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STUDIES ON THE BIOSYNTHESIS OF RETINOL AND

THE EFFECT OF PROTEIN IN RATS

by

Savitri Ramarao Kotecheri (Shenoy)

A Dissertation Submitted to the

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INTRODUCTION

One of the interesting phenomena in the field of nutrition today is that of interrelationships, which could be defined as a mutual influence or interference of two or more nutrients, on the metabolism in living systems. This phenomenon oftentimes necessitates a change in the requirement of one nutrient with respect to the other. One such instance is seen in the manner in which the dietary proteins and their amino acid constituents seem to affect the utilization of dietary carotene, the precursor of vitamin A.

Vitamin A is said to play a role in the metabolism of protein. It has been reported that vitamin A can ameliorate the negative effect of some imbalanced proteins (Bhattacharya and Esh, 1962). Similarly protein plays a wide variety of roles in the metabolism of vitamin A and of beta-carotene, the provitamin A.

Both the amount of dietary nitrogen source as well as the character of its amino acid composition appear to be determining factors with regard to the efficiency of carotene utilization. Yet the complexity of the relationship has frequently been a deterrent in the elucidation of the specific metabolic steps involved in the protein-carotene interaction. The understanding of this relationship is of particular significance because of the high incidence of xerophthalmia and keratomalacia attributable to vitamin A deficiency in children suffering from kwashiorkor, the syndrome of protein malnutrition. Moreover the characterization of this relationship might be beneficial for developing countries where carotene is often the only important source of vitamin A and where protein of low biological value is being consumed in suboptimal

quantities.

Previous work in this laboratory has shown that the amount of retinol stored in the liver of rats was a function of the amount of protein fed when the diet was supplemented with an adequate dose of carotene as provitamin A. Similar findings have been reported from other laboratories though the quantitative relationships vary with the experimental approach. However, in our laboratory this effect of protein was not demonstrable when dietary carotene was replaced by preformed retinol at similar levels based on the international unit.

The major steps involved in the utilization of carotene by the mammalian organisms are: absorption into the mucosal cell of the small intestine, conversion to vitamin A, transport in the circulatory system and deposition in hepatic and other tissues. Since under the specific experimental conditions employed in this laboratory, preformed vitamin A was not affected by protein, it is postulated that the interaction between protein and carotene occurs prior to the release of the newly formed vitamin A into the circulation. The interaction between protein and carotene is more pronounced when the two nutrients are fed simultaneously than when they are given post-absorptively with respect to each other.

Conclusions concerning carotene-protein interactions are derived from results of growth assays and liver and kidney storage tests. Data obtained with these methods are usually influenced by several chemical and physiological variables such as relative stability of the compounds under investigation, relative absorption rates, rate and control of transport,

turnover, utilization for maintenance.

The mechanism underlying the effect of protein on the utilization of carotene is not understood at present. It could be due to an increase in the efficiency of conversion of carotene to vitamin A and its esters in the intestinal mucosa by enhancing the rate of formation of metabolic products. This effect of protein on rate of formation of retinol products could be a direct influence, by increasing the activity of a yet unidentified enzyme system involved in the mechanism of carotene conversion. The effect might be indirect by simply protecting carotene against breakdown into non-utilizable products thus making carotene more available at the site of conversion. In either case a difference in the rate of carotene conversion as measured by the formation of retinol and its esters in animals fed different levels of protein should be apparent.

The present study was designed to test the hypothesis that protein enhances the rate of formation of retinol products from carotene at the site of conversion. Radio-active carotene was employed to investigate the effect of protein on the intestinal metabolism of the provitamin A.

One aspect of the problem has been the development of suitable techniques (a) for the necessary physical and chemical assays and (b) for procedures with respect to pretreatment of the animals and to surgical procedures which would simulate physiological conditions during carotene absorption. In developing the methodology it was found advisable to characterize the carotene-protein interrelationship with respect to time by an *in vivo* study using non-radio-active carotene.

REVIEW OF LITERATURE

The literature supporting this thesis will be discussed in two parts. The first part will cover studies which refer to the development of methodology. The second part will deal with several aspects of carotene metabolism with special reference to protein effect.

Part A - Methodology

The lack of precise information on carotene metabolism is due partly to the complexity of the physiological events, and partly to lack of suitable methods for studying the phenomenon. The use of labelled carotene has been helpful in assaying carotene and its metabolic products in the microquantities in which they are formed in mammalian tissues. The study of the phenomenon *in situ* in the intestines of animals and also *in vitro* with intestinal sections and mucosal homogenates, as well as cellular and sub-cellular fractions has further contributed to the understanding of this mechanism.

Synthesis of carotene

Virtually all green plants contain carotenoid pigments associated with chlorophyll, although the role played by the former in photosynthesis is still not well defined. Carotene also occurs in plant tissues without being accompanied by chlorophyll, as in fruits like tomatoes and peaches and roots such as carrots. The most abundantly occurring carotene isomer is beta-carotene and the all-trans species preponderates considerably in nature. Leaves of plants have been most frequently used to obtain

biologically synthesized carotene (Glover et al., 1948; Glover and Redfearn, 1953) although the material has also been extracted from algae and fungi (Strain et al., 1944). Carrots have often been the source of unlabelled carotene obtained for feeding studies (Thompson et al., 1949; Ganguly et al., 1959; Deshmukh and Ganguly, 1964).

The unavailability of radioactive all-trans beta-carotene produced commercially has made it necessary to evolve a simple and economic method of biosynthesis of provitamin A. Glover and Redfearn reported in 1953 the biosynthesis of radioactive beta-carotene from tomato leaves which had been exposed to labelled carbon dioxide. Later Willmer and Laughland (1957) used rye seedlings for the purpose. The algae Chlorella pyrenoidosa has also been employed (Olson, 1961a, 1964a) for the synthesis of biologically labelled carotene. The most common source for the production of biologically labelled beta-carotene today, used by Lilly et al. (1958) and others (Olson, 1964a; Krause, 1961; Huang and Goodman, 1964; Grain et al., 1965), is the fungus Phycomyces blakesleeanus which is grown in a culture medium containing ^{14}C-acetate. In this mold beta-carotene constitutes 95% of the total pigments. Mucor haemalis is the other mold usually employed in the synthesis of carotene, especially for the purpose of studying metabolic steps involved in its biosynthesis.

Yield and specific activity of the sample of ^{14}C-carotene is predominantly a function of a) total radioactivity and specific activity of the ^{14}C-source used, b) rate of the synthetic process, c) length of exposure of the metabolizing organism to the radioactive source and d) degree of dilution from pre-existing carotene. Thus Olson (1961a) reported

a yield of 15 mcg. of labelled carotene containing about 10^{-4} of the added radioactivity from 90-100 mg. of dry cells of *C. pyrenoidosa*. Huang and Goodman secured a specific activity of 23600 d.p.m./mg. in the sample of beta-carotene from *P. blakesleeanus*.

Extraction procedures

Plant tissues Booth (1957) reviewed work up to 1957, which included the different phases in the separation of carotene from other pigments. He also discussed the advantages and disadvantages involved in these procedures. The author has given a simple extraction method for effective and exhaustive extraction of carotene from plant sources. The tissue is ground with quartz in a mixture of petrol and acetone containing hydroquinone. The supernatant is decanted and washed thoroughly with distilled water to free it of acetone.

Since the present study deals with radioactive beta-carotene, the review will be restricted to work pertaining to the extraction procedures of biologically labelled beta-carotene.

Glover and Redfearn (1953) and later Laughland (1954) were among the first to report synthesis and extraction of carotene from plant materials exposed to $^{14}CO_2$. They recognized an unidentified compound of high specific activity which moves with beta-carotene on the column during chromatography after a simple solvent extraction procedure. Later it was found that this compound hampered the accurate determination of these samples by radioassay methods. Willmer and Laughland (1957) overcame this difficulty. Following saponification of the plant tissue, they extracted the unsaponifiable fraction and cooled it by passing it through dry ice to

remove sterols. After further purification they obtained a relatively pure sample of ^{14}C-carotene. However, the authors did not identify the compound which had hampered the assay in their previous work. Perhaps the compound was of steroid nature.

The much-quoted method described by Lilly *et al.* (1958) differs from the above in two ways. Whereas diethyl ether was employed for extraction purposes in the study of Willmer and Laughland (1957), Lilly *et al.* (1958) used petroleum ether (B.R. 35-37°C). Moreover the sterols were removed here by simply shaking the non-saponifiable extract with tri-calcium phosphate. The method has been widely used in the field with much success (Krause, 1961; Olson, 1961a; Lotspeich *et al.*, 1963; Huang and Goodman, 1965; Crain *et al.*, 1965).

Animal tissue Alkali digestion, followed by solvent extraction has been used to separate lipid material from animal tissues. These procedures have been followed by workers who were interested only in the non-saponifiable fraction of lipids such as carotene and/or vitamin A (Davis, 1933; Hjarde, 1950; Parrish and Smith, 1956; Olsen *et al.*, 1959; Sibbald and Hutcheson, 1960; Krause, 1961). These methods are still employed with mild modifications to extract total retinol from liver, kidney, and serum. The limitations of this method, which are disadvantageous in the study of carotene metabolism, are destruction of retinal and hydrolysis of retinyl esters. Both these compounds play an important role in carotene metabolism. The first is the suspected intermediate in the formation of vitamin A from carotene and the second is the predominant derivative of

vitamin A in all the tissues studied (Thompson et al., 1949; Olson, 1961a). The ester is used often as a criterion of the conversion process. Thus in the extraction of animal tissues for carotene and its metabolic products, attempts are made to conserve these compounds in the extract of intestines and contents, which are the organs under consideration for this type of study. Thompson and co-workers (1949) developed a method for the extraction of non-radioactive carotene and its content. In their procedure the material was homogenized in a Waring blender with a solvent mixture of ethanol, water, and hexane (35:15:100). On standing the two phases separated. With the second extraction of the aqueous layer the authors claim to have achieved a quantitative transfer of all the lipid material into the hexane phase without further manipulation. This method has been used repeatedly by these workers as well as by others (Ganguly et al., 1959; Deshmukh et al., 1964). However spontaneous separation of the phases is slow. Consequently Folch et al. (1951) have washed the lipid extracts thoroughly with water to speed up separation. Later work of this group (1959) showed that washing the lipid extracts with 0.2 volume of some salt solution, e.g. $CaCl_2$, followed by centrifugation may be more efficient in the removal of non-lipid contaminants as well as in the recovery of lipid materials in the solvent phase, than washing with water. As much as 99.4 - 99.7% recoveries of lipids have been obtained by this method. Addition of 0.2 volume of 2% calcium chloride solution to the extractant prior to centrifugation has been employed to wash lipid extracts of intestines and other tissues (Olson, 1961a; Huang and Goodman, 1965).

Commonly employed organic solvents or a mixture of these have been used for extraction purposes. Olson (1961a) used a mixture of hexane and ethanol (3:1) for extraction, while Huang and Goodman (1964) and Folch _et al_. (1959) employed a chloroform-methanol (2:1) system for the purpose. Optimum recoveries have also been reported by workers who used a simple method of extraction of lipids from tissue with chloroform, followed by repeated washing with water (Bligh and Dyer, 1959; Crain _et al_., 1965).

All the extraction methods reviewed appear to have given results which were acceptable to the user in the respective laboratories. Therefore, any one of the methods mentioned could probably be standardized and used.

Chromatography

Plant extracts When unlabelled carotene is isolated it is usually identified and assayed by means of spectrophotometric methods. Hence care is taken to remove any contaminants which may alter the absorption of light. However, in the purification of ^{14}C-carotene, which is estimated both by optical and radio assay methods, special attention is also given to remove any extraneous materials which may have ^{14}C-activity although they might not interfere with the optical properties of the compound under investigation. Chromotography has been used most frequently for the separation and purification of carotene from other plant pigments.

An extensive review covering all aspects of chromatographic procedures used in the separation of carotene from other pigments was published by Booth in 1957. The present review will therefore be limited to material

pertinent to the chromatographic separation of ^{14}C-carotene and its isomers.

Alumina has been the adsorbant of choice in the separation of carotene from other pigments because it is one of the most discriminating adsorbants known, retaining most of the pigments and passing only carotene. Most of the other adsorbants that are in vogue for this purpose fail to retain colored artifacts and non-carotene chromogens which are usually associated with beta-carotene in several biological materials. A certain balance between adsorbant and eluant is necessary for optimum recovery of the chromogen.

Willmer and Laughland (1957), using active alumina as adsorbant and petroleum ether as eluant, have secured pure radio beta-carotene. For this same adsorbant Olson (1961a) used increasing amounts of acetone in hexane as eluant with the fractional collection method; fractions 11 - 14 had maximum beta-carotene concentrations. Later the same author (1964a), using deactivated alumina (5 - 6% water) as adsorbant and 100% hexane as eluant, found similar satisfactory separation of carotene without using the fractional collection method. Not only is this method used for the separation of carotene from other pigments, but also for isolation of carotene from its metabolic products in the tissues. These results have been confirmed by Huang and Goodman (1965) with 6% alumina and hexane.

In order to avoid the need for treatment of the adsorbant, Lilly _et al._ (1958) and later Cain _et al._, (1965) have used bone meal which is softer than the untreated alumina. This adsorbant had been used earlier by Glover _et al._ (1948) for the separation of unlabelled carotene.

Where a sample of very high purity was required, as for instance in

the studies of metabolic pathways in the biosynthesis of beta-carotene, pre-treated silicic acid was used to separate and purify beta-carotene labelled with ^{14}C from P. blakesleeanus (Lotspeich et al., 1963). This method was followed later by Grain and his coworkers (1965). One hundred per cent hexane or light petroleum ether has been the eluant in these methods.

In all these papers, little mention is made of separation of beta-carotene from the alpha variety. This may be because in most of the studies under consideration the mold P. blakesteeanus was used. Beta-carotene constituted about 95% of the total pigments found in this mold.

Methods have been published for the separation of carotene isomers. The work of one laboratory will be cited here. Bickoff and his colleagues (1948, 1949) used a column of well-packed calcium hydroxide for the separation of all-trans alpha, all-trans beta, neo beta-u, and neo beta-B isomers of carotene from each other. Organic ethers such as p-cresyl methyl ether, p-chloro ethyl phenyl ether, p-, m-, or o-cresyl ethyl ether have been used successfully as eluants for fractional collection of different isomers.

The criteria of purity in the studies discussed have been constant specific activity to recrystallization or rechromatography, characteristic absorption spectrum of beta-carotene comparable to that obtained for pure synthetic compounds, or a combination of both.

Animal tissues

Phase separation, molecular distillation and other methods have been

employed to separate esters from the alcoholic form of vitamin A in animal tissue extracts. However, workers employing these methods were usually not concerned with the separation of carotenes from retinol and its derivatives. Recently thin-layer chromatography on a layer of alumina (Harashima, 1964; Olson and Hayaishi, 1965) and paper chromatography (Suzuki et al., 1959), have also been used to separate and detect small amounts of carotene and retinol derivatives.

The more commonly used methods for the separation of animal tissue components with vitamin A activity employ column chromatography. The review will be restricted to papers pertaining to this approach.

In column chromatography of animal tissue the nature of adsorbants and eluants as well as the amount of lipid present in the extracts have been shown to affect the separation of components.

Adsorbants As in the separation of plant pigments, alumina has been used as adsorbant in the separation of various retinol derivatives, the parent substance carotene and its breakdown products. A certain degree of retentive activity of alumina is necessary to obtain sufficient separation of the substances under investigation. Yet a relatively high activity results in increased loss of certain compounds like vitamin A, possibly due to excessive retention on the column. Thus alumina is softened or deactivated to a desirable degree by uniform addition of adequate amounts of water. Such deactivation, however, results in a reduced separation of the components, especially in the unsaponifiable matter (Lambertsen and Braekkan, 1958). Therefore the degree of activity has to be chosen carefully so that a compromise between separation and yield

will result. This means that small losses are inevitable which should be taken into consideration. For instance, efficiency of separation reported by Huang and Goodman (1965) was such that less than 3% of any compound was eluted in a fraction other than the one under inspection. After chromatography recoveries of beta-carotene and retinal exceeded 90% while those of retinyl esters and retinol amounted to only 85 - 90%.

Thompson and his co-workers (1949) developed a chromatographic technique to separate carotene, vitamin A and its ester form from each other. These workers used highly activated alumina as adsorbant. Immediately before adding the lipid extract, the column was weakened by the addition of 2-3 ml of 8% ethanol in hexane.

In the study regarding intestinal metabolism of ^{14}C-carotene, Olson (1961a) used previously deactivated (5 - 6%) alumina. Huang and Goodman (1965) have also used deactivated (Grade III - Woelm) alumina in separating different metabolites from ^{14}C-carotene. Alumina (Grade III - Woelm) is a product obtained by the addition of 6% water to grade I alumina. Softer adsorbants such as bone meal have been effective, especially in the removal of carotene from other lipid materials (Glover _et al._, 1948; Krause _et al._ 1954).

Eluants The second factor to be considered in chromatographic procedures is the nature of the eluant. Huang and Goodman (1965) used 100% hexane to elute carotene. However, small amounts of acetone in either petroleum ether or hexane have been effective in the resolution of carotene from its metabolites (Thompson _et al._, 1949; Parrish and Smith,

1956; Olsen et al., 1959; Sibbald and Hutcheson, 1960; Olson, 1961a). To separate carotene, retinyl esters and retinol, mixtures of hexane with 2% acetone, 20% acetone and 8% ethanol have been used in that order (Thompson et al., 1949). Almost complete recoveries have been stated for pure synthetic non-radio compounds alone, as well as for these compounds admixed with tissue extracts. In instances where retinyl esters were not present, as after saponification of the tissue under investigation, acetone-hexane mixtures have been used as eluants (Parrish and Smith, 1956; Olsen et al., 1959; and Sibbald and Hutcheson, 1960).

Thompson et al. (1949) mentioned difficulties encountered by biologically inactive artifacts resulting from degradation of carotene and vitamin A. These materials contaminated the retinol fraction which was assayed by physico-chemical methods. Olson (1961a) has been able to separate these breakdown products from retinol by using a different series of eluants. In addition he was also able to separate retinal from retinol. Thus for a column of 10 gm of deactivated alumina, the author used 80 ml of hexane for the elution of carotene, 80 ml hexane for retinyl ester, 50 - 60 ml of 1 - 2% acetone for retinal, 60 - 80 ml of 3 - 5% acetone for retinol and 20 ml of 100% acetone for the terminal polar fraction (TPF), used in this order. The TPF contains small amounts of artifacts of beta-carotene breakdown. Because of poor separation, the first two fractions had to be rechromatographed occasionally. The same method has been used repeatedly (Harashima, 1964; Olson and Hayaishi, 1965). Huang and Goodman (1964) have also been successful in separating retinol from TPF. In addition, by changing solvents they were able to avoid overlapping of the

carotene and retinyl ester fraction. Hexane, 15% benzene, 50% benzene, 100% benzene and 100% methanol were used as eluants for carotene, retinyl esters, retinal, retinol and TPF respectively.

Lipid The amount of total lipid present in the tissue extract under investigation is another factor determining successful separation and recoveries of components. Thompson et al. (1949) quoted results which indicated that the quantities of fat usually present in extracts of blood, small intestine and liver do not interfere with the chromatographic separation. Olson (1961a) obtained complete separation of all components concerned when the amount of lipid present was small. No mention, however, was made of the amounts involved. Huang and Goodman (1965) specifically mention a maximum load of 10 mg lipid per 1 gm of alumina used for efficient separation of the compounds.

Preparation of animals

Vitamin A status Decisions with respect to the vitamin A status of animals used for studies on carotene metabolism have often been made on the basis of the objective of the investigation. Thus short term studies involving the intestinal metabolism of non-radio active carotene have been conducted mostly with animals made vitamin A deficient by previous treatment (Sexton et al., 1946; Mattson et al., 1947; Sibbald and Olsen, 1958; Deshmukh and Ganguly, 1964). This approach was used so that the small quantity of vitamin A formed de novo in the intestines and its distribution in the other tissues could be determined accurately by chemical and physico-chemical methods. However when the problem concerned

the *in situ* metabolism in the intestine only, attempts were made to clear the intestines of any previous carotene or vitamin A without regard to liver stores or serum concentrations. Thus Glover *et al.* (1948) have shown that a vitamin A-free diet for 4 days was adequate for this purpose. Thompson and his co-workers (1949) maintained stock-colony rats on a vitamin A-deficient diet for 7 days and used these animals to study the several aspects of intestinal metabolism of unlabelled carotene. In the reinvestigation of these factors where radioactively labelled carotene was employed rats were often taken directly from the stock-colony. The presence of previously existing carotene or vitamin A stores did not hinder the radio assay methods employed (Krause *et al.*, 1954; Olson, 1961a; Crain *et al.*, 1965; Goodman *et al.*, 1965; Huang and Goodman, 1965). Exception to this is seen in the work of Willmer and Laughland (1957) who used vitamin A-deficient rats to study the tissue distribution of radioactive carbon following the administration of ^{14}C-beta-carotene.

Presence of food in the intestine In order to avoid complications due to the presence of other dietary constituents during the intestinal utilization of carotene a preliminary fasting period of 24 hours was introduced by several workers (Thompson *et al.*, 1949; Sibbald and Hutcheson 1960; Deshmukh and Ganguly, 1964; Ganguly *et al.*, 1959; Crain *et al.*, 1965). In addition Thompson and co-workers (1949) maintained their animals on a skim milk diet for 24 hours preceding the fast. This procedure prolonged the period during which solid food was absent from the intestine. At the same time the fast was not extended beyond the 24 hours preceding

the experiment. Shorter periods, 15 hours, (Glover et al., 1948) and 2-6 hours (Olson, 1961a) have also been employed for the same purpose. However, only a few workers have studied the metabolism of ^{14}C-carotene in rats during their digestive phase (Krause et al., 1954; Willmer and Laughland, 1957).

Whether food should be present or absent during the absorption of carotene depends on the objective of the study. Thus, when the investigators were interested in the metabolism of carotene during its intestinal phase, the dose was usually administered in a suitable medium without any accessory food (Glover et al., 1948; Ganguly et al., 1959; Sibbald and Hutcheson, 1960; Olson, 1961a; Crain et al., 1965; Huang and Goodman, 1965).

To simulate the normal physiological process of digestion under controlled conditions, Thompson and his coworkers (1949) fed orally 1 gm of vitamin A-free diet together with an oily solution of carotene to determine the latter's conversion to vitamin A in the natural course of events. Deshmukh and Ganguly (1964) followed the same procedure.

Effect of anaesthetics The use of anaesthetic during the administration of carotene has been the preferred procedure when the provitamin was injected intraduodenally. In an earlier study, an almost complete inhibition of the process of conversion of carotene to vitamin A was observed both in rats and pigs by Thompson and collaborators (1950) when soluble pentobarbitone (Nembutal) or cyclopropane were employed. The investigation suggested interference in the mechanism of conversion. Their preliminary work supported this thesis. A slight depression in carotene

conversion to vitamin A was seen in amphetamine-treated animals while pro-stigmin administration enhanced the conversion slightly. Later, Sibbald and Olsen (1958) showed that *in vivo* conversion of carotene to vitamin A in the ligated duodenal loops of chicks anaesthetized with sodium pentobarbital was possible. However, distention of duodenum with fluid and lack of transport of the newly formed vitamin A to other tissues were reported in this study. It was suggested that the latter effect might be due to an interference of the anaesthetic with the nervous system, which normally regulates absorptive processes.

In contrast, formation of vitamin A from beta-carotene in the rat proceeded well during ether anaesthesia (Olson, 1961a). Thus ether has been widely used as an anaesthetic during carotene administration by intraduodenal injection (Huang and Goodman, 1965; Crain et al., 1965).

The use of anaesthetics has been prevalent in preparation of animals for the removal of intestines for *in vitro* studies. Thompson *et al.* (1949) failed to detect the formation of vitamin A from carotene in isolated live intestines of rats, a failure which might have been attributable in part to the effect of anaestheia. Krause (1961) to avoid the use of an anaesthetic decapitated the animals to remove the intestines for the purpose of *in vitro* experiments. However, Olson (1964a) has demonstrated the conversion process in intestinal sections of rats which had been sacrificed under ether anaesthesia. The same anaesthetic has been used preliminary to work related to the conversion process in rat intestinal homogenates and their cellular fractions (Goodman *et al.*, 1965; Olson and Hayaishi, 1965).

Preparation and administration of carotene dose

Amount of carotene, the medium used for the preparation of the dose, and the mode of administration apparently affect the conversion of carotene to vitamin A in the intestines.

The common method of carotene administration in studies regarding its metabolism has been by means of oral feeding (Sexton et al., 1946; Mattson et al., 1947; Glover et al., 1947b; Deshmukh and Ganguly, 1964). When non-radioactive carotene was given orally, a large dose was used to study the formation of vitamin A in the intestines as well as in livers. The limitation of this method of feeding lies in the fact that waste occurs both in the container and in the mouth of the animal.

In order to avoid the losses occurring in the container and also in the mouth, stomach tube feeding has been used for the unlabelled form (Ganguly et al., 1959) as well as for the labelled form (Krause et al., 1954; Willmer and Laughland, 1957; Huang and Goodman, 1965).

Surgical methods have been developed to place small amounts of the chromogen in situ in the intestines for study of the metabolism of carotene in vivo. The advantage of this method is that losses of carotene occurring in the upper part of the alimentary canal are avoided. Furthermore it facilitates the studies of in situ metabolism of small quantities of provitamin over relatively short periods.

That vitamin A can be formed from small quantities of carotene in the ligated loop of the duodenum of chicks in vivo was shown by Sibbald and Olsen (1958). It was also reported that the intact blood supply to the intestine, but not the continuity of the alimentary canal, is essential for the mechanism of conversion. However, the same authors were unable

to observe transport of newly formed retinol from the intestinal wall to other tissues during a 4-hr period. The authors suggested that relocation did not take place due to the ligation of the intestine. Ligation might have prevented or inhibited peristalsis, which normally causes movement of lymph by exerting a rhythmic pressure upon the lymph vessels. In this experiment about 50% conversion took place in the intestine from 40 mcg of the non-radio active carotene. This value is surprisingly high compared to the other works.

Olson (1961a) made a thorough investigation of the intestinal metabolism of ^{14}C-carotene using the method of intraduodenal injection of micro quantities (5 - 20 mcg) of labelled carotene into the ligated intestinal loop in rats. He used a loop extending from the pylorus to mid-jejunum. The peritoneal cavity was opened by ventral midline incision. This was followed by an injection of ^{14}C-beta-carotene into the ligated loop of the upper half of the intestine. The incision was closed with wound clips. With this procedure the author was able to demonstrate the presence of newly formed radioactive retinol derivatives in the intestinal walls as well as the deposition of retinol in the liver in a period of 1 hour. The values found for hepatic retinol following this mode of administration were comparable to those obtained after stomach-tube feeding. Later Crain and his collaborators (1965) also used the same method to study the metabolism of ^{3}H ^{14}C-carotene and ^{14}C-retinol which were administered in a large quantity of 1.25 mg of carotene and 0.217 mg of retinol. These investigators were able to demonstrate appreciable activity in liver as well as in the intestines over a period of 6 hours.

Discrepancy between the results of Sibbald and Olsen (1958) and those of Olson (1961a) as well as Grain *et al*., (1965) could be attributed to two factors. The sensitivity of the radio assay methods could have been responsible for picking up the retinol activity in the liver of rats in the work with labelled carotene. This difference regarding the relocation of retinol formed could also be due to the species differences with respect to physiological processes.

Beta-carotene for oral- or stomach-tube feeding has usually been prepared in a lipid carrier such as corn or arachis oil. When the dose was injected, an aqueous phase was prepared. Solubilizing agents such as Tween 20, 40 or 80 have been used to disperse the carotene in the aqueous phase, (Willmer and Laughland, 1957; Sibbald and Olsen, 1958; Olson, 1961a). Olson has demonstrated that Tween 20 up to a concentration of 5% does not inhibit the formation of retinyl ester. Use of corn oil as a medium to disperse doubly labelled beta-carotene and singly labelled retinol for the purpose of intraduodenal injection was made by Grain and co-workers (1965).

Where large doses of carotene were fed orally the percentage of conversion had been small. The manner in which dosage influences the conversion of carotene was studied by Olson (1961a). At low levels (50 - 80 mcg) the conversion seemed to increase with the amount of carotene. When the level of carotene was increased to more than 100 mcg, the formation of retinyl esters tended to level off.

Preparation of tissues

Separation of contents from the intestinal walls   It has been necessary to separate the intestinal contents from intestinal wall in order

to identify the locus of conversion of carotene and to examine the partition of carotene and its metabolic products between the wall and content of the intestine. Methods in which the contents were either squeezed out or washed out in sequence with 30% alcohol, absolute alcohol and diethyl ether were abandoned by Thompson et al. (1949), since these methods were found to extract appreciable amounts of fat and fat-soluble materials from the intestinal cells into the contents. Another procedure in which saline in varying quantities was forced through the lumen to flush out the intestinal contents was used subsequently by these co-workers. Others have followed a similar procedure (Willmer and Laughland, 1957; Ganguly et al., 1959; Olson, 1961a; Deshmukh and Ganguly, 1964).

Thompson et al. (1949) also showed that removal of the intestinal contents after the death of the animal had resulted in leakage of vitamin A from the wall to the content of the intestine. Thus, results were misleading. Flushing the contents with the blood supply to the intestines intact was satisfactory in retaining the compounds in the original sites to a large extent. Olson (1961a), however, found interpretable results by washing the contents out after removal of the intestinal loop from the body of the rat. However the objectives of the two studies were different. While Thompson and his co-workers were interested in locating the site of carotene conversion, Olson (1961a) used the metabolic products in the intestinal walls to interpret the effect of various factors on the intestinal metabolism of carotene. Therefore the purpose of the experiment may determine the mode of separation of contents from the intestines.

Part B - Metabolism of Carotene

Intestinal metabolism

Conversion of carotene

Site Moore in 1930 proposed that the main site of biosynthesis of vitamin A from beta-carotene was the liver. The basis for this hypothesis was the appearance and storage of retinol in livers of rats fed carotene. However, supporting evidence for this theory was lacking. Furthermore, carotene fed orally was more effective in ameliorating the avitaminotic condition than was carotene given by parenteral administration by intrasplenic, intra-cardial, intra-peritoneal, and intravenous routes (Sexton et al., 1946; Lease et al., 1941).

Thus it was postulated that an extra-hepatic tissue could be the site of conversion. Sexton et al. (1946) further pointed to the possibility that intestinal tissue might be the locus of transformation of the provitamin. Direct proof, such as formation of retinol derivatives in the intestines after an oral dose of carotene, supported the thesis. Indirect evidence, such as the presence of retinol but not carotene in lymph after a similar treatment, was also presented. Intestinal conversion has been experimentally verified in goats, rats and also in pigs (Glover et al., 1947a, 1948; Goodwin and Gregory, 1948; Wiese et al., 1947; Thompson et al. 1949; 1950; Mattson et al., 1948). Even though efforts were made to find the exact location of this mechanism by fluorescence microscopy (Popper and Greenberg, 1941) the results were not encouraging.

Recently, investigations of Olson (1961a) regarding intestinal metabolism of ^{14}C-carotene in vivo in rats have confirmed the theory that

intestinal walls are the locus of conversion of carotene. The author also showed that the biosynthesis of retinol takes place in the mucosal cells of the intestinal walls and that the mechanism is most efficient in the upper one-third of the intestine.

Attempts have also been made to demonstrate and confirm the site of conversion of carotene by in vitro methods using cell homogenates. Earlier, workers had failed to demonstrate the reaction of biosynthesis of retinol in vitro (Glover et al., 1948; Bieri and Pollard, 1954; De and Sundarrajan, 1951; Worker, 1959; Olson, 1959; Kon and Thompson, 1951). These studies were carried out with non-radioactive substrates and were difficult to interpret since the amounts of metabolites formed were small. Using more sensitive methods, researchers have lately been able to show the formation of vitamin A from unlabelled beta-carotene by cell-free homogenates of rat intestinal mucosa (Suzuki et al., 1959) and cell hemogenates of cow duodenum (Reddy and Thomas, 1962). Radioactive beta-carotene has also been employed to show the synthesis of retinol by in vitro techniques (Olson, 1964a; Olson and Hayaishi, 1965; Goodman and Huang, 1965).

