1, J = 2 and 8 cps, 5 H), 2.32–3.33 (m, 18, Ar H), and 3.70 (s, 1, β H).

Anal. Calcd for $C_{28}H_{20}O_2$: C, 86.57; H, 5.19. Found: C, 86.44; H, 5.39.

3-(2-Hydroxybenzyl)flavone—A solution of 0.5 g of flavanone in 1 ml of salicylaldehyde was saturated with anhydrous hydrogen chloride, stoppered, and allowed to stand for 24 hr; 3 ml of methanol was then added. A crystalline solid separated. Recrystallization from ethanol gave 0.4 g (55%) of fine off-white crystals, mp 200–201°. The ultraviolet spectrum (EtOH) has λ_{max}^{sh} 307 mμ (ϵ 11, 600), 283 (13,800), and 241 (21,800). The infrared spectrum (CCl_4) shows significant adsorptions at 3100 (bonded OH), 1640 (C=O), 1615 (C=C), and 1230 cm^{-1} (=COC–). The nmr spectrum ($CDCl_3$, very dilute owing to insolubility) has signals at τ 0.3 (singlet, bonded OH, wt 1), 1.65 (perturbed pair of doublets, J = 8 cps, 5 H, wt 1), 2.0–3.1 (complex multiplet, Ar H, wt 12), and 6.07 (singlet, CH_2, wt 2). In deuterated dimethyl sulfoxide the signal at 0.3 is missing and is replaced by a singlet (wt 1) at τ 6.52.

Anal. Calcd for $C_{22}H_{16}O_3$: C, 80.47; H, 4.91. Found: C, 80.29; H, 5.11.

3-(2-Acetoxybenzyl)flavone.—A solution of 0.5 g of 3-(2-hydroxybenzyl)flavone, 3 ml of acetic anhydride, and 2 drops of phosphoric acid was refluxed for 5 min and then poured into 5 ml of water. An oil separated which soon solidified. An additional 50 ml of water was added, and the solid was removed by filtration and washed thoroughly with water. The solid was recrystallized from 20 ml of ethanol and gave 0.4 g (71%) of small white crystals, mp 143–144°. The infrared spectrum (CCl_4) has characteristic absorptions at 1770 (CH_3C=O), 1650 (C=O), 1625 (C=C), and 1215, 1198 cm^{-1} (=COC– and –COC=O). The nmr spectrum ($CDCl_3$) has signals at τ 1.82 (perturbed pair of doublets, J = 7.5 cps, 5 H, wt 1), 2.42–3.16 (complex multiplet, Ar H, wt 12), 6.12 (singlet, CH_2, wt 2), and 7.87 (singlet, CH_3CO, wt 3).

Registry No.—**2a**, 24467-41-2; **2b**, 24467-42-3; **2c**, 24467-43-4; **3a**, 24467-44-5; **3b**, 24467-45-6; **3c**, 24467-46-7; **5**, 24467-47-8; *trans*-3-(2-nitrobenzylidene)flavanone, 24467-48-9; 3-(2-hydroxybenzyl)flavone, 24467-49-0; 3-(2-acetoxybenzyl)flavone, 24467-50-3.

Oxidation of β-Carotene. Site of Initial Attack

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The reaction between β-carotene and molecular oxygen in toluene at 60° was investigated. A linear relation was found between the loss in β-carotene and time. The reaction rate increased with increasing temperature. The activation energy, E_a, for the oxidation of β-carotene was found to be 10.2 kcal/mol. Though free-radical initiators caused rate enhancement, the kinetics of the reaction and the light absorption characteristics of the reaction solution were altered. This indicated a difference in the mechanism of the reaction in the presence of free-radical initiators. The rate of loss of β-carotene was increased in the presence of cupric ions and decreased in the presence of diphenylamine. The products of the reaction were β-carotene 5,6-monoepoxide and its isomer, β-carotene 5,6,5′,6′-diepoxide, and β-carotene 5,8-monoepoxide and its isomer, β-carotene 5,8,5′,8′-diepoxide. A reaction mechanism was proposed.

