Phytochemistry, 1970, Vol. 9, pp. 2271 to 2280. Pergamon Press. Printed in England.

ISOLATION AND PURIFICATION OF CYTOCHROME c FROM SOME SPECIES OF HIGHER PLANTS

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(*Received* 13 *March* 1970)

Abstract—Cytochrome c was isolated and purified from dark-grown seedlings of mung bean, sunflower, sesame, castor oil and buckwheat. The major steps of the method employed were adsorption on Amberlite CG-50, concentration on Sephadex CM-50, molecular sieve chromatography on Bio-gel P-30 and Sephadex G-50, and ion-exchange chromatography on CM-52 Cellulose. Unlike previous methods for the purification of cytochrome c this method did not involve the use of organic solvents during the initial extraction, or fractional precipitation of the protein with ammonium sulphate. The Ampholine iso-electric focusing technique was also investigated as a possible method in the purification of cytochrome c from plant sources. The final yield of purified cytochrome c from the various species was in the range 0·2–0·45 mg/kg dry seeds.

INTRODUCTION

THE AMINO acid sequences of cytochromes c from numerous species have been determined and the information obtained has been used for phylogenetic comparisons.[1] The organisms investigated have included many classes of vertebrates, several invertebrates, bacteria, protozoa, fungi and yeasts.[2] In contrast this protein has only been isolated and purified from a relatively few species of higher plants such as wheat,[3] soybean[4] and rice,[5] and until recently the only information available on the primary structure of a higher plant cytochrome c was that for wheat germ.[3] Recently an investigation into the phylogeny of plants using the primary structure of their cytochrome c was instigated in this laboratory,[6] and the amino acid sequences of cytochrome c from mung bean (*Phaseolus aureus* L. = *Vigna radiata*),[7] sunflower (*Helianthus annus* L.),[6] castor-oil (*Ricinus communis* L.) and sesame (*Sesamum indicum* L.) (unpublished data) have been determined. Several other species of higher plants from widely separated taxa are currently being investigated.

In this communication we wish to describe the methods we have employed in the isolation and purification of cytochrome c from mung bean, sunflower, castor-oil, sesame and buckwheat (*Fagopyrum esculentum* Moench.).

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RESULTS AND DISCUSSION

The method used for the isolation and purification of cytochrome c is given in Fig. 1. It is a combination and modification of methods previously used with other materials.[8] However, the widely employed method of initially extracting the tissues with organic solvents[4,9,10] was omitted as it has been shown[8,11] that such treatment may lead to denaturation. Fractional precipitation with ammonium sulphate[4,5,9] was also omitted from the present procedure since Flatmark and Vesterberg[12] have shown that there is a danger of cytochrome c being deamidated in solutions of ammonium sulphate.

The initial extraction was carried out in the presence of ascorbic acid and EDTA at pH 4·6, in order to maintain cytochrome c in the reduced form and also to inhibit the oxidative polymerization of phenols. Furthermore, at pH 4·6, many other proteins, particularly the storage globulins of mung bean, were insoluble. Preliminary investigations at pH values greater or less than 4·6 gave extracts which filtered poorly and/or yielded cloudy filtrates.

Immediately after the initial filtration the pH of the filtrate was adjusted to 8 with 1 M Tris, since modifications of the protein are known to occur[8] at the lower pH. This adjustment of the pH necessitated a further filtration step since it led to the precipitation of alkali-insoluble proteins, calcium–magnesium phytate and other materials. A particularly copious and gelatinous precipitate was always obtained at this stage during the extraction of the seedlings of those species which have oil or fat as their major feed reserve, i.e. castor, sunflower, sesame.

It was found possible to adsorb the cytochrome c from the filtrate at pH 8 on columns of Amberlite CG-50 in the NH_4^+ (pH 10) form, as there was a sufficiently low cation concentration in the extract and Tris was not adsorbed on the CG-50. Although the cytochrome was adsorbed by the upper part of the resin column it was only rarely seen as a distinct red band (sunflower). The resin was washed after removal from the column by repeated stirring and decantation with several vol. of distilled water since this washing procedure removed considerable quantities of coloured impurities.

