Studies on Carotene Metabolism

A thesis presented for the Degree of Doctor of Philosophy

University of New Zealand

November 1949

William Anderson McGillivray

STUDIES ON CAROTENE METABOLISM

A Thesis Presented
for the
Degree of Doctor of Philosophy

University of New Zealand

November, 1949.

William Anderson McGillivray.

I N D E X

INTRODUCTION

Over twenty years ago, during the course of the early investigations into the chemistry and physiology of vitamin A, it was found that the potency of herbage was related to its carotene content and mainly as a result of the work of Moore, it was established that this carotene could be utilized by animals and converted into vitamin A. Since this recognition of the carotenes as provitamins, the problems of the mode and site of conversion in the animal body have aroused the interest of many workers.

Until recently it was considered that the liver was the main site of conversion. Apart from the somewhat equivocal results obtained from attempted in vitro conversions using liver preparations, this assumption was based mainly on the fact that the feeding of carotene to vitamin A-deficient animals resulted in the almost immediate appearance of the vitamin in the livers. At the same time little, if any, carotene appeared in the livers whereas the alimentary tracts contained relatively large amounts of carotene and little vitamin.

Recent observations however suggest that in certain animals at least, the conversion takes place in the wall of the intestine rather than in the liver. At the time this investigation was commenced it had been established by Deuel and his co-workers that the intestinal wall was the main site of conversion in the rat. It seemed possible that there might be species differences and it was resolved to determine the site of conversion in other animals, particularly ruminants, and to investigate the enzyme systems involved. During the past two years reports have appeared of work of a similar nature carried out by various teams and it is now established that the wall of the intestine is the

main site of conversion in a range of experimental animals and no evidence has so far been obtained of a secondary site. These findings have of necessity modified the course of this investigation.

Prior to the work of Deuel and his team, interest in aspects of carotene metabolism had been aroused in this Department through observations suggesting the relative non-availability to ruminants of carotene from certain pastures. This problem, together with other factors affecting the efficiency of conversion of carotene to vitamin A in ruminants, has also been studied. Since this investigation is continuing at the present time the results presented here are not complete in themselves.

Vitamin A or carotene metabolism studies had not previously been undertaken in this Department so that it was necessary to devote some time to perfecting procedures such as the surviving tissue technique and to establishing suitable methods of assay for vitamin A and carotene in the samples handled. During this preliminary work and while awaiting the arrival of necessary apparatus and chemicals, a short investigation into the vitamin content of New Zealand mutton and lamb was undertaken. This was an extension of work previously carried out and is described in an appendix to this thesis.

. . . . . .

C H A P T E R I

Methods of Estimation of Vitamin A and Carotene

In an investigation of this type it is essential that the methods of assay should be reliable and as accurate and reproducible as possible. Unsubstantiated claims by several workers to have effected the conversion of carotene to vitamin A by various methods, may, as will be discussed later, be attributed to doubtful methods of assay. It was considered necessary therefore to devote some time to a thorough examination of the methods available for the estimation of vitamin A and carotene occurring separately and together and in the presence of related substances, in particular their oxidative breakdown products.

A survey of the literature showed that, although many of the methods differed in small detail only, there were in some cases conflicting statements particularly with regard to the efficiency of the various extraction procedures and the stability of the vitamin and pro-vitamin under the different conditions employed.

The results of the present investigation are most conveniently discussed under the following headings :

(1) Comparison of methods of assay.

(a) The spectrophotometric method

(b) Colorimetric methods.

(2) Stability of vitamin A and carotene under the conditions likely to be encountered in the preparation of samples for assay.

(3) Extraction of the vitamin and separation into a non-aqueous solvent.

(1) Comparison of Methods of Assay.

It is generally recognised that vitamin A can be estimated by colorimetric, spectrophotometric and biological methods. The literature relating to these methods has reached formidable proportions but it is concerned mainly with the relative merits of the various methods in particular cases and the relationship between biological and non-biological assays rather than with details of the methods themselves. To this extent much of it is not relevant to this investigation. It has been fully reviewed in recent publications (e.g. 1, 2, 3, 4) and no attempt will be made here to discuss it in any detail. Briefly the problem is resolving itself into one of expression of results. There are available well established chemical and physical methods by which the vitamin A content of a wide range of materials can be estimated with a high degree of precision. Vitamin A and its esters have been obtained in a high state of purity and optical constants ($E^{1\%}_{1cm}$ 325 (or 328), 620 mu etc.) accurately determined. It is not difficult, therefore, to arrive at a reliable figure for the percentage vitamin A in a preparation. Difficulties are however experienced when it is attempted, as is demanded by convention, to convert these percentages to "vitamin A potencies" expressed either in International Units or United States Pharmacopoeia Units since the "conversion factors" in use (Bioassay/$E^{1\%}_{1cm}\lambda$ max.) vary widely. These variations appear to be attributable mainly to a lack of precision in the bioassays. By the definition of the International Unit of vitamin A as the biological activity of 0.6 micrograms of a sample of pure β carotene and the U. S. P. Unit as that of various reference oils, these biological methods of assay, of which the curative rat growth method (5) is at present official, are rendered

absolute. On this basis non-biological assay methods must therefore be regarded as relative only and their accuracy is limited to that of the biological methods. As has been pointed out by Gridgeman (2) this position is not satisfactory and the tendency at the present time seems to be to ascribe analytical finality at least to the physico-chemical methods and to use the biological methods mainly for the determination of the relative potencies of the various A-vitamers.

Now that pure crystalline vitamin A is available, it may be possible to remove much of the confusion at present existing, by expressing potencies on a percentage basis, that is, as milligrams or micrograms pure vitamin A rather than in International Units.

In general the agreement between biological and non-biological methods, within the limits of accuracy of the former, is good. A number of workers have found that where non-biological methods differ amongst themselves, the colorimetric methods agree most closely with biologically determined values (6, 7, 8) due to the presence of substances which absorb in the region in which spectroscopic readings are taken but which do not interfere with the colorimetric estimation. For this reason and because simpler, less expensive apparatus is required, colorimetric methods are used in many laboratories in preference to direct spectroscopic estimations.

It was apparent that in this investigation much of the material to be assayed for vitamin A would differ from samples normally handled, in containing little or no interfering materials apart from carotene and its breakdown products. In some respects therefore estimations would be simpler than those normally encountered; nevertheless the estimation of vitamin A in the presence of

these breakdown products does introduce special problems. From the point of view of simplicity the spectroscopic method appeared to offer most promise. At the same time it seemed desirable to have available a reliable colorimetric method to confirm spectroscopic findings and for use where the latter method was not applicable.

(a) The Spectrophotometric Method -

1. Standardisation of the Instrument.

The instrument used for the spectrophotometric measurements was a Beckman Model D U Photoelectric Quartz Spectrophotometer fitted with an Ultra Violet Accessory Set giving an effective wavelength range of 220 to 1000 mμ. The instrument arrived and was set up by the author during the course of the present investigation. Two similar models were available in adjacent laboratories and prior to the arrival of the above instrument, spectrophotometric estimations were carried out using these. The operating procedures followed were, in all cases, those recommended by the manufacturers. Cells were calibrated for differences in light transmission and these calibrations were checked at regular intervals. All extinction figures quoted have been corrected for these differences in transmission and related to a solution thickness of 1 cm. No attempt was made to compare the three instruments used beyond noting that at a number of points over the range 300 to 500 mμ the agreement in extinction values was better than ± 2%. It has been shown recently that the variations between instruments may be fairly large (9). In all critical cases therefore, where such instrument to instrument variations might affect results, estimations were repeated using the new instrument, and all results presented relate to this one model.

Attention has been drawn by many workers to the need for checking both the density and wavelength scales of

spectrophotometers. Because of its numerous intense lines the manufacturers recommend a mercury arc enclosed in an ultra violet transmitting envelope for checking the wavelength scale of the Beckman model. This lamp was not however available. Attempts were made to carry out the same type of calibration using the hydrogen lamp as supplied for transmission measurements in the ultra violet. Their closeness and low intensity makes resolution of most of the hydrogen lines difficult and uncertain. Where resolution could be effected (e.g., the lines at 379.8, 434.0, 486.1 mu) the wavelength drum readings agreed to within about 0.2 mu of the values recorded (10) for the apparently corresponding hydrogen lines.

A number of standards have been suggested for the calibration of the density scale on spectrophotometric apparatus used for the estimation of vitamin A, carotene and related substances. Both β carotene and vitamin A alcohol or its esters (or even B. . . Reference Oils) appear to be in common use for this purpose (1, 2). None of these substances can be regarded as satisfactory since, apart from the doubtful original purity of some of these preparations, their stability and hence the purity of any particular solution, is open to question. A number of more stable organic substances such as anthraquinone, salicylaldehyde (11) and 2 phenyl azo-p-cresol (12) have also been suggested. Although these substances possess the advantage that their position of maximum absorption coincides with that of vitamin A, unlike inorganic standards they cannot be obtained readily in a sufficiently high state of purity. The degree of precision in the construction of spectrophotometers such as the Beckman model however, suggests that the actual scale calibrations themselves can be accepted without checking and that the only errors likely to be encountered

ore those due to the mal-alignment of the scales resulting in a uniform displacement over the whole range. Provided therefore the scales are checked at a number of points over the range in use, it is not necessary to check at the particular wavelengths at which readings are to be taken.

Potassium chromate dissolved in dilute potassium hydroxide has been widely used as a spectrophotometric standard and as its optical constants have been determined with sufficient accuracy for checking a precise instrument such as the Beckman, it was used in this investigation. The solution gives two distinct peaks in the near ultra violet and appeared to adequately cover the range in which most determinations were to be made. Morton (11) recommends the use of a 0.003 N solution in 0.05 N potassium hydroxide as a standard and gives an absorption curve for this solution with molecular extinctions of 752, 3660, 203 and 4530 corresponding to the points of maximum and minimum absorption at 229, 272.5, 312.5 and 371.5 mu respectively.

> A solution of Analar Potassium chromate (0.003 N) was prepared in 0.05 N Analar potassium hydroxide, free from carbonate. The absorption of this solution was determined over the range 215 to 430 mu, readings being taken normally every 2 mu and every 0.5 mu near the points of maxima and minima. As extinctions near the peak at 371.5 mu were outside the normal range of the instrument, duplicate readings were taken on a sample of the solution diluted to 0.0015 N with 0.05 N hydroxide. As nearly as could be determined molecular extinctions calculated from the two solutions agreed exactly.

Extinction values obtained were converted to molecular extinction coefficients to give the curve shown in Figure I. In each case the maximum and minimum points occurred at the wavelengths quoted by Morton. As is shown in the Figure molecular extinctions also agreed closely at the two maxima. Agreement was not however so close at the minima due possibly to the presence of an impurity in the chromate sample. Any overall

absorption due to such an impurity would most markedly affect the regions of lowest absorption. In addition to the recorded maximum and minimum points, readings were taken from an enlarged copy of the figure given by Morton, at a number of wavelengths on the sloping portions of the curve. Experimentally determined molecular extinctions agreed to within ± 1.5% of these readings which are shown as crosses in Figure 1.

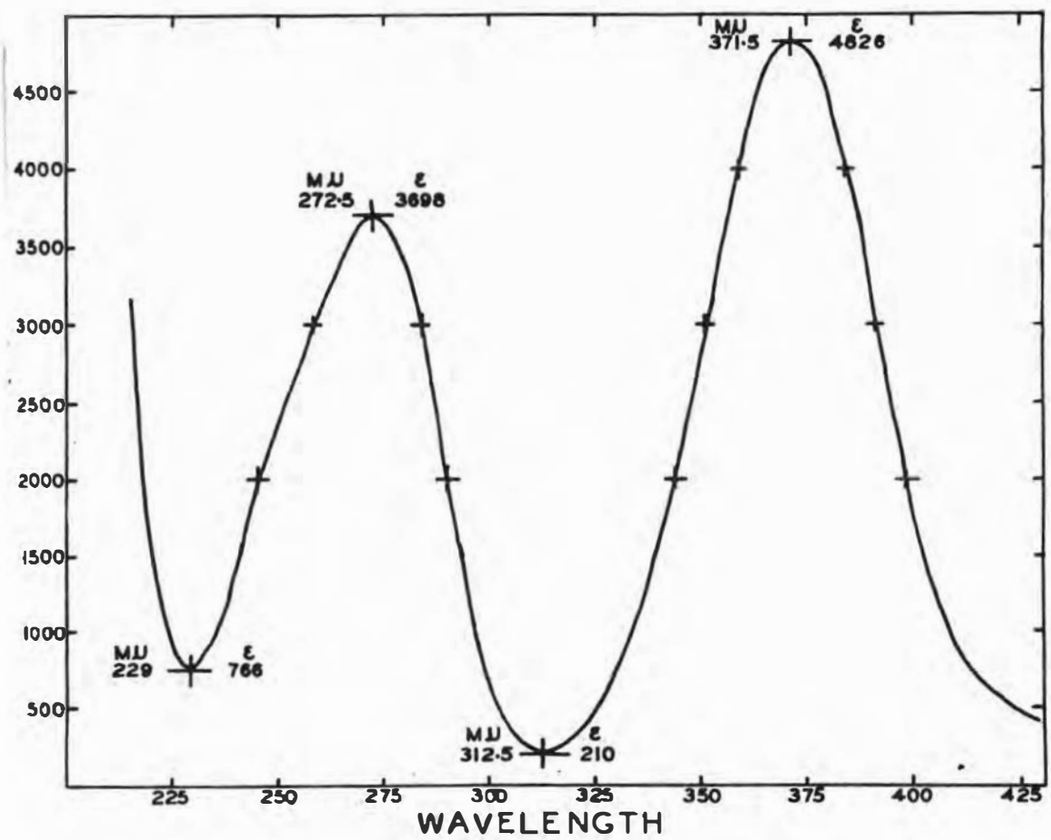


Fig.1 - Absorption curve for potassium chromate (0.003 N) in potassium hydroxide (0.05N). Crosses represent check points from Morton's curve (11).

. . . . . .

It appeared therefore that the instrument was correctly aligned and required no adjustment.

2. Choice of Solvent for Spectrophotometric Estimation of Vitamin A and Carotene.

A number of solvents have been suggested for the spectrophotometric estimation of vitamin A. These different solvents affect markedly the intensity of absorption and to some extent the shape of the absorption curve and the position of the maximum. The relative differences in intensity of absorption of solutions of Vitamin A in the more common solvents are listed by Gridgeman (1) together with certain of their advantages and disadvantages.

The solvents in most common use appear to be absolute ethanol, cyclohexane and isopropanol. As will be discussed later, petroleum ether has been found most satisfactory for extracting vitamin A and related products from aqueous solutions. Obviously the procedure would be simplified and losses due to destruction during evaporation of solvent avoided if petroleum ether could also be used as solvent for the estimation. The only reference to this solvent appears to be that of Baxter and Robeson (13) who found that the intensity of absorption is the same in this solvent as in ethanol. Petroleum ether is normally used as solvent for the estimation of the carotenoid pigments but it seemed possible that its variable composition might render it unsuitable in the near ultra violet. Since it is not a pure compound batches will differ in composition and on repeated distillation in solvent recovery, the lower boiling fractions will be lost.

Experiments were therefore carried out to investigate the effect of changes in the composition of the solvent on the intensity of absorption.

Solvents were prepared from a sample of petroleum ether of boiling range 45 - 70° as follows :

Solvents 1 - 3. Fractions of boiling ranges 49-52°C. 56.5 - 59.5°C and 63 - 65°C respectively, obtained by fractionating the sample.

Solvent 4. Residue from the above fractionation.

Solvent 5. Sample distilled once using short fractionating column, boiling range 48 - 66°C.

Solvent 6. Sample purified by repeatedly shaking with concentrated sulphuric acid, washing with alkali and distilling from calcium oxide as described by Chibnall (14).

Solvent 7. Original sample untreated.

Solvent 8. Solvent 3 plus 10% benzene (as might be obtained on eluting a chromatogram with petroleum ether and benzene).

Solvent 9. Solvent 3 shaken with equal volume of 50% aqueous ethanol (see later).

Solvent 10. A crude sample of petroleum ether untreated; boiling range 60 - 80°C.

Vitamin A alcohol was dissolved in the redistilled petroleum ether (solvent 5) to give a concentration of 360 ug/ml. Aliquots of this solution were diluted with the solvents described above to give a series of solutions containing 3.60 ug/ml. (and 1% of solvent 5 which may be neglected). The apparent vitamin A content of these solutions was estimated by measuring the extinctions at 325 mu taking $E^{1\%}_{1cm}$ (325 mu) as 1780 (see later). These measurements were made using a reference cell containing (a) distilled ether (solvent 5) and (b) the solvent corresponding to that in which the vitamin was dissolved.

The apparent vitamin A contents are listed in Table 1.

TABLE 1

No.	Solvent	Apparent Vitamin A Reference cell containing (a) Distilled ether (solvent 5)	(b) Corresponding solvent
		ug/ml	ug/ml
1.	Petroleum Ether - range 49 - 52°C	3.64	3.64
2.	Petroleum Ether - range 56.5 - 59.5°C	3.57	3.59
3.	Petroleum Ether - range 63 - 65°C	3.67	3.65
4.	Residue from distillation of samples 1, 2 and 3	4.62	3.55
5.	Original petroleum ether distilled once - range 48 - 66°C	3.60	3.60
6.	Petroleum Ether purified by treating with sulphuric acid, washing with alkali and distilling from CaO	3.64	3.62
7.	Original Petroleum Ether untreated	3.75	3.62
8.	Sample 3 plus 10% benzene	3.64	3.61
9.	Sample 3 shaken with equal volume of 50% aqueous ethanol	3.58	3.60
10.	Undistilled Petroleum Ether from extremely crude sample boiling range 60 - 80°C	5.09	3.38

It is clear that small variations in the composition of the solvent are not important even in the near ultraviolet. Certain of the solvents do contain an impurity which appears to interfere with the estimation of the vitamin but this can be allowed for by using the same sample in the reference cell and provided it is distilled before use, petroleum ether appears to be as satisfactory as the solvents in more common use for the spectroscopic estimation of vitamin A. Using solvents 1 - 9, similar estimations were made with different concentrations of vitamin A over the range 1.80 to 10.80 ug/ml. In all cases agreement between the solvents was of the same order as that shown in the Table.

As already mentioned petroleum ether has been used extensively as solvent for the estimation of carotene and it was used throughout this investigation both for vitamin A and its precursor.

3. Spectroscopic Constants and Absorption Curves.

Using both petroleum ether[1], and absolute ethanol as solvents, absorption curves were determined for vitamin A alcohol. The vitamin A alcohol used was a fresh sample[2] as supplied by the Eastman Kodak Company with a labelled $E^{1\%}_{1\,cm}$ value of 1750. The absorption curve was also determined for a sample of carotene[3] in petroleum ether[1].

Vitamin A. Vitamin A alcohol (21.5 mg.) was dissolved in absolute ethanol (prepared from 95% ethanol by standing over and distilling from freshly ignited calcium oxide[4]) to give a concentration of 215 ug/ml. This was further diluted with absolute ethanol to give a concentration of 6.0 ug/ml. The absorption of this solution and a solution of the same concentration in petroleum ether prepared from the vitamin A solution used in the previous experiment, was determined over the range 280 to 365 mu in steps of 2 mu except near the point of maximum absorption where readings were taken at 1 mu intervals. The two solutions were also diluted to give a range of concentrations between 2 and 10 ug/ml. and from the readings at 325 mu on these solutions with nominal band widths of 1.5 mm[4], $E^{1\%}_{1\,cm}$ values were calculated for vitamin A alcohol in both solvents.

Footnotes. 1. The term "petroleum ether" used throughout the thesis in this connection (i.e. as a spectroscopic solvent) refers to the redistilled product, boiling ran e approximately 42 - 66°C.

2. This sample had been despatched by Air Mail and was used immediately upon arrival.

3. This sample, also supplied by the Eastman Kodak Company consisted of a mixture of 90% β and 10% α carotene. Unless otherwise defined the term "carotene" refers to this mixture.

4. These band widths were used for all vitamin A and carotene estimations and necessitated setting the sensitivity control at approximately mid position. For measurements at other wavelengths the system adopted was to leave this control at about the same position and to alter the slit width as required, the final balancing being made by a slight alteration of the sensitivity control.

Carotene. Mixed carotene (42.6 mg) from a freshly opened tube was dissolved in petroleum ether to give a concentration of 85.2 ug/ml. The solution was further diluted to give a concentration of 4.26 ug/ml. and the absorption measured as for vitamin A over the range 225 to 500 mu. $E^{1\%}_{1cm}$ values were also calculated from readings taken on a series of dilutions containing from 1 to 7 ug/ml. at 450 mu with a nominal band width of 1.5 mu. (See footnote 4 previous page.)

The absorption curves obtained for vitamin A alcohol in petroleum ether and absolute ethanol are shown in Figure 2. Over the region investigated the curves for the two solvents are almost identical, the ethanol curve agreeing closely with published figures, for example those given recently by Morton and Stubbs (15).

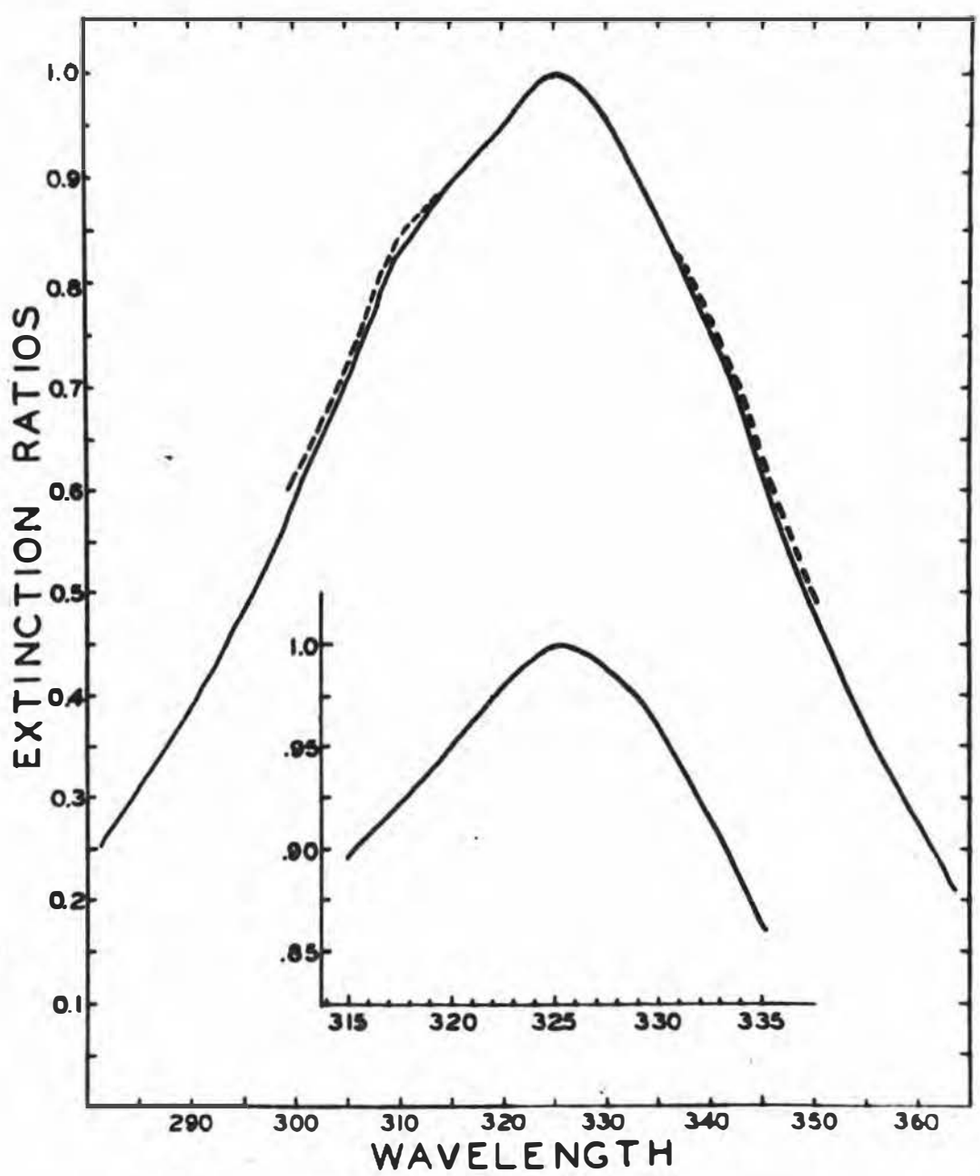


Fig.2 - Absorption curve for vitamin A alcohol in redistilled petroleum ether ——— and absolute ethanol - - - - (6.0 ug/ml.).

$E^{1\%}_{1cm}$ (325 mμ) values calculated from readings on solutions containing between 2 and 10 μg vitamin A per ml. (20 concentrations for each solvent) were 1735 ± 30 for absolute ethanol and 1725 ± 25 for petroleum ether. Within the limits of experimental error therefore, the values may be taken as identical for the two solvents and agree well with the figure of 1730 supplied by the manufacturers for the freshly prepared alcohol. There is still some controversy over the true $E^{1\%}_{1cm.}$ values for the vitamin but recent work (for example 16) suggests that it lies in the range 1780 ± 50. It is unlikely that the vitamin preparation used in this estimation was absolutely pure and the figure of 1780 has therefore been used in this investigation for vitamin A alcohol in absolute ethanol and in petroleum ether, concentrations being calculated in micrograms per millilitre by multiplying the observed extinction coefficient by the factor $10^4/1780$.

