FACTORS AFFECTING THE FORMATION OF VITAMIN A FROM B-CAROTENE IN VITRO USING DUODENUM AND OTHER TISSUES FROM DIFFERENT ANIMALS

Thesis for the Degree of Ph. D.

MICHIGAN STATE UNIVERSITY

Bandaru Sivarama Reddy

1963

THESIS

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presented by

Bandaru Sivarama Reddy

has been accepted towards fulfillment
of the requirements for

Ph.D. degree in Dairy

J. W. Thomas
Major professor

Date May 13, 1963

O-169

FACTORS AFFECTING THE FORMATION OF VITAMIN A FROM B-CAROTENE IN VITRO USING DUODENUM AND OTHER TISSUES FROM DIFFERENT ANIMALS

By

Bandaru Sivarama Reddy

AN ABSTRACT OF A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Dairy

1963

ABSTRACT

FACTORS AFFECTING THE FORMATION OF VITAMIN A FROM B-CAROTENE IN VITRO USING DUODENUM AND OTHER TISSUES FROM DIFFERENT ANIMALS

By Bandaru Sivarama Reddy

The study herein was conducted to obtain whether an in vitro system modified from several investigators was satisfactory for studying vitamin A formation from B-carotene using duodenum and other tissue homogenates from different animals. When fresh duodenal homogenates from different animals were incubated with aqueous carotene suspension and 0.25M sucrose for 3 hours at 37°C, a considerable amount of vitamin A was formed. Much more vitamin A was formed when 0.25M sucrose was used than when any of the more commonly used buffers were employed. The formation of small amounts of vitamin A were more readily detected and measured when the saponification was performed on the incubated mixture.

The conversion process appeared to be a rapid reaction. The tissue homogenized under nitrogen was more effective in vitamin A formation than the tissues homogenized under oxygen. The conversion process studied was apparently enzymic. The extent of vitamin A formation was greatly reduced when the homogenates were kept one hour or more before an addition of the carotene suspension. Conversion was greatly reduced by heat, KCN, urea, NO_3^- or iodoacetate but not appreciably reduced by $SO_4^=$, NaCl or $CO_3^=$.

Identification of vitamin A formed by this system was carried out on chromatographed tissue extract by various methods such as absorption spectra, changes in spectra after irradiation, maleic anhydride method, conversion to anhydrovitamin A and paper chromatography.

The small intestine especially the duodenum and liver were found to contain the most activity for conversion of carotene to vitamin A in all animal species tested. Many tissues were found to possess the ability to form small amounts of vitamin A from carotene.

The decrease in activity of vitamin A formation produced by NO_3^- or NO_2^- was somewhat specific. Large amounts of NO_3^- added to the in vitro system produced a marked inhibition of vitamin A formation whereas small amounts produced little or no effect. The extent of conversion was decreased by using duodenal homogenates from animals fed NO_3^- or NO_2^-.

Duodenal homogenates from hypothyroid and thyroidectomized animals considerably reduced the formation of vitamin A from B-carotene as compared to the duodenal homogenates from control and hyperthyroid animals, whereas no difference was observed between the duodenal homogenates from control and hyperthyroid animals. Addition of high levels of L-thyroxine or L-triiodothyronine to the in vitro system reduced the extent and efficiency of vitamin A formation. The inhibitory effect of 3 5' 3' L-triiodothyronine

on vitamin A formation was reduced to certain extent by the addition of isomers of thyroxine and triiodothyronine.

Little or no vitamin A was formed when carotene was incubated with hemolyzed blood or sedimented blood cells. Vitamin A formation was markedly reduced when these fractions were incubated with carotene and duodenal homogenates. The inhibitory factor was located in the sedimented blood cells.

When the duodenal homogenates were centrifuged at 5000 XG, 10,000 XG and 25,000 XG and the resulting supernatant and sedimented fractions were incubated with carotene, the supernatant fraction from a 5000 XG preparation contained more activity than that in the sedimented fractions from 15,000 XG and 25,000 XG preparation.

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ACKNOWLEDGEMENTS

The author wishes to express his gratitude to Dr. J. W. Thomas for his advice and guidance throughout the course of the investigation and for critically reading this manuscript. The author is also grateful for the critical reading and helpful suggestions of Dr. H. D. Hafs, Dr. J. Meites, and Dr. C. J. Pollard. The encouragement and suggestions of Dr. L. D. Brown and Dr. R. S. Emery are also deeply appreciated. The cooperation extended by the staff and the graduate colleagues of dairy cattle nutrition section is also acknowledged.

The author is indebted to Dr. C. A. Lassiter for the appointment of research assistantship thereby making this investigation possible.

The author wishes to thank his wife, Subhashini, for her unselfish help and encouragement during the preparation of this thesis.

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INTRODUCTION

During the last 40 years studies on the absorption of B-carotene and its conversion into vitamin A have occupied a large percentage of the investigations on fat soluable vitamins. Although the conversion of B-carotene to vitamin A is a well established process, the mechanism underlying the conversion is not understood at the present time. The formation of vitamin A from B-carotene *in vivo* has been well established. Numerous attempts have been made to demonstrate the formation of vitamin A from B-carotene *in vitro*, but the results are for the most part conflicting. Conversion of carotene to vitamin A *in vitro* has been claimed on the basis of conventional methods for vitamin A analysis while other investigators have criticized or seriously doubted such claims.

Feeds and forages containing high levels of nitrate have been implicated as a factor responsible for lowering of vitamin A status of animals receiving adequate levels of carotene. In addition there is an indication that dietary nitrate can adversely affect the normal functioning of thyroid gland and thereby impair the formation of vitamin A from carotene. Some of these results were somewhat conflicting.

It is indeed time consuming to study all of the above controversial topics *in vivo*. The principal aim of the studies reported in this thesis was to modify the *in vitro* media used by various investigators and to establish whether B-carotene could be converted into vitamin A in this *in vitro* system. It was also decided to isolate and identify any vitamin A thus formed using appropriate procedures. With the help of this *in vitro* system, it was then planned to study the

efficiency of vitamin A formation by various tissues, the effect of nitrate and other chemicals on vitamin A formation, and the interrelationship between thyroid status, nitrate and vitamin A formation.

REVIEW OF LITERATURE

The conversion of B-carotene to vitamin A, whether by central fission or by B-oxidation, may appear to be a very simple process. Although the conversion was discovered 30 years ago, the mechanism of vitamin A formation from carotenoids has not been elucidated very clearly nor in full detail.

Moore (128) was the pioneer in developing the idea that carotene was the precursor of vitamin A. The liver of rats depleted of vitamin A contained little or no blue "Lovibond Units" of vitamin A activity as compared to rats given carotene supplements orally for 16 - 26 days. Using the same procedure with chicks, Capper et al. (34) demonstrated that the carotene was not stored in the liver unchanged but was presumably converted into vitamin A.

These and other deductive experiments established the view that carotene was the precursor of vitamin A.

In vivo conversion of B-carotene into vitamin A.

According to the present concepts the small intestine plays a major role in the conversion of B-carotene into vitamin A in laboratory animals as well as in large animals. Early work of Moore (129) was interpreted to indicate that the liver was the organ in which the conversion occurred. The liver oils of rats invariably contained vitamin A at extremely high concentrations when diets containing large amounts of carotene were given for prolonged periods. Small amounts of unconverted pigments were also found in the liver oils of all rats which had received carotene. From this evidence it was deduced that vitamin A was formed in the liver.

With the aid of fluorescence microscopy, Popper (145) observed the appearance of vitamin A in the intestine, in the Kupffer cells of the

liver, in the epithelial cells of the adrenal cortex, in the tubular and Leydig cells of the testicle, in the granulosa and theca-lutein cells, and in the cortical stroma of the ovary. Although the information could have been used to indicate vitamin A formation in the intestine or other extrahepatic tissue, no such deductions were made by the author or others.

The possibility of conversion of carotene to vitamin A in extrahepatic tissue was suggested by Sexton et al. (157) in 1946. When the carotene was administered either intravenously or intraperitoneally to deficient rats the growth response was low as compared to that of rats given oral doses. Liver vitamin A was determined spectrophotometrically on petroleum ether extracts. These authors suggested the wall of intestine as a possible site of such conversion.

Vitamin A first appeared in the intestinal wall 1.5 hours after oral administration of carotene dissolved in oil to vitamin deficient rats (119). Analysis of liver and intestine three hours after dosing showed that they contained a total of 1.6 ug of carotene and 22.8 IU of vitamin A and 13.5 ug of carotene and 40.3 IU of vitamin A respectively. Vitamin A was determined by the Carr-Price reaction.

The results of Mattson et al. (119) were presented almost simultaneously with a note by Thompson et al. (175) who dosed pigs with B-carotene in arachis oil and found an increase in the vitamin A ester in blood plasma 3 - 7 hours later. Vitamin A was detected in the mesenteric lymph channels and in the wall of the intestine and its contents in greater quantities than in control pigs. Later the British workers made a thorough and comprehensive study of B-carotene disappearance in the small intestine using laboratory as well as large animals. In one

study when B-carotene was given by mouth to vitamin A deficient rats, vitamin A appeared within 15 minutes in the wall and contents of the small intestine, whereas the first increase in the blood and in the liver did not occur until 45 - 60 minutes had elapsed (176). Up to two hours after dosing there was more vitamin A in the small intestine than in the liver. Similar results were also found in pigs. Further evidence showed the appearance of vitamin A in the portal and systemic circulation of pigs and in the mesenteric lymph of pigs and rats two hours after carotene feeding (173). It was found that the vitamin A appeared almost exclusively in the wall of the intestine, 75% of it in the ester form. No conversion was observed in that part of small intestine preceeding the entrance of the common bile duct. These workers separated carotene, vitamin A alcohol and its ester by column chromatography from the ether extract and then used the Carr-Price reaction to estimate vitamin A content of the samples. These experiments indicated that the small intestine was the site of conversion of carotene to vitamin A and that the lymphatic system was a route of transport of the vitamin A so formed.

Ball and his co-workers (10) fed retinene (vitamin A aldehyde) and found that it was converted to vitamin A alcohol in the intestinal wall. These results gave added emphasis to the idea that the carotene might be converted into vitamin A in the intestinal wall. Further confirmation of extrahepatic conversion of carotene to vitamin A was advanced by Krause and Pierce (110). They fed carotene to rats without any hepatic circulation and found an increase in serum vitamin A 6 - 8 hours after administration of carotene.

Further evidence was obtained from the detailed investigations of Glover et al. (66). They fed depleted rats a diet rich in carotene and subsequently killed them a few hours later. These authors separated vitamin A from unsaponifiable matter by column chromatography and proved its identity by maximum absorption in the antimony trichloride reaction at 617 mu and in the ultraviolet light at 328 mu. When the absorption of carotene was maximal, the concentration of vitamin A present in the intestinal wall was comparable to that in the liver. These results helped to establish the view that the wall of small intestine was the site of transformation of carotene into vitamin A.

In goats provided with a thoracic canula, an increase in vitamin A levels of the lymph was noted after feeding carotene, indicating that vitamin A was formed from carotene in the gut wall and transported to the liver via the lymph (70). This work was further extended and it was demonstrated that the intestine or the intestinal wall was the site for the conversion in conscious rats (4). These authors separated vitamin A from unsaponifiable matter by column chromatography and determined vitamin A by antimony trichloride reaction and also by ultraviolet absorption at 328 mu. Klosterman et al. (100) demonstrated that when lambs were given carotene by mouth or allowed green grass for a short period, an increase of vitamin A in the blood serum occurred. When carotene was present in the intestine, vitamin A was also found in the intestinal wall. These observations coupled with the fact that no measurable amounts of carotene were found in the blood of normal sheep suggests that the carotene was converted into vitamin A during digestion and/or absorption rather than by liver.

Other experiments indicated that the intestine was the major site of formation of vitamin A in calves, goats, sheep and rabbits (103, 104). It was also established that the ingestion of carotene in Tween resulted in an earlier and greater increase in blood carotene and vitamin A than ingestion of carotene in oil using calves, rabbits and rats. Whatever the form or the vehicle of dispersion, carotene injected into calves was converted only to a very limited extent into vitamin A (103).

Simultaneously, Thompson et al. (174) and Cheng and Deuel (37) showed that the carotene was converted into vitamin A in the intestine of chicks. The former worker determined vitamin A by the Carr-Price reaction after separating vitamin A by column chromatography and the latter by the Carr-Price reaction on crude extracts.

Extensive studies by the Canadian workers demonstrated the conversion of carotene to vitamin A in vivo with chicks (161, 162, 163). In all these studies vitamin A in the sample was determined by the Carr-Price reaction after saponification, extraction and separation of carotene and vitamin A by column chromatography. Sibbald and Olsen (163) found that the vitamin A was formed from carotene in a ligated loop of the duodenum proximal to the entry of the bile and pancreatic ducts of the living chick. However, the relocation of vitamin A so formed was inhibited. It was suggested that this inhibition probably was due to pressure resulting from the secretion of fluid into the duodenal loop. In another study Sibbald and Hutcheson (161) found increased amounts of vitamin A in the duodenal wall using ligated duodenal loops in living chicks. When the blood supply to a duodenal loop was ligated the formation of vitamin A from carotene was prevented. They suggested that the blood supplied some factors for the converting mechanism and that

the absence of blood supply caused death of the tissue of the duodenal wall and hence inhibited its power of absorption and/or conversion. It was also shown that the minimum time necessary for the production of vitamin A from carotene injected into ligated duodenal loop of living vitamin A depleted chicks was 15 minutes (162).

Although the intestine has been reported to be the site of conversion some doubts still existed whether the actual locus of formation was in the wall or in the lumen or both. De and Sundararajan (43) reported that the whole of the small intestine became practically devoid of carotene in about 24 hours time when B-carotene in oil was administered to albino rats. The intestinal wall contained small amounts of carotene and also vitamin A. The intestinal lumen, on the other hand, contained large amounts of carotene but no vitamin A in most experiments. The vitamin A was estimated from ultraviolet absorption at 328 mu after chromatographic separation of the extracts. These authors claimed that the wall of small intestine appeared to be the site of conversion of carotene to vitamin A when carotene was fed.

The actual locus of intestinal conversion of carotene to vitamin A was investigated further. Greenberg (73) used histochemical techniques to visualize carotene and vitamin A in the unstained frozen tissue sections of the rat small intestine. After a meal containing carotene the first recognizable vitamin A was seen in 5 to 15 minutes and was first visualized within the intestinal lumen just adjacent to the tips of the villi. Subsequently the vitamin A was recognized histochemically within the mucosa even though small amounts of unchanged carotene are known to be absorbed (73). The data suggested that the conversion of carotene to vitamin A could occur as an extracellular process.

Although, most experiments had shown the small intestine to be a major site of conversion of carotene to vitamin A Belgian workers (105, 106) investigated the possibility that conversion could occur in the liver. Kowalewski and Henrotin (105) injected 25 mg of B-carotene emulsified in isotonic glucose solution into the portal vein and analyzed liver and blood one hour later. There was some indication of synthesis of vitamin A. In another study, Kowalewski et al. (106) investigated the role of portal and lymphatic circulations in the transport of carotene when administered into the intestine. The increase in both carotene and vitamin A in the blood and liver were greater after carotene had been given in an emulsified form than after it had been given in oil. When carotene was emulsified all of it was absorbed by the portal vein, but when it was given in oil a substantial portion was absorbed by the lymphatic duct. Carotene appeared to be absorbed rapidly into the portal vein when given in an aqueous emulsion.

The review of the literature pertaining to the formation of vitamin A from carotene in vivo, clearly indicated that the wall of the small intestine appeared to be the major site of conversion when the carotene was fed to laboratory as well as to large animals. The lymphatic system was a route of transport of the vitamin A so formed.

Conversion of carotene to vitamin A by the organs and body fluids other than intestine and liver.

Although such organs as the intestine and liver could convert B-carotene to vitamin A, early work on the site of conversion of intravenously administered carotene led to the conclusion that perhaps many tissues had the ability to form vitamin A from carotene. The question deciding the relative importance of the various possible sites for conversion of carotene is still undetermined after many experiments.

During recent years, several workers have investigated various aspects of the utilization of intravenously administered carotene by various animal species.

Klosterman et al. (100) found that colloidal carotene injected into the blood stream of sheep was rapidly removed with no increase in blood or liver vitamin A.

Eaton et al. (48) reported that in dairy calves both intravenous and oral administration of carotene resulted in higher blood plasma levels of carotene and vitamin A than in the controls. There was no real differences in vitamin A concentration of either liver or lungs in above two groups.

Warner and Maynard (183) found that intravenous injections of an aqueous dispersion of carotene increased plasma carotene and vitamin A levels significantly in dairy calves as estimated by independent analytical procedures. An ultraviolet examination of chromatographed, post injection, plasma extracts showed typical vitamin A absorption spectra.

Elliot (49) injected high carotene blood plasma intravenously to dairy calves and found no increase in liver vitamin A levels as compared with control calves. These results indicated that carotene in blood plasma was not converted to vitamin A in dairy calves.

Church et al. (38) found significant increases in plasma vitamin A after injecting a solubilized aqueous carotene in sheep. Carotene injections into calves caused no significant differences in plasma vitamin A values. The data obtained in these experiments indicated a difference between cattle and sheep in their ability to convert intravenously injected carotene to vitamin A. The results were contrary

to those reported by Klosterman et al. (100) with sheep and Eaton et al. (48) and Warner and Maynard (183) in dairy calves. One factor involved in this difference may have been the nature of the carotene dispersion.

Kon et al. (103) studied this problem further using animal species (rat or rabbit) whose blood does not contain carotenoids and a third (calf) that usually has large quantities of carotenoids in the blood. These experiments provided evidence for the formation of vitamin A from the injected carotene in rats and rabbits. By contrast, all their experiments with calves were negative in that they provided virtually no evidence that vitamin A was formed from injected carotene. Results indicated that a large part of the carotene that appeared initially in the circulation of rats and rabbits and in the lungs was rapidly destroyed, presumably by oxidative breakdown, with the simultaneous appearance of some vitamin A. These workers shared the view of Bieri and Pollard (20) that vitamin A might be one of the products of an oxidative chain and that its formation need not be confined to any particular site, though the lung might well prove to play an important role even if not a specific part.

Schuh et al. (154) demonstrated that intravenously administered carotene was well utilized by dairy calves. This was contrary to the results obtained by Church et al. (38) and Kon et al. (103).

Bieri and Pollard (19, 20) intravenously administered 1000 ug of aqueous B-carotene solubilized in Tween 40 to vitamin A deficient rats. Serum vitamin A levels reached a maximum of 134 ug% in six hours. Vitamin A was also found in liver, kidney, small intestine, lung and spleen. It was shown that ligating the bile duct did not interfere

with the conversion. Rats injected with carotene after surgical removal of the small intestine had vitamin A in the serum, liver, and kidney. These findings indicated that vitamin A could be formed from carotene in tissues other than the small intestine.

The experiments conducted by Bieri (17) showed conclusively that the chicken utilized circulating B-carotene and to a lesser extent cryptoxanthin as a source of vitamin A. It was proposed that those provitamins A which escaped conversion during absorption through the small intestine were still utilizable by the bird. Vitamin A was found in liver, kidney, and serum when carotene was injected intravenously into rats and rabbits.

Intravenously administered carotene emulsion was converted into vitamin A and the conversion was less efficient than that of aqueous carotene dispersion (123, 124, 126). These authors concluded that the conversion of the carotene molecule was initiated in the blood where there was rapid breakdown to an intermediate which was then taken up by the tissues and more slowly converted into vitamin A.

Attempts have been made to locate more precisely the site at which conversion of injected carotene occurred. Worker (190) demonstrated that the appearance of vitamin A in blood plasma after injection of a Tween dispersion of carotene was in no way affected by complete removal of the liver in rabbits, or by complete removal of the liver alone or both liver and viscera in rats.

Worker (192) concluded that the ability to convert injected carotene was an attribute not of any one tissue or organ but of many or perhaps of all cells. This belief was further supported by the results of preliminary in vitro experiments showing that many tissues including

blood possessed the ability to form small amounts of vitamin A upon incubation with carotene in Tween dispersed in physiological saline. The identification of vitamin A was carried out by absorption spectra and Carr-Price reaction after separating ether extracts on alumina.

In further endeavors to directly locate the site at which conversion of injected B-carotene into vitamin A occurred, Worker (193) attempted to demonstrate the in vitro conversion by carrying out perfusion and incubation experiments on a variety of tissue preparations. It was concluded that it was not possible to demonstrate conversion of carotene to vitamin A in vitro either in perfused organs such as intestine, kidney, liver or lung of the rat or guinea pig. Furthermore it was not possible to demonstrate in vitro conversion in the blood of the rat, guinea pig or sheep. These results contradicted the preliminary in vitro observations that many tissues including blood possessed the ability to form small amounts of vitamin A (190). Vitamin A was estimated by the $SbCl_3$ method as in the previous observations (48).

After conducting further experiments with rats, McGillivray (122) suggested a mechanism for formation of vitamin A from carotenoids that were injected intravenously in an aqueous dispersion. The suggestion was made that after intravenous injection a rapid break down of the carotene molecule to retinene occurred through a coupled oxidation with unsaturated lipids, possibly with hemoglobin as a catalyst. Retinene was then removed by the tissues from the oxidizing environment of the blood and reduced through the already established enzyme system to vitamin A. This vitamin A was then released slowly from the tissues into the blood from which it was removed by the liver.

Attempts have also been made to study the utilization of intramuscularly and subcutaneously injected aqueous dispersions of carotene. Tomarelli et al. (177) and Bieri and Sandman (21) administered aqueous dispersions of carotene intramuscularly to rats and found an effective conversion to vitamin A as demonstrated by the resumption of growth in vitamin A deficient animals. Further, Bieri and Sandman (21) showed that approximately 4 - 6 times as much carotene was required parenterally as orally for maximum growth and that aqueous carotene was also well utilized when given subcutaneously.

Working with chicks, Sibbald and Hutcheson (100) found that the crop did not possess the ability to convert B-carotene to vitamin A within a four hour period even though carotene might be absorbed by the crop wall in the presence of bile.

The metabolism of radioactive B-carotene was studied in the dog heart-lung preparation by Olson et al. (140) who showed that less than 0.05 ug vitamin A was formed from 5 to 20 ug B-carotene. It was concluded that the lung was probably not an important organ for conversion of B-carotene into vitamin A.

A possible relationship between vitamin A and red blood cells has been studied recently. Pollard and Bieri (144) indicated that a remarkable property of the hemolyzed blood from young animals was its ability to destroy vitamin A and carotene. Rabbit and rat blood were more active in this respect than the blood of mice, guinea pigs and chicks. The activity resided in the cells and was liberated upon rupture of the cells. The lack of a highly positive correlation between number of reticulocytes and the destructive activity suggested that the system was complex.

The information reviewed above clearly indicated that in most trials an aqueous dispersion of carotene administered parenterally was utilized for the formation of vitamin A. On the contrary, carotene in oil was not well utilized. This is one explanation for some of the conflicting reports concerning parenterally administered carotene as a precursor of vitamin A. The suggestion also was offered that the ability to convert carotene to vitamin A was an attribute of all cells.

Product formed when animals were given radioactive B-carotene.

Since the results obtained in the study of carotene conversion were influenced by physiological variables, radioactive B-carotene has been used to investigate the carotene metabolism to overcome some of the difficulties encountered using unlabeled carotene.

Krause et al. (109) found that the feeding of C^{14} labeled carotene resulted in the deposition of all of the absorbed C^{14} in the carotene-free, non-saponifiable fraction from the total animal. The highest concentration of C^{14} was found in the extrahepato-intestinal tissues and the least amount was recovered in the liver. The uptake of the carotene free, non-saponifiable fraction was higher at the end of 6 hours than 24 hours. In another study Krause and Saunders (111) demonstrated that the feeding of labeled C^{14} carotene resulted in the deposition of approximately 1.5% of absorbed C^{14} into liver vitamin A after 24 hours. They determined vitamin A by the method described by Glover and Redfearn (67) which involved the separation of vitamin A from carotene by column chromatography and measuring extinction at 328 mu. In the total animal, an average of about 15% of the absorbed C^{14} went into fatty acids and 40% into nonsaponifiable material. An average of about 5% of the absorbed C^{14} remained to be accounted for.

Fishwick and Glover (59) studied the metabolism of uniformly C^{14} labeled B-carotene in the rat. The results indicated that the formation of vitamin A resulted from oxidative fission of a terminal double bond in B-carotene, involving at least in part some other system than B-oxidation.

Wilmer and Laughland (187) determined the distribution of C^{14} in the tissues of the rat at various intervals up to 28 hours after administration of an oral dose of randomly labeled C^{14} B-carotene. During early stages of absorption, the activity was high in the intestine and blood. Increased activity was observed in other tissues when the metabolic stages became superimposed upon the absorption process. The adrenal glands exhibited the greatest concentration which was maintained throughout the experimental period of 28 hours. Following the adrenal glands in decreasing order of their activity were hypophysis, liver, intestine, kidneys, lungs, heart, spleen, blood and stomach.

Normal and vitamin A deficient animals were given 25 to 50 ug of C^{14} B-carotene dissolved in oil (182). The radioactive B-carotene could be detected neither in the lymph nor in the liver which, however contained about 1 to 8% of the total activity given. The radioactivity of the liver lipids was twice as high as that of vitamin A isolated therefrom. This could indicate an oxidative degradation of the B-carotene to acid compounds.

Using C^{14} labeled B-carotene, Olson (138, 139) has attempted a more direct study of B-carotene absorption and its conversion to vitamin A by rat intestine <u>in vivo</u>. The formation of vitamin A proceeded well when the C^{14} labeled B-carotene was administered by intraduodenal injection or by a stomach tube. A peak of radioactivity occurred in

the intestinal mucosa at 1 hour while in the liver it plateued at 3 to 5 hours. The separation of B-carotene and vitamin A derivatives were carried out by column chromatography and the identification and quantitative analysis of compounds were carried out by visible and ultraviolet absorption spectra, and the Carr-Price reaction. In the intestinal mucosa, vitamin A ester and carotene accounted for a very large share of the total radioactivity. Much smaller amounts of radioactivity occurred in retinene, vitamin A alcohol and the terminal polar fraction in that order. In the liver, vitamin A ester was found in maximal quantities. It was also found that the upper one-third of the intestine was most active in the vitamin A ester formation and that the middle one-third had little ability to carry out the conversion from B-carotene.

These studies presented a new approach to the problem of carotene to vitamin A conversion. The radioactive study clearly indicated that within a 24-hour period, B-carotene was metabolized extensively and appreciable amounts of radioactivity appeared in the non-saponifiable and acidic fractions as well as in liver vitamin A. Only a small portion was oxidized to CO_2. The finding that C^{14} from labeled B-carotene entered into compounds other than vitamin A supported the suggestion that the carotene was not entirely converted into vitamin A and it might be directed into other metabolic pathways. This finding supported the hypothesis of stepwise breakdown of B-carotene by B-oxidation or otherwise to give one molecule of vitamin A. On the contrary, in other studies, appreciable quantities of other radioactive products, non-polar or acidic, did not accumulate. Since vitamin A was also metabolized in the intestine to a small amount of acidic

products (138), the acidic fraction which was alleged to derive from B-oxidation of B-carotene might well have arisen from further oxidation of vitamin A and not directly from a cleavage reaction.

With further refinements this method has promise of elucidating the mechanism of formation of vitamin A from B-carotene.

In vitro conversion of carotene to vitamin A.

The conversion of carotene to vitamin A in an in vitro system has received only limited emphasis. Attempts have also been made to prepare active tissue that will carry out the reaction in vitro. During the past years claims for in vitro conversion of carotene to vitamin A using tissues have been made (55, 102, 121, 135, 136, 137, 141, 152, 167, 169, 185, 188) while negative results have been reported by others (3, 43, 46, 53, 149, 193).

Olcott and McCann (135) incubated rat liver thoroughly ground with sand, phosphate buffer (p^H 7.45) and ethyl laurate in which carotene was dissolved. They extracted the incubation mixture with ether and observed an absorption band at 325 mu after dissolving the dried sample in $CHCl_3$. It was reported that the agent responsible for transformation appeared to be an enzyme, provisionally called carotenase since it was destroyed by heat.

Pariente and Ralli (141) attempted to prepare carotenase from the livers of normal dogs and of dogs on a vitamin A deficient diet. They succeeded only once out of four trials. A liver extract was made using phosphate buffer at p^H 7.4 saturated with toluene. The extract was filtered and the filtrate was incubated with colloidal carotene for 36 hours at 37°C. They estimated vitamin A in the ether extracts with $SbCl_3$. It was claimed that carotenase was active in a solution of

p^H 7.4 but inactive in solutions of unfavourable acidity or alkalinity and that it was destroyed by cold. They concluded that in solutions of p^H 7.4 carotenase maintained its activity for a considerable period of time if kept at 37°C.

Euler and Klussman (55) obtained evidence for the formation of vitamin A by a positive antimony tricloride reaction when liver tissue from cows was incubated with colloidal carotene.

Some unusual evidence was reported by Wilson et al. (188) who fed carotene to rabbits and dropped pieces of liver in the melted paraffin after sacrificing the animals. These authors claimed that there was a disappearance of carotene and an appearance of vitamin A after autolyzing for 28 days.

Wiese et al. (185) presented evidence that the in vitro transformation of carotene to vitamin A occurred when the small intestine of vitamin A deficient rats were incubated in Ringer-Locke solution for 3 hours at 37°C. In these experiments, the incubated mixture was saponified, extracted and the vitamin A determined by the Carr-Price reaction.

In experiments with calf tissues Stallcup and Herman (107) demonstrated conversion of colloidal carotene into vitamin A using minced liver preparations and isolated intestinal loops after incubation for 3 hours at 37°C. Vitamin A was determined from the ether extracts by the Carr-Price reaction. McGillivray (121) incubated sections of intestines of sheep with carotene in Ringer-Locke solution maintained at 37°C and the formed product identified as vitamin A by colorimetric and spectophotometric methods.

Rosenberg and Sobel (152) estimated vitamin A in extracts of intestinal tissue by difference in absorption at 328 mu before and after ultraviolet irradiation. Subtraction of extinction of the postirradiation spectrum from the extinction of the preirradiation spectrum gave a curve which closely resembled that of pure vitamin A. Employing this method the conversion of carotene to vitamin A in the isolated intestinal loops of rats was demonstrated in vitro by incubating at 45°C for one hour.

A small, but definite amount of conversion of carotene to vitamin A was obtained when preparations of chick duodenum or liver homogenates were incubated with the aqueous suspension of carotene in Tween 60 (136). The two tissues would appear to be active as enzyme sources. The amount of vitamin A found in the liver preparations was actually greater than the duodenal preparations.

Japanese workers (102, 169) made an extensive study in vitro on metabolic pathways of carotene to vitamin A. Suzuki et al. (169) prepared intestinal homogenates from rats by grinding in a mortor with 0.25 M sucrose solution and incubated this mixture with an aqueous dispersion of B-carotene. One compound formed was identified as vitamin A ester using paper chromatography. In another study Koisumi et al. (102) used intestinal homogenates of vitamin B_1 deficient rats and found that B-carotene was convertible to vitamin A ester in the rats receiving thiamine as well as in rats that were slightly thiamine deficient, but complete failure of the conversion was observed in the severely deficient animals. The additive effects of various coenzymes revealed that the addition of FAD + DPN or DPN + cytochrome C contributed to the metabolic conversion of B-carotene to vitamin A, whereas

other combinations of coenzymes gave negative results. It was also demonstrated that FAD + DPN stimulated the *in vitro* formation of vitamin A from B-carotene even in B_1 deficient rat intestinal homogenates.

Olson (137) incubated longitudinally cut sections of washed rat intestine *in vitro* in Krebs-Ringer bicarbonate solution with glycocholate, under 95% O_2 - 5% CO_2 at 38°C for one hour. After one hour, the intestine was washed, homogenized in $CHCl_3$ - CH_3OH mixture. The hexane extract was chromatographed on alumina and vitamin A derivatives determined by ultraviolet absorption spectra and Carr-Price reaction. About 2 - 4 ug vitamin A ester was formed in each section.

In spite of all these positive results, many negative results have also been reported.

Ahmed (3) failed to observe the conversion of carotene to vitamin A by rat liver *in vitro*. Perfusion of an isolated organ also gave negative results.

Euler and Euler (53) using shark liver could not obtain vitamin A from carotene after incubation *in vitro*.

Rea and Drummond (49) were unable to confirm the work of Olcott and McCann (135). They were unable to effect the conversion by incubating carotene with liver tissues from vitamin A deficient rats and cats. Attempts to extract the enzyme carotenase were not successful.

Drummond and McWalter (46) claimed that they were again unsuccessful with liver incubations, even when the conditions were made more physiological by allowing rabbits to absorb carotene into their livers just before they were killed. De and Sundararajan (43) were unable

to demonstrate in the rat any increase in vitamin A after incubating whole intestine with carotene.

Workers (193) had made an extensive study using tissues from the rat, guinea pig, and sheep. He claimed that considerable attention was paid to detail and extreme care was taken at all times to ensure that the results obtained were not limited in any way by poor technique. Despite those precautions, he found that it was not possible to demonstrate conversion of carotene into vitamin A in vitro, either in the perfused intact animal or in tissue slices or homogenates of abdominal wall, intestine, kidney, liver or lung or in isolated perfused organs (intestine, kidney, liver or lung) of the rat or guinea pig or sheep. It was concluded on the basis of the results reported that either the enzyme involved must be extremely sensitive to non-physiological conditions or that the necessary cofactors, perhaps supplied from the circulation, were not present in isolated tissues in sufficient concentrations.

In some of the above reviewed cases, vitamin A was estimated solely by the antimony trichloride reaction without previous chromatography, the validity of which would appear open to question (167, 185). Others estimated vitamin A by the difference in absorption at 325 mu before and after ultraviolet irradiation, the validity of which may be questioned (152). In contrast to these reservations McGillivray (121) identified vitamin A from in vitro trials by colorimetrically and spectrophotometrically. However, in many cases negative results have been reported. Hence it is apparent from this review that results are somewhat conflicting and logical doubt does exist about the alleged in vitro formation of vitamin A.

Effect of nitrate and nitrite on the conversion of carotene to vitamin A.

The effect of nitrate and nitrite on the apparent conversion of carotene to vitamin A have been studied in vivo using various species of animals, but the results were for the most part conflicting.

Smith et al. (166) reported that the rats fed corn silage containing one percent of KNO_3 on a dry matter basis showed a reduced level of vitamin A in the plasma and liver and interpreted this to indicate a reduction in the rats of conversion of carotene to vitamin A.

In one study, Illinois investigators (93) found that dietary nitrate caused a rapid depletion of vitamin A in steers. In another similar study, Illinois workers (165) found that dietary nitrate exerted no significant effect upon liver vitamin A. Plasma vitamin A changed in the animals fed hay, hay + nitrate, silage and silage + nitrate from 30 ug% to 20, 18, 17, and 16 ug%, respectively. Dietary nitrate had no effect upon hemoglobin, methemoglobin, hemocrit nor upon plasma or liver contents of vitamin A in sheep.

Goodrich et al. (69) found that the effect of sodium nitrate on plasma vitamin A levels in sheep was uncertain, but liver vitamin A stores were significantly lowered by feeding of rations containing 3% sodium nitrate.

O'Dell et al. (134) found that dietary nitrate at the level of 0.3% of diet caused a rapid depletion of liver vitamin A in rats. After eight weeks xerophthalmia was noticed in these animals.

Holst et al. (86) added nitrite to sheep rations in the form of equal molar mixtures of sodium and posassium nitrite at the levels of 0.1 to 0.75%. Liver vitamin A was low in those animals fed NO_2.