Although the intestinal mucosa seems to be the major site of conversion, reports have indicated that it may also take place in other tissues. Thus in his review article on the biosynthesis of carotenoids and retinol, Olson (1964b) makes the following observation:

> However upon the removal of intestine and a number of other organs of the rat, the increase in retinol concentration in plasma and liver after intravenous injection of beta carotene was not affected appreciably. The lung and liver have been particularly mentioned as possible sites of conversion. However, in a dog heart-lung preparation, beta-carotene was not converted at an appreciable

> rate to retinol. Recently, the formation of retinol ester from beta-carotene has been demonstrated in the isolated perfused rat liver. In the rat the conversion rate for intestine was approximately twice as great per gram wet weight of tissue. Both organs converted carotene into retinol at a much faster rate than that required to satisfy the nutritional requirements....Thus whether organs other than the intestine and liver are capable of forming retinol from carotene is yet uncertain.

However, the rat intestine is apparently so efficient in converting carotene that in a healthy living rat other tissues are almost entirely devoid of carotene even after oral administration of a large dose of the provitamin A.

Mechanism The first product of cleavage of beta-carotene in the biosynthesis of retinol is supposed to be the aldehyde derivative, retinal. It is thought that retinal is next reduced by an alcohol dehydrogenase to retinol in the living system. The mechanism of conversion of carotene to retinol is explained on the basis of two theories. The first one is the central fission theory which states that one molecule of beta-carotene is split into two of retinal by a symmetrical cleavage at the central double bond. Structural configuration of the two molecules and also the experimental evidences are the basis for this theory (Koehn, 1948; Burns *et al.*, 1951; Olson and Hayaishi, 1965; Goodman and Huang, 1965). The attrition theory proposes that beta-carotene following oxidation at one end undergoes beta oxidation until retinal is formed. It is further stated that the methyl group on the beta-carbon atom of retinal prevents further oxidation of the molecule. This view has been supported by many experiments, particularly those designed to evaluate the biological activity of vitamin A and carotene. The studies are too numerous to give

due credit to all. In most of these experiments beta-carotene is only half as active as vitamin A on a weight basis. These investigations have also given the basis for the international unit of vitamin A activity. Still the phenomenon of carotene cleavage is controversial although a greater body of material is brought forth in favor of the central cleavage theory. Thus in the same much-cited review article, Olson (1964b) criticizing work published in this area up to 1964 summarizes as follows:

> The major pathway for the transformation of beta-carotene into retinol has been fairly well established as a central cleavage type of reaction, but the details of the reaction sequence are still unclear. As in the case of carotene biosynthesis, suitable cell-free systems have not been readily prepared and appreciable amounts of intermediates between beta-carotene and retinol do not accumulate. It seems likely that the conversion of partially degraded beta-carotene molecules such as the beta apo-carotenals into retinol proceeds in a manner analogous to beta-carotene cleavage rather than by stepwise oxidation of small two- or three-carbon entities.

In order to resolve the discrepancy between the central fission theory which implies equal biopotency between vitamin A and carotene on a weight basis, and the findings in nutritional experiments where the biopotency of carotene is only half that of vitamin A, one might postulate a reduced efficiency of uptake of carotene into the mucosal cells.

On the other hand, one might accept the postulation of Crain _et al_., (1965) who interpreted their data to indicate that some beta-carotene was metabolized by a process other than central fission.

Factors affecting conversion Certain factors are obligatory for the retinol formation from its precursor. In the investigations of Olson (1964a) a requirement of conjugated bile acid was demonstrated for the biosynthesis of retinol from beta-carotene, but not for the further

metabolism of the former. He propounded the theory that in addition to acting as emulsifiers for carotene, absorption of conjugated bile acids probably stimulates the formation of retinol by enhancing the absorption of beta-carotene by means of an interaction with the cell membrane of the intestinal mucosa. It appears that lack of bile acids in the lower intestine is the limiting factor for the conversion process in that portion of the gastro-intestinal tract, because the same author has demonstrated the formation of vitamin A from carotene at this point when exogenous sodium glycocholate was incorporated into the carotene dose. Another factor which has been found important for the conversion mechanism is the structural integrity of the mucosa, since the conversion does not take place in cyanide poisoned mucosa even though the non-specific binding of carotene to the mucosa occurs.

Recent work with cell-free homogenates employing radio beta-carotene has demonstrated that the reaction of conversion of carotene to retinol is enzymatic in nature. Thus Goodman and Huang (1965) have experimental evidences of the existence of an enzyme system present in the soluble fraction of the intestinal mucosal homogenates. The enzyme, which probably is a dioxygenase, requires molecular oxygen for the reaction. Bile acids and a lipid fraction from the microsomal fraction are obligatory in the formation of retinal, which is the primary product. Stoichiometry of the reaction favors the central cleavage theory.

Simultaneously and independently, Olson and Hayaishi (1965) also showed that similar enzyme systems are present in the high-speed supernatant fraction of intestinal, liver and kidney homogenates of rats.

Slight differences in requirements of the reaction medium in the two studies mentioned are seen perhaps due to differences in experimental conditions. Central fission is stressed here too, supporting this theory. Further work of Goodman et al., (1965) showed that the hydrogen atoms attached to the central carbon atoms are retained during the biosynthesis of retinol. Here beta-carbon labelled with tritium as well as with carbon 14 was used. Under the circumstances when the biopotency of beta-carotene is still a matter of controversy, this new enzyme is greeted with some interest. If central fission is the main mechanism of conversion of carotene, then in order to resolve the in vivo nutritional studies which have noted beta-carotene to be half as efficient as vitamin A, it could be postulated that the efficiency of uptake of carotene is such that half of the provitamin is lost.

Much evidence has accumulated to establish firmly that the retinal formed from carotene is converted to retinol in the mucosal cells of intestines. The reaction is mediated through a non-specific enzyme system identified with alcohol dehydrogenases requiring reduced nicotinanide adenine dinucleotide as a cofactor. This enzyme, known by the name of retinene reductase, is also found in the outer segments of the retina, in liver and in skin (Glover et al., 1948; Zachman and Olson, 1961).

It is assumed that the metabolic pathway of the retinol molecule is the same whatever the genesis of the moiety may be. This would mean that carotene converted to retinol would be metabolized in an identical way as the preformed retinol. Also it is a well accepted fact that the vitamin is esterified with long chain fatty acids in the mucosal cells before being

released into the lymphatic system (Eden and Sellers, 1948, 1949, 1950; Glover and Morton, 1948; Thompson et al., 1949; Kaiser and Kagan, 1956; Plack, 1959; Ganguly et al., 1959). It is further postulated that retinol is the molecular species that crosses the mucosal membrane from the lumen and that this step is energy dependent. The esters, if fed, are first hydrolyzed to alcohol in the intestinal lumen by the hydrolytic enzymes of pancreatic origin as well as those residing on the surface of the mucosal cell membrane. The absorbed alcohol is preferentially esterified with palmitic acid by a specific esterase situated in the microsomal fraction (Mahadevan et al., 1963a; Kaiser and Kagan, 1956). Mahadevan et al., 1963b, also showed that Tween 20 inhibits the hydrolysis of retinyl palmitate. In the mucosa as well as in the muscles of the intestine, the ester is the predominating derivative of retinol.

Extra-intestinal metabolism

General   Tissue distribution of ^{14}C-carotene fed to rats by intubation has been studied by Willmer and Laughland (1957). Interestingly enough, the adrenal gland received the highest percent of the carbon-14 dose, both in the saponifiable as well as in the non-saponifiable portion of the lipid extracts. Concentration of the activity was also higher compared to the other organs studied. The pituitary gland had more activity in the saponifiable than in the non-saponifiable extract. Liver concentrations increased for 10 hrs after dosing while blood concentration was maximum at 2 hours, indicating a balance between the intestinal absorption and hepatic storage. Although low amounts of $^{14}CO_2$ appeared in the be-

ginning, appreciable quantities amounting to 12% of the dose were formed over the 24 hour period. Krause and Sanders (1957) also tried to follow the fate of injected ^{14}C-carotene. Some of the activity of the injected ^{14}C-carotene was found in the fatty acid fractions. These workers failed to account for nearly 40% of the absorbed activity of labelled carotene. The only water-soluble fraction found to contain radioactivity was that obtained from the liver. Olson (1961a) in his balance study in rats could not account for about 20% of the injected radioactivity at 0 or 1 hour intervals. He suggested that some material might have been transported out of the intestines or might have been metabolized to compounds not extractable by Skelly-ethanol or chloroform-methanol solvents.

Goodman et al. (1965) studied the effect of intravenous injection of chylo-microns containing newly-absorbed labelled vitamin A. Even though liver was the recipient of the largest fraction, activity was also found in tissues such as adrenal, kidney, depot fat, small intestine and plasma.

Transport in lymph Drummond and his associates (1935) made the interesting observation that vitamin A esters are absorbed through the lymphatic system. Popper and Volk (1944) later confirmed this finding with their technique of fluorescent microscopy. Thompson et al. (1950) showed by cannulating the lymph of rats and pigs that retinol is exclusively transported as its ester in the lymph. Also, that no increase in vitamin A is seen in portal blood after the oral administration of retinol is cogent proof for the fact that lymph serves as the major pathway for the removal of vitamin A from the intestinal wall. This has been

shown in bullocks, sheep and rats (Eden and Sellers, 1948, 1949) and in goats (Goodwin and Gregory, 1948).

Goodman et al. (1965) fed labelled beta-carotene or retinol to rats with cannulated thoracic ducts. Washed chylomicrons from these animals were found to contain 82% of the radioactivity absorbed. Irrespective of the compound fed, the highest activity was recovered from the retinyl esters in the lymph. Moreover the authors showed that palmitate is the predominating derivative of the vitamin in lymph. Although palmitate appears to be the fatty acid used for esterification, stearate also is seen to an appreciable extent in lymph, the ratio of the two acids being 2:1.

Recently two reports have appeared which question the validity of the hypothesis that lymph is the only route by which retinol is transported. Murray (1961) observed an increase in retinol concentration in portal blood compared to systemic blood in rats whose lymphatic system had been blocked at the thoracic duct. This observation could not be confirmed in control rats, thus indicating an alternate pathway for the transport of retinol. Supporting evidence for the second non-lymphatic transport mechanism of vitamin A comes from Lawrence et al., (1966). Rats with cannulation of the lymph duct, fed ^{14}C-carotene of ^{14}C-retinol, had labelled retinyl esters in their livers.

Transport in blood After an oral dose or an intravenous injection of retinol, the ester form predominated in the blood of rats without a change in concentration of the alcohol, although in fasting blood the vita-

min existed almost exclusively in the form of alcohol with 10-17% of the total in the esterified form (Hoch and Hoch, 1946; Krinsky et al., 1958). These findings have been confirmed by others in rats and pigs (Thompson et al., 1949, 1950; Ganguly and Krinsky, 1953). Similar observations have been made in humans (Krinsky et al., 1958; Popper et al., 1948; Week and Sevigne, 1950). Retinol concentration in blood seems to be independent of the amount of total retinol derivatives in the liver, since serum has detectable amounts of vitamin A when liver stores are exhausted (Glover et al., 1947a; Ganguly and Krinsky, 1953; High and Wilson, 1956).

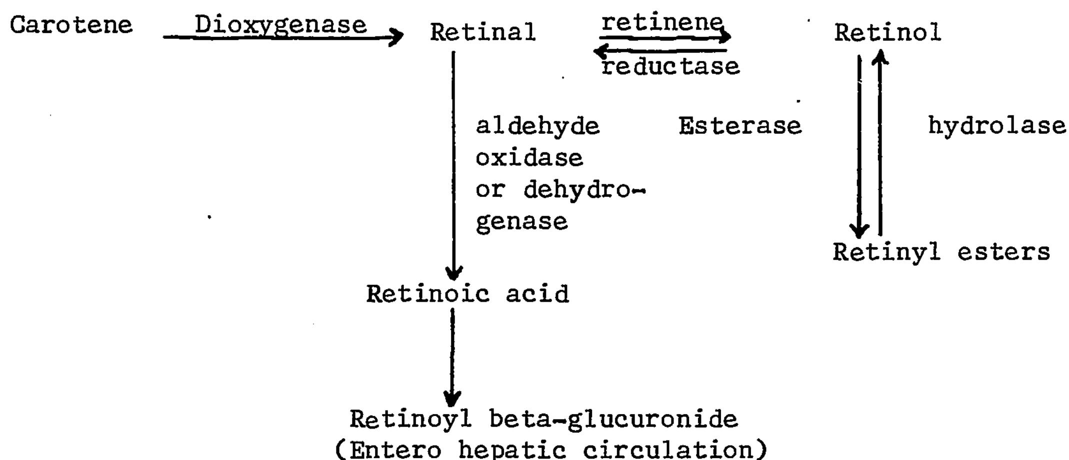
Retinol and its esters may be associated with separate proteins in the blood. It had been demonstrated that the esters were in the form of a complex with low density lipo-proteins, probably lipo-proteins S_f 0-10. (Dzialoszynski et al., 1945; Ganguly et al., (1952); Garbers, 1958; Garbers et al., 1958). However Krinsky et al., (1958) using more sophisticated methods of centrifugation showed that alcohol was not transported by lipo-protein, but by a protein of a density greater than 1.21 g/ml. Although this fraction was associated with serum albumin it was apparently not albumin itself. Later reports of Garbers et al., (1960) are in disagreement with this finding. Using labelled retinol, these investigators showed incorporation of about 64% of the radioactivity into the $alpha_1$-globulin fraction, and of the remainder into the $alpha_2$-globulin fraction, thus indicating the association of retinol with these fractions of plasma proteins.

For years it had been assumed that retinol was the active metabolite

of the vitamin. However recent work has shown that retinoic acid can replace retinol in general maintenance and growth of rats but not in visual functions, (Dowling and Wald, 1960). This indicates that the process retinal⟶retinoic acid is irreversible. Also the fact that animals maintained on retinoic acid are unable to store retinol or its esters in the liver lends further support to the finding that retinoic acid can not be reduced in the body.

For growth, retinoic acid was found to be half as active as the acetate form of vitamin A when fed orally, but the compound attained an activity of 140% when administered intravenously (Malathi *et al.*, 1963). This indicated that retinoic acid, if not by itself the active metabolite, might be easily converted to the active form. The observation of Thomas and Pitt (1960) that the acid was more effective in producing hyper-vitaminosis-A than was retinol acetate lends support to the theory. The acid was not detected in animal tissues until recently. But Jurkowitz (1962) demonstrated its presence in human plasma following the administration of a large oral dose. Recently Dunagin *et al.* (1964) succeeded in isolating retinyl beta glucoronide, the suspected metabolite of retinoic acid from the bile of rats. Furthermore, this metabolite seems to enter the enterohepatic circulation after reaching the intestines (Zachman and Olson., 1964).

Thus the apparent pathway of beta-carotene or retinol metabolism may tentatively be indicated by the following steps:



Storage in liver   As far as is known, there is no excretory pathway for vitamin A. It appears that the amounts that are not metabolized are stored in the system. About 90% of the total vitamin A of the body is stored in the liver. However, the figure undoubtedly will vary with the physiological and nutritional status of the animal. While liver has an almost unlimited capacity to store retinol, there appears to be an upper limit for the concentration of vitamin A in the blood and kidney. Therefore it appears that the major portion of retinol is stored in the liver. Gray et al. (1940) showed that the ester form predominates in this tissue, a finding which has been supported by later work of Ganguly and Krinsky (1953). Rats deficient in vitamin A if given a large dose of retinol showed increases in the ester fraction of the liver while the alcohol fraction remained unchanged. In addition to alcohol and ester, detectable amounts of the aldehyde form have also been found on rat livers (Mahadevan et al., 1963b).

Even though alcohol and ester factions of retinol coexist in the liver, their association with lipo-proteins, as well as their cellular distribution,

is widely different. For example, the supernatant of liver homogenates contained 86.2% of the total ester fraction and 52.3% of the total alcohol fraction, while 20.4% of the total alcohol fraction and only traces of the ester fraction were associated with the microsomal fraction (Ganguly and Krinsky, 1953). The ester in solution with fats was seen to be stored in the Kupffer cells, while the alcohol, complexed with a lipoprotein, was stored in the parenchymal cells (Glover and Morton, 1948; Krishnamurthy and Ganguly, 1956). However later findings of Krishnamurthy _et al_., (1958) suggested that the esters might also be associated with lipo-proteins. The basis for this suggestion was the observation that varying amounts of retinol were recovered when liver proteins were denatured by two different agents. Attempts by the same authors to demonstrate this effect _in vitro_ have failed.

Effect of protein on hepatic storage of retinol vitamin A supplement

It has been suggested by some authors, and denied by others, that vitamin A utilization as judged by hepatic storage, is influenced by protein intake. The difference in approaches to the study of the relationship may account for the divergent results.

The study of Dye and her co-workers (1945) dealt with suboptimal levels of vitamin A and graded levels of protein. They found no indications of differences in protein intake in the absence of adequate amounts of vitamin A. Later most of the studies on protein-vitamin A interrelationships have been carried out with adequate or excessive amounts of vitamin A. One of the approaches to the problem of vitamin A-protein interrelationships

in rats has been to feed adequate supplements of vitamin A over a period of time. During this period the animals were also given either a protein-free diet or graded amounts of protein ranging from suboptimal to adequate to excessive. Thus Rechcigl et al., (1962) fed rats diets containing 0 or 18% casein protein for 3 weeks. During this period the animals were given a constant daily intake of 30 I.U. of vitamin A. Although a loss of weight was seen in the animals maintained on the protein-free diet, liver stores of vitamin A were significantly higher in this group than in the controls. The authors suggested that the increased storage of retinol in the protein free groups was the result of decreased utilization of vitamin A with cessation of growth. Other workers failed to confirm these results in similarly conducted experiments (Murray, 1961; Mathew and Beaton, 1963). Murray (1961) compared groups of rats fed diets containing 0 or 20% casein protein for a 2-week period. A supplement of 100 I.U. of vitamin A was given three times a week during this period. The supplementation period here was shorter than the one used by Rechcigl et al., (1962) and thus might not have been sufficient for the manifestation of differences in hepatic stores between the two groups.

In a second method used to study the influence of protein on vitamin A metabolism, uniform hepatic stores of the vitamin are built up prior to the experiment by administering either a single or several large doses of retinol. The rate of mobilization of vitamin A from the liver under the influence of graded amounts of protein in the diet can then be studied under these conditions. Experiments of this type enable the investigator to draw conclusions as to the effect of protein on vitamin A utilization

in a situation uncomplicated by effects on absorption and transport. This approach has been used in rats by Murray (1961), Jagannathan and Patwardhan (1960), Deshmukh et al., (1964) and in pigs by Friend et al. (1961). Murray (1961) administered a large single dose of vitamin A to rats and then fed either a protein-free diet or one containing 20% casein protein for 2 or 4 weeks. Though he could not detect differences in total residual hepatic storage attributable to the two diets fed, he found, on further analysis of the data, that the rate of depletion of the existing vitamin A stores was reduced from 8 I.U./day to 5 I.U./day when protein was removed from the diet. However, this difference in depletion rate was evident during the last 2 weeks of the 4 week study only.

Rechcigl and co-workers (1962) followed a similar method for the assessment of the protein effect on liver stores of vitamin A. These authors administered a large dose of vitamin A acetate to rats two days prior to the experiment. The animals were then maintained on a vitamin A-free diet with graded levels of casein. The dietary regime was followed for 3 weeks. Highest vitamin A content was observed in the livers of rats fed a protein-free diet. With an increase in the dietary protein there was a progressive decrease in the amount of vitamin A left in the liver. The authors also compared proteins of different nutritive value. Residual hepatic stores of retinol increased as the biological value of the proteins decreased. In these experiments a direct relationship was observed between the rate of growth and vitamin A utilization, in that the faster growing animals metabolized larger amounts of vitamin A than the slower growing ones, thus leaving less for hepatic storage. The last finding

was a confirmation of earlier reports stating that the metabolic need for vitamin A was related directly to the rate of growth. (Baumann *et al.*, 1934; Baumann *et al.*, 1942; Vavich and Kemmerer, 1950; Arnrich and Morgan, 1954; Arnrich and Pederson, 1956). Graded levels of dietary protein were used with a constant intake of preformed vitamin A in studies of Baumann *et al.*, (1942); Jagannathan and Patwardhan (1960); Esh *et al.* (1960); and Anderson *et al.*, (1962).

A positive correlation between the extent of hepatic storage of retinol and the amount of dietary protein was demonstrated in these investigations. However, there seemed to be a certain optimum level of protein intake for maximum storage of retinol, since with higher amounts of protein the hepatic storage decreased. Thus the optimum level of protein was 18% of the diet in the investigations of Baumann *et al.*, (1942) and 12% in other works (Jagannathan and Patwardhan, 1960; Esh *et al.*, 1960).

A few studies could be quoted which refute the existence of a relationship between the dietary protein and the vitamin A stores formed in response to vitamin A feeding. Arnrich and Pederson (1956) showed that rats fed 11, 22 and 40 per cent casein protein in the diet with an adequate amount of pre-formed retinol showed no difference in the hepatic storage of retinol. These observations are supported by other reports (Mathew and Beaton, 1963; Ruffin, 1965). In these studies, however, an adequate and constant amount of vitamin A was given to the animals daily. This amount of the vitamin was slightly more than the vitamin A requirement for growing rats of similar weight recommended by the National Research Council of the National Academy of Sciences (1962).

The effect of dietary protein on the utilization of larger doses of vitamin A may be slightly different from that on smaller doses. MacMillan (1966) was not able to find differences in the amount of hepatic vitamin A in animals fed low levels of vitamin A (25 mcg/day). However, when the dose of vitamin A was increased 3 or 9 times, rats maintained on 20% protein stored more retinol in their livers than did those maintained on 10% casein protein. There was no further increase in retinol storage on increasing the level of protein to 40 per cent. This study was carried out over a period of 4 weeks. In a short term experiment of similar nature Deshmukh *et al.*, (1964) also showed a correlation between dietary protein and hepatic stores of vitamin A. They fed 5, 10, and 20% vitamin A free-protein diets to animals for 30 days and then gave a single large dose of 10 mg. of vitamin A acetate orally. They observed that the efficiency of absorption of retinol as seen by hepatic stores of vitamin A increased progressively with increase in dietary protein. Thus values of 2800, 3200 and 3500 mcg of vitamin A per liver were obtained for groups fed 5, 10 and 20 percent protein respectively.

The existence of species differences in the protein-vitamin A relationship is illustrated when studies dealing with chicks are compared to those in rats. In the rat experiments quoted, vitamin A utilization as measured by hepatic storage was independent of protein in some experiments and increased with the level of dietary protein in others. In contrast, a negative correlation was demonstrated between the quantity of protein fed and the magnitude of hepatic stores in chicks maintained on graded level of protein with a standard supplement of vitamin A (Olsen *et al.*, 1959;

Stoewsand and Scott, 1961). This variation in response between species indicates that a certain amount of caution should be used in applying data obtained in one species to another.

Carotene supplement

It was Fraps (1946) who first pointed out a relationship between dietary protein and carotene utilization. Substituting casein for corn meal in the diet, he noted that both the apparent digestibility of carotene and the resulting hepatic stores of retinol had improved. This influence of protein on the utilization of carotene has been shown in several laboratories.

Jagannathan and Patwardhan (1960) employed 3, 6, 12, 18 and 36 per cent protein in the diet and fed a constant dose of 30 mcg of carotene daily with one of these diets over a period of 4 weeks. They observed a trend towards increasing the hepatic storage of vitamin A with an increase in the dietary protein intake. However this effect was seen at low levels of protein intake only with the optimum at 12% protein for maximum storage of retinol. On the other hand Arnrich and Pederson (1956) showed such a relationship even at a higher level of dietary protein. They maintained rats on 11, 22, or 40% diets, fed either ad lib or in a restricted amount, with a supplement of 100 mcg carotene per day. They showed that the liver stores of retinol varied directly with the dietary intake of protein; animals fed the high protein diet had the largest amount of hepatic retinol. However, pair-fed animals on comparable levels had even greater hepatic stores than those fed ad lib. This difference could be attributed

to the restricted growth rate of the pair-fed animals, which might have resulted in a lower requirement for vitamin A for metabolic purposes. Later, Johnson and Arnrich (1960) demonstrated that a further increase in the dietary protein from 40 to 60 per cent had no further effect on hepatic storage. Apparently a level of 40 per cent protein in the diet was the optimum for maximum hepatic storage of vitamin A. The beneficial influence of protein on carotene utilization has been reported repeatedly from this laboratory (Brown, 1961; Hillers and Arnrich, 1964; Ruffin and Arnrich, 1966; MacMillan, 1966), as well as from others (Mathew and Beaton, 1963). In these studies, relatively low doses of carotene were administered. However the supplements were sufficiently large to give appreciable hepatic retinol, and compared in magnitudes to the recommendations of the National Research Council for the growing rat (1962). When MacMillan increased the carotene dose 3 and 9 times to excessive amounts, rats fed 20 and 40 per cent protein tended to store similar amounts of hepatic vitamin A from a given dose.

Supplementation of diets containing 5% casein with the addition of nitrogen in the form of non-essential amino acids also enhanced carotene utilization. However, this effect could be demonstrated at low levels of protein only (Brown, 1961).

A correlation between the dietary content of protein and the magnitude of hepatic stores of retinol has been shown in other species also. Thus Friend and co-workers (1961) and also Eaton _et al_. (1964) showed the relationship to exist in pigs and Anderson _et al_. (1962) in sheep.

The biological values of the protein under consideration also appear

to influence the utilization of carotene. James and ElGhindi (1953) compared zein, casein, lactalbumin and gluten at equal levels. They found that rats fed casein had the smallest livers, but the highest amount of vitamin A stored therein. Both the amount and the concentration of vitamin A in liver were greater in these animals than in those fed the other three proteins. This difference in hepatic stores of retinol in response to different protein sources has also been demonstrated in animals fed casein and gelatin (Raica et al., 1959), casein and fish meal (Deuel et al., 1946) or egg albumin and plant proteins (Kramer and Tarzan, 1958a). In experiments reported by Hillers (1963) casein protein was more efficient in promoting retinol storage from carotene than were either gluten or zein fed at comparable levels. Furthermore, even in instances where the additional nitrogen was not utilized for growth, increase in protein level in the diet resulted in higher hepatic stores of retinol from carotene.

Supplementing the cereal proteins gluten and zein with limiting essential amino acids has been shown to improve the hepatic stores of vitamin A. However, these levels of liver deposits did not approach those obtained with casein protein at comparable intakes of protein. That the addition of the limiting essential amino acids to the dietary protein at low levels of intake improves carotene utilization had previously been shown by Johnson (1959).

One of the limitations inherent in the practice of interpreting the extent of carotene utilization by the magnitude of hepatic retinol stores lies in the fact that no valid method exists which assesses the amount of vitamin A used for metabolic processes during the period of accumulative

storage. In order to overcome this problem Berger et al. (1962) conducted parallel studies to separate the effects of protein on the utilization of vitamin A for metabolic purposes from those related to carotene utilization. Using the data from the first phase of the study for the correction of the amount of retinol used for maintenance in the second, they concluded that carotene conversion did take place even on a protein-free diet. Employing the same experimental approach they confirmed previously cited studies which had shown that both the quality and the quantity of the protein determined the extent of carotene utilization as reflected in the hepatic storage of retinol.

In general, there seems to be good agreement between investigators that both the amount and the nature of the amino acid composition of the protein under consideration influence the utilization of carotene, although the controversy regarding the effect of protein on the utilization of pre-formed Vitamin A still exists.

Effect of protein on the intestinal metabolism of carotene

Little direct evidence can be found for the effect of dietary protein on the intestinal metabolism of carotene. However, circumstantial evidence has accumulated which indicates that the small intestine might be the locus for the protein-carotene interaction. Arnrich and Pederson (1956) and later Ruffin (1965) showed that while the quantity of protein in the diet had no effect on low levels of pre-formed vitamin A utilization the utilization of carotene was enhanced by increasing levels of protein. In these studies the vitamin A or carotene intake was slightly in excess of the daily requirement for growing rats as recommended by the Nutrition

Research Council (1962). The difference in behavior of dietary carotene and vitamin A led to the suggestion that proteins might exert their influence on the utilization of carotene prior to the release of newly formed retinol from the intestinal conversion site. Ruffin and Arnrich (1966) studied the effect of the presence or absence of dietary protein during the absorptive phase of carotene metabolism. Groups of animals were conditioned to eat their daily allotment of protein within a few hours. For the remaining part of the 24 hours period thay had access to a protein free diet. Half of the animals received their daily carotene dose with the protein supplement while the other half was dosed 12 hours later, during the post-absorptive period with respect to protein. Under these conditions of the experiment, simultaneous feeding of protein and carotene gave considerably greater vitamin A storage than did delayed supplementation with the carotene dose. However, there was still an effect from the level of protein in the diet even when carotene was fed 12 hrs after the protein supplement. This observation points to the possible existence of more than one mechanism in the protein-carotene interrelationship.

So far, there appears to be one study dealing with the direct approach to the problem of protein-carotene interaction in the intestine. Deshmukh and Ganguly (1964) maintained rats on diets containing 5, 10 or 20% protein, free of vitamin A, over a period of 30 days. After this period the animals were given 4000 mcg of carotene orally. Together with the carotene, the rats received 1 gm of their respective diets. The rate of carotene utilization for vitamin A synthesis was measured by the amount of retinol derivatives found in the intestinal walls, liver and serum after

different periods within a 24 hrs time span. In all intervals studied, the retinol fraction tended to increase with the increase in the dietary protein in all tissues under consideration. There was also an inverse relationship between the dietary protein and the unconverted carotene in the small intestine. This indicated that the dietary protein affects the conversion process of carotene to retinol in the intestines. In a subsequent publication (Deshmukh et al., 1965) the same investigators studied the metabolism of retinal under similar experimental conditions. More of the aldehyde form of the vitamin was absorbed unchanged in animals fed the lower levels of protein than in those maintained on higher protein intakes. From these results the authors postulated that protein insufficiency affects two steps in the overall process of carotene metabolism in the intestinal mucosa, namely oxidation of carotene to retinal and subsequent reduction to retinol.

Serum level of vitamin A and dietary protein

From epidemiological studies McLaren (1958) observed an inverse relationship between dietary protein and the serum level of vitamin A in humans. Since then, more evidence has been brought up in favor of this observation by investigations on humans as well as on animals. Friend and colleagues (1961) studied protein malnutrition in pigs. They showed a positive correlation (R = 0.76) between serum vitamin A and serum albumin levels. In contrast to uncomplicated avitaminosis, serum vitamin A levels in protein malnutrition could not be increased by the administration of exogeneous vitamin A. Anderson et al., (1962) observed similar

effects in sheep. Deshmukh et al., (1964) in a similar study on rats, showed that groups fed insufficient amounts of protein had lower concentrations of serum vitamin A and albumin than those fed adequate amounts of protein. In these investigations, the low levels of serum retinol in protein malnutrition could be corrected only by realimentation with an adequate amount of protein.

Arroyave's work with children suffering from kwashiorkor indicated that a similar relationship may exist in humans, (Arroyave et al., 1959). The patients had low levels of serum albumin and vitamin A while analysis of liver biopsy samples indicated the presence of appreciable amounts of hepatic vitamin A. Furthermore, the serum vitamin A level could not be raised by administering retinyl palmitate. But when the children were given skim milk without exogenous vitamin A, the serum vitamin A level rose to normal. In contrast, Ehrlich et al., (1964) were unable to draw conclusions regarding protein malnutrition and serum vitamin A levels. However, the protein intake of these subjects was not as low as that encountered in kwashiorkor.

Fecal excretion of carotene as related to protein nutrition

A large portion of dietary carotene is excreted unchanged in the feces (Moore, 1930; Ahmed, 1931; Johnson and Baumann, 1948). Several factors affect the fecal excretion of carotene, not always consistently. Kramer and Tarzan (1958b) recovered 80% of the ingested carotene in the feces of rats consuming carrots, while there was less than 50% recovery when the rats were fed the same amount of carotene in an oily solution.

Hepatic stores, however, were not affected consistently in both groups. The authors felt that under the experimental conditions, carotene utilization could not be gauged by fecal losses. Johnson (1959) drew similar conclusions from her work. Although she found an inverse relationship between fecal carotene and the dietary protein intake, the large differences in the magnitude of hepatic stores of retinol could not be explained on the basis of fecal carotene losses only.

Fecal losses of carotene have also been shown to vary with the nature of the diet. Thus Fraps (1946) showed that the bulk of the diet decreased the digestibility of carotene, as shown by fecal losses, without affecting the liver stores of vitamin A. James and ElGhindi (1953) found more vitamin A in the liver and less carotene in the feces of rats fed proteins of animal origin as compared to those that were fed plant proteins. Kramer and Tarzan (1958b) also demonstrated such a variation of fecal carotene in rats maintained on diets containing proteins of different biological value.

