Utilization of a natural β-carotene stereoisomers mixture from the fungus *Phycomyces blakesleeanus* as a source of vitamin A and β-carotene in rats' diet

P. Shlomai, A. Ben-Amotz, P. Margalith, and S. Mokady

Department of Food Engineering and Biotechnology, Technion; and Israel Oceanographic and Limnological Research, Tel-Shikmona, Haifa, Israel

Phycomyces blakesleeanus, *a β-carotene–producing fungi, has been tested as a source of vitamin A and β-carotene in the rat diet. The β-carotene in the fungi is composed of 80–90% of the all-*trans *stereoisomer, and the rest is the 9-*cis *stereoisomer. Female weanling rats were fed a vitamin A-deficient diet for 60 days. Thereafter, the rats were divided into groups and fed the vitamin A-deficient diet supplemented with synthetic crystalline β-carotene powder or fungal powder to levels of 12,24, and 48 mg β-carotene/kg diet. A diet supplemented with the 9-*cis*-rich* Dunaliella bardawil *powder to a level of 24 mg β-carotene/kg was also employed. Following a 10-day feeding period, samples were taken for plasma and liver analyses of retinol, retinyl esters, and β-carotenes.*

*Liver analyses revealed a higher conversion rate of β-carotene to vitamin A and a higher β-carotene accumulation in the rats fed the diets supplemented with fungal or algal powder, as compared to those supplemented with synthetic β-carotene. A significant correlation was found between the total β-carotene accumulated in the liver and the content of dietary 9-*cis *β-carotene.*

*The conversion of β-carotene to retinol in the liver was found to be affected by the dietary concentration of the carotenoid. The lower the β-carotene concentration in the feed, the higher the conversion to retinol. This inverse relation was also observed for total β-carotene accumulation, its 9-*cis*:all-*trans *ratio and for retinol:retinyl palmitate ratio in the livers.*

These studies demonstrate the possibility of using dried P. blakesleeanus mycelium *as a natural source of vitamin A and β-carotene in the rat diet.*

*The 9-*cis *β-carotene was shown to be of lower vitamin A biopotency than the all-*trans *β-carotene. However, its contribution to enhanced accumulation of vitamin A and β-carotene in rats' livers seems to be of high importance.*

Keywords: β-carotene stereoisomers; *Dunaliella bardawil*; *Phycomyces blakesleeanus*

Introduction

Numerous studies were published concerning the cultivation of the β-carotene–producing fungus *Phycomyces blakesleeanus,*[1] its biosynthetic pathways,[2] and genetic aspects.[3] A number of carotene super-producing mutants were developed for possible commercial use.[4] However, there is practically no published information concerning the nutritional value of this fungus.

Natural β-carotene, as found in *P. blakesleeanus*[5] and in many fruits and vegetables,[6] consists mainly of two stereoisomers. The major stereoisomer in *P. blakesleeanus* is all-*trans* β-carotene (80–90%) and the rest is composed of 9-*cis* β-carotene. Minor amounts of β-zeacarotene and phytoene are also present.

There is a growing interest in the production and

Address for reprint requests to Prof. S. Mokady at the Dept. of Food Engineering and Biotechnology, Technion, Haifa 32000, Israel.
Received March 28, 1991; accepted January 2, 1992.

utilization of natural β-carotene from microbial origin. Production on a commercial scale of the halotolerant algae *Dunaliella bardawil* as a natural β-carotene source is already carried out in a number of plants.[7]

Because β-carotene is produced as the major carotenoid only by a limited number of mycelial fungi,[1] it was of interest to study the possible nutritional use of *P. blakesleeanus* as a source of vitamin A in the diet of vitamin A-deficient rats, as compared with synthetic β-carotene and to the alga *D. bardawil,* in which the 9-*cis* β-carotene was previously found to enhance liver β-carotene accumulation.[8,9] Furthermore, the different ratio of 9-*cis*:all-*trans* stereoisomers as found in these β-carotene preparations[5,10] allowed us to gain additional information on the metabolic effects of the 9-*cis* β-carotene stereoisomer.