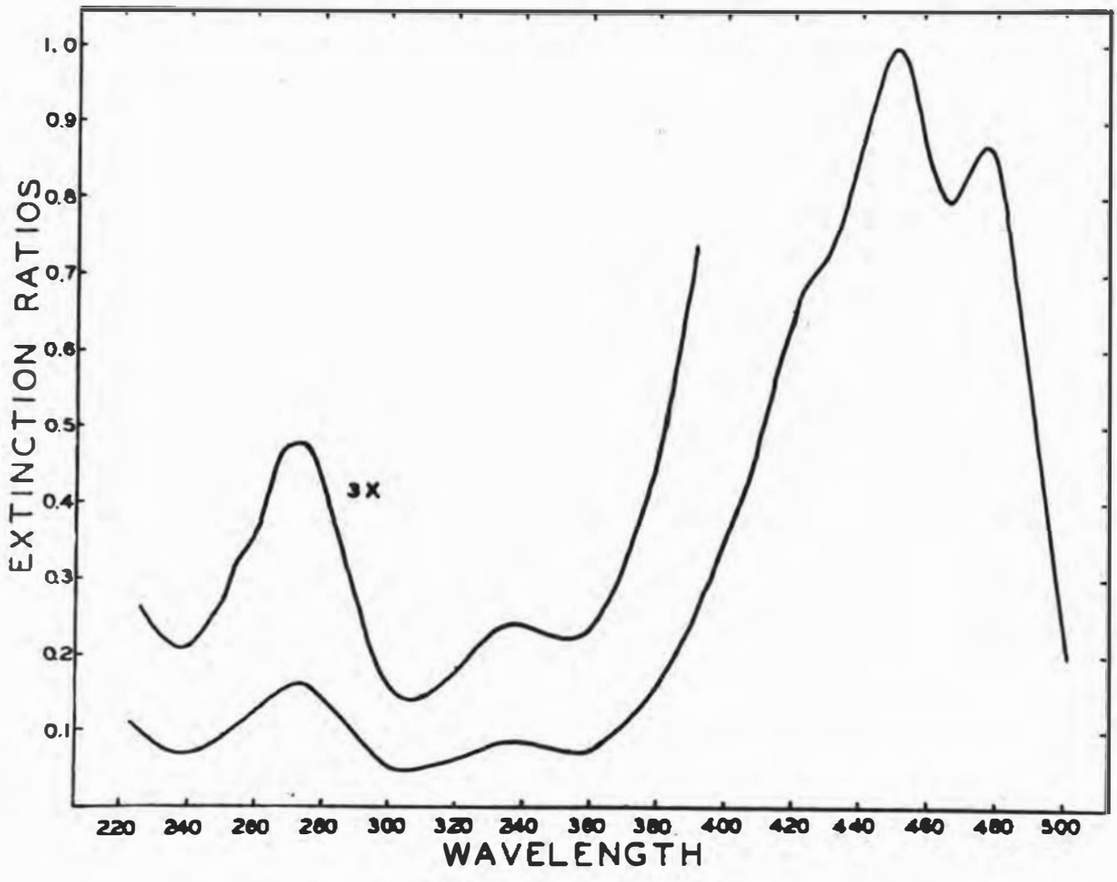


Fig. 3 - absorption curve for carotene (90% β and 10% α) in redistilled petroleum ether (4.26 and 12.78 μg/ml).

The curve for the carotene sample in petroleum ether is shown in Fig.3. The point of maximum absorption for the mixture occurred at 450 mu and the $E^{1\%}_{1cm}$ value calculated from readings at this wavelength on solutions containing between 1 and 7 ug/ml. (20 concentrations) was 2470 ± 35. Using the same solvent, Morton (11) gives a figure of 2500 at 450 mu for β carotene but no figure appears to be available for α carotene although the maximum is stated to occur at 447.5 mu. Carotene concentrations in ug/ml. have therefore been expressed in terms of the mixed sample by multiplying the observed extinction coefficient by the factor $10^4/2470$.

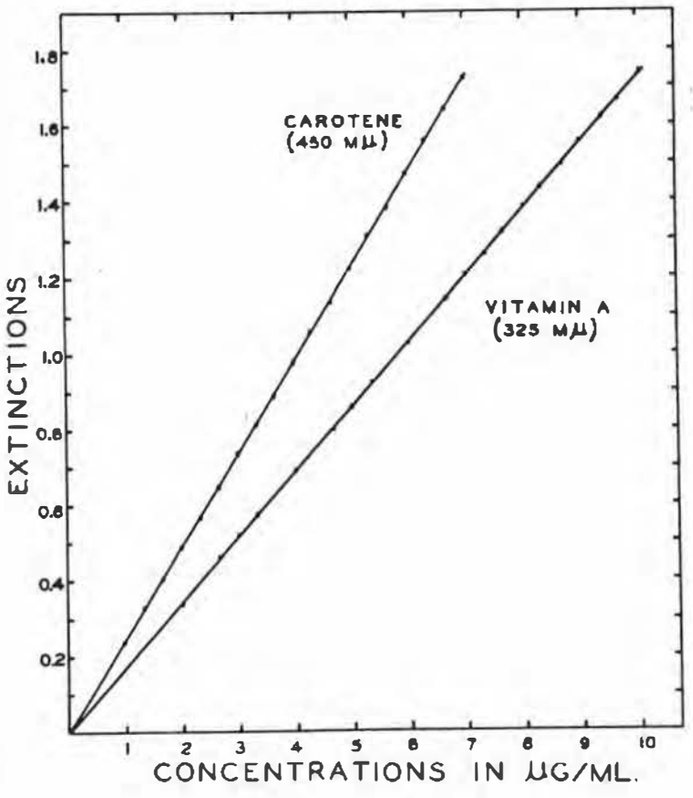


Fig.4 - Relationship between concentration and extinction readings for solutions of carotene and vitamin A in petroleum ether.

As shown in Figure 4, solutions of carotene and vitamin A in petroleum ether obey Beer's Law over the concentration range investigated. Linearity was not investigated at higher concentrations as there was a marked decrease in reproducibility at higher extinction readings. The optimum range for the instrument was

between about 0.3 and 1.0 density units. Rawlings and Wait (17) obtained the best reproducibility between 0.3 and 0.8 units.

4. Specificity of the Spectrophotometric Method for Vitamin A.

The direct estimation of vitamin A by determining the optical density of a solution at 325 mu is based on the assumption that no other substances present in the solution absorb at this wavelength. This assumption is not normally justified and it is usually necessary to make some allowance for this extraneous absorption. For example with fish liver oils the observed extinction is, in effect, reduced by about 10% when converted to International Units to allow for this lack of specificity.

The presence of interfering material can be detected from an examination of the shape of the absorption curve and to facilitate this Oser and co-workers (18) have suggested the plotting of "extinction ratios" (i.e. observed extinction at any wavelength divided by extinction at the wavelength of maximum absorption). This is a convenient system since it eliminates the effect of concentration on the apparent shape of absorption curves.

Most efforts to make a quantitative correction for the non-vitamin absorption have been directed towards destroying the vitamin and estimating residual absorption at $\lambda_{max.}$ for vitamin A. A method suitable for most solutions has been described by Little (19). Absorption at 328 mu is measured first on the original solution and again after destroying the vitamin A by irradiation with ultra violet light. Provided light of less than 300 mu is excluded there is little destruction of non-vitamin materials.

The method appears to give satisfactory results in many cases and a few determinations were carried out on

liver extracts using it. In most cases in the present investigation however, the interfering materials consisted of carotene and its oxidative breakdown products and, as would be expected, these are also affected by ultra violet light and the method was therefore abandoned.

From their observations of the shapes of absorption curves for various vitamin A containing materials, Oser and co-workers (18) suggested a method for detecting contamination based on measurements of absorptions at 300 (310 optional), 328 and 350 mμ. From these readings extinction ratios $E_{300m\mu}/E_{328m\mu}$, ($E_{310m\mu}/E_{328m\mu}$) and $E_{350m\mu}/E_{328m\mu}$ are calculated. These should not exceed 0.73, (0.91) and 0.65 respectively and if higher ratios are obtained it is an indication that there is sufficient extraneous material present to invalidate direct readings at 328 mμ. Obviously the nearer these values approach those for the pure vitamin, the greater the reliance which can be placed on the spectroscopic estimation.

This method has been widely used (3) for sorting out solutions to which direct spectroscopy may or may not be applied and it seemed that it might be extended to enable the irrelevant absorption to be estimated and allowed for quantitatively by comparing the extinction ratios obtained from measurements at three wavelengths with those calculated for pure vitamin A. A correction of this type can only be calculated by assuming that the absorption due to the interfering material is linear over the wavelength range selected or more correctly that the points on the absorption curve for the interfering materials corresponding to the wavelengths chosen are co-linear. This assumption is probably justified over a limited wavelength range and a method has been developed for estimating the amount of irrelevant absorption at 325 mμ.

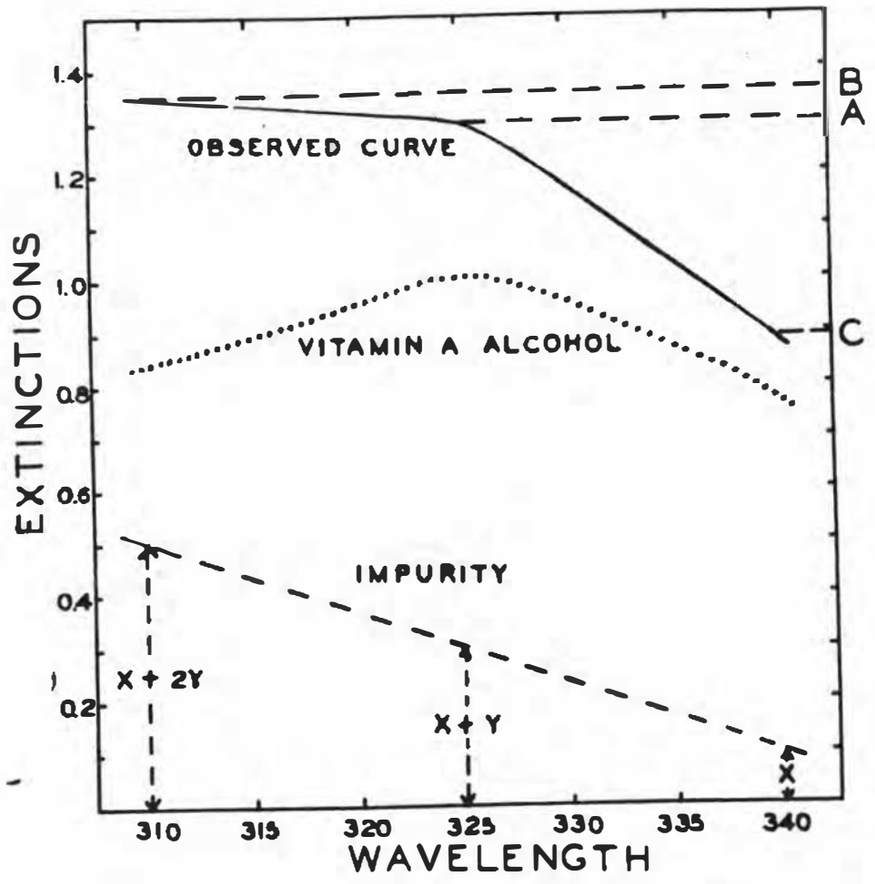


Fig.5 - The estimation of vitamin A in the presence of interfering materials.

. . . . . .

Considering vitamin A alcohol in absolute ethanol, a hypothetical case is illustrated in Figure 5. The dashed line represents an idealised curve for the interfering substances which together with the vitamin A present (dotted) give the absorption curve for the solution shown as a full line. 325, 310 and 340 mu have been selected as suitable wavelengths and readings giving extinction co-efficients denoted by the letters A,B and C respectively are taken at these wavelengths which represent $\lambda_{max.}$, ($\lambda_{max.}$ - 15 mu) and ($\lambda_{max.}$ + 15 mu). The absorptions due to the interfering substance at these wavelengths are denoted by (x + y), (x + 2y) and x respectively, so that the absorptions due to vitamin A are A - (x + y) at 325 mu, B - (x + 2y) at 310 mu and C - x at 340 mu.

The ratios E_{310mu}/E_{325mu} and E_{340mu}/E_{325mu} were

determined for a range of concentrations of vitamin A alcohol in absolute ethanol, giving mean values of 0.846 and 0.771 respectively. Substituting the above values it follows that :

$$B - (x + 2y) = 0.846\,(A - (x + y)) \quad \ldots \quad (1)$$

and $C - x = 0.771\,(A - (x + y)) \quad \ldots \quad (2)$

Adding these equations (1) and (2) gives the expression :

$$B + C - 2(x + y) = 1.617\,(A - (x + y)) \quad \ldots \quad (3)$$

which in terms of $(x + y)$, the irrelevant absorption at 325 mu, reduces to :

$$x + y = 2.60\,(B + C - 1.617A) \quad \ldots \quad (4)$$

The actual amount of irrelevant absorption may conveniently be read directly from a graph on which values of $B + C - 1.617A$ are plotted against $x + y$ and subtracted from reading A (taken at 325 mu) to give the extinction due to vitamin A. Alternatively equation (4) may be rewritten to give $(A - (x + y))$, the extinction due to the vitamin present, directly :

$$\text{viz.} \quad A - (x + y) = A - 2.60\,(B + C - 1.617A)$$

$$\text{whence} \quad A - (x + y) = 2.60\,(2A - (B + C))$$

and taking $E^{1\%}_{1\,cm}$ (325 mu) for vitamin A alcohol in absolute ethanol as 1780 this becomes :

corrected concentration of vitamin A in ug/ml.

$$= 10^4/1780 \times 2.60\,(2A - (B + C))$$

$$= 14.61\,(2A - (B + C))$$

This equation holds for vitamin A alcohol in absolute ethanol only, since the solvent and the state of the vitamin (free or esterified) affects both the position of $\lambda_{max.}$ and the shape of the curve, but similar equations with new constants can be derived for other solvents and for the vitamin in esterified forms. In the case of vitamin A alcohol in petroleum ether, for example, the ratios E_{310mu}/E_{325mu} and E_{340mu}/E_{325mu} were found to be 0.832 and 0.756 respectively.

Hence $B - (x + 2y) = 0.832 (A - (x + y))$

and $C - x = 0.756 (A - (x + y))$

which by similar calculation reduces to :

$$x + y = 2.428 (B + C - 1.588 A)$$

and corrected concentration of vitamin A in ug/ml

$$= 13.64 (2A - (B + C))$$

5. Application of three point correction procedure to the estimation of vitamin A in the presence of carotene and its breakdown products.

The estimation of vitamin A in the presence of carotene does not offer any particular difficulties since although carotene does absorb to some extent at 325 mu, this can readily be allowed for. The usual method is to measure the extinctions at 325 and 450 mu and from a knowledge of the ratio E_{325mu}/E_{450mu} for carotene to correct the reading at 325 mu for the absorption due to carotene at that wavelength. This correction is only valid however for relatively pure carotene since the ratio is increased by isomerisation or oxidative decomposition and where this has occurred, the use of the ratio calculated for pure carotene leads to erroneously high vitamin A figures. Correction figures vary and values for the absorption due to β carotene at 325 mu relative to that at 450 mu of from 5 - 10% have been reported. (54, 55, 56). Lehrenk, Lilker and King (57) suggest the figure of 5% estimated by extrapolating the results obtained with partially isomerised solutions, as a reliable ratio for pure β carotene. As shown in Figure 5, for the mixed carotene sample used in this investigation, the extinction at 325 mu was 6.15% of that at 450 mu and this correction was used for the spectroscopic estimation of vitamin A in the presence of undecomposed carotene. It became apparent early in the investigation that conditions under which conversion of carotene to vitamin A might be expected to occur, also favoured decomposition or isomerisation. It was necessary, therefore, that methods of assay should

be applicable to these mixtures. Although relatively pure vitamin could be obtained free from most interfering material by chromatography, it was desired to avoid this as a routine method due to manipulative difficulties and the problem, when only small amounts might be present, of locating the vitamin on the column amongst a number of bands, all of which, at low concentrations, showed similar fluorescence under ultra violet light and gave on the extruded columns, similar reactions with the Carr-Price reagent. At higher concentrations these difficulties are not so great and chromatography has been applied in special cases.

The validity of the original assumption that the absorption of interfering substances was linear over the range 310 to 340 mu was tested by estimating the recovery of known amounts of vitamin A added to solutions of partially decomposed carotene and by examining the absorption curves of oxidised and isomerised carotene. It is probable that the changes occurring in the absorption spectrum of carotene during decomposition will depend on the conditions. Carotene was therefore allowed to decompose under various conditions.

> A solution of carotene in peroxide-free ethyl ether[1] was exposed to dull light at room temperature for some weeks. Samples were withdrawn at intervals, the ether removed in a stream of nitrogen at room temperature, and the residue taken up in petroleum ether. The absorption spectra of these solutions were measured over the range 225 to 500 mu in steps of 5 mu.

The curves obtained are shown in Figure 6. It is apparent that although there is some departure from linearity over the range being considered, points on the curves

---

Footnote 1. The peroxide-free solvent was prepared immediately before use by distilling from charcoal and a few drops of strong caustic soda solution, ethyl ether which had been stored for several days in contact with these reagents.

corresponding to 310, 325 and 340 mμ are reasonably co-linear. The same was found to apply to the curves for isomerised carotene given by Zechmeister (20) and to readings taken at the three wavelengths on petroleum ether extracts of solutions of colloidal carotene which had been incubated for varying periods at 37°C. (see later.)

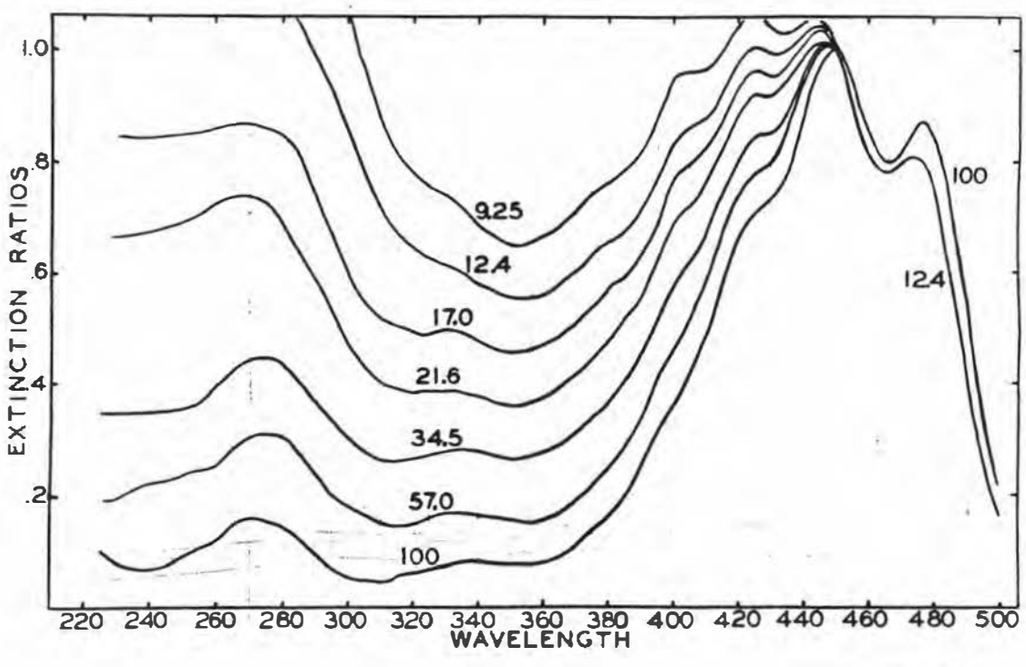


Fig.6 - Curves showing the decomposition of carotene at room temperature in dull light. Numbers above the curves represent apparent carotene remaining as estimated from the extinction reading at 450 mμ.

. . . . . .

Using the three point correction procedure, the recovery of vitamin A added to a number of partly decomposed or isomerised carotene solutions was investigated.

A mixture of carotene isomers was obtained by refluxing a solution in petroleum ether for one hour with a trace of iodine equivalent to about 2% of the carotene. Known amounts of vitamin A were added to this solution and to the solutions of oxidised carotene described above. Optical densities of these solutions were then measured at 310, 325 and 340 mμ.

Typical results selected from a number of determinations are shown in Table 2.

TABLE 2

Vit.A. added ug/ml	Readings 325 mu	310 mu	340 mu	Vitamin A recovered ug/ml Un-corrected	Corrected carotene only *	Corrected 3-point method
3.10	1.900	1.730	1.815	10.68	7.86	3.48
3.10	1.225	1.095	1.120	6.88	5.47	3.21
3.43	0.725	0.585	0.600	4.07	3.50	3.62
3.43	0.885	0.770	0.740	4.97	4.43	3.55
4.94	0.995	0.815	0.805	5.58	5.02	5.05
4.94	1.100	0.965	0.880	6.17	5.98	4.85
6.20	1.215	0.995	0.975	6.83	6.27	6.28
6.20	1.440	1.150	1.255	8.09	6.39	6.49
6.20	1.380	1.175	1.120	7.76	7.20	6.35
6.20	1.325	1.145	1.055	7.44	7.25	6.15
6.20	1.230	1.065	0.950	6.92	6.86	6.07
6.20	1.550	1.410	1.220	8.60	8.38	5.87
6.20	1.755	1.600	1.470	9.85	9.30	6.00

* - Corrected for carotene by subtracting from E_{325mu} , 6.15% of E_{450mu}.

It is apparent that satisfactory recoveries were obtained in all cases and it is considered that the method is suitable for the estimation of vitamin A in the presence of this type of interfering material. It is probable that the method could be extended to other types of interference and during the course of this investigation Morton and Stubbs (15) introduced a somewhat similar method for fish oils. In this method wavelengths are selected above and below $\lambda_{max.}$ such that for the pure vitamin, the absorption at these points is a definite fraction of the maximum. The fraction chosen is 6/7ths of the maximum and for vitamin A alcohol in ethanol, for example, readings are taken at 311, 325 and 335.5 mu. If absorbing impurities are present the ratios E_{311mu}/E_{325mu} and $E_{335.5mu}/E_{325mu}$ will be greater than 6/7ths and if the irrelevant absorption is not parallel to the wavelength axis, the two ratios will not be equal.

Morton and Stubbs' calculation first allows for this "drop" by proportion and then by solving an equation with one unknown, the absorption due to the vitamin A present is determined.

Since it is based on the same principle, the accuracy of this method must be of the same order as that of the method already described but as a result of the selection of wavelengths unsymmetrically placed about 325 mu, the calculation, even when reduced to nomogramic form (21) is somewhat more tedious.

(b) Colorimetric Methods

Since the original work of Drummond and Watson (22) established that the substance present in livers which gave a coloration with sulphuric acid was vitamin A, colour tests have been very thoroughly investigated. In 1925 Rosenheim and Drummond (23) showed that sulphuric acid could be replaced by a number of other reagents and shortly afterwards Carr and Price (24) introduced a concentrated solution of antimony trichloride in chloroform as a reagent for vitamin A. A number of other colour reactions have been proposed (50, 51) but this has remained the most widely used colorimetric reagent although it does suffer from a number of disadvantages. It is unstable and unpleasant to handle being extremely hygroscopic and corrosive, the colour produced, although intense, is transient commencing to fade almost immediately, the colour formation is liable to be inhibited by interfering substances and the reagent is not specific for vitamin A.

Despite this it has been used for the estimation of vitamin A in the non-saponifiable residues from a wide range of materials and has been found by the majority of workers to give satisfactory results. In attempts to overcome some of the disadvantages of the reagent, various

modifications to the original method have been suggested. Measurement of the colour developed by photo-electric means as suggested by Bonn and Evelyn (25) has greatly enhanced the precision of the method and the use of an internal standard as recommended by Oser and others (7) has eliminated sources of error due to the possible presence of colour inhibitors or promoters. Other modifications as described in recent reviews (26 and 27) do not appear to offer any additional advantages.

Particularly over the last few years attention has however been directed towards the introduction of a more satisfactory reagent. Some of the older methods have been re-investigated. Robin (28), for example, has reverted to the original sulphuric acid test in the form of the Liebermann-Buchard reagent and tri-chloracetic acid (29) has also been revived. So far none appear to offer any marked advantages over the Carr-Price reagent but with the more precise methods of colour measurement now available, further work in this direction might be of value.

The chief disadvantage of the Carr-Price reagent in the present investigation was, however, its reaction with carotene and its oxidation products. Johnson and Baumann(30) have shown that the intensity of the blue colour produced by carotene solutions depends on the state of oxidation of the sample. Expressing results as ug/of per 100 ug carotene, they have shown that as oxidation proceeds, the apparent vitamin A increases from 53.6 ug for pure carotene to 52.4 ug for heavily oxidised material. At the same time they note an increase in stability of the colour.

Kobel and Korbin (31) have introduced a new reagent in the form of glycerol 1,3 dichlorohydrin (G.D.) which appears to be superior in some respects to the antimony trichloride. When mixed with a solution of vitamin A in chloroform, the reagent gives a blue colour changing rapidly.

to a permanganate shade which is stable for from 2 to 10 minutes after mixing. The reagent is not hygroscopic and is non-corrosive. The colour intensity is however only about one quarter that of the Carr-Price reagent.

In a later publication (32) the same workers reported that some samples of SbCl$_3$ did not react without "activation" by distilling from antimony trichloride. More recently Penketh (33) has shown that the activating principle is H^+ and that the activation may be accomplished more simply by the addition of 2% hydrochloric acid. The activated reagent has been used for the estimation of vitamin A in a range of materials and has been found to give results in good agreement with those obtained spectroscopically and with the Carr-Price reagent (8, 34, 35, 36).

It appears however that as in the case of the Carr-Price reagent, SbCl$_3$ is affected by certain interfering materials which may inhibit the colour development. Allen, Wise and Jacobson (52) recently reported the occurrence in blood plasma of factors which interfere with the glycerol dichlorohydrin reaction with vitamin A and carotenoids while Bell and Kelley (35) experienced similar difficulties with extracts of fortified poultry mashes. Blood plasma also contains materials which interfere with the Carr-Price reaction (53) and recently when estimating vitamin A in mashes at the request of the Poultry Department, the author found that this reagent gave higher results and more complete recoveries of added vitamin after saponification, due apparently to the presence of inhibitors in the unsaponified extracts.

No information was available regarding the reaction of SbCl$_3$ with substances related to vitamin A and the carotenes (26) and it was thought that it might possess some advantages over the Carr-Price reagent in this respect.

A solution of carotene in peroxide-free ethyl ether was exposed to diffuse sunlight at room temperature. At intervals two samples were withdrawn simultaneously and evaporated separately to dryness in a stream of nitrogen at room temperature.

The residue from one sample was dissolved in petroleum ether and the absorption measured at 325 and 450 mu. The other residue was taken up in chloroform. 1 ml. of this solution was treated with 4 ml. of CDH activated shortly before use by the addition of 2% hydrochloric acid and the colour intensity estimated three minutes after mixing at 555 mu using the Beckman spectrophotometer. A further 1 ml. of the chloroform solution was treated with 8 ml. of a solution of antimony trichloride in chloroform (25% w/v). Owing to the corrosive nature of this reagent colour intensities were measured on a laboratory built colorimeter (37) rather than on the Beckman as the special Carr-Price reagent cells (38) were not available for this instrument. The colorimeter, which was of the direct reading type, was fitted with a red filter and calibrated using solutions of vitamin A in chloroform. The instrument was adjusted to read 100% transmission on a control cell containing chloroform. A cell containing the 1 ml. of chloroform solution together with two drops of acetic anhydride was moved into the light path and the reagent added from a rapid delivery pipette. Percentage transmission was measured three seconds after addition of the reagent.

The percentage carotene destroyed was estimated from the decrease in the readings at 450 mu and apparent vitamin A at different stages of decomposition was calculated from the results obtained by these three methods of estimation. The results are expressed in ug/100ug carotene in Figure 7. It is clear that CDH reacts with decomposition products in the same way as the Carr-Price reagent, over which it does not appear to possess any advantages in this respect.

The values obtained for apparent vitamin A per 100 ug of undecomposed carotene were 6.4 ug for the Carr-Price reagent, 16.8 ug for CDH and 8.52 ug for the spectroscopic method. For the colorimetric reagents, the increase in apparent vitamin A up to about 40% decomposition of carotene, is small. In this investigation, where vitamin A has been estimated by the Carr-Price reagent in the presence of undecomposed carotene, the above figure of 6.4 ug

per 100 ug carotene has been used.

