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Biosynthesis of retinol as affected by dietary protein and feeding patterns in carotene-fed rats

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BIOSYNTHESIS OF RETINOL AS AFFECTED BY DIETARY PROTEIN AND FEEDING PATTERNS IN CAROTENE-FED RATS

by

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INTRODUCTION

Vitamin A deficiency is still one of the major causes of blindness in the world (Olson, 1969) despite the fact that many populations have access to carotenoids, precursors of vitamin A, in local foods. Low serum levels of vitamin A, keratomalacia, or xeropthalmia have frequently been reported with kwashiorkor, the protein deficiency syndrome. An understanding of the links between level of protein nutrition and vitamin A deficiency symptoms would be helpful in combating the deficiency problems. Vitamin A deposition from β-carotene generally has been reported to be augmented with increasing dietary protein levels or with proteins of higher biological value. Thus investigation of the interrelationships between dietary protein and carotene metabolism seems particularly pertinent.

The mechanism of the effect of protein on carotene utilization has not been clearly defined. Studies from this laboratory have indicated that hepatic vitamin A deposition from an adequate dose of the provitamin is dependent on the amount of protein fed. Likewise the recovery from the gastro-intestinal tract of newly synthesized ^{14}C-retinyl esters is greater in animals fed 40% protein than 10% protein (Kotecheri, 1967).

Another report from this laboratory suggests that protein exerts its effect during the intestinal phase of carotene metabolism since vitamin A reserves in the liver were higher when carotene supplementation was given with the protein than

with the protein-free portion of the diet (Ruffin, 1965). Changes in efficiency of carotene utilization with protein could be due to an increased rate of conversion of carotene to retinal and subsequently to retinol or to an increased availability of carotene perhaps due to decreased formation of non-utilizable breakdown products.

The present experiments were designed to explore the effects on carotene metabolism of 1) dietary protein level and 2) the simultaneous presence of protein and carotene in the gastro-intestinal tract.

Parameters investigated were the formation of ^{14}C-metabolic products from labeled β-carotene *in vivo* and *in vitro* and the deposition of vitamin A reserves derived from carotene during a twenty-eight day feeding period.

REVIEW OF LITERATURE

Metabolism of Carotene and Vitamin A

Absorption

Carotene Digestibility of foodstuffs affects the absorption of carotene from the intestine. In the cells of plants the provitamins are found in fat solutions, in protein complexes, or in crystalline forms. Hence, during passage through the intestinal tract the plant cells must be broken down before absorption of carotene can occur (Thompson, 1965). Not surprisingly, studies have indicated that carotene is more easily utilized in well digested dietary fats and oily solutions than in vegetable sources (Moore, 1957; Thompson, 1965). Although carotene can be utilized in the absence of fat, animal and human studies have indicated that dietary fat exerts a beneficial effect on the absorption of provitamins (Thompson, 1965; Roels, 1968). Bile stimulates the absorption of carotene *in vitro* (Olson, 1964b) as do surface active agents such as the Tweens (Thompson, 1965). The effects of bile on dispersion of the provitamins are important; however, Olson (1964b) also suggests that conjugated bile acids may stimulate retinyl ester formation by enhancing β-carotene absorption through an interaction with the membrane of intestinal mucosal cells.

Natural or added antioxidants aid in the utilization of carotene (Moore, 1957). If high doses of carotene are given, the efficiency of absorption decreases when compared to the

efficiency of absorption of preformed vitamin A (Moore, 1957; MacMillan, 1966).

Nature and quantity of dietary protein, the integrity of the intestinal mucosa, and the hormonal and physiological state of the animal are additional factors which may influence the absorption of the provitamin (Olson, 1968).

Vitamin A Vitamin A occurs in foods chiefly as all-trans retinyl esters (Plack, 1965). These dietary retinyl esters appear to be totally hydrolyzed in the lumen before absorption (Mahadevan et al., 1963a; Ganguly, 1969). Mahadevan et al. (1961, 1963b) indicate that hydrolysis of the higher retinyl esters is stimulated by bile salts and that the hydrolytic enzyme might be located on the outer surface of the mucosal-cell membrane. David and Ganguly (1967) conclude that pancreatic hydrolase plays a significant role in the hydrolysis of dietary retinyl esters and Olson (1968) suggests that hydrolysis may occur either at the surface of the bulk lipid phase or in the micelle itself.

The lipid carrier affects the absorption of vitamin A since the vitamin is susceptible to oxidation by rancid fats. Susceptibility is reduced by the presence of antioxidants (Plack, 1965). Ames (1969) reports that in vitamin E-deficient rats oral supplementation with d-α-tocopherol increased the utilization of orally administered vitamin A approximately six-fold.

Bile salts and lecithin aid the absorption of vitamin A by dispersing the fat in the intestine (Plack, 1965), although the absorption of retinol is less dependent on the specific nature of the detergent than is the absorption of β-carotene (Olson, 1964b).

The amount of dietary protein may also affect the absorption of vitamin A. Low protein levels presumably decrease the activity of certain enzymes involved in vitamin A metabolism (Plack, 1965) and reduce the number of intestinal epithelial cells (Mahadevan et al., 1965).

Conversion of carotene

In 1930 Moore suggested that carotene behaved in vivo as a precursor of vitamin A (Moore, 1930). The site of this conversion and the exact mechanism have since received considerable attention in the literature.

Site In 1931 Moore assumed that the conversion of carotene was effected in the liver since vitamin A appeared in this organ after feeding β-carotene to vitamin A-deficient rats (Moore, 1931). Subsequent investigations revealed, however, that the intestine, and not the liver, is the primary site of conversion. This was firmly established by Thompson and associates (1949, 1950) who reported the presence of vitamin A in the intestine of vitamin A-deficient rats within five minutes after a carotene meal. The first increase of vitamin A in the liver and blood, however, occurred after this five-minute

interval, and in selected cases, appearance in these sites was experimentally prevented by diverting the lymph flow.

Rate of conversion of carotene to vitamin A is apparently not homogeneous throughout the length of the intestine. Thompson and coworkers (1949) dosed vitamin A-deficient rats with 4 mg of β-carotene in arachis oil and sacrificed the animals 0.5, 1, and 2 hours later. The second quarter of the small intestine had the highest content of vitamin A regardless of the time interval between dosing and autopsy. The upper quarter of the intestine contained considerable vitamin A, while the lower half had very little. In a second study Thompson et al. (1950) reported that the peak of β-carotene conversion occurred just proximal to the middle point of the small intestine. Olson (1961b) also found variations in the retinyl ester formation in different portions of the small intestine of the rat. One hour after intraduodenal injection of ^{14}C-β-carotene (suspended in Tween 20), the first third of the intestine contained the highest amount of ^{14}C-retinyl ester, the middle intestinal segment contained somewhat less labeled ester, and the last third of the intestine contained very little of the ^{14}C-retinyl ester.

Both the liver and the lung had been suggested as secondary sites for β-carotene conversion (Olson, 1964a). Zachman and Olson (1963) demonstrated the formation of retinyl ester from β-carotene in isolated perfused rat liver. Olson and Hayaishi

(1965) further reported conversion of β-carotene to retinal catalyzed by a cell-free enzyme system derived from rat liver. However, the maximum rate of carotene cleavage in the rat intestinal wall is 10 to 20 times that required to satisfy its nutritional requirements (Olson, 1961b) and apparently little carotene reaches the liver in a healthy animal even after a large dose of the provitamin (Thompson et al., 1949, 1950; Huang and Goodman, 1965). Olson and coworkers (1960) observed only negligible formation of retinol in dog heart-lung preparations and concluded that the lung is not an important organ for conversion of β-carotene into vitamin A.

Mechanism Karrer and coworkers originally postulated in 1930 that β-carotene is cleaved at the central 15-15' double bond to yield two molecules of retinol (Karrer et al., 1930). Glover and Redfearn (1954) proposed that terminal oxidation is the method of conversion with one molecule of retinol being formed per molecule of β-carotene through a series of β-apocarotenals. Olson (1961a, 1969) has summarized the development of these two major hypotheses.

Most recent studies support the central cleavage hypothesis with the conclusion that the terminal oxidation pathway is of minor importance in the gastrointestinal tract (Olson, 1964a, 1969; Goodman, 1969a). Olson (1961b) injected ^{14}C-β-carotene into rat duodenal-jejunal loops. Labeled retinyl esters accumulated in the mucosal cells while acidic products contained

less than 25 per cent of the radioactivity found in the retinyl ester fraction. If oxidation of one mole of β-carotene to one mole of retinol were the predominant pathway of conversion *in vivo*, the low molecular weight compounds and acidic labeled compounds recovered should approximate the amount of retinyl ester formed. Blomstrand and Werner (1967) fed labeled β-carotene to human volunteers and recovered 60-75% of the absorbed radioactivity as ^{14}C-retinyl esters. They concluded that in human intestinal mucosa the mechanism of vitamin A biosynthesis is central cleavage of β-carotene into two molecules of retinol.

Goodman and Huang (1965) investigated the conversion mechanism using 104,000 x *g* soluble supernatant fractions of rat mucosal homogenates. After incubation of the enzyme preparations with ^{14}C-β-carotene (about 1100 cpm per incubation flask), extraction, and chromatography on deactivated alumina, 535 ± 28 cpm were recovered in the β-carotene fraction while 460 ± 20 cpm were recovered in the retinal fractions. Thus, the retinal fraction contained more than 80% of the radioactivity which had disappeared from β-carotene. Goodman and Huang (1965, p. 880) cite their results as "not consistent with the possibility that only one molecule of retinol is formed from one molecule of β-carotene".

Lakshmanan *et al.* (1968) suggest that the rapid conversion of β-apo-carotenals to retinal can be explained by scission at the 15-15' position instead of through stepwise terminal

oxidation as postulated by Glover.

Inconclusive data from Crain et al. (1965) indicated that some β-carotene was converted to products other than retinol. A mixture of 1.2 mg of doubly-labeled $^{14}C,^{3}H$-β-carotene and 0.2 mg singly labeled 6,7-^{14}C-retinol was injected into ligated intestinal segments of rats. If β-carotene were converted only to retinol, proportional amounts of the absorbed labeled carbon from β-carotene and from retinol should have been stored in the liver. Crain and his coworkers calculated that 2.96 μmoles of labeled carbon should have been found in the liver from β-carotene instead of the observed 0.31 μmole and therefore concluded that some β-carotene was metabolized by a process other than central fission.

Goodman et al. (1966b) fed β-carotene uniformly labeled with ^{14}C and specifically labeled with ^{3}H at the 15 and 15' positions to rats. There was no loss of ^{3}H relative to ^{14}C during the cleavage reaction indicating that the hydrogen atoms attached to the central carbon atoms were completely retained during the biosynthesis of retinal. Complete retention of ^{3}H relative to ^{14}C was also found during the conversion of the β-carotene to retinal with homogenates of rat intestinal mucosa.

Goodman (1969a, p. 963) summarizes: "The reaction appears to be a dioxygenase reaction in which molecular oxygen reacts with the central two carbon atoms of β-carotene followed by the cleavage of the central double bond of carotene to yield two molecules of retinal."

The second step in the synthesis of retinol from carotene, the reduction of retinal to retinol, has been investigated by Fidge and Goodman (1968) with purified preparations from rat intestinal mucosa. Retinal reduction requires NADH or NADPH as cofactor and is stimulated by the addition of glutathione and inhibited by -SH inhibitors.

Olson (1961b) reported that the rate of retinyl ester formation *in vivo* increased linearly with the amount of β-carotene present to about 80 μg. Goodman *et al.* (1967) indicated that the β-carotene cleavage reaction *in vitro* displayed Michaelis kinetics with a V_{max} of 8.3×10^{-9} mole of retinal formed per hour and an apparent K_m of 3.3×10^{-6} mole (7 mg of protein partially purified by ammonium sulfate precipitations was used in each incubation). In their system the reaction rate was directly proportional to the amount of substrate as long as the β-carotene added to each 2 ml incubation mixture did not exceed 1.8 μg.

Olson (1961a) hypothesized that the rate limiting step in overall conversion resides at the absorptive step since the calculated amount of intracellular β-carotene is very low. Olson (1961a) also suggested that the increase in retinyl ester to a maximum concentration, followed by a decrease, indicates that the formation of the carrier lipoprotein and its secretion into the lymph may be a second rate-limiting step in the metabolism of β-carotene.

Factors essential for β-carotene cleavage Numerous workers (summarized by Olson, 1969; Goodman, 1969a) report that carotene cleavage requires molecular oxygen *in vitro*. Vartapetian and associates (1966) confirmed these observations *in vivo* when they fed β-carotene to vitamin A-deficient rats and found that the liver retinyl ester contained heavy oxygen which originated from atmospheric oxygen $^{18}O_2$.

Previously Thompson *et al.* (1950) failed to note conversion of β-carotene to retinyl ester in rat intestine between the pylorus and the entrance of the common bile duct. Olson (1960, 1961b) reported that the conversion of β-carotene (suspended in Tween 20) into retinyl ester in washed ligated loops of bile duct-ligated animals occurs only in the presence of bile or bile acids and stated that bile salts probably enhanced β-carotene absorption. He also hypothesized that the conversion of β-carotene to retinyl ester in the lower region of the small intestine was limited by the low concentration of bile salts as well as by the relative inability of the mucosal cells to catalyze the reaction.

When rat intestinal slices were incubated with β-carotene (suspended in Tween 40), formation of retinyl ester depended on the presence of bile or bile acids. Glycocholate also stimulated carotene cleavage by intestinal tissue of the hamster, chicken, and lamb but not by the opossum or turtle (Olson, 1964b). Olson reported that unconjugated acids, with the exception of cholate, were inactive and postulated that

> "although the physiochemical state of the solubilized carotene is undoubtedly important, the particular effectiveness of conjugated bile salts in stimulating carotene cleavage to retinol ester must be dependent on specific biological interactions with mucosal cells" (Olson, 1964b, p. 406).

Goodman and coworkers (1967) studied the carotene cleavage enzyme in a cell-free system derived from rat intestinal mucosa. Under these conditions, the bile salt requirement was highly nonspecific and could be satisfied by a variety of bile salts or synthetic detergents; in the absence of any bile salt or detergent, no cleavage enzyme activity was observed. The need for a detergent (or bile salt) was also observed in cell-free systems from hog or rabbit intestinal mucosa (Fidge et al., 1969; Lakshmanan et al., 1968).

Detailed in vitro investigations of cleavage enzyme activity in intestinal homogenate fractions from the rat (Goodman et al., 1967), the rabbit (Lakshmanan et al., 1968) and the hog (Fidge et al., 1969) indicate that the reaction has a slightly alkaline pH optimum in all three species. Quantitative differences were seen among the three species but all three enzymes were inhibited by ferrous iron chelators and sulfhydryl-binding compounds while being stimulated by the addition of thiols. These findings suggest the involvement of a metal ion and of one or more -SH groups of the enzyme in the cleavage reaction. The molecular weight of these enzymes appears to be in the 100,000 to 200,000 range.

Esterification

A number of recent studies indicate retinol formed from β-carotene is esterified and subsequently metabolized in the same manner as preformed retinol. Huang and Goodman (1965) report that the composition of rat lymph retinyl esters after test meals containing β-carotene was virtually identical with that seen after feeding preformed labeled retinol. They further reported that retinyl palmitate was the predominant ester reaching the lymph after feeding β-carotene.

Mahadevan and Ganguly (1961) fed rats retinol in widely varying fatty acid carriers. In all cases they found the intestinal muscles contained 79-93% of the retinyl esters as palmitate 2.5 to 3 hours after feeding. Mahadevan et al. (1963a) fed rats acetate, stearate, laurate, palmitate, and linoleate esters of retinol. Each ester was given in groundnut oil; the intestinal tissues were later analyzed for retinyl ester composition. Regardless of the ester fed, palmitate was the predominant ester in the mucosa, thus demonstrating that even the long-chain esters were hydrolyzed at the time of absorption.

The mechanism for the preferential esterification of retinol with palmitate is not yet clear. Ganguly (1967) suggested that some specific lipoprotein, which can more easily recognize the palmitate ester of retinol, picks up retinyl palmitate and transports it through the lymphatic system.

Presumably the other esters remaining in the mucosa would eventually be re-esterified with palmitate and then would be readily removed. Olson (1968) alternatively hypothesizes that the predominance of palmitate must reflect the specificity of the retinyl ester synthetase.

Transport of vitamin A

Huang and Goodman (1965) reported that more than 90% of the vitamin A in the lymph of rats is present as retinyl ester (mainly as the palmitate ester), while most of the remainder is present as retinol, retinal, or acidic compounds. Radioactivity recovered from the lymph as labeled β-carotene was insignificant confirming the reported inability of the rat to absorb unchanged β-carotene. Human lymph as analyzed by Goodman et al. (1966a) contains similar proportions of vitamin A substances, although man on the average can absorb slightly more dietary carotene than rats.

Ganguly (1960) reviewed reports on vitamin A transport and concluded that vitamin A was absorbed only through the lymphatic route and that transport of vitamin A through the portal route was not significant. Subsequent work, however, has led him to re-evaluate this conclusion and state that "vitamin A is not absorbed through the lymphatic system alone, significant amounts of it being absorbed through the portal route also" (Ganguly, 1969, p. 923).

Murray and Grice (1961) obstructed the normal lymph flow by ligation of the lymph duct and found retinol to be absorbed by the portal route. Lawrence et al. (1966) fed 5 mg retinyl-15-^{14}C palmitate-9,10-^{3}H (1.39 x 10^6 cpm of ^{14}C and 1.07 x 10^6 cpm of ^{3}H) to lymph-cannulated rats by stomach tube and recovered retinyl ester (7.87 x 10^4 cpm from ^{14}C and 2.53 x 10^2 cpm from ^{3}H) in the liver. They concluded that circulation routes other than the thoracic lymph are involved in transport of retinyl ester from the intestine to the liver. Olson (1968) criticizes their work and suggests that cannulation of the lymph duct had partially blocked the normal lymph flow and caused the retinyl ester to be absorbed via the portal route.

Fidge and coworkers (1968) introduced seven to fourteen µg of retinol, retinal, or retinoic acid into the duodenum of lymph- and bile duct-cannulated rats as a bile-lipid mixture resembling normal intestinal contents. After introduction of ^{14}C-retinoic acid, 90-95% of the recovered radioactivity was found in the bile while only 5-7% of the ^{14}C from retinoic acid was recovered in the lymph. This finding indicates that retinoic acid absorption occurs via the portal venous route. When ^{14}C-retinol or ^{14}C-retinal were given, 70-80% of the radioactivity recovered was in the lymph and 15-20% of the labeled compounds were recovered in the bile. Fidge and his associates (1968) concluded from this study that under normal conditions dietary retinal is reduced to retinol, esterified,

and transported via the lymphatics in a manner similar to dietary retinol. However, a small amount of retinal is apparently oxidized and transported similarly to retinoic acid by the venous portal route. After feeding ^{14}C-retinal more radioactivity was found in the bile when fat absorption was impaired (Fidge et al., 1968) which may explain the report of Crain et al. (1967) that following the administration of retinal-15-^{14}C to rats with ligated intestinal loops, portal blood contained 30-40% of the absorbed radioactivity.

The presence of an enzyme capable of oxidizing retinal to retinoic acid has been demonstrated in the rat intestinal mucosa (Crain et al., 1967; Deshmukh and Ganguly, 1967). Deshmukh and Ganguly (1967) suggest that in the presence of large amounts of retinal considerable retinoic acid can be formed in the intestine and that the retinoic acid may be very rapidly degraded, converted to an unknown derivative, or absorbed. Deshmukh and Ganguly (1967) also report the presence of retinal reductase in rat intestinal mucosa. Fidge et al. (1968) suggest that the relative importance of the lymphatic and portal pathways of retinal metabolism in vivo can vary with the condition of the animal.

In the intestinal lymphatics, transport of retinyl esters appears to be largely effected by the chylomicrons. Huang and Goodman (1965) fed test meals containing ^{14}C-β-carotene or ^{14}C-retinol to rats with cannulated thoracic ducts and

collected their chyle. Washed chylomicrons contained 82% of the radioactivity recovered in the lymph. The lymph normally enters the blood stream through the thoracic duct and the chylomicrons are removed from the vascular compartment by the liver (Goodman et al., 1965). During active absorption following the administration of either retinol or β-carotene, the major portion of retinyl esters and carotenoids in human serum has been found in the S_f10-400 lipoprotein fractions. A few hours later, the amount of retinyl ester in the S_f10-400 fraction decreased and the S_f0-10 fraction became the major carrier of retinyl esters and carotenoids (Krinsky et al., 1958; Cornwell et al., 1962).

Ganguly (1960) summarized numerous published studies and concluded that retinyl ester and retinol are transported in normal post-absorptive blood by separate lipoproteins. When retinyl ester or retinol is administered orally to rats, pigs, or humans, the increase of vitamin A in blood occurs in the ester fraction only. The retinol level of the blood is also independent of the liver reserve of retinol or retinyl ester (Ganguly, 1960).

The existence of a specific protein for binding plasma retinol has been postulated for more than a decade (Ganguly, 1960). Initially this transport protein was identified in the albumin fraction, α_1-globulin fraction, α_2-globulin fraction, the β-globulin fraction and the prealbumin fraction by various

workers (Olson, 1968). Alvsaker et al. (1967) postulated that tryptophan-rich prealbumin was the carrier protein for retinol in human serum. Their hypothesis was based on the demonstration of green fluorescence of the protein upon exposure to ultraviolet light. The fluorescence was shown to be due to the presence of retinol.

