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In vitro conversion of carotene to vitamin A

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IN VITRO CONVERSION OF CAROTENE TO VITAMIN A

by

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INTRODUCTION

The only known natural method of formation of vitamin A is by degradation of carotenoids. All higher animals require vitamin A and must acquire it either by ingesting the vitamin itself or by ingesting a carotenoid provitamin and converting it to vitamin A. Only plant life possesses the enzymatic systems required for carotenoid synthesis. Some of these plant enzymes are specific oxidases as is evidenced by the large quantities of oxidized carotenoids of specific structure that exist in nature, e.g. the xanthophyll series. However, one oxidation product of carotenoids, vitamin A, is unknown in plants.

It seems reasonable to conjecture that animal life possesses an enzyme or series of enzymes not found in plants that catalyze the conversion of provitamin carotenoids to vitamin A. If vitamin formation resulted from a non-specific oxidation process, it would seem strange that the vitamin is not found even in trace amounts in plant life where carotenoids abound but can rapidly be formed from small amounts of carotenoids taken in by animals.

Elucidation of the mode of action of this hypothesized enzyme system would explain the key reaction which is the source of all natural vitamin A. It might also be hoped that an understanding of this process would throw some light on a fruitful approach to studying the systemic mode of action of

vitamin A.

The problem of conversion of the provitamin carotenoids to vitamin A has been actively investigated for 30 years. During this time attention has been centered upon the conversion of beta-carotene because it has the most provitamin activity of all the carotenoids. No enzyme system yet has been isolated that will convert beta-carotene primarily because no in vitro system has been devised that would bring about the conversion.

The investigations described in the experimental section of this thesis were performed in an effort to develop such an in vitro system. Much of the work was devoted to testing prescribed analytical procedures and to developing a superior procedure for the detection and estimation of minute amounts of vitamin A formed in the presence of high concentrations of carotene and lipid.

The tissue used as a potential enzyme source was rat intestine. Reasons for the selection of rat intestine stem from the results of previous work (discussed in the literature review) that established the intestine as the site of efficient in vivo conversion.

In a biochemical system capable of converting carotene to vitamin A, it is necessary to present a water insoluble substrate in suitable form for enzyme catalysis to take place in an aqueous system. Synthetic and natural emulsifying

agents were tested for conversion-promoting effectiveness. Other factors that might have a profound influence on conversion, such as anaerobic and aerobic incubation, antioxidants for the preservation of labile carotene, and incubation time and temperature, also were investigated.

The studies reported herein were with *in vitro* systems with conditions varied to test the effectiveness of vitamin A formation from *beta*-carotene. The purpose was to develop an efficient *in vitro* converting enzyme system with long range goals of purification of the system for conversion in a biochemically defined system and elucidation of the mechanism of the conversion.

LITERATURE REVIEW

It has been 30 years since Moore (1) first established beta-carotene as a precursor of vitamin A by recording an increase in the vitamin A liver stores of rats receiving carotene. During the interval since this discovery to the present time a number of investigators have sought to elucidate the mechanism of the conversion of beta-carotene to vitamin A. The fact that the mechanism still remains obscure is due chiefly to the failure to develop *in vitro* systems capable of performing the conversion.

This review deals with research preliminary to and essential for the clarification of the mechanism of conversion of beta-carotene to vitamin A in living systems. The work to be reviewed includes that which established the formation of vitamin A from beta-carotene by *in vivo* and chemical means, that which established the site of *in vivo* conversion and factors influencing the conversion efficiency, and finally the work on the development of *in vitro* systems capable of converting carotene to vitamin A.

Several excellent reviews have been written on the subject of vitamin A formation from carotene. In 1951 Kon and Thompson (2) reviewed early work including the establishment of the intestine as the site of conversion. In 1956 Morton and Goodwin (3) provided a general review of vitamin A research while Lowe and Morton (4) published a more compre-

hensive review of the conversion problem in an article limited to several aspects of vitamin A research. In 1957 Moore's book, "Vitamin A", (5) which reviews all aspects of vitamin A including the conversion problem, was published.

In vivo Conversion

The first evidence of vitamin A formation from beta-carotene was obtained by Moore (1, 6, 7). Vitamin A depleted rats were given daily doses of carotene and sacrificed at intervals. Liver analyses showed increasing vitamin A stores with increasing length of time during which carotene was administered. Capper et al. (8) confirmed the results by demonstrating conversion in chickens. By taking small pieces of liver from rabbits by biopsy, Wolff et al. (9) found that the vitamin A content of liver increased after injections of carotene.

Early workers (10) hypothesized that the vitamin A activity of beta-carotene could be due to symmetrical fission at the central position of unsaturation with one molecule of beta-carotene generating two molecules of vitamin A. Much later Glover and Redfearn (11), noting that in vivo yield does not exceed 50% of the theoretical by central cleavage, presented evidence that initial oxidative attack occurs at a terminal double bond and that vitamin A is formed after successive oxidative degradations of apo-carotenals.

Chemical Conversion

The problem of central oxidative fission was investigated in purely chemical systems. The central double bond of beta-carotene might be considered most vulnerable to reagent attack because of its symmetrical and least sterically hindered position. Opposed to favorable stereochemical aspects, however, is the supposition that double-bond interaction in an extended system of conjugated double-bonds of this type would be expected to impart maximal single-bond character at the center and thus render this center less favorable to electrostatic orientation of the attacking reagent molecule (12, 13).

Controlled oxidations with chromic acid and with lead tetraacetate (14) and with permanganate (15) resulted in initial end oxidation of the beta-ionone ring to long chain carbonyl compounds but no vitamin A aldehyde. In 1945, however, with hydrogen peroxide Hunter and Williams (16) obtained small amounts of vitamin A aldehyde (0.5%). Five years later Wendler et al. (13), using osmium tetroxide as catalyst, obtained vitamin A in 30% yield with H_2O_2 as the oxidant.

Peroxidation of beta-carotene to form vitamin A aldehyde as an intermediate in vitamin A formation is consistent with the fact that vitamin A aldehyde has not been isolated from any animal organs except the eye. Glover et al. (17) were unable to recover vitamin A aldehyde after oral dosing except from stomach and intestinal contents. All absorbed vitamin A

aldehyde in the intestinal wall had been reduced to vitamin A alcohol and most of the alcohol had been esterified.

In vivo research has established that vitamin A is formed from beta-carotene in good yield. Chemical studies have shown that the conversion could be performed with inorganic systems also in good yield. The next step in understanding the in vivo process was to find the site of conversion so that more controlled studies could be made.

Site of Conversion

Liver

Work on establishing the site of conversion with in vivo and in vitro systems began as soon as Moore established beta-carotene as a provitamin. This was in 1930, 15 years before successful conversion of beta-carotene to vitamin A by chemical systems. Workers prior to 1947 believed the liver to be the site of conversion. Thus, for more than 15 years studies were performed using liver as the biochemical system. A number of reports of conversion with liver contributed to maintaining confidence that the liver was the site of conversion.

The first conversion attempts with liver were performed by von Euler and von Euler (18) with shark liver and by Ahmad (19) with mammalian liver. Both failed to demonstrate conversion. However in 1939 Olcott and McCann (20) claimed

separation of the "beta-carotenase" enzyme from liver tissue. Homogenized liver of a vitamin A deficient rat was incubated with an aqueous dispersion of beta-carotene in ethyl laurate. The spectrum of the nonsaponifiable extract showed a peak at 328 mu. Without beta-carotene no peak occurred. Incubation of beta-carotene with a toluene-water extract of a liver autolyzate showed the same spectral confirmation of vitamin A. The control in which the extract was boiled prior to incubation showed a spectrum with no peak at the vitamin A absorption maximum.

Von Euler and Klussmann (91) also claimed conversion and carotenase isolation from cow liver as did Parienti and Palli (92) from dog liver.

All three groups claimed conversion of carotene to vitamin A with a water soluble extract of liver tissue. Other workers were not able to verify these results. Rea and Drummond (93) repeated Olcott and McCann's experiment but found no vitamin A. Drummond and MacWalter (94) were unable to obtain conversion with homogenized liver or with liver that had absorbed intravenously injected carotene before removal of the organ. Woolf and Moore (95) pointed out analytical complications in spectrographic, colorimetric, and biological assays and suggested combinations of all these types of analyses for more convincing evidence of vitamin A formation.

In vivo experiments contributed additional evidence that

the liver is not the site of conversion. Drummond and MacWalter (26) injected colloidal carotene into the portal vein and observed the appearance of orange carotene granules in the Kupfler cells of the liver but no vitamin A formation. Other workers (27, 28) also obtained feeble evidence of conversion with carotene injected either intravenously or subcutaneously.

Intestine

The fact that only minute amounts of vitamin A were formed from injected carotene while large amounts were formed from oral dosages of carotene caused some to doubt the liver as the site of conversion. In one study Sexton et al. (28) found rats dying of vitamin A deficiency with enough beta-carotene deposited in the liver to provide adequate oral dosages to prevent deficiency for a year.

Mattson et al. (29) investigated the role of the intestine as the possible converting tissue. Vitamin A deficient rats were dosed with carotene dissolved in oil and sacrificed at intervals up to 6 hours after dosing. The livers and intestines were analyzed for vitamin A and beta-carotene. The results showed higher levels of vitamin A in the intestine than the liver at first. As the time interval increased between dosage and sacrifice liver levels increased. This suggested vitamin A formation in the intestine with subsequent

transfer to the liver. Glover et al. (30, 31) confirmed these results.

The most comprehensive work on the intestine as the site of conversion was performed by Thompson et al. (32, 33, 34). The appearance of vitamin A in the wall of the small intestine before appearance in the liver and blood was confirmed. Vitamin A was detected in the wall of the small intestine as soon as 15 minutes after dosing. The appearance of vitamin A in the lymph of the pig and the rat was detected by ultraviolet spectrophotometry and fluorescence under ultraviolet irradiation. Vitamin A in the contents of the small intestine was found to be transferred from the wall after death. Sectional analysis of the small intestine showed the highest concentration of vitamin A to be in the middle sections. Most of the vitamin A formed was esterified.

Alexander and Goodwin (35) demonstrated in rats conversion of carotene to vitamin A in intestines cannulated with polyethylene tubing. Mattson (36) confirmed his earlier results with Mehl and Deuel by purifying the vitamin A in the non-saponifiable intestinal extract. The vitamin was separated from carotene by partitioning between methyl alcohol and light petroleum, then further purified by absorption chromatography on magnesia. The ultraviolet absorption spectrum of the fluorescent band from the magnesia column was characteristic of vitamin A with absorption maximum at 328 mu.

These experiments contributed strong evidence that the intestine is the site of conversion. Cheng and Deuel (37) and Thompson et al. (38) confirmed the conversion in the intestine of the chick. Work also was performed to determine whether conversion takes place in the lumen before absorption or in the wall after absorption. The early work of Thompson et al. (30), which established the intestine as the site of conversion, suggested the wall as the site of conversion. Sibbald and Hutcheson (39) reported that conversion occurs in the wall of a ligatured duodenal loop of the chicken. They also found that continuity of the alimentary canal was not essential for conversion but that the blood supply to the duodenal wall was essential for vitamin A formation from beta-carotene. This suggests that conversion occurs in the intestinal wall.

Greenberg (40) came to a different conclusion. Working with unstained frozen tissue sections and identifying vitamin A and carotene by fluorescence, he found that vitamin A was first recognizable 5 to 15 minutes after a carotene meal within the intestinal lumen just adjacent to the tips of the villi. His data suggested that the conversion is an extracellular process. More evidence is needed to determine whether conversion is effected in the lumen or the wall.

Other sites

The large number of failures to convert carotene to vitamin A in the liver during the period from 1930 to 1947

led to the search for other sites of conversion. The studies discussed in the previous section, beginning in 1947, established conversion in the intestine. However, more recent work has indicated the intestine is not the only possible site of conversion although it appears to be the most important.

As early as 1946 Tomarelli (41) found that intramuscular injections of beta-carotene and alpha-tocopherol dispersed in Tween 80 were as effective in curing vitamin A deficiency as equivalent amounts of carotene given orally.

Bieri and Sandman (42) confirmed that intramuscularly injected carotene relieves vitamin A deficiency in rats although they did not find this route as effective as oral administration. They also found that carotene dissolved in oils is ineffective in relieving vitamin A deficiency but when dispersed in Tween 40 it becomes effective. Bieri and Pollard (43, 44) demonstrated that conversion is not dependent on the small intestine. In one experiment vitamin A was formed in rats with the bile duct ligated and severed before carotene was injected. This prevented the transport of injected carotene by the bile to the gut. In another experiment dispersed injected carotene was converted to vitamin A even though the small intestine, kidneys and most of the liver had been removed before injection.

Kon et al. (45) also found in rats that intravenously injected carotene dispersed in Tween 40 gave rise to increased

blood and liver levels of vitamin A. Worker (46) found that conversion of intravenously administered carotene dispersed in Tween 40 to vitamin A was not adversely affected in hepatectomized-eviscerated rats. In even more drastic terminal experiments, McGillivray et al. (47) reported that the formation of vitamin A from an aqueous dispersion of carotene administered intravenously was unaffected by the complete removal of the liver, stomach, small intestine, large intestine, pancreas, kidneys, adrenals, and gonads. Worker (48) found that the increase in blood levels of vitamin A 5 minutes after carotene injection was as great in rats with lungs removed as in those which were decapitated as in controls.

Extraintestinal conversion has not been as conclusively observed in other species. Eaton et al. (49) reported limited conversion in dairy calves, while Kon et al. (45) found little evidence of conversion. Klosterman et al. (50) found no increase in vitamin A in the blood of vitamin A deficient lambs after intravenous injection of carotene dispersed in Tween 20. However Church et al. (51, 52) found a significant increase with an intravenous injection of solubilized, aqueous carotene preparation. Kirschman and MacVicar (53) found that intravenously administered carotene dispersed in Tween 40 was converted in ileectomized sheep.

Hentges et al. (54) noted a disappearance of the symptoms of vitamin A deficiency in pigs when water-miscible carotene

preparations were administered by both intravenous and intramuscular injection. Kon et al. (45) obtained conversion in rabbits and Bieri and Edwards (55) in chicks by injecting carotene dispersed in Tween 40. Bonfante (56, 57) porposed that conversion can occur in the lungs, a conclusion drawn after he demonstrated that oxen allowed to absorb an aerosol (water containing carotene, 50 mg. per 100 ml) showed a decrease in blood carotene content and an increase in the vitamin A content of the blood.

The observations cited above strongly suggest that other organs besides the small intestine are capable of transforming carotene into vitamin A, especially if the carotene is colloidally dispersed by a surface active agent. In most cases the conversion by injected routes did not approach the efficiency of the oral route. The small intestine appears to be the most important site of conversion, but other sites also possess the capacity to convert carotene to vitamin A.

Factors Influencing Conversion

Claims have come from many laboratories that certain cofactors have a profound effect on the in vivo efficiency of the conversion of carotene to vitamin A. The literature on this subject was reviewed thoroughly by Lowe and Morton (4). Only a brief general discussion will be undertaken here. Lowe and Morton's review should be consulted for a detailed pre-

sentation and original references.

Vitamin A formation from carotene has been linked with the level of certain hormones (thyroxine, insulin and adrenal hormones), with several vitamins (tocopherols and vitamin B_{12}), with phosphate and with antibiotics.

Evidence showing that thyroxin increases conversion of carotene to vitamin A and that compounds having antithyroid activity (such as thiouracil) inhibit conversion is balanced by evidence from other laboratories showing that these compounds have no effect on the conversion.

The literature on insulin effects also suffers from contradictory reports. However, in 1953, Rosenberg and Sobel (58) demonstrated that vitamin A deficient rats stored four times as much vitamin A, after carotene administration by stomach tube, as similarly treated deficient rats with alloxan diabetes. Diabetes was induced by subcutaneous injections of alloxan. Diabetic rats stored orally fed vitamin A as well as non-diabetic rats, indicating poorer absorption of carotene by the diabetic rat. It was not proved that insulin could reverse this effect.

Regarding adrenal hormones, Clark and Colburn (59) have demonstrated that cortisone treatment impairs formation of vitamin A from carotene and decreases depletion time for vitamin A starved rats.

Most of the vitamin work on the effect of tocopherol

level on vitamin A formation has led to the conclusion that small amounts of vitamin E have a synergistic effect on vitamin A formation from carotene while larger amounts have an inhibitory effect (60). This work also showed that tocopherols had no effect on carotene or vitamin A absorption and that they markedly protected carotene from oxidative destruction. Presumably at low levels the tocopherol role is that of an antioxidant that suppresses non-specific oxidative degradation of carotene but at higher levels suppresses the oxidative process involved in the conversion of carotene to vitamin A.

This role is not conclusive, however, since there are several non-conforming reports in the literature. Bieri (61) found that when beta-carotene was dispersed in aqueous Tween 40 and injected into rats dosed with large amounts of tocopherol no inhibition of conversion occurred. Unless the enzyme-converting system is different when carotene is administered by parenteral routes this finding does not support earlier studies.

Teppel (62, 63) has shown that alpha-tocopherol and other antioxidants with *in vivo* vitamin E activity effectively inhibit *in vitro* oxidation of unsaturated fatty acids and concurrent oxidative destruction of vitamin A catalyzed by hematin compounds.

There is some evidence that vitamin B_{12} is involved in

the conversion of carotene to vitamin A. The results of numerous studies as to the role, if any, of phosphate on conversion are contradictory and do not encourage generalization. Experimentation has indicated that several antibiotics, including aureomycin and chlortetracycline, enhance conversion. This area of investigation is reported in detail in the review by Lowe and Morton (4).

In vitro Conversion Studies

The work reviewed in this section was performed in order to find a biochemical system of known components capable of converting carotene to vitamin A. The in vitro work was performed with intestinal tissue and began about the time, 1947, the intestine was shown to be the primary site of conversion. Earlier in vitro studies with liver tissue carried out when the liver was believed to be the site of conversion were discussed above in the section on liver.

Concurrent with the report establishing the small intestine as the site of conversion, Deuel's group presented evidence for in vitro conversion in the small intestine of vitamin A depleted rats (64).

> The animals were stomach tubed with one ml of carotene solution and sacrificed immediately. The carotene concentration was 310 µg/ml and the solution was stabilized with "Tween" containing 0.5% of alpha-tocopherol.
>
> The carotene was immediately washed from the stomach into the intestine with Ringer-Locke

> solution and the intestine removed after ligation at the pylorus and ileocaecal valves. The intestines were incubated in Ringer-Locke solution under aneerobic conditions for 3 hours at 37°C; they were then removed from the bath, the contents flushed out with 0.9% saline and the washings discarded. The intestinal wall was saponified, extracted and the vitamin A determined by the Carr-Price reaction. (64, p. 76)

The results of 18 such experiments showed vitamin A formation in every case and an average of 8.7 I.U. (2.6 ug) per intestine. The controls showed 4 to 6 I.U. of vitamin A per intestine. However, these did not show the color characteristic of vitamin A but were amber and did not fade on standing.