Preliminary experiments showed that the most efficient elution of the adsorbed cytochrome c was achieved by stirring the resin for 1 hr at room temp. with 0·5 N NaCl, whilst the pH was maintained at $8 \pm 0{\cdot}1$ by the addition of 2 N NaOH. It was important to maintain this pH as cytochrome c became very strongly bound to the resin below pH 6·5.

Following reduction with ascorbate, the eluted cytochrome c was dialysed against 0·05 M NaH_2PO_4–NaOH buffer, pH 8·0, for 18 hr at 2°. Under these conditions the losses of cytochrome c were very small; however, preliminary experiments showed considerable losses of cytochrome c if the dialysis was carried out against distilled water. With the majority of the species investigated it was necessary to remove the proteins precipitated during dialysis by centrifugation.

The reduced protein was adsorbed and concentrated from the dialysate by passage through columns of CM-50 Sephadex equilibrated with 0·05 M buffer, pH 8·0. The cytochrome c was strongly adsorbed as a deep red band in the top 2–3 cm of the column and a considerable number of impurities which absorbed at 280 nm were not retained by the column; these were washed down the column after adsorption of the cytochrome c by passing 3–4 l. of 0·05 M buffer, pH 8. It was found necessary to add a little ascorbate to this washing buffer to main-

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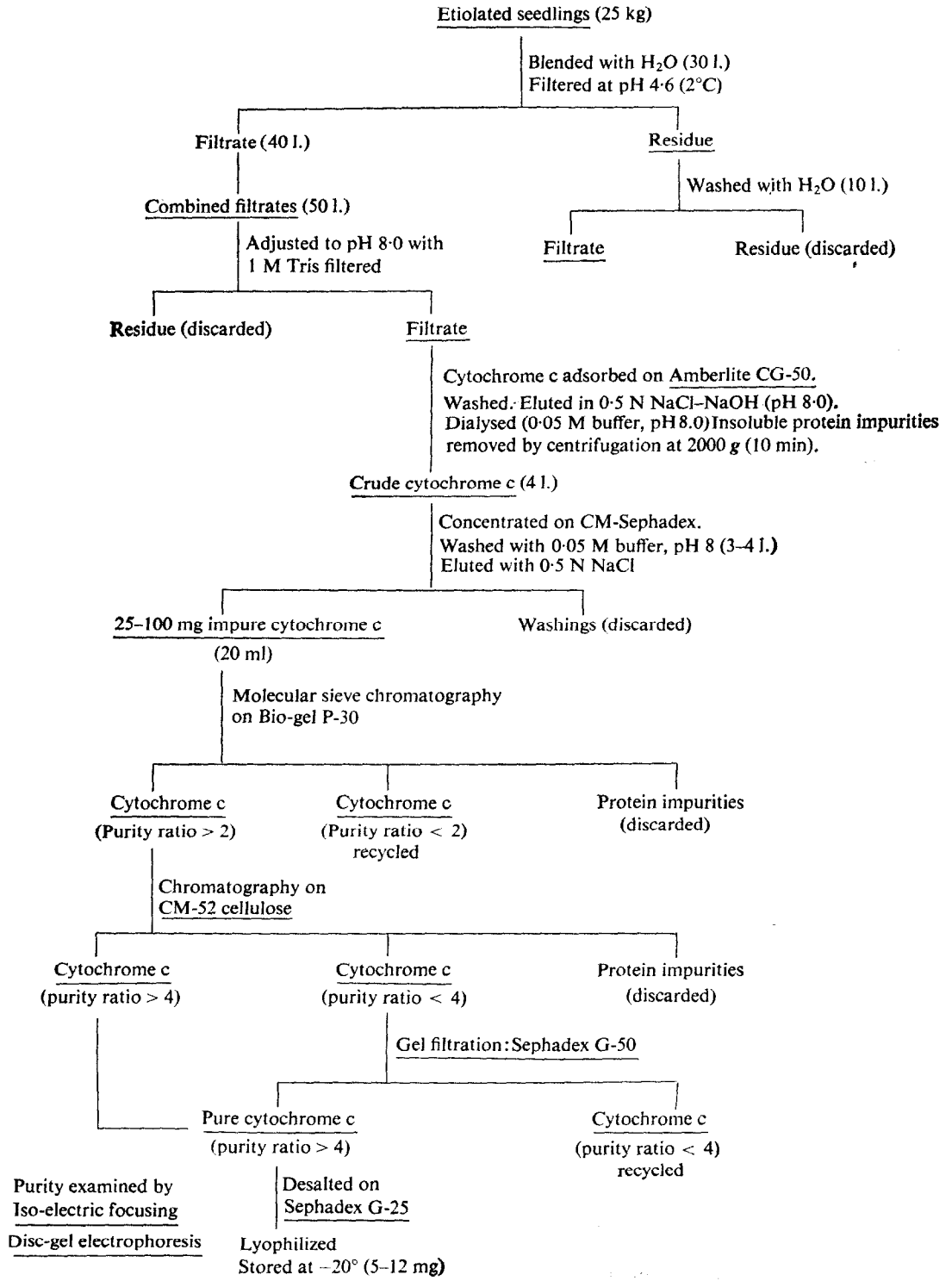

