Doklady Biochemistry and Biophysics, Vol. 400, 2005, pp. 7–13. Translated from Doklady Akademii Nauk, Vol. 400, No. 1, 2005, pp. 119–125.

BIOCHEMISTRY, BIOPHYSICS, AND MOLECULAR BIOLOGY

Pools of ^{14}C-Malic Acid as a Substrate for Pyruvate Production for the DOXP/MEP Pathway of Biosynthesis of Carotenoids in Chloroplasts

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Presented by Academician V.L. Sviderskii May 15, 2004

Received May 26, 2004

After publishing the data on the existence of a new pathway of carotenoid biosynthesis in chloroplasts, which was termed the DOXP/MEP pathway (by the names of the key biosynthesis products), it became clear that the idea of T.W. Goodwin regarding generalization of the acetate–mevalonate (AC/MVA) pathway of biosynthesis of carotenoids, steroids, and other isoprenoids in the cytosol and chloroplasts was inconsistent [1–3]. The absence of critical consideration of ample experimental data on the synthesis of isoprenoids in the cytosol and chloroplasts in representatives of different taxonomic groups and the assurance of Russian and foreign researchers in the impeccability of Goodwin's school have delayed almost by 40 years the progress in studies on the biosynthesis and renewal of carotenoids in chloroplasts [1–4]. The discovery of interaction between 3-phosphoglycerol aldehyde (3PhGA) and pyruvate (PYR) to form 1-deoxy-*D*-xylulose-5-phopsphate (DOXP), with subsequent transformation of the latter to 2-*C*-methyl-*D*-erythritol-4-phosphate (MEP) [1–4], put the end to the long-term supremacy of the idea of Goodwin and allowed the pathways of isopentenyl pyrophosphate (IPP) synthesis in the cytosol and chloroplasts to be distinguished. This gave rise to further studies of the renewal of carotenoid pool in chloroplasts and search for new substrates for their biosynthesis [5]. An analysis of the pathways of designing contradictory and sometimes just fitted studies on the biosynthesis of carotenoids during the past 50–60 years may be very interesting for the Institute of Natural History. With regard for new data, the clarification of the pathways of biosynthesis, renewal, and conversions of violaxanthine (Viol), neoxanthine (Neo), anteraxanthine (Ant), zeaxanthine (Zea), lutein (Lut), carotene (Car), and lutein epoxide, further studies should be performed at a new level. For this reason, when assessing the renewal of carotenoids, it is necessary to study the time course of incorporation of various key metabolic products in chloroplasts, which are related to the formation of PYR and 3PhGA pools.

It is known that, in the course of photosynthesis in N4 plants, rapid decarboxylation of

phosphoenol pyruvate (PEP) via oxaloacetic acid (OAA) leads to the production of malate (Mal) and aspartate (Asp), which may be involved in Calvin's cycle, in which they undergo decarboxylation with subsequent regeneration of PYR [6]. Thus, it can be assumed that the label from Mal will be incorporated into PYR, and the detached carbon atom will be incorporated through phosphoglyceric acid (PGA) into 3PhGA. In addition, malate may be incorporated into the citric-acid cycle, yielding α-ketoglutaric acid (α-KGA). The possibility of implication of the latter in the biosynthesis of carotenoids of chloroplasts was demonstrated earlier [5].

In this work, we studied the possibilities of incorporation of radioactive label from ^{14}C-malic acid (Amersham, no. CFB-42) into the main carotenoids of chloroplasts in plants with the C4 pathway of photosynthesis. The study was performed with three- to four-week seedling of *Zea mays, Sorghum sudanense, Amaranthus retroflexus*, and leaves of *Bryophyllum, Miskanthus sinensis*, and *Atriplex hortensis.*

Cut leaves were injected with radioactively labeled ^{14}C-malic acid [7] and allowed to incubate in Petri dishes in a radioactively labeled solution in the light (1500 μE m^2 s^{-1}). Xanthophyls and Car were isolated from material fixed in liquid nitrogen using radiochemical purification, as described earlier [8–10]. Pigment preparations were stored in liquid nitrogen. Radioactivity was determined on an Intertechnique scintillation counter (France). To suppress the biosynthesis of carotenoids, experiments were performed in the presence of fosmidomycin, which blocks the DOXP/MEP stage of recombination during IPP production [4, 5]. Characteristics of high-order derivative spectra (D^{IV}–D^{XII}) of isolated preparations were used as additional criteria of their purity. Xanthophyll and Car solutions and in polar

