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Nucleic acid changes during soybean leaf senescence.

Chit-Fong Lynn
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NUCLEIC ACID CHANGES DURING SOYBEAN *LEAF SENESCENCE*

BY

CHIT-FONG LYNN

A Thesis

Submitted to the Faculty of Graduate Studies through the Department of Biology in Partial Fulfilment of the Requirement for the Degree of Master of Science at the University of Windsor

WINDSOR, ONTARIO, CANADA

1970

Approved:

321808

ABSTRACT

Generally, losses in chlorophyll, protein and nucleic acids have been shown to occur during leaf senescence. Attempts have been made to chemically regulate senescence in excised and intact plants and plant parts, with growth regulators, like kinetin, gibberellic acid and more recently with several growth retardants. In this study the retardant, (2-chloroethyl) trimethylammonium chloride (CCC), at a concentration of 100ppm delays senescence, by controlling chlorophyll, protein and nucleic acid matabolism. The MAK column chromatography of nucleic acids of soybean leaves in the control shows that the labile ribosomal RNA degrades most and fast and that there is preferential incorporation of ^{32}Pi into soluble RNA fraction. Profiles of nucleic acids of treated leaves show delay in RNA degradation. A distinct, newly isolated fraction, not previously referred to in literature, is obtained. Further work is necessary to determine the nature of this fraction. A high concentration of CCC shows reduced rate of RNA synthesis. DNA-RNA remains relatively stable during senescence.

ACKNOWLEDGEMENTS

I wish to acknowlege the scientific training and the financial assistance given by Dr. D. T. N. Pillay during the course of this work and in the prepararion of this manuscript.

Special thanks are extended to Drs. D. des S. Thomas and M. M. Abdel-Sayed, both of Department of Biology, and Dr. G. W. Kosicki, Department of Chemistry, all of the University of Windsor for their crictical scrutiny in reviewing this thesis.

Finally I sincerely express my thanks to Mr. G. S. Sotoros for his technical assistance and encouragement during this research.

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INTRODUCTION

Senescence or ageing in leaves is characterized by a gradual decrease in metabolic activity, which manifests itself in yellowing and declines in the levels of chlorophyll, protein and nucleic acids. Recently several reports by Richmond & Lang (1957) ; Osborne (1962); Shaw & Manocha (1965) ; Fletcher & Osborne (1966); Sacher (1967) ; Beevers (1967) and Wollgiehn (1967) have described the many changes in the above variables in excised leaves or leaf discs as well as in other plant organs.

However, little information is available on ageing in intact plants or plant organs. Investigations into the causes of ageing of higher plants are complicated because the higher plant is not a uniform organism and because the individual organs do not age simultaneously. Therefore, simple analysis of the metabolism of ageing leaves will scarcely give the answer to this question. It is necessary to either hasten or retard this ageing process by different methods to gain some insight into the causes of ageing.

Many proposals have been made to account for the accelerated deterioration of excised leaves (Chibnall 1939). It was Chibnall (1954), who suggested that leaf

integrity might be controlled by hormonal materials, normally supplied by the roots, which would be depleted in a detached leaf and senescence would ensue. This hypothesis was supported by the works of Richmond & Lang (1957) and Kende (1964) who showed that kinetin could retard senescence of detached leaves. Similarly, observations of Fletcher & Osborne (1966) and by Beevers (1967) supported the proposition that senescence of detached leaves might be a function of hormonal status, and thus any manipulations which varied hormonal level of the tissue might alter the rate of senescence. The hormones used in these studies include auxin, kinetin and GA[1].

Recently, several growth retarding chemicals such B-995, CCC[2], and AMO-1618 have been shown to influence senescence in excised leaves (Halevy & Wittwer 1965;

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[1]Abbreviations : GA, gibberellic acid ; B-995, N-dimethylaminosuccinamic acid ; AMO-1618, 2'-isopropyl-4'-(trimethylammonium chloride)-5'-methylphenyl piperidine carboxylate ; CCC, (2-chloroethyl)trimethylammonium chloride ; s-RNA, soluble RNA ; DNA, deoxyribonucleic acid ; RNA, ribonucleic acid ; s1-RNA, 4s transfer RNA; s2-RNA, 5s RNA, or transfer-like RNA; lr-RNA, light ribosomal RNA; hr-RNA, heavy ribosomal RNA; m-RNA, messenger RNA ; TB-RNA, tenaciously or tightly bound RNA ; TCA, trichloro-acetic acid ; RNAase, ribonuclease; r-RNA, ribosomal RNA.

[2]Kindly supplied by American Cyanamid Co., Agricultural Division, Princeton, N.J.

Harada 1966 ; Kesseler et al. 1967 ; Beevers 1967 ; Pillay & Mehdi 1968). Plants treated with retardants generally show a darker green color of leaves and also have some influence on the chlorophyll breakdown and senescence.

Since the processes of ageing are regulated at the molecular level, it was of interest to study the nucleic acid changes in senescing soybean leaves and their relation to a growth retardant, CCC.

MATERIALS AND METHODS

Preparation of the Material

Soybean seeds (Glycine max L.) var "Harosoy 63"* were surface sterilized with 6% Javex for 20 minutes and germinated in the dark at 28°C for 2-3 days. Later they were transferred to growth chambers under a 16 hour photoperiod. Before studing the nucleic acid metabolism in senescing leaves, it was desirable first to determine the nucleic acid changes in leaves of different stages and ages of growth. For this, two sets of leaf samples were used. In one case only primary leaves were collected from plants in different stages of growth, i.e. 8, 13, 28 and 35 days old. In the second case, plants were grown under controlled environmental conditions as described above for 5 weeks. Leaves of different ages, designated as budding, green mature, yellowing and yellow leaves, were harvested for analyses.

For studies involving chemical regulation of

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*Kindly supplied by Research Station, Canada Department of Agriculture, Harrow, Ontario.

leaf senescence, the growth retardant, (2-chloroethyl) trimethylammonium chloride, Cycocel or CCC was used. When the primary leaves were fully expanded, 50ml of CCC_{100ppm} or 50ml of $CCC_{1000ppm}$ were supplied to each pot with 4 plants, 4 times in a period of one week, on days 1, 3, 5, and 7. Control plants were supplied with tap water. Leaves of different ages (budding, green mature, yellowing and yellow) were harvested after 35 days.

Leaf samples were rinsed several times with distilled water and blotted dry with paper towels before subjecting to chemical analyses.

Chlorophyll determination

Chlorophyll was extracted from 1 gm leaf samples with 10ml of 80% ethanol in a boiling water bath for 7 minutes (repeated 3X). For complete extraction leaves were ground with washed and ignited sea sand in a mortar, filtered with Whatman #1 filter paper and washed twice with 5ml ethanol. Washings and the initial extract were combined and made up to 50ml. Chlorophyll was estimated by determining the O.D. at 665mμ with a Bausch & Lomb Spectronic 20 colorimeter.

Protein determination

Protein was determined by the method of Lowry et al.(1951). Soybean leaves of 1 gm fresh weight were homogenized in 10ml cold solution (vol./wt.) containing 0.4M sucrose, 0.005M EDTA and 0.05M Tris-HCl, pH 7.6, in a Sorvall Omni- Mixer for 1 minute. The homogenate was filtered through 4 layers of cheese cloth and centrifuged at 1,200xg for 10 minutes. 1 ml of 10% TCA was added to 1 ml of above supernatant to precipitate the protein. It was then centrifuged at 15,000xg for 15 minutes. The precipitate was dissolved in 5ml of 0.1N NaOH. A 1ml aliquot was used for determination of protein.

To 1 ml aliquots of extract, 5 ml of reagent f* were added and incubated for 25 min. at 25°C, following which 0.5 ml of reagent d* was added and diluted to 10 ml with distilled water and incubated at room temperature for 30 min. O.D. was determined at 750mu with DB-G spectrophotometer and the concentration of protein was expressed as mg/gm fresh weight.

Nucleic Acids

(A) Incorporation of ^{32}Pi into soybean leaves

About 7-10 gm of freshly harvested leaves

*Reagents f and d (see Appendix IV)

of different ages and/or stages were incubated in 40-50 ml of a medium containing 1% sucrose; 10^{-4}M NH_4citrate, pH 6.0; 25µg/ml chloramphenicol and 0.5mCi ^{32}Pi; for 3 hours in the dark at 30°C. Following incubation the leaves were washed several times with cold phosphate buffer and cold distilled water.

(B) Extraction and Purification of RNA

Nucleic acids were extracted by a phenol-tris-buffer method described by Cherry et al. (1965). All steps were carried out in cold room or on ice in a container. Leaves incorporated with ^{32}Pi were homogenized in a solution, containing 40 ml 0.01M Tris buffer pH 7.6; 0.06M KCl; 0.01M $MgCl_2$; 1 ml bentonite (40 mg; see Appendix II); 4.6 ml 11% Dupanol and 65 ml phenol pre-equilibrated with Tris-HCl, in a Sorvall Omni-Mixer. The tissue was homogenized for 1 min. at full speed first and then gently stirred for another minute. This was repeated three times, followed by centrifugation at 20,000xg for 10 min. The aqueous layer was carefully removed with a syringe. To this aqueous layer, 1 ml bentonite and 1 volume of phenol was added and then shaken occasionally for about 5 min. The mixture was centrifuged at 20,000xg for 10 min. and the aqueous layer removed. The phenol treatment was repeated with

½ volume of phenol for 5 min., followed by centrifugation at 20,000xg for 10 min. and removal of the aqueous layer. Potassium acetate was added to the aqueous layer to a concentration of 0.2M. After mixing with 2 volumes of cold 95% ethanol, the solution was left at -10°C for 2 or more hours. The RNA precipitate was collected by centrifugation at 30,000xg for 20 min. The supernatant was decanted and the RNA was dissolved in 5-10 ml 0.05M sodium phosphate buffer, pH 6.7. The RNA solution was dialyzed overnight against 0.05M sodium phosphate buffer, pH 6.7 in the cold with two buffer changes. After dialysis, 0.1 ml of the RNA solution was diluted to 5 ml with 0.05M sodium phosphate buffer and an absorption spectrum from 220mμ to 300mμ was determined.

(C) Separation of Purified RNA on a Methylated-Albumin Kieselguhr column

The methylated albumin kieselguhr (MAK) columns were prepared essentially by the method of Mandell & Hershey (1960; see Appendix I). About 25-30 O.D. units of purified RNA were added in 40 ml starting buffered saline (0.3M) to the column. When the liquid level nearly reached the kieselguhr, 20 ml of starting buffered saline were added. When this liquid almost touched the kieselguhr, another 10 ml

of starting buffered saline were added. The RNA was eluted from the column with a linear gradient of 0.3M to 1.5M NaCl in 0.05M sodium phosphate buffer, pH 6.7. The eluate from the column was collected in 5 ml fractions. The U.V. absorbancy of each fraction at 260mμ was recorded with a Unicam SP800-Spectrophotometer. For measurements of radioactivity an aliquot of 0.2 ml from each fraction was added to 5 ml of scintillation fluid (Appendix III) in each vial and the radioactivity was determined in a Nuclear-Chicago scintillation counter (Model 6850).