FIG. 1. FLOW SHEET FOR ISOLATION AND PURIFICATION OF CYTOCHROMES C FROM SOME SPECIES OF HIGHER PLANTS.

tain the cytochrome in the reduced form. In sunflower and sesame large amounts of coloured impurities which had a higher affinity for the CM-Sephadex than the reduced cytochrome c were left adsorbed to the upper part of the column after the cytochrome had migrated downwards during the washing procedure. These impurities were removed by pipetting off the upper 3–4 cm of the column prior to the elution of the cytochrome in 0·5 N NaCl.

After dialysis against 0·05 M buffer, pH 8, the vol. (300 ml) of the eluted cytochrome c was reduced by adsorption and elution from a further small column of CM-50 Sephadex. The amount of impure cytochrome c obtained in this concentrated (20 ml) solution varied from 25 to 100 mg depending on the species under investigation, and the purity ratio E410 nm/E280 nm observed at this stage was very variable (Table 1).

Considerable purification was then achieved for cytochrome c from most species by molecular sieve chromatography on Bio-gel P-30. This method was particularly useful for the purification of cytochrome c from castor, buckwheat and sesame. In preparations from

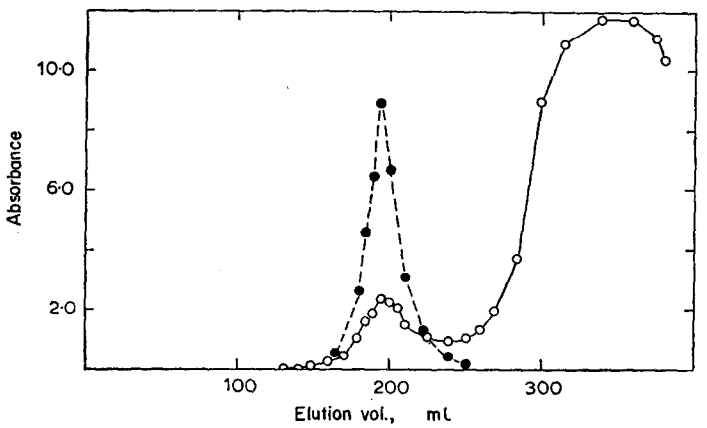

FIG. 2. ELUTION PROFILE OF PARTIALLY PURIFIED CYTOCHROME C FROM SESAME DURING MOLECULAR SIEVE CHROMATOGRAPHY ON A COLUMN OF BIO-GEL P-30 (3·1 × 80 cm).

Elution was carried out with 0·05 M NaH_2PO_4–NaOH buffer, pH 8·0. Absorbance at 280 nm (—○—), absorbance at 410 nm (—●—).

sesame seedlings the major impurity removed by gel filtration on Bio-gel P-30 (Fig. 2) was a yellow compound with a mol. wt. smaller than that of cytochrome c. In contrast, the preparations from buckwheat yielded a grey-coloured impurity of high mol. wt., together with a pale-yellow compound of smaller mol. wt. than cytochrome c. In all species investigated it was found necessary to subject those fractions containing relatively impure cytochrome c to several passages through the gel in order to obtain sufficient material of higher purity (Table 1). Preliminary experiments revealed that it was important to carry out all the gel filtration steps (Bio-gel P-30, and Sephadex G-50) with 0·05 M buffer, pH 8·0, as considerable losses (25–35%) of cytochrome c occurred when distilled water alone was used.

