THE CAROTENOIDS OF DURUM WHEAT

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In Partial Fulfillment of the

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by

J. B. Lier

Department of Plant Science

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A dissertation submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

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Major Professor: L. J. La Croix, Department of Plant Science.

ABSTRACT

The carotenoid pigments present in the grain of Triticum durum Desf. cv. Hercules were examined. The developing seed contains the carotenoid pigments of α-carotene, β-carotene, lycopene, intermediate xanthophylls and lutein. The mature seed contains the xanthophyll lutein localized in the seed endosperm.

To allow for comparative studies of biosynthetic pathways of pigments, while eliminating genetic effects, it was considered desirable to have varying levels of mature seed pigment within one variety of durum wheat. CPTA (chlorophenylthiotriethylamine) known to influence pigment synthesis in fruit of other species, was applied to durum wheat by spraying at various stages of plant growth to obtain such material.

Preliminary experiments showed that Hercules seeds germinated on solutions of CPTA produced red coleoptiles, due to the formation of the carotenoid lycopene. First leaves showed red tips, while subsequent plant development appeared normal. Injection of CPTA solutions in the stem of Hercules plants led to harvested seed with increased pigment levels. CPTA was then applied to plants in the field by spraying at various stages of growth. Harvested grain showed a significant increase in pigment, steroid and protein content. Highest pigment levels were obtained with applications of 40 or 56 oz per acre one week after flowering.

Interactions between protein, pigment and steroid synthesis were observed by statistical analyses of levels of these constituents in flour obtained from seeds of CPTA sprayed and non-sprayed plants. Such interactions were most apparent when data are expressed on a per seed basis

and analyses were on large and small seed classes.

For pigment analyses, a method, using methanol as an extraction solvent, was developed, while for steroid analyses, a method used to determine cholesterol levels in sera was adapted to determine β-sitosterol levels in grain.

The development pattern of carotenoid pigments in the durum wheat seed was studied by measuring the levels of β-carotene and lutein through the growing season in seeds of normal and induced high pigment Hercules. β-carotene was present in significant amounts during two periods. The first coincided with chloroplast development up to ten days after flowering, while the second began with the onset of endosperm development between two and three weeks after flowering. Lutein on the other hand was synthesized throughout seed development. No significant differences for these pigments were detected between sprayed and non-sprayed Hercules up to 33 days after flowering. Thus the consistent increase in lutein levels of mature seed observed in sprayed durum wheat was indicative of lutein synthesis later than 33 days after flowering.

Pigment development patterns indicated two complements of carotenoids formed in the seed. The first one was associated with chloroplast containing tissue, while the other was located in the endosperm. It was concluded that lutein is initially synthesized in the chloroplast and synthesis continues till 3 to 4 weeks after flowering, resulting in a relatively constant lutein level in the seed. Further degradative losses were then balanced by the onset of lutein synthesis in the endosperm. In this manner lutein levels in the seed remained essentially unchanged till about 4-5 weeks after flowering. After this time the major synthesis of lutein in the endosperm took place, giving rise to different lutein levels in sprayed and non-

sprayed durum wheat.

A more detailed pattern of pigment synthesis was developed from ^{14}C incorporation studies. These showed that a pool of lycopene was built up in the seeds between two and three weeks after flowering. Only after this pool was formed, was there a tendency towards the formation of intermediate xanthophylls and finally the xanothophyll lutein. Thus the lutein of the endospermic complement of carotenoids did not start to form till at least four weeks after flowering.

The action of CPTA in producing mature seeds with increased pigment levels, appeared to be through its action on the lycopene pool. Seeds of CPTA sprayed durum wheat plants contained a larger lycopene pool. Indications were that in the final stages of maturation all lycopene in the seed was converted to lutein, thus producing a higher concentration of lutein in the mature seed of CPTA sprayed durum wheat.

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INTRODUCTION

The carotenoid lutein is the major pigment in durum wheat and contributes to the desirable yellow color of its products.

Durum wheat varieties with relatively high levels of this pigment have been developed by plantbreeders. So far, the biochemical aspects of pigment synthesis in durum wheat have not been investigated and for that reason form the subject of this study.

The two main objectives were: (1) to obtain one durum wheat variety with two different pigment levels by chemical treatment of the growing plant and (2) to use such material for a comparative biochemical study of different aspects of grain pigment biosynthesis.

The thesis accordingly has been divided into two parts. In Part I several field experiments are described, designed to test the action of CPTA on durum wheat. The mature seeds were analysed for such constituents as pigment, protein and steroid. The experiments were so designed as to facilitate statistical analysis of the results, and an exhaustive statistical analysis was carried out. In Part II experiments are described, designed to test the action of CPTA on the developing durum wheat seed. The object here was to study the mode of synthesis of lutein. It was felt that a comparative study of treated and non-treated durum wheat would shed new light on this problem.

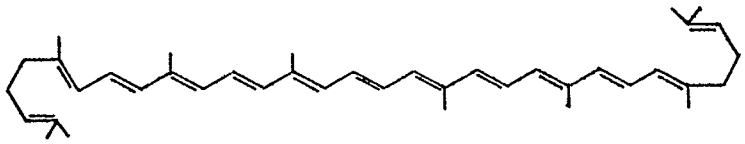
LITERATURE REVIEW

Yellow pigmentation is a desirable characteristic of durum wheat products. In olden times it was noted that the durum wheats grown around the Mediterranean Sea could be used to make macaroni of superior quality. Since this macaroni had a yellow color, yellow pigmentation became associated with quality and has remained so to this day.

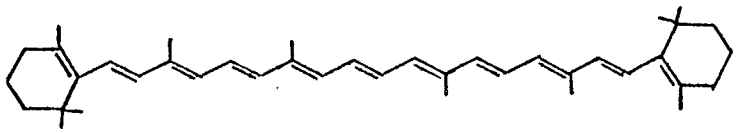
In contrast bread wheat flours are bleached to produce a white product, symbolizing purity.

The yellow pigments present in the wheats are carotenoids. The carotenoids can be divided into three classes:

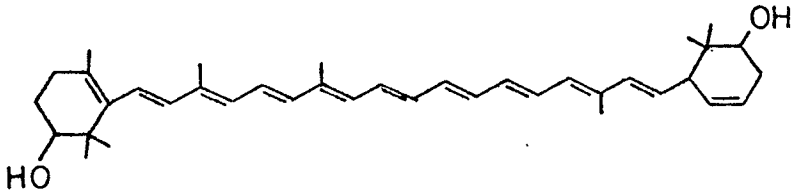
1. Acyclic hydrocarbons e.g. lycopene



2. Cyclic hydrocarbons e.g. β-carotene



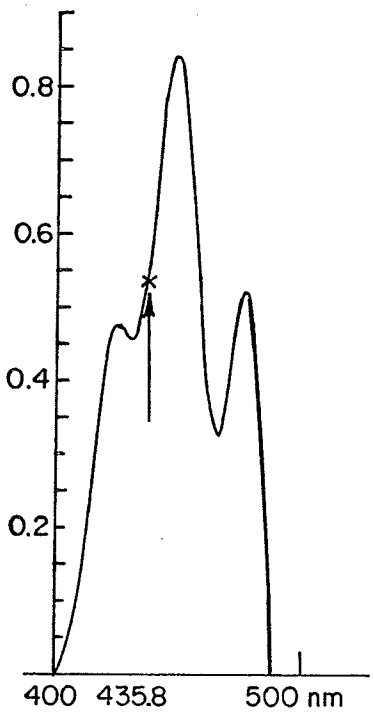
3. Oxygenated products of 1) and 2) e.g. lutein



The first two classes together are called carotenes, while the third class is usually referred to as the xanthophylls.

The light absorpition properties of these pigments provide the best indication of the chromophoric system present. Most carotenoids have a conjugated double bond system and show a typical three peak spectrum in the 400-500 nm range, e.g. lutein.

Figure I

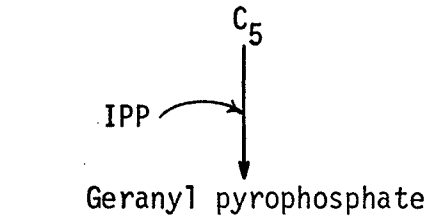


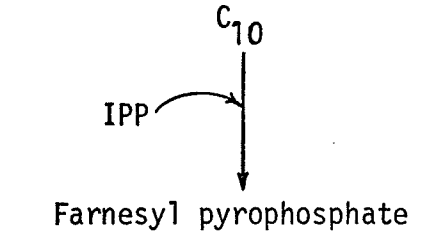
The carotenoids of wheat have not been widely investigated. A reason for this is that investigations of chloroplast carotenoids usually centre on spinach and bean as source material, while investigations of biosynthetic aspects of carotenoid synthesis are usually performed on bacteria, molds or algae. Many such investigations are described in "Chemistry and Biochemistry of Plant Pigments" (1). Most of the information available on wheat carotenoids can be found in the A.A.C.C. monograph, "Wheat: Chemistry and Technology" (2).

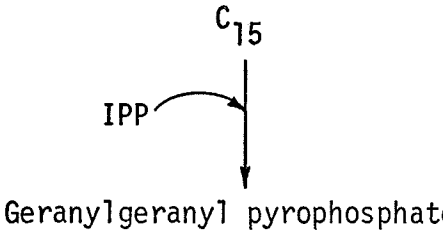
Most of the carotenoids have a C_{40} skeleton and can be considered to be a build up of eight isoprene units. A generalized scheme for the biosynthesis of a carotene is shown in the following diagram.

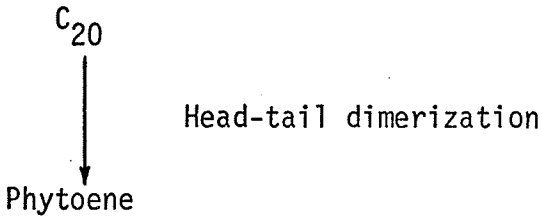
Mevalonic acid (M.V.A.)

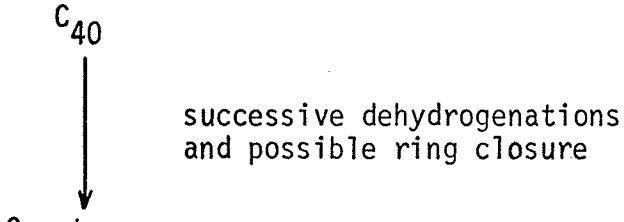
Isopentenyl pyrophosphate (IPP)











C_{40}

Xanthophylls are produced from C_{40} precursors by insertion of oxygen. Using ^{18}O Yamamoto et al (3) proved that in Chlorella the oxygen in lutein is derived from molecular oxygen and not from water. Whether oxygen is inserted before or after ring closure seems a matter of controversy. Costes (4) fed labelled phytoene, lycopene and β-carotene to isolated chloroplasts and found an incorporation into lutein of 16.5% for phytoene and lycopene but only 4.5% for β-carotene. Indeed the pathway from phytoene to the native carotenoid might well be under individual genetic control.

The function of carotenoids is still not completely understood. In the chloroplast they probably serve a protective function in transferral of excessive energy, absorbed by the chlorophyll molecule, thus preventing chlorophyll destruction (1). In the immature grain carotenoids are likely present in the chloroplasts, these are located in the so called "cross layer" of the pericarp (5). These parenchymous cells are empty in the mature grain and the carotenoids thus are presumably broken down with the chlorophyll.

Lutein is present in the mature endosperm of durum wheat. Further investigations will be necessary to show whether this lutein is derived from a new complement of carotenoid synthesis.

The yellow pigment composition of bread wheats and durum wheats differs as shown by Lepage and Sims (6). Durum wheats contain mainly free lutein while bread wheats have a preponderance of lutein esters (Table I).

TABLE I

Spectrophotometric determination of wheat flour carotenoids in water-saturated n-butanol at 450 mm.[a]

Fraction	Carotenoids	Average M.W.	Relative weight percent	
			Mindum %	Thatcher %
A	Lutein diester	1084	5.3	31.9
B	Lutein monoester	826	9.8	46.5
C	Free lutein	568	84.8	21.6

[a] Reference (6).