In view of the fact that the oxidative reactivity of the β-carotene molecule may be influenced by both an electronic factor and a stereochemical factor, as was suggested by Zechmeister, *et al.*,[2] the size and reactivity of the oxidizing agent would be expected to play a predominant role. The reaction site would not only be a function of the inherent reactivity (electron density) of the β-carotene molecule but also a function of the size and reactivity (stability) of the attacking reagent. This is shown by the results of the oxidation reactions of β-carotene in the presence of the various metal oxides (and metal oxide catalysts)[3–5] and by peroxides alone or with enzyme catalysis.[6–8] The oxygen in metal oxides or in the form of peroxy radicals is in an activated (reactive) state and thus might nullify the inherent differences in reactivities in the various parts of the β-carotene molecule. From a stereochemical consideration oxidation with metal oxides, for example OsO_4 and H_2O_2 or $KMnO_4$, involves the transitory formation of a five-membered intermediate.[9] The formation of such a space-requiring transition state would favor those sites of least steric hindrance. These might give the most stable transition state even though these sites may not be the centers of the highest electron density.

Oxidation of β-carotene with molecular oxygen has two unique characteristics. (1) Owing to the smaller size of the oxygen molecule, steric hindrance is not important. (2) Owing to the relative unreactivity of the oxygen molecule compared with peroxy radicals, the competitive reactivity between different carbon atoms could be retained.

The oxidation of β-carotene with molecular oxygen may therefore reflect the inherent reactivity of the β-carotene molecule. Also, the reaction of β-carotene with oxygen could illustrate the mechanism of the uncoupled enzymatic oxidation of β-carotene *in vivo*[10] by way of model systems.

Results and Discussion

The rate of loss of β-carotene is shown in Figure 1. A straight line passing through the origin was obtained. This indicates an overall zero-order reaction kinetics. Thus

$$\beta\text{-carotene} + O_2 \rightleftharpoons [\beta\text{-carotene}\cdot O_2]^* \xrightarrow{k_0} \text{products}$$

$$\frac{d(P)}{dt} = k_{obsd} = k_0[\beta\text{-carotene}\cdot O_2]^*$$

(1) Faculty of Agriculture, University of Khartoum, Khartoum North, Sudan.

(2) L. Zechmeister, A. L. Le Rosen, W. A. Schroeder, A. Polgar, and L. Pauling, *J. Amer. Chem. Soc.*, **65**, 1940 (1943).

(3) (a) B. A. Gross and R. W. McFarlane, *Science*, **106**, 375 (1947); (b) P. Meunier, *C. R. Acad. Sci., Paris*, **231**, 1170 (1950).

(4) P. Karrer and L. Solmssen, *Helv. Chim. Acta*, **20**, 682 (1937).

(5) P. Karrer, L. Solmssen, and T. Gugetmann, *ibid.*, **20**, 1020 (1937).

(6) R. F. Hunter and N. E. Williams, *J. Chem. Soc.*, 554 (1945).

(7) P. Karrer and E. Jucher, *Helv. Chim. Acta*, **28**, 427 (1945).

(8) M. Elahi, Ph.D. Thesis, University of New South Wales, Kensington, N. S. W., Australia, 1967.

(9) R. Wiberg and K. Saegebarth, *J. Amer. Chem. Soc.*, **79**, 2822 (1957).

(10) D. S. Goodman, *Amer. J. Clin. Nut.*, **22**, 963 (1969).

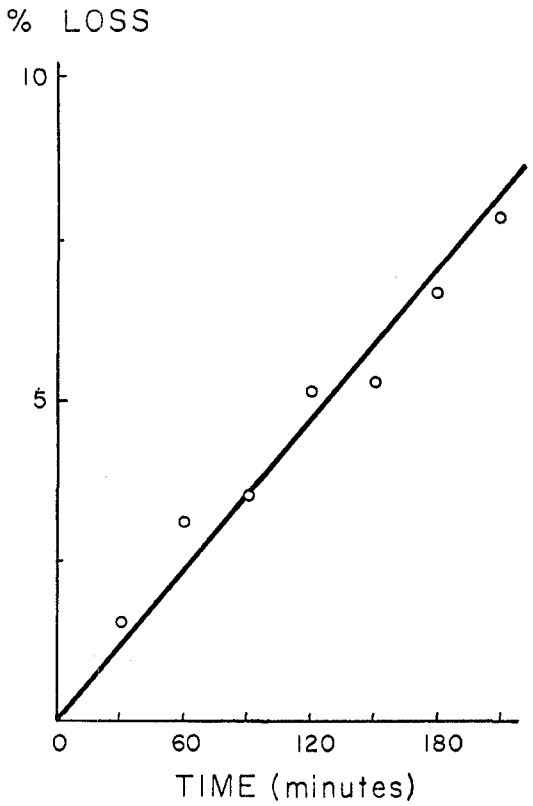


Figure 1.—Degradation of β-carotene with oxygen. Rate of loss of β-carotene.