Ion-Exchange Column Chromatography on CM-52 Cellulose

For the complete purification of cytochrome c from sunflower and buckwheat an additional step involving ion-exchange column chromatography on CM-52 Cellulose was found to be necessary. Figure 3 shows a chromatogram of partially purified cytochrome c from buckwheat on a small column of CM-52 Cellulose. Three peaks of cytochrome c were eluted

TABLE 1. COMPARATIVE YIELDS AND PURITIES OF CYTOCHROME C AT THE DIFFERENT STEPS OF PURIFICATION FROM 100 kg OF DRY SEEDS

Step	Mung bean		Sunflower		Castor		Sesame		Buckwheat	
	mg	pI	mg	pI	mg	pI	mg	pI	mg	pI
Crude extract after Amberlite CG-50 (16–20 l.)	160–200	<0·01	250–320	<0·01	600–800	0·3	250–350	<0·01	160–280	0·1
CM-50 Sephadex (100–300 ml)	140–170	0·28–0·36	200–250	0·05	250–360	1·2–1·5	100–140	0·05	130–160	0·9–1·3
Bio-gel P-30 (recycled several times)	55	2·3	120	1·8	180	2·6	48	3·1	60	2·2
CM-52 Cellulose	—		42	2·9	—		—		38	4·3
Sephadex G-50 (recycled several times)	35	4·0	30	3·9	52	4·6–4·7	26	4·2	32	4·4
Final yield after desalting with Sephadex G-25	30	4·0	24	3·9	45	4·7	20	4·2	28	4·4

pI (purity index) = E410 nm/E280 nm.

by the increasing linear gradient of phosphate buffer employed. The cytochrome c in peaks II and III was found to be almost completely pure (purity index E410 nm/E280 nm = 4·3) whilst peak I contained other proteins. Although the cytochrome c was applied to the column in the reduced form, spectrophotometric examination of the eluted peaks revealed that peaks II and III were the reduced and oxidized forms of the protein respectively. This separation of the reduced and oxidized forms during column chromatography on CM-cellulose has been described by Kadenbach and Urban,[13] who routinely treated their preparations with ferricyanide prior to chromatography in order to obtain a single peak of oxidized cytochrome c. No attempt to oxidize the cytochrome c with ferricyanide was made during this procedure as preliminary experiments revealed that this step led to the formation of multiple forms of the protein. It is probable that the impure cytochrome c eluted in peak I was one of the deamidated forms described by Flatmark[14] and observed by many other workers during chromatography on cation-exchange resins.[8]