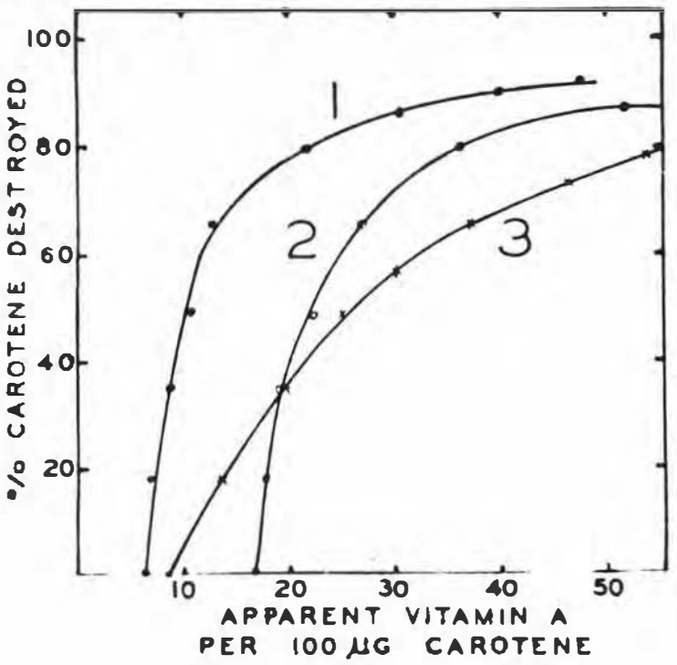


Fig.7 - Apparent vitamin A at different stages of decomposition of a solution of carotene as estimated by the Carr-Price reagent (curve 1) Glycerol dichlorohydrin (curve 2) and spectroscopically (curve 3).

During the course of this work unexpected difficulties were encountered in the use of the CPH reagent. It appears that the colour intensity and stability depend on the past history of the reagent and although reproducible results can be obtained by adopting a uniform technique and by repeatedly checking against a vitamin A standard, the reagent may not be as satisfactory as at first imagined. The reagent had been used previously in this laboratory for the estimation of vitamin A in the livers of sheep and cattle (39) As supplied [1] it gave almost no colour with vitamin A but when distilled from antimony trichloride it gave a relatively stable violet colour, $E^{1\%}_{1cm}$ (550 mu) 960 as determined on a Coleman Universal spectrophotometer Model 11 (Kobel and Berbin (32) obtained values of 1100

Footnote 1. The reagent used was Practical Grade CPH supplied by the Eastman Kodak Company.

to 1270 using the same instrument). The same activated sample, after storage for 8 to 10 months in a dark bottle, was tested prior to use in this experiment and was found to give a colour of lower intensity than before and of greatly reduced stability. Re-activation by again distilling from antimony trichloride increased the intensity to approximately its original level but the colour remained stable for only about two minutes and then faded fairly rapidly.

A second batch of GDH gave, on activation, a stable violet colour, $E^{1\%}_{1 cm.}$ determined as before, 910. After storage for only a few weeks there was a detectable decrease in stability. A further sample of the same batch was activated by the addition of 2% hydrochloric acid. The colour in this case was measured at 555 mu using the Beckman Spectrophotometer. It was found to be stable for about five minutes after which it faded slowly. The $E^{1\%}_{1 cm.}$ (555 mu) value was 1020 which is still considerably lower than Sobel and Werbin's figure of 1420 for this instrument (32). Penketh (33) has reported that on standing, the activating effect of the H^+ becomes more marked giving a greater intensity but lower stability of colour. This increased intensity with activated samples on standing has also been observed in this investigation but it appears to be followed, on storage for a few weeks, by a marked decrease in intensity together with the reduced stability.

It is apparent that the conditions governing the reaction between activated glycerol dichlorohydrin and vitamin A require further investigation and where a colorimetric method was required the Carr-Price reagent has been used. All such estimations were carried out using the photoelectric colorimeter and where colour development might be affected by interfering materials, an internal standard was employed.

Although their original paper is not available, it appears that other workers have also criticised the GDH reagent (40) but have found that it increases the sensitivity of the Carr-Price reagent. This observation has not been confirmed, the addition of GDH in the proportion recommended making little difference to the Carr-Price beyond a slight reduction in colour stability.

"Acid earths" have come into prominence recently as reagents for vitamin A. The reaction of "Super filtrol" which is derived from the aluminium silicate mineral montmorillonite, has been reported by several workers (41, 42, 43) and Lowman (41) has suggested that the reaction might be used for the quantitative estimation of vitamin A. Activated bentonite has also been suggested (44). These earths, when shaken with solutions of vitamin A in non-polar solvents, give an intense blue colour. The colour is stable but cannot be eluted as such from the particles of the solid and can only be estimated by comparison with colour standards or by means of a reflection type colorimeter. From the point of view of accuracy, such methods would probably compare favourably with subjective colorimetric estimations but reflection measurements are not as reliable as objective transmission readings. The reaction involved is apparently a dehydration of the vitamin on the surface of the particles due to the action of adsorbed sulphuric or hydrochloric acid and is therefore comparable to the reaction with most colorimetric reagents resulting in the formation of anhydro vitamin A (45). Complex formation between this substance and the adsorbed acid results in the appearance of the blue colour and elution with polar solvent gives a yellow solution presumably containing anhydro vitamin A. The reaction between vitamin A and the "Super Filtrol" suggests a rapid indirect method for the estimation of the vitamin particularly in the presence of large amounts of interfering

substances.

The absorption of the solution in petroleum ether is measured at λ max. for vitamin A and for anhydro vitamin A. The solution is then treated with an activated earth which adsorbs the vitamin and possibly other impurities. The vitamin is converted to the anhydro form and as it is unlikely that other materials normally associated with vitamin A would be affected, elution with a polar solvent gives a solution containing the impurities unchanged and the vitamin A in the anhydro form. The absorption of this solution is then measured at the same two wavelengths.

From these readings and a knowledge of the absorption of pure vitamin A and anhydro vitamin A it would be possible to calculate the amount of vitamin A originally present. The method appears a promising one but the difficulty so far has been the complete elution of the anhydro vitamin A and this is still being investigated.

(2) <u>Stability under the Conditions likely to be encountered in the Preparation of Samples for Assay.</u>

It is generally conceded that both vitamin A and carotene are unstable to heat and light and most workers recommend that all manipulations should be carried out in dull light and that undue heat should be avoided. Widely different interpretations are however placed on these provisos in different laboratories. Estimations are carried out in illuminations ranging from dull red light with all its attendant difficulties to bright, unshaded daylight while operations such as solvent evaporation are carried out at temperatures ranging from the boiling point of the solvent at normal pressures without any precautions down to removal at room temperatures

under reduced pressure in a stream of nitrogen. Each step of a normal extraction procedure was therefore investigated to determine the amount of light and heat permissible without risk of destruction of the vitamin.

(a) Saponification

5 ml. samples of a solution of vitamin A in ethanol were subjected to the following treatments -

1. Heated in a boiling water bath with 100 ml. of 10% aqueous potassium hydroxide for one hour.

2. Refluxed with 100 ml. of 10% ethanolic potash for one hour.

In no case was any destruction of the vitamin detectable provided light was excluded. Repeating the experiments with carotene solutions gave similar results.

(b) Effect of Exposure to Light.

A solution consisting of a mixture of vitamin A and carotene in petroleum ether was exposed to diffuse sunlight of approximately 1000 foot candles intensity for some hours. Samples were withdrawn at intervals and estimated spectroscopically for carotene and vitamin A.

In a similar experiment solutions of vitamin A in petroleum ether were placed at different distances from a window through which diffuse sunlight was shining. Samples were assayed at intervals for vitamin A.

Light intensities were measured at the times of sampling using a General Electric Photoelectric Exposure Meter.

The resultant decomposition of the solutions is shown in Figures 8 and 9. No attempt was made to control the light intensity, the object of the experiment being merely to obtain an idea of the amount of light permissible without undue decomposition.

These results refer to pure materials and the rates of destruction will obviously be different with partly oxidised samples or in the presence of anti-oxidants. However it would appear that in most cases the loss of both vitamin A and carotene will be

negligible provided the product of the time of exposure in hours by the light intensity in foot candles does not exceed a value of about 100 at room temperature. It is not necessary therefore to take the extreme precaution of working in dull red light but at the same time large losses will occur unless the normal laboratory illumination is considerably reduced. Further, since the rate of decomposition increases with temperature, samples require more complete protection during saponification and evaporation of solvents. 50 foot candles has been found to be a convenient level of illumination while handling carotene and vitamin A solutions, and these may safely be exposed to this intensity for at least two hours without loss.

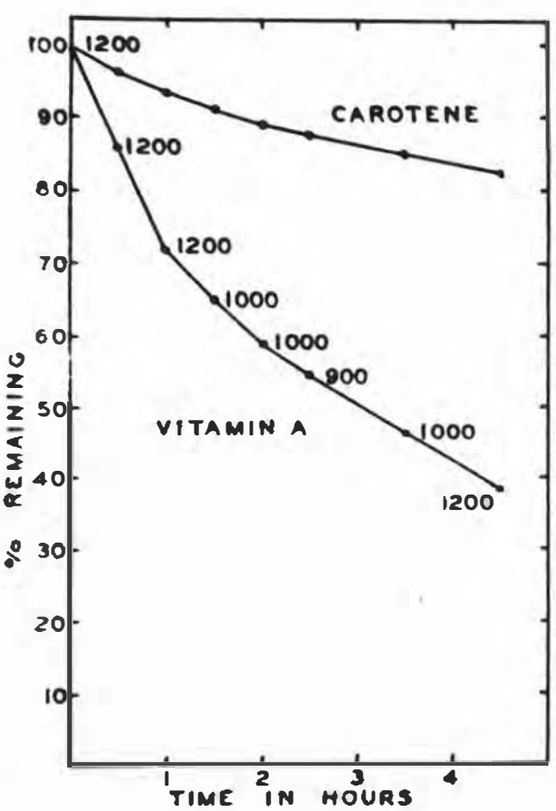


Fig. 8

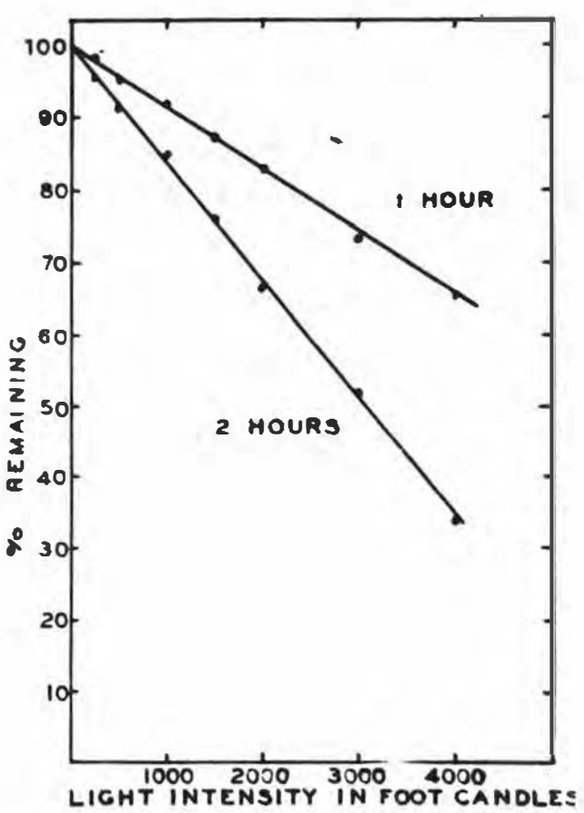


Fig. 9

Fig. 8 - Decomposition of a solution of vitamin A and carotene in petroleum ether by light at room temperature. Figures represent approximate light intensities at times of sampling in foot candles.

Fig. 9 - Decomposition of a solution of vitamin A in petroleum ether by light at room temperature.

(c) Evaporation of the olvent

After extracting the vitamin from the saponification mixture it is generally necessary to evaporate the ether solution to dryness and to take the residue up in a suitable solvent for assay.

Solutions of vitamin A and carotene in peroxide-free ethyl ether were evaporated to dryness under the various conditions described in Table 3. The residues were taken up in petroleum ether and vitamin A and carotene estimated spectroscopically.

T A B L E 3

Method of Evaporation	Average Recovery as % Vitamin A	Carotene
Dull red light at room temperature in a stream of nitrogen.	100	100
Dull red light at 55°C and the last traces removed in a stream of nitrogen at room temperature	100	99
Dull red light at 55°C and taken to dryness on water bath without Nitrogen.	96	96
Dull red light to dryness on boiling water bath (5 mins. heating)	94	89
Fairly bright light (1500 foot candles) and taken to dryness at 55°C. Exposure to light and heat 16 minutes.	83	94
Fairly bright light (1500 foot candles) and taken to dryness on boiling water bath. Exposure to light and heat 5 minutes.	80	84

From the results it is apparent that the temperature of the water bath is not important provided the last traces of the solvent are removed at room temperature in a stream of nitrogen. Apparently it is the film of material left on the flask which is most susceptible to oxidation or destruction by heat. It is also necessary to carry out the evaporation in very dull light.

(3) Extraction of the Vitamin and Separation into a Non-aqueous Solvent.

At the start of this investigation it appeared that vitamin assays would be carried out on a number of organs particularly livers. This aspect of the investigation was not proceeded with but the results of preliminary experiments are briefly reported here since they are of some interest in themselves.

(a) Extraction.

It was found that saponification offered the most convenient and efficient method of extracting tissues. Provided heating is continued until all the material passes into solution both ethanolic and aqueous potash are equally effective. Solution is normally more rapid, however, in the ethanolic medium and may be accelerated by using the Waring Blendor to suspend the tissue in the alcohol. If this method is used, the Blendor jar must be filled with nitrogen and precautions taken to exclude all oxygen during the comminution otherwise heavy losses of vitamin are liable to occur. These losses are not confined to the actual blending time but have been found to continue at the rate of up to ten percent per hour on standing at room temperature following comminution.

(b) Distribution of the Vitamin through the Liver.

Both from the point of view of the physiology of the vitamin and of determining the minimum sample which may be regarded as representative, it is of interest to know the distribution through the liver. Assays were therefore carried out on a large number of samples taken from various parts of a sheep liver. Narrow slices were cut from the liver and divided into sections weighing between 1 and 2 grams. Vitamin content of these sections was determined by means of the Carr-Price reagent using the method of Callup and Hoeffer (46). The dis-

tribution of these samples with their vitamin content in micrograms per gram fresh weight is shown in Figure 10.

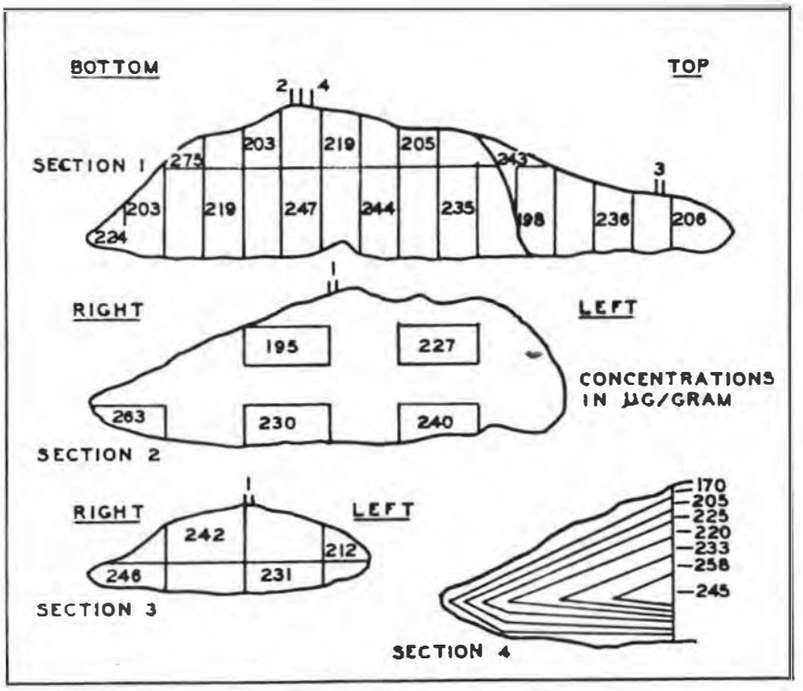


Fig.10 - Distribution of vitamin A through a sheep's liver. Figures represent concentrations in ug/gm. fresh weight.

. . . . . .

In addition a number of random samples of various weights were also assayed. The weights taken and the variations in vitamin content are shown in Table 4.

T A B L E 4

Sample	Vitamin content Mean ug/gm	S.D.
Random samples described in Fig.10	226	25
12 1 gm cores selected at random	236	48
12 5 gm cores selected at random	231	18
12 samples ranging from 10 to 50 gms	240	7
Whole liver minced and estimated - 6 estimations.	238	6

From the results obtained it would appear that there are fairly large variations of a somewhat random nature from point to point with a tendency for a greater concentration towards the interior of the liver. Further, it would seem that assays carried out on less than 10 grams

of material do not give a true indication of the vitamin A content in the liver.

(c) Comparison of Methods for the Estimation of Vitamin A in Liver Extracts.

The vitamin A content of extracts prepared from three sheep livers was estimated using a number of the methods already discussed. As is shown in Table 5, the methods gave results in close agreement.

TABLE 5

Method	Vitamin A content of solutions 1	2	3
	ug	ug	ug
Direct estimation at 325 mu using 3 point correction	26.45	15.9	10.6
Destructive irradiation technique	25.8	15.3	10.2
Glycerol dichlorohydrin method	24.0	15.8	10.6
Carr-Price reagent - using Tintometer	25.2	16.0	11.0
Carr-Price reagent - photo-electric colorimeter	24.6	15.0	10.3

Each result is the mean of at least three determinations.

(d) Extraction of Vitamin A and Carotene into a Non-aqueous Solvent.

The usual method for extracting the vitamin from the saponification mixture is by shaking repeatedly with peroxide-free ethyl ether. This method does not lend itself readily to the assay of large numbers of samples since, as described by workers such as Oser and others (7), it involves a tedious series of manipulations, viz. repeated extractions with ether, the washing of these combined extracts and their drying and evaporation to dryness. Other methods are simpler, dispensing with a number of the steps (e.g. Davies (47)) but give low recoveries of the vitamin. As an alternative to ethyl ether, Kimble (48) suggested a method for blood based on a single extraction with petroleum ether and this method has been applied to liver extracts by Gallup and Roeffler (46). Provided the solution to be extracted is adjusted to

contain approximately 50% ethanol and is shaken for about two minutes with an equal volume of petroleum ether, the vitamin is quantitatively extracted into the epiphase and since there is no change in the relative volumes of the layers, it may be estimated by withdrawing an aliquot of the ether. This ether contains a trace of ethanol but as shown in Table 1, is nevertheless a satisfactory spectroscopic solvent. It has been found that the ethanol, water, ether ratios are not critical and may be varied by at least 10% without appreciably affecting the relative volumes or the efficiency of extraction. After shaking there is a rapid separation of the two layers giving a clear epiphase which may be estimated immediately without preliminary drying. The reports by earlier workers (49,27) that petroleum ether was not a satisfactory extractant and that the vitamin could be completely recovered only after a large number of extractions, can only be attributed to the use of alcohol, water, ether ratios widely different from those recommended here. The efficiency of this single extraction was checked for a range of concentrations of carotene and vitamin A.

> Standard solutions of carotene and vitamin A were prepared in ethanol. These were diluted to give a series of solutions containing from 2 to 10 ug vitamin A and 1 to 7 ug carotene per ml. 10 ml. aliquots of these solutions were mixed with an equal volume of water and shaken with 20 ml. of petroleum ether. Samples of the epiphasic layers were withdrawn and estimated spectroscopically for vitamin A or carotene.

Recoveries averaged 99% for both carotene and vitamin A with a deviation of ± 2%. The method has been used extensively throughout this investigation and has proved most satisfactory. It is simple and convenient and has given reliable and reproducible results. It breaks down however in the presence of more than about 5 mg. of fat per ml. of ether, and the percentage of vitamin extracted decreases rapidly as the amount of fat increases.

ince many of the samples to be assayed did contain more than this amount of fat, efforts were made to overcome this disadvantage.

The fats may readily be removed from solution after saponification to free acids by precipitation as insoluble soaps. It was found necessary to shake the soap with ether since a portion of the vitamin is adsorbed on the precipitate and at this stage a number of the soaps tended to remain in suspension in the ether layer. This problem was overcome by the use of zinc salts as precipitants. The zinc soaps leave a clear ether layer, but, in common with most other soaps, are soluble in ether and give an absorption in the 325 mu region. This absorption is not linear over the range 310 to 340 mu so that a correction cannot be applied. Copper also gives a satisfactory precipitate and in spite of its pro-oxident properties does not cause any decomposition of the vitamin during the short time of contact. The copper soap is readily removed from solution in the ether by shaking with dilute ammonia but the copper soaps are so soluble in the ether that there is sufficient ammonium soap formed in this reaction to interfere with the extraction of the vitamin. The vitamin A passes into the ether with the copper soaps but on shaking this solution with ammonia some of the vitamin is re-extracted into the hydrophase due to the presence of relatively large amounts of ammonium soaps. The method does not therefore appear capable of extension to fat-containing mixtures.

. . . . . .

CHAPTER II

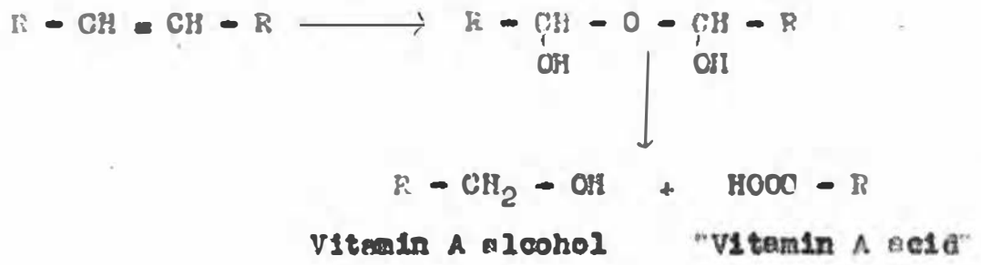
The Chemical Conversion of Carotene to Vitamin A

Little is known of the mechanism of the conversion of carotene to vitamin A in the animal body. Without considering recently reported biological evidence which will be discussed later, the structure of vitamin A suggests that it is formed through the fission of the central double bond of β carotene, one molecule of which would then give one or two molecules of the vitamin depending on the course of the reaction. Alternatively the break could occur at any other double bond, giving one long chain molecule which then undergoes degradation until vitamin A is formed. A reaction of this latter type was suggested by Morton (58) in 1940. This would appear reasonable for a symmetrical molecule such as β carotene but it would be difficult to explain the biological activities of carotene containing only one β ionone ring on this assumption. In these unsymmetrical molecules, a preferential attack on one or other terminal ring system would be expected rendering the carotenes biologically inactive if the attack were on the β ionone ring or of activity equal to β carotene if the other ring were concerned. As has been pointed out by Hunter (59), in the oxidation of α carotene with alkaline permanganate, it is the β ionone ring which is attacked yielding an apocarotinal containing the α ionone ring (60). α carotene might be expected to behave similarly during the process of in vivo conversion and therefore be inactive. In addition chemical theory is in favour of fission at the central bond (59).

Experiments involving the chemical fission of this central bond are therefore of interest since they may suggest possible courses for the in vivo conversion. Hunter and Williams (61) brought about this degradation by means of hydrogen peroxide to yield the aldehyde,

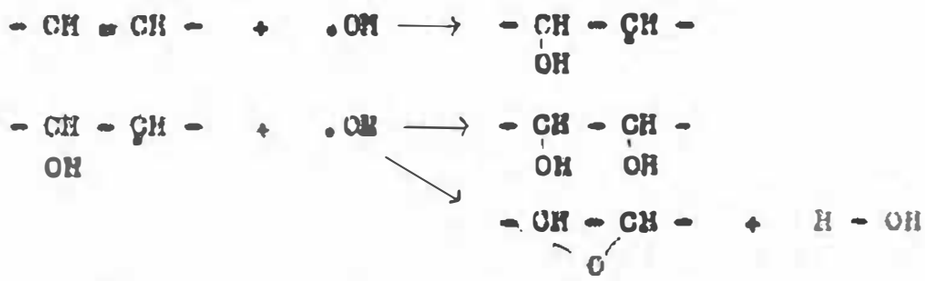
retinene. The optimum temperature for the peroxide oxidation was 38 to 40°C and Hunter (62) considers the use of hydrogen peroxide a close approach to biological conditions. He attributes the extremely poor yields obtained to resonance since in extended conjugated systems each double bond loses some of its double-bonded character to the neighbouring single bonds and the effect should increase towards the centre of the system (63). If indeed the in vivo conversion does proceed through similar reactions, it is possible that the geometrical configuration of the β carotene molecule or some form of induced polarisation may render the central bond more susceptible to attack and so increase the yield to at least the 50% normally obtained biologically. Higher homologues of retinene did not yield retinene on treatment with hydrogen peroxide (62) indicating that the aldehyde is formed by fission of the central bond of carotene and not by successive degradation. If retinene is formed, it should be readily reducible to vitamin A in the animal body and recently Glover, Goodwin and Morton (64) have demonstrated that this reduction can occur.

Goss and McFarlane repeated the hydrogen peroxide oxidation of carotene using osmium tetroxide as catalyst (65) and found that the reaction proceeded to vitamin A alcohol in yields of 30 to 40% of the theoretical. They consider that in the presence of the catalyst the central bond is broken by simultaneous epoxide and di-glycol formation to give a compound identical with that postulated for the Cannizzaro intermediate. This intermediate, according to Fredenhagen and Bonhoeffer (66) then undergoes rearrangement prior to fission yielding one molecule of vitamin A and one molecule of "vitamin A acid".



Vitamin A alcohol "Vitamin A acid"

Oxidations with hydrogen peroxide at low temperatures are considered to involve the free hydroxyl radicle, .OH, and it is probable that the oxidation of unsaturated hydrocarbons, which is accelerated by catalysts such as osmium tetroxide, may also take place through the same free radicle, its immediate precursor in this case being the relatively unstable per-acid (67). Thus it seems likely that the central bond might be attacked by the hydroxyl radicles to give a glycol. Further in autoxidations involving free hydroxyl radicles it is not uncommon for chain breaking reactions to occur resulting in the formation of di-glycols or epoxides.



It is possible therefore that these two reactions could occur together giving the suggested intermediate. If the course of the reaction is as suggested by Goss and McFarlane, it should be possible to isolate from the reaction mixture, a compound which has been referred to as "vitamin A acid , in approximately the same quantity as the vitamin A alcohol. This acid has been synthesised by Arens and van Dorp (68). It has a maximum absorption at 343 mu with a log E value of about 4.65.

This suggests an alternative course for the in vivo conversion and because of this and the unusual type of reaction involved, the work of Goss and McFarlane was

repeated. The reactions were carried out at a temperature of 35 to 40°C, the original workers having failed to record their temperature.