Lepage and Sims measured pigment concentrations by taking O.D. readings at 450 nm in a water saturated n-butanol extract. In the approved AACC method (7) 435.8 nm is used as the wavelength of measurement. O.D. readings at 450 nm are to be preferred, since they are taken at the high plateau of the lutein spectrum, while 435.8 nm is located on the downward slope (Fig.I). The approved AACC method for pigment determinations is based on the studies of Binnington and Geddes (8,9,10,11) who used 435.8 nm as the wavelength of measurement, because they used a mercury arc light and one of the main lines in its spectrum is at 435.8 nm. Measurements are converted to amounts of β-carotene even though there is little if any β-carotene present in a mature seed sample.

Chen and Geddes (12) determined the distribution of carotene, xanthophyll and xanthophyll ester in a durum wheat and found, respectively, 7.6; 84.8 and 7.6 % of the total. These results could compare with those of Lepage and Sims if the carotene fraction, which was a petroleum ether extract, was equal to the lutein diester of Lepage and Sims, and if the xanthophyll

ester fraction equalled Lepage and Sims monoester fraction.

Chen and Geddes also determined the carotenoid content of the kernels of Thatcher wheat at different stages of development. These results are given in Table II.

TABLE II

Carotenoid content and distribution in Thatcher Wheat at successive stages of development.[a]

(Total carotenoid content, mg per 100 tillers; dry wt.)

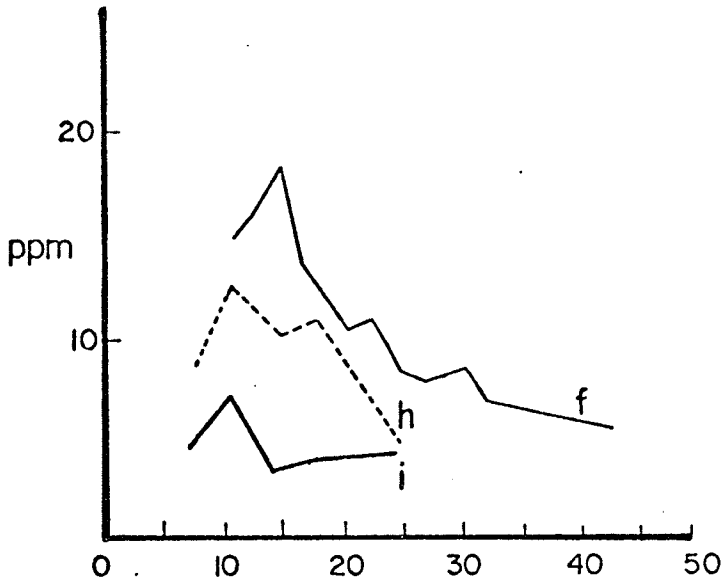
	Days after Blossoming								
	7	10	13	16	19	22	25	28	30
Kernel	0.1	0.59	1.15	0.96	1.24	1.07	0.57	0.38	0.16
Rachis	3.2	3.98	2.95	2.44	2.14	1.97	1.06	0.69	0.30
Stems & Leaves	10.88	19.99	18.45	16.58	13.69	12.66	8.58	4.44	2.11

[a] Reference (12)

A similar study was undertaken by Bouguerra (13) on durum wheat. His results are reproduced in Figure 2.

The data presented in Figure 2 were interpreted as showing an increase in carotenoid pigments up to approximately 12 days after flowering after which their total quite rapidly decreased. Little significance was attached to the second peak occurring about 20 days after flowering. This second peak occurred in the kernels but not in the rachis or the stem and the leaves. This possibly denotes the new synthesis of carotenoids in the endosperm mentioned before.

FIGURE 2



Days after flowering.

Legend

Development of carotenoid pigments in durum wheat cv D.981 through the growing season.

f - Total carotenoids in p.p.m. determined by a colorimetric method.

h - Total β -carotene in p.p.m. determined by chromatography and spectrophotometry.

i - Total lutein in p.p.m. determined by chromatography and spectrophotometry.

The occurrence of this secondary peak in two such disparate experiments reduces the chance of it being caused by environmental factors and requires further investigation.

Environmental control exerts a definite influence on carotenoid synthesis in durum wheat (14). Of the environmental control factors, light and temperature are probably the most important. It is a well known fact that etiolated seedlings contain only small amounts of carotenoids. Ripened fruits often contain large amounts of carotenoids and the process of fruit ripening is influenced by both light and temperature (1,15).

Chemical control over carotenoid synthesis is a subject that has provided the stimulus to much new research in this field during the sixties.

Examples of this are: Goodwins work on diphenylamine (1); Coggins et al (16) on the action of CPTA*; Burns (17) on the action of herbicides; Mummery and Valadon (18) on antibiotics; Poling et al (19) on β-(diethylamino)-ethoxy, -propoxy and -butoxy-benzene; Batra et al (20) on nicotine and CPTA.

Coggins et al (16) showed that they could induce the formation of lycopene in grapefruits and tomatoes. Even though there are no reports of lycopene being present in durum wheat, such a chemical could possibly stimulate the formation of its native carotenoid lutein. Batra's work (20) seems to point against this premise since they show that nicotine and CPTA both inhibit ring closure between lycopene and β-carotene.

* Chlorophenyl thiotriethylamine

When one works with individual carotenoids extreme care should be taken during the extraction and separation procedures because the carotenoids are quite labile. A report by Nelson and Livingstone (20) showed that the carotenoids can be shielded from destruction by the addition of ethoxyquin (6-ethoxy-2, 2, 4-trimethyl-1, 2-dihydroquinoline).

PART I

THE EFFECT OF CHLOROPHENYLTHIOTRIETHYLAMINE (CPTA) ON DURUM WHEAT c v HERCULES

MATERIALS AND METHODS

Hercules, a Canadian variety of durum wheat was used for this study. The action of CPTA on Hercules durum wheat was studied in three different experiments as follows:

Experiment I. This experiment was conducted during the summer of 1971. The experimental design was a four replicate split plot, with plots consisting of 9 rows, 9 ft in length. CPTA was applied as an aqueous solution by means of a pressure sprayer calibrated to deliver 20 gal/acre. Three rates of chemical 8, 16 and 32 oz/acre of active ingredient were applied, at the three leaf stage, at the boot stage and four days after flowering, while the check plots received no treatment. Total pigment content of Brabender milled flour was determined as well as 1000 kernel weight.

Experiment II. This experiment was conducted during the summer of 1972. Rates of CPTA application were 0, 24, 40 and 56 oz/acre. Unsprayed check plots were also provided to test the actions of the wetting agent (Triton X-100) alone, if any. The spray was applied at two stages, one week and two weeks after flowering. The harvested grain was analysed for total pigment of ground grain obtained from a Wiley Mill.

Experiment III. This experiment was conducted during the summer of 1972. The experimental design was a four replicate latin square. A new formulation of CPTA was used in the experiment, providing CPTA in the amine form. The chemical was applied one week after flowering at rates of 24, 40 and 56 oz/acre of active ingredient. Check plots received no treatment. The harvested

grain was separated into large and small seeds by sieving with a 5/64 x 3/4 sieve. Data obtained in this experiment included protein, pigment and steroid content of Buhler milled flour.

Analysis.

Pigment analysis: ten ml of methanol (purified over aluminum oxide) was added to 1.0 - 1.5 g of flour or ground seed in a screw-capped vial. The contents were mixed by swirling and left overnight, mixed again in the morning and left to settle. The supernatant was decanted into a cuvette and its spectrum was determined between 550 nm and 400 nm on a Carey Spectrophotometer. Optical density at 442 nm was used for calculation of pigment content. Pigment content was expressed in parts per million. This method of pigment analysis was developed because the approved AACC method (7) has some inherent flaws (6). If methanol is used as an extraction solvent instead of water saturated n-butanol, optical density readings are higher and the ratio of solvent to sample weight is not as critical.

With methanol as solvent, pigment determinations can be carried out on samples of 10-15 seeds, as well as on larger size samples. Accordingly, for this study methanol was chosen over water saturated n-butanol as the extraction solvent in pigment determinations. Pigment values obtained by the AACC method, though not absolute, are well established and have the merit of relative proportionality. Accordingly, pigment values have been so calculated as to conform with values obtained by the AACC method. AACC method pigment values are calculated by using an absorptivity of 1.6632, i.e. a solution of 1.0 mg pigment in 100 ml of solvent has an optical density at 435.8 nm of 1.6632 (8).

To obtain pigment values that conform to standard method values, an absorptivity of Hercules extracts in methanol of 2.1249 was used. This absorptivity was calculated from the formula:

$$1.6632 \times \frac{\text{O.D. at 442 nm (methanol extract 2 g/10 ml)}}{\text{O.D. at 435.8 nm (butanol extract 2 g/10 ml)}}$$

Steroid analysis: Fifteen ml of a solution of 10% glacial acetic acid in chloroform was added to 2.0 - 3.0 g of flour in a screw-capped vial. The contents were mixed by swirling and left overnight, remixed in the morning and left to settle. Ten ml of the supernatant was transferred to another vial and evaporated to dryness. The residue was taken up in 1 ml of glacial acetic acid, brought to the boiling point and left to cool. Five ml colour reagent was then added and after 10 min the optical density of the resulting solution was read at 662 nm. The colour reagent was prepared as follows: 1.45 g of p-toluene sulfonic acid was added to 600 ml of acetic anhydride and 400 ml of glacial acetic acid. To 25 ml of this solution, 5 ml of concentrated sulfuric acid was added in portions with cooling. A sample of β-sitosterol was used to obtain a standard curve. Results are expressed in mg percent.

This method to determine β-sitosterol levels in grain was adapted from a method used to determine serum cholesterol.(23)

Protein analysis: Protein levels were determined by the macro Kjeldahl procedure.

RESULTS

Results of each of the three field experiments will be presented separately.

Experiment I. Spraying with CPTA after flowering induced a significant increase in pigment content of the harvested seed (Table III). A rate of application of 32 oz/acre after flowering gave a pigment content of 6.5 ppm in the harvested seed, 20% above pigment content of the control sample. The harvested grain was also analysed for 1000 kernel weight. Statistical analysis showed that this seed characteristic was not significantly affected by either the rate or the time at which CPTA was applied.

TABLE III

Effect of CPTA on mean pigment content of Hercules durum wheat in p.p.m.

Experiment I.

Time of spraying	No treat.	Rate of CPTA spray (oz/acre) 8	16	32	Average
Three leaf stage	5.2^{a*}	5.2^{a}	5.5^{ab}	5.1^{a}	5.25^{A}
Boot stage	5.1^{a}	5.1^{a}	5.2^{a}	5.4^{ab}	5.20^{A}
Post flowering	5.7^{ab}	5.6^{ab}	5.9^{bc}	6.5^{c}	5.93^{B}

*Lower case letters, comparisons of 4 treatments at 3 stages of spraying with CPTA. Upper case letters, comparisons at 3 stages of spraying with CPTA. Means with like letters do not differ significantly at the 1% level by Duncan's multiple range test.

Experiment II. The results of this experiment were three-fold. It showed that the wetting agent did not influence pigment content, that the time of spraying should be approximately one week after flowering for optimum effect of CPTA on pigment content and, that a rate of spray of approximately 56 oz/acre should near maximize pigment increase (Table IV). CPTA treatment at 56 oz/acre increased seed pigment content as compared to lower treatment rates, but only when the treatment was applied one week after flowering.

TABLE IV

Effect of CPTA on mean pigment content of Hercules durum wheat in p.p.m.

Experiment II.

Time of spraying (weeks after flowering)	No treat.	Rate of CPTA spray (oz/acre) 0*	24	40	56
One week	5.7^{a**}	5.7^{a}	7.0^{b}	7.1^{b}	7.6^{c}
Two weeks	6.0^{a}	5.9^{a}	6.3^{b}	6.6^{c}	6.6^{c}

* Water and wetting agent only.

** Lower case letters, comparisons of 5 treatments within each stage of spraying with CPTA. Means with like letters do not differ significantly at the 1% level by Duncan's multiple range test.

Experiment III. Spraying with CPTA in the amine form did not result in larger pigment increases than those obtained in other experiments. (Table V). Spraying with CPTA also positively affects both protein and steroid content. The effect predominates in the smaller size kernels.

TABLE V

Effect of CPTA on mean protein, steroid and pigment content of Hercules durum wheat flour.

Experiment III.

Seed Size	Content	No treat.	Rate of CPTA spray (oz/acre) 24	40	56
Large	Steroid	99^a	99^a	104^b	103^a
	Protein*	15.0^a	15.2^a	15.2^a	15.1^a
	Pigment	5.3^a	6.2^b	6.5^c	6.6^c
Small	Steroid	100^a	104^a	109^b	104^a
	Protein	14.0^a	14.4^b	14.7^c	14.6^{bc}
	Pigment	6.3^a	7.5^b	7.7^b	7.7^b

*Protein content in % (N x 5.7 and 0% m.b.)
Pigment content in p.p.m. 0% m.b.
Steroid content in mg/100 g 0% m.b.