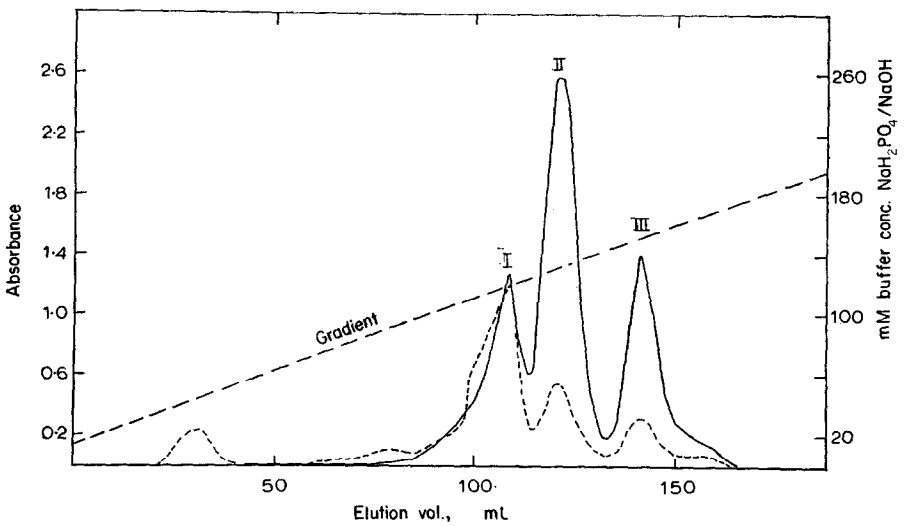

FIG. 3. PROFILE OF BUCKWHEAT CYTOCHROME C ELUTED FROM A COLUMN OF CM-52 CELLULOSE (1 × 10 cm) WITH A LINEAR GRADIENT OF NaH_2PO_4–NaOH BUFFER, pH 7·2.
(– – – –), Absorbance at 280 nm; (———), absorbance at 410 nm.

Iso-electric Fractionation of Cytochrome c by Ampholine Electrofocusing

During initial experiments on the purification of cytochrome c from mung bean the Ampholine iso-electric focusing technique was investigated as a possible step in the purification procedure.

It was not possible to electrofocus the impure cytochrome c preparations obtained after the Amberlite CG-50 and CM-50 Sephadex steps as these preparations contained relatively large amounts of other proteins which precipitated out in the column during the 6-day electrofocusing period leading to a disturbance of the iso-electric gradient and contamination of the cytochrome c fractions.

However, some purification of the cytochrome was achieved when preparations of higher purity index E410 nm/E280 nm = 2·3 were subjected to the electrofocusing. The purity

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index of the pooled cytochrome c fractions after the focusing was 2·8. Polyacrylamide disc electrophoresis of such preparations before and after electrofocusing revealed the presence of a major band of protein impurity which was completely removed by the iso-electric fractionation.

A further difficulty encountered in this method was the tendency of the cytochrome c to migrate into the cathode solution during prolonged focusing owing to the proximity of the pI for the protein and the cathode solution. The pI of cytochrome c is near the pH limit of the ampholyte carriers currently available (pH 10).

This technique was not used routinely in the purification procedure for the other species investigated as the alternative column chromatography and gel filtration steps outlined above proved to be quicker and entirely satisfactory.

Yields and Purities of Cytochrome c

Table 1 shows the comparative yields and purities of the cytochromes c at the different stages in the purification procedure. It can be seen that the final yield of purified cytochrome c (purity index > 3·9) from the various species varied from 0·2 to 0·45 mg/kg of dry seeds, which is similar to that reported for other higher plants by earlier workers.[4] Although absorption ratios were used as an index of purity, in addition samples of each purified cytochrome c were subjected to polyacrylamide gel disc electrophoresis at pH 4·3. In every case the final preparations yielded a single band of protein.

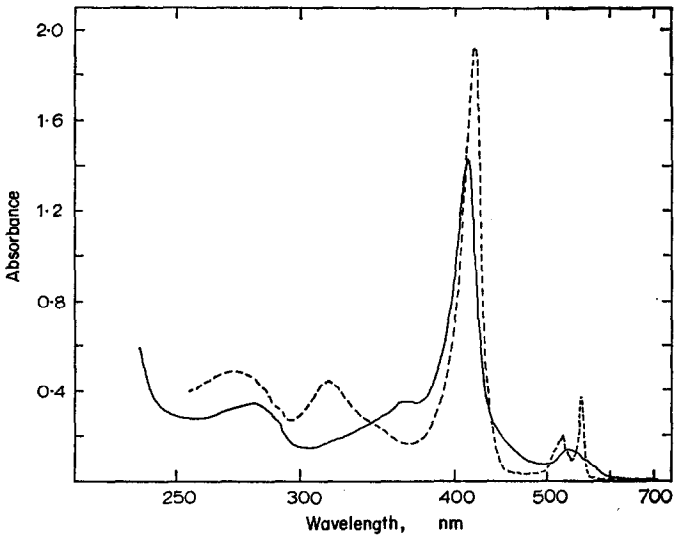

FIG. 4. ABSORPTION SPECTRUM OF CYTOCHROME C FROM BUCKWHEAT IN 0·05 M NaH_2PO_4–NaOH BUFFER, pH 8·0.