β carotene (50 mg) was dissolved in peroxide-free ethyl ether previously dried over anhydrous sodium sulphate. The solution was added to anhydrous sodium sulphate (about 10 gms.) in a flask fitted with a reflux condenser and stirring device and heated to about 40°C. Osmium tetroxide (about 20 mg) was added followed after about 15 minutes by the dropwise addition of hydrogen peroxide (2 ml. 30% solution) through the condenser with constant stirring. The heating was continued until the colour was discharged (about 10 minutes) and sufficient sodium bicarbonate solution was then added to destroy the unreacted peroxide. When the effervescence had ceased, alcohol containing a small amount of aqueous potash was added. After thorough shaking the lower layer was discarded, the ether washed several times with water, dried over anhydrous sodium sulphate and evaporated to dryness under nitrogen. The residue was taken up in petroleum ether and its absorption measured.

Typical curves for the product are shown in Figure 11.

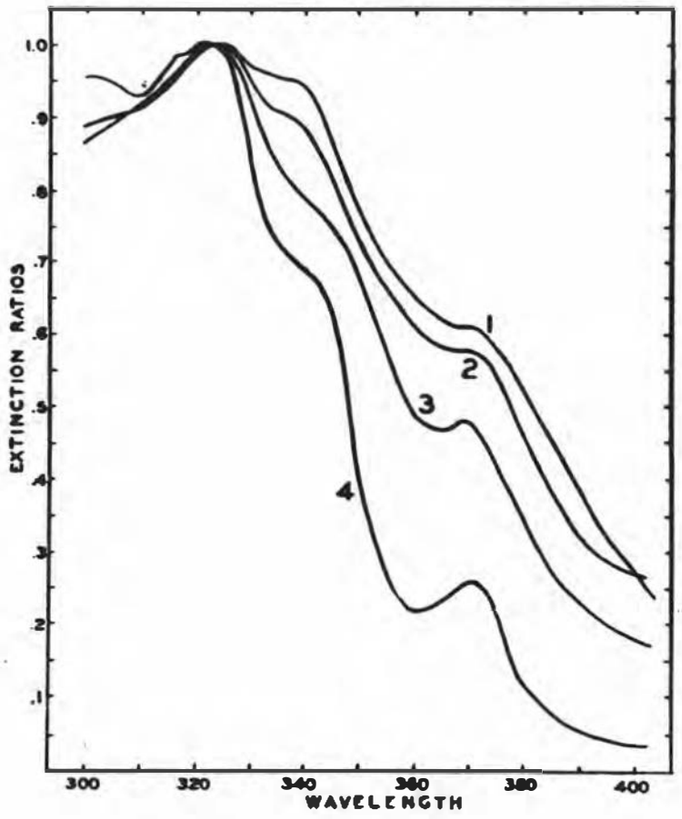


Fig.11 - Typical curves for the product of the oxidation of β carotene with hydrogen peroxide and osmium tetroxide.

. . . . . .

The curves are all of the same general shape showing a sharp maximum at about 325 mu, a point of inflection at about 335 to 340 mu and a secondary maximum or point of inflection at about 370 mu. The curves also agree with that published by Gose and McFarlane, the small differences being due probably to slight variations in the reaction procedures. The products gave intense blue colours with the Carr-Price reagent but the intensity was not as great as would have been expected assuming the absorption at 325 mu to be entirely vitamin A, and the blue colour faded more slowly than that due to vitamin A alone. Further the curves obtained are atypical of the vitamin indicating the presence of other substances absorbing in the region of 325 mu.

The product was therefore chromatographed on alumina (B.D.H. for Chromatography) to yield three rather diffuse bands. When the extruded column was painted with the Carr-Price reagent, all three bands gave colours very similar to that given by Vitamin A. The bands were eluted, dissolved in petroleum ether and their absorption curves measured as shown in Figure 12. It was apparent that the zone giving the absorption curve(1) contained most of the vitamin A and rechromatographing this solution gave a more definite zone whose absorption curve (4) closely resembled that of vitamin A with a maximum at 326 - 327 mu. The blue colour obtained on treating this solution with the Carr-Price reagent appeared to be of the same hue as that produced by the pure vitamin and faded at about the same rate. It is probable that certain losses of the vitamin occurred during the purification but even allowing for these, it seems that the claim of 30 to 40% conversion of carotene to vitamin A based on the Carr-Price colour is high and a figure of 15% would be more accurate.

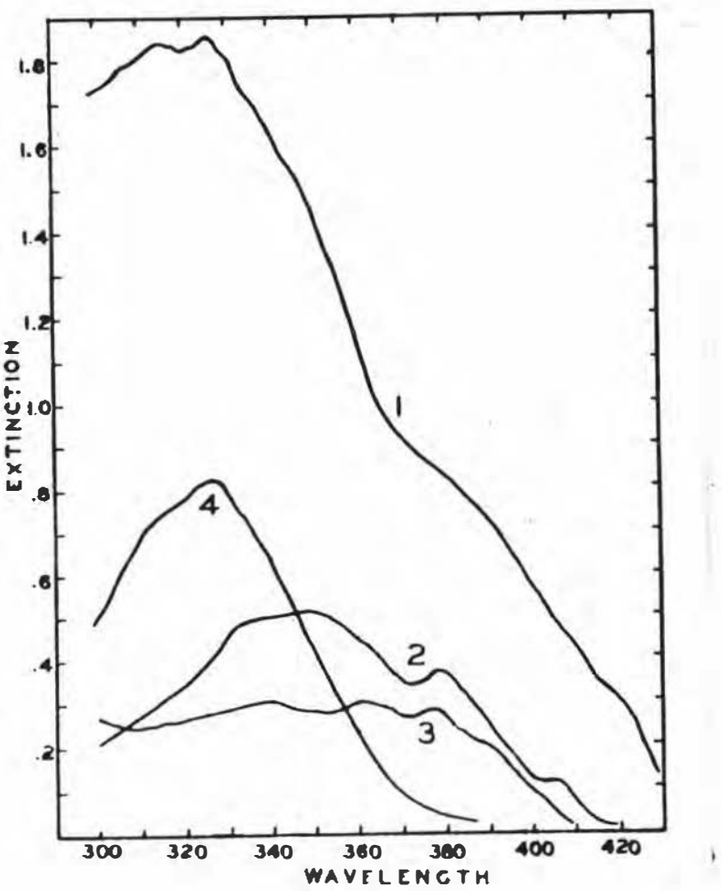


Fig.12 - Product of the oxidation of β carotene with hydrogen peroxide and osmium tetroxide chromatographed on alumina. The absorption due to the three main zones is shown in Curves 1, 2 and 3. Curve 4 shows the absorption of the main zone obtained by re-chromatographing solution 1.

The course of the reaction may be followed from Figure 13 which shows the absorption spectra of samples taken at intervals during the oxidation. It is apparent that little action takes place until after the addition of the hydrogen peroxide when there is a rapid increase in the absorption in the near ultra violet giving a curve somewhat similar to that of the final product. The treatment with bicarbonate and potash does however alter the shape of the curve slightly due apparently to the removal of some acid material. The residue from these washing was therefore extracted several times withethyl ether, acidified with hydrochloric

acid and again extracted twice with ether. The ether from these latter extractions was dried over anhydrous sodium sulphate, evaporated to dryness, the residue taken up in petroleum ether and the absorption measured. The product showed a general absorption over the range investigated (300 to 400 mμ) without the appearance of any peak in the 343 mμ region. Chromatography of this mixture gave a series of poorly defined bands which were not investigated further beyond noting that one of the main bands when eluted and dissolved in petroleum ether gave an absorption maximum at about 369 mμ and another gave a maximum at about 317 mμ.

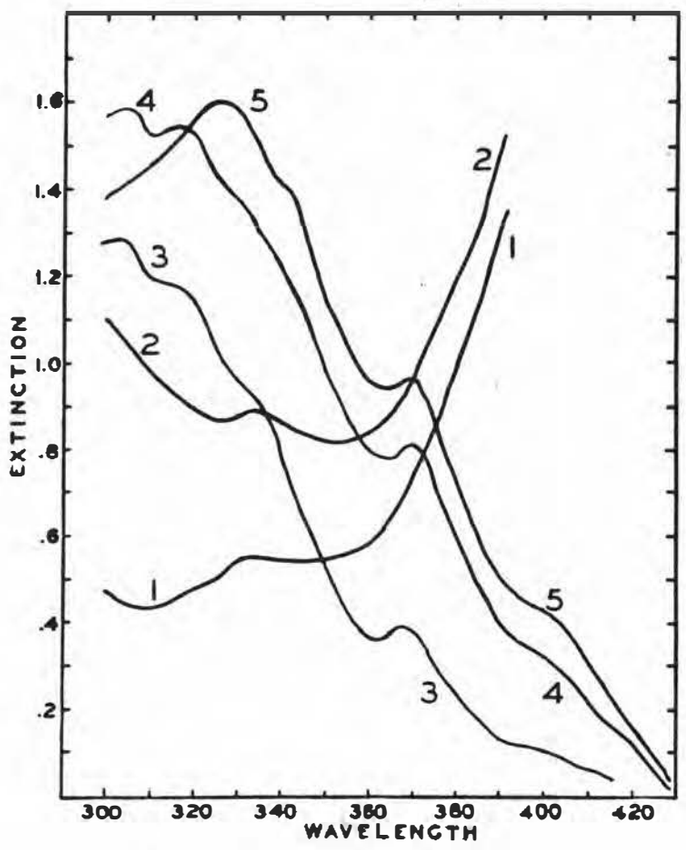


Fig. 13 - Stages in the oxidation of β carotene with hydrogen peroxide and osmium tetroxide. 1 - β carotene, 2 - heated to 40°C for 15 mins. with osmium tetroxide, 3 - 15 mins. after addition of hydrogen peroxide, 4 - excess peroxide destroyed by sodium bicarbonate, 5 - final product after shaking with potash solution.

Even if the whole of the absorption at 343 mu had been due to the vitamin A acid, however, it would have been equivalent to less than half the vitamin A isolated. Unless the acid is unstable under the conditions of the experiment due to the carbonyl group conjugated with the double bonds, these observations offer no support for Goss and McFarlane's suggested mechanism.

Vanadium pentoxide replaces osmium tetroxide in a number of hydrogen peroxide oxidations while copper sulphate is another catalyst frequently employed. Reactions similar to that already described for osmium tetroxide were carried out using these reagents. Almost no reaction occurred with copper sulphate and with vanadium pentoxide the reaction appeared to follow a different course yielding mainly products absorbing below 300 mu with little absorption in the 325 mu region.

It had been assumed that the product of the reaction is in fact vitamin A. This is based on its absorption maximum at about 325 mu and its reaction with the Carr-Price reagent. There is the possibility, however, that the product might be the di-glycol formed by the addition of two hydroxy groups to the central double bond of β carotene.

$$R - CH = CH - R \quad + \quad 2.OH \longrightarrow R - \underset{OH}{CH} - \underset{OH}{CH} - R$$

This compound does not appear to have been prepared but it would be expected to have physical properties very similar to those of vitamin A. Maximum absorption for the glycol would be in the 325 mu region since only conjugated double bonds affect the position of $\lambda_{max.}$ (11), the only effect of the second set of five conjugated double bonds separated from the first by three single bonds being to approximately double

$\lambda_{max.}$ In addition the glycol would probably react similarly with colorimetric reagents so that it would be difficult to distinguish it from vitamin A.

It should be possible to distinguish chemically between the glycol and vitamin A and the simplest method appeared to be by oxidising the compound with periodic acid which is specific for glycols. If the compound is the glycol formed by the addition of two hydroxy groups across the central double bond of β carotene it should yield on treatment with periodic acid two molecules of retinene (vitamin A aldehyde) whereas on similar treatment vitamin A would be unaffected (69).

> Periodic acid (10 mg) dissolved in a few ml. of ethanol was added to an ethyl ether solution of the crude product (approximately 6 mg. calculated as vitamin A) obtained from the oxidation of β carotene using osmium tetroxide and hydrogen peroxide. After standing 12 hours at room temperature, the reaction mixture was extracted several times with dilute aqueous potash, washed with water and dried over anhydrous sodium sulphate.

The absorption spectrum of this solution did not differ significantly from that of the original peroxide oxidation product. The peak in the 325 mμ region was unchanged and there was no increase in absorption in the region of λ max. for retinene.

It appears therefore that the product is not the glycol and is most probably vitamin A as originally suggested by Coss and McFarlane (65) although the mechanism of the reaction has not yet been elucidated.

As will be discussed later the glycol is of some interest as a possible intermediate in the conversion of carotene to vitamin A in the animal body and attempts were made to prepare it by other methods with a view to determining its pro-vitamin activity.

In the elucidation of the structure of the carotenes

extensive use has been made of various oxidising agents (e.g. 70). Neutral potassium permanganate in acetone is commonly used as a mild reagent for the formation of glycols from ethylenic compounds but does not appear to have been applied to the carotenes. It was suggested (71) that this reagent at low temperatures might preferentially attack the central double bond of β carotene to give the required glycol.

> β carotene (100 mg.) was dissolved in redistilled acetone (200 ml.) and the solution cooled to -5°C. 10 ml. of an aqueous solution of potassium permanganate (2 mg/ml.) was added and the mixture held at -5°C for 48 hours. After filtering off the brown precipitate of manganese dioxide which formed during the course of the reaction, the acetone was removed in a stream of nitrogen and the residue taken up in petroleum ether. This solution absorbed heavily below about 300 mu but gave no indication of a peak in the 325 mu region.
>
> The solution was chromatographed on an aluminium oxide column and the chromatogram developed with petroleum ether containing 5% benzene. The extruded column was painted with antimony trichloride solution and the bands giving a coloration with this reagent mechanically separated and eluted with ethanol.

About 60% of the carotene was recovered unchanged and none of the products showed absorption peaks above 300 mu. It appeared therefore that little if any of the glycol had been formed in the reaction.

Towards the end of this investigation an alternative synthesis was attempted with more promising results. Hypoiodous acid, formed by the interaction in moist ethyl ether of iodine and mercuric oxide, reacts readily with unsaturated compounds to give a hydroiodation product (72) which may be converted to the di-glycol by treatment with aqueous potash or sodium acetate (73).

β carotene (26.8 mg.) was dissolved in moist peroxide-free ethyl ether and the solution cooled to about 10°C. Freshly precipitated mercuric oxide (20 mg.) was added followed by the dropwise addition with continuel shaking of a solution of iodine (12.7 mg.) also in ethyl ether. A rapid reaction took place and after about one hour most of the carotene colour had been discharged and no free iodine could be detected.

After filtering the solution was extracted several times with aqueous potassium iodide to remove mercuric iodide, washed with water, dried and evaporated to dryness. The residue was taken up in ethanol containing 5% sodium acetate and the solution boiled under reflux for one hour. On cooling water was added to reduce the alcohol concentration to about 50% and the solution extracted with petroleum ether.

This petroleum ether solution showed a general absorption in the near ultra violet with a broad but clearly defined maximum at about 335 mμ. The reaction is being investigated further in an attempt to increase the yield of this compound and to effect its purification.

. . . . . .

C H A P T E R III

The Conversion of Carotene to Vitamin A in the Animal Body.

(a) The Site of Conversion

The role of the carotenes as precursors of vitamin A was established about 20 years ago by early workers in this field (74-79). In 1931, Moore (74) reported a high vitamin A storage in livers of rats fed a carotene-rich diet. At the same time the livers contained little carotene but the converse was true of the alimentary tracts which contained large amounts of carotene and little vitamin. These observations, which have been amply confirmed by subsequent workers, were considered by Moore to indicate the liver as the site of conversion of carotene to vitamin A. This conclusion is consistent with many other findings reported in the literature. For example, damage or poisoning of the liver usually results in decreased hepatic storage of vitamin A (80, 81) and an impaired ability to convert the carotene to the vitamin (82). It was considered that the transfer of the provitamin across the wall of the intestine depended on the formation of a water-soluble, diffusable complex with bile acids (80, 83). The carotene was then transported in the form of a colloidal solution (84) to the liver where its conversion to vitamin A through the action of the enzyme "carotenase" was assumed to occur (e.g. 85).

No direct evidence of a satisfactory nature has, however, been advanced in support of this conclusion. If the conversion does occur in the liver through enzyme action, it should be possible to demonstrate the formation of vitamin A in vitro by incubating carotene with whole livers or liver extracts. In 1931 Olcott and McConn (86) claimed to have effected the conversion by incubating colloidal carotene with fresh rat liver tissue

or with an aqueous liver extract. They noted a disappearance of carotene and the formation of an absorption band with a maximum at 328 mμ characteristic of vitamin A. These changes did not take place when the "carotenase" had been inactivated by heating. These findings were confirmed by Parienti and Kelli (87) who detected by the Carr-Price reaction, the formation of vitamin A when colloidal carotene was incubated with minced dog liver and von Euler and Klussmann (88) working with extracts of cow livers reported an increase in the absorption at 328 mμ on incubation with carotene. More recently Wilson, Ahmad and Mazumder (89) obtained positive results with rabbit livers undergoing anaerobic autolysis as indicated by the disappearance of carotene and the formation of vitamin A estimated colorimetrically. At the same time, other workers, using similar techniques, have been unable to demonstrate any in vitro conversion of carotene to vitamin A. Thus Ahmad (90) and Rae and Drummond (91) could not detect the formation of vitamin A on incubating carotene with cat liver preparations and negative results were also reported by Drummond and MacWalter (92) for rabbit livers even when the liver cells were allowed to take up carotene from the circulatory system prior to incubation.

In the absence of direct evidence to the contrary however, the liver remained, until recently, the generally accepted site of conversion and the discrepancies between the results obtained by the various workers have been attributed mainly to species differences. These differences in carotene metabolism are well recognised (94) and the negative results with cat livers, for example, could be explained by the inability of these animals under normal conditions to utilise carotene even when it is added to a vitamin-deficient diet (85). The original

work of Olcott and McCann (86) was criticised by Woolf and Moore (93) who showed that the formation of vitamin A had not been conclusively established. In all other cases where positive results have been claimed, at the most only traces of vitamin A appear to have been formed and these in vitro experiments cannot be regarded as providing conclusive evidence of conversion in the liver.

Efforts to demonstrate the conversion in the liver by experiments of a different type also gave conflicting results. In 1930 Wolff, Overhoff and Eckelen (95), using a method of partial hepatectomy, claimed to have demonstrated the in vivo conversion of carotene to vitamin A in the liver of rabbits. A portion of the liver from vitamin A-deficient rabbits was removed and assayed for the vitamin using antimony trichloride. The animals then received an injection of carotene into the circulation and after three days the vitamin content of the residual liver tissue was determined and was found to be considerably higher than the initial sample. These findings were confirmed by Drummond and MacWalter (92) but more recent investigations into the physiological value of carotene administered otherwise than by the oral route have given conflicting results. Thus Lease, Lease, Steenbock and Baumann (96) found that vitamin A-deficient rats could utilise intraperitoneally or subcutaneously injected colloidal carotene or oily solutions of carotene as indicated by restoration of growth and the cure of ophthalmia, but the amounts required were ten to one hundred times as great as when the carotene was given orally. No liver storage resulted from large intakes of parenteral carotene and particles of the injected pigment, which were only partially available to the animals, were found at the sites of injection and in other tissues. In contrast vitamin A was

utilised about as efficiently when injected as when given orally. These workers note that much of the injected carotene could not be traced; it was not converted into vitamin A or excreted. According to Tomarelli, Chaney and Bernhart (97) however, intramuscularly injected carotene can be utilised provided it has been water-stabilized (e.g. by solution in "Tween 80"), and there is a rapid transport from the site of injection and efficient conversion. Similar results were reported by Kith (98) and by Vinet, Plessier and Raoul (99) but conflicting results were obtained by Roklina, Belakhovski and Sodrova (100).

The findings of Lease and co-workers have been confirmed by Sexton, Mehl and Deuel (101). These workers, in an extensive investigation also using rats, extended the previous observations by demonstrating that the deposited carotene was unaltered and was stereochemically identical with the original material. From their findings they considered that the conversion of carotene to vitamin A was an extra-hepatic function and suggested the wall of the intestine as a possible site.

It is indeed difficult to explain the non-availability of the injected carotene if conversion does occur in the liver but the possibility remained that although the carotene did reach the liver, it might do so in a condition or manner unsuitable for conversion to the vitamin. The carotene in the blood is considered to exist in the form of a colloidal solution (84) so that the state of the carotene in the plasma sol used by Deuel and his co-workers should be similar to that in normal blood; but even when this solution is injected into the heart, the carotene accumulates unchanged and in a relatively non-available form in the liver. At the same time there is evidence of the existence in the

blood of some species of a carotene-protein complex (101, 102) and possibly only carotene in this form could be converted to the vitamin in the liver. In addition only carotene which reaches the liver in low concentrations might be metabolised. As has been reported recently for vitamin A (103), higher concentrations might be taken up and retained in the Kupfer cells just as any other foreign substance (104). The absence of carotene from the blood and liver tissue of a number of species does however constitute strong evidence against the liver as the main site of conversion. If carotene is transported to the liver and transformed there to vitamin A, it should be present in appreciable amounts in the blood of all species and to a certain extent in the livers, unless the process of conversion is sufficiently rapid to prevent the accumulation of any provitamin.

In a subsequent publication, Matteon, Mehl and Deuel (105) showed that following the oral administration of carotene to vitamin A-deficient rats, the vitamin first appeared in the intestinal wall and that for approximately four hours the quantity in the wall of the intestine was greater than in the liver. The source of this vitamin in the wall of the intestine could not have been the liver since following vitamin supplementation, the wall of the intestine contains little vitamin even when hepatic stores were high. During the process of carotene absorption there was no increase in the provitamin content of the livers whereas that of the intestinal walls did increase. These observations clearly indicated the wall of the intestine as the site of conversion and this was confirmed by Wiese, Mehl and Deuel (106) who effected the in vitro conversion of

carotene to vitamin A in the intestines of vitamin A-deficient rats. These workers used a surviving-tissue technique in which the intestines were removed from rats and incubated with colloidal carotene under anaerobic conditions in Ringer-Locke solution. In all cases some vitamin A, as detected by the Carr-Price reagent, was formed.

These observations proved that for the rat at least, the main site of conversion of carotene to vitamin A was the wall of the intestine and the evidence so far presented was against any appreciable conversion taking place elsewhere. This idea of conversion in the wall of the intestine was not however entirely new. In 1941 Popper (107), using fluorescent microscopy, investigated the rate of appearance of vitamin A in various organs of vitamin A-depleted rats following oral administration of carotene. In a number of cases he was able to detect characteristic vitamin A fluorescence in the intestinal wall before the liver and suggested that conversion could occur in the former organ although he considered the liver to be the main site. The fact that small amounts of carotene administered by routes other than the oral appear to be converted might be explained by an excretion of this carotene into the lumen of the intestine from which it could be re-absorbed and converted in the ordinary way into Vitamin A. Sexton Mehl and Neuel (101), in suggesting such a mechanism, showed that traces of intraperitoneally administered carotene could be recovered in the gastrointestinal tract and the faeces.

Thus prior to the commencement of this investigation early in 1948, it had been established that in the rat the wall of the intestine and not the liver was

the main site of conversion of carotene to vitamin A but this has not been confirmed for any other species. The possibility of species differences could not be overlooked and as one aspect of this study of the carotene metabolism of ruminants, it was of interest to determine whether the wall of the intestine was also the site of conversion in herbivorous animals which are entirely dependent for their vitamin A on the biologically active carotenoid pigments present in herbage.

In this investigation only limited facilities have been available for handling experimental animals and it has been necessary to restrict most of the observations to sheep but the site of conversion in cattle has also been investigated since these animals appear to differ markedly from other herbivora in their absorption and utilization of carotene. Thus the blood plasma of sheep contains, per 100 ml., 25 - 35 ug of vitamin A but only about 1 - 2 ug of carotene and these levels are normally independent of carotene intake. Although the vitamin A levels in the blood of cattle are of the same order, the carotene content may be as high as 1.5 mg/100 ml and both the vitamin A and carotene levels vary with the dietary provitamin intake.

Assuming the conversion to occur in the liver, the carotene must be transported to that organ from the intestine either directly in the portal blood or indirectly by the lymphatic route and the systemic circulation. In the latter case it should be possible to show the presence of carotene in the intestinal lymph and the systemic blood plasma would also be expected to contain some carotene, the actual level depending on the rate of absorption by the intestine and the rate of removal from the systemic circulation by the liver. If however, the portal route is involved, carotene should be present in the portal blood

but its presence in, or absence from, the systemic plasma would depend on the efficiency with which it is removed from the portal blood by the liver. Unless the rate of absorption of the pigment by the intestine exceeds that at which it is removed by the liver, no carotene will reach the systemic circulation.

On the other hand, if conversion occurs in the wall of the intestine, the vitamin A formed will be transported from there to the liver by either the portal or lymphatic route or both, and the carotene will not appear in the blood or lymph unless the rate of absorption exceeds the rate of conversion to the vitamin. This excess carotene may be transformed to vitamin A at a secondary site of conversion or it may be treated merely as a waste product and decomposed in various tissues.

It was apparent that a comparison of the carotene and vitamin A levels in portal and systemic blood and in intestinal and non-intestinal lymph, would supply information regarding the site of conversion and the mode of transport of the vitamin or provitamin from the intestine and experiments were therefore carried out to determine these levels.

(1) Conversion of Carotene to Vitamin A in the Sheep.

The comparison of portal and systemic plasma levels was made on an anaesthesised sheep and in order to accentuate any differences, an excess of readily absorbable carotene was provided by injecting the provitamin in a colloidal state into the lumen of the intestine.

Preparation of Colloidal Carotene

A saturated solution of carotene (ca.20 mg) was prepared in ethanol containing synthetic α tocopherol (ca.5 mg) as anti-oxidant. The solution was run slowly into distilled water which had been boiled for some time to expel dissolved oxygen and cooled to about 70°C under nitrogen. The colloidal solution was boiled under reduced pressure

to remove the alcohol, cooled and filtered.

Collection of Blood Samples

A sheep was anaesthesised by the intravenous injection of sodium pentothal (1 gm.), an incision made along the right costal arch and the colloidal solution containing approximately 20 mg of carotene injected into the lumen of the intestine at various points along the jejunum. Blood samples were taken at intervals from the portal and jugular veins and citrated. Anaesthesia was maintained during the latter stages of the experiment by ether inhalation.

Determination of Carotene and Vitamin A.

The plasma samples were extracted using the method described by Kimble (108). In order to detect small amounts of carotene, up to 40 ml samples of plasma were used. The petroleum ether extract was evaporated to dryness in a stream of nitrogen, the residue taken up in 5 ml petroleum ether and the carotene content estimated. An aliquot of this solution was then evaporated to dryness, the residue dissolved in chloroform and vitamin A estimated colorimetrically using the photoelectric method and internal standard already described.

The vitamin A levels in the portal and systemic plasma samples are shown in Table 6. Carotene could not be detected in measureable amounts in any of the samples. The limit of the method described for carotene in blood is considered to be about 2 ug./100 ml of plasma.