RESULTS

Chlorophyll Changes

Loss of chlorophyll in senescing soybean leaves is shown in Table 1. The chlorophyll content was determined for three stages of growth, i.e. green mature, yellowing and yellow stage. These stages are found from the apex to the bottom of a plant. In the control, 80% of chlorophyll is lost between the green mature and the yellowing stages, and only 3% of the original content is left in the senescing yellow primary leaves. Treatment of plants with CCC (100ppm) effectively delays the onset of senescence and preserves 17% of chlorophyll content in the yellow primary leaves.

Protein Changes

As chlorophyll content decreases, losses in protein levels also occur in senescing leaves. Data presented in Table (2) show that control plants lose more than 50% of protein from green mature to yellow leaves. However, the loss in protein in CCC (100ppm) treated plants is minimal. It appears that the growth

Table 1

The chlorophyll content* in different ages of soybean leaves.

Treatment	Sample	Chlorophyll content OD 665mu
H_2O	Green Mature	1.425
	Yellowing	0.288
	Yellow	0.045
CCC (100ppm)	Green Mature	1.450
	Yellowing	0.860
	Yellow	0.255

*Results are given as OD665 after extracting one gram fresh weight of leaves with ethanol and making up to 50 ml.

Table 2

Changes of protein content* in various ages of soybean leaves.

Treatment	Sample	Protein content mg/gm fresh weight
H_2O	Green Mature	1.550
	Yellowing	1.300
	Yellow	0.800
CCC (100ppm)	Green Mature	1.650
	Yellowing	1.625
	Yellow	1.100

*The determination is by the method of Lowry et al. (1951).

Table 3

Effect of age on the incorporation of ^{32}Pi into various nucleic acid fractions in soybean primary leaves.

RNA fraction from MAK column	Specific Activity, CPM/ml/OD			
	Budding	Green Mature	Yellowing	Yellow
soluble	4,300	5,945	19,000	39,333
DNA-RNA	3,303	1,100	1,540	3,070
light ribosomal	3,000	2,043	9,333	32,600
heavy ribosomal	2,860	3,394	9,888	37,258
messenger	5,227	4,851	-	34,499

Table 4. Effect of soybean leaf age of both water control and CCC (100ppm) treatment on the incorporation of ^{32}Pi into various nucleic acid fractions.

RNA fraction from MAK column	Specific Activity, CPM/ml/OD					
	Green Mature		Yellowing		Yellow	
	control	CCC	control	CCC	Control	CCC
soluble	4,893	5,690	16,505	14,889	8,867	43,476
DNA-RNA	1,400	1,174	2,174	2,515	4,250	21,324
light ribosomal	1,563	1,654	4,325	4,886	4,400	9,180
heavy ribosomal	1,988	2,190	4,413	4,451	7,863	9,504
messenger	—	2,907	11,814	9,679	—	2,417
VII	1,573	—				

Table 5

Effect of soybean leaf age of CCC (100ppm) treatment on the incorporation of ^{32}Pi into various nucleic acid fractions.

RNA fraction from MAK column	Specific Activity, CPM/ml/OD	
	Dark Green Mature	Bleaching Yellowish Green
soluble	3,952	1,464
DNA-RNA	1,700	380
light ribosomal	1,174	583
heavy ribosomal	1,131	679
messenger	1,554	1,205

retardant, CCC, somehow arrests the loss of protein during senescence.

Nucleic Acids

A. Nucleic Acid Changes in Primary Leaves in Different Stages, and in Leaves of Different Ages.

The nucleic acids from soybean leaves were fractionated on MAK columns into six RNA components in the following order:- I, s1-RNA ; II, s2-RNA; III, DNA-RNA; IV, lr-RNA; V, hr-RNA, and VI, m-RNA. The nucleic acid fractions (Fig. 1 a) follow the pattern as in other plant material, and are similar to the ones characterized and reported by Cherry et al.(1965). The results presented here, are concerned mainly with changes in nucleic acid fractions in (A) soybean leaves of different ages (budding, green mature, yellowing, and yellow) and of (B) primary leaves in different stages of senescence.

Profiles presented in Figs. 1 (a), (b) and 2 (a), (b) indicate that based on U.V. absorbancy, the s-RNA (s1 and s2) fraction increases from the budding stage to the green mature stage and declines in the yellowing and yellow stages of the leaf (Figs. 1 c, d and 2 c, d). DNA-RNA fraction appears to be more stable

Fig. 1. Fractionation of nucleic acids from soybean primary leaves of different ages on MAK column :-

(a) 8 days old (c) 28 days old

(b) 13 days old (d) 35 days old

7 gm fresh weight of leaves were incubated in a medium (45 ml) containing 1% sucrose, 10^{-4}M NH_4citrate, pH 6.0, 25 µg/ml chloramphenicol and 0.5mCi ^{32}Pi for 3 hours in the dark at 30°C. Extraction and purification of total nucleic acid from the leaves were as described by Cherry et al. (1965). The leaves were homogenized on ice in a mixture containing 40 ml 0.01M Tris-Hcl, pH 7.6, 0.06M KCl, 0.01M $MgCl_2$; 1 ml (40 mg) bentonite; 4.6 ml 11% Dupanol and 65 ml phenol pre-equilibrated with Tris buffer. The aqueous layer was drawn off after centrifugation and reextracted twice with phenol. Nucleic acids were precipitated with 2 volumes of cold ethanol in the presence of potassium acetate, dissolved in 0.05M sodium phosphate, pH 6.7 and dialyzed overnight against the same buffer at 4°C. Purified total nucleic acid was fractionated on MAK—column with a linear gradient of NaCl from 0.3M to 1.5M in 0.05M phosphate buffer, pH 6.7. Fractions containing 5 ml were collected and assayed for absorbancy at 260mµ and for radioactivity.

The nucleic acids were fractionated in the following order :- I, s1-RNA; II, s2-RNA; III, DNA-RNA; IV, lr-RNA; V, hr-RNA, and VI, m-RNA.

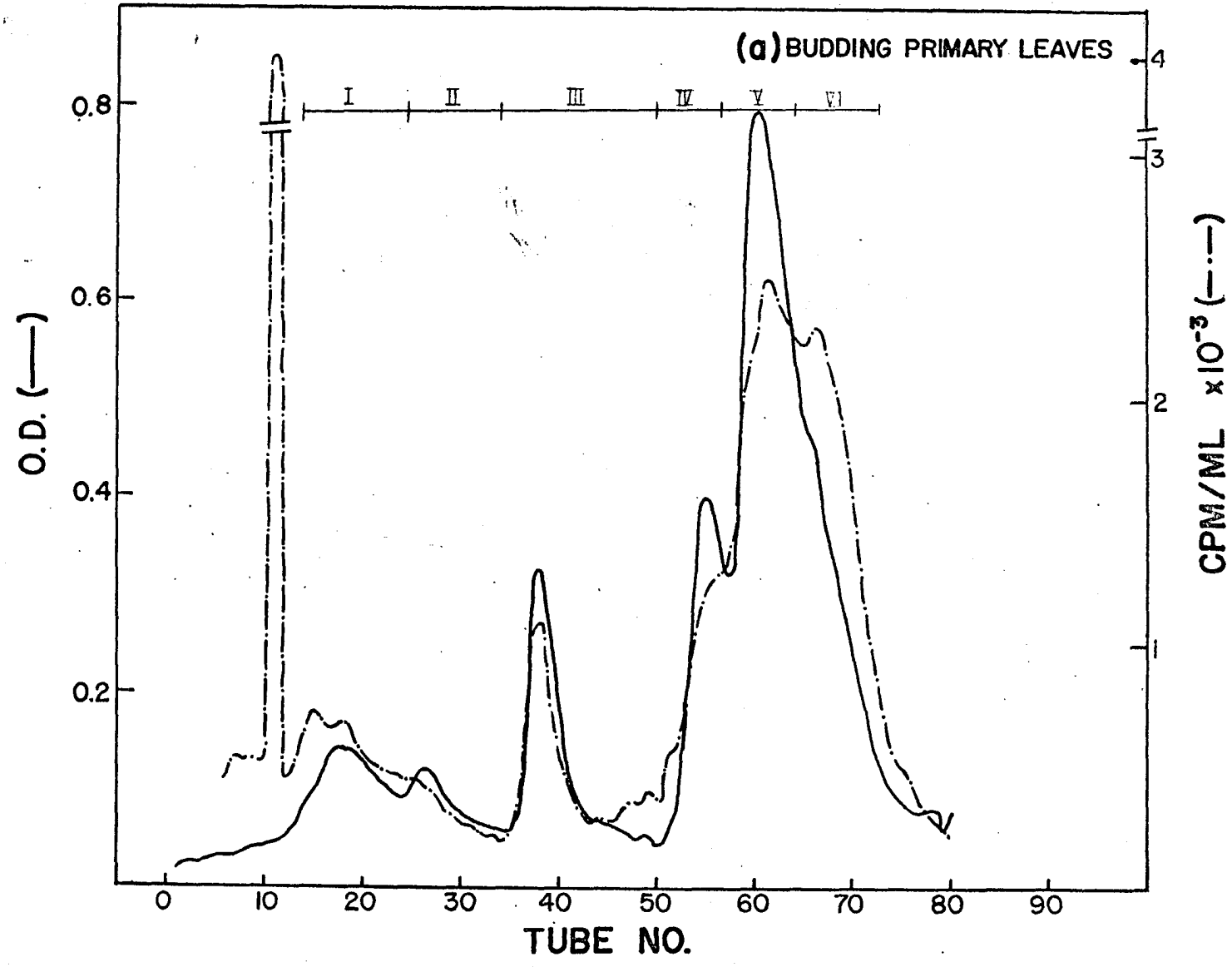

Fig. 1. Fractionation of nucleic acids from soybean primary leaves of different ages on MAK column:-