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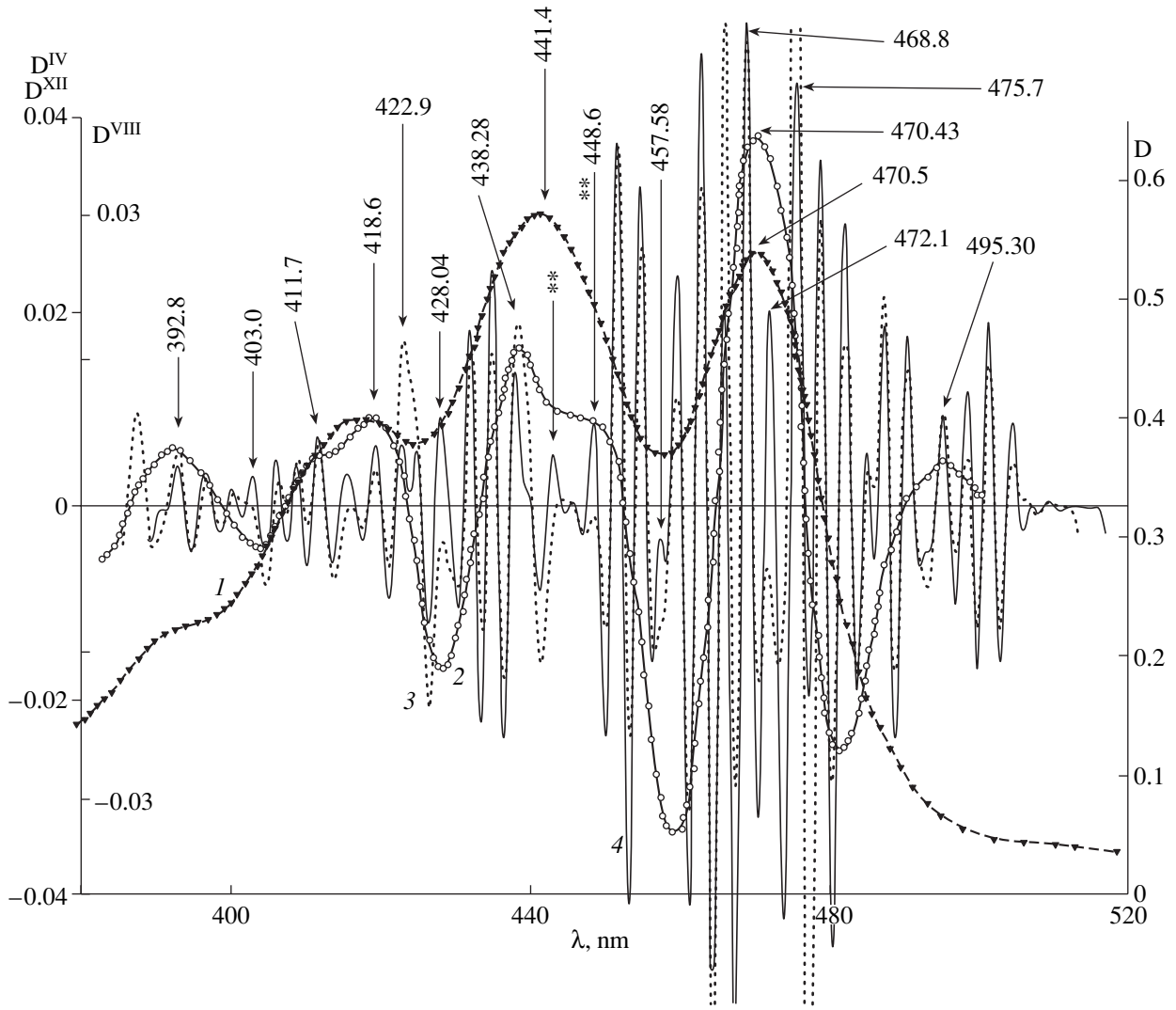