Emerick and Olson (52) found that feeding of 0.5% $NaNO_2$, but not 3.0% $NaNO_3$ resulted in reduction in the amount of vitamin A stored in liver when vitamin A was administered orally, but not when administered by subcutaneous injection. They suggested the effect might be due to the detrimental action of nitrite on preformed vitamin A within the digestive tract. Both nitrite and nitrate significantly lowered the liver storage of vitamin A with the greatest effect resulting from nitrite feeding. They proposed two possible mechanisms for the effect of nitrite on the liver vitamin A storage. The nitrite ion had an effect on thyroid function which was necessary for conversion of carotene to vitamin A. The nitrate effect was also the result of a lowered absorption of carotene.

Pugh et al. (146) conducted in vitro experiments to study carotene and vitamin A destruction by nitrite. Incubations were performed at 37°C for four hours using aqueous dispersions of B-carotene and KNO_2 having molar ratios ranging from 1 : 0.5 to 1 : 100 and with p^H ranging from 1 to 7. Destruction of carotene was greatest at p^H 1 to 3 followed by an abrupt decrease to lesser destruction at p^H 5 to 7. The amount of B-carotene destroyed increased rapidly as the molar ratio of nitrite to carotene increased.

Weichenthal et al. (184) found that feeding 1% KNO_3 in the diet appeared to have no effect on plasma vitamin A levels.

Hale et al. (75, 76) studied the effect of ration, energy level and nitrate on liver vitamin A depletion in fattening steers fed a low carotene ration. A high TDN (71.3%) ration resulted in a significantly larger vitamin A depletion than did a low TDN (54.3%) ration. The

addition of 1% KNO_3 in the diet reduced liver vitamin A stores, but the reduction was not significant.

The possible interrelationship between nitrate, vitamin A and thyroid gland or iodine has been studied in vivo.

Bloomfield et al. (24) injected 3 and 100 uc of carrier-free I^{131} to rats and sheep, respectively, which were fed a nitrate containing diet for one week. They found that 0.31 and 0.92% dietary nitrate when consumed by rats and sheep, respectively, could affect the normal iodine metabolism of the thyroid gland and recommended that animal rations suspected of containing nitrates be supplemented with adequate amounts of iodine and vitamin A.

Yadav et al. (196) fed groups of rats on four vitamin A deficient rations containing low iodine (0.2 ug/ g), low iodine-nitrate (1.5% of KNO_3), adequate iodine (2 ug/ g) and adequate iodine-nitrate until they were deficient and found that the incidence of xerophthalmia was 45.5, 75.0, 0.00 and 33.0% respectively. The liver vitamin A after a depletion period was highest in the group fed adequate iodine and lowest in the group fed the iodine-nitrate diet. The thyroid weights were increased in the groups fed nitrate.

Bloomfield et al. (25) fed a diet containing 1.5% KNO_3 to sheep 6 days prior to injection of 100 uc I^{131}. The thyroidal uptake of I^{131} was increased in this group. They speculated that dietary nitrate induced an increase in pituitary TSH.

In another study, Bloomfield et al. (26) found that rats maintained in a normal environment showed a decreased thyroidal I^{131} uptake after 7 hours on a 2.5% nitrate supplemented diet but were able to overcome this effect after 2 weeks. The rats could not compensate for

a nitrate load under cold stress. Although the information reviewed above is somewhat conflicting, there appears to be some kind of relationship between vitamin A, dietary nitrate, and thyroid function. Most results indicated that the addition of NO_3^- to the ration or feeding of rations having a high nitrate content had an adverse effect upon the animal's vitamin A status. The decreased storage of vitamin A in the liver and low serum levels of vitamin A from orally administered carotene in the presence of dietary NO_3^- or NO_2^- suggested a detrimental action of NO_3^- or NO_2^- on provitamin A. The conclusion that nitrate ion has an effect on thyroid function and that a normal functioning thyroid gland is necessary for conversion of carotene to vitamin A suggested a possible mechanism for the effect of NO_3 on vitamin A storage.

Importance of bile acids on the conversion of carotene to vitamin A.

There are many complicating factors, both chemical and physiological, which will either enhance or inhibit the formation of vitamin A from carotene *in vivo* as well as *in vitro*. One of the most important natural substances which aid the absorption process is bile. The bile and its salts have high emulsifying properties. This could be important in the utilization of B-carotene inside the intestine.

Drummond and McWalter (47) recognized the importance of bile for the absorption of carotene and showed that carotene could form a complex with desoxycholic acid.

Altschule (5) showed microscopic evidence of epithelial lesions of vitamin A deficiency in infants with severe protracted jaundice due to congenital atresia of the bile ducts in spite of the fact that they were receiving diets adequate in vitamin A. Conclusions were drawn that

the deficiency occurred as a result of a failure to absorb vitamin A from the gastro-intestinal tract due to the absence of bile.

Using vaginal smear as the criterion for the conversion of carotene to vitamin A, Greaves and Schmidt (71) demonstrated that irrespective of the channel used in administering the carotene, little or no vitamin A was formed in the vitamin A deficient icteric rats. In some attempts, carotene could be made effective by the simultaneous administration of sodium desoxycholate or sodium glycodesoxycholate.

An interesting experiment was conducted by Bernhard et al. (15) who showed that the absorption of vitamin A through the thoracic lymph duct was lowest when bile was withdrawn from the bile duct as compared to control rats. The administration of natural bile of rats through the fistula increased the absorption of vitamin A. The bile pigments greatly retarded the oxidation of vitamin A in vitro.

Vahouny et al. (180) studied the comparative effects of various bile acids on the intestinal absorption of cholesterol. Cholanic, lithocholic and desoxycholic acids with 0, 1 and 2-hydroxyl radicals, respectively, produced no increase in lymph cholesterol over control groups. Conjugated bile salts, glycocholate and taurocholate gave comparable and significant elevations in total lymph cholesterol. Cholic acid with two free hydroxyl groups and an unconjugated carboxyl radical was the most effective bile acid in promoting cholesterol absorption from the intestine. It is possible that these results may be applied to the absorption of carotene and vitamin A.

A study on the requirement for bile acids in the intestinal conversion of carotene to vitamin A in vivo and in vitro has been made by Olson (137, 138, 139). The experiments in vivo indicated that ligation

of bile duct prevented vitamin A formation in washed duodenal loops, but that the addition of either rat bile or sodium glycocholate to B-carotene suspensions allowed the conversion to occur. It was also found that cholic acid was somewhat less effective and 5% deoxycholic acid destroyed the mucosa and allowed no vitamin A formation. Under normal conditions the upper two-thirds of small intestint formed more vitamin A than the lower third of the small intestine. The addition of glycocholate to a B-carotene suspension stimulated vitamin A formation in all portions of intestine. In addition, when longitudinally cut sections of washed rat intestine were incubated in vitro with glycocholate, vitamin A ester formation also took place. On the other hand, the formation in vitro of vitamin A ester from Tween solutions of C^{14}-vitamin A alcohol or C^{14}-retinene did not require glycocholate. Bile did not function merely as a general emulsifying agent in this case, but possessed a more specific function which was due mainly to the cholanic acid structure and was enhanced by conjugation (138).

However, Sibbald and Olsen (163) found sodium glycocholate neither improved nor retarded the rate of conversion at a level of 22/100 ml of solution.

It is evident that the conjugated bile salts do not solely act as emulsifying agents, but also function in a more specific manner. It seems likely that bile salts primarily enhance B-carotene absorption leading to vitamin A formation. Further, it appears that specific types of bile salts having 3 hydroxyl groups such as taurocholate and glycocholate were involved in stimulation of vitamin A formation more than other types of bile salts as indicated by in vitro experiments conducted by Olson (137).

The importance of tocopherol on the utilization of carotene and vitamin A.

One of the earliest and most frequent proposals for the mechanism of action of tocopherol has been that it served primarily as an intracellular antioxidant, preventing the oxidation of compounds such as unsaturated fats, vitamin A and ascorbic acid. The tocopherols are now recognized to be the most important natural stabilizing compound for vitamin A and carotene. Synergistic action of tocopherols on vitamin A deposition in the tissues has also been known for some time (131). There was also the suggestion that the tocopherols increased the efficiency of utilization of carotene. In opposition to this suggestion, there was also another idea that small doses of tocopherols had little effect on the utilization of carotene whereas larger doses decreased the storage of vitamin A (91).

Moore (131) was the first to demonstrate the synergistic action of tocopherols on the deposition of vitamin A in the tissues. Rats fed a diet deficient in vitamin E had much lower liver reserves of vitamin A than control animals receiving the same diet supplemented by wheat germ oil concentrate or dl- alpha tocopherol.

The mechanism by which tocopherols potentiated the action of vitamin A was not entirely clear. Davies and Moore (42) found that the reserves of vitamin A were used up much more rapidly in rats restricted to a diet deficient in both vitamins A and E, than in control animals given dl - alpha tocopherol. Prolonged deficiency of vitamin E led to a secondary deficiency of vitamin A as indicated by the disappearance of this vitamin from the liver. They concluded that increased storage of vitamin A accompanying the administration of tocopherol was probably to be ascribed to its antioxidant action.

Sherman (164) demonstrated that carotene administered to vitamin A deficient rats was destroyed in the gastro-intestinal tract by the simultaneous feeding of unsaturated fatty acid esters. The gastro-intestinal destruction of carotene was prevented by feeding alpha tocopherol. It appeared to them that in the absence of alpha tocopherol there was a physiological antagonism between unsaturated fatty acids and carotene which resulted in the inefficient utilization of carotene.

Quackenbush (147) believed that in promoting a biological response to carotene the tocopherol functioned as an antioxidant in the gestro-intestinal tract rather than as a compound regulating some phase of metabolism in the tissues. Daily supplements of 5 ug of carotene in ethyl linolate failed to produce growth in rats deficient in vitamin A but when a distillate from soybean oil was given simultaneously growth resulted. The protective factor in the distillate was apparently tocopherol since an equivalent amount synthetic alpha tocopherol and soybean tocopherol produced a similar response.

The synergistic action of vitamin E on both carotene and vitamin A was confirmed by Guggenheim (74) who found that the daily administration of vitamin E in combination with 100 IU of vitamin A increased the liver storage of vitamin A. Vitamin E increased both utilization of vitamin A and carotene and the fecal excretion of carotene. Apparently vitamin E acted by protecting carotene and vitamin A against oxidation in the intestine.

The sparing and synergistic action of vitamin E were referred to as covitamin E activities by Hickman and co-workers (79, 80, 81).

Natural vitamin E (mixed tocopherols) enhanced the growth promoting power of vitamin A alcohol, vitamin A acetate, and USP reference oil. The vitamin A activity of carotene was markedly influenced by the tocopherol intake of the experimental rats. Approximately 0.5 mg of natural mixed tocopherols was the optimum daily dose with which to demonstrate the sparing action of vitamin E on carotene. Synergism was largely lost when vitamin E and A were administered on alternate days. Furthermore, the "covitamin E" action was lost when the tocopherol was given parenterally, while vitamin A was given orally. The data indicated that sparing action was due to repression of oxidation in and/or near the gastrointestinal tract.

The results obtained by Rao (148) supported the evidence shown by Hickman et al. (79, 80, 81). The wide variations in growth response of rats to 1 ug of B-carotene in ground nut, olive and coconut oils were reduced when pure alpha tocopherol was added to oils low in tocopherol.

The covitamin E action on vitamin A was confirmed by Lemley et al. (113) who introduced another concept. The favorable effect of tocopherol on the storage of vitamin A in the liver of the rat was diminished by extending the supplementing period from three days to three months and by raising the dietary intake of vitamin A. The "covitamin E" activity of tocopherols might have been more pronounced if smaller quantities of vitamin A had been employed. The storage of vitamin A in the liver of rats was increased when tocopherols were given with vitamin A for more than 3 months. The results of these workers were in contrast to the concept of intestinal action of the tocopherol as proposed by Hickman et al. (79).

An interesting observation was made by Tomarelli and Gyorgy (178) who found that rice bran extracts contained a factor which could further support the action of tocopherol by increasing the growth response with small does of carotene. The rice bran extract was effective in that it delayed destruction of tocopherol in a linoleic acid solution.

Although there was considerable evidence that alpha tocopherol increased the storage of liver vitamin A and potentiated the action vitamin A, some workers did not find any beneficial effect of alpha tocopherol (9, 61, 98).

In 1940, Bacharach (9) was unable to demonstrate any difference between the vitamin A content of the livers of rats receiving vitamin E as tocopherol in normal curative or prophylactic doses and those of negative control rats receiving no supplement.

Fraps and Meinke (61) determined the relative values of carotene in foods as measured by storage of vitamin A in livers of rats. These investigators did not find any increase in storage of vitamin A by addition of tocopherol in rats.

Kemmerer et al. (98) noted that alpha tocopherol fed with spinach did not increase the utilization of carotene for liver storage of vitamin A in rats.

Embree (50) listed the reasons for these divergent results. The laboratory animals on fortified diets which contained several percent fresh or hydrogenated vegetable oils already contained more tocopherols than most human diets or the diets of many farm animals. Addition of more vitamin E to such an experimental diet was, for most purposes, superfluous. He was critical of the diet used by Kemmerer et al. (98)

because both test and control diets already contained enough vitamin A to afford optimum utilization of the carotene.

Herbert and Morgan (77) reported that the addition of 0.5 mg alpha tocopherol daily to the diet of partially depleted rats receiving 35 to 129 ug of vitamin A daily for 14 or 28 days produced no significant change in the liver stores of vitamin A. Addition of 0.5 ug alpha tocopherol supplement daily to the diet of similar rats given 24 to 17 ug of carotene in oil daily for 14 or 28 days produced a significant increase in liver vitamin A. Above these levels no effect was found. The vitamin A content of livers was determined by the Carr-Price reaction.

Although there seemed to be considerable evidence in favor of tocopherol increasing the efficiency of utilization of carotene and enhancing its value as a source of vitamin A, there were some reports which indicated that large doses of tocopherols have detrimental effect on vitamin A storage.

Johnson and Baumann (91) found that alpha tocopherol did not interfere with the storage of vitamin A when the vitamin itself was fed, but the stores of vitamin A due to B-carotene were lowered significantly when 5 to 10 mg of alpha tocopherol were fed with carotene. Tocopherol injected intraperitoneally also appeared to interfere somewhat with the utilization of ingested carotene, but tocopherol fed eight hours after the carotene failed to interfere with the storage of vitamin A. In another study, Swick and Baumann (171) found that high doses of alpha tocopherol and alpha tocopheryl acetate diminished the storage of vitamin A in the livers and kidneys of rats fed moderate amounts of B-carotene.

A daily dose of 2 mg of tocopherol significantly diminished the efficiency of utilization of B-carotene (30).

High and co-workers (83) reported that other antioxidents might also possess a variable effect on carotene metabolism. In rats large amounts of tertiary butylhydroquinone and octylhydroquinone decreased the utilization of carotene for tissue deposition of vitamin A, whereas small amounts of octyhydroquinone increased vitamin A deposition. Large amounts of octylhydroquinone were more effective than small amounts in inhibiting the oxidative decomposition of carotene in vitro. Large amounts of vitamin E were likewise effective. These investigators postulated that nonspecific impairment of oxidative processes occurred in and near the alimentary tract when large amounts of these substances were administered with carotene, thus interfering with the enzymatic conversion of carotene to vitamin A.

In further work High et al. (82) found that large amounts (10 mg/day) of alpha tocopheryl acetate were without effect on the utilization of preformed vitamin A and did not interfere with the absorption of carotene in the alimentary tract. On the other hand, alpha tocopheryl acetate protected carotene markedly against oxidative decomposition in vitro. These experiments supported the view that the locus of antioxidants as well as vitamin E was in the intestinal wall or at the site of conversion of carotene to vitamin A, and that the mode of action was either directly or indirectly involved in the enzymatic conversion of carotene to vitamin A.

An interesting observation was made by Dicks et al. (44). Calves partially depleted of their tocopherol and vitamin A stores were fed alpha tocopherol ranging from 1 to 25 mg per 100 pounds body weight per

day and three levels of vitamin A (10, 100 or 1000 ug) for 4 weeks. The tocopherol resulted in an increase in utilization of vitamin A at 1000 ug level, a decrease in utilization at 100 ug level and an inappreciable change at 10 ug level.

Reports also have appeared on the effect of tocopherol on the utilization of intravenously administered carotene. McGillivray and Worker (125) demonstrated that simultaneous intravenous administration of tocopherol and carotene in rats inhibited the conversion of carotene into vitamin A. Even as little as 1 mg alpha tocopheryl acetate decreased the amounts of both vitamin A alcohol and ester forms in the liver. It was possible that the inhibitory effect of tocopherol on the mechanism of conversion was apparent only when tocopherol and carotene were administered simultaneously.

Further investigations by McGillivray and Worker (126) showed that high levels of alpha tocopherol in the blood and tissues inhibited the conversion of carotene to vitamin A. On the basis of results obtained with intravenous injections of tocopherol previously to, simultaneously to and subsequently to intravenous administration of carotene, these authors concluded that carotene underwent a rapid breakdown possibly in the blood to an imtermediate which was then more slowly converted to vitamin A. The diverse information reviewed above appeared to be puzzling. The results of different investigators could have been influenced by the severity of the deficiency of vitamin E to which the animals were exposed. Similar results could not be expected when tocopherol was required to correct a deficiency as when tocopherol was given to reinforce levels which were already adequate in the diet. Also the large doses of tocopherol could inhibit one of the steps from carotene

to vitamin A formation, while in smaller doses tocopherol could enhance the formation of vitamin A from carotene. Small doses of tocopherol could prevent some destructive oxidation of carotene.

Interrelationships between thyroid status and vitamin A.

During the past thirty years a considerable volume of literature has accumulated on the interrelationships between vitamin A and thyroid status. Most observations were from experimental studies although some clinical observations have also been reported. Some evidence indicated that the thyroid gland regulated the conversion of carotene to vitamin A. Claims were made that the thyroid gland influenced the absorption of carotene.

The possible relationships between vitamin A and thyroid were first indicated by clinical studies. Wold (189), in 1932, collected specimens of human liver from 957 cases at autopsy and found that there appeared to be some relationship between certain chronic diseases and low vitamin A status.

Logaras and Drummond (116) studied the influence of thyroid and vitamin A on metabolic rate and found that the thyroxine treatment increased the storage of vitamin A as compared to control pigs. Clinical investigations (130) showed that high reserves of vitamin A were found in exophthalmic goiter, although the small number of cases made the authenticity of these results questionable.

Johnson and Baumann (90) depleted rats of vitamin A and at the same time brought them into either hypo-or hyperthyroid state. These rats were fed vitamin A daily for 15 days. Within the dosage limits employed the ability to store preformed vitamin A was approximately the same in hypo 'or hyperthyroid rats as in normal rats.

Heimer et al. (78) intensively studied the effect of thyroid hormone on the storage of vitamin A and found that vitamin A storage in the liver of rats on a vitamin A free diet was highest in thyroidectomized animals, intermediate in the thyroxine supplemented animals, and lowest in the controls. From this it appeared that the storage was affected in some way by the thyroid hormone. The greater the thyroid activity the lower was the storage of vitamin A.

Morgan and White (133) found that when 1.68 mg of vitamin A was fed to rats for 42 days there was no significant difference in total vitamin A per liver between controls and animals fed dessicated thyroid gland.

Bamji and Sundaresan (11) studied the effect of hypothyroidism and hyperthyroidism on the absorption of retinal (vitamin A aldehyde) from the intestine of rats and on the level of liver vitamin A. The rate of absorption of retinal was faster in hyperthyroid rats than in controls. Hyperthyroid rats were observed to have more vitamin A in livers if analyzed two hours after the last dose of retinal, whereas they stored less vitamin A if analyzed five days after the last dose. This was probably due to greater utilization. Conversely, the hypothyroid rats had consistently higher liver reserves of vitamin A than the controls whether analyzed two hours or five days after the last dose of retinal, probably due to poorer utilization of their reserves of vitamin A.

Reciprocal relationship or antagonism between the metabolism of vitamin A and thyroxine was proposed by many research workers.

Fasold and Peters (57) concluded that hypervitaminosis A in rats could be prevented or curved by thyroxine injection. Conversely a solution of vitamin A in arachis oil or arachis oil alone prevented

the toxic action of thyroxine and permitted the storage in the liver of both carotene and vitamin A but not normal amounts of fat and glycogen.

Sherwood et al. (159) demonstrated that cod liver oil produced a depletion of colloid in the thyroid of rats but had no effect when its vitamin A content was removed by heat and oxidation.

Abelin (1, 2) found that the ingestion of a vitamin A preparation by rats decreased the rise in basal metabolism resulting from thyroid administration. The action of excess doses of thyroid in lowering the growth rate was also partially counteracted by administration of vitamin A. The latter also increased muscle glycogen. The thyroxine and vitamin A were antagonistic because of their opposite influences on lipid and carbohydrate metabolism.

Sure and Buchanan (168) confirmed the results of Abelin (1, 2) and also demonstrated that a diet containing 50% dried skim-milk powder furnished a sufficient amount of the vitamin B-complex, some of which are antithyrogenic in experimental hyperthyroidism. However such a ration containing 10% butterfat and supplemented with four drops of cod liver oil per animal per day did not provide a sufficient amount of vitamin A to counteract the rapid catabolism produced by oral administration of thyroxine.

Greaves and Schmidt (72) reported that the antagonism between these two compounds was confined to the central nervous system. The vitamin A requirement of the rat might be increased by the administration of thyroxine or dessicated thyroid tissue. In thyroidectomized rats the need for vitamin A was decreased.

Schulze and Hundhausen (155) showed that large amounts of vitamin A produced an antithyroid action. Histological examination of the

thyroid glands of the rats showed a somewhat increased activity for the rats deprived of vitamin A. The results also indicated a low thyroid stimulating hormone (TSH) in the pituitary gland of rats receiving large amounts of vitamin A and a high content of TSH in the pituitary glands of the rats deprived of vitamin A.

Coplan and Samson (40) found that the thyroid glands of both male and female rats on vitamin A deficient diet showed hyperplasia of epithelial cells similar to that observed in rats on an iodine deficient diet for a short period. The degeneration of the epithelial cells seemed to be a specific effect of vitamin A deficiency.

Logan (115) found that patients with both cretinism and some form of malignent disease had a I^{131} uptake ranging from 21.2 to 68.2% After treatment with 50,000 IU vitamin A per day for three weeks the uptake was reduced from 14.8 to 32.8%.

The mechanism of antagonism was studied by Belasco and Murlin (14) who reported that vitamin A antagonized the active thyroid principle, possibly by inhibiting thyroid function since vitamin A depressed thyroid tissue respiration in vitro but not that of liver or kidney. The vitamin A was able to take up the available iodine in body tissues by virtue of its double bonds. The organic iodide thus formed could antagonize hyperthyroidism similar to other inorganic or organic iodine compounds exclusive of thyroxine or thyroglobulin. Thus iodinated vitamin A might effect the storage of colloid either by acting on the anterior pituitary gland or on the thyroid gland directly and consequently causes a lower oxygen uptake of the thyroid.

Working on energy metabolism, Sadhu and Brody (153) demonstrated that excess vitamin A depressed metabolic rate and reduced thyroid size of normal, thiouracil treated and thyroxine treated rats.

The effect of thyroid activity on the metabolic requirement for vitamin A was also investigated. Cooper et al. (39) determined the growth response to suboptimal levels of vitamin A in chicks fed thyroprotein or thiouracil. They found that hyperthyroid chicks showed smaller gains than normal at all levels of vitamin A fed (25, 50, 75 and 100 IU per day). The hypothyroid chicks were heaviest at the 25 and 50 IU levels. However hypothyroid chicks did not increase in weight as the levels of vitamin A increased.

According to Blaizot and Benac (23) the average oxygen consumption was 10 to 25% greater in the vitamin A deprived rats than in normal rats. The increase in oxygen consumption was an earlier sign of deficiency than cessation of growth or xerophthalmia. This indicated that thyroxine aggravated the effects of avitaminosis.

Frapes et al. (60) showed in pigs that insufficient (0.0 IU/lb ration) and excessive (6400 IU/lb ration) intakes of vitamin A lowered the rate of secretion of thyroxine. The effect of vitamin A on thyroid secretion was thought to be direct and not a reflection of its effect on growth.

The evidence which was reviewed has indicated that there was antagonism or at least some kind of interaction between vitamin A and thyroid gland activity or thyroxine.

However Baumann and Moore (13) showed that injected thyroxine failed to counteract the hypervitaminosis A in rats receiving excess dietary vitamin A. Animals receiving both thyroxine and excess vitamin

A consumed less food, lost weight more rapidly and died earlier than those receiving either agent alone. The injection of thyroxine alone produced a temporary decrease in food intake followed by a marked increase. They did not find any evidence for a specific antagonism between thyroxine and vitamin A.

Sheets and Struck (158) found that the effect of small or massive doses of vitamin A on the metabolic rate of rats was questionable. Vitamin A did not show any effect in animals fed thyroid powder or in thyroidectomized animals.

A very interesting finding was reported by Hochstadt and Malkiel (84) who found that the inhibitory factor was not the vitamin A and could be separated from the vitamin A. Ether extracts prepared from blood of normal individuals were tested for vitamin A and also for their power to inhibit the protective action of thyroxine in acetonitrile poisoned mice. Little of the latter activity but some vitamin A was found in the serum extracted for a one hour period. After longer extraction periods the extracts contained no vitamin A which was presumably oxidized but had considerable power to inhibit thyroxine.

A review of all the evidence indicates that there is some kind of interrelationship, possibily antagonistic, between thyroid status and vitamin A in animals. It is interesting to note that vitamin A can decrease the metabolic regulatory action of thyroxine. Although this activity forms a strong case for specific antagonism, the degree of specificity of this influence remains undefined. However there was higher reserves of liver vitamin A in hypothyroidism due to poorer utilization of the liver vitamin A reserves whereas in hyperthyroidism

there were lower reserves of liver vitamin A due to efficient utilization of liver vitamin A reserves.

The effect of the thyroid gland on the conversion of carotene to vitamin A.

There seems to be some evidence that the thyroid gland is directly or indirectly concerned in the conversion of carotene to vitamin A. Numerous reports have been appearing on this subject and several of them are in disagreement. As early as 1907 VonNoorden (181) suggested that the thyroid gland might be involved in carotene metabolism. He observed that carotenemia might be associated with certain metabolic disorders.

Fasold and Heidemann (56) made an interesting observation that after thyroidectomy the carotene content of goat milk increased and the vitamin A content was decreased although goat milk normally contained vitamin A but low carotene. This was attributed to inability to transform carotene into vitamin A in the absence of the thyroid gland.

Drill and Truant (45) demonstrated that in rats fed vitamin A free diets supplements of carotene prevented the occular changes characteristic of vitamin A deficiency in control rats but failed to prevent xerophthalmia in similar thyroidectomized rats. This indicated that the carotene was not being utilized in the animals. Daily injections of thyroxine to the animals allowed them to survive, gain weight and to be cured of their xerophthalmia.

Johnson and Baumann (90) found that when carotene was fed to the hyperthyroid animals they accumulated larger stores of vitamin A than normal rats receiving equivalent amounts of carotene. However rats receiving thiourea or thiouracil stored very little vitamin A. The administration of thyroxine neutralized the effects of both thiourea

and thiouracil and increased the ability of animals to convert carotene to vitamin A. It was suggested that altered carotene metabolism associated with thyroid dysfunction was not due to changes in the basal metabolic rate per se, but was brought about by some other physiological action of the thyroid gland.

Kelly and Day (97) fed rats a vitamin A deficient diet plus 0.5% of thiouracil or 0.6% thyroglobulin. All animals were given B-carotene in wheat germ oil as a single dose. The thyroid status affected the conversion of carotene to vitamin A as indicated by the content of vitamin A found in the liver. The investigators proposed that the amount of vitamin A present in the tissues may result from two opposite effects of thiouracil: 1) impairment of carotene conversion to vitamin A and 2) increased retention of vitamin A in the liver once it was deposited there.

Cama et al. (33) confirmed the observation reported by Johnson and Baumann (90) and Kelly and Day (97). In addition these investigators also confirmed the results of Worker (191) that thyroid activity had little influence on the conversion of intravenously administered carotene to vitamin A.

Kowlewski et al. (107, 108) studied the effect of thyroidectomy and of thyroxine on the utilization of carotene administered intravenously. These investigators found that the thyroidectomy increased and prolonged the rise of carotene in plasma and suppressed the increase of plasma vitamin A in normal and thyroid treated dogs. When observations on thyroidectomized dogs were repeated after adequate treatment with thyroxine carotene disappeared from the serum and vitamin A increased temporarily as in normal animals.

Swick et al. (172) noted a very slight increase in liver stores of vitamin A in hyperthyroid animals as compared to controls. Depleted pigs were given either thiouracil or iodinated casein and their subsequent responses to carotene or vitamin A determined. Essentially similar stores of vitamin A were found in the livers and kidneys of the three groups with a suggestion of increased stores in hyperthyroid animals that were free from diarrhea.

Further evidence for an unequivocal relationship between thyroid status and the ability of the organism to convert B-carotene to vitamin A came from the reports of Serif and Brevik (156). Liver store of vitamin A was increased in hyperthyroid rats and decreased in hypothyroid rats as compared to control animals. This data indicated that butyl 4-hydroxy - 3, 5-diiodobenzene (BDH) functioned as an inhibitor of the process whereby thyroxine was degraded to an active hormone presumably triiodothyronine. Thus BDH could inhibit the action of exogenous thyroxine but not the effects of exogenous triiodothyronine. This led to the conclusion that the effective form of thyroid hormone was triiodothyronine and that thyroxine per se possessed no activity on the conversion of carotene to vitamin A.

Further evidence that the thyroid hormone stimulated the formation of vitamin A from carotene was shown by Kaplansky and Balaba (95) using an *in vitro* system. Iodinated casein acted as a catalyst *in vitro* by increasing conversion at p^H 7.3. If the iodinated casein was heated, it lost this property. Lowry and Lowry (117) and McGillivray (120) were unable to confirm this report.

Although most of the above reports have added support to the hypothesis that the thyroid gland influenced the conversion of B-carotene to vitamin A, several other reports have been appeared with opposite results.

Remington et al. (150) reported that oral dosages of carotene were equally as effective as vitamin A in curing xerophthalmia in thyroidectomized rats.

Using growth as the criteria Wiese and his co-workers (186) found that although the extent of maximum growth after vitamin A or carotene feeding was markedly depressed by hypothyroidism, the point of 50% response was unaltered either with the vitamin A or carotene feeding. This data indicated that low levels of carotene and vitamin A were about equally well utilized by hypothyroid rats.

Similarly, Bieri and Schultze (22) could not demonstrate any effect of thiouracil on vitamin A concentration in the serum, liver and kidneys of the rats fed or injected with aqueous despersions of carotene.

Arnrich and Morgan (8) found that administration of small doses of carotene produced greater amounts of vitamin A in the livers of the hypothyroid rats than in the normal controls. Extending the studies to dogs, Arnrich (7) found similar increases in liver vitamin A concentrations in normal and thiouracil treated young mature dogs following the ingestion of carotene. However thiouracil administration caused a rise in concentration of blood vitamin A as compared with the level found in normal controls.

Worker (191) showed that thyroidectomy or hyperthyroidism had no direct effect on the conversion of intravenously administered carotene

to vitamin A as observed by similar blood levels and liver storage of vitamin A in treated groups.

Recently Boguth and Sari (27) observed that the conversion of carotene to vitamin A was somewhat independent of thyroid status. Hypophysectomized and vitamin A depleted rats given 100 ug B-carotene daily recovered from symptoms of deficiency in five days.

Horvat and Merril (87) administered 0.4 mg of 3 5' 3' - triiodo-D-thyronine (D-T_3) daily for up to 21 days to patients with myxedema and found a decreased level of serum carotenoids. Vitamin A levels were not consistently affected. Thyroidectomized rats deprived of vitamin A 5-10 days received 45 ug D-T_3 daily per Kg. body weight and 515 ug of carotene per day by mouth for four days before they were slaughtered. This treatment did not affect storage of vitamin A in liver. About 20% of the carotene was recovered in the feces with or without D-T_3. These results indicated no evidence that D-T_3 enhanced vitamin A formation from B-carotene. In these experiments the experimental period was rather short and previous dietary history may not have been adequately standardized.

There is also considerable evidence to show that the thyroid hormone has a significant role in the intestinal absorption of carotene. The results of some experiments have suggested that the thyroxine increased and thiouracil decreased the efficiency of absorption.

Cama and Goodwin (32) showed that thiouracil inhibited

and desiccated thyroid stimulated the absorption of B-carotene from the intestinal tract in rats. Fecal excretion of carotene was decreased 18% in rats given desiccated thyroid compared to controls. Administration of thiouracil increased excretion an average of 31%. Desiccated thyroid counteracted the inhibition of Thiouracil when administered together with Thiouracil.

Chanda et al (35) reported that digestibility of carotene by cows and goats was markedly increased by thyroxine and reduced by thiouracil injections. Further evidence that the thyroid might influence the absorption of carotene was shown by Chanda and Owen (36). When cows were deprived of carotene for 15 days the milk yields were not affected but the concentration of vitamin A and carotene in the milk decreased. When a carotene containing diet was fed the concentrations of both carotene and vitamin A increased at rates which were enhanced by thyroxine and diminished by Thiouracil. The effects of discontinuing thiouracil were similar to those of giving thyroxine.

These conflicting reports reviewed demonstrate that the thyroid gland either directly or indirectly has some effect on carotene and vitamin A metabolism. But the degree and specificity of this influence was uncertain. The different results obtained by all these investigators were not readily understood. Most probably, the conditions used were not identical. It is very difficult to completely thyroidectomize animals and a small piece of thyroid gland

remaining inside would be sufficient to make the experimental results erroneous. Other possible explanations for obtaining conflicting results may be the medium in which carotene was dispersed since this influences the carotene absorption and different properties of thyroxine and triiodothyronine have different activities in normal animals of different strains and species.

Mechanism of conversion of B-carotene to vitamin A.

The mechanism of vitamin A formation is not very well understood. There are two hypothesis on the mechanism of the conversion of B-carotene to vitamin A; the direct central cleavage hypothesis to yield two molecules of vitamin A (96) and the B-oxidation mechanism to yield one molecule of vitamin A (67).

Most of the studies to date were based on measurements such as growth, liver vitamin A, blood or lymph vitamin A and nutritional balance. It is evident that these results can be affected by several variables such as stability and rate of absorption, transport, turnover, etc. To overcome this variability C^{14} labeled B-carotene has recently been used in an effort to more directly study the mechanism of conversion (138).

Kuhn et al. (112) and Hunter (89) supported the theory of central fission based on biological activity of the isomers of carotene. B-carotene which could theoritically yield two molecules of vitamin A, was twice as effective for growth promotion as alpha and gamma carotenes which

could yield only one molecule of vitamin A. Lutein, zeaxanthia and violaxanthin had no growth promoting activity.

Koehn (101) investigated the relative biological potencies of pure B-carotene, vitamin A alcohol and acetate for the rat under identical dietary conditions in the presence of adequate alpha tocopherol. Similar growth of vitamin A depleted rats was obtained when receiving daily supplements of either 1 ug of B-carotene or vitamin A alcohol. Similar results were obtained by feeding B-carotene and vitamin A acetate at stoichiometrically equivalent levels. The date indicated that B-carotene was quantitatively converted into vitamin A and supported the theory that the conversion of B-carotene into vitamin A in vivo was by central cleavage to form two molecules of vitamin A and not by an oxidative reaction that inactivates one half of the carotene molecule.

Johnson and coworkers (92) showed that B-carotene was about two-thirds as active as vitamin A on a weight for weight basis in the chick as observed from growth responses when fed by dropper with 0.5 mg of alpha tocopherol daily. Evidence supporting the central fission theory also came from the studies of Burns et al. (30).

