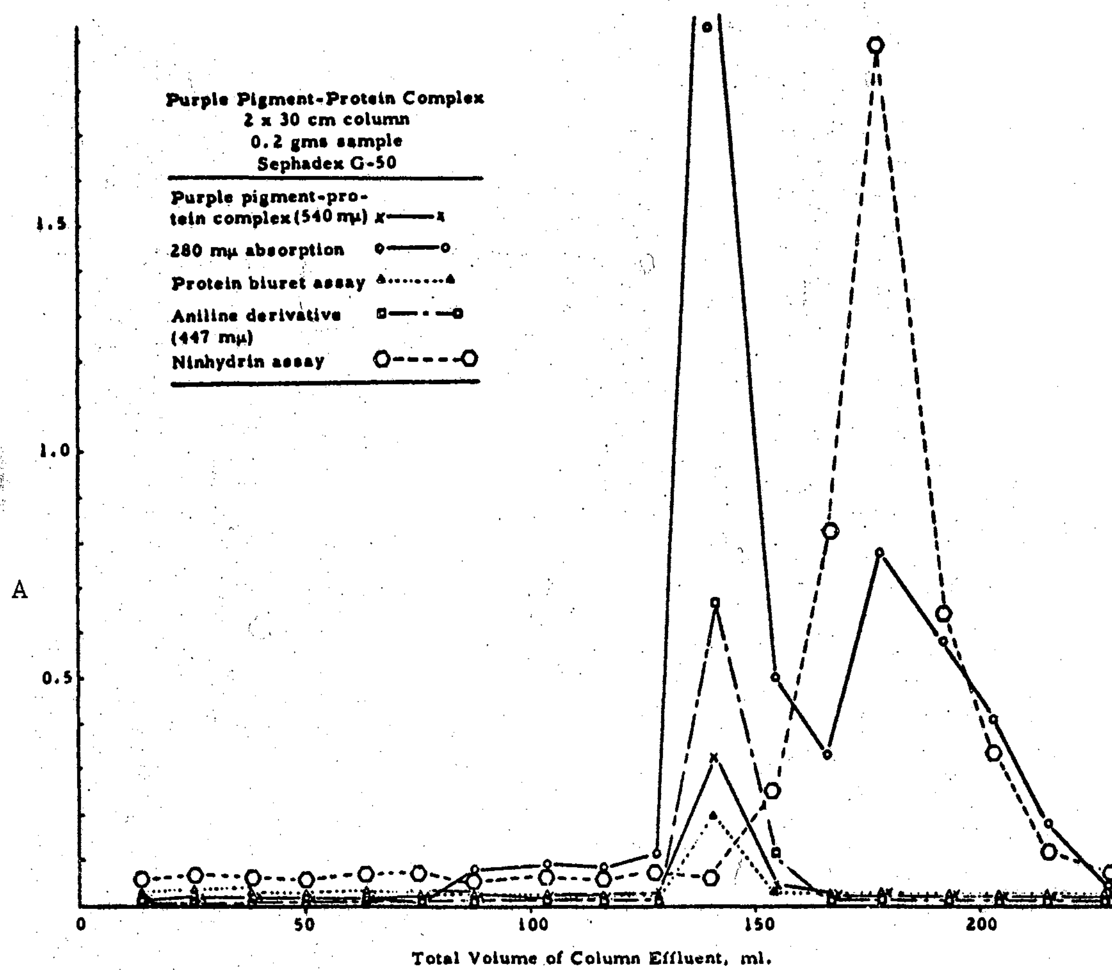


Figure 4. Sephadex Chromatography of Gossypurpurin-Glycoprotein Complex (crude isolate).

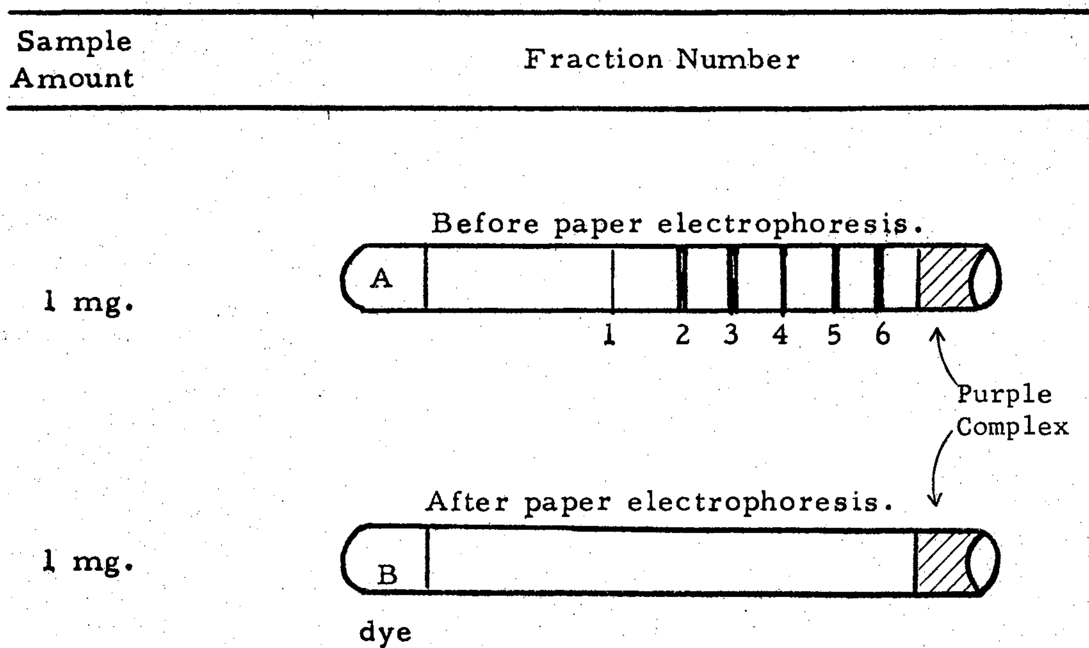


Figure 5. Polyacrylamide Gel Electrophoretic Separation of Blue Protein Complex

pigment protein isolate before paper electrophoresis. There was only one band visible, "B", after paper electrophoresis purification. The purple-protein was assumed to be pure at this point. The purple-protein complex was found to be contaminated with other proteins which cannot be separated with a Sephadex column. These were removed by the paper electrophoresis step.