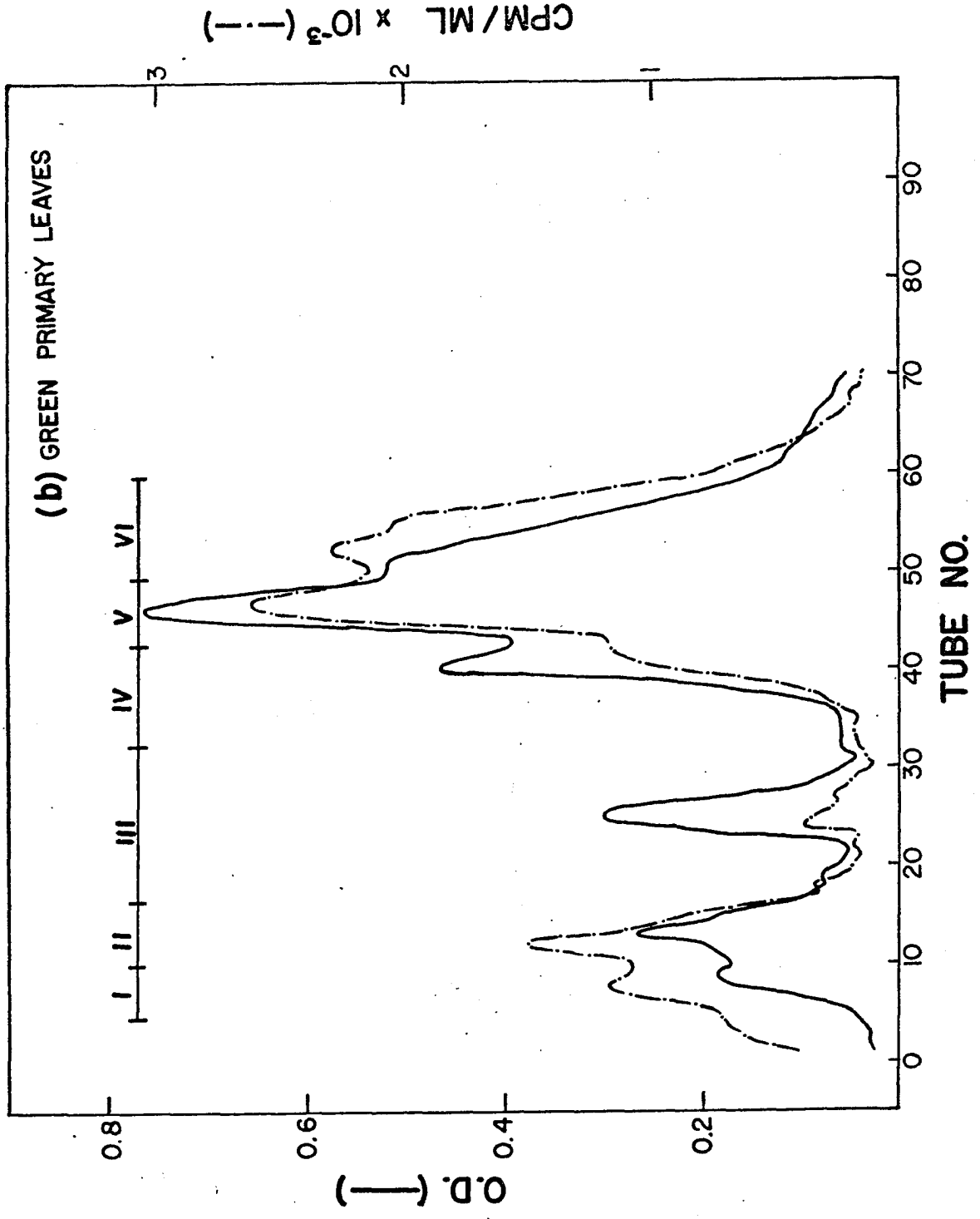

Fig. 1. (b) 13 days old

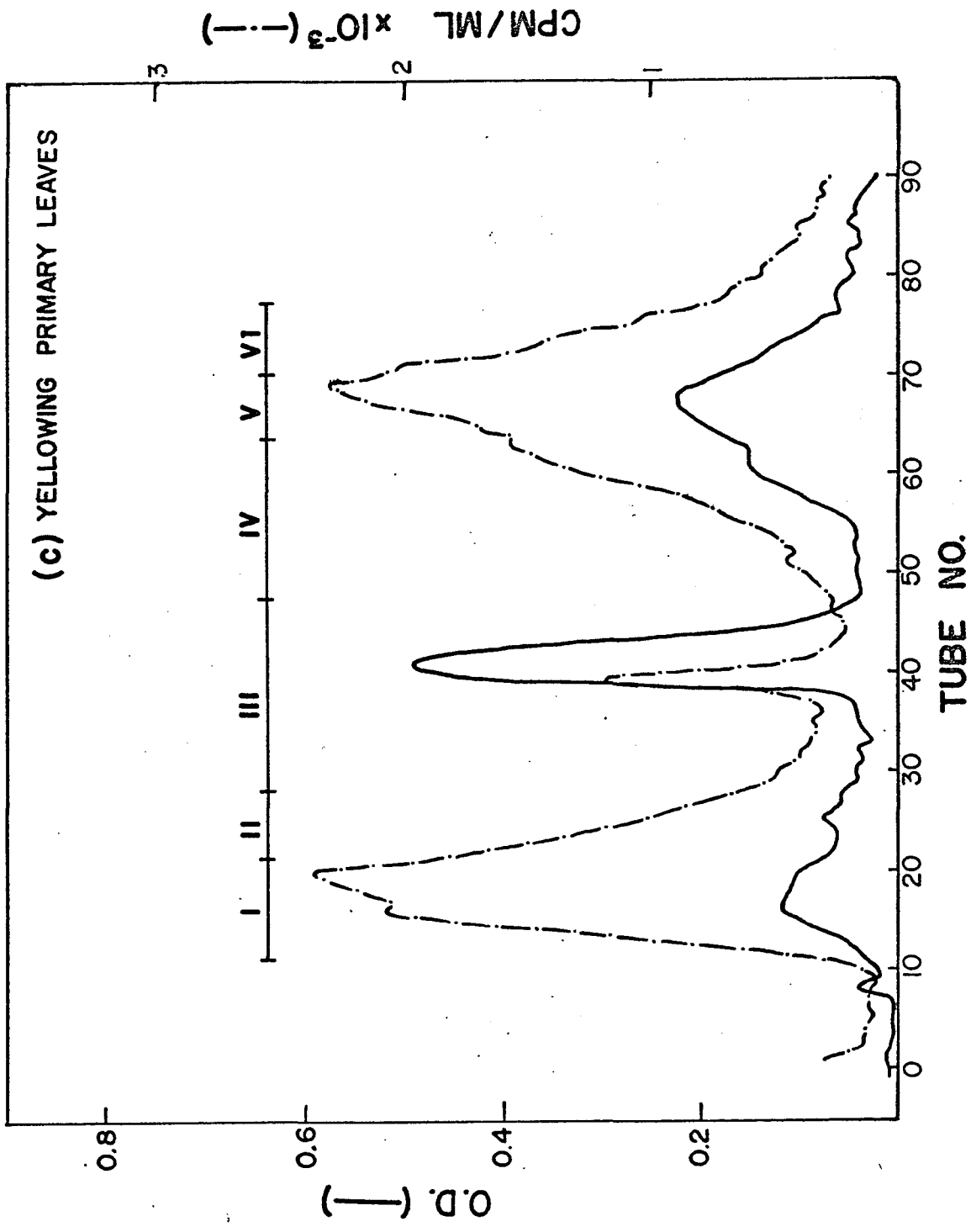

Fig. 1. (c) 28 days old

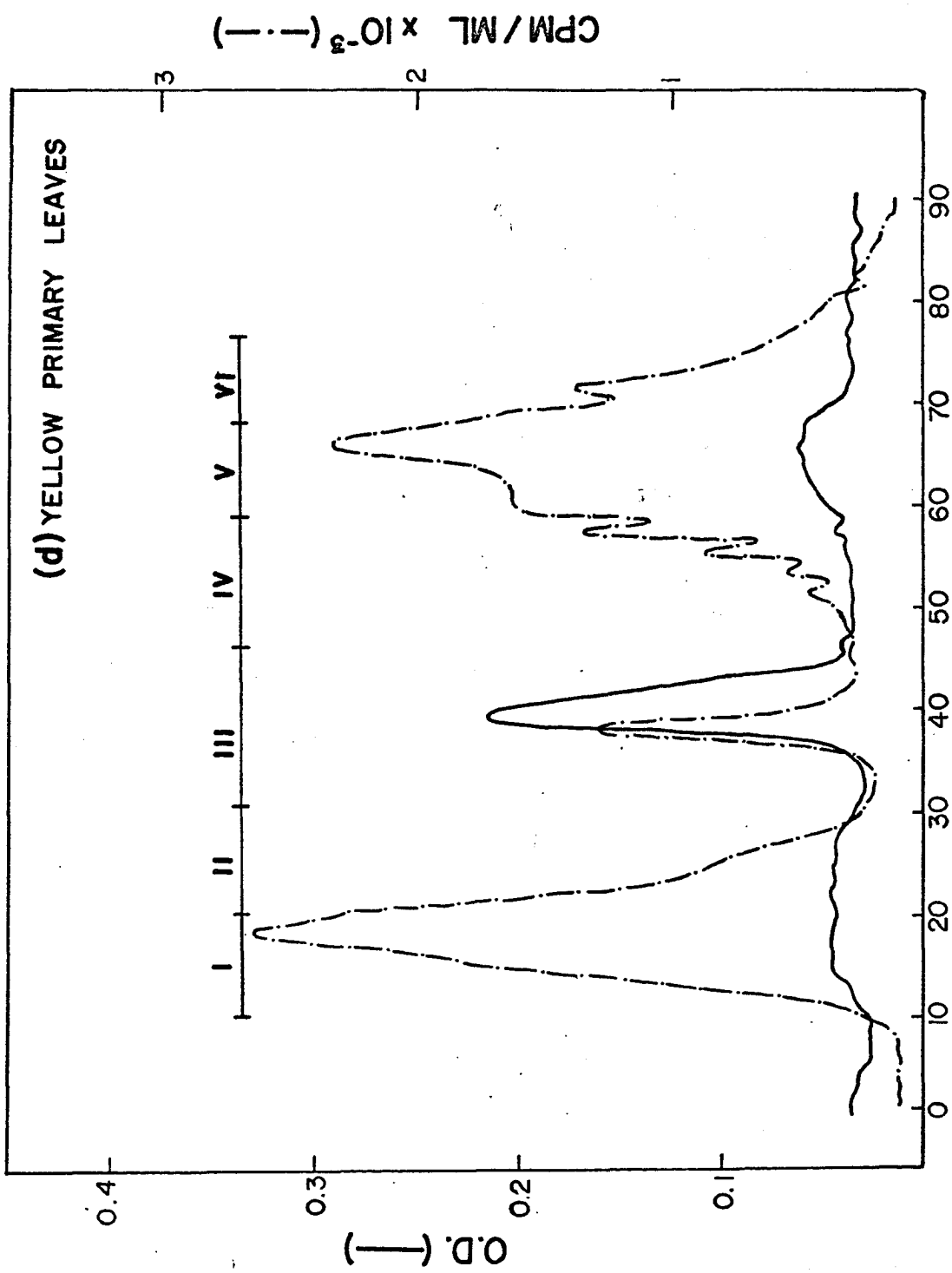

Fig. 1. (d) 35 days old

Fig. 2. MAK column fractionation of nucleic acids of soybean leaves of different ages:-

(a) Budding

(b) Green Mature

(c) Yellowing

(d) Yellow

The nucleic acids were extracted with Tris-phenol-buffer from the soybean leaves after incubation for 3 hours in the dark, centrifuged, dissolved, dialyzed and fractionated with NaCl. 5 ml aliquots were collected and assayed for U.V. absorbancy and for radioactivity (details as described in Fig. 1).