In 1968 Goodman and coworkers (Raz et al., 1968; Kanai et al., 1968; Goodman, 1969b) made a major contribution to the understanding of post-absorptive transport of retinol by delineating the plasma protein carrier. They collected plasma from volunteers who had been injected intravenously with retinol-15-^{14}C and reported that retinol-binding protein is a distinct plasma protein with a hydrated density greater than 1.21. The purified transport protein had α_1 mobility on electrophoresis and a molecular weight near 21,000-22,000. According to this study there is one binding site for retinol per molecule of retinol binding protein, and the usual concentration of the protein in plasma is 3-4 mg per 100 ml. Retinol-binding protein appears to circulate in the plasma as a complex with a large protein which has prealbumin mobility on electrophoresis. The formation of the complex between retinol-binding protein and prealbumin may stabilize the interaction of retinol with retinol-binding protein as well as preventing the glomerular filtration of the retinol-binding protein molecule. Raz et al. (1968) subsequently reported that the plasma prealbumin which

forms a complex with retinol-binding protein appears to be the same protein fraction which binds thyroxine. Addition of enough thyroxine to saturate prealbumin did not impair the ability of prealbumin to complex with retinol-binding protein. This suggests that the interaction between retinol-binding protein and prealbumin involves a site on the prealbumin molecule separate from the site of the thyroxine-prealbumin interaction.

Vitamin A deposition

The liver, under normal conditions, is the primary site for vitamin A deposition (Moore, 1957). Goodman et al. (1965) injected chylomicrons containing labeled vitamin A intravenously into normal rats and studied the tissue distribution of the radioactivity. At least two-thirds of the ^{14}C in injected chylomicrons was removed from the vascular compartment by the liver. Substantial amounts of the remaining ^{14}C were found in the kidneys and in the total depot fat. Plasma contained 20% of the recovered ^{14}C 17 minutes after injection but only 1-4% of the radioactivity after longer time intervals indicating that plasma does not serve as a reservoir for deposition of vitamin A. Willmer and Laughland (1957) fed ^{14}C-β-carotene to vitamin A-deficient rats and found after various time intervals that the total radioactivity recovered was greatest in the liver followed by the intestines, adrenal glands, blood, kidneys, lungs, heart, hypophysis, stomach and

spleen. The concentration of ^{14}C per gram of tissue was greatest in the adrenal glands. Goodman et al. (1965) did not find large quantities of radioactivity in the adrenals and suggested that the findings of Willmer and Laughland might have been due to their use of vitamin A-deficient rats.

With the exception of blood and the retina, vitamin A in the body exists mainly in its esterified form (Moore, 1957) and predominately as palmitate (Mahadevan and Ganguly, 1961). Goodman and associates (1965) reported that three hours after intravenous injection of chylomicrons into rats the labeled retinyl esters contained 85-90% saturated fatty acids and that retinyl palmitate composed 70-75% of the labeled esters. Mahadevan and Ganguly (1961), Futterman and Andrews (1964), and Mahadevan et al. (1964) also indicated that retinyl palmitate is the principle ester of retinol deposited in the liver. Numerous reports indicate that most retinyl ester is stored in the Kupffer cells of the liver (Ganguly, 1967; Olson, 1968).

The factors which control the storage and release of retinol by the liver have not been identified, but animals placed on vitamin A-deficient diets maintain a relatively constant level of plasma retinol until their liver stores are almost depleted (Dowling and Wald, 1958; Olson, 1968).

Enterohepatic circulation

Numerous reports indicate that, in bile duct-cannulated rats, water-soluble derivatives of vitamin A are excreted in

the bile after the intraportal, intraduodenal, or intrajugular injection of ^{14}C-labeled retinoic acid, retinal, or retinol (Zachman and Olson, 1964; Dunagin et al., 1964; Zachman et al., 1966a; Emerick et al., 1967; Lippel and Olson, 1968a, 1968b; Fidge et al., 1968).

Zachman and coworkers reported that forty per cent of the labeled carbon from retinoic acid injected intraportally appeared in rat bile within six hours and 60% of the ^{14}C was excreted into the bile within 24 hours. Less than 1% of the radioactivity injected as ^{14}C-retinoic acid appeared in the liver (Zachman and Olson, 1964; Zachman et al., 1966a). When retinoic acid was given intraduodenally, 92-95% of the recovered radioactivity was found in the lymph within 24 hours (Fidge et al., 1968). When ^{14}C-retinol or ^{14}C-retinal was injected intraportally, 25-35% of the dose was excreted in the bile in the first 24-hour period and 25-35% was stored in the liver as retinyl ester (Zachman et al., 1966a). Metabolites of ^{14}C-retinol and ^{14}C-retinoic acid were also found in the bile of chicks, guinea pigs (Zachman et al., 1966b) and rabbits (Olson, 1969) after intraportal injections of the labeled compounds.

Zachman and Olson (1965) showed that water-soluble metabolites of ^{14}C-retinol were formed in isolated perfused rat liver. After one hour of perfusion, approximately 10% of the perfusate activity appeared in the bile. This rate of

production of metabolites was similar to the rate observed *in vivo* and Zachman *et al.* (1966a) suggest that the liver is probably the major site of their formation.

Retinol derivatives travel between the liver and the gastrointestinal tract in an enterohepatic circulation (Olson, 1968). When rat bile containing ^{14}C-retinol metabolites was placed in the intestinal loop of another rat, 30% of this radioactivity was again excreted into the bile (Zachman and Olson, 1964; Zachman *et al.*, 1966a). Semilogarithmic plots of bile radioactivity against time of cannulation indicated that some reabsorption of metabolites normally occurred since rate of excretion in uncannulated rats was only one-half the rate in cannulated animals (Zachman *et al.*, 1966a).

Goodman *et al.* (1965) recovered only 8-9% of the radioactivity in bile of rats which had been intravenously injected with chylomicrons containing ^{14}C-retinyl ester. Zachman and coworkers (1966a) suggest that the smaller amount of radioactivity in the bile in this study was the result of ^{14}C being injected as an ester. They further postulate that the extent of oxidation of retinol to retinal and of retinal to retinoic acid may be important in determining the quantity of bile metabolites from each substrate.

The major metabolite of retinoic acid in rat bile appears to be retinoyl-β-glucuronide (Dunagin *et al.*, 1965; Dunagin *et al.*, 1966). When ^{14}C retinal was injected intraportally

into rats, a minimum of 6% of the injected dose appeared as free or conjugated retinoic acid (Dunagin et al., 1966). Nath and Olson (1967) injected rats with large doses of retinol and determined the labeled compounds in bile after 6, 15, and 30 days. Approximately 0.3 μg of ^{14}C was excreted per ml of bile at all time intervals. Over eighty per cent of the recovered radioactivity had an R_F similar to retinoyl-β-glucuronide on thin-layer chromatography.

Lippel and Olson (1968a, 1968b) demonstrated that several of the non-polar products previously reported in rat bile were actually breakdown products of β-glucuronide which had been produced during the isolation procedures. However in Roberts and DeLuca's work some $^{14}CO_2$ was released during metabolism of [14] or [15]^{14}C-retinoic acid. They concluded that retinoyl-β-glucuronide could not account for all the biliary excretion products of retinoic acid (Roberts and DeLuca, 1967a, 1967b; DeLuca and Roberts, 1969).

The enterohepatic circulation of vitamin A derivatives is apparently not a conservation mechanism (Olson, 1969) since the rate of excretion of labeled retinoic acid derivatives in the feces closely paralleled their rate of secretion into bile (Nath and Olson, 1967).

Excretion

Previous concepts of vitamin A metabolism included the idea that the vitamin could not be excreted and was, therefore,

stored in the liver. Actually, a number of derivatives of vitamin A had simply escaped detection until the use of labeled compounds, and it now seems that in the rat most metabolites of vitamin A are excreted in the feces (Olson, 1968). A greater percentage of a dose of retinoic acid is excreted in rat feces than is excreted from equivalent doses of retinol (Roberts and DeLuca, 1967b). Urine is a second important route for excretion of vitamin A derivatives. When retinol and retinoic acid were labeled in different positions, 8-40% of the total dose was excreted in the urine (Wolf et al., 1957; Roberts and DeLuca, 1967b; Nath and Olson, 1967; Sundaresan and Therriault, 1967, 1968). Larger percentages of labeled carbon from [6,7]-^{14}C- were excreted than when the [14] or [15]-labeled compound was injected (Roberts and DeLuca, 1967b; Sundaresan and Therriault, 1967, 1968).

Roberts and DeLuca have demonstrated the decarboxylation of retinoic acid in vitro with liver, kidney and intestinal slices (Roberts and DeLuca, 1967a; DeLuca and Roberts, 1969). They suggest three different pathways of metabolism in vivo based on their work with retinoic acid and retinyl acetate labeled in various positions with ^{14}C. Pathway I includes urinary and fecal products still containing all carbon atoms. This pathway includes retinoyl-β-glucuronide and probably represents approximately 60% of the vitamin A metabolism. Pathway II involves terminal decarboxylation of retinoate and

Pathway III entails removal of the last two carbon atoms of the isoprenoid chain as CO_2. Pathway II may be a precursor of Pathway III.

Active form

The active form of vitamin A is still in doubt. Retinoic acid will maintain growth but is not stored in the liver (Dowling and Wald, 1960), will not function in the visual cycle (Dowling and Wald, 1960), and will not maintain normal reproduction (Ganguly, 1967). Wald (1960) and Heller (1968) have demonstrated that 11-cis retinal combines with an ε-amino group of opsin-bound lysine to form rhodopsin in the visual process. Olson (1969) speculates that retinal may be the active form of the vitamin in stimulating growth and maintaining the normal reproductive process. He does not eliminate the possibility that decarboxylation may produce a biologically active molecule but cites a study from Coward and J. Thompson (1966) demonstrating that undegraded retinol and retinyl esters are the major form of vitamin A found in rat tissue after administration of small doses of retinyl acetate.

Effects of Protein Level on the Metabolism of Vitamin A and β-carotene

Absorption of preformed vitamin A

Mahadevan et al. (1965) reviewed the literature on absorption of vitamin A and concluded that secretion of many pancreatic enzymes, such as lipase, trypsin, and amylase, is

reduced with diets deficient in protein. They postulated that pancreatic vitamin A esterases would probably be similarly affected. Protein malnutrition also interferes with normal bile formation. Since bile salts activate the enzyme which hydrolyzes the higher retinyl esters, decreased secretion of bile directly affects vitamin A absorption (Mahadevan et al., 1965).

Deshmukh et al. (1964) fed a single massive dose (10 mg) of retinyl acetate in groundnut oil to young rats which had been maintained on 5, 10, or 20% protein (casein) diets. One hour after dosing, the concentrations of retinol in the intestine, blood, and liver were highest in the group fed 20% protein and least with 5% protein. However, differences in liver vitamin A deposition among the groups receiving the three dietary protein levels were not as apparent at longer intervals after dosing. Deshmukh and co-workers suggest that with reduced protein intake the fraction of vitamin A absorbed from a single dose is not greatly diminished but that the rate of absorption is slowed. The alcohol-ester ratio of the intestinal contents in the Deshmukh study was always highest with the 20% protein diet and least with the 5% level of dietary protein. This suggests that the hydrolytic enzyme is more active in the group fed 20% protein. Deskmukh and coworkers (1964) also assayed pancreatic and intestinal (mucosa) retinyl ester hydrolases and synthetases. Activity of these enzymes

(expressed as μg of retinol or retinyl ester formed per mg of whole homogenate protein) was reduced with 5% protein diets to 25-50% of the levels found in tissues of rats fed 20% protein.

Deshmukh et al. (1965) also studied the absorption of retinal in rats. Young animals with low reserves of vitamin A were fed 5 mg retinal in groundnut oil. Reduction of retinal to retinol and its deposition in the liver as retinol or retinyl esters decreased with reduced protein intake.

Nir and coworkers (1967) confirmed the relationship between dietary protein intake and absorption of preformed vitamin A in another species. They fed vitamin A-depleted chicks a single dose of 11,000 I.U. of retinyl palmitate and reported that absorption of the vitamin as measured by plasma vitamin A levels was slower in chicks which had been maintained on a 10% protein diet than in those maintained on a 30% protein diet. When chicks were dosed with an equivalent dose of vitamin A alcohol, absorption was equally efficient at both dietary protein levels. Seemingly the uptake of retinol into the mucosal cell is not dependent on protein. Hydrolysis of retinyl palmitate, however, appears to require an adequate level of dietary protein. In the same study Nir et al. also assayed retinyl ester hydrolase and synthetase from pancreatic and mucosal tissue. Both enzymes displayed decreased activity in tissues derived from protein-depleted chicks although the retinyl ester synthetase was less affected than the hydrolase

in these experiments. The apparent discrepancy between retinyl ester synthetase activity *in vivo* and *in vitro* can be resolved if the levels of the enzyme *in vivo* are higher than necessary to esterify all the retinol absorbed into the mucosal cell. Then, a decrease in potential synthetase activity in tissues from protein-deficient chicks would not cause an obligatory decline in plasma vitamin A levels.

Absorption and conversion of carotene

Mahadevan *et al.* (1965) have indicated several ways in which protein malnutrition could affect absorption and conversion of β-carotene. Protein malnutrition, for example, leads to extensive atrophy of the cells of the intestine which may lead to impaired uptake of all products of digestion. Furthermore, lipid absorption may be reduced by low lipase levels characteristically found with low protein diets. Finally, bile secretion is abnormal in protein-deficient humans and bile is essential for the conversion of β-carotene to retinol. Jagannathan and Patwardhan (1960a) were not able to demonstrate a difference in hepatic retinol in β-carotene supplemented rats fed 12, 18, or 30% protein (casein) diets for four weeks. However, hepatic retinol was significantly lower in groups fed 6% protein diets than in the 12, 18 or 30% groups. These workers later studied utilization of hepatic deposits and indicated that an increased metabolic requirement for vitamin A in animals fed 30% protein diets might explain

the relatively low value for hepatic deposition found in these animals (Jagannathan and Patwardhan, 1960b).

Arnrich and Pederson (1956) reported that utilization of carotene suspensions, as measured by liver and kidney vitamin A deposition in rats previously depleted of vitamin A, increased progressively as casein comprised 11, 22 or 40% of the diet. When preformed vitamin A was fed, similarly vitamin A-depleted rats on the low protein diet stored as much vitamin A in their livers as the animals fed 40% casein. Since it was not possible to demonstrate that protein intake affects utilization of preformed vitamin A, Arnrich and Pederson concluded that the effect of protein intake occurred in the absorption or conversion of carotene rather than in the utilization of vitamin A formed by the conversion process. Ruffin (1965) likewise fed 10, 20, or 40% protein (casein) diets to young vitamin A-depleted rats and reported that utilization of carotene was enhanced by increasing levels of protein while the quantity of protein in the diet had no effect on dietary vitamin A utilization. Ruffin and Arnrich (1966) also reported that utilization of carotene was affected by 10 and 20% mean dietary protein levels even when the carotene supplement was fed separately from the protein.

Mathews and Beaton (1963) used a similar design but measured vitamin A serum levels instead of hepatic deposits. They administered 6 µg vitamin A, 12 µg vitamin A, or 24 µg carotene daily for fourteen days to rats which had been

maintained on 4% or 20% casein vitamin A-depletion diets for five weeks. Rats on the 4 and 20% casein diets showed almost identical serum retinol response to administered vitamin A while animals maintained on the 4% casein diet and supplemented with carotene had significantly lower fasting serum retinol levels than the group which received a 20% casein diet with carotene supplementation. Mathews and Beaton suggest that in animals on a 4% casein diet there is interference with the absorption or conversion of carotene.

Friend et al. (1961) also indicate that low liver reserves of retinol in protein malnourished pigs may be due to reduced conversion of carotenoids or to reduced absorption or both.

Tissue levels of retinol reflect not only the efficiency of carotene conversion but also the subsequent metabolism and utilization of the vitamin. Deshmukh and Ganguly (1964), therefore, endeavored to isolate intestinal processes by examining the amounts of vitamin A formed in the intestine from a given amount of β-carotene. Vitamin A-depleted, young rats were given 5, 10, or 20% casein diets for about 30 days. At 0, 1, 2, 3, 4, 6, 12, or 24-hour intervals after a 3 mg dose of β-carotene in groundnut oil (mixed with 1 g of the respective diet), the liver, blood, stomach, intestinal walls and intestinal contents were analyzed for vitamin A and β-carotene content. At almost all time intervals after the administration

of β-carotene, the vitamin A content of the intestinal wall, blood, and liver progressively increased with higher protein levels of the diet. The carotene content of the intestinal wall and intestinal contents decreased with increasing protein intakes. The differences in carotene and in vitamin A recovered from the intestinal wall were greater between groups receiving 5 and 10% protein diets than between those animals receiving 10 and 20% protein diets. Deshmukh and Ganguly suggest that the increased carotene content of the small intestinal wall and contents in rats fed low-protein diets reflect the reduced conversion of β-carotene to vitamin A and would be further evidenced by the reduced absorption and storage of the newly-formed vitamin.

Deshmukh and Ganguly (1964) also fed 0.4 mg of β-carotene in groundnut oil daily for seven consecutive days to rats which had previously received 5, 10, or 20% protein diets for 30 days. On the 8th day after carotene supplementation began, the animals were autopsied and tissues were analyzed for β-carotene and vitamin A content. Again, the vitamin A content of the intestinal wall, blood, and liver progressively increased with higher dietary protein content while the carotene content of the intestinal wall was inversely related to the dietary protein content.

Kotecheri (1967) studied the intestinal phase of carotene metabolism with the use of radioactive carotene. She fed 10

and 40% protein diets to vitamin A-depleted rats for four weeks. Subsequently ^{14}C-β-carotene was injected into the duodenum of the animals one hour after they had received a one gram test meal of their respective diets. After a two and one-half hour metabolic period, the mean retinyl ester recovered from the intestinal wall in the group receiving 10% protein was 1.87% of the injected dose, while the corresponding value for the group which had been fed 40% protein was 5.18%.

Gronowska-Senger and Wolf (1970) recently reported another study which indicates that the enzyme which converts β-carotene to retinal is dependent on dietary protein intake. Young rats were fed 0, 5, 10, 20, or 40% protein diets with casein replacing sucrose. Aliquots of the 96,000 x g supernatant of intestinal mucosa from each of the animals were incubated with β-carotene. A low protein diet (0-5%) resulted in a low activity of the enzyme (expressed as μmoles of retinal formed per total mucosa, per mg of protein, or per mg of DNA) although maximum activity was reached with the 10% protein intake rather than with 20 or 40% protein diets. Their studies demonstrated that the higher specific activity of the carotene cleavage enzyme isolated from rats fed a 10% protein diet was not due to decreased secretion of digestive enzymes from the pancreas, or to the presence of a cofactor in animals fed 10% dietary protein, or to the presence of an inhibitor in rats receiving 40% dietary protein. Polysome profiles indicated that *in vitro*

the mucosa from animals fed 10% protein may synthesize protein more efficiently than intestinal preparations from rats receiving 20 or 40% protein.

The results of Gronowska-Senger and Wolf (1970) are in contrast to those obtained in vivo by Kotecheri (1967) who found higher retinyl ester formation in animals fed 40% protein than in those fed 10% protein. In vivo the presence of higher levels of protein in the intestine may promote conditions favorable to β-carotene cleavage or may slow degradation of β-carotene allowing more retinyl ester to be formed.

Dowling and Wald (1958) and Friend et al. (1961) have established that hepatic reserves of vitamin A, provided they remain above a minimum level, are of little importance in regulating the amount of circulating retinol. Ascarelli (1969) suggests that protein supply is probably the most important limiting factor in determining level of vitamin A in the blood. The following studies indicate that both absorptive and post-absorptive phases of vitamin A transport are affected by dietary protein level.

Absorptive Rodbell et al. (1959) reported that the protein portion of chylomicra is manufactured in the intestinal mucosa. Thus absorption and transport of retinyl ester in the intestinal lymph system could conceivably be impeded by the diminished synthesis of the protein portion of chylomicra with dietary protein insufficiency.

Cravioto et al. (1959) have indicated that lipoprotein levels in the blood are reduced in kwashiorkor. Since newly absorbed retinyl esters are carried by low density lipoproteins in the blood, transport of the vitamin could be impaired with low dietary protein levels.

Studies with rats and with humans have confirmed the impaired transport of dietary retinyl esters in dietary protein insufficiency. Deshmukh et al. (1964), for instance, dosed young rats maintained on 5, 10, or 20% casein diets with 10 mg of retinyl acetate in groundnut oil. Within one hour of dosing, retinyl ester concentrations were highest in the 20% casein group and least in the 5% casein-fed rats. The same trend was observed at two and three hour intervals after dosing with retinyl acetate.

Arroyave et al. (1959) examined children with kwashiorkor in Central America. When the children were admitted to the hospital, oral administration of 75,000 µg of retinyl palmitate produced no measurable effect on serum vitamin A levels; but after only five days of dietary treatment with skimmed milk, an equal dose of retinyl palmitate produced large increases in serum vitamin A. Arroyave and coworkers suggest that therapeutic doses of skimmed milk may restore pancreatic lipase to satisfactory levels in three to four days. They further indicate that the low serum vitamin A levels found in kwashiorkor may be due to a generalized failure to absorb fat-soluble factors.

Post-absorptive In the post-absorptive state, retinol is transported by a high-density plasma protein which complexes with pre-albumin. Retinol transport in the fasting blood, as in the absorptive state, has been reported to vary with dietary protein levels in rats, pigs, chicks, and humans.

Deshmukh et al. (1964), for example, built hepatic vitamin A reserves in adult rats by giving a dose of 10 mg of retinyl acetate. The animals were then depleted of vitamin A reserves for several weeks on 5, 10, or 20% vitamin A-free casein diets. Serum retinol concentrations were directly correlated with albumin levels. When protein-depleted rats were refed with a 20% casein diet, both albumin and retinol levels increased to normal values (Deshmukh et al., 1964, 1965).

Mathews and Beaton (1963), also studying rats (mean initial wt = 183 g), fed 20% casein, 4% casein, or protein-free diets and orally supplemented the animals with vitamin A in corn oil for six weeks. When no dietary protein was given, both fasting serum retinol levels and serum protein levels were significantly lower than in comparable animals fed 4 or 20% casein. Serum albumin, as separated by starch gel electrophoresis, appeared to be reduced more than other protein zones. Rats fed 4% or 20% casein diets did not show significant differences between serum retinol levels or between serum protein levels.