An inverse relationship between fecal carotene and hepatic vitamin A stores as well with dietary protein intake has been demonstrated in rats (Jagannathan and Patwardhan, 1960; Deshmukh and Ganguly, 1964). However, such a correlation could not be shown from studies in this laboratory (Hillers, 1963).

Moore (1957) attributed the descrepancies in the results of fecal losses of carotene to two factors. The first one is the difference in the physical and chemical state of the pigment in the dietary source and that in the feces. The other is the efficiency of the extraction procedures employed, which might easily-recover more carotene from the feces than

from the dietary source thus leading to a negative balance of carotene. Since experimenters interested in the chemical steps of carotene metabolism have reported the presence of unidentifiable breakdown products, one may add to Moore's two factors another one, the destruction of carotene in the gastrointestinal tract. Therefore, the interpretation of results on the basis of fecal carotene losses should be made with a certain amount of discretion.

DEVELOPMENT OF METHODS

Section I: Synthesis, Isolation, and Purification of Biologically Labelled Beta-Carotene

Tobacco plants were used for the synthesis of labelled carotene since the leaves contain appreciable amounts of beta-carotene[1] (about 40 mcg. of beta-carotene per gram of leaf tissue).

The procedure for production of all-trans beta-carotene labelled with ^{14}C- was developed through successive trials with various modifications made necessary to secure an adequate yield of beta-carotene with a high specific activity. A total of 4 trials had to be run in order to obtain sufficient amounts of labelled carotene for the whole study.

The final procedure together with the modifications involved in specific steps, is described below:

Photosynthesis

Five tobacco plants (N. tabacum var Samsuri) each nearly 12" high with 3 - 4 young but not tender leaves were selected for the purpose.

Size, number, and state of maturation of leaves: Size, number, and state of maturation of leaves were found to be of importance in determining the final yield and the specific activity of the sample of beta-carotene. On the basis of previous work in this laboratory, 3 - 4 leaves per plant were used since a higher number of leaves had resulted in the synthesis of labelled carotene of low specific activity. In trial no. 2 where leaves were big and mature, the yield of the sample was good but the specific activity was low. In trial no. 3, where tender and young leaves were used, a sample

[1]Dr. S. A. Aronoff, Biochemistry Department, Iowa State University, Ames, Iowa. Plant feeding experiment. Private communication. 1966.

of low yield and high specific activity was obtained.

The plants were kept in darkness for 24 hrs. prior to feeding of labelled carbon dioxide, to facilitate efficient photosynthesis. They were then introduced into a large glass growth chamber (2' X 2' X 2'). Two auxiliary lamps with 150 watt bulbs each were used above the chamber to produce a uniform light effect. Five millicuries of barium carbonate[1] ($Ba^{14}CO_3$) were introduced into the air-tight chamber. Carbon dioxide was liberated from the carbonate by the addition of 2 ml. of 10% perchloric acid. A pump was used to circulate the gas through the chamber effectively. The amount of radioactivity in the chamber was recorded at intervals with a G. M. tube and counter, attached to the apparatus as shown in the flow diagram (Figure 1). The plants were allowed to respire in this atmosphere until all the carbon dioxide was absorbed by the plants, i.e., until all the activity of carbon dioxide was reduced to a constant. The operation required about three hours.

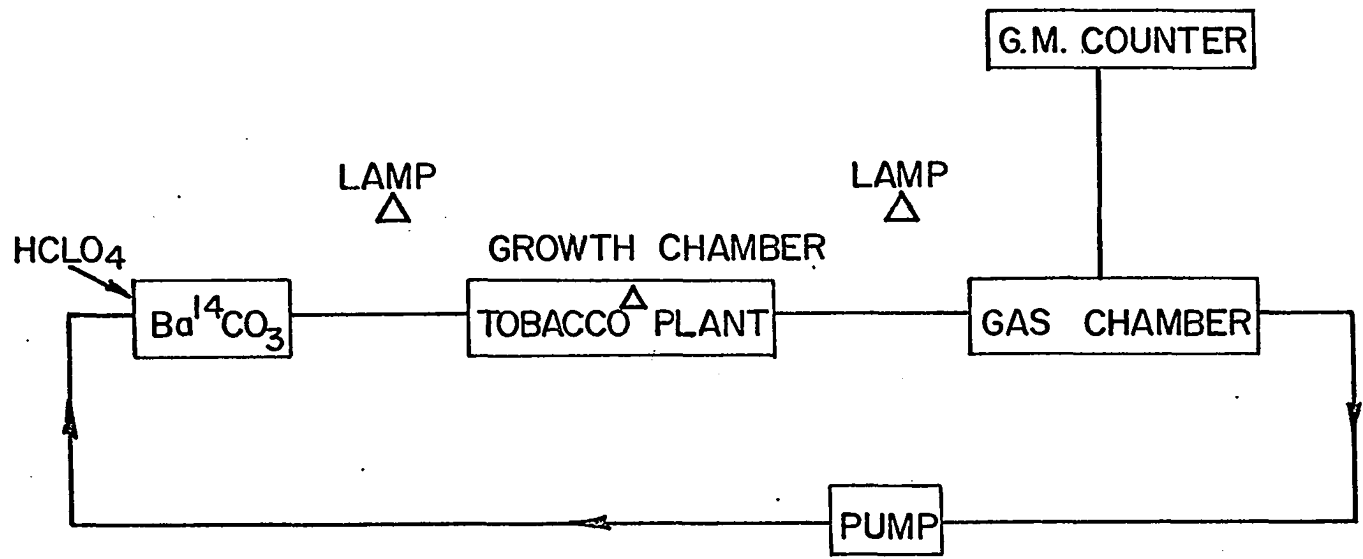
Extraction of carotene

The extraction procedures were an extension of those described by Willmer and Laughland (1957). The extraction and purification were done in a room with no direct light source. The development of the procedure adapted in our laboratory will be described below with the modifications involved in the steps under consideration. Quantities are given for 10 gm. of leaf tissue.

1. Remove leaves with a sharp razor blade and weigh.
2. Introduce 50 ml. acetone per 10 gm. leaf tissue in a Waring blender. Add a few mg. of hydroquinone (quinol) followed by

[1]Nuclear Chicago Inc.

Figure 1. Diagram of apparatus used for photosynthesis of ^{14}C-carotene.


G.M. COUNTER
LAMP
LAMP
GROWTH CHAMBER
HCLO4
Ba14CO3
TOBACCO PLANT
GAS CHAMBER
PUMP

the leaves. Homogenize for one minute in an atmosphere of nitrogen. Add 15 - 30 ml. of petroleum ether[1], B. R. 60 - 70°C, (skelly B) from a squeeze bottle and allow the particles to settle for one minute. Prepare a 500 ml. separatory funnel covered with black cloth or tin foil containing a layer of 20 ml. skelly B over 50 ml. of a half-saturated aqueous solution of ammonium sulphate. Decant the supernatant from the Waring blender into the separatory funnel. Wash repeatedly with distilled water, transferring the washings to the separatory funnel, until the solvent layer is colorless.

3. Repeat the extraction procedure, step no. 2 on the residue, i.e., adding acetone, quinol, and nitrogen before blending each time and extracting repeatedly with skelly B, transferring the supernatant to the separatory funnel used in step no. 2 until no color remains in the leaf particles. If not enough room is left in the separatory funnel, distribute the extracts in two separatory funnels of 500 ml. capacity each.
4. Add 100 ml. distilled water to the separatory funnel. Displace air with nitrogen gas, swirl gently and allow a few minutes for the separation of the layers. Discard the aqueous layer.
5. Dry the skelly B extract over a few gms. of anhydrous sodium sulphate, swirl and transfer it to an evaporation flask (500 ml.

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[1]Petroleum ether B. R. 60 - 70°C. Distilled over solid KOH. Barton Solvents, Des Moines, Iowa. The term skelly B will be used, henceforth, for this solvent.

capacity). Concentrate under vacuum in a flash evaporator to about 5 ml. Maintain the water bath at 50°C. Use nitrogen to enter the evaporator when releasing the vacuum.

6. Saponify the solvent extracts in the dark for 18 hrs. at room temperature by the addition of 100 ml. of absolute ethanol and 2 ml. of 50% KOH/10 gm. of leaf tissue. Extract the non-saponifiable fraction thoroughly with skelly B (100 ml.) Wash the extract with distilled water to free it from alkali as tested by phenolphthalein. Dry over anhydrous sodium sulphate.

   Prevention of trailing: In the first attempt at synthesis saponification: The leaf extract obtained in step 5 was passed through a well-packed column of sucrose: supercel: (1/1 (v/v)) to separate the chlorophylls from the carotenoids. Trailing of the chlorophyll band into the carotenoids prevented a satisfactory separation of the fractions. Introduction of saponification allowed the chlorophylls to enter the aqueous phase of the saponified materials during solvent extraction.

7. Reduce the volume to about 20 ml. by evaporating under reduced pressure in an atmosphere of nitrogen. Cool to -60°C by placing the flask in dry ice. Pass the solution through dry ice according to the technique of Glover, Goodwin, and Morton (1948). This step removes all the sterol from the mixtures.

   Removal of steroids: In trial no. 1 analysis of an aliquot of the synthesized carotene by chromatographic measures had

resulted in the recovery of 35 - 36% of the activity in the carotene fraction, but of a recovery of about 94.5% of beta carotene as determined by spectrophotometric analysis. (Table 1) This suggested that some extraneous material of high specific activity, possibly sterols, was moving with carotene during chromatography. The above method of cooling was successful in removing this extraneous material.

8. Concentrate the mixture of carotenoids further to about 1 - 2 ml. in a 30 ml. beaker under a stream of nitrogen.

Chromatography

Deactivation of adsorbant Pipette 7 ml. of distilled water into a dry flask with ground glass stopper and evenly distribute the water on the glass surface by gentle rotation. Add 93 gm. of fresh aluminum oxide (Woelm Neutral - grade I).[1] Shake well until lumps and moist spots are no longer observed. After standing for 2 hours the water will be evenly distributed throughout the mass. A homogeneous adsorbant of approximately grade III activity in a free flowing condition will be obtained. Activity is retained for a month. About 10 gm. alumina should be used for 10 gm. leaf tissue. More than one column may be necessary to purify all the carotene.

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[1] Alupharm Chemicals. New Orleans, La.

Table 1. Analysis of stock solution of ^{14}C-carotene from the first run of synthesis

Dose no.	Amount analyzed		Amount recovered Fr. I (carotene)				Amount recovered Fr. III (retinol)	
	mcg.	c/m	mg.	c/m	% mg.	% c/m	c/m	% c/m
1	10.49	35552	9.84	13040	94	36.7	18850	53
2	7.25	28500	6.89	9993	95	35.1		
				Ave.	94.5	35.9		

<u>Procedure:</u>

1. Pour in one pouring, a suspension of approximately 5 gm. of deactivated alumina in 5 ml. skelly B into a 1 X 15 cm. chromatography tube to form a column of 5 - 6 cm. high. Allow skelly B to drain by gravity only. Before the column has run dry, add the sample. Follow with 25 ml. skelly B to elute all the carotene. Collect the eluate in a 50 ml. beaker.

   This operation not only removes any remaining chlorophyll in solution but also the xanthophylls, and thus facilitates the next chromatographic step on calcium hydroxide, since fewer pigments are left for separation.

2. Concentrate the mixture of carotenes eluted as above to about 5 ml. under a stream of nitrogen.

3. Prepare a column of calcium hydroxide to a height of 6" in a glass column of 1" X 12". Layer it with an inch of anhydrous sodium sulphate. Use suction to pack this column. Tamp frequently to get a tight packing.
4. Add 10 ml. of skelly B to the column without removing suction. Before the column has run dry, add the mixture of carotenes, followed by 35 ml. of 2% acetone to separate the carotenes on the column. The main orange colored band of all-trans beta carotene will be seen following the narrow band of the alpha variety. Elute and collect the beta carotene band in a large test tube. Use about 250 ml. of 1.5% p-methyl anisole for the eluant.

   The arrangement for chromatography is shown in Figure 2.
5. Evaporate the eluate to dryness to remove p-methyl anisole, under reduced pressure. Allow the nitrogen to enter the evaporator to release the vacuum. Immediately add skelly B and make up the volume to 25 ml. per 10 gm. leaf tissue.

The beta-carotene obtained by the procedures described above served as the stock solution of beta carotene. The concentration was determined spectrophotometrically with a Beckman spectrophotometer, model DU. An aliquot of the stock solution was counted on a cupped copper planchet of 1¼" diameter on a gas flow counter with Geiger operation.[1] The specific activity of the sample obtained was thus determined.

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[1]Model C 110 B, Nuclear Chicago Inc., Courtesy Dr. S. A. Aronoff, Biochemistry Dept., I.S.U., Ames, Iowa.

Identification

The all-trans beta-carotene was rechromatographed on deactivated alumina to constant specific activity. It was identified by comparing the characteristic absorption spectrum of this material with that of synthetic beta-carotene[1] sample (Figure 3).

Preparation of a dose of labelled carotene to be administered to animals

On the day of the experiment a suitable aliquot of the stock solution was diluted with crystalline carrier beta-carotene to give the sesired specific activity. This sample was freshly chromatographed on deactivated alumina immediately before use.

A solution containing about 20 mcg. of beta-carotene was evaporated in a two dram vial under a stream of nitrogen; 0.1 ml. of acetone was added followed by 0.1 ml. of Tween 20[2] (Polyoxyethylene (20) sorbitan monolaurate) for solubilization and 0.8 ml. of Kreb's Ringer solution[3] was added to make one ml. of carotene dose. The concentration of the dose was determined spectrophotometrically.

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[1]General Biochemicals, Inc., Chagrin Falls, Ohio.

[2]Generous gift of Atlas Chemical Industries Inc., Wilmington, Delaware.

[3]Kreb's Ringer solution was prepared freshly by combining the following solutions in proportions stated:

1. 100 ml. of 0.154 M. sodium chloride solution
2. 4 ml. of 0.154 M. potassium chloride solution
3. 3 ml. of 0.110 M. calcium chloride solution
4. 1 ml. of 0.154 M. dihydrogen potassium phosphate solution
5. 1 ml. of 0.154 M. magnesium sulphate solution
6. 21 ml. of 0.154 M. sodium bicarbonate solution.

Figure 2. Arrangement for chromatography of biosynthesized ^{14}C-carotene.

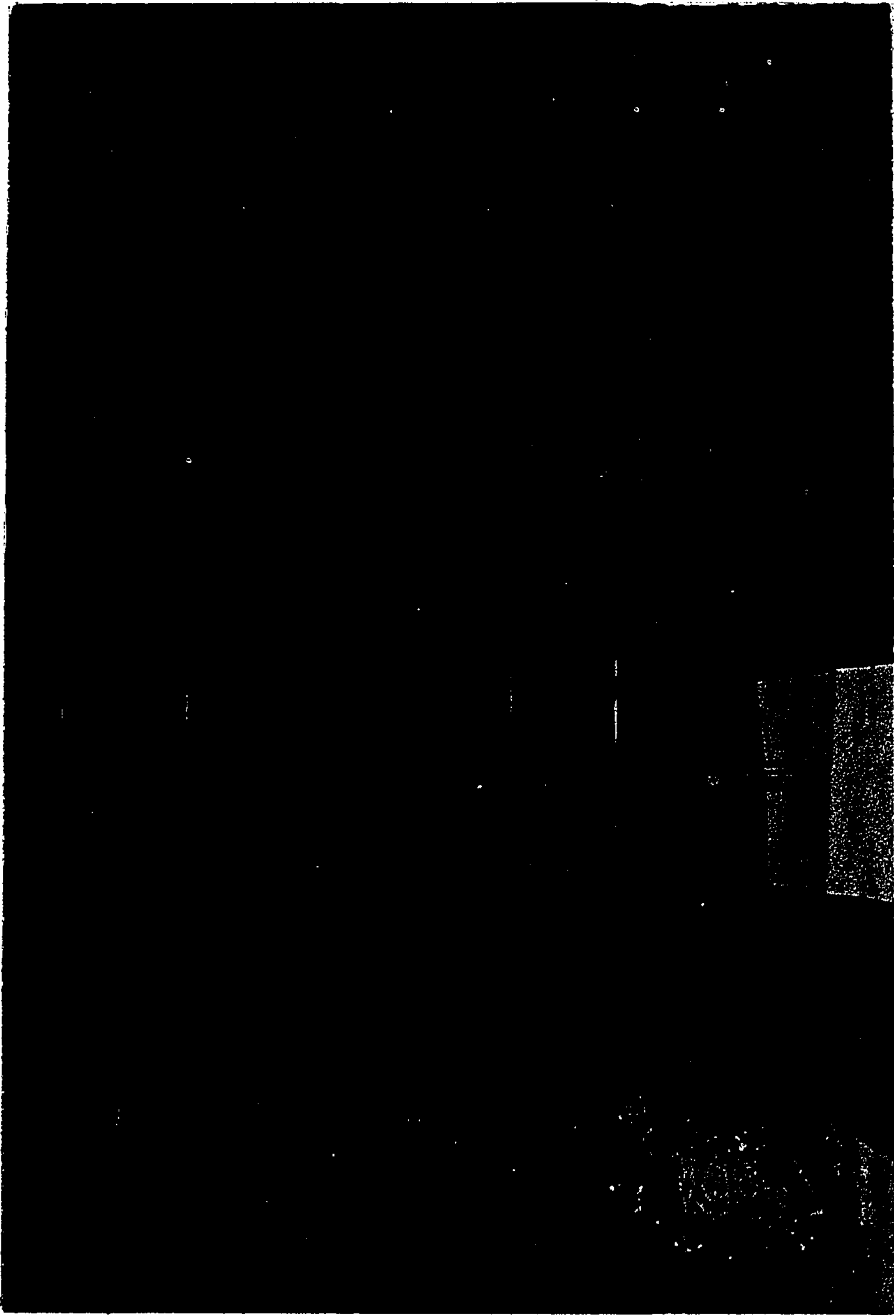

Figure 3. Absorption spectra of beta-carotene.
A: standard synthetic beta-carotene
B_1 B_2 Beta-carotene synthesized by tobacco leaves (N. tabacum var Samsuri) following purification by chromatography.
These samples came from two different batches.

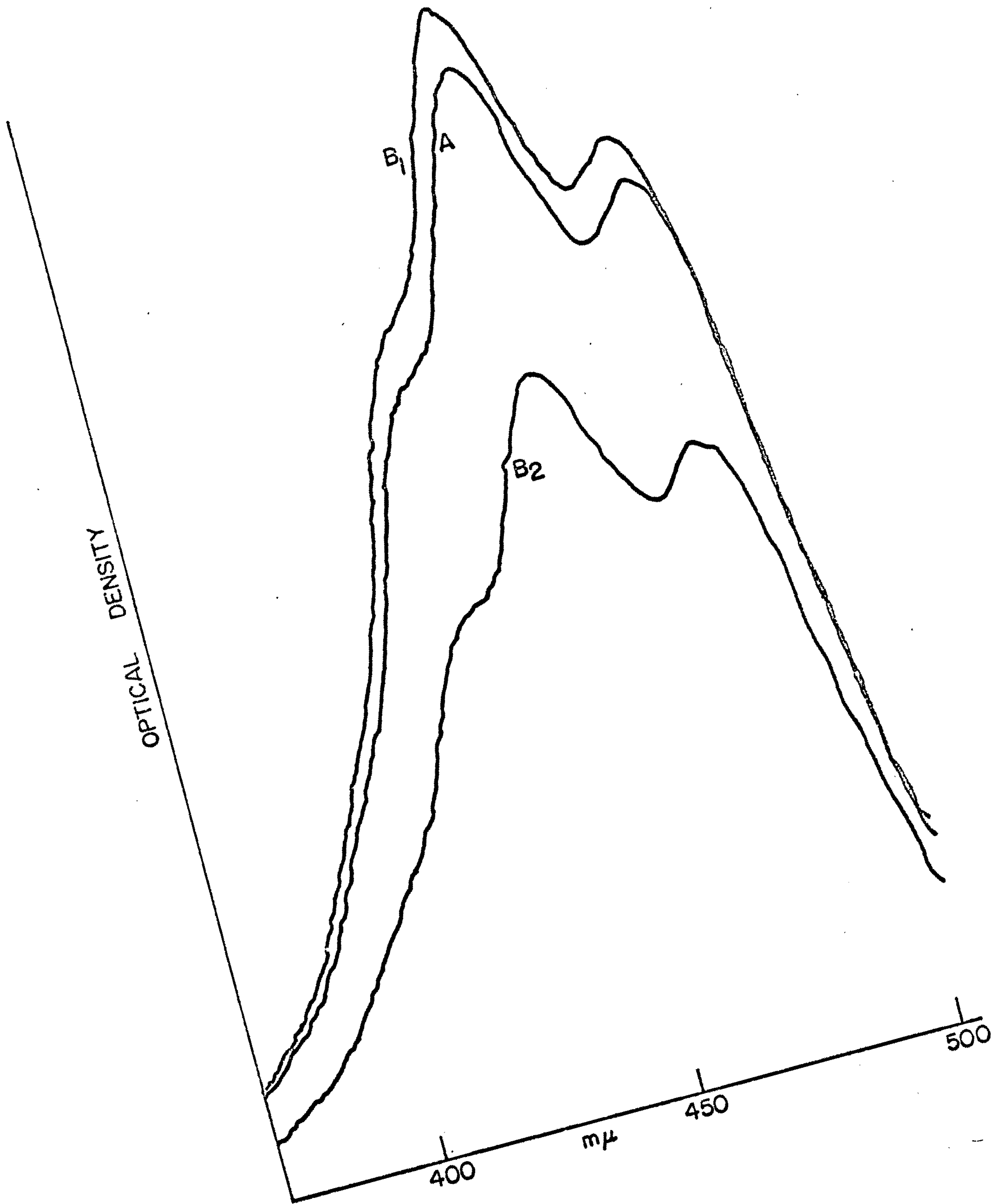
B1
A
B2
OPTICAL DENSITY
400
mμ
450
500

Table 2. Yield and/or specific activities of all trans beta-carotene obtained in different runs of synthesis

Serial no.	$Ba^{14}CO_3$ used	Specific activity	Time taken	No. of leaves/ plant	Wt. of leaves	Total yield of carotene	Amt. of carotene/ gm tissue	Total radioactivity incorporated	Specific activity
	mc.		hrs.		gm.	mcg.	mcg.	c/m	c/m/mcg.
Non radio-active									
1	---	---	---	4	3.4	131.4	38.6	---	---
2	---	---	---	6	5.4	198.5	36.8	---	---
Radio-active									
I	1.0		3	3	5.2	--	--	---	1451
II	5.0		4	4	62.5	1823	29.2	1020000	560
III	5.0		3	3	35.4	405	11.4	12285000	3033
IV	5.0		3	3	45.1	1350	30.0	3305556	2449

The results of the different trials in synthesis as to yield and specific activity in each run are recorded in Table 2.

Section II: Extraction Procedures for the Tissues

The same extraction procedures were followed throughout the study. These were different for different tissues. They will be discussed separately for each. Attempts made early in the investigation to modify certain of the steps of the method had to be abandoned for reasons described in those steps.

The procedure that was followed is described in the following outline.

I. <u>Tissues of gastro intestinal tract:</u>

1. <u>Intestinal walls:</u> All manipulations were carried out in semi-darkness. Nitrogen was used in handling samples wherever possible.

   a. Remove the intestine from the abdominal cavity of the rat as outlined in the section describing the animal experiment.

   b. Cut the tissue into approximately 1 inch pieces into a 500 ml. erlenmeyer flask containing 50 ml. of a 3:1 solvent mixture of skelly B and ethanol. Add non-radioactive carriers of 100 mcg. beta-carotene, 500 mcg. retinyl palmitate and 100 mcg. retinol to the flask.

      Attempts were made to use chloroform: methanol system of solvents in place of skelly B: ethanol for extracting the tissues. But this method had to be abandoned because of low recovery of carotene obtained therein.

(Results in Appendix, Table 24). Synthetic carriers had not been added to the tissue extracts in Series I and II for reasons stated in the section on chromatography.

c. Transfer the mixture to a stainless steel blender[1] of capacity 360 ml. Homogenize for 1 minute. Allow the sediment to settle.

d. Filter with suction. Collect the filtrate in a suction flask. Wash the blender twice with 10 ml. portions of the same solvent mixture; transfer the washings to the funnel. Wash the residue on the filter paper twice with 10 ml. portions of the solvent system. Collect the washings in the same suction flask.

e. Transfer the filtrate to a 125 ml. centrifuge separatory funnel.[2] Wash the flask twice with solvent. Transfer the washings to the separatory funnel. This procedure should yield approximately 100 ml. of filtrate.

f. Allow the two layers to separate for 5 minutes. Drain the lower aqueous layer. One extraction is usually sufficient to extract all the materials. However, wherever necessary, as judged by yellow color in aqueous layer, make another extraction of the latter and add to the solvent layer. Add

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[1] Waring blender.

[2] Centrifuge Lubri-flo Tube. Fisher and Porter Co.

10 ml. of a 2% calcium chloride solution. Centrifuge for 5 minutes at 1000 r.p.m. Discard the lower layer. Dry the solvent extract by adding a few grams of anhydrous sodium sulphate.

On the basis of previous work in this laboratory, it had been recommended to use one litre of distilled water to wash the lipid extracts. This procedure proved to be cumbersome in our trial experiments. Emulsions were invariably formed and were almost impossible to break. Also the recoveries of compounds under investigation were low and inconsistent. (Results in Appendix, Table 25.)

g. Transfer the lipid extract to a 250 ml. evaporating flask. Concentrate to a volume of approximately 5 ml. under reduced pressure in a flask evaporator. Maintain the water bath at 50°C. Allow nitrogen to enter the evaporator to release the vacuum.

h. Transfer the sample quantitatively to a 30 ml. beaker using skelly B for rinsing.

i. Evaporate to a small volume (1 - 2 ml.) on a water bath maintained at 50°C. under a stream of nitrogen.

j. Chromatograph this concentrate on a column of alumina to separate the various fractions. This will be later described in the section on chromatography.

2. <u>Stomach</u>: Whenever necessary, the stomachs were frozen in liquid

nitrogen and stored. If so, they were first brought to room temperature. The rest of the procedure is the same as that described for the intestinal wall.

3. Colon: The procedure for the extraction of colon was the same as the one followed for the intestinal wall. Since the interest was mainly in the intestinal metabolism of carotene, colons were not scheduled to be chromatographed. The addition of non-radioactive synthetic carriers was therefore omitted in the analysis of thé colon.

II. Intestinal Contents:

The procedure for the intestinal contents was slightly different in the beginning stages of the operation.

a. Prepare an erlenmeyer flask containing 50 ml. of the solvent system of skelly B : ethanol :(3 : 1) and the added carriers of non-radioactive carotene (100 mcg.), retinyl palmitate (500 mcg.) and retinol (100 mcg.)

b. Flush out the contents from the intestines directly into the erlenmeyer flask.

c. Transfer the mixture to a stainless steel blender of capacity of 360 ml. and homogenize for 15 seconds.

   Homogenization for one minute resulted in emulsions which were almost impossible to break.

The rest of the procedure is the same as that for the intestinal walls. Continue with step 'd' under intestinal walls.

III. <u>Liver</u>:

Livers were extracted by a modification of the method of Gallup and Hoefer (1946).

1. Remove liver from the abdominal cavity. Rinse with distilled water and dry with filter paper. Put immediately into a tared beaker containing hot 5% aqueous KOH. Weigh by difference.

   In series I livers were not washed. This may account for the appreciable amounts of radioactivity in livers of zero hour controls.

2. Cut the organ into small pieces and heat for 1½ hrs. on a water bath maintained just below boiling. Ensure complete disintegration of tissues.

3. Cool the sample. Make to a volume of 25 ml. with 5% KOH. If necessary, freeze the sample and bring to room temperature just before analysis. This was done when hepatic vitamin A was determined by Carr-Price reaction.

4. Transfer to a centrifuge separatory funnel. Add 25 ml. of 95% ethanol followed by 50 ml. of petroleum ether[1] B.R. 28° - 38°C, (skelly A). Shake.

5. Centrifuge to separate the lipid extract from the aqueous layer.

6. Drain off the lower aqueous layer. Add a few grams of anhydrous sodium sulphate to dry the extract.

7. Evaporate the solvent to dryness in a flash evaporator and dissolve the residue immediately in skelly B.

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[1]Petroleum ether B.R. 28°-38°C. Barton Solvents, Des Moines, Iowa. The term skelly A will be used, henceforth, for this solvent.

In the first experiment liver tissue was saponified by adding alcoholic potash (5 gm. KOH in 100 ml. of ethanol) followed by extracting the retinol with skelly B. In later experiments saponification method had changed because of two reasons:

a) Incomplete disintegration of the tissue, as judged by visual inspection.

b) The same sample could not be used for the Carr-Price reaction when necessary.

IV. Blood:

The method of Roels and Trout (1959) was used for the extraction of vitamin A from serum.

1. Transfer the blood from the syringe (described on p. 108) to a 15 ml. test tube. Allow it to stand for 60 minutes.
2. Centrifuge the blood for 30 minutes at 1000 r.p.m.
3. Store the serum under nitrogen gas, if necessary, in a freezer.
4. Bring it to room temperature just before extraction.
5. Transfer 1 ml. of serum to a 15 ml. test tube. Add 1 ml. of freshly prepared 0.1N KOH in 90% ethanol.
6. Saponify the mixture for 20 minutes at 60°C in a water bath. Cool.
7. Add 2 ml. of skelly A. Shake well by hand for 10 minutes. Centrifuge for 1 minute at 500 r.p.m.
8. Transfer the skelly layer to a 30 ml. beaker.

9. Repeat steps 5 and 6 with the aqueous layer remaining in step 7. Transfer the solvent extract to the same beaker. (Step 8)

Section III: Chromatography of the Tissue Extracts

The chromatographic procedures described by Goodman and co-worker (1965) with slight modifications were adapted for all the experiments except the first one. The procedure allowed for the separation of beta-carotene, retinol and retinyl ester from each other as well as from their breakdown products. By using a suitable support, eight to ten chromatographic separations could be carried out conveniently at the same time. This modified method will be described first in detail. The deviations of the procedure used in the first experiment and the need to modify these will be discussed later.

Deactivation of adsorbant

Pipette 7 ml. of distilled water into a dry flask with ground glass stopper and evenly distribute the water on the glass surface by gentle rotation. Add 93 gm. of fresh aluminum oxide (Woelm - Neutral grade I)[1] shake well until lumps and moist spots are no longer observed. After standing for 2 hours, the water is evenly distributed throughout the mass. A homogeneous adsorbant of approximately grade III activity in a free flowing condition is obtained. Activity is retained for a month.

Modification regarding the activity of adsorbant had been introduced previously. Goodman and co-worker (1965) and Olson (1961a,

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[1]Alupharm Chemicals, New Orleans, Louisiana.

had used alumina columns of Grade III activity containing 6% water (V/W) for chromatography. But in our preliminary studies this column was found to be retentive, more so in the case of retinol and its esters than in the case of carotene. Using a 7% alumina column, better recoveries were obtained. Comparisons of recoveries of compounds on these two columns separately is given in the Appendix, Table 26. Synthetic compounds in solution in suitable proportion were used for this purpose.

Procedure

1. Pour in one pouring a suspension of approximately 5 gm. of deactivated alumina in 5 ml. skelly B into a 1 X 15 cm. chromatography tube to form a column 5 - 6 cm. high. Allow skelly B to drain by gravity only.
2. Before the solvent head disappears at the top of the column, pour the sample of tissue extract under investigation over the column of alumina and allow it to percolate by gravity only.

   The maximum load of lipid per gm. of alumina should be 10 mg.
3. Rinse the beaker twice or thrice with skelly B, keeping the volume as small as possible. Pour the rinsings onto the column.
   - Larger volumes would result in diffusion of the band.

Elution and collection

Precaution: Do not allow the column to get dry until all the

eluates have been collected.

1. Elute different fractions with different eluants. The order of the eluants and volumes of each is given below.

   a. Fraction I - 20 ml. of skelly B to elute beta carotene

   b. Fraction II - 20 ml. of 15% benzene in skelly B to elute retinyl esters

   c. Fraction III - 50 ml. of 100% benzene to elute retinol

   d. Fraction IV - 30 ml. of 8% ethanol in skelly B to elute the miscellaneous breakdown products in the lipid extracts.

2. Collect each fraction in separate receivers placed just below the column. Wash the tip of the column before changing receivers. Collect the washings in the same receiver.