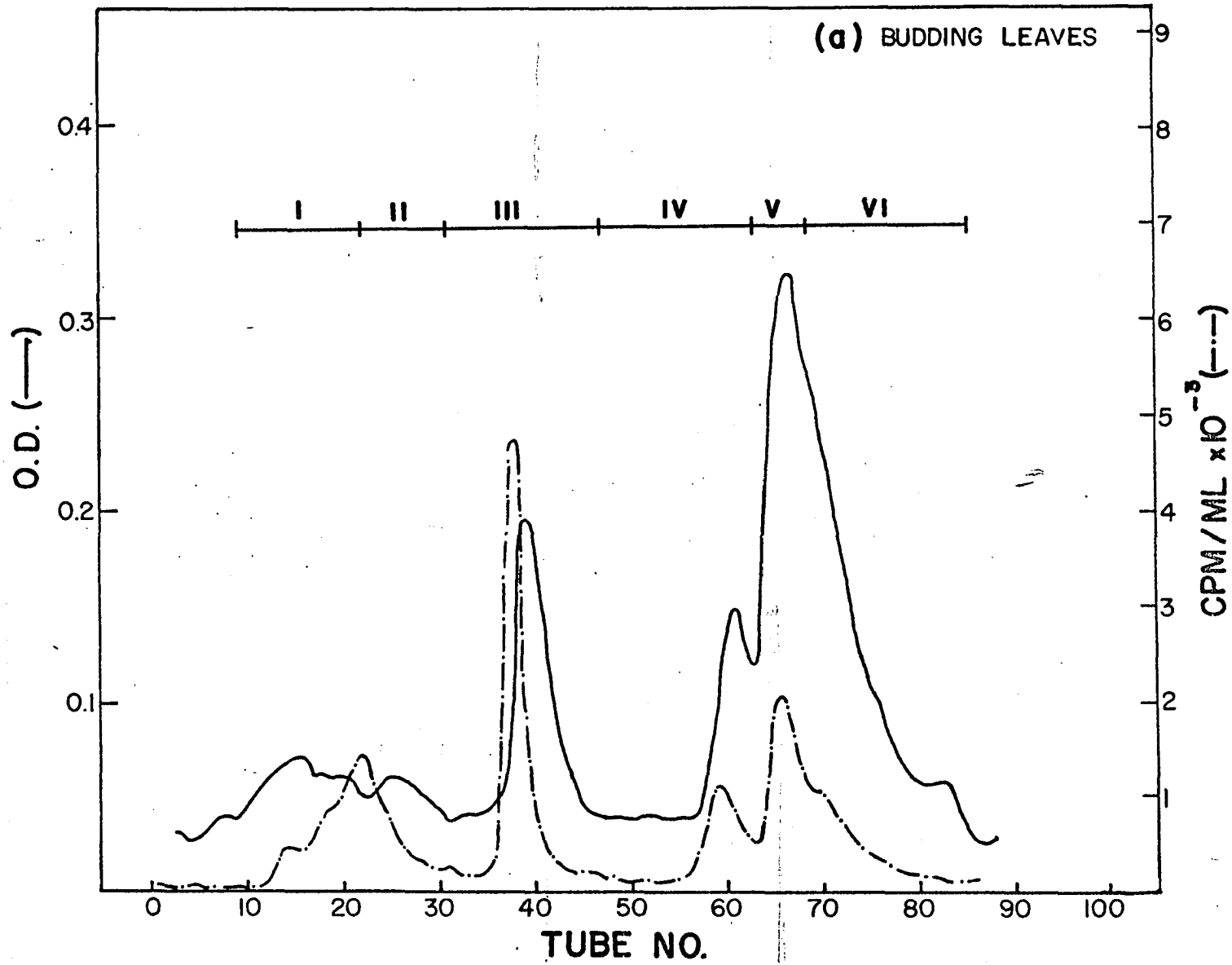

Fig. 2. MAK column fractionation of nucleic acids of soybean leaves of different ages:-