In 1937, Holmes and Corbet (85) crystallized vitamin A in the form of pale yellow needles from the liver oils of three different species of fish and found that its biological potency for growth was that of B-carotene on a weight for weight basis. Mead et al. (127) confirmed these results by

using a biological assay method. Since the potency of vitamin A alcohol and B-carotene was 3181 and 1670 IU/ mg respectively, vitamin A itself was twice as active as B-carotene on weight for weight basis.

In another nutritional study it was shown by Hume (88) that B-carotene seemed to be no more effective, mole for mole, than vitamin A. The investigator emphasized that if 0.3 ug of vitamin A has the same activity as 0.6 ug of B-carotene then the molecule of B-carotene does not split into two molecules of vitamin A as was long supposed. Further, Zechemister et al. (196) applying resonance theory suggested that the central double bond of a conjugated system will be more stable than a terminal one and hence less susceptible to attack. The theory might be applied to B-carotene.

Von Euler et al. (54) showed that B-apo-8′-carotenal was biologically active which provided support for the terminal oxidation theory.

Glover and Redfearn (67) prepared possible intermediates of B-carotene by stepwise degradation from one end such as B-apo-8′, B-apo-10′, and B-apo-12′-carotenals and administered them to vitamin A deficient rats. All were transformed to vitamin A. These results indicated that the carotenals were oxidized by a type of B-oxidation to form vitamin A aldehyde. These investigators suggested that the provitamins A were first formed into vitamin A aldehyde by stepwise oxidation and was then reduced to vitamin A alcohol.

Fazakerley and Glover (58) found that B-apo-carotenoic acids were biologically active for stimulating growth in vitamin A deficient rats.

Glover (64) an exponent of the stepwise oxidation theory slightly modified the original theory and concluded that there is an oxidative attack at more than one position in the chain. The true nature of the conversion process is far from clear, but the work with synthetic B-apo-carotenoids has eliminated the normal B-oxidation system as originally being responsible. Oxidative enzymes are probably responsible for carotene degradation.

Most of the above studies were conducted with intact animals, and consequently many of the deductions about the absorption and cleavage of B-carotene were based by necessity on observations which were indirectly related to the problem being studied. This was particularly true of biological methods such as measurements based on growth, vitamin A levels in liver, blood or lymph and nutritional balance. All of these were affected by several variables such as relative stability, absorption rate, transport, uptake by tissues, rate of turn over and metabolism etc. Hence it is not surprising that considerable controversy exists regarding the influence of various factors on B-carotene cleavage, and that no definite conclusion can be forthcoming from this method of attack.

Further evidence for stepwise oxidation theory was

provided by Suzuki et al. (169). Studies on metabolic conversion of B-carotene to vitamin A in vitro by rat tissue homogenates revealed that there were two metabolic intermediates, one with an absorption maxima 296 mu in ethanol which was identified as B-ionone, and the second with an absorption maxima at 365 mu in petroleum ether which was identified as a vitamin A aldehyde-protein complex.

The work attempted by Olson (139,140) represented a new approach to the problem of carotene to vitamin A conversion. He used C^{14} labeled B-carotene. Although these experiments were not conclusive, the central cleavage mechanism was favored as the major pathway for B-carotene metabolism in the intestinal mucosa. Appreciable amounts of acidic and polar products which would be expected from stepwise cleavage in the mucosa were not found.

The information reviewed above clearly indicated that the true nature of the conversion process is still far from clear. Glover and his colleagues (64,142) found that B-apo-carotenals and B-apo-carotenoic acids, which possessed but one B-ionone ring, were biologically active in stimulating the growth of deficient rats and that radioactive acidic products as well as vitamin A were present 24 hr after radioactive B-carotene was fed to rats. These authors favored the B-oxidation theory for vitamin A formation. Olson (138) who favored central fission theory showed that appreciable quantities of acidic or non polar

products did not accumulate in his studies with rats. The possibility exists that the acidic fractions which appeared during B-carotene cleavage might well arise from further oxidation of vitamin A and not from the direct cleavage reaction since vitamin A was also metabolized in the intestine to form a small amount of acidic products. The two hypotheses are somewhat contradictory to each other, and the present information is not adequate enough to delineate the pathway for vitamin A formation.

EXPERIMENTAL PROCEDURE

Animals, diets and treatments

Dairy cattle, sheep, rabbits, rats, swine and chicks were used in these experiments. Tissues were obtained from cattle, rabbits, rats, chickens and pigs fed experimental diets and from cattle, sheep and rabbits fed normal diets.

All types of dairy cattle used in the experiments. The calves fed experimental diets were reared to 7 days of age under a normal situation. At 7 days of age a mixture of 0.7 lb dried skim milk plus 0.6 lb dried whey reconstituted with water per day per 100 lb of body weight was fed. When the calves attained two seeks of age, they were given free access to carotene dificient diet which was made up of dried beet pulp, soybean flakes, soybean meal, oats and barley, mineral mixture and vitamin D. The substitute milk feeding was discontinued when calves were two to three months of age. Hay was never fed. All eight calves grew at a normal rate and care was taken to prevent drastic depletion of their vitamin A stores. Tissues from these calves were used to study carotene conversion. Some of these calves and other normal calves were fed $NaNO_3$ at the rate of 1% of diet (experiment 11) three weeks prior to slaughtering.

In the nitrite studies (experiment 6), one Jersey and one Holstein yearling heifer were each fed nitrite and one

Jersey heifer was kept as control. All three received high concentrate diet low in carotene and KNO_2 was added to the diet of the two heifers in gradually increasing levels to reach the rate of 13 gm per 100 lb body weight. These animals were depleted of the vitamin A reserves to the extent that they had slight papilledema.

Calves used in the experiments to study interrelationships between thyroid status and nitrate on carotene conversion were divided into control, hyperthyroid and hypothyroid groups with two in each group. All calves received a normal calf ration of hay, grain and milk throughout the experimental period. In addition the hyper - and hypothyroid groups received by capsule 1 g of thyroprotein and 10 g of thiouracil daily, respectively (Experiments: 22, 23, 24, 25, 26 and 27).

Other cattle used in these studies received normal rations and were considered to be non-depleted.

Sheep used in these experiments were maintained on various barn dried hays as the only feed for several months prior to slaughtering and carotene intake was assumed to be normal (Experiments 13, 14, and 43).

Rabbits of the Dutch Belted breed were generally maintained on a normal diet consisting of commercial rabbit pellets fed ad libitum. However, in experiment 9 they were transferred from this diet to alfalfa hay and then to a low carotene diet consisting of spelt plus trace mineralized salt and calcium phosphate. After some time on this diet

the rabbits were divided into three dietary groups; (1) control group (spelt), (2) control diet plus 2% NO_3^-, and (3) control diet plus 0.3% NO_3^-. At the same time two extra rabbits were fed the normal diet.

Three groups of three rabbits each were used in experiment 12. Group one was maintained on normal diet consisting of commercial rabbit pellets, group two received this normal diet plus 2% NO_3^- and group three was fed normal diet plus 0.5% NO_3^-. The NO_3^- used was in the form of $NaNO_3^-$.

Some of the other rabbits used in this study were depleted of their vitamin A reserves by feeding rolled oats, mineralized salt and calcium phosphate (Experiments: 36, 37, 57, 61 and 63).

Rabbits used to study interrelationship between thyroid status and nitrate on carotene conversion (Experiments: 29, 30, 31 and 32) were reared on the commercial rabbit pellet diet. They were divided into four groups of three each. They were designated as (1) control, (2) hypothyroid, (3) hyperthyroid and (4) thyroidectomized. The control and thyroidectomized groups were fed the commercial rabbit pellets while the hyper- and hypothyroid groups received this diet plus .03% thyroprotein or 0.2% thiouracil, respectively.

Rats used in these experiments were cross breds obtained from the Animal Husbandry Department of Michigan State University. Non-depleted rats of both sexes were used for experiment 28 and vitamin A depleted rats of both sexes were

used for experiment 34. The dams of the latter rats used were maintained on a normal diet. After littering the pups received dams' milk until weaning and when ready to eat solid food their diet was changed to a vitamin A deficient diet which contained the following composition:

Ground milo	68.9%
Soybean oilmeal, solvent (50% C.P.)	28.0%
Dicalcium phosphate	1.8%
Calcium carbonate	0.6%
B-vitamins, Dawes Forbee (2-4-9-90)	0.1%
Dawes vitamin B_{12} supplement (6 mg/lb)	0.2%
Trace mineralized salt	0.4%
Irradiated yeast 9F(9000IU of vitamin D_2/gm)	5.0%

The B-vitamin, Dawes Forbee (2-4-9-90) contained the following:

Riboflavin	2.00 g
Niacin	9.00 g
Choline chloride	90.00 g

Soybean meal added to make up to one pound.

Under these conditions there was little storage of vitamin A in the liver. The intestine was completely devoid of vitamin A.

Berkshire young pigs (Experiments 40, 41) were of both sexes and were obtained from the piggery of the Animan Husbandry Department, Michigan State University. They were reared normally until six weeks of age when they were then

kept on vitamin A deficient diet. At the time of slaughtering they were eight weeks old. The vitamin A deficient diet had the following composition:

Ground milo	78.338%
Soybean oil meal (50% C.P.)	19.000%
Dicalcium phosphate	0.700%
Lime stone	1.100%
Trace mineralized salt high in zinc	0.500%
B-vitamins, Dawes Forbee (2-4-9-90)	0.100%
Irradiated yeast 9F(9000 IU vitamin D_2/g)	2.000%
Dawes B_{12} supplement (6 mg/lb)	0.150%
Antibiotic, protrap	0.100%
Zinc oxide	0.012%

Chicks (Experiment 35) were cockerels obained from the Poultry Department, Michigan State University. The day old Cobb's strain White Rock Chicks were obtained from hens fed the usual breeder mash and had a normal carryover of vitamin A from the dam. The birds were then fed the following diet which was adequate in all known nutrients except vitamin A:

Milo	60.0%
Soybean oil meal (44% C.P.)	34.0%
Lime stone	01.5%
Salt (Iodized salt + 10 mg manganese sulphate)	0.5%
Vitamin premix	2.0%

The vitamin premix contained the following:

Riboflavin	200.0 mg
DL - Calcium pantothenate	500.0 mg
Niacin	1250.0 mg
Choline chloride	2000.0 mg
vitamin B_{12}	0.5 mg
Procaine pencillin	200.0 mg
Vitamin D_3	40,000 IU
Soybean meal	2.0 lb

The experimental diet was fed ad libitum along with fresh tap water for four weeks.

Reagents and solutions

All organic solvents were redistilled before using. ACS grade petroleum ether (bp. 30-60) and acetone were distilled over KOH pellets with the first and last 10% discarded. Chloroform was purified by distillation with the first and last portions discarded as above. Ethyl alcohol, 95%, used in these experiments was aldehyde free. Care was taken to use only pure anhydrous granular Na_2SO_4 which does not absorb vitamin A. Ethyl ether used here met all ACS specifications for purity. Benzene was redistilled to make it moisture free. Other chemicals such as KOH, $SbCl_3$, etc., were reagent grade commercial products.

Deactivated Alumina

Alumina for chromatographic purposes was Merck's reagent grade chromatographic AI_2O_3 (No. 71707). Deactivated alumina was prepared according to the method of Olson (138)

with modification. One hundred g of Al_2O_3 was shaken with 6 ml of water in 500 ml of petroleum ether for 3 hr at room temperature. The material was filtered with suction and the alumina was spread on a filter paper and dried by gentle raking with a glass rod for 15 - 30 min. This was immediately stored in a tightly closed amber colored bottle. Care was taken not to over dry the material since this made it too active.

Eluting solutions.

The eluting solutions used in the chromatographic separation of compounds formed after incubating with B-carotene were as follows:

1. Petroleum ether,
2. 3% acetone in petroleum ether, and
3. 8% acetone in petroleum ether.

Antimony trichloride (Carr-Price) reagent.

The $SbCl_3$ reagent was made by the method described by Parrish (142) with slight modification. Chloroform was added to 100 gm of reagent grade $SbCl_3$ to make 500 ml. To this 15 ml of acetic anhydride was added, the mixture refluxed for one-half hour, cooled, filtered and stored in a tightly stoppered amber colored bottle. The solution had to be clear and free from cloudiness.

Solution for removing antimony trichloride from cuvettes.

The cuvettes were washed first with 40% Hcl, then with detergent solution, distilled water and finally with alcohol.

Vitamin A standard solution.

Vitamin A acetate dissolved in cottonseed oil in a gelatin capsule with a potency of 30 mg vitamin A g/oil and also pure vitamin A alcohol obtained from Hoffman-Roche Co., Inc. were used to make a standard curve after dissolving in $CHCl_3$.

Carotene standard solution.

Pure 100% B-carotene was dissolved in petroleum ether and used to prepare the standard curve.

Carotene dispersion for incubations.

The B-carotene used in the incubation experiments was 100% pure purchased from Eastman Kodak Co. A suspension of carotene was prepared by adding a weighed amount of B-carotene to 50 ml of distilled water, shaken well and the agitating by passing nitrogen into it; then 5 ml of Tween 80 was added and the mixture was shaken vigorously to make a uniform suspension. Five grams of sodium glycocholate was dissolved in the suspension by stirring with a glass rod. Then the suspension was diluted to 100 ml with distilled water. All the operations were carried out in a closed room where there was no chance of direct light entering. Carotene suspensions were always made immediately before use and any remaining suspension discarded.

Physiological buffers.

All physiological buffers used in these experiments were made in the conventional manner. Krebs-Ringer solution was prepared as described by Umbreit et al. (179). Phosphate

buffer, Tris buffer and veronal buffer were made as described by Gomori (68). To simplyfy the preparation and handling of the solutions, it was found convenient to make up the concentrated stock solutions of all ingredients which were stored at 5° C. When needed the ingredients were mixed and diluted to make up the buffer at the required P^H and used immediately. The 0.25 M sucrose solution and 0.9% normal saline solution were made the evening before use and stored at 5° C.

Hormones.

I-and D-thyroxine, and I- and D-triiodothyronene were donated by Smith Kline & French Labs; 3 5′ 3′- triiodothyronine was donated by Warner-Lambert Research Institute. All the solutions of required concentration were made just before use or on the previous night and stored at 5° C.

Procedure.

All experiments conducted and their main purposes are shown in Appendix table 1.

In vitro experiments using tissue homogenates.

Experimental animals used in this study were fasted overnight before slaughtering. Thus, when slaughtered less solid material was present in the small intestine and washing of lumen was facilitated. Some tissues were also obtained from various animals at the slaughter house and previous treatment of these animals was not always ascertained. In all cases the animals were thoroughly bled out, the abdominal cavity was opened immediately and the duodenum and

the organs were removed as quickly as possible. The duodenum was washed and completely flushed once with cold 0.9% physiological saline solution. The same procedure was followed when other portions of the intestinal tract were used. Flushing out with saline solution removed most of the intestinal contents. When the other organs were used they were removed immediately and rinsed in cold 0.9% physiological saline solution. There was lot of fat and fascia adhering to the outer wall of intestines from calves, heifers, sheep and pigs and these materials were separated from the tissue with a sissors. The lumen of the duodenum (or intestine) was opened by splitting it lengthwise before they were cut into small pieces to facilitate homogenization. Other organs were cut into small pieces prior to homogenization in a waring blender with freshly prepared cold 0.25 M sucrose solution. A 2:1 ratio of tissue weight to solution volume was used except in unusual cases. All the above operations were carried out as rapidly as possible. In our experience, speed of operation was an essential factor. Aliquots of homogenate each weighing 8 to 12 g were placed in low actinic 250 ml flasks, containing 2 ml of 0.25 M sucrose solution and in some trials 2 ml of various experimental chemical solutions.

When the other organs were used, they were homogenized in the same manner and similarly used. The samples were then incubated for three hours at 38^{o} C in an incubator. Incubation time were varied from 5 min to 4 hours to study

the influence of incubation times on vitamin A formation and carotene disappearance (Experiment 44, 45, 46). All the procedures were conducted in the dark or in dim light.

Incubation of different portions of the duodenum

In experiments 17 and 18, calf duodenum was split length wise after rinsing and flushing with 0.9% saline and one portion homogenized. The mucosa was separated from the muscular layer for the other half and these two portions were homogenized separately. These three portions were then incubated with a carotene dispersion as described earlier.

Incubation of heated duodenal homogenates

In experiment 11, the flasks containing calf duodenal homogenates and 2 ml of 0.25 M sucrose were placed in boiling water for 1, 2 and 4 min. Internal temperatures were not observed. A similar procedure was followed using sheep duodenal homogenates in experiment 13, and steer duodenal homogenates in experiment 56. However, in the last two experiments the flasks were removed from the bath at 1, 2 and 4 min after the temperature of the contents in the flasks reached 90°C. Then the heated homogenates were cooled to 20°C and incubated with the carotene dispersion as usual.

Incubation of blood and its components with or without duodenal homogenates

Blood from heifers or steers was drawn from the jugular vein either into test tubes containing anticoagulant such as sodium citrate or oxalate or into test tubes containing no anticoagulant. From this serum, plasma and sedimented

cells were separated. Hemolyzed whole blood was made by vigorously shaking whole blood with distilled water for 1 min. An aqueous dispersion of carotene was incubated for 3 hr with 2 ml each of whole blood, hemolyzed whole blood, sedemented cells, plasma and serum in the low actinic flasks at 38°C as usual. In certain experiments tissue homogenates were incubated with carotene dispersion and 2 ml each of the above blood components separately.

In some experiments (No. 57, 58, 59) the sedimented portion was mixed with equal volume of distilled water, centrifuged at 27,000XG and the sediment and supernatant of each tube ranging in volume from 0.25 to 2 ml were incubated separately with the carotene dispersion and the tissue homogenates. In other experiments the sediment was further diluted with an equal volume of distilled water, centrifuged at 27,000 XG and the sediment and supernatent of each ranging in volume from 1 to 2 ml were incubated as above.

Incubation of duodenal homogenates with different levels of various chemicals

Various solutions were prepared just before use or made the previous evening and stored at 5°C for use the next day. The different levels of various ions and compounds used in these experiments are given in Table 1 and will be evident in the section on Results.

Incubation of duodenal homogenates using different buffers

This experiment was designed to compare a 0.9% NaCl solution, a phosphate buffer at P^H 7.4, a Krebs-Ringer

solution, a Tris buffer at P^H 7.2, a veronal buffer at P^H 7.4 and a 0.25 M sucrose solution as the incubation media. After flushing the duodenum with 0.9% NaCl solution, the tissue was homogenized and incubated with each of the buffers separately as previously described for 0.25 M sucrose.

Separation of activity by differential centrifugation of homogenates

An attempt was made to concentrate the activity from the homogenates of liver and duodenum of depleted rabbits by differential centrifugation. The tissue under investigation was homogenized with cold 0.25 M sucrose solution. A 1:1.5 ratio of tissue weight to solution volume was used. The homogenate was transferred into three centrifuging tubes in equal portions and centrifuged for 15 min at 5°C at 5000 XG, 10,000 XG, and 25,000 XG, respectively. The supernatant fluid was separated carefully from the sediment and these two fractions were used as enzyme sources. Then the incubation was carried out using either 1 to 4 ml of supernatant fluid or 1 to 4 g sediment, 2 ml 0.25 M sucrose and 2 ml aqueous dispersion of carotene at 38°C for three hours as usual. The total volume of incubation mixture was always kept constant for each degree of centrifugation.

Incubation of isolated intestinal loops

In this study the incubation of isolated intestinal loops obtained from rabbits was carried out *in vitro* to investigate the effect of the adding nitrate solution directly into the loop on the formation of vitamin A from carotene.

TABLE 1

Incubation of duodenal homogenates with different levels of various chemicals

Experiment no.	Compounds or ions used	Amount added/ g. tissue
3-6, 8-15, 20	NO_3^-	0.2 to 1006 u moles
3-5	NO_2^-	54 to 536 "
10,11,21,25-27	$SO_4^=$, $CO_3^=$, NaCl	50 to 500 "
22-27, 38, 39	L-Thyroxine, L-Triiodothyronine	0.00001 to 1.0 ug
42	D-Thyroxine, L-Triiodothyronine	0.1 "
25-27	Fresh thyroid gland	1 g
25, 27	Fresh adrenal gland	1 g
27	Thiouracil	1 to 10 mg
42	3 5'3'-LTriiodothyronine	10 ug
55, 56	DL-Alanine, DL-Proline, L-Tryptophan	2×10^{-2} M*
54	p-Chloromercuric benzoate	10^{-5} to 10^{-3} M*
24, 54	Iodoacetate, KCN	10^{-5} to 10^{-3} M*
24, 54	Urea	4 to 8 M*
54, 55	p-Dinitrophenol	10^{-5} to 10^{-3} M*
54	Antimycin, Hydroxylamine	10^{-5} to 10^{-3} M*

* Final concentration in the flask

The rabbits were killed and the abdominal cavity was opened immediately. The small intestines to be used were quickly removed and placed in the cold 0.9% NaCl solution. The contents of small intestine were flushed once with cold 0.9% NaCl solution. The intestinal portion to be used was weighed rapidly on a balance and one end of it was tied off. The 1 ml of aqueous carotene dispersion and 1 ml of 0.25 M sucrose solution were injected into the lumen and the other end tied. These loops were placed in low actinic flasks containing 0.25 M sucrose solution and incubated for 3 hr at 38°C. The control loops not containing carotene suspension were incubated simultaneously. When effect of nitrate was studied 3 sections of equal length were made from the same intestine by ligating at 4 places. The following combinations were injected into the three loops:

Loop1- - 1 ml of 0.25 M sucrose only,

Loop2- - 1 ml of 0.25 M sucrose plus 1 ml aqueous carotene dispersion,

Loop3- - the above combination as in loop 2 plus 0.5 ml NO_3^- solution.

The intestinal sections were then placed in low actinic flasks containing about 50 ml of 0.25 M sucrose solution and incubated at 38°C for 3 hr. After incubation, the sections were removed from the flasks and loops were separated by cutting with scissors. The sucrose solution in the flask was discarded. The loops with contents were then homogenized

with 20 ml of 1 N KOH in alcohol and saponification was carried out at 60°C for 20 min. The usual procedure of extraction and determination of carotene and vitamin A was performed as described on page 70.

In vivo conversion of B-carotene to vitamin A using intestinal loops

The experimental procedure followed was essentially that of Olson (138) with some modifications. The rabbits were fasted overnight before the operation. Prior to performing laparotomy the hair on the abdominal region at the site of operation was clipped with scissors or with clippers. The animals were anesthetized by giving subcutaneous injection of 1.6 g of urethane per Kg body weight dissolved in 0.85% saline solution. The laparotomy was performed by a ventral midline incision of about 2 in. The blunt end of a forceps was used to expose the small intestine and three loops were formed by ligating near the pyloric end and distally at three more places. The three loops thus formed were approximately of equal length. Double ligatures were made between the segments used. Injections of 1 ml of 0.25 M sucrose and 0.5 ml of the carotene dispersion were made into the first loop and the above combination plus 0.5 ml of nitrate were made into the third loop. Sucrose alone was injected into second loop which served as control to estimate the endogenous carotene and vitamin A. After injection the small intestine was returned to its original position within the abdominal cavity. The muscular layer

was sutured with silk thread and the incision on the skin was closed with small wound clips. The animals were then returned to their respective cages. One and one-half hours later the rabbits were slaughtered and the intestinal loops were removed and externally rinsed in 0.9% NaCl solution. The three loops with contents were then homogenized separately with 20 ml of 1 N KOH in 95% alcohol and saponified at 60°C for 20 min. Following this, the conventional procedure of extraction and determination of carotene and vitamin A was carried out as described below.

Saponification and extraction

After incubation the reaction was stopped by adding 20 ml of 1 N KOH in 95% ethanol and placing the flask in an oven at 60 to 65°C for 20 min. This mixture was cooled to room temperature and extracted once very thoroughly with a 50 ml of petroleum ether by intermittent shaking during a period of 30 min. From this stage on the operation was carried out in a cold room. The ether layer was decanted into a 150 ml beaker and washed with 10 - 20 ml of distilled water to free it from alkali. The ether layer was then transferred into an Erlemeyer flask containing 5 g of anhydrous Na_2SO_4 and agitated gently to remove traces of water. The mixture was usually kept for a short time in a dark cold room before aliquots were taken for carotene and vitamin A quantitation and characterization. With each incubation trial a sample of carotene in duplicate without added tissue was carried through the entire procedure except

that the sample was kept at room temperature. A sample of homogenate without added carotene was also carried through the entire procedure. These values were used as the amount of carotene added and as the amount of endogenous carotene and vitamin A present in the tissue. Endogenous levels of vitamin A and carotene were determined by the method of Davies (41) with appropriate modifications.

Estimation of carotene

A standard carotene curve was made using serial dilutions of B-carotene in petroleum ether and immediately measuring absorbancy at 440 mu with the Beckman model B spectrophotometer. This same aliquot was then evaporated under nitrogen and used to determine the amount of antimony trichloride reaction product due to carotene as described below. The procedure was performed on every control sample of carotene.

Estimation of vitamin A

Vitamin A was estimated by the Carr-Price reaction using two different standard vitamin A preparations. With all experimental samples the same aliquot used to determine carotene was used to estimate vitamin A. After reading absorbancy at 440 mu the solution was evaporated under a stream of nitrogen keeping the test tube warm. The tube was cooled and 0.6 ml of $CHCl_3$ added to the residue. Two ml of $SbCl_3$ reagent was added quickly and the absorbancy was read at 620 mu in a Beckman model B spectrophotometer

within 8 to 10 sec after adding the $SbCl_3$ reagent. Under these conditions reproducibility was good and the color was measured very near its peak intensity. The clear blue color produced was without turbidity and faded very rapidly. This is typical of vitamin A in the Carr-Price reaction. Carotene produced a blue color that faded more slowly than vitamin A. The oxidation products of carotene have been reported to produce a nonfading grey to blue color which is not characteristic of vitamin A in the Carr-Price reaction (193). Vitamin A values obtained by the Carr-Price reaction were always corrected for the carotene present.

Chromatographic separation of carotene and vitamin A derivatives

Some information indicates that the Carr-Price reaction is relatively non-specific for vitamin A and certain carotenoid oxidation products are known to have a much greater specific $SbCl_3$ - 620 mu absorption than carotene (132). This makes it desirable to characterize the individual compounds in the extract of the incubated media after chromatographic separation. Many methods were available for the separation of carotene and vitamin A derivatives. Chromatographic separation of the lipids in the ether extract was performed using the procedure outlined by Olson (138) with some modifications.

The chromatographic column was prepared in a 10 x 300 mm chromatogram tube by pouring a suspension of 10 g of

deactivated alumina in petroleum ether into the tube. It was packed by gravity with slight tapping of the tube. It was desirable to form the column with one pouring to avoid air bubbles. The petroleum ether was allowed to run out and the whole column was again washed with 10 ml of petroleum ether.

Meanwhile, the lipid extract was evaporated to a small volume in the oven at 50 - 60°C until a viscous oily residue was formed. At this phase it was removed from the oven and final evaporation was carried out under nitrogen. Finally it was diluted to 1 ml with petroleum ether.

The concentrated ether extract was added to the column just before the top of the column ran dry. Five ml of petroleum ether was then slowly added. When the solvent had just about all drained into the column the eluting solutions were added. Successive 10 ml eluate fractions were collected into clean test tubes. B-carotene was eluted with 80 ml petroleum ether; vitamin A ester with an additional 70 ml of petroleum ether; vitamin A aldehyde with 60 ml of 3% acetone in petroleum ether and vitamin A alcohol with 80 ml of 8% acetone in petroleum ether. No suction was applied to the columns at any stage and the eluents were allowed to percolate slowly through it. The column was examined regularly with the ultraviolet lamp when tissue extracts were chromatographed to observe the flourescence of the vitamin A alcohol band. Complete elution of

vitamin A alcohol was indicated when the last ml of eluate collected showed no vitamin A florescence. The carotene eluate was colored and all others were colorless. Immediately after the fractions were collected all the tubes were stored at 5°C for further identification and quantitative analysis.

The identification and quantitative analysis of products from the column fraction were performed in several ways as outlined in Fig. 1 and below.

Carr-Price Reaction

The Carr-Price reaction was employed on all 10 ml fractions collected in elution tubes in the same order as collected. The fraction in each tube was evaporated under nitrogen in the dark and the residue immediately dissolved in 0.6 ml of $CHCl_3$. The Carr-Price reaction is somewhat non-specific on any crude extract but when performed on chromatographed products where carotene and vitamin A derivatives have been separated this reaction can be used to quantitatively and qualitatively indicate the presence of the above products.

Absorption spectrum

Another method employed to identify and to quantitate the carotene and vitamin A derivatives was by pooling the carotene fractions, the vitamin A ester fractions, the vitamin A aldehyde fractions and the vitamin A alcohol fractions separated on the column and measuring the ultra-

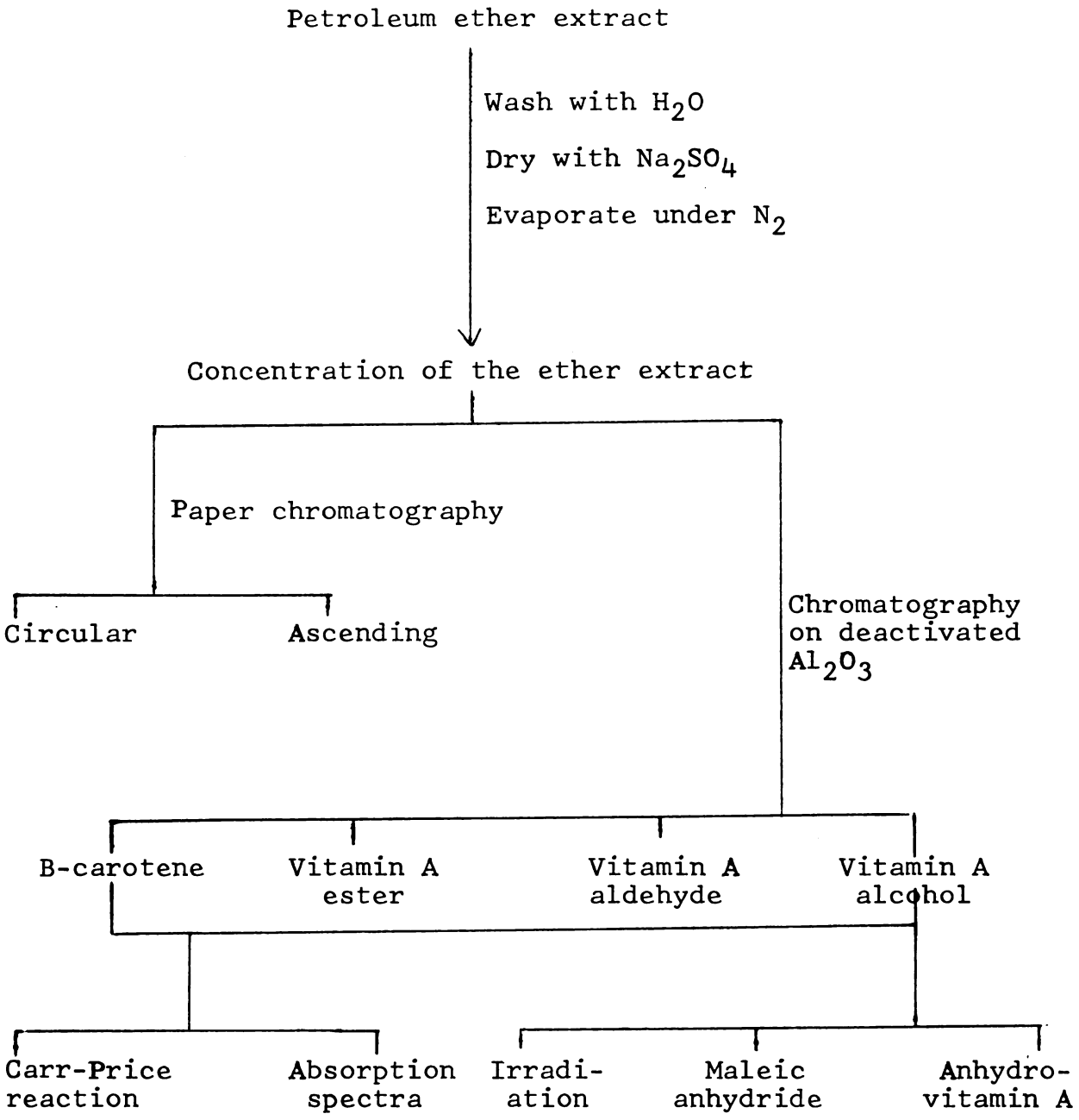


Figure 1

A flow sheet of methods for the isolation and identification of vitamin A derivatives from extracts of incubations with B-carotene suspension and intestinal homogenates in vitro.

violet absorption spectra on one aliquot and the Carr-Price reaction on another aliquot. The split pooled aliquots of carotene and vitamin A derivatives were evaporated under nitrogen and diluted to 3-4 ml with petroleum ether for measurement of absorption spectra. Simultaneously, known concentrations of standard vitamin A alcohol were similarly prepared.

Ultraviolet absorption spectra on appropriate samples were measured using a Beckman model DU Spectrophotometer and an automatic recording Beckman Model DK-2 spectrophotometer. The absorption maxima of B-carotene, vitamin A ester, vitamin A aldehyde and vitamin A alcohol in petroleum ether have been reported to be at 450 mu, 324 mu, 365 mu, respectively (31).

Irradiation of vitamin A alcohol

Modification of the destructive irradiation technique of Bessey et al (16) was used on an eluted aliquot of vitamin A alcohol and on standard vitamin A alcohol of a known concentration. Five ml of the pooled vitamin A alcohol sample and 5 ml of standard vitamin A alcohol were taken in separate clean test tubes. The absorption spectra were measured on both samples. The solutions were placed in test tubes and exposed to an ultraviolet light source for 36 hr in a dark room. One sample from a tissue extract and one pure vitamin A alcohol sample were also irradiated. A green fluorescence was seen from both solutions during the

early period of irradiation. After irradiation they were remade to the 5 ml mark and the absorption spectrum again determined. This technique was used on two different samples at two different times.

Absorption spectrum of blue color produced by Carr-Price reaction

Further characterization of vitamin A alcohol fraction was carried out by measuring the absorption spectra of the blue color produced by the Carr-Price reaction. An aliquot of the eluate using 8% acetone in petroleum ether was evaporated under nitrogen and the residue was immediately dissolved in 1 ml $CHCl_3$. The direct absorption spectra was measured in the Beckmann DK-2 spectrophotometer using this $CHCl_3$ solution and 2 ml of Carr-Price reagent in the region of 500-800 mu. The complete absorption curve was rapidly recorded so that 620 mu peak was reached soon after the time of maximum color development. The absorption spectra of the Carr-Price reaction product was measured on the unchromatographed original ether extract from the incubation mixture and on the standard vitamin A alcohol.

Maleic anhydride reaction method

Further characterization of vitamin A alcohol fraction was carried out by the maleic anhydride reaction method slightly modified from that outlined by Robeson and Baxter (151). The principle of this procedure was based on the fact that maleic anhydride reacts with the conjugated double

bonds in vitamin A to form a product which gives no blue color with $SbCl_3$. The speed of this reaction varies among the several isomers. An aliquot of pooled vitamin A alcohol fractions was evaporated under nitrogen the residue immediately dissolved in 5 ml of benzene and transferred into a 10 ml volumetric flask. The flask was filled to the mark with 10% maleic anhydride in benzene. The mixture was shaken well and stored in the dark at 20°C. The Carr-Price reaction was performed on a 0.6 ml aliquot at 4, 8, 12 and 16 hr after addition of maleic anhydride. A sample of the same solution which had not been treated with maleic anhydride and a sample of 10% maleic anhydride in benzene alone were also analyzed by the same times. From this, the percentage of initial vitamin A potency was calculated. The recovery value "R" was obtained by determining the content of vitamin A after a predetermined developing time in a solution with and without the addition of maleic anhydride (151). At the end of 48 hours the maleic anhydride treated and untreated samples were evaporated under nitrogen and dissolved in 1 ml of $CHCl_3$. The absorption spectra produced by the Carr-Price reagent with the maleic anhydride treated sample and with the untreated sample were determined using a Beckmann model DK-2 spectrophotometer.