TABLE 6

Time in hours after injecting carotene	Plasma Vitamin A ug/100 ml	
	Jugular	Portal
0	28	25
½	32	44
1	-	37
1½	22	-
2	28	-
3	24	-

Collection of Lymph Samples

Intestinal and non-intestinal lymph glands were removed from a number of sheep immediately following slaughter. Three groups of similar pasture-fed animals were used and sufficient were included in each group to provide about 5 ml of both types of lymph. In each group the intestinal glands, which included duodenal, jejunal and ileal, were combined and as much lymph as possible collected from them. In the same way, samples of non-intestinal lymph were collected from various other glands mainly submaxillary and pharyngeal from the same animals.

Preliminary estimations of carotene and vitamin A were carried out using a method similar to that already described for blood plasma. Poor recoveries of vitamin A added as internal standard indicated the presence of colour inhibitors in amounts greater than encountered in blood plasma, and for the lymph samples the Kimble method was modified to include saponification as described by Parrish, Wise and Hughes (53).

As might be expected by its absence from the blood plasma, no carotene could be detected in any of the lymph samples. The vitamin A content is shown in Table 7.

TABLE 7

Group	Lymph vitamin A ug/100 ml. Intestinal	Non-intestinal
1	118	38
2	84	35
3	102	34

From these results it appears that no appreciable amount of carotene is transported from the intestine by either the portal or lymphatic routes. Since during the absorption of carotene the vitamin A level is higher in the intestinal than the non-intestinal lymph and in the portal than in the systemic blood, it must be concluded that the wall of the intestine is the main site of conversion and that the vitamin formed there is transported to the liver for storage by both the lymphatic and portal routes. This site has been confirmed by in vitro experiments similar to those described for rats

by Wiese, Mehl and Deuel (106).

In carrying out these experiments it is necessary to consider the conditions which must be satisfied to establish with any degree of certainty the conversion of carotene to vitamin A. Obviously the mere disappearance of carotene is no indication of conversion to the vitamin since conditions under which the in vitro transformation might be expected to occur also favour oxidative decomposition. Similarly an increase in the absorption at 325 mu or in the colour given by the Carr-Price reagent does not necessarily indicate vitamin A since nonbiologically active decomposition products also give these reactions (see Figure 7).

It is considered that the following conditions must be satisfied before the formation of vitamin A can be established conclusively:

(1) As a preliminary it should be possible to detect the presence of vitamin A by a method specific for the vitamin in the presence of decomposition products.

(2) The vitamin should be separated from the reaction mixture in a sufficiently pure state that the formation of an absorption band characteristic of vitamin A with a peak at about 325 mu can be demonstrated.

(3) A colorimetric estimation of the vitamin should give a figure in agreement with that obtained spectrophotometrically and the absorption curve for the product of the colorimetric reaction should be similar to that produced with the pure vitamin.

Deuel and his co-workers estimated the vitamin A formed by the Carr-Price reagent. In a subsequent publication Mattson (109) drew attention to the non-specificity of this reagent and repeated part of the work of Mattson, Mehl and Deuel (105). In these

experiments he was able to establish the identity of the vitamin formed by its fluorescence, by its absorption curve and by the formation of a single fluorescing band in a mixed chromatogram with pure vitamin A.

In the experiments reported here the three point correction procedure was used to detect the formation of vitamin A. In a number of cases the mixture obtained was chromatographed to give a relatively pure product which was identified by its absorption curve and its reaction with glycerol dichlorohydrin. CbH was used in preference to antimony trichloride since the transitory nature of the colour produced by the latter reagent renders measurement of its absorption curve difficult in spectrophotometers of the Beckman type.

Incubation of Tissues

Intestines were removed from sheep as rapidly as possible following slaughter and placed in a bath containing Ringer-Locke solution[1] maintained about 37°C. Sections (approximately 2 to 3 feet in length) were removed from the jejunum and the contents flushed out with Ringer-Locke solution. Colloidal carotene (about 20 ml) was introduced into one section from each animal and after ligation at the ends, the tissue was incubated anaerobically in Ringer-Locke solution for 2 to 3 hours at 37°C. Corresponding sections immediately above and below that incubated were taken as controls and assayed immediately for Vitamin A.

Estimation of vitamin A

After incubation the colloidal carotene was flushed out with 0.9% saline. The sections were comminuted in the Waring Blendor under nitrogen with ethanol containing 5% potassium hydroxide. The suspension was then refluxed until a clear

---

Footnote 1 - The Ringer-Locke solution employed had the following composition : Sodium chloride 0.9%; Potassium chloride 0.042%; Calcium chloride 0.024%; Sodium bicarbonate 0.05%; Magnesium chloride 0.02%; Glucose 0.05%.

solution was obtained (10 to 15 mins.) and the vitamin A extracted into petroleum ether using the method already described for liver samples. Vitamin A was estimated in the control sections by the same method.

In these experiments it was found that the stability of the carotene solutions varied with the method of preparation and a uniform procedure was adopted.

Preparation of Colloidal Carotene

The apparatus shown in Fig.14 has been found convenient for the preparation of small quantities of colloidal carotene. The required amount of carotene is dissolved in the minimum quantity of peroxide-free ethyl ether and added to about 50 ml of ethanol in flask A. After isolating the flask from the rest of the apparatus by tap B, the ether is removed from the solution by gentle heat applied through an electrically heated coil of resistance wire dipping below the surface of the solution. Slight suction is applied at C and ethanol is added from D to maintain the volume at about 50 ml. Approximately 200 ml of distilled water was placed in flask E and boiled for some time by means of a similar electrically heated coil to

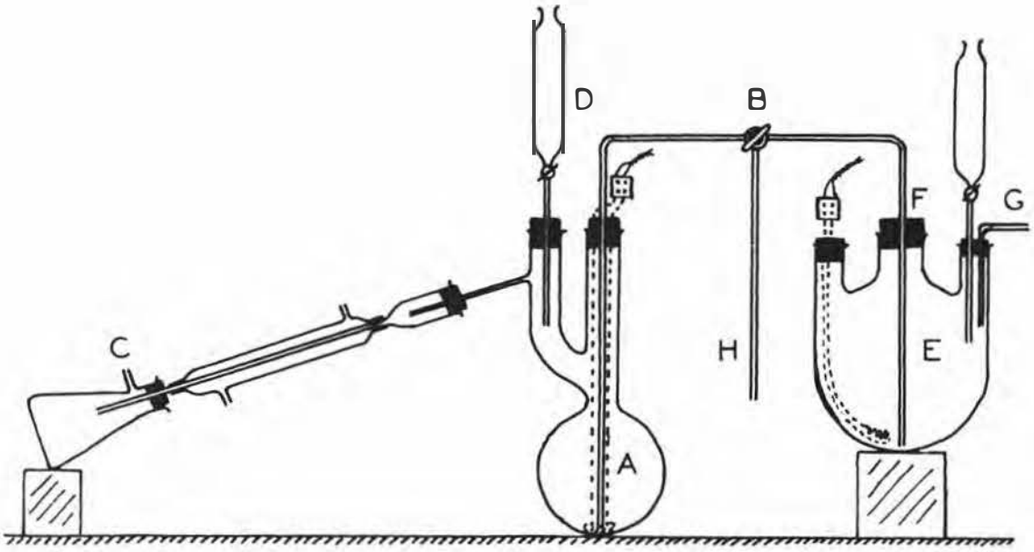


Fig.14 - Apparatus used for the preparation of colloidal carotene.

expel as completely as possible dissolved air. The water is allowed to cool to about 70°C and the solution of carotene in ethanol is introduced slowly through the capillary tube F by opening tap B and applying gentle suction at C. Tap B is again closed and the solution in E boiled under reduced pressure to remove ethanol. The colloidal carotene is allowed to cool in the flask and is withdrawn as required through H by introducing nitrogen at C. The solutions were usually prepared to contain about 40 ug carotene per ml.

Provided precautions were taken to exclude air it was found possible to produce relatively stable colloidal solutions using this apparatus. In some cases α tocopherol equal to about one quarter the weight of the carotene was included as an anti-oxidant.

It was not possible to deplete the sheep of vitamin A before slaughter or even to maintain them for a short time on a carotene-free diet. In most cases therefore, carotene absorption was proceeding at the time of slaughter and the sections contained appreciable quantities of vitamin A and carotene before incubation. A large number of experiments were carried out using sections of this type and, omitting preliminary experiments, the incubated tissues showed consistent increases in vitamin A content of up to 15%. Although it was considered that this was clear evidence of conversion in the wall of the intestine, the increases were small and the results less convincing than those reported by Wiese, Mehl and Deuel (106) with vitamin A-depleted rats.

It was possible however to carry out a few experiments on intestines from sheep which had been fed a poor quality hay of low carotene content for some time prior to slaughter. Control sections taken from six of these sheep averaged 7.2 ug of vitamin A. Following incubation with carotene the vitamin A content of similar sections ranged from 8.5 to 27.3 ug with an average value of 18.9 ug representing an increase of 162%.

The identity of the vitamin A formed in these experiments was confirmed spectroscopically and colorimetrically.

> The ether extracts containing the crude vitamin A from the incubated sections were combined, washed with water to remove traces of ethanol, dried over anhydrous sodium sulphate and evaporated to about 20 ml under reduced pressure. The absorption curve of this solution was measured and it was then chromatographed on a column of 1:1 magnesium oxide - Hyflo Supercel as described by Mattson (109). The chromatogram was developed with petroleum ether containing 5% benzene and the vitamin A band, detected by its fluorescence under ultra violet light, separated mechanically and eluted with petroleum ether saturated with ethanol. After measuring its absorption curve the solution was evaporated to dryness and the residue taken up in chloroform. The vitamin A content of this solution was estimated colorimetrically using activated glycerol dichlorohydrin. In addition the absorption curve of the coloured compound formed with SbCl was also measured.

The absorption curve for the original petroleum ether extract is shown in Figure 15, curve 1.

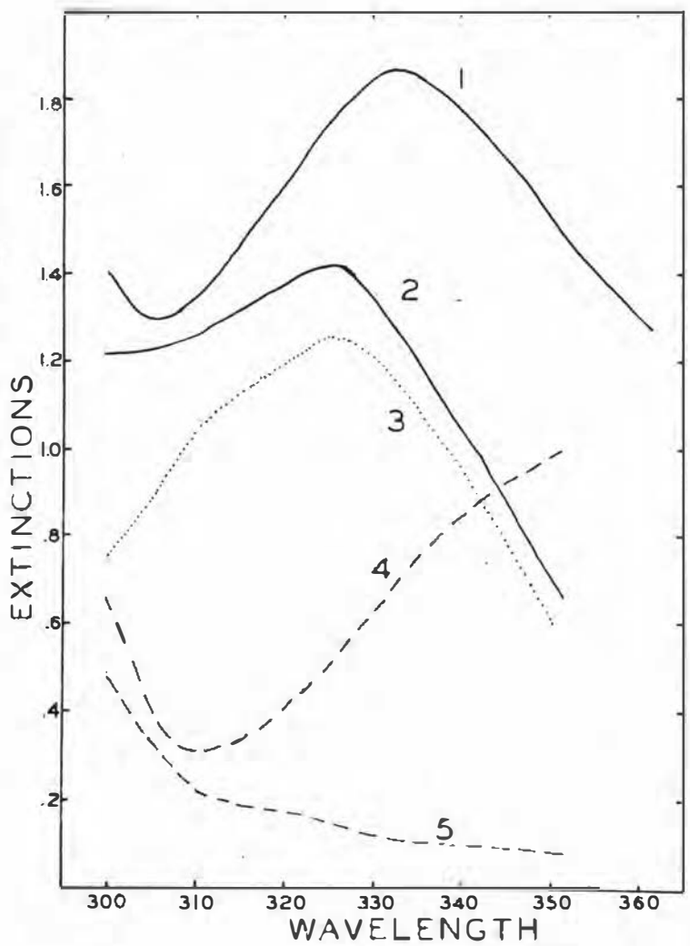


Fig.15 - Vitamin A formed on incubating intestines with carotene.

Curve 2 gives the absorption of the purified solution obtained after chromatography. This curve resembles that of vitamin A and application of the three point correction procedure gave an apparent total vitamin A content of 88 ug (78% recovery) as illustrated by curve 3. Curves 4 and 5 represent the impurity apparently present in the original and purified solutions respectively. The total vitamin A content estimated colorimetrically with CBH was 88 ug (86% recovery) and the absorption curve of the product agreed closely with that reported for pure vitamin A (32).

It may be concluded that in sheep as in rats, the wall of the intestine is the main site of conversion of carotene to vitamin A. Shortly after this work was carried out other workers reported confirmation of the findings of Deuel and his co-workers for rats and similar results for other species. The intestinal conversion in rats has been reported by Glover, Goodwin and Morton (110, 111) and by Thompson, Ganguly and Kon (112). These latter workers also found the mesenteric lymphatics and intestinal walls and contents of pigs dosed with carotene some hours before slaughter contained much larger quantities of vitamin A than control pigs. Similar results have also been obtained with rats and pigs by Thompson, Breuds, Cowie, Ganguly and Kon (113) who have also demonstrated the efficient absorption of colloidal carotene. These workers mention that as early as 1939 the intestine had been suggested by Wagner (114) as the site of conversion in whales. The results so far reported for rats have been obtained with vitamin-depleted animals but recently Krause and Pierce (122) have shown that non-depleted rats without hepatic circulation were able to convert carotene to vitamin A illustrating that the transformation is an extra-hepatic function and that the

site of conversion is not altered by the deficiency state of the animals used by other workers.

Failure to detect carotene in the portal or systemic blood plasma of sheep has been confirmed by Goodwin and Gregory (115) who extended the observations to goats and rabbits. These workers also showed that the vitamin A content of the lymph withdrawn from goats provided with a thoracic cannulae increased after feeding carotene indicating conversion in the wall of the intestine.

This later work has been summarised in recent reviews (116, 117).

Regarding the transport of vitamin A from the intestine to the liver, it now appears that the lymphatic route is the more important one. Eden and Kellers (118) found a marked increase in the vitamin A content of the duodenal, jejunal and ileal lymph following oral administration of emulsified halibut-liver oil to bullocks. At the same time, no increase in the portal as compared with the systemic blood vitamin level was noted. As pointed out by Goodwin and Gregory (115) an experiment of the type already described in which portal and systemic vitamin A and carotene levels were compared in an anaesthetised sheep, is open to the criticism that absorption processes in general are retarded by anaesthesia and trauma of several hours duration. The differences in levels noted during the early stages of the experiment (Table 6) are however significant, and are supported by the observations of Goodwin and Gregory who, using conscious goats, found slightly higher levels in the portal than in the systemic plasma following carotene administration. It is possible therefore that some transport occurs by both routes and the vitamin A absorption may in fact parallel that of fats (119).

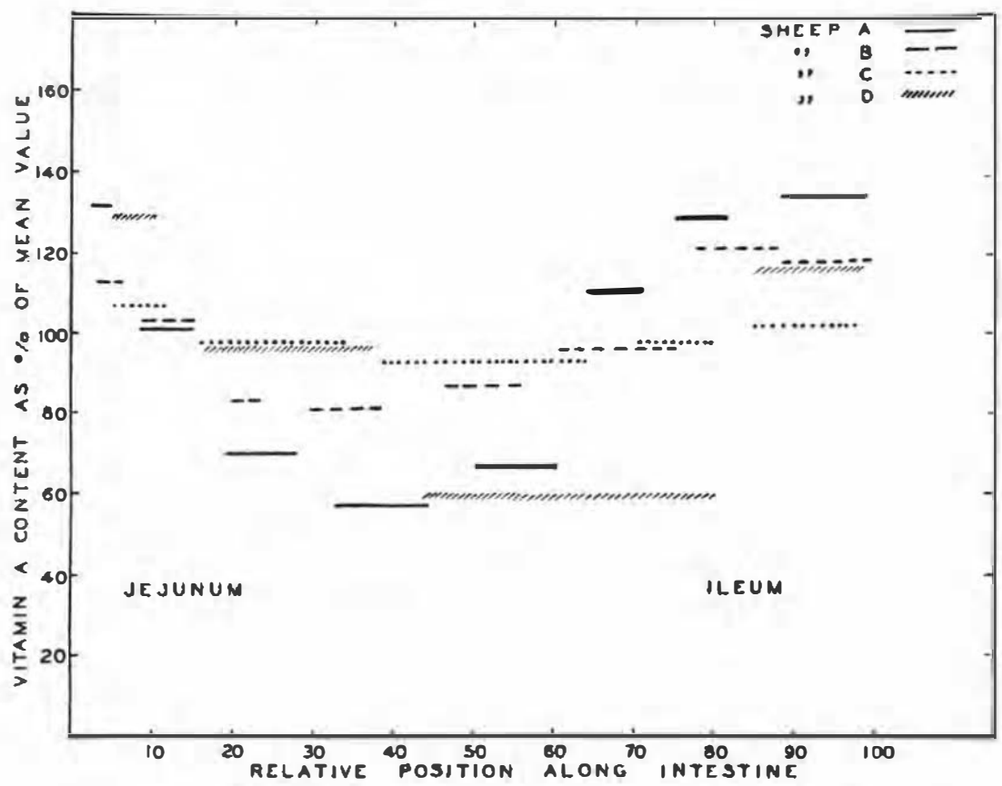


Fig. 16. - Relative vitamin A potencies of intestinal lymph.

In an attempt to determine whether the conversion could occur at any point along the intestine or was limited to a particular region, whole intestines were incubated with colloidal carotene using a method similar to that already described for the short sections. These experiments gave variable results due possible to injury to portions of the tissue caused during handling. It was possible however, to obtain an indication of where carotene absorption and conversion normally occurred by estimating vitamin A in the intestinal lymph glands.

> Duodenal, jejunal and ileal lymph glands were removed from 4 pasture fed sheep immediately following slaughter. Insufficient lymph could be obtained from each gland for assay so the assumption was made that the ratio of lymph to gland tissue was relatively constant and whole glands were assayed. After grinding with sand each gland was assayed separately for vitamin A using the method already described for liver samples.
>
> For plasma and lymph samples it was found that provided the extracts had been saponified, results agreeing to within about ± 8% of the Carr-Price figures could be obtained using the three point correction procedure and this spectroscopic method was therefore used for the lymph glands.

The results obtained are shown in Figure 16. Since only relative figures were required the average vitamin A content of the glands from each sheep in micrograms per gram was calculated and a comparison between sheep made by expressing the vitamin A concentration (ug/gm) in each gland as a percentage of the mean for the animal. These percentages are plotted against the relative position of the gland along the intestine also expressed as a percentage.

Owing to the variations in size and number of the glands their relative positions are plotted as lines, the length of which indicate the relative sizes of the glands. The low level of vitamin A and the small size of a number of the glands limits the reliance which can be placed on individual assays but the results obtained from

the four sheep indicate that although there is some carotene absorption over the whole intestine, maximum absorption occurs in the upper and lower portions.

Efforts to repeat the in vitro conversion experiments using emulsions of carotene in place of the colloidal solutions were unsuccessful. The carotene was dissolved in a number of fats or fatty acids and solutions dispersed in water. Experiments were carried out over the pH range 6.0 to 8.5 using various emulsifying systems in particular the oil-oleic acid-sodium carbonate and oleic acid-bile salt-monostearate systems described by Frazer, Schulman and Stewart (120). No conversion of carotene to vitamin A could be established and difficulty was experienced with decomposition of the carotene. This accelerated decomposition in the presence of fats and fatty acids led to the experiments described in Chapter 4.

Attempts have also been made to effect the conversion of carotene using tissue slices, minced tissues and homogenates prepared from the intestines using the methods described by Umbreit, Burris and Stauffer (121). The tissue preparations were incubated with colloidal carotene at pH values between 5.5 and 7.5 but in no case has it been possible to establish the formation of vitamin A. With the tissue slices there was some evidence of conversion of carotene to vitamin A but in quantities too small to allow conclusive identification of the products and it was not possible to increase the yield. In view of the negative results these experiments are not reported in detail.

(2) Conversion of Carotene to Vitamin A in Cattle

No recent investigation has been reported into the site of conversion of carotene to vitamin A in cattle. Eden and Sellers (118) have shown that in cattle the lymph

draining from the small intestine is the main pathway by which orally administered vitamin A reaches the general circulation. It seems reasonable to assume that if vitamin A is formed from carotene in the intestinal wall it will be transported by the same route and the vitamin A levels in intestinal and non-intestinal lymph were therefore compared.

> As already described for sheep, samples of intestinal and non-intestinal lymph were obtained from a pasture-fed bullock immediately following slaughter. These were assayed for vitamin A using the modified Kimble method. Sufficient lymph was obtained to carry out the assays in duplicate.

The intestinal lymph samples contained 142 and 159 ug vitamin A per 100 ml and the non-intestinal 48 and 60 ug per 100 ml. It is apparent that in cattle as in other species vitamin A is formed in the wall of the intestine. As might be expected from its level in the tissues and blood plasma, carotene was also present in the intestinal lymph, the two samples assayed averaging 345 ug per 100 ml.

In cattle as distinct from other species where little or no carotene passes across the wall of the intestine, the possibility of a secondary site of conversion must be considered. In view of this possible species difference, the in vitro experiments of Olcott and McCann (86) and Wilson, Ahmad and Mazumdar (89) were repeated using cow livers. Contrary to the early findings of von Euler and Klussmann (88), the formation of vitamin A could not be demonstrated in any of these experiments.

If there is no secondary site of conversion in cattle, the carotene in the tissues may not represent, as is frequently supposed, a large potential reserve of vitamin A on which the animal may draw but may be merely a waste product.

(b) Mode of Conversion

Following the demonstration of the structural relationship between vitamin A and β carotene, Karrer, Helfenstein and Wehrli (123) suggested that the transformation in the animal body occurred through a symmetrical cleavage of the provitamin molecule, one molecule of β carotene giving rise to two molecules of vitamin . This view was generally accepted until crystalline vitamin A was prepared and its biological potency was determined by a number of workers (13, 124, 125, 126), found to be approximately double that of carotene.

One international unit of vitamin A is defined as the biological activity of 0.6 ug pure β carotene, i.e. 1 ug β carotene is equivalent to 1.67 I.U. vitamin A. If the cleavage of the β carotene molecule is symmetrical to give two molecules of vitamin A, 1 ug β carotene would give 1.07 ug vitamin A and the biological potency of this quantity of vitamin A must be 1.67 I.U. or 1 ug vitamin A should be equivalent to 1.56 I.U.

If the cleavage is asymmetrical one molecule of β carotene would give only one molecule of vitamin A. 1 ug β carotene would give 0.54 ug vitamin A and this would be equivalent to 1.67 I.U. or 1 ug vitamin A should be equivalent to 3.12 I.U.

Thus from theoretical considerations the biological potency of vitamin A should be equal to or double that of β carotene depending on whether the cleavage of the provitamin molecule is symmetrical or asymmetrical. From the experimentally determined vitamin A potencies Mead, Underhill and Seward (125) concluded that either an asymmetrical split is involved or that the β carotene is not converted quantitatively into

vitamin A in the animal body and favoured the former explanation.

As already mentioned cleavage of the central double bond of β carotene is supported by chemical theory and by the biological activities of the asymmetrical carotenes. In addition, if one molecule of β carotene gives rise to only one molecule of vitamin A, the experimentally determined biological potencies of 3 - 3.5 I.U. per ug vitamin A could only be explained by assuming complete utilisation of the β carotene. There is ample evidence however, of incomplete utilisation of β carotene under bio-assay conditions (1).

Attention has frequently been drawn to the necessity for an adequate vitamin E intake in bio-assays. The effect of tocopherol on the utilisation of vitamin A and carotene has been reviewed by Bickman (127) who concluded that tocopherol exerts a greater synergistic effect on β carotene than on vitamin A. The lower biological activity of β carotene could therefore be due to insufficient vitamin E and Koehn (128) has recently shown that with an adequate tocopherol intake rats can convert β carotene quantitatively to vitamin A. This observation supports the theory of in vivo conversion of β carotene to vitamin A by cleavage of the central double bond.

Hunter (62) has suggested that the in vivo conversion proceeds through the oxidation of the central double bond to retinene or a product afterwards converted to this. Glover, Goodwin and Morton (64) have shown that retinene administered orally to rats is rapidly absorbed and converted to vitamin A in the wall of the intestine and consider that the in vivo transformation of β carotene to vitamin A is more likely to proceed through oxidation of the former to retinene and reduction

of this to vitamin A than by direct hydrolytic fission.

The oxidation of β carotene to retinene may proceed through intermediates such as a di-glycol.

$$R - CH = CH - R \longrightarrow R - \underset{OH}{CH} - \underset{OH}{CH} - R \longrightarrow 2\ R - CH = O$$

β carotene — di-glycol — retinene

$$\downarrow$$

$$2\ R - CH_2OH$$

vitamin A

It is for this reason that attempts have been made to prepare this glycol since the enzyme systems involved in the transformation can be studied most conveniently through the various intermediates.

. . . . . .

CHAPTER IV

The Stability of Carotene towards Oxidation

During the course of the work described in the previous chapter it was found that carotene was unstable in the presence of various fats, fatty acids and their esters. As it was desired to investigate the in vitro conversion of carotene to vitamin A using emulsions such as might be encountered in the intestine, the stability of carotene in solutions containing these substances and the effect of various anti-oxidants was investigated. In order to simplify the procedure and to obtain results more rapidly, the decomposition was investigated under accelerated conditions at 100°C with forced aeration. Liquid paraffin was chosen as an inert solvent, the substances under test being added to various concentrations of carotene in this solvent. A number of experiments were also carried out using a sample of fully hydrogenated coconut oil as solvent. In both cases it was found that the stability of the carotene in the controls was extremely high and varied with the carotene concentration. The problem of the instability of the carotene emulsions was largely overcome by the use of hydrogenated coconut oil as solvent but the abnormal behaviour of the solutions of carotene in paraffin and the hydrogenated oil was investigated further.

In recent years considerable attention has been paid to the stability of vitamin A in various solvents and under different conditions of storage and the spectral changes which occur on irradiation and oxidation have been very fully investigated (130). The behaviour of carotene under similar conditions has a more limited practical application and except in relation to the keeping quality of carotene-containing natural fats

such as butterfat, has not been studied to any extent. The early work of McNicholas (56) appears to be the only detailed study of the decomposition by irradiation but was concerned mainly with the initial and latter stages of the destruction. Decomposition at higher temperatures in pure solvents does not appear to have been investigated in any detail.

Preparation of Solutions

The carotene used in this investigation was a mixture of β carotene (89%) and α carotene (10%) as prepared by the British Chlorophyll Co. It was used as supplied without further purification. A sample from a freshly opened phial was dissolved in peroxide-free ethyl ether, and aliquots of this solution added to pharmaceutical grade liquid paraffin (as defined in the British Pharmacopoeia 1932) to give the required concentrations of carotene. The ether was removed by warming under reduced pressure. Solutions in hydrogenated cocomit oil[1] were prepared in a similar manner.

Determinations

Apparent carotene was determined spectroscopically by measurement of the absorption at 450 mμ of aliquots of the solutions suitably diluted with petroleum ether. Absorption spectra were also determined in a number of cases, readings being taken normally at 5 mμ intervals and at 1 mμ intervals in regions of rapid change in absorption.