Molecular Weight and Physical Properties of Purified Complexes

Examination of the purple-protein complex in the Beckman Model E Analytical Ultracentrifuge showed sedimentation rapidly at low speed. An S value of 470 (by comparison to urease, M. W. 500,000, S value = 19) for this complex gave a molecular weight of approximately 60,000,000. This type of "banding" for the complex as shown in Figure 6 is usually conducted with density gradient studies. In this case an indication of a molecular weight range for the complex was found. Earlier work indicated a polydesperse nature for the purple complex. Although this is not established, molecules of gossypurpurin-protein may be associated through hydrogen bonding.

The yellow-protein complex shows a single peak in the ultracentrifuge, Figure 7, no protein contaminants by electrophoretic methods and a single band from the Sephadex G-75 gel filtration column. The complex was assumed to be a single molecular species at this point. The determination of molecular weight by sedimentation velocity gave an S value of 6 which is equivalent to a molecular weight of 100,000. A better estimate of molecular weight was obtained by rechromatography of the pure yellow complex on Sephadex G-75. A plot of log molecular weight versus

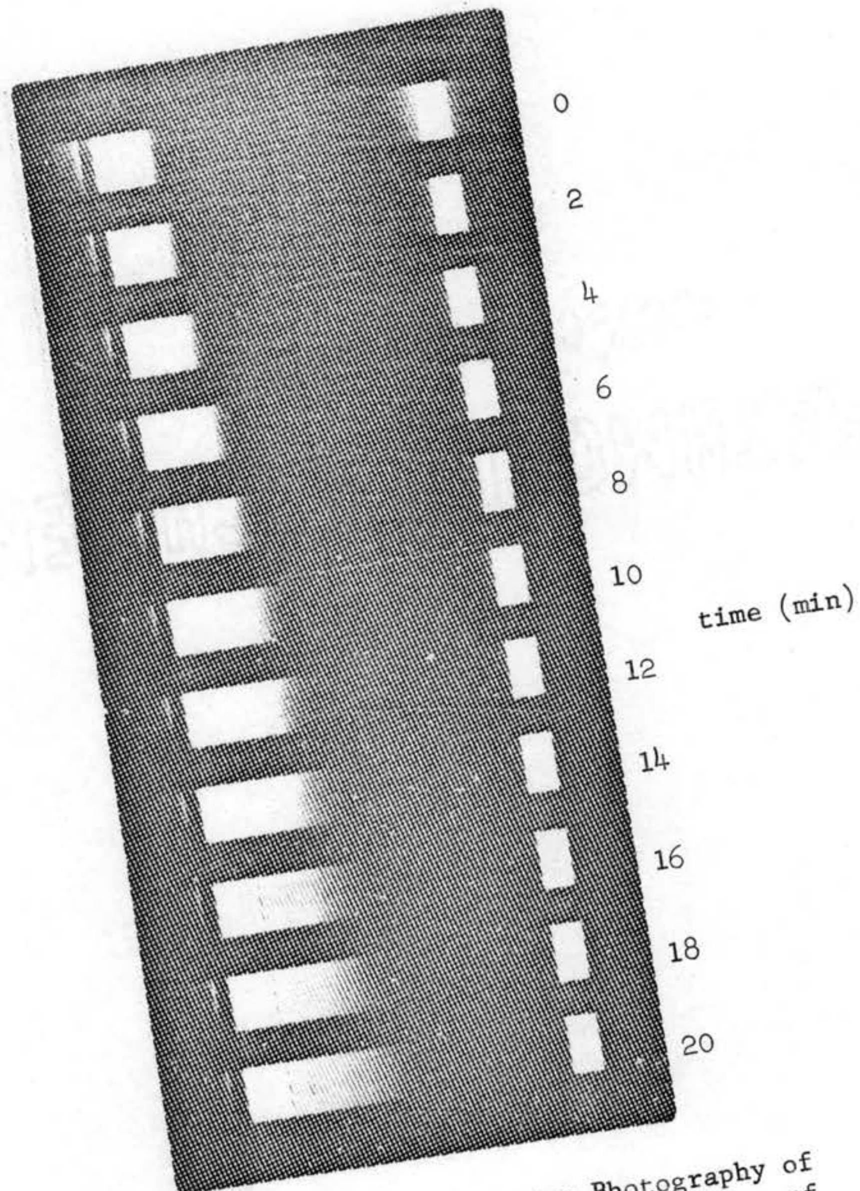


Figure 6. Ultraviolet Absorption Photography of Successive Stages in the Banding of Gossypurpurin-glycoprotein (0.50 g/dl) in Water at 20°C. The Exposures Were Taken at Intervals of 20 Minutes at 7,928 rpm in a Beckman Model E Analytical Ultracentrifuge.