Materials and methods

Fungi

Phycomyces blakesleeanus NRRL 1555(−) was grown in 10 l fermentors as described previously.[5] The mycelia were collected by filtration, dried in a vacuum oven at 50–60° C, and powdered. The dry fungal powder contained about 2.2 mg β-carotene/g.

Algae

Dunaliella bardawil was grown in media containing 1.5–2.5 M NaCl in outdoor cultures under natural illumination, as previously described.[7,11] The alga were collected by centrifugation and spray dried. The dry algal powder contained about 30 mg β-carotene/g.

Synthetic β-carotene

Synthetic β-carotene (C9750) was obtained as crystalline powder from Sigma Chemical Co. (St. Louis, MO, USA).

Animals and diets

Female weanling rats of the Charles River CD strain were fed a vitamin A-deficient diet containing 100 g protein (derived from vitamin-free casein)/kg ad libitum. The diet was designed according to the Association of Official Analytical Chemists,[12] but was lacking vitamin A. At the end of a 60 day-feeding period the vitamin A content in the livers of the depleted rats was negligible (<5 μg). The rats were divided into groups of six, housed in individual cages and fed the vitamin A-deficient diet supplemented with the various β-carotene preparations: synthetic β-carotene powder at levels of 12, 24, and 48 mg/kg diet; fungal powder to a level of 12, 24, and 48 mg β-carotene/kg diet; algal powder to a level of 24 mg β-carotene/kg diet. Followiing a 10-day feeding period, the rats were sacrificed by CO_2 asphyxiation and blood was withdrawn by heart puncture over EDTA. The livers were removed, weighed, and stored at −18° C. The blood samples were centrifuged (4500 rpm, 20 min) and the plasma was stored at −18° C until assayed.

Analytical determinations

The extraction of the dietary β-carotene preparations and rat livers were carried out as described.[5,13] The vitamin A (as retinol and retinyl esters) and β-carotene were determined spectrophotometrically at 326 nm ($E^{1\%}_{1cm} = 1600$) and 476 nm ($E^{1\%}_{1cm} = 2262$), respectively.

High performance liquid chromatography (HPLC)

The HPLC analysis was performed using a Waters system equipped with 510 and 501 pumps, U6K injector, and 490 Programmable Multiwavelength Detector (Waters, Associates, Inc., Milford, MA, USA). The latter was set at 450 nm for the detection of carotenoids and at 326 nm for the detection of retinol and retinyl esters. The two outputs were read simultaneously by using Waters System Interface Module and Waters 840 Data and Chromatography Control Station on a Digital 350 professional computer. The HPLC system was attached to a flow-through spectrophotometer (model 8452A Diode Array, Hewlett-Packard, Rockville, MD, USA), and each peak of interest was scanned spectrally.

A stainless steel column of 25 cm × 4.6 mm (i.d.) packed with C18 reversed phase material of 5 μm particle size (Vydac TP 201 54, The Separation Group, Hesperia, CA, USA), protected by a 5-cm guard column (C18 ODS, Shimadzu, Kyoto, Japan), was used for carotenoids, retinol, and retinyl esters analysis. Elution was performed with an isocratic solvent, methanol:acetonitrile (9:1), at a flow rate of 1 mL/min.

Standards for retinol and retinyl esters were obtained from Sigma Chemical Co. and β-carotene stereoisomers were obtained from Ten-Tec Ltd. (Haifa, Israel).

Statistical analysis

Data were expressed as the mean ± the standard deviation (SD) and analyzed by one-way analysis of variance (ANOVA). If the ANOVA showed a significant value ($P < 0.01$), Fisher's least significant difference test for multiple comparison was applied.

Results

Animal growth and development

No significant differences were observed in the weight gain, food intake, or relative liver weight of the rats from the various dietary groups.