Fig. 1. The absorbance spectrum of violaxanthine solution in ethanol, which was saponified and then sequentially purified in six times (*1*), and its fourth (*2*), eighth (*3*), and twelfth (*4*) derivatives. The arrows with two asterisks indicate the characteristic sites of the spectra, where the use of the twelfth derivative allows better distinguishing the specific features of the spectrum that are poorly resolved in the eighth derivative. Ordinate shows the optical density (expressed in arbitrary units).

and nonpolar solvents were analyzed spectrophotometrically on a UV-VIS Specord 40 spectrophotometer (Carl Zeiss, Jena, Germany), which prints minima and maxima of the spectral curve, thereby making it more convenient for initial monitoring of the process of digitizing. The absorption spectra and their fourth-order derivatives (D^{IV}) were digitized using the Graph Digitizer 2.16 software. Further differentiation of spectra and the plotting of data were performed using the Microcal Origin 6.1 and Spectra Calc software, respectively.

Figures 1–4 show the absorption spectra of carotenoids and their high-order derivatives that were not published earlier. Data shown on Fig. 1 (curve *2*) indicate that even D^{IV} reliably reveals five absorption maxima in the absorption spectrum of Viol in ethanol (EtOH), whereas only three distinct maxima can reliably distinguished on the spectral curve *D*. The advantages of the D^{VIII} spectrum are obvious: the number of bands that can be distinguished on the spectral curve become greater almost in six times. The D^{XII} spectrum is especially worth mentioning. Recording the D^{XII} spectrum allows to distinguish and specify the positions of extrema of bands on the wavelength scale. In particular, this applies to those bands that are less pronounced in the D^{VIII} spectrum (specifically, 403.0, 418.6, 428.04, 448.6, 457.58, 472.1, 475.7, 484.97, and 492.88 nm). The properties of the D^{XII} spectrum are manifested most distinctly in the major absorption maximum (shown with arrows with two asterisks at 441.4–443.13 and 448.6 nm).

For reliable pigment identification, spectral characteristics of the pigment in polar and nonpolar solvents are usually used. Figure 2 shows the spectra of Viol in