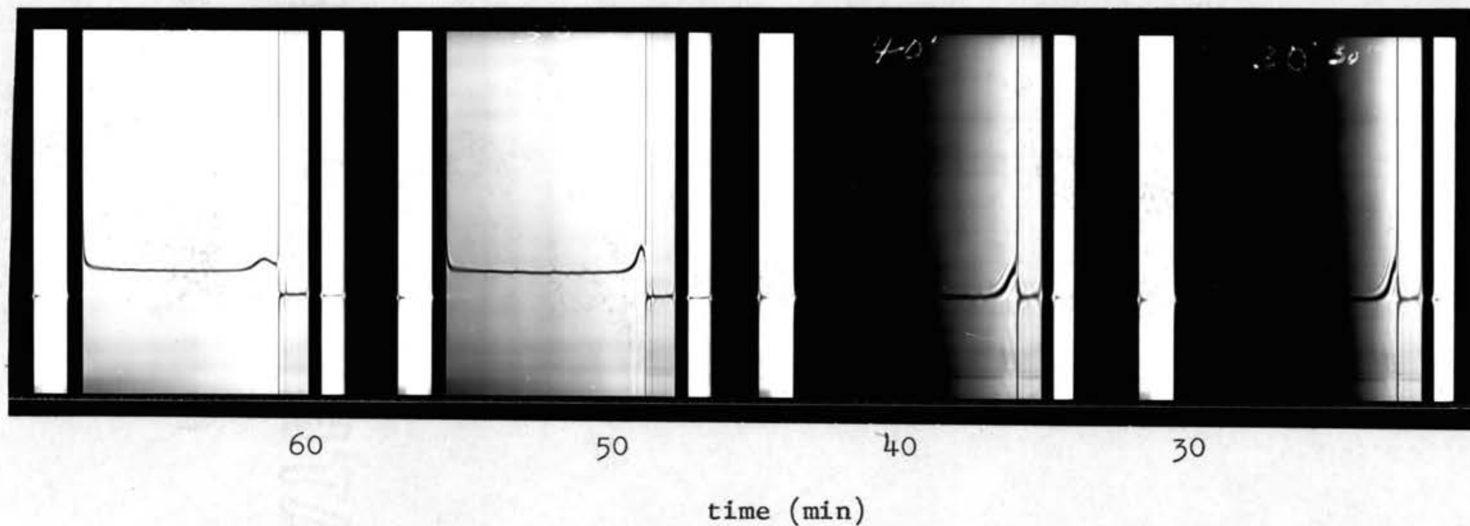


Figure 7. Sedimentation Pattern of Gossypol-Glycoprotein (0.50 g/dl) in Water in An-D Cell Temperature 20°C, 57,980 rpm. Direction of Sedimentation from Right to Left. The Photographs were Taken 30, 40, 50, and 60 minutes After Reaching Maximum Speed.

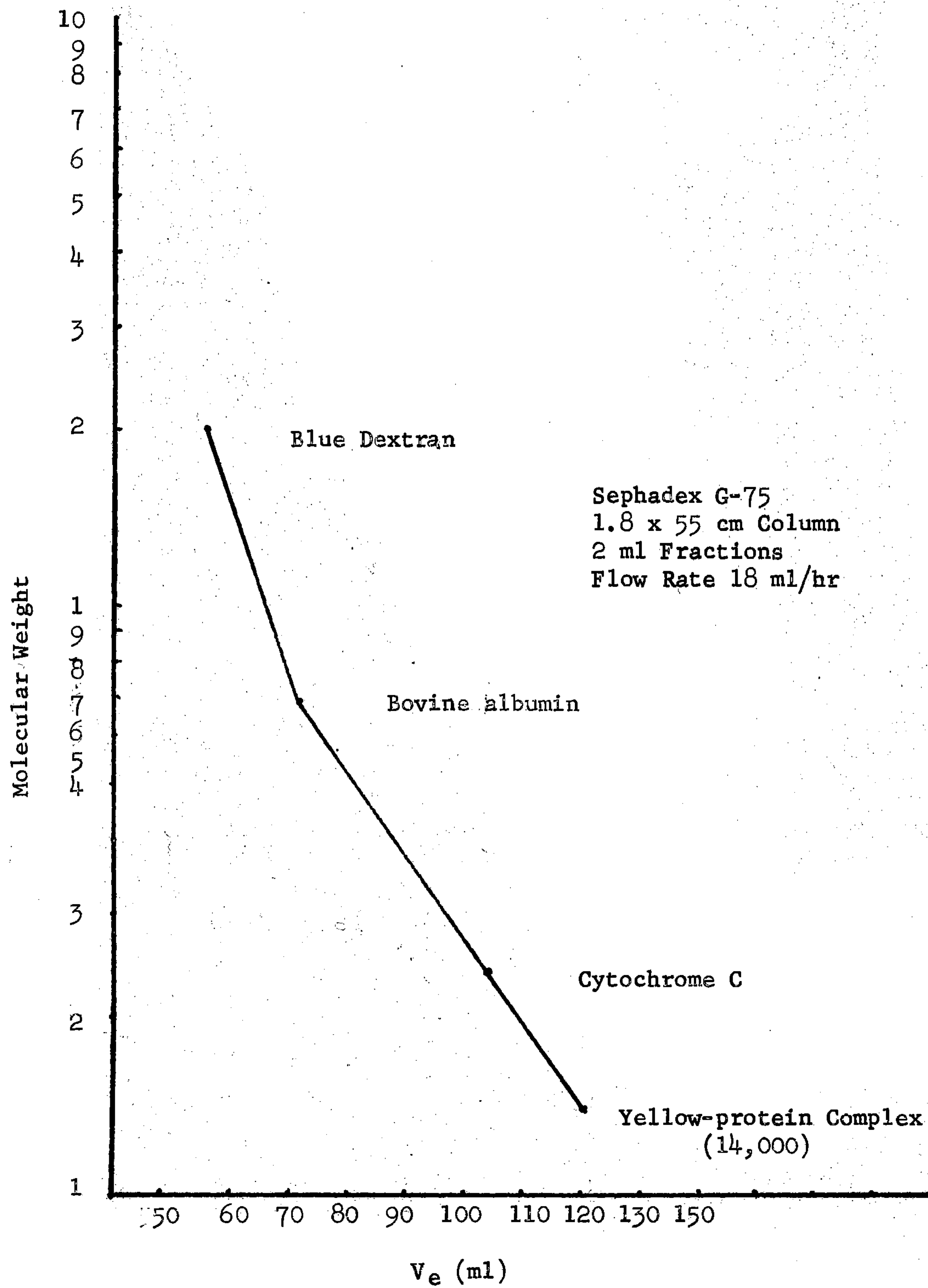


Figure 8. Determination of the Molecular Weight of the Gossypol Complex with Sephadex G-75