The slope of the straight line is the observed rate constant k_{obsd}.

The overall zero-order kinetics must mean that the rate of the reaction is independent of the concentration of β-carotene in the presence of excess oxygen. The reaction between oxygen and β-carotene may go through an activated dipole association complex, formed in an equilibrium step. This activated complex may then decompose into products or go back to free oxygen and β-carotene.

It is important to notice that there is no lag phase, which is observed in the autoxidation of fats, and that the reaction is not autocatalytic; that is, there is no buildup of a reactive intermediate that decomposes into species that will further catalyze the reaction.

In order to obtain information about the energetics of the reaction between β-carotene and oxygen the effect of temperature on the reaction rate was studied. The reaction rate constants, k_{obsd}, determined at 60, 70, 80, and 90°, are, respectively, 3.93×10^{-2}, 6.12×10^{-2}, 10.54×10^{-2}, and 14.03×10^{-2}. Thus the rate of the reaction increases with increasing temperature.

When the logarithms of the rate constant are plotted against the reciprocal of the absolute temperature, a straight line relationship is obtained. From the slope of the line and using the relationship

$$\log k = -E_a/2.3RT$$

where k = the rate constant, E_a = activation energy, R = the gas constant (1.987 cal/mol), and T = the absolute temperature, the activation energy is calculated to be 10.20 kcal/mol.

The activation energy reported[11] for the autoxidation of linoleic acid is 15.2 kcal/mol and 17.2 kcal/mol for its ethyl ester. Thus the activation energy for the oxidation of β-carotene is 5.0 kcal lower than that of the free acid and 7.0 kcal lower than that of the ester. The low activation energy for the oxidation of β-carotene indicates that this reaction is more favored than the autoxidation reaction of linoleic acid. This difference in reactivity, however, can be attributed to the greater stability of an allylic (carbon 4) radical in the β-carotene molecule, which is stabilized over 11 double bonds compared with only two double bounds in the linoleic acid and ester. The possibility for the formation of an allylic radical and hence a free-radical mechanism for the destruction of β-carotene by oxygen remains open.

To further investigate the possibility of free radical participation in the oxidation of β-carotene, the reaction was run in the presence of catalytic amounts of free radical initiators. N-Bromosuccinimide (NBS) is a specific reagent for the preferential production of allylic radicals[12] *via* a free-radical chain mechanism.[13,14] Thus

$$RH \xrightarrow{NBS} R\cdot \text{ (carbon 4)} \xrightarrow{O_2} ROO\cdot \xrightarrow{RH} ROOH \longrightarrow \text{products}$$

When the data obtained in the presence of 2×10^{-4} M N-bromosuccinimide is plotted as per cent loss in β-carotene against time in minutes, a straight-line relationship is obtained with a positive intercept. The ratio of $k_{obsd}(NBS)/k_{obsd}(control) = 6.5$. $k_{obsd}(NBS)$ is the observed rate constant for the oxidation of β-carotene in the presence of N-bromosuccinimide and $k_{obsd}(control)$ is the observed rate constant for the oxidation of β-carotene with oxygen alone. Thus the oxidation of β-carotene in the presence of N-bromosuccinimide is 6.5 times faster than the control.

If the oxidation in the presence of N-bromosuccinimide proceeds through the formation of an allylic radical (carbon 4), then it would be expected that the relative reactivities of β-carotene and a vitamin A derivative would be similar. When the loss in vitamin A acetate brought about by oxidation in the presence of NBS is plotted against time, a curve is obtained and the slope of the tangent gives the observed rate constant. The ratio of $k_{obsd}(\beta\text{-carotene} + NBS)/k_{obsd}(\text{vitamin A acetate} + NBS) = 1.4$. Thus β-carotene is slightly more reactive than vitamin A acetate. This could be attributed to the extra six double bonds in β-carotene which will participate in the stabilization of an allylic radical.