In a subsequent study, 100 g rats were depleted of retinol and were fed for seven weeks 20% casein, 4% casein, or protein-

free diets ad libitum (Veen and Beaton, 1966). Restriction of dietary protein to 4% of the diet reduced plasma albumin, and thus plasma total protein, significantly below that of control animals. A further lowering of plasma albumin and total protein occurred in the group fed the protein-free diet. When increasing amounts of retinol were fed, plasma levels of retinol reached a plateau which indicated saturation of the retinol transport system. The plasma level of retinol at which saturation was evident depended upon the amount of dietary protein fed, and significant correlations of plasma retinol with albumin were exhibited. Veen and Beaton (1966) hypothesized that the protein which transports retinol is a subfraction of albumin.

Friend et al. (1961) obtained fasting blood samples from pigs fed low protein, low protein plus 5% casein or low protein plus 20% casein diets. In the protein-malnourished pigs, oral dosing with retinyl ester did not increase the low serum concentration of retinol as long as the serum albumin (as determined by paper electrophoresis) level remained low. When serum albumin concentrations were increased by raising the dietary protein intake, serum retinol also increased. The correlation ($r = 0.76$) between serum retinol and serum albumin was highly significant. Friend and his coworkers concluded that dietary protein deficiency limits the production of a serum protein fraction carrying vitamin A.

A correlation between plasma albumin and plasma retinol has also been demonstrated in chicks (Nir and Ascarelli, 1967). Ascarelli (1969, p. 917) suggests that

> "if we assume the synthesis of the complexing pre-albumin parallels the synthesis of the whole albumin fraction, then we may better understand such claims.. that there is a significant and positive correlation between vitamin A and albumin in plasma..".

Finally, in human studies, Gopalan et al. (1960) compared the serum retinol levels of apparently healthy children with those suffering from kwashiorkor. Serum retinol levels of the group with kwashiorkor were significantly lower than those of the control group despite the fact that the kwashiorkor group did not display clinical signs of vitamin A deficiency. When the children with kwashiorkor were treated with a high protein diet, a significant increase in serum retinol levels was observed although no vitamin A supplement had been administered. Arroyave and coworkers (1961) suggest that the sudden appearance of retinol in the circulation following protein therapy is due to mobilization of liver reserves. They reported that serum retinol did not increase with protein therapy in two children whose hepatic reserves were negligible. Arroyave et al. (1963) also indicate that the increase in retinol parallels the increase in serum protein and albumin concentrations.

Arroyave (1969) summarized results from his laboratory and suggested that in severe protein deficiency, due to the lowering of plasma protein carriers of the vitamin, the vitamin

A of the diet does not reach the liver and that whatever liver reserves exist are not available to the other tissues of the body for utilization.

Deposition of vitamin A

Studies of the effects of dietary protein on vitamin A deposition can be divided into two categories: those feeding preformed vitamin A and those feeding β-carotene. Reports, particularly in the category dealing with preformed vitamin A, are discrepant making the data difficult to interpret.

Preformed vitamin A Mahadevan et al. (1965) in a review of the literature, summarize and state that a low protein diet leads to reduced deposition of vitamin A in the liver.

Jagannathan and Patwardhan (1960a), for instance, fed 35 I.U. of vitamin A daily for four weeks to rats maintained on 3, 6, 12, 18, or 30% casein diets. The rats fed protein at the 3 or 6% level deposited significantly less hepatic vitamin A than did animals which received 12 or 18% protein in the diet. Animals receiving 30% dietary protein accumulated less vitamin A in the liver than did those fed 12% protein; however, in a subsequent study Jagannathan and Patwardhan (1960b) reported that vitamin A was utilized at a significantly higher rate in animals fed 30% dietary protein than in those fed 6, 12, or 18% protein. Thus they postulate in the second study (Jagannathan and Patwardhan 1960b, p. 788) that "increasing the dietary protein beyond a particular limit perhaps results in an

increase in the requirement of vitamin A for some metabolic functions connected with the quantity of ingested protein".

Ruffin (1965) fed 10%, 20%, and 40% protein (casein) diets to rats and supplemented the animals with 18 µg retinyl acetate. She found, on the other hand, that hepatic reserves of vitamin A were not significantly different at the three levels of dietary protein studied. These results confirmed similar work by Arnrich and Pederson (1956) in which they reported that hepatic deposition of vitamin A did not increase linearly with dietary protein when preformed vitamin A was used for supplementation.

Mathews and Beaton (1963) and Rechcigl et al. (1962) have also observed that liver reserves of vitamin A did not differ greatly in rats maintained on different levels of protein intake.

Deshmukh and Ganguly (1964) conclude from their work and from reviewing the literature that although absorption of vitamin A is slowed with a reduced protein intake, mobilization and utilization of the vitamin reserves are also retarded on a lower protein intake and that the net effect is one of similar hepatic deposition of vitamin A with a range of dietary protein intakes.

Work by MacMillan (1966) may clarify some of the apparent discrepancies of reports on deposition of vitamin A. MacMillan's data indicate that with medium (248 I.U./day for

28 days) or high (712 I.U./day for 28 days) levels of vitamin A supplementation an increase in hepatic reserves occurred as protein level comprised 10% or 20% of the diet. Excessive levels of dietary protein (40%) did not significantly increase hepatic vitamin A deposition above the level obtained with 20% dietary protein. With low (83 I.U./day for 28 days) levels of vitamin A supplementation, however, no effect on vitamin A reserves was observed as dietary protein levels were increased from 10% to 20% or 40%. Thus, level of dietary protein appears to be without effect on the utilization of doses of preformed vitamin A which are adequate for optimum biological performance; however, when supplementation levels are much higher, the effect of protein nutriture is evident.

β-carotene The following reports suggest that deposition of hepatic vitamin A from β-carotene is reduced when inadequate dietary protein is fed. Levels of dietary protein higher than the optimum for growth may augment hepatic vitamin A deposition beyond that obtained with a diet "adequate" in protein.

Jagannathan and Patwardhan (1960a) fed young rats diets containing 6, 12, 18, or 30% protein (casein) and supplemented daily with 30 μg of β-carotene for four weeks. Hepatic storage of vitamin A was significantly lower in animals receiving protein at the 6% level than in those fed the higher levels of protein. No significant differences in liver reserves were

found among groups fed 12, 18, and 30% dietary protein.

Arnrich and Pederson (1956) investigated hepatic and renal vitamin A deposits in rats previously depleted of vitamin A and then fed 11, 22, or 40% dietary protein. In animals supplemented with β-carotene, they found that vitamin A reserves were progressively greater as dietary protein was increased. Ruffin (1965) and Kotecheri (1967) also reported that hepatic vitamin A reserves derived from β-carotene increased as protein fed was raised from 10% to 20% or 40% of the diet. Kotecheri (1967), however, reported that supplementation with β-carotene for at least four weeks was necessary before rats maintained on the high protein intake had significantly larger hepatic reserves than those animals fed the adequate level of protein. Johnson and Arnrich (1960) demonstrated that an increase in dietary protein from forty to sixty per cent had no further effect on storage of hepatic vitamin A in rats supplemented for four weeks with β-carotene.

Berger et al. (1962) investigated hepatic and renal deposition of vitamin A and concluded that the efficiency of carotene recovery is affected by the level of dietary protein. After correcting for utilization of vitamin A at both protein levels, they calculated carotene recovery to be 64.6% of the ingested dose with an 18% casein diet and 41.8% of the dose with 9% dietary casein. Berger et al. (1962) stress the importance of determining the amount of vitamin A metabolized

at different levels of dietary protein when evaluating the efficiency of carotene conversion by liver and kidney storage.

Jagannathan and Patwardhan (1960b, p. 785) caution that

> "the amount of vitamin A storage found in the liver is normally the sum total of the effect of two processes, both of which can vary according to the composition of the diet: (1) the degree of absorption of the vitamin from the gastro-intestinal tract resulting in the accumulation of it in the liver; (2) the rate of utilization of the stored vitamin by the organism for its metabolic functions resulting in its withdrawal from the storage organs".

Utilization of vitamin A

Utilization of vitamin A is usually studied by administering a large single dose or several sizeable doses of vitamin A to normal animals to build uniform liver reserves of the vitamin. Animals are then placed on experimental diets and depletion of the existing stores is measured.

Mahadevan et al. (1965) reviewed the literature and concluded that when utilization of vitamin A is separated from the effects of absorption, animals receiving the lowest quantity of dietary protein had the largest liver reserves while those receiving higher protein diets retained less vitamin A in their livers.

Jagannathan and Patwardhan (1960b), for instance, fed 6, 12, 18, or 30% protein (casein) diets to young rats with high initial vitamin A reserves. After four weeks on the diets, all animals had lost substantial amounts of vitamin from the liver.

Total vitamin A in the liver decreased from 614 I.U. for the animals fed a 6% protein diet to 536 I.U. for the animals receiving 30% dietary protein. The higher retention of hepatic vitamin A at the 6% protein level was attributed to a lowered metabolic requirement for the vitamin due to slower growth at this protein level. Jagannathan and Patwardhan (1960b) postulate that feeding higher levels of dietary protein may increase the requirement for vitamin A for some metabolic functions connected with the quantity of protein ingested.

Rechcigl and coworkers (1962) also studied utilization of hepatic and renal vitamin A in rats given a single large dose of vitamin A acetate and then fed protein-free, 6% casein, 12% casein or 18% casein diets. After three weeks on the experimental diets, hepatic vitamin A totaled 317 µg, 303 µg, 268 µg, and 241 µg respectively. Thus, vitamin A depletion or utilization was least in animals receiving a protein-free diet and greatest in rats receiving the 18% casein diet. Renal vitamin A, however, increased as higher levels of dietary protein were fed. A linear relationship apparently existed between rate of growth and vitamin A utilization at the different levels of dietary protein.

Excretion

In the rat most metabolites of vitamin A are excreted in the feces (Olson, 1968). Reports, however, conflict on whether dietary protein level has a significant effect on the rate of

excretion of these metabolites.

Jagannathan and Patwardhan (1960a) measured fecal excretion of β-carotene in young rats supplemented with the provitamin and maintained on 6, 12, 18, or 30% protein (casein) diets. Excretion of β-carotene was maximum with 6% protein and minimum with the 30% protein diet. They concluded that absorption of β-carotene from the gastro-intestinal tract was poor in the group fed low protein.

Deshmukh and Ganguly (1964) fed a total of 2.8 mg of β-carotene in groundnut oil during a seven-day period to rats receiving 5, 10, or 20% protein diets. Rats on the 5% protein diet excreted 2079 µg β-carotene in the feces, those on the 10% protein level excreted 1787 µg, and the animals receiving 20% dietary protein excreted 1321 µg of β-carotene. Deshmukh and Ganguly state that the reduction in the formation of vitamin A could be accounted for by the increased recovery of carotene from the gastro-intestinal tract of rats fed lower protein diets.

Johnson (1959), on the other hand, fed rats 5, 20, 40, and 60% casein diets and dosed the animals three times weekly for four weeks with a total weekly supplement of 372 µg of carotene. Fecal excretion of carotene by rats fed 60% casein was approximately 25% less than excretion by rats fed 5% casein. However, these variations in fecal losses were not of sufficient magnitude to account for the relatively large differences among the groups in hepatic vitamin A deposition.

Excretion of the recently isolated water-soluble derivatives of vitamin A has not been studied in relation to protein intake, so more data are needed in this area.

Effects of Feeding Pattern on Metabolism

Cohn and Joseph (1968, p. 94) state that

> "differences in the periodicity of consumption of the diet (feeding frequency and rate of ingestion of the diet) by an animal are accompanied by enzymatic adaptations to the magnitude of the load of nutrients requiring disposition per unit of time".

Different feeding frequencies and feeding patterns have been used to investigate changes in rate of lipid metabolism, in nitrogen secretion, in pancreatic enzyme activity, in intestinal absorption, in glycogen storage, in food efficiency and in general physical activity.

Leveille (1967) reported enhanced lipogenesis in meal-fed rats as compared to nibbling rats. Fatty acid synthesis was approximately 200 times higher in adipose tissue and nine times higher in liver of the meal-fed animals than of nibbling animals. Isolated adipose tissue from meal-fed rats also showed an enhanced ability to synthesize lipids from acetate *in vitro* (Hollifield and Parson, 1962a; Beaton *et al.*, 1964; Leveille and Hanson, 1965; and Leveille, 1970).

Wu and Wu (1950) observed a number of years ago that the amount of urinary nitrogen decreased in man as the daily number of feedings increased. Cohn *et al.* (1962) force-fed rats once daily to total daily food intakes equal to those of *ad libitum*

control animals. They observed that the force-fed animals excreted about 37% more urea nitrogen over a seven-day period than pair-fed animals eating ad libitum. In another study (Cohn et al., 1965) where diets containing ^{15}N-labeled protein were used, force-fed animals excreted more dietary ^{15}N as urea than did ad libitum controls. Cohn and coworkers (1962, 1965) suggest that an organism can utilize a limited load of dietary protein per unit of time for anabolic purposes. When this limit is exceeded, non-utilized nitrogen is excreted in the urine as urea. In space-fed animals, as opposed to nibbling animals, more of the carbon chain of ingested amino acids seems to be deposited as fat.

Snook and Meyer (1964) found that pancreatic enzyme activity responded very rapidly to dietary protein. Rats which had been previously maintained on a 15% casein diet were fed either a protein-free or a 15% whole-egg diet. After only one feeding, tryptic, chymotryptic, total proteolytic and amolytic activities were reduced with the protein-free diet and elevated with the whole-egg protein diet. Dietary protein appeared to stimulate pancreatic enzyme secretion directly since enzyme activity was altered after only one feeding of the test meal. In a subsequent study with casein and egg proteins Snook (1968), however, was not able to demonstrate significant differences in levels of pancreatic enzymes between groups of rats fed at twelve hour intervals or ad libitum. She questioned

whether feeding frequency *per se* affects pancreatic enzyme activity since her results indicated that the interaction between feeding frequency and dietary protein, rather than feeding frequency alone, affected the various pancreatic constituents[1].

That the "stress" of infrequent large meals causes the digestive tract to increase in size and thus expand its absorptive area has been postulated as a major reason for the effects of meal feeding on metabolism (Leveille, 1970). In one study Leveille and Chakrabarty (1968) reported that the intestinal weight per 100 grams body weight of meal-fed rats was 40% higher than that of nibbling rats. In the same study glucose absorption per 100 grams body weight was similarly higher in meal-fed rats lending support to the suggestion that absorptive area increases with infrequent feeding (*i.e.* meal feeding).

Utilization of absorbed glucose also appears to be more efficient by meal-fed rats than by control (nibbling rats). Leveille and Chakrabarty (1967) reported that rate of glycogen deposition in liver did not differ between meal-fed and nibbling rats. Adipose tissue, however, could accumulate considerable quantities of glycogen in meal-fed rats, while

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[1]Snook (1968, p. 358) commented, however, that it was difficult to standardize the two methods of feeding since the time lapse between eating and removal of the pancreas could be determined more exactly in space-fed rats than in those eating *ad libitum*.

virtually no accumulation was observed in adipose tissue of nibbling rats. Leveille and Hanson (1965) noted that isolated adipose tissue from meal-eating rats converted more glucose to glycogen than did tissue from nibbling rats.

Food efficiency differences (measured by weight gain) between meal-fed and nibbling rats have been investigated by several workers. Hollifield and Parson (1962b), for example, reported that after 10 weeks rats accustomed to a feeding period of two hours per day were more than 30% heavier than animals consuming like quantities of food ad libitum. Cohn et al. (1965) likewise reported that rats force-fed once daily weighed more than the control animals when food consumption was equalized between both groups. Leveille (1970), similarly, indicated that meal-fed animals gained weight as rapidly as animals eating ad libitum even though the meal-fed animals were consuming only 75-80% as much of the identical diet.

Leveille and O'Hea (1967) investigated means by which meal-feeding alters basal energy metabolism. While they found no differences in basal oxygen consumption or heat production between meal-fed and control rats, they did find that the energy expenditure of meal-fed animals was lower by as much as 57% during one period. On the basis of these results, they suggest that the apparent greater food efficiency of meal-fed animals may result from less physical activity and the ensuing reduced energy expenditure. Heggeness (1969), on the other hand, inferred that differences in food efficiency and body

composition associated with intermittent feeding may be partially due to modification of thyroid activity. He found that animals fed ad libitum showed a transient increase in resting oxygen consumption while the resting metabolic rate of meal-fed animals showed no comparable increase.

Although the effects of feeding pattern on general aspects of metabolism are being investigated in detail, very little research has been reported specifically on the effect of spaced feeding of the protein source on carotene or vitamin A utilization.

Fraps (1946) reported that in rats hepatic storage of vitamin A was reduced when the ration was withheld after carotene was fed. He suggested that the presence of digested food nutrients may have aided in storage of vitamin A in the liver.

Faruque and Walker (1970) reported that in lambs the amount of administered retinyl palmitate absorbed and retained in the liver appears to be controlled by the amount of protein in the diet at the time of administration. Pair-fed lambs supplemented with retinyl palmitate in a 5% protein diet for 24 hours had lower retinol reserves when autopsied 24 hours later than did animals given similar amounts of retinyl palmitate dissolved in a 25% protein diet. Since both groups of animals received the 25% protein diet before and after the 24-hour supplementation period, Faruque and Walker (1970, p. 21) conclude that "it was hardly possible that any of the lambs

were protein-deficient in the accepted sense, though immediate biochemical changes must have occurred".

Ruffin (1965) investigated simultaneous feeding of carotene with the protein or with the protein-free portion of the diet using rats. More hepatic vitamin A was deposited when carotene was fed simultaneously with the protein than when carotene feeding was delayed (Mean daily intakes of 10% or 20% protein were examined.).

Thus a number of studies concur that meal feeding, as opposed to the more natural nibbling pattern in rats, effects significant changes in metabolism. A number of other studies indicate that hepatic deposition of vitamin A also appears to vary with the feeding pattern of the protein source.

At this point, however, it is not possible to clearly delineate the effects of feeding frequency per se from the potential enhanced absorption and/or conversion of β-carotene due to the presence of protein and to the increase of enzymes in the gastro-intestinal tract. Hence further studies must be designed to treat these variables independently.

EXPERIMENTAL METHODS

Preparation of Biologically Labeled β-carotene

Photosynthesis

Labeled β-carotene was synthesized by the method of Kotecheri (1967). A modification was the use of excised leaves in a "lollipop" arrangement as described by Aronoff (1956). Three or four leaves were placed in a beaker of water and inserted into a glass growth chamber. Five millicuries of barium carbonate[1] ($Ba^{14}CO_3$) were introduced into the air-tight chamber, and $^{14}CO_2$ was liberated from the carbonate by the addition of 10% perchloric acid. The leaves were allowed to respire in this atmosphere until all the carbon dioxide was absorbed, i.e., until the activity of carbon dioxide was reduced to a constant.

Extraction and purification

Extraction and purification procedures used in this laboratory have been described in detail by Kotecheri (1967). Fat soluble components of the leaves were extracted and dried. The lipid extract was then saponified and passed through dry ice to remove sterols according to the technique of Glover, Goodwin, and Morton (1948). This extract was concentrated and

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[1]Amersham-Searle, Corp., Des Plaines, Illinois.

chromatographed on deactivated alumina (7%)[1] to remove xanthophylls. The eluate was further concentrated and chromatographed on a calcium hydroxide column to separate the isomers of carotene. After the all-trans β-carotene was rechromatographed on deactivated alumina (7%) to constant specific activity, the yield from two trials was determined as 73,000 dpm/μg and 60,000 dpm/μg. Radioactivity was measured by gas-flow counting[2] and concentration by spectrophotometry[3]. These stock solutions were stored under nitrogen at -20°C. A comparison of the absorption spectrum of the synthesized material with that of synthetic all-trans β-carotene[4] is presented in the Appendix, Figure 2.

Preparation of labeled β-carotene dose for individual experiments

On the day of each experiment a suitable aliquot of the stock solution was diluted with carrier β-carotene to give the desired specific activity. This sample was freshly chromatographed on alumina (7%) immediately before use. The concentration of the dose was determined spectrophotometrically.

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[1]Woelm Neutral, Alupharm Chemicals, New Orleans, La.

[2]Model 1043 Low Background Planchet Sampler Changer, Model D47 Gas Flow Detector, Model 8703 Decade Scaler, Amersham-Searle Corp., Des Plaines, Ill.

[3]Model 240 Gilford Instrument Laboratories, Inc., Oberlin, Ohio.

[4]General Biochemicals, Chagrin Falls, Ohio.

For Experiments I and II solutions containing the specific quantity of β-carotene used as a dose for an individual animal were evaporated under a stream of nitrogen. One-tenth ml of acetone was added followed by 0.1 ml of Tween 20 (Polyoxyethylene (20) sorbitan monolaurate)[1] for solubilization. Eight-tenths ml of Kreb's Ringer solution was added to make one ml of carotene dose.

In Experiment III a quantity of β-carotene solution sufficient to serve as substrate for all enzyme incubations on a given day was evaporated under a stream of nitrogen. Acetone was added so that 0.9 μg of β-carotene was contained in 50 μl of acetone.

Animal Studies

Animals

Weanling male albino rats of the Wistar strain were used in all experiments. These animals were bred in our laboratory and were placed in separate, suspended wire-meshed cages. Weight records were kept during the depletion and experimental periods, and food intake was recorded during the experimental period.

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[1]Gift of Atlas Chemical Industries, Inc., Wilmington, Delaware.

Diets

The composition of diets is given in Tables 1, 2, and 3. The fat and mineral salt content of the diets remained constant, while casein replaced sucrose as the protein level of the diets was increased.