Glover et al. (31) in 1948 also attempted in vitro conversion with intestine. They used beta-carotene in peanut oil and lecithin rather than tocopherol. The analysis for vitamin A was both by the Carr-Price test modified for carotene contribution and by taking ultra violet absorption spectrum corrected by the Morton Stubbs method (65, 66). No vitamin A formation was detected. However, 5 hours were allowed to pass between dosing and sacrifice, perhaps time enough for most of the carotene to pass out of the gut wall. Also the animals were aneasthetized before the intestines were removed.

Stallcup and Herman (67) in 1950 reported in vitro conversion of Tween 80 dispersed carotene to vitamin A with whole sections of small intestine and also with minced liver. The method of analysis was the Carr-Price reaction.

The following year McGillivray (68) obtained evidence of conversion with excised, ligated sections of sheep intestine. In six experiments an average of 7.2 µg of vitamin A was found in the controls and 18.8 µg in the test sections incubated with beta-carotene dispersed in aqueous Tween 80. After incubation, the intestines were flushed, saponified and extracted with petroleum ether. The non-saponifiable extract was chromatographed on a magnesia-Hyflo-Supercel column. Vitamin A was measured by two methods. Activated glycerol dichlorohydrin was used (69) and the absorption spectrum was taken.

As in many of the earlier studies on the conversion problem, confirmation was not forthcoming. Kon and Thompson (?) in their 1951 review mentioned briefly that they were unable to effect conversion by perfusion of surviving rat intestine. A paper from India (70) the same year reported failure to demonstrate the *in vitro* conversion of carotene by rat intestine. In 1953, Rosenberg and Sobel (71) repeated the work of Wiese *et al.* (64) but obtained no evidence of conversion. Instead of the characteristic blue, a brownish color was obtained with antimony trichloride and greenish-yellow instead of the magenta color with activated glycerol dichlorohydrin.

In a careful study in 1953, Bieri and Pollard (72) found little or no vitamin A formation. In the manner of previous investigations they incubated Tween-dispersed carotene various lengths of time in rabbit, rat and calf small intestine.

After saponification and extraction, removal of sterols, and chromatography on alumina, vitamin A estimation was made by three different procedures; the Carr-Price test, activated glycerol dichlorohydrin, and the absorption spectrum. These workers claim that 1 μg of vitamin A can be identified by this scheme but in eight experiments with rat intestine only one indicated vitamin A formation, 1.4 μg. In several experiments a flattened absorption maximum appeared at the vitamin A maximum (328 mμ) but the color tests were negative. Results also indicated no vitamin A formation with rabbit and calf small intestine. Homogenates of liver tissue to which was added pyruvate, coenzyme I, ATP, liver and pancreatin tissue, pancreative bile salts, and blood also failed to convert carotene to vitamin A.

However, another report in 1953 indicated vitamin A formation. Rosenberg and Sobel (71), after being unable to confirm the results of Wiese et al. (64), found vitamin A formation by a different method of analysis. The experimental procedure of Wiese et al. was used except that vitamin A was analyzed by the destructive irradiation method that Bessey et al. (73) had employed for serum vitamin A analysis. Results obtained with 10 rat intestines showed a mean vitamin A formation of 4.24 μg compared with 2.6 μg in the experiments by Wiese et al.

In 1957, Rogers (74) provided evidence that the photo-

sensitive material that led Rosenberg and Sobel to report vitamin A formation was not vitamin A but a pseudo vitamin A occurring in the intestines of vitamin A deficient rats. This unidentified material was distinguished from vitamin A by paper chromatography. Rogers found no evidence of vitamin A formation in an extensive investigation of fractions of homogenized rat intestines incubated with carotene dispersed in synthetic detergents and bile salts in the presence of a number of additives tested as potential cofactors.

Evidence for conversion was again presented from a Canadian laboratory (75) in 1959. Small but definite amounts of vitamin A formation were detected when aqueous Tween 60 dispersions of carotene were incubated in chick duodenum and with chick liver homogenate. The amount of conversion appeared to be proportional to the amount of provitamin added. About 1 percent of the carotene was accounted for as vitamin A. Slightly superior efficiency of conversion was observed with the liver homogenate. The Carr-Price test was used to identify vitamin A and these workers have expressed some concern that contamination by carotene pigments could have contributed to the vitamin A levels found.

In 1960, Olson (76) provided evidence that sodium glycocholate is required in the intestinal conversion of beta-carotene to vitamin A. His method of analysis made use of radioactive beta-carotene. Radioactive vitamin A was iden-

tified by chromatographic characterization on alumina. Vitamin A was characterized first as the ester and then as the alcohol, aldehyde, and semicarbazone.

Olson had previously claimed (77) a bile requirement when he found that washed ligated intestinal loops converted carotene to vitamin A only when the bile duct was not ligated. When bile salts were added instead of natural bile, sodium glycocholate was found to be as effective as natural bile and sodium cholate about 30% as effective. Sodium oleate, sodium deoxycholate, cephalin and Tween 20 were ineffective.

As in other claims of conversion the amount of vitamin A formed was small, less than one µg. However, in this case small doses of beta-carotene (10 µg in the presence of sodium glycocholate) were used so that better than 5% conversion was observed in ligated intestinal loops and 2.5% with intestinal slices. It was not demonstrated that this conversion efficiency would hold at higher dosage levels. Olson suggests that glycocholate, and perhaps other bile salts, are specifically required for the conversion of beta-carotene to vitamin A in the intestine.

MATERIALS AND METHODS

The chemicals that were required during the course of this investigation of the conversion of beta-carotene to vitamin A are listed below:

Crystalline beta-carotene (Nutritional Biochemicals Corp., Cleveland, Ohio). All trans-crystalline beta-carotene was employed as the vitamin A precursor. Oxidized degradatory products that accumulated during storage were removed by chromatography on alumina in the presence of gamma tocopherol (78).

Dispersible beta-carotene (courtesy of Hoffmann-La Roche Inc., Nutley, N. J.). Dispersible beta-carotene provided a water soluble precursor without the disadvantages of a non-physiological dispersing agent.

Crystalline vitamin A (Nutritional Biochemicals Corp., Cleveland, Ohio). Vitamin A was required to develop a feasible analytical process and to measure the level of vitamin A that could be detected under experimental conditions.

Tocopherols (Nutritional Biochemical Corp., Cleveland, Ohio). Alpha and gamma tocopherol were used to protect carotene from non-specific oxidation during incubation and chromatography (78).

Tweens (Atlas Powder Co., Wilmington, Dela.), Tweens (20, 40, and 80), which have varying degrees of hydrophilicity, were utilized as a means of colloidally dispersing caro-

tene in an aqueous medium.

Bile salts (Bacto-Bile Salts, Difco Co., Detroit, Michigan). A mixture of bile salts was used as a dispersing agent.

Soybean oil was used as a carotene solubilizing agent.

Skellysolve A was purified by passing through an alcolated silica gel column.

Skellysolve B was purified by shaking repeatedly with concentrated sulfuric acid, washing with water and drying with sodium sulfate followed by distillation. Skellysolve B used in later experiments was purified by passing through an alcolated silica gel column.

Cyclohexane (Distillation Products Industries, Rochester 3, N. Y.)

Kerosene (Fisher Scientific Co., St. Louis, Mo.). Kerosene with no appreciable absorption above 300 mμ was required.

Xylene (J. T. Baker Chem. Co., Phillipsburg, N. J.).

Chloroform (Mallinckrodt Chemical Works, St. Louis, Mo.). Chloroform was used in the Carr-Price test.

Isopropanol (Distillation Products Industries, Rochester 3, N. Y.).

Ethanol, 95% was purified by distillation over KOH.

Hydrogen peroxide (J. T. Baker Chem. Co., Phillipsburg, N. J.).

Ringer-Locke solution contained the following percentages of salts (w/v): NaCl 0.7%, KCl 0.03%, $CaCl_2$ 0.026%, $NaHCO_3$

0.003%.

Vitamin A test diet (Nutritional Biochemicals Corp., Cleveland, Ohio).

Alumina (Mallinckrodt Chemical Works, St. Louis, Mo.). Brockman No. 1 alumina was mixed with water to obtain the desired activity.

Silica gel (courtesy of Davidson Chemical Co., Baltimore, Md.). A commercially activated dessicant 28 to 200 mesh was most satisfactory.

Methods

Depletion of the rats

In all the early experiments the animals were maintained on a Nutrition Biochemicals Vitamin A Test Diet until pronounced signs of deficiency appeared, e.g. loss of weight and keratinization of epithelial cells of skin and eyes. This required from 3 weeks to 3 months depending on the vitamin A potency of the pre-experimental diet and the age of the rat when placed on the deficient diet. Male rats were used in all cases. Later it was observed that no vitamin A could be detected in the intestine after several days on the test diet although liver and blood supplies were still high. Some animals were then maintained on the deficiency diet for as short a period as 1 week before sacrifice.

Sacrifice of animals and tissue preparation

Because of the inhibitory effect of anaesthetics on enzyme systems (33) the animals were sacrificed by a sharp blow at the base of the skull followed by decapitation. The peritoneal cavity was opened as quickly as possible and the small intestine was bathed in Ringer-Locke's solution while being stripped of lymphatic tissue. The tract was tied off, then excised at the posterior end near the caecum. The anterior end was cut at the pyloric sphincter. After syringal injection of the sample the anterior end was tied off.

Several methods were used to prepare homogenates. Some homogenates were prepared by grinding the intestine submerged in Ringer-Locke's solution in a Potter-Elvehjem tissue homogenizer. The tube was submerged in an ice bath to dissipate heat generated by friction. Nevertheless by this method considerable heat was generated where the tissue was crushed between the wall of the tube and the rotating plunger. Heat denaturation during grinding was minimized by first freezing with liquid nitrogen then crushing the brittle intestine in a mortar. Another method of homogenizing was performed with a Serval omni-mixer. This cutting procedure produced less heat and required less time than the use of the Potter-Elvehjem tissue homogenizer.

Preparation of the beta-carotene substrate

In a number of experiments anion detergents (Tweens) were used as dispersing agents. Concentrations were varied from 0.01% to 10%. The higher concentrations have the advantage of dispersing larger amounts of carotene and the lower concentrations minimize the emulsion formed during extraction and the amount of contaminant which interferes with spectrophotometric and colorimetric analyses. The maintenance of a low Tween level also safeguards the possibility of conversion in case this non-physiological compound disturbs metabolic processes. In other experiments a mixture of bile salts was used as the dispersing agent.

In some experiments carotene solubilized in soybean oil and partially emulsified with aqueous Tween was used. The oil emulsion did not hold more than a few minutes without shaking except in the region of the interface between oil and aqueous Tween.

Tocopherol was the only antioxidant employed. In vivo studies (79) established a greater conversion of carotene to vitamin A when administered in conjunction with vitamin E than when the carotene was administered without antioxidant protection. Gamma-tocopherol was used instead of alpha-tocopherol when carotene was purified on an alumina column because of the report of Lambertsen and Braekkan (78). Both carotene and tocopherol were dissolved in a volatile organic

solvent, usually ethyl ether, and added to the Ringer-Locke solution containing the dispersing agent. The ether was evaporated in a stream of nitrogen at 40-50°C.

Several other additives were tried in hopes of promoting the conversion. Since one mechanism of conversion has been hypothesized to be peroxidative and since several chemical systems have been devised that convert beta-carotene into vitamin A aldehyde by oxidation with hydrogen peroxide in the presence of an inorganic catalyst, one experiment was devoted to testing the effect of various levels of hydrogen peroxide on the *in vitro* system. Glucose was included in some experiments as an energy source and in one experiment phosphate buffers, pH 6.0-7.5 were substituted for Ringer-Locke's solution.

Incubation

The incubation of the carotene dispersion in the ligated excised intestine or with ground homogenates or tissue slices was carried out in a water bath at constant temperature. Most incubations were run at 37°C. Several were conducted at 40°C and several at 45°C. Incubation times were varied from a few minutes up to 2 hours. The earlier experiments were conducted under nitrogen simulating anaerobic conditions in the lumen of the small intestine. Later experiments were conducted in an air atmosphere or under oxygen with whole intestine to

prolong metabolism in the tissue and to approximate conditions in the wall of the intestine rather than in the lumen. The incubation of whole intestine was performed in Ringer-Locke solution and the major part of homogenates in Ringer-Locke solution with dispersing agent, carotene and other additives.

Saponification

Due to the presence of intestinal fatty material and to the character of the dispersing agent, extraction with a non-polar solvent was not feasible without saponification because of the formation of stable emulsions. Several experiments were performed without saponification but the loss caused by incomplete separation in the extraction, due to emulsion, and the masking of spectrophotometric and colorimetric techniques by the preponderance of lipid material precluded the omission of the saponification step. Saponification in the deep freeze and at room temperature proved insufficient to overcome the same difficulties. It was necessary to reflux in 90% ethanol in the presence of base for one half hour to obtain a fairly clean emulsion-free separation.

Purification

The saponificate was diluted by a factor of two with water and extracted with a non-polar solvent, usually Skellysolve A or B. The non-saponifiable materials, including

carotene and vitamin A, are contained in the non-aqueous fraction. The non-saponifiable extract was then washed with 50% ethanol followed by second washes with water. Various means, depending on the method of analysis, were used to further purify vitamin A. Analysis by destructive irradiation was performed without further purification.

The descending paper chromatographic method developed by Kaiser and Kagan (80) effectively separates carotene from vitamin A. The original procedure was modified by using an opaque tank flushed with nitrogen so that development proceeded in the dark in the absence of air. This system uses Munktell 20 filter paper. The solvent, isopropanol-water (1:1), moves vitamin A at the solvent front while carotene remains at the origin.

Capillary columns of alumina of varying degrees of activity were tested also for capacity to separate beta-carotene and vitamin A. A silica gel column with absorbed methanol as the stationary phase and Skellysolve A as the moving phase (81), which had proved successful in separating carotenes from xanthophylls, was tested as a means of separating the hydrocarbon, beta-carotene, from the mono-alcohol, vitamin A.

Analysis

Sensitive methods for the identification and measurement of vitamin A have been developed. The ultraviolet spectrum

absorption maximum is at 325 mμ in hexane with a molar extinction coefficient of 52,000. Spectral curves were measured in the region of the wavelength of maximum absorption, but the presence of impurities absorbing in this region limited spectral evidence to a qualitative interpretation.

In order to eliminate the spectral contribution of impurities a destructive irradiation method was tested. The procedure developed by Bessey and Lowry (73) for blood analyses was followed. This method is based on the fact that vitamin A is rapidly and completely destroyed by ultraviolet light. The assumption is made that other compounds present are not spectrally altered by the irradiation treatment. Thus the difference obtained by subtracting the absorption reading at 328 mμ after irradiation from the reading before irradiation will serve as a measure for vitamin A.

In this procedure the non-saponifiable fraction is taken to dryness and redissolved in a 1:1 mixture of kerosene-xylene. The kerosene must be deficient in highly unsaturated hydrocarbons so that absorption by the solvent above 300 mμ does not interfere with the determination. The spectrum is taken in quartz microcells (2.5x10x25mm) using a diaphram with a 1.2 mm aperture inserted in the Beckman model DU spectrophotometer. The solution is then placed in quartz tubes, internal diameter 2 mm, and irradiated with a General Electric B-H4 mercury discharge lamp. Fan cooling is provided

during irradiation. The spectrum is taken again after irradiation and the difference spectrum calculated. The difference spectrum over the 300 to 400 mμ region gives a vitamin A curve in the absence of interfering impurities.

The Carr-Price reagent was used for many of the quantitative determinations as well as for qualitative identification on paper. The distinctive blue complex formed by the reaction of vitamin A with antimony trichloride in chloroform has an absorption peak at 617 mμ with an extinction coefficient of 145,000. However, this reagent reacts with beta-carotene and other carotenoids to form complexes which have absorption maxima in and near the blue region but with smaller extinction coefficients. The transient existence of the blue complex and the corrosive and deliquescent properties of antimony trichloride make this reagent a difficult one with which to work. However, the sensitivity and specificity of this reagent have disposed chemists to place more reliance on results achieved with antimony trichloride than other colorimetric methods of determination.

In these experiments concentrations were calculated from standard curves prepared for vitamin A and beta-carotene. Samples were dissolved in 1 ml of chloroform and 9 ml of antimony trichloride in chloroform (1 lb. $SbCl_3$ in 1500 ml of $CHCl_3$) were dispensed from a rapid delivery pipette into a tube in a Coleman Junior Spectrophotometer. Readings were

taken 7 seconds after commencement of delivery at a wavelength setting of 620 mμ.

EXPERIMENTAL AND RESULTS

The experiments described herein are arranged according to the method of analysis used. This arrangement corresponds closely with the chronology of the work. The early experiments were analyzed by the destructive irradiation method (73). Because the concentration of the dispersing agent was considered important, a study was then made correlating beta-carotene dispersibility with Tween concentration. Experiments were subsequently performed using a paper chromatographic separation procedure. This method was later abandoned in favor of a column separation technique.

Except for the study relating Tween concentration to carotene dispersibility, all the experiments described in this section deal with testing analytical methods used for conversion experiments and actual experiments on the *in vitro* conversion of beta-carotene to vitamin A.

Rats of the Wistar strain were used for the experiments analyzed by the destructive irradiation method, whereas rats of the Sprague-Dawley strain were employed for the remaining experiments. The animals used were in an extreme state of vitamin A deficiency except in the column chromatographic experiments where rats were sacrificed while in a mild deficiency state induced by at least 7 days on a vitamin A deficient diet.

Analysis by Destructive Irradiation

Preliminary studies

The first analyses for vitamin A formation by intestinal action on beta-carotene were carried out by the destructive irradiation method (73). Before intensive studies were initiated it was necessary that information be obtained on the effects of irradiation on vitamin A and beta-carotene. For best results the absorption of vitamin A in the 370 to 390 mμ region should be completely destroyed by irradiation. Beta-carotene, which would be present in comparatively large amounts, should undergo no irradiation-induced absorption change through this region.