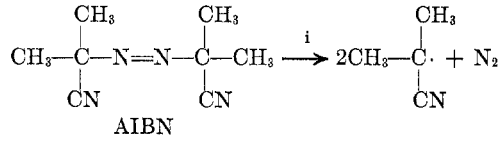
Though use of N-bromosuccinimide results in rate enhancement, the reaction is accompanied by a shift (6 mμ) of the overall absorption spectra of the β-carotene reaction mixture to longer wavelengths, loss of the fine structure, and finally the reduction to a single peak.

Though this by itself gives evidence for the incorporation of oxygen at the 4 and/or the 4′ positions as carbonyl groups and thus lengthens the conjugated chain, this behavior is not observed in the oxidation of β-carotene with oxygen alone. Further, on examination

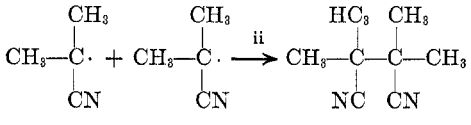
(11) J. L. Bolland and G. Gee, *Trans. Faraday Soc.*, **42**, 236 (1946).
(12) A. Wohl, *Chem. Ber.*, **52**, 51 (1919).
(13) G. Bloomfield, *J. Chem. Soc.*, 114 (1944).
(14) D. H. Heye, *Annu. Rept. Progr. Chem.*, **41**, 184 (1944).

of the reaction products in the absence of NBS, no 4-keto-β-carotene or 4,4′-diketo-β-carotene could be found. Petracek and Zechmeister[15] obtained these compounds by oxidizing β-carotene with NBS. The evidence is therefore that the reaction catalyzed by NBS is quite different from that brought about by oxygen alone and that the 4,4′ positions are probably not the sites of oxygen attack.

This is confirmed by the use of azobisisobutyronitrile (AIBN) as an initiator. Compared with NBS, AIBN is an efficient free-radical initiator and does not bring about side reactions. With catalytic amounts of AIBN, Figure 2, the initial rate of loss of β-carotene increases continuously with time, indicative of a chain process not observed in the oxidation of β-carotene with oxygen alone. Furthermore the destruction of β-carotene in the presence of AIBN is subject to catalysis by stearic acid. In the presence of (2×10^{-3} *M*) stearic acid (molar ratio of β-carotene:AIBN:stearic acid 1:1:10) the rate of loss of β-carotene increased by a factor of 2.5. The effect of stearic acid might be a dilution effect retarding the recombination between radicals to form nonradical species. Thus



AIBN



Reaction ii is subject to dilution effects.

If the oxidation of β-carotene does not occur through hydrogen abstraction at the 4,4′ positions, then the activation energy of 10.20 kcal/mol is too small and hence would exclude any such process occurring for all other hydrogens in the molecule. That is to say, the oxygen molecule does not attack a preformed free radical or assist in hydrogen abstraction to form one. The possibility, however, remains that the process might involve (a) the addition of the oxygen molecule across a double bond in a single step (this is unlikely because it will result in higher reaction order kinetics) or (b) the activation of the oxygen molecule through electron donation in a process possibly involving a dipole association product.

To obtain more information about the nature of such association between oxygen and a double bond in β-carotene the effect of free-radical inhibitors on the reaction was studied. The addition of such inhibitor, diphenylamine in excess (molar ratio of ten diphenylamine to one β-carotene), after allowing the reaction to proceed for 80 min, completely stops the loss of β-carotene. If the reaction is initiated in the presence of 2×10^{-4} *M* diphenylamine a lag phase (22 min) is introduced and the rate is reduced by 48%. This behavior in the presence of diphenylamine indicates the presence of free-radical character in the associated complex. This is further evidence against addition of oxygen across a double bond in a single step. Diphenylamine might decompose the β-carotene–oxygen associated complex.