elution volume, Figure 8, shows a molecular weight of 14,000 by comparison to bovine albumin and cytochrome C. It is established that glycoproteins in solutions are less dense with a larger volume than normal proteins. These "hydrated" proteins do not follow the normal patterns of behavior observed for proteins in the two types of molecular weight determination. More work is needed here to establish the relation of the two complexes with each other. Treatment of the pure purple complex with 10% N, N-dimethylformamide gave a subunit with a yellow color plus a purple complex. Treatment of the purple complex with 100% N, N-dimethylforamide releases the pigment and the colorless glycoprotein precipitate shows a migration in disc electrophoresis. Subunit structure of the purple complex remains to be established but the yellow-complex appears to be one unit in the purple complex.

Table I summarizes the physical properties of the two pure complexes. Both are soluble in water and insoluble in acetone-5% water. The solubility in 10% trichloroacetic acid (TCA) and a 50:50 mixture of TCA, 10% and ethanol indicates a higher protein content for the yellow complex and a higher carbohydrate content for the purple complex. Molecular weight data and electrophoretic migration is also shown in this table.

Chemical Properties of Purified Complexes

The pigments of both pigment-protein complexes are dissociated by dioxane. The purple-protein complex was completely dissociated by dimethylformamide (100%) but only partial dissociation of the yellow-protein was observed. Table II indictes that there are two types of bonding, hydrogen-bonded pigment and Shiffs base, present in the yellow-

TABLE I

PHYSICAL PROPERTIES OF PIGMENT COMPLEXES

Item	Gossypurpurin-Glycoprotein (Purple)	Gossypol-Glycoprotein (Yellow)
<u>Solubility</u>		
Water	Soluble	Soluble
TCA* (10%)	Partially Soluble	Insoluble
TCA (10%) + Ethanol (50/50)	Insoluble	Insoluble
Acetone, 5% Water	Insoluble	Insoluble
<u>Electrophoretic Mobility</u>		
Zone (Disc) Electrophoresis	0.0 cm	0.5 cm
Paper Electrophoresis	0.0 cm	2.0 cm
<u>Molecular Weight</u>		
Gel Filtration	>200,000	14,000
Sedimentation Velocity	60,000,000	100,000

* TCA = trichloroacetic acid

protein complex, but in purple-protein complex only hydrogen-bonded pigment is indicated. A positive Molisch and Biuret test shows carbohydrates associated with protein. The negative, test tube, ninhydrin test shows the isolates are free of amino acids and peptides.

When the purple-protein complex was treated with 1,4-dioxane, a deep blue colored dioxane solution was obtained. The dissociation was rapid but a longer time was needed (6 hrs) to completely remove all of the pigment from the complex. The absorption spectrum of the purple compound in chloroform solution showed maxima at 566 mμ, at 530 mμ and at 370 mμ, as illustrated in Figure 9. This spectrum was similar to the absorption spectrum of synthetic gossypurpurin. The absorption spectrum of the aniline derivative of this purple compound in chloroform solution showed maxima at 440 mμ. Figure 10 compares the absorption spectrum of the aniline derivative of the blue pigment with dianilinogossypol. The spectra are similar.

Gossypurpurin has been reported to be very unstable in all organic solvents (principally polar) in which it is soluble. It is reported to decompose to give a yellow pigment which is identical with pure gossypol. This work was repeated and the absorption spectrum shows a maxima at 367 - 370 mμ (Figure 11). The absorption spectrum of the yellow compound had a shoulder at 370 mμ; this may be due to the compound being oxidized since the sample was stored over 6 months.

Properties of the Pigments from the Complexes

A green precipitate was obtained when the purple compound was treated with glacial acetic acid, in contrast to the yellow precipitate obtained with gossypol. Concentrated sulfuric acid reacted with the

TABLE II

CHEMICAL PROPERTIES OF PIGMENT COMPLEXES

Item	Gossypurpurin-Glycoprotein (Purple)	Gossypol-Glycoprotein (Yellow)
Color Tests		
Molish	Positive	Positive
Biuret	Positive	Positive
Ninhydrin	Negative	Negative
Pigment Dissociation		
Dioxane	Complete	Complete
Dimethylformamide (100%)	Complete	Partial
Dimethylformamide (10% in water)	Partial	--
Color (normal)	Purple (Blue-red)	Yellow
Strong Base	Yellow	Brown
Strong Acid	Red	Yellow