Table 1. Composition of diets - Experiment I

Ingredients	0% Protein	20% Protein	30% Protein	60% Protein
Casein[a]	0.0	22.7[b]	33.1[c]	66.2[c]
Sucrose[d]	87.0	64.3	53.9	20.8
Corn oil[e]	9.0	9.0	9.0	9.0
Mineral salts[f]	4.0	4.0	4.0	4.0

[a]Vitamin Free Test Casein, General Biochemicals, Chagrin Falls, Ohio.

[b]Lot #680479 - 88.1% protein (Depletion period diet).

[c]Lot #682207 - 90.6% protein.

[d]Powdered sugar containing 3% starch, obtained locally.

[e]Mazola, Best Foods, Englewood Cliffs, N.J.

[f]Hawk-Oser Salt Mix, General Biochemicals, Chagrin Falls, Ohio.

Table 2. Composition of diets - Experiment II

Ingredients	0% Protein	15% Protein	20% Protein	30% Protein	60% Protein
Casein[a]	0.0	16.6[b]	21.9[c]	33.1[b]	66.2[b]
Sucrose[d]	87.0	70.4	65.1	53.9	20.8
Corn oil[e]	9.0	9.0	9.0	9.0	9.0
Mineral salts[f]	4.0	4.0	4.0	4.0	4.0

[a]Vitamin Free Test Casein, General Biochemicals, Chagrin Falls, Ohio.

[b]Lot #682207 - 90.68% protein.

[c]Lot #910028 - 91.25% protein (Depletion period diet).

[d]Powdered sugar containing 3% starch, obtained locally.

[e]Mazola, Best Foods, Englewood Cliffs, N.J.

[f]Hawk-Oser Salt Mix, General Biochemicals, Chagrin Falls, Ohio.

Table 3. Composition of diets - Experiment III

Ingredients	0% Protein	20% Protein	30% Protein
Casein[a]	0.0	22.3[b]	33.4[b]
Sucrose[c]	87.0	64.7	53.6
Corn oil[d]	9.0	9.0	9.0
Mineral salts[e]	4.0	4.0	4.0

[a]Vitamin Free Test Casein, General Biochemicals, Chagrin Falls, Ohio.

[b]Lot #681423 - 89.75% protein.

[c]Powdered sugar containing 3% starch, obtained locally.

[d]Mazola, Best Foods, Englewood Cliffs, N.J.

[e]Hawk-Oser Salt Mix, General Biochemicals, Chagrin Falls, Ohio.

Supplements

Vitamin supplements were prepared at the beginning of each experiment for the entire period and were stored in dark glass bottles. All supplements were stored at 4°C except the carotene solution which was held at room temperature to prevent separation.

Water-soluble vitamins The water-soluble vitamins were dissolved in 20% ethanol. One milliliter of the solution satisfied the daily requirement of the rat. Composition of this vitamin solution is given in Table 4.

Fat-soluble vitamins The vitamin D-tocopherol supplement was prepared by dissolving one gram of vitamin D_2 stock solution[1] and two grams of liquid dl-α-tocopherol[2] in cottonseed oil[3] and making the mixture to 100 g with the same oil. Two drops of this solution contained approximately one mg of α-tocopheroal and 25 I.U. of vitamin D_2.

Carotene The carotene supplements were prepared by dissolving weighed quantities of carotene (85% beta and 15% alpha) in a small quantity of chloroform. Cottonseed oil was

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[1]The vitamin D_2 stock solution was prepared by dissolving 125 mg of vitamin D_2 crystals (obtained from General Biochemicals, Chagrin Falls, Ohio) in 2 ml of chloroform and adding cottonseed oil to 100 g.

[2]General Biochemicals, Chagrin Falls, Ohio.

[3]Hunt-Wesson Foods, Inc., Fullerton, Calif.

Table 4. Composition of the water-soluble vitamin mixture

Vitamin[a]	Dosage per day (mcg)	Vitamins per 2 liters of 20% ethanol (mg)
Thiamine HCl	20.0	40.0
Riboflavin	39.0	78.0
Pyridoxine HCl	20.0	40.0
Folic acid	20.0	40.0
Calcium pantothenate	97.0	194.0
Para-amino benzoic acid	97.0	194.0
Vitamin B_{12}	0.2	0.4[b]
Biotin	2.0	4.0[c]
Niacin	64.0	128.0
Inositol	2.4 mg	4.8 g
Choline HCl	7.0 mg	14.0 g

[a]All vitamins were obtained from General Biochemicals, Chagrin Falls, Ohio, with the exception of niacin which was obtained from Nutritional Biochemicals Corp., Cleveland, Ohio.

[b]400 mg of 0.1% vitamin B_{12} triturated in mannitol.

[c]400 mg of 1% biotin triturated in dextrin.

added until the daily dose was contained in either two or three drops of the solution depending on the experiment. Concentration was determined at 451 mμ with a Gilford spectrophotometer.

Retinyl acetate A weighed amount of crystalline retinyl acetate[1] was dissolved in cottonseed oil[2]. Cottonseed

[1]General Biochemicals, Chagrin Falls, Ohio.

[2]Wesson, Hunt-Wesson Foods, Inc., Fullerton, California.

oil was added until the daily dose was contained in three drops of the solution. The concentration of the supplement was determined at 328 mμ in a Gilford spectrophotometer.

Depletion period

All animals were subjected to a vitamin A-depletion regimen of three weeks. A 21-day period has been found adequate to deplete weanling animals of existing hepatic stores of vitamin A without producing deficiency symptoms.

Three-week-old animals weighing about 50 g were fed ad libitum a diet containing 20% protein (Table 1) for two weeks. The animals were conditioned to the experimental feeding pattern during the third week of depletion by daily feeding of first a protein-free diet and then a diet containing protein. The concentration of casein in the protein diet was increased so that the mean protein intake was still equivalent to 20% of the daily diet.

Animals received one ml water-soluble vitamins and 2 drops vitamin D-tocopherol supplement daily. During the final week of depletion the supplements were divided so that the rats received one-half ml water-soluble vitamins and one drop fat-soluble supplement with each of the two feedings.

Feeding pattern

The feeding pattern was designed so that all groups of rats would be post-absorptive with respect to their previous

diet when they received a carotene or retinyl acetate supplement and the new portion of diet. Food intake was limited to 12 g per day and after the first week of the experimental period, most animals consumed their diets within three hours.

The feeding pattern is illustrated for the three experiments in Tables 5, 6, and 7. Symbols used to identify groups of rats and diets were as follows:

C/PF = carotene with protein-free diet

C/P = carotene with protein diet

A/PF = retinyl acetate with protein-free diet

A/P = retinyl acetate with protein diet

PF = protein-free diet

P = protein diet.

Experimental period

After the depletion period, animals were placed on the four-week experimental regimens outlined below. Littermates were randomly assigned within each protein level in Experiments I and II, i.e., one animal received carotene with the protein-free diet and its littermate received carotene with the protein diet. In Experiment III littermates were compared within the carotene supplementation and within the retinyl acetate supplementation.

In Experiment I the carotene supplement was 70 µg per day while in Experiment II it was 53 µg per day. Twenty-four µg of retinyl acetate or 66 µg of carotene were fed in Experiment III.

Table 5. Feeding pattern. Experiment I

Symbol	Protein intake equivalent to % of diet	24-hour pattern			
		8:00 am	12:00	5:00 pm	9:00 pm
20 C/PF	20	Fast	4 g PF plus carotene[a]	Fast	8 g 30% P
20 C/P	20		4 g PF		8 g 30% P plus carotene
40 C/PF	40		4 g PF plus carotene		8 g 60% P
40 C/P	40		4 g PF		8 g 60% P plus carotene

[a]All animals received their carotene supplement with 1 ml water-soluble vitamins and 2 drops vitamin D-tocopherol mixture.

Table 6. Feeding pattern - Experiment II

Symbol	Protein intake equivalent to % of diet	24-hour pattern			
		8:00 am	12:00	5:00 pm	9:00 pm
10 C/PF	10	Fast	4 g PF plus carotene[a]	Fast	8 g 15% P[a]
10 C/P	10		4 g PF		8 g 15% P plus carotene
20 C/PF	20		4 g PF plus carotene		8 g 30% P
20 C/P	20		4 g PF		8 g 30% P plus carotene
40 C/PF	40		4 g PF plus carotene		8 g 60% P
40 C/P	40		4 g PF		8 g 60% P plus carotene

[a]All animals received 1/2 ml water-soluble vitamins and 1 drop vitamin D-tocopherol each time they were fed.

Table 7. Feeding pattern - Experiment III

Symbol	Protein intake equivalent to % of diet	24-hour pattern			
		8:00 am	12:00	5:00 pm	9:00 pm
20 C/PF	20	Fast	4 g PF plus carotene[a]	Fast	8 g 30% P[a]
20 C/P	20		4 g PF		8 g 30% P plus carotene
20 A/PF	20		4 g PF plus vitamin A acetate		8 g 30% P
20 A/P	20		4 g PF		8 g 30% P plus vitamin A acetate

[a]All animals received 1/2 ml water-soluble vitamins and 1 drop vitamin D-tocopherol supplement each time they were fed.

Autopsy and Preparation of Tissue

All animals were autopsied on the 29th day of the experimental period. After an overnight fast, the animals were given vitamin supplements devoid of carotene or retinyl acetate and one gram of the diet which they normally received with the carotene or retinyl acetate supplement. At specified intervals following the test meals, surgical procedures were begun or the rats were sacrificed.

Surgical and autopsy procedures

Experiment I One hour after receiving the test meal, each rat was anaesthetized with ether. A dose of 16 µg of labeled β-carotene (S.A. = 5700 dpm) was injected intraduodenally near the pyloric sphincter. The incision was closed with surgical clips and the animal was returned to its cage for two and one-half hours. After this interval, the rat was reanaesthetized with sodium pentobarbitol injected intraperitoneally. The intestine above the midpoint of the mesentary was removed, and the intestinal content was flushed out with warm isotonic saline and collected. The intestine, stomach, liver, and kidneys were removed and prepared for chemical analysis as discussed under preparation of tissues.

Experiment II Two and one-half hours after the test meal, each rat was anaesthetized with ether. A dose of 10 µg of labeled β-carotene (S.A. = 10,300 dpm) was injected

intraduodenally near the pyloric sphincter. The incision was closed with wound clips, and the rat was returned to its cage for one hour. A one-hour metabolism period was selected for this study because Olson (1961b) reported that the vitamin A ester content of the intestinal mucosa rose to a maximum at the one-hour interval after intraduodenal administration of β-carotene. The animal was then reanaesthetized with ether. Blood was removed from the ascending abdominal artery and allowed to clot at refrigerator temperature. Twenty-four inches of the intestine was removed and the warm isotonic saline rinsings of this portion were collected. The lower intestine, stomach, liver, and kidneys were also removed and prepared for analysis as discussed below.

Experiment III Animals were given test meals three hours before they were anaesthetized with ether and sacrificed. The inferior vena cava was severed to insure uniform drainage of blood from the liver. In rats which had been supplemented with β-carotene, the upper twenty-four inches of the intestine was removed and rinsed with ice-cold saline. This segment of the intestine was placed on a chilled glass plate for immediate removal of the mucosa. The liver and kidneys were removed from all animals and prepared for chemical analysis.

Preparation of tissue

Gastro-intestinal tract All gastro-intestinal tissue analyzed on the day of autopsy was cleaned of visible fat and

minced into flasks containing 50 ml of 3:1 petroleum ether[1] and ethanol. A mixture of nonradioactive carriers comprised of 100 µg of β-carotene, 500 µg of retinyl palmitate, and 100 µg retinol was added to each flask. Intestinal rinsings were handled in the same manner as the gastro-intestinal tissue.

In Experiment I the upper intestine and intestinal contents were prepared for extraction immediately while the stomach and lower intestine were frozen in liquid nitrogen for later analysis. When the frozen tissues were analyzed, they were brought to room temperature and were then treated by the same procedure as the unfrozen tissues.

In Experiment II all tissues of the gastro-intestinal tract were analyzed on the day of autopsy. The upper 12 inches of the intestinal tract was labeled the upper intestine, the next 12 inches was the middle intestine, and the remainder was called the lower intestine. In this experiment the intestinal rinsings were combined with the stomach for analysis.

Variability had made interpretation of the results of Experiment I difficult, so in Experiment II two sets of littermates fed on the same protein level were autopsied on the same day. Intestinal tissue, stomachs, and intestinal contents from rats on the same feeding pattern were each pooled before extraction.

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[1]Petroleum ether B.R. 60-70°C. Barton Solvents, Des Moines, Iowa.

Liver and kidneys The liver and kidneys were immediately removed, weighed and minced in hot 5% aqueous KOH. When the tissues were reduced to a homogeneous suspension, they were stored at -20°C.

Serum After clotting at refrigerator temperature, blood was centrifuged at 1000 r.p.m. for 20 minutes. The serum and a few of the packed red blood cells were poured to another tube and re-centrifuged for 15 minutes at the same speed. Serum was stored in glass vials at -20°C.

Mucosal supernatant A modification of the method of Goodman et al. (1967) was used for the assay of carotene dioxygenase. The intestine was cut lengthwise, rinsed again with ice-cold saline and scraped with a microscope slide. The mucosal scrapings were placed in a weighed glass homogenizer. These manipulations were performed at 4°C. The scrapings were weighed and were then homogenized with a teflon pestle at high speed in 6 ml of 0.1 M potassium phosphate buffer, pH 7.7, containing 30 mM nicotinamide and 4 mM $MgCl_2$. After centrifugation of the homogenate for 20 minutes at 4°C, the 2000 x g supernatant was removed and held in the cold until added to the incubation flasks.

Analytical Procedure

Extraction of tissue

All manipulations were carried out in semi-darkness. Nitrogen was used in handling samples wherever possible.

Intestinal tract In Experiment II the intestinal contents and rinsings were added to the stomach before homogenization, and this combination was treated as the stomach. Extraction procedures for the remaining gastro-intestinal tissues were identical to those of Kotecheri (1967).

Incubation medium In Experiment III, the 2 ml assay medium was extracted by rinsing the incubation flask into 40 ml of petroleum ether[1]:ethanol (3:1) in a small stainless steel blender. The flask was rinsed repeatedly into the blender with the same solvent mixture and nonradioactive carriers containing 40 µg β-carotene, 100 µg retinyl esters, 100 µg retinal, 100 µg retinol and 40 µg retinoic acid were added. The remainder of the extraction procedure was the same as that described by Kotecheri (1967) for the intestinal walls.

Chromatography

Suitable aliquots of each tissue extract were chromatographed on deactivated alumina[2] (7%) columns one cm in diameter. The methods used were modifications of procedures discussed by Olson (1961b), Huang and Goodman (1965), and Kotecheri (1967).

The maximum load of lipid for achieving good separation was 10 mg per gram of alumina. Most alumina columns used were prepared with five grams of alumina. When it was necessary to

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[1] B.P. 60-70°C.

[2] Woelm Neutral, Alupharm Chemicals, New Orleans, Louisiana.

use an aliquot of the extract which contained more than 50 mg lipid, the column was increased to 7.5 g of alumina and the volume of each eluant was increased proportionally. Whenever there was a possibility of lipid overload on a column and thus poor separation of the β-carotene and retinyl esters, two or three drops of the eluate between these two fractions were collected in a tube containing antimony trichloride in $CHCl_3$ as an indicator. If a blue color resulted, the β-carotene and retinyl ester fractions were evaporated and rechromatographed on a fresh alumina column.

Experiments I and II The order of elution and the volume of eluants required for a 5 g alumina column were as follows:

1. Fraction I - 20 ml of hexane to elute β-carotene
2. Fraction II - 20 ml of 15% benzene in hexane to elute retinyl esters
3. Fraction III - 50 ml of benzene to elute retinol
4. Fraction IV - 30 ml of 8% ethanol in hexane to elute the miscellaneous breakdown products.

In Experiment II extracts of the liver were also chromatographed. Since these samples had been saponified, elution of Fraction II was omitted.

The β-carotene fraction was eluted directly into a volumetric flask. Other fractions were evaporated to dryness in a vacuum or under nitrogen and were made to a small volume. Aliquots of each fraction were plated on 1-1/4" cupped copper

planchets and assayed for radioactivity in the low background gas flow counter.

Experiment III In this experiment 5 g of alumina was used in all columns, and the order and volume of eluants was as listed below:

1. Fraction I - 20 ml of hexane to elute β-carotene
2. Fraction II - 20 ml of 15% benzene in hexane to elute retinyl esters
3. Fraction III - 20 ml of 50% benzene in hexane to elute retinal
4. Fraction IV - 30 ml of methanol to elute retinol and the miscellaneous breakdown products.

Enzyme assay

Carotene dioxygenase was assayed by a modification of the method of Goodman et al. (1967). The enzyme was isolated as described in the section on preparation of tissue. Protein concentration of the supernatant fraction was determined by the biuret reaction (Henry, 1964). In our laboratory the rate of reaction was found to be linear between 4 and 9 mg added supernatant protein (See Appendix, Table 16).

Enzyme preparations in 25-ml Erlenmeyer flasks were incubated with ^{14}C-β-carotene (S.A. 16,700 dpm) in a shaker incubator. Each incubation flask contained potassium phosphate buffer, pH 7.7, 200 μmoles[1]; nicotinamide, 30 μmoles[1];

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[1]These figures include the non-protein components of the homogenate fraction.

magnesium chloride, 4 μmoles[1]; glutathione, 10 μmoles, sodium glycocholate, 12 μmoles; α-tocopherol, 0.25 mg added in 25 μl of acetone; ^{14}C-β-carotene, 0.9 μg in 0.05 ml acetone; and the supernatant fraction (containing 5-9 mg protein). The supernatant fraction was added last to each flask.

The reaction was allowed to proceed in the dark at 37°C for 30 minutes with room air as the gas phase. After the incubation period, the preparations were treated as described under extraction procedures.

Carr-Price analysis

Hepatic and renal vitamin A were assayed by a modification of the method of Gallup and Hoefer (1946). This modification has been used extensively in our laboratory and is described by MacMillan (1966). Optical density readings were taken at 620 mμ with a Beckman B spectrophotometer[2] twelve seconds after the Carr-Price reagent began to enter the spectrophotometer tube.

Serum analysis

Serum was analyzed by a modification of the method of Bessey et al. (1946). One ml of serum and one ml of alcoholic KOH were mixed in a stoppered centrifuge tube and placed in a water bath at 60°C for 25 minutes. After cooling, retinol was

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[1]These figures include the non-protein components of the homogenate fraction.

[2]Beckman Company, Fullerton, California.

extracted into 1 ml of kerosene-xylene (1:1) and read at 328 mμ with the Gilford spectrophotometer. Samples were irradiated for 50 minutes and readings were again taken in the spectrophotometer. An aliquot of the kerosene-xylene was plated to determine radioactivity.

Statistical Methods

A split plot experimental design was used for this series of experiments. In Experiments I and II each dietary protein level (main plot) was divided into two supplementation patterns (sub-plots). In Experiment III type of vitamin supplement comprised the main plot with the same supplementation patterns as sub-plots (Cochran and Cox, 1968).

Analysis of variance and the t test were performed on all data. In Experiment II where additional values were obtained at one protein level for hepatic and renal deposition, a least squares analysis with proportional sub-class numbers was used (Snedecor and Cochran, 1967).

RESULTS

Animals studied in this series of experiments were conditioned to a feeding program in which the daily diet was given in two time-spaced portions: a protein-free portion and a protein portion. Two factors are operant in Experiments I and II. One is the dietary protein level. The other is the pattern of supplementation, i.e., whether the source of vitamin A was given with the protein or protein-free portion of the diet. A split-plot design, with level of dietary protein as the whole units and pattern of supplementation as the sub-plots, was used for statistical analysis.

In Experiment III animals fed adequate protein diets were supplemented with carotene or with retinyl acetate utilizing the pattern of supplementation from Experiments I and II. A split-plot design was also used for analysis of these data. Probability values mentioned in the text were obtained with the two-tailed t test unless otherwise noted.[1]

The present studies indicate that both dietary protein level and presence of protein in the gastro-intestinal tract (as controlled by spaced feeding) are significant variables in various aspects of β-carotene metabolism. These two factors are discussed separately.

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[1]Probability values for tests of significance are tabulated in the Appendix.

Growth

Mean initial weights, taken at the end of the preliminary depletion period, were between 138 and 139 g for the four groups in Experiment I. In Experiment II mean initial weights varied from 132 to 136 µg; and in Experiment III the mean initial weight range was 126 to 130 g (Tables 8 and 9). To control the rate of growth as a variable food intake was restricted to 12 g per day for all animals.

Dietary protein level

Growth differences due to dietary protein level were of physiological significance only in Experiment II where two groups of rats received inadequate amounts (10%) of protein. The mean weight gain for these animals was 78 g (Table 8).

When animals received 20 or 40% protein in Experiment I, their mean weight gain was 117 or 116 g respectively. In Experiment II animals gained 104 and 108 g on corresponding protein regimens (Table 8). (The differences in weight gain observed here may be due partially to the fact that animals in Experiment I were refed amounts of diet equivalent to what they spilled while those in Experiment II were not.) Animals in Experiment III had a mean weight gain of 98 g on a 20% protein diet (Table 9).

Despite the fact that none of the groups reached their growth potential with the restricted intakes, animals fed 10% protein demonstrated additional growth inhibition which indicated a mild protein deficiency condition.