(———), Spectrum of oxidized cytochrome c; (– – – –), spectrum of cytochrome c reduced with a minute crystal of $Na_2S_2O_4$.

Figure 4 shows the absorption spectrum of oxidized cytochrome c from buckwheat in the range 250–600 nm and of the reduced cytochrome c in the range 300–600 nm. The absorption maxima were found at 279, 360, 410 and 530 nm in the oxidized form, and at 416, 521 and 550 nm in the reduced form. The absorption spectra of the cytochromes c obtained from the other plant species were essentially similar to that for buckwheat.

TABLE 2. COMPARISON OF SPECTRAL PROPERTIES OF CYTOCHROMES C FROM SOME SPECIES OF HIGHER PLANTS (R = REDUCED FORM; O = OXIDIZED FORM)

Spp.	550 nm (R)/ 280 nm (O)	416 nm (R)/ 410 nm (O)	410 nm (O)/ 280 nm (O)	416 nm (R)/ 550 nm (R)	Ref.
Horse heart	1·20	1·20	4·6	4·4	15
Wheat germ	1·16	1·20	3·7–3·8	4·4	3
Soybean	1·0	1·20	4·0	4·5	4
Rice	0·96	1·30	3·5–4·0	4·76–4·87	5
Castor	1·22	1·25	4·9	5·5	
Mung bean	0·88	1·18	4·0	5·3	
Sesame	1·0	1·25	4·2	5·2	
Sunflower	0·92	1·20	3·9	5·0	
Buckwheat	1·10	1·34	4·4	5·4	

Table 2 gives the ratios of the absorbancies of the various maxima in the oxidized and reduced states for the cytochromes c purified during this investigation. Also included for comparative purposes are the ratios previously obtained by other workers for horse heart,[15] wheat germ,[3] soybean[4] and rice.[5] It can be seen that the spectral ratios are quite similar. However, it is perhaps of interest to note that the γ reduced (410 nm)/α reduced (550 nm) ratios of the plant cytochromes c isolated during this investigation (5·0–5·5) were somewhat higher than those observed for this protein from animal sources (4·4), but not as high as those reported for bacteria[16] and algae[17] (5·0–7·1).

The cytochromes c isolated and purified during this investigation were all obtained from the metabolically active tissues of germinating seedlings. These tissues were chosen as suitable starting materials as the seeds are readily available, can be stored, and are biochemically conservative. In addition, as the seeds were germinated in the dark, the complication of separating the mitochondrial cytochrome c from the photosynthetic cytochromes was avoided.

The results of this investigation suggest that it may be possible to obtain sufficient cytochrome c for sequence purposes from seedlings of many species of higher plants.

EXPERIMENTAL

Plant materials. Seeds in quantities of 100 kg were obtained from the following sources: mung bean from Husscin & Co., Newcastle upon Tyne; sunflower from Tyneside Seed Stores, Gateshead; buckwheat from Wearside Seed Company Ltd., Sunderland; castor oil as a generous gift from the British Oil & Cake Mills, Hull, and sesame from Frank Fehr & Co. Ltd., London E.C.3. Samples of these seeds are being grown in the University Botanic Garden and voucher specimens will be deposited in the Herbarium.

The seeds were soaked for 24 hr in running tap water prior to being germinated in the dark under a constant fine spray of water at 23–25° for 3–9 days.

Chemicals. Chemicals and reagents used were of the highest purity readily available.

Extraction. The etiolated seedlings were grown and extracted in four to five separate batches of 20–25 kg fr. wt. Each batch was subdivided into smaller quantities of approximately 2 kg, each of which were blended for 3 min in a large (5 l.) Waring blender with 3 l. of pre-chilled (2–4°) distilled water to which was added 30 g ascorbic acid and 1 g EDTA. The pH of the homogenate was adjusted to pH 4·6 ± 0·1 with 2 N HCl.