Decomposition under forced aeration

The stability of the various solutions was studied in a Swift Oxidation Tester (132) under forced aeration at 100°C. Large test tubes containing about 30 ml of solution were placed in copper thimbles dipping into a bath of boiling water. Inlet tubes drawn to fine tips extended to the bottom of these test tubes and through these air was blown at a constant excess pressure of about 15 mm mercury. Aliquots were withdrawn at intervals, weighed, diluted to suitable concentrations with petroleum ether, and their optical densities measured.

Footnote 1 - Prepared and supplied by Abels Ltd., Auckland, iodine value 0.1.

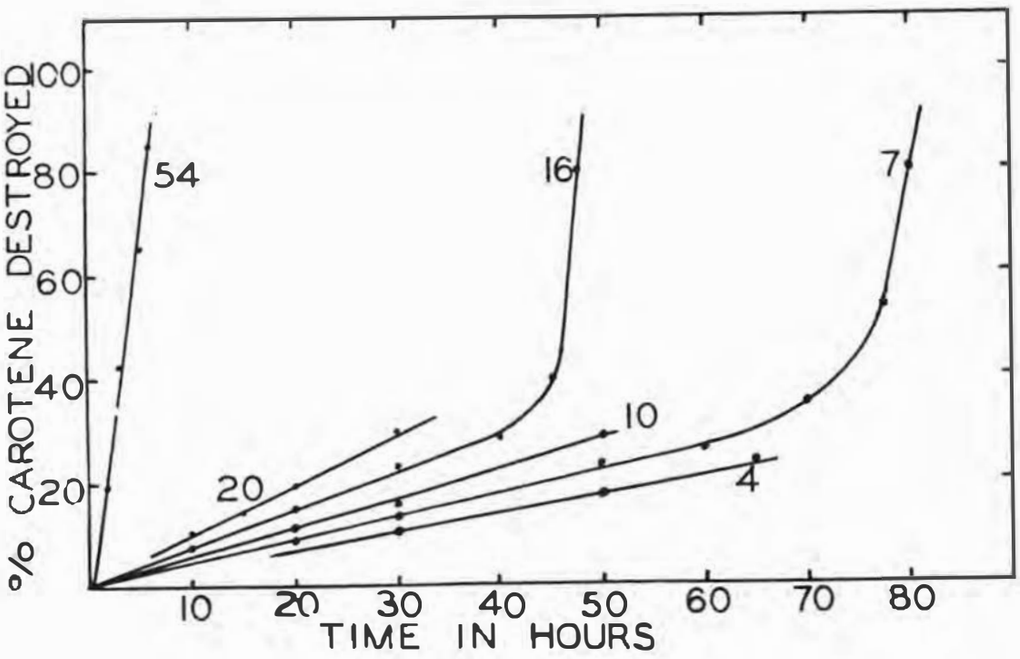


Fig. 17

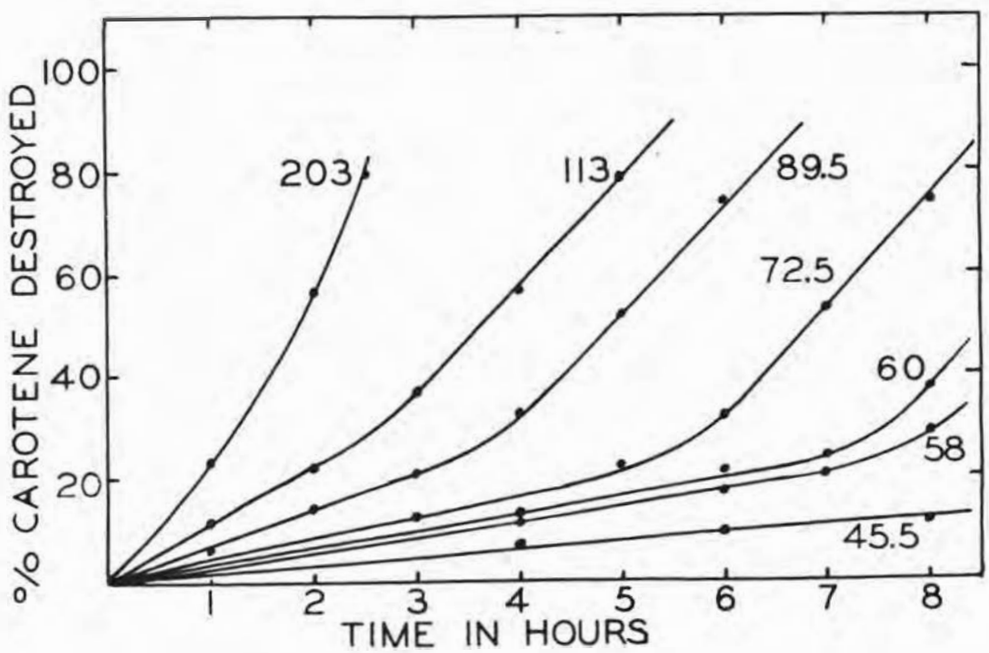


Fig. 18

Curves showing rates of decomposition of carotene in liquid paraffin. Figures indicate initial carotene concentrations in ug/ml.

. . . . . .

Using paraffin as solvent, decomposition rates were determined for a range of carotene concentrations from 4 to 203 ug/ml. (Figures 17 and 18) The results

shown in the two figures are not entirely comparable since it was necessary to use two different samples of paraffin and the second sample used for the higher concentrations (Fig.18) gave a slightly longer induction period than the first. The decomposition curves are all however of the same general shape showing an induction period, the length of which depends on the carotene concentration, followed by a rapid autocatalytic decomposition.

From these results it was apparent that an extremely effective anti-oxidant was present. Schibsted (14) has described a method for the purification of petroleum ether by shaking repeatedly with concentrated sulphuric acid, refluxing with caustic soda and distilling from lime. A sample of the paraffin (dissolved in petroleum ether to lower the viscosity) was treated in this way except that the distillation was omitted. Using the product as solvent the decomposition rates were measured for a series of carotene concentrations from 4 to 50 ug/ml. Complete destruction of the carotene took place in 4 to 6 hours without any apparent relationship between destruction time and carotene concentration. Comparable results were obtained using as solvent, paraffin which had been subjected to aeration at 100°C for several days.

The decomposition in hydrogenated coconut oil was not so fully investigated due to difficulty in obtaining further supplies of a suitably hydrogenated product. The results obtained did however indicate that the induction period depends again on the carotene concentration. The decomposition curves differed somewhat from those obtained using liquid paraffin and were similar in shape to those reported for vitamin A in shark liver oil (130). The induction periods with

the hydrogenated oil were much longer than with paraffin and the autocatalytic stage less rapid and decreasing in velocity markedly towards the end of the reaction. A solution containing 100 ug/ml, for example, showed an induction period of approximately 50 hours and complete destruction of the carotene only after a further 20 hours. Solutions below 50 ug/ml. had not reached the end of their induction periods after 84 hours continuous aeration at 100°C. Typical curves are shown in Figure 19. The wider scatter of points is probably due to a varying air pressure through the tubes resulting from sudden fluctuations in water pressure in the main operating the air pump encountered at the time these latter experiments were carried out.

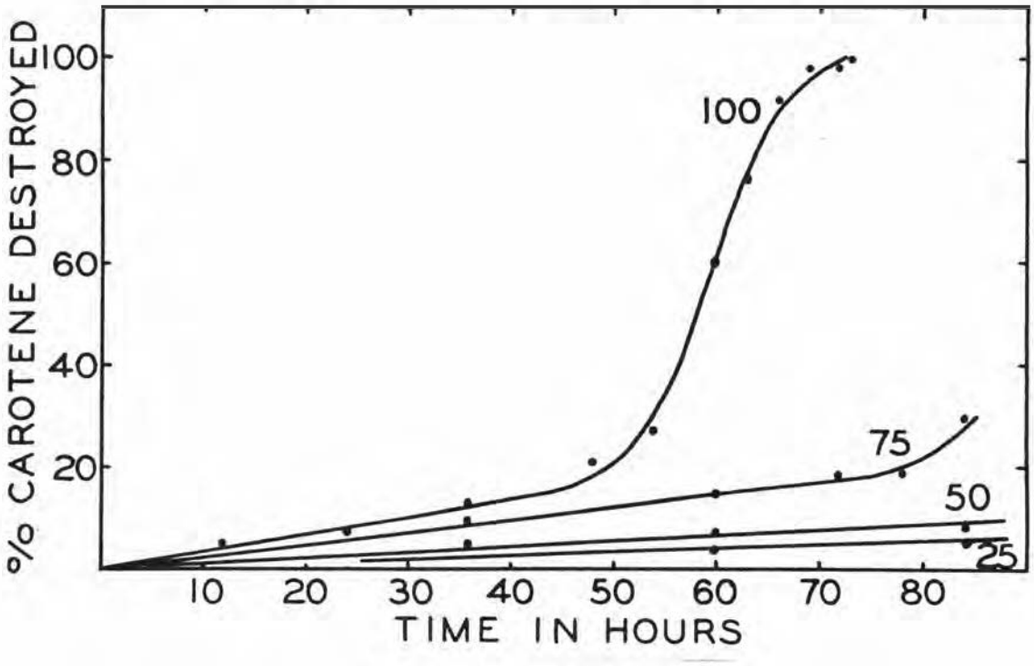


Fig.19 - Curves showing rates of decomposition of carotene in fully hydrogenated coconut oil. Figures indicate initial carotene concentrations in ug/ml.

. . . . . .

Small quantities of saturated fatty acids or their esters exert a marked effect on the stability of carotene in paraffin solution. Baumann and Steenbock

(134) have recorded the rapid destruction of carotene in the presence of esters such as ethyl stearate and in the present investigation it was found that the addition of 5% of purified methyl stearate to a paraffin solution of carotene reduced the induction time to about one fifth while an equivalent amount of purified saturated fatty acid such as myristic or stearic reduced it to about 1/12th. The effects of the addition of various substances on the decomposition curves are shown in Figure 20.

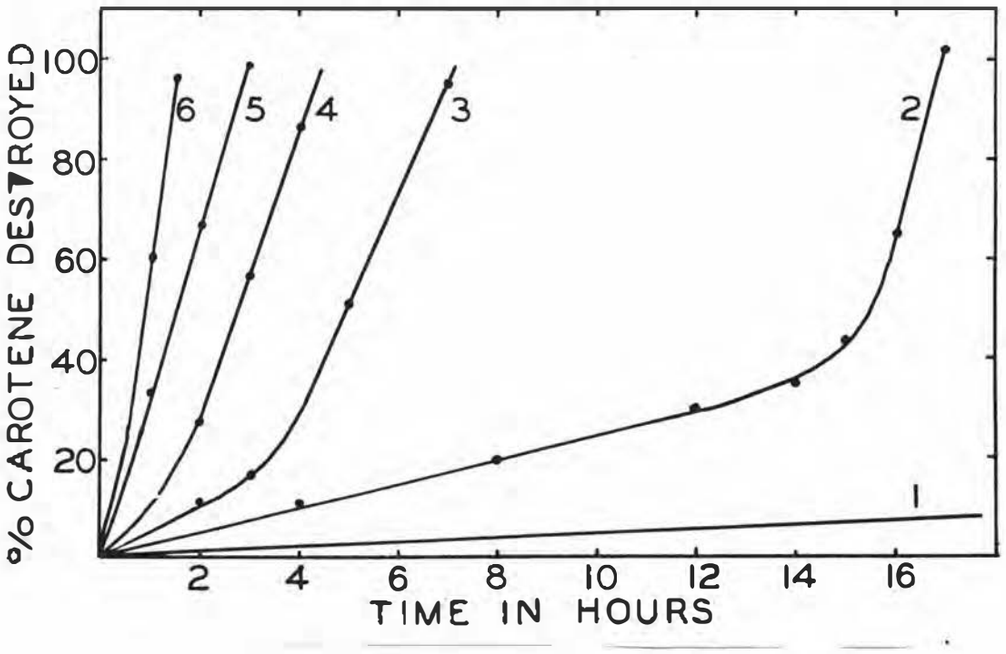


Fig.20 - Curves showing the effect of various substances on the rate of decomposition of carotene in paraffin. 1, Control; 2, 5% methyl stearate; 3, 5% stearic acid; 4, 5% myristic acid; 5, 10% paraffin subjected to aeration at 100°C for several days; 6, 50 ug % benzoyl peroxide. Initial carotene concentration 7 ug/ml in all cases.

. . . . . .

The spectral changes occurring during the destruction of carotene at 100°C in liquid paraffin are shown in Figure 21. This solution contained initially 113 ug/ml. The changes taking place during high temperature oxidation are of the same general type as those noted during the slow decomposition of a

solution of carotene in ethyl ether in dull light at room temperature (Figure 6). During the latter stages of decomposition however, there is a more rapid increase in the extinction ratios in the shorter wavelengths at the high temperature.

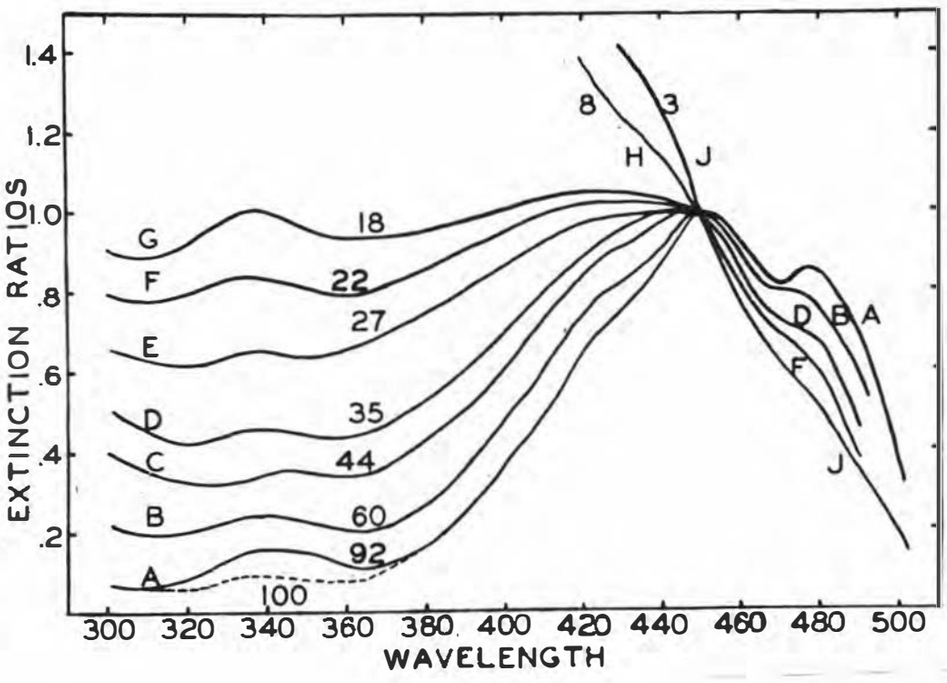


Fig.21 - Spectral changes occurring during the decomposition of carotene at 100°C in liquid paraffin. Figures show percentage carotene remaining at each stage.

. . . . . .

From the results obtained it appears that both solvents contain naturally-occurring anti-oxidants and the stability of the carotene depends on its concentration. In the case of liquid paraffin the destruction of 25 to 30% of the carotene corresponds to the end of the induction period and may be taken as a measure of the stability of the carotene. Taking 30% destruction as the end of the induction period the stability is directly proportional to the logarithm of the carotene concentration up to 50 µg/ml. as shown in Figure 22. Above this concentration there is a rapid deviation from linearity.

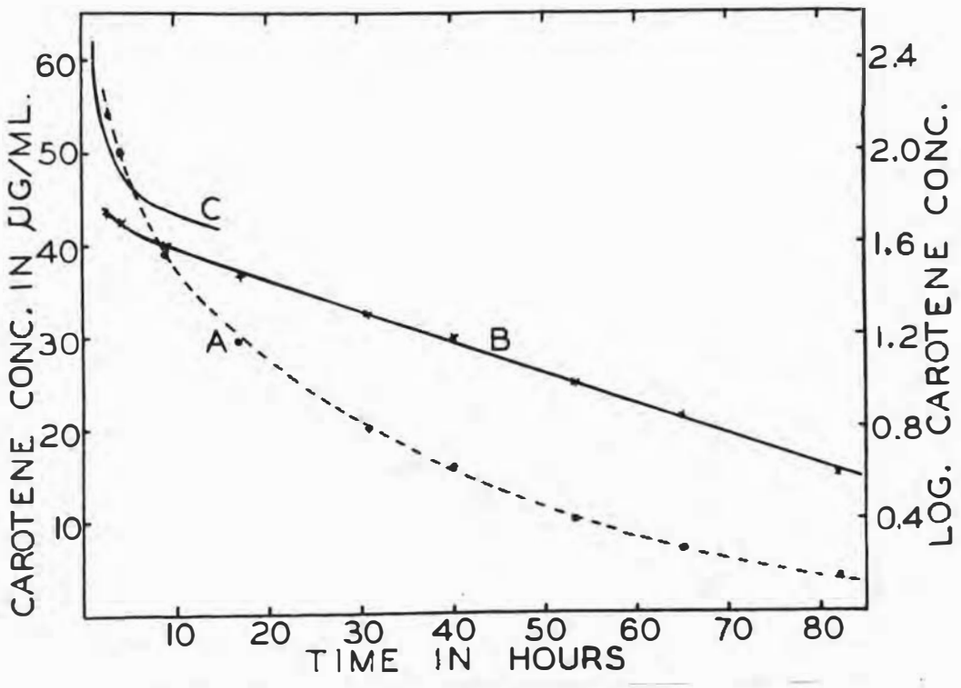


Fig.22 - Relationship between initial carotene concentration and time for 30% destruction. Times from Figure 17 plotted against carotene concentration (curve A) and log. concentration (curve B) and times from Fig.18 against log. concentration (curve C).

. . . . . .

Difficulties have been reported in obtaining stable solutions of carotene in oils. While the stability in liquid paraffin may not at present be of more than academic interest due to the poor absorption of paraffin under normal conditions (135), it is suggested that consideration should be given to the use of hydrogenated coconut oil as a solvent for carotene for feeding trial and similar work. In addition to the high stability of carotene in this solvent, its other physical properties make it highly suitable for this purpose.

The conditions of decomposition of the carotene in these experiments differed entirely from those already investigated (chapter I) but it is of interest to note that the absorption in the region 310 to 340 mu is still relatively linear.

C H A P T E R V

Thyroid Function and Carotene Metabolism

As a result of work over the last 20 odd years, a number of aspects of vitamin A metabolism have been found to be interrelated with thyroid function. The relationship between thyroidal activity and carotene metabolism has also been extensively investigated but with conflicting results.

The early literature is concerned mainly with clinical observations suggesting the association of carotinaemia or high plasma carotene levels with the hypothroid state. More recently controlled laboratory experiments have given equivocal results, some workers reporting that the formation of vitamin A from carotene is impaired in a hypothyroid condition and enhanced in hyperthyroidism, while others find that the thyroid state has no effect on the conversion.

Hoorden (136) was the first to suggest the association of carotinaemia with metabolic disturbances. This observation has been confirmed by subsequent workers (137, 138, 139) and it appears that carotinaemia, although an uncommon condition, is frequently associated with hypothyroidism and that when the thyroid disfunction is alleviated, carotene plasma levels fall. Of similar significance is the observation that the low vitamin A levels in the blood of cretins is only slightly raised by the ingestion of carotene (140). Wohl and Feldman (141) from studies on dark adaption concluded that all types of thyroid disfunction reduced vitamin A reserves. On the other hand the effect of a hyperthyroid state is illustrated in the abnormally high vitamin A reserves found by Moore (142) in the livers of patients dying from thyrotoxicosis.

In these early observations carotinaemia appears to have been taken as indicative of inability to convert carotene to vitamin A but when unaccompanied by low serum vitamin A level or symptoms of vitamin deficiency, this assumption would hardly appear to be justified. Carotinaemia in itself is probably a harmless condition, being merely an abnormally high level of carotene in the blood. Since yellowing of the skin is frequently the first symptom of carotinaemia, it is commonly used as a synonym for xanthosis cutis and, as is pointed out by Bricknell and Prescott (85) both of these conditions may result from dietetic causes such as the ingestion of excessive quantities of carrots or similar carotene-rich vegetables. On the other hand they may arise from pathological conditions such as myxoedema, tuberculosis, diabetes and abnormal fat metabolism. The attaching of undue importance to the association of carotinaemia and thyroid hypofunction has also been criticised by Drill (143) who, in a review, points out that the latter condition is frequently accompanied by hepatic disorders which are also encountered in some cases of non-hypothyroid carotinaemia. It is possible therefore that liver damage alone is responsible for the carotinaemia and that the same condition might give rise to abnormal vitamin A storage or utilisation.

At the same time other evidence suggests a relationship between thyroid function and carotene metabolism. Kunde (144) reports the appearance of xerophthalmia in rabbits maintained for 8 to 12 months following thyroidectomy on a diet adequate for normal animals. It has also been claimed that cats rendered thyrotoxic can, in contrast to normal animals, effect the conversion of carotene to vitamin A (145) while thyroidectomized guinea-pigs store only carotene and

not vitamin A in their livers (146). Other workers have found an increase in the carotene and a decrease in the vitamin A content of goats' milk following thyroidectomy (147, 148).

The results of more recent studies are not however in agreement. In these experiments animals have been rendered hypothyroid by thyroidectomy or by treatment with thiourea or thiouracil while the hyperthyroid condition has been achieved by administration of thyroxine or thyroxine-containing preparations. Drill and Truent (149) found that supplementation of a vitamin A-deficient diet with the preformed vitamin prevented the appearance of xerophthalmia in both control and thyroidectomised rats but that a carotene supplement was effective for the controls only. Similarly ocular symptoms of a vitamin A deficiency cannot be relieved in thiouracil treated animals by carotene unless thyroid powder is administered with it (150). Contrary to these results Remington, Harris and Smith (151) found the ability of rats to utilise carotene in the cure of xerophthalmia unimpaired by thyroidectomy.

Liver storage tests have also given conflicting results. Using vitamin A-deficient rats, one group of which had been rendered hypothyroid by thiouracil treatment, Wiese, Neuel and Kehl (152) estimated vitamin A in the livers at various intervals following carotene administration. The livers averaged the same amount of vitamin A and treatment with thiouracil appeared to make no difference to the ability of the animals to convert carotene to vitamin A. In similar experiments Johnson and Baumann (153) found that rats rendered hyperthyroid by feeding desiccated thyroid tissue or hypothyroid by thiourea or thiouracil treatment were

able to absorb and store preformed vitamin A as efficiently as normal rats. In the case of carotene however, the hyperthyroid rats accumulated larger reserves of vitamin A than normal, whereas the stores in the livers of hypothyroid rats were smaller. These findings are largely supported by the results of experiments of a similar nature carried out by Kelley and Day (154). Johnson and Baumann (153) also showed that the enhanced ability to convert carotene to vitamin A was not due to the increased metabolic rate alone since di-nitro phenol which also raises the basal metabolism, was found to be without effect on the conversion.

The metabolic rate does however effect the rate of depletion of liver stores of vitamin A by animals on a deficient diet, since with the same initial reserves, hypothyroid rats survive longer than normal rats (151, 155), while the survival time of hyperthyroid rats is shorter (163). The significance which can be attached to liver storage tests is therefore limited by the fact that the thiouracil retards the utilisation of the stored vitamin A, possibly due to a lower maintenance requirement associated with the lower metabolic rate. This effect is super-imposed on any direct bearing the hypothyroid state may have on the efficiency of conversion of carotene so that the liver vitamin A may represent the difference between the two opposing effects of the hypothyroid state; firstly the possibly impaired conversion resulting in less vitamin A available for storage and secondly the better retention of the stored vitamin. Thus the relative results obtained with normal and thiouracil-treated vitamin deficient rats, could depend on the length of time during which carotene supplements were fed and the time between the supplements

and slaughter. Immediately following the feeding of carotene the effect of any impaired conversion would be most marked, the vitamin A content of livers from hypothyroid animals being low. If the feeding period or the time between feeding and slaughter is extended, the second effect may equal or exceed the first, the better retention of vitamin A resulting in a liver storage in the hypothyroid animals equal to or greater than the normal or hyperthyroid. The results obtained by Kelley and Day (154) would seem to bear out this hypothesis.

Hypothyroidism appears to have no effect on the absorption of preformed vitamin A and Riese, Mohl and Deuel (156) have recently compared the efficiency of utilisation of carotene and vitamin A at low levels by thiouracil-treated and normal vitamin A-deficient rats. These workers found that the relative effectiveness of vitamin A and carotene in promoting growth of hypothyroid rats did not differ materially from that found in normal rats. They concluded that if the conversion of carotene to vitamin A is associated with the thyroid state, it is not seriously impaired in severely hypothyroid rats and that if the thyroid hormone is essential for the conversion it must be effective in very small amounts.

Recently, while investigating the effect of the thyroid state on the milk production of dairy cows, Campbell and McDowell (157) observed that contrary to the results obtained with goats (147), (148), the feeding of large quantities of thiourea and thiouracil did not influence either the carotene or vitamin A content of the milk. (This work is so far unpublished but consisted of feeding a Friesian cow 10 gms. thiourea daily for 14 days followed by 15 gms. of thiouracil

daily for 7 days. During and immediately following the feeding period, samples of butterfat were assayed for carotene and vitamin A. Levels of both remained relatively constant throughout and of the same order as in the fat of an untreated control cow of the same breed. Only the two animals were used but a gross effect outside normal seasonal or lactational trends was expected.)

It was considered that experiments of a different type would furnish additional evidence on this problem of the possible association between thyroid function and carotene metabolism. Assuming that the thyroid does exert an influence it could do so by acting directly on the enzyme system concerned in the conversion, or by affecting the rate of absorption of the carotene or its stability in the digestive tract. Whatever the mode of action, hyperthyroidism would be associated with an increase in the vitamin A content of the blood while a hypothyroid state would result in a decrease. Positive changes of this type could be taken as further evidence of the effect of the thyroid on the conversion, but the significance of negative results would, as in the case of liver storage tests, be limited by the fact that these trends might be offset or completely masked by the apparent effect of metabolic rate on vitamin A requirements as illustrated by differences in utilisation of liver stores.

As already discussed, in certain species such as sheep, goats and rabbits, the conversion of carotene to vitamin A is normally so efficient that little or no carotene appears in the blood or is stored in the tissues. With these animals in a hypothyroid state, if the effect is directly on the enzyme system, the drop in vitamin A might be associated with the appearance of unconverted carotene in the blood. This

would not be the case if the action is one involving rate of absorption or stability. On the other hand if the hyperthyroid state does increase the rate of absorption of carotene, it might also result in the appearance of carotene in the blood due to the rate of absorption exceeding that of conversion. This again would not be the case if stability of carotene is involved or if the action is on the enzyme system. On analogy with other species, the appearance of carotene in the blood would result in some accumulation of the pigment in the tissues, particularly the liver. Findings of this type depending on the presence or absence of carotene would be more significant than those depending on changes in the relative concentrations of vitamin A and carotene in the blood or tissues. Whether or not positive results could be expected, would depend, in addition to the severity of the thyroid disfunction, on the relative normal efficiencies of the processes of absorption and conversion of carotene. Unless the two processes are of the same order, large changes could occur in either, without the appearance of carotene in the blood. The more closely the efficiency of the process of carotene absorption approaches that of conversion, the more marked would be the influence of hypo- and hyperthyroidism. With sheep it would appear that the rate of conversion just keeps pace with the absorption since even under normal conditions traces of carotene may be found in the livers and the increased absorption associated with high levels of feeding results in the appearance of some carotene in the blood (158).