Lower case letters, comparisons of 4 treatments for each constituent within seed size. Means with like letters do not differ significantly at the 1% level by Duncan's multiple range test.

Quality analyses: The question remained whether this induced high pigment Hercules would compare favorable with the untreated Hercules in the macaroni making process. Samples of each were therefore tested for their spaghetti-making quality*. The treated Hercules showed a higher pigment content as expected. Other test results were the same, except for protein content, which was shown to be 1.0% higher in the semolina of treated Hercules.

To test whether this difference in protein content was due to the action of CPTA, all samples in Experiment III were analysed for protein content and the data subjected to statistical analysis. CPTA significantly increased the protein content of Hercules durum wheat (Table V).

* Courtesty Grain Research Laboratory, Winnipeg, Canada.

DISCUSSION

In preliminary experiments, it seemed that CPTA showed a negative effect on normal carotenoid synthesis in durum wheat. When durum wheat was germinated in solutions of CPTA, it showed red coleoptiles. The red colour was due to the formation of lycopene. This seemed like an inhibition of ring closure, so that no β-carotene could be formed. However, when the first leaf emerged, it showed only a red tip, while all subsequent leaves were normal in appearance. This could indicate that the action of CPTA was only of short duration, but further experiments showed a long lasting effect of CPTA. If solutions of CPTA were injected in the stem after heading the harvested seeds showed an increased pigment content. As a result of these preliminary findings, the field trials described above were undertaken and a 20-30% increase in pigment level was obtained.

It is difficult to see by which mechanism CPTA could influence both protein and pigment. With regard to pigment, if CPTA affects the mevalonate pool, it should exert an influence on steroid as well as pigment as both share the same pathway up to farnasylpyrophosphate. The influences on protein might be secondary, through the formation of lipoprotein, as steroid and carotenoid are increased. A three component multiple regression analysis might give an indication whether this is the case. To be able to do this multiple regression analysis, the samples in Experiment III were analysed for steroid content as well.

The results of this steroid analysis showed that CPTA treatment signifi cantly increased the steroid level of the harvested seed. Where the pigment increase was 20-30%, the steroid increase was smaller, approximately 4%. The mevalonate pool does not appear to control relative rates of steroid and pigment synthesis since differing percentage increases were observed. If their

synthesis is compartmentalized they should be independent of each other, e.g. C^{14} mevalonate fed to green seedlings does not appear in carotenoids of the chloroplast, since the chloroplast membrane is impermeable to mevalonate. In the endosperm, however, steroid and pigment might be expected to draw from the same mevalonic acid pool, but, if a co-factor like NADPH regulates the level of steroid synthesis independent of carotenogenesis, an increase in the mevalonate pool might be expected to increase the carotenoid level markedly, while only slightly increasing the steroid content.

The multiple regression analysis was carried out on the result of triplicate analyses for the three constituents on all samples of Experiment III and showed, in the overall picture, significant correlation coefficients: positive between pigment and steroid, negative between protein and steroid, and negative between protein and pigment (Table VI).

TABLE VI

Correlation coefficients for the data obtained in Experiment III.

Class	Correlation coefficient	Interaction		
		Pigment/ steroid	Pigment/ protein	Steroid/ protein
Overall	Direct	+0.50*	-0.36*	-0.29*
	Partial	+0.44*	-0.25	-0.14
Seed size large	Direct	+0.12	-0.07	+0.12
	Partial	+0.13	-0.08	+0.13
Seed size small	Direct	+0.43*	-0.15	-0.29
	Partial	+0.41*	-0.02	-0.26

*Significant at the 1% level, measured by the t test. n= 96 overall
n= 48 for seed sizes

However, the significance of these overall correlation coefficients is artificial, since they represent a straight line relationship between two groups of values, those for large seeds and those for small seeds. Therefore, changes occur when the data are analysed on the basis of two seed size classes. No significant correlation coefficients were present in the analysis for large seeds while the picture for the small seeds was similar to the overall picture (n decreases from 96 to 48).

That no significant correlation coefficients were observed in the analysis of large seeds must be due to the large amount of extra storage protein present in these seeds. This extra storage protein could mask any functional relationship that might exist between the three constituents. On a per weight basis protein increased with seed size while steroid and pigment declined. If these values were recalculated on a per seed basis, the concentration of all three increased with seed size (Table VII). Thus, there is a large amount of protein in the large seed that could not be related to pigment and steroid increases and therefore, gave rise to negative correlation coefficients between them in the overall picture. The positive correlation coefficient for steroid and pigment likely means that the action of CPTA is on an early precursor of carotenoid synthesis. These aspects need further investigation.

TABLE VII

Protein, steroid and pigment content of Hercules durum wheat flour of different seed sizes

Seed size	Steroid mg %	Protein %	Pigment ppm	Steroid ug/seed	Protein mg/seed	Pigment ug/seed
Large	99	15.0	5.3	57	8.6	0.30
Small	100	14.0	6.3	41	5.7	0.26

PART II

A COMPARATIVE BIOCHEMICAL STUDY OF DIFFERENT ASPECTS OF GRAIN PIGMENT BIOSYNTHESIS

Experiment IV: This experiment was designed to follow the development of pigments in the seeds of durum wheat c v Hercules. Randomized selections of 200 heads were collected from a durum wheat plot at three day intervals after flowering. The heads were frozen, freeze-dried and stored at -20°C for later analysis.

Experiment V. This experiment was designed to compare pigment development in the seeds of CPTA treated and non-treated durum wheat c v Hercules.

Nine plots of durum wheat were selected. Three of these were sprayed with CPTA (56 oz/acre) at one week after flowering and three others were sprayed two weeks after flowering. When flowering was general one day was chosen to tag heads, which had begun anthesis, sufficient in number for all sample collections. At two day intervals after flowering 25 tagged heads of each of the nine plots were collected and combined by treatment. The heads were frozen, freeze-dried and stored at -20°C for later analysis.

Experiment VI. This experiment was designed to follow pigment development in durum wheat seed by introducing ^{14}C into the biosynthetic pathway by assimilation of labelled CO_2 through photosynthesis. Six plots of durum wheat were selected. Three of these were sprayed with CPTA (56 oz/acre) one week after flowering. To facilitate sample collection a sufficient number of heads were tagged that flowered on one day. At two day intervals after flowering 4 tagged heads of each plot were collected and combined by treatment. The awns and stems were cut back so the heads could be placed in vials with water within a glass tank. The tank was then flushed with air, made free of CO_2 by scrubbing through a NaOH bath,and closed. Within the tank a vial containing $Na_2{}^{14}CO_3$ was tipped over into a lactic

bath to liberate $^{14}CO_2$. Photosynthesis was then allowed to proceed by illumination with a bank of fluorescent lights. Light intensity within the tank was approximately 60 ft candles. After 24 hours one gram of $BaCO_3$ was tipped into the lactic acid to produce excess cold CO_2 and chase $^{14}CO_2$ into the seed. The tank was then left for another 24 hour period. At the end of these two days the tank was opened and the heads were removed. Of each treatment 4 heads were immediately sampled,while the others were left outside the tank exposed to light. Another 4 heads of each treatment were sampled after a 24 hour interval and the last heads were sampled after another 24 hour period. Samples when taken were immediately frozen, then freeze-dried and stored at -20°C for later analysis.

Analysis:

(a) Weight: The heads were taken from storage and threshed. The seeds were collected, counted and weighed.

(b) Chlorophyll: The samples were ground in a Wiley mill (60 mesh screen). Aliquots of ground sample were extracted with methyl alcohol overnight. Spectra were determined (between 750 and 600 nm) on the supernatant with a Carey spectrophotometer. Chlorophyll content was calculated from the formula (1). Total chlorophyll (mg/l) = 25.5 OD650 + 4.0 OD665.

(c) Carotene and Lutein: In experiments IV and V ground seeds were exhaustively extracted with methanol containing approximately 0.1% ethoxyquin. The methanol extracts were flash evaporated and taken up in petroleum ether. These petroleum ether solutions were added to aluminum

oxide columns. The columns were developed to yield fractions of carotene and lutein. Spectra were determined (between 550 and 400 nm) on the two eluates. Maximum O.D. for the two peaks between 440 and 450 nm was used to calculate carotene and lutein content.

Column Chromatography.

Aluminum oxide activity I was used for column chromatography. Best results were obtained if the columns were pre-conditioned as follows. The glass columns were filled with a solution of 1% H_2O, 1% CH_3OH and 0.1% ethoxyquin in acetone. The solution was allowed to drain slowly, while aluminumoxide was added and allowed to settle. The columns were then washed with several changes of acetone, till no more ethoxyquin could be observed in the eluate. The columns were then dried over night at 90°F in a forced air oven and were ready for use.

When petroleum ether extracts were applied to these columns all carotenoids were retained at the top. With ethoxyquin present, carotenes could be eluted first with 0.2% acetone in petroleum ether, while ethyoxyquin could be removed by successive washings with 2%, 5%, 10% and 50% acetone in petroleum ether. Lutein could then be collected by washing the columns with acetone.

It was found in trials preliminary to experiment VI, that traces of ethoxyquin interfered in scintillation counting. For that reason the use of ethoxyquin was omitted in that experiment. In order to still have protection against carotenoid loss, excess carotenoid was added to each sample. First of all a 10 ml beaker full of carrot flour was added to each sample before extraction was started. Then after methanol was added to start extraction some lutein was added to the mixture. This lutein was

obtained by extracting 500 gr of Hercules durum wheat flour with methanol and separating lutein from the extract by column chromatography as in the experiment. The lutein fraction was flash evaporated, redissolved in 50 ml of methanol and divided over eight samples.

Columns treated without the use of ethoxyquin showed the following elution patterns:

0.2%	acetone in petroleum ether elutes	$\alpha + \beta$	carotene
0.5%	acetone in petroleum ether elutes	ξ	carotene
1%	acetone in petroleum ether elutes		lycopene
5%	acetone in petroleum ether elutes		pigment X_1
10%	acetone in petroleum ether elutes		pigment X_2
100%	acetone elutes		lutein

Assay of Radioactivity

The eluates of different carotenoid fractions obtained in Experiment VI were assayed for radioactivity. The eluates were flash evaporated and then taken up in petroleum ether and transferred to a counting vial. The solutions were then treated with chlorine gas, to destroy all color, and left to evaporate to dryness. This destruction of color facilitated counting by eliminating color quenching.

One of three different scintillation cocktails depending on availability, was then added and the samples were counted in a Nuclear Chicago Model 724 liquid scintillation spectrometer. Observed counting rates were converted to absolute values using the Channels Ratio Method. The three different scintillator solutions used were:

I. Aquasol, obtained from New England Nuclear,

II. 2,5-diphenyloxazole (PPO) 10 gr plus 1,4-bis-2-(5-phenyloxazolyl)-benzene (POPOP) 100 mg in toluene 1L.,

III. Napthalene 50 g, PPO 10 g, POPOP 100 mg in dioxane:ethyl cellosolve (5:1) 1L.

RESULTS

Experiment IV

Freeze-dried seed weight increased rapidly during the growing season. A maximum was reached approximately 24 days after flowering, after which the weight decreased slightly and then levelled off (Table VIII, Fig.3).

When calculated as a percentage, seed chlorophyll content showed a steady decline during the growing season. Chlorophyll content per seed however showed an increase up to approximately 9 days after flowering, after which it steadily declined (Table IX, Fig.4).

Seed carotene content, expressed as percentage of seed weight showed a development pattern similar to that found by other investigators (12,13) i.e. a peak at approximately 9 days after flowering with a retardation in the decline at approximately 21 days after flowering. On a per seed basis, however, one can distinguish two definite periods of synthesis at 9-12 days after flowering and at approximately 21 days after flowering (Table X, Fig.5). Seed lutein content showed great variability both on a per weight and per seed basis. Lutein synthesis seemed to lag behind carotene synthesis by a week or more (Table XI, Fig.6).