Separate solutions of vitamin A and beta-carotene in 1:1 kerosene-xylene were prepared. A 1:1 mixture of the vitamin A solution and the beta-carotene solution also was prepared. The concentration of the vitamin A solution was 4.2 μg per ml; the beta-carotene solution, 4.5 μg per ml. The concentrations were halved in the mixture. Absorbancies were measured with the Beckman Model DU Spectrophotometer with diaphram inserted. Measurements were made on the solutions in quartz microcells before and after irradiation in capillary quartz tubes. Absorbancies were measured from aliquots of these solutions at 330 mμ for all aliquots and at 480 mμ for aliquots which contained beta-carotene. The data are listed

Table 1. Effect of time of irradiation on absorbancy of solutions of vitamin A, beta-carotene and a mixture containing both

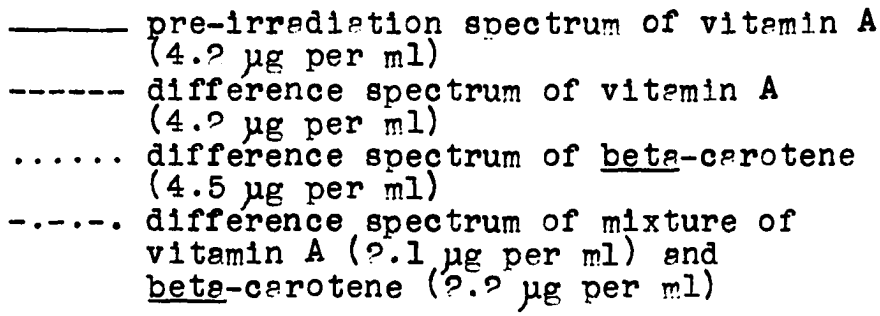
Time (min.)	Vitamin A	Vitamin A and beta-carotene		Beta-carotene	
	330 mμ	330 mμ	460 mμ	330 mμ	460 mμ
0	0.656	0.361	0.486	0.081	0.982
5	0.263				
10	0.133				
20	0.036	0.071	0.206	0.096	0.510
35	0.021	0.059	0.110	0.088	0.355
50	0.010	0.072	0.081	0.078	0.257

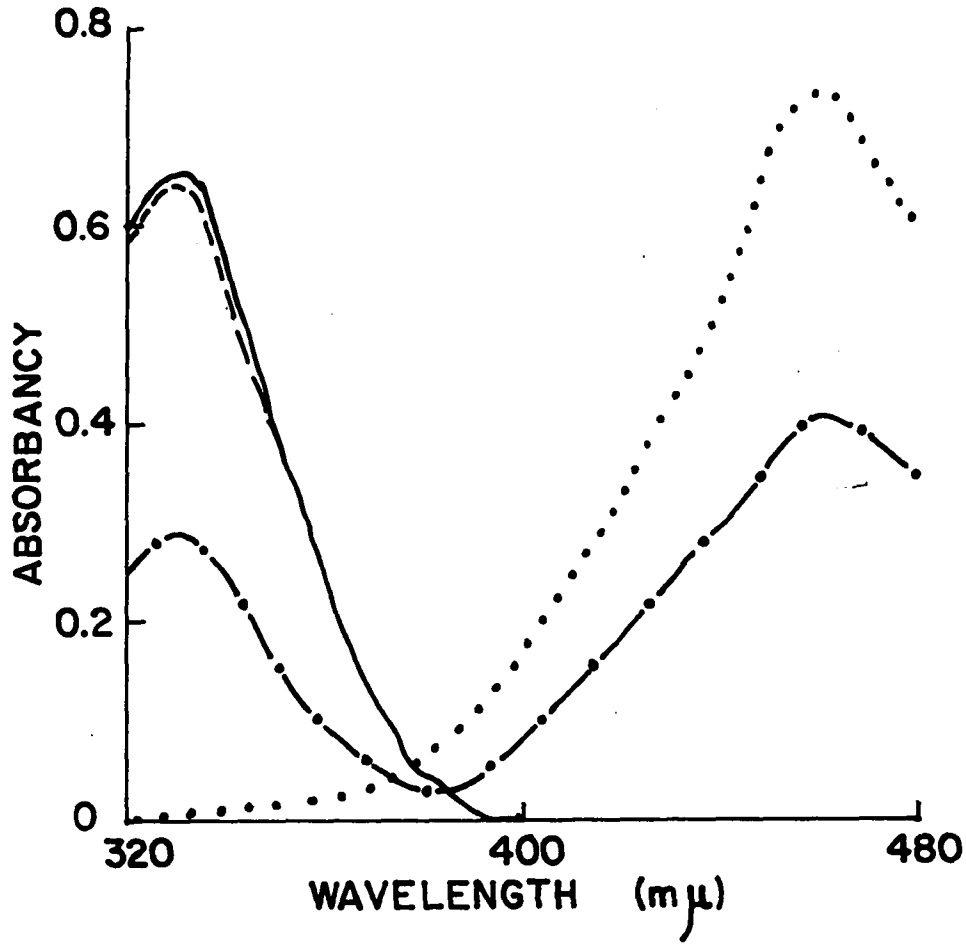
in Table 1.

The absorption of vitamin A was reduced almost 95% after 20 minutes of irradiation while only a very small change was observed in absorption by carotene at the same wavelength. Measurements at the carotene absorption maximum showed that carotene destruction was considerable, although not as complete as vitamin A destruction.

The spectrum was taken for aliquots of each of the three original solutions over the range of 320 to 480 mμ. Aliquots then were irradiated for periods of 20, 35, and 50 minutes. Figure 1 shows the difference spectra of the three solutions after 50 minutes of irradiation along with the pre-irradiation

Figure 1. Absorption spectrum of vitamin A and the difference spectra (post-irradiation spectrum subtracted from pre-irradiation spectrum) of vitamin A and beta-carotene in 1:1 kerosene-xylene




0.8
0.6
0.4
0.2
0
ABSORBANCY
320
400
480
WAVELENGTH (mμ)

spectrum of vitamin A.

The difference spectrum of vitamin A is almost superimposable on the spectrum of vitamin A. Also, the difference spectrum of a solution containing both vitamin A and beta-carotene is almost identical with the vitamin A spectrum in the 320 to 380 mμ range, especially from 320 to 360 mμ. The resemblance decreases from 360 to 380 mμ as the beta-carotene difference spectrum becomes significant and modifies the decline of the difference spectrum of the mixture. Thus the vitamin A difference spectrum is almost identical with the absorption spectrum of vitamin A over the range of 320 to 360 mμ when vitamin A is subjected to irradiation in the presence of beta-carotene.

Conversion studies on non-deficient intestinal tissue

In order to ascertain whether or not normal intestinal levels of vitamin A can be detected by this method, an assay was conducted on non-deficient rats. The rat intestines were carried through the procedure to be used for carotene to vitamin A conversion studies in deficient rats.

Three young (6 weeks old) rats of the Wistar strain were starved overnight, sacrificed by a blow on the head, and then decapitated. After excision the small intestines of rats 1 and 2 were injected with 0.5 ml of 5% Tween 80 containing 250 μg of alpha-tocopherol. The intestine of rat 3 was

injected with 0.5 ml of a 5% Tween 80 solution which contained 250 µg of alpha-tocopherol and 150 µg of beta-carotene. The latter was employed to ascertain whether the assay would proceed as well in the presence of a substantial amount of beta-carotene and to see if a higher level of vitamin A could be observed. The intestines were tied off and incubated 2 hours in Ringer-Locke solution at 45°C (71) in a water bath.

The contents were rinsed out with 20 ml of 0.9% NaCl and the intestines refluxed for 15 minutes with 1 ml of 50% aqueous KOH in 9 ml of absolute ethanol. An equal volume of water was added and the saponificate extracted with Skellysolve A. The petroleum ether fraction was evaporated to dryness on a water bath at 40°C under nitrogen and redissolved in 2 ml of a 1:1 mixture of kerosene-xylene. Absorbancies were measured before and after irradiation for 35 minutes covering the range from 320 to 390 mµ.

Using 1570 as $E^{1\%}_{1cm}$ for vitamin A in kerosene-xylene (71), the amounts of vitamin A in the intestines incubated without added carotene were 9.2 and 2.6 µg, respectively, while the amount in the intestine incubated with carotene was 4.6 µg. Obviously these data do not present evidence of conversion of beta-carotene to vitamin A. Since these rats were on a non-deficient diet, the carotene intake during the period preceding sacrifice may possibly account for the disparity in levels of vitamin A found.

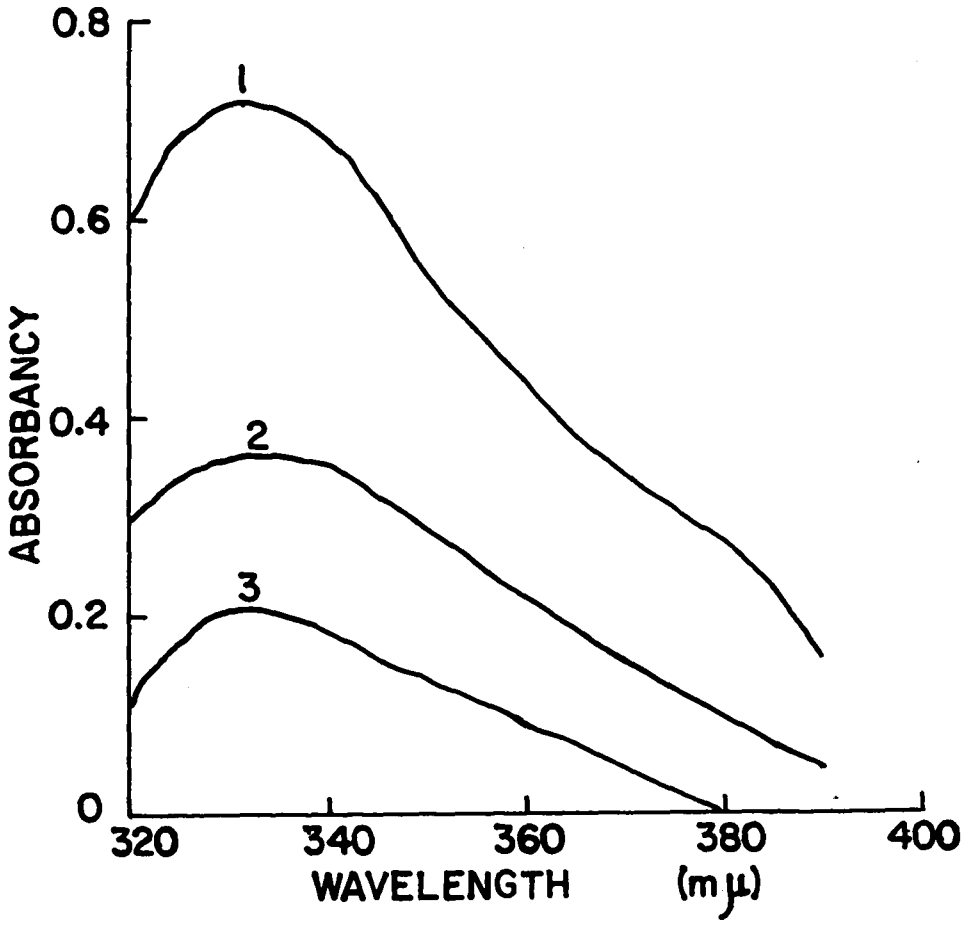
The shapes of the difference spectra from 320 to 390 mμ are reasonable facsimiles (Figure 2) of the vitamin A spectrum obtained on extracts from intestines to which no carotene was added. However, the difference spectrum resulting when an intestine was incubated with beta-carotene showed a broad peak extending from 330 to 340 mμ. Irradiations for 20 and 50 minutes of aliquots from this intestinal extract showed the same broad peak. Zechmeister and Polgar (82) have shown that prolonged heating causes cis inversion of all-trans beta-carotene accompanied by the appearance of a cis peak at 340 mμ. Bieri (83) demonstrated that the cis peak is partially destroyed by irradiation. Thus, carotene isomerization during saponification (refluxing) could give a false estimation of vitamin A by contributing to the difference spectrum at 330 mμ.

The results of these tests made it evident that unambiguous identification of small amounts of vitamin A formed in vitro must be made with vitamin A deficient animals, with blank runs exhibiting at least a uniform reproducible difference spectrum or preferably no difference spectrum at all. Also, saponification at reflux temperature, 80°C, should be avoided because of possible carotene isomerization.

Figure 2. Difference spectra of extracts of rat small intestine

1 and 3 - Extract of intestine not deficient in vitamin A

2 - Extract of intestine not deficient in vitamin A incubated with 150 µg of beta-carotene


0.8
0.6
0.4
0.2
0
ABSORBANCY
1
2
3
320
340
360
380
400
WAVELENGTH (mμ)

Conversion Studies on Vitamin A Deficient Intestinal Tissue

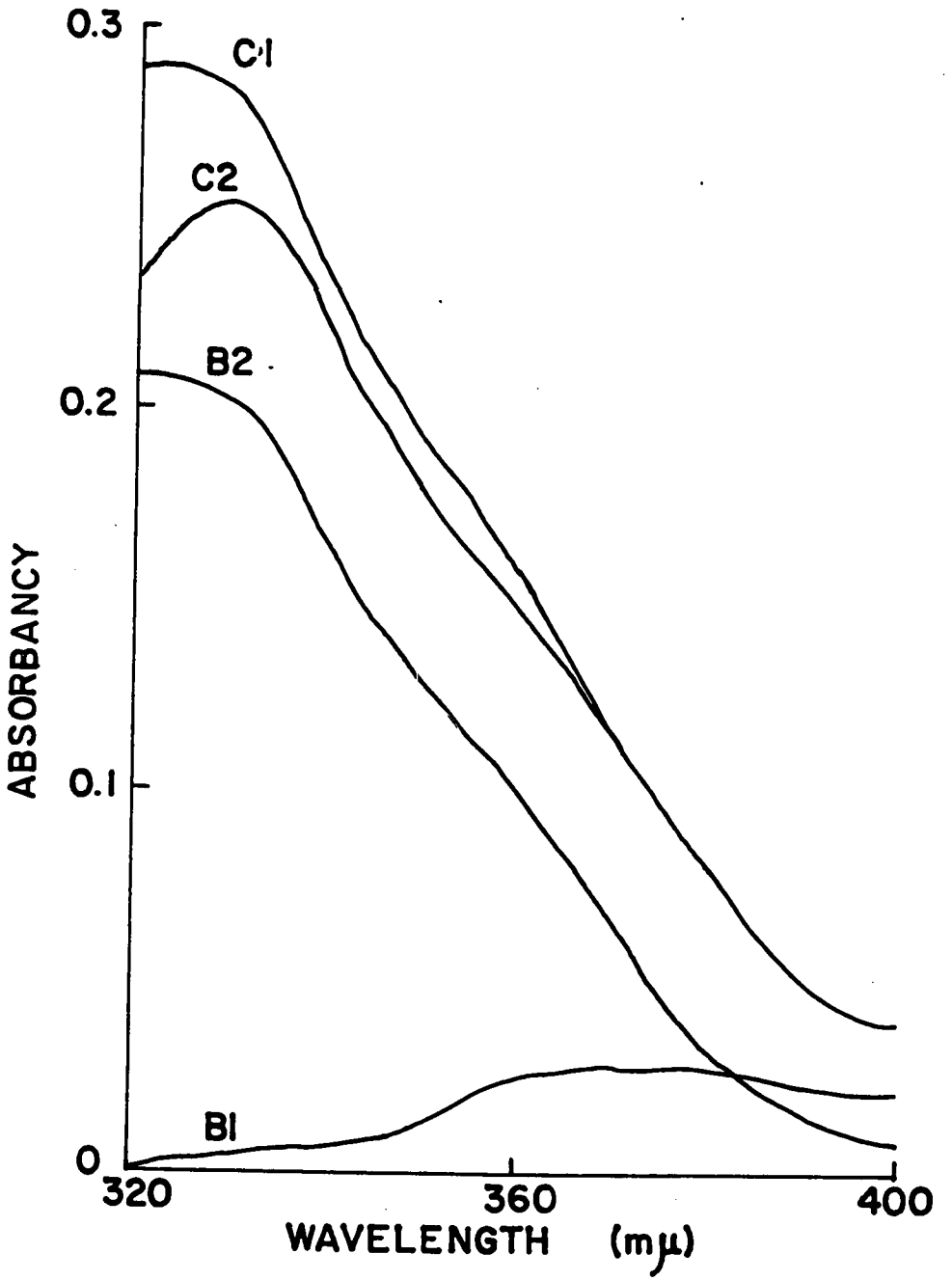
Whole intestine

Dispersion of carotene in Tween An experiment to study conversion of beta-carotene to vitamin A in deficient animals was performed. Four weanling rats (5 weeks old) of the Wistar strain were placed on the vitamin A test diet and maintained on this diet for 4 weeks. At the end of this time marked deficiency symptoms had developed. The animals were sacrificed and the same procedure was followed as with the non-deficient rats. Two of the intestines were injected with carotene and two were not. After incubation, saponification was conducted over-night at room temperature. Irradiation of the extracts was for 40 minutes. The difference spectra are shown in Figure 3. The intestines devoid of carotene were lableled B_1 and B_2 and those with carotene C_1 and C_2.

One of the carotene-supplied intestines, C_2, exhibited a difference spectrum closely approximating the spectrum of vitamin A over the range measured and indicating the formation of 3.3 µg of vitamin A. The other carotene-supplied intestine, C_1, deviated in the 300 to 325 mµ region by a continuous, though modified, rise in absorbancy as the wavelength decreased. Inspection of the spectrum of one of the carotene-free intestines, B_2, shows the same type of spectrum

Figure 3. Difference spectra of extracts of small intestines from vitamin A deficient rats

C_1 and C_2 - Incubated with 150 µg of beta-carotene
B_1 and B_2 - Controls incubated without carotene


0.3
C1
C2
B2
0.2
ABSORBANCY
0.1
B1
0
320
360
400
WAVELENGTH (mμ)

in this region as carotene supplied intestine, C_1. The other intestine that was not supplied with carotene, B_1, exhibited a non-interfering spectrum with almost no absorption in the critical range, 320 to 340 mμ, and very low level absorption from 340 to 400 mμ.

It is apparent from results with the control, B_2, that some light sensitive material obscured vitamin A and could be mistaken for vitamin A, since photo destruction of this material occurred in the region of the vitamin A peak and a difference spectrum which was identical to that of vitamin A except in the 320 to 330 mμ region was obtained. Succeeding tests also showed a spectrum similar to that observed with B_2 in most cases. Irradiation studies on solutions of various Tweens and tocopherol showed no change in light absorption above 320 mμ.

Use of Water-Dispersible Carotene

In order to obviate the extraction difficulties and the non-physiological nature of a Tween system, an experiment was performed using a commercially prepared water dispersible beta-carotene. This experiment was performed with whole intestine and with ground homogenates.

