this instability and indicate that carotene determinations are of importance in quality evaluation.

Acknowledgment

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BIOASSAY OF CAROTENOIDS

Vitamin A Activity of Beta-apo-8′-carotenal

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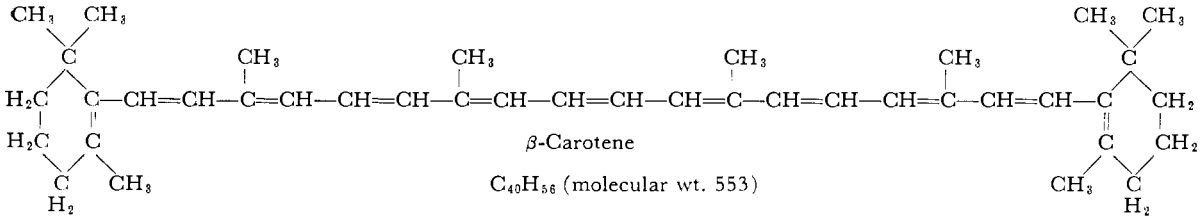
β-Apo-8′-carotenal in oil solution was assayed for vitamin A activity in a series of U.S.P. vitamin A curative rat-growth assays. Combining the data from two assays yielded an average potency of 1,200,000 U.S.P. units of vitamin A per gram of all-*trans* β-apo-8′-carotenal with a range of 1,070,000 to 1,330,000 units at $P = 0.05$ or 72 ± 8% of the activity of all-*trans* β-carotene on a weight basis. Dry stabilized beadlets of β-apo-8′-carotenal were also assayed in three separate tests. Within the limits of the assay procedure no significant difference was seen in vitamin A potency. The possibility is discussed that β-apo-8′-carotenal is an intermediate product in the biological conversion of β-carotene to vitamin A.

Euler, Karrer, and Solmssen (*4*) reported β-apo-8′-carotenal to be the main product resulting from the partial oxidation of β-carotene with calcium permanganate. Daily supplements of 5 γ fed to vitamin A–deficient rats stimulated growth. Glover and Redfearn (*6*) prepared β-apo-8′-, β-apo-10′-, and β-apo-12′-carotenal and fed them to vitamin A–deficient rats. All were transformed into vitamin A. It was noted that β-apo-10′-carotenal undergoes β-oxidation to β-apo-12′-carotenal. The structural relationship of vitamin A, β-carotene, and β-apo-8′-carotenal is shown on page 391. The commonly accepted vitamin A potency of β-carotene is 1,667,000 I.U. per gram as compared to 3,333,000 for vitamin A alcohol. On the basis of the formulas of the two molecules, conversion of β-carotene to vitamin A has been postulated to proceed via hydrolytic cleavage at the central double bond. Theoretically this would be expected to yield two molecules of vitamin A from one of β-carotene. However, attempts to carry out this unusual reaction chemically have generally been very inefficient. Conversion in the animal body under most conditions yields only one rather than two molecules of vitamin A as indicated by the relative potencies given above, which are based on numerous bioassays. Under certain conditions, however, a much more efficient conversion, approaching closely the theoretically possible two molecules of vitamin A, has been reported (*3, 7, 11*).

The stepwise oxidation of β-carotene from one end, yielding successively β-apo-8′-carotenal, β-apo-10′-carotenal, β-apo-12′-carotenal, and subsequently vitamin A aldehyde, which is reduced to vitamin A, has been postulated by Glover and Redfearn (*6*) as a possible mode of its conversion to vitamin A. In support of this they observed that the hitherto unidentified substances related to the carotenoids found by Festenstein (*5*) in horse intestine had spectroscopic and chromatographic properties identical to those of the β-apo-10′- and β-apo-12′-carotenals. Winterstein (*16*) isolated β-apo-8′-carotenal from oranges, tangerines, and spinach and demonstrated the identity of the naturally occurring substance with synthetic β-apo-8′-carotenal.

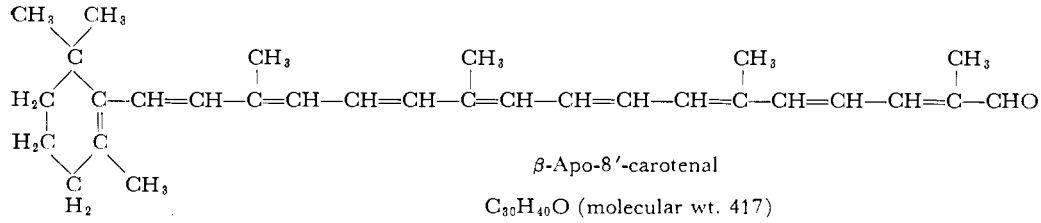
Isler *et al.* (*8*) synthesized β-apo-8′-carotenal as part of an intensive program to produce synthetically many of the naturally occurring carotenoids. This carotenoid has very desirable properties for coloring foods and beverages. Also, as a feed ingredient, it has been reported by Steinegger, Streiff, and Zeller (*14*), Steinegger and Zanetti (*15*), and Marusich, Kadin, and Bauernfeind (*13*) to be an efficient pigmenter for coloring egg yolks uniformly with a pleasing yellow equal to that obtained with high quality ingredients rich in natural xanthophylls.