β-Carotene stereoisomers in the diet

The HPLC analysis of the synthetic β-carotene, the fungal powder, and the algal powder showed that the 9-*cis* stereoisomer accounts for 8.4%, 16.8%, and 51.2%, respectively *(Figure 1).*

Utilization of β-carotene as a source for vitamin A

The utilization of the β-carotene from the fungal and algal sources versus the synthetic source was evaluated by measuring spectrophotometrically the total vitamin A stored (as retinol and retinyl esters) in the rats' livers and plasma *(Table 1).*

The percentage of conversion of β-carotene to vitamin A was significantly higher in the livers of rats that consumed the fungal and algal β-carotene, as compared with those fed on the synthetic β-carotene. As was already shown,[14] lower β-carotene concentra-

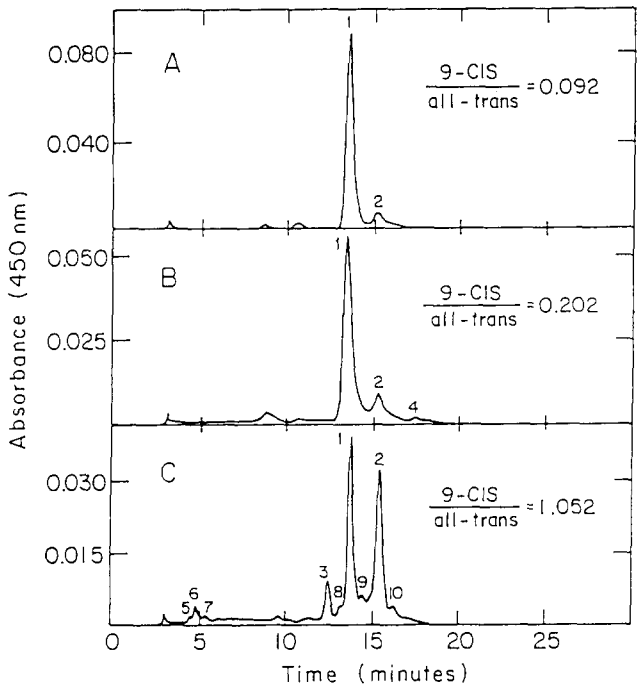

Figure 1 Carotene analysis by high-performance liquid chromatography of (A) synthetic β-carotene, (B) an extract of *Phycomyces blakesleeanus,* and (C) an extract of *Dunaliella bardawil.* 1, all-*trans* β-carotene; 2, 9-*cis* β-carotene; 3, all-*trans* α-carotene; 4, all-*trans* β-zeacarotene; 5, chlorophhyll *b;* 6, lutein; 7, zeaxanthin; 8, 9,13-*dicis* β-carotene; 9, 9,9-*dicis* β-carotene; 10, 13-*cis* β-carotene. For chromatographic conditions see p. 6,7.

tions in the diet yielded higher conversion percentages to retinol. This was significant with synthetic β-carotene, and was also detected with the fungal β-carotene *(Table 1).*

The ratio between the level of retinol to retinyl palmitate in the livers of the rats from the various dietary groups *(Table 1)* was determined by HPLC *(Figure 2).* Similarly, the ratio between retinol:retinyl palmitate was affected by the dietary concentration of β-carotene. Higher levels of dietary β-carotene led to a lower ratio of retinol:retinyl palmitate.

The vitamin A level in the plasma of the rats from all dietary groups was essentially similar, except for the group fed the highest level of fungal β-carotene, which was significantly higher *(Table 1).*

β-*Carotene accumulation in liver*

The accumulation of β-carotene in rats' livers and the 9-*cis*:all-*trans* ratio is given in *Table 1* and *Figure 3,* respectively.