In the experiment with whole intestine, 0.40 g of water-dispersible beta-carotene, which was 2.4% beta-carotene (9.6 mg beta-carotene), was suspended in 10 ml of Ringer-Locke

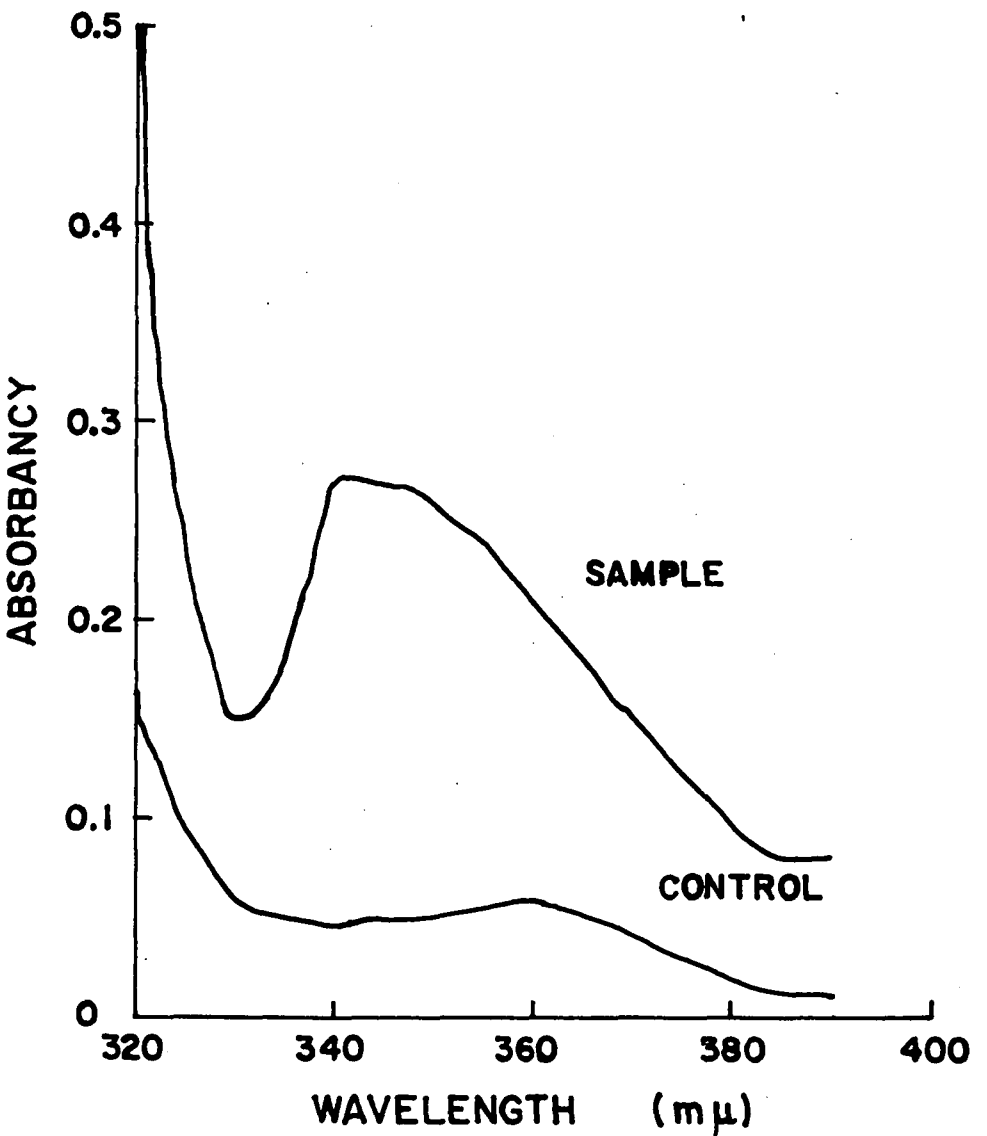
solution and injected into a freshly excised intestine of a vitamin A deficient rat. Incubation and extraction were performed as described previously with non-deficient animals. The destructive irradiation method was used for vitamin A analysis. The experiment was performed with two animals with almost identical results. An increase of irradiation time from 20 to 40 minutes altered only the magnitude of the difference spectrum. The shape remained the same. An intestine, into which no water-dispersible carotene was injected, served as a control.

The difference spectrum of a sample and the control is shown in Figure 4. A minimum in the curve appeared where the maximum for vitamin A occurs. The rise beyond 330 mμ is characteristic of the cis peak for beta-carotene. The rise below 330 mμ is characteristic of the substance that appears in deficient rat intestine giving a false estimation of vitamin A except for the fact that there was no leveling in the 320-325 mμ region. The shape of the curve and the magnitude at 330 mμ discounts the formation of vitamin A.

Homogenate preparations in Ringer-Locke solution and phosphate buffer at pHs 6.5, 7.0, and 7.5 also showed no vitamin A formation from the water-dispersible beta-carotene. The water-dispersible beta-carotene was not used as a vitamin A precursor in further studies with the destructive irradiation method.

Figure 4. Difference spectra of extracts of vitamin A deficient rat small intestine incubated with water-dispersible beta-carotene

sample - Incubated with 9.6 mg of beta-carotene
control - Incubated without beta-carotene


0.5
0.4
0.3
0.2
0.1
0
ABSORBANCY
SAMPLE
CONTROL
320
340
360
380
400
WAVELENGTH (mμ)

The only results which suggested vitamin A formation from beta-carotene were obtained with Tween-dispersed beta-carotene in excised whole intestine. These results were not clear-cut since a number of vitamin A deficient intestines in the absence of beta-carotene showed on irradiation a difference spectrum similar to that of vitamin A.

Homogenates

In studies with homogenate of rat intestine the difference spectrum arising from components other than vitamin A could be cancelled by a control, while the variable results achieved with whole intestine controls did not afford a reliable blank determination. Also, since conversion of beta-carotene to vitamin A by homogenates is a step in the direction of enzyme purification and mechanism study, an experiment was designed to attempt conversion of Tween-dispersed beta-carotene to vitamin A with homogenate of rat intestine.

The small intestines of two vitamin A deficient rats were excised, rinsed with an injection of 15 ml of Ringer-Locke solution and homogenized in a Potter-Elvehjem homogenizer. A suspension of 0.0330 g of beta-carotene in 100 ml of 10% Tween 80 in Ringer-Locke solution was prepared along with 0.1 M phosphate buffers of pH 6.5, 7.0, and 7.5 Six Warburg flasks containing materials described in Table 2 were incubated for 2 hours at 37°C in a water bath with continuous

Table 2. Contents of flasks incubated in conversion studies with intestinal homogenates

Flask[b]	Intestinal homogenate (ml)	Beta-carotene suspension[a] (ml)	10% Tween 80 (ml)	Ringer-Locke (ml)	0.1 M phosphate buffers (ml)		
					pH 6.5	pH 7.0	pH 7.5
1	2	5		5			
2	2	5			5		
3	2	5				5	
4	2	5					5
5	2		5	5			
6		5		5			

[a]In 10% Tween 80.

[b]Small amounts, 5 to 10 mg, of alpha-tocopherol were added to each of the flasks.

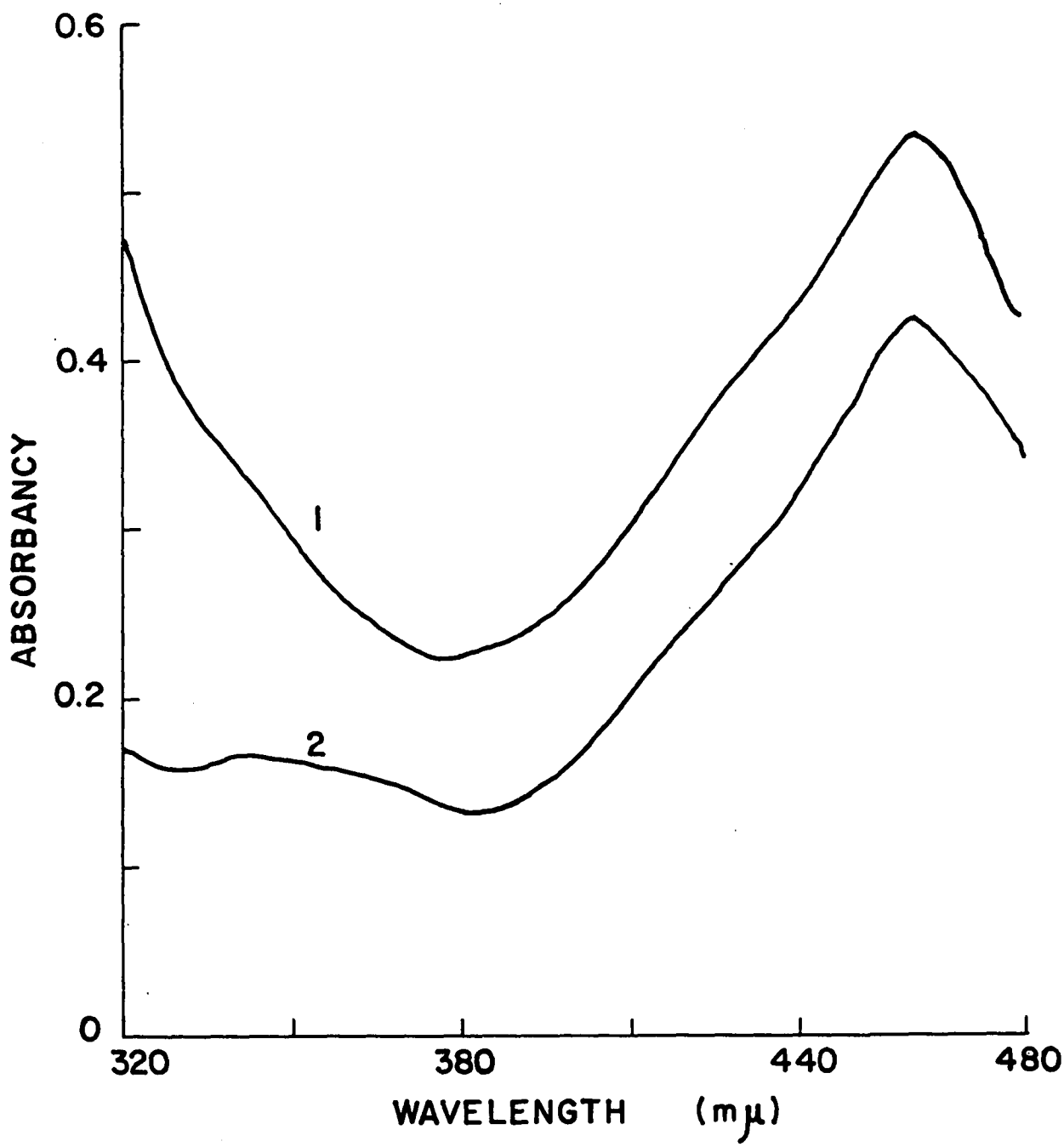
shaking and exposure to air.

After incubation the samples were saponified in the refrigerator for 3 days with 1 ml of 55% KOH and 9 ml of ethanol. The samples were then extracted with Skellysolve A. Formation of emulsions made the extraction difficult. The Skellysolve A extracts were evaporated to dryness and taken up in several ml of kerosene-xylene 1:1. The spectra were measured from 320 to 480 mμ.

Figure 5 shows the pre-irradiation spectrum and the difference spectrum of the extract from flask 2 incubated with pH 6.5 phosphate buffer. The difference spectra of all the other samples were similar to the one depicted. All were relatively flat throughout the 320 to 380 mμ region. The rise in the pre-irradiation spectrum as the wavelength decreased was due to material extracted from the homogenate. All samples showed this rise except that from flask 6 which contained no homogenate. The flat difference spectrum rules out vitamin A formation since the $E^{1\%}_{1cm}$ for pure vitamin A increases from almost zero at 380 mμ to 1570 at 328 mμ.

These analyses have failed to demonstrate conversion of beta-carotene into vitamin A with homogenates; earlier studies showed very limited conversion with whole intestine. Because of the variability of controls and the formation of a compound in deficient rat intestine with a spectrum almost identical to the spectrum of vitamin A, the applicability of the

Figure 5. Pre-irradiation spectrum (curve 1) and difference spectrum (curve 2) of an extract from vitamin A deficient rat small intestinal homogenate incubated with beta-carotene at pH 6.5


0.6
0.4
0.2
0
ABSORBANCY
1
2
320
380
440
480
WAVELENGTH (mμ)

destructive irradiation method seems to be of doubtful value for demonstrating vitamin A formation from beta-carotene by excised rat intestine.

Effect of Tween Concentration on Dispersion of Carotene

The problem of supplying a water-insoluble substrate in utilizable form in an aqueous medium confronts the experimentor where carotene is the metabolite. It was found that carotene disperses in smaller particles when dissolved first in a volatile organic solvent followed by mixing with the dispersing agent and volatilizing the organic solvent from the dispersion than when carotene crystals are dispersed directly in the aqueous dispersing agent. The question also arose as to the relationship of the degree of dispersion to the concentration of the dispersing agent. Aqueous Tween solutions covering a wide range of Tween concentrations have been used in biological experiments.

In an *in vitro* system it is important that the dispersing agent does not act as a metabolic poison. Although the problem is complex, it is possible that high concentrations of surface active compounds may inhibit enzyme action more than low concentrations. On the other hand, it is necessary that the dispersing agent has sufficient action to suspend the substrate in small aggregates.

It seemed desirable, therefore, to investigate the dis-

persion of carotene in Tween to determine whether the dispersing action is effective at low Tween concentrations.

The following aqueous solutions (w/v) of Tween 80 were prepared: 0.01%, 0.05%, 0.1%, 0.5%, 1%, 5%. To 10 ml of each solution 100 µg of beta-carotene in 1 ml of ether was added. The ether was removed by bubbling nitrogen through the mixture at 45°C. The absorption spectra of the beta-carotene suspensions in Tween 80 were then determined over the range of 320 to 550 mµ with the aid of a Beckman model DU spectrophotometer. Each suspension was measured against a blank containing the same percent Tween 80 without beta-carotene.

Figure 6 shows the absorption spectrum of beta-carotene in each of the aqueous Tween mixtures and also the absorption spectrum of the same amount of beta-carotene in true solution in Skellysolve B. The spectrum in Skellysolve B was measured on a solution containing 0.7 µg/ml and corrected to the absorption at 10 µg/ml, assuming that the Beer-Lambert law is valid.

Duysens (84) has shown that the absorption spectrum of suspensions is flattened due to the fact that the decrease in absorption is more pronounced at wavelengths of high absorption than at wavelengths of low absorption. The flattening of the absorption spectrum with corresponding decrease in extinction coefficient is an index of molecular aggregation.

Figure 6. Absorption spectra of beta-carotene (10 µg/ml) in true solution and in suspension in varying concentrations of aqueous Tween 80

curve	solvent or dispersing agent
1	Skellysolve B
2	5% Tween 80
3	1% " "
4	0.5% " "
5	0.1% " "
6	0.05% " "
7	0.01% " "

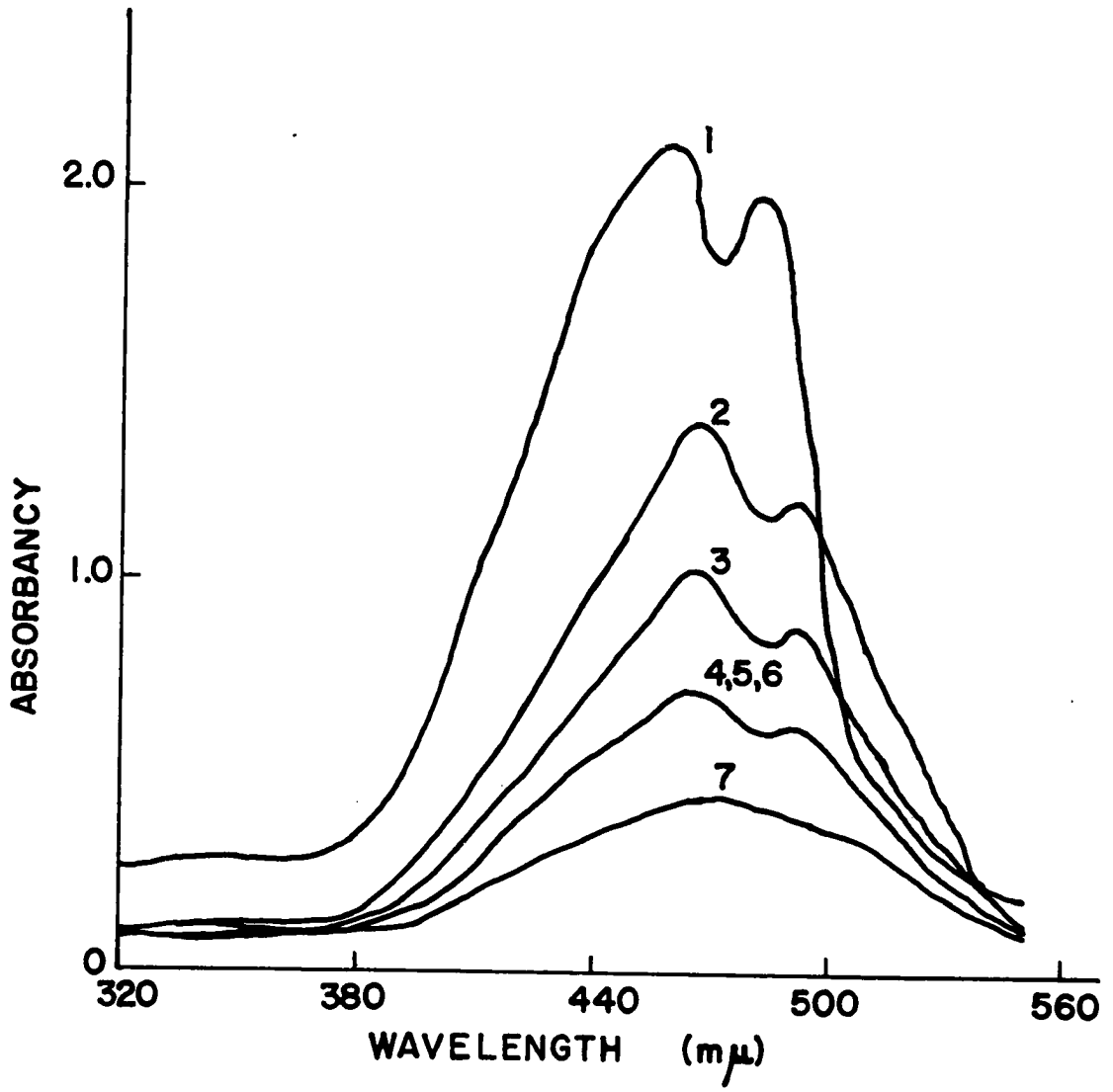

2.0
1.0
0
ABSORBANCY
1
2
3
4,5,6
7
320
380
440
500
560
WAVELENGTH (mμ)

Figure 6 shows that the degree of dispersion increases with Tween concentration but that the relationship is not linear.