Structural Formulas



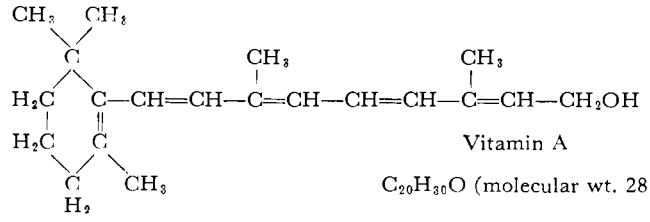
β-Carotene

$C_{40}H_{56}$ (molecular wt. 553)



β-Apo-8′-carotenal

$C_{30}H_{40}O$ (molecular wt. 417)



Vitamin A

$C_{20}H_{30}O$ (molecular wt. 286)

In view of such potential applications of β-apo-8′-carotenal in foods and feeds, its vitamin A activity has been determined quantitatively by means of the U.S.P. rat curative growth test. Because this carotenoid resembles β-carotene and many other carotenoid pigments in being readily destroyed by oxidative processes, it has been found desirable to stabilize it by incorporation into gelatin-sugar beadlets similar to the vitamin A and carotene beadlets which have gained wide acceptance in the food and feed fields. Therefore dry beadlets containing β-apo-8′-carotenal were included in the present rat bioassay in addition to oil solutions of the pure crystalline material synthesized by the method of Isler *et al.* (*8*).

Chemical Assay of β-Apo-8′-carotenal

Crystalline all-*trans* β-apo-8′-carotenal or oil solutions thereof are assayed by dissolving in petroleum ether (Skellysolve B), making the appropriate dilutions, and reading in a Beckman DU spectrophotometer at the maximum at 454 to 455 mμ. An $E^{1\%}_{1cm.}$ of 2640 is used in calculating the potency of the sample.

In the assay of mixtures of cis and trans stereoisomers of β-apo-8′-carotenal, such as are present in the dry beadlet preparations used in this study, a somewhat different procedure is used. The method of assay utilizes the technique described by Bunnell, Driscoll, and Bauernfeind (*2*) for β-carotene preparations, which involves isomerization to an equilibrium mixture. The procedure for assay is the same as reported in this paper, except that the sample is read in the Beckman spectrophotometer in petroleum ether (Skellysolve B) at the maximum at 454 to 455 mμ and potency is calculated using an $E^{1\%}_{1cm.}$ of 2530.

The latter extinction is the value for the stereoisomeric mixture determined experimentally by iodine isomerization. This value gives the best approximation of the total β-apo-8′-carotenal content that can be made without the elaborate and time-consuming separation and individual determination of all the stereoisomers.

Biological Assay of β-Apo-8′-carotenal

An oil solution of β-apo-8′-carotenal and stabilized gelatin-sugar beadlets containing 2.53% β-apo-8′-carotenal were compared to the U.S.P. vitamin A reference oil in two successive assays. The same beadlet preparation plus a second containing 7.18% β-apo-8′-carotenal was tested in a third assay using both the U.S.P. vitamin A reference oil and a dry stabilized vitamin A beadlet as the standard. For all assays, the oil solutions of β-apo-8′-carotenal and vitamin A standard were dosed orally. Each rat was given the appropriate dosage in 0.1 ml. of cottonseed oil every fourth day for 4 weeks using a 1-ml. tuberculin syringe with a blunt-tipped needle. The β-apo-8′-carotenal beadlets were fed in the diet in all assays, as were the vitamin A beadlets in the third assay. An amount of both substances equivalent to that dosed orally in 0.1 ml. was added to 40 grams of the U.S.P. vitamin A–free ration. The rats were given access to feed *ad libitum* and feed intake was recorded. The levels fed, number of rats per group, growth responses, and calculated potencies of β-apo-8′-carotenal are shown in Table I for the three assays.

Discussion of Results

The potencies obtained for β-apo-8′-carotenal in the three assays are in good agreement. To determine whether overall average potencies and error limits could be calculated, the data for the three assays of the beadlets and those for the two assays of oil solution were tested for homogeneity by the X^2 test (*1*). As the potencies obtained in the individual assays were found to be homogeneous at the 5% level, the data were combined. The composite vitamin A value obtained for the stabilized beadlets is 1,070,000 U.S.P. units per gram of β-apo-8′-carotenal with a range of 950,000 to 1,190,000 units at $P = 0.05$. In oil solution, 1 gram of β-apo-8′-carotenal has vitamin A activity equal to 1,200,000 U.S.P. units with a range of 1,070,000 to 1,330,000 units.