Experiments were therefore carried out using normal, hypo- and hyperthyroid sheep. Owing to the limited facilities available for handling experimental

animals and the small amount of carotene concentrate available at the time, a pilot experiment using three sheep only was carried out with a view to repeating the work later with larger numbers of animals once dosage levels had been established and if promising preliminary results were obtained. To investigate the possible effect of the thyroid state on the stability of the carotene in the digestive tract, its apparent digestibility (i.e. carotene absorbed plus carotene destroyed in the digestive tract) was also determined in each case using chromium oxide as a reference substance.

Animals and Feeding

Three mature ewes of approximately the same weight (180 lbs.) were used as experimental animals. They were fed throughout on medium quality meadow hay. On 21/6/48, following a prefeeding period of 9 days, all animals received 2.00 gms. per day of a carotene concentrate[1] containing approximately 2% carotene. In addition, from the same date animal 2 received 5.0 gms. per day of iodinated casein[2] and animal 3 received 5.0 gms. thiouracil, animal 1 acting as a control. Blood samples were also taken at the start of the experiment from the jugular vein and citrated. This treatment was continued for 7 days when further blood samples were taken. From this date (28/6/48) the thyroprotein to animal 2 was increased to 8.0 gms. per day and the thiouracil to animal 3 to 6.0 gms. per day and the three animals received in addition 3.000 gms. of chromium oxide per day. The various materials to be administered were placed in a number of small, soluble gelatin capsules which were forced down the animals' throats. On 2/7/48 further blood samples were taken. Animal 2 died suddenly on this date due apparently to the effects of excessive doses of thyroprotein. The liver was removed immediately and assayed for vitamin A and carotene. Approximately 100 gm. samples of freshly voided faeces were collected twice daily from the remaining two animals on 4/7/48 and 5/7/48 and the animals were slaughtered on 6/7/48. Further blood samples were taken at slaughter and the livers were removed. A sample of faeces was also taken from the rectum.

Footnote 1 - Supplied by the British Chlorophyll Co.

Footnote 2 - Prepared and supplied by the Dairy Research Institute, Palmerston North.

Methods of Assay

Hay, carotene concentrate and faeces were assayed for carotene using the method of Moore (159). Carotene and vitamin A were estimated in the blood plasma using the method of Kimble (108) modified by the inclusion of the saponification step as recommended by Parrish, Wise and Hughes (53). The method of Gallup and Hoeffer (46) already discussed was used for liver samples. The whole livers were minced and 10 gram samples of the well mixed product used for assay. Chromium as Cr_2O_3 was determined in the oxide and the faeces using the method described by Barnicoat (129). All determinations were carried out in duplicate.

None of the plasma samples contained measurable quantities of carotene and no definite trends were found in the vitamin A content, the small fluctuations being of an erratic nature. Only small variations were found in the vitamin A content of the livers. The three livers did contain small amounts of carotene but only of the same order as had been found in livers of sheep fed normally on pasture or on hay and concentrates (unpublished results) and as reported for pasture-fed animals by Peirce (158). The figures obtained in this experiment are shown in Table 8.

TABLE 8

Sheep	Vitamin A Blood Plasma ug/100 ml.				Livers ug/gm	Carotene Livers ug/gm
	21/6/48	28/6/48	2/7/48	6/7/48		
1	28	32	26	31	202	2.0
2	33	30	29	-	285*	3.7*
3	26	28	31	30	336	3.6

* Assayed following death of animal on 2/7/48.

The carotene content of the hay was 15.8 ug/gm dry matter, giving with the concentrate, a carotene intake of 47 mg. per day or a carotene to Cr_2O_3 ratio in the feed of 15.7 mg/gram Cr_2O_3. The ratios determined in the faeces from animals 1 and 3 together

with the calculated apparent digestibilities are shown in Table 3. It is of interest to note that apparent digestibilities of the same order were obtained with other sheep fed the hay either alone or supplemented with 15 mg. vitamin A per day.

T A B L E 2

Ratio Carotene to Cr_2O_3 in ug/gm.			
Feed		15.7	
		Sheep 1	Sheep 2
Faeces 4/7/48	a.m.	11.2	12.1
	p.m.	9.6	13.6
5/7/48	a.m.	13.6	12.9
	p.m.	9.9	10.2
6/7/48		12.8	12.9
Average excretion		11.4	12.3
Apparent digestibility per centage*		42%	38%

* Calculated from the formula;

Apparent digestibility percentage of nutrient = $100 \cdot \frac{f-0.8e}{f}$

where f = ratio nutrient to Cr_2O_3 fed

e = ratio nutrient to Cr_2O_3 excreted. (129)

As will be shown later, the carotene excreted may not represent, as has been assumed here, the difference between the carotene ingested and that absorbed or destroyed in the digestive tract. Samples were taken at various points along the tract and assayed for chromium and carotene to obtain an indication of where the destruction of the carotene, if any, was occurring. Due to sampling errors or inefficient mixing of the oxide with the feed in the upper portion of the digestive tract, widely different ratios were obtained. The results are not therefore recorded here but carotene changes in the digestive tracts of normal animals will be discussed later.

The effect of thiouracil on the stability of carotene was also investigated by incubating solutions of colloidal carotene with varying concentrations of thiouracil for from 4 to 12 hours at 37°C. In no case was the carotene decomposition greater than in the controls incubated under the same conditions but without thiouracil.

From these experiments it seems unlikely that thiouracil decreases the stability of carotene in the digestive tract. As already noted, any changes in plasma or liver vitamin A due to variations in the conversion of carotene would be counter-balanced by the apparent effect of the thyroid state on vitamin A requirements so that only positive results would have any real significance. The same applies to the failure to detect carotene in the plasma or to any extent in the livers. Carotene in the blood of the hypothyroid animal for example would have indicated an action directly on the enzyme system involved in the conversion, and while a negative result could be explained by assuming no relationship of this type between thyroid function and carotene conversion, it could also result from a decreased conversion associated with decreased absorption of carotene.

There can be no doubt that the animals were in hypo- and hyper-thyroid states. The respiration rate of animal 2 rose to 1½ times normal during the course of the thyroprotein treatment following by its death from symptoms of typical hyperthyroidism. For sheep, one gram of thiourea per 100 lbs. live weight is considered sufficient to produce thyroid hypertrophy (131) and animal 3 received over 3 times this amount of thiouracil for the last 7 days. It can be concluded therefore that the carotene metabolism of sheep in a

hypo- or hyper-thyroid condition does not differ detectably from the normal; if the conversion of carotene to vitamin A is influenced by the thyroid state, the effect does not appear to be on the enzyme system involved or on the stability of the provitamin. It is quite possible however that the absorption of carotene may be affected since Althausen and Stockholm (164) have shown that absorption processes are retarded in the hypothyroid state.

It was intended to repeat this work using more animals on a higher carotene intake (about 2 grams per day from pasture) and possibly a higher thiouracil dosage level. Before this could be done however a preliminary report appeared of work by Goodwin (160) of a similar type. Rabbits were used in this investigation and while on a carotene rich diet were fed 500 mg. thiouracil per day. (on a body weight basis this is about five times the level used for the sheep.) The results were the same as those obtained in this investigation. No carotene was detected in the plasma and vitamin A levels in these and similar animals treated with desiccated thyroid were normal. Goodwin concludes from his findings that the primary action of thiouracil must be on the absorption of carotene. In view of these findings the work with sheep was not repeated.

More recently Allen, Rice and Jacobson (161) fed iodinated casein to calves at a level of 1.5 gms/ 100 lbs. body weight but found little or no effect on the vitamin A and carotene plasma when the calves were receiving adequate carotene. At higher levels of thyroprotein, bodily health deteriorated before any

changes in plasma concentrations were noted. There was no evidence of increased conversion and at threshold levels of carotene intake the increased metabolic rate lowered the plasma levels. Thiouracil at the rate of 5 - 10 gms/100 lbs. gave slight increases in plasma vitamin A but of questionable significance. On the other hand, although no information as to dosage is available, Herrick, Andrews, Beeson and Harper (162) working with lambs, report that "thiourea and high levels of thiouracil appeared to interfere with the conversion of carotene to vitamin A".

.....

C H A P T E R VI

Thyroprotein and the In Vitro Conversion of Carotene

In view of the possible effect of the thyroid on carotene metabolism, claims by Russian workers to have effected the conversion of carotene to vitamin A using thyroid extracts and iodinated proteins are of considerable interest. In 1940 Balaba (165) reported the in vitro transformation using thyroglobulin, thyroid extract and fresh minced thyroids and later Koplansky and Balaba (166) found that iodinated casein was also effective. The activity of these substances was destroyed on boiling and their action was likened to that of the so called "carotinase". Koplansky and Balaba considered that in the animal body carotene is converted into vitamin A in the thyroid gland " as well as in the liver " and suggested that the activity of the enzyme system responsible for the transformation depended on the presence of the thyroxine grouping.

No attempt appeared to have been made by other workers to confirm these findings and since the evidence so far presented is against any direct action of the thyroid on the mechanism of conversion of carotene to vitamin A, the experiments of Koplansky and Balaba were repeated.

These workers incubated colloidal carotene with various concentrations of iodinated casein over a range of pH values. That vitamin A was formed under these conditions was concluded from the disappearance of carotene and the increase in apparent vitamin A as estimated by the Carr-Price reagent. The identity of the product was established by "the zone of absorption at 328 mu characterising vitamin A ". The activity of

the thyroprotein increased up to a concentration of 30 mg. per 100 ml when a condition of equilibrium was reached with 45 to 50% of the carotene transformed to vitamin A. The optimum pH is given as 7.3 although this appears to be merely the pH at which the recovery of carotene was lowest, no figure being given for vitamin A formed.

As already discussed the disappearance of carotene is in itself no indication of conversion to vitamin A and even although it has been established that, in similar experiments, some vitamin A is formed, it cannot be assumed that the difference between the carotene added and recovered after incubation represents the vitamin A formed. In the absence of experimental details it is difficult to assess the value of the Carr-Price estimation or of the spectroscopic characterisation of the vitamin A supposedly formed but on the evidence presented it is doubtful if the presence of the vitamin was conclusively established. In repeating the experiments therefore particular attention was paid to the identity of the product.

> Iodinated casein was prepared from skim milk using the method and quantities described by Kaplansky and Salabs. Combined iodine estimated by the method of Harrington (167) in a sample after dialysis was found to be 1.2%.
>
> Colloidal carotene was prepared as already described and iodinated casein dissolved in weak alkali added to give a concentration equivalent to 30 mg. per 100 ml colloidal carotene. The pH was adjusted to 7.3 with acetic acid and the solution incubated for two hours at 37°C.
>
> After incubation the unchanged carotene and the products were extracted into non-aqueous solvent by adding an equal volume of ethanol and shaking vigorously with two volumes of petroleum ether (water-ethanol-ether ratio of 1:1:2). The extraction of the colloidal solution was facilitated by the addition of about 5% sodium sulphate to the aqueous layer.
>
> Carotene was estimated by the absorption at 450 mμ and vitamin A spectroscopically

using the three point correction procedure and colorimetrically with the Carr-Price reagent.

The solution contained initially 385 ug carotene and of this 266 ug or approximately 70% as estimated from the absorption at 450 mu was recovered. No vitamin A could however be detected spectroscopically and there was no indication of a peak at 325 mu. During incubation there was a marked increase in the absorption in the shorter wave lengths with the development of a small peak at 335 mu characteristic of neo-β carotene, indicating decomposition and isomerisation of the carotene. The vitamin A content estimated colorimetrically was 22.5 ug (corrected for carotene present) but since decomposition had occurred little reliance could be placed on this figure. The absorption at 325 mu, also corrected for carotene only, was equivalent to a total apparent vitamin A content of about 38 ug.

The experiment was repeated a number of times using varying quantities of iodinated casein but with similar results.

The combined petroleum ether solutions from these experiments were evaporated to small volume and chromatographed on a column of 1:1 magnesium oxide - hyflo supercel. The column was developed with petroleum ether containing 5% benzene. After elution of the carotene no fluorescence could be detected on the column. Elution with petroleum ether saturated with ethanol gave a solution absorbing relatively strongly below 300 mu but giving only a general decreasing absorption above that wavelength with no peak in the 325 mu region.

It is apparent therefore that no appreciable amount of vitamin A was produced in these experiments. Decomposition and isomerisation however occurred, in some cases to a marked extent, and if conclusions were based entirely on the Carr-Price reaction and direct

spectroscopic estimations at 325 mu, it could be erroneously assumed that vitamin A was formed.

The possibility that this failure to detect conversion of carotene to vitamin A might be due to a low thyroxine content in the iodinated casein used could not be overlooked. The method described by Kaplansky and Nelaba gives a product of low combined iodine content and the experiments were therefore repeated using other preparations.

> Iodinated casein was prepared using the method described by Reineke and Turner (168). Conditions were chosen to give the highest possible thyroxine content. 20 mg of Mn_3O_4 was used as a catalyst and iodine was added equivalent to 6 atoms per mole of tyrosine (calculated from the protein content of the skim milk). Vigorous stirring of the solution was maintained.
>
> Two samples prepared by this method had combined iodine contents of 5.1 and 6.3%.

The incubation of colloidal carotene was repeated with these preparations but the results obtained were similar to those already described. The amount of decomposition which occurred during incubation varied but was considerably greater than in control samples incubated under similar conditions either without the addition of thyroprotein or with an equivalent amount of casein. After boiling the activity of the iodinated casein was reduced but the decomposition was still greater than with controls.

Kaplansky and Nelaba found the lowest carotene recoveries at pH 7.3. The effect of pH on carotene decomposition was therefore investigated.

> Iodinated casein dissolved in weak alkali was added to a series of carotene solutions. A phosphoric, acetic, boric acid buffer was added and the pH of the solutions adjusted to values from 4 to 11 with caustic soda. The solutions were incubated for 2 hours

and the products extracted as before.

Carotene decomposition was estimated by the ratio $E_{325\ m\mu}/E_{450\ m\mu}$ since this ratio can readily be converted to apparent vitamin A per 100 ug carotene.

The effect of pH on the decomposition of carotene measured in this way is shown in Figure 23 curve 1. These findings are in agreement with those of Kaplansky and Belaba. The results were not entirely reproducible, individual ratios varying by as much as 20% but the curves were of the same general shape and the points plotted represent the mean of a number of experiments.

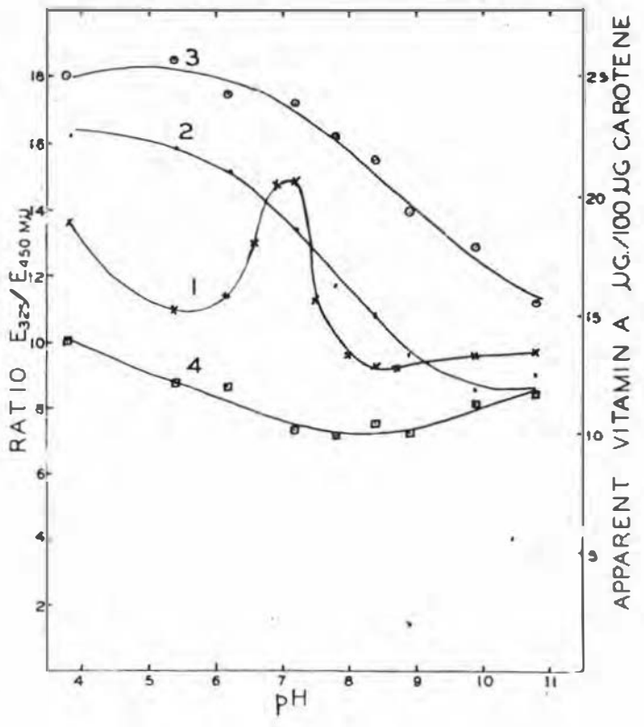


Fig.23 - Effect of pH on the decomposition of colloidal carotene solutions in the presence of iodinated casein (curve 1), iodine (curve 2) and hypoiodous acid (curve 3). Curve 4 shows the decomposition of the control solution.

. . . . . .

It seemed unlikely that the isomerisation and decomposition of the carotene would be caused by the thyroxine present in the protein. Obviously, only a part of the iodine in the iodinated casein is combined with tyrosine and other iodo compounds present might be responsible for the changes which occur on incubation. Carotene in petroleum ether solution is readily isomerised by iodine (20). Any free iodine in the thyroprotein would be removed by dialysis (169) but the possibility of loosely bound or adsorbed iodine reacting similarly on colloidal solutions was investigated.

> A series of solutions of colloidal carotene containing iodine equivalent to 5% of the carotene present were prepared and the pH adjusted to values from 4 to 11. The solutions were incubated for 2 hours at 37°C, iodine destroyed by thiosulphate and the products extracted into petroleum ether as before.

In iodinated proteins a portion of the combined iodine may be attached directly to nitrogen. If these N-iodo compounds exist there is a possibility of I^+ ions being formed and the effect of these ions on carotene was also investigated by incubating a colloidal solution with hypoiodous acid.

> Hypoiodous acid was prepared by shaking a solution of iodine with an excess of freshly precipitated mercuric oxid . After filtering the solution was added to colloidal carotene to give an iodine concentration again equivalent to about 5% of the carotene. pH values were adjusted as before and the solutions incubated for 2 hours.

The effect of iodine and hypoiodous acid on the decomposition of carotene at different pH values is shown in Figure 23, Curves 2 and 3 respectively. The effect of pH in the absence of other substances is shown in Curve 4.

It is apparent that although considerable decomposition does occur in the presence of iodine and

hypoiodous acid particularly at the lower pH values, the increased decomposition with iodinated casein at pH about 7 cannot be attributed to the presence of free iodine or I^- ions alone.

The work of Salaba (165) with minced thyroids was also repeated but again no vitamin A could be detected although as before the carotene decomposition was greater in the presence of the thyroid preparations than in the controls.

It has been confirmed therefore that, as reported by the Russian workers, there is a disappearance of carotene on incubation with thyroprotein and thyroid extracts but it is suggested that the carotene is isomerised and decomposed without the formation of vitamin A. In the experiments reported here, the decomposition of carotene and the formation of "apparent vitamin A" as estimated colorimetrically was considerably less than reported by Koplansky and Salaba.

From a preliminary report of work similar to that described above, it appears that Cama and Goodwin (170) have also been unable to demonstrate any conversion of carotene to vitamin A.

Although their original paper is not yet available, Di Bella, Osima and Bellini Camurri (171) have found that thyroxine does affect the stability of carotene, which would appear to support the conclusions reached in this investigation.

. . . . . .

C H A P T E R VII

The Availability of Carotene from Herbage

Interest in the carotene metabolism of herbivora was aroused through a number of observations suggesting the relative non-availability of carotene from herbage during certain periods of the year. Clinical observations have suggested conditions of vitamin A deficiency in cattle even when the provitamin content of the feed was considerably in excess of requirements. Thus during a particularly dry summer young stock on the Massey College farm developed symptoms which were ascribed to a vitamin A deficiency and which responded to oral and intramuscular administration of carotene. These animals were on a diet of hay and a limited amount of poor quality pasture giving a calculated carotene intake of 2 to 3 times the normal recommended level. These observations are however, unsupported by evidence of low hepatic stores and low plasma levels of vitamin A and their significance is further limited by the lack of adequate controls.

Strongest evidence in support of a reduced "effective" carotene intake during summer months is provided by the decrease in the carotene and vitamin A content of butterfat during this period.

The vitamin and provitamin content of New Zealand butterfat has been investigated by several workers (172, 173) and since, in contrast to conditions in Britain and America, the cows are pasture fed throughout the year, the potency of the butterfat is an indication of the amount of available carotene in the pasture. In all cases marked seasonal variations have been reported. The vitamin A potency is highest in late winter and spring (August to October) and

decreases gradually through the spring and early summer reaching a minimum value in late summer (February). The potency then increases again through the autumn. Figure 24, which is an idealised curve drawn from results obtained over a number of years by the Dairy Research Institute (173) shows the type of variation encountered.

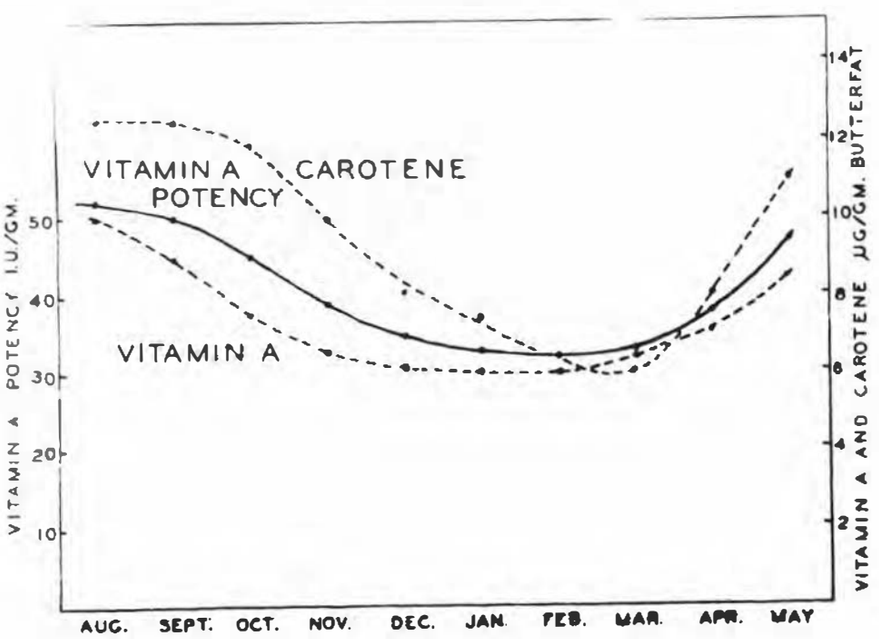


Fig.24 - Seasonal variation in the vitamin A potency of New Zealand butterfat.

. . . . . .

These variations are seasonal rather than lactational since no appreciable differences in vitamin A potency have been found (during the months of February and March) between the fat from "spring-calvers" and "autumn-calvers" (172).

A similar seasonal trend is apparent when total vitamin A secretion is considered. Figure 25 shows this variation in total vitamin A secretion calculated from the product of average monthly fat production (174) by average monthly vitamin A potency of the fat (173) for the corresponding district.

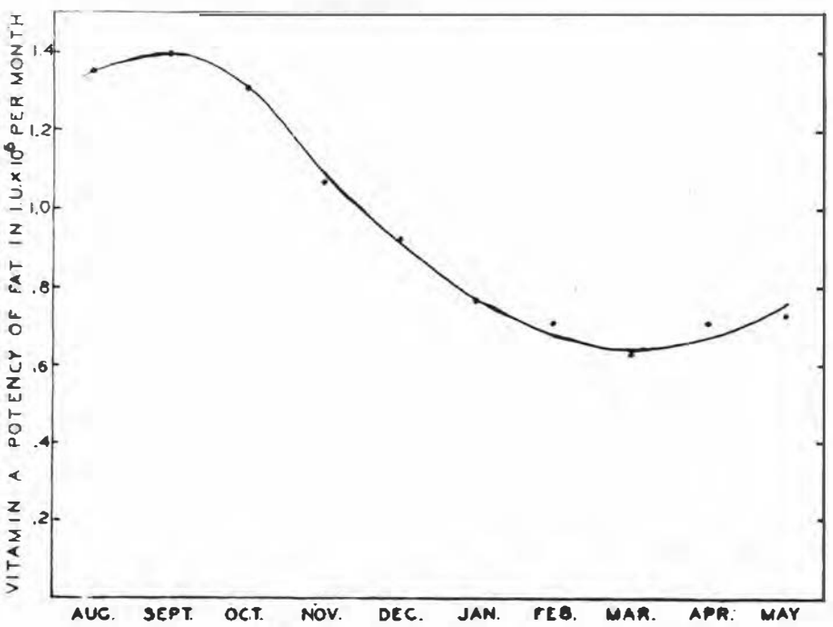


Fig.25 - Seasonal variations in total vitamin A secretion.

. . . . . .

It would appear therefore that there must be a decrease in the carotene absorption during the summer months.

Wilbur, Hilton and Hauge (175) have investigated the vitamin A requirements of dairy cattle for the production of fat of high vitamin A potency. They found that the vitamin A potency of the fat increased with provitamin intake until a maximum of 36 to 37 Sherman-Munsell units per gram was reached with a daily carotene intake of 300 mg regardless of the source. Taking the Sherman-Munsell unit as approximately 1.4 I.U. (176) this gives a maximum potency of 52 I.U. per gram which is about the highest level found in New Zealand butterfat. The minimum potency of New Zealand butterfat is about 30 I.U. per gram or 21 Sherman-Munsell units which would appear to correspond to a carotene intake of about 150 mg per day.

Assuming for pasture fed cows a daily intake of dry matter of 10 to 12 kilograms, the carotene con-

tent of New Zealand pasture must decrease from at least 25 to 30 ug/gm. dry matter in the late winter and spring to 12 to 15 ug/gm. in mid-summer.

No information appears to be available regarding variation in the carotene content of New Zealand pasture throughout the year but an investigation is at present being carried out in this Department (181). Results are not yet available for the summer period but figures for selected pastures from June to November indicate an average value of about 550 ug/gm. dry matter with a deviation of about 100 ug.

The carotene content of grasses has however been extensively investigated by British workers (e.g. 177, 178, 179). The results show that the carotene content decreases with maturity and for average grasses the levels fall from a maximum of about 600 ug/gm. during the spring flush to about 150 ug/gm. in mid-summer. Clovers decrease from about 450 ug/gm. to 300 ug/gm. over the same period. This carotene content is significantly correlated with the protein or non-protein-nitrogen (180). From the British figures, and assuming as Cawley's preliminary figures (181) indicate, that a similar correlation exists between carotene and protein, it seems unlikely that the provitamin content of average New Zealand pasture would fall below the British minimum of 150 ug/gm. This is ten times the figure expected from the vitamin A potency of mid-summer butterfat, and it appears therefore that as compared with the carotene from the various sources used as Wilbur, Hilton and Hauge (175), the bulk of the provitamin from summer pasture is not utilised.

It was thought that this non-availability of

the carotene might be associated with the high fibre or low protein content of summer pasture and experiments were planned to investigate the effects of various levels of fibre (and lignin) and protein in the diet on the utilisation of carotene by sheep. Carotene absorption is dependent on adequate fat in the diet and although there appears to be little seasonal variation in the lipid content of grasses (182), feeds were also planned to give a range of fat contents. Carotene-free basal diets were planned and it was proposed to estimate the apparent digestibility (i.e. provitamin absorbed or decomposed in the alimentary tract) of carotene supplements added either in the form of concentrates in oil or as carotene-rich dried grass.