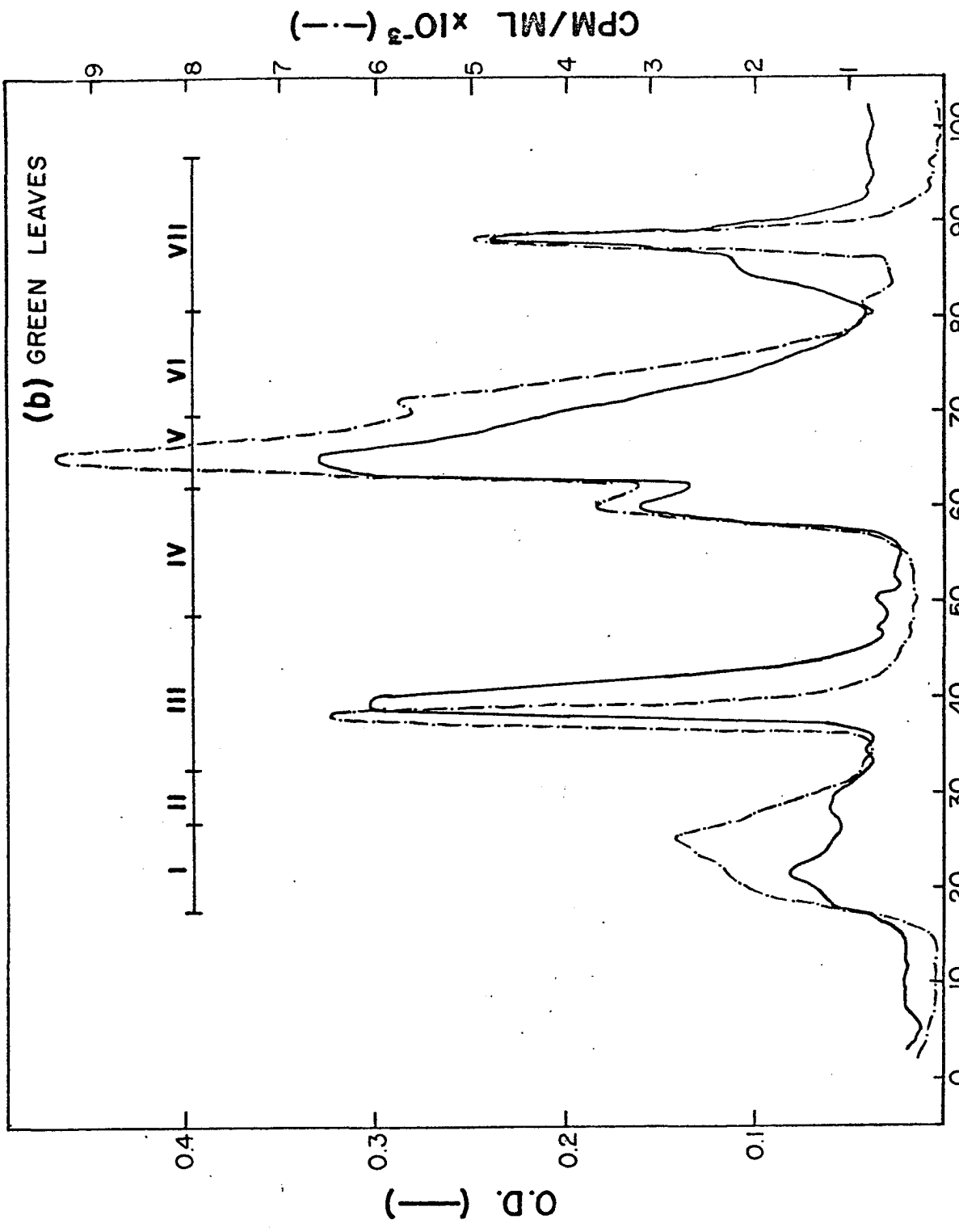

Fig. 2. (b) Green Mature

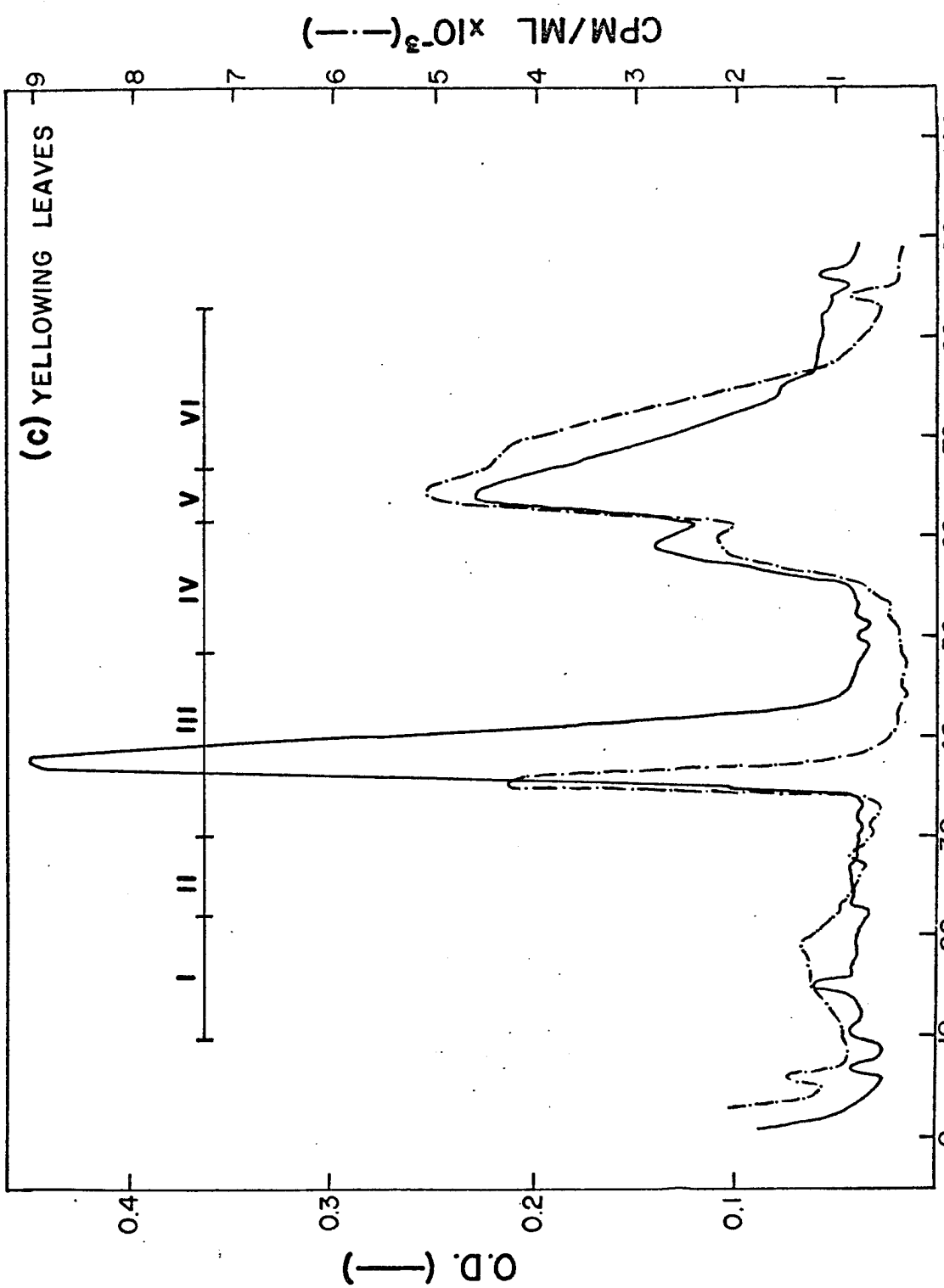

Fig. 2 (c) Yellowing

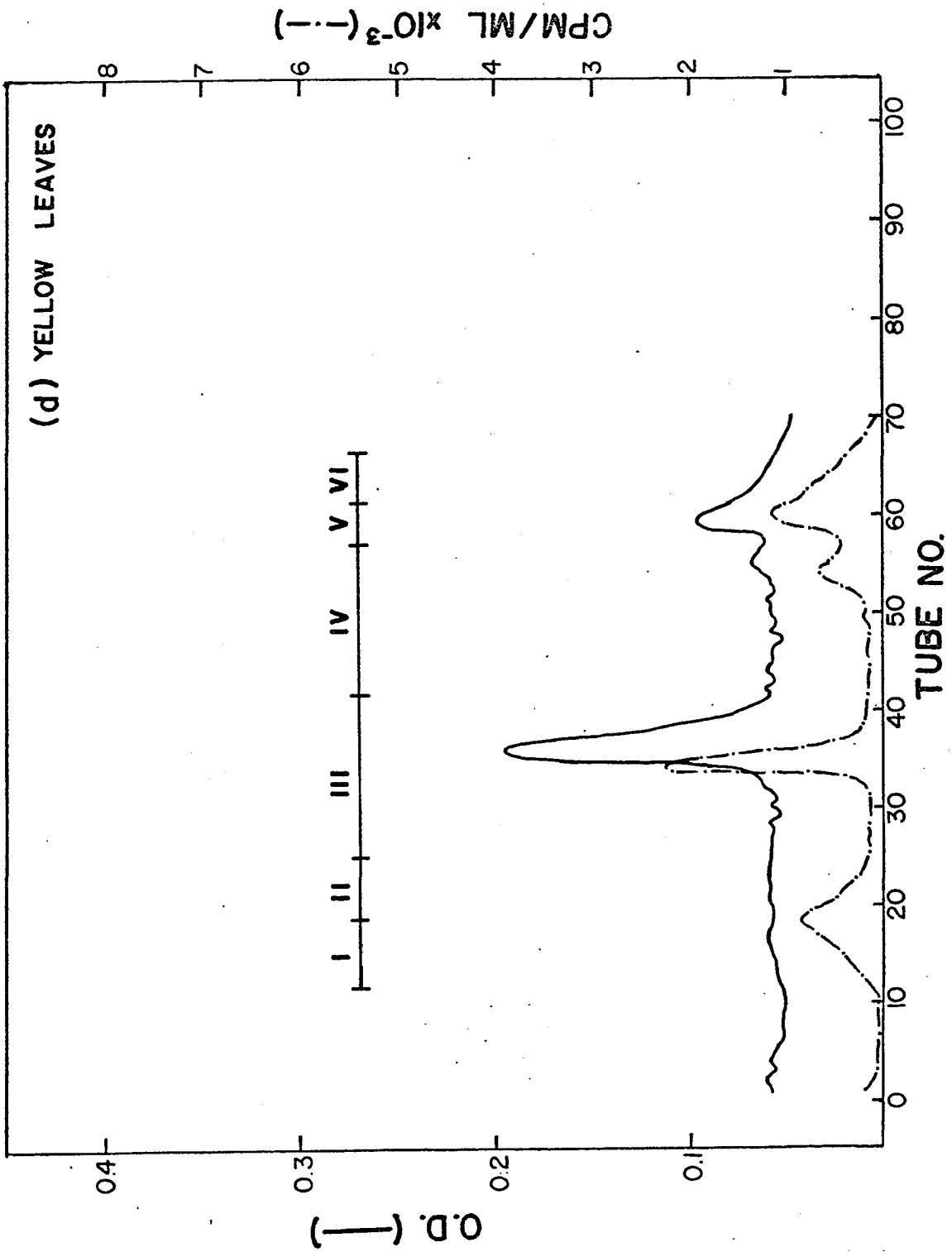

Fig. 2. (d) Yellow

Fig. 3. MAK column fractionation of nucleic acids of soybean leaves of different ages, both control and CCC (100ppm) treated plants:-

(a) Green Mature

(b) Yellowing

(c) Yellow

The nucleic acids were extracted with Tris-phenol-buffer from the soybean leaves after incubation for 3 hours in the dark, centrifuged, dissolved, dialyzed and fractionated with NaCl. 5 ml aliquots were collected and assayed for U.V. absorbancy and for radioactivity (details as described in Fig. 1).

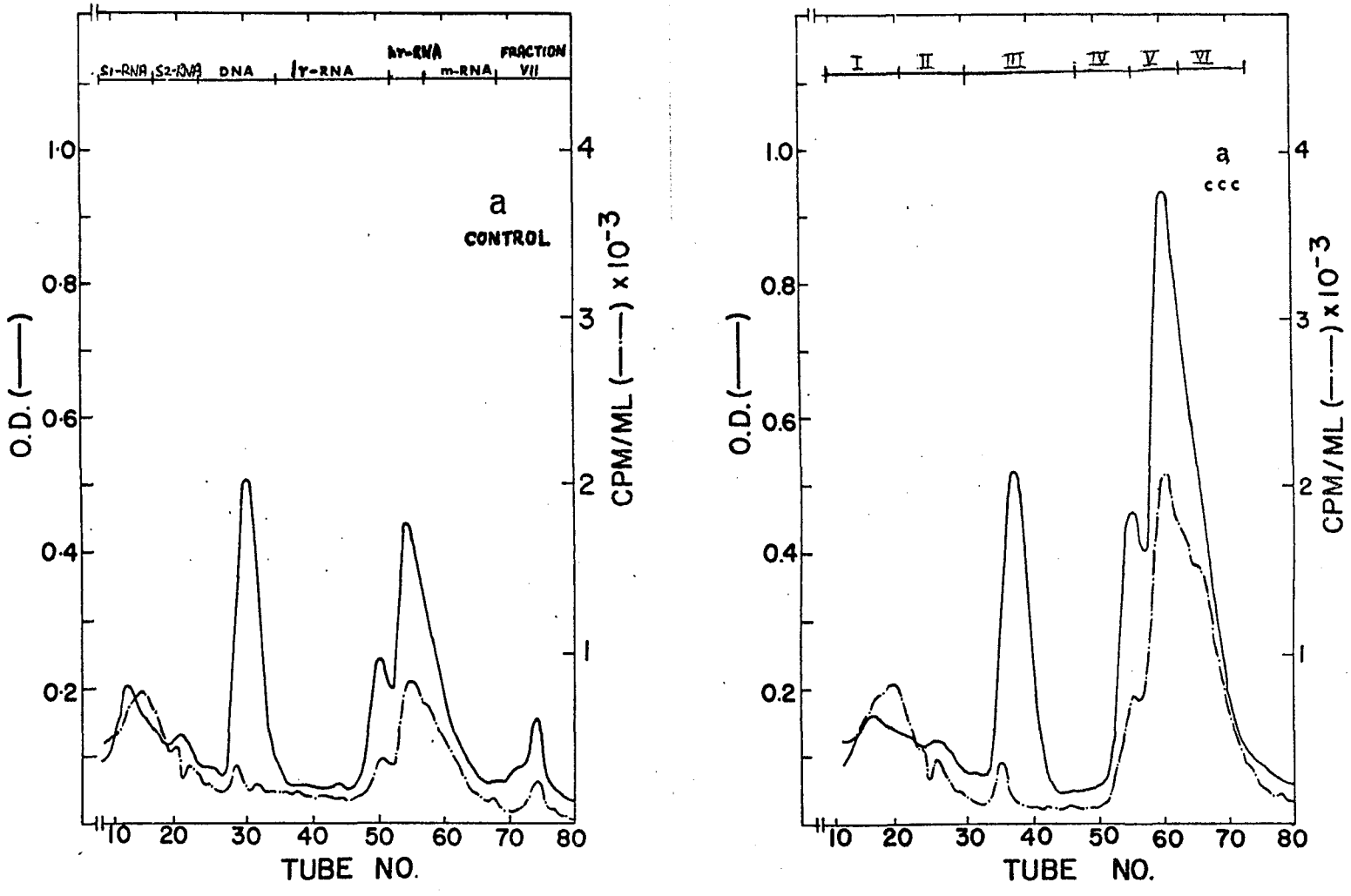

Fig. 3. MAK column fractionation of nucleic acids of soybean leaves of different ages, both control and CCC (100ppm) treated plants:-