It is of interest to compare these values with the vitamin A activity of all-*trans* β-carotene, which has yielded an average potency of 1 I.U. per 0.58 γ in repeated rat-growth assays in this laboratory under the conditions used in the present

Table I. Vitamin A Activity of β-Apo-8′-carotenal in Oil Solution and Stabilized Beadlets

Supplement	Mode of Administration	No. of Rats Surviving	Supplement per Rat per 4 Days	Weight Gain in 28 Days, G.	Calcd. Vitamin A Activity, U.S.P. Units per Gram β-Apo-8′-carotenal: Potency	Range of potency at P = 0.05
Assay 1			*U.S.P. Units*			
U.S.P. vitamin A reference oil	Oral	6	3.2	25		
		10	4.7	37		
		13	6.9	51		
			Micrograms[a]			
Oil solution of β-apo-8′-carotenal	Oral	10	3.8	31	1,150,000	960,000–1,400,000
		12	5.6	44		
		14	8.3	84		
Stabilized beadlets β-apo-8′-carotenal (2.53%)	In diet	8	3.1[b]	33		
		12	5.0	52	1,000,000	700,000–1,400,000
		14	8.4	75		
Assay 2			*U.S.P. Units*			
U.S.P. vitamin A reference oil	Oral	34	4.1	34		
		27	6.0	42		
		34	8.9	65		
			Micrograms			
Oil solution of β-apo-8′-carotenal	Oral	30	4.9	44		
		31	7.2	66	1,200,000	1,080,000–1,340,000
		35	10.7	85		
Stabilized beadlets β-apo-8′-carotenal (2.53%)	In diet	12	4.3	34		
		16	6.6	49	1,060,000	940,000–1,190,000
		32	11.5	85		
Assay 3			*U.S.P. Units*			
Stabilized vitamin A beadlet	In diet	14	4.9	26		
		17	7.4	42		
		21	12.4	68		
U.S.P. vitamin A reference oil	Oral	15	4.2	14		
		20	6.1	39	107[c]	91–125%
		11	9.0	58		
			Micrograms			
Stabilized beadlets β-apo-8′-carotenal (2.53%)	In diet	8	5.1	25		
		10	7.8	44	940,000	760,000–1,160,000
		9	12.4	63		
Stabilized beadlets β-apo-8′-carotenal (7.18%)	In diet	10	5.1	19		
		11	8.0	40	930,000	830,000–1,110,000
		12	13.1	74		

[a] Weight of β-apo-8′-carotenal in supplement.
[b] Av. per 4 days calculated from total feed intake over 28-day assay period for all in-diet feeding.
[c] % of in-diet standard response.

assays (*12*). This value is very close to the International Standard value of 1 I.U. per 0.6 γ. In oil solution all-trans β-apo-8′-carotenal has 72% (range at $P = 0.05$: 64 to 80%) of the activity of all-trans β-carotene. The beadlets in which the β-apo-8′-carotenal is partially stereoisomerized are 64% (range at $P = 0.05$: 57 to 71%) as active as all-trans β-carotene. While the differences between the oil solution and beadlet form are not statistically significant, the somewhat lower activity of the beadlets could be due to the presence of less active stereoisomers formed in manufacturing. This difference may also be influenced by the fact that the accuracy and precision of the assay procedure are less for the beadlets than for oil solutions of the pure trans isomer.

The conversion of β-carotene to vitamin A via β-apo-8′-carotenal would require cleavage of the β-ionone ring from one end of the molecule. This has been achieved chemically by Karrer and his coworkers (*9, 10*) by permanganate treatment. The fact that β-apo-8′-carotenal has considerable vitamin A activity has led to speculation (*6*) that conversion of β-carotene to vitamin A in vivo may proceed by such cleavage of one ionone ring followed by β-oxidation to vitamin A aldehyde, which is reduced to vitamin A. If β-apo-8′-carotenal were totally converted to vitamin A, it would have a potency of 2,280,000 I.U. per gram. The actual vitamin A potency in the rat as determined in the present assays is only 53% of this theoretical value. The fact that all-trans β-apo-8′-carotenal has less vitamin A activity than all-trans β-carotene even on a weight basis suggests the existence of an important route or routes of conversion of β-carotene to vitamin A other than that postulated above. The formation under certain conditions (*3, 7, 11*) of more than one molecule of vitamin A from one molecule of carotene would require an alternate metabolic pathway.