Identification of vitamin A by conversion to anhydrovitamin A

Embree (51) has pointed out that vitamin A treated with dry alcoholic HCl formed anhydrovitamin A; a compound which

had characteristic absorption bands at 350, 368, and 389 mu and produced a blue color with $SbCl_3$. The formation of anhydrovitamin A is less subject to interference and misinterpretation than are other vitamin A derivatives. The method of Budowski and Bondi (29) was used to form anhydrovitamin A from vitamin A alcohol.

An aliquot of chromatographed vitamin A alcohol fraction from a composite tissue extract and standard vitamin A alcohol in petroleum ether were evaporated separately under nitrogen and dissolved each in 3 ml of redistilled benzene. These served as stock solutions.

Toluene - p - sulfonic acid in benzene was used as a catalyst to prepare anhydrovitamin A. Toluene - p - sulfonic acid weighing 15 mg was refluxed with 100 ml of redistilled benzene until dissolved. Then 10 ml of solvent was distilled off to evaporate any moisture present. The solution was allowed to cool, readjusted to 100 ml volume with benzene and used as the catalyst or dehydrating agent.

One ml of the above stock solutions was mixed with 4 ml of the catalyst solution. After 1 min the catalyst was neutralized by shaking with 1 g of dry Na_2CO_3. The solution was allowed to settle. The absorption spectra was then measured in a Beckmann DK-2 spectrophotometer in the region 340 - 500 mu against standard vitamin A alcohol. The readings thus obtained represented the changes in absorbancy caused by dehydration. Also the absorption spectra of the

anhydro-derivative and the original vitamin A alcohol were measured against benzene for both the standard and the experimental sample. After measuring absorption spectra these samples were evaporated under nitrogen and dissolved in 1 ml of $CHCl_3$. The absorption spectra of the Carr-Price reaction product was measured immediately. Standard vitamin A alcohol was treated by the same procedure.

Identification of vitamin A by paper chromatography

In order to more adequately characterize the products formed, the ether extract of the incubated media was subjected to two different paper chromatographic procedures.

Paper partition chromatography. This procedure was similar to that of Suzuki (170) with minor modifications. In order to separate small amounts of different forms of vitamin A and carotene from the ether extract (unsapanifiable portion) the technique of one dimentional ascending paper partition chromatography was employed. The strips of chromatographic paper (Whatman No. 54) was impregnated with a $Ca(H_2PO_4)_2$ solution (100 g per liter) at room temperature in a clean glass tray. After three hours, the salt solution was decanted and a 2N NH_4OH solution was poured on the wet filter paper in a glass tray. The paper was soaked for one-half hour. Then the paper was thoroughly washed for one-half hour with distilled water or until it did not give a basic reaction or an odor of ammonia. A sample of the ether extract from a tissue-carotene incubation was evapor-

ated under nitrogen and separate drops of the viscous concentrate thus obtained were applied on the paper strips. Standards were run simultaneously.

The atmosphere in the chromatographic tank was saturated with the vapor of the developing solvent (petroleum ether). The strips were then developed by the ascending technique with petroleum ether as solvent for 2 to 3 hours. The strips were dried in a hot air oven and then sprayed with antimony trichloride to detect colored areas.

Circular paper chromatography. This chromatographic technique was described by Giri and Rao (62,63) and Mahadevan et al. (118) and was used with slight modifications. This technique separated vitamin A alcohol and carotene fairly well. Circular Whatman No. 1 filter paper (24 cm) was first impregnated with 10% paraffin oil in petroleum ether and then dried for 24 hr at room temperature to remove the solvent. A small circle of about 4 cm diameter was drawn with a lead pencil and a slit cut at the center. In order to irrigate the chromatogram with solvent, a detachable tail was made using a 0.5 x 4 cm strip of filter paper and this was introduced into the slit. The solvent used for developing the chromatogram was butanol - acetic acid - water (4:1:5). The viscous concentrate from ether extracts and known compounds were spotted separately at different places on the circle drawn with the pencil. The filter paper with its tail end was kept in a pyrex dish in such a

way that the tail was immersed in developing solvent contained in a small beaker which was kept underneath the paper in the center of a pyrex dish. The outer of perimeter of the filter paper rested on the rim of the pyrex dish. The entire apparatus was kept in a chromatographic tank which was already saturated with the solvent. The chromatogram was allowed to develop in the dark for 12 hr. Frequent examinations were made using an ultraviolet lamp.

Determination of carotene and vitamin A in plasma

Carotene and vitamin A in the blood were determined by a modification of the method described by Kimble (99). Usually 10 ml of plasma (or serum) was placed in a thick walled test tube. An equal volume of 95% ethanol was added and the mixture was well shaken. Then 10 ml of petroleum ether (b.p 30-60°C) was added, the tube corked and the contents shaken for 5 - 8 min on a mechanical test tube shaker. The corks used were encased with aluminum foil so that solvent would not extract extraneous material from the corks. The tubes were centrifuged for 1 min at 480 XG to obtain good separation of petroleum ether from the alcohol plasma mixture or allowed to stand at 40°C and 5 ml of petroleum ether layer was then pipetted into a cuvette or into a standardized test tube for reading the carotene concentration. When pipetting care was taken not to touch the sides of the test tube with the pipette tip. The concentration of carotene was measured in the Beckmann B spectrophotometer

at 440 mu with petroleum ether as a blank. Then the petroleum ether extract in the cuvette was then evaporated under nitrogen and dissolved with 0.6 ml $CHCl_3$. Measurement of vitamin A was made by the antimony trichloride reaction in the Beckmann model B spectrophotometer as described elsewhere.

Determination of carotene and vitamin A in liver.

The usual alkali digestion method as described by Davies (41) with some modification was used to determine carotene and vitamin A in the liver. Five g of liver preferably obtained from several different areas were weighed into a beaker. The liver sample was minced well with scissors and 10 ml of 5% KOH added. This was transferred into an Erlemeyer flask and the mixture digested on a steam bath for 30 min. The digested solution was cooled to room temperature transferred to a separating funnel and the Erlemeyer flask was rinsed with 5 ml distilled water. Then 5 ml of ethanol was added and the mixture was shaken. To this 40 ml of peroxide free ethyl ether were added and the material was shaken for 1 min. The layers were allowed to separate and the lower layer was drawn into another separatory funnel and reextracted with 10 ml of ethyl ether as before. The first extract was washed first by shaking with 5 ml of water and then gently shaken with 50 ml of water. The same wash water was used to wash the second extract. The two extracts were combined. Two more washings were

performed first by using 10 ml of wash solutions made of from 1 g of KOH, 10 ml ethanol and 90 ml distilled water. The second 10 ml of wash solution was made up of 1 ml concentrated HCl and 100 ml ethanol diluted to 1 liter with distilled water. The extract was dried by passing through a layer of anhydrous Na_2SO_4 and diluted to 50 ml with ethyl ether. Five ml of this ether extract was transferred into a cuvette and the concentration of carotene and vitamin A were measured in the same way as already described.

RESULTS

PRELIMINARY EXPERIMENTS

The preliminary experiments were conducted to study whether an incubating media modified from several investigators (121, 137, 169, 193) was satisfactory for studying vitamin A formation in vitro. In these trials when fresh duodenal homogenates of rabbits and Holstein cow were incubated a considerable amount of vitamin A was formed. The extent of vitamin A formation in nine samples of duodenal homogenates from animals of these two species is shown in Table 2. Vitamin A formed per gram tissue presented in the table was corrected for endogenous vitamin A. The amount of vitamin A formed was about 14 and 12 times the endogenous level in rabbits and cow, respectively. Vitamin A formed per flask was about 11 ug when duodenal homogenated from rabbits were used and about 7 ug when duodenal homogenate from cow were used. The amount of carotene unrecovered was similar in both trials. The extent of vitamin A formed was more in tissue from rabbits than in tissue from the cow. About 4 and 2.6% of unrecovered carotene was found as vita-A expressed on a weight basis. The results clearly indicated that 0.25 M sucrose solution plus an aqueous carotene suspension maintained physiological conditions well enough to allow formation of measurable amounts of vitamin A in vitro. The homogenates from cow duodenum contained some

TABLE 2

Extent and variation in formation of vitamin A after incubating carotene with duodenal homogenates (Experiment numbers 1 and 2)

Tissue source and item	Carotene used/ g tissue	Carotene unrecovered	Vitamin A formed	
			per g tissue	per ug carotene unrecovered
	ug	%	ug	M.ug
Rabbit				
Av. of 9 samples	112	36.41	1.63	40.09
St. error		0.26	0.16	1.28
Coef. of variation		7.00	28.90	28.70
Holstein cow				
Av. of 9 samples	112	36.41	1.06	25.74
St. error		0.24	0.02	0.47
Coef. of variation		5.90	15.00	16.00

Av = Average; St. = Standard; Coef. = Coefficient

pieces of fat and fascia that were not homogenized into fine particles but the rabbit tissue had a more homogenous appearance. However, the coefficients of variation were less for the tissues from the cow. The variation and standard error were not excessive, but in all trials duplicate samples were incubated simultaneously and the average value used.

Effect of addition of glycocholate and saponification of tissue

In studying B-carotene cleavage by longitudinally cut sections of washed rat intestines in vitro, Olson (137) observed that sodium glycocholate and perhaps other bile salts were an absolute requirement for vitamin A formation. In the present study 5% sodium glycocholate was incorporated into the aqueous carotene suspension in 5% Tween 80 and incubated with rabbit duodenal homogenates and with duodenal loops at 38°C for 3 hr. The addition of glycocholate to a carotene suspension increased vitamin A formation to 119% of control when duodenal homogenates were used. These results somewhat confirmed the observations of Olson (137). When duodenal loops were used the addition of glycocholate was without any beneficial effect. The lack of any beneficial effect by the addition of sodium glycocholate to the duodenal loops may be explained on the basis that the duodenal loops already contained some bile. In general an aqueous carotene suspension containing 5% sodium glycocholate and 5% Tween 80 were employed for all subsequent

work. In two experiments (No. 10, 11), the effectiveness of petroleum ether to extract vitamin A from the incubated tissue following saponification was compared to incubated tissue not saponified using duodenal homogenates from calves. The results indicated that the extraction of vitamin A was not complete in the non-saponified sample. Only 54 to 66% of the vitamin A extracted following saponification was found by extracting the non-saponified samples. The saponification procedure left only small particles of residue in the mixture, whereas when not saponified the mixture contained large masses of tissue. It appeared desirable to saponify all samples in order to more thoroughly extract the vitamin A formed. The vitamin A ester was probably hydrolyzed to vitamin A alcohol by saponification. For all results given saponification was employed prior to ether extraction.

Effect of altering carotene to tissue ratio and incubation time

The manner in which the ratio of carotene to tissue influenced vitamin A formation was studied. Samples of calf duodenal homogenates were incubated for 3 hours with various levels of carotene. A summary of two such experiments are presented in Table 3. The results clearly showed that large amounts of carotene added to homogenates would increase the total amount of vitamin A formed. Vitamin A formed was corrected for endogenous vitamin A. Vitamin A

TABLE 3

Effect of altering carotene to tissue ratio on amount of vitamin A formed

Tissue source	Expt. no.	Carotene incubated/ g tissue	Vitamin A formed	
			per g tissue	As % of carotene unrecovered
		ug	ug	%
Calf fed deficient diet	10	0	0.03*	---
		401	2.47	1.7
		134	1.29	2.6
		67	0.71	2.4
		34	0.43	3.9
		17	0.23	3.5
Calf fed 1% NO_3	11	0	0.01*	---
		291	1.69	2.8
		98	0.64	3.1
		49	0.49	4.1
		24	0.26	3.4
		12	0.14	4.2

* = Endogenous level of vitamin A in the tissue

formed per g tissue ranged from 2.4 ug down to 0.23 ug, when carotene incubated ranged from 401 to 17 ug/g tissue. The efficiency of vitamin A formation expressed as per cent of carotene unrecovered tended to increase as the level of carotene decreased in the incubating media. Similar results were obtained in experiment 11 using tissue from a calf fed the carotene deficient diet. These results indicated that when comparing the amount of vitamin A formed between different experiments the carotene to tissue ratio in the homogenates must be considered.

The curves in Fig. 2 indicated that formation of vitamin A was practically linear up to about 40 - 60 ug carotene/g tissue. At higher carotene levels the vitamin A formation continued to increase with the amount of incubated carotene but at a greatly reduced rate.

The results of previous experiments showed that 30 - 40% of carotene incubated could not be recovered and disappeared from the incubating mixture. This suggested that during incubation a considerable amount of carotene was destroyed. At the same time it was possible that a small quantity of vitamin A could also be destroyed. Moreover, an incubation time which was optimum for maximum recovery of vitamin A with the least destruction of carotene appeared to be desirable. Therefore the effect of incubation time on formation of vitamin A was studied. Duodenal homogenates from calf and cows were incubated for periods varying from

TABLE 4

Effect of incubation time on the extent of formation of vitamin A and the recovery of carotene using duodenal homogenates from non-depleted animals

Tissue source and Expt. no.	Incubation time	Carotene incubated/ g tissue	Carotene recovered	Vitamin A formed per g tissue activity		as % of carotene unrecovered
	min.	ug	%	ug	%	%
Calf 44	0	0.0	--	0.03*	---	----
	30	14.1	86	0.47	107	23.2
	60	"	78	0.45	102	14.3
	120	"	76	0.44	100	12.7
	180	"	67	0.44	100	9.5
	240	"	41	0.43	98	5.1
Cow 45,46	0	0.0	--	0.07*	---	----
	5	10.2**	74	0.10	24	3.9
	15	12.6	68	0.18	46	4.4
	30	"	63	0.39	102	8.9
	45	"	56	0.37	98	7.5
	60	"	58	0.37	97	7.6
	120	"	52	0.38	100	6.8
	180	"	50	0.38	100	6.6
	240	"	44	0.35	92	5.2

* = Endogenous vitamin A level in the tissue

** = Expt. 46 only, others average of 45 and 46

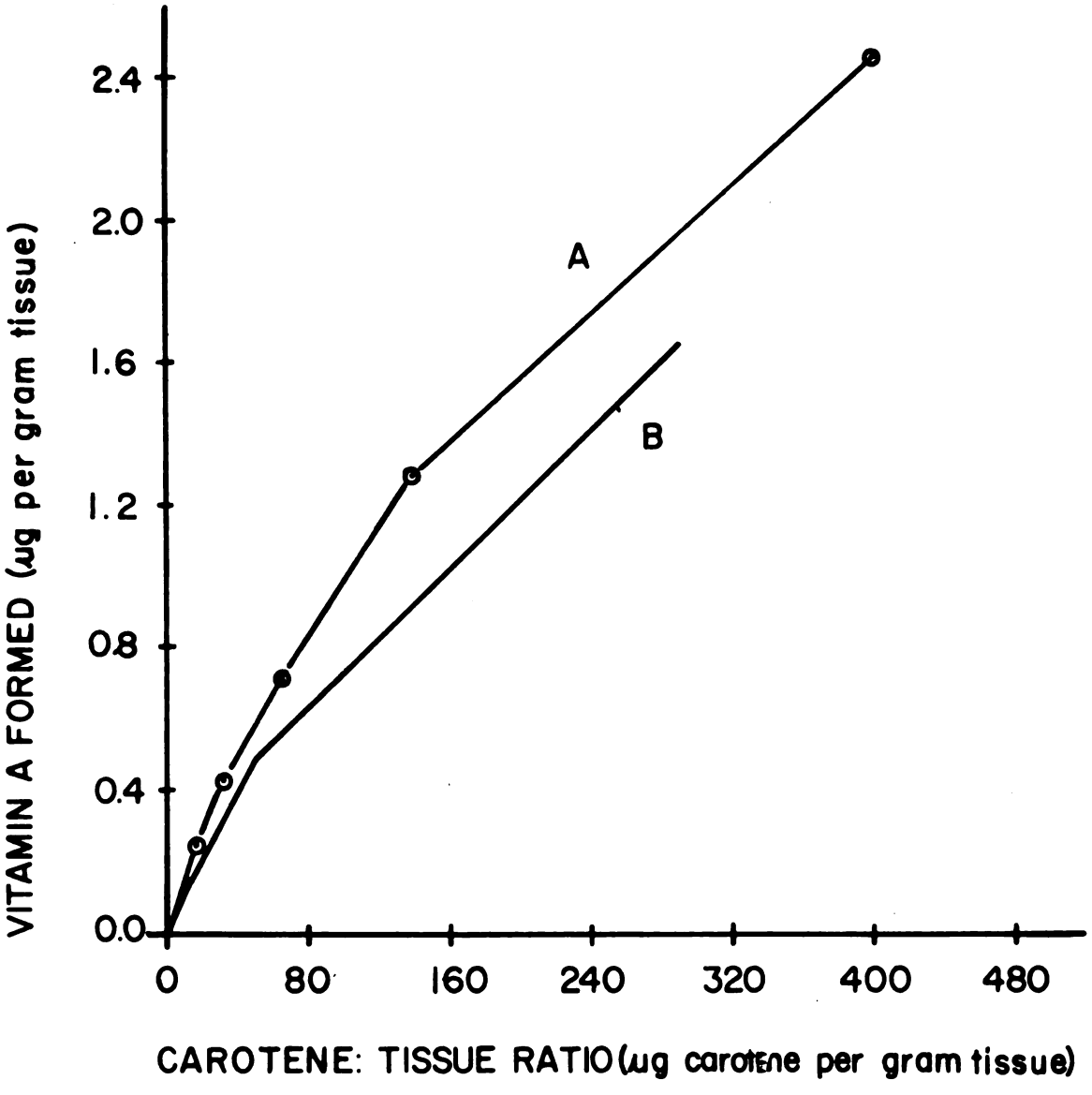


Figure 2. Effect of altering carotene to tissue ratio on vitamin A formation

A--Tissue from calf fed deficient ration
B--Tissue from calf fed 1% NO_3^- ration

5 to 240 min at 38°C. Results are summarized in Table 4. The results indicated that even after 5 min of incubation a small amount of vitamin A was formed (Experiment 45). Maximum vitamin A formation was attained in 30 min and decreased very slightly thereafter. In experiment 44, using calf duodenal homogenates, the recovery of carotene was 86% at 30 min incubation and then decreased progressively to 41% at 240 min. Similar results were noted with tissues from a cow where the recovery was 74% at 5 min and 44% at 240 min. The efficiency of vitamin A formed (expressed as per cent of carotene unrecovered) also progressively decreased from 5 to 240 min of incubation. The conversion process appears to be a very rapid reaction since vitamin A was formed after 5 min of incubation. The results also indicated that increasing incubation time beyond 30 min did not have any appreciable effect on the amount of vitamin A formation.

Efficiency of various buffers

The usefulness of 0.25 M sucrose, 0.9% NaCl, Krebs-Ringer solution, phosphate buffer pH 7.2, veronal buffer pH 7.4 as diluting and incubating media for the formation of vitamin A from B-carotene were compared. The results of such experiments are presented in Table 5. Sucrose was most effective and veronal buffer was least effective. Efficiency of vitamin A was also maximal when 0.25 M sucrose solution was used and minimum when veronal buffer was used.

TABLE 5

Influence of various buffers as incubating media on the formation of vitamin A from carotene

Tissue source and Expt. no.	Buffers used	Carotene incubated/	Vitamin A formed per g tissue	Vitamin A formed as % of carotene unrecovered
		ug	ug	%
Calf 44		0	0.08*	---
	0.25 M sucrose	14	0.44	9.5
	0.9% NaCl	"	0.14	2.7
	Krebs-Ringer	"	0.18	3.1
	Phosphate buffer	"	0.17	3.0
	Veronal buffer	"	0.08	1.3
Cow 47, 48		0	0.21*	---
	0.25 M sucrose	14	0.45	9.2
	0.9% NaCl	"	0.16	3.0
	Krebs-Ringer	"	0.35	7.2
	Phosphate buffer	"	0.15	2.8
	Veronal buffer	"	0.06	1.1
	Tris buffer	"	0.10	1.5

* = Endogenous level of vitamin A in the tissue

The data indicated that 0.25 M sucrose did not function merely as an agent which supplied essential nutrients and optimum P^H for the reaction to proceed *in vitro*, but probably had a more specific action. The data indicated that of the several buffers tried a 0.25 M sucrose solution was superior.

Effect of homogenizing tissue in waring blender or in an omni mixer and using nitrogen or oxygen

An experiment was conducted to study whether homogenizing tissue either in a waring blender or in an Omni Mixer* affected vitamin A formation. Duodenum and liver were each homogenized separately and incubated with B-carotene at 38°C. The results showed that homogenizing tissue in an Omni Mixer with powdered glass beads reduced vitamin A formation to 60% compared to the tissue that was homogenized in a waring blender. The decrease in activity by homogenizing in an Omni Mixer may have been due to an effect of the powdered glass beads. It may be possible that homogenization with glass beads in an Omni Mixer might have destroyed nuclei, mitochondria and/or other particulates since powdered glass beads at high speed usually disrupt these subcellular particles (28). Homogenization without powdered glass beads in the waring blender may have minimized this disruption. It appears possible that *in vitro* formation of

*Serval Omni Mixer Homogenizer

vitamin A from B-carotene was maximum when the destruction of nuclei, mitochondria and other cell particulates was minimal.

In another study, rabbit intestinal tissue was homogenized separately in the waring blender as usual and in an Omni Mixer under oxygen and nitrogen. In this procedure, the homogenizing liquid and head space in the tightly enclosed Omni Mixer was saturated with the gas before homogenization. These homogenates were then incubated with the carotene suspension at 38°C and the extent of vitamin A formation measured. Tissues homogenized in an Omni Mixer under nitrogen formed more vitamin A than the same tissue homogenized under oxygen. The extent of vitamin A formation and the recovery of carotene were increased under the influence of nitrogen as compared to oxygen. It was also observed that the extent of vitamin A formation was more with the tissue homogenized in the waring blender than with the tissue homogenized in an Omni Mixer under oxygen. These results indicated destruction of carotene and vitamin A occurred under the influence of oxygen probably due to oxidation. These effects were less pronounced under nitrogen.

Effect of heating and other treatments of tissue

The results of heating the homogenates are summarized in Table 6. In experiment No. 60 using steer duodenal homogenates, the activity (vitamin A formed/g) was reduced

TABLE 6

Effect of heating duodenal homogenates on vitamin A formation from carotene

Tissue source and expt. no.	Heating time	Temperature observed	Carotene incubated	Carotene recovered	Vitamin A formed	
					per g tissue	Activity
	min.	°C	ug	%	ug	%
Calf fed 1% NO_3 diet 11	0	--	24	67	0.26	100
	1	--	"	60	0.13	49
	2	--	"	59	0.14	55
	4	--	"	57	0.14	52
Steer 38	0	--	15	63	0.53	100
	1	90	"	43	0.06	11
	2	"	"	46	0.03	6
	3	"	"	46	0.04	8
	5	"	"	46	0.03	6
Sheep 13	0	--	31	68	0.36	100
	1	90	"	56	0.06	17
	2	"	"	57	0.05	14
	4	"	"	54	0.05	14

from 100% down to between 11.3 and 5.7%. This procedure reduced the activity to 14 - 17% for sheep tissue. When the heated duodenal homogenates from the calf in experiment No. 11 were incubated, the activity was reduced to about 50% of unheated homogenates. These values were higher than those for experiments No. 11 and 13. The temperature of the homogenates was not determined in experiment No. 11. This may account for the high activity even after heating the homogenates. In all cases carotene recovery ranged from 43 to 60% in heated samples and 63 - 68% in unheated samples.

In another trial (Experiment 33), duodenal tissue was obtained from the pigs which were subjected to the usual procedure of scalding the entire body surface on the open flame. Interval between slaughtering and homogenization of the duodenum was more than one hour. When these duodenal homogenates were incubated the activity was nil.

In the light of these results it is clear that heating of the homogenates inactivated the enzyme or factors which were responsible for conversion of carotene to vitamin A.

In another trial, the homogenates were left standing at room temperature ($\pm$ 22°C) for 1, 2 and 3 hours before adding carotene and incubating. This procedure reduced the activity to 25, 8 and 8% of the control value, respectively. In some preliminary experiments when the interval between slaughtering and homogenization of the tissue was too long

(± one hour) the formation of vitamin A from carotene was nil. These results indicated that the time between slaughtering animals and incubating homogenized tissue was very important in in vitro studies.

ISOLATION AND IDENTIFICATION OF VITAMIN A DERIVATIVES FROM INCUBATED MATERIAL

The Carr-Price reaction is relatively non-specific for determining vitamin A since certain carotenoids and their oxidation products give some blue color with $SbCl_3$ (132). Some oxidation products of carotenoids which give interfering reactions with $SbCl_3$, produce a non-fading grey to blue color (193). The ether extract that was extracted from the incubated mixture gave typical vitamin A blue color that faded in a few seconds in all Carr-Price determinations.

The ether extract from the incubated tissue was subjected to column chromatography on deactivated Al_2O_3 and portions of eluate were used for identification in various ways. The results using this eluate are given below.

Carr-Price reaction on column eluates

The Carr-Price reaction was employed on all successive 10 ml fractions collected. The results are shown in Table 7. The fractions in tubes No. 1 - 8, 9 - 16, 17 - 21 and 22 - 29 should contain B-carotene, vitamin A ester, aldehyde and alcohol, respectively, since the standard B-carotene, vitamin A ester, aldehyde and alcohol were eluted in this order. The Carr-Price reaction products were princi-

TABLE 7

Absorbancy of Carr-Price reaction product on successive portions eluted from a deactivated alumina column

Tube no.	Optical density 620 mu	Tube no.	Optical density 620 mu
1	0.000	16	0.000
2	0.010	17	0.000
3	0.010	18	0.005
4	0.020	19	0.005
5	0.050	20	0.000
6	0.025	21	0.000
7	0.015	22	0.060
8	0.005	23	0.150
9	0.000	24	0.250
10	0.000	25	0.420
11	0.005	26	0.695
12	0.010	27	0.810
13	0.005	28	0.120
14	0.000	29	0.040
15	0.000		

pally found in tubes No. 22 - 29 which contained vitamin A alcohol. The Carr-Price reaction optical density exhibited by carotene fractions was very low (tubes No. 1 - 8). Little or no vitamin A activity was found in the ester and aldehyde fractions in tubes No. 9 - 16 and 17 - 21, respectively. The major components of tubes No. 1 - 8 and 22 - 29 were tentatively identified as B-carotene and vitamin A alcohol respectively. These results also indicated that any vitamin A ester formed was hydrolyzed to vitamin A alcohol by the saponification process and that any vitamin A aldehyde that may have been formed was small or was destroyed by saponification.

Absorption spectra of B-carotene and vitamin A derivatives after separation on deactivated alumina

The absorption spectra of each of the four pooled fractions (above) were measured in Beckmann Model DU spectrophotometer. The B-carotene fraction exhibited an absorption maximum at 449 mu. The ester fraction did not contain vitamin A since the incubation mixture was saponified. Thus no absorption spectra were found for this fraction. Similarly no spectra was noted for the aldehyde fraction. Ultraviolet absorption spectra of the vitamin A alcohol fraction and standard vitamin A alcohol in petroleum ether are shown in Figure 3. Both vitamin A alcohol fraction and standard vitamin A alcohol exhibited a pronounced peak at 325 mu. This absorption maxima was similar to that published

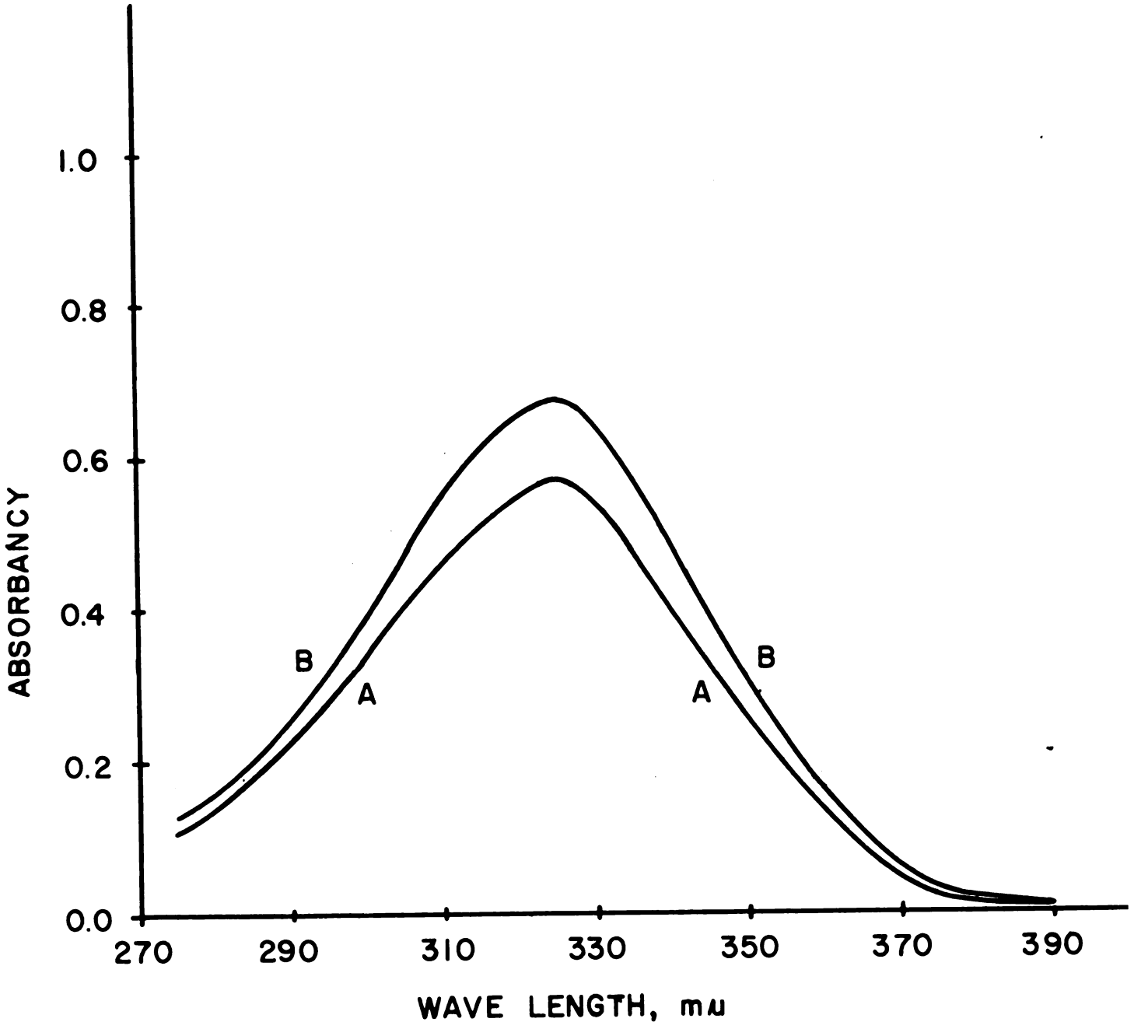


Figure 3. Absorption spectra of vitamin A alcohol fraction from ether extract of incubated mixture containing duodenal homogenates and B-carotene (ether extract was chromatographed on deactivated alumina).

A--Vitamin A alcohol fraction
B--Standard vitamin A alcohol

for vitamin A alcohol (31) suggesting that the ether extract of incubated mixture contained vitamin A alcohol.

A definative experiment (No. 62) was conducted using homogenates from rabbit intestine and muscle to isolate and identify vitamin A formed from B-carotene by various methods. The ether extracts of the incubated tissue with and without carotene added were subjected to column chromatography on deactivated alumina, and fractions collected as described above. The ultraviolet absorption spectra of the vitamin A alcohol fraction was measured using a Beckmann Model DK-2 recording spectrophotometer, on the extracts of muscle and duodenal tissue incubated with and without carotene. A standard vitamin A alcohol was also used. These absorption curves are shown in Figure 4. Standard vitamin A alcohol and vitamin A alcohol fraction from the duodenum-carotene incubation mixture gave characteristic spectra with absorption maxima at 325 mu. The absorption spectra obtained on the vitamin A alcohol fraction from muscle and from duodenum (endogenous level) and from the muscle - carotene incubation mixture did not have the characteristic absorption of vitamin A. Vitamin A if present was in too low a concentration to be detected in these samples.

Irradiation of the vitamin A alcohol fraction

In this experiment no attempt was made to quantitatively estimate the vitamin A alcohol fraction. Although this procedure has been used to determine serum vitamin A (16)

Figure 4.

Absorption spectra of vitamin A alcohol fractions in petroleum ether chromatographed from ether extracts of incubated mixture.

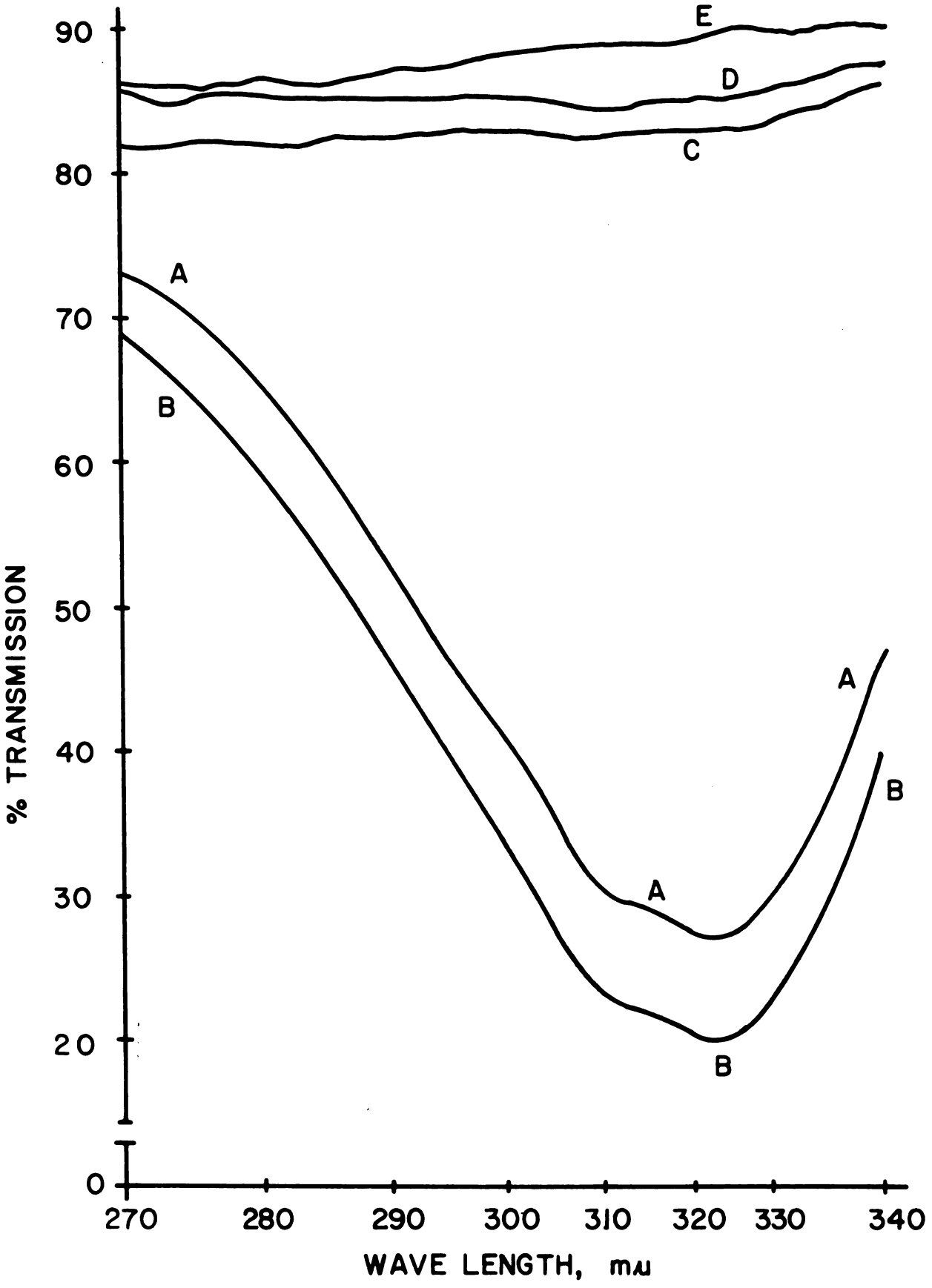
A--Duodenum + B-carotene

B--Standard vitamin A alcohol

C--Duodenum (endogenous)

D--Muscle + B-carotene

E--Muscle (endogenous)


90
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% TRANSMISSION
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270
280
290
300
310
320
330
340
WAVE LENGTH, mμ

Figure 4

Bieri and Pollard (18) showed that this method was of little value for vitamin A in tissue extracts. The irradiation technique was employed here to help identify vitamin A alcohol in the chromatographed ether extract from an incubated mixture. Figure 5 shows the ultraviolet absorption spectra of vitamin A alcohol fraction and standard vitamin A alcohol before and after irradiation with ultraviolet light for 36 hr. Before irradiation both solutions had a characteristic absorption spectra with the pronounced peak at 325 mu. After irradiation the characteristic absorption spectra was not present. The extract from another incubated mixture was chromatographed and the vitamin A alcohol fraction and standard vitamin A alcohol were irradiated for 24 hr. This procedure destroyed 75% of the vitamin A in both solutions.