TABLE VIII

Development of seed weight of a sample of durum wheat c.v. Hercules during the growing season 1971

Days after flowering	Number of seeds	Seed dry wt (mg)	
		Total	Per seed
3	500	3150	6.2
6	500	5590	11.2
9	3476	5170	15.0
12	3932	8830	22.4
15	2879	8760	30.6
18	4240	1473	34.7
21	5153	2015	39.0
24	5216	2357	45.2
27	4554	1889	41.5
30	5832	2447	41.8

FIGURE 3. Freeze-dried weight of developing seed at various times after flowering. Experiment IV.

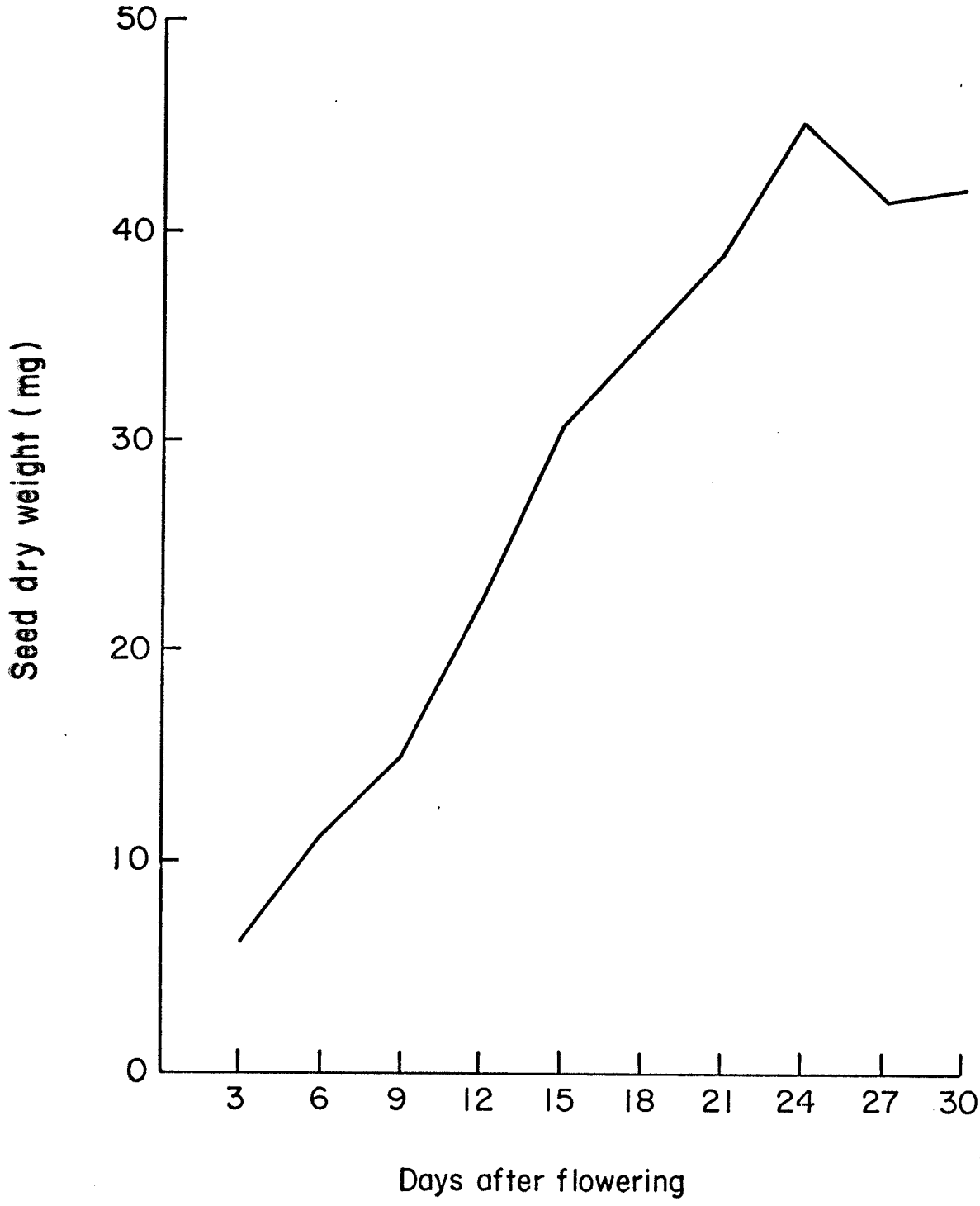

50
40
30
20
10
0
Seed dry weight (mg)
3
6
9
12
15
18
21
24
27
30
Days after flowering

TABLE IX

Chlorophyll content of Hercules durum wheat seed at successive stages of development during the growing season 1971

Days after flowering	Total chlorophyll	
	ug per g. dry wt.	ug per seed
3	455	2.8
6	316	3.4
9	286	4.3
12	170	3.8
15	114	3.5
18	96	3.3
21	70	2.7
24	58	2.8
27	41	1.7
30	30	1.3

FIGURE 4. Chlorophyll content of developing seed at various times after flowering. Experiment IV.

Broken line: Chlorophyll ug x 10^{2}/g D.W.

Solid line: Chlorophyll ug/seed.

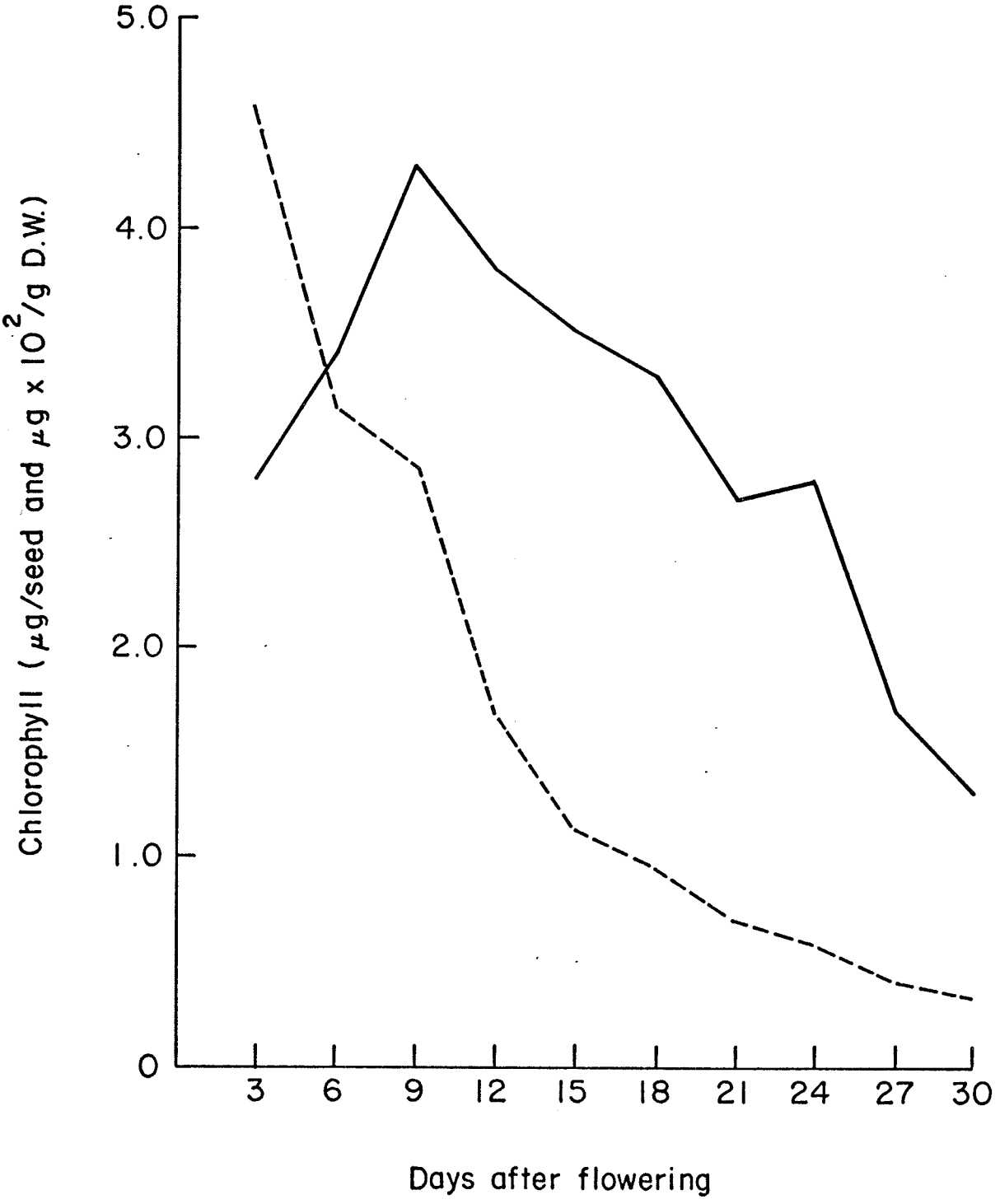

5.0
4.0
3.0
2.0
1.0
0
Chlorophyll (μg/seed and μg x 10²/g D.W.)
3
6
9
12
15
18
21
24
27
30
Days after flowering

TABLE X

Carotene content of Hercules durum wheat seed at successive stages of development during the growing season 1971

Sampling days after flowering	Total + β-carotene	
	ug per g. dry wt.	ug per seed
3	5.7	0.035
6	3.0	0.034
9	7.6	0.114
12	5.0	0.112
15	2.0	0.061
18	2.3	0.080
21	2.6	0.101
24	1.6	0.072
27	1.5	0.062
30	0.8	0.032

FIGURE 5. Carotene content of developing seed at various times after flowering. Experiment IV.

Broken line: Carotene ug/g D.W.

Solid line: Carotene ug x 10^{-2}/seed.

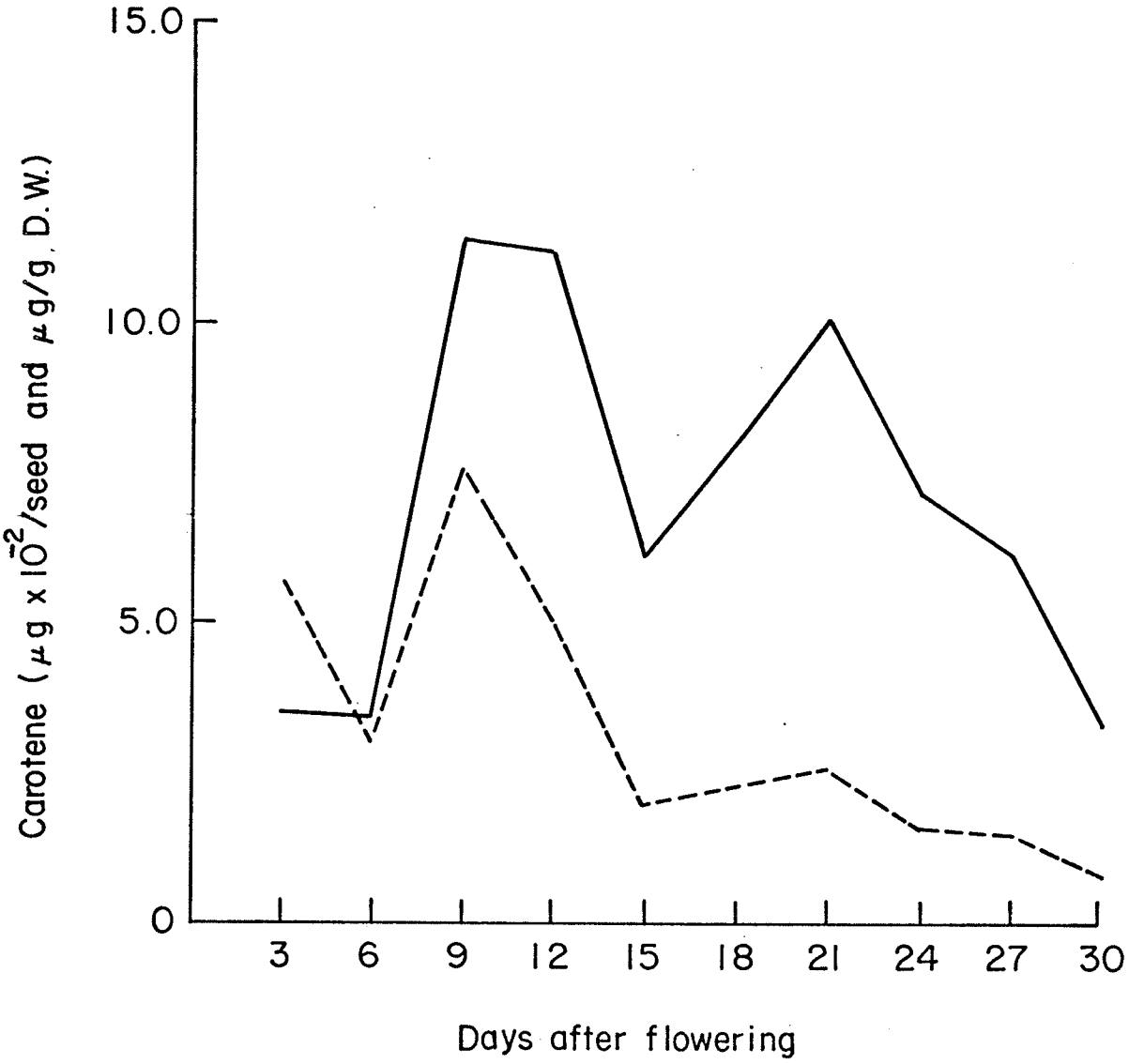

15.0
10.0
5.0
0
Carotene (μg x 10⁻²/seed and μg/g. D.W.)
3
6
9
12
15
18
21
24
27
30
Days after flowering