Table 8. Mean values for body weight, weight gain, and hepatic and renal vitamin A for young vitamin A-depleted rats fed carotene (Experiments I and II)

Experiment symbol	No. of rats	Carotene/ day	Body weight		Weight gain	Vitamin A		
			Initial	Final		Hepatic	Renal	Hepatic + renal
		µg	g	g	g	µg	µg	µg
Experiment I								
20 C/PF	13	70	139	253	114	75	18	93
20 C/P	13	70	138	258	120	132	16	148
40 C/PF	12	70	138	254	116	98	16	114
40 C/P	12	70	139	256	117	166	18	184
20 PF, P[a]	26	70	138	256	117	104	17	120
40 PF, P[a]	24	70	138	254	116	132	17	149
PF - 20, 40[a]	25	70	138	252	114	87	17	104
P - 20, 40[a]	25	70	138	257	118	149	17	166

Experiment II								
10 C/PF	12	53	133	212	79	42	19	61
10 C/P	12	53	136	213	77	34	20	54
20 C/PF	16	53	135	238	103	74	13	87
20 C/P	16	53	133	237	104	114	9	123
40 C/PF	12	53	134	241	107	102	8	110
40 C/P	12	53	132	240	108	177	7	184
10 PF, P[a]	24	53	134	212	78	38	20	58
20 PF, P[a]	32	53	134	238	104	94	11	105
40 PF, P[a]	24	53	133	240	108	140	8	147
PF 10, 20, 40[a]	40	53	134	230	96	73	13	86
P 10 , 20, 40[a]	40	53	134	230	96	108	12	120

[a]Arithmetic mean of above group means.

Table 9. Mean values for body weight, weight gain, and hepatic and renal vitamin A for young vitamin A-depleted rats fed retinyl acetate or carotene. Experiment III

Symbol	No. of rats	Dose/ day	Body weight		Weight gain	Vitamin A		
			Initial	Final		Hepatic	Renal	Hepatic + renal
		µg						
20 A/PF	8	24	126	231	105	313	13	326
20 A/P	8	24	127	224	97	348	11	359
20 C/PF	8	66	126	220	94	107	11	118
20 C/P	8	66	130	227	97	160	14	174
A - PF, P[a]	16	24	126	227	101	330	12	342
C - PF, P[a]	16	66	128	224	96	134	12	146

[a]Arithmetic means of above group means.

With growth as a criterion, 20% protein seems to be the optimum dietary level, (under the conditions of slightly restricted intakes) since an increase in protein intake to 40% did not increase weight gain of the animals. Any effect on metabolism of carotene by the 40% protein level, therefore, is not related to the rate of growth.

Presence of dietary protein in the gastro-intestinal tract

Within each experiment the fluctuation in weight gain based on feeding pattern was inconsequential (Tables 8 and 9). With the levels of carotene fed, growth of the animals was not dependent on vitamin A supply. Therefore a difference in weight gain due to the presence or absence of protein in the intestinal tract at the time of supplementation was not expected.

Vitamin A Reserves

For the purposes of this section, the term, total vitamin A deposition, includes both hepatic and renal stores. Renal deposition, as well as hepatic must be considered since the former constituted 30% or more of total vitamin A reserves in rats fed the low protein diet (Experiment II).

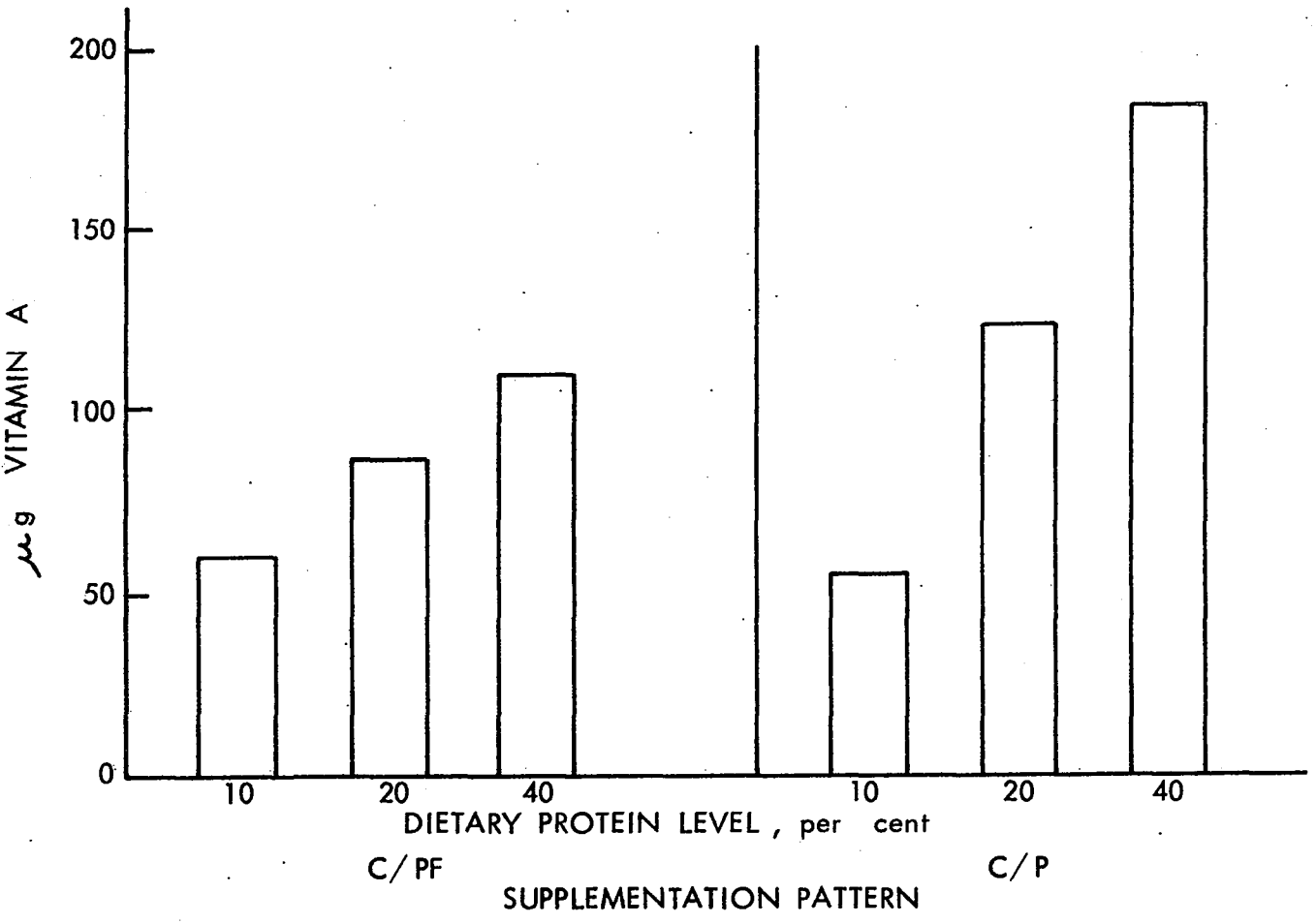
The main plot effects, i.e. dietary protein levels, were consistent across each sub-plot grouping, or supplementation pattern. Likewise, the treatment of sub-plot effects was consistent within the main plot groupings. Therefore the effects of dietary protein level and supplementation pattern were treated independently.

Dietary protein level

In Experiment I total vitamin A deposition increased significantly (120 µg to 149 µg respectively) as protein intake changed from adequate to excessive ($p < 0.05$)(Table 8). Vitamin A reserves also increased in Experiment II as each higher protein level was fed (58 µg, 105 µg, 147 µg)(Table 8). The increased vitamin A reserves from β-carotene with higher levels of dietary protein confirmed previous work from our laboratories (Ruffin and Arnrich, 1966; MacMillan, 1966; and Kotecheri, 1967), which indicated that vitamin A deposition was augmented as dietary protein comprised 10, 20 or 40% of the diet.

Vitamin A deposition was significantly increased ($p < 0.005$) between groups fed 10 and 40% protein even when the supplement was fed separately from the protein diet (Figure 1, Table 8). Thus, utilization of carotene appeared to be affected by the nutritional state of the animal without the presence of protein at the site of conversion. Ruffin (1965), likewise, reported that animals with 20% protein intakes had significantly larger vitamin A reserves than mildly protein-deficient animals despite the fact that both groups received carotene supplements with protein-free diets. With the slightly altered feeding plan used in the present study, differences in vitamin A deposition were not confirmed between the 10 and 20% protein levels unless carotene was given with the protein diet.

Figure 1. Mean vitamin A deposition of groups fed 70 µg of carotene per day with the protein-free (C/PF) or with the protein (C/P) portion of the diets Experiment II


200
150
100
50
0
µg VITAMIN A
10
20
40
10
20
40
DIETARY PROTEIN LEVEL , per cent
C/PF
C/P
SUPPLEMENTATION PATTERN

Serum vitamin A levels were 28, 33, and 35 µg per 100 ml respectively for 10, 20, and 40% dietary protein in Experiment II. Statistically there was no difference in these values. Dowling and Wald (1958) reported that serum vitamin A levels do not fall until hepatic reserves are substantially depleted. Since the 10% protein level, used in the present study, allowed vitamin A deposition, normal serum levels would be expected even in the mildly protein-deficient group.

Presence of dietary protein in the gastro-intestinal tract

Highly significant differences in vitamin A deposition in response to presence or absence of protein in the gastro-intestinal during carotene supplementation were observed in Experiment I (F test, $p < 0.0005$), (Table 8). Groups given carotene with the protein-free diet deposited a mean of 104 µg while those supplemented with the protein diet had vitamin A reserves of 166 µg. Likewise, in Experiment II (Table 8), vitamin A deposition by animals fed adequate or excessive protein was significantly higher ($p < 0.001$) when the supplementation was given with a protein rather than a protein-free diet. Mean deposition by groups 20 C/P and 40 C/P (154 µg) exceeded deposition by corresponding groups on the C/PF feeding pattern (99 µg) by 54% (Experiment II)(Table 8). Vitamin A deposition by animals fed adequate protein confirmed the enhancing effect of simultaneous presence of protein and carotene in the gastro-intestinal tract demonstrated by Ruffin (1965). That absolute values for vitamin A reserves were lower in Experiment II than in Experiment I can be attributed to lower daily carotene doses

in Experiment II.

When 10% dietary protein was fed in Experiment II, vitamin A reserves appeared to be unaffected by the feeding pattern. Ruffin (1965) reported increased vitamin A deposition with the presence of protein in the intestinal tract even with mildly protein-deficient rats.

She, however, used a slightly different feeding pattern. This problem will be considered in detail in the discussion section.

In Experiment III simultaneous presence of a vitamin supplement and protein in the intestinal tract was investigated with 70 I.U. vitamin A or 100 I.U. carotene in rats fed 20% protein. It was hoped that similar levels of reserves would be produced with the two supplements. However, vitamin A reserves of 326 and 359 µg were observed with groups 20 A/PF and 20 A/P respectively ($p < 0.01$) while groups 20 C/PF and 20 C/P deposited 118 and 174 µg of vitamin A respectively ($p < 0.001$). The relative magnitude of increase between groups 20 C/PF and 20 C/P was greater (47% increase) than the limited effect in groups supplemented with retinyl acetate (10% increase).

Feeding pattern appeared to produce no significant differences in serum vitamin A levels in Experiment II. If dietary protein is not extremely low and if hepatic vitamin A reserves are adequate, serum vitamin A levels are maintained within a narrow range (Ganguly, 1967). Therefore it was improbable that the feeding pattern would produce a change in serum levels.

Metabolism of ^{14}C-β-carotene

Experiments I and II included *in vivo* investigations of the effects of presence of protein in the intestine on the metabolism of ^{14}C-β-carotene in the rat. Dietary protein level was an additional variable in Experiment II. Animals were fed 1 g of the diet that they normally received with carotene supplementation one hour before the intestinal injection of ^{14}C-β-carotene for Experiment I and two and one-half hours before injection in Experiment II. Metabolic products of ^{14}C-β-carotene were examined two and one-half hours and one hour following the intestinal injection of β-carotene in Experiments I and II respectively. The feeding and injection sequences were designed so that conditions in the gastro-intestinal tract during the metabolic period would simulate those present if the carotene supplementation had been oral.

Experiment III included an *in vitro* study of carotene cleavage enzyme activity in mucosal homogenates from rats pre-fed an adequate protein diet (20%) for four weeks and accustomed to the same spaced-feeding regimen used in the other studies. Three hours prior to autopsy the rats were fed 1 g of the diet they normally received with carotene supplementation.

Metabolic products of ^{14}C-β-carotene cleavage, such as retinyl esters, retinal, retinol, and polar fractions, as well as the unconverted β-carotene are reported when pertinent in

subsequent sections. Values for these compounds are expressed as per cent of injected dose (Experiments I and II) or as per cent of recovered ^{14}C (Experiment III).

Here, again, effects of dietary protein level and supplementation pattern are treated independently. Probability values mentioned in the text were obtained with the two-tailed t test unless otherwise designated.

Dietary protein level

Dietary protein level (the main plot effect) was a variable in labeled β-carotene studies in Experiment II. Metabolism of ^{14}C-β-carotene was investigated only in animals fed 20% protein in Experiment I. The need to analyze samples rapidly made it physically impossible to treat both levels of protein in that experiment.

Intestinal wall Retinyl ester formation in the intestinal wall was examined extensively in this study. This chromatographic fraction has been found to be the best index of carotene conversion during active metabolism (Olson, 1961b; Huang and Goodman, 1965). The intestine was divided into three segments and each was analyzed individually in order to localize the most active areas of β-carotene conversion.

With 10 or 40% levels of dietary protein, unconverted β-carotene recovered from the upper intestine was 8.1 or 4.0% of the injected dose respectively ($p < 0.05$) (Table 10). Dietary protein level, however, did not cause significant changes in

Table 10. Radioactivity recovered as β-carotene from the intestinal wall expressed as percentage of injected dose. Experiment II

Group	Upper intestine	Middle intestine	Lower intestine	Whole intestine
10 C/PF	8.7	5.7	0.8	15.2
10 C/P	7.5	2.8	0.5	10.8
20 C/PF	6.4	6.8	1.8	15.1
20 C/P	4.2	7.0	1.0	12.2
40 C/PF	4.6	8.7	1.0	14.4
40 C/P	3.5	6.0	2.9	12.5
10 - PF, P[a]	8.1	4.2	0.6	13.0
20 - PF, P[a]	5.3	6.9	1.4	13.6
40 - PF, P[a]	4.0	7.4	2.0	13.4
PF - 10, 20, 40[a]	6.6	7.1	1.2	14.9
P - 10, 20, 40[a]	5.0	5.3	1.5	11.8

[a]Arithmetic mean of the above group means.

retinyl esters (Table 11) or in other chromatographic fractions of the upper intestine.

Retinyl ester recovery from the wall of the middle intestine was 2.6%, 5.5%, or 8.2% of the injected β-carotene dose as protein comprised 10, 20, or 40% respectively of the diet (Table 11). The F test indicated that the effect of

Table 11. Radioactivity recovered as retinyl ester from the intestinal wall expressed as percentage of injected dose. Experiment II

Group	Upper intestine	Middle intestine	Lower intestine	Whole intestine
10 C/PF	7.9	2.9	0.0	10.9
10 C/P	5.9	2.3	0.1	8.3
20 C/PF	6.6	4.8	0.1	11.4
20 C/P	6.7	6.2	0.0	13.0
40 C/PF	7.5	6.0	0.1	13.6
40 C/P	6.7	10.3	0.4	17.4
10 - PF, P[a]	6.9	2.6	0.0	9.6
20 - PF, P[a]	6.6	5.5	0.1	12.2
40 - PF, P[a]	7.1	8.2	0.2	15.5
PF - 10, 20, 40[a]	7.3	4.6	0.1	12.0
P - 10, 20, 40[a]	6.4	6.3	0.2	12.9

[a]Arithmetic mean of the above group means.

protein intake was highly significant ($p < 0.01$) although the *t* test showed a significant difference only between the animals fed 10% and 40% protein ($p < 0.005$). Recovery of unconverted β-carotene from the middle intestine was greater in groups with higher protein intakes (F test, $p < 0.01$) (Table 10). It is

interesting to note that this increase was apparent despite the concurrent higher conversion of β-carotene to retinyl ester in animals consuming larger quantities of protein.

Total recovery of radioactivity in the middle intestine was 7.2, 13.0, or 16.2% of the injected dose respectively as protein made up 10, 20, or 40% of the diet (Table 12). The F value for the effect of protein on total ^{14}C recovery was highly significant ($p < 0.0005$).

Only very small amounts of radioactivity were recovered from the lower portion of the intestine in each group. Yet, conversion of β-carotene to retinyl ester (Table 11) was increased as higher levels of dietary protein were fed (F test, $p < 0.025$). Neither recovery of unconverted β-carotene (Table 10) nor recovery of total radioactivity (Table 12) from the lower intestine exhibited significant differences due to dietary protein level.

Effects of dietary protein level on the metabolism of ^{14}C-β-carotene in the intestinal wall seemed to manifest themselves chiefly in the middle intestine, which is the area suggested by Thompson et al. (1949) as the most active area of carotene conversion. ^{14}C-β-carotene recovery from the middle intestinal wall was higher in groups fed 40 than 10% protein which suggested that more β-carotene was adsorbed in this region of the intestine by rats consuming greater quantities of dietary protein. Since β-carotene recovery in the upper intestine

Table 12. Total radioactivity recovered from the intestinal wall expressed as percentage of injected dose. Experiment II

Group	Upper intestine	Middle intestine	Lower intestine	Whole intestine
10 C/PF	17.4	9.1	0.8	27.4
10 C/P	14.0	5.3	0.6	20.0
20 C/PF	13.8	12.1	2.0	27.9
20 C/P	11.7	13.9	1.1	26.7
40 C/PF	12.9	15.4	1.1	29.5
40 C/P	10.8	16.9	3.5	31.2
10 - PF, P[a]	15.7	7.2	0.7	23.7
20 - PF, P[a]	12.8	13.0	1.6	27.3
40 - PF, P[a]	11.8	16.2	2.3	30.4
PF - 10, 20, 40[a]	14.7	12.2	1.3	28.3
P - 10, 20, 40[a]	12.2	12.0	1.7	26.0

[a]Arithmetic mean of the above group means.

decreased slightly between the 10 and 40% levels it is possible that the injected dose moves down the intestine at a faster rate in rats consuming the higher protein levels. More movement of β-carotene down the intestine from the pyloric sphincter would increase the potential for adsorption to mucosal surfaces and would promote passage of carotene to the areas cited as

most active for conversion.

When the intestine was considered as a whole, total recovery of unconverted β-carotene was similar at all dietary protein levels examined (13.0 - 13.6%) (Table 10). Animals fed 10, 20, and 40% dietary protein showed conversion of 9.6, 12.2, and 15.5% of the injected dose to retinyl esters respectively (F test, $p < 0.001$) (Table 11). Differences in retinyl ester formation between animals fed adequate and excessive dietary protein were likewise significant ($p < 0.05$) indicating that the differences seen with protein were not dependent on the fact that one group was slightly protein deficient. Level of dietary protein also produced a significant increase in the total radioactivity recovered from the intestine (F test, $p < 0.025$) with higher protein levels.

Stomach and intestinal contents In Experiment II with protein as 10, 20, or 40% of the diet, β-carotene recovered from the stomach and intestinal contents decreased progressively from 31.2 to 26.6 to 23.4% of the injected dose (F test, $p < 0.025$) (Table 13). Seemingly, less β-carotene was adsorbed to the mucosal surface in rats consuming the lower protein diets and thus more of the injected dose was regurgitated into the stomach or flushed out of the intestine when it was rinsed at autopsy.

Total gastro-intestinal tract When intestinal wall, stomach, and intestinal contents were considered together in Experiment II, retinyl ester formation was 10.2, 12.7, or

Table 13. Total radioactivity recovered from the stomach and intestinal contents expressed as percentage of injected dose. Experiment II

Group	β-carotene	Other fractions	Total
10 C/PF	29.7	2.2	31.9
10 C/P	32.6	2.3	34.9
20 C/PF	23.8	1.8	25.6
20 C/P	29.3	1.9	31.2
40 C/PF	23.7	1.7	25.4
40 C/P	23.1	1.7	24.8
10 - PF, P[a]	31.2	2.2	33.4
20 - PF, P[a]	26.6	1.8	28.4
40 - PF, P[a]	23.4	1.7	25.1
PF - 10, 20, 40[a]	25.7	1.9	27.6
P - 10, 20, 40[a]	28.3	2.0	30.3

[a]Arithmetic mean of the above group means.

16.2% of the injected dose in animals receiving 10, 20, or 40% protein respectively (F test, $p < 0.001$). β-carotene recovery tended to decrease with higher dietary protein and the difference between the 10% protein group and the 40% protein group was very nearly significant at the 0.05 level. The increased retinyl ester formation in groups fed higher levels of dietary

protein no doubt accounts for disappearance of some of the β-carotene. Total recovery of radioactivity from the gastro-intestinal tract did not vary significantly with dietary protein intake. Instead, the changes observed in the gastro-intestinal tract were due to distribution of 1) unconverted ^{14}C-β-carotene and its metabolic products and 2) radioactivity in different segments of the intestine.

Liver Amounts of radioactivity recovered in the liver after the one-hour metabolism period of Experiment II were very small. The effect of dietary protein, however, was highly significant (F test, $p < 0.01$) with recovered hepatic retinol as 0.4, 0.6, or 0.7% respectively of the injected dose with the 10, 20, and 40% protein levels.

Presence of dietary protein in the gastro-intestinal tract

Intestinal wall The intestine in Experiment I was divided approximately at the midpoint into two sections labeled upper and lower. In Experiment II the upper twelve inches of the intestinal tract was designated the upper intestine; the next twelve inches was the middle intestine; and the remainder was called the lower intestine. Thus, the intestinal segment designated upper in Experiment I was actually comparable to the upper intestine plus portions of the middle intestine in Experiment II. After the two and one-half hour metabolic period of Experiment I, retinyl ester recovery from the upper intestine was 5.1% in group 20 C/PF and 9.4% in group 20 C/P

($p < 0.025$). Retinyl ester formation in the whole intestinal wall in Experiment I, likewise, was greater in animals fed protein rather than protein-free diets before autopsy ($p < 0.05$). However, in Experiment II (after a one-hour metabolic period) presence of dietary protein in the intestine at the time of β-carotene injection affected retinyl ester formation only in the lower intestine for rats fed 10 or 20% protein (Table 11). Since the metabolic period was two and one-half hours in Experiment I versus one hour in Experiment II, the time factor may explain the apparent discrepancy in the data for animals receiving adequate protein.