(a) Green Mature

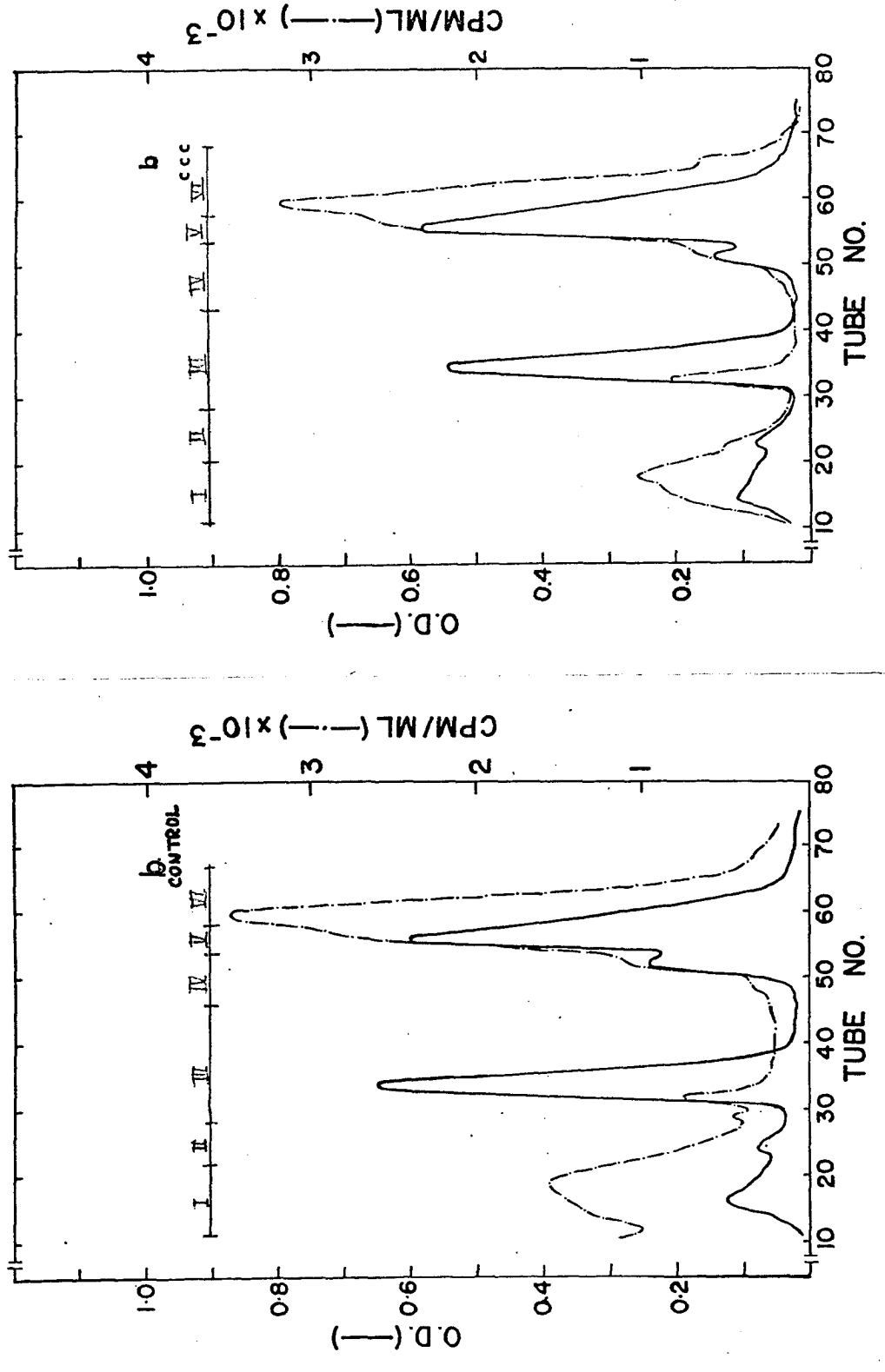

Fig. 3. (b) Yellowing

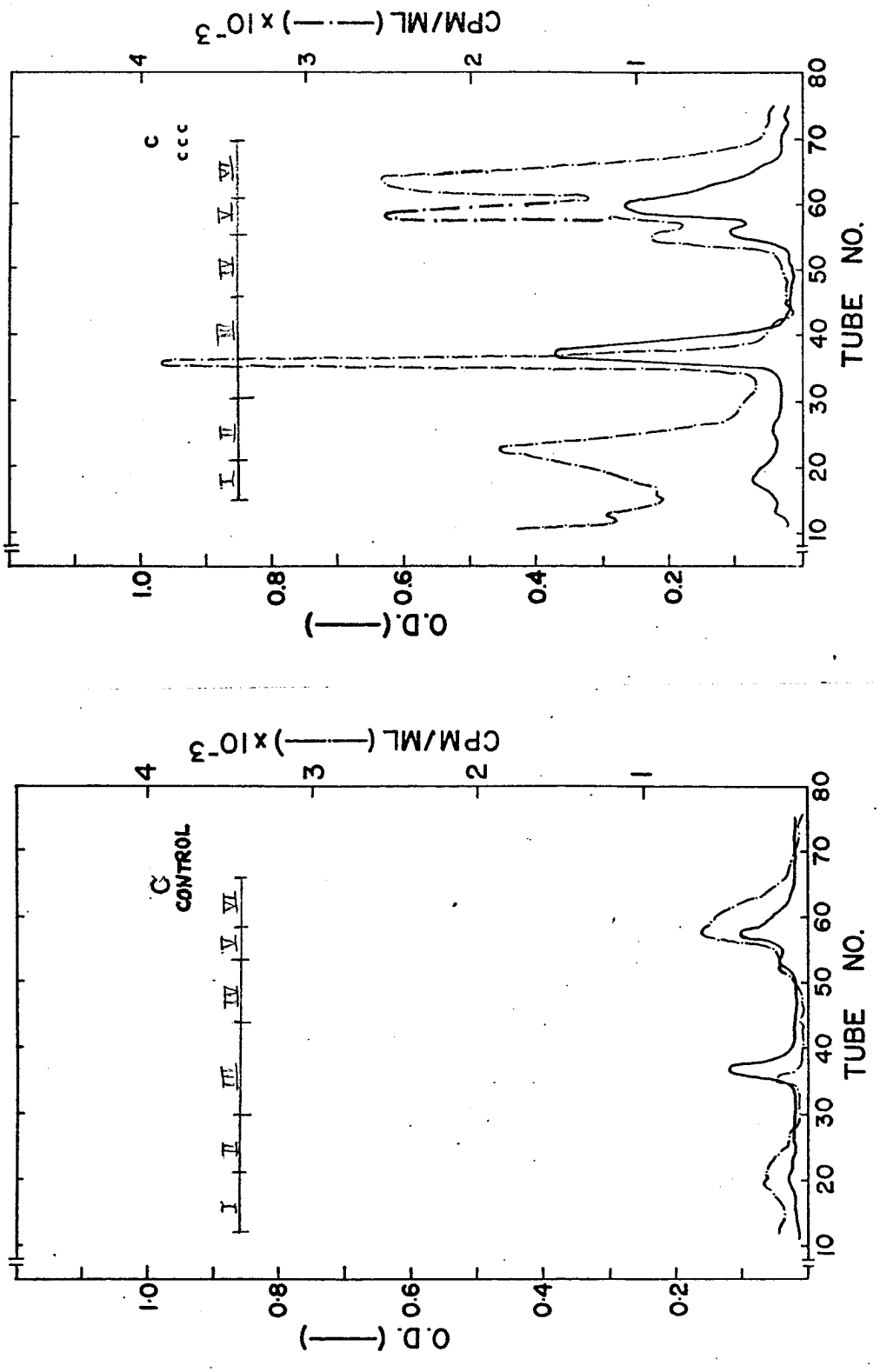

Fig. 3. (c) Yellow

Fig. 4. MAK column fractionation of nucleic acids of green mature leaves from both control and CCC (100ppm) treated plants, after a week's delay in sampling.

The nucleic acids were extracted with Tris-phenol-buffer from the soybean leaves after incubation for 3 hours in the dark, centrifuged, dissolved, dialyzed and fractionated with NaCl. 5 ml aliquots were collected and assayed for U.V. absorbancy and for radioactivity (details as described in Fig. 1).

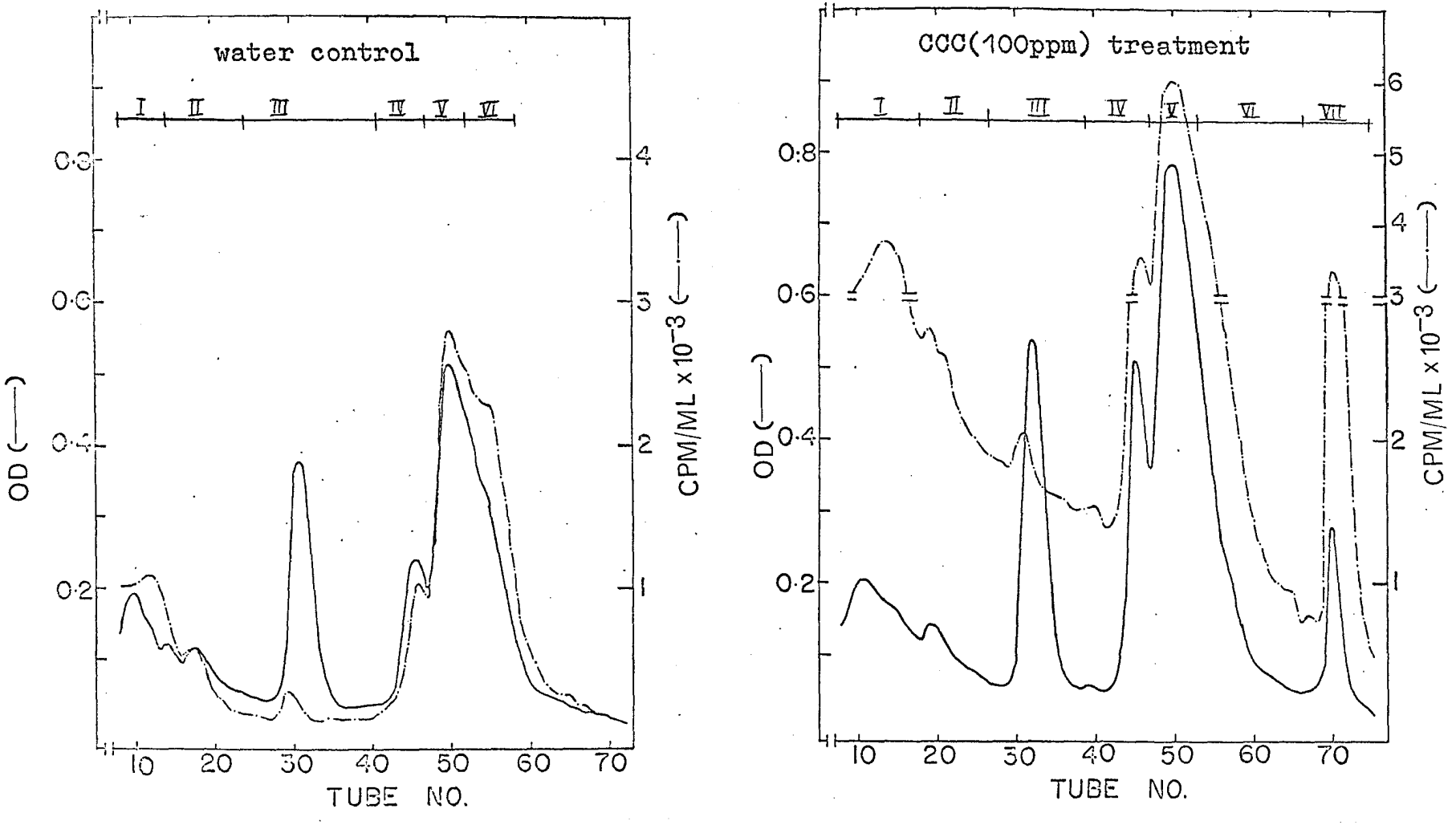

Fig. 4. MAK column fractionation of nucleic acids of green mature leaves from both control and CCC (100ppm) treated plants, after a week's delay in sampling.