TABLE XI

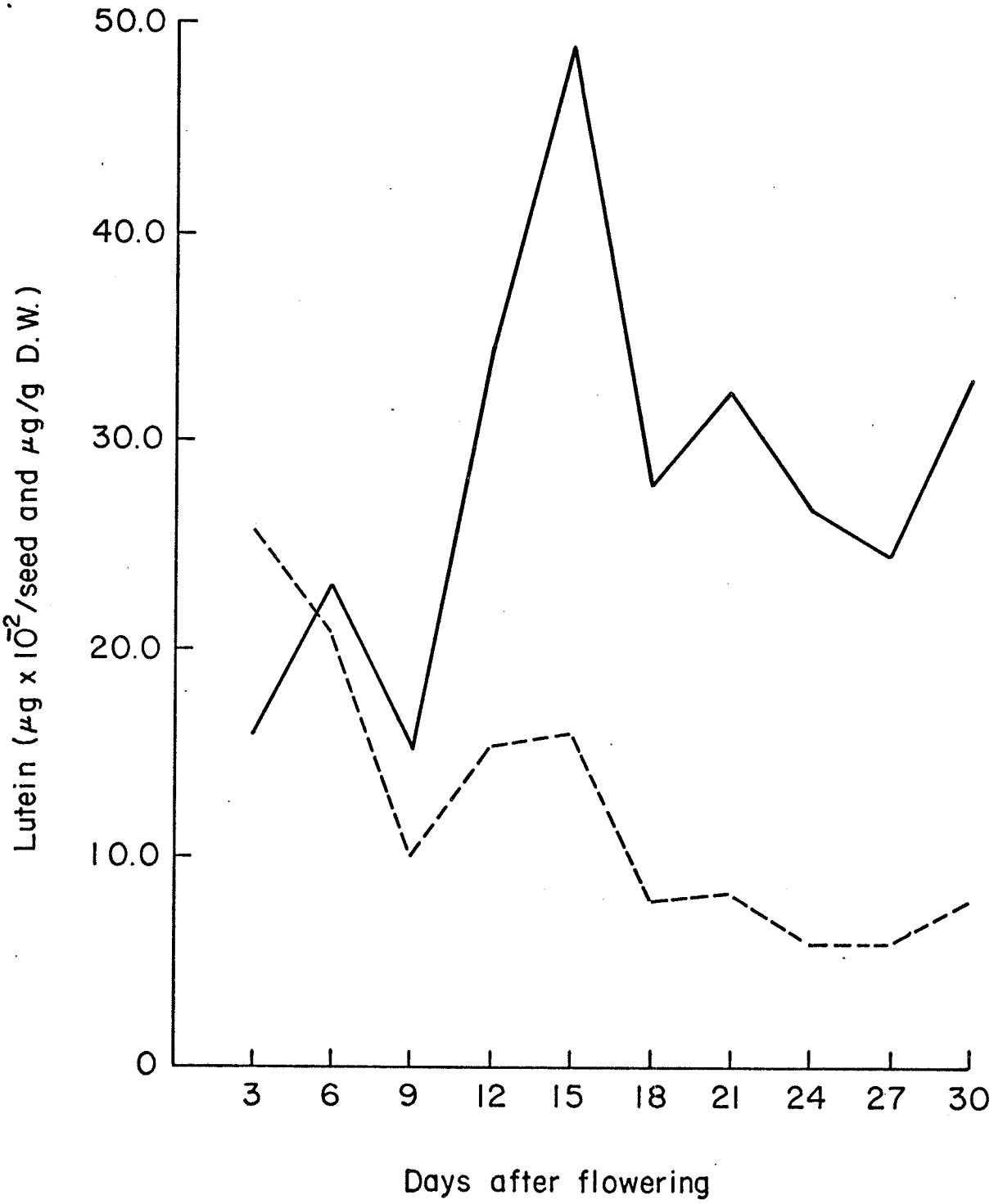
Lutein content of Hercules durum wheat seed at successive stages of development during the growing season 1971

Sampling days after flowering	Total Lutein	
	ug per g. dry wt.	ug per seed
3	25.8	0.160
6	20.8	0.233
9	10.1	0.152
12	15.4	0.345
15	16.0	0.490
18	8.0	0.278
21	8.3	0.324
24	5.9	0.267
27	5.9	0.245
30	7.9	0.330

FIGURE 6. Lutein content of developing seed at various times after flowering.
Experiment IV.

Broken line: Lutein ug/g D.W.

Solid line: Lutein ug x 10^{-2}/seed.


50.0
40.0
30.0
20.0
10.0
0
Lutein (μg x 10⁻²/seed and μg/g D.W.)
3
6
9
12
15
18
21
24
27
30
Days after flowering

Experiment V

Seed chlorophyll content showed a development pattern similar to that shown in Experiment IV. CPTA spraying promoted seed chlorophyll synthesis (Fig.7). The peak of chlorophyll synthesis in this case was at approximately 15 days after flowering. The delay relative to Experiment IV was probably due to environmental factors.

Seed carotene content as in Experiment IV showed two periods of synthesis. The first occurred at ten days after flowering, the second between 17 and 23 days after flowering. Spraying with CPTA two weeks after flowering delayed the destruction of carotene formed during the second period of synthesis (Fig.8).

Seed lutein content seemed to be the most stable of the three pigments with values fluctuating between 0.2 and 0.3 ug per seed. Periods of synthesis and decline alternate during the growing season. Spraying with CPTA at one week after flowering raised the basic level of lutein for about one week and again at about four weeks after flowering. Spraying with CPTA at two weeks after flowering induced a marked increase in lutein content between 21 and 29 days after flowering. At 33 days after flowering, however, lutein levels in all three samples were similar (Fig.9).

FIGURE 7. Chlorophyll content of developing seed at various times after flowering. Experiment V.

(ug/seed)

non-sprayed

sprayed one week after flowering (CPTA 56 oz/acre)

sprayed two weeks after flowering (CPTA 56 oz/acre)

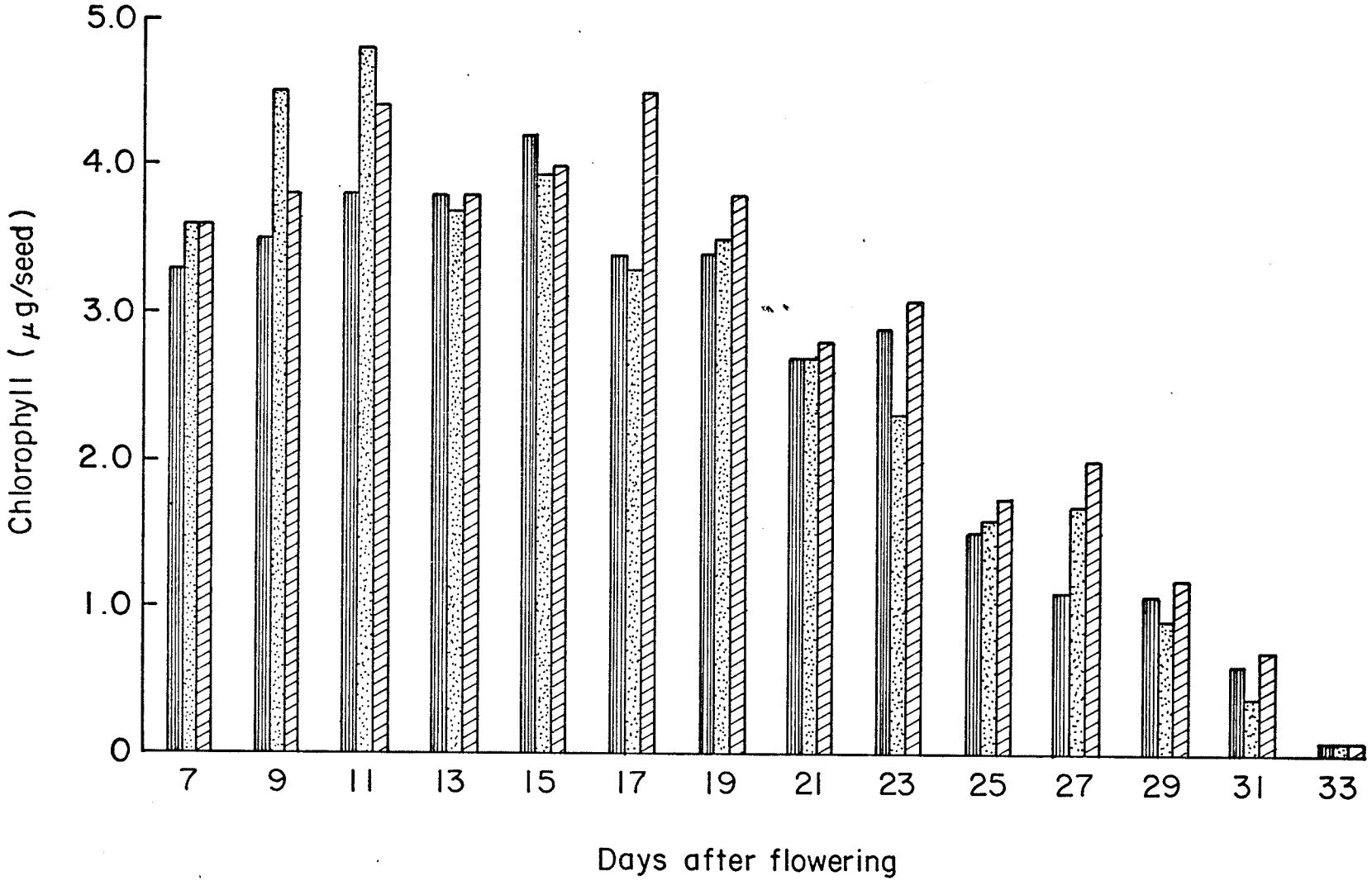

5.0
4.0
3.0
2.0
1.0
0
Chlorophyll (μg/seed)
7
9
11
13
15
17
19
21
23
25
27
29
31
33
Days after flowering

FIGURE 8. Carotene content of developing seed at various times after flowering. Experiment V.
(ug x 10^{-2}/seed)


non-sprayed.


sprayed one week after flowering (CPTA 56 oz/acre)


sprayed two weeks after flowering (CPTA 56 oz/acre)