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ENZYME INHIBITION

The Trypsin Inhibitor of Alfalfa

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A trypsin inhibitor was isolated from alfalfa meal by extraction with water, dialysis, and precipitation with ethyl alcohol. A kinetic study indicated it to be a noncompetitive inhibitor. It was inactivated slowly by heat, and amino acids were liberated from it by acid hydrolysis. Its properties indicate a polypeptide or a noncoagulable protein.

KENDALL (*3*) reported that in vitro hydrolysis of casein by trypsin is inhibited by aqueous extracts of the fresh forage of alfalfa, ladino clover, and soybeans. Beauchene and Mitchell (*1*) detected a similar inhibition of trypsin by extracts of commercial dehydrated alfalfa, indicating that the inhibitory substance withstands the heat of the dehydration process.

A trypsin inhibitor in alfalfa meal conceivably could be partially responsible for the growth depression which has been observed when alfalfa meal is added to broiler rations at levels of 10% or more. Investigation of this possibility will require isolation of the inhibitor in sufficient quantity for in vivo studies. This report is concerned with a method of isolating the trypsin inhibitor and attempts to determine its chemical nature.

Experimental

The inhibitory activity of extracts of alfalfa meal, and of the fractions obtained from them, was determined by measuring the decrease in the amount of amino acids and peptides released during the in vitro digestion of casein by trypsin when the extracts or fractions were present. Amino acids and peptides were determined by a modification of the method of Spies and Chambers (*6*), which depends on the reaction of these substances with cupric ions under proper conditions to produce complexes having a blue color.

Measurement of Inhibition. A modification of the procedure of Beauchene and Mitchell (*1*) was used.

A 3% solution of casein in pH 8.4 phosphate buffer was prepared and adjusted to pH 8.4 with dilute sodium hydroxide. Five milliliters of the casein solution were placed in each of four 25-ml. beakers, 2 ml. of water were added to beakers 1 and 2, and 2 ml. of the inhibitor solution were added to beakers 3 and 4. The pH was adjusted to 8.4, if necessary, by means of a Beckman pH meter equipped with microelectrodes. To beakers 1 and 3 was added 1 ml. of a solution containing 30 mg. of trypsin per 100 ml. of water. A portion of the trypsin solution was heated to boiling, and 1 ml. of this solution was added to beakers 2 and 4. The following mixtures thus were obtained:

Beaker	Mixture
1	Casein, trypsin
2	Casein, inactivated trypsin
3	Casein, trypsin, inhibitor
4	Casein, inactivated trypsin, inhibitor

The beakers were covered with small watch glasses and placed in a water bath maintained at 37° C. After 4 hours, 5 ml. of the solutions were placed in test tubes and the undigested casein was precipitated by adding 5 ml. of a 10% trichloroacetic acid solution. The samples were filtered and the filtrates were adjusted to pH 7 with 40% sodium hydroxide. Five milliliters of each filtrate were placed in 15-ml. graduated centrifuge tubes and 3 ml. of a suspension of copper phosphate in pH 9.1 borate buffer were added. The contents of the tubes were mixed, allowed to stand 5 minutes, and centrifuged at 2000 r.p.m. to sediment the excess copper phosphate. The intensity of the blue color of the supernatant solutions was measured at 620 mμ with a Beckman DU spectrophotometer. A standard curve was prepared by carrying known quantities of alanine through the procedure.

Isolation of Trypsin Inhibitor. Preliminary extraction studies were performed by extracting 25-gram portions of commercial dehydrated alfalfa meal in a Waring Blendor with 200 ml. of various solvents. After blending for 10 minutes, the samples were filtered through a cloth held in a Büchner funnel. When organic solvents were used, the solvent was removed by evaporation on a steam plate under a current of air from an electric fan, and the residue was extracted with water. The final volume in each case was adjusted to 200 ml. and the solutions were centrifuged.

Dialyzability of the inhibitor was determined by placing a water extract in a cellophane dialysis bag and dialyzing against distilled water for several days. The water was changed frequently, and the dialyzates so obtained were combined and concentrated to the same volume as the original extract.

The inhibitory activities of the various extracts are shown in Table I, from which it is apparent that the inhibitor was soluble in water but was not extracted by acetone or 95% ethyl alcohol. However, aqueous ethyl alcohol extracted the substance, with a progressive increase in inhibition as the alcohol concentration was decreased. The dialyzed extract was as inhibitory as the undialyzed extract, while the dialyzate was noninhibitory. These properties were utilized in the procedure which was adopted for isolation of the inhibitor.

Concentrates of the inhibitor were prepared from both commercial dehydrated alfalfa meal and alfalfa dried at 50° C. in a laboratory oven. Twenty-