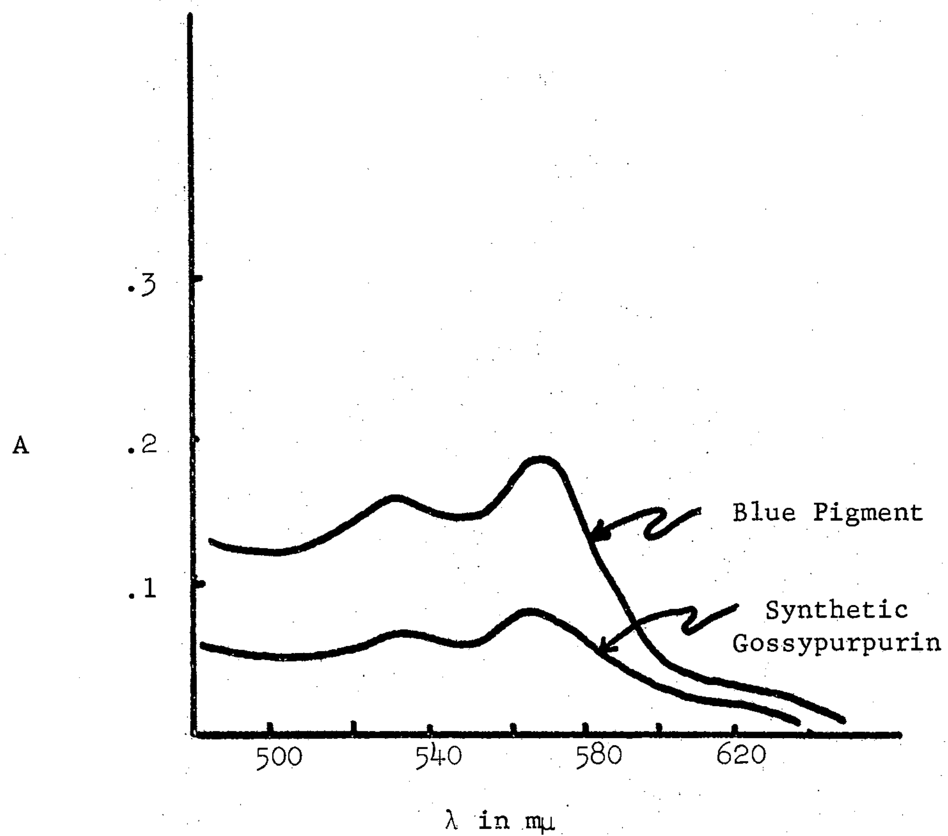


Figure 9. The Visible Spectrum of the Blue Pigment and the Synthetic Gossypurpurin

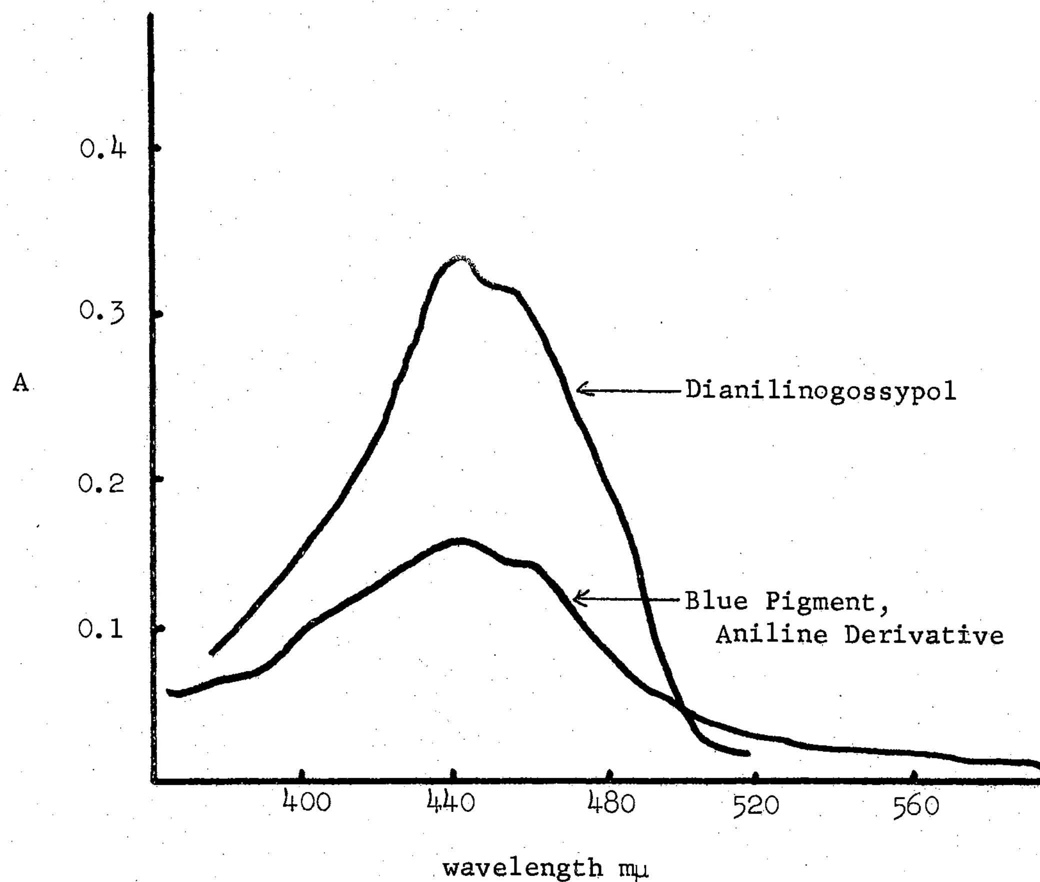


Figure 10. The Visible Spectrum of the Aniline Derivative of the Blue Pigment and Dianilinogossypol