Presence of protein in the intestine during the metabolism of ^{14}C-β-carotene increased ^{14}C-retinyl ester formation in the middle ($p < 0.05$), lower ($p < 0.001$), and whole intestinal wall ($p < 0.05$) for animals with 40% protein intakes. Retinyl ester recovery from the whole intestine was 13.6% in group 40 C/PF and 17.4% in group 40 C/P ($p < 0.05$) (Table 11). If the presence of protein in the gastro-intestinal tract enhances the conversion of β-carotene to vitamin A, the most obvious changes would be expected between groups 40 C/PF and 40 C/P where differences in dietary composition were the greatest.

Stomach and intestinal contents In Experiments I and II, neither β-carotene nor other chromatographic fractions of the stomach and intestinal contents showed significant differences due to the presence or absence of dietary protein (Table 13). These results indicate that the presence of protein in

the gastro-intestinal tract does not exert a specific effect on adsorption of β-carotene to the mucosal wall.

Total gastro-intestinal tract Feeding a protein or protein-free diet before injection of β-carotene did not produce significant differences in recovery of unconverted β-carotene or total radioactivity from the gastro-intestinal tract in Experiments I or II. In Experiment I, retinyl ester formation in rats fed adequate protein was 11.2% or 8.3% respectively of the injected dose in the presence or absence of protein ($p < 0.05$) (Table 14). After a one-hour metabolic period (Experiment II), retinyl ester formation was not affected by supplementation pattern at the 10 or 20% protein levels. However, rats fed excessive protein formed retinyl esters equal to 18.1% or 14.2% of the injected dose respectively in the presence or absence of protein ($p < 0.05$). The increases in retinyl ester recovery from the total gastro-intestinal tract are reflections of those reported and discussed previously for the whole intestinal wall.

Liver Recovery of radioactivity in the liver in Experiments I and II was not consistently influenced by feeding a protein or protein-free diet prior to injection of β-carotene. Olson (1961) indicated that radioactivity in the liver reached a maximum level in 3 to 5 hours after the intestinal injection of labeled β-carotene. If longer metabolic periods had been examined in this study, differences in radioactivity in the liver might have corresponded to the changes seen with

Table 14. Radioactivity recovered as retinyl ester from the gastro-intestinal tract expressed as percentage of injected dose. Experiment I

Group	Upper intestine	Lower intestine	Whole intestine	Gastro-intestinal tract
20 C/PF	5.1	2.7	7.8	8.3
20 C/P	9.4	1.3	10.7	11.2

supplementation pattern after the carotene feeding study.

Mucosal preparation In Experiment III, an in vitro assay of the carotene cleavage enzyme derived from mucosal scrapings indicated that activity (expressed as retinal formation per 24 inches of intestine or per gram of intestine) was significantly greater when the enzyme preparation was obtained from rats pre-fed protein diets than from rats fed protein-free diets ($p < 0.025$). Since this was a cell-free system, the increased retinal formation could not have been due to greater absorption of carotene. It is possible that the products of protein digestion stimulated synthesis of the carotene cleavage enzyme. In addition, the carotene cleavage enzyme is enhanced by the addition of thiols (Goodman et al., 1967). The influx of amino acids during active absorption could provide a source of -SH groups and thus increase enzyme activity.

DISCUSSION

Numerous investigators have indicated that protein malnutrition adversely affects the absorption and conversion of β-carotene (Mahadevan, et al. 1965). In addition, Arnrich and Pederson (1956), Ruffin and Arnrich (1966), MacMillan (1966), and Kotecheri (1967) have reported increased deposition of vitamin A from a wide range of doses of β-carotene as protein comprises 40% rather than 20% of the diet for four weeks. In confirmation vitamin A deposition in the present studies was lower in carotene-supplemented rats fed 10% dietary protein than in those fed 20 or 40% protein despite reduced vitamin A demands of the mildly-deficient (10%) animals for growth. Furthermore, mean vitamin A deposition after the twenty-eight day feeding period continued to rise as the protein intake exceeded the need of the animals (20% vs. 40%).

In the present study serum vitamin A levels in animals fed 10, 20 or 40% protein diets appeared to be similar regardless of the level of dietary protein. Mathews and Beaton (1963) suggest that with a 4% protein diet there is interference with the absorption or conversion of carotene and that this interference is reflected in serum levels. Dowling and Wald (1958), however, reported that when hepatic vitamin A reserves exist serum levels of the vitamin are maintained. In these experiments even rats fed mildly-deficient protein diets had

accumulated vitamin A reserves. Arroyave et al. (1961) examined another aspect of the relationship between protein nutriture and serum vitamin A levels. They reported that in severe protein deficiency plasma carriers of the vitamin may drop so precipitously that whatever liver reserves exist are not available for utilization. In the present study, however, only a mild protein deficiency condition existed and the decline of serum protein levels presumably would not have been of consequence.

Since vitamin A deposition over a period of weeks reflects not only the absorption and conversion of β-carotene but also the subsequent metabolism and utilization of the vitamin, the conversion of ^{14}C-β-carotene to labeled products was also examined during a short metabolic period on the day of autopsy. The middle intestine has been reported to be a very active area for carotene conversion (Thompson et al., 1949) and in the present study effects of dietary protein on the metabolism of ^{14}C-β-carotene were most perceptible in this area. The markedly increased retinyl ester formation in the middle intestine as the diet contained 10, 20, or 40% protein may have been abetted by increased adsorption of β-carotene to the mucosal surface, by a greater number of mucosal cells, or by increased levels of the β-carotene cleavage enzyme.

Results from this study confirm those of Kotecheri (1967) who indicated that retinyl ester formation in vivo was significantly higher in animals fed 40% than 10% protein. However,

since Kotecheri (1967) compared only animals fed deficient and excessive protein, further information was needed to ascertain whether the changes she reported were, in fact, due to the excessive protein intake or whether they were created by comparison with deficient animals. In the present experiments, retinyl ester formation by groups fed 40% protein was significantly higher than in groups with an adequate protein intake. Thus, the effect of excessive protein was not an artifact created by comparison with protein-deficient animals.

^{14}C-retinyl ester formation in Experiment II showed a high correlation ($r = 0.75$) with total deposition of vitamin A in liver and kidney resulting from 28-day supplementation. Apparently the more rapid ^{14}C-retinyl ester formation and hepatic deposition with higher dietary protein are also manifested in the feeding study.

A second aspect of this study was investigation of the effects of protein in the intestinal tract at the time of carotene supplementation. A feeding pattern was designed so that animals would be either actively absorbing amino acids or would be post-absorptive with respect to protein. In Experiments I and II carotene supplementation with the protein portion of the diet produced significantly higher vitamin A reserves than did supplementation with the protein-free portion in animals fed adequate or excessive protein. Apparently at these protein levels, the presence of protein in the small

intestine enhances the absorption and/or conversion of carotene. When protein was inadequate, however, no significant differences in total vitamin A deposition were seen. The results at the 10% protein level varied from those of Ruffin (1965) who reported that more hepatic vitamin A was deposited when carotene was fed simultaneously with the protein than when carotene feeding was delayed for rats receiving either 10 or 20% protein. The feeding pattern used by Ruffin was slightly different from the pattern used in the present studies which may have contributed to the discrepancy. She fed small portions of a concentrated protein source and nine hours later allowed animals access to the remainder of the diet for 15 hours. Groups with protein intakes equivalent to 10% received 2.7 g more protein-free diet (and 2.7 g less of the concentrated protein source) than did animals with adequate protein intakes. Since there were no enforced fasting periods, animals consuming the larger quantities of protein-free diet may not have been strictly post-absorptive when the carotene supplement (with protein diet) was given. Thus bile or lipase levels in the intestine may have remained higher causing more vitamin A deposition from the subsequent carotene supplement.

In Experiment I ^{14}C-retinyl ester formation in rats fed 20% protein was significantly enhanced by the presence of protein in the gastro-intestinal tract at the time of ^{14}C-β-carotene injection. These results were not confirmed in

comparable groups from Experiment II; however, the difference in length of the metabolic period between Experiments I and II may explain the variation in these data.

Effects of feeding pattern on ^{14}C-β-carotene metabolism were seen in tissues from rats with 40% protein intakes but not in their counterparts fed 10 or 20% protein. This was the case for several parameters. The difference in protein and sucrose constituents of the diets fed prior to injection of ^{14}C-β-carotene was much more pronounced between groups 40 C/PF and 40 C/P than between groups at the adequate or mildly-deficient protein levels (Table 15). For instance, animals on 10% protein diets with the C/PF or C/P feeding regimen received 870 milligrams or 720 milligrams of sucrose from a one-gram meal respectively. Comparable groups of rats fed excessive protein received 870 mg (C/PF) or 270 mg (C/P) of sucrose. Thus, it is not surprising that differences could be detected more easily in animals consuming 40% dietary protein.

Table 15. Composition of one gram test meals

Constitutent	Mean daily protein intake equivalent to			
	0 mg	10% mg	20% mg	40% mg
Protein	0	150	300	600
Sucrose	870	720	570	270
Corn oil	90	90	90	90
Minerals	40	40	40	40

Numerous factors, such as micelle formation, membrane transport, and activity of enzymes involved in vitamin A formation affect the conversion of carotene to vitamin A. One of these systems, i.e., the carotene cleavage enzyme, was examined in vitro in Experiment III. Activity of the enzyme was higher from animals fed protein rather than protein-free diets prior to autopsy. These findings suggested that the carotene conversion process was affected by the presence of products of protein digestion.

If protein exerts an effect only on the intestinal conversion of carotene, for instance, on the carotene cleavage enzyme, a spaced-feeding regimen should produce no change in the utilization of preformed vitamin A. In past experiments no enhancement of preformed vitamin A utilization had been observed with increasing dietary protein, contrary to the effects of protein on carotene. Therefore, it was decided to go further and assess the effects of pattern of supplementation on both dietary retinyl acetate and carotene (Experiment III). The animals fed retinyl acetate simultaneously with protein had higher vitamin A reserves than those in which supplementation occurred with the protein-free diet. One potential effect of protein might be to increase hydrolysis of retinyl esters. Ascarelli (1969), suggested that vitamin A acetate is so quickly hydrolyzed (in the chicken) that it is physiologically

similar to a vitamin A alcohol injection. However, since the rapidity of hydrolysis of retinyl acetate in the rat has not been verified, the possibility remains that the products of protein digestion may stimulate the intestinal hydrolase. Another contingency is that effects observed with retinyl acetate may simulate the effects of presence of dietary protein in the gastro-intestinal tract on the transport or deposition of retinol newly-formed from β-carotene. Although the magnitude of difference in vitamin A deposition between the two carotene-supplemented groups was much greater than between the groups supplemented with retinyl acetate, it appears that protein may interact at more than one point in vitamin A and carotene metabolism.

In the following section an attempt will be made to summarize information on carotene metabolism from this and other laboratories and to give proper perspective to relationships of tissue vitamin A levels and intestinal conversion processes. Absorption and conversion are two key aspects of carotene metabolism which are potentially influenced by the nutritional state of the animal with respect to protein.

Since the calculated amount of intracellular β-carotene in the intestine is very low, it is conceivable that absorption is the rate-limiting step in carotene conversion (Olson, 1961a). This is further supported by observations of 1) a generalized

failure to absorb fat-soluble factors in extreme protein deficiency conditions (Arroyave et al., 1959) 2) a fall in levels of lipase with protein deficiency (Mahadevan et al., 1965) and 3) a decreased bile flow in protein malnutrition (Mahadevan et al., 1965). In addition, absorption from the intestine may be diminished by atrophy of intestinal cells. Factors related to absorption which influence the increased vitamin A deposition from carotene with higher protein levels have been less thoroughly investigated. Perhaps higher levels of lipase or of other pancreatic enzymes (Snook and Meyer, 1964), may affect the absorption of the provitamin favorably.

The present study suggests an increase in ^{14}C-retinyl ester formation during short metabolic periods as protein composes a higher percentage of the diet. Numerous reports have noted increased vitamin A reserves from β-carotene with higher levels of dietary protein (Arnrich and Pederson, 1956; Ruffin and Arnrich, 1966, MacMillan, 1966; and Kotecheri, 1967). Of course, it is very difficult to relate the intestinal absorption and conversion of carotene to hepatic reserves since vitamin A deposition is an accumulative process encompassing not only absorption and conversion but also transport and utilization of the vitamin. Since vitamin A is continually released from the liver for metabolic purposes, it seems that turn-over studies are needed before a thorough understanding of effects of dietary protein on hepatic deposition can be achieved.

In studies of vitamin A deposition, an effect of protein was not observed when retinyl acetate was fed: therefore the increased hepatic deposition from carotene was assumed to be a response of the absorption and/or conversion process to dietary protein (Arnrich and Pederson, 1956; Ruffin and Arnrich, 1966; MacMillan, 1966). However, in the present study when retinyl acetate was fed simultaneously with or without protein, there was a slight but significant stimulation of vitamin A deposition with the presence of protein in the digesta. Perhaps the increased vitamin A reserves observed when retinyl acetate was metabolized in the presence of protein were due to stimulation of the hydrolase or of uptake by the mucosal cell. Or, the enhancement may have been due to increased transport rate and deposition of the vitamin. Particularly if the latter postulation were correct, it is difficult to understand why overall protein intake, as well as the presence of protein in the gastro-intestinal tract, did not increase vitamin A deposition in the dosage range examined (Ruffin and Arnrich, 1966). Possibly increased metabolic needs for vitamin A with higher protein levels normally obliterate all beneficial effects of protein on utilization of the preformed vitamin. Since the protein in the intestine is more concentrated with the spaced feeding regimen, the effects may thus be more apparent.

Differences with supplementation pattern were more pronounced in carotene-fed groups than in groups space-fed and

given retinyl acetate, so it appears protein has effects on carotene beyond those shown for retinol. Using the same reasoning as above, the unique effects of protein on carotene metabolism may predominate over the increased metabolic demands for vitamin A usually seen with higher protein levels. The result, then, would be higher net deposition of vitamin A from carotene with increasing protein levels.

Carotene utilization has been reported to be enhanced by increasing the amount of nitrogen in the diet even when the additional nitrogen is from a very poor quality protein and not utilized for growth (Hillers, 1963). A protein that is of too low biological value to be utilized for growth may also not be optimum for enzyme synthesis. Perhaps the increased vitamin A deposition cited in this study is concomitant with amino acid absorption regardless of the suitability of these amino acid mixtures for protein synthesis.

Interpretation of the mechanism of effect of protein on carotene conversion is limited by the many factors which could influence the reaction. Among these factors are levels of the carotene cleavage enzyme and activity of the cleavage enzyme system. In the present study, on the C/PF regimen animals with excessive (40%) protein as compared to deficient (10%) intakes stored more vitamin A despite the fact that carotene was given with the protein-free portion of diet. Since carotene and protein were not in the intestinal tract simultaneously in the

feeding studies discussed above, a change in level of the carotene cleavage enzyme with overall protein nutrition of the animal may be implicated. However, it is not possible to separate effects of absorption, transport, and utilization from those of enzyme level in a feeding study, so no firm conclusions can be drawn. Gronowska-Senger and Wolf (1970) reported, contradictorily, that *in vitro* higher conversion of β-carotene to retinal was observed with mucosal preparations from fasted rats maintained on mildly deficient rather than adequate or excessive protein diets. Their study indicates that the enzyme levels do not increase with higher protein intakes. Additional studies of the effects of protein level on the carotene cleavage enzyme and related enzyme systems are definitely needed in order to resolve discrepancies between the *in vivo* and *in vitro* studies.

Goodman *et al.* (1967) have purified and partially characterized the carotene cleavage enzyme. They reported that activity of the enzyme *in vitro* was increased by the addition of thiols. Data from the present study as well as from Ruffin (1965) indicate a marked enhancement of carotene utilization when carotene and protein were fed simultaneously. Some products of protein digestion, such as the sulfur containing amino acids, may increase the activity of the carotene cleavage enzyme. When the carotene cleavage enzyme was assayed *in vitro*, the preparations from rats pre-fed protein rather than protein-

free diets converted more ^{14}C-β-carotene to ^{14}C-retinal. Since this was an isolated system, it seems that products of protein digestion must directly affect the activity of the enzyme.

Of course, activity of the carotene cleavage enzyme will decrease if carotene is degraded rapidly to non-utilizable products. Kotecheri (1967) recovered more of the injected dose from animals fed excessive protein than from those fed 10% protein. Some of the difference in recovery may be attributed to increased degradation of carotene in rats fed low protein diets. A time-sequence study (rather than a single metabolic period) of the effects of protein on formation and excretion of water-soluble derivatives of vitamin A would be necessary to clarify the rate of degradation of carotene.

SUMMARY AND CONCLUSIONS

Effects on carotene metabolism of 1) dietary protein level and of 2) the simultaneous presence of protein and carotene in the gastro-intestinal tract were explored in the present studies. Male, weanling rats were depleted of existing vitamin A reserves and littermates were assigned to the two feeding patterns within the split-plot experimental design.

Effects of dietary protein level on hepatic and renal vitamin A deposition from carotene were measured after the 28-day feeding period in Experiments I and II. Presence or absence of protein in the gastro-intestinal tract during vitamin supplementation was concurrently a variable in all studies. Vitamin A deposition was enhanced with higher levels of dietary protein. Deposition was also increased with the simultaneous feeding of protein and carotene in rats with adequate or excessive protein intakes. Rats consuming a deficient (10%) protein diet, however, showed no response to supplementation pattern.

In Experiment III vitamin A deposition from carotene or from retinyl ester was compared in rats fed adequate protein to ascertain the effects of the supplementation pattern on the utilization of the preformed vitamin. Deposition of vitamin A from both sources increased when protein were present in the intestinal tract although the effect was of much greater magnitude in carotene-supplemented animals.

On the day of autopsy, the formation of ^{14}C-products from labeled β-carotene during short metabolic periods was investigated *in vivo* and *in vitro*. The metabolism of injected ^{14}C-β-carotene in the presence or absence of dietary protein was examined in animals fed adequate protein in Experiment I. Retinyl ester formation in the intestinal wall was significantly higher in animals fed protein test meals prior to the injection. In Experiment II both the effects of dietary protein level and of supplementation pattern were considered. Mean retinyl ester formation in the intestinal wall increased significantly as protein comprised 10, 20, or 40% of the diet. Enhanced retinyl ester formation due to the presence of protein in the intestine was observed in animals fed 40% protein.

In Experiment III the carotene cleavage enzyme, obtained from intestinal mucosal scrapings, was assayed. Activity of the enzyme (expressed as retinal formation per 24 inches of intestine or per gram of intestine) was significantly greater when the enzyme preparation was obtained from rats pre-fed protein rather than protein-free diets.

In these studies, vitamin A reserves were enhanced by increasing levels of dietary protein. The vitamin A reserves increased despite the decreased demands of the mildly-protein deficient animals for growth. Retinyl ester formation likewise increased with each increment in protein intake. Thus, protein intakes above those needed for optimum growth, under the

conditions of this experiment still augmented retinyl ester formation. The middle intestine was found to be the most active site of conversion of β-carotene to retinyl esters one hour after the injection of the provitamin into the upper duodenum. Deposition in the liver of ^{14}C products from β-carotene after only one hour of metabolism was very slight, but the animals with higher protein intakes had more of the newly-formed vitamin.

Deposition of vitamin A in animals with adequate or excessive protein intakes is also increased with the simultaneous ingestion of protein and carotene. Animals with a mild protein deficiency, however, showed no changes in vitamin A reserves in response to pattern of supplementation. When conversion of ^{14}C-β-carotene was examined, the effect of presence of protein in the gastro-intestinal tract was most pronounced with mean daily intakes of 40% protein. The in vitro assay of the carotene cleavage enzyme indicated that the products of digestion of protein directly affect the activity of the enzyme system.

The effect of protein on vitamin A deposition, however, is apparently due not only to its presence in the intestine during the biosynthesis of retinol. Increased vitamin A deposition was observed in animals fed excessive protein compared to those fed deficient protein diets even when the supplement was given with the protein-free portion of the diet. Also, deposition

of vitamin A from retinyl acetate was increased with the presence of protein in the gastro-intestinal tract. Although the magnitude of increase in response to protein in the intestine was much smaller in groups supplemented with retinyl acetate than with carotene, it still appears that the presence of protein during the digestive phase affects more than one aspect of carotene metabolism.