The total level of β-carotene accumulated in the livers of the rats fed fungal β-carotene was significantly higher than that in the livers of animals that consumed the synthetic β-carotene. When the accumulation of β-carotene in the livers of the rats fed 24 mg β-carotene/kg diet was compared, the storage of the algal β-carotene was found to be significantly higher than that of the fungal source *(Table 1).* A statistically significant correlation between total β-carotene accumulated in the liver *(Table 1)* and the percentage of 9-*cis* stereoisomer in the dietary β-carotene can be observed in *Figure 4.* The higher the concentration of β-carotene in the diet, the lower the accumulation in the liver. This was more pronounced in the case of synthetic β-carotene *(Table 1).*

The ratio of 9-*cis*:all-*trans* β-carotene in the rats' livers *(Table 1)* was found to be significantly higher than the ratio present in the dietary β-carotene sources *(Figure 1),* except for the algal β-carotene–fed group. It is noteworthy that this ratio is the highest in the group fed synthetic β-carotene *(Table 1),* despite the very low content of 9-*cis* in this source *(Figure 1).* The inverse relationship between the dietary level of β-carotene and this ratio can be detected here as well.

To enable comparison between the three β-carotene sources (using the diets containing 24 mg β-carotene/kg diet), the different 9-*cis*:all-*trans* ratio in these sources had to be taken into consideration. Therefore, an additional parameter was introduced: the ratio be-

Table 1 β-Carotene intake, its utilization as vitamin A source, and its accumulation in rats*· †

			Recovered as vitamin A (retinol and retinyl esters)						
			in liver‡			in plasma‡	Accumulated as β-carotene in liver‡		
Diet supplementation	Dietary β-carotene (mg/kg diet)	β-carotene consumed† (μg)	μg	% conversion	Retinol§ R. palmitate	ng/ml	μg	% of intake	9-*cis* all-*trans*
β-carotene	12	1704[a] ± 58	68.3[a] ± 7.9	4.02[b] ± 0.54	0.579[b] ± 0.399	77[a] ± 14	4.81[a] ± 0.39	0.282[c] ± 0.024	2.043[d] ± 0.196
	24	3366[b] ± 111	111.6[b] ± 16.3	3.31[ab] ± 0.41	0.305[ab] ± 0.084	62[a] ± 9	6.41[ab] ± 1.51	0.190[b] ± 0.039	1.263[c] ± 0.014
	48	7066[c] ± 410	180.9[c] ± 28.4	2.56[a] ± 0.29	0.157[a] ± 0.056	69[a] ± 25	7.60[b] ± 1.24	0.107[a] ± 0.015	1.142[bc] ± 0.466
P. blakesleeanus	12	1768[a] ± 45	127.8[b] ± 17.6	7.23[d] ± 1.04	0.244[ab] ± 0.101	76[a] ± 20	6.30[ab] ± 0.89	0.356[d] ± 0.053	1.228[bc] ± 0.083
	24	3544[b] ± 132	255.3[d] ± 17.0	7.21[d] ± 0.43	0.119[a] ± 0.030	69[a] ± 17	10.87[c] ± 1.01	0.307[d] ± 0.033	0.535[ab] ± 0.051
	48	6994[c] ± 199	426.9[e] ± 28.9	6.10[c] ± 0.41	0.127[a] ± 0.051	120[b] ± 17	19.18[d] ± 1.58	0.274[c] ± 0.024	0.435[a] ± 0.095
D. bardawil	24	3642[b] ± 92	277.3[d] ± 24.1	7.60[d] ± 0.51	0.124[a] ± 0.043	65[a] ± 18	17.46[d] ± 1.42	0.479[e] ± 0.031	1.311[c] ± 0.349

*Values are expressed as mean ± SD. Means with different superscripts in each column are significantly different ($P < 0.01$).
†Vitamin A-deficient rats were fed on the specified diets, containing the various β-carotene sources, for 10 days; for details, see Materials and methods.
‡n = 5–6 observations per group.
§n = 3–4 observations per group.