Fig. 5. Photograph of soybean plants, 25 days after treatment with CCC.

200 ml of CCC, 100ppm or 1000ppm were supplied in one week to the soybean plants when the primary leaves were fully expanded. 50 ml of each solution were supplied on days 1, 3, 5, and 7; control plants were supplied with tap water.

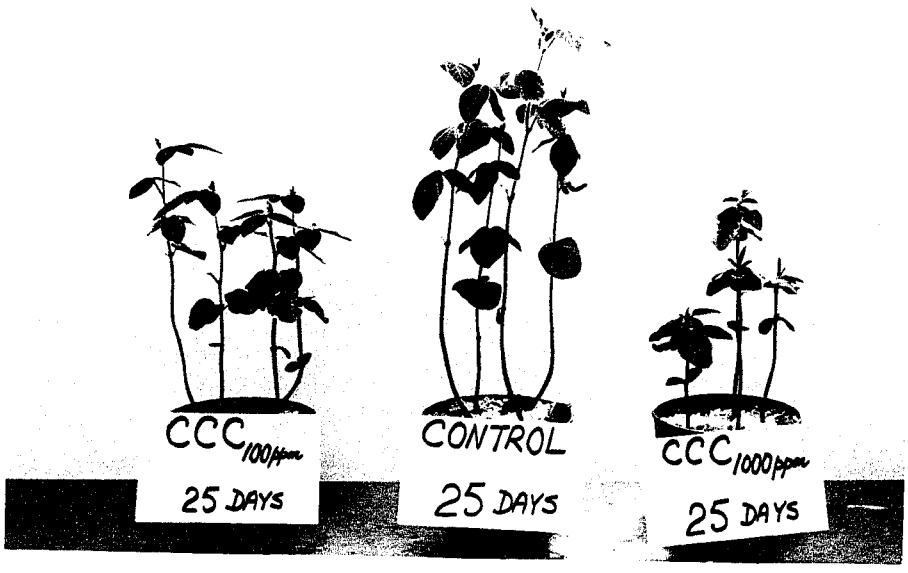

Fig. 6. MAK column fractionation of nucleic acids of CCC (1000ppm) treated leaves of different ages:-

(a) Green Mature

(b) Yellowing

The nucleic acids were extracted with Tris-phenol-buffer from the soybean leaves after incubation for 3 hours in the dark, centrifuged, dissolved, dialyzed and fractionated with NaCl. 5 ml aliquots were collected and assayed for U.V. absorbancy and for radioactivity (details as described in Fig.1).

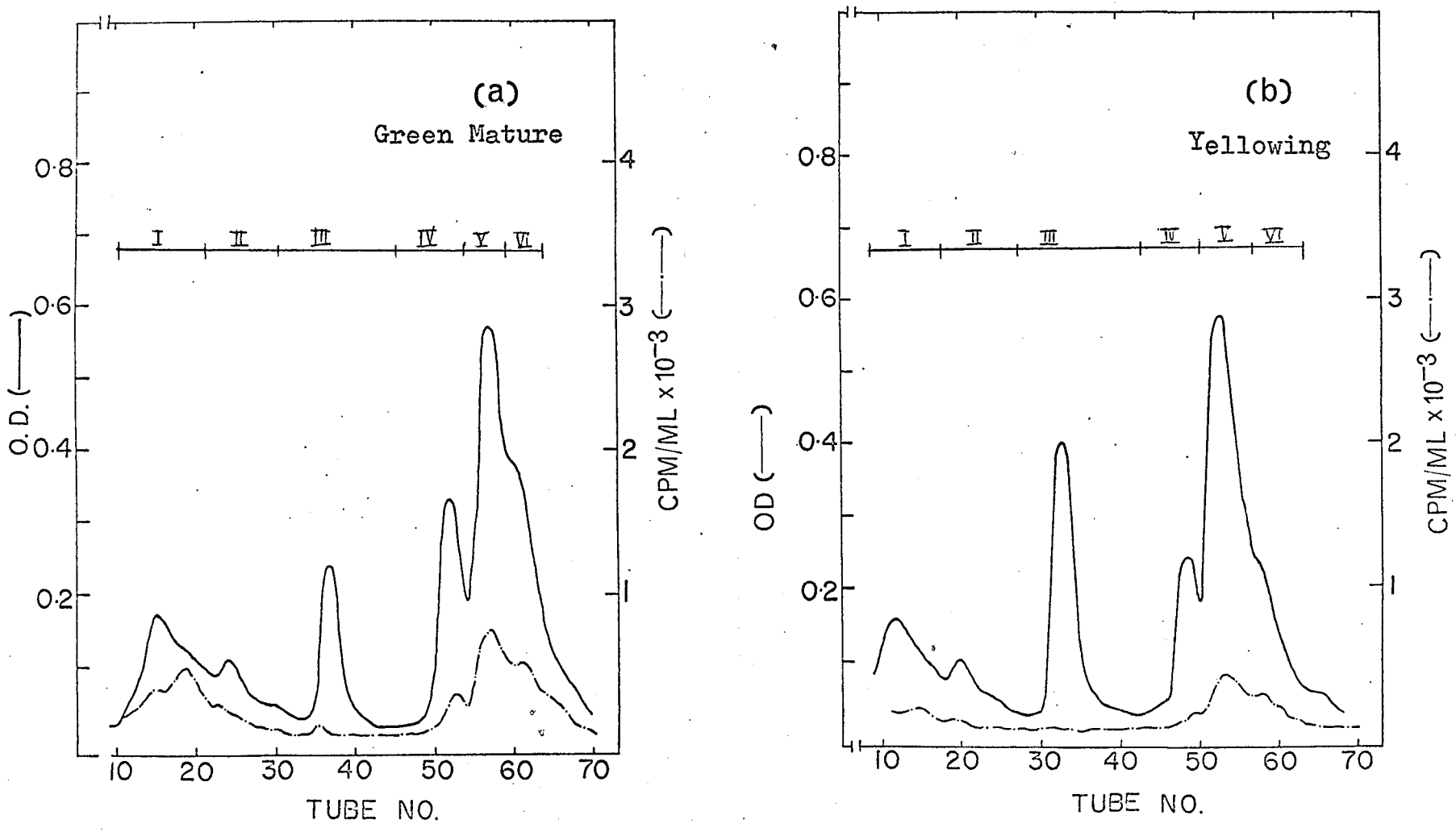

Fig. 6. MAK column fractionation of nucleic acids of CCC (1000ppm) treated leaves of different ages:-

(a) Green Mature

(b) Yellowing

in all the stages. But, sometimes the ^{32}Pi incorporation in this fraction is not observed in the green mature leaves (Fig. 1 b). According to Cherry (1969) this could be due to the non-metabolized DNA. Degradation of lr-RNA and hr-RNA is fast commencing with the yellowing stage of leaf (Table 3). ^{32}Pi incorporation is observed in all RNA fractions, in all ages and stages of leaves, especially in the young mature leaves with a distinct peak of higher molecular weight fraction, i.e. m-RNA (Figs. 1 b, and 2 b).

Data presented here show that the s-RNA fraction preferentially incorporates more ^{32}Pi in the yellowing and yellow leaves (Figs. 1 c, d and 2 c, d). Apparently this is a characteristic of senescing tissue and probably results from RNAase degradation and thus increases the relative amount of s-RNA. A similar observation was reported by Wollgiehn (1967).

The only difference between nucleic acid profiles of either primary leaves in different stages of senescence or leaves of different ages, is that, in the green mature trifoliate leaves, there is a distinct, newly isolated peak after hr-RNA, which is designated as fraction VII. Otherwise, the nucleic acid metabolism in these two sets of experimental material is more or less the same.

B. Effect of CCC on Nucleic Acids

From preliminary studies, it was concluded that the nucleic acid species in primary leaves collected from different stages of plant growth, or leaves collected from different ages, show no significant difference in the profiles. Therefore, in this work with CCC, only leaf samples of different ages were used, especially to study the relationship between age of leaf and CCC treatment.

Profiles in Fig. 3 (a) indicate no obvious difference between control and CCC treated green mature leaves, except that fraction VII is found in control green leaves. However, a marked difference is observed in the yellow leaves, since more nucleic acid synthesis is still going on (by ^{32}Pi incorporation) in the CCC treated leaves. In Fig. 3 (b) the profile for control and CCC treatment in yellowing leaves does not show any significant difference, but data on specific activity in Table (4) indicate definite differences in the nucleic acid metabolism in these two treatments ; CCC treatment may have some significance in enhanced RNA synthesis. Results presented in Fig. 3 (c) for yellow leaves show some dynamic differences between CCC treated and control plants; while the ability to synthesize RNA is lost in the control, the

RNA synthesis as shown by the incorporation of ^{32}Pi is still going of in CCC treatment (Table 4).

It should be pointed out that the fraction VII appears again in the control green leaves and not in the CCC treatment. But it is observed that after a week's delay in sampling, this fraction is present in the CCC treatment but not in the control (Fig. 4). It is difficult to predict the exact cause(s) for this, except to speculate that the metabolic processes in CCC treated plants may be slowed down a little.

While the overall length of internodes is not greatly reduced at the harvest time (Fig. 5), yet the ^{32}Pi incorporation in CCC (100ppm) treated leaves is relatively higher than the control.

Plants treated with a higher concentration of CCC, e.g. 1000ppm, show a quite different pattern of nucleic acid metabolism. The incorporation of ^{32}Pi into RNA in green leaves is low and decreases considerably in so called yellowing leaves (Figs. 6 a and b). There is little or no ^{32}Pi incorporation in either s-RNA or DNA-RNA. However, a distinct, rapidly labeled fraction, presumably m-RNA is obtained after the hr-RNA fraction (Table 5).