   In series I only 3 fractions had been collected. The order and volumes of eluants for the three fractions had been:

   a. Fraction I - 16 ml. of skelly B to elute carotene

   b. Fraction II - 16 ml. of 15% benzene in skelly B to elute retinyl esters

   c. Fraction III - 30 ml. of 8% ethanol in skelly B to elute retinol.

   This method had been found adequate in the separation of the three synthetic compounds as shown by percent recoveries in Table 3. But difficulties in terms of added carriers and eluants were met in dealing with radioactive carotene as described below:

Table 3. Recovery of pure synthetic carriers of beta-carotene, retinyl esters and retinol after extraction and chromatography on alumina

Serial number	Amt. of synthetic compound added			Amount recovered			% recovery		
	Fraction I Carotene	Fraction II R.esters	Fraction III Retinol	Fraction I Carotene	Fraction II R.esters	Fraction III Retinol	Fraction I Carotene	Fraction II R.esters	Fraction III Retinol
	mcg.	mcg.	mcg.	mcg.	mcg.	mcg.	%.	%	%
1	155.3	265.3	110.2	153.5	252.5	100	98.8	95.2	90.7
2	155.3	265.3	110.2	149.5	260.4	108	96.2	98.1	93.5
3	155.3	265.3	110.2	151.5	246.5	97.4	97.5	92.9	88.4
						Av.	97.5	95.4	90.8

Added carriers: In the preliminary studies, it was intended to add relatively large amounts of non-radioactive carriers of beta-carotene, retinyl palmitate and retinol to the tissues before the extraction and chromatographic procedure. This was planned in view of the fact that the added carriers of compounds under consideration would facilitate the separation of small amounts of newly synthesized radioactive compounds from the injected beta-carotene. In a pilot study conducted with animals injected with radioactive beta-carotene, it was found that the beta-carotene added as carrier to the tissues, as judged by the color on the column, could not be eluted satisfactorily. Thus addition of carriers had been omitted in the first two experiments. Later, it was shown (Table 5) that the carriers could be used successfully if lower amounts of carrier carotene were incorporated and volumes of eluants were changed. Therefore, non-radioactive compounds -- approximately 100 mcg. retinol -- were used again with the tissues before extraction in the last three series.

Eluants: It was found advisable to alter the order and volume of eluants slightly for the following two reasons:

Table 4. Recovery of non radioactive synthetic carriers of beta-carotene, retinyl palmitate and retinol after extraction and chromatography on alumina

Serial number	Fraction I Carotene	Fraction II R.esters	Fraction III Retinol	Fraction I Carotene	Fraction II R.esters	Fraction III Retinol	Fraction I Carotene	Fraction II R.esters	Fraction III Retinol
	mcg.	mcg.	mcg.	mcg.	mcg.	mcg.	%	%	%
1	110.4	536.6	110.5	105.3	514.8	102.0	95.4	97.8	92.3
2	110.4	536.6	110.5	105.3	514.8	103.7	95.4	97.8	93.8
3	110.4	536.6	110.5	104.9	514.8	107.1	95.0	97.8	96.9

Table 5. Recovery of added carriers of synthetic compounds to the intestinal walls and contents of animals injected with approximately 20 mcg. ^{14}C-carotene after extraction and chromotography on alumina

Serial no. and tissue	Carriers added			Carriers recovered			% recovery[a]		
	Fraction I Carotene	Fraction II R.esters	Fraction III Retinol	Fraction I Carotene	Fraction II R.esters	Fraction III Retinol	Fraction I Carotene	Fraction II R.esters	Fraction III Retinol
	mcg.	mcg.	mcg.	mcg.	mcg.	mcg.	%	%	%
1 Content	109.4	519.8	95.2	110.6	476.2	74.8	101.1	91.6	78.6
2 Intestine	109.4	519.8	95.2	108.5	466.3	75.5	99.2	89.7	79.3
3 Content	109.4	519.8	95.2	110.0	478.2	76.6	100.5	92.0	80.5
4 Intestine	109.4	514.8	95.2	99.4	472.3	76.6	91.0	90.9	80.5

[a] In all these fractions, radioactive beta-carotene (injected and unutilized) as well as newly synthesized retinyl esters and retinol are present.

a) Even though the elution of carotene seemed to be satisfactory as tested by percent recovery, a small fraction of this appeared to diffuse into the next fraction of retinyl ester, as shown by variable but appreciable amount of radioactivity, in that fraction in zero hour control group in series I (Table 6) where it had not been anticipated. This contamination could easily lead to a large error in the relatively small amount of retinyl ester formed which was to be taken as the criterion of the metabolism.

b) Invariably, in zero hour controls in series I activity was seen in the retinol fraction. This was suspected to be due to the artifacts produced by the breakdown of carotene. Thompson et al. (1949) had also observed this phenomenon. When large amounts of non radioactive retinol were used for chromatographic purposes, this small amount of artifact was not noticeable. But, it was suspected that in series I Fraction III of the chromatographic eluates (Table 6) had been contaminated with the labelled breakdown products which would hamper the determination of the small amounts of retinol expected.

Accordingly, it was decided to follow the chromatographic procedure employed by Goodman and co-worker (1965) with one modification. The last fraction of breakdown products was eluted with 8% ethanol in our experiment,

Table 6. Radioactivity recovered in each chromatographic fraction of different tissue extracts, in percentage of injected dose of ^{14}C-carotene (values represent mean of 10 animals/ group ± standard errors), Series I

Group symbol	Extract	Fraction I carotene	Fraction II Retinyl esters	Fraction III Retinol	Total
0-20 C	Small intestine-wall	29.7±4.2[a]	1.22± 0.27	2.46±1.14	33.4±3.7
	Small intestine contents	55.0±5.1	2.13± 0.58	1.84±0.35	59.0±5.2
	Small intestine+ contents	84.7±4.4	3.38± 0.68	4.28±1.15	92.2±5.1
	Liver	-	-	-	0.9±0.3
	Total	-	-	-	93.0±4.9
1-20 C	Small intestine-wall	20.8±2.7	9.29± 2.21	2.66±1.30	33.1±4.9
	Small intestine contents	46.3±3.4	1.56± 0.57	1.82±0.29	49.7±4.3
	Small intestine+ contents	67.1±4.9	10.86± 2.27	4.80±1.35	82.8±6.1
	Liver	-	-	-	0.7±0.2
	Total	-	-	-	83.5±6.1

[a]Standard error of the mean.

Table 6. (Continued)

Group symbol	Extract	Fraction I Carotene	Fraction II Retinyl esters	Fraction III Retinol	Total
3-20 G	Small intestine-wall	19.3±3.2	9.91±1.24	2.16±0.69	31.4±3.6
	Small intestine contents	39.4±4.2	2.35±0.62	2.62±0.36	44.4±3.8
	Small intestine+ contents	58.8±4.1	12.27±1.27	4.77±0.84	75.8±3.9
	Liver	-	-	-	1.6±0.6
	Total	-	-	-	77.2±3.7
0-40 G	Small intestine-wall	23.6±6.4	1.05±0.23	1.01±0.31	25.7±6.6
	Small intestine contents	68.7±6.0	1.06±0.23	1.94±0.29	71.7±6.1
	Small intestine+ contents	92.4±8.3	2.11±0.33	2.93±0.51	97.4±8.5
	Liver	-	-	-	0.6±0.2
	Total	-	-	-	97.7±8.6

Table 6. (Continued)

Group symbol	Extract	Fraction I Carotene	Fraction II Retinyl esters	Fraction III Retinol	Total
1-50 C	Small intestine-wall	16.9±2.5	5.79±1.83	0.49±1.92	25.7±2.3
	Small intestine contents	57.9±4.8	1.07±0.18	3.07±0.74	62.1±4.1
	Small intestine+ contents	74.9±4.6	6.84±1.88	6.09±1.90	87.7±4.7
	Liver	-	-	-	0.6±0.2
	Total	-	-	-	88.1±4.8
3-40 C	Small intestine-wall	13.6±2.1	7.55±1.45	1.09±0.23	22.2±3.0
	Small intestine contents	50.7±3.5	1.46±0.21	2.82±0.26	55.2±3.5
	Small intestine+ contents	64.6±3.0	9.02±1.47	3.91±0.41	77.5±2.5
	Liver	-	-	-	0.8±0.2
	Total	-	-	-	78.2±2.51

while Goodman and co-worker used 100% methanol.

Concentration

1. Evaporate each of the fractions to dryness separately in a flash evaporator. Allow only nitrogen to enter the evaporator to release vacuum.
2. Take up the residue immediately in skelly B, transfer to a 5 ml. volumetric flask and make up to volume with the same solvent.

Tests of validity of the chromatographic method as applied to various materials

Preliminary work in this study had shown that 100% of the added carriers of non radioactive synthetic beta carotene, retinyl palmitate and retinol could never be obtained after the extraction and chromatographic separation. Hence a standard of recovery had to be set. Goodman and co-worker (1964) had recovered more than 90% of added beta-carotene, and 85-90% of added retinyl ester and retinol in their procedure. However in this study, recoveries of more than 90% in the case of added beta-carotene and retinyl palmitate, but only 80% in the case of retinol could be obtained. Since these recoveries had been consistent in many runs, it was decided to take these values as satisfactory recoveries for later experiments and discussions.

1) Pure synthetic compounds: Mixtures of non radioactive carotene, retinol and retinyl palmitate were passed in triplicate through chromatographic columns and eluted as described above. Table 4 shows the separation to be quantitative in terms of recoveries based on the criteria given above.

2) Analysis of a dose of ^{14}C-carotene: A dose of approximately 20 mcg. of ^{14}C-carotene was passed through the alumina column in triplicate and different fractions eluted as described above. Table 7 shows the recoveries of radioactivity in different fractions. As can be seen, negligible amounts of radioactivity were obtained in fraction II and III showing that (1) elution of carotene was complete and (2) the retinol fraction was uncontaminated with the miscellaneous breakdown products.

Table 7. Recovery of radioactivity (c/m) and % in different chromatographic fractions after passing a dose of carotene through a column of alumina

Serial no.	Dose	Fraction							
	c/m	Carotene c/m	Retinyl ester c/m	Retinol c/m	TPF c/m	Carotene %	Retinyl ester %	Retinol %	TPF %
1	20900	18780	-	95	570	90.0	-	0.5	2.7
2	20660	19265	70	90	225	93.2	0.3	0.4	1.1
3	17900	17170	110	130	400	95.9	0.6	0.7	2.2

3) Tissue extracts when non radioactive carotene had been injected: The walls and contents of the small intestines of a vitamin A-free rat injected with 300 mcg. of non radioactive carotene were flushed with saline immediately and extracted separately as described in Section II. Pure retinol and retinyl palmitate were added as carriers during the extraction. The lipid extracts were chromatographed. Table 8 shows satisfactory recoveries of the added compounds.

Table 8. Recovery of carriers of retinyl ester and retinol added to vitamin A-free tissues after extraction and chromatography on alumina

Tissue	Amount added			Amount recovered		% recovered	
	Fraction I[a] Carotene	Fraction II R. esters	Fraction III Retinol	Fraction II R. esters	Fraction III Retinol	Fraction II R. esters	Fraction III Retinol
	mcg.	mcg.	mcg.	mcg.	mcg.	mcg.	mcg.
Content		165.3	135.7	150.5	124.8	91.0	92.0
	297.6						
Intestine		165.3	135.7	154.4	121.0	93.4	89.2

[a]Injected into the loop of small intestine and flushed out immediately.

4) Tissue extracts when a radioactive dose had been injected: Table 5 shows the recovery of the added carriers of beta-carotene, retinyl ester and retinol from the intestinal walls and the intestinal contents of two rats injected with approximately 20 mcg. of labelled beta carotene. The animals were autopsied one hour later. Recoveries were consistent and were greater than 90% in the case of carotene and retinyl ester and about 80% in the case of retinol.

Section IV: Assay Procedures

Spectrophotometric analysis

Wherever necessary, the concentrations of solutions were determined by means of spectrophotometric analysis using a Beckman spectrophotometer model DU with a tungsten lamp.

Carotene was measured at 451mμ, sensitivity 2 and a slit-width of 0.16.

Retinol and retinyl esters were assayed at 328mμ, sensitivity 4 and a slit-width of 0.3.

Estimation of vitamin A in liver by Carr-Price reaction

The hepatic vitamin A was determined by a modification of the Carr-Price reaction, as described by Gallup and Hoeffer (1946). The solutions were covered with a black cloth throughout the analysis to protect them from light. The method is outlined below.

1. Bring the saponified samples to room temperature, if necessary.
2. Transfer in duplicate 5 ml. aliquots of liver homogenate to 40 ml. constricted-neck centrifuge tubes with ground glass stoppers.

3. Add to these 5 ml. of 95% ethanol and 10 ml. of skelly A. Stopper the tubes.

4. Shake for 2 minutes by hand to extract the non-saponified lipid material.

5. Centrifuge for one minute at low speed.

6. Pipette duplicate aliquots of 0.5, 1.0, 2.0, or 3.0 ml. of the skelly A layer into calibrated Beckman tubes.[1]

7. Evaporate the samples under a stream of nitrogen in a water bath of less than 40°C.

8. Add to each tube 1 ml of chloroform immediately after evaporation.

9. Adjust the Beckman spectrophotometer model B to a wavelength of 620 mμ. Set the transmission at 100% with a blank tube containing 1 ml of chloroform, 9 ml of a saturated solution of antimony trichloride in chloroform (Carr-Price reagent) and 1 drop of acetic anhydride.

10. Insert the tube (step 8) into the spectrophotometer. Add 1 drop of acetic anhydride to the tube. Follow this by 9 ml of Carr-Price reagent from a calibrated rapid delivery pipette.[2]

11. Set an electric timer for 15 seconds, as the reagent begins to enter the colorimeter tube.

12. Obtain the O.D. reading at the end of 15 seconds. This timing is necessary since the color fades rapidly.

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[1]Beckman Co., Fullerton, California.

[2]Scientific Glass Apparatus Co., Bloomfield, New Jersey.

The optical density readings were converted to mcg. of vitamin A by means of a standard curve prepared by reacting quantitative dilutions of vitamin A acetate dissolved in chloroform with the Carr Price reagent. Readings were made 15 sec. after the addition of the reagent. A correction factor was applied to convert the vitamin A acetate values to equivalent vitamin A alcohol values. These were used throughout the study.

Counting of radioactivity

Radioactivity of the sample was in most instances determined using a gas flow counter[1] with Geiger operation and with automatic sample changer. Whenever a manual gas flow counter had been used, necessary corrections were made to convert the efficiency to that of the automatic counter. (Conversion factor - M = 1.8 A).

A suitable aliquot (250 µl) of the solution under investigation was pipetted onto a cupped copper planchet (1¼" in diameter) and allowed to dry. The time taken for a set number of counts (640 counts) was recorded.

Smaller aliquots (100 µl) from a larger volume were used for counting purposes in series I. In some cases, recoveries of total radioactivity fed were found to be more than 100%. This was possibly due to the magnification of errors in pipetting. Hence in later experiments the samples were made up to a smaller volume (5 ml.) and a larger aliquot (250 µl) of this was taken for counting. The results seemed to be satisfactory.

Statistical analysis

For the first four series, a student's 't' test was used to compare the groups. Analysis of variance was used in the statistical treatment of

[1]Model C 110B; Nuclear Chicago, Courtesy of Dr. S. A. Aronoff, Biochemistry Department, I.S.U., Ames, Iowa.

data in series V with regard to hepatic storage over different periods. Comparisons by the two factor factorial method with split plot design were made in interpreting the values for accumulated hepatic stores. However, for the intestinal metabolism of ^{14}C-carotene a student's 't' was once again used to compare the two groups.

Section V: Animal Studies

Four series of animal experiments were conducted for the development of the method. The results of each series were used as a basis for the next study. Two additional experiments designed to support some of the results of the above experiments are discussed in Appendix A. The four trials described in this section served for the development of a suitable method for the study of the protein effect on carotene utilization following intra-duodenal ^{14}C-carotene dosage.

Criteria of ^{14}C-carotene metabolism in the intestines were:

a. Recovery of radioactivity in the retinyl ester fraction of the intestinal wall as percentage of the injected dose of ^{14}C-carotene.

b. Recovery of radioactivity in the non-saponifiable fraction of liver as percentage of the injected dose of ^{14}C-carotene.

Amount of newly formed retinyl ester found in the intestinal wall was chosen as a measure of the conversion of carotene in the intestine among the various metabolites of retinol, because it had been shown that this derivative was the predominant one in all the tissues studied, (Olson, 1961; Huang and Goodman, 1965). It had also been shown that this fraction

was least contaminated by breakdown products of beta-carotene while the reverse was true of the retinol fraction (Olson, 1961).

Animals

In all series male albino rats of the Wistar strain were used. In the first series animals born and raised in our laboratory were employed. For the rest of the study, weanling animals were purchased from Simonsen Laboratories[1] and further reared in our laboratory. However, all the animals came from the same original stock (Simonsen Laboratories).

Animals were placed in separate wire-meshed cages. Food intake and weight records were kept during the experimental periods.

Diets and supplements

The composition of different diets and supplements are given in Tables 9 and 10. The level of casein in the diet was altered at the expense of sucrose.

A mixture of water-soluble vitamins was prepared by dissolving weighed amounts of the same in 20% ethanol. The concentration was adjusted to satisfy the daily requirement of water soluble vitamins with one ml. of solution. Each animal received either one ml. of this solution per day or two ml. on alternate days, as indicated in each experiment.

The vitamin E - vitamin D supplement was prepared by dissolving two gm. of the dl-alpha tocopherol[2] and one gm. of vitamin D[3] stock solution

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[1]White Bear, Minnesota.

[2]General Biochemicals Inc.

[3]The vitamin D stock solution was prepared by dissolving 25 milligrams of vitamin D crystals in 2 milliliters of chloroform and adding enough cottonseed oil to give 100 grams.

in cotton seed oil and making the mixture to 100 gm. with the same oil. Two drops of this solution contained roughly one mg. of alpha tocopherol and 25 I.U. of vitamin D.

The carotene dose for oral feeding was prepared by dissolving a weighed amount of carotene (85% beta and 15% alpha) in a small quantity of chloroform. The solution was made to volume with cotton seed oil. The concentrations were so adjusted that the daily dose was contained in either two or three drops of solution, depending on the experiment. The concentrations are given for each series.

Table 9. Composition of protein-containing diets

Ingredients	10% casein protein	20% casein protein	40% casein protein
	%	%	%
Casein[a]	11.5	23.0	46.0
Sucrose[b]	75.5	64.0	41.0
Fat[c]	9.0	9.0	9.0
Mineral salts[d]	4.0	4.0	4.0

[a]General Biochemicals, Inc.

[b]Powdered sugar containing 3 per cent starch, obtained locally.

[c]Crisco.

[d]Hawk Oser Salts.

Table 10. 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	4.8
Choline HCL	4.8	9.6

[a]All vitamins were obtained from General Biochemicals, Inc.

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

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

Depletion period

In all the experiments, except in series I, the animals were first subjected to a vitamin A-depletion period of three weeks. It has been shown that this period was adequate to deplete the animals of existing hepatic stores of retinol without producing deficiency symptoms.

The depletion period was identical for all the experiments. Three week old animals weighing about 50 gm. were fed *ad libitum* a diet containing 20% vitamin A-free casein protein for three weeks. During this period they were given a supplement of water soluble vitamins, vitamin D, and tocopherol every alternate day. The diet was completely devoid of any source of vitamin A.

After the depletion period rats were assigned to different groups as described for each individual series. Table 11 gives a summary of the treatments used in different series.

Series I The first experiment was designed to meet the original objective, that is to find more direct evidence for the protein effect on the intestinal metabolism of carotene in rats given an intraduodenal injection of ^{14}C-carotene. The specific aims were to evaluate the effect of two different levels of casein protein: 20 and 40% of protein in the diet on:

1. The degree of absorption of biologically labelled carotene from the lumen of the intestine.
2. The extent of conversion of carotene into vitamin A in the walls of the intestine.
3. The amount of retinol deposited in the liver from the uptake of biologically labelled carotene.

Intestinal contents were separated from the intestinal wall to determine the partition of carotene between these two. This facilitated the assay procedures for the small amount of retinol formed in the intestinal wall.

Table 11. Group symbols and treatments used in series I to IV

Series	Treatment	Group symbol	No. rats/ group	% casein protein in diet	Oral carotene dose	24 hr. pre-trtmt.	Carotene injection into	Exogeneous protein in loop	Autopsy after dose in hours	Tissues analyzed	Fractions assayed
I	1	0-20C	10	20	40 mcg/ alt. day	24 hrs. fasting	Ligated loop	Casein hydrolysate and albumin injected	0	Intestinal walls Intestinal contents Liver	I Carotene II Retinol ester III Retinol
	2	1-20C	10	20					1		
	3	3-20C	10	20					3		
	4	0-40C	10	40					0		
	5	1-40C	10	40					1		
	6	3-40C	10	40					3		
II	1	1-20C	8	20	120 mcg/ alt. day	Restricted feeding for 24 hrs	Ligated loop	None	1	Intestinal walls Intestinal contents Liver	I Carotene II Retinol ester III Retinol IV Breakdown products
	2	1-40C	8	40					1		
III	1	1-20C	10	20	120 mcg/ alt. day	15 hrs fast then o gm. diet 2 hrs before expt.	Loop without ligation	Natural food	1	Intestinal walls Intestinal contents Liver Stomach Colon	I Carotene II Retinol ester III Retinol IV Breakdown products
	2	1-40C	10	40					1		

Table 11. (Continued)

Series	Treatment	Group symbol	No. rats/ group	% casein protein in diet	Oral carotene dose	24 hr. pre-trtmt.	Carotene injection into	Exogeneous protein in loop	Autopsy after dose in hours	Tissues analyzed	Fractions assayed
IV	1	5-10C	7	10	120 mcg/ alt. day	15 hrs. fast, then 1 gm. diet 2 hrs. before expt.	Loop without ligation	Natural food	5	Intestinal walls Intestinal contents Liver Stomach Colon Blood	I Carotene II Retinol ester III Retinol IV Breakdown products
	2	5-40C	7	40					5		

Two different intervals of time were used: one and three hours following intraduodenal injection of ^{14}C-carotene. One group served as zero hour controls.

Methodology

Design Sixty animals were used for the experiment. Ten animals were assigned to each of the six treatments.

1. 20% casein protein diet - to be sacrificed after 0 hour
2. 20% casein protein diet - to be sacrificed after 1 hour
3. 20% casein protein diet - to be sacrificed after 3 hours
4. 40% casein protein diet - to be sacrificed after 0 hour
5. 40% casein protein diet - to be sacrificed after 1 hour
6. 40% casein protein diet - to be sacrificed after 3 hours.

Pairs of animals were used for treatments 1 and 2 and treatments 4 and 5. The others were random samples.

Procedure Five week old animals weighing between 120 and 190 gm. from the stock colony were grouped according to the above plan. They were fed *ad libitum* a purified diet free of vitamin A containing either 20 or 40% casein protein. The diet was supplemented with the vitamin mixtures and 40 mcg. of carotene every alternate day.

After 6 days on this dietary regime, the rats were fasted for 24 hours. Following the fast, surgery and necropsy were conducted as follows:

1. Anaesthetize the rat with ether
2. Make a small incision on the ventral side just off the midline
3. Open the abdominal cavity gently

4. Locate the duodenum. Ligate the duodenum between pyloric sphincter and entrance of the bile duct with cotton thread.
5. Deflect the small intestine to operator's right. Locate the small edge of the mesentery holding the small intestine together.
6. Locate the center point of the mesentery edge and follow it out to the intestine. Ligate at this point.
7. Inject the upper portion of the intestine just distal to the first ligature with a dose of carotene. Follow it with the protein formula.

The purpose of the protein formula was to simulate the naturally occurring dietary constituents of the intestinal contents during ^{14}C-carotene absorption. The formula was an aqueous solution of casein hydrolysate and bovine albumin fraction V. The bovine albumin fraction V was substituted for casein, since the latter was insoluble in water. Rats who had received 20% casein protein in their previous diet were given 0.2 gm./ml. of protein mixture, and the other group received twice the amount of protein. Total solid content was 0.5 gm./ml. with the addition of either 0.3 or 0.1 gm. dextrose. The composition of the protein formula at two different levels was as follows:

	Quantities per ml. of solution	
	Low level	High level
Casein hydrolysate	0.1 gm.	0.2 gm.
Bovine albumin fraction V	0.1 gm.	0.2 gm.
Dextrose	0.3 gm.	0.1 gm.
	0.5 gm.	0.5 gm.

8. Replace the intestines within the body cavity, close the skin of the peritoneum with surgical clips and return the animal to the cage for a specified interval of time. Start the time period with the injection of the radioactive carotene. Record the time when the rat comes out of anesthesia. After the specified time interval of 0, 1, or 3 hours reanesthetize the animal with ether.
9. Locate the posterior ligature.
10. Cut the intestine just anterior to the ligature and clip an artery forceps to one edge to hold the end of the intestine in a 500 ml. erlenmeyer flask which contains 50 ml. of a solvent mixture of skelly B and ethanol (3:1).
11. Using a no. 18 hypodermic needle of 2" length, force 30 ml. of isotonic saline, maintained at 37°C through the intestine from the anterior end. Collect the flushings in a separate erlenmeyer flask. Treat as described in the extraction procedure.
12. Remove the ligated intestinal loop without stretching. Do not allow any of the washings remaining in the intestine to enter the erlenmeyer flask containing the intestinal contents. Measure the length of the loop. It should be 18 to 24" in length.
13. Collect the intestine into another erlenmeyer flask containing 50 ml. of a mixture of skelly B and ethanol (3:1) and treat as described under extraction procedure.
14. Remove the liver. Saponify as described in the extraction procedure. Freeze and store if necessary and analyze later.

15. Rinse the syringe with skelly-ethanol (3:1) and return the washings to the vial which had contained the dose injected into the animal. Determine radioactivity of the residual carotene and subtract from the original dose. This will give the radioactivity injected into the intestine.

The intestinal wall and the intestinal contents were assayed for unconverted carotene, retinol, and retinol esters. In case of the livers, only the retinol fractions were assayed for radioactivity since no activity had been seen in the carotene fraction in a pilot study.

Results and discussion Results of this series were not considered reliable since the chemical analysis in terms of chromatography was unsatisfactory. This aspect has been discussed in detail in the section on chromatography. However, carotene analysis, as seen by the percent activity recovered in fraction I from the chromatographic column, seemed to be consistent in zero hour controls. This is seen in the small standard errors of the mean (Table 6). The mean percent recovery of radioactivity in the carotene fraction in the loop of the small intestine plus contents of groups 0-20-C was 84.7 ± 4.4. The comparable value for group 0-40-C was 92.4 ± 8.3. The difference was statistically insignificant.

Large and variable quantities of injected carotene were found to be mechanically adsorbed on the intestinal wall even in zero hour controls, (Table 6). Olson (1961a) had also made similar observations. This phenomenon made the interpretation regarding the distribution of unconverted carotene impossible. Hence it was decided to omit these control groups in later experiments.

Since casein hydrolysate had not been tested as a source of protein in previous experiments, it was thought desirable to explore the effect of casein hydrolysate in the diet on the utilization of carotene. A feeding study was conducted in which the effect of feeding either hydrolyzed or unhydrolyzed casein were compared. The criterion of carotene utilization was the magnitude of hepatic storage following a 4-week refeeding period with carotene. The details of this study are given in Appendix A, Table 28). The results indicated that the animals fed whole casein in their diet stored more than twice (230 mcg.) as much vitamin A as did those fed casein hydrolysate (99 mcg.). The reason for this adverse effect of casein hydrolysate is not known. Consequently, the idea of dosing with the protein formula containing casein hydrolysate was abandoned.

Series II The second experiment was conducted in a similar manner as the first one with the following changes:

1. In order to reduce the variability among the animals due to the fluctuation in previous hepatic storage of vitamin A, all rats were subjected to a depletion period of three weeks as described on page 91. This was deemed necessary, since it was thought that the pre-vitamin A status of the animal might have a bearing on the intestinal metabolism of carotene.
2. The exogenous protein formula was not administered in the intestinal loop during ^{14}C-carotene absorption for reasons discussed above.
3. The chemical analysis in terms of chromatographic measures was

altered as discussed on page 72.

Methodology Weanling rats, eight litters of two animals each, were purchased from Simonsen Laboratories. After a depletion period of three weeks, they were divided into two groups, one receiving 20% casein protein in the diet and the other 40%. Pairs were used for the two treatments. Vitamin supplements together with 120 mcg. of carotene were given to animals every alternate day during this repletion period so that the last dose of non-radioactive carotene was fed 48 hours prior to the experiment. Paired comparison was used to analyze the data.

The animals were on this regime for two weeks. Food jars were removed 4 hours before surgery, so that the small intestine would be free of solid dietary materials during the operation. A dose of approximately 20 mcg. of ^{14}C-carotene containing 3 mg. of dextrose was injected into the ligated loop of upper intestine as in the previous trial.

After an interval of 1 hour, the intestinal contents and intestinal walls were analyzed for the unconverted carotene, retinol, retinyl esters, and the miscellaneous breakdown products. The total activity of the non-saponifiable fraction of the livers was also determined.

Results and discussion Table 12 shows the per cent radioactivity of the injected dose recovered in different fractions from the chromatographic columns. Group 1-40 C had more than twice the amount of retinyl ester present in the intestinal walls than did group 1-20 C. However, due to the large standard error, a significant difference between the two groups could not be demonstrated. Whereas 9.04$\pm$3.42 per cent of the

Table 12. Radioactivity recovered in each chromatographic fraction of different tissue extracts, in percentage of injected dose of ^{14}C-carotene (Values represent mean of 8 animals/group ± standard errors), Series II

Group symbol	Extract	Fraction I Carotene	Fraction II Retinyl ester	Fraction III Retinol	Fraction IV Misc. break-down prod.	Total
1-20C	Small Intestine-wall	12.7±2.4[a]	4.18±2.57	0.70±0.17	0.34±0.12	17.9±2.5
	Small intestine contents	41.4±4.5	1.20±0.17	1.71±0.20	0.76±0.12	45.1±4.4
	Small intestine+ contents	53.9±5.1	5.38±2.62	2.38±0.30	1.05±0.14	63.0±4.2
	Liver	-	-	-	-	0.3±0.1
	Total	-	-	-	-	63.1±4.2
1-40C	Small intestine-wall	16.4±3.2	9.04±3.42	0.88±0.17	0.31±0.10	26.6±5.9
	Small intestine contents	42.3±6.0	0.90±0.20	1.09±0.23	0.32±0.07	44.6±6.2
	Small intestine+ contents	58.6±4.4	9.94±3.55	2.02±0.27	0.63±0.14	71.2±5.1
	Liver	-	-	-	-	0.4±0.1
	Total	-	-	-	-	64.5±6.9

[a]Standard error of the mean.

injected dose had been recovered in the ester fraction of the intestinal; wall of group 1-40C, only 4.03±2.57 per cent had been recovered in group 1-20C.

The amount of labelled retinol deposited during the period had also been low. Only 0.3±0.1 per cent of the injected dose was recovered in the livers of group 1-20C and 0.4±0.1 per cent in group 1-40C. This difference again was not significant. It was obvious here that attempts should be made to:

1. Reduce the variability within the groups
2. Increase the hepatic deposition of the newly formed retinol which was to be taken as one of the parameters of carotene metabolism.

The low hepatic deposition of retinol during the experimental period, could have been due to a reduced rate in the digestive processes, caused by the ligation of the intestine during carotene absorption. Sibbald and Olsen (1958) had also failed to relocate the retinol biosynthesized from carotene in the ligated intestinal loop of chicks in other tissues. Their interpretation was that peristalsis was inhibited by ligation of the intestine and that this might have hampered the movement of lymph and thus normal digestion.

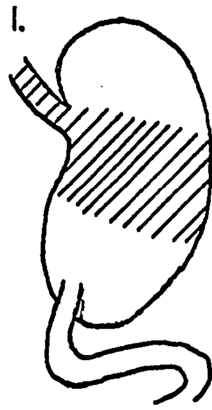
In the next experiment it was decided to inject ^{14}C-carotene intraduodenally without the ligature of the loop.