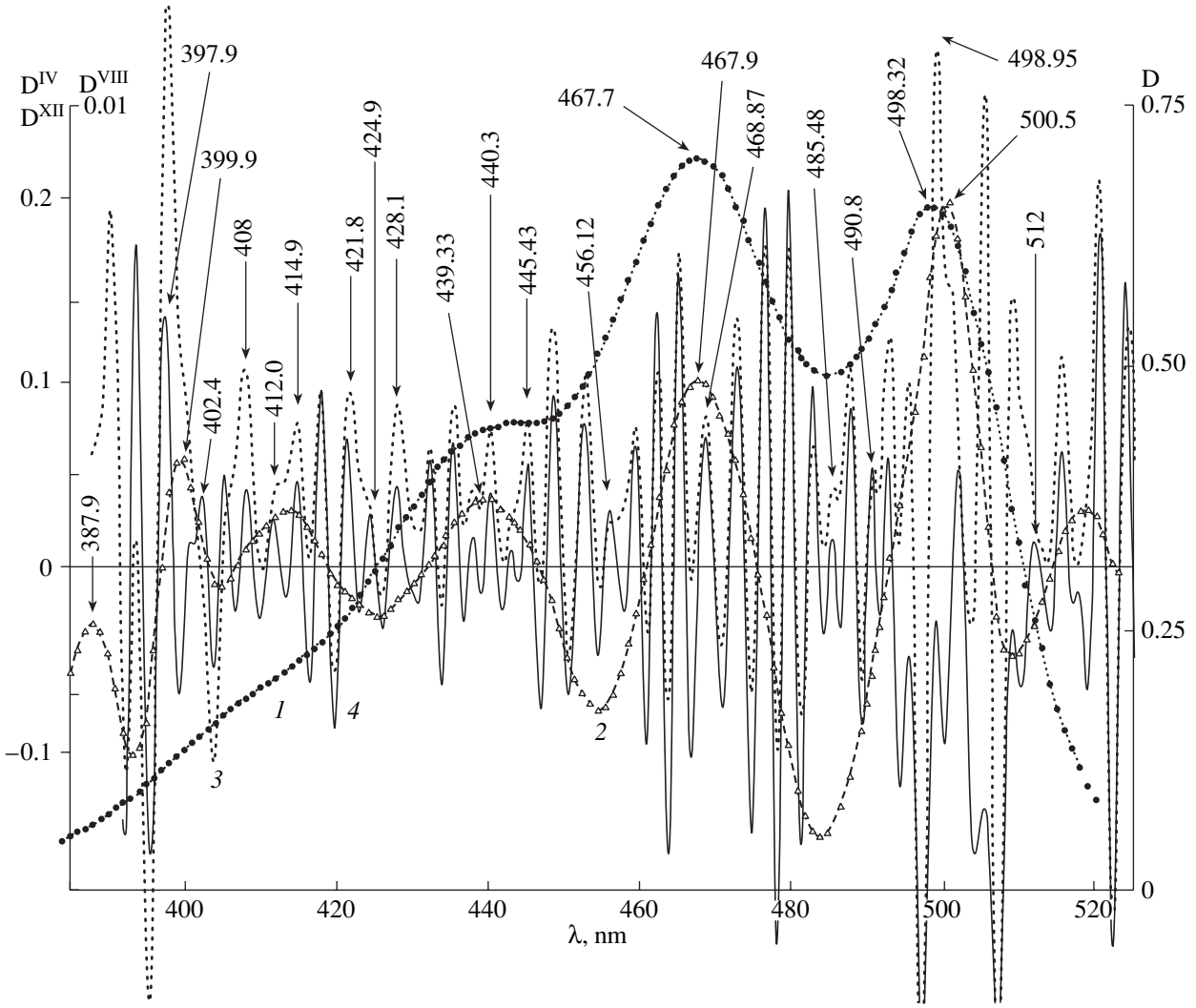

Fig. 2. The absorbance spectrum of violaxanthine solution in carbon disulfide, which was saponified and then sequentially purified in six times (*1*), and its fourth (*2*), eighth (*3*), and twelfth (*4*) derivatives. For designations, see Fig. 1.

carbon bisulfide (CS_2); curve *2* illustrates the advantages of the D^{IV} spectrum for identification of slight changes in the D spectrum. Similarly, according to the data shown in Fig. 1, the D^{VIII} spectrum allows specifying the absorption maxima that are not pronounced in the D^{IV} spectrum. A lesser half-width of bands on the D^{XII} spectrum allows determining more precisely the positions of unpronounced maxima and arms of the D^{VIII} spectrum, located at 399.9, 402.4, 412.0–414.9, 421.8, 424.9, 438.07–439.33–440.3, 443.2–445.43, 456.12–459.71, 485.48–487.73, 498.95–501.92, 512.0–515.39, and 517.7 nm. Taking into account the small size of pictures in the journal, it is more reasonable to display scanned figures and to analyze the specific features of one or another band at a high resolution on a monitor. The aforementioned considerations regarding Viol also apply to Lut. Figure 3 shows the absorption spectra of Lut and their fourth- to twelfth-order derivatives in freshly distilled carbon tetrachloride. Four distinct peaks easily detectable on the D^{IV} spectrum illustrate advantages of recording the fourth-order derivative spectrum. Absorption curves of the D^{VIII} spectrum have a lesser half-width (Fig. 3, curve *3*); they allow the positions of the major maxima to be determined more precisely. Similarly to Figs. 1 and 2, some regions on the spectral curve D^{XII} on Fig. 3 are resolved much better than those on D^{VIII}, on which these bands are latent or expressed only as a tendency. These are bands at 401.1, 408.0, 410.58, 416.8, 418.9, 421.9, 425.5, 428.0, 432.93, 453.2, 462.0, 464.6, 467.3, 471.7, 488.9, 498.1, 501.3, 504.4, 507.4, and 509.6 nm. Note the change in the contour of the spectral curve at 446 nm upon the transition from D^{IV} to D^{XII}.