The homogenate (50–60 l.) was then filtered through Whatman No. 6 filter papers in ten 28-cm Buchner funnels at 2–4°. A mild vacuum of *ca.* 400 mmHg was employed to facilitate this filtration process. The dry filter residues were washed by the addition of distilled water (1 l. to each funnel). The clear filtrate obtained was generally pale yellow in colour. This filtrate was then adjusted to pH 8·0 by the addition of 1 M

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tris-(hydroxymethyl) aminomethane and allowed to stand for 2 hr at 2–4°; the resulting precipitate was removed by filtration.

Adsorption of Cytochrome c on Amberlite CG-50

The filtrate at pH 8·0 was passed through three to four columns (6 cm × 28 cm) of Amberlite CG-50 (100–200 mesh) in the NH_4^+ form (pH 10), at a flow rate of 400–500 ml/hr. Approximately 15 l. of filtrate was passed through each individual column. The adsorption process was carried out at 2–4° temp.

After adsorption the resin was removed from the columns and washed by stirring and decantation with distilled water until the supernatant was colourless. The individual batches of resin were then stirred with 350 ml 0·5 N NaCl whilst sufficient 2 N NaOH was added to maintain the pH at 8·0. After stirring for 1 hr at room temp. the suspended resin was poured back into the columns and the supernatant collected. The resin was washed with 0·5 N NaCl (*ca.* 700 ml) until the washings exhibited no further absorption at 550 nm as determined with a hand low-dispersion spectroscope (R. & J. Beck, London).

The supernatant and washings containing cytochrome c were combined and 0·2 g/l. of ascorbic acid added. The combined eluates (4 l.) were then dialysed against 60 l. of 0·05 M NaH_2PO_4–NaOH buffer (pH 8·0) at 2–4° for 18 hr.

Concentration of Cytochrome c on CM-Sephadex

Following dialysis the solution containing cytochrome c in 0·05 M buffer at pH 8 was passed through a column of CM-50 Sephadex (6 cm × 25 cm) buffered at pH 8·0 with 0·05 M NaH_2PO_4–NaOH and at a flow rate of 250 ml/hr. A bright red band of cytochrome (2–3 cm in depth) was adsorbed at the top of the column. The column was then washed through with 3–4 l. of 0·05 M buffer, pH 8·0, containing 0·1 g/l. of ascorbic acid. During this washing process the band of cytochrome c migrated down the column approximately 10–15 cm. In the extractions of sesame and sunflower a brown band of impurity was left at the top of the column following this washing procedure. This impurity was largely removed by pipetting off the upper 3–4 cm.

The cytochrome c was eluted with 0·5 N NaCl. Following dialysis against 0·05 M buffer, pH 8·0, the eluate containing cytochrome c (200 ml) was further concentrated by passage through a small column of CM-Sephadex (3·1 × 15 cm) buffered at pH 8·0. The cytochrome was eluted in approximately 30 ml of 0·5 N NaCl and the volume further reduced to 10 ml by rotary evaporation at 30°.

Molecular-sieve Chromatography on Bio-gel P-30

The cytochrome c was completely reduced by the addition of 10 mg of ascorbic acid and then applied in 0·05 M NaH_2PO_4–NaOH buffer, pH 8·0, to a column of Bio-gel P-30 (3·1 × 80 cm) (50–150 mesh, Bio-Rad Labs., Richmond, California, U.S.A.). Elution was performed with the same buffer at a rate of 15–20 ml/hr. Fractions of 1·5 ml were collected on a fraction collector and assayed spectrophotometrically at 280 and 410 nm. Those fractions having a 410 nm/280 nm absorption ratio of greater than 1·8 were combined and concentrated by rotary evaporation. All other fractions containing cytochrome c were combined, concentrated and recycled again through the molecular sieve.

Chromatography on CM-52 Cellulose

The partially purified cytochrome c from buckwheat and sunflower was further purified on a column of CM-52 Cellulose (1 cm × 10 cm). The column was initially equilibrated with 15 mM Na-Phosphate buffer, pH 7·2. The cytochrome c in the reduced form was applied in the same buffer and, after adsorption, the column was eluted with a linear gradient of Na-Phosphate buffer from 15–300 mM (pH 7·2 and 150 ml of each).