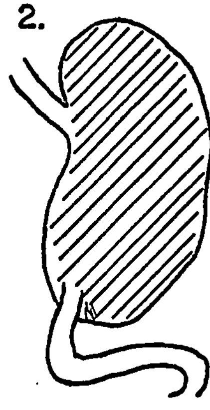
Series III The procedure followed in series III was the same as the one described for series II with the following modification:

The dose of carotene was injected into the duodenum without ligation of the intestinal loop. This mode of administration made it possible to have the customary ingesta in the intestine during ^{14}C-carotene absorption. For this purpose food was withheld for 15 hours. This not only cleared the gastrointestinal tract of previous material but also induced the animals to consume a test meal offered subsequently within 30 minutes. The test meal consisted of 1 gm. of the same diet fed previously to the animals. It was offered 2 hours prior to surgery. Basis for choosing this 2 hour-time interval were observations obtained from a pilot study where the passage of food through the gastrointestinal tract was followed for a period of 3 hours. Figure 4 shows the presence of food in different parts of the tract at different periods of time. Since the presence of food had been noted in the small intestine between the second and third hour, it was decided to inject labelled carotene 2 hours after the test meal and allow a period of 1 hour for absorption. Thus it was hoped that all the animals would be in a similar physiological state with respect to the digestive process at the beginning of the experiment. This modification made analysis of the stomach and colon for radioactivity essential. A pilot study had shown that without this ligature animals tended to regurgitate some intestinal contents into the stomach. In addition, the passage of some of the intestinal contents into the colon one hour after the operation had been observed.

Figure 4. Schematic presentation of passage of test meal through the digestive tract in 4 rats killed at intervals. Shaded areas represent digesta:

1. 30 min. after feeding
2. 60 min. after feeding
3. 120 min. after feeding
4. 180 min. after feeding.


1.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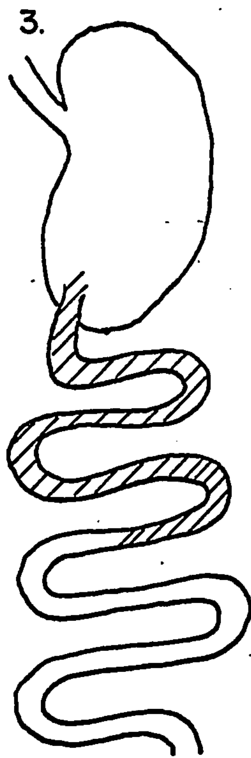
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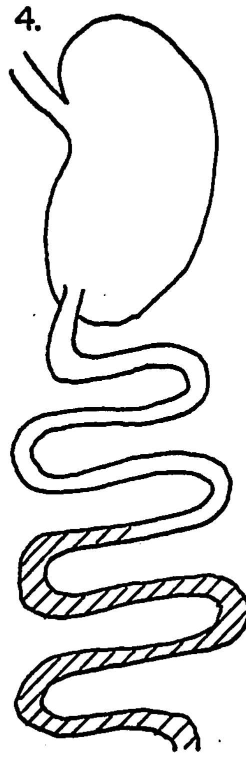
4.

<u>Methodology</u> Twenty animals were pretreated in a similar manner to the ones in series II. The rats were randomly assigned to two groups maintained on 20 and 40% protein in the diet. Data was treated for group comparison.

On the 15th day, all the animals were fasted overnight for 15 hours. After that 1 gm. of the test meal together with a supplement of vitamins devoid of carotene was given. Two hours after feeding, ^{14}C-carotene was injected into the duodenum, just below the pyloric sphincter without the ligature. The procedure was as follows:

1. Anaesthetize the rat with ether
2. Make a small incision on the ventral side just off the midline
3. Open the abdominal cavity gently
4. Locate the stomach and the duodenum
5. Holding the stomach gently with a pair of blunt forceps, inject slowly a dose of ^{14}C-carotene into the duodenum just below the pyloric sphincter. See that the needle is directed toward the jejunum and not towards the stomach.
6. If the organs have protruded out of the cavity during this operation replace them into the abdomen with gentle pressure.
7. Close the abdominal skin with surgical clips and return animal to cage. Start time period with the injection of the radioactive carotene.

   Record the time when rat comes out of anaesthesia.

   2½ hours later:
8. Reanesthetize the animal with ether.

9. Open up the abdominal cavity.

10. Ligate the duodenum between the pyloric sphincter and the entrance of the bile duct with cotton thread.

11. Deflect the small intestine to operator's right. Locate the small edge of mesentary holding the small intestine together.

12. Locate the center point of the mesentary edge and follow it out to the intestine. Ligate with a thread. Cut the intestine just anterior to the ligature and immediately clip an artery forcep to one edge to hold the end of the intestine into a 500 ml. erlenmeyer flask which contains 50 ml. of a solvent mixture of skelly B and ethanol (3:1).

13. Using a no. 18 hypodermic needle of 2" length, force 30 ml. of isotonic saline, maintained at 37°C., through the intestine from the anterior end. Collect the flushings in a separate erlenmeyer flask. Prepare for chemical analysis.

14. Remove the ligated intestinal loop without stretching. Do not allow any of the washings remaining in the intestine to enter the erlenmeyer flask containing intestinal contents. Measure the loop. It should be 18-24" in length.

15. Collect the intestine into another erlenmeyer flask containing 50 ml. solvent mixture of skelly B and ethanol (3:1) and treat as described in the extraction procedure.

16. Remove the stomach and treat as described in the extraction procedure under stomach.

Stomach could be frozen and stored in liquid nitrogen until the time of analysis.

17. Remove the liver. Saponify as described in the extraction procedure. Freeze and store if necessary and analyze later.
18. Remove the colon. Put it into another 500 ml. erlenmeyer flask containing 50 ml. skelly B and ethanol (3:1) and treat as described in the extraction procedure.
19. Rinse the syringe with skelly-ethanol (3:1) solvent system and return the washings to the vial which had contained the dose injected into the animal.

    Determine the radioactivity of the residual carotene and subtract from the original dose. This will give the radioactivity injected into the intestine.

Results and Discussions The percentage radioactivity of the injected dose recovered in each of the fractions of the chromatographic column or the total activity of the tissues studied are shown in Table 13.

Animals in group 1-20 C had a mean intestinal retinyl ester content of 6.26 ± 1.97 whereas those of group 1-40 C had 7.30 ± 2.63. The difference was insignificant.

Group 1-20 C stored a mean of 1.22 ± 0.27 per cent of the injected dose in their livers and group 1-40 C, a mean of 0.85 ± 0.21. Again the difference was not significant. The increase in liver storage found here compared to that in series II could have been due to the unligated intestinal state and/or the natural food present in the intestines during ^{14}C-carotene absorption. Individual variability in hepatic storage seemed to be less than that found in series II as shown by smaller standard errors.

Series IV Up to this phase in the study the levels of protein used had been limited to either adequate or excessive amounts, that is 20 or 40% of the diet. Results of a pilot study (Appendix, Table 27) using liver vitamin A storage following supplementation with carotene over two weeks had indicated that the effect of protein could not be demonstrated between animals fed 20 and 40% protein in their diet. Consequently in series IV, 10 or 40% casein protein was incorporated into the experimental diets. Furthermore, the intestinal metabolism of ^{14}C-carotene was studied over a period of 5 hours taking hepatic retinol as the parameter of carotene utilization. The total radioactivity in the non-saponifiable fraction of serum was also determined in this experiment.

Methodology Seven pairs of animals were pretreated like those in series II, except that each of the pair was maintained on either 10 or 40% casein protein in the diet. Seventeen hours before surgery the rats were given a dose of 60 mcg. carotene in the vitamin supplements. It was hoped that feeding the substrate might induce any enzyme which might be involved in the conversion process. The 48 hour period used previously in series II and III might have been too long to maintain enzyme activity without substrate.

After an overnight starvation of 15 hours a test meal consisting of 1 gm. diet and the vitamin supplements without carotene was offered to all animals. Two hours later, a dose of approximately 20 mcg. of ^{14}C-carotene was injected into the duodenum without ligation. After an interval of 5 hours blood was collected by heart puncture. The intestinal content was flushed out with saline and collected. Stomach, intestine, colon, and liver were removed. All the tissues were prepared for chemical analysis. The

Table 13. Radioactivity recovered in each chromatographic fractions of different tissues, in percentage of injected dose of ^{14}C-carotene (Values represent means of 10 animals/group together with the standard errors of the mean), Series III

Group	Tissue	Fraction I Carotene	Fraction II Ret.esters	Fraction III Retinol	Fraction IV Breakdown prod.	Total
1-20C	Small intestine wall	14.0±1.3[a]	6.26±1.97	1.44±0.12	1.66±1.40	23.4±2.7
	Small intestine cont.	38.2±4.7	1.19±0.17	1.80±0.23	0.72±0.37	41.9±4.8
	Stomach	11.3±2.4	0.65±0.17	0.51±0.12	1.88±1.47	13.0±2.8
	Stomach + small int.+ contents	59.7±5.2	7.88±2.27	3.74±0.3	2.69±1.53	74.2±6.2
	Liver	--	--	--	--	1.2±0.3
	Colon	--	--	--	--	0.4±0.1
	Serum	--	--	--	--	--
	Total act. recovered	--	--	--	--	78.7±6.3
1-40C	Small intestine wall	16.1±2.6	7.30±2.63	1.75±0.41	0.25±0.08	25.5±3.4
	Small intestine cont.	42.4±7.6	1.21±0.26	1.79±0.24	0.74±0.15	46.2±7.9
	Stomach	9.1±3.8	0.40±0.24	0.73±0.37	0.57±0.22	10.9±4.6
	Stomach + small int.+ contents	67.7±6.1	8.91±2.65	4.36±0.49	1.56±0.20	82.6±6.0
	Liver	--	--	--	--	0.9±0.2
	Colon	--	--	--	--	0.3±0.1
	Serum	--	--	--	--	--
	Total act. recovered	--	--	--	--	83.7±6.0

[a]Standard error of the mean.

procedure here was the same as the one used in series III.

Results and discussion The data in Table 14 show that group 5-10 C stored on an average 2.8 ±0.43 per cent of the injected dose in the liver, while the corresponding value of group 5-40 C was 4.4 ±0.64. Even though variability had decreased, the difference was found to be significant only at the 10% level with a student's 't' test.

The intestinal retinyl ester values in per cent of the injected dose were 1.83±0.74 for group 5-10 C and 1.66±0.70 for group 5-40 C. As would be expected, these values were much lower, five hours after the injection of the dose, than those obtained after shorter time intervals in previous experiment.

The total unconverted carotene in the stomach and small intestine in the two groups and the total activity found in colon in the two groups at the end of 5 hours show vast variability in digestive processes of individual animals as indicated by the large standard errors (Table 14).

The serum retinol values, in percentage of injected dose, were 1.56 and 1.70% for groups 5-10 C and 5-40 C respectively. The difference between the two values was negligible.

Modifications suggested for series V Previous work in this laboratory (Hiller, 1963; Ruffin, 1965) had shown that animals fed 40% casein protein in the diet had significantly more vitamin A stores than the ones fed 20% casein protein, and those fed 10% protein in the diet had still less vitamin A stores than the ones fed 20% over a period of 4 weeks. Since it was suspected that hepatic deposition was an accumulative effect of the level of protein, the length of the feeding period and also the amount of

Table 14. Radioactivity recovered in each chromatographic fraction of different tissues, in percentage of injected dose of ^{14}C-carotene (Values represent means of 7 animals/group together with the standard errors of the mean)

Group	Tissue	Fraction I Carotene	Fraction II Ret.esters	Fraction III Retinol	Fraction IV Breakdown prod.	Total
5-10C	Small intestine wall	15.2±5.1[a]	1.83±0.74	0.55±0.14	0.24±0.06	17.8±4.9
	Small intestine cont.	13.1±4.0	0.50±0.20	0.75±0.17	0.86±0.14	15.2±4.0
	Stomach	2.9±1.0	0.39±0.2	0.05±0.03	0.29±0.27	3.6±1.1
	Stomach + small int.+ contents	30.5±5.1	1.98±0.5	1.19±0.27	1.42±0.38	30.1±6.3
	Liver	---	---	---	---	2.8±0.4
	Colon	---	---	---	---	14.4±6.0
	Serum	---	---	---	---	1.6±0.7
	Total act. recovered	---	---	---	---	54.0±5.3
5-40C	Small intestine wall	7.2±3.2	1.66±0.70	0.80±0.27	0.22±0.10	9.9±3.6
	Small intestine cont.	7.3±3.9	0.41±0.13	0.31±0.12	0.54±0.25	8.6±3.9
	Stomach	2.0±0.8	0.10±0.10	0.16±0.08	0.28±0.13	2.6±0.8
	Stomach + small int.+ contents	16.5±5.7	2.17±0.81	1.26±0.20	1.06±0.28	21.1±5.9
	Liver	---	---	---	---	4.4±0.7
	Colon	---	---	---	---	20.3±6.7
	Serum	---	---	---	---	1.7±0.1
	Total act. recovered	---	---	---	---	49.3±9.1

[a]Standard error of the mean.

carotene fed, it was planned to feed the animals for 4 weeks and use animals fed either 10 or 40% protein.

Variability in the rate of passage of ingesta in the digestive tract was apparent, as indicated by values for unconverted ^{14}C-carotene in the small intestine and the colon after a period of five hours (series IV). Therefore it was thought desirable to inject ^{14}C-carotene one hour after feeding the test meal instead of waiting for 2 hours.

Since one of the objectives of the study was to characterize carotene metabolism in terms of the amount of retinyl ester formed from ^{14}C-carotene in the intestinal wall and of retinol accumulated in the liver, conditions were selected which would give the best compromise for the two parameters. Olson (1961a) had shown in his experiments that the peak period of conversion of ^{14}C-carotene injected introduodenally was one hour, as determined by the presence of retinyl esters in the intestine. In our study, liver deposition in one hour had been very low. Although appreciable amounts of retinol were found in the liver after 5 hours, retinyl ester in the wall of the intestines had decreased considerably. An experiment to determine the rate of formation of retinyl ester formed from a dose of carotene at different intervals of time had not been done, since such an experiment would not have been valid due to the variability among the animals. Thus an interval of two and a half hours was chosen as the time interval for the study of carotene absorption. It was hoped that this interval would be a satisfactory compromise yielding appreciable quantities of metabolic products in the intestinal walls as well as in the liver.

Summary of Development of Methods

A suitable method for producing and purifying biologically labelled beta-carotene from tobacco plants exposed to $^{14}CO_2$ was developed. Size of plant, number and relative maturity of leaves were factors to be considered. Plants, 12" high, with 3-4 young but not tender leaves, per 1 mc. of $Ba^{14}CO_3$ were used, giving optimum yields combined with highest specific activity.

From a number of published procedures concerned with extraction and purification of non-radioactive beta-carotene, a suitable method was developed for the isolation of biologically labelled carotene from other labelled metabolites of the plant. Extraction of the plant tissue with a skelly B/acetone mixture followed by saponification and further extraction of the non-saponifiable material comprised the extraction step. The radioactive contaminants, probably sterols, were next removed by cooling and filtering the extract through dry ice.

Subsequent purification was achieved by double chromatography on alumina and on calcium hydroxide. The former separated carotenes from other plant pigments while the calcium hydroxide column isolated beta-carotene from its geometric isomers. Thus a relatively pure sample of beta-carotene labelled with Carbon-14 was obtained. The purity was checked by comparing the absorption spectrum of the biosynthesized sample with that of pure synthetic beta-carotene.

Lipid extracts of animal tissues were prepared by homogenization with a mixture of skelly B/ethanol (3:1) and subsequent washing of the solvent

layer with 2% calcium chloride solution.

Separation of unconverted beta-carotene and metabolic products of the injected carotene from the tissues was achieved by column chromatography. A column of 7% alumina was found to be effective in the separation of beta-carotene, retinyl esters, retinol, and the miscellaneous breakdown products in tissue extracts. These compounds were eluted with skelly B, 15% benzene, 100% benzene, and 8% ethanol respectively. Recoveries of more than 95% were obtained for synthetic samples of beta-carotene and retinyl esters, but recovery of synthetic retinol was only 90%. When synthetic carriers of beta-carotene, retinyl esters, and retinol were added to animal tissue extracts, recoveries were reduced to approximately 90% for beta-carotene and retinyl esters and 80% for retinol. These values were taken as criteria for satisfactory separation of the compounds under investigation for subsequent series of animal experiments.

Four animal series were conducted in order to develop suitable techniques for the evaluation of the degree of absorption and/or conversion of ^{14}C-carotene in rats fed two different levels of casein protein in the diet. Various combinations of pretreatments and surgical procedures were explored with regard to their suitability in simulating physiological conditions during absorption.

Low yields in the biologically active metabolic products of carotene from the upper intestinal tract, low levels of ^{14}C-labelled hepatic retinol and excessive variability among animals subjected to various treatments led to a number of modifications of the original procedure. In the first series ^{14}C-carotene had been injected into the ligated loop of the upper small intestine together with casein hydrolysate-albumin mixtures compounded

to simulate nitrogenous material present during carotene absorption. Young stock animals maintained for a brief period on experimental diets before the *in vitro* experiment had been used in the first series.

Major modifications developed in series II to IV dealt with a) dietary pretreatment, b) ligation of the intestine, c) presence of the digesta during carotene absorption, and d) selection of a suitable period for the intestinal carotene metabolism. Supported by results from previous studies, the dietary pretreatment was changed to include periods of several weeks duration of controlled hepatic retinol accumulated in the presence of varying protein intakes. Modifications for b) and c) were developed simultaneously. Because of possible interference with the digestive sequences in the alimentary canal as a result of intestinal block, the ligation was omitted and analysis of the stomach for regurgitated material and of the colon for material that had escaped absorption were included in the procedure. This change from the original procedure allowed the presence of digesta from test meals at the site of absorption of duodenally injected ^{14}C-carotene. This step simulated the physiological processes of digestion of dietary carotene.

Data from series I were not interpreted because methods of analysis needed modification. Data from series II through IV failed to reflect differences between dietary pretreatment. In some instances means between groups were similar, in others variabilities within groups were too great for interpretation. Though means differed in some, the recovery of biologically active products from ^{14}C-carotene metabolism had been low. Thus the results were used to formulate recommendations for the final ex-

periment. These included:

1) Introduction of a 4-week preliminary feeding period.

2) Comparison of groups maintained on either inadequate or excessive protein intakes.

3) Omission of the intestinal ligation.

4) Use of a test meal.

5) Use of observation times of 2.5 hours.

6) Support for the _in vitro_ study of ^{14}C-carotene metabolism with a feeding experiment with protein and time of supplementation as variables.

EFFECT OF PROTEIN ON BIOSYNTHESIS OF RETINOL

Studies conducted previously in this laboratory which had established that the utilization of carotene measured by hepatic retinol storage was dependent on protein intake had been based on a refeeding period of four weeks. Data were not available to support the assumption that the protein-carotene relationship could also be demonstrated after two weeks of refeeding. Yet it had been assumed that time of repletion was not a factor to be considered. Therefore in planning the four experiments described in the section on Development of Methods (series I - IV) a two week- rather than a four-week refeeding period had preceded the *in vitro* experiment with radioactive carotene. Subsequently data obtained in a pilot study (Appendix, Table 27) had indicated that the carotene-protein interrelationship might be time dependent, since groups fed adequate and excessive amounts of protein seemed to have similar hepatic retinol stores after two weeks of refeeding, while previously the four week refeeding period had consistently shown large differences in carotene utilization in response to these two levels of protein intake.

In an attempt to clarify the relationship involved it was decided to combine in series V the tracer *in vivo* experiment with a feeding study where time of feeding as well as protein intake would be the variables.

The objectives of series V were to investigate:

1. The utilization of non-radioactive carotene fed over different periods of time to groups of rats maintained on graded levels of casein in the diet.

2. The metabolism of ^{14}C-carotene during the digestive phase in vitamin A depleted rats refed with non-radioactive carotene for four weeks, and maintained on two different levels of casein.

To meet the first objective it was planned to correlate the magnitude of hepatic retinol stores with the length of the repletion period as well as with the three levels of dietary protein, representing inadequate, adequate and excessive intakes of this nutrient. The relative rates of hepatic retinol deposition for different protein intakes over one, two, three and four week periods were to be determined.

To meet the second objective two groups of rats from the first phase of the study, those fed inadequate and excessive levels of protein for four weeks (groups 10C-4 and 40C-4, Table 15) were to be used to explore the metabolism of ^{14}C-carotene _in vivo_. The method described under Development of Methods, section V, was to be followed with proper modifications. The same parameters as those explored previously were to be used for evaluation of intestinal metabolism.

Procedure

Description of animals, diets, and supplements, as well as mode of depletion of vitamin A stores are found under development of methods, section V.

One hundred twenty six male weanling rats[1] were purchased in litters of three each. All animals were depleted of their stores of vitamin A for

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[1]Simonsen Laboratories, White Bear, Minnesota.

three weeks. Following depletion, the animals were assigned to one of the dietary regimes. Litters were taken as blocks receiving 10, 20, or 40% protein in the diet. The litter-mates were randomly assigned to the three groups so that each diet was fed to one of the rats of each litter. During this regime, the animals received a dose of 110 mcg. of carotene with their daily supplement of vitamins. Animals were sacrificed after 1, 2, 3, or 4 weeks of repletion with carotene. For each period 10 or 12 litters were selected at random. Thus a randomized block design involving a factorial treatment combination was obtained. A split plot design with time as main plot and protein as sub plot was used to analyze the data statistically.

The group symbols together with the treatment received by the group are given in Table 15.

Animals sacrificed after 1 - 3 weeks of refeeding

After an overnight fast of 15 hrs. the rats were offered 1 gm. of their respective diets together with 20 mcg. of carotene in the vitamin supplements. Four and one-half hours after the test meal an injection of sodium pentobarbital (50 mcg./100 gm. body weight) was given and the animals were sacrificed by rupture of the hepatic vein. Livers were removed and prepared for chemical analysis of vitamin A by the Carr-Price reaction.

Animals sacrificed after 4 weeks of feeding

Autopsy procedures for rats killed after 4 weeks of refeeding differed slightly from those autopsied after 1 - 3 weeks, since the two groups fed

Table 15. Group symbols and treatments used in series V (All animals fed 110 mcg. labelled carotene during repletion period)

Treatment	Group symbol	No. animals/ group	% casein protein in diet	Exp. period repletion (weeks)	24 hr. pretreatment	Carotene injection into	Nature and amt. of carotene	Exogenous protein in loop	Autopsy after dose (hrs)	Tissues analyzed	Fractions assayed
1	10C-1	10	10	1	15 hr. fast then 1 gm. diet 4½ hrs before sacrifice	oral feeding	Non-radioactive 20 mcg	Natural food	4.5	Liver	Retinol
2	20C-1	10	20	1							
3	40C-1	10	40	1							
4	10C-2	10	10	2							
5	20C-2	10	20	2							
6	40C-2	10	40	2							
7	10C-3	10	10	3							
8	20C-3	10	20	3							
9	40C-3	10	40	3							
10[a]	10C-4	12	10	4	15 hr. fast then 1 gm. 1 hr. before surgery	loop without ligation	radioactive 20 mcg		2.5	Intest. Walls Intest. cont. Liver Stomach Colon Serum	I Garotene II Retinyl-ester III Retinol IV Breakdown products
11	20C-4	12	20	4							
12[a]	40C-4	12	40	4							

[a]These groups were used for radioactive experiments.

10 and 40 per cent protein in the diet (10C-4 and 40C-4) were to be used for the experiment with radioactive carotene. It was assumed that the procedures used did not affect the hepatic retinol which had accumulated over the 4-week period of supplementation. This assumption seemed justified on the basis of the small amounts of retinol recovered in the liver a few hours following injection of 20 mcg. of carotene (Series IV).

After an overnight fast of 15 hours all rats were offered 1 gm. of their respective diets together with vitamin supplements devoid of carotene. One hour later, the rats were anaesthetized with ether. A dose of approximately 20 mcg. of either labelled or non-radioactive carotene was injected intraduodenally without ligation of the intestines. Two and one-half hours later, the animals were reanaesthetized. Group 20C-4 which was not used for the experiment with radioactive carotene was sacrificed by rupture of the hepatic vein. The livers of these animals were then removed and prepared for chemical analysis of vitamin A. In groups 10C-4 and 40C-4, blood was drawn by heart puncture. The intestinal contents were flushed out and collected. Intestinal walls, stomach, colon and liver were removed and were treated as described for series IV.

For groups 10C-4 and 40C-4 the experimental procedure used was that described for series IV with modifications. In the present series, however, an aliquot of the saponified liver was used for the chemical determination of vitamin A by the Carr-Price reaction. Another aliquot was extracted and subsequently chromatographed on alumina to determine the ^{14}C-retinol content. Fraction II and III were eluted together since the sample had been saponified.

It was expected that activity of the serum of individual rats would be too low for quantitative estimation. Consequently, it seemed advisable to pool samples for counting. Equal aliquots of serum from 4 sets of 3 rats per group were pooled, saponified, extracted and chromatographed on alumina to separate beta-carotene, retinol and the terminal polar fraction.

Results and Discussion

Rate of growth

Mean initial weights, taken at the end of the preliminary depletion period, were approximately 142 gm. for all twelve groups, with a range from 135 to 149 gm. Means for initial weights of any three groups, scheduled to be autopsied at the same time, did not vary more than 5 gm from each other (Table 16). This design put the main effect on protein intake and subjugated the effect of length of feeding period. Sets of groups refed for either 1 or 3 weeks were heavier initially than those refed for 2 or 4 weeks. This fluctuation in mean initial weights puts a limitation on the interpretation of the data with respect to time.

Since food intake had been slightly restricted with reference to the animals' potential *ad libitum* consumption, gains in weight over the four week experimental period did not reflect the optimum which could have been attained on the different protein levels. However the slight restriction imposed made it possible to eliminate the energy value of the dietary intake as a variable in the study. In spite of the restriction imposed, rats receiving adequate amounts of protein (that is either 20 or 40 per cent protein in the diet) showed a higher overall rate of growth than those maintained on an inadequate protein intake (Table 17, Figure 5).

Table 16. Mean values for body and liver weights, and hepatic vitamin A for young vitamin A depleted rats fed carotene

Symbol	No. rats	Mean values for body and liver weights - repletion period				Hepatic vitamin A	
		Initial g	Final g	Gain g	Liver g	Total	Concentration
10C-1	10	146	156	10	5.5	116	21.2
20C-1	10	143	168	25	6.4	197	31.0
40C-1	10	144	166	21	6.6	192	29.2
10C-2	10	140	166	27	6.1	195	32.5
20C-2	10	137	187	50	7.5	323	43.4
40C-2	10	137	188	51	7.3	355	49.9
10C-3	10	146	198	52	6.5	211	32.1
20C-3	10	149	227	77	8.3	417	50.8
40C-3	10	148	225	77	8.4	463	54.9
10C-4	12	135	202	67	6.5	391	60.4
20C-4	12	140	238	99	8.2	604	75.0
40C-4	12	136	243	107	8.9	811	94.3

Table 17. Mean values for gain in weights for all animals in the study

Protein in diet	Gain in weight in grams Period of repletion in weeks			
	1[a]	2[b]	3[c]	4[d]
10%	12±0.69[e]	31±1.35	53±1.71	67±2.9
20%	23±0.68	51±1.74	77±1.50	99±2.92
40%	22±0.84	51±2.24	80±2.29	107±2.6

[a]42 animals/group.

[b]32 animals/group.

[c]22 animals/group.

[d]12 animals/group.

[e]Standard error of the mean.

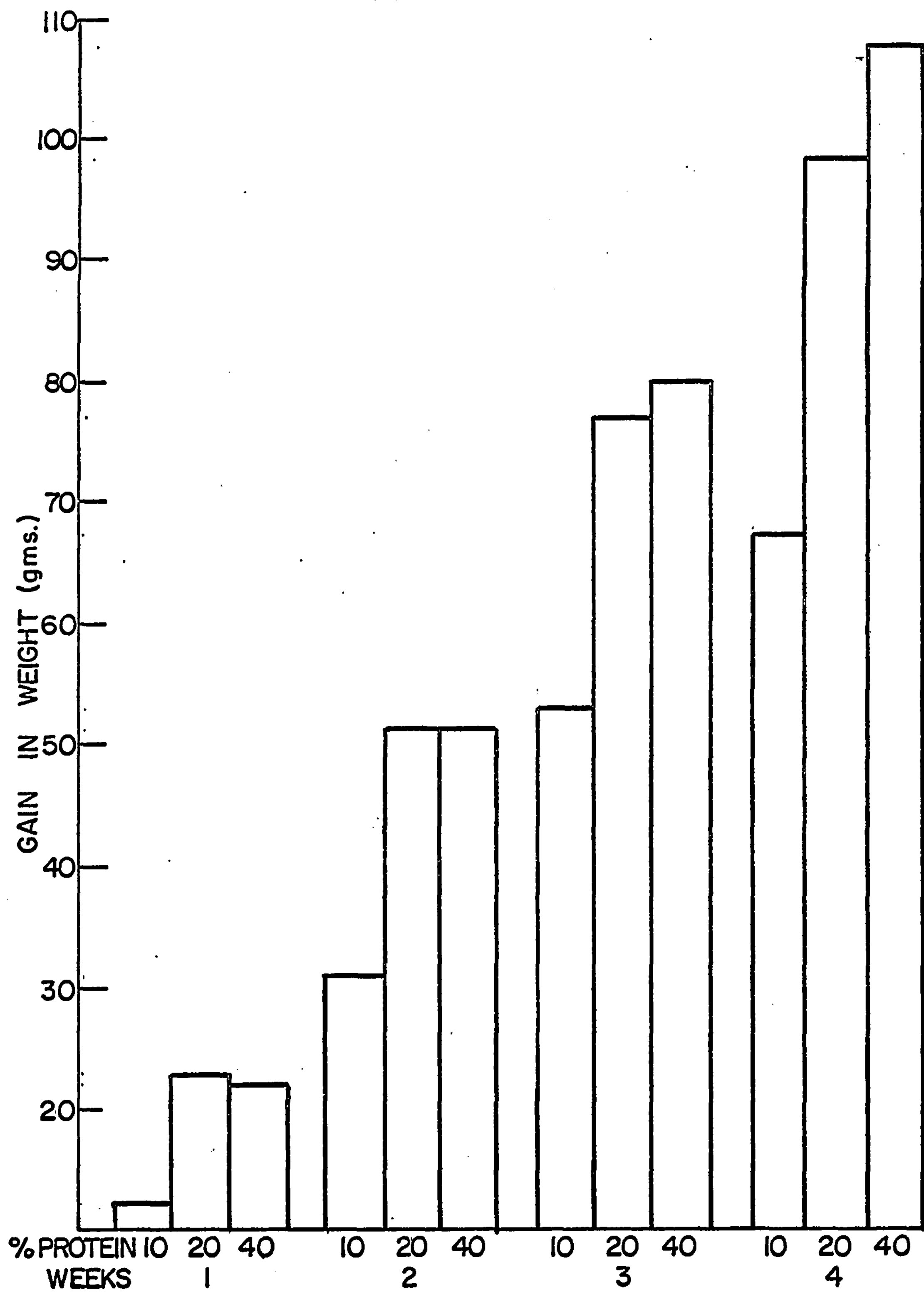
The difference was apparent after the first week, and was maintained throughout the whole period.

Groups receiving 20 or 40 per cent protein in the diet made similar gains for the first three weeks. For instance mean gains for animals fed either 20 or 40% protein in the diet were almost identical with values of 77 and 80 gm. respectively for the three week period.

However in comparable groups kept on the experiment for four weeks, total gain was slightly but significantly greater in group 40C-4 compared to that of group 20C-4. Reasons for the difference in growth rate of these two groups is not apparent.

Under conditions of moderate food restriction, optimum response in terms of rate of growth to the dietary protein level may be attained with

Figure 5. Mean gain in weight of groups of rats fed 3 different levels of protein for 1, 2, 3 or 4 weeks.


GAIN IN WEIGHT (gms.)
110
100
90
80
70
60
50
40
30
20
% PROTEIN 10 20 40 10 20 40 10 20 40 10 20 40
WEEKS 1 2 3 4

a ration furnishing approximately 20 per cent of casein protein. This conclusion is based on the fact that in similarly designed studies carried out in this laboratory, growth rates were identical in groups fed either 20 or 40 per cent casein protein in their diets, (Johnson, 1959; Hillers, 1963; Ruffin, 1965). Occasional exceptions were also seen, when the excessive levels of dietary protein caused a slight additional stimulation to the rate of gain (MacMillan, 1966). However it seems reasonable to assume that the optimum of protein intake for growth under the specific conditions of this type of experiment is close to the 20 per cent level.

Hepatic stores of animals fed graded levels of protein over different periods of repletion

Table 18 shows the mean hepatic stores of vitamin A of different groups. The analysis of variance (Table 19) indicates that the effect of protein, of time of repletion as well as of the interaction between these two factors is significant. Therefore it is apparent that variables, protein and time of repletion, individually influence the utilization of carotene as seen by the magnitude of hepatic stores of vitamin A. These two variables when considered together interact while exerting their influence. The two factors will be considered separately first.