Figure 4 shows the spectra of Car in methanol (MtOH). All the above considerations regarding the specific features of the derivative spectra D^{IV} and D^{VIII} apply to the absorption spectrum of carotene as well. Note the splitting of the band corresponding to the peak

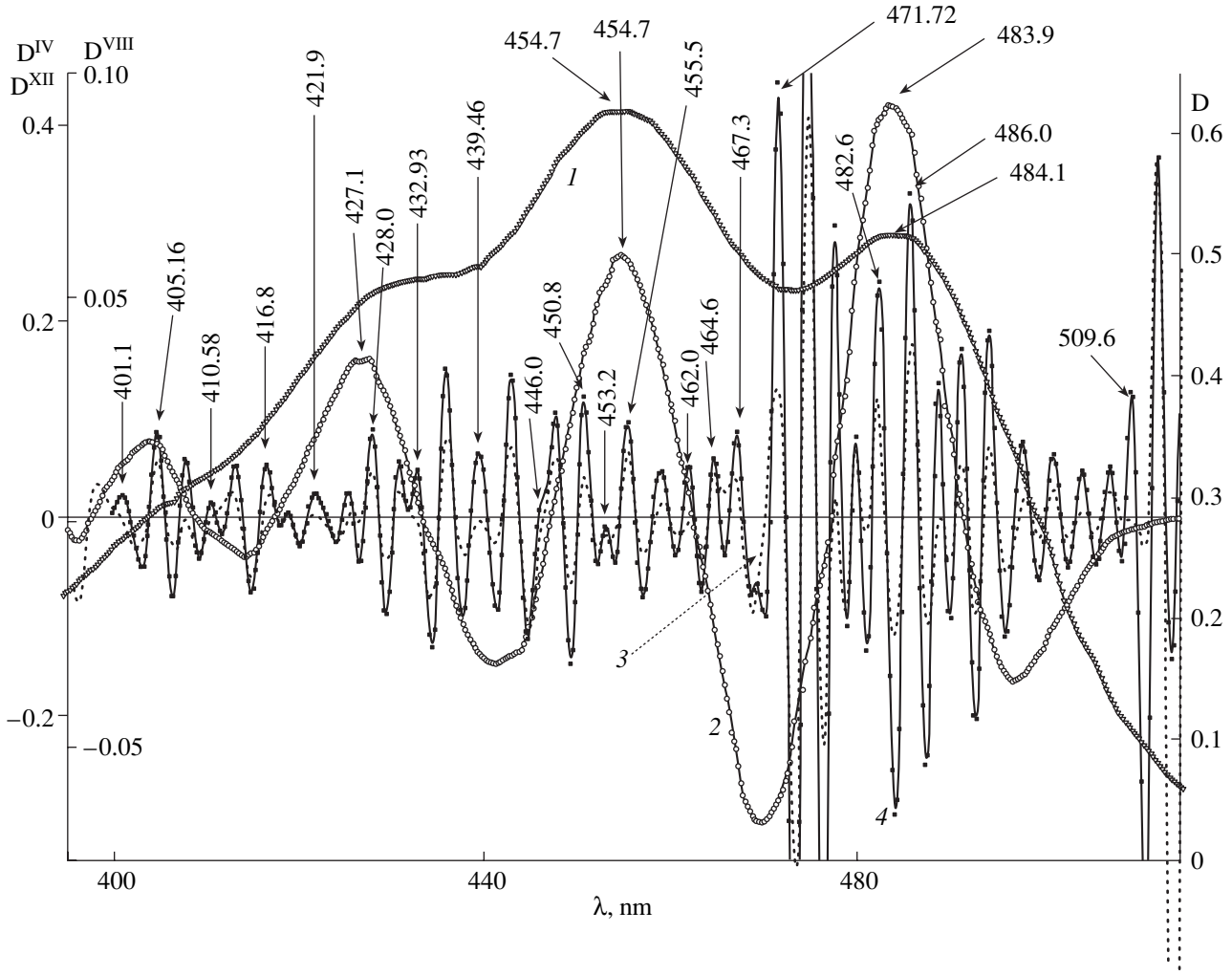

Fig. 3. The absorbance spectrum of lutein solution in carbon tetrachloride, which was saponified and then sequentially purified in six times (*1*), and its fourth (*2*), eighth (*3*), and twelfth (*4*) derivatives. For designations, see Fig. 1.

at 410.8–410.75**–410.78 nm, as well as the expression of latent bands on the D^{VIII} spectrum (at 417.5, 435.6–437.8, 440.1, 444.9, 450.9, 457.65–459.7, 467.1, 492.3, 498.2, and 507.4 nm).