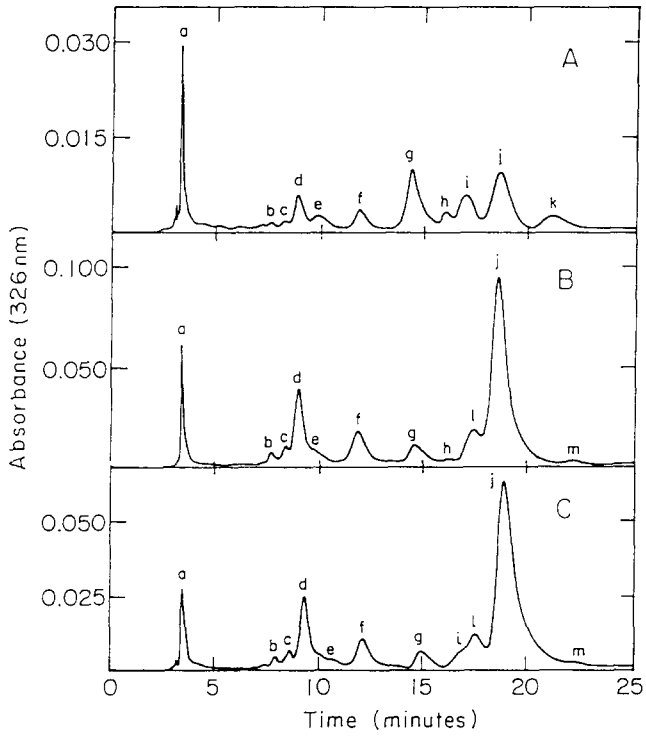

Figure 2 Retinol and retinyl esters analysis by high-performance liquid chromatography of liver extracts of rats fed a vitamin A-deficient diet supplemented with (A) synthetic β-carotene; (B) powdered *Phycomyces blakesleeanus*, and (C) spray-dried *Dunaliella bardawil*. The level of β-carotene in all diets was 24 mg/kg. a, all-*trans* retinol; j, all-*trans* retinyl palmitate; b,c,d,e,f,g,h,i,k,l,m are unidentified retinoids, presumed to be retinyl esters. For chromatographic conditions see Results. For details of dietary treatment see Materials and methods.

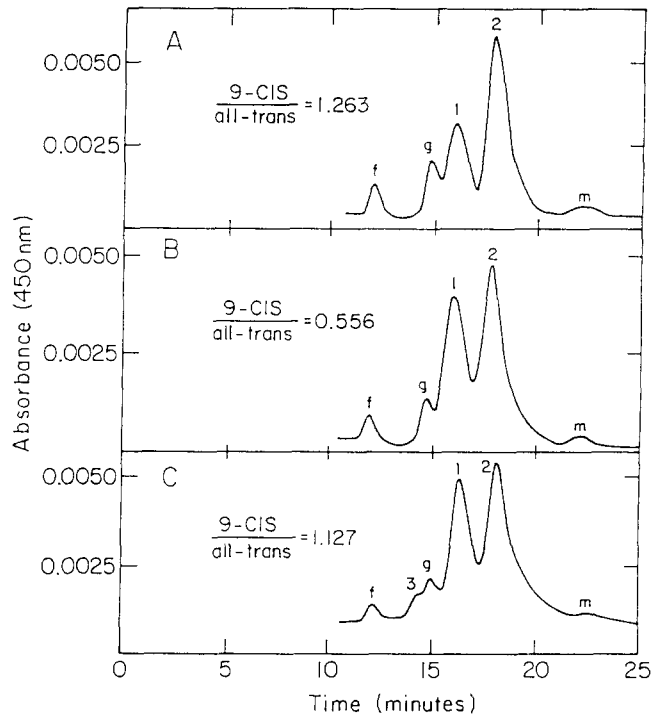

Figure 3 Carotene analysis by high-performance liquid chromatography of liver extracts of rats fed a vitamin A-deficient diet supplemented with (A) synthetic β-carotene; (B) powdered *Phycomyces blakesleeanus*, and (C) spray-dried *Dunaliella bardawil*. The level of β-carotene in all diets was 24 mg/kg. 1, all-*trans* β-carotene; 2, 9-*cis* β-carotene; 3, all-*trans* α-carotene; f,g,m are unidentified retinyl esters. For chromatographic conditions see Results. For details of dietary treatment see Materials and methods.

tween 9-*cis*:all-*trans* in the diet to 9-*cis*:all-*trans* in the rats' livers. A statistically significant correlation between this parameter and the dietary β-carotene level is presented in *Figure 5*.