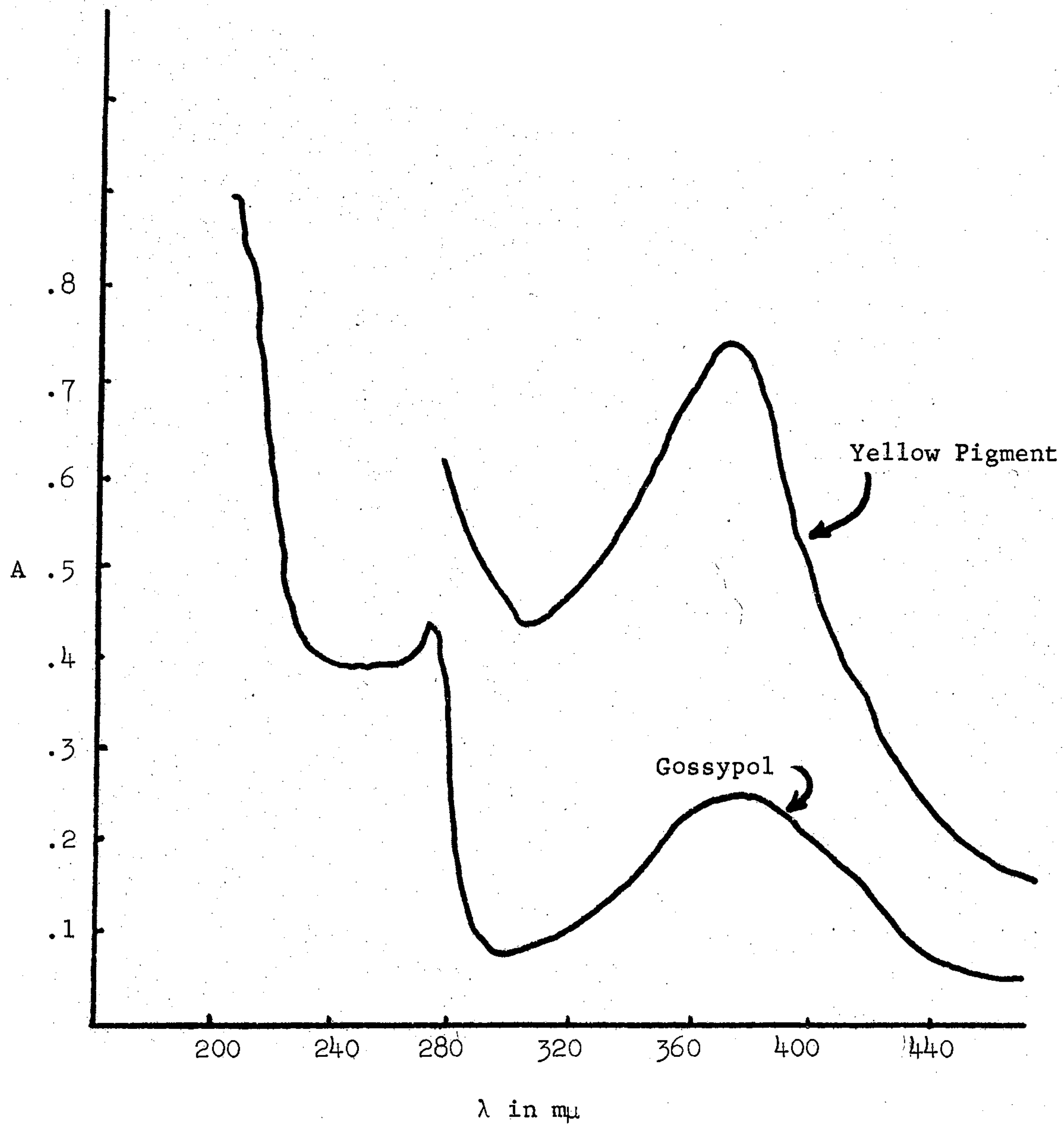


Figure 11. The Visible Spectrum of the Yellow-Pigment and Gossypol

purple compound to produce a yellow-green color which becomes orange after ten minutes, in contrast to the dark-red color developed immediately with gossypol. The reported chemical properties of gossypurpurin are similar to the "free" blue pigment.

The qualitative test of the dissociated compound of the yellow-protein complex with a chloroform solution of antimony trichloride formed a brilliant red color. Concentrated sulfuric acid with the same pigment formed a red solution. These results were very similar to the qualitative tests for gossypol.

Composition of Purified Complexes

Typical chromatograms of the amino acids of the two pigment-protein complex hydrolysates were obtained from the amino acid analyzer. Both hydrolysates showed normal peaks for the amino acids by this procedure. Glutamic acid, aspartic acid, leucine and glycine were the major compounds present. Table III and IV show the amino acid composition of the two complexes as calculated from the amino acid analyzer data. There is some destruction of certain amino acids in a 6 N HCl hydrolysis but aspartic and glutamic acids are fairly stable under these conditions (43). These results indicate that the amino acid compositions are similar in some respects.

Table V shows the carbohydrate present in the two complexes obtained from paper chromatography. The yellow-protein complex appears to contain more sugar than the purple-protein complex from gas chromatography (Figures 12, 13) but this is not indicated by solubility characteristics. Some hexosamine may have been destroyed by the longer period (20 hrs) of hydrolysis in 2 N HCl at 105°C (43).

TABLE III

AMINO ACID ANALYSIS OF GLYCOPROTEIN-GOSSYPURPURIN COMPLEX OF COTTONSEED PIGMENT GLAND MATERIAL

Amino Acid	μ moles/mg Glycoprotein-Gossypurpurin	mg of Amino Acid Residue per g Glycoprotein Gossypurpurin
Methionine Sulfoxide	Trace	Trace
Aspartic Acid	0.054	7.182
Threonine	0.034	4.046
Serine	0.040	4.200
Proline	0.034	3.910
Glutamic Acid	0.080	11.760
Glycine	0.040	3.000
Alanine	0.047	4.183
Valine	0.020	2.340
Half-Cystine	0.007	0.840
Isoleucine	0.034	4.454
Leucine	0.054	7.074
Glucosamine	Trace	Trace
Tyrosine	0.013	2.354
Phenylalanine	0.013	2.145

TABLE IV

AMINO ACID ANALYSIS OF GLYCOPROTEIN-GOSSYPOL COMPLEX FROM COTTONSEED PIGMENT GLAND MATERIAL

Amino Acid	μ moles/mg Complex	mg of Amino Acid Residue per g Glycoprotein-Gossypol Complex
Methionine Sulfoxides	Trace	Trace
Unknown*	0.094	12.314
Aspartic Acid	0.080	10.640
Threonine	0.027	3.213
Serine	0.027	2.835
Proline	0.020	2.300
Glutamic Acid	0.067	9.849
Glycine	0.080	6.000
Alanine	0.054	4.806
Valine	0.020	2.340
Half-Cystine	Trace	Trace
Methionine**	0.0067	0.998
Isoleucine	0.014	1.834
Leucine	0.020	2.620
Tyrosine	0.020	3.620
Phenylalanine	0.020	3.300

* Identification indicates hydroxyproline, β hydroxy aspartic, glucosaminic acid, or O-phospho-5-hydrosy lysine. (calculation on the basis of hydroxyproline molecular weight).