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APPENDIX

Figure 2. Absorption spectra of β-carotene

A: standard synthetic β-carotene

B: β-carotene synthesized by tobacco leaves following purification by chromatography

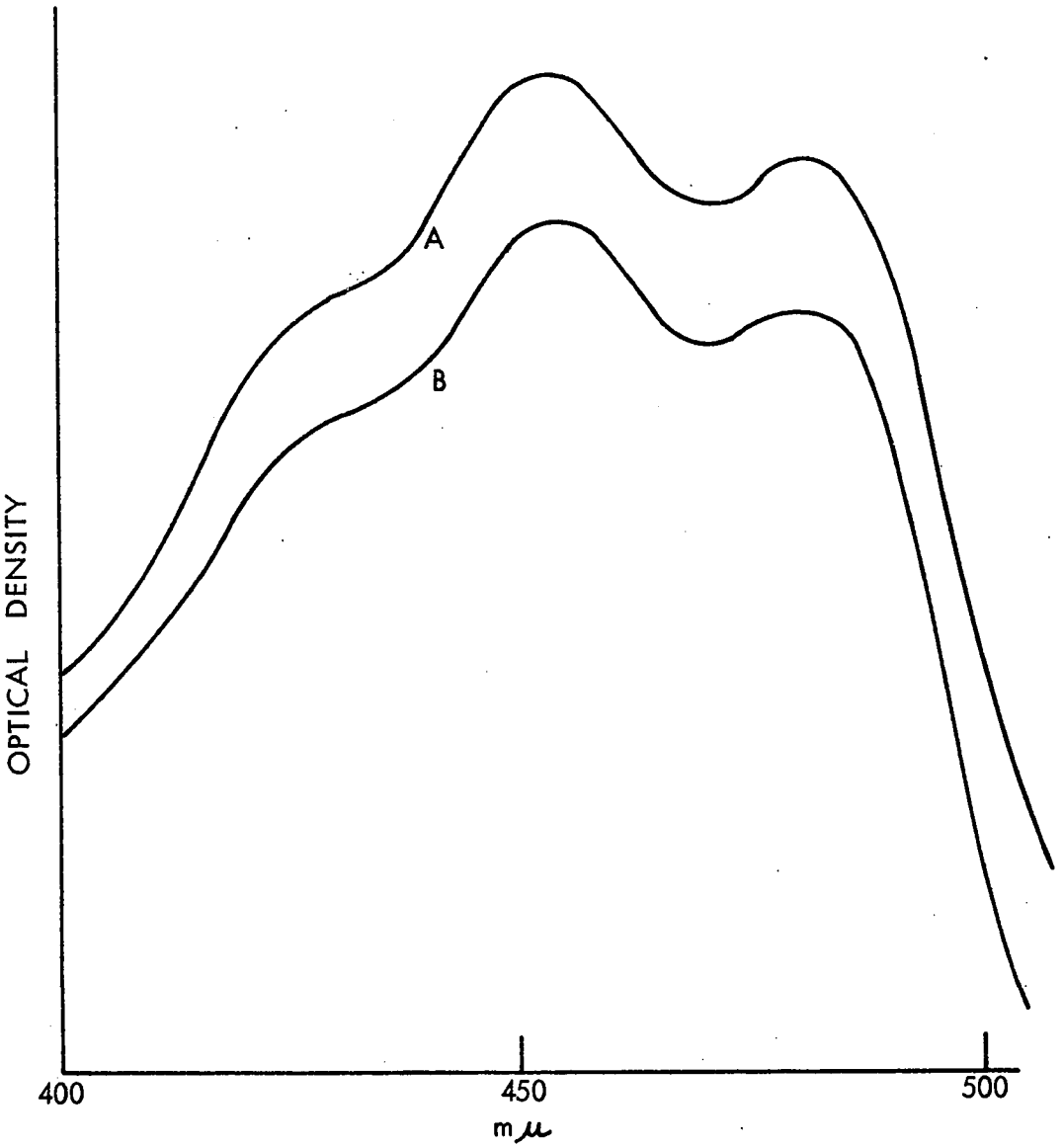
OPTICAL DENSITY
A
B
400
450
500
mμ

Table 16. Formation of ^{14}C-retinal from ^{14}C-β-carotene over a range of supernatant protein concentrations. Experiment III

Trial	Supernatant protein/flask mg	Retinal cpm
10 A	4.7	98
10 B	7.7	157
10 C	7.7	152
10 D	10.8	274
32 A	4.4	116
32 B	6.7	176
32 C	6.7	185
32 D	8.9	229

Table 17. Body weights, food intake, organ weights, and retinol levels. Experiment I

Group symbol	Rat no.	Body weight		Food intake		Organ weights		Retinol levels	
		Initial	Final	Protein-free diet	Protein	Liver	Kidney	Hepatic	Renal
		g	g	g	g	g	g	mcg	mcg
20 C/PF	11	133	249	108	224	10.6	1.9	72	28
	15	134	243	112	224	10.1	2.1	68	14
	18	144	265	112	224	9.9	2.2	77	22
	24	137	240	90	221	9.3	1.8	80	13
	30	141	272	112	224	11.4	2.1	112	10
	32	141	255	111	224	9.8	2.1	51	10
	37	138	255	112	224	9.7	2.0	20	38
	48	142	259	110	223	10.3	1.8	75	10
	50	139	247	111	223	10.4	2.1	62	17
	53	137	243	112	223	9.6	1.9	108	16
	58	139	264	111	223	9.9	2.0	56	24
	59	137	258	111	223	10.4	1.9	132	23
	63	139	241	111	224	9.4	1.7	65	15
Mean		139	253	109	223	10.1	2.0	75	18
20 C/P	12	130	259	99	224	10.3	2.0	126	16
	16	147	275	112	224	9.2	2.2	167	17
	17	130	257	97	224	9.4	2.0	76	17
	25	162	283	112	224	10.3	2.1	213	4
	29	133	256	112	224	9.6	1.8	117	11
	33	137	252	96	224	9.3	2.0	114	7
	38	134	250	106	224	10.4	2.0	67	19
	47	127	244	97	223	9.3	1.8	136	36
	49	140	264	112	223	9.7	1.9	144	14
	54	149	264	111	224	9.7	2.1	146	17

Table 17 (Continued)

Group symbol	Rat no.	Body weight		Food intake		Organ weights		Retinol levels	
		Initial	Final	Protein-free diet	Protein	Liver	Kidney	Hepatic	Renal
		g	g	g	g	g	g	mcg	mcg
	57	128	253	112	223	9.9	2.2	148	23
	60	147	255	108	224	9.4	2.0	205	16
	64	133	248	107	223	9.0	1.8	51	17
Mean		138	258	106	224	9.7	2.0	132	16
40 C/PF	9	149	266	112	224	11.6	2.5	190	4
	14	136	251	111	224	11.8	2.8	164	13
	20	126	241	93	216	10.2	2.2	103	13
	22	146	246	109	218	10.0	1.9	146	5
	27	138	243	107	223	9.0	2.2	44	22
	28	139	270	105	224	10.6	2.4	105	14
	35	136	268	95	224	11.0	2.3	106	22
	42	147	265	111	224	11.1	2.4	50	14
	45	132	255	109	222	10.6	2.2	80	34
	52	143	259	111	221	11.0	2.5	126	11
	56	136	247	111	223	11.2	2.4	78	20
	61	133	233	108	233	9.7	2.1	81	25
Mean		138	254	107	222	10.6	2.3	98	16

Table 17 (Continued)

Group symbol	Rat no.	Body weight		Food intake		Organ weights		Retinol levels	
		Initial	Final	Protein-free diet	Protein	Liver	Kidney	Hepatic	Renal
		g	g	g	g	g	g	mcg	mcg
40 C/P	10	153	273	112	224	11.8	2.6	200	17
	13	125	240	104	224	11.1	2.4	133	12
	21	145	271	109	224	11.3	2.3	132	9
	23	139	247	87	219	9.4	2.2	302	7
	26	135	251	90	224	10.6	2.2	143	25
	31	136	265	105	224	10.7	2.1	157	32
	36	143	261	86	224	10.9	2.3	140	11
	41	139	277	112	224	15.1	2.8	139	32
	46	143	255	108	224	9.9	2.2	179	9
	51	126	226	77	221	9.9	2.2	163	17
	55	144	255	112	223	10.7	2.4	164	21
	62	138	257	108	223	9.9	2.2	141	26
Mean		139	256	101	223	10.9	2.3	166	18

Table 18. Body weights, food intake, organ weights, and retinol levels. Experiment II

Group symbol	Rat no.	Body weight		Food intake		Organ weights		Retinol levels	
		Initial	Final	Protein-free diet	Protein diet	Liver	Kidney	Hepatic	Renal
		g	g	g	g	g	g	mcg	mcg
10 C/PF	103	118	191	92	221	7.4	1.3	50	7
	104	142	220	111	223	8.0	1.5	55	16
	110	134	212	104	223	7.3	1.4	42	24
	113	117	191	89	216	6.2	1.2	27	24
	120	124	203	100	221	6.7	1.4	26	19
	122	138	216	106	223	7.4	1.4	41	8
	143	140	219	95	223	7.6	1.4	12	39
	144	146	221	103	223	7.9	1.6	15	22
	151	127	217	111	223	8.0	1.4	94	11
	153	121	208	106	222	6.9	1.3	63	20
	156	142	220	108	221	7.9	1.4	73	15
	158	143	224	108	223	7.5	1.5	12	17
Mean		133	212	103	222	7.4	1.4	42	19
10 C/P	102	125	205	102	221	6.8	1.4	20	29
	105	133	199	99	220	6.7	1.4	56	10
	111	137	217	104	222	7.6	1.6	3	17
	112	139	211	97	221	6.2	1.5	15	38
	119	131	203	93	220	6.2	1.9	22	23
	121	142	218	104	221	6.9	1.5	48	20
	142	138	220	102	221	7.0[a]	1.5[a]	5[a]	41[a]
	145	145	215	103	221	6.7	1.5	30	17
	152	123	212	108	222	7.3	1.4	120	7
	154	132	215	104	221	6.4	1.2	28	11
	155	140	216	108	221	7.1	1.4	33	19
	157	149	230	108	223	7.0	1.5	31	12
Mean		136	213	103	221	6.8	1.5	34	20

[a]Missing data calculated.

Table 18 (Continued)

Group symbol	Rat no.	Body weight		Food intake		Organ weights		Retinol levels	
		Initial	Final	Protein-free diet	Protein diet	Liver	Kidney	Hepatic	Renal
		g	g	g	g	g	g	mcg	mcg
20 C/PF	78	131	244	108	223	9.0	2.0	80	23
	82	141	244	101	223	9.7	1.9	38	17
	92	134	229	104	217	8.4	1.6	102	4
	94	135	226	111	223	8.1	1.9	51	12
	124	131	241	110	223	9.4	2.0	97	11
	127	132	229	108	222	8.6	1.7	52	13
	129	146	249	106	222	8.9	1.9	63	3
	132	137	246	110	222	8.8	1.8	51	9
	137	153	259	108	223	8.9	1.9	28	16
	141	141	243	99	217	8.6	1.8	72	7
	147	124	244	104	220	9.8[a]	1.8[a]	104[a]	10[a]
	150	130	232	111	222	8.4[a]	1.6[a]	196[a]	13[a]
	159	145	241	111	223	8.4	1.7	27	21[a]
	161	122	231	108	222	9.6	1.8	74	25
	167	126	217	104	216	7.2	1.6	90	12
	170	126	235	110	222	8.8	1.8	63	11
Mean		135	238	107	221	8.8	1.8	74	12
20 C/P	79	130	226	104	223	8.8	1.9	85	15
	81	139	243	111	222	9.2	1.8	148	10
	91	138	232	106	221	8.5	1.7	136	6
	95	137	224	111	223	8.2	1.6	100	11
	123	122	226	97	219	8.0	2.2	110	3
	126	126	230	106	223	8.1	1.7	140	3
	130	138	236	103	222	8.8	1.9	91	3
	131	144	243	112	223	8.3	1.9	117	8
	138	132	239	104	221	8.8	2.0	80	9
	139	148	250	100	222	8.6	2.0	60	6
	148	131	241	106	220	8.8	2.0	94	7

Table 18 (Continued)

Group symbol	Rat no.	Body weight		Food intake		Organ weights		Retinol levels	
		Initial	Final	Protein-free diet	Protein	Liver	Kidney	Hepatic	Renal
		g	g	g	g	g	g	mcg	mcg
	149	127	236	109	222	8.1	1.7	236	5
	160	130	238	111	221	8.4	2.0	127	17
	162	131	256	110	223	9.4	2.2	90	25
	168	134	240	111	221	7.7	1.6	85	10
	169	126	228	107	219	8.5	1.6	125	9
Mean		133	237	107	222	8.5	1.9	114	9
40 C/PF	85	136	241	105	223	9.5	2.1	131	8
	89	150	250	110	223	9.8	2.0	72	4
	97	149	243	110	222	8.8	2.2	100	3
	100	137	235	108	210	10.0	2.4	85	8
	106	119	233	98	222	9.4	2.4	96	10
	108	123	232	106	220	8.6	1.8	162	5
	116	118	248	104	220	9.3	2.1	51	17
	118	139	253	103	222	9.9	2.2	83	11
	134	141	234	106	222	8.7	1.8	102	8
	135	134	242	105	222	9.2	2.0	68	6
	163	119	223	94	222	8.5	1.8	101	9
	166	133	257	100	219	9.4	1.8	178	6
Mean		134	241	104	221	9.3	2.0	102	8

Table 18 (Continued)

Group symbol	Rat no.	Body weight		Food intake		Organ weights		Retinol levels	
		Initial	Final	Protein-free diet	Protein	Liver	Kidney	Hepatic	Renal
		g	g	g	g	g	g	mcg	mcg
40 C/P	86	132	229	101	215	8.8	1.9	201[a]	6
	90	138	245	108	213	9.5	2.2	136	6
	98	152	258	108	223	9.8	2.4	147	6
	99	121	228	91	218	9.0	2.0	155	13
	107	139	237	94	202	8.2	2.2	208	2
	109	113	236	97	211	9.1	2.3	246	10
	114	125	231	103	220	9.0	1.9	189	6
	117	132	246	106	221	8.9	2.2	158	5
	133	131	230	101	222	9.1	1.7	159	6
	136	133	250	100	222	10.1	2.2	224	10
	164	137	247	110	221	9.1	2.0	143	6
	165	127	237	106	210	8.9	2.0	158	5
Mean		132	240	102	216	9.1	2.1	177	7

Table 19. Body weights, food intake, organ weights, and retinol levels. Experiment III

Group symbol	Rat no.	Body weight		Food intake		Organ weights		Retinol levels	
		Initial	Final	Protein-free diet	Protein	Liver	Kidney	Hepatic	Renal
		g	g	g	g	g	g	mcg	mcg
20 C/PF	180	131	195	82	157	7.5	1.6	73	5
	184	118	207	55	214	7.9	1.9	147	12
	187	122	193	67	174	7.4	1.4	132	19
	191	125	221	98	218	9.6	2.0	163	9
	198	127	245	93	222	9.7	2.0	66	18
	202	133	235	98	223	9.9	1.8	63	8
	209	116	232	82	223	10.1	1.6	77	13
	211	140	231	90	217	9.9	2.1	134	5
Mean		126	220	83	206	9.0	1.8	107	11
20 C/P	179	128	187	96	138	7.6	1.6	138	2
	183	126	219	52	218	7.6	1.9	197	12
	188	130	217	91	196	8.3	1.9	180	5
	192	130	215	100	204	8.4	2.0	230	10
	199	134	244	88	224	9.2	1.9	145	29
	203	131	234	98	221	9.6	2.2	152	7
	210	131	244	91	224	10.4	1.9	130	5
	212	131	257	88	221	9.3	1.9	112	40
Mean		130	227	88	206	8.8	1.9	160	14

Table 19 (Continued)

Group symbol	Rat no.	Body weight		Food intake		Organ weights		Retinol levels	
		Initial	Final	Protein-free diet	Protein	Liver	Kidney	Hepatic	Renal
		g	g	g	g	g	g	mcg	mcg
20 A/PF	181	126	242	82	229	9.7	2.1	324	11
	186	131	219	78	184	8.1	1.7	351	9
	189	129	211	74	202	7.5	1.9	380	17
	194	131	224	72	206	8.0	2.0	363	7
	197	121	243	108	212	10.6	1.9	256	20
	201	125	238	98	224	11.0	2.0	220	27
	205	132	243	84	220	9.4	1.9	307	3
	207	117	226	86	221	11.4	1.8	301	8
Mean		126	231	85	212	9.5	1.9	313	13
20 A/P	182	138	224	76	216	8.2	2.0	355	2
	185	136	235	73	215	8.6	1.9	387	8
	190	132	207	90	193	8.4	2.0	375	10
	193	128	213	69	204	7.5	2.0	425	12
	196	121	214	112	188	9.0	1.9	371	5
	200	121	235	99	220	10.2	2.0	232	32
	206	109	224	94	216	8.5	1.8	301	10
	208	131	244	108	220	10.2	2.1	337	10
Mean		127	224	90	209	8.8	2.0	348	11

Table 20. Serum retinol levels. Experiment II

Group	Rat no.	Retinol µg %	Group	Rat no.	Retinol µg %	Group	Rat no.	Retinol µg %
10 C/PF	104	28	20 C/PF	127	33	40 C/PF	89	38
	143	38		137	38		100	32
	144	23		141	32		106	29
	151	43		147	44		108	29
	153	17		159	23		116	48
	156	26		161	38		118	41
				167	22		134	45
				170	32		135	60
							166	34
Mean		29			33			40
10 C/P	111	33	20 C/P	130	18	40 C/P	86	33
	112	41		131	36		90	32
	145	26		139	46		98	19
	152	29		148	27		99	29
	154	13		149	40		107	24
	155	21		160	34		109	38
				162	35		117	38
				168	25		133	40
				169	37		136	18
Mean		27			33			30

Table 21. Percentage of injected ^{14}C-β-carotene recovered in chromatographic fractions of gastro-intestinal tissues. Experiment I

Group symbol	Rat no.	Small intestine-wall Fraction[a] I	II	III	IV	Small intestine-contents Fraction[a] I	II	III	IV
20 C/PF	37	0.7	1.7	1.6	0.1	0.4	0.0	0.1	0.1
	48	12.8	8.7	0.4	1.1	18.4	0.1	0.1	0.3
	50	2.7	6.0	0.4	0.2	3.2	0.2	0.3	0.1
	53	1.6	5.7	0.5	0.0	0.7	0.0	0.2	0.0
	58	0.1	4.8	0.2	0.0	0.1	0.1	0.1	0.1
	59	2.6	3.5	0.4	0.3	5.8	0.1	0.4	0.1
	63	7.1	5.5	0.4	0.1	14.0	0.3	0.8	0.0
Mean		3.9	5.1	0.6	0.3	6.1	0.1	0.3	0.1
20 C/P	38	8.9	2.4	1.2	0.3	17.4	0.3	0.7	0.0
	47	19.7	21.3	1.1	0.2	22.4	1.1	0.9	1.5
	49	2.1	14.2	0.6	0.1	2.2	0.4	0.2	0.4
	54	6.7	11.9	0.4	0.0	7.9	0.2	0.5	0.0
	57	0.7	9.8	0.7	0.0	0.8	0.2	0.2	0.0
	60	0.3	2.0	0.2	0.0	7.6	0.1	0.4	0.1
	64	3.5	4.0	0.2	0.0	12.1	0.2	0.6	0.0
Mean		6.0	9.4	0.6	0.1	10.1	0.4	0.5	0.3

[a]Fraction I = β-carotene
Fraction II = Retinyl esters
Fraction III = Retinol
Fraction IV = Breakdown products.

Table 21 (Continued)

Group symbol	Rat no.	Stomach Fraction[a] I	II	III	IV	Large intestine Fraction[a] I	II	III	IV
20 C/PF	37	14.9	0.6	0.6	0.1	20.6	0.8[b]	1.5	0.0
	48	11.0	0.1	0.2	0.2	0.4	10.7[b]	0.4	1.0
	50	17.0	0.6	1.1	0.1	20.6	1.2	1.1	0.1
	53	4.6	0.2	0.5	0.1	12.8	1.8	1.7	0.3
	58	10.2	0.2	0.2	0.0	19.0	4.1	1.2	0.1
	59	25.7	1.0	0.5	0.1	0.1	0.0	0.2	0.1
	63	13.5	0.1	0.3	0.1	1.5	0.0	0.1	0.1
Mean		13.8	0.4	0.5	0.1	10.7	2.7	0.9	0.2
20 C/P	38	28.6	0.3	0.9	0.0	0.3	0.0	0.0	0.1
	47	1.4	0.0	0.1	0.2	0.2	0.0	0.0	0.0
	49	0.0	0.2	0.2	0.1	13.1	1.1	1.6	0.1
	54[b]	16.7	0.2	0.4	0.1	3.1	0.3	0.2	0.0
	57[b]	8.1	0.0	0.2	0.0	14.7	4.2	0.9	0.0
	60	5.7	0.3	0.2	0.1	12.8	3.1	1.4	0.1
	64	21.7	0.3	1.1	0.0	0.6	0.2	0.2	0.0
Mean		11.7	0.2	0.4	0.1	6.4	1.3	0.6	0.0

[b]Missing values calculated.

Table 22. Percentage of injected ^{14}C-β-carotene recovered in chromatographic fractions of liver. Experiment I

Group symbol	Rat no.	Liver				Group symbol	Rat no.	Liver			
		Total	B.C.	Ret.	PF			Total	B.C.	Ret.	PF
20 C/PF	37	9.3	0.7	7.1	0.2	20 C/P	38	2.7	0.0	2.1	0.0
	48	7.3	0.8	5.1	0.0		47	11.2	1.4	9.0	0.2
	50	8.9	0.7	6.7	0.1		49	17.7	0.8	15.1	0.4
	53	12.8	1.8	8.0	0.2		54	10.0	0.3	8.1	0.2
	58	15.8	0.6	13.0	0.1		57	15.5	0.5	13.3	0.1
	59	9.6	0.9	8.3	0.1		60	21.7	0.6	16.7	0.5
	63	9.6	0.8	6.9	0.2		64	3.1	0.2	2.4	0.1
Mean		10.5	0.9	7.9	0.1			11.7	0.5	9.5	0.2

Table 23. Percentage of injected ^{14}C-β-carotene recovered in chromatographic fractions of gastro-intestinal tissues. Experiment II

Group symbol	Rat no.	Upper intestine Fraction[a] I	II	III	IV	Middle intestine Fraction[a] I	II	III	IV
10 C/PF	103-104	5.1	11.1	0.8	0.0	4.1	3.6	0.2	0.1
	110-113	17.2	13.0	0.9	0.0	5.5	0.9	0.3	0.0
	120-122	9.0	6.7	0.6	0.1	4.6	1.0	0.2	0.0
	143-144	8.3	7.1	0.9	0.1	4.9	2.1	0.6	0.1
	151-153	7.2	6.8	0.7	0.1	5.5	5.4	0.3	0.1
	156-158	5.6	2.9	0.3	0.1	9.7	4.6	0.5	0.2
Mean		8.7	7.9	0.7	0.1	5.7	2.9	0.4	0.1
10 C/P	102-105	8.2	5.0	0.4	0.0	1.8	4.3	0.1	0.0
	111-112	11.6	5.3	0.6	0.0	3.0	0.1	0.1	0.0
	119-121	13.8	8.8	0.8	0.1	5.3	0.3	0.2	0.0
	145	2.8	6.4	0.9	0.2	2.5	0.2	0.5	0.1
	152-154	1.0	3.5	0.2	0.0	2.1	8.6	0.3	0.0
	155-157	7.5	6.6	0.5	0.0	2.2	0.1	0.1	0.0
Mean		7.5	5.9	0.6	0.0	2.8	2.3	0.2	0.0
20 C/PF	124-127	6.1	6.3	0.7	0.1	6.1	3.8	0.4	0.1
	129-132	9.2	5.4	0.6	0.1	5.6	0.8	0.2	0.0
	137-141	7.4	7.5	0.8	0.2	6.1	5.1	0.5	0.1
	147	11.4	8.7	1.0	0.2	7.6	1.6	0.4	0.1
	159-161	0.7	5.7	0.5	0.0	8.0	9.0	0.7	0.1
	167-170	3.7	5.9	0.5	0.1	7.4	8.4	0.6	0.1
Mean		6.4	6.6	0.7	0.1	6.8	4.8	0.5	0.1

[a]Fraction I = β-carotene
Fraction II = Retinyl esters
Fraction III = Retinol
Fraction IV = Breakdown products.