Discussion

The fungal β-carotene was found to be more efficient than the synthetic β-carotene, as expressed by the amount recovered in the liver as vitamin A *(Table 1)* or accumulated as β-carotene *(Table 2)*. Its vitamin A biopotency was found to be similar to that observed for the algal β-carotene *(Table 1)*.

The negative effect of high β-carotene levels in the conversion of β-carotene to retinol were previously attributed[14,15] to some rate-limiting processes, such as the uptake of β-carotene or its enzymatic conversion, occuring in the intestinal mucosa. Such mechanisms cannot fully explain these results, and therefore, an additional explanation is offered: because the all-*trans* stereoisomer tends to crystallize easily, an increment in its level in the diet will decrease its availability. In contrast, the 9-*cis* β-carotene that is present in the natural sources probably prevents such crystallization by acting as a solvent for the all-*trans* stereoisomer.[8,9] This may explain the larger drop in the conversion of

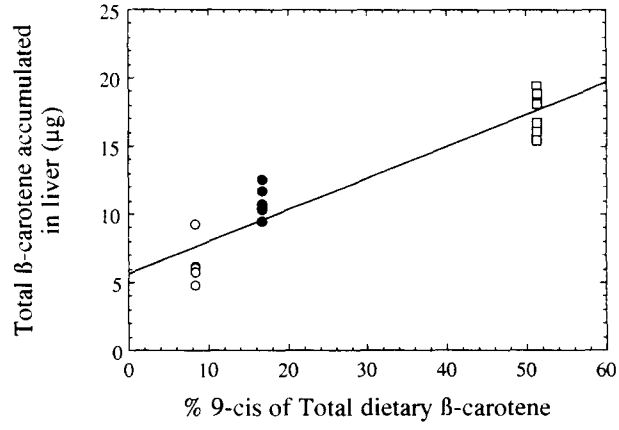

Figure 4 Linear correlation between total β-carotene accumulated in the liver of rats fed vitamin A-deficient diets supplemented with the various β-carotene sources to a level of 24 mg β-carotene/kg (y) versus the percentage of *9-cis* stereoisomer in the dietary β-carotene (x). Synthetic β-carotene, (○); fungal β-carotene, (●); and algal β-carotene, (□). Regression equation and correlation coefficient were: $y = 5.646 + 0.235\ x$, $n = 16$, $r = 0.934$.

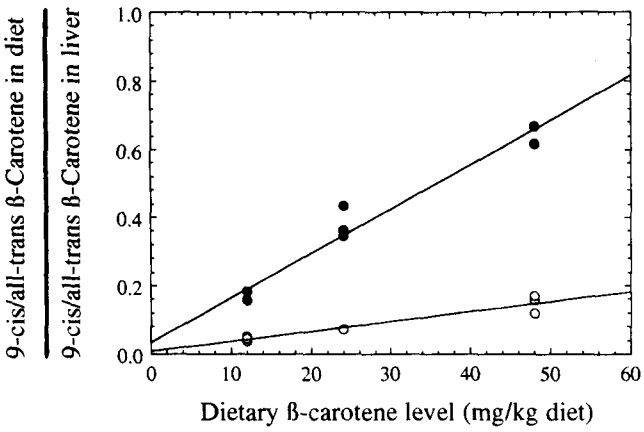

Figure 5 Linear correlation between the ratio of 9-*cis*:all-*trans* β-carotene in diet to that in liver (y) versus dietary β-carotene level (x). Synthetic β-carotene, (○); regression equation and correlation coefficient: y = 0.006 + 0.003 x, n = 9, *r* = 0.952. Fungal β-carotene, (●); regression equation and correlation coefficient: y = 0.032 + 0.013 x, n = 8, *r* = 0.977.