The results of this procedure furnished further indirect evidence that the product formed by incubation of carotene suspension with duodenal homogenates was actually vitamin A and that none was formed when muscle was similarly incubated.

Absorption spectra of Carr-Price reaction products.

The absorption spectra of the Carr-Price reaction products was measured in a Beckmann DK-2 spectrophotometer. This was performed on a composite tissue extract before and after column chromatography and a standard vitamin A alcohol. It was necessary to add $SbCl_3$ reagent quickly and have a sufficient

Figure 5.

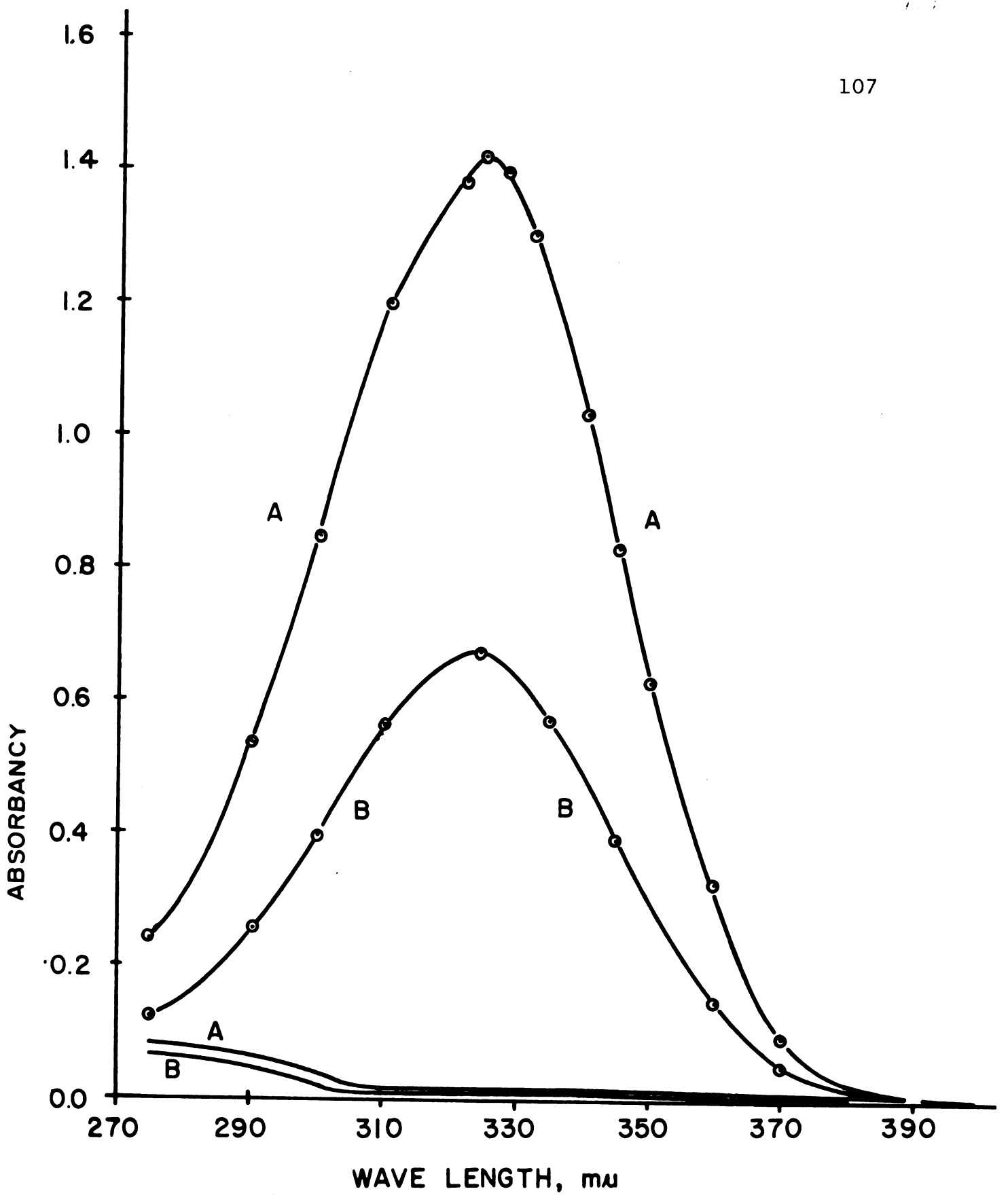
Absorption spectra of vitamin A alcohol fraction before and after ultraviolet irradiation.

A 0—0 Vitamin A alcohol fraction from tissue extract before irradiation

A —— Vitamin A alcohol fraction from tissue extract after irradiation

B 0—0 Standard vitamin A alcohol before irradiation

B —— Standard vitamin A alcohol after irradiation


1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
ABSORBANCY
A
A
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B
A
B
270
290
310
330
350
370
390
WAVE LENGTH, mμ

Figure 5

concentration of vitamin A present. Figure 6 shows that the absorption spectra of these 3 products had a maximum absorption at 620 mu. Another observation made was that the blue color produced with all samples faded rather quickly which is characteristic of the reaction between vitamin A and the Carr-Price reagent. The ether extract which was not subjected to column chromatography produced an absorption curve similar to that of standard vitamin A alcohol but with a lower and somewhat broader maximum absorption curve. In another trial, vitamin A was estimated in the ether extract of an incubated mixture by the Carr-Price reaction and also by measuring extinction at 325 mu after chromatographing this same sample. Vitamin A present per ml of ether extract was 0.14 ug when the vitamin A was determined by the Carr-Price reaction and 0.16 ug when it was determined by measuring extinction at 325 mu. Similar results were obtained when vitamin A was estimated by the Carr-Price reaction and by measuring extinction at 325 mu on a concentrated vitamin A alcohol fraction. Vitamin A present per ml of the concentrated vitamin A alcohol fraction was 0.804 ug as determined by the former method and 0.780 ug by the later method. These results indicated that the vitamin A determinations made on the crude or on the chromatographed ether extracts from tissue incubations by the Carr-Price reaction actually represented vitamin A present. Any value obtained by the Carr-Price reaction has

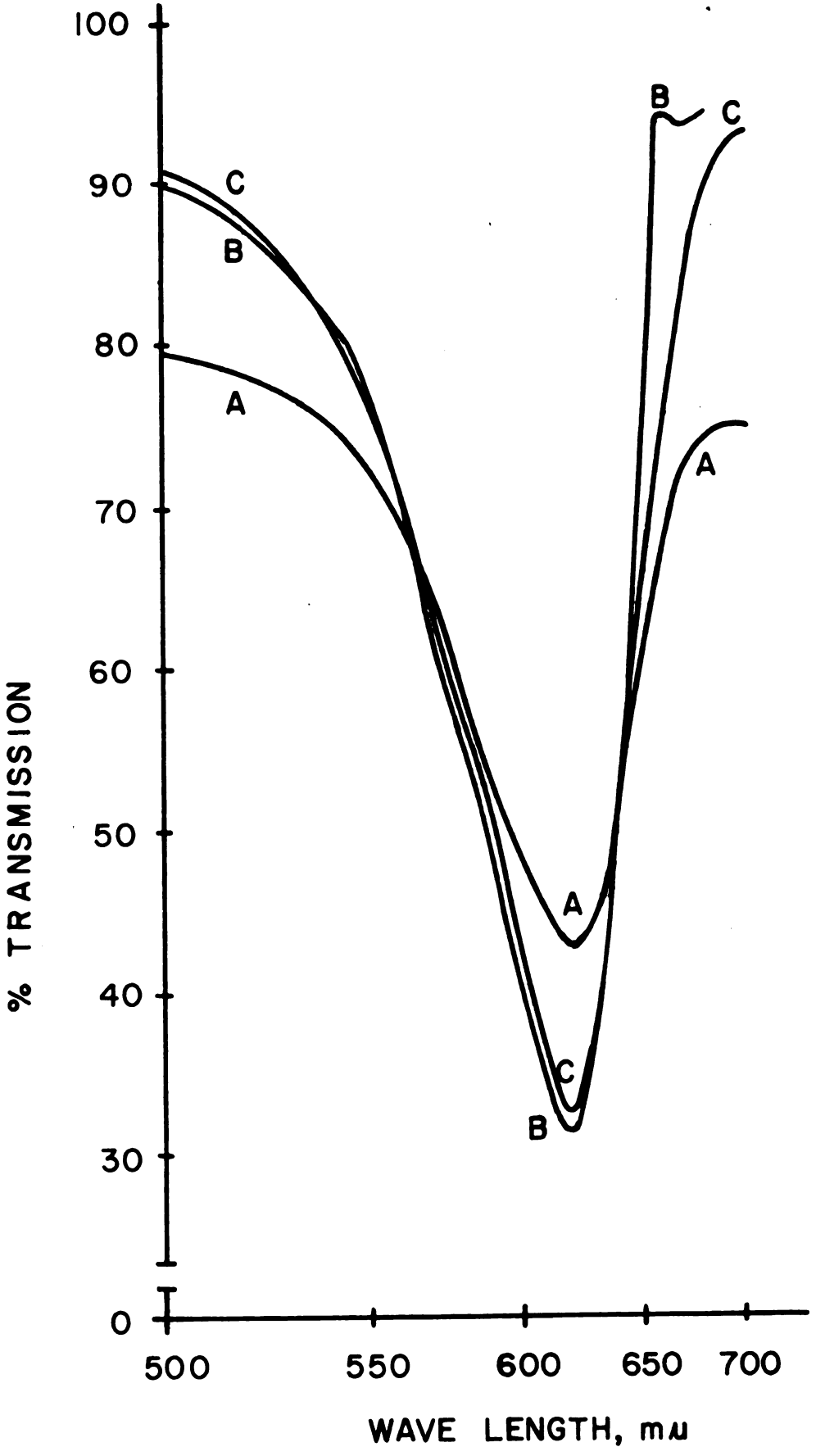
Figure 6

Absorption spectra of Carr-Price reaction products of tissue extract before and after chromatographic separation on deactivated alumina.

A--Tissue extract

B--Vitamin A alcohol fraction after chromatographing

C--Vitamin A alcohol fraction after chromatographing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% TRANSMISSION
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WAVE LENGTH, mμ
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C
A
B
C
A
B
C

Figure 6

very close estimates of the true vitamin A content in the extract.

Maleic anhydride reaction method

Although the maleic anhydride reaction has been used to determine the per cent of neovitamin A (151), this method was used in this study to indicate the presence of vitamin A in two different extracts from incubated mixtures. One sample was a composite of several extracts and the other was the extract from the duodenal-carotene incubation in experiment 62.

Maleic anhydride reacted rapidly with the vitamin A alcohol fraction and standard vitamin A alcohol in Benzene. The results are shown in Table 8. The percentages of initial vitamin A potency after 16 hr were 2 and 94 for maleic anhydride treated and untreated vitamin A alcohol fraction, respectively, and 2.3 and 96 for maleic anhydride treated and untreated standard vitamin A alcohol. The recovery value "R" was determined by the formula (6):

$$\frac{100 \text{ X units in treated solution}}{\text{Units in untreated solution}}$$

The recovery values "R" for the vitamin A alcohol fraction and for the standard vitamin A alcohol were estimated to be 2.2 and 2.4%, respectively. At the end of 48 hours maleic anhydride treated and untreated tissue extracts and vitamin A alcohol solutions were evaporated under nitrogen and dissolved in 1 ml of $CHCl_3$. The absorption spectra produced

TABLE 8

Percent of initial potency of samples with and without maleic anhydride treatment at different intervals and "R" values

Source of material	Treatment	Hours and % initial potency					R
		0	4	8	12	16	
							%
Chromatographed tissue extract*	Treated	100	24.5	16.3	4.1	2.0	2.16
	Untreated						
Standard vitamin A alcohol	Treated	100	26.9	15.4	6.9	2.3	2.39
	Untreated	100	98.1	96.2	97.0	96.2	
Chromatographed tissue extract*	Treated	100	36.5	8.7	5.2	2.6	2.82
	Untreated	100	95.2	92.0	96.0	92.0	

* Two different samples done at different times

by the Carr-Price reaction was measured in the Beckmann DK-2 spectrophotometer (Fig. 7). The vitamin A alcohol fraction which was not treated with maleic anhydride had a characteristic absorption spectra and a pronounced peak at 620 mu. The maleic anhydride treated sample gave little or no peak at 620 mu, which indicated the destruction of vitamin A alcohol and formation of a non-chromaphoric add-juct with maleic anhydride.

The vitamin A alcohol fraction (representing the edogenous vitamin A from this experiment #62) was subject-ed to the same procedure. Maleic anhydride treated and un-treated samples were reacted with $SbCl_3$ and initial optical densities were 0.008 and 0.009, respectively. After 16 hr of treatment no blue color was produced by these two samples with $SbCl_3$ and the optical density recorded was 0.000. These results indicated that the endogenous level of vitamin A in the duodenum was barely detectible. This extract gave no peak at 620 mu when the above absorption spectra was measured on Carr-Price reaction product.

The results of these experiments suggested that the ex-tract from two different saponified incubation mixtures con-tained vitamin A and that the endogenous duodenal level of vitamin A was practically negligible.

Conversion to anhydro vitamin A

Ultraviolet absorption spectra of a vitamin A alcohol fraction and standard vitamin A alcohol before and after

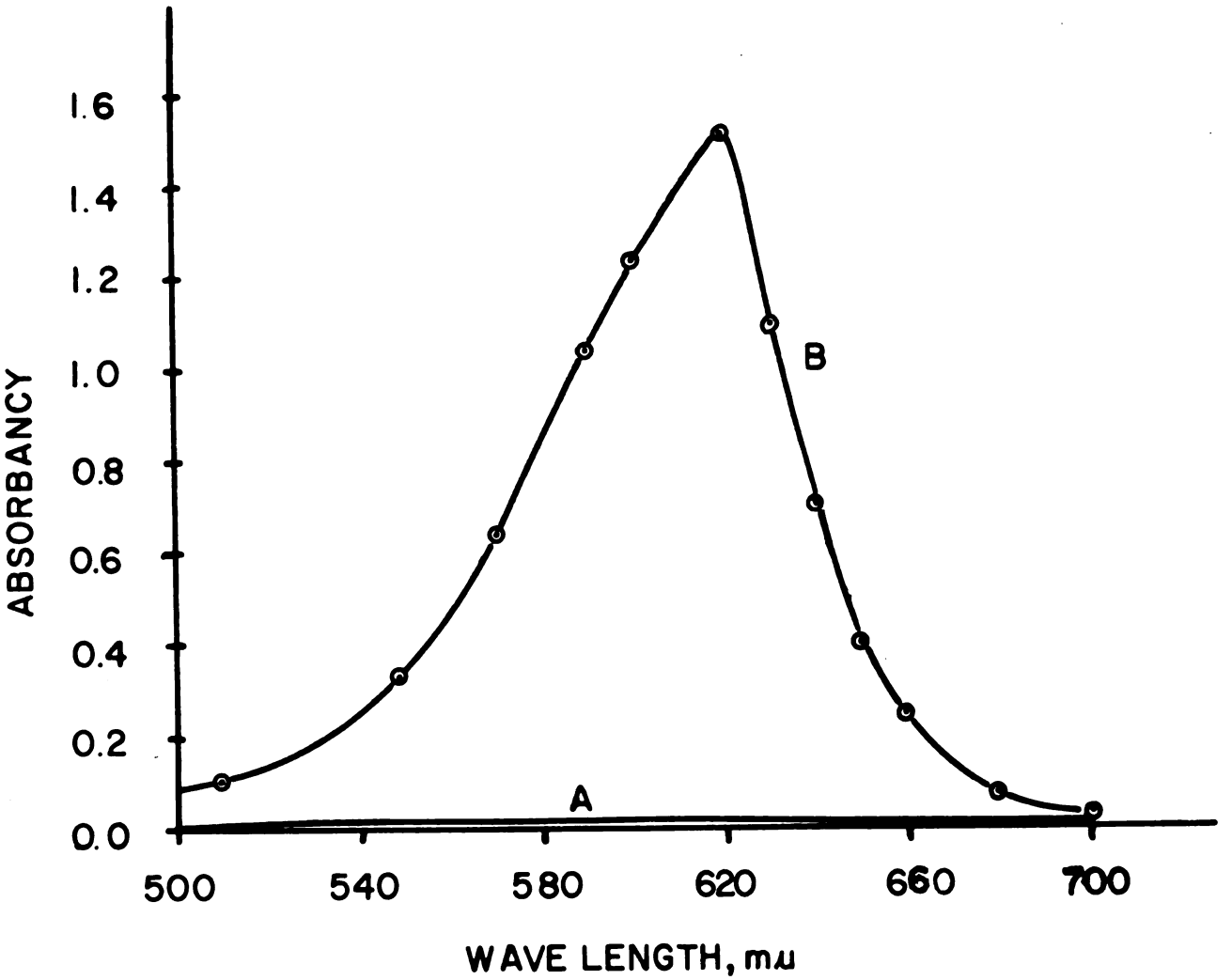


Figure 7. Absorption spectra of Carr-Price reaction product of maleic anhydride treated and untreated vitamin A alcohol fraction.

A--Sample treated with maleic anhydride
B--Sample untreated with maleic anhydride

conversion to anhydrovitamin A are shown in Figure 8. The spectra of the vitamin A alcohol fraction and standard vitamin A alcohol in benzene had maximum at 331 mu. Using benzene as the solvent shifts the absorption nearly 6 - 7 mu compared to petroleum ether as a solvent (29). Anhydrovitamin A made from the vitamin A alcohol fraction and the standard vitamin A alcohol exhibited absorption maxima at 358, 377 and 399 mu and minima at 364 and 346. All these absorption maxima were consistent with those reported by Budowsky and Bondi (29). The source of the chromatographed vitamin A alcohol fraction used in this trial was a composite of ether extracts from several incubation mixtures. Identification of vitamin A alcohol by conversion to anhydrovitamin A was performed using 2 different composite samples at 2 different times and once using extracts from a given incubation (Experiment 62). All 3 gave similar results.

The increase in absorbancy or decrease in per cent transmission caused by dehydration was also measured by determining absorption spectra of anhydrovitamin A using the standard vitamin A alcohol in benzene in the control cuvette. Figure 9 shows this absorption spectra of anhydrovitamin A made from the vitamin A alcohol fraction and from standard vitamin A alcohol. The "difference" absorption spectra of anhydrovitamin A made from the vitamin A alcohol fraction was similar to that made from standard vitamin A alcohol. The "difference" absorption spectra has absorption

Figure 8

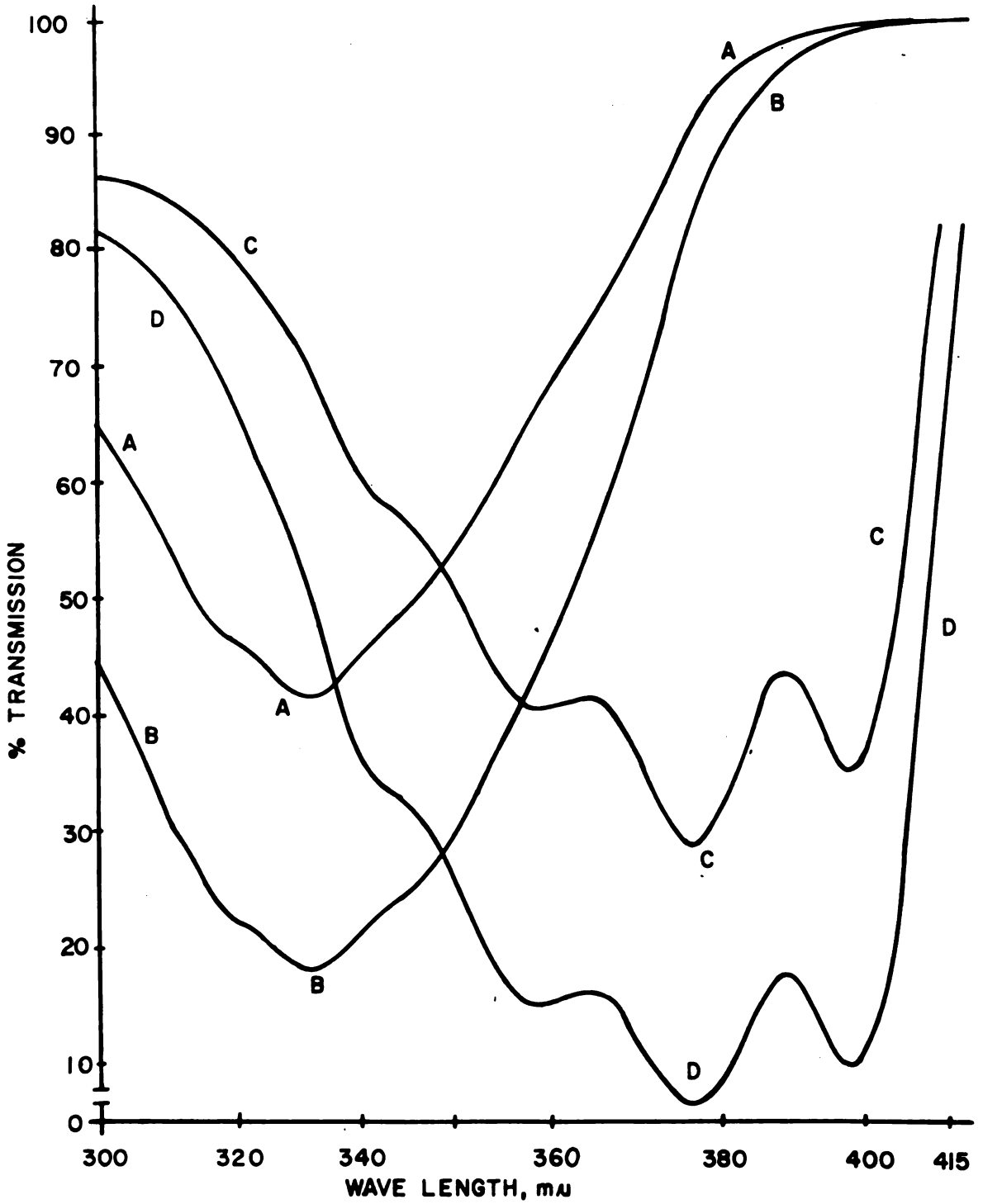
Absorption spectra of vitamin A alcohol fraction in benzene before and after conversion to anhydrovitamin A.

A--Vitamin A alcohol fraction from ether extract of duodenum and B-carotene incubation mixture

B--Standard vitamin A alcohol

C--Anhydrovitamin A from Sample A

D--Anhydrovitamin A from Sample B


100
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80
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10
0
% TRANSMISSION
300
320
340
360
380
400
415
WAVE LENGTH, mμ
A
B
C
D

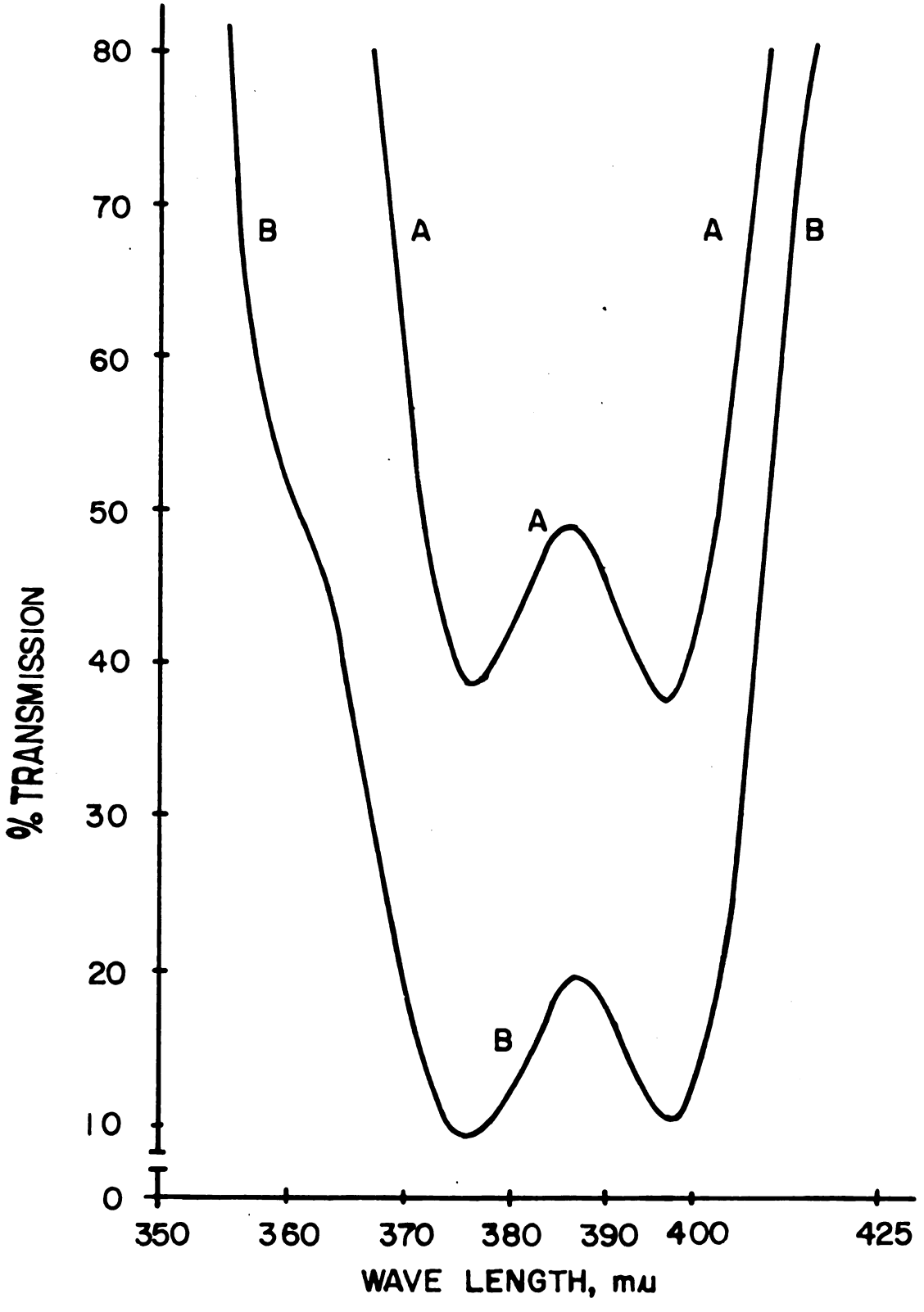
Figure 8

Figure 9

"Difference" absorption spectra of anhydrovitamin A made from tissue extract and from standard vitamin A alcohol.

A--Anhydro derivative from vitamin A alcohol fraction of chromatographed ether extract

B--Anhydro derivative from standard vitamin A alcohol


80
70
60
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40
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20
10
0
% TRANSMISSION
B
A
A
B
A
B
350
360
370
380
390
400
425
WAVE LENGTH, mμ

Figure 9

maxima at 377 and 399 mu. These results were consistent with published values (29).

After measuring absorption spectra, the above samples were each evaporated under nitrogen and dissolved in 1 ml $CHCl_3$. The absorption spectra of the Carr-Price reaction products were then measured. Figure 10 shows the characteristic absorption spectra obtained with vitamin A alcohol and their anhydro derivatives. In all samples, the maximum absorption was observed at 620 mu which is characteristic of the Carr-Price reaction products with both vitamin A and anhydrovitamin A (31).

The conversion of vitamin A in the chromatographed tissue extracts to anhydrovitamin A and its identification offers strong evidence for the formation of vitamin A in the incubated tissue.

In experiment 62 ether extracts from the incubated mixture of carotene plus intestinal homogenates and from the incubated mixture of intestinal homogenate alone (designated as endogenous) were chromatographed separately on deactivated alumina and fractions collected. Vitamin A alcohol fraction was converted to the anhydrovitamin A derivative. The anhydro derivative of the vitamin A alcohol fraction extracted from the incubated mixture of carotene plus intestinal homogenate had the characteristic absorption spectra. The anhydro derivative of the vitamin A alcohol fraction from the incubated mixture of intestinal

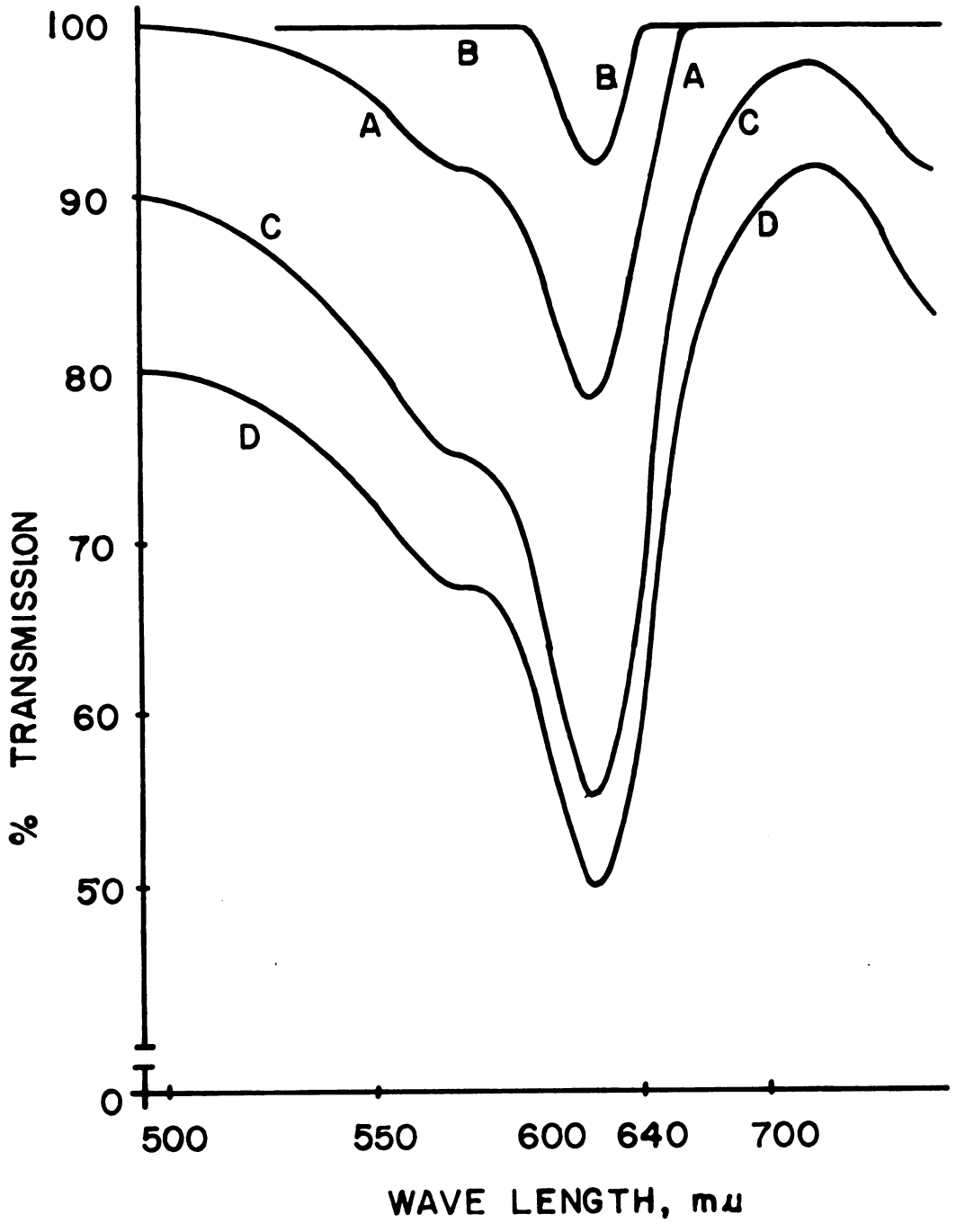

Figure 10. Absorption spectra of Carr-Price reaction products of vitamin A alcohol fraction before and after conversion to anhydro-vitamin A.

A--Vitamin A alcohol fraction after chromatography of ether extract from tissue
B--Anhydro derivative of Sample A
C--Standard vitamin A alcohol
D--Anhydro derivative of Sample C

homogenate alone (endogenous) showed no absorption spectra. This evidence clearly indicates formation of vitamin A from B-carotene by intestinal homogenates in vitro.

Identification of metabolic intermediates from B-carotene to vitamin A by paper chromatography

In order to more adequately identify the products formed after incubation of B-carotene with duodenal homogenates, combined ether extracts from several incubated mixtures were concentrated under nitrogen and a viscous concentrate obtained. This concentrate was tested by paper partition chromatography (170) and circular paper chromatography (118, 63) for the presence of vitamin A derivatives and carotenoids. Standard vitamin A derivatives and carotene were also run simultaneously with the unknown sample. The location of B-carotene was visualized by its yellow color before spraying with $SbCl_3$ in the paper partition chromatographic procedure. Vitamin A alcohol was identified after spraying with $SbCl_3$. R_F values of about 0.85 were found for standard B-carotene and B-carotene from the concentrated ether extract. The R_F values of about 0.10 were obtained for standard vitamin A alcohol and for vitamin A alcohol from the concentrated ether extract.

The separation of B-carotene and vitamin A alcohol was also achieved using circular paper chromatography. With the aid of an ultraviolet lamp and standards, the presence of B-carotene and vitamin A alcohol in the concentrated

ether extract was identified. The vitamin A alcohol band exhibited fluorescence whereas B-carotene showed a dark band without any fluorescence when exposed to an ultraviolet lamp. The results of these experiments provided substantial evidence for the presence of vitamin A alcohol in the extracts of incubated tissue.

FORMATION OF VITAMIN A BY VARIOUS ORGANS

The homogenates of duodenal tissue from cattle, sheep and pigs still contained some portions of unground tissue and were not as homogenous as desirable, whereas the homogenized tissue from rabbits, rats and chicks were reasonably homogenous mixtures. Mahadevan et al. (118) observed that during absorption, vitamin A could cross the mucosal cells to appear in the intestinal muscles either as alcohol or as a higher fatty acid ester. According to these observations muscle was not involved in formation of vitamin A from carotene (118).