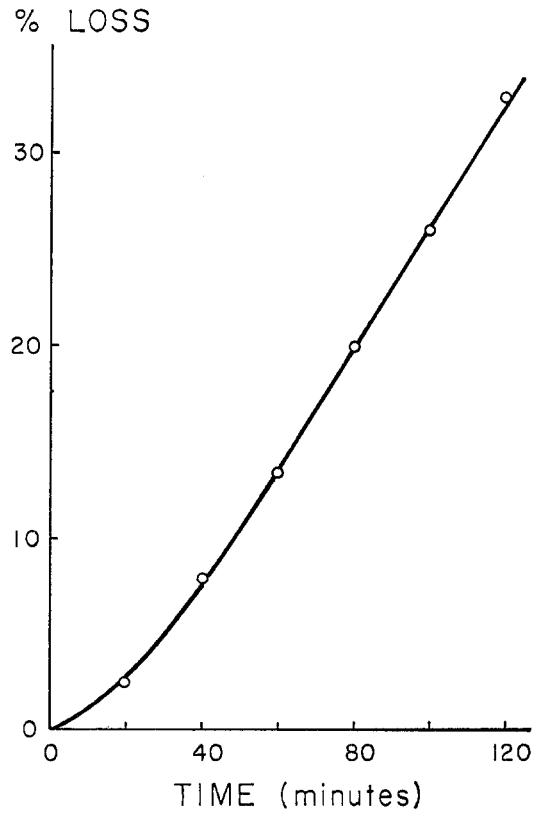


Figure 2.—Effect of azobisisobutyronitrile (2×10^{-4} *M*) on rate of loss of β-carotene.

The reaction between β-carotene and oxygen is subject to metal ion catalysis. Cupric stearate causes a rate enhancement of 4.3-fold. Cupric ions presumably stabilized the association between β-carotene and oxygen.

The products of the oxidation of β-carotene are shown in Table I. Tentative identification is given together with the evidence for the identification.

The predominant formation of epoxides is further evidence that the initial site of the oxygen attack on the β-carotene molecule occurs at the terminal double bonds. This is in agreement with the predictions of the oxidation of a conjugated system, since in a conjugated system the highest electron density is found in the terminal double bonds, with a progressive depletion of electron density as the central double bond is approached. It is therefore reasonable that reactions requiring high electron density would occur at the terminal double bonds.

Central Bond Cleavage.—As has been mentioned before, β-carotene and a number of carotenoids are converted into vitamin A in the animal body. In the mechanism of the conversion of β-carotene into vitamin A, the most obvious expectation is that *in vivo* this takes place by a hydrolytic fission of the β-carotene molecule as follows.

$$\beta\text{-carotene} + H_2O \longrightarrow 2(\text{vitamin A})$$

However, the absence of any evidence for such a hydrolytic fission and the failure of all attempts to bring about such a conversion *in vitro* makes the case

(15) F. J. Petracek and L. Zechmeister, *J. Amer. Chem. Soc.*, **78**, 1427 (1956).

TABLE I

R_f VALUES OF BANDS SEPARATED FROM OXIDATION OF β-CAROTENE WITH OXYGEN (TIME, 60 MIN)

Compound	R_f (% acetone) 3	R_f (% acetone) 5	Color with HCl
β-Carotene	0.94	0.97	No color
β-Carotene 5,6-monoepoxide	0.88	0.93	Blue
β-Carotene 5,6-monoepoxide isomer	0.82	0.93	Blue
β-Carotene 5,6,5′,6′-diepoxide	0.77	0.90	Blue
β-Carotene 5,8-monoepoxide	0.42	0.47	No color
β-Carotene 5,8-monoepoxide isomer	0.40	0.47	No color
Polyene carbonyl	0.37	0.38	No color
β-Carotene 5,8,5′,8′-diepoxide	0.34	0.30	No color

(i) β-Carotene
- (a) Decreased R_f value
- (b) Epiphasic in phase test
- (c) Spectral properties: maxima (in hexane) at 475, 446, and 423 mμ corresponding to the recorded spectrum of Tsukida and Zechmeister:[a] 475, 446, and 423 mμ

(ii) β-Carotene 5,6-monoepoxide isomer
- (a) Decreased R_f value
- (b) Epiphasic in phase test
- (c) Spectral properties: maxima (in hexane) at 473, 444, and 423 mμ

(iii) β-Carotene 5,6,5′,6′-diepoxide
- (a) Decreased R_f values
- (b) Epiphasic in phase test
- (c) Spectral properties: maxima (in hexane) at 468, 440, and 417 mμ (Tsukida and Zechmeister:[b] 470, 440, and 417 mμ)

(iv) β-Carotene 5,8-monoepoxide
- (a) Decreased R_f value
- (b) Epiphasic in phase test
- (c) Spectral properties: maxima (in hexane) at 450, 427, and 404 mμ (Elahi:[8] 451, 426, and 440 mμ)

(v) β-Carotene 5,8-monoepoxide isomer
- (a) Decreased R_f value
- (b) Epiphasic in phase test
- (c) Spectral properties: maxima (in hexane) at 448, 427, 404, and 500 mμ. The 427mμ peak is indicative of the 5,8-epoxide