Figure 7 shows the change in absorption at the wavelength of maximum absorption (460 mμ) as a function of the log of Tween 80 concentration. There is little change in absorption through the concentration interval of 0.05% to 0.5% Tween 80. Apparently 0.05% aqueous Tween 80 disperses carotene as effectively as 0.5%.

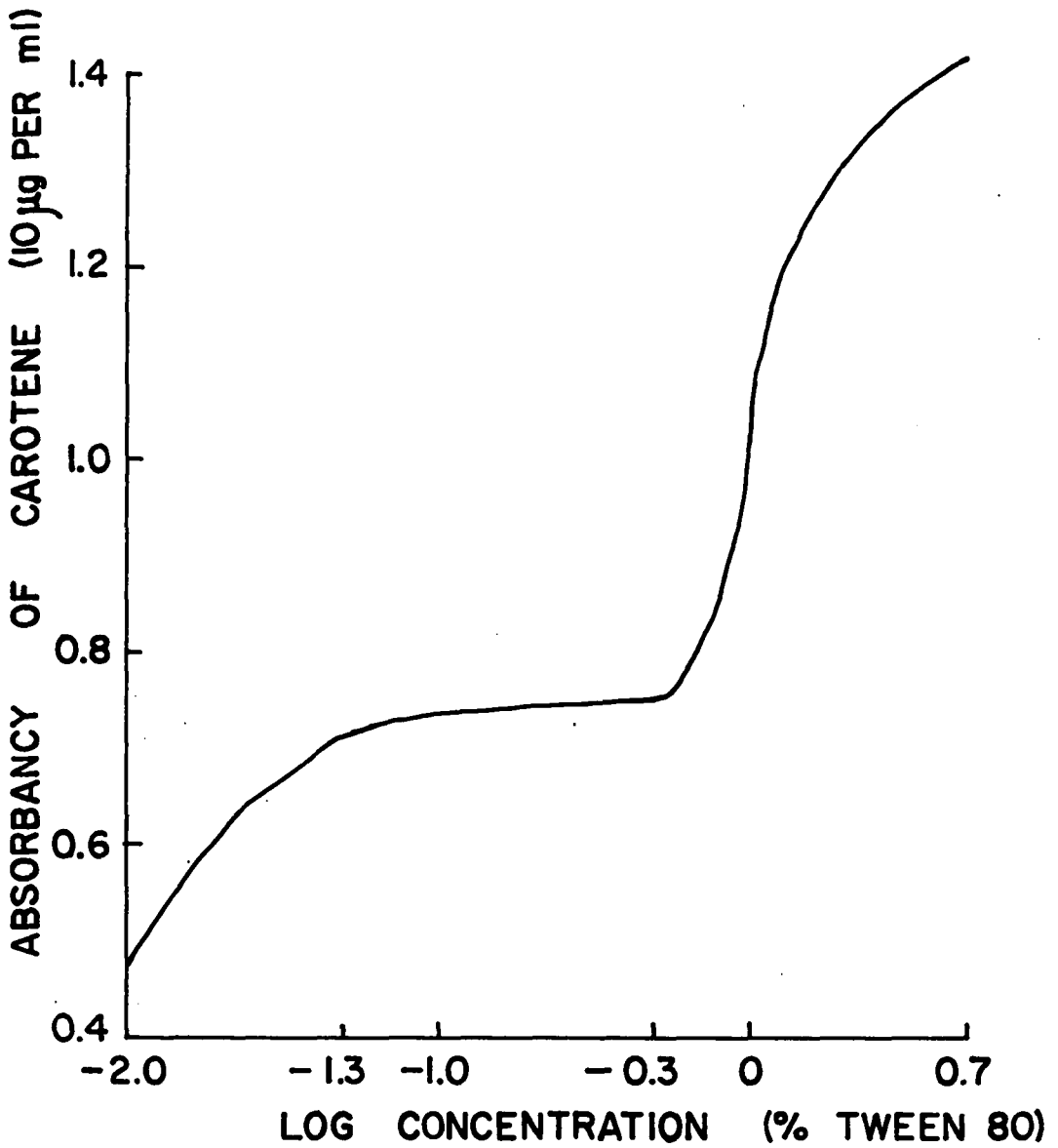
At a Tween 80 concentration of 0.01% there was very limited absorption. Reference to Figure 7 shows that the two characteristic maxima at 463 mμ and 487 mμ have flattened into a smoothly rounded line devoid of fine structure. Carotene shows no absorption spectrum in pure water because it is completely insoluble. The lowest concentration of Tween 80 that gave a differentiable carotene spectrum was 0.05%. To noticeably improve dispersion it was necessary to increase Tween 80 concentration to 1%, a 20-fold increase.

Separation by Paper Chromatography

Preliminary studies

The procedure developed by Kaiser and Kagan (80) for the separation of micro amounts of vitamin A alcohol from vitamin A esters looked particularly applicable to the present problem. By this procedure micro amounts of vitamin A alcohol, vitamin A acetate and vitamin A palmitate are separated

Figure 7. Absorbancy of beta-carotene at 460 mμ as a function of the logarithm of Tween 80 concentration


ABSORBANCY OF CAROTENE (10 µg PER ml)
1.4
1.2
1.0
0.8
0.6
0.4
-2.0
-1.3
-1.0
-0.3
0
0.7
LOG CONCENTRATION (% TWEEN 80)

cleanly by descending paper chromatography using Munktell 20 filter paper and a solvent system of 1:1 isopropanol and water. Development requires 3 to 4 hours, the strips are dried in warm air, and the vitamin and its esters are identified by spraying with antimony trichloride in chloroform. The alcohol moves at the solvent front, the acetate moves about half the distance between the origin and the solvent front, and the palmitate remains at the origin. One to 2 µg mixtures are completely resolved.

In the present study it was necessary to separate vitamin A alcohol, which would be the only form of the vitamin present after saponification, from beta-carotene, a lipophilic compound that would be expected to remain at the origin with this solvent system.

Mixtures of vitamin A, vitamin A acetate and beta-carotene were chromatographed by the Kagan-Kaiser procedure modified to the extent that the paper strips were dried in a stream of nitrogen rather than in hot air. The vitamin A alcohol traveled at the solvent front, vitamin A acetate moved with an R_f of 0.6, and beta-carotene remained at the origin.

Loading tests showed the system capable of resolving up to 16 µg of vitamin A from beta-carotene. Larger amounts were incompletely resolved after the solvent front had moved 15 to 25 cm.

It was observed that the color of the vitamin A spot on

the paper was not the deep blue characteristic of the Carr-Price complex, but displayed a purple to pink tone. Washing the filter paper in versene and in solvent prior to chromatogram development did not aid in giving the typical blue color.

Elution and spectral measurement of the vitamin A spots showed that major destruction of vitamin A had occurred when the complex gave the purple color. This information was obtained by spotting two chromatograms each with 1.7 µg of vitamin A. Both were developed at the same time in the same tank until each solvent front had moved 20 cm. One chromatogram was treated with antimony trichloride in chloroform, giving the purple color at the "vitamin A" spot. The other was cut and eluted and the spectrum of the extract was measured from 320 to 390 mµ. A control was spotted on paper and eluted without development. The developed chromatogram showed more than a 10-fold decrease in vitamin A concentration.

Since vitamin A is a very labile compound susceptible to oxidation and photo destruction, it is probable that in the highly exposed state on the filter paper oxidation occurs much faster than in solution or crystalline form.

Samples of 1 and 2 µg of vitamin A were spotted on the Munktell 20 filter paper, exposed to the atmosphere for various time intervals, and treated with antimony trichloride in chloroform. Results are presented in Table 3.

Table 3. Color of the vitamin A complex with antimony trichloride on Munktell 20 filter paper as affected by time of exposure

Time (min.)	1 µg	2 µg
0	blue	blue
5	blue	blue
10	blue-purple	
15	purple	blue-purple
20	lighter purple	
30	fading purple-pink	
70	very faint pink	
105		light purple
270		very faint purple
1380		colorless

It is apparent from the data in Table 3 that decomposition took place rapidly when small amounts of vitamin A were absorbed on the filter paper and allowed to dry. The somewhat slower decomposition in the chromatograph jar was probably due to oxidation at a slower rate in the solvent saturated atmosphere.

All subsequent chromatograms were developed in opaque battery jars made free of oxygen by flushing with nitrogen that had been purified by passing through vanadyl sulfate

according to the procedure of Meites and Meites (85). The chromatogram strip was placed in the jar prior to flushing. (Figure 8 shows the apparatus.) The jar was then flushed with nitrogen for 15 minutes before the solvent was introduced.

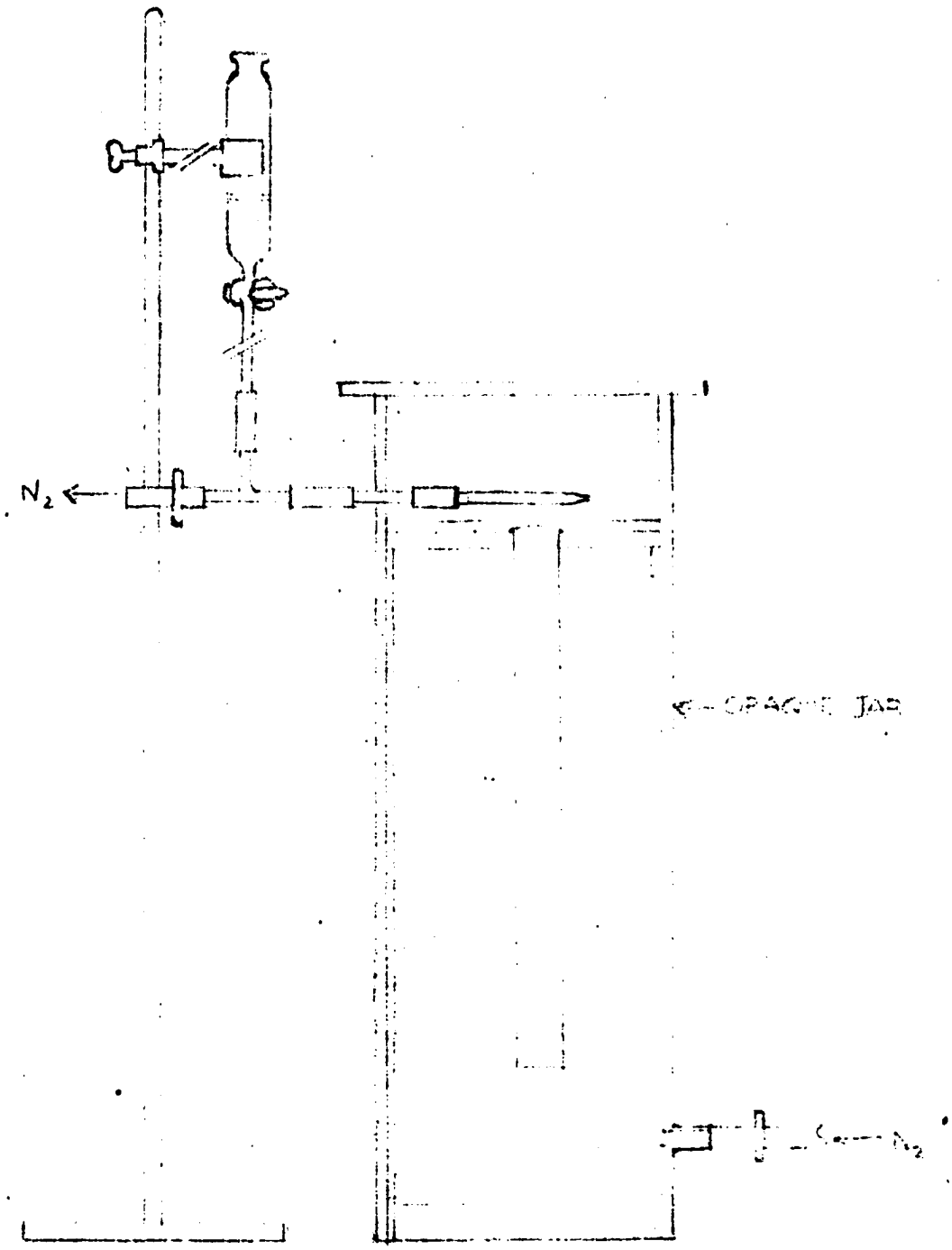
Flushing the jar upset the equilibrium between liquid and vapor phase. Even with large sheets of filter paper lining the inside of the jar it took more than a day for saturation to occur as indicated by the reduced rate of movement of the solvent front with corresponding increase in time of development. Consequently the chromatograms were developed in a non-equilibrated system. The R_f value of vitamin A, however, was unaffected.

Some decomposition was still observed after modifying the procedure to eliminate atmospheric oxidation and photodestruction. Washing the filter paper in ethanol, isopropanol-water 1:1, versene, and deionized water did not prevent this destruction.

Tween 20 and the antioxidant, alpha-tocopherol, were tested in an attempt to prevent destruction. Several drops of an aqueous dispersion of 5% Tween 20 and 0.3% alpha-tocopherol were placed on a chromatogram with 1.7 µg of vitamin A. This chromatogram was developed in conjunction with a chromatogram spotted with the same levels of Tween 20 and vitamin A but no alpha-tocopherol.

Tween 20 decreased the rate of movement of vitamin A,

Figure 8. Apparatus for the development of descending paper chromatograms


N_2
N_2

probably due to a reduction in the rate of extraction of vitamin A by the solvent. The area on the paper covered by vitamin A was larger when Tween was present. This was due, at least in part, to the fact that the oil-like Tween spreads more readily than volatile organic solvents when applied to the paper. Complete resolution of vitamin A from beta-carotene was easily obtained for amounts of vitamin A up to 15 ug when the solvent front moved 20 to 30 cm, providing the Tween was confined to a small area during application.

The chromatograms described above were treated with the Carr-Price reagent after the solvent front had moved 20 cm. The characteristic blue color was obtained at the vitamin A and the beta-carotene spots. Another chromatogram using alpha-tocopherol in the absence of Tween indicated that some decomposition had occurred.

When performing elutions for spectral measurements of the compounds developed on the Munktell 20 filter paper it was found necessary to elute with Skellysolve A rather than ethanol. Ethanol washings extracted from the paper some components absorbing above 300 mμ to the extent that small amounts of vitamin A were obscured. Petroleum ether (Skellysolve A) washings of the Munktell 20 filter paper showed no absorption above 300 mμ.

The effects of Tween 20 and alpha-tocopherol on the recovery of vitamin A from paper chromatograms were studied

further, using two pairs of chromatograms. Several drops of 5% Tween 20 and 0.3% alpha-tocopherol in aqueous dispersion were applied at the origin along with 15 µg of vitamin A to one of each pair. Only the 15 µg of vitamin A was applied to the other chromatogram of each pair. After development until the solvent front had moved 29 cm one chromatogram of each pair was treated with antimony trichloride and the vitamin A spot of one of each pair was eluted with 5 ml of Skellysolve A and the spectrum of the eluate measured over the range of 270 to 360 mµ.

The chromatograms to which Tween and alpha-tocopherol were applied showed the blue Carr-Price color test characteristic of vitamin A and a recovery of 10 µg as estimated from the known extinction coefficient at 325 mµ. The chromatograms containing only vitamin A gave a pinkish-purple spot with antimony trichloride but failed to show the characteristic vitamin A spectrum, indicating severe degradation of vitamin A.

A study of the recovery of vitamin A from Munktell 20 paper in the presence of Tween 20 was made, using 5 ml of Skellysolve A as the eluting solvent. It was discovered that vitamin A was incompletely recovered from chromatograms containing Tween and also that the Tween was incompletely removed from the paper. Treatment of eluted strips of filter paper with antimony trichloride showed traces of vitamin A

still on the paper. Thus, vitamin A is partitioned between the eluting solvent, Skellysolve A, and the emulsifying agent, Tween 20. Recovery of the applied vitamin A varied from 60 to 80% after elution from developed or undeveloped chromatograms containing Tween 20 and alpha-tocopherol.

The time required to develop chromatograms 25 to 30 cm under the non-equilibrated conditions, caused by flushing the chromatographic jar with nitrogen, was about 24 hours. An experiment was performed to discover whether vitamin A was appreciably degraded during the prolonged time of development. Two chromatograms were spotted with Tween 20, alpha-tocopherol and 12 μg of vitamin A. After 6 hours the solvent fronts had moved 12.5 cm. At this time one of the chromatograms was removed. The vitamin A spot was eluted with Skellysolve A and the absorption spectrum measured. The result was a recovery of 9.7 μg of vitamin A. After 22 hours the solvent front of the remaining chromatogram had moved 30 cm. Elution and spectral study showed that 9.0 μg of vitamin A were recovered. Thus decomposition was not extensive when Tween 20 and alpha-tocopherol were applied with vitamin A.

It was found that beta-carotene does not form the blue complex with the Carr-Price reagent in the presence of Tweens. Vitamin A, however, does give the characteristic blue complex in the presence of Tweens. It was observed further that a qualitative identification of vitamin A in the presence of

beta-carotene on a paper chromatogram could be made after applying Tween 20 to the area to be tested before treatment with the Carr-Price reagent.

Development of chromatograms containing moderate amounts of Tween 20 at the origin resulted in the transfer of Tween on the paper leaving beta-carotene at the origin. The carotene then gave a blue color with the Carr-Price reagent.

Conversion studies with homogenates

Vitamin A was measured by a modification of the method of Kaiser and Kagan (80). The non-saponifiable extract was spotted on a strip of Munktell 20 filter paper which was placed in an opaque battery jar. The system was flushed for 15 minutes with nitrogen before introduction of the developing solvent. Approximately 2 cm of solvent, 1:1 isopropanol-water was maintained in the bottom of the jar, and the inside of the jar was lined with Whatman No. 1 filter paper to hasten saturation of the atmosphere within the jar. The developing solvent was introduced into the trough in the closed jar (see Figure 8) without the introduction of oxygen other than that from air dissolved in the 10 to 15 ml of solvent used for development of the chromatogram. The chromatogram was developed, then dried in a stream of nitrogen. The developed area of the chromatogram was treated with antimony trichloride in chloroform and the colored areas were marked and measured.

Tween-dispersed carotene

In prior experiments the use of the Potter-Elvehjem homogenizer resulted in some heating at the site of crushing of the intestinal wall between the teflon plunger and the glass wall of the homogenizer. It was postulated that enzyme denaturation could have occurred before the heat was dissipated by the ice water bath surrounding the homogenizer. Thus in one experiment liquid nitrogen was used to freeze the intestine prior to grinding.