Effect of protein

As the level of protein increased from 10 to 20 per cent of the diet rats stored significantly larger amounts of retinol in their livers through out the repletion period, that is after 1, 2, 3 and 4 weeks (Figure 6). At the end of 4 weeks, rats fed low levels of protein, had a mean value

Table 18. Mean hepatic vitamin A values

Protein in diet	Period of repletion in weeks Mean of hepatic vitamin A in mcg. 1	2	3	4
10%	116±15.4[a]	195±19.0[a]	211±21.0[a]	391±26.9[b]
20%	197±20.0[a]	323±33.5[a]	417±37.5[a]	604±45.5[b]
40%	192±17.6[a]	355±28.2[a]	463±40.5[a]	811±41.5[b]

[a]10 animals/group.

[b]12 animals/group.

Table 19. Analysis of variance for hepatic vitamin A values

Source of variation	Degrees of freedom	Mean square	"F" values
Time of repletion (A)	3,	111420	58.37**
Time of repletion within litters	38	19088	
Percent protein in the diet (B)	2	608074	46.03**
Interaction AB	6	63740	4.82**
Error	76	13211	

**Significant at the 1% level.

Figure 6. Mean hepatic retinol of groups of rats fed 3 different levels of protein for 1, 2, 3 or 4 weeks.

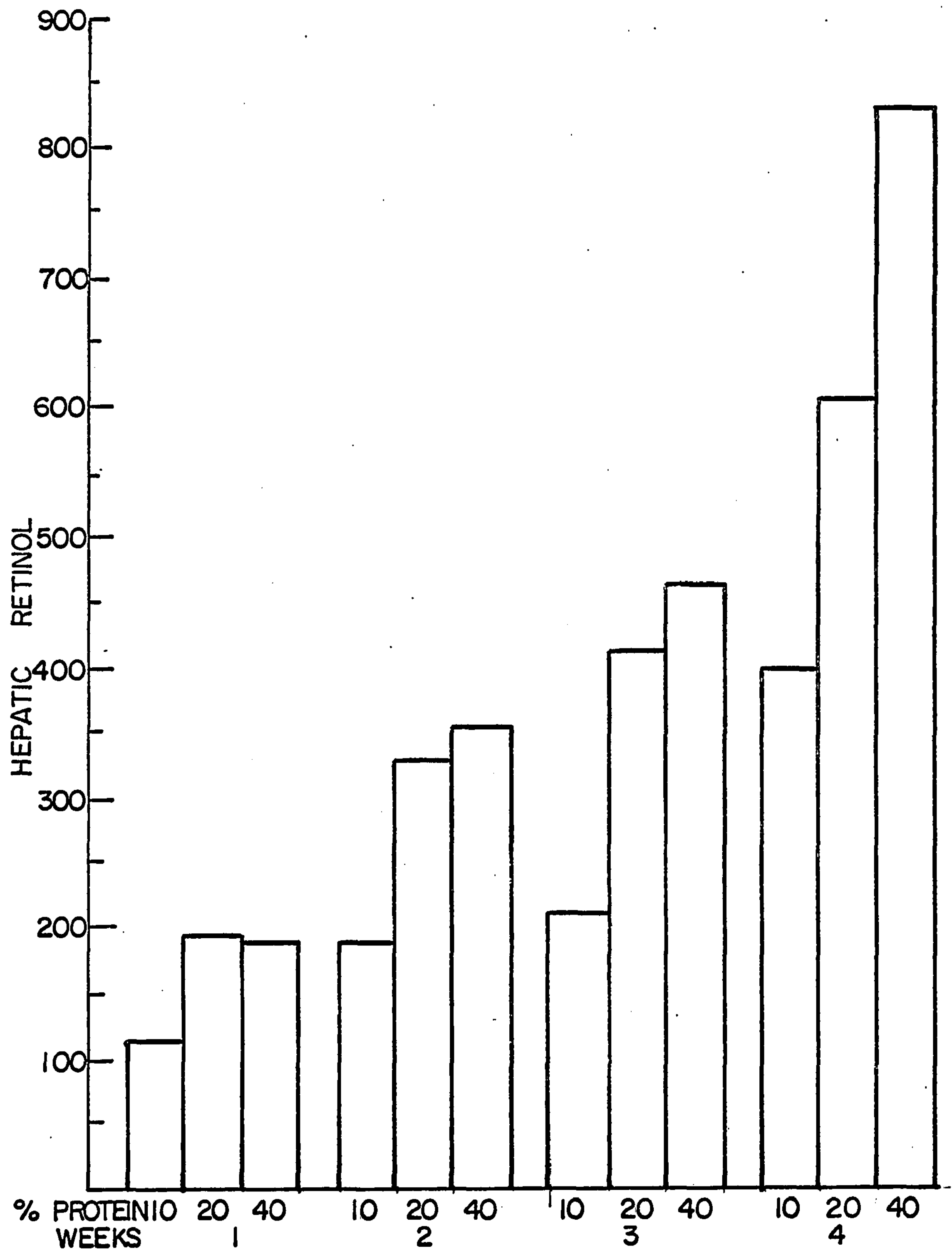

900
800
700
600
500
400
300
200
100
HEPATIC RETINOL
% PROTEIN 10 20 40 10 20 40 10 20 40 10 20 40
WEEKS 1 2 3 4

Table 20. P values for hepatic vitamin A for Table 18

Groups compared		P value
10C-1	20C-1	0.01
20C-1	40C-1	-[a]
10C-1	40C-1	0.01
10C-2	20C-2	0.01
20C-2	40C-2	-[a]
10C-2	20C-2	0.01
10C-3	20C-3	0.01
20C-3	40C-3	-[a]
10C-3	40C-3	0.01
10C-4	20C-4	0.01
20C-4	40C-4	0.01
10C-4	40C-4	0.01
10C-1	10C-2	0.01
10C-2	10C-3	-[a]
10C-3	10C-4	0.01
20C-1	20C-2	0.01
20C-2	20C-3	0.1[a]
20C-3	20C-4	0.01
40C-1	40C-2	0.01
40C-2	40C-3	0.05
40C-3	40C-4	0.01

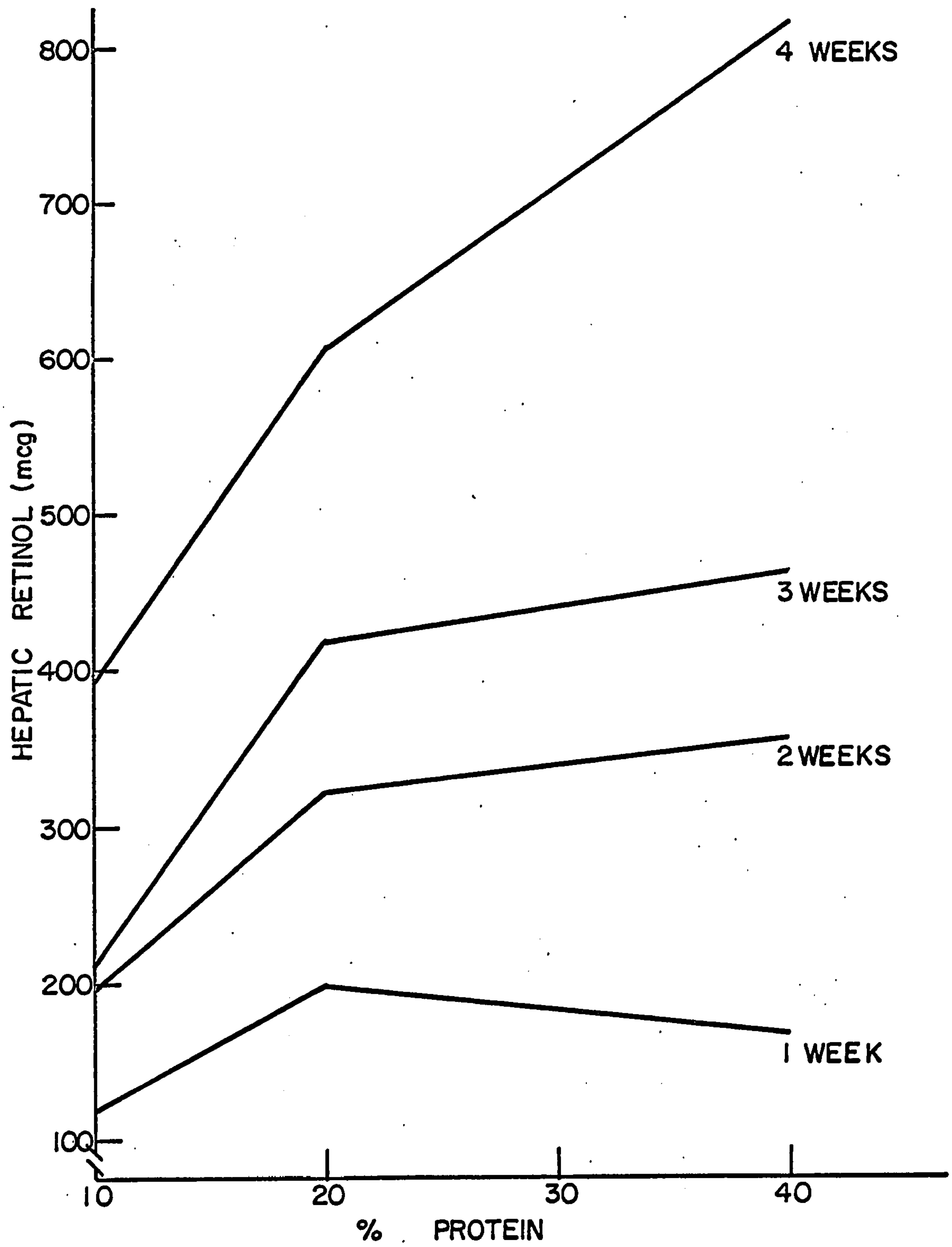
[a]Not significant.

of 391 mg. for hepatic retinol while the corresponding value for group 20C-4 was 604, an increase of 50%. Throughout the study an increase in hepatic stores ranging from 50-100% was observed when the protein intake was increased from inadequate to adequate amounts.

On the other hand no such increase was seen in the hepatic stores for the first 3 weeks when the level of protein was increased from 20 to 40 per cent in the diet. The values were 197 and 192, 323 and 355, and 417 and 463 mg. respectively for groups fed adequate and excessive amounts of protein over 1, 2 and 3 weeks of repletion and differences were not significant (Table 20). A different picture was seen at the end of the 4th week. Rats maintained on the high protein intake had significantly larger hepatic stores of retinol than those fed the adequate level; the values for the two groups were 811 and 604 mg. respectively. However, there was a change in the slope of the curve relating protein intake and hepatic vitamin A (Figure 7) for groups maintained on repletion for 4 weeks. A steep slope with respect to hepatic stores between groups fed inadequate and adequate amount was observed while the slope had considerably decreased between groups fed 20 and 40 per cent protein in the diet.

It has been shown consistently in this laboratory that at the end of a 4-week repletion period, the response of hepatic stores of vitamin A were almost linear over the whole range of protein intakes (Brown, 1961; Hillers, 1963; Ruffin, 1965). In most of these studies approximately 50 mcg. of carotene had been used as the daily supplement, an amount within the range of the recommended dose for growing rats (National Academy of Science National Research Council, 1962). The fact that a dose of 110 mcg.

Figure 7. Mean hepatic retinol of groups of rats fed 3 different levels of protein for 1, 2, 3 or 4 weeks.



HEPATIC RETINOL (mcg)
800
700
600
500
400
300
200
100
4 WEEKS
3 WEEKS
2 WEEKS
1 WEEK
10
20
30
40
% PROTEIN

carotene per day was used in the present study might explain the decrease in slope between 20 and 40 per cent protein. Recently MacMillan (1966) has reported a similar decrease in slope when the dose of carotene increased 3 or 9 times over the control level of 45 mcg. Apparently there is a decrease in carotene utilization when carotene is consumed in relatively large amounts (Olson, 1961a).

Time of repletion

For a given time period the accumulative carotene dose for the three dietary groups had been the same. Therefore liver stores could be compared directly. The interpretation of the significance of changes in hepatic vitamin A stores with time is complicated by the fact that the accumulative dose had increased. To facilitate comparison over time, data were expressed as a ratio of total hepatic retinol to total carotene administered X 100 (Table 21). No clear pattern seems to emerge except the one already referred to in relation to protein as a variable, that is consistently poor utilization of carotene by rats maintained on inadequate protein intake for all periods. Furthermore, the lack of differentiation between groups fed 20 or 40% protein in the diet for the first three weeks and the significant improved carotene utilization of rats on high protein intake for four weeks, has also been discussed. It is tempting to speculate what might have caused a relative decline in the utilization figures during the third week of the experiment. But as indicated earlier the three groups refed for three weeks had higher initial weights than corresponding groups refed for 2 or 4 weeks.

Table 21. Per cent carotene utilization $\frac{\text{hepatic retinol (mcg)}}{\text{carotene dose (mcg)}}$ X 100

Protein in diet	Efficiency of carotene, % dose			
	Period of repletion in weeks			
	1	2	3	4
10%	15[a]	13[a]	9[a]	13[b]
20%	25[a]	21[a]	18[a]	20[b]
40%	24[a]	23[a]	20[a]	26[b]

[a]10 animals/group.

[b]12 animals/group.

In spite of differences in initial weights growth rates had been almost identical at the end of three weeks for animals fed the same diet; for example, groups 10C-3 and 10C-4 gained 55 and 52 gm. respectively during the three week period. Since hepatic retinol constitutes a balance between the vitamin A entering the system from the dietary source and that metabolized for metabolic purposes, it is possible that the relatively heavy rats in groups fed for three weeks showed decreased net utilization when compared to corresponding dietary groups repleted for 4 weeks. It does not seem wise to explore the data further for reasons given above, without confirmation of the findings in another experiment. However, the original premise that a feeding period of at least 4 weeks is needed for the demonstration of the protein effect between groups fed adequate and excessive amounts is still valid.

Intestinal metabolism of ^{14}C-carotene

In this part of the study which is concerned with the intestinal metabolism of labelled carotene, recoveries of activities in individual tissues will be discussed. Different chromatographic fractions will be dealt with when necessary.

Table 22 shows the radioactivity recovered in each chromatographic fraction of different tissues in per cent of the injected dose of ^{14}C-carotene. This method of reporting the results facilitates the comparison of the data since radioactivity received by individual animals, though accurately determined for each rat, varied slightly. Unless otherwise stated, numerical values for the radioactive fractions are given in per cent in the discussion. These percentages are defined as the amount of radioactivity recovered of the injected dose.

Intestinal wall The intestinal wall is thought to be the major site of biosynthesis of retinol from carotene in rats (Thompson *et al.*, 1949; Olson, 1961a). Retinyl esters are the predominating derivatives in the tissue during active metabolism (Olson, 1961a; Huang and Goodman, 1965). The amount of retinyl esters in this tissue in terms of percentage of the injected dose has been used here as one of the major criteria of carotene conversion.

The retinyl ester fraction of the intestinal wall in group 10C-4 had a mean value of 1.97% at the end of the experimental period of two and one half hours. The corresponding value for group 40C-4 was 5.18% and was statistically significantly higher.

Table 22. Radioactivity recovered in each chromatographic fraction of different tissues, in percentage of injected dose of ^{14}C-carotene (Values represent means of 12 animals/group together with the standard errors of the mean), Series V

Group	Tissue	Fraction I Carotene	Fraction II Retinyl ester	Fraction III Retinol	Fraction IV Breakdown prod.	Total
10C-4	Small intestine wall	16.6±2.7[a]	1.97±0.74	0.80±0.08	0.70±0.30	19.7±3.0
	Small intestine cont.	16.9±2.1	0.49±0.19	0.68±0.11	0.32±0.11	18.6±2.2
	Stomach	8.4±2.6	0.17±0.07	0.47±0.14	0.30±0.08	9.3±2.8
	Stomach + small intestine	42.0±3.2	2.62±0.81	1.97±0.18	1.02±0.18	47.6±3.8
	Liver	0.5±0.1	----	5.87	0.44±0.09	6.1±1.0
	Colon	----	----	----	----	0.7±0.3
	Serum	trace	----	1.05[b]	trace	----
	Total activity recovered	----	----	----	----	54.0±3.4
40C-4	Small intestine wall	16.7±2.1	5.18±1.37	0.83±0.09	0.32±0.09	24.2±2.4
	Small intestine cont.	21.1±1.6	0.54±0.13	0.71±0.09	0.43±0.14	22.7±1.7
	Stomach	12.2±3.3	0.18±0.06	0.29±0.07	0.26±0.06	13.0±3.3
	Stomach + small intestine	52.0±4.1	5.89±1.57	1.90±0.14	1.00±0.20	62.0±4.5
	Liver	1.1±0.2	----	9.90	0.55±0.11	10.1±1.6
	Colon	----	----	----	----	1.7±1.0
	Serum	trace	----	1.15[b]	trace	
	Total activity recovered	----	----	----	----	72.9±3.6

[a]Standard error of the mean

[b]Interpolated means.

Activities recovered in retinol, fraction III, in the intestinal wall were identical in both groups. Since retinal was not separated in the chromatographic column, it was probably eluted with the retinol fraction (Huang and Goodman, 1965). Thus the value for retinal plus retinol in this tissue was 0.8% for both groups. Since the values for the combined retinal and retinol fraction amounted to less than 1.0%, the same trend is seen whether total retinol derivatives or retinyl esters are compared between the two groups. Values for the total retinol derivatives were 2.77% and 6.01% for group 10C-4 and 40C-4 respectively (P 0.05).

Fraction I which represented unconverted carotene recovered from the intestinal tissue was almost identical in the two groups under investigation. The two values were 16.6 and 16.7% respectively. Interpretation of the significance of the amount of carotene recovered from this tissue is difficult, since it is not possible to differentiate between the fraction of carotene absorbed by the intestinal mucosal cells and that mechanically adsorbed onto the intestinal wall. It was shown in series I that, even when injected carotene was flushed out of the intestines immediately after injection, 20 - 30% of the injected carotene was recovered from the intestinal wall (Table 6). The only possible interpretation of this finding was that mechanical adsorption had taken place. Similar observations have been reported by Olson (1961a). Therefore unconverted carotene will only be considered in terms of the total carotene recovered from the upper part of the gastrointestinal tract.

The radioactivity recovered from breakdown products was relatively small. Values for fraction IV were 0.7% in group 10C-4 and 0.3% in group

40C-4. Individual variations were great. In applying a student's 't' test there was no significant difference.

Small intestine and stomach Since carotene was injected intraduodenally the activity in the stomach was probably entirely due to regurgitation of the intestinal contents. Appreciable amounts of radioactivity in retinol derivatives recovered from this organ further suggested that the regurgitation process had occurred throughout the experimental period. Hence it would also be invalid to consider the activity in the intestinal contents separately. Consequently, it was thought logical to combine values for the contents of small intestine and stomach for the interpretation of total utilized carotene and of total metabolic products.

The activity of the different products in this part of the digestive tract was in general a reflection of the relative magnitude of the products in the intestinal wall. Thus the total amount of retinyl esters formed in the combined tissues and contents was 2.62% after two and one-half hours in group 10C-4, and 5.89% in group 40C-4 (P 0.05). Likewise, the percentage of the activity recovered in the combined retinol derivatives in this part of the digestive tract was significantly higher in group 40C-4 than in group 10C-4. Values were 4.59 and 7.79% for the two groups respectively (P 0.05).

The total carotene fraction of the combined tissues and contents accounted for 52% of the dose in animals that had consumed the higher protein diet while the corresponding value was 42% in the other group.

Total TPF in the upper gastrointestinal tract was of low magnitude and identical in the two groups with a value of 1.0% for both. This means that the formation of hexane-extractable breakdown products exclusive of retinol derivative was similar in the two groups.

Appreciable but variable amounts of activity have also been recovered from the retinol derivatives of the intestinal contents. This trend is observed in the stomach also. This was possibly due to sloughing off of the mucosal cells either during the period of carotene absorption or during the removal of the contents with saline. Similar observations have been made by Thompson et al. (1949) even after correcting for the so-called artifacts.

Small and variable quantities of hexane-extractable radioactive compounds were recovered from the colon. Values of 0.7% and 1.65% were obtained for groups 10C-4 and 40C-4 respectively. The difference between the two groups was statistically insignificant. On fractionating the colon extract on alumina in a pilot study, no activity had been obtained in fractions II and III indicating the absence of retinol derivatives in this part of the intestines. Variable amounts of activity were recovered in the carotene and/or TP fractions from this tissue. Therefore it was thought unnecessary to chromatograph the colon.

In series IV total activity ranging from 14 - 20% had been recovered from the colon after five hours following the injection of the dose. That less than 2% were found in the present experiment is probably explainable by the difference in the time periods used. Apparently a period of 2.5 hours was short enough to prevent passage of any significant portion of the dose into the colon.

Deshmukh and Ganguly (1964) have recently studied the intestinal metabolism of non-radioactive carotene. These workers demonstrated a direct relationship between dietary protein and the formation of retinol products. Animals fed 5, 10, and 20% protein in the diet and given 4,000 mcg. carotene orally, had formed 6.5, 15.2, and 19.5 mcg. of total vitamin A in the intestines after an interval of three hours. Unlike the results found in the present study, an inverse relationship was demonstrated between dietary protein and the unconverted carotene in the intestines after different intervals of time up to 24 hours. After 3 hours, the same three groups had 725, 572, and 515 mcg. of residual carotene in their intestinal contents indicating that residual carotene had decreased while the formation of intestinal vitamin A had increased. There are differences in the experimental conditions of the two studies which might account in part for the discrepancy between results. The major difference is found in the size of the dose which makes comparison difficult. For instance values for the amounts of intestinal retinol expressed as percent of the dose were 0.16, 0.4, and 0.5% for the three levels of protein used by Deshmukh and Ganguly (1964) while in the present study, the relative conversion gave values of 2.77, and 6.01 for the same tissue for 10 and 40% protein.

<u>Liver</u> The non-saponifiable fraction of the hepatic lipid extracts was chromatographed in this study to obtain a measure of activity incorporated into the retinol fraction of the liver during the experimental period. Since saponification had preceded chromatography, the retinyl ester fraction was not eluted separately.

Recovery of the activity in the total non-saponifiable fraction of the livers of groups 10C-4 was 6.1%. This amount was significantly lower than the corresponding amount for group 40C-4. The value for the latter was 10.1%. After chromatography, the respective values for retinol for the group 10C-4 and 40C-4 were 5.9% and 9.9% (P 0.05). These data indicate that animals on the high protein diet had stored more hepatic retinol than those on the low protein diet even though the absorptive period was short. This result might be correlated with an enhanced rate of formation of retinol at the site of conversion in groups fed excessive levels of protein. Almost 100% of the activity of the non-saponifiable lipid extract of the liver resided in the retinol fraction thus indicating that retinol was the main product of non-saponifiable compounds in the liver from the conversion of carotene. This hepatic storage of newly formed retinol should be the balance of that biosynthesized from carotene, and that utilized for metabolic purposes during the experimental period.

<u>Serum</u> As described in the procedure, serum samples had been pooled to obtain sufficient activity for counting. In order to express the activity in the serum as per cent of the injected dose, certain assumptions had to be made in the calculations. It is realized that these values represent rough estimates at best, but they may serve in a semi-quantitative way to give some idea of the magnitude of the activity represented by the amounts in blood. It was assumed that rats had 6 ml. of blood/100 gm. body weight. The pooled values for serum activity and for body weight were employed to calculate the total serum activity per

set of 3 animals. From this interpolated value, the percentage of the 3 injected doses incorporated into the total retinol fraction of the serum was determined. The mean value for these 4 sets of pooled samples gave a rough estimation of the serum retinol values for the 12 animals comprising the group. On this basis, both groups under consideration had similar estimates of activity in the serum. The mean value for group 10C-4 was 1.05% while that for group 40C-4 was 1.15% of the total activity injected.

Total activity recovered Since the activity recovered in the serum was small and identical in both groups and since the values were only semiquantitative for reasons given earlier, it was decided to disregard the serum values and to base total activity recovered on values obtained from intestines and their contents, stomach, colon and liver.

A mean value of 54.3±3.4 per cent of the injected dose was recovered in the four organs mentioned in animals receiving the inadequate amount of protein while that recovered in the group on the high protein diet was significantly higher with a value of 73.8±3.6 per cent. Most of the fraction came from the total unconverted carotene in both groups.

In two and one-half hours carotene will be metabolized into different products, utilizable as well as nonutilizable by the system. These products are probably distributed throughout the tissues. Only a few tissues were analyzed in this study. Furthermore the activity which might have been lost from the body via the lungs and kidneys was also not recovered. Under these circumstances, where a balance study was not conducted, results should be interpreted with due credit to the undetermined fractions as well as the irrecoverable ones. The irrecoverable fractions would be those

which were not extracted by the hexane-ethanol solvent system. The unconverted fraction was larger in magnitude in group 10C-4 than in group 40C-4. In other words more of the degraded carotene had not been accounted for in rats fed the low level of protein than in those receiving the high protein diet under similar experimental conditions. Olson (1961a) has shown that even after a zero hour interval following ^{14}C-carotene injection about 20% of the activity could not be accounted for. He considered the possibility that some material was either transported out of the intestine or was metabolized to compounds not extracted by hexane-ethanol. The results reported here do suggest that the degree of degradation of carotene to unrecovered compounds was retarded in the presence of high amounts of dietary protein, since the recoveries of unconverted carotene had been slightly greater in group 40C-4 than in group 10C-4.

Recoveries of carotene were quantitative within the limits of the methods used, since the intestinal wall acts as a barrier to the absorption of carotene, and since all tissues of the gastro intestinal tract were analyzed for unconverted carotene. Formation of metabolic products of carotene as per cent of "carotene disappeared" was therefore calculated as follows:

$$\text{Percentage of the fraction} = \frac{\text{Fraction under consideration X 100}}{\text{Injected dose - unconverted carotene in upper part of gastrointestinal tract}}$$

Results obtained by this method (Table 23) show basically the same trends as those obtained by the first method of calculation. Thus the retinyl ester values of the intestinal wall were 3.9% and 11.2% for groups 10C-4 and 40C-4 respectively. Similarly hepatic retinol values were 9.8% and

Table 23. Radioactivity recovered in each chromatographic fraction of different tissues in percentage of disappeared carotene (injected dose - total recovered carotene)

Group	Tissue	Fraction II retinyl ester	Fraction III retinal	Fraction IV TPF	Unfractionated
10C-4	Intestine wall	3.90±1.52[a]	1.44±0.20	0.60±0.17	—
	Intestine contents	0.87±0.60	0.60±0.21	1.23±0.21	—
	Stomach	0.32±0.14	0.86±0.25	0.48±0.11	—
	Intestinal wall cont. + stomach	5.09±1.70	3.56±0.44	1.68±0.41	—
	Liver	—	9.82±1.46	—	10.68±1.47
	Colon	—	—	—	1.09±0.12
40C-4	Intestine wall	11.20±3.15	1.88±0.22	1.07±0.12	—
	Intestine contents	1.24±0.13	1.87±0.14	0.59±0.20	—
	Stomach	0.43±0.20	0.88±0.32	1.00±0.32	—
	Intestinal wall contents and stomach	13.02±3.51	4.62±0.92	2.56±0.72	—
	Liver	—	17.73±2.05	—	18.50±1.71
	Colon	—	—	—	3.38±1.79

[a]Standard error of the mean.

7.7% for the two groups respectively.

Therefore, on a comparative basis, one might suggest that animals maintained on a high plane of protein nutrition had utilized relatively smaller amounts of carotene to form comparatively greater amounts of retinyl esters, the major product of carotene conversion. On the other hand, groups with low protein intakes had utilized more carotene for the purpose of the formation of a relatively small quantity of retinyl ester. This suggests that the efficiency of the mechanism at work is enhanced with an increase in protein intake.

Assuming that all animals under investigation had had their peak period of conversion in the intestine at the same time, some interpretation could be made from the data obtained. The degree of conversion of carotene appears to have increased in the group fed excessive amounts of protein. This could be interpreted, in two ways, on the basis of recent reports.

Goodman and Huang (1965) and also Olson and Hayaishi (1965) have pointed to the possible existence of a dioxygenase enzyme system present in the soluble fraction of the intestinal mucosal homogenates. This enzyme, according to the reports, mediates the reaction of biosynthesis of retinol from its precursor, carotene. Since several enzymes are shown to be influenced by the protein nutrition of the animal it is possible that this enzyme belongs to the same category. If so, animals fed inadequate amounts of protein might have had insufficient amounts of this enzyme with a resulting low degree of carotene conversion.

Suzuki _et al._ (1959) have shown that beta-carotene is degraded to beta-ionone, and retinal-protein complex which then is reduced to retinol

and later, its esters. However the nature of the protein moiety was not defined by the authors. If the formation of this retinal-protein complex is an obligatory step in the biosynthesis of retinol, dietary protein could play a role in the formation of this compound. Animals on a high plane of nutrition might have formed more of the retinal-protein complex and thus retinol than those maintained on a low level of protein. Thus efficiency could be due to a direct influence of protein on the rate of conversion of carotene. It could also be due to an indirect influence of protein such as protecting the chromogen against non-utilizable breakdown products. This tendency is also apparent based on amounts of unconverted carotene and the total activity recovered.

The concentrations of serum vitamin A in both groups did not differ. If distribution of vitamin A in the extra hepatic tissues were a reflection of the concentration in the blood, extra hepatic tissue distribution of retinol would also be similar in both groups. On the basis of these assumptions the ratio of hepatic to intestinal retinol derivatives were calculated for each group. At the end of 2.5 hours this ratio was 2.1 for group 10C-4, but only 1.6 for group 40C-4. If both groups had utilized the newly formed vitamin A in an identical fashion, this ratio should have been approximately the same for both groups. That the ratio decreased from 2.1 to 1.6 with an increase in protein intake would be indicative of a greater rate of utilization of newly formed retinol for metabolic purposes in rats fed the higher protein level. This result, though based on many assumptions, is not contradictory to the well-documented fact that rats on higher protein intakes metabolize larger amounts of vitamin A

than do those consuming low protein diets (Rechcigl et al., 1962; Jagannathan and Patwardhan, 1960).

Regardless of mechanism, this study has provided some direct evidence to show that protein influences the biosynthesis of retinol from beta-carotene in the intestinal walls.

Data from the first phase of series V confirmed the previously established fact that utilization of carotene, measured by hepatic retinol stores is enhanced with an increase in dietary protein. However the data also pointed to the time dependence of this relationship in the region of adequate to high protein intake, since the feeding period had to be extended to at least four weeks before any enhancement of hepatic retinol storage with high protein intakes could be demonstrated. That part of the protein effect is exerted at the intestinal level of carotene metabolism was shown in the second phase of the experiment where a high protein intake had stimulated the formation of retinol derivatives from beta-carotene in the intestinal wall. The enhanced rate of intestinal metabolism of carotene in rats fed 40% protein in the diet is in accord with the presence of increased total radioactivity of the retinol fraction of the liver in those animals, 2.5 hours after dosing, as well as with the increased accumulation of total hepatic retinol after 4 weeks of supplementation.

Enzymes involved in the conversion process of carotene as well as the stability of the chromogen in the intestines against degradation to non-utilizable products may be protein dependent.

Indirect evidence of an increased metabolic demand for vitamin A with elevated protein intakes is based on data from the second phase of the study where the ratio

$$\frac{\text{total retinol derivatives in liver}}{\text{total retinol derivatives in intestinal wall}}$$

decreased with an increase in dietary protein. However, even after an apparent greater metabolic demand for the newly formed vitamin A with increased protein intakes, the resultant stores still reflected the relative conversion rates in the intestines.

The fact that at least 4 weeks of dietary treatment were required before the protein effect could be detected between adequate and excessive intakes may be partly due to the complexity of the relationship between protein and carotene/vitamin A metabolism. The animals were approximately 60 days old at the end of the 3rd week of supplementation. At this age male rats of the strain used here are sexually mature. Additional data related to nitrogen retention, to the components of growth increment, in forms of fat and protein, and to levels of anabolic hormones might offer clues for possible shifts in the animals' metabolism coinciding with the emergence of the protein effect on carotene utilization between intermediate and high protein intakes.

SUMMARY AND CONCLUSION

The purpose of the work reported here was to extend available information concerning the influence of dietary protein on carotene metabolism. The problem was investigated in a two-phase study. One phase was concerned with the characterization of the protein-carotene interrelationship over different periods of time. The object was to explore trends in the rate of hepatic retinol deposition following carotene supplementation as influenced by protein intake as well as time of repletion. The second phase involved comparisons of the effect of two different levels of dietary protein on the degree of conversion of ^{14}C-carotene during the intestinal phase of carotene metabolism. In the course of the investigation it became necessary to develop methods suitable for the study of the second phase. The development of methods concerned physico-chemical and physiological aspects of experimentation.