(vi) Polyene carbonyl
- (a) Decreased R_f value
- (b) Epiphasic in phase test
- (c) Spectral properties: single peak at 378 mμ (in hexane)

(vii) β-Carotene 5,8,5′,8′-diepoxide
- (a) Decreased R_f value
- (b) Mainly epiphasic in phase test
- (c) Spectral properties: maxima (in hexane) at 426, 401, and 381 mμ corresponding to the recorded spectrum (Elahi:[8] 426, 401, and 381 mμ)

[a] K. Tsukida and L. Zechmeister, *Arch. Biochem. Biophys.*, **74**, 408 (1958).

for this mechanism doubtful. Clover, *et al.*,[16] administered vitamin A aldehyde orally and parentally to vitamin A depleted rats and found that it was converted to vitamin A in the gut wall. This suggested that the transformation of β-carotene to vitamin A *in vivo* is more likely achieved by oxidation of β-carotene to vitamin A aldehyde, which is then rapidly reduced to vitamin A, rather than by hydrolytic fission. Further, this process implies that the reaction site is the central double bond, and regardless of the mechanism of the reaction the enzyme system would have to accomplish this conversion at the position of least electron density, that is, the least reactive double bond. The information obtained from the degradation of β-carotene with oxygen shows that direct cleavage of a double bond is an energetically feasible process (10–20 kcal/mol) at those sites with high electron density, namely the terminal double bonds. Therefore, it appears that the function of the enzyme system in the enzymatic oxidative cleavage of β-carotene must be to minimize or oppose the electron density depletion from the center of the molecule.

(16) J. Glover, T. W. Goodwin, and R. A. Morton, *Biochem. J.*, **43**, 109 (1948).

Experimental Section

Materials. Chemicals.—All-*trans* crystalline β-carotene was a gift from Hoffmann-La Roche. All-*trans* vitamin A acetate was a commercial material. Cupric stearate, N-bromosuccinamide, and azobisisobutyronitrile (AIBN) were commercial materials. AIBN was recrystallized from methanol, mp 105–106°. Diphenylamine was recrystallized from petroleum ether, mp 54–55°.

Solvents.—Petroleum ether refers to the fraction which distilled at 60–80°. Toluene analytical reagent, bp 110, was used. Spectroscopic grade *n*-hexane was used for spectral determinations.

Adsorbent.—Aluminium oxide thin layer chromatography plates were obtained from Brinkmann Instruments Inc., N. Y. Absorbent thickness was 250 μ. Neutral alumina supplied by Baker Chemical Co. of N. J. was used for chromatography.

Methods.—Spectra were measured using silica cells on a Cary recording spectrophotometer, Model 14.

Partition Test.—Partition tests were done by shaking a hexane solution with an equal volume of 95% methanol and determining the ratio of the concentrations in hexane by estimating (spectrophotometrically at λ_{max}) the concentration remaining in hexane.

Oxidation of β-Carotene.—β-Carotene (5 mg) was dissolved in 50 ml of toluene. The solution was incubated in a thermostat at 60° in the dark. A slow stream of oxygen was passed through the solution. Samples were withdrawn at regular time intervals and the concentration of the residual β-carotene was determined spectrophotometrically at the wavelength of maximum absorption, 464 mμ.

Identification of Major Reaction Products.—The reaction mixture was applied under a stream of nitrogen along a straight line on an alumina thin layer plate (250-mμ thickness) about 3 cm from the bottom. Drying was completed under a stream of nitrogen. The chromatogram was developed by the ascending technique empolying one of the following systems: (1) 1% acetone in petroleum ether, (2) 3% acetone in petroleum ether, and (3) 5% acetone in petroleum ether. The chromatogram was developed until the solvent front reached a distance of 15 cm from the applied mixture. Bands were removed from partially dry chromatograms, extracted with acetone, and rechromatographed for better separation. The R_f values were measured from the center of the band.

After removal from the plate, bands were again extracted with acetone and the solvent was evaporated to dryness under reduced pressure at low temperature while the products were protected from exposure to light. The absorption spectra were obtained after redissolving the products in spectroscopic hexane.

Registry No.—β-Carotene, 116-32-5.

Acknowledgment.—This work was supported in part by Public Health Service Grant No. AM 11665.