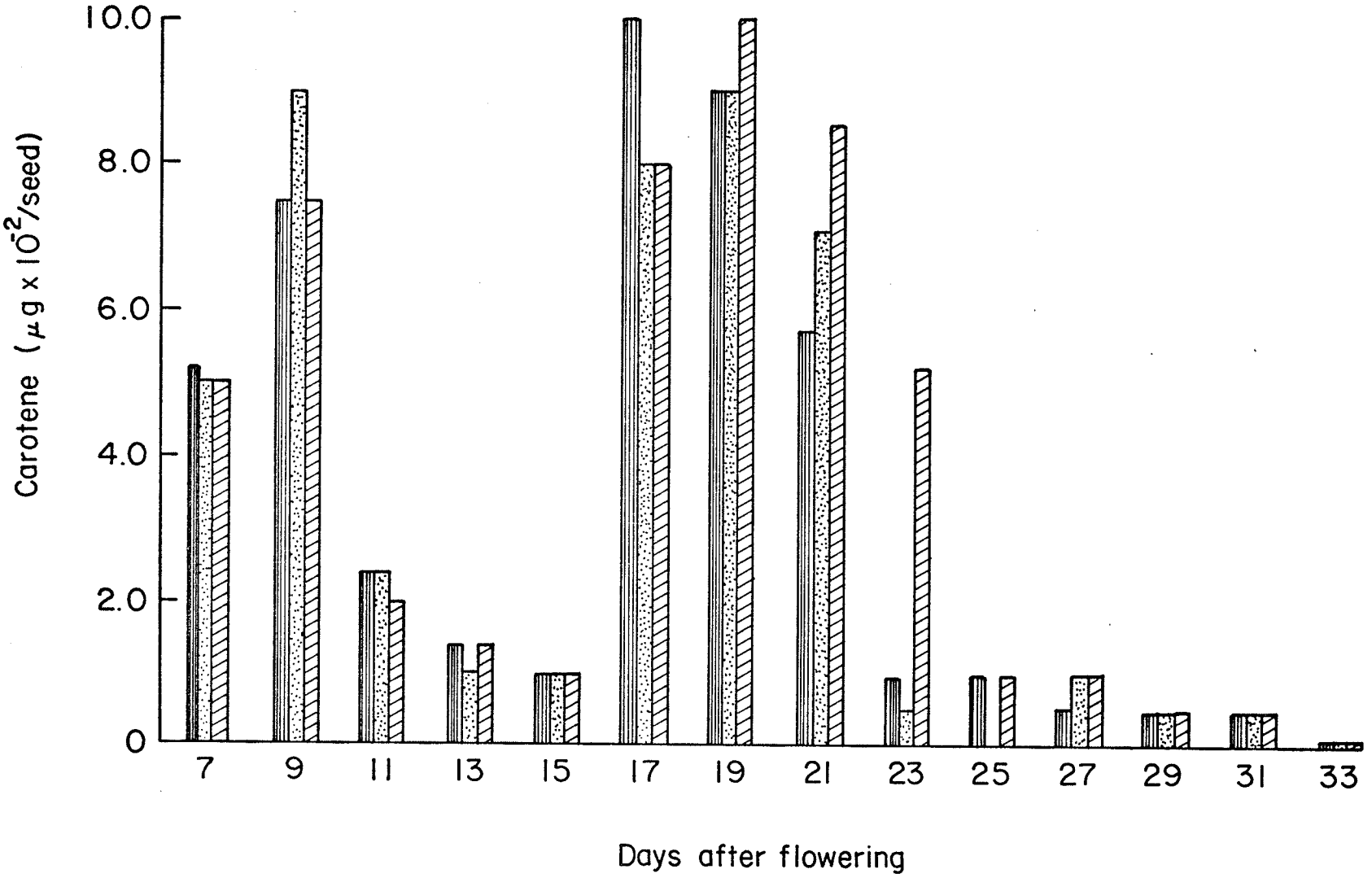

10.0
8.0
6.0
4.0
2.0
0
Carotene (μg x 10⁻²/seed)
7
9
11
13
15
17
19
21
23
25
27
29
31
33
Days after flowering

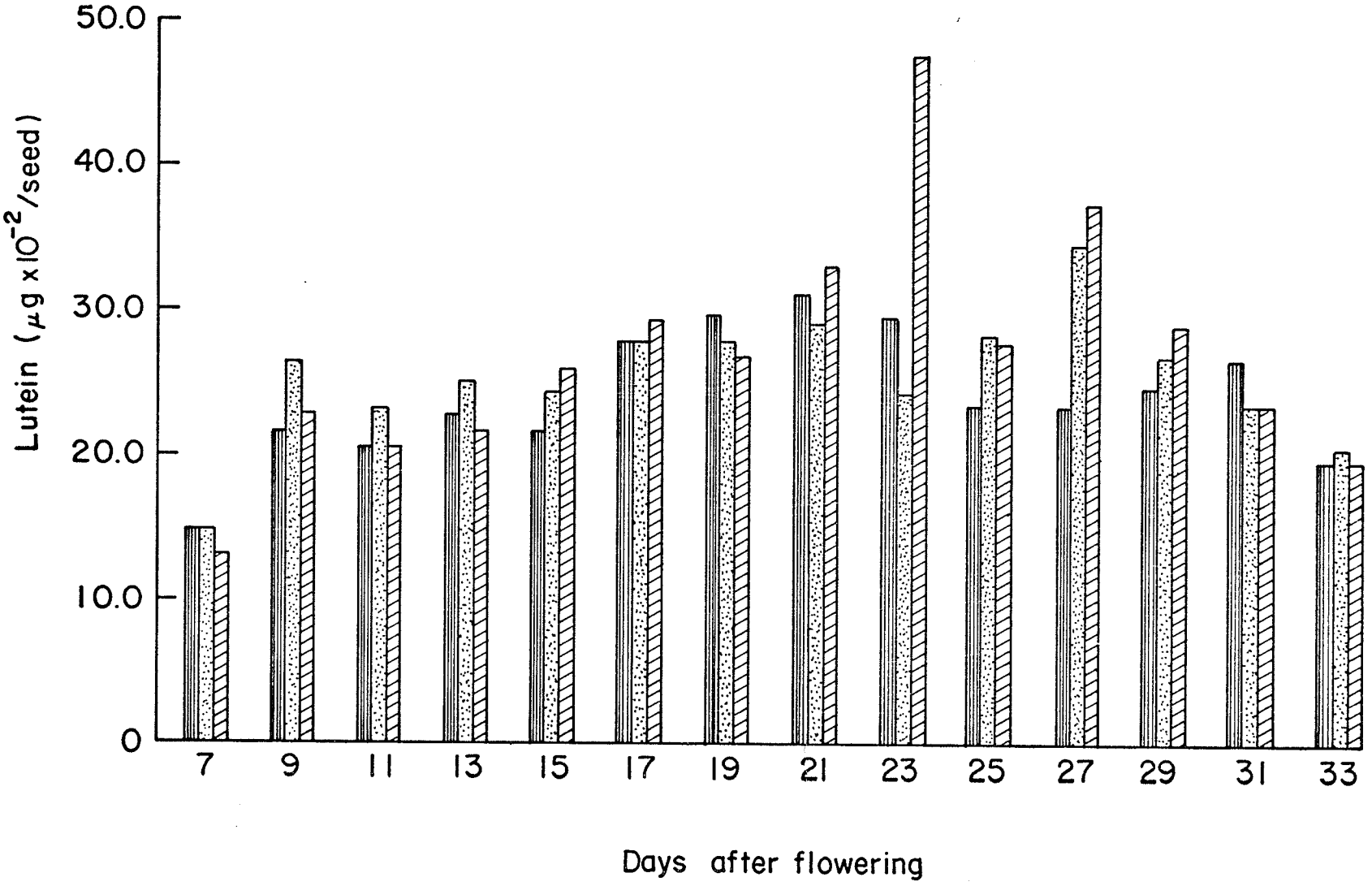
FIGURE 9. Lutein content of developing seed at various times after flowering.

(ug x 10^{-2}/seed)

non-sprayed

sprayed one week after flowering (CPTA 56 oz/acre)

sprayed two weeks after flowering (CPTA 56 oz/acre)


50.0
40.0
30.0
20.0
10.0
0
Lutein ($\mu g \times 10^{-2}$/seed)
7
9
11
13
15
17
19
21
23
25
27
29
31
33
Days after flowering

Experiment VI.

An assay for radioactivity in the different fractions of carotenoids showed that $^{14}CO_2$ was incorporated in all of these (Tables XII to XX).

Total radioactivity determined ranged from 300 - 16000 dpm, or approximately 0.1 - 5.0% incorporation. When the total dpm's in seven fractions of carotenoids was 300, a fraction containing 9% of the total had a radioactivity twice that of background. Reliability of counts less than this is questionable, nevertheless whole percentage numbers were assigned to such fractions, as the results were obtained from 40 minute counts and judged to show presence of some radioactivity. However, in the final analysis of data little importance was attached to fractions containing less than 10% of total d.p.m.'s. The fraction percentages of total d.p.m.'s obtained over each three day period were averaged and these results were graphically represented in Figures 10 and 11.

α + β Carotene: The fractions of total counts in all carotenoids going into α + β carotene were very small from 13-21 days after flowering. They then rose to about 10-15 percent (Figs. 10 and 11).

ζ - Carotene: The percentage of total counts going into ζ -carotene was low from 13-17 days after flowering, then rose to about 25% at 19-21 days after flowering. At 23-25 days after flowering it was very low again, then increased to about 15% at 29 days after flowering (Figs. 10 and 11).

TABLE XII

Percentage distribution of radioactivity incorporated from $^{14}CO_2$ into seven different carotenoids by Hercules durum wheat seed at 13-16 days after flowering.

Carotenoid	No treatment				Treatment (CPTA 56 oz/acre)			
	day 14	day 15	day 16	Average	day 14	day 15	day 16	Average
α -carotene	-	9	7	5	2	1	5	3
β -carotene	4	7	8	6	6	2	5	4
ξ -carotene	9	6	8	8	11	2	4	6
Lycopene	4	57	34	32	3	2	13	6
Pigment X_1+X_2*	66	18	6	30	45	33	26	35
Lutein	18	4	36	19	24	60	47	43
Total d.p.m. incorporated	333	305	345		1102	1282	835	

Total $^{14}CO_2$ fed = 300,000 d.p.m./sample.

* Unidentified pigments. Probably intermediate Xanthophylls.

TABLE XIII

Percentage distribution of radioactivity incorporated from $^{14}CO_2$ into seven different carotenoids by Hercules durum wheat seed at 15-18 days after flowering

Carotenoid	No treatment				Treatment (CPTA 56 oz/acre)			
	day 16	day 17	day 18	Average	day 16	day 17	day 18	Average
α -carotene	6	4	-	3	2	1	1	1
β -carotene	3	3	1	2	2	5	1	3
ξ -carotene	16	10	-	9	8	15	-	8
Lycopene	15	38	37	30	42	36	81	53
Pigment X_1	4	4	37	15	2	9	2	4
Pigment X_2	11	9	6	9	8	10	2	7
Lutein	45	33	19	32	35	25	14	25
Total d.p.m. incorporated	342	678	946		608	886	2768	

Total $^{14}CO_2$ fed = 300,000 d.p.m./sample

TABLE XIV

Percentage distribution of radioactivity incorporated from $^{14}CO_2$ into seven different carotenoids by Hercules durum wheat seed at 17-20 days after flowering.

Carotenoid	No treatment				Treatment (CPTA 56 oz/acre)			
	Day 18	Day 19	Day 20	Average	Day 18	Day 19	Day 20	Average
α -carotene	-	-	-	-	-	1	1	1
β -carotene	4	3	3	3	3	2	3	3
ξ -carotene	3	3	3	3	1	17	16	11
Lycopene	69	73	78	73	65	61	64	63
Pigments X_1,X_2	7	5	3	5	6	4	3	4
Lutein	18	16	13	16	25	14	13	17
Total d.p.m. incorporated	388	1132	1298		1923	3328	2375	

Total $^{14}CO_2$ fed = 300,000 d.p.m./sample

TABLE XV

Percentage distribution of radioactivity incorporated from $^{14}CO_2$ into seven different carotenoids by Hercules durum wheat seed at 19-22 days after flowering

Carotenoid	No treatment				Treatment (CPTA 56 oz/acre)			
	Day 20	Day 21	Day 22	Average	Day 20	Day 21	Day 22	Average
α -carotene	-	-	-	-	1	2	-	1
β -carotene	4	2	7	4	3	4	5	4
ξ -carotene	34	22	19	25	8	38	40	29
Lycopene	31	52	33	39	59	40	37	45
Pigments X_1,X_2	14	9	9	11	10	9	10	10
Lutein	17	15	31	22	19	8	7	11
Total d.p.m. incorporated	1104	730	410		2594	4802	6742	

Total $^{14}CO_2$ fed = 300,000 d.p.m./sample

TABLE XVI

Percentage distribution of radioactivity incorporated from $^{14}CO_2$ into seven different carotenoids by Hercules durum wheat seed at 21-24 days after flowering.