DISCUSSION

The characteristic losses of chlorophyll, protein and RNA which occur during senescence in soybean leaves, are in agreement with previous reports by Osborne (1962) ; Fletcher & Osborne (1966) and Beevers (1967). Lower concentrations of CCC, i.e. 100ppm, preserve chlorophyll and protein (Tables 1 and 2) and delay senescence. Higher concentrations of CCC, i.e. 1000-2000ppm, cause considerable foliar damage and enhance senescence as reported by Pillay & Mehdi (1968).

The phenomenon of senescence or ageing of leaves is capable of manipulations. Considerable efforts have been made by hormonal regulation to manipulate these changes in ageing leaves. Kinetin, GA and retardants have been shown to affect rates of senescence. These observations are difficult to interpret using the current concepts for the mode of action of growth regulators. The reports of Halevy & Wittwer (1965); Harada (1966) ; Kesseler *et al.* (1967) and Pillay & Mehdi (1968) show that application of retardants delay senescence. GA treatment accelerated senescence of *Phaseolus* leaves (Halevy & Wittwer 1965) and soybean

leaves (Pillay & Mehdi 1968). However Fletcher & Osborne (1966) and Beevers (1967) have shown GA to be capable of delaying senescence in Taraxacum and Nasturtium leaf discs respectively. Therefore for those investigators that claim GA delays senescence, the finding that CCC retards senescence is difficult to reconcile, since it is reported that this compound interferes with GA biosynthesis (Ninnemann et al., 1964; Kende et al., 1965 and Harada & Lang, 1965). In view of above, one might expect an impeded GA biosynthesis would reduce GA level in tissue and thus lead to an accelerated senescence. Ruddat & Pharis (1966) used this rationale to explain the accelerated senescence of soybean leaves following AMO-1618 treatment. However, it is significant that Reid & Carr (1967) reported that treatment of peas with CCC resulted in the production of an altered complex of GA-like compounds in the plants. According to Beevers (1967), if this occurred in Nasturtium leaf discs following CCC treatment it is forseeable that the " abnormal " GA-like compounds might retard senescence. Based on the results obtained in this study, we are in no position to either support this contention or discredit it at this time ; further work is necessary to resolve these suggestions.

The declining capacity for protein synthesis

during senescence has been associated with a decreased capacity for RNA synthesis. The most discussed concept is that hormones (Osborne, 1962, 1965; Fletcher & Osborne, 1966 and Sacher, 1965) regulate the DNA-dependent RNA synthesis to control repression and derepression. It also appears that the requirement for RNA synthesis to delay senescence must involve specific types of RNA. Though CCC or kinetin treated tissues (Beevers 1967) remained green, they showed a decline in RNA level. Results presented on the basis of U.V. absorbancy, confirms this observation. Presumably there must be a continued synthesis of the correct species of RNA to maintain cellular activity even though total RNA content declines. In order to decide whether the ageing of leaves has its origin in disturbances of the nucleic acid metabolism, it must be determined, if during ageing, all RNA fractions are influenced in the same way, or if several RNA fractions are specifically influenced. For example, one could assume a specific degradation of the ribosomes or a cessation of messenger RNA synthesis leading to a disturbance of enzyme synthesis.

Results presented in this thesis indicate some alterations in the different nucleic acid fractions after separation on MAK columns. The incorporation of ^{32}Pi is influenced in all fractions in the nearly the same way

in CCC treated (100ppm) and control leaves. Profiles in Figs. 3 (b) and (c) show a retarded rate of RNA breakdown in the yellowing and yellow leaves of CCC treated plants. The r-RNA (fractions IV and V; Figs. 1 c, d and 2 c, d) is very labile and decreases faster than s-RNA (fractions I and II), or the DNA-RNA (fraction III) which is more stable during ageing (Table 3). This observation is in agreement with the findings of Wollgiehn (1967). The m-RNA, found at a later age of the leaves, might be responsible for the synthesis of several hydrolytic and proteolytic enzymes as observed by Balz (1966) in the tobacco leaves; McHale & Dove (1968) in the tomato leaves; Cherry (1967) in the peanut cotyledons and Anderson & Rowan (1967) in the tobacco leaves

The newly isolated fraction VII has to be charaterized by further investigation, since the existing literature does not have a comparable fraction. While this fraction is repeatedly obtained in the control green mature leaves, it is not obtained in the CCC treatment (Fig. 3 a). But a week's delay in sampling the material, this fraction is only obtained in the CCC treated green mature leaves but not in control (Fig. 4). This observation allows one to speculate this delay in appearance of fraction VII, the nature of its composition and its role in senescence. It should be pointed out that

the results obtained do not permit any comparison with the TB-RNA, previously reported by Tester & Dure (1967); Cherry (1969) and Johri & Varner (1970) which normally does not elute in this range with NaCl, but elutes with either sodium lauryl sulfate (SLS) or NH_4OH. However, a suggestion could be made regarding a possible correlation between the retarded rate of RNA synthesis and the rate of RNA breakdown. Plants treated with CCC (1000ppm) show little ^{32}Pi incorporation in dark green mature trifoliates, which is further decreased in the yellowing leaves (Fig.6 and Table 5). Reduction in RNA synthesis might be due to retardation of growth by CCC at higher concentrations.

The data provide evidence that the retardant, CCC, does influence senescence in plants through alterations of nucleic acid metabolism which is consitent with the suggestions of Humphries (1968) and Rennert & Knypl (1967).

SUMMARY

Senescence in intact soybean leaves is accompanied by a depletion in chlorophyll, protein and RNA. Chemical regulation of leaf senescence by the growth retardant, CCC (100ppm), has resulted in the preservation of chlorophyll, protein and a delay in the degradation of RNA components.

The MAK column chromatography of nucleic acid fractions shows most of the RNA losses in the soluble and ribosomal fractions. The DNA-RNA fraction remains more stable in all stages of leaf development. In yellowing leaves, the s-RNA fraction preferentially incorporates more ^{32}Pi; this probably results from RNAase degradation and thus there is an increase in the relative amount of s-RNA. The so called m-RNA peak in yellowing and yellow leaves may be responsible for the production of several hydrolytic and proteolytic enzymes. In addition to the six RNA fractions normally obtained on MAK column chromatography, another fraction VII is obtained. This fraction could be considered as entirely a new one, as no comparable fraction has been reported in literature so far. The appearance of this

fraction VII in 5 weeks old control leaves and in 6 weeks old CCC (100ppm) treated leaves leads one to suggest a correlation between retardation of RNA synthesis and RNA degradation. Higher concentration of CCC (1000ppm) reduces the rate of RNA synthesis.

APPENDIX I

The Methylated Albumin Kieselguhr (MAK) columns were prepared according to the method of Mandell & Hershey (1960):-

A. Methylation of Protein

Five grams bovine serum albumin " fraction V " (Sigma Chemical Co.) were suspended in 500 ml absolute methanol ; 4.2 ml concentrated HCl was added and the bottle tightly sealed and incubated at 37°C for 5 days in the dark with periodical shakings. At the end of incubation time the precipitate was collected by centrifugation at 5,000xg for 10 minutes, and washed with 50 ml methyl alcohol 3 times (collected at 20,000xg). It was further washed twice with anhydrous ether (30 ml and 50 ml respectively), and collected by centrifugation at 12,100xg for 10 minutes. At the termination of washings, it was allowed to evaporate over KOH. A 1% solution of the methylated protein was made in distilled water for the column. (The 1% protein solution should have a pH not less than 4.5)

B. Preparation of Kieselguhr

150 gm Kieselguhr (diatomaceous earth) available as " Hyflo Super Celite " (John-Manville Products Corp., N.Y. City) was suspended in 600 ml of 1 M NaOH overnight. The diatomaceous earth was filtered through Buchner funnel, washed with 450 ml 1 M NaOH, suspended overnight again in 300 ml 1 N HCl, filtered and washed with 450 ml 1 N HCl. To remove the residual acidity, the Kieselguhr was washed with distilled water; resuspended twice in distilled water to remove fine particles and finally dried in an oven.

C. Preparation of MAK

A suspension of 10 gm Kieselguhr in 50 ml of 0.1 M NaCl-0.05 M sodium phosphate buffer, pH 6.7 was boiled, cooled immediately and followed by adding 2.5 ml methylated albumin while stirring.

D. Preparation of MAK-column

The MAK-column used for analysis of nucleic acids was composed of 3 layers in a 2.5 x 25 cm glass column equipped with a porous glass disc, The first layer was made by suspending 1 gm of standard grade paper powder in 20 ml of 0.1 M NaCl-0.05 M sodium phosphate buffer, pH 6.7. When the liquid level nearly touched the powder, the freshly prepared MAK was added

gently to the column with a pipette and the excess MAK was washed down with 0.1 M buffered saline. The third layer was made by adding 1 gm Kieselguhr, boiled and cooled in 10 ml 0.1 M buffered saline. The column was washed with 200-250 ml 0.1 M buffered saline under presure. At the end of washing, some buffered saline was left on top of the Kieselguhr to prevent the column becoming dry. The column was ready for use immediately or stored in the cold for a maximum of 2 days.

APPENDIX II

Bentonite was prepared essentially according to the method of Fraenkel-Conrat et al. (1961).

Forty grams bentonite (Fisher Scientific Co., Toronto) were suspended in distilled water overnight. The suspension was centrifuged at 2,500 rpm for 15 min. The supernatant was collected and centrifuged again at 8,500 rpm for 20 min. The supernatant was decanted and the residue resuspended in 0.1 M pH 7.0 Versene for 48 hours at 25°C. The sediment was collected by centrifuging at 2,500 rpm and discarded. The supernatant was centrifuged again at 8,500 rpm. This precipitate was suspended in 0.01 M acetate buffer, pH 6.0 for 30 min., followed by centrifuging at 8,500 rpm. The precipitate collected was freeze-dried. The bentonite was prepared to give 40 mg/ml with 0.1 M acetate buffer, pH 5.0.

APPENDIX III

The scintillation fluid used for counting aqueous samples composed of toluene, ethylene glycol monomethyl ether, PPO (2,5-diphenyloxazole) and POPOP (p-bis[2-(5-phenyloxazole)] benzene) in the following composition: 1000 ml, 700 ml, 4 gm, and 50 mg respectively.

APPENDIX IV

Protein was determined by the method of Lowry *et al.* (1951). Reagents for the determination were as follows :-

a. 4% Na_2CO_3 in 0.1 N NaOH
b. 4% Na tartrate
c. 2% $CuSO_4 \cdot 5H_2O$
d. Phenol-Reagent (Folin-Ciocalteau) 1:1 dilution
e. Bovine serum albumin (0.1 mg/ml in 0.1 N NaOH)
f. Mix reagents a, b, and c, 100:1:1 in respective order shortly before use.

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