Thus, taken together with the previously published data on the derivative spectra of Viol in acetone, CCl_4, and methanol [5, 11], Lut in methanol and CS_2 [4], carotene in ligarine and CCl_4 [4, 5], and neoxanthine in acetone [5], the results of this study provide reliable basis for identification of carotenoids during the isolation and purification of them, when determining the pathways of label incorporation into certain pigment fractions.

The table summarizes the results of studies on the time course of incorporation of ^{14}C-Mal into the fractions of different carotenoids as a function of the duration of exposure to a labeled substrate. Based on these data, the following conclusions can be made. First, the radioactive label from Mal is obviously incorporated into the major carotenoid fractions of chloroplasts. Second, the time of incorporation of the radioactive label into pigments is short and corresponds to the time characteristic of Mal metabolism in chloroplasts in the C4 plants [12]. Third, a rapid increase in the specific radioactivity (SR) of pigments during the first 3–25 min decreased between the first and second hours of exposure. The increment in SR of Viol, Neo, Lut, and Car by the second hour of exposure in the mean of six first experiments was 8.3, 13.5, 17.5, and 18.1%, respectively (table). This is indicative of a rapider saturation of Viol pools with the radioactive label and, therefore, a rapider renewal of pool of this pigment, which is the key xanthophyll in the violaxanthine cycle of light reactions [10]. The second place with respect to renewal rate belongs to Neo—an intermediate pigment in the violaxanthine cycle [13, 14]. The renewal of Lut and Car, the final compounds of Viol reduction, proceeds more slowly at similar rates. The results obtained are in agreement with the data on the incorporation of radioactively labeled CO_2 and glucose [4, 10] and α-KGA [5]