Two vitamin A deficient rats were sacrificed by decapitation. The small intestines were excised and rinsed in Ringer-Locke solution. The intestines were frozen in liquid nitrogen and pulverized in a morter. The pulverized frozen tissue was incubated with a beta-carotene substrate prepared by dispersing 3 mg of beta-carotene and 5 mg of alpha-tocopherol in 1 ml of Tween 80 and diluting to 100 ml with Ringer-Locke solution. One g of egg albumen was added to the carotene substrate mixture in order to flood the medium with protein substrate, thus perhaps protecting any "carotenase" enzyme from protease degradation. Dispersion of the substrate suspension was improved further by use of a Potter-Elvehjem homogenizer. Fifty ml of the resulting suspension and the pulverized intestine described above were incubated, with constant stirring, in a 125 ml round-bottomed flask for 2 hours at 37°C.

The contents were extracted with Skellysolve A, but emulsion formation prevented distinct layer formation. The Skellysolve A was separated by differential freezing with liquid nitrogen. After the Skellysolve A extraction, the residue was filtered and the intestinal particles were saponified with 5% KOH in 95% ethanol at room temperature for 24 hours. The saponificate was diluted with an equal volume of water and extracted with Skellysolve A. Each Skellysolve A fraction was washed with water several times and evaporated to dryness under nitrogen. The residues were taken up in several drops of absolute ethanol and chromatographed on Munktell 20 paper according to the procedure described above.

On the chromatogram of the extract made prior to saponification carotene was identified at the origin by treatment with antimony trichloride in chloroform. Vitamin A was not identified. A faint green spot with an R_f of 0.5 was observed, probably indicating some carotenoid-like product.

Neither vitamin A nor beta-carotene was identified on the chromatogram of the non-saponifiable extract of the intestinal material. The absence of beta-carotene suggests that it was not absorbed by the homogenized tissue during incubation, although other possibilities exist. If any carotene had been absorbed, it may have been metabolized or severely degraded. The extraction prior to saponification should not have extracted bound carotene since Skellysolve A extractions

in this work have been ineffective in extracting carotene from cellular material that has been neither saponified nor treated with alcohol.

The results of this experiment indicated that no vitamin A was formed after incubation with tissue that had been frozen in liquid nitrogen.

Another method for the preparation of small intestine homogenates was tested. The small intestines of three vitamin A deficient rats were excised and transferred to the small Serval omni-mixer vessel which contained 30 ml of Ringer-Locke solution. The vessel was attached to the mixer and then immersed in an ice bath. The mixture was homogenized at full speed for 5 minutes. The resulting homogenate was incubated 20 minutes at 37°C under nitrogen with 100 ml of the beta-carotene suspension prepared as follows.

Ten mg of beta-carotene and 10 mg of alpha-tocopherol were dispersed in 0.5 ml of Tween 80 to which was added 10 ml of ethyl ether. The ether was removed by passing a stream of nitrogen through the suspension maintained in a water bath at 37°C. The Tween dispersed carotene was then diluted to 500 ml with Ringer-Locke solution forming a clear yellow suspension homogenous in appearance.

After incubation an equal volume of 95% ethanol was added to the mixture, then several Skellysolve A extractions were performed. The Skellysolve A extracts were combined, evapo-

rated to dryness under nitrogen and the residue was taken up in 400 µl of ethyl ether. One hundred µl of the ether solution was chromatographed on Munktell 20 paper as outlined above. The remainder was tested by the destructive irradiation method. No vitamin A was found by either method of analysis. A similar experiment wherein incubation time was extended to 2 hours also showed no vitamin A formation.

Dispersion of carotene in bile salts

A mixture of bile salts (Bacto-bile salts) was tested as a dispersing agent in conversion studies. Preliminary experiments showed that vitamin A was effectively extracted and identified with antimony trichloride after suspension in bile salts. Spectrophotometric identification of vitamin A was corrected for absorption by bile salts since the spectrum of the bile salts extract (cyclohexane) showed absorption in the 330 mµ region where vitamin A absorption is at a maximum. No absorption due to bile salts was found in the carotene absorption maximum region at or near 450 mµ.

Bacto-bile salts were used as the dispersing agent in the following experiment. Two hundred µg of beta-carotene were dispersed in 40 ml of Ringer-Locke solution containing 40 mg of bile salts. The resulting mixture was incubated 30 minutes at 37°C under nitrogen with the homogenate of a vitamin A deficient rat small intestine prepared by grinding with a

Serval omni-mixer. Paper chromatographic analysis showed no vitamin A formation. A similar experiment with the addition of 200 µg of alpha-tocopherol also demonstrated no vitamin A formation.

Separation by Column Chromatography

The paper chromatographic method of analysis was found to be unsatisfactory when large amounts of Tweens and bile salts remained in the unsaponifiable extract. For this reason and because the use of vegetable oils to solubilize carotene was planned for future work, a method of separation of vitamin A and beta-carotene was needed that would be accurate in the presence of considerable amounts of fatty materials.

Alumina, magnesia, and calcium hydroxide have been used extensively in column chromatography of carotenoid pigments (86). However, invariably some destruction occurs on the column due to irreversible absorption (78). The problem under investigation requires quantitative separation of very small amounts of vitamin A, several µg, from appreciably larger amounts of beta-carotene and efforts were made to develop appropriate techniques.

Preliminary studies

Alumina No vitamin A was recovered when 10 µg samples were passed through columns of Brockman No. 1 alumina.

Even the use of capillary columns, ? mm inside diameter and 6 cm in length, did not prevent severe loss.

Partial inactivation of the alumina by mixing with water (5 and 10% by weight) decreased the absorptive power of the alumina to the extent that milligram portions of beta-carotene and microgram portions of vitamin A were not resolved.

Partition chromatography using silica gel A type of column chromatography, partition chromatography, offered another means of separation. Purcell (81) has demonstrated the separation of carotenoid hydrocarbons, monohydroxy carotenoids, and polyhydroxy carotenoids from each other. This separation was accomplished by partitioning between an immobile methanol phase absorbed on silica gel and a moving petroleum ether phase.

Vitamin A would be expected to partition as a monohydroxy carotenoid. This was tested and confirmed as follows. A column 1 cm in diameter was packed with silica gel to a height of 8 cm. Methanol was allowed to percolate through the column by gravity flow. The column was then stoppered and maintained under methanol for 1 hour. Fifty ml of Skellysolve A was then passed through the column to wash out unabsorbed methanol. Two hundred μg of beta-carotene and 10 μg of vitamin A dissolved in Skellysolve A were introduced on the column. Skellysolve A was passed through the column until 50 ml were collected (Fraction 1). Fifty ml of 10% ethyl ether in

Skellysolve A were then passed over the column (Fraction 2). Spectral analysis showed that 192 µg of beta-carotene was recovered from fraction 1 and 9 µg of vitamin A was recovered from fraction 2. Resolution was complete.

In all subsequent conversion experiments the silica gel column was used to separate vitamin A and beta-carotene.

Analytical methods Vitamin A and beta-carotene fractions from the silica gel column were evaporated to dryness under nitrogen, then redissolved in chloroform. Each fraction was analyzed spectrophotometrically in the Beckman Model DU spectrophotometer and colorimetrically by the Carr-Price method in the Coleman Junior spectophotometer.

Beta-carotene was estimated quantitatively from its absorption at 463 mµ. Vitamin A could not be estimated quantitatively from the absorption at 335 mµ because impurities absorbing in this region were present. The shape of the absorption spectrum from 300 to 380 mµ was examined and used as qualitative evidence to establish the presence or absence of vitamin A.

Colorimetric analyses were performed by reacting 1 ml of the chloroform solutions with 9 ml of antimony trichloride in chloroform dispensed from a rapid delivery pipette into a test tube in the Coleman Junior spectrophotometer. Absorption readings were taken 7 seconds after commencement of delivery from the pipette. Concentrations of vitamin A and beta-

carotene were determined from standard curves prepared by performing the operations just described on 1 ml chloroform aliquots of vitamin A and of beta-carotene solutions of known concentrations. Figures 9 and 10 represent the standard curves prepared for vitamin A and beta-carotene, respectively.

Conversion studies using intestinal tissue

Effect of H_2O_2 in homogenates It has been postulated (87) that beta-carotene is oxidized by hydrogen peroxide at the central double bond in the first step of the conversion to vitamin A. This oxidation has been carried out successfully in chemical systems using inorganic catalysts such as OsO_4 (13). It is postulated that the release of catalase in intestinal tissue homogenates does not permit the H_2O_2 accumulation sufficient to oxidize beta-carotene.

An experiment was performed to test for vitamin A formation from beta-carotene in the presence of added amounts of H_2O_2 to homogenates of small intestines from vitamin A deficient rats. Each homogenate was prepared by grinding a vitamin A deficient rat small intestine with 5 ml of Ringer-Locke solution. The bulk of the intestine increased the volume to slightly over 10 ml after homogenation. To the 10 ml of homogenate was added 10 ml of 0.1% aqueous Tween 20 which contained 1 mg of dispersed beta-carotene. Levels of 15, 45, and 1000 ppm of H_2O_2 were added to separate flasks containing the

Figure 9. Calibration curve for the estimation of vitamin A with the Carr-Price reagent

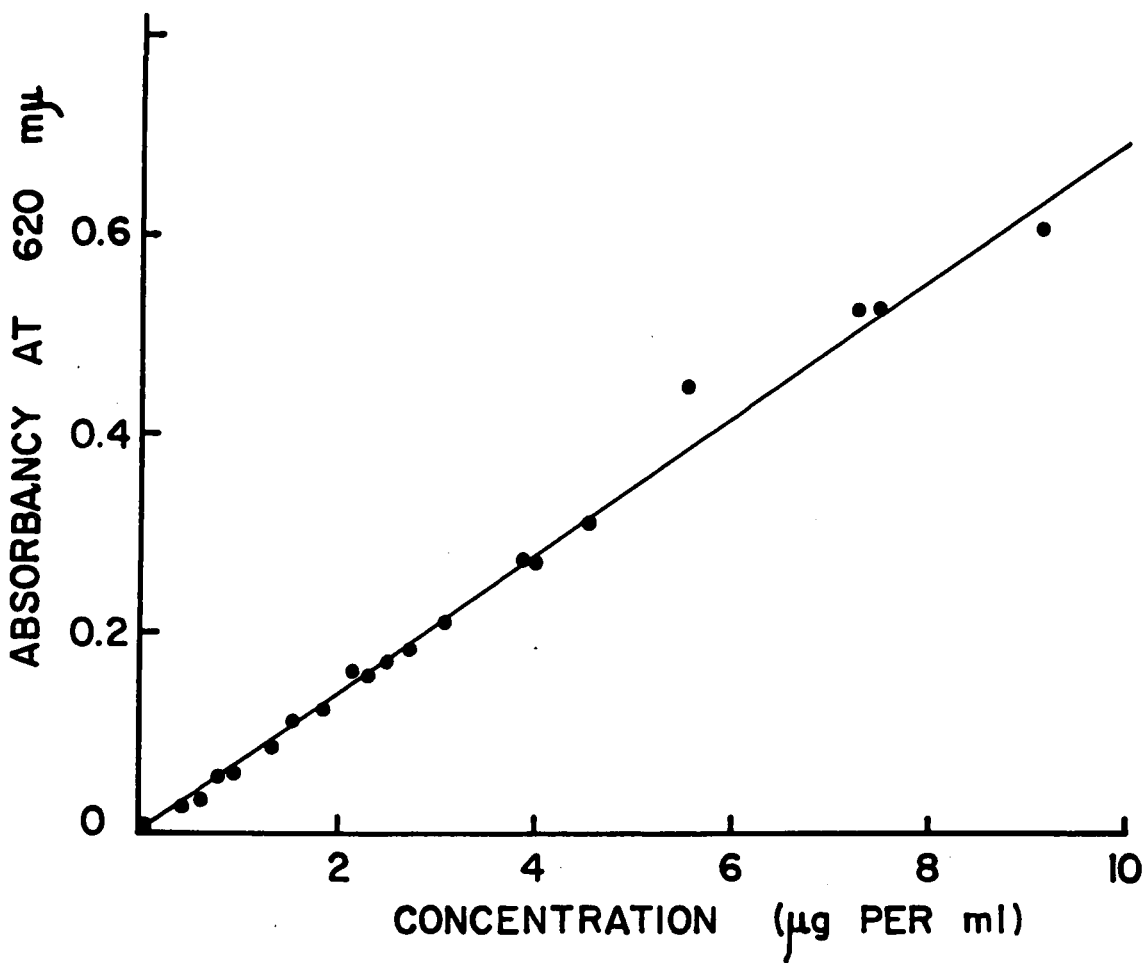
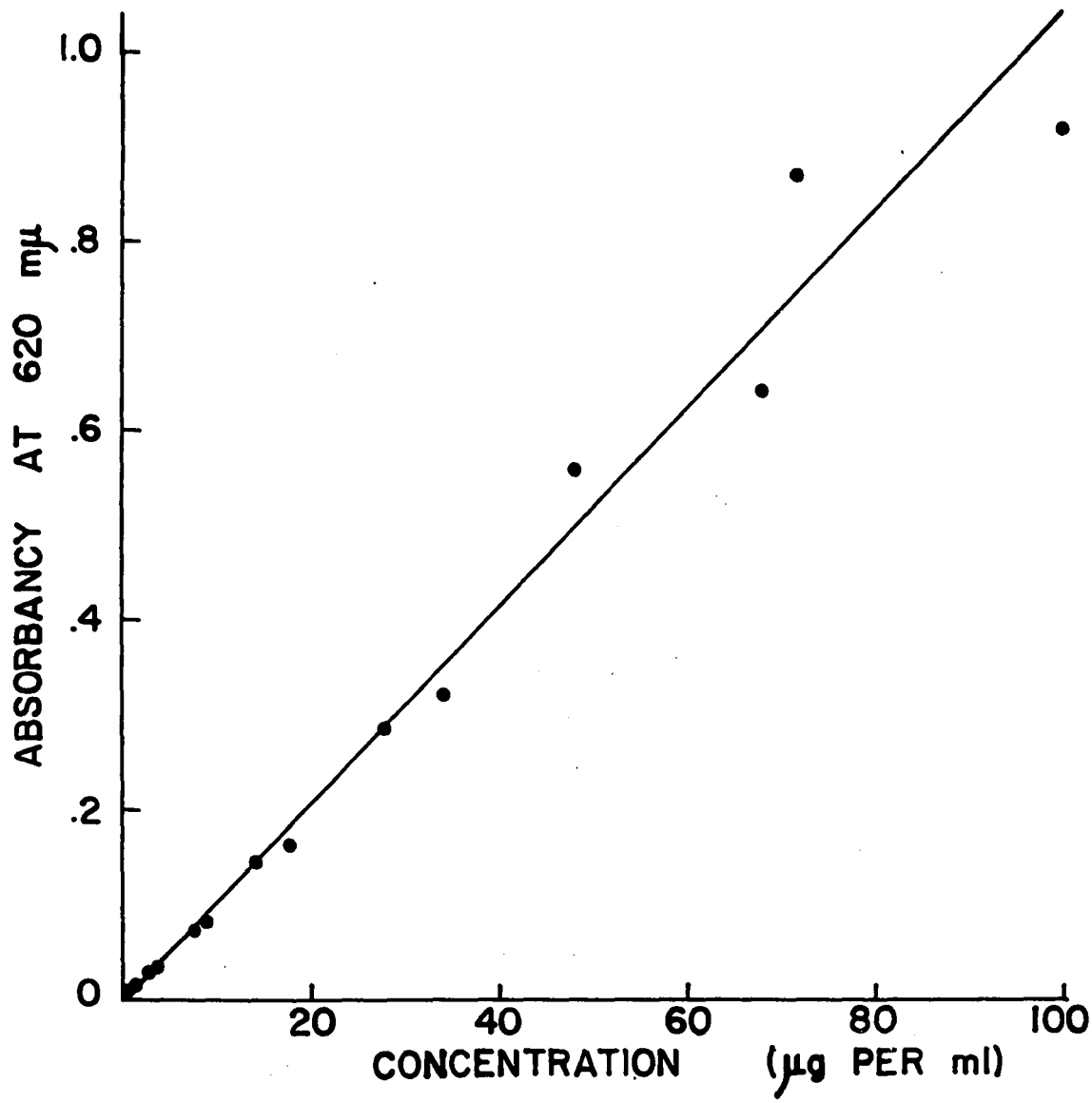

ABSORBANCY AT 620 mμ
0
0.2
0.4
0.6
2
4
6
8
10
CONCENTRATION (μg PER ml)

Figure 10. Calibration curve for the estimation of beta-carotene with the Carr-Price reagent


1.0
.8
.6
.4
.2
0
ABSORBANCY AT 620 mμ
20
40
60
80
100
CONCENTRATION (μg PER ml)

homogenate mixtures prepared above. The flasks then were incubated at 37°C for 30 minutes in a nitrogen atmosphere. Following incubation each homogenate mixture was saponified 30 minutes at 80°C after the addition of 10 ml of 50% aqueous KOH and enough ethanol to make the mixture 85% in ethanol. After dilution with an equal volume of water, the saponificate was extracted with portions of Skellysolve A until the extracts were colorless. The Skellysolve extracts were combined, washed with water and then dried. Each extract was then passed through the silica gel-methanol column.

Four fractions (Table 4) were collected from the column. Each fraction was 50 ml in volume unless the eluant had not become colorless. If the eluant was still colored, the volume of the fraction was increased beyond 50 ml to the amount necessary to elute all colored material in the band emerging

Table 4. Fractional elution of carotenoids from silica gel column (81)

Fraction	Eluting solvent	Compounds present in eluate
1	Skellysolve A	Hydrocarbons (beta-carotene)
2	10% ethyl ether in Skellysolve A	Vitamin A and some monohydroxy carotenoids
3	50% ethyl ether in Skellysolve A	Monohydroxy carotenoids
4	Methanol	Polyhydroxy carotenoids

from the column.

Each fraction was evaporated to dryness under nitrogen and redissolved in chloroform. The absorption spectrum of each fraction was measured from 300 to 550 mμ. The Carr-Price test also was performed on each fraction.

The results of these experiments are summarized in Table 5. These data show that H_2O_2 at all levels tested resulted in marked destruction of beta-carotene and no formation of vitamin A.