Gel Filtration Chromatography on Sephadex G-50

The combined fractions of reduced cytochrome c, having an absorption ratio 410 nm/280 nm greater than 1·8, were applied in 0·05 M buffer to a column of Sephadex G-50 (3·1 × 80 cm). The protein was eluted with the same buffer at a flow-rate of 60 ml/hr and collected in fractions of 2·5–3·0 ml. All fractions having an absorption ratio 410 nm/280 nm greater than 4·0 were combined, desalted and lyophilized. Fractions containing impure cytochrome c were recycled again through Bio-gel P-30 and/or Sephadex G-50.

Isoelectric Fractionation by Ampholine Electrofocusing

Isoelectric fractionation of the various cytochrome preparations was carried out by means of the LKB Electrofocusing System. The anode solution contained 0·1 ml conc. H_2SO_4 in 40 ml of water, and the cathode solution contained 0·1 g diaminoethane, 0·2 g morpholine, and 48 g sucrose in 56 ml of water. The stock ampholyte solution contained 100 μl of ethanolamine, 100 μl diethanolamine, 100 μl of diaminoethane, 400 mg arginine (free-base), 10 ml of 8 % (w/v) ampholine compounds, pH 7–10, and 1 ml of 8 % (w/v) ampholine compound (LKB Produckter AB, Stockholm, Sweden), pH 5–8, in water (total vol. 50 ml). For the preparation of the linear ampholyte gradient two solutions were prepared from the stock ampholyte solution. The less-dense

solution contained 12·5 ml of stock solution made up to 220 ml with water and the dense solution contained 37·5 ml of stock solution and 100 g sucrose made up to 155 ml with water. The linear gradient was pumped into the column until half the column was filled, when the pump was stopped and the sucrose concentration at that stage estimated by means of a hand refractometer. An appropriate amount of sucrose was added to the sample of cytochrome c, which had been previously dialysed against mM NH_4OH. This sample, 80 ml, was then pumped into the column, followed by the remainder of the ampholyte gradient. The gradient was stopped about 1 cm below the upper electrode and the anode solution was pumped into the column. The whole apparatus was maintained at 2–4°. The power supply was initially regulated to supply 5 mA at 400 V and subsequently raised to 1 kV (maximum power load 6 W). The complete electrofocusing took approximately 6 days. The cytochrome c was observed as a single red band towards the bottom of the column. The presence of other contaminating proteins was revealed by means of a u.v. lamp. The eluate from the column was collected by means of a fraction collector and the fractions containing cytochrome c pooled and dialysed against distilled water.

Polyacrylamide Gel Electrophoresis

Disc electrophoresis in polyacrylamide gel was performed at pH 4·3 using the method described by Reisfeld *et al.*[18] 60–100-μg quantities of protein were applied to the gels. The gels were stained with 0·05% Coomassie Brilliant Blue in 12·5% (w/v) trichloroacetic acid in water for 30 min and destained by washing with 12·5% (w/v) trichloroacetic acid.

Assay of Cytochrome c

The purity and quantity of cytochrome c was estimated spectrophotometrically using silica cells of 1 cm light path. The cytochrome c content was estimated from the α-absorption by using the mammalian cytochrome c extinction coefficient of 27·8 mM^{-1} cm^{-1} and assuming a mol. wt. of 13,000.

Desalting of Purified Cytochrome c

Solutions containing purified cytochrome c in the reduced form were desalted by passage through small columns of Sephadex G-25 (2 × 15 cm) in distilled water. All samples of pure cytochrome c were lyophilized and sealed in glass tubes which were stored at −20°.

Acknowledgements—The authors are greatly indebted to Mr. G. Bainbridge, Mr. P. Little and Mr. D. Caird for much technical assistance. Grateful acknowledgement is also made for the generous gifts of seeds from Dr. D. R. Williams of Silcock & Lever Feeds Ltd., and from Dr. A. R. Claridge and Dr. D. A. S. Barnes of the British Oil & Cake Mills Ltd., Hull. Thanks are due to the Nuffield Foundation for financial assistance. This work was also supported in part by the award of an S.R.C. Research Studentship to J. A. M. R.

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