** Not found in purple complex.

TABLE V

PAPER CHROMATOGRAPHIC EXAMINATION OF CARBOHYDRATES IN BLUE AND YELLOW-PROTEIN COMPLEXES

Purple-Protein Complex	R_f Value	
Sugar	Solvent A*	Solvent B*
Arabinose	0.29	0.31
Hexosamine	0.19	0.20
Disaccharide	0.14	0.10
Yellow-Protein Complex		
Arabinose	0.29	0.31
Glucosamine	0.17	0.17
Mannosamine	0.18	0.19
Disaccharide	--	--

* Solvent A: pyridine : butanol : water (3:2:1)

* Solvent B: butanol : acetic acid : water (4:1:5)

Tracings of typical gas chromatographic separations of trimethylsilyl derivatives from 2 N HCl hydrolysates of both pure complexes are shown in Figures 12 and 13. This data was used mainly for identification.

A summary of the compositon of the two pure complexes is given in Table VI.

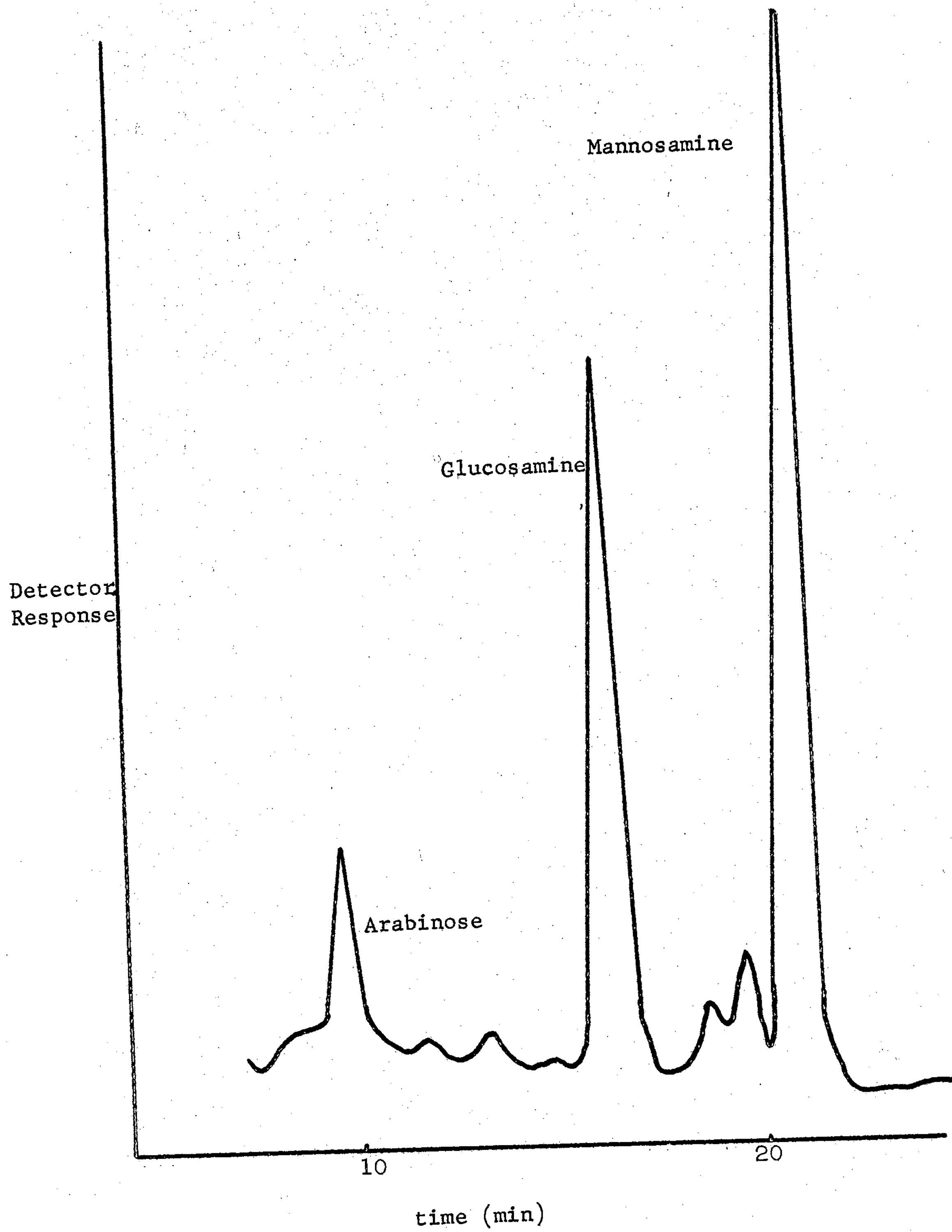


Figure 12. Gas Chromatography of TMS Derivatives of Gossypol-Glycoprotein Complex