Table 5. Effect of H_2O_2 added to intestinal tissue homogenates on recovery of beta-carotene and vitamin A formation

Flask	H_2O_2 added (ppm)	Beta-carotene recovered (%)	Vitamin A formed (μg)
1	1000	12.1	0
2	45	18.2	0
3	15	22.7	0

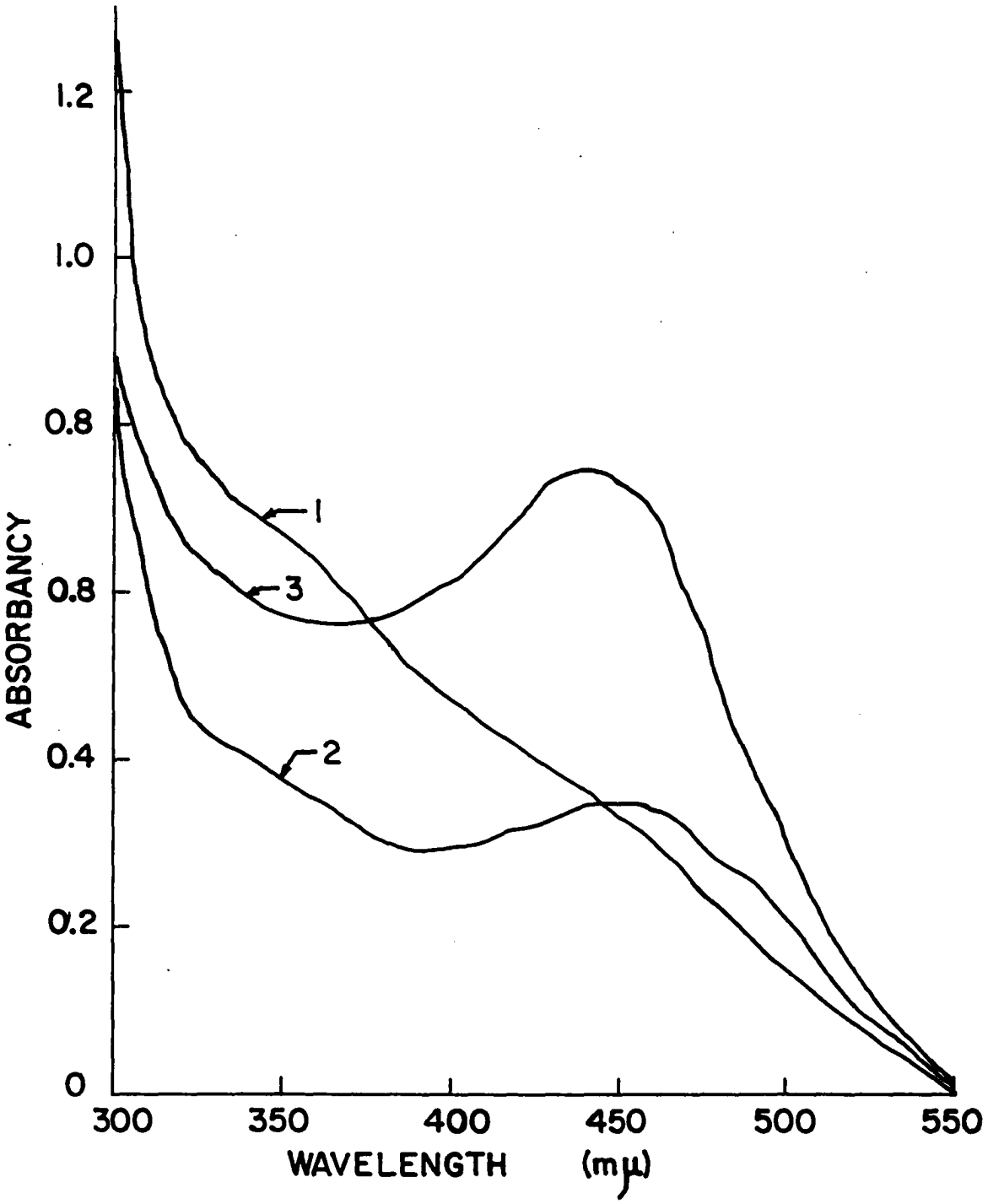
The spectra of the fractions show progressive degradation of beta-carotene with increasing levels of H_2O_2. The spectrum of fraction 1 in each case was that of beta-carotene. Fraction 3 and fraction 4 indicated that beta-carotene had undergone extensive degradation. These fractions show continuous decreasing absorption above 300 mμ.

Fraction 2, which should contain the vitamin A, was the most interesting when examined as a function of H_2O_2 concentration (Figure 11). In the presence of 1000 ppm of H_2O_2, evidence of carotenoid maxima above 400 mμ had disappeared, but at lower levels (45 and 15 ppm of H_2O_2) absorption maxima existed in the 440 to 480 mμ range. This evidence suggested that oxidation had occurred with retention of a long series of conjugated double bonds.

None of the absorption spectra offered evidence for the formation of vitamin A. The Carr-Price test with fraction 2 from the experiment in which 15 ppm of H_2O_2 was used did show a blue color that would amount to the formation of 1 μg of vitamin A. However, examination of Figure 11 shows oxygenated carotenoids present in this fraction and no spectral evidence for vitamin A. Also, the shade of blue in the Carr-Price test appeared more characteristic of the blue obtained with carotene than the deeper blue characteristic of vitamin A. The results of this experiment failed to demonstrate vitamin A formation from beta-carotene incubated with rat intestinal homogenates in the presence of varying amounts of H_2O_2.

Whole intestine Additional conversion studies were performed on excised rat small intestines. Several alterations were made in experimental conditions and metabolite preparations: (a) In most of the experiments soybean oil was used to solublize carotene. (b) The oil was dispersed with

Figure 11. Absorption spectra of monohydroxy carotenoid fraction from vitamin A deficient rat small intestinal homogenates incubated with beta-carotene in the presence of varying concentrations (curve 1, 1000 ppm; curve 2, 45 ppm; curve 3, 15 ppm) of H_2O_2


1.2
1.0
0.8
0.8
0.4
0.2
0
ABSORBANCY
1
3
2
300
350
400
450
500
550
WAVELENGTH (mμ)

Tweens or bile salts. (c) Glucose was introduced as an energy source in several experiments. (d) An aerobic atmosphere (air or O_2) was provided. (e) Gamma-tocopherol was substituted for alpha-tocopherol.

In the experiments in which soybean oil was used beta-carotene and gamma-tocopherol were first dissolved in ether, then mixed with the oil. The ether then was removed by evaporation under nitrogen. The oil was dispersed in the aqueous medium containing the dispersing agent and glucose. Dispersion was enhanced by grinding in the Potter-Elvehjem homogenizer, but permanent emulsions could not be maintained. The resultant mixture, 10 ml in volume, was injected into the excised small intestine at the pyloric juncture after tying off the posterior end of the small intestine near the caecum. The anterior end was tied off and the intestine incubated in Ringer-Locke solution. Incubation was accompanied by intermittant agitation in order to restore the emulsion. The experimental data are summarized in Table 6.

After incubation the contents were rinsed out of each intestine with 20 ml of Ringer-Locke solution. The contents and the tissue were analyzed separately for vitamin A and beta-carotene. Saponifications were performed for 30 minutes at 80°C in ethanolic KOH. Four fractions were eluted from the silica gel column and analyzed spectrophotometrically and by the Carr-Price test as described in the previous experi-

Table 6. Conditions for *in vitro* studies with excised intestines

Experiment	Incubation Time (min)	Incubation Temp. (°C)	Oil[a] (ml)	Carotene added (mg)	Dispersant (%)	Gamma-tocopherol (mg)	Glucose (%)	Atmosphere
1	30	37		1	0.1 Tween 20		1	air
2	60	40	1	2.5	0.01 bile salts			air
3	60	39	1	4	3 bile salts			air
4	50	39	1	3	5 Tween 80	6		air
5	75	39	1	5	5 Tween 80	8	0.1	O_2
6	60	37	1	5	5 Tween 80	8	0.1	O_2
7[b]	60	39	1	1	10 Tween 80	8	0.1	O_2

[a]Soybean oil.

[b]Conditions for each of five intestines that subsequently were combined for analysis.

ment.

The results tabulated in Table 7 show that vitamin A was identified in one experiment. In this experiment the ultraviolet absorption spectrum was measured in the Skellysolve-ether fraction taken from the column (fraction 2). A flattened shoulder was found in the 325 mμ region where vitamin A

Table 7. Results of in vitro studies with excised intestines

Experiment	Vitamin A in wall (μg)	Vitamin A in contents (μg)	Carotene in wall (μg)	Carotene in contents (μg)
1	0	0	1	90
2	0	0	--	124
3	0	0	24	148
4	0	0	5	160
5	0	0	8	740
6	0	0	8	536
7[a]	4	0	25	--

[a]Analysis of a combination of five intestines.

absorbs maximally. However, absorption increased as wavelength decreased below 320 mμ. This evidence suggested the presence of vitamin A along with considerable amounts of impurities, but was by no means conclusive identification of vitamin A.

The Carr-Price test then was performed on this fraction after evaporation to dryness under nitrogen and redissolving in chloroform. The Carr-Price test gave the characteristic blue color indicative of vitamin A. Caution must be exercised in interpreting this evidence since higher homologues of vitamin A, apo-carotenols, also form a blue complex with antimony trichloride. (Beta-carotene itself was eliminated from this fraction by chromatography.)

In this experiment the ultraviolet absorption spectrum declined rapidly as wavelength increased beyond 330 mμ. In fact, the absorption spectrum was typical of vitamin A beyond 330 mμ. This fact eliminated the possibility of identifiable levels of compounds, such as the apo-carotenols, which have absorption maxima beyond 330 mμ. Thus, the evidence obtained from the results of the two methods of analysis strongly suggested that vitamin A was present.

The conditions under which this experiment was performed were different than conditions of the early experiments and slightly different than those for the other experiments tabulated in Table 6. The substrate, beta-carotene, was solubilized in soybean oil and the oil was dispersed in 10% aqueous Tween 80, the highest concentration of dispersing agent used in any experiment. An antioxidant, gamma-tocopherol, and an energy source, glucose, were provided and the incubation was conducted under oxygen.

Five intestines were incubated under the conditions specified in experiment 7, Table 6. These five intestines were pooled before analysis. Since 4 μg of vitamin A were identified by the Carr-Price test, the average vitamin A formation per intestine was 0.8 μg. It is possible that in the immediately preceding experiments, which were performed under similar conditions with one intestine, amounts of vitamin A could have been formed that were less than 1 μg and escaped detection.

The results (Table 7) indicate that beta-carotene destruction was extensive in all experiments and that in only one experiment was vitamin A formation detected. Vitamin A was identified in the intestinal wall but not in the intestinal contents.

There was very little carotene in the intestinal wall and this did not correlate with the total carotene introduced into the intestine. It is possible that some or all of the small amounts of beta-carotene found in the intestinal wall were present because of incomplete washing of the contents from the intestine.

DISCUSSION

Evaluation of Methods of Analysis

Three methods were tested for effectiveness of analysis for vitamin A in extracts from intestinal tissue containing lipids and considerable amounts of added beta-carotene and emulsifying agents. An interpretation of the findings and an evaluation of the usefulness of each method are discussed below.

A method for analyzing for vitamin A by destroying the vitamin with ultraviolet irradiation was described by Little (88) and successfully applied to vitamin A analysis in serum by Bessey et al. (73). The procedure for applying this method is outlined in the "Methods" section of this thesis.

The accuracy of this destructive irradiation method depends on two conditions. First, vitamin A must be sufficiently degraded by irradiation so that the absorption spectrum of the vitamin A degradation products produced by irradiation treatment displays little or no absorption above 300 mμ. Secondly, no other light sensitive materials that change in absorption capacity in the region of the vitamin A absorption maximum, 328 mμ, may be present with vitamin A.

If these conditions hold, the difference spectrum obtained by subtracting the post-irradiation spectrum from the pre-irradiation spectrum will give the true vitamin A absorption

spectrum. This is true because the pre-irradiation spectrum will be composed of vitamin A absorption plus absorption of other compounds in the solution. The post-irradiation spectrum will be composed only of the absorption of the other compounds since absorption of these was unchanged by the irradiation treatment while the absorption of vitamin A was reduced to zero.

Once the validity of these two conditions is established vitamin A may be estimated by merely taking the absorption difference between the pre- and post-irradiation spectra at the absorption maximum for vitamin A.

The first condition was tested and confirmed for solutions of vitamin A subjected to irradiation (Table 1). The results show vitamin A absorption destruction to be 95% after 20 minutes of irradiation. Figure 1 depicts the absorption spectrum (pre-irradiation spectrum) and difference spectrum of vitamin A. It can be seen that the difference spectrum is almost superimposed on the absorption spectrum in the region of maximum absorption. Thus, the method is capable of vitamin A estimation within several percent of the true value. This compared favorably to most methods of analysis because these methods require additional purification steps where losses occur.

In experiments on the conversion of beta-carotene to vitamin A substantial amounts of the substrate, beta-carotene,

are required. If conversion efficiency is not high beta-carotene will be present in the non-saponifiable extract with vitamin A. Furthermore, beta-carotene is destroyed by irradiation. In order to satisfy the second condition it is essential that beta-carotene destruction does not alter absorption in the region of the vitamin A absorption maximum at 328 mμ.

The results from irradiated solutions of beta-carotene are contained in Table 1 and the spectra are depicted in Figure 1. These demonstrate considerable destruction of absorption by beta-carotene in the region of maximum absorption (453 mμ) but no change in absorption in the region of the vitamin A absorption maximum (328 mμ). Also Table 1 and Figure 1 show that when a solution containing both vitamin A and beta-carotene was irradiated, beta-carotene destruction had no effect on the difference spectrum in the critical region of the vitamin A absorption maximum, 328 mμ. Therefore, the conclusion was drawn that the presence of beta-carotene does not invalidate this destructive irradiation method as a means of estimating vitamin A.

The applicability of the destructive irradiation method was further examined to make certain that the minute amounts of vitamin A present in non-deficient rat intestine could be identified. Curves 1 and 3 (in Figure 2) were obtained in this study. Both curves approximate the absorption spectrum

of vitamin A. These results suggest that the destructive irradiation method is applicable to the measurement of normal intestinal levels of vitamin A.

The absorption difference spectrum (curve 2) of the extract from a normal rat intestine incubated with beta-carotene also is shown in Figure 2. The absorption maximum region has been broadened to extend from 330 mμ to 340 mμ. Vitamin A has a narrow absorption maximum region in kerosene-xylene (1:1) from 328 to 330 mμ. A cis isomer of beta-carotene, however, has an absorption peak at 340 mμ (82). Moreover, the absorption of the cis isomer is destroyed by irradiation (83). All-trans beta-carotene was used as the substrate in the present work and the saponification of the gut was performed at a refluxing temperature of 80°C. At this temperature some cis inversion undoubtedly occurred (82) leading to an overestimation of vitamin A. To prevent cis inversion of beta-carotene saponification must not be done at reflux temperature.

This limitation severely reduced the convenience of this method of analysis. Saponification at lower temperatures was found to be less complete and, due to the presence of significant amounts of emulsifying agents, extraction of the non-saponifiable fraction was made more difficult because of the formation of stable emulsions.

The destructive irradiation method was then investigated

as a means of analyzing for vitamin A formed from beta-carotene in vitamin A deficient rat intestine. Rosenberg and Sobel (71) had found *in vitro* formation of vitamin A of more than 4 μg per intestine by this method of analysis. The procedure of Rosenberg and Sobel was followed except that the carotene substrate was injected directly into the excised anterior end of the small intestine after ligation and excision of the posterior end rather than by stomach tube followed by squeezing into the intestine. This modification was made because Thompson *et al.* (33) had reported observable levels of *in vivo* conversion in 15 minutes after oral dosage. Also, controls incubated in the absence of carotene were tested whereas Rosenberg and Sobel did not report controls.

The difference absorption spectra (Figure 3) obtained from the non-saponifiable extracts of two vitamin A deficient intestines incubated with carotene (C_1 and C_2) and two intestines incubated without carotene (B_1 and B_2) deserve comment. It appears that vitamin A formation occurred in the intestines incubated with carotene although the spectrum of C_1 is not representative of vitamin A in the 320 to 325 mμ region. However, inspection of one of the blank determinations, B_2, shows a curve that appears an exact replica in contour of C_1. Analysis of other vitamin A deficient rat intestines with no additives showed B_2 type difference spectra. The light sensitive material then is present in the intestine and not a

degradatory product of Tween or alpha-tocopherol. None of the rat intestines analyzed that were not vitamin A deficient showed this phenomenon. Thus, it appears that the material with a light sensitive vitamin A-like spectrum arises in vitamin A deficient rat intestine.

The presence of this "pseudo vitamin A" does not satisfy the second condition described above. With another material present displaying an absorption difference spectrum similar to vitamin A, the difference between the pre- and post-irradiation spectrum at 328 mμ can not be taken as evidence of vitamin A formation. The conclusion then is that the difference spectrum method of analysis for vitamin A is not feasible for conversion studies of beta-carotene to vitamin A by vitamin A deficient rat intestine.

This conclusion does not support the findings of Rosenberg and Sobel (71) but does support the evidence presented by Rogers (74). He found that a compound with a vitamin A-like spectrum exists in vitamin A deficient rat intestine and may be distinguished from vitamin A by chromatography.

The Carr-Price reagent (antimony trichloride in chloroform) has been the most used and most highly regarded method, aside from biological assays, of estimating vitamin A. However, carotenes as well as vitamin A form a blue complex with the Carr-Price reagent. Carotenes may be estimated also by the absorption spectrum at 450 mμ and the amount found by this

method subtracted from the results of the Carr-Price colorimetric test. However, when large amounts of carotene and only small amounts of vitamin A are present better accuracy can be obtained by separating carotene from vitamin A before colorimetric analysis.

Paper chromatographic methods offer a good means of separating minute amounts of compounds. In conversion studies, however, because of the poor conversion ratio, substantial amounts of carotene are present but only small amounts of vitamin A. Thus a paper chromatographic method with widely differing R_f values for carotene and vitamin A is required in order to resolve the two compounds when considerable amounts of carotene are present. The paper chromatographic separatory method of Kaiser and Kagan (80) looked particularly applicable to this problem because by this method beta-carotene, the component present in comparatively large amounts, does not move while vitamin A moves at the solvent front.

The results reported in the previous section confirmed this method to be an effective one for separating vitamin A from beta-carotene without loss due to decomposition providing the following modifications were made: (a) The chromatogram should be developed in a nitrogen atmosphere in the absence of light (Figure 8). (b) Alpha-tocopherol dispersed in Tween 20 should be applied with vitamin A at the chromatogram origin.

(c) The developed chromatogram should be dried in a stream of nitrogen rather than in a stream of hot air.

The effectiveness of the paper chromatographic method was reduced, however, in experiments in which large amounts of emulsifying agents (Tweens and bile salts) and oils were used. It was practically impossible to saponify completely enough to prevent large quantities of these substances from being extracted into the non-saponifiable fraction. The result was that when the Skellysolve solvent was evaporated vitamin A and carotene remained solubilized in several drops of oil or Tween. The oil residue spread readily when applied to the paper chromatogram forming a large spot at the origin from which the pigments were not resolved. Thus, a paper chromatographic separation of carotene and vitamin A is not feasible in the presence of large amounts of lipoid material unless saponification followed by extraction is repeated several times. It would seem preferable to avoid repeated saponification, if possible, when attempting to recover labile compounds such as carotene and vitamin A.