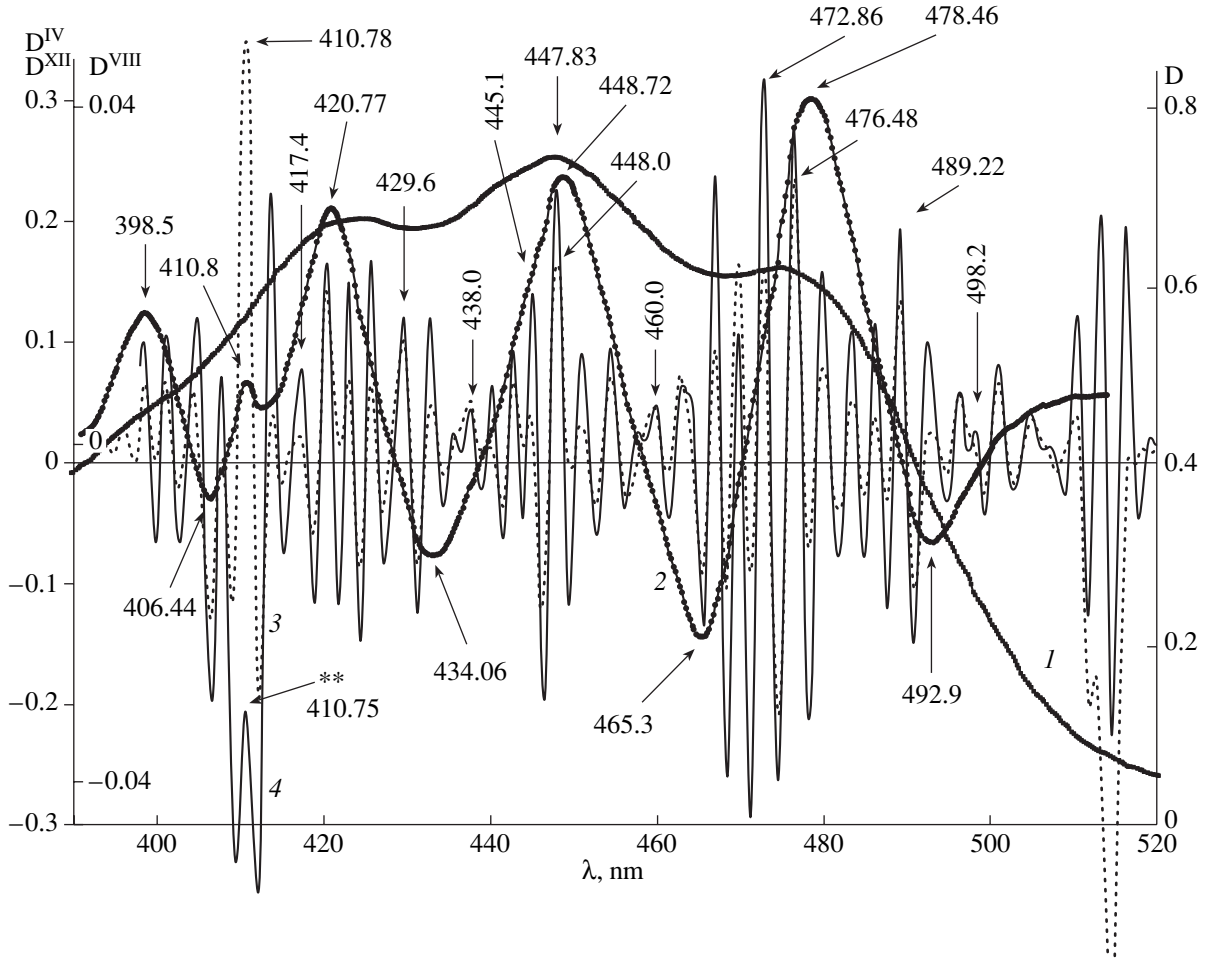

Fig. 4. The absorbance spectrum of carotene solution in methanol, which was saponified and then sequentially purified in six times (*1*), and its fourth (*2*), eighth (*3*), and twelfth (*4*) derivatives. For designations, see Fig. 1.

into carotenoids. The data presented in the table also show that, similarly to the previous results [4, 5], fosmidomycin did not completely inhibit the synthesis of carotene and xanthophylls in the study objects. Apparently, as we discussed earlier, this is related to the specific features of the C4 plants, which possess a second decarboxylating system in combination with additional Mal pools [4]. Indeed, *Z. mays* and *S. sudanense* are classified in group I of the C4 plants [6, 12], the chloroplasts of mesophyll in which have a granular structure, less pronounced than that of chloroplasts of the coat. The role of Mal as a source of CO_2 in the reactions of Calvin's cycle upon its transfer from mesophyll to chloroplasts to form PYR from the backbone of Mal is most pronounced in group I plants. In addition to PEP formation, PYR may interact with 3-PhGA of mesophyll, yielding the substrate for IPP production. In this case, $^{14}CO_2$ released is then incorporated through ribulose diphosphate (RuDP) into 3-PhGA of coat cells. This pathway represents the second branch whereby IPP can be produced, along which the exogenous radioactive label is incorporated into IPP. In the other group of C4 plants, in which *A. retroflexus* and *Atripex hortensis* can be classified, mitochondria of coat cells play an additional role in the course of Asp and Mal decarboxylation to form PYR and release CO_2. The latter also interacts with RuDP, thereby providing for the second branch of IPP formation [6]. An easy exchange with Mal between the cytoplasm and chloroplasts and its rapid conversion into OAA was reported by U. Heber, G. Krause, and K. Santarius [6]. This process is accompanied by a typical compartmentalization of reactions in coat cells. In addition, part of PYR pools is transferred from the cytoplasm of coal cells to the cytoplasm of mesophyll cells (via formation of alanine and regeneration of PEP). Thus, it cannot be ruled out that it is the interaction between the two branches of IPP biosynthesis enables a rapid incorporation of the label from Mal into xanthophyll pools. The rates of Mal metabolism and Viol desepoxidation are commensurable [6, 12]. Malate can be also released from mitochondria, followed by its oxidation to OAA in the cytoplasm and subsequent formation of PYR and PEP. Apparently, the Mal-mediated shuttle mechanism related to OAA for-