Carotenoid	No treatment				Treatment (CPTA 56 oz/acre)			
	Day 22	Day 23	Day 24	Average	Day 22	Day 23	Day 24	Average
α -carotene	-	1	1	1	1	1	-	1
β -carotene	2	3	3	3	2	5	3	3
ξ -carotene	2	43	6	17	16	54	2	24
Lycopene	56	31	62	50	37	24	64	42
Pigment X_1	3	2	4	3	9	3	9	7
Pigment X_2	17	6	9	11	16	6	11	11
Lutein	19	13	16	16	19	7	10	12
Total d.p.m. incorporated	1637	6923	2087		1592	1592	16390	

Total $^{14}CO_2$ fed = 300,000 d.p.m./sample

TABLE XVII

Percentage distribution of radioactivity incorporated from $^{14}CO_2$ into seven different carotenoids by Hercules durum wheat seed at 23-26 days after flowering.

Carotenoid	No treatment				Treatment (CPTA 56 oz/acre)			
	Day 24	Day 25	Day 26	Average	Day 24	Day 25	Day 26	Average
α -carotene	1	3	-	1	1	1	1	1
β -carotene	14	12	13	13	11	8	9	9
ξ -carotene	2	6	2	3	2	1	2	2
Lycopene	2	5	4	4	5	3	7	5
Pigment X_1	13	23	36	24	17	23	19	20
Pigment X_2	65	47	42	51	61	61	61	61
Lutein	4	4	2	3	4	2	1	2
Total d.p.m. incorporated	2048	1556	9530		2323	7766	1797	

Total $^{14}CO_2$ fed = 300,000 d.p.m./sample

TABLE XVIII

Percentage distribution of radioactivity incorporated from $^{14}CO_2$ into seven different carotenoids by Hercules durum wheat seed at 25-28 days after flowering.

Carotenoid	No Treatment				Treatment (CPTA 56 oz/acre)			
	Day 26	Day 27	Day 28	Average	Day 26	Day 27	Day 28	Average
α -carotene	1	2	1	1	1	1	-	1
β -carotene	6	7	8	7	8	7	9	8
ξ -carotene	5	3	3	4	4	3	2	3
Lycopene	6	4	18	9	7	11	19	12
Pigment X_1	17	20	12	16	21	17	7	15
Pigment X_2	62	62	56	59	57	60	62	60
Lutein	3	2	3	3	2	2	2	2
Total d.p.m. incorporated	1459	2666	1669		2418	4112	5239	

Total $^{14}CO_2$ fed = 300,000 d.p.m./sample

TABLE XIX

Percentage distribution of radioactivity incorporated from $^{14}CO_2$ into seven different carotenoids by Hercules durum wheat seed at 27-30 days after flowering.

Carotenoid	No treatment				Treatment (CPTA 56 oz/acre)			
	Day 28	Day 29	Day 30	Average	Day 28	Day 29	Day 30	Average
α -carotene	3	11	-	5	4	4	4	4
β -carotene	18	12	11	14	8	10	18	12
ξ -carotene	29	14	12	19	26	6	11	14
Lycopene	35	24	27	29	32	37	34	34
Pigment X_1	4	4	-	3	-	1	2	1
Pigment X_2	8	7	4	6	4	5	12	7
Lutein	30	29	45	35	27	38	19	28
Total d.p.m. incorporated	445	481	352		542	1443	1696	

Total $^{14}CO_2$ fed = 300,000 d.p.m./sample

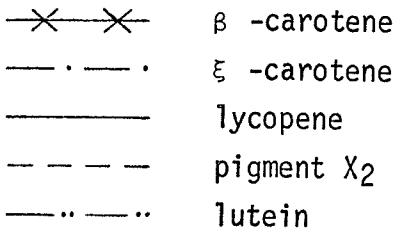
TABLE XX

Percentage distribution of radioactivity incorporated from $^{14}CO_2$ into seven different carotenoids by Hercules durum wheat seed at 29-32 days after flowering.

Carotenoid	No treatment				Treatment (CPTA 56 oz/acre)			
	Day 30	Day 31	Day 32	Average	Day 30	Day 31	Day 32	Average
β -carotene	8	13	10	10	10	7	12	10
ξ -carotene	14	11	11	12	19	12	18	16
Lycopene	37	29	36	34	38	38	30	35
Pigments X_1,X_2	22	31	23	25	22	27	24	24
Lutein	19	16	20	18	16	16	16	16
Total d.p.m. incorporated	302	246	276		317	576	1379	

Total $^{14}CO_2$ fed = 300,000 d.p.m./sample

FIGURE 10. Percent distribution of incorporated ^{14}C in several pigment fractions of developing seed of durum wheat at various times after flowering.
Experiment VI.



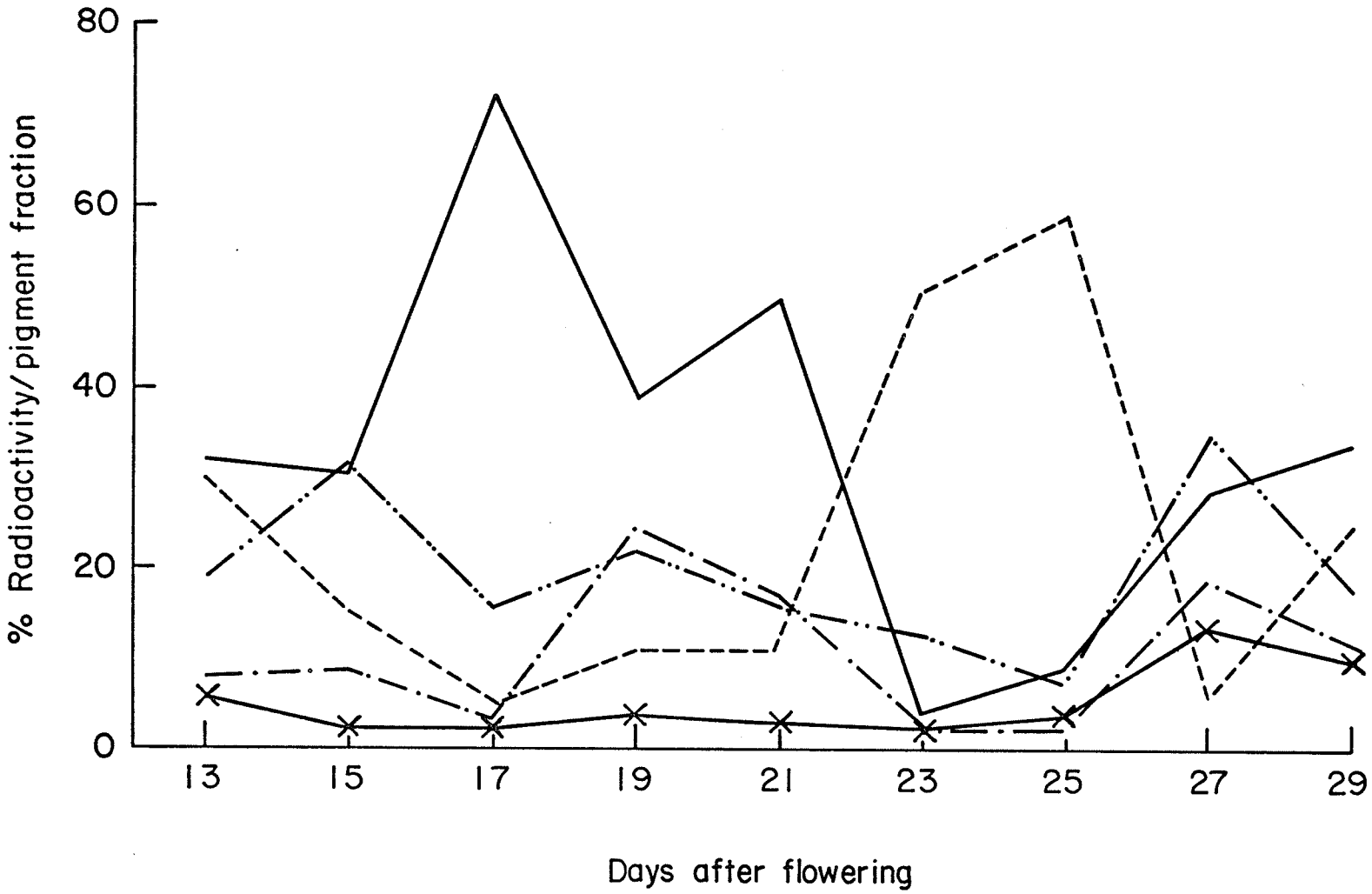
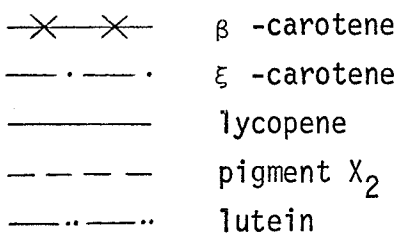
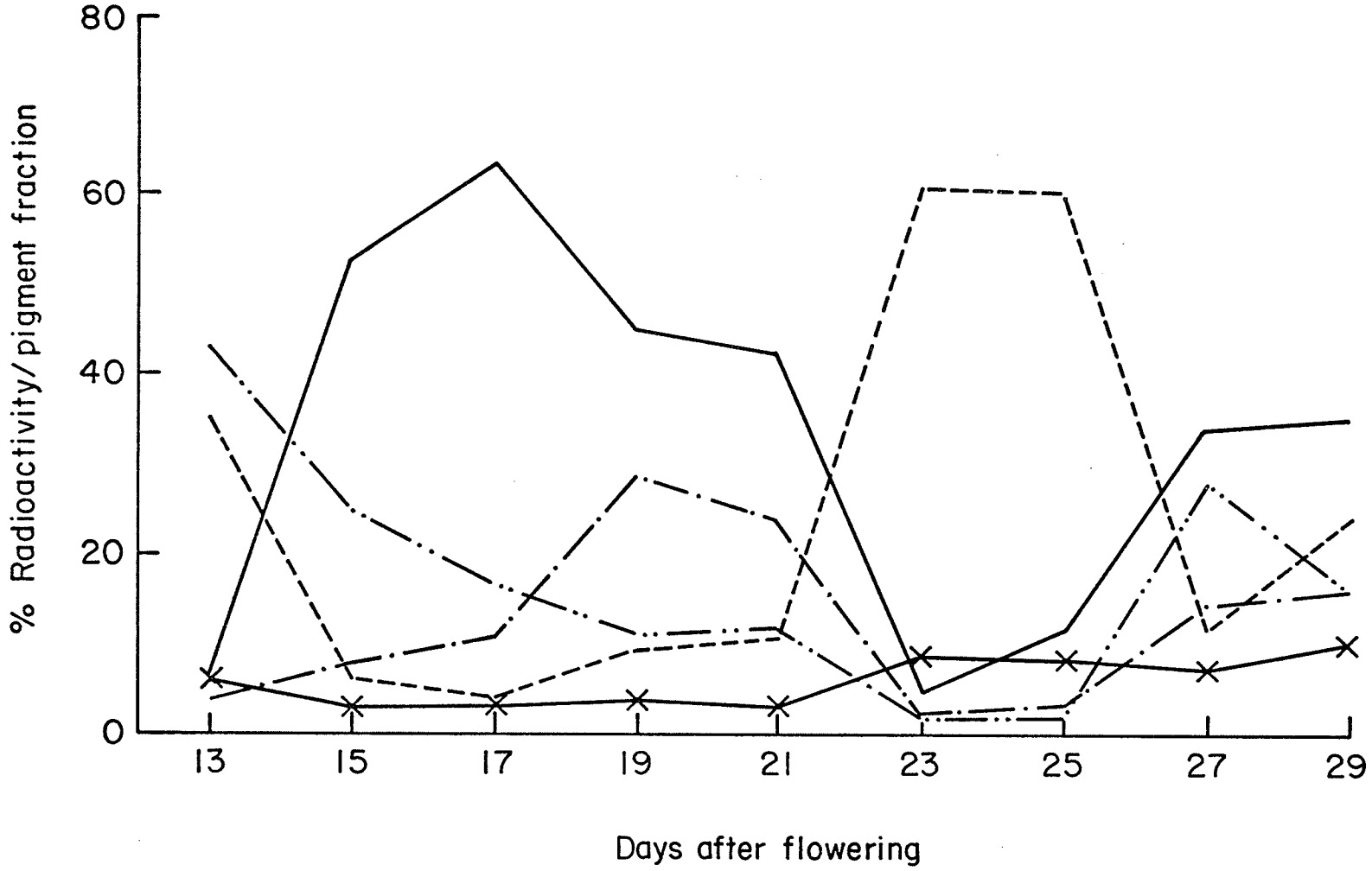

80
60
40
20
0
% Radioactivity/pigment fraction
13
15
17
19
21
23
25
27
29
Days after flowering

FIGURE 11. Percent distribution of incorporated ^{14}C in several pigment fractions of developing seed of CPTA treated durum wheat at various times after flowering.