Table 23 (Continued)

Group symbol	Rat no.	Lower intestine Fraction[a] I	II	III	IV	Stomach + intestinal contents Fraction[a] I	II	III	IV
10 C/PF	103-104	1.0	0.0	0.1	0.0	30.8	0.4	1.0	0.1
	110-113	0.3	0.0	0.0	0.0	27.7	0.8	1.4	0.0
	120-122	0.3	0.0	0.0	0.0	48.1	0.6	1.5	0.1
	143-144	1.0	0.0	0.1	0.0	26.4	1.1	2.6	0.1
	151-153	1.9	0.1	0.0	0.0	23.7	0.8	1.0	0.2
	156-158	0.2	0.1	0.0	0.0	21.4	0.7	0.6	0.2
Mean		0.8	0.0	0.0	0.0	29.7	0.7	1.4	0.1
10 C/P	102-105	0.0	0.0	0.0	0.1	33.7[b]	0.1[b]	1.3[b]	0.2[b]
	111-112	0.3	0.0	0.1	0.0	34.1	0.4	1.1	0.0
	119-121	1.2	0.1	0.1	0.0	28.7	0.4	1.2	0.0
	145	0.1	0.1	0.1	0.1	45.0	0.6	4.0	0.2
	152-154	0.5	0.2	0.0	0.0	25.7	0.6	1.4	0.3
	155-157	0.8	0.0	0.1	0.0	28.4	0.5	1.0	0.4
Mean		0.5	0.1	0.1	0.0	32.6	0.4	1.7	0.2
20 C/PF	124-127	0.7	0.0	0.0	0.0	30.2	0.4	1.3	0.4
	129-132	4.0	0.1	0.0	0.1	27.6	0.4	0.7	0.2
	137-141	1.6	0.1	0.1	0.0	22.5	0.8	1.7	0.0
	147	1.4	0.1	0.0	0.1	17.1	0.7	0.9	0.3
	159-161	3.2	0.1	0.1	0.0	23.7	0.7	0.8	0.3
	167-170	0.2	0.0	0.0	0.0	21.5	0.5	0.9	0.2
Mean		1.8	0.1	0.0	0.0	23.8	0.6	1.0	0.2

[b]Missing values calculated.

Table 23 (Continued)

Group symbol	Rat no.	Upper intestine Fraction[a] I	II	III	IV	Middle intestine Fraction[a] I	II	III	IV
20 C/P	123-126	5.7	7.7	0.7	0.1	9.5	8.1	0.7	0.1
	130-131	5.0	6.2	0.7	0.0	6.7	8.2	0.6	0.1
	138-139	6.7	8.9	1.1	0.2	10.4	1.0	0.6	0.2
	148-149	3.7	8.5	0.7	0.1	6.0	5.0	0.3	0.1
	160-162	3.9	6.1	0.6	0.1	7.1	7.2	0.6	0.1
	169	0.5	2.7	0.2	0.0	2.4	8.0	0.6	0.0
Mean		4.2	6.7	0.7	0.1	7.0	6.2	0.6	0.1
40 C/PF	85- 89	7.7	7.9	0.8	0.1	4.7	5.5	0.5	0.1
	97-100	5.3	9.9	0.8	0.0	10.0	3.0	0.3	0.1
	106-108	3.5	7.2	0.8	0.0	4.4	10.6	1.0	0.1
	116-118	4.4	7.7	1.0	0.0	12.7	6.2	0.7	0.1
	134-135	5.7	6.7	0.6	0.1	8.7	4.5	0.3	0.1
	163-166	1.2	5.6	0.5	0.0	11.9	6.2	0.8	0.2
Mean		4.6	7.5	0.8	0.0	8.7	6.0	0.6	0.1
40 C/P	86- 90	1.1	4.3	0.2	0.0	5.3	13.5	0.4	0.1
	98- 99	4.4	4.5	0.4	0.0	9.0	10.0	0.5	0.2
	107-109	2.6	9.8	0.8	0.0	5.3	11.6	0.6	0.1
	114-117	11.9	11.9	1.1	0.1	6.8	1.0	0.3	0.1
	133-136	0.4	3.9	0.3	0.0	5.3	17.0	0.7	0.1
	164-165	0.8	5.9	0.4	0.0	4.6	8.5	0.5	0.1
Mean		3.5	6.7	0.5	0.0	6.0	10.3	0.5	0.1

Table 23 (Continued)

Group symbol	Rat no.	Lower intestine Fraction[a] I	II	III	IV	Stomach + intestinal contents Fraction[a] I	II	III	IV
20 C/P	123-126	0.9[b]	0.1[b]	0.1[b]	0.0[b]	21.2[b]	0.4[b]	1.3[b]	0.1[b]
	130-131	3.1[b]	0.1[b]	0.0[b]	0.1[b]	33.1[b]	0.3[b]	1.0[b]	0.1[b]
	138-139	0.3	0.0	0.0	0.0	15.2	0.3	1.2	0.0
	148-149	0.6	0.0	0.1	0.0	36.0	0.5	1.8	0.4
	160-162	0.5	0.0	0.0	0.0	28.6	0.7	1.0	0.2
	169	0.5	0.1	0.0	0.0	41.5	0.5	1.8	0.2
Mean		1.0	0.0	0.0	0.0	29.3	0.4	1.4	0.2
40 C/PF	85- 89	1.3	0.0	0.1	0.0	19.5	0.8	0.6	0.3
	97-100	0.4	0.0	0.1	0.0	34.0	0.7	0.6	0.2
	106-108	2.0	0.2	0.0	0.0	20.8	0.5	1.2	0.1
	116-118	0.3	0.1	0.0	0.0	17.4	0.6	1.0	0.1
	134-135	0.7	0.1	0.0	0.0	26.8	0.4	0.7	0.1
	163-166	1.3	0.1	0.1	0.0	23.7	0.8	1.5	0.0
Mean		1.0	0.1	0.0	0.0	23.7	0.6	0.9	0.1
40 C/P	86- 90	0.9	0.3	0.1	0.0	23.2	0.6	1.0	0.1
	98- 99	3.0	0.5	0.2	0.0	21.4	0.5	0.6	0.1
	107-109	10.0	1.0	0.2	0.1	21.6	0.4	1.2	0.2
	114-117	0.7	0.1	0.0	0.0	29.6	1.0	0.7	0.2
	133-136	1.6	0.6	0.1	0.0	20.5	0.8	0.9	0.1
	164-165	1.3	0.1	0.0	0.0	22.4	0.6	1.3	0.0
Mean		2.9	0.4	0.1	0.0	23.1	0.6	1.0	0.1

Table 24. Percentage of injected ^{14}C-β-carotene recovered in chromatographic fractions of liver and kidney. Experiment II

Group symbol	Rat no.	Liver				Kidney	Group symbol	Rat no.	Liver				Kidney
		Total	B.C.	Ret.	PF				Total	B.C.	Ret.	PF	
10 C/PF	103	0.3	0.1	0.3	0.0	0.0	10 C/P	102	0.1	0.1	0.2	0.1	0.0
	104	---				0.0		105	0.1				0.0
	110	0.6	0.1	0.4	0.0	0.0		111	0.3	0.1	0.4	0.2	0.0
	113	0.1				0.0		112	0.1				0.0
	120	0.6	0.1	0.6	0.1	0.0		119	0.2	0.0	0.3	0.0	0.0
	122	0.2				0.0		121	0.2				0.0
	143	0.2	0.0	0.4	0.1	0.0		145	0.5	0.1	0.2	0.2	0.0
	144	0.5				0.0		150	---				---
	151	0.1	0.0	0.4	0.0	0.0		152	0.4	0.1	0.4	0.0	0.0
	153	0.6				0.0		154	0.3				0.0
	156	0.2	0.0	0.5	0.0	0.0		155	0.1	0.1	0.2	0.1	0.0
	158	0.6				0.0		157	0.1				0.0
Mean		0.4	0.0	0.4	0.0	0.0			0.2	0.1	0.3	0.1	0.0

Table 24 (Continued)

Group symbol	Rat no.	Liver Total	B.C.	Ret.	PF	Kidney	Group symbol	Rat no.	Liver Total	B.C.	Ret.	PF	Kidney
20 C/PF	124	0.4	0.1	0.4	0.0	0.0	20 C/P	123	0.7	0.2	0.8	0.0	0.0
	127	0.3				0.0		126	0.3				0.0
	129	0.2	0.1	0.3	0.0	0.0		130	0.4	0.1	0.5	0.0	0.0
	132	0.0				0.0		131	0.2				0.0
	137	0.2	0.1	0.7	0.0	0.0		138	0.1	0.0	0.3	0.0	0.0
	141	0.8				0.0		139	0.3				0.0
	147	0.3	0.1	0.3	0.1	0.0		148	0.1	0.0	0.6	0.1	0.0
								149	0.7				0.0
	159	0.3	0.2	0.6	0.0	0.0		160	0.0	0.1	0.4	0.0	0.0
	161	0.4				0.0		162	0.5				0.0
	167	0.3	0.0	0.8	0.1	0.0							
	170	1.0				0.0		169	1.9	0.2	1.2	0.1	0.0
Mean		0.4	0.1	0.5	0.0	0.0			0.5	0.1	0.6	0.0	0.0
40 C/PF	85	0.2	0.0	0.5	0.1	0.0	40 C/P	86	0.0	0.1	0.5	0.0	0.1
	89	0.4				0.0		90	0.7				0.0
	97	0.2	0.0	0.3	0.0	0.0		98	0.7	0.1	0.8	0.0	0.0
	100	0.3				0.0		99	0.3				0.0
	106	0.8	0.2	0.8	0.2	0.0		107	0.6	0.0	0.7	0.1	0.0
	108	0.3				0.0		109	0.4				0.0
	116	---	0.2	0.9	0.1	0.0		114	0.6	0.2	0.6	0.0	0.0
	118	0.8				0.0		117	0.3				0.0
	134	0.2	0.1	0.2	0.0	0.0		133	0.5	0.2	1.2	0.0	0.0
	135	0.2				0.0		136	0.9				0.0
	163	0.5	0.3	0.7	0.0	0.0		164	0.4	0.2	0.8	0.0	0.0
	166	0.4				0.0		165	0.8				---
Mean		0.4	0.1	0.6	0.1	0.0			0.5	0.1	0.8	0.0	0.0

Table 25. ^{14}C-retinal formation per 24" intestine. Experiment III

Group symbol	Rat no.	Weight mucosa from 24" intestine	Supernatant protein/total intestinal mucosa	^{14}C-retinal formed/total intestinal mucosa
		g	g	$\mu M \times 10^{-3}$
20 C/PF	180	1.57	0.16	1.71
	184	1.43	0.11	1.84
	187	1.25	0.16	0.99
	191	1.79	0.22	4.47
	202	2.03	0.22	3.12
	209	2.19	0.30	11.98
	211	1.98	0.26	3.50
Mean		1.75	0.20	3.94
20 C/P	179	1.05	0.15	2.11
	183	1.68	0.14	3.65
	188	1.55	0.21	2.43
	192	1.68	0.19	5.51
	203	1.65	0.18	3.48
	210	2.25	0.27	13.90
	212	1.70	0.19	10.79
Mean		1.65	0.19	5.98

Table 26. Vitamin A deposition - Significance levels for analysis of variance (Table 8). Experiments I and II

Source of variation	Hepatic p<	Renal p<	Hepatic + renal p<
		Experiment I	
Protein	N.S.[a]	N.S.	0.05
Carotene supplementation pattern	0.0005	N.S.	0.0005
Interaction	N.S.	N.S.	N.S.
		Experiment II	
Protein	0.0005	0.0005	0.0005
Carotene supplementation pattern	0.0005	N.S.	0.0005
Interaction	0.0005	N.S.	0.0005

[a]N.S. = not significant.

Table 27. Vitamin A deposition - Significance levels for t test (Table 8). Experiments I and II

Groups compared	Experiment I			Experiment II		
	Hepatic p<	Renal p<	Hepatic + renal p<	Hepatic p<	Renal p<	Hepatic + renal p<
10 vs. 20	--[a]	--	--	0.001	0.001	0.001
10 vs. 40	--	--	--	0.001	0.001	0.001
20 vs. 40	N.S.[b]	N.S.	0.05	0.05	N.S.	0.005
C/PF vs. C/P	0.001	N.S.	0.001	0.001	N.S.	0.001
10 C/PF vs. 10 C/P	--	--	--	N.S.	N.S.	N.S.
20 C/PF vs. 20 C/P	0.001	N.S.	0.001	0.001	0.05	0.001
40 C/PF vs. 40 C/P	0.001	N.S.	0.001	0.001	N.S.	0.001
10 C/PF vs. 20 C/PF	--	--	--	0.05	0.05	N.S.
10 C/PF vs. 40 C/PF	--	--	--	0.001	0.001	0.005
20 C/PF vs. 40 C/PF	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
10 C/P vs. 20 C/P	--	--	--	0.001	0.001	0.001
10 C/P vs. 40 C/P	--	--	--	0.001	0.001	0.001
20 C/P vs. 40 C/P	N.S.	N.S.	0.05	0.001	N.S.	0.001

[a] -- indicates group was not evaluated in Experiment I.

[b] N.S. = not significant.

Table 28. Vitamin A deposition - Significance levels for analysis of variance and *t* test (Table 9). Experiment III

Analysis	C/PF vs. C/P			A/PF vs. A/P		
	Hepatic	Renal	Hepatic + renal	Hepatic	Renal	Hepatic + renal
	p<	p<	p<	p<	p<	p<
F test	0.005	N.S.[a]	0.001	0.05	N.S.	0.05
t test	0.001	N.S.	0.001	0.01	N.S.	0.01

[a]N.S. = not significant.

Table 29. Recovery of ^{14}C-retinyl esters from the gastro-intestinal tract - Significance levels for analysis of variance and *t* test (Table 14). Experiment I

Analysis	Upper intestine	Lower intestine	Whole intestine	Gastro-intestinal tract
	p<	p<	p<	p<
F test	N.S.	N.S.	N.S.	N.S.
t test	0.025	N.S.	0.05	0.05

[a]N.S. = not significant.

Table 30. Recovery of ^{14}C from the gastro-intestinal tract - Significance levels for analysis of variance (Tables 10-13). Experiment II

Source of variation	Upper intestine	Middle intestine	Lower intestine	Whole intestine	Stomach & intestinal contents	Gastro-intestinal tract
	p<	p<	p<	p<	p<	p<
			^{14}C-β-carotene			
Protein	N.S.[a]	0.01	N.S.	N.S.	0.025	N.S.
Carotene supplementation pattern	N.S.	0.05	N.S.	N.S.	N.S.	N.S.
Interaction	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
			^{14}C-retinyl esters			
Protein	N.S.	0.01	0.025	0.001	--[b]	0.001
Carotene supplementation pattern	N.S.	N.S.	0.025	N.S.	--	N.S.
Interaction	N.S.	N.S.	0.025	0.05	--	0.01
			Total ^{14}C			
Protein	N.S.	0.005	N.S.	0.025	0.025	N.S.
Carotene supplementation pattern	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
Interaction	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.

[a] N.S. = not significant.

[b] -- indicates group was not evaluated.

Table 31. Recovery of ^{14}C-β-carotene from the gastro-intestinal tract - Significance levels for t test (Tables 10 and 13). Experiment II

Groups compared	Upper intestine	Middle intestine	Lower intestine	Whole intestine	Stomach & intestinal contents	Gastro-intestinal tract
	p<	p<	p<	p<	p<	p<
10 vs. 20	N.S.	0.025	N.S.	N.S.	N.S.	N.S.
10 vs. 40	0.05	0.005	N.S.	N.S.	0.025	N.S.
20 vs. 40	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
C/PF vs. C/P	N.S.	0.05	N.S.	N.S.	N.S.	N.S.
10 C/PF vs. 10 C/P	N.S.	0.05	N.S.	N.S.	N.S.	N.S.
20 C/PF vs. 20 C/P	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
40 C/PF vs. 40 C/P	N.S.	N.S.	0.05	N.S.	N.S.	N.S.
10 C/PF vs. 20 C/PF	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
10 C/PF vs. 40 C/PF	N.S.	0.05	N.S.	N.S.	N.S.	N.S.
20 C/PF vs. 40 C/PF	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
10 C/P vs. 20 C/P	N.S.	0.01	N.S.	N.S.	N.S.	N.S.
10 C/P vs. 40 C/P	N.S.	0.025	0.025	N.S.	N.S.	N.S.
20 C/P vs. 40 C/P	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.

[a]N.S. = not significant.

Table 32. Recovery of ^{14}C-retinyl esters from the gastro-intestinal tract - Significance levels for *t* test (Table 11). Experiment II

Groups compared	Upper intestine	Middle intestine	Lower intestine	Whole intestine	Gastro-intestinal tract
	p<	p<	p<	p<	p<
10 vs. 20	N.S.[a]	N.S.	N.S.	0.05	0.05
10 vs. 40	N.S.	0.005	0.01	0.001	0.001
20 vs. 40	N.S.	N.S.	0.025	0.025	0.025
C/PF vs. C/P	N.S.	N.S.	0.05	N.S.	N.S.
10 C/PF vs. 10 C/P	N.S.	N.S.	N.S.	N.S.	N.S.
20 C/PF vs. 20 C/P	N.S.	N.S.	N.S.	N.S.	N.S.
40 C/PF vs. 40 C/P	N.S.	0.05	0.001	0.05	0.05
10 C/PF vs. 20 C/PF	N.S.	N.S.	N.S.	N.S.	N.S.
10 C/PF vs. 40 C/PF	N.S.	N.S.	N.S.	N.S.	N.S.
20 C/PF vs. 40 C/PF	N.S.	N.S.	N.S.	N.S.	N.S.
10 C/P vs. 20 C/P	N.S.	N.S.	N.S.	0.025	0.025
10 C/P vs. 40 C/P	N.S.	0.005	0.001	0.001	0.001
20 C/P vs. 40 C/P	N.S.	N.S.	0.001	0.025	0.025

[a] N.S. = not significant.

Table 33. Recovery of total ^{14}C from the gastro-intestinal tract - Significance levels for *t* test (Tables 12 and 13). Experiment II

Groups compared	Upper intestine	Middle intestine	Lower intestine	Whole intestine	Stomach & intestinal contents	Gastro-intestinal tract
	p<	p<	p<	p<	p<	p<
10 vs. 20	N.S.[a]	0.001	N.S.	N.S.	N.S.	N.S.
10 vs. 40	N.S.	0.001	N.S.	0.01	0.025	N.S.
20 vs. 40	N.S.	0.025	N.S.	N.S.	N.S.	N.S.
C/PF vs. C/P	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
10 C/PF vs. 10 C/P	N.S.	N.S.	N.S.	0.05	N.S.	N.S.
20 C/PF vs. 20 C/P	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
40 C/PF vs. 40 C/P	N.S.	N.S.	0.05	N.S.	N.S.	N.S.
10 C/PF vs. 20 C/PF	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
10 C/PF vs. 40 C/PF	N.S.	0.025	N.S.	N.S.	N.S.	N.S.
20 C/PF vs. 40 C/PF	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
10 C/P vs. 20 C/P	N.S.	0.005	N.S.	0.05	N.S.	N.S.
10 C/P vs. 40 C/P	N.S.	0.001	0.025	0.005	N.S.	N.S.
20 C/P vs. 40 C/P	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.

[a] N.S. = not significant.

Table 34. Retinol recovery from liver - Significance levels for analysis of variance and t test. Experiment II

	p value <
F test	
Protein	0.01
Carotene supplementation pattern	N.S.[a]
Interaction	N.S.
t test	
10 vs. 20	0.025
10 vs. 40	0.005
20 vs. 40	N.S.
C/PF vs. C/P	N.S.
10 C/PF vs. 10 C/P	N.S.
20 C/PF vs. 20 C/P	N.S.
40 C/PF vs. 40 C/P	N.S.
10 C/PF vs. 20 C/PF	N.S.
10 C/PF vs. 40 C/PF	N.S.
20 C/PF vs. 40 C/PF	N.S.
10 C/P vs. 20 C/P	0.025
10 C/P vs. 40 C/P	0.005
20 C/P vs. 40 C/P	N.S.

[a]N.S. = not significant.

Table 35. Retinal formation by carotene cleavage enzyme (mucosal supernatant) - Significance levels for analysis of variance and t test. Experiment III

Analysis	Retinal/24" intestine p<	Retinal/g intestine p<
F test	N.S.[a]	N.S.
t test	.025	.025

[a]N.S. - not significant.