The possibility existed that mucosa only would make a more homogenous homogenate as well as provide a more potent source of active tissue. Two experiments (No. 17, 18) were conducted to compare the activity of duodenal mucosa, the muscular layer and the usual combination.

The extent of vitamin A formation was greater by whole duodenum (usual combination) than with either of the two portions. The vitamin A formed per gram of whole duodenum,

mucosa and muscular layer was 0.40, 0.34 and 0.06 ug respectively. The percentage of carotene recovered from the incubation mixture was essentially the same for all these tissue sources (68, 70 and 70% respectively).

Various tissues from depleted and normal animals were homogenized and incubated with the carotene suspension. The experiment number and animal source are indicated below.

Animal species	Status	Experiment No.
Rabbits	Vitamin A depleted	36, 37
Rats	"	34
Pigs	"	40, 41
Chicks	"	35
Calves	"	16, 21
Rats	Normal	28
Sheep	Normal	43

The tissues used and the results obtained are given in Tables 9, 10, and 11. The results presented in Table 9 indicated that the tissues used from rabbits, rats, pigs, chicks and sheep formed some vitamin A. In the case of depleted rabbits and pigs, the liver was the most active followed by the upper small intestine and lower small intestine in that order. In depleted rabbits the kidney was more active than the large intestine. The kidney and large intestine were equally active in the depleted pigs. In the

TABLE 9

Vitamin A formation from carotene by various tissue homogenates from vitamin A depleted rabbits, pits, rats, and chicks compared to non-depleted rats and sheep

	Vitamin A formed/g tissue					
	Depleted			Non-depleted		
Species Expt. no.	Rabbits 36,37	Rats 34	Pigs 40,41	Chicks 35	Rats 28	Sheep 43
Tissue						
Upper small intestine	0.66	0.46	0.32	0.26	0.47	0.42
Lower small intestine	0.52	0.44	0.21	0.21	0.19	0.37
Large intestine	0.28	0.25	0.19	0.27	0.15	0.36
Liver	0.74	0.38	0.42	0.24	0.24	----
Kidney	0.43	0.39	0.18	0.11	0.28	0.28
Lung	0.09	0.07	0.09	0.18	0.11	0.07
Muscle	0.05	0.06	0.05	0.08	0.07	----
Heart	0.09	0.00	0.05	0.05	0.04	0.04
ug of carotene incubated per g tissue	14.29	17.34	14.00	15.19	19.28	20.00
Endogenous liver (ug of vitamin A/g)	0.65	0.15	3.83	0.10	0.97	----

case of depleted and non-depleted rats, the small intestine was the most active of all the tissues and the liver and kidney were equally active and more active than the large intestine. The results with chicks showed that small intestine, large intestine and liver were approximately equal in their ability to form vitamin A. In normal rats the lower small intestine and large intestines were equally active and both were less active than the upper small intestine. In normal sheep, the upper small intestine was the most active followed by the lower small and large intestines. Homogenates of lung, muscle and heart tissue from all the above animals were probably capable of forming very minute amounts of vitamin A (less than 0.1 ug vitamin A per g tissue). The data of Olson (140) indicated that less than 0.05 ug vitamin A was formed from 5 - 20 ug carotene in heart-lung preparation of a dog. This data indicated that muscle, lung and heart tissue are possibly not important organs for vitamin A formation.

Efficiency of vitamin A formation by various tissues expressed as per cent of carotene unrecovered is shown in Table 10. In all species the upper small intestine was one of the most efficient tissues for converting carotene to vitamin A. Only in pigs was the liver more efficient than the upper small intestine. The efficiency of lung, muscle and heart tissue was extremely low ($<1\%$) and was the least of all tissues studied. The very low value in

TABLE 10

Efficiency of vitamin A formation by various tissue homogenates expressed as % carotene unrecovered

Species Expt. no.	Vitamin A formed as % of carotene unrecovered					
	Depleted			Non-depleted		
	Rabbits 36,37	Rats 34	Pigs 40,41	Chicks 35	Rats 28	Sheep 43
Tissue						
Upper small intestine	16.9	11.6	3.0	3.1	5.8	4.9
Lower small intestine	9.7	12.3	1.9	2.9	2.4	3.7
Large intestine	4.9	2.5	1.8	2.4	2.1	4.1
Liver	6.6	2.3	3.2	3.0	4.4	---
Kidney	6.8	3.7	1.6	0.3	3.2	2.2
Lung	0.7	0.4	1.0	0.6	1.0	0.6
Muscle	0.6	0.8	0.7	0.7	1.2	---
Heart	0.6	0.0	0.6	0.6	0.4	0.4

extent and efficiency for kidney from chick is not consistent with the other species. Usually the efficiencies of the lower small and large intestine were somewhat similar except in the case of rats.

Vitamin A formation from carotene by various tissue homogenates from depleted calves is shown in Table 11. Duodenum was most active and jejunum and liver were only slightly less active in extent of vitamin A formation. Ileum and kidney were about equally active but were less active than jejunum and liver. The colon was least active of all the intestinal segments. As observed in other animals, lung, heart and muscle were capable of forming only traces of vitamin A. Lymph glands, rumen, omasum and abomasum were in this same category. The results also indicated that tissue from the duodenum and jejunum were the most efficient in vitamin A conversion followed by liver, kidney and ileum. The other tissues tested were verylow in their efficiency of vitamin A conversion.

The data from these studies indicated that several segments of the intestinal tract can form some vitamin A but that the small intestine, especially the duodenum, is the major site in the gastrointestinal tract. The liver and kidney are both very active in this respect while other tissues in the body possess a very limited ability to form vitamin A from carotene.

TABLE 11

Vitamin A formation from carotene by various tissues homogenates from depleted calves (Experiments 16, 21)

Tissue	Carotene incubated/ g tissue	Vitamin A formed	
		per g tissue	as % of carotene unrecovered
	ug	ug	%
Rumen	33	0.11	1.4
Omasum	"	0.10	2.4
Abomasum	"	0.10	0.8
Duodenum	"	0.63	7.8
Jejunum	"	0.52	7.3
Ileum	"	0.27	3.8
Colon	"	0.17	1.7
Liver	"	0.55	5.9
Kidney	"	0.31	4.3
Lung	"	0.16	1.8
Heart	"	0.14	1.4
Diaphragm muscle	"	0.10	1.0
Lymph gland	"	0.12	1.0
Endogenous liver (ug vitamin A/g)	--	0.16	---

EFFECT OF NO_3^- ION ON VITAMIN A FORMATION IN VITRO AND IN VIVO

These experiments were designed to explore the direct effect of nitrate on the formation of vitamin A in the *in vitro* system described previously. The effect of nitrate on vitamin A metabolism when added to the diet and when introduced into intestinal loops was also studied.

Effect of adding NO_3^- to intestinal loops in vitro and in vivo

Isolated intestinal loops were used both *in vitro* and *in vivo* to study the effect of adding NO_3^- on vitamin A formation. The results are given in Table 12. Some vitamin A was formed in all these trials. The results of experiment 7 clearly showed that there was more vitamin A formed by *in vivo* loops than in loops taken from another rabbit and incubated *in vitro* (1.19 Vs 0.66 ug). The addition of 1600 u moles of NO_3^-/g tissue inside the *in vitro* loop reduced the conversion to 41% of control whereas addition of same amount of NO_3^- inside the loops incubated *in vitro* reduced the conversion to only 88% of control. Thus the extent of conversion was reduced more by NO_3^- addition into the *in vivo* system than in the *in vitro* system. When higher levels of NO_3^- (8000 u moles/g tissue) were introduced into a loop of calf intestine and incubated *in vitro* the extent of conversion was reduced to 47% of control (Experiment 8). The efficiency (ug vitamin A formed ÷ ug carotene unrecovered)

TABLE 12

Effect of adding NO_3^- on vitamin A formation from B-carotene using intestinal loops *in vivo* and *in vitro*

Tissue source and expt. no.	NO_3^- injected/ loop	Carotene incubated/ g tissue	Vitamin A formed per g tissue		as % of carotene unrecovered
				activity	
	u moles	ug	ug	%	%
In vivo					
Rabbits 7	0	0	0.15*	<<	---
	0	50	1.19	100	9.6
	1600	50	0.49	41	2.1
In vitro					
Rabbits 7	0	0	0.16*	--	---
	0	50	0.66	100	6.6
	1600	50	0.58	88	4.4
In vitro					
Depleted calf 8	0	0	0.00*	--	---
	0	26	0.45	100	3.3
	8000	26	0.21	47	3.3

* = Endogenous level of vitamin A in the tissue

of the conversion process was 9.6% in vivo and only 6.6% in vitro (Experiment 7). Nitrate reduced this efficiency to 2 and 4%, respectively. The efficiency was 3.3% with loops from the calf with and without NO_3^- addition. Addition of NO_3^- reduced the extent of and the efficiency of vitamin A formation by intestinal loops under in vitro and in vivo condition.

Effect of NO_3^- on vitamin A formation by tissue from depleted animals

Variable levels of NO_3^- ranging from 0.2 to 1000 u moles/g tissue were added to intestinal homogenates from rabbits, sheep and calves that had been fed normal rations and to homogenates from rabbits, pigs and calves fed rations low carotene and/or vitamin A. The results obtained with normal and depleted animals are shown in Tables 13 and 14, respectively. The main results with normal and depleted animals were similar and will be discussed together. All values for vitamin A formed per gram of tissue as presented in the tables were corrected for endogenous vitamin A. The statistical significance is also shown in these tables. Averages with superscripts of same number represent a homogenous group. Lower case for p is equal to 0.05 and capital letter for p is equal to 0.01. The addition of 100 - 650 u moles NO_3^-/g tissue reduced the activity to about 35 - 48% of control in the case of normal and depleted rabbits. The inhibiting action of NO_3^- in depleted rabbits may probably be

TABLE 13

Effect of adding different levels of NO_3^- to the incubation media on vitamin A formation from B-carotene by duodenal homogenates from normal animals

Tissue source and Expt. no.	NO_3^- added/ g tissue	Carotene incubated/ g tissue	Vitamin A formed per g tissue		Vitamin A formed as % of carotene unrecovered
				activity	
	u moles	ug	ug	%	%
Rabbits	0	0	0.10*	--	---
	0	32	0.66	100	6.6
9,12	100	"	0.28	42	3.9
	400	"	0.32	48	2.7
Calf	0	0	0.08*	--	---
	0	23	0.45^{A}	100	6.7^{BC}
15	1000	"	0.23^{E}	51	2.5^{F}
	500	"	0.29^{D}	64	3.6^{E}
	250	"	0.30^{D}	67	3.7^{E}
	50	"	0.31^{CD}	69	4.1^{DE}
	10	"	0.35^{BC}	78	4.9^{D}
	5	"	0.39^{BC}	87	6.1^{C}
	2	"	0.44^{A}	98	7.4^{AB}
	0.2	"	0.45^{A}	100	7.9^{A}
Sheep	0	0	0.05*	--	---
	0	26	0.39	100	5.3
13, 14	1000	"	0.40	103	4.1
	500	"	0.38	97	4.4
	50	"	0.16	41	1.8
	10	"	0.22	56	2.1
	2	22	0.35	90	4.0
	0.2	"	0.41	105	4.9

* = Endogenous vitamin A in the tissue

Averages with superscripts of same number represent a homogenous group.

Capital letter for p = 0.01.

TABLE 14

Effect of adding different levels of NO_3^- to the incubation m media on vitamin A formation from B-carotene by duodenal homogenates from depleted animals

Tissue source and expt. no.	NO_3^- added/ g tissue	Carotene incubated/ g tissue	Vitamin A formed		
			per g tissue		
				activity	as % of carotene unrecovered
	u moles	ug	ug	%	%
Rabbits 9	0	0	0.04*	--	---
	0	59	0.96	100	5.4
	650	"	0.34	35	1.9
Pigs 40,41	0	0	0.11*	--	---
	0	14	0.32	100	3.0
	1000	"	0.19	59	1.7
	50	"	0.23	72	2.1
Calf 8,10, 20-a	0	0	0.02*	--	---
	0	26	0.59^{A}	100	8.0^{A}
	1000	"	0.08^{D}	14	0.8^{C}
	500	"	0.20^{C}	34	1.5^{C}
	50	"	0.19^{C}	32	1.3^{C}
	10	30	0.34^{B}	58	3.0^{BC}
	5	"	0.55^{A}	93	5.6^{AB}
	2	"	0.55^{A}	93	5.7^{AB}
	0.2	"	0.53^{A}	90	5.5^{AB}

* = Endogenous level of vitamin A in the tissue

Averages with superscripts of same number represent a homogenous group.

Capital letter for p = 0.01.

slightly more than in normal animals. The efficiency of vitamin A formation was 33 - 66% of control. In the case of depleted pigs, addition of 1000 u moles NO_3^-/g tissue reduced the activity to 59% of control and 50 u moles NO_3^- reduced the activity to 72% of control.

A marked difference in activity was noticed between depleted and normal calves when higher levels of NO_3^- were added to the intestinal homogenates. With tissue from depleted calves (Table 14) addition of high levels (50 - 1000 u moles/g tissue) of NO_3^- to the incubating media reduced vitamin A formation (activity) to 14 - 34% of control values. On the other hand with tissue from normal calves similar amounts of NO_3^- reduced the activity only to 51 - 69% of control values (Table 13). The efficiency of vitamin A formation was decreased to a greater extent using tissue from depleted calves than in tissue from normal calves when the higher levels of NO_3^- were added to the incubating media ($p = 0.01$). The addition of 10 u moles of NO_3^- to the incubating media decreased the amount and efficiency of vitamin A formation more for tissue from depleted than from normal calves ($p = 0.05$). The addition of 0.2 - 5.0 u moles of NO_3^- reduced activity to about the same extent in tissue from depleted and normal calves. However, this low level of NO_3^- reduced efficiency in tissue from depleted animals (Table 14) but actually increased efficiency in tissues from normal animals (Table 13).

The data for sheep (Table 13) did not show the same trends as the other species. High and low NO_3^- levels had little effect but the intermediate level (10 - 50 ug/g) reduced extent and efficiency of conversion. No explanation for this discrepancy can be given at this time.

The addition of higher levels of NO_3^- to the incubating media reduced the percent of carotene recovered from 77 down to 68% and from 70 down to about 63% in the case of normal rabbits and calves, respectively. On the other hand with the tissues from depleted animals these amounts of NO_3^- reduced the percent of carotene recovered to about 55% compared to 70% for the control tissue.

Addition of NO_3^- to the incubating media containing intestinal homogenates from rabbits, calves and pigs produced a decrease in the extent and efficiency of vitamin A formation from carotene. In general, the level of NO_3^- added and the reduction of activity and efficiency of conversion were positively related. Of special interest was the observation that levels of NO_3^- from 10 to 1000 u moles/g tissue adversely affected the extent and efficiency of vitimin A formation to a greater extent in depleted calves than in normal calves.

Comparison of inhibitory effect of NO_3^- and NO_2^- in vitro

The results on the comparative effects of NO_3^- and NO_2^- added to the in vitro system are shown in Table 15. The statistical significance of these results is also shown in

TABLE 15

Comparison of the inhibitory effect of NO_3^- and NO_2^- on vitamin A formation from B-carotene by duodenal homogenates

Tissue source and expt. no.	NO_3^- or NO_2^- added/g tissue	Carotene incubated/ g tissue	Vitamin A formed per g tissue		Vitamin A formed as % of carotene unrecovered
				activity	
	u moles	ug	ug	%	%
Rabbits 3, 5	0	0	0.16*	--	---
	0	46	1.60	100	11.9
	340 NO_3	"	0.51	32	5.6
	34 NO_3	"	0.62	39	6.4
	500 NO_2	"	0.44	28	4.6
	50 NO_2	"	0.49	31	4.6
Bull 4	0	0	0.06*	--	---
	0	44	0.93^{A}	100	11.9^{A}
	400 NO_3	"	0.54^{B}	63	8.6^{B}
	40 NO_3	"	0.63^{B}	68	8.4^{B}
	540 NO_2	"	0.61^{B}	66	9.0^{B}
	54 NO_2	"	0.61^{B}	66	9.3^{B}

* = Endogenous level of vitamin A in the tissue

Averages with supercripts of same number represent a homogenous group.

Capital letter for p = 0.01.

the table. The results with rabbit tissue indicated that the addition of 340 u moles NO_3^- or 500 u moles NO_2^-/ tissue reduced the activity (vitamin A formed/g tissue) to 32 or 28% of control whereas 34 u moles NO_3^- or 50 u moles NO_2^- reduced the activity to 39 or 31% of control, respectively. Activity was reduced to about 66% of control when 40 - 400 u moles NO_3^- or 54 - 540 u moles NO_2^-/g tissue were added to incubating media containing bull duodenal homogenates (Experiment 4). There was little difference in the effect of NO_3^- or NO_2^- in the efficiency of vitamin A formation and the recovery of carotene. However species difference was observed. The efficiency of vitamin A formation and the recovery of carotene were greater in tissue from bulls than in tissue from rabbits after NO_3^- and/or NO_2^- addition ($p = 0.01$). Both NO_3^- and NO_2^- had approximately the same inhibitory effect on the formation of vitamin A from carotene by duodenal homogenates.

Effect of feeding NO_3^- on vitamin A and carotene levels in blood and liver

The amounts of carotene and vitamin A in blood plasma and liver of rabbits fed NO_3^- are summarized in Table 16. The data indicated that there was less carotene in the blood plasma and liver of rabbits fed 2% dietary NO_3^- than in those fed either 0.3, o.5% or no NO_3^-. The statistical significance varied with the each experiment as indicated in Table 16. When the rabbits received a carotene low ration the addition

TABLE 16

Effect of dietary NO_3 on carotene and vitamin A in blood plasma and liver of rabbits fed two different diets

Dietary treatment	Carotene		Vitamin A	
	Blood plasma	Liver	Blood plasma	Liver
	u/100 ml	ug	u/100 ml	ug
Experiment 9				
Deficient ration	6.3[a] (1.8)	25.7 (10.7)	0.60 (0.02)	19.9 (4.8)
" + 0.3% NO_3^-	3.3[a] (1.1)	55.5 (27.0)	0.37 (0.33)	20.9 (4.1)
" + 2.0% NO_3^-	0.5[b] (0.7)	16.8 (14.7)	0.09 (0.01)	12.9 (9.0)
Experiment 12				
Normal ration	26.7[a] (5.5)	160.0[a] (19.0)	27.6[Aa] (1.3)	1872 (297)
" + 0.5% NO_3^-	8.9[b] (10.2)	37.0[b] (36.0)	17.1[Bb] (2.1)	1056 (539)
" + 2.0% NO_3^-	4.0[b] (3.5)	21.0[b] (20.0)	12.9[Bc] (1.4)	359 (87)

Averages with superscripts of same number represent a homogenous group.

Lower case for p = 0.05.
Capital letter for p = 0.01.

Figures in parenthesis represent standard deviations

of either 0.3 or 2.0% NO_3^- reduced the carotene level of blood plasma as compared to control animals. Addition of 2.0% NO_3^- to the diet of rabbits reduced the total liver carotene as compared to control animals (Experiment 9). The total liver carotene in rabbits fed 0.3% NO_3^- ration was more than either in control animals or in animals fed 2.0% NO_3^-. When rabbits were fed normal ration, the addition of NO_3^- (0.5 or 2.0%) reduced both the blood concentration of carotene and total liver carotene stores ($p = 0.01$, Experiment 12).

The decrease in plasma carotene concentration was more when 0.5% NO_3^- was added to a normal ration than when 0.3% NO_3^- was added to a low carotene ration. When 2.0% NO_3^- was fed the percentage decrease was more with the normal diet than with the low carotene diet.

There was less vitamin A in blood plasma of rabbits fed 2.0% NO_3^- than in those fed the lower levels of NO_3^- and less in those fed 0.3 or 0.5% NO_3^- than in those fed no NO_3^-. The statistical significance of the difference is indicated by the superscript letters following the average values in Table 16. In experiment 12 vitamin A levels in blood were reduced significantly ($p = 0.01$) by feeding 0.5 or 2.0% NO_3^- in the diet. The differences in blood plasma values between the 0.5 and 2.0% NO_3^- diet were significant at $p = 0.05$ level.

The liver storage of vitamin A was decreased more by

2.0% NO_3^- in the diet than by 0.3 or 0.5% NO_3^-. The low level of NO_3^- produced a lower liver storage of vitamin A than was found in the animals fed no NO_3^-. The statistical significance of this difference is indicated in Table 16. The values given for liver in this table are ug of vitamin A / liver and not merely concentration (ug vitamin A/g liver). Differences between total liver storage and concentration were similar but the latter were somewhat more homogenous within each treatment group. Concentrations of vitamin A in the livers of rabbits fed 0.5, 2.0% and no NO_3^- were 23, 17 and 27 ug/g for experiment 12. Each value was different from any other value at P = 0.05.

Effect of feeding NO_3^- or NO_2^- on in vitro vitamin A formation by tissues

Vitamin A formation from carotene was studied to assess the effect of dietary NO_3^- or NO_2 using duodenal homogenates from (1) rabbits fed normal ration with and without NO_3^-, (2) rabbits fed deficient ration with and without NO_3^-, (3) calves fed a deficient ration with and without NO_3^-, and (4) yearling heifers fed a deficient ration with and without NO_2^-. The results are presented in Table 17. The statistical significance of the difference is indicated by the superscript letters following the average values. The data indicated that duodenum from all animals fed either NO_3^- or NO_2^- converted carotene to vitamin A, but to a smaller degree than control animals not fed either NO_3^- or NO_2^-. All vitamim

TABLE 17

Extent of formation of vitamin A from B-carotene by duodenal homogenates of animals fed deficient or normal ration with and without added nitrate or nitrite

Tissue source and expt. no.	Ration	Carotene incubated/ g tissue	Vitamin A formed per g tissue		
				activity	as % of carotene unrecovered
		ug	ug	%	%
Rabbits 9	Deficient diet	60	0.96 a	100	5.4 a
	" + 0.3% NO_3^-	49	0.53 b	55	8.3 a
	" + 2.0% NO_3^-	74	0.46 b	48	5.0 a
Calves 8,10,11	Deficient diet	26	0.59 a	100	8.0 A
	" + 1% NO_3^-	24	0.26 b	44	3.4 B
Heifer 6	Deficient diet	56	0.85 A	100	8.6 A
	" + 13g NO_2/ cwt	56	0.38 B	44	5.6 B
Rabbits 12	Normal diet	27	0.57 Ba	100	6.4 B
	" + 5.0% NO_3^-	"	0.39 Bb	68	5.0 C
	" + 2.0% NO_3^-	"	0.31	54	3.3

Averages with superscripts of same number represent a homogenous group.

Lower case for p = 0.05.

Capital letter for p = 0.01.

A values shown in the table were corrected for endogenous vitamin A. The addition of 0.3 or 0.5% NO_3^- to dificient and normal rabbits reduced the activity (vitamin A formed/g tissue) to 55 and 68% of respective control animals not fed NO_3^-. Similarly the addition of 2.0% NO_3^- in the diet reduced the activity to 48% and 54%, respectively. Addition of dietary NO_3^- at 1% level to calves and NO_2^- at 13 g/CWt to yearling heifers reduced the activity of the duodenal homogenates to 44% of respective control tissues from animals not fed either NO_3^- or NO_2^-. In all trials the feeding of NO_3^- or NO_2^- caused the tissue to form less vitamin A than the same tissue from non-NO_3^- fed animals.

The percentage of carotene recovered was similar in the animals fed NO_3^- or not fed NO_3^-. The efficiency of vitamin A formation decreased with the amount of NO_3^- fed except with the rabbits in experiment 9. In the experiment 9, the amount of tissue obtained from the rabbits was less than anticipated. Hence this insufficient tissue changed the carotene : tissue ratio. In experiment 12 these difficulties were foreseen and planned for. The efficiency of vitamin A formation was considerably reduced by feeding NO_2^- to heifers (Experiment 6). The degree of inhibition in the tissue was more in the calves fed NO_3^- than in the rabbits fed NO_3^-.

Effect of adding NO_3^- to intestinal homogenates from animals fed NO_3^- or NO_2^-

Vitamin A formation was studied when different levels of NO_3^- were added *in vitro* to intestinal homogenates from rabbits in experiment 9 and 12. The results are summarized in Table 18. The experiment was planned to add equal quantities of NO_3^- to all incubation mixtures in experiment 9. However this was not accomplished because the tissue obtained was less than anticipated in some cases. The urgency of continuing the experiment without delay to get maximum and comparable activity outweighed the anticipated difference in NO_3^- : tissue ratio. In experiment 12 these difficulties were foreseen and planned for. The previous results (Tables 13, 14) indicated that the inhibitory effect produced by 50 u moles NO_3^- was similar to that produced by 500 u moles NO_3^-.

The vitamin A formed was corrected for endogenous vitamin A. The addition of 645 u moles NO_3^-/g tissue to the incubating media containing intestinal homogenates from animals fed the deficient ration produced a greater decrease in activity than did a level of 403 u moles NO_3^-/g tissue from animals fed normal ration (35 Vs 56%, $p = 0.15$). Similarly, the same type of trand was observed when the results were expressed as efficiency of vitamin A formation. The addition of 538 - 808 u moles NO_3^-/g tissue to intestinal homogenates from depleted rabbits fed no NO_3^-, 0.3% NO_3^- or 2.0% NO_3^-

TABLE 18

Effect of adding different levels of NO_3^- to the incubation medium on vitamin A formation from B-carotene by intestinal homogenates from rabbits fed NO_3^-

Expt No.	Treatment	NO_3^- added/g tissue	Carotene incubated/ g tissue	Vitamin A formed per gm tissue		As % of carotene unrecovered
				Activity		
		u moles	ug	ug	%	%
9*	Deficient diet + no NO_3^-	0	59	0.96^{A}	100	5.4
"	"	645	"	0.34^{B}	35	1.9
"	Deficient diet + 0.3% NO_3^-	0	49	0.53^{Ba}	100	8.3
"	"	538	"	0.32^{B}	60	2.8
"	Deficient Diet + 2.0% NO_3^-	0	74	0.46^{B}	100	5.0
"	"	808	"	0.38^{B}	82	2.2
12**	Normal diet + no NO_3^-	0	27	0.57^{A}	100	6.4
"	"	403	"	0.32^{Ba}	56	2.6
"	"	101	"	0.32^{Ba}	56	2.5
"	Normal diet +0.5% NO_3^-	0	"	0.39^{C}	100	5.0
"	"	403	"	0.30^{Ba}	76	3.3
"	"	101	"	0.30^{Ba}	76	3.1
"	Normal diet +2.0% NO_3^-	0	"	0.31^{Ba}	100	3.3
"	"	403	"	0.27^{Bb}	86	2.6
"	"	101	"	0.28^{Bb}	90	2.8

* = Endogenous vitamin A level was 0.04 ug.
** = Endogenous vitamin A level was ranged from 0.02 to o.15 ug.

reduced the activity to 35, 60 and 82% of controls, respectively. When 100 - 400 u moles NO_3^-/g tissue were added to intestinal homogenates from normal rabbits fed no NO_3^-, 0.5% NO_3^- or 2.0% NO_3^- the activities were 56, 76 and 86 - 90% of control. The efficiency of vitamin A formation had a similar trend.

In general, a study of data in Table 18 revealed that the addition of high levels of NO_3^- to intestinal homogenates from the depleted or normal animals produced a larger decrease in vitamin A formation than did equivalent NO_3^- levels to homogenates from the above animals fed NO_3^- ($p = 0.01$). Furthermore, the inhibitory effect of NO_3^- added to in vitro system was more pronounced in depleted animals than in normal animals.

The effects of adding various levels of NO_3^- to the incubating media containing duodenal homogenates from a calf fed NO_3^- and two yearling heifers fed NO_2^- are summarized in table 19. The addition of low levels (0.2 - 20 u moles/g tissue) of NO_3^- to the incubating media containing homogenates from a depleted calf fed 1.0% NO_3^- was without an appreciable effect on extent and efficiency of vitamin A formation. The addition of 480 u moles NO_3^-/g tissue to duodenal homogenates from heifers fed the deficient ration and the deficient ration plus NO_2^- reduced the activity of duodenal homogenates to 53 and 74%, respectively. Similarly the addition of 480 u moles NO_3^- to the duodenal homogenates

TABLE 19

Effect of adding different levels of NO_3^- to the incubating media on vitamin A formation from B-carotene by duodenal homogenates from calves and heifers fed NO_3^- or NO_2^-

Tissue source and expt. no.	Treatment	NO_3^- added/ g tissue	Carotene incubated/ g tissue	Vitamin A formed per g tissue		
					activity	as % of carotene unrecovered
		u moles	ug	ug	%	%
Calf 11	Deficient diet + 1% NO_3	0	0	0.01*	--	---
	"	0	24	0.26	100	3.4
	"	20	"	0.24	92	2.5
	"	10	"	0.26	100	3.0
	"	5	"	0.26	100	2.9
	"	2	"	0.25	95	3.1
	"	0.2	"	0.20	100	3.3
Yearling heifer 6	Deficient diet + no NO_2	0	0	0.00*	--	---
	"	0	56	0.85	100	8.6
	"	480	"	0.45	53	4.9
	Deficient diet + 13 g NO_2/cwt	0	0	0.00*	--	---
	"	0	0	0.38	100	5.3
	"	480	0	0.28	74	3.9

* = Endogenous level of vitamin A in the tissue

from NO_2^- fed heifers reduced the efficiency of vitamin A formation to 75% of control, whereas the addition of the same amounts of NO_2^- to the duodenal homogenates from the heifer not fed NO_2^- reduced the efficiency to 58% of control. The results with yearling heifers tended to support the findings with rabbits (Table 18) that the addition of high levels of NO_3^- to the in vitro system produced a greater decrease in vitamin A formation in tissue from those not fed NO_3^- than did equivalent NO_3^- levels in the media containing duodenal homogenates from animals fed NO_3^- (in this case NO_2^-).

THYROID STATUS AND VITAMIN A FORMATION

Effect of adding L - thyroxine (L - T_4) and L - triiodothyronine (L - T_3) to in vitro system

Experiments were conducted to study the effect on vitamin A formation of adding high levels of L-thyroxine (L-T_4) and L-triiodothyronine (L-T_3) to duodenal homogenates from depleted calf. The results are shown in Table 20. Addition of 0.125 to 6.25 ug/g tissue of either L-T_4 or L-T_3 reduced the amount of vitamin A formed/g tissue from 0.41 ug to 0.19 to 0.27 ug ($p = 0.01$). This amounted to a reduction in activity to 46 to 66% of control. Accompanying this reduction in vitamin A formation was a marked decrease in efficiency of vitamin A formation as compared to control (1.9 to 2.5% Vs 12.7%, $p = 0.01$).

TABLE 20

Effect of adding high levels of L-thyroxine ($L\text{-}T_4$) and L-triiodothyronine ($L\text{-}T_3$) on vitamin A formation from B-carotene by duodenal homogenates from a carotene depleted calf (Expt. 8)

$L\text{-}T_4$ or $L\text{-}T_3$ added/ g tissue	Carotene incubated/ g tissue	Vitamin A formed		
		per g tissue		as % of carotene unrecovered
			activity	
ug	ug	ug	%	%
0	0	0.00*	<-	-----
0	26	0.41	100	12.7
6.250 $L\text{-}T_4$	"	0.22	52	1.9
0.625 "	"	0.24	59	2.2
0.125 "	"	0.24	59	2.5
6.250 $L\text{-}T_3$	"	0.27	66	2.0
0.625 "	"	0.21	51	2.0
0.125 "	"	0.19	46	2.0

* = Endogenous vitamin A in tissue

In experiments 38 and 39, the duodenal homogenates from normal steers were incubated with low levels of L-T_4 and L-T_3. The effect of such levels on vitamin A formation from carotene are summarized in Table 21. Vitamin A values presented in this table were corrected for endogenous vitamin A. The addition of 0.1 and 0.01 ug L-T_4/g tissue to the duodenal homogenates reduced the vitamin A formation (per cent of activity) to 82 - 84% of control, whereas the efficiency of vitamin A formation was reduced slightly (4.6 - 5.1% Vs 6.8%). Similar results were obtained by adding 0.1, and 0.001 ug L-T_3/g tissue to the duodenal homogenates. When 0.001, 0.0001 and 0.00001 ug L-T_4/g tissue or 0.0001 and 0.00001 ug L-T_3/g tissue were added to the duodenal homogenates, the activity and efficiency of vitamin A formation were somewhat similar to the control. The differences between values are not statistically significant at 5% level.

Effect of T_4 analogue on vitamin A formation

In search of an explanation for the reduced vitamin A formation by high levels of L-T_4 and L-T_3, an experiment was conducted to determine whether a T_4 analogue such as 3 5′ 3′ L-triiodothyronine (3 5′ 3′ L-T_3) had any effect on vitamin A formation from carotene. The results of such an experiment using both liver and duodenal homogenates from depleted calf are presented in Table 22. The analysis of variance of this data is shown in Appendix Table 11. When

TABLE 21

Effect of adding low levels of L-T_4 and L-T_3 on vitamin A formation from B-carotene by duodenal homogenates from steers (Expt. 38, 39)

L-T_4 or L-T_3 added/ g tissue	Carotene incubated/ g tissue	Vitamin A formed per g tissue		
			activity	as % of carotene unrecovered
ug	ug	ug	%	%
0	0	0.11*	--	---
0	17	0.44	100	6.8
0.1 L-T_4	"	0.36	82	4.6
0.01 L-T_4	"	0.37	84	5.1
0.001 L-T_4	"	0.43	98	6.1
0.0001 L-T_4	"	0.42	95	6.4
0.00001 L-T_4	"	0.43	98	6.5
0.1 L-T_3	"	0.38	86	5.0
0.01 L-T_3	"	0.39	89	5.9
0.001 L-T_3	"	0.39	89	5.9
0.0001 L-T_3	"	0.45	102	6.4
0.00001 L-T_3	"	0.45	102	6.4

* = Endogenous level of vitamin A in the tissue

TABLE 22

Effect of adding isomers of L-T_4 and L-T_3 and combinations of them with 3 5' 3' L-T_3 on vitamin A formation from B-carotene* by duodenal and liver homogenates from depleted calf (Expt. 42)

L-T_4 or T_3 added/g tissue	3 5'3' L-T_3 added/g tissue	Vitamin A formed					
		Duodenum			Liver		
		per g tissue		as % of carotene unrecovered	per g tissue		as % of carotene unrecovered
			activity			activity	
ug	ug	ug	%	%	ug	%	%
0	0	0.03**	--	---	0.04**	--	---
0	0	0.67	100	11.4	0.62	100	11.8
0.01 D-T_4	0	0.45	67	7.5	0.45	73	8.1
" L-T_4	0	0.45	67	7.6	0.49	70	7.4
" D-T_3	0	0.48	72	7.7	0.47	76	6.3
" L-T_3	0	0.48	72	8.0	0.49	79	6.7
0	10	0.39	58	5.5	0.36	58	5.1
0.01 D-T_4	"	0.46	69	7.4	0.47	76	6.5
" L-T_4	"	0.45	67	7.6	0.47	76	6.3
" D-T_3	"	0.44	66	6.3	0.48	77	6.0
" L-T_3	"	0.45	67	6.0	0.47	76	6.0

* = 23 ug of carotene incubated/g tissue
** = Endogenous level of vitamin A in the tissue

0.01 ug of either D-T_4 of D-T_3/g tissue was added to the duodenal and liver homogenates, the extent and efficiency of vitamin A formation was similar to their L-isomers. All vitamin A values were corrected for endogenous vitamin A. An addition of 10 ug of 3 5′ 3′ L-T_3/g tissue to liver as well as duodenal homogenates produced a marked reduction of vitamin A formation to 58% of control. In addition 3 5′ 3′ L-T_3 reduced the efficiency of vitamin A formation from 11% down to 5%. On the other hand, when D-T_4, L-T_4, D-T_3 and L-T_3 were added in combination with 3 5′ 3′ L-T_3 to the duodenal and liver homogenates the activity (vitamin A formed/g tissue) was somewhat higher than the activity observed after incubating with 3 5′ 3′ L-T_3 alone. The activity was increased by such treatment to about 68% from 58% in the case of duodenal homogenates and to about 76% from 58% in the case of liver homogenates.