From a series of four trials, a method was developed to produce and purify biologically synthesized radioactive beta-carotene. Leaves of tobacco plants, exposed to $^{14}CO_2$ for 3 - 4 hours were extracted with a skelly B:acetone mixture followed by saponification and further extraction of the non-saponifiable matter with skelly B. After removing the sterols by cooling, the solvent extract was subjected to double chromatography - on alumina and on calcium hydroxide. Thirty to thirty-five mcg. of pure beta-carotene per gram of leaf tissue with a specific activity up to 3033 cpm/mcg. could be obtained under the conditions specified by this procedure.

Extraction with a skelly B:ethanol (3:1) solvent system followed by

washing with a 2% calcium chloride solution was found most effective for quantitative extraction of lipids from animal tissues. Subsequent chromatography of the lipid extract on 7% alumina gave good separation of beta-carotene and its biologically-active metabolites. Recoveries ranged from 80-90 per cent for retinol, retinyl esters and beta carotene added as carriers to tissues of the gastro-intestinal tract.

One problem in evolving a suitable procedure was to find methods which would simulate as closely as possible the physiological conditions during carotene absorption and conversion. Modifications worked out are concerned with the dietary treatment preceding the radio tracer experiment, manipulation of the intestinal tissue _in situ_ and simulation of the intestinal contents during carotene absorption in intact animals. The following procedure was evolved on the basis of explorations concerning these points.

A dose of ^{14}C-carotene was injected into the unligated loop of the upper intestine in animals maintained on controlled dietary regimes for a few weeks. This method of administration was more effective, with respect to relocation of radioactivity in livers, than the one in the original procedure where the ^{14}C-carotene dose was injected into a ligated loop of the intestine. The change from the original procedure permitted the presence of the ingesta from a test meal in the intestine during ^{14}C-carotene absorption. This process simulated most closely the physiological process of absorption of dietary carotene. Experimentation with different absorption periods suggested a 2.5 hour time interval as the best compromise between optimum levels of intestinal metabolites and measurable activity in hepatic retinol. In order to evolve this method, four animal

experiments were conducted in sequence. These were of a preliminary nature. The results of each sequence formed the basis for planning the subsequent series. Radioactivity incorporated into newly formed retinyl esters in the intestinal wall and into the lipid fraction of the liver were considered as main criteria of the metabolism of duodenally injected ^{14}C-carotene.

The need for modifications in analytical procedures was indicated after the completion of series I. Data from series II to IV failed to show significant differences between dietary treatments for reasons such as low recoveries of intestinal fractions, low level of hepatic ^{14}C-retinol and large standard errors indicating excessive individual variability. Consequently results of series I to IV were not interpreted.

Series V constitutes the major experiment where the methods evolved in the preceding series were used. Two approaches to the study of carotene metabolism were combined in one experiment. Intakes of 10, 20 and 40 per cent casein protein in the diet were compared for their effect on carotene utilization for repletion periods lasting from 1 to 4 weeks. Hepatic retinol accumulated during these periods was taken as the parameter of carotene utilization. Two groups from this study fed 10 to 40 per cent casein protein for 4 weeks were further used to explore the influence of dietary protein on the conversion of ^{14}C-carotene in the intestines during the absorptive phase.

The results of series V may be summarized as follows:

The magnitude of hepatic retinol increased over one, two, three and

four weeks of repletion when the dietary intake of protein was raised from 10 to 20 per cent. When the intake was further increased to 40 per cent, there was no further enhancement in hepatic retinol for the first three weeks. At the end of four weeks, however, hepatic vitamin A content of animals fed excessive amounts of protein had risen significantly over that of rats on an adequate protein intake.

The fluctuation in mean initial weights made the interpretation of results with respect to the time effect on carotene utilization difficult. Furthermore, due to the changing need for vitamin A as well as for protein for metabolic activity during growth, it is not easy to assess the influence of protein on the utilization of carotene in growing animals from data on net storage of hepatic vitamin A. During the growth period, an interaction between time of repletion and dietary protein might be expected especially if the period of observation extends beyond the period of rapid development.

The rate of intestinal metabolism of ^{14}C-carotene measured by ^{14}C-retinyl ester formation in the intestine was doubled when the dietary intake of protein was increased from inadequate to excessive levels. Though the protein effect has thus been demonstrated at the intestinal site of conversion, the data give no information on the mechanism. Implication of interactions at the molecular level analogous to reports from other laboratories have been discussed.

The recently formed ^{14}C-retinol in liver of the group fed 40 per cent protein had increased by approximately 170% over that of animals maintained on 10 per cent protein. The decrease in the ratio of ^{14}C-

retinol derivatives of the liver to those of the intestinal wall with increasing protein intake might be attributed to increased utilization of ^{14}C-retinol for metabolic purposes in the group maintained on excessive amounts of protein. In spite of the increased demand for vitamin A by the system, animals fed high protein diets still had relatively large amounts of hepatic ^{14}C-retinol. This may indicate that the enhancing effect of protein on the anabolism of vitamin A from beta-carotene outweighs that on the catabolism of vitamin A for metabolic purposes.

Irrespective of the mechanism, some direct evidence is presented here to demonstrate the enhancing effect of dietary protein on the utilization of carotene as judged by the retinol biosynthesis in the intestines as well as the accumulated retinol stores in the liver.

BIBLIOGRAPHY

Ahmad, B.
1931 The fate of carotene after absorption in the animal organism. Biochemical Journal 25: 1195.

Anderson, T. A., F. Hubbert, C. B. Roubicek and R. E. Taylor
1962 Influence of protein depletion on vitamin A and carotene utilization by vitamin A deficient sheep. Journal of Nutrition 78: 341.

Arnrich, L. and A. F. Morgan
1954 The utilization of carotene by hypothyroid rats. Journal of Nutrition 54: 107.

Arnrich, L. and D. J. Pederson
1956 Dietary protein and carotene utilization. Federation Proceedings 15: 212.

Arroyave, G., F. Veteri, M. Behar and N. S. Scrimshaw
1959 Impairment of absorption of vitamin A palmitate in severe protein malnutrition (Kwashiorkor). American Journal of Clinical Nutrition 7: 185.

Arroyave, G., D. Wilson, H. Castellanos and N. S. Scrimshaw
1961 Vitamin A transport in severe protein malnutrition. Federation Proceedings 20: 372.

Arroyave, G., D. Wilson, C. Contreras and M. Behar
1963 Alterations in serum concentration of vitamin A associated with the hypoproteinemia of severe protein malnutrition. Journal of Pediatrics 62: 920.

Baumann, C. A., E. G. Foster and P. R. Moore
1942 The effect of dibenzathracine, of alcohol, and of other agents on vitamin A in the rat. Journal of Biological Chemistry 142: 597.

Baumann, C. A., B. M. Riising and H. Steenbock
1934 Fat soluble vitamins. XLIII. The absorption and storage of vitamin A on the rat. Journal of Biological Chemistry 107: 705.

Berger, S., M. Rechcigl, Jr., J. K. Loosli and H. H. Williams
1962 Protein quality and carotene utilization. Journal of Nutrition 77: 174.

Bhattacharya, R. K. and G. C. Esh
1962 Interrelationship of dietary protein and vitamin A in metabolism. 5. Protective influence of vitamin A during amino acid imbalance. Annals of Biochemistry and Experimental Medicine 22: 147.

Bickoff, E. M. and C. R. Thompson
1949 Separation of geometric isomers of beta-carotene on calcium hydroxide. Journal of Association of Official Agricultural Chemists 32: 775.

Bickoff, E. M., L. M. White, A. Bevenue and K. T. Williams
1948 Isolation and spectrophotumetric characterization of four carotene isomers. Journal of Association of Official Agricultural Chemists 31: 633.

Bieri, J. G. and C. F. Pollard
1954 Studies of the site of conversion of beta-carotene injected intravenously into rats. British Journal of Nutrition 8: 32.

Bligh, E. G. and W. J. Dyer
1959 A rapid method of total lipid extraction and purification. Canadian Journal of Biochemistry and Physiology 37: 911.

Booth, V. H.
1957 Carotene: its determination in biological materials. Cambridge, England, W. Heffer and Sons Ltd.

Brown, R. G.
1961 Studies on the relationship of dietary carotene and nitrogen sources in rats. Unpublished M.S. thesis. Ames, Iowa, Library, Iowa State University of Science and Technology.

Burns, M. J., S. M. Hauge and F. W. Quackenbush
1951 Utilization of vitamin A and carotene by the rat. I. Effects of tocopherol, Tween and dietary fat. Archives of Biochemistry and Biophysics 30: 341.

Crain, F. D., F. J. Lotspeich and R. F. Krause
1965 Metabolism of beta-carotene. Proceedings of the Society for Experimental Biology and Medicine 119: 606.

Davis, A. W.
1933 The colorimetric determination of vitamin A by the alkali digestion method. Biochemical Journal 27: 1770.

De, N. K. and A. R. Sundarrajan
1951 The site of in vivo conversion of carotene to vitamin A. Indian Journal of Medical Research 39: 479.

Deshmukh, D. S. and J. Ganguly
1964 Effect of dietary protein content on the intestinal conversion of beta-carotene to vitamin A in rats. Indian Journal of Biochemistry 1: 204.

Deshmukh, D. S., P. Malathi and J. Ganguly
1964 Studies on metabolism of vitamin A. 5. Dietary protein content and metabolism of vitamin A. Biochemical Journal 90: 98.

Deshmukh, D. S., S. K. Murthy, S. Mahadevan and J. Ganguly
1965 Studies on metabolism of vitamin A absorption of retinal (vitamin A aldehyde) in rats. Biochemical Journal 96: 377.

Deuel, A. J., Jr., M. C. Hrubetz, C. H. Johnston, H. S. Rollman and E. Geiger
1946 Studies on the nutritive value of fish proteins. Journal of Nutrition 31: 187.

Dowling, J. E. and G. Wald
1960 The biological function of vitamin A acid. National Academy of Science Proceedings 46: 587.

Drummond, J. C., M. E. Bell and E. T. Palmer
1935 Observations on the absorption of carotene and vitamin A. British Medical Journal 1: 1208.

Dunagin, P. E.
1964 Identification of free and conjugated retinoic acid as a product of retinol metabolism in rat _in vivo_. Biochemica et Biophysica Acta 90: 432.

Dye, M., I. Bateman and T. Porter
1945 The effect of the level of protein in the diet on the utilization of vitamin A. Journal of Nutrition 29: 341.

Dzialoszynski, L. M., E. M. Mystkowski and C. P. Steward
1945 The mode of occurrence of carotene and vitamin A in human blood plasma. Biochemical Journal 39: 63.

Eaton, H. D., W. B. Boucher and P. C. Shaw
1964 Vitamin A in serum and liver of weanling pigs fed a protein deficient diet and fixed intake per unit of liver weight of carotene in oil. Journal of Nutrition 82: 343.

Eden, E. and K. C. Sellers
1948 The absorption of vitamin A. Biochemical Journal 42: xlix.

Eden, E. and K. C. Sellers
1949 The absorption of vitamin A in ruminants and rats. Biochemical Journal 44: 264.

Eden, E. and K. C. Sellers
1950 Hydrolysis and esterification of vitamin A during absorption. Biochemical Journal 46: 261.

Ehrlich, S. T., B. R. Farthing and D. S. Moschette
1964 Metabolic patterns in preadolescent children. XI. Response of vitamin A and carotene serum levels to dietary protein and vitamin A. Journal of Nutrition 84: 389.

Esh, G. C., S. Battacharya and F. M. Som
1960 Studies on the utilization of vitamin A. Part IV. Influence of the level of protein on the storage and utilization of vitamin A. Annals of Biochemistry and Experimental Medicine 20: 15.

Folch, J., I. Ascoli, M. Lees, J. A. Meath and F. N. LeBaron
1951 Preparation of lipid extracts from brain tissue. Journal of Biological Chemistry 191: 833.

Folch, J., M. Lees and G. M. Sloane-Stanley
1959 A simple method for the isolation and purification of total lipids from animal tissues. Journal of Biological Chemistry 226: 497.

Fraps, G. S.
1946 Effect of bulk, casein, and fat in the ration on the utilization of carotenes by white rats. Archives of Biochemistry 10: 485.

Friend, C. J., C. R. Heard, B. S. Platt, R. J. C. Stewart and M. R. Turner
1961 The effect of dietary protein deficiency on transport of vitamin A in blood and its storage in the liver. British Journal of Nutrition 15: 231.

Gallup, W. D. and J. A. Hoefer
1946 Determination of vitamin A in liver. Industrial and Engineering Chemistry, Analytical Edition 18: 288.

Ganguly, J. and N. I. Krinsky
1953 Absence of relationship between vitamin A alcohol levels in plasma and in liver of rats. Biochemical Journal 54: 177.

Ganguly, J., N. I. Krinsky, J. W. Mehl and H. J. Deuel, Jr.
1952 Studies of the distribution of vitamin A as ester and alcohol and of carotenoids in plasma proteins of several species. Archives of Biochemistry and Biophysics 38: 275.

Ganguly, J., S. Krishnamurthy and S. Mahadevan
1959 The transport of carotenoids, vitamin A, and cholesterol across the intestines of rats and chickens. Biochemical Journal 71: 756.

Garbers, C. F.
1958 Transport of vitamin A ester in rat serum. Nature 182: 1018.

Garbers, C. F., J. Gillman and M. Peisach
1958 The association of vitamin A alcohol with rat serum proteins. South African Journal of Medical Science 23: 34.

Garbers, C. F., J. Gillman and M. Peisach
1960 The transport of vitamin A in rat serum with special reference to the occurrence of unidentified metabolites of vitamin A in the rat. Biochemical Journal 75: 124.

Glover, J., T. W. Goodwin and R. A. Morton
1947a Studies in vitamin A. 2. The relationship between blood vitamin A levels and liver stores in rats. Biochemical Journal 41: 97.

Glover, J., T. W. Goodwin and R. A. Morton
1947b Conversion of beta-carotene into vitamin A in the intestine of the rat. Biochemical Journal 41: xiv.

Glover, J., T. W. Goodwin and R. A. Morton
1948 Studies on vitamin A conversion of beta-carotene into vitamin A in the intestine of the rat. Biochemical Journal 43: 512.

Glover, J. and R. A. Morton
1948 The administration, storage and metabolism of vitamin A. Biochemical Journal 43: xii.

Glover, J. and E. R. Redfearn
1953 Biosynthesis of ^{14}C-beta-carotene. Biochemical Journal 54: 8.

Glover, J. and E. R. Redfearn
1954 The mechanism of the transformation of beta-carotene in vitamin A in vivo. Biochemical Journal 58: 15.

Goodman, D. S. and H. S. Huang
1965 Biosynthesis of vitamin A with rat intestinal enzymes. Science 149: 879.

Goodman, D. S., H. S. Huang and T. Shiratori
1965 Vitamin A and carotenoids. 2. Tissue distribution and metabolism of newly absorbed vitamin A in the rat. Journal of Lipid Research 6: 390.

Goodman, D. S., H. S. Huang and T. Shiratori
1966 Mechanism of the biosynthesis of vitamin A from beta-carotene. Journal of Biological Chemistry 241: 1929.

Goodwin, T. W. and R. A. Gregory
1948 Studies in vitamin A. 7. Carotene metabolism in herbivores. Biochemical Journal 43: 505.

Gray, E. LeB, K. C. D. Hickman and E. F. Brown
1940 The state of vitamin A in the liver of the rat after feeding various forms of the vitamin. Journal of Nutrition 19: 39.

Harashima, K.
1964 Conversion of beta-carotene to vitamin A by rat intestinal sections. Biochemica et Biophysica Acta 90: 211.

High, E. G. and S. S. Wilson
1956 Studies on vitamin A ester; alcohol partition between the liver, kidneys, and plasma of rats. Archives of Biochemistry and Biophysics 62: 163.

Hillers, V. N.
1963 Effects of different dietary proteins on carotene utilization in young rats. Unpublished M.S. thesis. Ames, Iowa, Library, Iowa State University of Science and Technology.

Hillers, V. N. and L. Arnrich
1964 Effects of different dietary proteins on carotene utilization in young rats. Federation Proceedings 23: 293.

Hjarde, W.
1950 Spectrophotometric determination of vitamin A by means of chromatography. Acta Chemica Scandinavica 4: 628.

Hoch, H. and R. Hoch
1946 The state of vitamin A in human serum. British Journal of Experimental Pathology 27: 316.

Huang, H. S. and D. S. Goodman
1964 Intestinal absorption and metabolism of ^{14}C-beta-carotene in the rat. Federation Proceedings 23: 270.

Huang, H. S. and D. S. Goodman
1965 Vitamin A and carotenoids. 1. Intestinal absorption and metabolism of ^{14}C-labelled vitamin A alcohol and beta-carotene in the rat. Journal of Biological Chemistry 240: 2839.

Jagannathan, S. N. and V. N. Patwardhan
1960 Dietary protein in vitamin A metabolism. Part I. Influence of level of dietary protein on the utilization of orally fed preformed vitamin A and beta-carotene in the rat. Indian Journal of Medical Research 48: 775.

James, W. H. and I. M. ElGhindi
1953 Utilization of carotene 1. As affected by certain proteins in the diet of growing albino rats. Journal of Nutrition 51: 97.

Johnson, R. M. and C. A. Baumann
1948 Relative significance of growth and metabolic rate upon the utilization of vitamin A by the rat. Journal of Nutrition 35: 703.

Johnson, V.
1959 The utilization of carotene as affected by variations in dietary nitrogen in rats. Unpublished M.S. thesis. Ames, Iowa, Library, Iowa State University of Science and Technology.

Johnson, V. and L. Arnrich
1960 Utilization of carotene as affected by variations in dietary nitrogen in young rats. Federation Proceedings 19: 412.

Jurkowitz, L.
1962 Determination of vitamin A acid in human plasma after oral administration. Archives of Biochemistry and Biophysics 98: 337.

Kaiser, E. and B. M. Kagan
1956 Separation of vitamin A alcohol and vitamin A esters by paper chromatography. Archives of Biochemistry and Biophysics 63: 118.

Koehn, C. J.
1948 Relative biological activity of beta-carotene and vitamin A. Archives of Biochemistry 17: 337.

Kon, S. K. and S. Y. Thompson
1951 Site of conversion of carotene to vitamin A. British Journal of Nutrition 5: 114.

Kramer, M. and R. Tarzan
1958a Studies on carotene metabolism. I. The effect of different dietary proteins on carotene metabolism. Internationale Zeitschrift für Vitamin Forschung 28: 289.

Kramer, M. and R. Tarzan
1958b Studies on carotene metabolism. II. The biological value of natural carotene sources and oily carotene solutions. Internationale Zeitschrift für Vitamin Forschung 28: 295.

Krause, R. F.
1961 In vitro metabolism of C-14 labeled beta-carotene. Proceedings of the Society for Experimental Biology and Medicine 107: 363.

Krause, R. F., M. O. Coover and L. T. Powell
1954 Conversion of C-14 carotene to a non-saponifiable substance or substances in the rat. Proceedings of the Society for Experimental Biology and Medicine 85: 317.

Krause, R. F. and P. L. Sanders
1957 Metabolism of C^{14} labelled beta-carotene in the rat. Proceedings of the Society for Experimental Biology and Medicine 95: 549.

Krinsky, N. I., D. J. Cornwell, and J. L. Oncley
1958 The transport of vitamin A and carotenoids in human plasma. Archives of Biochemistry and Biophysics 73: 233.

Krishnamurthy, S. and J. Ganguly
1956 Effect of blocking the reticulo-endothelial system on the storage of vitamin A ester and alcohol in the liver of the rat. Nature 177: 575.

Krishnamurthy, S. S. Mahadevan and J. Ganguly
1958 Association of vitamin A ester and vitamin A alcohol with proteins in rat liver. Journal of Biological Chemistry 233: 32.

Lambertsen, G. and O. R. Braekkan
1958 The losses of vitamin A during chromatography. Acta Chemica Scandinavica 12: 360.

Laughland, D. H.
1954 The biosynthesis of ^{14}C-beta-carotene and its use in metabolic studies with chickens. World's Poultry Congress, 10th, Edinburgh, 1954 [Proceedings 4]: 157.

Lawrence, C. W., F. D. Craine, F. J. Lotspeich and R. F. Krause
1966 Absorption, transport, and storage of retinyl 15-^{14}C palmitate 9-10-3H in the rat. Journal of Lipid Research 7: 226.

Lease, J. G., E. J. Lease, H. Steenbock and C. A. Baumann
1941 The comparative physiological value of injected carotene and vitamin A. Journal of Laboratory and Clinical Medicine 27: 502.

Lilly, V. G., H. L. Barnett, R. F. Krause and F. J. Lotspeich
1958 A method of obtaining pure radioactive beta-carotene using phycomyces blakesleeanus. Mycologia 50: 862.

Lotspeich, F. J., R. F. Krause, V. G. Lilly and H. L. Barnette
1963 Asymmetrical incorporation of ^{14}C-acetate into beta-carotene biosynthesized by phycomyces blakesleeanus. Proceedings of Society for Experimental Biology and Medicine 114: 444.

MacMillan, J. B.
1966 Interaction of dietary protein with graded amounts of vitamin A or carotene in rats. Unpublished M.S. thesis. Ames, Iowa, Library, Iowa State University of Science and Technology.

Mahadevan, S.
1963 Studies on metabolism of vitamin A. 3. The mode of absorption of vitamin A esters in the living rat. Biochemical Journal 88: 531.

Mahadevan, S., S. Krishnamurthy and J. Ganguly
1959 The mode of absorption of vitamin A across the intestine of rats. Archives of Biochemistry and Biophysics 83: 371.

Mahadevan, S., S. K. Murthy and J. Ganguly
1959 Studies of vitamin A esterase. 4. The hydrolysis and synthesis of vitamin A esters by rat intestinal mucosa. Biochemical Journal 79: 416.

Mahadevan, S., P. Seshadri Sastry and J. Ganguly
1963a Studies on metabolism of vitamin A. 3. The mode of absorption of vitamin A esters in the living rat. Biochemical Journal 88: 531.

Mahadevan, S. P., Seshadri Sastry and J. Ganguly
1963b Studies on metabolism of vitamin A. 4. Studies on the mode of absorption of vitamin A by rat intestine _in vitro_. Biochemical Journal 88: 534.

Malathi, P., K. Subbarao, P. Seshadri Sastri and J. Ganguly
1963 Studies on metabolism of vitamin A. 1. The biological activity of vitamin A acid in rats. Biochemical Journal 87: 305.

Mathew, J. and G. H. Beaton
1963 Vitamin A and carotene utilization in protein-deprived rats. Canadian Journal of Biochemistry and Physiology 41: 543.

Mattson, F. H.
1948 The site of conversion of carotene to vitamin A. Journal of Biological Chemistry 176: 1467.

Mattson, F. H., J. W. Mehl and H. J. Deuel, Jr.
1947 Studies on carotenoid metabolism. VII. The site of conversion of carotene to vitamin A in the rat. Archives of Biochemistry 15: 65.

McLaren, D. S.
1958 Hypovitaminosis A. VI. The pathogenesis of hypovitaminosis A. Federation Proceedings 17: 136.

McLaren, D. S., M. Tchalian and Z. A. Ajans
1965 Biochemical and hematologic changes in the vitamin A deficient rat. American Journal of Clinical Nutrition 17: 131.

Moore, T.
1930 Vitamin A and carotene. V. The absence of liver oil vitamin A from carotene. VI. The conversion of carotene to vitamin A _in vivo_. Biochemical Journal 24: 692.

Moore, T.
1957 Vitamin A. New York, New York, Elsevier Publishing Company.

Murray, T. K.
1961 Effect of protein free diet in the vitamin A storage and depletion in the rat. Canadian Journal of Biochemistry and Physiology 39: 1099.

Murray, T. K. and H. C. Grice
1961 Absorption of vitamin A after the thoracic duct ligation in the rat. Canadian Journal of Biochemistry and Physiology 39: 1101.

National Academy of Science-National Research Council
1962 Nutrient requirements of laboratory rat. Nutrient Requirements of Domestic Animals 10: 68.

Olsen, E. M., J. D. Harvey, D. C. Hill and H. D. Branion
1959 Distribution of carotene and vitamin A in the tissues of chicks. Poultry Science 38: 688.

Olson, J. A.
1959 The conversion of radioactive beta-carotene to vitamin A in the rat. Federation Proceedings 18: 296.

Olson, J. A.
1961a The conversion of radioactive beta-carotene into vitamin A by the rat intestine in vivo. Journal of Biological Chemistry 236: 349.

Olson, J. A.
1961b The absorption of beta-carotene and its conversion into vitamin A. Journal of Clinical Nutrition 9: 1.

Olson, J. A.
1964a The effect of bile and bile salts on the uptake and cleavage of beta-carotene into retinol ester (vitamin A ester) by intestinal slices. Journal of Lipid Research 5: 402.

Olson, J. A.
1964b The biosynthesis and metabolism of carotenoids and retinol (vitamin A). Journal of Lipid Research 5: 281.

Olson, J. A. and O. Hayaishi
1965 The enzymatic cleavage of beta-caortene into vitamin A by soluble enzymes of rat liver and intestine. Proceedings of National Academy of Sciences 54: 1364.

Parrish, D. B. and H. A. Smith
1956 Report on vitamin A in mixed feeds. Journal of Association of Official Agricultural Chemists 39: 126.

Plack, P. A.
1959 The conversion of 11-*cis* into all-*trans* vitamin A in the rat. British Journal of Nutrition 13: 111.

Popper, H. and R. Greenberg
1941 Visualization of vitamin A in rat organs by fluorescence microscopy. Archives of Pathology 32: 11.

Popper, H., F. Steigmann, A. Dubin, H. A. Dyniewicz and F. P. Hesser
1948 Significance of vitamin A alcohol and ester partitioning under normal and pathological circumstances. Society for Experimental Biology and Medicine Proceedings 68: 676.

Popper, H. and B. W. Volk
1944 Absorption of vitamin A in the rat. Archives of Pathology 38: 71.

Raica, N., Jr., M. G. Vavich and A. R. Kemmerer
1959 The effects of several milk components and similar compounds on the utilization of carotene by the rat. Archives of Biochemistry and Biophysics 83: 376.

Rechcigl, M., Jr., S. Berger, J. K. Loosli and H. H. Williams
1962 Dietary protein and utilization of vitamin A. Journal of Nutrition 76: 435.

Reddy, B. S. and J. W. Thomas
1962 Effect of nitrate on the *in vitro* conversion of beta-carotene to vitamin A. Journal of Dairy Science 45: 683.

Roels, O. A. and M. Trout
1959 A method for the determination of carotene and vitamin A in human blood serum. American Journal of Clinical Nutrition 7: 197.

Ruffin, M. R.
1965 Carotene-protein interrelationships in rats: variations in feeding patterns and carotene utilization. Unpublished M.S. thesis. Ames, Iowa, Library, Iowa State University of Science and Technology.

Ruffin, M. R. and L. Arnrich
1966 The effect of different feeding patterns on carotene-protein interrelationships. Federation Proceedings 25: 546.

Sexton, E. L., J. W. Mehl and H. J. Deuel, Jr.
1946 Studies of carotenoid metabolism. VI. The relative provitamin A activity of carotene when introduced orally and parenterally in the rat. Journal of Nutrition 31: 299.

Sibbald, I. S. and L. M. Hutcheson
1960 The conversion of beta-carotene to vitamin A <u>in vitro</u>. Poultry Science 39: 99.

Sibbald, I. S. and E. Olsen
1958 The conversion of beta-carotene to vitamin A in the duodenum of the chick. Poultry Science 37: 1465.

Strain, H. H., W. M. Manning and G. Hardin
1944 Xanthophylls and carotenes of diatoms, brown algae, dinoflagellates, and sea-anemones. Biological Bulletin 86: 169.

Stoewsand, G. W. and M. L. Scott
1961 Effect of protein on utilization of vitamin A in the chick. Society of Experimental Biology and Medicine Proceedings 106: 635.

Suzuki, T., I. Koizumi and Y. Sahashi
1959 On metabolic pathways of carotenes to vitamin A in animals. I. Possible intermediates in the metabolic conversion of beta-carotene to vitamin A. Journal of Vitaminology (Kyoto) 5: 102.

Thomas, J. N. and G. A. J. Pitt
1960 Vitamin A acid and hyper-vitaminosis A. Nature 188: 672.

Thompson, S. Y., R. Brande, M. E. Coats, A. T. Corvies, J. Ganguly and S. K. Kon
1950 Further studies of the conversion of beta-carotene to vitamin A in the intestine. British Journal of Nutrition 4: 398.

Thompson, S. Y., J. Ganguly and S. K. Kon
1949 The conversion of beta-carotene to vitamin A in the intestine. British Journal of Nutrition 3: 50.

Vakil, V. K., O. A. Roels and M. Trout
1964 Storage and transport of vitamin A in relation to protein intake. British Journal of Nutrition 18: 217.

Vavich, M. G. and A. R. Kemmerer
1950 Factors influencing the utilization of carotene for storage of vitamin A. Journal of Nutrition 40: 605.

Vavich, M. G., J. W. Shill, N. Raica and A. R. Kemmerer
1955 Effect of non fat milk on the utilization of carotene and vitamin A. Archives of Biochemistry and Biophysics 45: 310.

Week, E. F. and F. J. Sevigne
1950 Vitamin A utilization studies. III. The utilization of vitamin A alcohol, vitamin A acetate and vitamin A natural esters by humans. Journal of Nutrition 40: 563.

Wiese, C. E., J. W. Mehl and H. J. Deuel, Jr.
1947 Studies on carotene matabolism. VIII. The *in vitro* conversion of carotene vitamin A in the intestine of the rat. Archives of Biochemistry 15: 75.

Willmer, J. S. and D. H. Laughland
1957 The distribution of radioactive carbon in the rat after the administration of randomly labelled C^{14}-beta-carotene. Canadian Journal of Physiology 35: 819.

Worker, N. A.
1959 Studies on the *in vitro* conversion of beta-carotene into vitamin A in tissues from the rat, guinea pig and sheep. British Journal of Nutrition 13: 400.

Zachman, R. D. and J. A. Olson.
1961 A comparison of retinene reductase and alcohol dehydrogenase of rat liver. Journal of Biological Chemistry 236: 2309.

Zachman, R. D. and J. A. Olson
1964 Formation and enterohepatic circulation of water soluble metabolites of retinol (vitamin A) in the rat. Nature 201: 1222.

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APPENDIX A

Table 24. Comparison of solvent mixtures for extraction procedure

Serial no.	Carotene recovered in $CHCl_3$:CH_3OH:2:1 phase	Carotene recovered in skB:Ethanol:3:1 phase
	mcg.	mcg.
1	8.88	21.5
2	9.12	23.2

Table 25. Adequacy of washing with 2% calcium chloride solution compared to that of distilled water

	Serial no.	Amount added			Amount recovered			% recovered		
		Fraction			Fraction			Fraction		
		I Garotene	II R.ester	III Retinol	I Garotene	II R.ester	III Retinol	I Garotene	II R.ester	III Retinol
		mcg	mcg	mcg	mcg	mcg	mcg	%	%	%
Washing with distilled H_2O	1	936	209.9	173	846.4	183.2	128	90.4	87.3	74
	2	1208	280.2	190	1160.3	245.5	110.1	96.0	88.0	58
2% calcium chloride solution	1	110.4	536.6	110.5	105.3	514.8	102	95.4	97.8	92.3
	2	110.4	536.6	110.5	105.3	514.8	103.7	95.4	97.8	93.8
	3	110.4	536.6	110.5	104.9	514.8	107.1	95	97.8	96.9

Table 26. Comparison of recoveries of added carriers of beta-carotene, retinyl palmitate and retinol after chromatographing on 6% alumina column, with those on 7% alumina column

% alumina	Serial number	Added			Recovered			% recovery		
		Fraction			Fraction			Fraction		
		I Carotene	II R.esters	III Retinol	I Carotene	II R.esters	III Retinol	I Carotene	II R.esters	III Retinol
		mcg.	mcg.	mcg.	mcg.	mcg.	mcg.	%	%	%
6%	1	986	415.8	398.5	891	211	307.4	90.4	67.2	77.3
		986	415.8	398.5	889	238.6	342	90.2	75	86
		986	374.3	278.8	955.8	346.5	233.2	96.5	92.5	83.5
		986	374.2	278.8	937.6	361.4	249.6	95	96.5	89.5

APPENDIX B

During the course of the development of methods, results obtained in series I to IV were substantiated with those from two feeding studies with non radioactive carotene. In these experiments hepatic storage of retinol over a given period of time was taken as a measure of the metabolism of orally fed carotene.