β-carotene to retinol, as found in the group fed synthetic β-carotene, which was practically devoid of the 9-*cis* stereoisomer.

To estimate the extent of the utilization of vitamin A we suggested the use of the ratio of retinol:retinyl palmitate in the liver. Vitamin A is stored in the liver as retinyl palmitate, which is hydrolized to retinol according to demand.[16] Some free retinol is always present in fairly constant levels, and therefore, a high ratio of retinol:retinyl palmitate points to a high utilization of vitamin A. The values of this ratio obtained in the present study *(Table 1)* are consistent with the explanation we suggested above for the percentage conversion.

The high demand for vitamin A in the depleted animals is also expressed in the ratio of the 9-*cis* and the all-*trans* β-carotene stereoisomers found in the liver *(Table 1)*. When this demand cannot be fully met, such as in the case of the low dietary β-carotene levels, the utilization of the all-*trans* stereoisomer is almost complete. Because the 9-*cis* stereoisomer is being utilized as a source for vitamin A to a lesser extent,[17] it accumulates in the liver, resulting in a high 9-*cis*:all-*trans* ratio as compared to this ratio in the β-carotene preparations used in the diets. Elevation of the dietary β-carotene levels leads to a lower percentage conversion of β-carotene to vitamin A, resulting in an increase in the accumulation of the all-*trans* stereoisomer in the liver. Therefore, the 9-*cis*:all-*trans* β-carotene ratio in the liver decreases until it reaches a constant value, which is similar to this ratio in the diet, in high dietary β-carotene levels. This effect is more pronounced in rats fed synthetic β-carotene, which has a lower availability as a vitamin A source than the fungal-originated β-carotene *(Figure 5)*.

There is very limited information in the literature on the importance of the 9-*cis* stereoisomer to the bioavailability of β-carotene. The occurrence of the 9-*cis* β-carotene in the fungus *P. blakesleeanus* may provide additional evidence of the unique role of the 9-*cis* stereoisomer as was suggested previously in the studies with *D. bardawil*.[8,9,18] Indeed, the amount of total β-carotene accumulated in the liver of rats was found to be highly correlated with the percentage of 9-*cis* β-carotene in the diet, regardless of its origin *(Figure 4)*.

In conclusion, the results of this study show that the fungus *P. blakesleeanus* can be an efficient source of vitamin A in the rat diet. Although the 9-*cis* β-carotene stereoisomer was shown to be of lower vitamin A potency than the all-*trans* stereoisomer, its contribution to enhanced availability and accumulation of vitamin A and β-carotene in rats' livers seems to be of considerable importance, especially in view of the accumulating data on the nature of β-carotene as an anti-cancer agent.[19–23]

References

1 Goodwin, T.W. (1980). *The biochemistry of Carotenoids*, 2nd ed., vol 1, Chapman and Hall, London, UK

2 Bramley, P.M. and Mackenzie A. (1988). Regulation of carotenoid biosynthesis. In *Current Topics in Cellular Regulation*, vol. 29, p. 291–343, Academic Press, New York, NY, USA

3 Cerda-Olmedo, E. (1985). Carotene mutants of *Phycomyces*. *Methods Enzymol.* **110,** 220–243

4 Murillo, F.J., Calderon, I.L., Lopez-Diaz, I., and Cerda-Olmedo, E. (1978). Carotene-superproducing strains of *Phycomyces*. *Appl. Environ. Microbiol.* **36,** 639–642