Interrelationship between thyroid status of animals and vitamin A formation

In another trial duodenal homogenates from hyperthyroid, hypothyroid and control calves and rabbits were incubated with a carotene suspension to elucidate the effect of thyroid status on vitamin A formation from carotene in vitro. The results are summarized in Table 23. The results indicated that there was a relationship between thyroid status and the ability of animals to convert carotene to vitamin A. The avility of duodenal homogenates from

TABLE 23

Effect of thyroid status of calves and rabbits on vitamin A formation from B-carotene by duodenal homogenates (Expts. 22-27, 29-32)

Thyroid status of animal	Vitamin A formed							
	Calves				Rabbits			
	Endogenous/g tissue	per g tissue		as % of carotene unrecovered	Endogenous/g tissue	per g tissue		as % of carotene unrecovered
			activity				activity	
	ug	ug	%	%	ug	ug	%	%
Control	0.11	0.63^{A}	100	10.5^{A}	0.09	1.13^{a}	100	14.9
Thyroprotein	0.09	0.62^{A}	98	11.0^{A}	0.13	1.15^{a}	102	11.3^{A}
Thiouracil	0.07	0.42^{B}	67	5.0^{B}	0.09	0.73^{b}	65	9.1^{BCb}
Thyroidectomized	----	----	--	----	0.17	0.71^{b}	63	8.6^{Cb}

Averages with superscripts of same number represent a homogenous group.

Lower case for p = 0.05.

Capital letter for p = 0.01.

hyperthyroid and control calves to form vitamin A from carotene was approximately equal. Similar results were obtained when duodenal homogenates from hyperthyroid and control rabbits were incubated with carotene. On the other hand, when the duodenal homogenates from hypothyroid calves were incubated, the activity was reduced to 67% of activity of duodenal homogenates from control calves ($p = 0.01$). The efficiency of vitamin A formation was also greatly reduced from 10.5% down to 5.0% ($p = 0.01$). Duodenal homogenates from rabbits which were made hypothyroid either by feeding thiouracil or by thyroidectomy showed a depressed activity to about 64% of activity of duodenal homogenates from control animals ($p = 0.05$). The efficiency of vitamin A formation was also reduced from 15% down to about 9% ($p = 0.01$). A high percentage of the carotene incubated was destroyed when the duodenal homogenates from the hypothyroid calf were incubated with carotene.

Using duodenal homogenates from the above hypothyroid, hyperthyroid and control animals, vitamin A formation was studied as effected by L-T_4 and L-T_3 additions with and without combinations of 10 and 500 u moles NO_3^-. The results are shown in Tables 24 and 25. The results in Table 24 indicated that the addition of 0.5 and 0.1 ug of L-T_4 or L-T_3/g tissue to the duodenal homogenates of control, hyperthyroid and hypothyroid calves reduced the activity to 72 - 77%, 56 - 76% and 69 - 81%, respectively.

TABLE 24

Effect of adding L-T_4 or L-T_3 with or without nitrate on vitamin A formation by duodenal homogenates from control, hyperthyroid and hypothyroid calves and rabbits

L-T_4 or L-T_3/ g tissue	NO_3^-/g tissue	vitamin A formed						
		Calves			Rabbits			
		Control	Thyro-protein	Thio-uracil	Control	Thyro-protein	Thio-uracil	Thyroid-ectomized
ug	u moles	ug	ug	ug	ug	ug	ug	ug
0	0	0.63	0.62	0.42	1.13	1.15	0.73	0.71
0	500	0.24	0.18	0.19	0.35	0.63	0.49	0.22
0	10	0.52	0.53	0.33	1.02	1.01	0.66	0.64
0.5 L-T_4	0	0.48	0.40	0.31	1.01	1.05	0.64	0.69
0.1 "	0	0.48	0.47	0.35	0.97	0.99	0.66	0.64
0.5 L-T_3	0	0.45	0.42	0.30	1.05	1.01	0.66	0.66
0.1 "	0	0.49	0.47	0.30	1.05	1.01	0.66	0.60
0.5 L-T_4	500	0.35	0.21	0.20	0.44	0.66	0.60	0.42
0.1 L-T_4	500	0.32	0.23	0.19	0.48	0.61	0.58	0.36
0.5 "	10	0.46	0.42	0.31	1.01	0.99	0.66	0.52
0.1 "	"	0.44	0.38	0.32	----	----	----	----
10 mg thio-uracil	0	----	0.30	----	----	0.23	0.39	----
Thyroid gland*	0	0.13	0.16	0.20	----	----	----	----
Adrenal gland*	0	0.08	0.26	----	----	----	----	----

* = Thyroid gland and adrenal gland were obtained from animal, the tissues of which were used to test the activity

TABLE 25

Effect of adding L-T_4 or L-T_3 with or without nitrate on the efficiency of vitamin A formation from B-carotene by duodenal homogenates from control, hyperthyroid and hypothyroid calves and rabbits

L-T_4 or L-T_3/ g tissue	NO_3^-/g tissue	Vitamin A formed as % carotene unrecovered						
		Calves			Rabbits			
		Control	Thyro-protein	Thio-uracil	Control	Thyro-protein	Thio-uracil	Thyroid-ectomized
ug	u moles	%	%	%	%	%	%	%
0	0	10.9	11.0	5.0	14.9	11.3	9.1	8.6
0	500	3.3	2.6	1.6	3.0	4.3	3.3	1.9
0	10	7.7	9.6	3.5	12.0	8.6	5.4	7.2
0.5 L-T_4	0	7.8	6.2	3.6	12.0	8.6	5.2	10.2
0.1 "	0	8.2	8.1	4.1	11.9	8.6	5.6	7.6
0.5 L-T_3	0	8.3	6.9	3.6	12.3	8.2	5.5	9.5
0.1 "	0	7.6	6.8	3.6	11.7	8.4	5.3	7.0
0.5 L-T_4	500	4.6	2.8	2.0	4.4	5.2	4.3	4.8
0.1 "	"	4.6	3.0	2.1	4.5	5.2	4.5	3.2
0.5 "	10	7.6	7.0	3.4	11.5	9.0	5.6	3.1
0.1 "	"	6.8	4.3	3.6	----	---	---	---
10 mg thio-uracil	0	---	3.5	---	----	1.6	1.9	---
Thyroid gland	0	1.3	1.6	1.4	----	---	---	---
Adrenal gland	0	0.7	2.7	---	----	---	---	---

When 10 u moles NO_3^-/g tissue were added to duodenal homogenates from the above animals activity was reduced to 83, 86 and 78% of control in the three groups of animals, respectively (Table 24). Addition of 500 u moles NO_3^- to the incubation mixture reduced the activity to about 34, 29 and 45% of control in the three groups of animals, respectively. When 10 u moles NO_3^- + 0.5 or 0.1 ug L-T_4 were added, the activity was slightly reduced as compared to either NO_3^- alone or L-T_4 alone. Incubation of 500 u moles NO_3^- + 0.5 or 0.1 ug L-T_4 with duodenal homogenates of the above animals increased the activity of vitamin A formation compared to the activity obtained by adding the same level of NO_3^- alone, but slightly reduced the activity compared to the activity produced by same level of L-T_4 alone. The data also indicated that the addition of approximately 1 g of homogenized normal thyroid gland to the duodenal homogenates reduced the activity markedly in control and hyperthyroid animals to about 25% of control whereas in hypothyroid animals this addition reduced the activity to about 50%. Approximately a 50% inhibition of vitamin A formation was observed by the addition of 10 mg thiouracil to incubating media, containing duodenal homogenates from hyperthyroid calves. The addition of 0.5 g of homogenized adrenal gland to incubating media containing duodenal homogenates from control and hypothyroid animals reduced the activity to about 13 and 42%, respectively.

Similar results were obtained when the intestinal homogenates from control, hyperthyroid, hypothyroid and thyroidectomized rabbits were incubated with various combinations of L-T_4 and NO_3^- (Table 24). There was one minor difference between hypothyroid and thyroidectomized rabbits. The addition of 500 u moles NO_3^-/g tissue to duodenal homogenates reduced the activity of vitamin A formation in thyroidectomized rabbits to 31% whereas in hypothyroid rabbits this addition reduced activity to about 55% of control. Another point of interest observed with rabbits was that the addition of thiouracil to the <u>in vitro</u> system reduced vitamin A formation more in the system containing duodenal homogenates from hyperthyroid animals than in hypothyroid animals. An analysis of variance of the data (Table 24) is shown in Appendix table 111.

The data in Table 25 presents the efficiency of vitamin A formation from the above treatments. A study of data in this table revealed the similar effects as indicated in Table 24. Addition of 500 u moles NO_3^-/g tissue to the incubating mixture containing duodenal homogenates reduced the efficiency of vitamin A formation more in the control and hyperthyroid animals than in the hypothyroid animals ($p = 0.01$). The addition of L-T_4 to the <u>in vitro</u> system containing 500 u moles NO_3^-/g of duodenal homogenates from the above animals slightly retarded the reduced efficiency produced by the addition of 500 u moles NO_3^- alone.

Effect of amino acids with and without L-T_4 on vitamin A formation

In most experiments a considerable reduction in vitamin A formation occurred when high levels of L-T_4 were added to the in vitro system containing duodenal homogenates. This decrease in activity by high levels of L-T_4 indicated that L-T_4 probably acted either as a tissue inhibitor or in some other manner to reduce the conversion of carotene to vitamin A. Barker (181) and Lindsay and Barker (184) found that the Qo_2 of kidney slices in vitro was considerably increased by the addition of certain amino acids (DL-alanine and L-proline) plus L-T_4 when compared to L-T_4 alone. DL-alanine, L-proline or L-tryptophan at final concentrations of 2×10^{-2} M with and without 0.01 ug L-T_4/g tissue were added to incubating media containing duodenal homogenates from steers. The results are shown in Table 26. The addition of 0.01 ug L-T_4/g tissue reduced the activity of vitamin A formation to 68% of control whereas the addition of 2×10^{-2} M of DL-alanine and L-proline each were without effect in reducing vitamin A formation. However, 2×10^{-2} M L-tryptophane reduced the activity to 82% of control. When DL-alanine and L-proline were added separately to the incubation media containing duodenal homogenates and 0.01 ug L-T_4/g tissue, the activity of vitamin A formation was increased from 68% to 79 - 83% of control. L-tryptaphane did not appreciably change the inhibitory effect of L-T_4. The data

TABLE 26

Effect of adding different amino acids with and without the combination of L-thyroxine on vitamin A formation from B-carotene by duodenal homogenates from steers (Expt. 55,56)

L-T_4 added/g tissue	Amino Acid in flask	Carotene incubated/ g tissue	Vitamin A formed per g tissue		As % of carotene unrecovered
				Activity	
ug		ug	ug	%	%
0	0	0	0.08*	-	-
0	0	14	0.51	100	9.3
.01	0	"	0.35	68	6.4
0	2×10^{-2} M Dl-alanine	"	0.50	98	9.4
0	2×10^{-2} M L-proline	"	0.45	95	8.5
0	2×10^{-2} M + L-Tryptaphane	"	0.42	82	5.9
.01	2×10^{-2} M DL-alanine	"	0.42	83	7.3
"	2×10^{-2} M L-proline	"	0.40	79	6.4
"	2×10^{-2} M + L-Tryptaphane	"	0.37	72	4.3

* = Endogenous level of vitamin A in tissues

also indicated that L-tryptaphane increased the destruction of carotene in vitro and thereby reduced the efficiency of vitamin A formation to 4.3% compared to 9.3% for control. An analysis of variance of the data (Table 26) is shown in Appendix table IV.

The data indicate that DL-alanine and L-proline reduced the inhibitory effect of L-T_4 at the tissue level.

EFFECT OF IONS AND ENZYME INHIBITORS

The results of series of experiments to determine the effect of ions such as $SO_4^=$, Cl^- and $CO_3^=$ on vitamin A formation from B-carotene by duodenal homogenates from calves are presented in Tables 27 and 28. The results in Table 27 indicated that the addition of 50-500 u moles $SO_4^=$, $CO_3^=$ as sodium salts or NaCl/g tissue to the duodenal homogenates from the normal calves reduced the activity from 100% down to 76 - 85% of control. In comparison, the addition of 500 u moles NO_3^-/g tissue reduced the activity to 27% and 10 u moles NO_3^- reduced the activity to 77% of control. Thus NO_3^- at the level of 10 u moles/g tissue had about the same inhibitory effect as 50-500 u moles of either $SO_4^=$, $CO_3^=$ or NaCl. The efficiency of vitamin A formation was 2% when 500 u moles NO_3^-/g tissue was added to duodenal homogenates compared to 6.1 to 7.1% when that level of $SO_4^=$, $CO_3^=$ or NaCl was added. The efficiency was 9.4% when none of these ions were added. Thus in this trial, all ions decreased extent

TABLE 27

Formation of vitamin A from B-carotene when various ions were added to duodenal homogenates

Tissue source and expt. no.	Ions added/g tissue	Carotene incubated/ g tissue	Vitamin A Formed		
			per g tissue		as % of carotene unrecovered
				activity	
	u moles	ug	ug	%	%
Calves 21/25, 27	0	0	0.09*	--	---
	0	22	0.66^{A}	100	9.4^{A}
	500 NO_3^-	"	0.18^{B}	27	2.0^{B}
	10 "	"	0.51^{A}	77	6.8^{A}
	500 $SO_4^=$	"	0.55^{A}	83	7.1^{A}
	50 "	"	0.55^{A}	83	6.9^{A}
	500 NaCl	"	0.55^{A}	83	6.5^{A}
	50 "	"	0.55^{A}	83	6.2^{A}
	500 $CO_3^=$	"	0.50^{A}	76	6.1^{A}
	50 "	"	0.56^{A}	85	6.9^{A}

* = Endogenous vitamin A level in the tissue

Averages with superscripts of same number represent a homogenous group.

Capital letter for p = 0.01.

TABLE 28

Formation of vitamin A from B-carotene when various ions were added to duodenal homogenates from calves fed nitrate or thiouracil

Tissue source and expt. no.	Ions added/ g tissue	Carotene incubated/ g tissue	Vitamin A formed per g tissue		Vitamin A formed as % of carotene unrecovered
				activity	
	u moles	ug	ug	%	%
Calf fed 1% NO_3 11	0	0	0.01*	--	---
	0	24	0.26[A]	100	3.4
	20 NO_3^-	"	0.24[A]	93	2.5
	6.5 $SO_4^=$	"	0.24[A]	93	3.7
	65 "	"	0.26[A]	100	3.3
	10.7 NaCl	"	0.25[A]	96	3.3
	107 "	"	0.24[A]	93	3.3
	10.4 $CO_3^=$	"	0.26[A]	100	3.5
	104 "	"	0.25[A]	96	3.0
Calf fed thiouracil 26	0	0	0.05*	--	---
	0	16	0.44[A]	100	3.0
	500 NO_3^-	"	0.16[B]	37	1.0
	10 "	"	0.38[A]	85	2.5
	500 $SO_4^=$	"	0.43[A]	98	2.8
	50 "	"	0.40[A]	91	2.8
	500 NaCl	"	0.36[A]	82	2.5
	50 "	"	0.39[A]	87	2.6
	500 $CO_3^=$	"	0.34[A]	77	2.3
	50 "	"	0.38[A]	85	2.7

* = Endogenous vitamin A in the tissue

Explanation of superscripts is shown in Table 16.

and efficiency of vitamin A formation with NO_3^- being much more effective than $SO_4^=$, $CO_3^=$ or NaCl.

The results obtained by incubating duodenal homogenates from hypothyroid and NO_3^- fed calves with the above ions are presented in Table 28. The addition of either 10 u moles NO_3^-, 50 - 500 u moles of $SO_4^=$, $CO_3^=$ or NaCl/g tissue to duodenal homogenates from the above animals reduced the activity of vitamin A formation from 100% down to 77 - 98% of control, whereas 500 u moles NO_3^- reduced the activity to 37% of control. The efficiency of vitamin A formation was not changed appreciably except when 500 u moles NO_3^-/g tissue were added. The results obtained when duodenal homogenates from a calf fed 1.0% NO_3^- in the diet were somewhat different from the above results. Nitrate ion at the level of 20 u moles/g tissue had practically the same effect as 6.5 - 65 u moles $SO_4^=$, 10.7 - 107 u moles NaCl or 10.4 - 104 $CO_3^=$ when added to the in vitro system.

Thus considering the results as whole it appeared that $SO_4^=$, NaCl and $CO_3^=$ had a limited effect on vitamin A formation from carotene, but that NO_3^- had considerably more inhibitory effect on the extent and efficiency of vitamin A formation from carotene.

The effects of some enzyme inhibitors and protein denaturing agents on vitamin A formation from carotene by duodenal homogenates are presented in Table 29. A concentration of 10^{-5} M p-chloromercuric benzoate or 10^{-5} - 10^{-3}

TABLE 29

Effect of various inhibitors on vitamin A formation from β-carotene by duodenal homogenates from a heifer (Expt. 54)

Inhibitors	Carotene incubated/ g tissue	Vitamin A formed		
		per g tissue		as % of carotene unrecovered
			activity	
	ug	ug	%	%
None	0	0.10*	--	---
None	11	0.49	100	16.5
10^{-3} M p-chloromercuric benzoate	"	0.28	57	5.6
10^{-5} M "	"	0.38	78	10.9
10^{-3} M iodoacetate	"	0.13	27	3.6
10^{-5} M "	"	0.14	29	4.9
8 M Urea	"	0.08	16	1.6
4 M "	"	0.13	27	2.8
10^{-3} M p-dinitrophenol	"	0.38	78	12.2
10^{-5} M "	"	0.38	78	10.1
10^{-3} M Antimycin A	"	0.29	59	6.1
10^{-5} M "	"	0.33	67	7.8
10^{-3} M Hydroxylamine	"	0.34	69	7.3
10^{-5} M "	"	0.33	67	10.4
10^{-3} M KCN	"	0.06	12	1.4
10^{-5} M "	"	0.10	20	2.9

* = Endogenous level of vitamin A in the tissue

M p-dinitrophenol reduced the activity of vitamin A formation from 100% down to 80% of control, whereas 10^{-3} M p-chloromercuric benzoate, 10^{-5} M - 10^{-3} M antimycin A and 10^{-5} - 10^{-3} M hydroxylamine reduced the activity to 60 - 70% of control. A concentration of 4 - 8 M urea, 10^{-5} - 10^{-3} M iodoacetate and 10^{-5} - 10^{-3} M potassium cyanide reduced activity to 12 - 30% of control when added to duodenal homogenate. The efficiency of vitamin A formation was also reduced in about the same order by these inhibitors as indicated by the fact that 10^{-3} M potassium cyanide in the in vitro system reduced the efficiency of vitamin A formation to 1.4% compared to 16.5% for control. The finding that respiratory poisons of tissue such as potassium cyanide greatly reduced the vitamin A formation indicated that the cytochrome oxidase or a similar system may be involved in the formation of vitamin A from carotene. The fact that iodoacetate considerably reduced the formation of vitamin A revealed that alcohol dehydrogenase or some thiol enzyme may be involved in the cleavage of carotene to vitamin A.

The effects of adding alpha tocopherol to the in vitro system containing liver or duodenal homogenates and carotene suspension are summarized in Table 30. dl-Alpha tocopherol ranging from 0 - 1000 ug/g tissue was added to the incubating media. The addition of 50 ug of dl-alpha tocopherol/g tissue to duodenal homogenates from a calf and a sheep slightly increased vitamin A formed/g tissue

to 120 - 140% control (Experiment 42, P = 0.05)(Experiment 43, p = 0.01). The percent of carotene recovered was slightly increased by adding low levels of alpha tocopherol as compared to control. The efficiency of conversion was also increased 1.5 times more than control. Liver homogenates from the calf gave the same type of trend in extent and efficiency of vitamin A formation as did duodenal homogenates.

When high levels of alpha tocopherol (500 - 1000ug/g tissue) were added, the percentage recovery of carotene and efficiency of vitamin A formation were considerably reduced (Experiment 60, p = 0.01). But the extent of vitamin A formation was not reduced. These results clearly revealed that low levels of alpha tocopherol (25 - 50 ug/g tissue) considerably increased the efficiency of vitamin A formation. At high levels (500 - 1000 ug/g tissue) alpha tocopherol was not beneficial. Furthermore, at high levels alpha tocopherol reduced the efficiency of vitamin A formation from B-carotene.

EFFECT OF BLOOD AND ITS FRACTIONS ON VITAMIN A FORMATION

Experiments with normal as well as depleted rabbits indicated that a considerable amount of carotene was destroyed when homogenates of unwashed liver which contained clots of blood was incubated with a carotene suspension.

TABLE 30

Effect of dl-alpha tocopherol on vitamin A formation from B-carotene by duodenal and liver homogenates

Tissue source and expt. no.	dl-alpha tocopherol /g tissue	Carotene		Vitamin A formed	
		incubated/ g tissue	recovered	per g tissue	as % of carotene unrecovered
	ug	ug	%	ug	%
Depleted calf 42					
Duodenum	0	0	--	0.03*	---
"	0	23	74	0.67[a]	11.4[A]
"	50	"	79	0.79[b]	16.9[B]
Liver	0	0	--	0.04*	---
"	0	23	77	0.62	11.8
"	50	"	84	0.81	21.7
Sheep 43					
Duodenum	0	0	--	0.15*	---
"	0	20	58	0.42[A]	4.9[A]
"	50	"	64	0.59[B]	8.0[B]
Steer 60					
Duodenum	0	0	--	0.10*	---
"	0	15	63	0.53	9.4[C]
"	25	"	71	0.56	12.6[A]
"	50	"	66	0.55	10.7[B]
"	100	"	67	0.49	9.7[C]
"	500	"	50	0.54	6.4[D]
"	1000	"	34	0.52	5.2[E]

* = Endogenous vitamin A in the tissue

Explanation of superscripts is shown in Table 16.

Hence these experiments were conducted to study the effect of adding blood and its fractions on vitamin A formation in the in vitro system. The results are shown in Tables 31, 32 and 33.

The extent of vitamin A formed and carotene recovered after incubating blood and its fractions with the carotene suspension are shown in Table 31. In experiment 51 when hemolyzed blood, sedimented blood cells, plasma and serum of heifers were each incubated with the carotene suspension the percentage recovery of carotene after incubation was similar in all cases, ranging from 93 to 98%. Little or no vitamin A was formed. In experiment 53, sedimented blood cells were mixed with an equal volume of water and centrifuged at 27000 XG. The resulting clear supernatant solution and the sediment were used as indicated in Table 31. In this experiment the percentage of carotene recovered was very low (44 - 54%) when 2 ml of hemolyzed blood, supernatant solution or sediment were incubated with the carotene suspension. Carotene recovery was highest (74%) using 2 ml plasma or 1 ml of this supernatant while recovery was intermediate (64%) using 1 ml of the sediment. The destructive action of blood was associated with the sedimented blood cells while the destructive action of plasma was very low and similar to duodenal tissue homogenates. The destructive action on carotene also depended on the quantity of supernatant solution and the sedimented blood cells used.

TABLE 31

Formation of vitamin A after incubating various blood fractions with the carotene suspension

Tissue source and expt. no.	Blood fractions incubated	Carotene		vitamin A formed/ flask
		incubated/ flask	recovered	
		ug	%	ug
Heifer	2 ml hemolyzed	109	94.2	-0.02
51	" sedimented blood cells	"	98.2	-0.08
	" plasma	"	98.2	0.10
	" serum	"	92.6	0.00
Steer	2 ml hemolyzed blood	73	54.9	0.46
53	" plasma	"	73.1	0.40
	" supernatant*	"	48.1	0.24
	1 ml supernatant*	"	74.2	0.28
	2 ml sediment*	"	44.6	0.16
	1 ml "	"	63.7	0.02

* = Sedimented blood cells were mixed with equal volume of water and centrifuged at 27000 X G. Supernatant fluid was disignated as supernatant and sedimented portion was designated as sediment.

The extent of vitamin A formation in each incubation flask was very low. This clearly indicated that blood was probably not an important system for conversion of carotene to vitamin A and that the carotene destroying system was located in the sedimented blood cells. These results indicated that blood cells might contain factor(s) responsible for carotene destruction.

Because of the destructive action of carotene in sedimented blood cells experiments were conducted to study the effect of incubating blood and its fractions with duodenal homogenates from sheep and steers on vitamin A formation. The results are shown in Table 32. The addition of whole hemolyzed blood and sedimented blood cells to the duodenal homogenates greatly reduced carotene recovery ($p = 0.01$). More carotene was destroyed when these blood components were added to tissue homogenates than when either component was incubated separately. Addition of plasma obtained from oxalated or citrated blood and serum did not alter carotene recovery as compared to controls which contained only duodenal homogenates and the carotene suspension. Vitamin A formed per g tissue was reduced to 17 - 35% of control by the addition of whole blood, hemolyzed blood and sedimented blood cells to the duodenal homogenates. When serum and plasma were added vitamin A formation was reduced to about 80 - 90% of control. The efficiency of vitamin A formation was not materially changed from the

TABLE 32

Effect of incubating different fractions of blood with duodenal homogenates on the formation of vitamin A from carotene

Tissue source and expt. no.	Blood fraction added	Carotene incubated/ g tissue	Carotene recovered	Vitamin A formed per g tissue		Vitamin A formed as % of carotene unrecovered
					activity	
		ug	%	ug	%	%
Sheep 13	None	31	68	0.36	100	3.7
	1 ml Whole blood	"	45	0.06	17	0.4
	2 ml "	"	51	0.08	21	0.5
Steer 49,50	None	15	64	0.43	100	8.1
	2 ml Whole blood*	"	32	0.14	35	1.9
	2 Sedimented blood cells	"	34	0.14	25	1.1
	" Hemolyzed blood cells	"	31	0.11	25	1.0
	" Serum	"	62	0.34	78	6.8
	" Plasma(oxalate)	"	61	0.35	82	6.1
	" Plasma(citrate)	"	58	0.39	90	6.6

* = Value obtained from Expt. No. 49

control by the addition of either serum or plasma but when whole blood, hemolyzed blood or sedimented blood cells were added the efficiency was considerably reduced ($p = 0.01$).

Two attempts were made to wash the sedimented blood cells with water in order to remove the factor(s) responsible for the destruction of carotene and for inhibiting the formation of vitamin A. The sedimented blood cells were further centrifuged at 27,000 XG after diluting with an equal volume of water. All these fractions were added to usual incubation. The results of two such trials are presented in Table 33. The carotene destroying system was more pronounced in the sedimented portion than in supernatant solution irrespective of quantity used. There was little difference between the carotene destroying capacity of the supernatant solution, the original sedimented blood cells or the original hemolyzed blood when each was incubated with duodenal homogenates. Vitamin A formed per g of tissue was reduced to about 20% of control using sedimented blood cells or hemolyzed blood. Activity was reduced to 65 - 80% in case of the supernatant solution and to about 57% in case of sediment. The smaller amount of sediment and supernatant had less effect than did the larger amounts of these materials. The efficiency of vitamin A formation was also reduced to about the same degree. All above vitamin A values were corrected for endogenous vitamin A. The results indicated that the factor responsible

TABLE 33

Effect of incubating different fractions of blood and centrifuged sedimented cells and supernatant fluid with duodenal homogenates on the formation of vitamin A from B-carotene

Tissue source and expt. no.	Blood fraction added	Carotene		Vitamin A formed		
		Incubated/ g tissue	recovered	per g tissue	activity	as % of carotene unrecovered
		ug	%	ug	%	%
Heifer 52,53	None	11	74	0.43	100	15.0
	2 ml sedimented cells	13	44	0.10	20	1.4
	" hemolyzed blood	11	47	0.10	23	1.7
	" plasma	"	74	0.36	84	10.2
	" supernatant*	"	53	0.28	66	5.7
	1 ml "	"	58	0.32	78	8.0
	0.5 ml "	13	49	0.32	65	4.9
	0.25 ml "	"	52	0.39	80	6.4
	2 ml sediment*	11	36	0.15	36	2.2
	1 ml "	"	42	0.18	43	3.1
	0.5 ml "	13	36	0.15	31	1.9
	0.25 ml "	"	35	0.28	57	3.4

* = Sedimented cells were mixed with equal volume of water and centrifuged at 27000 X G. Supernatant fluid was designated as supernatant and sedimented portion was designated as sediment.

for inhibition of vitamin A formation and the carotene destruction may be found in the sedimented blood cells and that washing these cells with water and further centrifugation removed some of the inhibitory effect but not all of it.

VITAMIN A FORMATION BY CELL FRACTIONS OF DUODENAL HOMOGENATES

Duodenal homogenates were slightly diluted and centrifuged at 5000 XG and 25,000 XG to obtain information on distribution of the activity that forms vitamin A. The results are shown in Table 34. In experiments 57 and 58 the supernatant from the 5000 XG fraction contained the most activity for formation of vitamin A from carotene when expressed on dry matter basis. The sediment of the 15,000 and 25,000 XG fractions both contained about 89% as much activity as the sediment of the 5000 XG fraction. The supernatant portion of the 15,000 XG and 25,000 XG fractions had the least activity of all cell fractions. However even these fractions with the least activity contained more activity on a dry matter basis than the original homogenates per se.

Different results were obtained with cell fractions of rabbit duodenal homogenates in experiment 59. Here only the 15,000 XG centrifugation was performed. Unlike the results obtained in experiment 57 and 58, the supernatant

TABLE 34

Vitamin A formation from B-carotene by centrifuged fractions of duodenal homogenates

Tissue source and expt. no.	Form of tissue	Carotene incubated/ g tissue	Vitamin A formed		
			per g or ml	per g D.M.	activity on D.M. basis
		ug	ug	ug	%
Rabbit(depleted	Homogenates	0	0.06	0.38	---
57,58	"	13	0.29	1.93	100
	Supernatant 5000 X G	0	0.09	0.87	---
	"	35	0.50	5.08	300
	Sediment 5000 X G	0	0.08	0.59	---
	"	23	0.49	3.57	202
	Supernatant 15000 X G	0	0.05	0.57	---
	"	33	0.28	2.76	144
	Sediment 15000 X G	0	0.09	0.52	---
	"	33	0.69	4.69	261
	Supernatant 25000 X G	0	0.02	0.23	---
	"	25	0.28	2.81	158
	Sediment 25000 X G	0	0.11	0.74	---
	"	38	0.78	5.07	268
Rabbit(depleted)	Homogenates	0	0.03	0.17	---
59	"	27	0.67	3.88	100
	Supernatant 15000 X G	0	0.03	0.43	---
	"	35	0.90	12.89	332
	Sediment 15000 X G	0	0.00	0.00	---
	"	53	1.11	6.29	162

portion from the 15,000 XG preparation contained twice as much activity as the sediment portion. It is difficult to explain the discrepancy. However, if activity was expressed on a volumeteric or fresh weight basis the sediment from the 15,000 XG preparation contained more activity than the supernatant from 15,000 XG preparation in all these experiments. The error might be in estimating the dry matter content of cell fractions in experiment 59. At present, this is the only possible explanation that can be given for this discrepancy. Under the conditions of these experiments these results indicated that the supernatant material from the 5000 XG preparation contained the most vitamin A forming activity followed by the sedimented portions from preparation of 15,000 XG and 25,000 XG. The supernatant fractions from the 5000 XG and 25,000 XG preparations contained the least activity.

An attempt was also made to prepare acetone powder from duodenum, kidney and liver of calves (Experiment 19, 20). The whole tissue was disintegrated in 10 volumes of acetone at -15°C in a waring blender for 3 minutes in the cold room and the precipitate obtained was allowed to settle for 10 minutes. The supernatant aqueous acetone was decanted. The material was filtered with suction. The precipitate was washed twice by 3 volumes of acetone at -15°C and once with diethyl ether at -15°C. The acetone powder thus prepared was dried and stored over $CaCl_2$ at

-15°C until it was used.

The acetone powder was suspended in an equal (w/v) amount of phosphate buffer at P^H 7.2 and incubated with the aqueous carotene suspension using a phosphate buffer as incubating media. The results of two such experiments showed that the vitamin A formed per gram acetone powder was very little or negligible.

DISCUSSION

One of the purposes of this investigation was to establish whether carotene could be converted into vitamin A in an *in vitro* system. The results indicate that 0.25 M sucrose solution and aqueous carotene suspension maintained physiological conditions well enough to allow the formation of vitamin A from B-carotene *in vitro* by tissue homogenates. In all experiments the amount of vitamin A formed ranged from 10 to 18 times the endogenous level in duodenum and 8 to 16 times the endogenous level in liver. Even in other tissues such as kidney and large intestine the amount formed after incubation was about 5 times the endogenous level.

During the past years, numerous attempts were made to show *in vitro* formation of vitamin A from B-carotene by the small intestine and liver. As mentioned in the review of literature there were successful and unsuccessful attempts to form vitamin A *in vitro*. In the light of the success observed in this study it is not easy to explain negative results. The difference may have been due to (1) incubating media, (2) better extraction following saponification of incubated media, or (3) preservation of activity in tissue used or other less obvious reasons. Considerable effort was made throughout the experiments so that the results obtained would not be attributable to poor technique or open to criticism in some other manner. The

extraction procedure employed to isolate the carotene and vitamin A from incubated media were all standard procedures with some minor modifications. The capability of vitamin A formation by intestinal homogenates was largely dependent on the incubating solution used. Much more vitamin A was formed when 0.25 M sucrose was used than when any of the more commonly used buffers were employed. Besides furnishing essential nutrients during the in vitro reaction the 0.25 M sucrose solution may have had a more specific action or effect. Other investigators (43, 103, 104, 193) used either phosphate buffer, 0.9% saline or Krebe-Ringer buffer as incubating solutions.

Saponification of the incubated mixture allowed a more complete extraction of vitamin A. The formation of small amounts of vitamin A were thus more readily detected and measured. In studies by other investigators (43, 103, 104, 193) saponification was not employed and hence the possibility exists that whatever small amounts of vitamin A were formed escaped extraction and detection.

The accuracy of determining the efficiency of conversion in early experiments may not have been as precise as desirable. Rather high levels of carotene were used in early experiments. Carotene not recovered was measured by difference which has certain inherent inaccuracies. In order that the carotene recovered might be satisfactorily determined, low amounts of carotene were used for incubation

so as to make extraction and determination of carotene easier and more accurate. The term "vitamin A formed as percent of carotene unrecovered" was used to express the efficiency of vitamin A formation.

Altering carotene to tissue ratio influenced vitamin A formation. These results demonstrated that with low levels of carotene (20 - 40 ug/g tissue) the formation of vitamin A was linear. As level of carotene increased (400 ug/g tissue) vitamin A formation also continued to increase, but with greatly reduced efficiency. Similar results have been obtained in vivo by Olson (138).

The observations of various investigators (71, 103, 137) conclusively demonstrated that the bile was necessary for conversion of carotene to vitamin A both in vitro and in vivo. The results of present in vitro studies indicate that the incorporation of sodium glycocholate into the carotene suspension increased the vitamin A formation to over 120% of control using duodenal homogenates. The possibility exists that the bile salts (or more specifically sodium glycocholate) did not function merely as emulsifying agents (138). When the bile duct was ligated or when the entire intestine was removed the vitamin A formation from intravenously injected carotene suspension in Tween 40 was similar to that in animals not so treated (20). This leads to the idea that bile salts or sodium glycocholate do not act solely as emulsifying agents, but might

also function in a more specific manner. Olson (138) suggested that conjugated bile acids possessed a more specific function which was located mainly in the cholanic acid structure but was enhanced by conjugation. Bernhard et al. (15) showed in vitro that bile greatly retarded oxidation of the vitamin A. On these assumptions it appeared that the more specific action of conjugated bile salts may be the retardation of vitamin A oxidation and this may be due to the cholanic acid structure.