Separation of beta-carotene and vitamin A by partitioning between Skellysolve A and methanol on a silica gel column was found to be superior to other methods investigated. This procedure was simple to perform. The time required to separate the non-saponifiable extract into four fractions was of the order of 1 hour whereas paper chromatography in a non-

equilibrated system required 12 to 24 hours. The percent recovery of beta-carotene and vitamin A was 90% or better. Separation of these two components was complete.

A criticism that might be leveled at this method of separation is that other monohydroxy carotenoid products besides vitamin A would not be separated from vitamin A on the column. Although this is true, other monohydroxy beta-carotene degradation products have not been isolated from animal biological systems except for the report by Glover and Redfearn (11) on findings of Festenstein. If compounds of this type, such as the apo-carotenols, are present, they can be identified readily by their absorption spectra.

The findings during the course of conversion studies reported herein have led to the development of the following procedure for purification and estimation of vitamin A formed from beta-carotene incubated with intestinal tissue.

After incubation the contents of the intestines are rinsed out with 10 ml of 0.9% NaCl. The intestinal tissue is then saponified in the presence of excess 55% aqueous KOH and enough ethanol to make the mixture 85% in ethanol. The mixture is refluxed 30 minutes (reflux temperature should be near 80°C). The mixture is then diluted with an equal volume of water and extracted with 50 ml portions of Skellysolve A until the extract is colorless. The extracts are then combined and washed with water until the washings no longer turn

pink with phenolphthalein. The extract is dried over sodium sulfate and evaporated under nitrogen to a volume of approximately 50 ml. The extract is then passed through a silica gel column containing absorbed methanol (prepared as outlined in the previous section). Fifty ml of Skellysolve A are passed through after the extract and collected in the same fraction (this fraction contains recovered beta-carotene). Fifty ml of 10% ethyl ether in Skellysolve A are then passed through the column and collected as the next fraction (this fraction contains vitamin A). (Fractions containing polyalcohol carotenoids can be eluted according to the procedure described in the previous section.) The vitamin A fraction is then evaporated to dryness under nitrogen and taken up in 3 ml of chloroform. The spectrum of the solution is measured from 300 to 500 mμ with the Beckman model DU spectrophotometer or the Cary Recording Spectrophotometer. One ml of the chloroform solution is then reacted with 9 ml of the Carr-Price reagent in a test tube contained in the Coleman Junior spectrophotometer. The absorbancy is measured at a wavelength setting of 620 mμ. The contents are analyzed by the same procedure.

By use of this procedure vitamin A can be separated from beta-carotene and polyoxygenated degradatory products of carotene. Two methods of analysis confirming one another serve to affirm the presence of vitamin A when it is identified.

One method alone offers much less certainty.

The danger of analyzing for vitamin A by an inconclusive method was emphasized as early as 1939 by Woolf and Moore (25) and again by Bieri and Pollard (72) in 1953 and Rogers (74) in 1957. All these workers, by applying more careful methods of analysis, were unable to confirm the results of earlier workers that had led to claims of *in vitro* conversion of *beta*-carotene to vitamin A.

Uncertainties in various analytical techniques should be recognized. The Carr-Price colorimetric test forms blue complexes with carotenoids as well as with vitamin A. The ultraviolet spectrum (in all cases examined) contained impurities absorbing in the region of the vitamin A absorption maximum, 335 mμ in chloroform, to the extent that small amounts of vitamin A absorbed enough only to form a shoulder on the curve that was descending sharply as wavelength increased. Thus, without further purification vitamin A can be estimated only qualitatively from the absorption spectrum.

A combination of the two analyses lends greater significance to the identification of vitamin A if the following line of reasoning is valid. It is postulated that limited oxidative degradation of *beta*-carotene leads to the formation of mono- and poly-apo-carotenols. Acids, if formed, would not be extracted into the non-saponifiable extract. The findings of Glover *et al.* (17) indicate that aldehydes formed are

quickly reduced to alcohols in intestine as well as other body tissue, probably due to the action of alcohol dehydrogenase. The non-saponifiable extract should contain then only carotenoid degradation products that are mono- and poly-alcohols. The partition column chromatographic procedure separates the mono-alcohols from the poly-alcohols.

The absorption spectrum of the mono-alcohol fraction should indicate only apo-carotenols and vitamin A resulting from beta-carotene degradation. The long chain apo-carotenols have absorption maxima beyond vitamin A where irrelevant absorption is less. The presence of small quantities of these apo-carotenols would be reflected by a rise in absorption at wavelengths beyond 330 mμ and less than 500 mμ.

Thus, when by the foregoing procedure the characteristic blue color indicative of vitamin A is obtained from the Carr-Price color test and the absorption spectrum shows evidence of vitamin A but not of apo-carotenols, the evidence for vitamin A formation is strong.

The Conversion Problem

The ultimate goal of conversion studies reported here and by others as well was to isolate a biochemical system of known components capable of converting beta-carotene to vitamin A and to determine the mechanism of conversion by studies with the isolated system. Although little progress has been

made with *in vitro* conversion studies, speculations have been tendered proposing the mechanism of conversion.

In 1931, Karrer *et al*. (10, 89) suggested that *beta*-carotene is cleaved symmetrically by addition of two molecules of water at the central double bond to give two molecules of vitamin A alcohol. This "hydrolytic central fission" process is now unattractive biochemically (4) and a specific "*beta*-carotenase" enzyme such as the one reported partially purified by Olcott and McCann (20) would not be expected today to do more than catalyze the first step in the conversion process.

In 1946, Hunter (87) suggested the initial step was by oxidative attack at the central double bond forming first vitamin A aldehyde with subsequent reduction to the alcohol. Chemical conversions with hydrogen peroxide (13, 16) gave weight to this hypothesis as did the finding (17) that vitamin A aldehyde was rapidly reduced to the alcohol on entering the gut wall.

In 1943, Glover and Redfearn (11) proposed that initial oxidation occurs at a terminal double bond and then proceeds by a process of *beta* oxidation until vitamin A aldehyde is formed. Oxidation proceeds no further because vitamin A aldehyde has a methyl group in the *beta* position. These workers also found that vitamin A was formed *in vivo* in vitamin A deficient rats when apo-carotenols (higher homo-

logues of vitamin A) were fed to vitamin A deficient rats. Later work by Glover and Fazakerley (90) and Redfearn (91) showed that a higher homologue of vitamin A, C_{25}, also with a beta methyl group was converted to vitamin A. This lead Glover and Fazakerley to suggest that an enzyme system, which removed five terminal carbons as a unit, was available.

A conclusive answer as to which, if any, of these proposals depicts the means by which vitamin A is formed from beta-carotene does not appear to be forthcoming from *in vivo* experimentation. One reason for this opinion is that intermediates between beta-carotene and vitamin A have not been detected, except for one finding that of two unidentified carotenoids in horse intestine. Glover and Redfearn (11) point out that these compounds have identical spectroscopic and chromatographic properties to two apo-carotenals. Aside from this, beta-carotene dosages have yielded only vitamin A along with undegraded beta-carotene. It appears then that the initial attack may be a slow conversion step and that once the initial step has occurred succeeding steps could occur rapidly until vitamin A is formed. If this is correct, mechanism elucidation through isolation and identification of intermediates successively formed during the *in vivo* conversion process does not appear to be likely.

Radioactive labeling of the beta-carotene or other provitamin carotenoid molecules does not offer an easy solution

to the problem. There is no position or positions at which a carotene could be labeled which would distinguish between central fission or stepwise degradation from one end merely by examining the sites of labeling and activity of the resulting vitamin A.

Yields of vitamin A should distinguish between central cleavage (two molecules of vitamin A per beta-carotene molecule) and stepwise degradation from a terminal double bond (one molecule of vitamin A per molecule of beta-carotene). The weight of evidence favors the 1:1 conversion ratio (92). The International Standard sets 0.6 μg of beta-carotene biologically equivalent to 0.3 μg of vitamin A. Two laboratories, however, have reported 2:1 conversion in the presence of tocopherol (79, 93).

Other explanations can account for no better than a 1:1 conversion ratio even if central fission occurs. Lack of absorption accounts for the loss of some beta-carotene. Severe degradation accounts for other losses. Willmer and Laughland (94) and Krause and Sanders (95), after dosing rats with randomly labeled beta-carotene and analyzing at various times, found considerable activity in other fractions besides the non-saponifiable fraction. These results were confirmed in this laboratory where it was found that radioactivity was located in the fatty acid, cholesterol ester, and neutral fractions as well as the non-saponifiable fraction.

In view of the difficulties encountered in designing *in vivo* experiments capable of giving conclusive evidence of the conversion mechanism, the *in vitro* approach seemed the better one and was undertaken in this investigation. Isolation of the system involved should answer the problem as to whether a specific enzyme "carotenase" exists and converts *beta*-carotene by central cleavage to vitamin A or whether a series of enzymes that perform other functions, such as the enzymes that degrade fatty acids by *beta* oxidation, also convert carotene to vitamin A by a similar process.

The experimental plan was to establish conditions such that conversion of *beta*-carotene to vitamin A would be accomplished first in excised whole intestine, then in tissue homogenates, and finally in homogenate fractions. Only in the final experiment with whole intestine was vitamin A formation observed; the amount found was very small. Homogenate conversion of *beta*-conversion to vitamin A was not accomplished. Results obtained during the course of this investigation will be discussed with regard to important controlling conditions of *in vitro* experiments.

Most *in vitro* conversion studies have been performed under nitrogen, presumably to simulate anaerobic conditions in the lumen of the small intestine and also in homogenate experiments to protect *beta*-carotene from atmospheric oxidation. Atmospheres of nitrogen, air, and oxygen were used

during the course of this investigation. In one experiment (Figure 3) some evidence of conversion was obtained with a nitrogen atmosphere. However, since the results were obtained by the destructive irradiation method of analysis the evidence of conversion is of doubtful value. In no other case was evidence of vitamin A formation obtained with incubation under a nitrogen atmosphere.

More definite evidence of conversion was obtained in an experiment performed under an oxygen atmosphere (see experiment 7, Table 7). This work, however, does not establish the requirement of an oxygen atmosphere for *in vitro* vitamin A formation or the role of the atmosphere in the conversion process. It may be that certain physiological processes essential to the conversion process that take place *in vivo* can operate *in vitro* for a short time in the presence of oxygen to produce low levels of vitamin A.

In various experiments aqueous Tweens and bile salts were used as dispersing agents for *beta*-carotene. In addition, a commercially prepared water-dispersible *beta*-carotene was tested. Tweens also were used to disperse soybean oil in which *beta*-carotene had been solubilized; the only method that produced evidence of conversion.

The dispersing agent most used by previous investigators has been one of the Tweens. Since one group of investigators (45) has reported toxicity due to Tweens, a study was per-

formed to correlate Tween concentration with degree of dispersibility of beta-carotene (see Figures 6 and 7). It was found that degree of dispersibility increased with increasing Tween concentration, although the rate of increase of carotene dispersion varied markedly at different Tween concentrations. Only a minute increase in dispersion was found as Tween concentration was increased from 0.05% to 0.5% but marked increases in carotene dispersion were observed as Tween concentration was increased at other levels. Possibly the critical micelle concentration of the aqueous Tween system is near 0.05% Tween.

Later findings (experiment 7, Table 6) suggested that conversion was promoted at a much higher Tween concentration (10%). In this experiment, however, the beta-carotene was solubilized in oil and the oil dispersed in small globules in the high Tween concentration. It is likely, considering the results indicating conversion, that this method of dispersion presented the metabolite beta-carotene for absorption by the intestinal wall more adequately than other methods.

In all experiments only small amounts of carotene were recovered from the intestinal wall, indicating poor absorption of the provitamin. It is possible that failure to solve the problem of presenting a water insoluble substrate in an aqueous system in a manner so that it can be absorbed by the gut wall or acted upon by an enzyme system without inhibiting

the enzyme system accounts for the failure to achieve *in vitro* conversion.

Other factors which may influence conversion of *beta*-carotene to vitamin A were tested and varied during the investigation. Antioxidants, either *alpha* or *gamma*-tocopherol, were used in conjunction with *beta*-carotene in most experiments to preserve carotene from non-specific oxidation during incubation. Incubation times were varied from 20 minutes to 2 hours and incubation temperatures varied from 37°C to 45°C. Glucose was provided as an energy source in several experiments.

Evidence for conversion was obtained when the *beta*-carotene substrate, solubilized in soybean oil dispersed by Tween 80 in Ringer-Locke solution, also contained *gamma*-tocopherol and glucose. Incubation was conducted 1 hour at 39°C under oxygen in a bath of Ringer-Locke solution. Peristaltic action continued throughout the incubation period.

All other combinations of cofactors and conditions reported in the experimental section did not give convincing evidence of vitamin A formation. However, further work is needed to establish whether the low level of conversion observed, 0.8 μg per intestine, can be effected in the absence of or alteration of any of the conditions listed above.

Homogenates of rat intestine were prepared by several methods and incubated with *beta*-carotene dispersed in Tweens

or bile salts in aqueous systems of Ringer-Locke solution or phosphate buffers. Additives such as alpha-tocopherol, albumen, and H_2O_2 were tested. Incubations were conducted for 20 minutes to 2 hours at temperatures from 37°C to 45°C under atmospheres of nitrogen or air. No evidence was obtained for vitamin A formation in any of the experiments.

In vivo conversion of beta-carotene to vitamin A approaches a 1:1 conversion ratio as the values for biological potency of each established as an International Standard shows. In vitro reports of conversion have been at a very low level (several μg). Moreover, the validity of the analytical method used has been questioned by later workers unable to repeat the results (see Literature Review). Evidence for conversion obtained in one experiment during the course of this investigation, like others, showed very poor conversion efficiency (1:500). Work in this investigation with tissue slices (unreported) and homogenates did not demonstrate vitamin A formation.

Obviously factors present in the in vivo system have not been properly duplicated in the in vitro system. Inadequate subdivision of beta-carotene in an aqueous system could be the causal factor for the extremely limited conversion. Olson (77) has recently suggested that a specific requirement of sodium glycocholate is necessary for conversion of carotene to vitamin A, but his results with sodium glycocholate show

vitamin A formation at less than 1 μg.

It may be that the factors necessary to promote vitamin A formation from beta-carotene in the living animal are rapidly depleted in the in vitro system. The findings of Sibbald and Hutcheson (39) that blood supply to the intestinal tract was essential to conversion in vivo but that continuity of the tract was not required suggested that the blood may furnish the needed factors. However, intestinal homogenates incubated in blood did not effect conversion.

Conversion reported in the experimental section of this thesis took place in vitro but in a whole excised small intestine manifesting peristaltic action and provided with an energy source and an oxygen atmosphere. The small amount of conversion observed in this metabolizing environment could be due to short-time duplication of a complex series of essential relationships in the living animal. In vitro conversion in cell-free homogenates may be a long step from in vitro conversion in such a pseudo "living" intestine.

It is still not known whether a specific "carotenase" enzyme exists or whether a multiple enzyme system is required to form vitamin A from beta-carotene. Conversion studies (Figure 3) with rats not deficient in vitamin A did not give evidence of conversion and hence did not suggest that the converting enzyme system is an inducible one.

The first step of progress in future work should be an

improvement in the *in vitro* ratio of conversion of *beta*-carotene to vitamin A. Vitamin A formation above the microgram level is necessary before confidence can be developed that the essential factors for the conversion process are available for isolation. This appears necessary notwithstanding the fact that experimental evidence is weighty on the testing of cofactors to promote conversion in *in vivo* and *in vitro* systems, but has not established any essential cofactors as yet (see Literature Review).

Results obtained in this investigation suggest that conversion studies should be attempted with variations on a substrate containing oil solubilized, Tween dispersed *beta*-carotene along with an antioxidant and an energy source incubated in an oxygen atmosphere.

SUMMARY

Experiments were performed on the *in vitro* conversion of *beta*-carotene to vitamin A by excised vitamin A deficient rat small intestine and homogenates thereof. In conjunction with these experiments methods of purification and analysis of microgram quantities of vitamin A were tested.

An *in vitro* conversion experiment which gave evidence of low level (0.8 µg per intestine) vitamin A formation was performed under the following conditions. *Beta*-carotene and *gamma*-tocopherol were solubilized in soybean oil and the oil solution was dispersed in 10% Tween 80 in glucose-containing Ringer-Locke solution. The resultant mixture was incubated in excised vitamin A deficient rat small intestine for one hour at 39°C under an oxygen atmosphere.

In this and other experiments a number of additives were presented with *beta*-carotene in an attempt to promote vitamin A formation. Among those tested were various Tweens, bile salts, and a water-dispersible gelatin preparation of *beta*-carotene, *alpha*- and *gamma*-tocopherols, albumen, hydrogen peroxide and glucose. Incubation baths were usually composed of Ringer-Locke solution. Incubation times and temperatures were varied and atmospheres of nitrogen, air, and oxygen were tested. Under no set of conditions, except those specified above, was vitamin A formation in whole intestine detected.

Intestinal homogenates were prepared in crushing-type and cutting-type tissue homogenizers and by pulverizing after freezing in liquid nitrogen. These homogenates were incubated in Ringer-Locke solution and in phosphate buffers with beta-carotene and combinations of the additives and conditions listed above. In no case was vitamin A formation effected by homogenates.

Several methods of purification were tested extensively. The destructive irradiation method of vitamin A estimation was found to be unreliable for conversion studies with vitamin A deficient rat intestine. A light sensitive material extracted into the non-saponifiable fraction, and with a difference spectrum similar to that of vitamin A, was found in vitamin A deficient rat intestine.

A paper chromatographic procedure was developed that separated vitamin A from beta-carotene without loss due to decomposition. However, this method of separation was found to be infeasible in the presence of the quantity of lipoid material that was taken into the non-saponifiable extract in conversion experiments in which dispersing agents were used.

A method of analysis was developed that met the problems imposed by *in vitro* conversion studies of carotene to vitamin A and gave reliable estimations of vitamin A at the microgram level. By this method vitamin A was separated from beta-

carotene and most carotene degradation products in the non-saponifiable extract by partition chromatography on a silica gel column containing absorbed methanol. On the purified vitamin A fraction it was necessary to measure the absorption spectrum and perform the Carr-Price color test as well in order to achieve a clear-cut quantitative estimation.

A study on the dispersion of carotene in Tween as a function of Tween concentration showed that degree of dispersion of carotene increased, but not linearly, with Tween concentration. A Tween concentration range was located in which change in degree of dispersion of carotene was minimal.

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