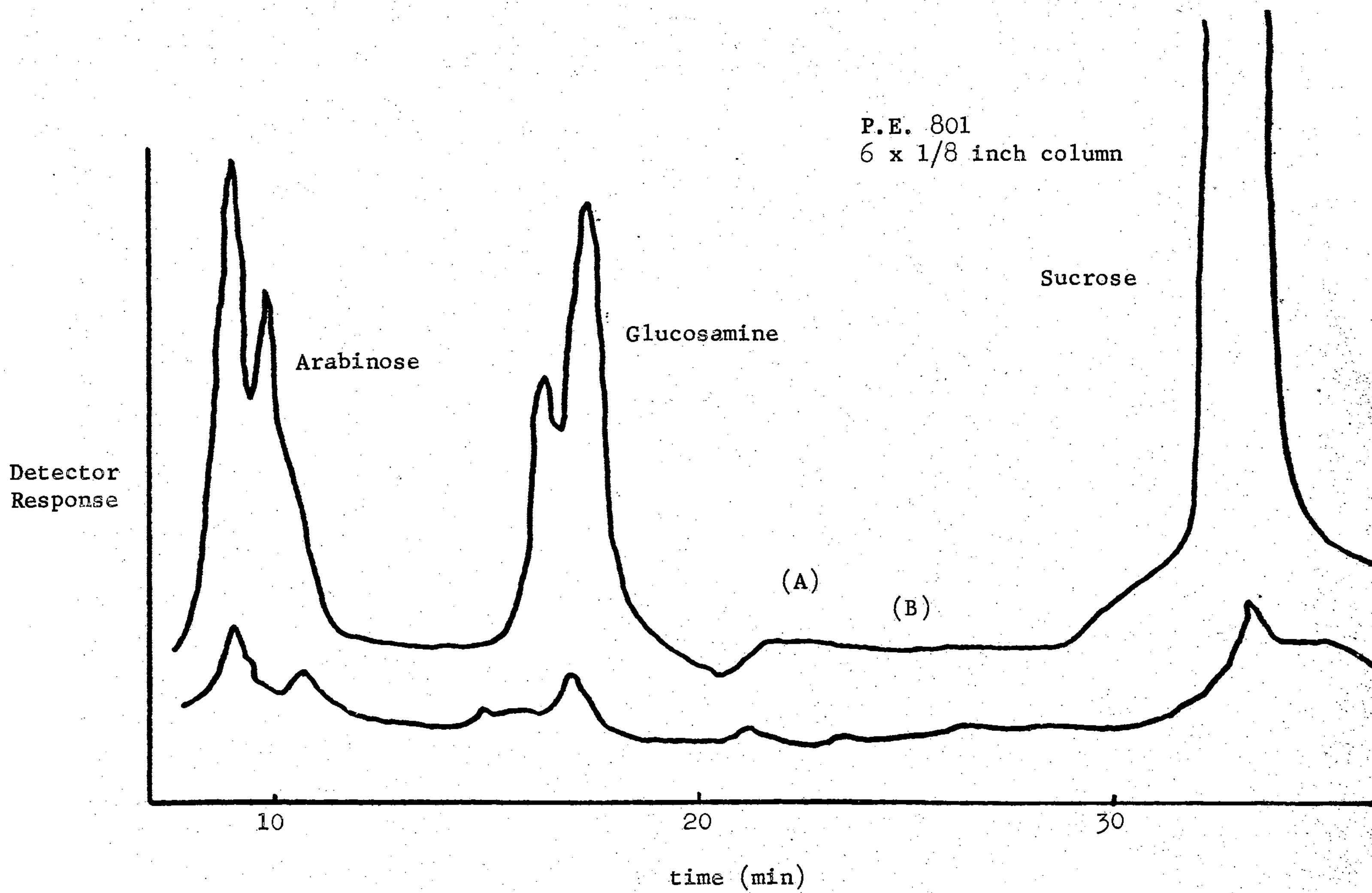


Figure 13. Gas Chromatography of TMS Derivatives (A) Purple-Protein and (B) Reference Compounds

TABLE VI

SUMMARY OF COMPLEX COMPOSITION

Item	Gossypurpurin-Glycoprotein	Gossypol-Glycoprotein
Pigments	Gossypurpurin	Gossypol
Carbohydrates	Arabinose Glucosamine A Disaccharide	Arabinose Glucosamine Mannosamine
Protein		
(ℓ) Acidic and Neutral Amino Acids	5.75 gm/100 gm	6.67 gm/100 gm
Ammonia	0.67 gm/100 gm	0.70 gm/100 gm

CHAPTER V

SUMMARY

Two purified pigment-glycoprotein complexes have been isolated from cottonseed pigment gland material. The pigment gland material was prepared from cold hexane extracted cottonseed meal therefore these compounds are naturally occurring constituents of the cottonseed pigment gland. A gossypol-glycoprotein complex shows a molecular weight of about 14,000 by gel filtration. This compound appears to be associated with a high molecular weight gossypurpurin-glycoprotein complex. The molecular weight of the purple gossypurpurin glycoprotein has not been determined but it is over 200,000 by gel filtration experiments.

Data from the amino acid analysis of the two pure pigment-glycoprotein complexes show a similar composition. A high aspartic and glutamic acid content was observed. Hydroxyproline was indicated as a constituent. The pure yellow complex contained the pigment gossypol while the blue complex contained gossypurpurin. Both complexes contain arabinose and glucosamine. Mannosamine was found in the yellow complex. The two complexes appear to be associated by hydrogen bonding during isolation.

The usual difficulties in obtaining quantitative carbohydrate and amino acid composition data for glycoproteins were encountered in this research. It is believed that this is the first report of the characterization of naturally occurring pigment-protein complexes.

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VITA

Chung-Min Chang

Candidate for the Degree of

Master of Science

Thesis: CHARACTERIZATION OF TWO NATURALLY OCCURRING GLYCOPROTEIN-PIGMENT COMPLEXES FROM COTTONSEED PIGMENT GLANDS

Major Field: Chemistry

Biographical:

Personal Data: Born in Shantung, China, February 7, 1919.

Education: Attended the National Northwestern Agricultural College, Shansi, China, and received the Bachelor of Science degree in 1946 with a major in Agricultural Chemistry; completed requirements for the Master of Science degree in May, 1967.

Professional Experience: Chemist at Taiwan Industrial Research and Development Institute, Taipei, Taiwan, from January 10, 1949 to June 30, 1953. Chemist at Taiwan Agricultural Chemical Works, Kaohsiung, Taiwan; worked at vegetable oil extraction and refining plant from July 1, 1953 to July 31, 1964. Graduate research assistant, Department of Biochemistry, Oklahoma State University.