The conversion process appeared to be a very rapid reaction based on the appearance of vitamin A within 5 min after incubating B-carotene with duodenal homogenates. The reaction from B-carotene to vitamin A alcohol or ester may occur so rapidly that intermediary product such as vitamin A aldehyde could not be easily identified. The data indicates that increasing the incubation time beyond 30 min did not increase the net amount of vitamin A formation. Maximum vitamin A formation occurred at 30 min and decreased slowly thereafter. This observation may not be attributable to a feed back mechanism since the recovery of B-carotene at 30 min was high and the recovery was least at 240 min. More likely this observation may be attributable to the destruction of these compounds after incubating beyond 30 min. The data indicate that tissue homogenized under nitrogen was more effective in vitamin A formation than the tissue homogenized under oxygen. These results

clearly suggest that the oxidative destruction of carotene and vitamin A was less under nitrogen.

The conversion process studied was apparently enzymatic. In a preliminary experiment the tissue was obtained about 1 hour after slaughter and the extent of conversion was negligible. The extent of vitamin A formation was greatly reduced when the homogenates were kept 1 - 2 hours before addition of the carotene suspension. Further the data clearly indicate that heating homogenates inactivated the enzyme or factors which were responsible for conversion of carotene to vitamin A. Duodenal tissue from the pigs which were subjected to scalding the entire body surface on open flame did not show any activity. This process might have inactivated the enzyme(s) or factor(s). The finding, that the tissue respiratory poison KCN considerably reduced the formation of vitamin A, indicates that cytochrome oxidase system may also be involved in the conversion process. The fact that iodoacetate reduced the formation of vitamin A reveals that some type of alcohol dehydrogenase or some kind of thiol enzyme may be involved in the cleavage of carotene to vitamin A. All the above experimental findings initiate that a type of enzyme is involved in this process. Although these experiments are not entirely conclusive, these results provide considerable evidence for the existence of an enzyme system responsible for vitamin A formation from B-carotene.

The positive results obtained on in vitro conversion of carotene to vitamin A (167, 185) have been questioned when only the $SbCl_3$ method was used for vitamin A determination. However, Rosenberg and Sobel (152) determined vitamin A formed in vitro by intestines with the ultraviolet irradiation technique proposed by Bessey et al. (16). The application of this method to intestinal vitamin A determination was shown to be of little use (18). Moore (132) indicated that the determination of vitamin A in the presence of carotene and its oxidation products requires special and careful techniques.

The chromatographed extract from incubated tissue had an ultraviolet absorption spectra indistinguishable from that of standard vitamin A alcohol whether in petroleum ether or benzene (29, 31). These spectra are consistent with those given for free vitamin A alcohol by Budowsky and Bondi (29). The endogenous vitamin A alcohol fraction from tissue did not have an absorption spectra characteristic of vitamin A alcohol. This ruled out the possibility that vitamin A found after incubation of the carotene suspension with duodenal homogenates was of endogenous origin.

The absorption spectra of the Carr-Price reaction products were similar for (1) the ether extract which was not subjected to column chromatography on deactivated alumina; (2) the vitamin A alcohol fraction separated after chromatography; and (3) standard vitamin A alcohol.

However, the reduced absorbancy of the Carr-Price reaction product of the unchromatographed extract would indicate that the amount of vitamin A in tissue extract was underestimated by the Carr-Price reaction as used in these experiments.

The behavior of vitamin A alcohol fraction and standard vitamin A alcohol with and without pretreatment of maleic anhydride furnished further evidence that the extracts from saponified and incubated mixtures contained vitamin A. The absorption spectra of the blue color produced with Carr-Price reaction by standard vitamin A alcohol and vitamin A alcohol fraction without pretreatment with maleic anhydride were similar and the absorption spectra of maleic anhydride treated samples were also similar. These findings together with similar recovery values ("R") for vitamin A alcohol fraction from the chromatographed extract and standard vitamin A alcohol give support to the interpretation that the ether extracts of the incubated mixtures contained vitamin A.

The ultraviolet absorption spectra (absorption maxima and minima) determined for the anhydro derivative made from vitamin A alcohol fraction and standard vitamin A alcohol were consistent with those reported by Budowsky and Bondi (29). These results were further supported by the finding that the "difference" absorption spectra of anhydrovitamin A made from vitamin A alcohol fraction was similar to that

made from standard vitamin A alcohol.

The destructive irradiation technique furnished evidence that vitamin A alcohol occurred in the extracts after incubating the carotene suspension with duodenal homogenates. The postirradiation absorption spectra of vitamin A in serum obtained by Bessey et al. (16) was different from the postirradiation absorption spectra of vitamin A observed in these trials. The difference may be due to extent of irradiation. In these studies irradiation was performed for 36 hours, whereas Bessey et al. (16) used only 60 min of irradiation. Irradiation for 36 hours destroyed all the vitamin A while irradiation for 24 hours did not destroy completely.

Identification by absorption spectra, paper partition chromatographic and circular paper chromatographic procedures demonstrated the presence of vitamin A alcohol was present in ether extracts of the incubated mixtures. The R_F values for B-carotene and vitamin A alcohol in this study were slightly different from the values obtained by Suzuki et al. (170) who used Toyo filter paper No. 50. The different papers and possible differences in impregnation process may have caused the small difference in R_F values.

The results of all these identification studies indicate that vitamin A was formed during the incubation process. None was formed when only duodenum or muscle were

incubated. The conclusion appears justified that the necessary factor(s) or enzyme(s) responsible to form vitamin A from carotene are present in duodenal and other tissue homogenates.

In previous investigations information regarding the conversion of carotene to vitamin A by various tissues had been obtained from studies on the accumulation of B-carotene and vitamin A in various tissues of intact animals after administration of B-carotene and from an examination of the vitamin A content of blood and various tissues (17, 19, 20, 123, 124, 126). Such procedures give only indirect evidence on vitamin A formation since the presence of carotenoids and vitamin A in the various tissues is not necessarily related to the site of conversion.

The invitro technique used in the present studies appears to be a satisfactory procedure to directly investigate the role of various tissues in the formation of vitamin A from B-carotene. Bieri and Pollard (19) and Kon et al. (103) showed that an aqueous suspension of carotene in Tween was more efficiently utilized in the formation of vitamin A than colloidal carotene. For this and other reasons an aqueous carotene suspension in Tween was always used in the present studies. The results of experiments reported here indicate that the small intestine, especially the duodenum, was one of the major sites of conversion of carotene to vitamin A in many of the animal species

tested. The data also indicate that the enzyme(s) or factor(s) effecting the formation of vitamin A from carotene were found in organs other than the small intestine. From a comparison of formation of vitamin A by the different organs tested it is apparent that the liver in addition to the small intestine is capable of playing an important role in carotene conversion. The third major organ involved in carotene conversion appeared to be the kidney except in chickens. Species differences in the extent of vitamin A formation from B-carotene by the small intestine, liver and kidney were also observed. The results obtained with upper small intestine and liver of chicks are in agreement with Olson et al. (136) who showed that chick duodenum and liver appeared to be about equally active as a source of enzymes required for the conversion of carotene to vitamin A. Olson (138) showed that in rats the first third of the intestine was most active, the middle third was somewhat less active, and the last third of the intestine was a very poor enzyme source for vitamin A formation. The results reported here furnish further evidence that the upper small intestine was most active in rats. In the vitamin A deficient mammals in which carotenoids and vitamin A were at a minimal level in the blood, both the small intestine and liver could play an important role in the formation of vitamin A from B-carotene.

Most of the work concerning the relative efficiency

of vitamin A formation by various organs from different animal species has been conducted in the intact animals (17, 103). This work indicated the existence of a species difference in the relative efficiency of vitamin A formation by the various organs. The results reported here provide evidence for species difference in the efficiency and/or extent of vitamin A formation from B-carotene.

Lung, muscle, heart, lymph gland, rumen, omasum and abomasum were consistently found to form only traces of vitamin A. Evidently the enzyme(s) or factor(s) responsible for the formation may be also found in these organs. Several investigators (20, 190, 192) demonstrated that complete removal of liver, kidney, stomach, intestine, pancreas, lungs, head, thyroid, parathyroids or adrenals did not effect the increase of vitamin A in the blood plasma after intravenous injection of B-carotene. The conclusion was made that the ability to convert carotene to vitamin A was not an attribute of any one tissue or organ, but of many or perhaps all organs and/or cells. Thus, many tissues besides the small intestine and liver were thought to possess the ability to form small amounts of vitamin A from carotene. The results obtained in this study offer positive proof for this idea.

The decrease in activity produced by NO_3^- or NO_2^- introduced into <u>in vivo</u> or <u>in vitro</u> loops considerably reduced the formation of vitamin A from B-carotene. The results

with rabbits also indicate that the extent of conversion was reduced more in *in vivo* loops than *in vitro* loops. The intestinal loops from rabbits were hemorrhagic when NO_3^- was added into them in experiment 9. Pollard and Bieri (144) have shown that vitamin A and B-carotene were rapidly destroyed by the hemolyzed blood cells. The hemorrhagic condition of *in vivo* loops after introduction of NO_3^- may be responsible for the decreased formation of vitamin A from carotene when compared to *in vitro* loops. These *in vivo* loops could have contained a sufficient amount of blood to depress vitamin A formation from B-carotene. The small intestine from the NO_2^- or NO_3^- fed animals were hemorrhagic. This hemorrhagic condition of the small intestine could have contributed in part to depress the vitamin A formation from carotene.

The addition of high levels of NO_3^- (50 - 1000 u moles/g tissue) to the incubating media containing intestinal homogenates from various animals species resulted in a decrease in the extent and efficiency of vitamin A formation from B-carotene. The degree of inhibition was related to the status of the animal whether it was depleted of vitamin A or normal. The inhibitory action of NO_3^- in depleted animals was more than in normal animals. High levels of added NO_3^- reduced the vitamin A formation to 14 - 34% of normal using tissues from a depleted calf but reduced it only to 51 - 69% of normal using tissues from a normal calf.

Thus in animals in which the carotenoid and vitamin A levels in the body were high (normal animals) the inhibitory effect of NO_3^- on vitamin A formation was less pronounced than in animals in which the carotenoid and vitamin A levels in the body were low (depleted animal). This may have implications to present day animal husbandry practice.

O'Dell et al. (134) reported that a diet containing 0.3% of KNO_2 caused a more rapid depletion of liver vitamin A in rats and that xerophthalmia developed after 8 weeks on the NO_3^- diet. In the present study the rabbits fed either 0.5% or 2.0% NO_3^- in the diet for nearly 6 weeks did not show any visible vitamin A deficiency symptoms. Even the rabbits fed the carotene low ration plus dietary NO_3^- at the 2.0% level did not show any visible deficiency symptoms.

Vitamin A levels in the blood plasma of rabbits fed the carotene deficient ration were reduced significantly ($p = 0.01$) by feeding either 0.5 or 2.0% dietary NO_3^-. Rabbits fed the normal diet reacted similarly. The data in this study on the blood vitamin A levels after NO_3^- feeding differed from the results reported by Weichenthal et al. (184), but are consistent with those reported by Smith et al. (165), using rats. The liver storage of vitamin A was decreased more by 2.0% NO_3^- in the diet than by 0.3 or 0.5% NO_3^-. The low level of NO_3^- produced a lower liver storage of vitamin A than was in animals fed no NO_3^-. This data indicated that the liver storage of vitamin A was negatively

related to the level of NO_3^- fed. These results are in agreement with the data obtained in sheep by Smith et al. (166) and Goodrich et al. (69). The results obtained with animals fed a carotene low diet suggest that dietary NO_3^- decreased the liver vitamin A level probably increasing the rate of loss of vitamin A from liver. The results with animals fed a normal diet suggest that the dietary NO_3^- decreased liver stores of vitamin A either by reducing the absorption of vitamin A from the intestine, or by a more rapid depletion of vitamin A from the liver, or by reducing the conversion of carotene to vitamin A. The possibility that the nitrate effect may be the result of increased destruction of vitamin A in the digestive tract of animals should not be overlooked. Hence, the data with live animals indicate that the influence of dietary NO_3^- intake on the vitamin A level of liver and blood plasma may be through many mechanisms rather than any single mechanism.

The addition of NO_3^- to the incubating media containing the intestinal homogenates from the animals fed either NO_3^- or NO_2^- produced a smaller inhibitory effect than when NO_3^- was added to the media containing the intestinal homogenates from the animals fed a diet not containing NO_3^- (Tables 17, 18). Another noteworthy effect observed in these experiments was that feeding either NO_3^- or NO_2^- to the animals resulted in a reduction of the amount of vitamin A formed by duodenal homogenates from these animals when compared to

homogenates from animals not fed NO_3^-. Apparently the tissue from NO_3^- fed animals contained some inhibitory compound that could cause a reduction in the effect of additional NO_3^- added *in vitro*. Furthermore, washing the tissue from a NO_2^- fed animal when extensively washed with 0.9% saline before homogenization did not change the activity of the tissue from the heifers fed NO_2^- or of tissue from a heifer fed a normal ration. Evidently the NO_3^- or other factor that exerts the inhibitory effect in animals fed NO_3^- is bound to or inside the tissue. The results of the present investigation indicate that dietary NO_3^- or NO_2^- exerted an inhibitory effect on the enzyme(s) or factor(s) in tissue responsible for the vitamin A formation. When the NO_3^- was added to the media containing this already affected tissue (from NO_3^- fed animals) the inhibitory effect did not proceed beyond a certain extent. The data also suggest that the inhibitory effect of NO_3^- added to the *in vitro* system was more pronounced in the depleted animals than in the normal animals.

In vitro and *in vivo* studies performed on the same animals suggested that nitrate caused a reduction in the extent and efficiency of vitamin A formation from carotene. The results obtained with other ions such as $SO_4^=$, NaCl, and $CO_3^=$ indicate that these ions had considerably less inhibitory effect than NO_3^- (Tables 27, 28). Hence a decrease in activity produced by either NO_3^- or NO_2^- must be somewhat

specific.

The data suggest that an addition of high levels (0.01 - 6.25 ug/g tissue) of L-T_4 or L-T_3 reduced the extent and efficiency of vitamin A formation from B-carotene in vitro. On the other hand, an addition of low levels of L-T_3 (0.0001 - 0.00001 ug/g tissue) to the incubating media contained duodenal homogenates slightly increased the vitamin A formation from B-carotene. In order to interpret the effect of a hormone on an enzyme system in terms of a physiological process in the intact animal the concentration of the hormone used for eliciting the in vitro response must not be very high as compared to physiological level of the hormone in live tissues. In these experiments 0.1 - 6.25 ug of L-T_4 or L-T_3/g tissue appeared to be a very high concentration since 10^{-5} - 10^{-4} M of L-T_4 was found to be 100 - 1000 times the concentration of the circulating hormone and probably 10,000 - 100,000 times that found in tissues such as skeletal muscle (143). In order to obtain responses to thyroid hormones in an enzyme system, a concentration of 10^{-4} to 10^{-5} M was generally required (143). In the experiments mentioned the levels of L-T_4 or L-T_3 used were higher than physiological levels. The 0.0001 - 0.00001 ug level were sufficiently moderate enough to allow the reaction to proceed without any inhibitory effect. Addition of amino acids such as DL-alanine and L-proline reduced the inhibitory effect of L-T_4 on vitamin A formation

and slightly improved the vitamin A formation in vitro. Barker and his co-workers (12,114) showed that the Qo_2 of kidney slices in vitro was considerably increased by adding certain amino acids as L-alanine or L-proline + L-T_4 compared to L-T_4 alone. In Barker's work as well as this work the tissues activity was increased by the addition of DL-alanine or L-proline while L-tryptaphan behaved as an inhibitor. When L-tryptaphan was added to the incubating media the formation of vitamin A and the recovery of carotene from the incubated media were very low. The reasons for the possible specific action of L-tryptaphan are not known.

These studies clearly suggest that the inhibitory effects produced by L- and D-isomers of T_4 or T_3 on the formation of vitamin A were similar. Pitt-Rivers and Tata (143) stated that D- and L-T_4 which have very different biological activities in vivo but were found to possess similar properties and to about the same extent in vitro. The results of these experiments substantiate the above statement (143) that activities of L- and D-T_4 in vitro were similar.

A thyroxine analogue inhibitor such as 3 5' 3' L-T_3 depressed vitamin A formation from B-carotene by intestinal homogenates. Addition of T_4 or T_3 isomers reduced the inhibitory effect of 3 5' 3' L-T_3 on vitamin A formation but only to a limited extent.

The data presented in Tables 23, 24 indicate that there was a relationship between thyroid status of animal and the ability of tissue of that animal to convert carotene to vitamin A. Duodenal homogenates from hypothyroid and thyroidectomized animals, reduced considerably the formation of vitamin A from carotene as compared to control and hyperthyroid animals, whereas no difference was observed between tissue of control and hyperthyroid animals. The data concerning hypothyroid and thyroidectomized animals is in agreement with Johnson and Baumann (90), Serif and Brevik (156), Kowlewski et al. (107, 108) and Kelly and Day (97). All these workers used living animals and measured liver and plasma vitamin A levels as a criteria for comparison. Bieri and Schultze (22) observed no significant difference in vitamin A storage in normal or thiouracil treated rats fed carotene. The reasons for these differences are not immediately apparent.

A study of data in Tables 23 and 24 indicate that the vitamin A formation was still possible in tissue from thyroidectomized or from animals fed thiouracil. This poses the question as to the necessity of the thyroid hormone for vitamin A formation. This data also suggest that the excess of thyroid hormone in the system (hyperthyroid status) does not increase or decrease net formation of vitamin A from B-carotene by tissues.

Apparently there is an interrelationship among thyroid

status of the animal, nitrate and vitamin A formation. Addition of high levels of NO_3^- to the incubating intestinal homogenates produced a large reduction in the amount of vitamin A formed from hyperthyroid animals. This level of NO_3^- produced an intermediate level of reduction in tissues from control animals and the least reduction in tissues from hypothyroid animals. The reason for the lesser inhibitory activity of NO_3^- on the tissues from hypothyroid animals may be that this tissues already contained an inhibitory factor and the further addition of an inhibitory factor (such as NO_3^-) only produced limited decrease in vitamin A formation. A slight improvement of vitamin A formation was observed by tissues from all animals when L-T_4 was added to the media containing higher levels of NO_3^-. These results demonstrate the unequivocal relationship between thyroid status of the tissue and nitrate on vitamin A formation.

The data in Table 30 indicate that the lower levels of alpha tocopherol considerably increased the efficiency of vitamin A formation and that the higher levels reduced the efficiency of vitamin A formation from B-carotene. This finding is in direct agreement with Johnson and Baumann (91), and Swick and Baumann (171) although these workers conducted the experiments on live animals. McGillivray and Worker (126) also showed that the high levels of alpha tocopherol in the blood and tissues achieved by the intravenous injec-

tion of alpha tocopherol inhibited the conversion of intravenously injected carotene to vitamin A.

The data concerning the effect of blood on the vitamin A formation indicate that the blood was probably not an important system for the conversion of carotene to vitamin A. The amount of vitamin A formed by blood was nil or extremely very small. The results of McGillivray (122) and Mc Gillivray and Worker (126) can be interpreted to indicate that following intravenous carotene administration the conversion of carotene was initiated in the blood where there was a rapid breakdown to an intermediate product that was then taken up by the tissues and more slowly converted into vitamin A. Zackman and Olson (195) were unable to isolate any intermediate from the blood and showed that the formation of vitamin A ester from B-carotene was greater than from the vitamin A aldehyde. In our studies only a very small quantity of vitamin A was formed after incubating blood and B-carotene. Two opposing forces namely the formation of vitamin A from B-carotene (122, 126), and destruction of vitamin A and B-carotene (144) may have acted simultaneously to produce only a slight increase of vitamin A which was identified in the incubation mixture.

Several trials conducted by Pollard and Bieri (144) indicated that there was a destruction of carotene when incubated <u>in vitro</u> with the hemolyzed whole blood. The data in this trial are in agreement with the results of Pollard

and Bieri (144). The data on washing and centrifuging the sedimented blood cells indicate that the blood cells contained factor(s) responsible for carotene destruction. Even after washing and centrifuging the sedimented blood cells at 27,000 XG the carotene destructive system was more pronounced in the sedimented portion.

Because of the destructive action of blood cells on carotene and vitamin A the effect of incubating blood and its fractions with the duodenal homogenates on vitamin A formation was conducted. The conversion of carotene to vitamin A was markedly reduced when centrifuged sedimented cells at 27,000 XG were incubated with the duodenal homogenates plus carotene. Washing these cells removed only a small portion of the inhibiting effect. The failure to remove the inhibitory factor completely by washing the sedimented cells twice suggested that the system was complex. The present studies indicate that the majority of the inhibitory factor was in the sedimented blood cells from blood.

The data on the distribution of activity responsible for carotene conversion in the cell fractions of duodenal homogenates indicate that the supernatant fraction of the 5000 XG preparation contained the most activity followed by sedimented fractions at 15,000 XG and 25,000 XG preparation. Different kinds of results were obtained in experiments 50 and 59. This discrepancy may be explained on the basis

that the degree of homogenization before centrifugation may not be the same in both experiments. In the experiment 59, the sedimented fraction of the 15,000 XG contained more activity than supernatant fraction of the 15,000 XG preparation if the activity was expressed on a fresh basis which agreed with the results of experiment 57. Hence the error may have been in estimating the dry matter content of cell fractions in experiment 59. Olson (138) observed that all the radioactivity from intestine incubated with B-carotene was present in the sedimented fraction when the intestinal homogenates prepared by mild homogenization in waring blender when centrifuged at 15,000 XG. When vigorous homogenization was used the radioactivity was found in the surface layer rather than in the sediment. This may offer another possible answer for the difference in results obtained in experiments 57 and 59.

SUMMARY

Duodenal homogenates from dairy cattle, and rabbits were used in an *in vitro* system to determine whether an incubating media modified from several investigators was satisfactory for studying vitamin A formation from B-carotene *in vitro*. This *in vitro* system has been shown to convert B-carotene into vitamin A. The amount of vitamin A formed was about 14 and 12 times the endogenous level in rabbits and cows respectively. Much more vitamin A was formed when 0.25 M sucrose was used than when any of the more commonly used buffers were employed. Saponification of the incubated mixture allowed a more complete extraction of vitamin A and the formation of small amounts of vitamin A *in vitro* were thus more readily detected and measured. The addition of glycocholate to a carotene suspension slightly increased vitamin A formation when duodenal homogenates were used. The extent of conversion increased as the amount of carotene added increased whereas the efficiency of vitamin A tended to increase as the level of carotene decreased in the incubating media. The conversion process appeared to be a very rapid reaction based on the appearance of vitamin A within 5 min after incubating B-carotene with duodenal homogenates.

The results suggested that the oxidative destruction of carotene and vitamin A was less under nitrogen as indicated by the fact that the tissue homogenized under nitrogen

was more effective in vitamin A formation than the tissue homogenized under oxygen.

The conversion process was apparently enzymic. Conversion was minimal or zero when the interval from slaughter to incubation was one hour or more. Conversion was greatly reduced by heat, KCN, urea, NO_3^- or iodoacetate but was not appreciably reduced by $SO_4^=$, NaCl or $CO_3^=$. Isolation and identification of vitamin A formed by this system was carried out on tissue extract after column chromatography on deactivated alumina. Presence of vitamin A was confirmed by various methods such as absorption spectra, changes in spectra after irradiation, maleic anhydride method, conversion to anhydrovitamin A and paper chromatography.

The small intestine especially the duodenum and liver were found to contain the most activity for the conversion of carotene to vitamin A in dairy cattle, sheep, rabbits, pigs, rats and chicks. The kidney also contained considerable activity except in chickens. Lung, muscle, heart, lymph gland, rumen, omasum and abomasum were found to form only traces of vitamin A. These results provided some evidence for a species difference in the efficiency and/or extent of vitamin A formation from B-carotene.

The decrease in activity of vitamin A formation produced by NO_3^- or NO_2^- was somewhat specific. Feeding 0.3 or 0.5% NO_3^- to rabbits generally reduced carotene and vitamin A in blood plasma and liver while feeding a level of 2.0%

produced a marked decrease in these constituents. In the in vitro system the extent of conversion was decreased by using duodenal homogenates and from animals fed NO_3^- or NO_2^- as well as when NO_3^- or NO_2^- was added to the in vitro media. Large amounts of NO_3^- added to the in vitro system produced a marked inhibition of vitamin A formation whereas small amounts produced little or no effect. Nitrate added in vitro to the duodenal homogenates from NO_3^- fed animals exerted a smaller inhibitory effect than when it was added to duodenal homogenates from animals fed no NO_3^-.

There seemed to be some kind of interrelationship between thyroid status of the animals, dietary NO_3^- and vitamin A formation. High levels of NO_3^- added to the in vitro system containing duodenal homogenates produced a large reduction in the amount of vitamin A formed from hyperthyroid animals. This level of NO_3^- produced an intermediate reduction in tissues from control animals and least reduction in tissues from the hypothyroid animals. Duodenal homogenates from hypothyroid and thyroidectomized animals considerably reduced the formation of vitamin A from carotene as compared to the duodenal homogenates from control and hyperthyroid animals, whereas no difference was observed between the duodenal homogenates from control and hyperthyroid animals. Vitamin A forming step was not entirely under the control of the thyroid hormone as even in the absence of thyroid hormone (hypothyroid or thyrodectomized), some vitamin A

was formed.

Addition of high levels of L-thyroxine (L-T_4) or L-triiodothyrodnine (L-T_3) to the in vitro system reduced the extent and efficiency of vitamin A formation. Addition of DL-alanine or L-proline reduced the inhibitory effect of L-T_4 on vitamin A formation. L-tryptaphan behaved as an inhibitor when added to the in vitro system containing duodenal homogenates. Addition of L- and D-isomers of T_4 and T_3 produced a similar inhibitory effect on the formation of vitamin A. Addition of T_4 and T_3 isomers reduced the inhibitory effect of 3 5' 3' L-T_3 on vitamin A formation to certain extent.

Little or no vitamin A was formed when carotene was incubated with blood. Considerable amounts of carotene were destroyed after incubation with hemolyzed blood or sedimented blood cells. Little or no vitamin A was formed when sedimented blood cells or hemolyzed blood were incubated with B-carotene. The conversion of carotene to vitamin A was markedly reduced when hemolyzed blood, sedimented blood cells or centrifuged sedimented cells form a 27,000 XG preparation were incubated with duodenal homogenates and carotene. These studies indicated that the inhibitory factor was located in the sedimented blood cells.

The data on the distribution of activity responsible for carotene conversion in the cell fractions of duodenal homogenates indicated that the supernatant fraction from a

5000 XG preparation contained more activity than that in the sedimented fractions from a 15,000 XG and 25,000 XG preparation.

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APPENDIX

APPENDIX TABLE 1

Purpose of various experiments conducted and tissues used

Expt. no.	Animal	Dietary treatment	Tissue used	Purpose of experiment
1	Rabbit	Normal	Small intestine	Extent of conversion
2	Holstein cow	"	Duodenum	"
3	Rabbit	"	Small intestine	Effect of NO_2^-, NO_3^- and L-T_4
4	Guernsy bull	"	Duodenum	"
5	Rabbit	"	Small intestine	Effect of NO_3^-, NO_2^-, L-T_4 and glycocholate and conversion in loops
6	Heifer	Deficient	Duodenum	Extent of conversion and effect of NO_3^-
7	"	" + KNO_2	"	"
	"	"	"	"
7	Rabbit	Normal	Small intestine loops	Effect of NO_3 <u>in vivo</u>
	"	"	"	Effect of NO_3 <u>in vitro</u>
8	Calf	Deficient	Duodenum	Conversion in lower gut, effect of NO_3^-, L-T_4 and L-T_3
9	Rabbit	Deficient	Small intestine	Effect of NO_3^-
	"	"+0.3% NO_3^-	"	"
	"	"+0.2% NO_3^-	"	"
	"	Normal	"	"
10	Calf	Deficient	Duodenum	Comparison between saponification and non saponification, effect of NO_3^-, $SO_4^=$, $CO_3^=$ and NaCl. Effect of heating homogenates and altering carotene : tissue
11	Calf	Deficient + 1% NO_3^-	"	"
12	Rabbit	Normal	Small intestine	Effect of NO_3^-
	"	"+0.5% NO_3^-	"	"
	"	"+2.0% NO_3^-	"	"

APPENDIX TABLE 1 (Continued)

Expt. no.	Animal species	Dietary treatment	Tissue used	Purpose of experiment
13	Sheep	Normal	Duodenum	Effect of NO_3^-, heating and blood
14	"	"	"	Effect of NO_3^-, KCN, urea and iodoacetate
15	Calf	"	"	Effect of NO_3^- and aging tissue
16	"	Deficient	Various tissues	Conversion by various tissues
17,18	"	Normal	Duodenum	Conversion by portions of duodenum
19,20	"	"	"	Conversion by acetone powder
21	"	Deficient	Various tissues	Effect of $SO_4^=$, $CO_3^=$ and NaCl. Conversion by various tissues
22,25	"	Normal	Duodenum	Effect of NO_3^-, L-T_4, L-T_3, L-T_4 + NO_3^- and L-T_3 + NO_3^-
23,26	"	'4 Thiouracil	"	"
24,27	"	'4Thyroprotein	"	"
28	Rats	Normal	Various tissues	Conversion by various tissues
29	Rabbits (Thyroidectomised)	"	Small intestine	Same as experiment 22
30	Rabbits	"	"	"
31	"	'4Thiouracil	"	"
32	"	'4Thyroprotein	"	"
33	Pigs	Normal	Duodenum	Extent of conversion
34	Rat	Deficient	Various tissues	Conversion by various tissues
35	Chick	"	"	"
36,37	Rabbits	"	"	"
38,39	Steer, heifer	Normal	Duodenum	Effect of L-T_4 and L-T_3
40,41	Pig	Deficient	Various tissues	Conversion by various tissues and effect of NO_3^-

APPENDIX TABLE 1 (Continued)

Expt. no.	Animal species	Dietary treatment	Tissue used	Purpose of experiment
42	Calf	Deficient	Duodenum, liver	Effect of D-T_4, D-T_3, L-T_4 L-T_3 and 3 5'3' L-T_3
43	Sheep	Normal	Various tissues	Conversion by various organs and effect of NO_3^-
44	Calf	"	Duodenum	Effect of varying incubations time and various buffers
45,46	Cow	"	"	Effect of varying incubation time
47,48	"	"	"	Effect of various buffers
49,50	Steer, heifer	"	"	Effect of blood fractions
51	Heifer	"	"	Incubation of blood fractions and carotene
52,53	Heifer, steer	"	"	Effect of washed and centrifuged
54	Heifer	"	"	Effect of various enzyme inhibitors
55,56	Steer	"	"	Effect of amino acids and L-T_4
57,58 59	Rabbit	Deficient	Small intestine	Conversion by cell fractions
60	"	"	"	Effect of heating and tocopherol
61	"	"	"	Comparison of homogenization in waring blender and omni-mixer
62	"	Normal	"	Effect of homogenization under NO_2 and O_2 and identification of vitamin A formed
63	"	Deficient	Small intestine, liver	Extent of conversion in extremely deficient animal

APPENDIX TABLE II

ANALYSIS OF VARIANCE OF DATA ON THE EFFECT OF ADDING ISOMERS OF L-T_4 AND L-T_3 AND COMBINATION OF THEM WITH 3 5'4' L-T_3 ON VITAMIN A FORMATION FROM B-CAROTENE (TABLE 22).

Duodenum

Source of Variation	D.F.	Sum of Squares	Mean Square	F	Signif.
Treatment*	1	0.0230	0.0230	12.11	0.01
Thyroid compounds**	4	0.0170	0.0043	2.26	n.s.
T X Tc	4	0.0580	0.0145	7.63	0.01
Between cells	9	0.0980	0.0109	5.74	0.01
Error	10	0.0190	0.0019		
Total	19	0.1170			

Liver

Source of Variation	D.F.	Sum of Squares	Mean Square	F	Signif.
Treatment*	1	0.0140	0.0140	9.33	0.05
Thyroid compounds**	4	0.0020	0.0005	0.33	n.s.
T X Tc	4	0.0540	0.0135	9.00	0.01
Between cells	9	0.0700	0.0078	5.20	0.01
Error	10	0.0150	0.0015		
Total	19	0.085			

* With or without adding 3 5'3' L-T_3

** With or without adding L- and D-T_4 or L- and D-T_3

APPENDIX TABLE III

ANALYSIS OF VARIANCE OF DATA ON THE EFFECT OF ADDING $L\text{-}T_4$ OR $L\text{-}T_3$ WITH OR WITHOUT NITRATE ON VITAMIN A FORMATION BY DUODENAL HOMOGENATES FROM CONTROL HYPERTHYROID AND HYPOTHYROID ANIMALS (TABLE 24).

Calves

Source of Variation	D.F.	Sum of Squares	Mean Square	F	Signif.
Status	2	0.404	0.2020	12.63	0.01
Thyroxine level	2	0.082	0.0410	2.56	n.s.
Nitrate level	2	1.046	0.5230	32.69	0.01
S X T	4	0.019	0.0048	0.30	n.s.
T X N	4	0.158	0.0395	2.47	0.05
N X S	4	0.061	0.0153	0.96	n.s.
S X T X N	8	0.034	0.0043	0.27	n.s.
Error	81	1.300	0.0160		
Total	107	2.144			

Rabbits

Source of Variation	D.F.	Sum of Squares	Mean Square	F	Signif.
Status	3	0.4119	0.1373	274.6	0.01
Thyroxine level	2	0.0014	0.0007	1.4	n.s.
Nitrate level	1	0.8855	0.8855	1777.1	0.01
S X T	6	0.0110	0.0018	3.6	n.s.
T X N	2	0.0467	0.0234	46.8	0.01
N X S	3	0.1818	0.0606	121.2	0.01
Error	6	0.0030	0.0005		
Total	23	1.5413			

APPENDIX TABLE IV

ANALYSIS OF VARIANCE OF DATA ON THE EFFECT OF ADDING DIFFERENT AMINO ACIDS WITH AND WITHOUT COMBINATION OF L-T_4 ON VITAMIN A FORMATION FROM B-CAROTENE (TABLE 26).

Vitamin A Formed Per Gram Tissue

Source of Variation	D.F.	Sum of Squares	Mean Square	F	Signif.
Amino acids	3	0.0198	0.0061	19.41	0.01
Thyroxine	1	0.0666	0.0666	195.88	0.01
Experiments	1	0.0001	0.0001	0.29	n.s.
A X T	3	0.0136	0.0045	13.23	0.01
T X E	1	0.0007	0.0007	2.06	n.s.
E X A	3	0.0043	0.0014	4.12	0.05
A X T X E	3	0.0006	0.0002	0.58	n.s.
Error	16	0.0054	0.00034		
Total	31	0.1111			

Vitamin A Formed as % Carotene Unrecovered

Source of Variation	D.F.	Sum of Squares	Mean Square	F	Signif.
Amino acids	3	51.2312	17.0771	35.48	0.01
Thyroxine	1	37.4112	37.4112	77.73	0.01
Experiments	1	14.3112	14.3112	29.73	0.01
A X T	3	1.6713	0.5571	1.16	n.s.
T X E	1	2.7613	2.7613	5.74	0.05
E X A	3	7.5013	2.5004	5.20	0.05
A X T X E	3	4.9012	1.6337	3.39	0.05
Error	16	7.7000	0.4813		
Total	31	127.4887			

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