Experiment I:

Purpose: The objective of this experiment was to assess the value of casein hydrolysate as a dietary protein source in the carotene conversion process. The information was needed since casein hydrolysate was used as part of the exogenous protein formula in the original procedure for series I.

Pretreatment: Eight litters of 2 males each were depleted of their hepatic retinol stores as described previously. They were then divided into two groups. Litter mates received either 20% unhydrolyzed or hydrolyzed casein protein in their diet. Diets were fed for four weeks, and were supplemented with 120 mcg carotene on alternate days.

Experimental: On the 29th day, the rats were given an injection of sodium pentobarbital[1] (50 mg./100 gm. body weight). The livers were removed after rupturing the hepatic vein and prepared for chemical analysis of vitamin A.

Results: Table 27 gives the data of this experiment. The animals fed 20% casein protein in diet had stored a mean of 230 mcg. of vitamin A

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[1] Sodium nembutal, Abbott Laboratories, No. Chicago, Illinois.

Table 27. Body weights, liver weights and the hepatic vitamin A (Experiment I)

Group symbol	Rat no.	Initial wt.	Final wt.	Liver wt.	Total vit. A
	g.	gm.	gm.	gm.	mcg.
20C	1	143	236	9.6	295
	9	145	261	9.4	202
	13	137	234	9.2	136
	21	124	232	9.6	218
	25	145	238	8.6	224
	29	138	232	9.3	256
	33	151	258	8.9	236
	37	166	262	9.6	271
Mean		144	244	9.3	230±17
20C	2	159	252	10.3	118
	10	149	250	9.9	54
	14	134	218	9.0	37
	22	140	218	9.9	127
	26	141	224	10.2	122
	30	120	200	10.4	154
	34	164	260	10.8	120
	38	158	228	12.6	57
Mean		146	231	10.4	99±15

in their livers whereas the ones fed 20% casein hydrolysate had only a mean of 99 mcg. of vitamin A. The difference was significant at the 1% level. This pointed out that the casein hydrolysate in the diet might have inhibited the rate of carotene metabolism in terms of its conversion or storage in liver. On the basis of these results the exogenous protein formula was omitted in later experiments.

Experiment II:

Purpose: The purpose of this experiment was to test the effectiveness of reducing the feeding period to 2 weeks in repletion studies with non radioactive carotene.

In studies preceeding the present report a 4 week repletion period with carotene had been used. Hepatic retinol laid down had been a linear function of protein from inadequate to adequate to excessive levels of intake. Since in the studies on intestinal metabolism rats were refed for only two weeks prior to the *in vivo* experiment, a pilot experiment was conducted in which hepatic vitamin A storage was determined following carotene ingestion over a two week refeeding period with adequate and excessive protein intakes.

Pretreatment: Ten litters of two males each were depleted of hepatic stores of retinol as described previously. Litter mates received either 20 or 40% casein protein in the diet for a period of two weeks, together with mcg carotene daily.

Experimental: See experiment.

Results: The data of experiment presented in Table 28 shows that the animals fed 20% casein in diet had a mean of 112 mcg. of hepatic

Table 28. Body weights, liver weights and the hepatic vitamin A (Experiment II)

Group symbol	Rat no.	Initial wt.	Final wt.	Liver wt.	Total vit. A
		gm.	gm.	gm.	mcg.
20C	3	157	216	10.4	174
	7	150	209	9.7	151
	11	138	192	8.0	110
	15	158	210	9.7	98
	19	145	194	9.2	127
	23	142	200	10.1	82
	27	131	190	8.9	99
	31	138	192	9.4	96
	35	153	216	9.6	105
	39	166	213	10.6	73
Mean		147.8	203	9.6	112±10
	4	150	216	9.9	151
	8	162	224	9.8	163
	12	135	194	9.5	130
	16	148	200	9.2	104
	20	156	205	10.1	184
	24	123	186	8.4	120
	28	150	203	9.4	124
	32	131	180	9.9	92
	36	159	215	9.2	99
	40	154	219	9.8	144
Mean		147	204	9.5	131±10

Vitamin A compared to the ones on 40% regime, who had a mean value of 131 mcg. This difference, however, was not significant. Results were later confirmed in series V. A discussion of the implication is found there.

APPENDIX C

Table 29. Radioactivity recovered in each chromatographic fraction of different tissues in percentage of injected dose of ^{14}C-carotene (Series I)

Group symbol	Rat no.	Small intestine-wall Fraction I Carotene	Small intestine-wall Fraction II R.ester	Small intestine-wall Fraction III Retinol	Small intestine-contents Fraction I Carotene	Small intestine-contents Fraction II R.ester	Small intestine-contents Fraction III Retinol	Liver
1-20C	2	26.8	11.86	4.98	41.4	0.07	3.92	1.84
	7	33.7	19.91	1.13	47.0	0.83	1.59	0.30
	10	17.4	1.98	0.00	68.7	0.04	1.20	0.00
	16	22.8	15.66	13.22	42.1	1.45	2.10	1.15
	25	26.8	10.58	3.04	46.0	6.12	1.54	0.95
	27	18.7	18.60	1.94	40.4	0.83	2.08	1.11
	33	7.7	3.74	0.95	57.9	0.45	1.25	0.48
	35	29.3	4.47	1.31	46.8	2.27	2.00	0.70
	39	11.0	4.88	1.80	56.8	1.50	1.95	0.49
	42	13.7	1.24	1.41	15.9	2.07	0.55	0.00
Mean		20.8	9.29	2.98	46.3	1.56	1.82	0.70
0-20C	1	14.4	1.3	9.8	68.0	5.3	0.9	1.37
	6	23.7	1.4	8.5	85.8	1.2	1.5	0.00
	9	33.4	0.0	0.6	49.8	0.0	1.2	0.00
	15	39.4	2.0	1.3	61.3	1.6	2.4	1.23
	24	25.6	1.7	0.7	41.8	0.4	2.5	0.49
	26	30.2	0.1	0.1	52.3	3.9	1.7	0.82
	32	47.4	1.0	0.1	30.1	0.0	0.2	0.43
	34	50.6	0.7	0.3	46.9	2.8	1.9	0.56
	38	21.8	2.7	2.7	46.2	4.0	4.2	1.46
	43	10.3	1.3	0.5	67.8	2.1	1.9	0.00
Mean		29.7	1.22	2.46	55.0	2.13	1.84	0.64
3-20C	3	13.7	13.1	0.7	32.6	1.3	2.0	3.38
	8	26.1	18.4	0.8	49.2	1.3	0.0	0.00
	11	8.3	5.5	2.0	49.3	1.4	3.7	0.32
	17	39.0	6.4	8.0	15.0	6.4	2.7	5.55
	46	8.9	10.5	1.2	57.0	2.5	3.2	0.37
	50	16.5	10.6	1.8	40.3	2.1	3.4	0.93
	51	8.9	10.6	2.5	21.9	5.4	2.4	2.00
	54	28.7	6.0	1.0	39.0	1.6	1.9	0.09
	55	24.0	7.3	2.7	38.3	0.8	3.7	0.29
	78	19.1	10.7	0.9	51.8	0.7	3.2	0.98
Mean		19.3	9.91	2.16	39.4	2.35	2.62	1.39

Table 29. (Continued)

Group symbol	Rat no.	Small intestine-wall Fraction K Carotene	II R.ester	III Retinol	Small intestine-contents Fraction I Carotene	II R.ester	III Retinol	Liver
0-40C	22	71.2	0.8	2.4	83.8	0.1	2.6	1.63
	26	16.1	0.6	0.1	62.5	1.9	0.8	0.68
	36	44.9	1.4	1.0	40.5	2.2	1.1	0.67
	40	15.3	0.9	3.0	54.4	0.9	3.0	0.50
	59	31.5	1.1	1.1	47.7	0.5	0.5	0.00
	63	8.3	1.6	0.0	88.7	0.5	2.3	0.02
	66	10.3	2.6	1.3	82.0	1.1	2.4	0.00
	71	7.3	0.1	0.3	53.4	0.5	1.3	0.22
	72	14.4	0.6	0.5	82.5	1.2	2.8	0.00
	75	17.0	0.8	0.4	91.6	1.7	2.6	0.00
Mean		23.6	1.05	1.01	68.7	1.06	1.94	0.37
1-40C	23	20.1	4.2	3.7	84.8	0.7	2.9	1.63
	29	14.9	6.5	1.6	43.1	0.9	1.7	0.62
	37	33.0	0.8	0.9	39.1	0.9	1.9	0.69
	41	5.5	6.6	13.3	63.2	1.9	9.5	0.74
	60	14.1	2.4	4.5	52.7	0.1	1.1	0.00
	62	24.8	1.2	0.0	58.3	1.6	2.7	0.41
	67	8.2	2.8	1.6	82.4	0.7	2.9	0.28
	70	12.8	7.8	1.3	49.1	0.9	2.0	0.00
	73	15.6	20.8	1.6	49.1	1.3	3.0	0.04
	76	19.7	4.8	1.7	57.2	1.7	3.0	0.00
Mean		16.9	5.79	3.02	57.9	1.07	3.07	0.44
3-40C	48	8.8	7.6	0.3	54.9	2.4	3.2	0.72
	49	21.1	4.4	0.8	46.5	2.0	2.3	0.04
	53	5.4	6.3	2.7	46.2	0.6	4.4	0.36
	57	11.0	2.8	0.6	61.3	0.8	1.9	0.19
	68	8.5	3.6	0.6	73.6	1.6	3.7	0.00
	69	5.9	4.3	1.4	56.2	1.2	2.1	0.14
	74	19.0	17.8	1.4	40.9	1.5	2.5	1.87
	77	23.9	7.4	1.2	46.2	1.1	3.1	0.70
	80	16.9	10.5	1.0	49.3	0.9	2.9	1.43
	81	15.5	10.8	0.9	34.5	2.5	2.1	1.89
Mean		13.6	7.55	1.09	51.0	1.46	2.82	0.73

Table 30. Radioactivity recovered in each chromatographic fraction of different tissues in percentage of injected dose of ^{14}C-carotene

Group symbol	Rat no.	Small intestine-wall Fraction				Small intestine-contents Fraction				Liver
		Carotene	Retinyl esters	Retinol	Breakdown prod.	Carotene	Retinyl esters	Retinol	Breakdown prod.	
1-20C	43	8.1	2.01	1.10	0.34	52.1	0.86	1.74	1.12	0.22
	47	10.3	3.04	0.59	0.41	65.5	0.55	2.64	0.75	0.84
	49	19.6	0.41	1.10	0.04	42.3	1.01	1.66	0.26	0.00
	51	24.9	1.32	0.37	0.00	36.2	0.84	1.09	1.28	0.05
	53	12.5	0.24	0.52	0.90	44.8	1.30	.97	0.48	0.28
	55	13.4	0.99	0.31	0.00	32.2	1.55	1.18	0.88	0.00
	57	7.0	3.48	0.17	0.66	27.4	2.00	2.18	0.81	0.52
	59	5.6	1.96	1.46	0.40	30.7	1.47	2.20	0.49	0.17
Mean		12.7	4.18	0.70	0.34	41.4	1.20	1.71	0.76	0.26
1-40C	44	11.9	1.82	0.44	0.66	54.0	0.63	0.97	0.54	0.27
	48	11.9	5.63	0.79	0.47	39.6	1.12	1.47	0.43	0.08
	50	11.5	0.52	1.05	0.10	42.1	0.05	2.08	0.00	0.65
	52	37.2	25.2	1.85	0.22	6.6	1.03	0.11	0.00	0.55
	54	12.0	3.46	1.04	0.00	30.9	0.90	0.91	0.37	0.00
	56	22.3	0.67	0.19	.69	54.6	0.56	1.25	0.36	0.00
	58	10.8	19.68	0.97	0.07	53.9	1.99	1.52	0.36	0.13
	60	13.7	15.36	0.71	0.28	56.3	0.90	0.42	0.50	1.27
Mean		16.4	9.04	0.88	0.31	42.2	0.90	1.09	0.32	0.37

Table 31. Radioactivity recovered in each chromatographic fraction of different tissues in percentage of injected dose of ^{14}C-carotene

Group symbol	Rat no.	Small intestine-wall Fraction I Carotene	Small intestine-wall Fraction II Ret. ester	Small intestine-wall Fraction III Retinol	Small intestine-wall Fraction IV Breakdown prod.	Small intestine-contents Fraction I Carotene	Small intestine-contents Fraction II Ret. ester	Small intestine-contents Fraction III Retinol	Small intestine-contents Fraction IV Breakdown prod.	Stomach Fraction I Carotene	Stomach Fraction II Ret. ester	Stomach Fraction III Retinol	Stomach Fraction IV Breakdown prod.	Liver	Colon
1-20C	75	15.6	1.18	1.31	0.00	55.3	1.41	3.64	1.06	9.48	0.89	0.63	1.36	0.58	0.27
	79	8.0	7.91	1.91	14.03	37.7	1.03	1.52	0.89	13.6	1.74	1.07	1.41	0.65	0.51
	82	22.2	8.58	1.06	0.38	64.8	0.88	2.26	1.35					0.74	0.28
	85	17.3	15.79	1.73	0.43	30.8	1.54	1.44	0.81	9.7	0.69	0.35	0.27	2.29	0.16
	88	12.7	15.38	1.23	0.59	30.2	2.18	1.96	0.80	7.1	0.10	0.75	0.15	2.31	0.92
	89	16.0	1.26	2.10	0.87	47.1	0.59	1.79	0.60	3.2	0.66	0.00	0.00	1.30	0.00
	91	13.8	0.01	1.35	0.21	38.03	1.27	1.49	0.01	25.7	0.71	0.87	1.22	1.20	0.00
	109	8.9	0.00	1.03	0.00	27.9	0.67	1.03	0.66	10.0	0.00	0.54	0.00		0.33
	112	14.7	1.89	1.04	0.00	12.9	1.69	1.49	0.69	3.29	0.37	0.05	0.17	0.38	0.15
	113	11.2	10.60	1.68	0.10	37.0	0.66	1.33	0.37	14.8	0.66	0.31	0.12	0.18	1.26
Mean		14.0	6.26	1.44	1.66	38.2	1.19	1.80	0.72	11.3	0.65	0.51	0.52	1.07	0.39
1-40C	74	20.4	1.07	2.01	0.14	56.7	1.13	2.13	1.15	8.5	0.79	1.19	0.71	0.44	0.08
	77	16.7	0.45	1.07	0.13	73.3	1.78	2.68	1.26	0.14	0.00	0.00	0.00	2.01	1.19
	80	6.3	12.63	5.04	0.76	10.3	0.75	1.23	0.89	14.7	0.30	1.10	0.98	1.80	0.67
	83	10.8	26.23	2.35	0.31	58.6	1.46	2.72	0.79	0.48	0.00	0.00	0.20	0.54	0.28
	86	19.7	8.08	0.92	0.09	40.6	2.61	0.94	1.38	3.57	0.02	0.11	0.08	0.31	0.43
	90	9.36	9.58	0.65	0.16	78.1	1.27	2.04	0.40	7.00	0.00	0.79	0.34	0.68	0.00
	92	13.1	0.27	0.93	0.00	18.2	0.14	0.88	0.25	39.1	2.40	3.76	2.27	0.31	0.09
	110	30.3	0.46	1.59	0.02	17.5	0.25	1.12	0.38	0.77	0.00	0.07	0.79	0.54	0.22
	114	27.6	12.05	2.16	0.69	28.6	2.19	1.45	0.00	0.89	0.12	0.87	0.30	1.16	0.12
	115	7.2	2.21	0.78	0.20	42.5	0.54	2.68	0.87	16.17	0.39	0.43	0.00	0.42	0.18
Mean		16.13	7.30	1.75	0.25	42.4	1.21	1.79	0.74	9.13	0.40	0.73	0.57	0.82	0.33

Table 32. Radioactivity recovered in each chromatographic fraction of different tissues in percentage of injected dose of ^{14}C-carotene, Series IV

Group symbol	Rat no.	Small intestine-wall				Small intestine-contents			
		Fraction				Fraction			
		Carotene	R.esters	Retinol	Break-down prod.	Carotene	R.esters	Retinol	Break-down prod.
5-10C	95	5.7	0.33	0.41	0.38	24.4	0.34	0.35	0.22
	96	41.3	0.28	0.00	0.26	0.7	0.00	0.78	1.30
	100	2.4	1.22	0.52	0.23	0.8	1.02	0.00	0.85
	103	10.0	5.70	1.07	0.19	22.7	1.11	1.11	0.74
	104	13.6	3.23	0.62	0.10	15.1	0.06	0.57	1.08
	107	9.4	1.03	0.27	0.09	22.4	0.96	0.96	0.67
	108	24.3	1.00	0.93	0.45	5.8	0.00	1.45	1.18
Mean		15.2	1.83	0.55	0.24	13.1	0.50	0.75	0.86
5-40C	93	2.8	0.34	0.72	0.58	0.3	0.27	0.51	0.00
	94	5.6	0.56	0.41	0.00	27.9	0.52	0.03	0.23
	98	1.1	2.03	0.39	0.07	0.4	0.61	0.83	0.17
	101	2.3	1.71	0.77	0.08	0.3	0.10	0.05	0.67
	102	11.3	5.60	0.40	0.27	13.5	1.01	0.47	0.00
	105	25.2	0.44	2.36	0.55	8.0	0.08	0.00	0.95
	106	2.5	0.96	0.58	0.00	0.8	0.26	0.27	1.82
Mean		7.2	1.16	0.80	0.22	7.3	0.41	0.31	0.54

Group symbol	Rat no.	Stomach				Liver	Colon	Serum
		Fraction						
		I	II	III	IV			
		Carotene	R.esters	Retinol	Break-down prod.			
5-10C	95	0.3	0.00	0.00	0.00	2.53	26.40	3.02
	96	0.0	0.00	0.00	1.62	2.40	29.92	7.01
	100	3.4	0.15	0.09	0.11	5.06	36.85	2.01
	103					2.32	1.46	1.36
	104	2.6	0.27	0.18	0.00	3.03	0.34	3.33
	107	4.7	1.08	0.03	0.00	1.36	0.44	2.19
	108	6.4	0.89	0.00	0.00	2.98	5.19	2.91
Mean		2.9	0.39	0.05	0.29	2.81	14.37	3.12
5-40C	93	0.8	0.00	0.00	0.61	4.20	3.61	3.15
	94	1.8	0.00	0.08	0.64	6.33	50.17	3.33
	98	1.2	0.56	0.00	0.00	5.95	26.81	1.94
	101	0.7	0.12	0.63	0.00	2.90	24.22	5.66
	102	3.7	0.00	0.12	0.00	3.43	7.48	2.73
	105	0.1	0.08	0.00	0.35	5.95	0.35	4.43
	106	5.9	0.00	0.32	0.49	2.05	30.22	2.39
Mean		2.0	0.10	0.16	0.29	3.07	14.23	3.38

Table 33. Radioactivity recovered in each chromatographic fraction of different tissues in percentage of injected dose of ^{14}C-carotene

Group symbol	Rat no.	Small intestine-wall Fraction				Small intestine-contents			
		K Garotene	II R.ester	III Retinol	IV Break-down prod.	I Garotene	II R.ester	III Retinol	IV Break-down prod.
10G-4	1	16.9	0.19	0.91	0.01	13.4	0.23	0.04	0.26
	25	5.0	0.08	0.77	0.00	10.7	0.60	0.46	0.09
	31	7.0	0.34	0.59	0.06	29.3	0.39	0.88	0.22
	46	10.8	6.33	1.23	0.19	14.4	0.38	0.83	0.92
	49	2.6	0.32	0.96	0.48	21.2	0.22	0.42	1.58
	61	24.7	0.73	1.23	0.99	19.3	0.58	0.35	0.99
	73	35.0	0.16	0.85	0.31	3.4	0.27	0.24	0.49
	82	15.3	0.07	0.36	0.00	14.5	0.60	0.99	0.00
	109	18.8	5.78	0.75	0.62	10.6	0.68	0.82	0.21
	112	15.6	3.26	0.82	0.23	27.6	0.60	0.76	0.00
	118	26.4	5.79	0.42	0.55	17.2	0.85	1.37	0.08
	127	21.0	0.53	0.67	0.35	21.6	0.42	0.97	0.31
Mean		16.6	1.97	0.80	0.32	16.9	0.49	0.68	0.43
40G-4	3	13.9	1.11	0.98	0.00	26.0	0.41	0.60	0.00
	27	17.9	1.76	0.85	0.00	19.5	0.14	0.48	0.00
	33	29.7	1.75	0.64	0.56	24.3	0.21	1.14	0.18
	48	15.3	12.39	0.72	0.15	20.0	0.32	0.72	0.29
	51	10.4	0.16	1.01	0.76	25.5	0.59	0.20	1.14
	63	8.0	-	0.83	0.67	15.3	0.34	0.81	0.86
	75	5.3	5.65	0.64	3.80	11.1	0.64	0.27	0.52
	84	17.9	0.63	0.56	0.27	32.6	1.51	1.14	0.00
	111	14.6	8.50	0.95	0.10	16.9	0.50	0.60	0.07
	114	28.2	4.01	0.86	0.35	18.9	0.09	0.76	0.01
	120	20.4	9.73	0.37	1.29	22.3	1.48	0.76	0.10
	129	18.8	11.24	1.55	0.44	20.8	0.35	0.94	0.69
Mean		16.7	5.18	0.83	0.70	21.1	0.54	0.71	0.32

Table 33. (Continued)

Group symtol	Rat no.	Stomach Fraction I Caro-tene	Stomach Fraction II R.ester	Stomach Fraction III Reti-nol	Stomach Fraction IV Break-down prod.	Liver Before chromo-tography	Liver Fraction I Caro-tene	Liver Fraction III Reti-nol	Liver Fraction IV Break-down prod.	Colon
10G-4	1	2.1	0.57	0.94	0.34	6.10	0.50	3.92	0.82	0.00
	25	4.0	0.00	0.28	0.11	4.77	0.56	3.77	0.33	3.94
	31	2.7	0.00	0.14	0.13	5.07	0.13	4.74	0.35	0.17
	46	29.3	0.65	1.36	0.67	0.81	0.00	1.00	0.08	0.51
	49	2.1	0.00	0.38	0.24	14.03	0.78	13.08	0.42	1.15
	61	7.3	0.00	0.38	0.24	6.19	0.46	5.76	0.28	0.65
	73	11.3	0.00	0.35	0.08	7.66	0.25	7.66	0.66	0.29
	82	11.7	0.12	0.46	0.18	5.47	0.66	4.79	1.07	0.35
	109	22.3	0.51	0.55	0.28	7.17	0.26	6.62	0.15	0.20
	112	5.5	0.00	0.00	0.36	2.78	0.39	2.98	0.52	0.80
	118	0.00	0.13	1.00	0.41	4.85	1.54	4.75	0.10	0.26
	121	2.9	0.03	0.03	0.26	8.96	0.05	9.07	0.47	0.16
Mean		8.4	0.17	0.47	0.26	6.16	0.47	5.87	0.47	0.71
40G-4	3	11.9	0.33	0.41	0.48	11.08	2.33	8.54	1.04	0.02
	27	27.9	0.00	0.38	0.59	4.74	1.04	4.40	0.12	2.46
	33	27.2	0.09	0.70	0.60	3.22	0.28	3.03	0.43	0.52
	48	0.8	0.04	0.00	0.00	16.8	1.86	14.48	0.58	12.93
	51	3.0	0.00	0.00	0.02	16.8	0.68	20.73	0.47	0.88
	63	14.6	0.00	0.62	0.33	12.05	1.48	11.62	0.24	0.77
	75	-	-	-	-	-	1.33	20.74	1.23	0.31
	84	1.9	0.00	0.26	0.07	6.12	1.17	5.63	1.03	0.34
	111	10.0	0.34	0.21	0.30	7.04	1.68	5.60	0.92	0.16
	114	2.6	0.06	0.16	0.07	8.71	1.48	8.75	0.09	0.27
	120	24.8	0.75	0.52	0.71	3.91	0.23	4.11	0.29	0.68
	129	9.2	0.32	0.01	0.06	11.50	0.13	11.14	0.18	0.40
Mean		12.17	0.18	0.29	0.30	9.27	1.14	9.90	0.55	1.65

Table 34. Body weights, liver weights, and hepatic vitamin A levels, Series V

Group symbol	Rat no.	Initial wt. gm.	Final wt. gm.	Gain in wt. gm.	Wt. on autopsy day gm.	Liver wt. gm.	Vit. A/ liver mcg	Vit. A/ gm. liver mcg.
10G-1	7	160	168	8	162	6.6	76	11.5
	19	160	168	8	167	5.4	175	32.4
	22	163	172	9	167	5.9	55	9.3
	40	121	140	19	136	4.2	76	18.1
	52	142	152	10	165	6.7	174	26.0
	79	141	150	9	146	5.0	53	10.6
	88	150	158	8	153	5.2	170	32.7
	97	145	154	9	151	5.2	126	24.2
	106	137	448	11	147	5.5	142	25.8
	115	142	150	8	147	5.3	112	21.1
Mean		146	156	99	154	5.5	116	21.2
20G-1	8	132	164	32	159	6.5	192	29.5
	20	147	173	26	171	6.1	171	28.0
	23	153	179	26	174	6.7	300	44.8
	41	157	181	24	184	6.6	316	47.9
	53	128	152	24	151	5.4	167	30.9
	80	151	172	21	169	7.3	144	19.7
	89	150	173	23	172	6.1	214	35.1
	98	138	162	24	161	6.0	127	21.2
	107	138	167	29	167	6.7	186	27.8
	116	137	158	21	157	6.3	156	24.8
Mean		143	168	25	167	6.4	197	31.0
40G-1	9	152	183	31	177	7.5	210	28.0
	21	146	172	26	165	6.9	231	33.5
	24	160	186	26	179	8.0	208	26.0
	42	148	173	25	171	6.7	304	45.4
	54	127	146	19	148	5.3	155	29.2
	81	168	180	12	174	7.2	88	12.2
	90	141	162	21	159	6.3	197	31.3
	99	146	163	17	160	6.8	189	27.8
	108	136	154	18	153	6.1	170	27.9
	117	118	136	18	134	5.5	167	30.4
Mean		144	166	21	162	6.6	192	29.2

Table 34. (Continued)

Group symbol	Rat no.	Initial wt. gm.	Final wt. gm.	Gain in wt. gm.	Wt. on sutopsy day gm.	Liver wt. gm.	Vit. A/ liver mcg.	Vit. A/ gm. liver mcg.
10C-2	10	134	175	41	172	5.7	302	23.1
	16	152	168	16	174	6.2	105	53.0
	34	127	161	34	151	5.6	225	16.9
	58	115	139	24	137	5.7	152	40.2
	64	136	174	38	174	6.3	221	26.7
	76	169	187	18	190	6.7	156	35.1
	85	127	147	20	141	5.2	167	23.3
	94	150	180	30	178	6.9	250	32.1
	103	157	177	20	175	6.0	230	36.2
	121	132	156	24	158	6.2	143	38.3
Mean		140	166	26.5	165	6.1	195	32.5
20C-2	11	134	189	55	187	6.9	470	38.7
	17	120	171	51	160	6.0	119	68.1
	35	123	180	57	178	7.6	449	19.8
	59	112	172	60	169	6.3	422	59.1
	65	148	200	52	201	8.0	332	67.0
	77	168	200	32	209	8.4	293	41.5
	86	145	188	43	195	8.1	232	34.9
	95	150	198	48	207	7.9	349	28.6
	104	147	193	46	195	8.1	262	44.2
	122	124	175	51	180	7.8	302	32.3
Mean		137	187	49.5	188	7.5	323	43.4
40C-2	12	134	174	40	170	6.1	393	29.9
	18	114	183	69	161	6.2	157	64.4
	36	136	190	54	186	7.0	436	25.3
	60	100	137	37	142	5.3	407	62.3
	66	144	199	55	198	8.3	321	76.8
	78	165	213	48	218	8.7	374	38.7
	87	124	184	60	183	7.0	356	43.0
	96	160	210	50	218	8.7	422	50.9
	105	147	189	42	197	7.2	428	48.5
	123	149	200	51	199	8.5	254	59.4
Mean		137	188	50.6	187	7.3	355	49.9

Table 34. (Continued)

Group symbol	Rat no.	Initial wt. gm.	Final wt. gm.	Gain in wt. gm.	Wt. on autopsy day gm.	Liver wt. gm.	Vit. A/ liver mcg.	Vit. A/ gm. liver mcg.
10G-3	4	154	204	50	207	6.7	178	23.3
	13	140	190	50	186	5.9	173	28.3
	37	141	208	67	207	6.8	235	16.3
	43	144	203	59	202	6.3	274	29.3
	55	130	175	45	177	5.4	88	26.6
	67	145	203	58	206	6.6	242	36.7
	70	139	195	56	198	6.5	264	40.6
	91	163	210	47	212	7.4	310	41.9
	100	136	190	54	191	6.3	147	34.6
	124	168	205	37	203	7.1	201	43.5
Mean		146	198	52.3	199	6.5	211	32.1
20G-3	5	153	235	82	238	9.2	421	39.2
	14	126	203	77	202	7.7	480	43.3
	38	150	228	78	230	8.3	537	23.3
	44	127	219	92	217	7.4	502	62.3
	56	138	219	81	222	8.1	189	45.8
	68	153	236	83	237	8.8	317	36.0
	71	177	254	77	256	8.5	522	61.4
	92	164	234	70	236	8.4	536	63.8
	101	146	218	72	212	7.9	310	64.7
	125	160	220	60	224	8.3	359	67.8
Mean		149	227-	77	227	8.3	417	50.8
40G-3	6	162	234	72	240	9.1	382	45.9
	15	156	231	75	234	8.0	364	58.6
	39	142	239	97	240	8.1	599	32.3
	45	146	232	86	232	8.7	631	45.5
	57	131	206	75	210	7.5	242	42.0
	69	144	206	62	209	7.7	415	53.9
	72	162	251	89	253	9.8	542	55.3
	93	153	228	75	229	8.4	583	69.4
	102	134	204	70	206	7.9	363	74.0
	126	147	216	69	217	8.6	504	72.5
Mean		148	225	77	227	8.4	463	54.9

Table 34. (Continued)

Group symbol	Rat no.	Initial wt. gm.	Final wt. gm.	Gain in wt. gm.	Wt. on autopsy day gm.	Liver wt. gm.	Vit. A/ liver mcg.	Vit. A/ gm. liver mcg.
10C-4	1	116	173	57	167	5.9	314	53.2
	25	131	198	67	196	5.7	441	77.4
	31	132	204	72	197	7.2	329	45.7
	46	130	196	66	189	6.7	432	64.5
	49	127	205	78	198	6.3	264	41.9
	61	102	192	90	188	5.8	289	49.8
	73	160	226	66	228	8.2	362	44.1
	82	159	225	66	226	6.9	580	84.1
	109	144	218	74	218	6.7	324	48.4
	112	157	218	61	218	6.9	453	65.7
	118	122	180	58	174	5.8	410	70.7
	127	139	193	54	192	6.3	496	78.7
Mean		135	202	67.4	199	6.5	391	60.4
20C-4	2	152	253	101	254	9.0	420	46.7
	26	129	250	121	252	7.9	809	102.4
	32	146	250	104	250	8.5	624	73.4
	47	140	241	101	242	8.1	536	66.2
	50	132	237	105	238	8.1	435	53.7
	62	130	228	98	226	7.8	620	79.5
	74	154	244	90	248	9.8	422	43.1
	83	119	202	83	202	6.7	638	95.2
	110	152	258	106	258	8.8	587	66.7
	113	146	238	92	237	7.7	863	112.1
	119	137	227	90	230	8.2	472	57.6
	128	139	231	92	230	7.9	820	103.8
Mean		140	238	98.6	239	8.2	604	75.0
40C-4	3	155	272	117	276	11.2	678	60.5
	27	138	244	106	240	8.9	761	85.5
	33	109	200	91	196	6.6	774	117.3
	48	143	252	109	252	9.2	1112	120.9
	51	124	226	102	225	7.7	936	121.6
	63	116	223	107	220	7.2	955	132.6
	75	170	268	98	274	11.0	639	58.1
	84	150	264	114	270	10.3	674	65.4
	111	124	247	123	244	8.2	615	75.0
	114	153	252	99	252	10.0	904	90.4
	120	125	240	115	243	8.8	869	98.8
	129	127	232	105	230	7.7	809	105.1
Mean		136	243	107	244	8.9	811	94.3