Time course of changes in the specific radioactivity of violaxanthine (Viol), neoxanthine (Neo), lutein (Lut), and β-carotene (Car) depending on the duration of exposure to malic acid

Experimental conditions and study object	Pigment fractions	Duration of exposure to malic acid (min); data are expressed in counts/100 s per μg carbon in pigment molecule (n = 5; SEM, 12%)					
		3	10	15	25	60	120
1. Cut leaves of *Z. mays* in 10 ml of infiltration solution containing 1 mCi malic acid	Viol	675	823	1342	1846	2200	2400
	Neo	530	726	987	1432	1657	1956
	Lut	275	374	630	976	1278	1780
	Car	245	346	645	924	1345	1830
2. Cut leaves of *S. sudanensis* in 10 ml of infiltration solution containing 1 mCi malic acid	Viol	579	920	1458	1926	2435	2657
	Neo	497	823	1134	1489	1897	2365
	Lut	324	435	775	1112	1576	1987
	Car	289	443	780	976	1489	1968
3. Cut leaves of *A. retroflexus* in 10 ml of infiltration solution containing 1 mCi malic acid	Viol	714	856	1468	2234	2469	2564
	Neo	587	846	1223	1765	1998	2345
	Lut	364	431	694	1198	1778	2134
	Car	296	415	812	1154	1653	1876
4. Leaves of *A. hortensis* in 10 ml of infiltration solution containing 1 mCi malic acid	Viol	645	879	1376	1987	2365	2678
	Neo	611	934	1342	1933	2314	2524
	Lut	411	498	832	1297	1897	2233
	Car	312	465	932	1314	1968	2295
5. Leaves of *Bryophyllum* sp. in 10 ml of infiltration solution containing 1 mCi malic acid	Viol	324	579	1124	1658	1799	1935
	Neo	287	473	882	1436	1767	1879
	Lut	224	362	637	873	1236	1465
	Car	239	376	689	936	1344	1567
6. Leaves of *M. sinensis* in 10 ml of infiltration solution containing 1 mCi malic acid	Viol	578	890	1567	1874	2279	2476
	Neo	467	1012	1263	1679	1926	2265
	Lut	443	521	711	1387	1911	2022
	Car	357	497	968	1421	1879	2189
7. Leaves of *Z. mays* in 10 ml of infiltration solution containing 1 mCi malic acid and fosmidomycin	Viol	92	130	324	375	427	456
	Neo	88	170	193	346	391	412
	Lut	65	129	145	211	245	268
	Car	64	131	156	221	254	246
8. Leaves of *S. sudanense* in 10 ml of infiltration solution containing 1 mCi malic acid and fosmidomycin	Viol	112	145	156	390	435	467
	Neo	79	95	187	326	411	467
	Lut	49	88	156	263	289	297
	Car	55	123	144	223	267	298
9. Leaves of *A. hortensis* in 10 ml of infiltration solution containing 1 mCi malic acid and fosmidomycin	Viol	145	187	297	356	423	435
	Neo	98	178	221	365	422	455
	Lut	66	97	167	243	287	321
	Car	72	101	165	236	273	318

mation facilitates the conversion of Mal into Asp; through Ala formation, it is also involved in the production of PYR in mesophyll cells. Thus, chloroplasts of the C4 plants have several opportunities of rapid incorporation of the ^{14}C label into carotenoid backbones, which are related to utilization of different substrates [4, 5], one of which is Mal. The processes of biosynthesis of carotenoids are tightly related to their redox transformations in cells of animals and plants [10, 13–15]. This especially concerns the renewal of pools of light-

mediated reaction of Viol desepoxidation [14, 15] and zeaxanthine pools. The latter is thought to play a protective role during energy dissipation [3, 13].

Thus, this was the first study to present high-order derivative spectra for Viol in EtOH and CS_2, Lut in CCl_4, and Car in MtOH. A rapid incorporation of ^{14}C from exogenous radioactively labeled malic acid into Viol, Neo, Lut, and Car. It was shown that, during the first minutes of exposure to the radioactive substrate, the label was primarily incorporated into Viol and Neo.

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