5 Shlomai, P., Ben-Amotz, A., and Margalith, P. (1991). Production of carotene stereoisomers by *Phycomyces blakesleeanus*. *Appl. Microbiol. Biotechnol.* **34,** 458–462

6 Bushway, R.G. (1986). Determination of α- and β-carotene in some raw fruits and vegetables by high performance liquid chromatography. *J. Agric. Food Chem.* **34,** 409–412

7 Ben-Amotz, A. and Avron, M. (1989). The biotechnology of mass culturing *Dunaliella* for products of commercial interest. In *Algal and cyanobacterial Biotechnology*, (R.C. Cresswel, T.A.V. Rees, and N. Shah, eds.) p. 91–114, Longman, London, UK

8 Ben-Amotz, A., Mokady, S., Edelstein, S., and Avron, M. (1989). Bioavailability of a natural isomer mixture as compared with synthetic all-*trans*-β-carotene in rats and chicks. *J. Nutr.* **119,** 1013–1019.

9 Mokady, S., Avron, M., and Ben-Amotz A. (1990). Accumulation in chick-livers of 9-*cis* versus all-*trans* β-carotene. *J. Nutr.* **120,** 889–892

10 Ben-Amotz, A., Lers, A., and Avron, M. (1988). Stereoisomers of β-carotene and phytoene in the alga *Dunaliella bardawil*. *Plant Physiol.* **86,** 1286–1291

11 Ben-Amotz, A., Katz, A., and Avron, M. (1982). Accumulation of β-carotene in halotolerant algae: purification and characterization of β-carotene-rich globules from *Dunaliella bardawil* (Chlorophyceae). *J. Phycol.* **18,** 529–537

12 Association of Official Analytical Chemists (1984). *Official Methods of Analysis*, 14th ed., p. 877–878. Association of Official Analytical Chemists, Arlington, VA, USA

13 Ben-Amotz, A., Mokady, S. and Avron, M. (1988). The β-carotene-rich alga *Dunaliella bardawil* as a source of retinol in a rat diet. *Brit. J. Nutr.* **59,** 443–449

14 Brubacher, G.B. and Weiser, H. (1985). The vitamin A activity of β-carotene. *Internat. J. Vit. Nutr. Res.* **55,** 5–15

15 Bauernfeind, J.C. (1981). *Carotenoids as Colorants and Vitamin A Precursors*, p. 645–648, Academic Press, New York, NY, USA

16 Goodman, D.S., Huang, H.S., and Shiratori, T. (1965). Tissue distribution and metabolism of newly absorbed vitamin A in the rat. *J. Lipid Res.* **6,** 390–396
17 Zechmeister, L. (1962). *cis-trans-Isomeric Carotenoids, Vitamin A and Arylpolyenes,* Springer-Verlag, Vienna, Austria
18 Mokady, S. and Ben-Amotz A. (1991). Dietary lipid level and the availability of β-carotene of *Dunaliella bardawil* in rats. *Nutr. Cancer* **15,** 47–52
19 Gey, K.F., Brubacher, G.B., and Stahelin, H.B. (1987). Plasma levels of antioxidant vitamins in relation to ischemic heart disease and cancer. *Amer. J. Clin. Nutr.* **45,** 1368–1377
20 Hennekens, C.H. and Eberlein, K. (1985). A randomize trial of aspirin and β-carotene among U.S. physicians. *Prev. Med.* **14,** 165–168
21 Menkes, M.S., Comstock, G.W., Vuilleumier, J.P., Hesling, K.J., Rider, A.A., and Brookmeyer, R. (1986). Serum β-carotene, vitamins A and E, selenium and the risk of lung cancer. *New Eng. J. Med.* **315,** 1250–1254
22 Mathews-Roth, M.M. (1982). Antitumor activity of β-carotene, canthaxanthin and phytoene. *Oncology* **39,** 33–37
23 Ziegler, R.G. (1989). A review of epidemiological evidence that carotenoids reduce the risk of cancer. *J. Nutr.* **119,** 116–122