Experiment VI.




80
60
40
20
0
% Radioactivity/pigment fraction
13
15
17
19
21
23
25
27
29
Days after flowering

Lycopene: This carotenoid seems of a pivotal nature in the formation of the total carotenoid complement of durum wheat. Between 15 and 23 days after flowering from 50-70% of all counts went into lycopene (Figs.10 and 11). During this period incorporation rates were very high. At 21 days after flowering total counts reached values of 16,000 d.p.m. which is an incorporation rate of about 5% (Table XVI), at day 23 the percentage of counts in lycopene was very small, then rose again to about 35% at day 29 (Figs. 10 and 11).

Pigments X_1 and X_2: These pigments could not be readily identified from their spectral characteristics. Their elution pattern suggests that they are intermediate xanthophylls, between lycopene and lutein. These pigments have been combined part of the time (Tables XII, XIV, XV and XX). In Figures 10 and 11 only X_2 is graphically represented. Its contributions to total counts was most important at the start of the experiment, day 13 after flowering and at day 23 and 25 after flowering.

Lutein: The percentage of counts going into this pigment was quite significant all through the growing season. Only at 23 and 25 days after flowering was there a very small incorporation into lutein (Figs. 10 and 11).

DISCUSSION

Interpretation of pigment data is facilitated if results are expressed as ug per seed rather than parts per million (p.p.m.). This is especially true for pigment data obtained during the growing season. Periods of pigment synthesis are easily discerned in the graphical representation of experimental results, if pigment data are expressed in concentrations per seed.

On the basis of concentrations of chlorophyll determined in Experiments IV and V, there appeared to be only one period of synthesis, after which this pigment gradually disappeared. Carotene on the other hand was clearly formed during two different periods. The first one is likely associated with the carotenoid complement of the chloroplast, and disappears with chloroplast degradation. The second period of synthesis could be the beginning of the formation of a new carotenoid complement in the endosperm.

In the case of lutein, there seemed to be many periods of synthesis. Peaks of synthetic activity at 21 days and 30 days after flowering could be associated with the formation of the carotenoid complement in the endosperm. Since endosperm development is actively occurring three to four weeks after flowering. These peaks occurred in both experiments. The period of synthesis at 6 days after flowering observed in Experiment IV was not recorded in Experiment V where collection of samples was not started till 7 days after flowering. The high peak at 15 days after flowering observed in Experiment IV was not present in Experiment V, this seems to be an environmental influence. If lutein synthesis lags behind carotene synthesis by a week or more, this lutein synthesis is

still associated with the chloroplast complement of carotenoids.

The environmental influence mentioned above was probably caused more by soil conditions than by other environmental factors. In Experiment IV plants were under early stress, stands were sparse and dwarfed, due to heavy, non-friable clay.

The plant stress conditions observed in Experiment IV led to an early degradation of seed chlorophyll. Concomitant with this early decline in chlorophyll, there was a rise in carotene content. Whether these two are correlated is not known, but since lutein synthesis follows carotene synthesis in every instance, it seems quite possible that the high peak of lutein synthesis at 15 days after flowering is directly correlated to the increased carotene content. If this is the case, carotene is either an intermediate in lutein synthesis, or a byproduct of a build up of early intermediates in lutein synthesis. Such a hypothesis could possibly be proven by labelling carotene or another intermediate with $^{14}CO_2$ incorporation, and observing whether this label shows up in lutein (Experiment VI).

The action of CPTA on durum wheat during the growing season seems to indicate that in the final analysis the amount of lutein present in a mature seed is determined by a late synthesis of lutein, i.e. after 33 days beyond flowering. At 33 days after flowering lutein levels in sprayed and non-sprayed samples are at the same level and do not show a 20% difference as observed in mature seed samples.

The design of Experiment VI did not necessarily guarantee incorporation of $^{14}CO_2$ into seed pigments, however, incorporation rates of up to 5% were obtained (Tables XII - XX).

Incorporation took place into pigments never suspected to be present in the durum wheat seed. In Experiments IV and V these were not observed in column chromatography, since they were obscured by the presence of ethoxyquin.

During the fractionation of the pigments in Experiment VI it was noted that large concentrations of lycopene were built up in the seeds up to about 20 days after flowering and these persisted till the end of the experiment. These high levels of lycopene were observed both during column chromatography and in the counting vials before destruction of the pigment with chlorine gas. It was also noted that CPTA spraying increased this lycopene pool considerably. At the time that this lycopene pool was building up, incorporation rates into lycopene were high, culminating at day 21-24 (Table XVI), where one of the CPTA sprayed samples contained about 10,000 d.p.m.'s in the lycopene fraction. This was an incorporation rate of about 3% indicating that lycopene formation could form an important part of the anabolic process at this stage of seed development.

When the lycopene pool had grown to a considerable size, conversion of intermediates to lycopene became less rapid and ^{14}C accumulated in ζ-carotene (Figs. 10 and 11) which thus can be considered an intermediate in lycopene synthesis. After 21 days lycopene, though present, was essentially unlabelled and radioactivity in ζ-carotene dropped, at the same time counts accumulated in two unknown pigments which might be intermediate xanthophylls. This seemed to indicate that the enzymes necessary for the formation of these unknown pigments had been induced by the high lycopene concentrations.

The formation of a lycopene pool before synthesis of other carotenoids takes place has been noted before. To wit the experiments described by Goodwin in reference (1) page 157, "The aquatic fungus Rhizophlyctis rosea synthesizes lycopene during the early stages of growth whilst older cultures contain both lycopene and γ-carotene; γ-carotene synthesis begins only after lycopene has reached a steady maximum level. Furthermore, if [2-^{14}C] MVA is added to a medium freshly inoculated with R. rosea and removed when the lycopene level has reached its maximum, then the γ -carotene eventually isolated is unlabelled, although the lycopene is strongly labelled; on the other hand, if the addition of MVA is delayed until lycopene synthesis is at its maximum then the isolated γ-carotene is strongly labelled and the lycopene essentially unlabelled (B.H. Davies, quoted by Goodwin)".

As a lycopene pool seemed to be necessary for the formation of pigment X_1 and X_2 so a pool of these pigments seemed to be necessary before lutein synthesis was started (Figs. 10 and 11). At day 27 the counts in pigment X dropped substantially and lutein became much more radioactive.

The conversion to lutein was not rapid, since at this time all intermediates showed some activity. Indeed it seemed that a steady state had been achieved by day 29.

Comparing day 13 with day 29 after flowering there is a striking similarity. This would suggest that day 13 is near the end of the cycle wherein the chloroplast complement of carotenoids is produced. Lutein synthesis continues for some time after the 13th day and thus will likely continue for some time after the 29th day. Since no lycopene is present

in the mature seed, it is likely that the whole lycopene pool is finally converted to lutein. Since the seeds of CPTA treated plants have a greater lycopene pool, they could end up with more lutein in the mature seed.

One could suggest on the basis of the curve for β-carotene in Figures 10 and 11 that it might be a by-product in lutein synthesis rather than an intermediate, which would solve the problem posed in the discussion of Experiments IV and V. However, this does not explain why there is not a high incorporation rate into β-carotene at about 20 days after flowering, when β-carotene is at its second formation peak. A possible explanation could be that this β-carotene is formed from lycopene present in the pool, because at this time the lycopene pool is no longer labelled and β-carotene formed from it would be non-radioactive. This suggests an approach to future experiments in this area. If the lycopene pool were to be made highly radioactive at about 17 days after flowering in a large number of heads in the field, samples could then be taken all through the growing season to observe what happened to the label.

If β-carotene is formed directly from lycopene in the pool, it should become quite radioactive at about 21 days after flowering. Pigment X_1 and X_2 on the other hand should never become very active in this case. Lutein should become quite active between 29 days after flowering and maturity if eventually all the lycopene in the pool is converted to lutein.

Other future experiments should be directed towards the establishment of concentrations for all postulated intermediates in lutein synthesis

throughout the growing season, and towards establishing their purity, i.e. that radioactivity of a fraction is not caused by another labelled compound with co-chromatographs with the particular pigment.

Another area of future research could be the influence of environment on final lutein levels in the mature seed. Such an influence might be at the time of lycopene synthesis. Since the action of CPTA seems to indicate that it raises the lutein level in mature seed through its effect on the lycopene pool, a similar mode of action might be induced by environmental conditions. A correlation study between environmental factors, such as light and temperature, at 16-22 days after flowering, and pigment levels in the mature seed might prove this point. It has been observed that there are areas in the country that consistently produce durum wheat with high pigment levels (22). It would be interesting to look at climatic conditions in these areas in this critical period. Environmental factors could also influence the final conversion of lycopene to lutein, and harvest conditions might be of importance in this regard. If lycopene is not converted to lutein and remained as such in the seed one would observe a brownish tinge in the flour and pigment extract and such conditions have been observed (22).

In the discussion of Experiment III it was stated that the action of CPTA was likely on an early precursor of lutein. Results of Experiment VI indicate that this precursor might be lycopene.

GENERAL DISCUSSION

This study was undertaken with the object of establishing the biochemical mode of synthesis of the major pigment of durum wheat, lutein. It was felt that if one variety of durum wheat with two different pigment levels were available, a comparative biochemical analysis of these could lead towards the solution of the problem of pigment synthesis.

A chemical approach was followed to obtain one variety of durum wheat with two different pigment levels. Spraying durum wheat c.v. Hercules with CPTA during the growing season had the desired effect, of raising the pigment level of the mature seed significantly. It was felt that CPTA might stimulate the formation of the major carotenoid of durum wheat, lutein, since it stimulated the formation of the major carotenoid in tomatoes, lycopene. Even though this turned out to be the case, our experiments show that CPTA actually stimulates the formation of lycopene and increases its pool,since in the seed the final conversion of lycopene to lutein leads to higher lutein levels in the mature seed. It must be noted that when the experiments were started the presence of lycopene in the durum wheat seed was not even suspected.

The correlation study between pigment,steroid and protein now fits well in the total picture. The formation of the lycopene pool between 2 and 3 weeks after flowering coincides with the start of endosperm development. Steroids and protein are involved, at this time, in the conversion of endosperm from a free nuclear state to a cellular form.

If the mevalonic acid (MVA) pool is high at this time, more steroid and carotenoid will be formed. However if steroid draws from the pool through a regulating system it could only be marginally affected and most of the extra MVA would go into carotenoid formation.

When the lycopene pool reaches near maximum size, the conversion of MVA to lycopene slows down and ε-carotene the intermediate in its synthesis builds up in concentration. At three weeks after flowering and coincident with the lycopene pool having reached a maximum size, an endosperm developmental sequence results in a direct conversion of MVA through its intermediates into lutein. This reaction sequence appears to require a high level of intermediates X_1 and X_2 as evidenced by the increase in radioactivity of these prior to lutein synthesis. This process takes another week, so that the formation of lutein present in the endosperm does not start till 4 weeks after flowering. At this point a steady state is achieved lasting at least till 33 days after flowering, since the amount of lutein present in sprayed and non-sprayed samples at this time is the same. In the final maturing of the sample it seems that the fresh supply of MVA becomes exhausted and the lycopene pool is used for lutein formation. Since sprayed samples have a greater lycopene pool, they end up with more lutein in the mature seed.

If endosperm lutein formation does not start till 4 weeks after flowering, all lutein present in the seed up to that point must exist within the chloroplast complement of carotenoids. And again all carotenes and intermediates in lutein synthesis present in the seed up to approximately 12 days after flowering exist within the chloroplast carotenoids. Since the steady states at 13 and 29 days after flowering appear similar, the

whole chloroplast complement of carotenoids could be formed along identical lines to that of the endosperm complement. However since the chloroplasts are present in the pericarp cross layer, and these cells are empty at maturity, they apparently senesce and their carotenoid complement is apparently destroyed and makes no contribution to the mature seed.

The amount of β-carotene present at different stages of the growing season, 7-12 and 17-21 days after flowering, seems to be no more than a by-product in the overall picture of pigment synthesis. The formation of β-carotene at 17-21 days after flowering, at the time the lycopene pool reaches close to maximal size, suggests it could result from a direct conversion of lycopene within the pool.

This discussion suggests that the premise on which this study was started, i.e. the use of a comparative biochemical analysis, seems to have been a valuable one.

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