Difficulties were experienced with palatability of the feeds and during the pre-feeding period, preliminary digestibility experiments were carried out with other sheep fed on lucerne hay. Carotene was determined in the feed and faeces using the method described by Moore (159). It was found however, that the carotene excreted ranged from approximately 80 to 160% of the quantity ingested. These anomalous results were attributed to errors in the determination of carotene in the faeces and led to the investigation of methods of carotene assay described in Chapter VIII. It has not been possible to complete the investigation into the effect of the composition of the feed on the utilisation of carotene.

Working with rats, Frape (183) found that an increase in the fibre content of the diet reduced the apparent absorption of carotene. The basal diet contained 59% starch and replacing half of this with cotton-seed hulls, increased the bulk of the faeces by 10 times and reduced the apparent percentage

digestibility of a daily 60 ug dose of carotene from 80 to 40%. Replacing half the starch with agar, gave equally bulky faeces but the absorption of carotene was unaffected. Similarly, varying the protein content of the feed from 18 to 36% had no effect on the absorption of carotene.

A further factor which might affect the availability of the provitamin is the tocopherol content of the pasture since an adequate level of vitamin E is essential for efficient utilisation of carotene. A low tocopherol content would affect both the absorption of the carotene and the stability of the vitamin A and carotene in the blood plasma and tissues.

Harris, Swanson and Hickman (184) found that the feeding of tocopherol at a level of 1 gram per day did not increase the vitamin A potency of the milk fat although it did increase the percentage of fat and the total output of fat. It is possible however, that the basal diet already contained sufficient tocopherol for maximum utilisation of the carotene. When vitamin A was fed to the cows the carotene content of the milk fat decreased by 33% but the feeding of vitamin A and tocopherol caused a decrease in carotene content of only 23%. These observations, which have been confirmed by other workers, (185) suggest that the carotene absorption or stability in the tissues was decreased in the presence of vitamin A but that these effects were offset to some extent by the tocopherol.

Experiments have therefore been commenced to investigate any seasonal changes in the tocopherol content of pasture. The plot selected is predominantly ryegrass and is cut at regular intervals. The samples have been assayed for total tocopherol content using

the method of Wall and Kelley (186). Only two representative samples have so far been assayed. The total tocopherol content of a sample collected in mid-September was 0.23 mg/gm. dry matter and of the second sample collected at the end of November, 0.19 mg/gm. Estimations were carried out using the Beckman spectrophotometer, the instrument being standardised against synthetic alpha tocopherol[1].

The tocopherol assays will be continued at more regular intervals over the summer period and if any significant differences are found between spring and summer pasture, tocopherol will be fed in mid-summer to a group of cows to bring their vitamin E intake up to the spring level, and the vitamin A potency of the butterfat from these animals compared with that of untreated controls.

Footnote 1 - Supplied by the Eastman Kodak Co.

. . . . . .

CHAPTER VIII

The estimation of Carotene in Plant Material and Faeces

The apparent excretion of 80 to 160% of ingested carotene by sheep mentioned in the previous chapter led to the conclusion that a pigment other than carotene was being estimated in the faeces or possibly that the method used was giving incomplete extraction of the carotene in the feed. Published methods for the estimation of carotene were therefore investigated with a view to overcoming this difficulty and determining the most suitable method for use with the particular materials being assayed. Due possibly to the wide range of materials in which it is necessary to estimate carotene, a large number of methods for its assay have been described over the last few years. Although these methods vary widely, most are still in use in different laboratories resulting in conflicting results due in many cases to the extension of methods to materials other than those for which they were designed.

Although the actual techniques differ the methods for extracting the pigments fall, in general, into two main groups; those requiring a preliminary saponification and those in which this step is omitted or follows extraction of the pigments with organic solvents. Once in the solution, the carotene is separated from the other pigments either by partition between immiscible liquids or by adsorption on solids and estimated colorometrically or spectrophotometrically.

Complete chromatographic analysis involving separation of the various carotene isomers is not practical for routine analysis nor is it of great value in assessing vitamin A potency in view of the recog-

nised variation in the absorption of carotenes from various sources and its dependence on the presence of anti-oxidants and other undetermined factors (187 - 193). Only methods of estimating "total carotenes" consisting of α and β carotene and traces of γ and possibly various neo carotenes need therefore be considered.

(a) Methods of Extraction

Heating the material with alkali is an initial step in most of the earlier methods and is still followed by many workers as a convenient method of disrupting the tissues and allowing the solvent to come into more intimate contact with the pigments. Saponification methods have been reported using both aqueous (194, 195) and more recently alcoholic (196, 197) potash, the material being heated or refluxed for periods of from half to two hours with potassium hydroxide of varying concentrations. The extracted pigments are then transferred to petroleum ether either directly by shaking the alcoholic solution repeatedly with petroleum ether (196, 197) or via other extractants such as ethyl ether (195). While saponification is necessary at some stage if physic separation is to be followed, it has largely given place to direct solvent extraction.

A range of solvents and several different procedures have been suggested for this direct extraction. Willstatter and Stoll (198) in their classical method used acetone. The same solvent has been used with modification by subsequent workers under different conditions, e.g. by slow maceration with the cold solvent (199, 200), or more rapidly by grinding the material with the solvent (201) or by the use of the Blendor. Zscheer (202) and Wall and Kelley (203) used

hot extraction with a mixture of acetone and petroleum ether and a Soxhlet apparatus with 30% acetone in petroleum ether has been recommended both in the tentative A.O.A.C. method (204) and in the Derby and Devitt (205) modification of this method.

Other solvents employed include methanol and petroleum ether mixtures (206), di-acetonol both hot, followed by petroleum ether (207) and cold in a Blendor (208), n-butanol (209), methanol (210), methanol and ethyl ether (211), pyridine (212), hot ethanol alone (214) or followed by petroleum ether (215) and ethanol and petroleum ether mixtures (203, 216, 217).

(b) Separation of Carotene from other Pigments

Whatever the method of extraction the carotene is normally accompanied by other pigments, mainly chlorophyll, xanthophylls and xanthophyll esters, and although attempts have been made (218) to estimate the carotenes in the presence of other pigments by measurements at a number of wavelengths, it is general to separate the carotenes from the other constituents. Borodin (219) first reported that carotenoid pigments could be separated into alcohol-soluble and ether-soluble fractions and from these observations Willstatter and Stoll (198) developed their method for removing non-carotene pigments. This original method in which a solution of the pigments in petroleum ether is shaken repeatedly with aqueous methanol which removed the other pigments leaving the carotenes in the ether, has been only slightly modified by subsequent workers and is still widely used. Hegsted, Porter and Peterson (215) used aqueous di-acetonol in place of methanol while orthophosphoric acid (220) and β-methyl pentane -/>

δ-diol (221) have also been suggested for special purposes.

Alternatively to phasic separation, the interfering pigments may be removed by adsorption on solids which either do not adsorb the carotenes or from which they may readily be eluted. As examples of these techniques, several methods involving grinding or triturating with calcium oxide or hydroxide (222, 223, 224) or treatment with barium hydroxide (201) have been reported and Frape and his co-workers (225) have developed relatively simple methods in which a petroleum ether solution of the pigments is shaken with specially prepared adsorbants which remove all non-carotene pigments. Most workers have however effected the separation on chromatographic columns packed with various adsorbants.

The more commonly used of these adsorbants include soda ash (226), Micron Brand Magnesium Oxide mixed with soda ash or Johns - Manville Hyflo Super-Cel (200, 203) heat treated siliceous earths such as Hyflo Super-Cel, Celite 501, 535 or 545 (227), alumina (228) di-calcium phosphate (159, 229) and tri-calcium phosphate or defatted bone meal (213).

Discussion of Methods

Published methods, of which those quoted serve as examples, were considered with a view to obtaining a rapid but accurate method of carotene assay applicable, if possible, to all the materials likely to be handled in this investigation, viz. fresh grass, dried materials such as hay, concentrates and dehydrated grasses, and faeces. Dried materials and faeces present special difficulties due to the possible presence of oxidation products of the carotenoids which

may give rise during extraction to artifacts absorbing in the region of the carotene maximum.

As has been pointed out by Booth (228) errors in carotene estimations up to the colorimetric stage may arise in the following ways :

(a) Losses before extraction begins
(b) Destruction during and after extraction
(c) Incomplete extraction
(d) Manipulative losses
(e) Incomplete removal of other pigments, and
(f) Chromogenesis and isomerisation.

Losses from certain of these causes will be negligible provided reasonable precautions are taken. Thus losses due to (a) are minimised by avoiding damage to fresh tissues, storage at low temperatures and addition of enzyme inhibitors such as cyanide during grinding, and general precautions such as exclusion of light as outlinedin chapter I, will reduce losses due to (b). However many of the methods reviewed contain inherent defects which will give rise to unavoidable errors of the types listed. For example, losses due to (d) can be reduced by careful technique but are inevitable in methods involving steps such as repeated extractions, washings and transference of carotene solutions. Carotene may be readily isomerised or oxidised by heat (208, 230) so that methods involving hot solvents or concentration by heat of carotene solutions are liable to increase errors due to (e) and (f). Zechmeister (20) has reported 10. isomerism of carotene on refluxing a solution in petroleum ether for 15 - 60 minutes.

It seems reasonable to assume that methods involving saponification with hot alkali may introduce the same errors and if oxidation products of the carotenoids are present (as in dried materials and faeces), epiphasic pigments may be produced (228).

Treatment with hot alkali may also give rise to errors due to (b) and (c) since several workers have reported destruction of the carotene by the hot alkali (228, 231) and Lease and Mitchell (232) and Frape and others (233) have found that the alkali isomerises the carbohydrate in certain materials to form a resinous film which renders the carotene non-extractable by organic solvents.

Distribution between two immiscible solvents as a method of removing interfering pigments may in certain cases give rise to serious errors due to (e) and phasic separation has largely been replaced by adsorption methods. Although Miller (234) has reported the removal of appreciable amounts of carotene in the hydrophase, phasic separation methods appear to give a satisfactory separation of carotene from xanthophylls provided the epiphase is washed until the hydrophase is colourless and the hydrophase contains sufficient water to render the carotene relatively insoluble in it (at least 8% by volume in the case of methanol). Materials containing epiphasic xanthophyll esters must however be saponified prior to phasic separation. In addition to the disadvantages of saponification already discussed, more serious errors may be introduced when it is applied to dried or stored materials since treatment of these with alkali may result in the formation from carotenoid oxidation products of a yellow artifact which is not removed by aqueous methanol (228) and is therefore estimated as carotene.

Other workers have shown that phasic separation is not applicable to dried materials or faeces due to these epiphasic pigments present in the materials or produced during saponification. Thus Kamasarma and Nakin (235) and Frape, Kemmerer and Greenberg (225) found a yellow epiphasic pigment in the faeces of rats

fed a carotene-free diet; Whitnah and others (236) found with cows an apparent excretion of over 100% of ingested carotene as estimated by distribution between petroleum ether and aqueous methanol; Cusckenbush and others (237) found that similar pigments developed in A.I.V. silage but Hegsted and others (215) were able to remove these by replacing the methanol with di-acetonol; and Hartman and others (238) noted the differences between biological values and chemical values (obtained by phasic separation methods) for the potency of lucerne hay, and subsequent workers (239) showed the presence of 11 - 32% of non-carotene pigments in the petroleum ether layer.

Concerning the various adsorbents for chromatographic separation, opinion is fairly evenly divided regarding the relative merits of "straight through" adsorbents, i.e. those which adsorb only non-carotene pigments allowing the carotene to pass through with the solvent, and other stronger adsorbants which remove all pigments from the petroleum ether solution and from which the carotene must be eluted with a more polar solvent. The weaker adsorbents do however appear to possess some advantages for routine work in that most of the carotene passes through with the original solvent and the column only requires rinsing with a small quantity of the same solvent resulting in little dilution. The same advantage, viz. avoidance of dilution, has been claimed for the stronger adsorbants since irrespective of the volume of the original pigment solution, the carotene is obtained in a small and definite volume of elutant. However this is an apparent advantage only, since the volume of the

the pigment solution obtained from most methods of extraction is considerably less than the volume of polar solvent required to completely elute the carotene. For routine work the adsorbant should be sufficiently porous to allow a rapid rate of flow; it should be easily packed into the columns and the packing should not be affected, e.g. should not channel or contract from the walls, when drawn dry of solvent; the columns should not require protection from atmospheric moisture and carbon dioxide; and the adsorbant should be readily obtainable, consistent and usable with little or no special activation, if possible "straight from the bottle".

Selection of Methods

From a consideration of the available methods for plant materials and the criticisms of those, it appeared that the cold solvent methods such as those described by Zscheile and Kelley (203) and Moore and Ely (216) for extracting the pigments followed by separation on a column of Hyflo Super-Cel as suggested by Wilkes (227) were most readily applicable to the range of samples to be handled and least subject to the foregoing sources of error.

However the Association of Vitamin Chemists in a recent publication (3) recommends, in addition to the method of Moore and Ely, a saponification method and the Soxhlet extraction method also described by Zscheile and Kelley, (203) and during the course of the present investigation, Derby and Hewitt (205) reported a comparison of the Zscheile and Kelley cold extraction method, their modified A.O.A.C. method and a method involving digestion with alcoholic potash. Although the Association of Vitamin Chemists points out the possibility of isomerisation of carotene in the Soxhlet

method, Derby and DeWitt obtained the highest results with their modified .O.A.C. method which also involves the use of hot solvents. Methods of extraction, using cold solvents in the Waring Blendor, were therefore compared with the A.O.A.C. method as modified by Derby and DeWitt. Although alkaline digestion has been so strongly criticised it does offer a convenient method and it seemed possible that the errors attributed to it might be due to the somewhat drastic conditions usually employed, both as regards duration of heating and strength of alkali. Bathurst (240) has found the method satisfactory provided the time of heating does not exceed about ten minutes and a digestion method using mild conditions was therefore investigated.

Methods

Representative samples of the types of material to be investigated were assayed by the following methods :

Method 1

The .O.A.C. method (204) as modified by Derby and DeWitt (205) except that the extraction was carried out in a Soxhlet apparatus in place of the Bailey-Walker extractor which was not available.

Method 2

The method of extraction in a Blendor using a "foaming mixture" of petroleum ether and ethanol as described by Moore and Ely (216).

Method 3

In this method, which is an adaption of the previous one, and in method 4, two modifications to standard procedures were introduced :

(1) During the initial extractions definite volumes of solvents were used and precautions were taken to prevent loss by evaporation. Aliquots of the solutions were then taken, so avoiding the necessity for repeated washings or re-extractions of the marc.

(2) Repeated extractions of the alcoholic solution of the pigments with petroleum ether to remove all the carotene were avoided by adding water equal to the volume of ethanol present and extracting with twice this volume of petroleum ether. It has already been shown (Chapter I) that under these conditions the one extraction with ether removes all the carotene and that the two layers separate sharply with no volume change. Preliminary experiments supported by the carotene recovery figures cited below show that other materials present do not affect the distribution and the method is equally applicable to plant extracts. More of the non-carotene pigments may be removed by a higher concentration of alcohol in the hydrophase but with concentrations greater than about 60% it was found that some carotene was retained and re-extraction with further petroleum ether was necessary. Further 50% alcohol gives the sharpest separation of layers without volume changes.

Up to 10 gms. of the material, depending on the carotene content, was comminuted in a sealed jar on the Waring Blendor for five minutes with 100 - 200 ml. of a foaming mixture consisting of a 25 - 35% solution of petroleum ether in 95% ethanol. Dried samples were first moistened with a small quantity of water as the foaming mixture does not completely extract dry materials. After comminution the residue was allowed to settle, an aliquot of the supernatant liquid withdrawn and water and petroleum ether added to give approximately equal volumes of ether and 50% ethanol.

The ether/alcohol ratio required to give a foaming mixture depends on the moisture content of the sample. Since an aliquot of the solution is to be taken later the estimation is simplified if there is a definite volume of water present. For most materials it was found convenient to add to the weighed sample in the Blendor jar, sufficient water to bring the total amount of water present (including the moisture originally in the sample) to 5 ml. This moist mass was then extracted with 135 ml of a foaming mixture containing 26% petroleum ether (100 ml ethanol and 35 ml ether). In this case the total volume of extractant is 140 ml and 40 ml forms a convenient aliquot containing 10 ml ether, 28.6 ml ethanol and 1.4 ml water. To give the correct ratios of ether, alcohol and water, this requires the addition of a further 47.2 ml petroleum ether and 27.2 ml water. There is however some latitude in the ratios and it is more convenient to add 50 ml ether and about 25 ml water, the carotene in the original sample being now contained in the equivalent of 210 ml ether.

Where the moisture content of the sample itself exceeds 5 gms., the 100 ml. 95% ethanol is replaced by 95 ml. absolute alcohol and the moisture content adjusted to 10 ml. by the addition of water to give the same total volume of 140 ml.

After addition of the ether and water, the two phases were shaken vigorously and after standing for a few seconds the bottom layer was discarded. Prior to chromatography a portion of the petroleum ether solution was washed twice by shaking gently with several times its own volume of water to remove traces of ethanol.

Method 4

Up to 5 gms. of the sample were digested by refluxing for 10 minutes with 50 ml. ethanol and 5 ml. 50% potassium hydroxide solution. (This avoids using alcoholic potash which decomposes on standing) The solution was cooled rapidly and poured through a pad of glass wool. To an aliquot consisting of half the total volume (i.e. 27½ ml. plus half the volume of water in the sample), 50 ml. of petroleum ether was added followed by 20 ml. water. Using these volumes the carotene present in the sample is contained in 100 ml. petroleum ether and a portion of this solution was washed with water as in Method 3.

The washed petroleum ether solutions of the pigments obtained by these four methods were dried over anhydrous sodium sulphate. In each case 20 to 30 ml. aliquots of these solutions were chromatographed on lightly packed Hyflo Super-Cel columns using the apparatus illustrated in Figure 26. Petroleum ether was then drawn through the column until colourless washings were obtained. With the particular columns in use about 20 ml. was adequate for complete removal of carotene. With some samples a yellow artifact moved down the columns fairly rapidly and in these cases it was necessary to elute all the pigments after each determination. This was most conveniently carried out by running through 20 - 30 ml ethyl ether. After washing with an equal volume of petroleum ether and drawing dry of solvent, the column was again ready for use.

To check the completeness of separation of other pigments from carotene on the Hyflo Super-Cel column, this adsorbent was compared with other more commonly employed materials. Petroleum ether solutions of the pigments obtained by method 3 were run through columns of di-calcium phosphate, bone meal

and a 3:1 mixture of Hyflo Super-Cel and magnesium oxide prepared and used as described by Moore (241), Zerm (213) and Hall and Kelley (203) respectively. Phasic separation was also compared with the Hyflo column. Aliquots of the ether solutions obtained by method 4 were repeatedly shaken with equal volumes of 90% methanol (90 ml. methanol plus 10 ml. water), until the hydrophane was colourless.

In all cases carotene was estimated in petroleum ether solution using the Beckman Spectrophotometer.

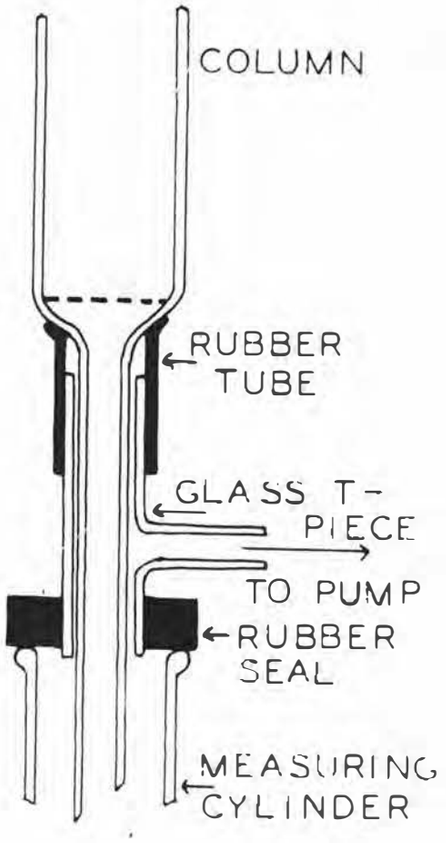


Fig.26 - Apparatus for chromatographic separation of carotene.

. . . . . .

Results

The carotene content of a number of samples

as determined by these four methods is shown in Table 10. The results represent the average of at least three determinations.

TABLE 10

Carotene contents of various samples expressed in ug/gm dry matter.

Material	Method 1	2	3	4
Fresh grass	-	718	708	682
Dried grass 1	-	-	352	334
" " 2	356	340	345	322
Faeces (Sheep) 1	-	42.4	41.5	43.2
" " 2	-	-	22.0	21.5
Hay 1	23.4	23.1	23.3	22.8
" 2	15.9	15.2	15.7	14.4
" 3	18.8	-	19.1	19.0
" 4	-	-	14.8	14.4
Ratios %	100	99	99	96

Carotene losses in methods 3 and 4 were checked by extracting samples with solvents containing known amounts of carotene. Recoveries are listed in Tables 11 and 12.

TABLE 11

Carotene recoveries using method 4.

Sample	Carotene in sample	Carotene added	Total Carotene	Carotene Found	% Recovery
	ug	ug	ug	ug	
Hay 4	72.0	22.4	94.4	91.2	96.5
" 4	72.0	44.8	116.8	116.0	99
" 4	72.0	112.0	184	177	96
" 4	72.0	224	296	280	95
Dried Grass 1	334	224	558	525	94
" " 1	334	112	446	436	98
				Average -	96.5

TABLE 12

Carotene recoveries using Method 3

Sample	Carotene in sample	Carotene added	Total Carotene	Carotene found	% Recovery
	ug	ug	ug	ug	
Dried Grass 1	352	97	449	438	98
" " 1	352	194	546	538	98
" " 1	352	291	643	648	100
" " 1	352	388	740	728	98
Hay 4	74.0	18.2	92.2	93.1	101
" 4	74.0	36.4	110.4	108.8	98.5
" 4	74.0	91.0	165	169	102
-	-	194	194	192	99
-	-	291	291	290	100
-	-	388	388	391	101
			Average		99.5

TABLE 13

Reproducibility of results using Method 3

Assay Number	Hay 3 (ug.	Fresh Grass er gram dry matter)	Dried Grass
1	18.8	720	348
2	18.9	682	363
3	19.5	738	355
4	19.0	693	342
5	19.4	690	345
6	19.2	711	361
Average	19.1 ± 2%	706 ± 5%*	352 ± 3%

* Larger deviation probably due to sampling errors.

Table 10 shows that contrary to the findings of Derby and Dewitt (205) results obtained by the four methods agreed closely. Method 4 does tend, however, to give slightly lower and less reproducible results and the low recoveries by this method (Table 11) indicate destruction of carotene by the hot

alkali in the presence of other plant material although previous work (Chapter I) has shown that carotene alone is not destroyed to any appreciable extent by similar treatment.

Apart from the possible sources of error mentioned already, method 1 is not applicable to fresh materials or faeces without preliminary drying and since it does not appear to extract carotene any more completely than the other methods, it was not investigated further.

Method 3 gives good recoveries of added carotene (Table 12) and consistent results (Table 13) and was therefore used for all subsequent routine determinations.

A further check on the completeness of extraction of carotene by this method was obtained by re-extracting the residues from a number of extractions. These combined residues were washed on a sintered glass filter with "foaming mixture" until the filtrate was colourless and re-extracted both on the blender with fresh foaming mixture and in a Soxhlet apparatus by method 1. In many cases any further carotene was too small to be measured and in no case did it exceed 2% of the amount estimated on the first extraction. Typical examples are shown in Table 14.

TABLE 14.

Completeness of extraction by method 3.

Material	Carotene by first extraction	Re-extracted by Method 3		Re-extracted by Method 1	
	ug/gm.	ug/gm.	%	ug/gm.	%
Exp 3	19.1	0.22	1.5	0.18	1.0
" 4	14.8	0.1	1	0.12	1.0
Dried Grass 1	352	15	0.5	10	0.5
Faeces 1	41.5	0.59	1.5	-	-

Although the 2% foaming mixture was found to be satisfactory for most samples, it may be necessary to vary its composition in certain cases. The effect of such variations was investigated by extracting a sample of hay (estimated without addition of water) with different ethanol-petroleum ether mixtures. The results in Table 15 indicate that over a wide concentration range the efficiency of extraction is unaltered.

TABLE 15

Effect of varying the concentration of the foaming mixture. 5 gm. Hay 3 extracted dry with 150 ml. foaming mixture of various concentrations.

Concentration of petroleum ether in mixture	Carotene found ug/gm.
25	18.7
30	19.2
35	18.8
40	18.7
45	19.1
50	18.8
	Average 18.9

As has been reported by a number of workers, the adsorbants tested gave almost identical results with quantitative recoveries of carotene. The rate of flow is however most rapid with Hyflo Super-Cel and this adsorbant appears to be one of the few which fulfil the requirements already listed being simple to use and less variable than the other materials.

Phasic separation is compared with adsorption on a column of Hyflo Super-Cel in Table 16. It is apparent that as was expected, much of the material under consideration contains epiphasic non-carotenoid pigments which invalidate the results obtained from

phasic separation and this relatively simple method was not therefore applicable. For fresh samples however it is probably as accurate as adsorption methods. When distributed between petroleum ether and 90% methanol the non-carotene pigments normally encountered were found to have a distribution coefficient of about 9 to 1 in favour of the hydrophase. To avoid repeated washings it is necessary to use a large volume of methanol and it was found that if the ether layer is washed with an equal volume of 90% methanol saturated with petroleum ether, no volume changes occur and no carotene passes into the hydrophase. Two such washings reduce the non-carotene pigment in the epiphase to about 1% of the original level.

TABLE 16

Separation of carotene from other pigments by distribution between immiscible solvents compared with chromatographic separation on a column of Hyflo Super-Cel.

Material	Apparent carotene in ug/gm as estimated by -		
	Phasic Separation	Hyflo Super-Cel	Phasic Separation followed by Hyflo Super-Cel
Hay 2	15.8	14.4	14.4
" 3	19.6	19.0	19.1
Dried Grass 2	328	322	330
Faeces 1	47.5	43.2	42.8
Fresh Grass 1	675	683	672
" " 2	485	478	482
" " 3	380	385	380

. . . . . .

C H A P T E R IX

The Apparent Synthesis of Carotene in the Intestine

In the proposed investigation into the effect of the composition of the feed on carotene utilisation described in Chapter VII, it was intended to estimate carotene absorption by determining its apparent digestibility. If however, appreciable decomposition of the carotene occurs in the digestive tract, the actual absorption may bear no relationship to apparent digestibility. It is of interest therefore to know what changes in carotene content, relative to an inert reference substance, occur in the digestive tract, and these changes were investigated in a number of pasture-fed sheep.

Lignin was considered to be the most satisfactory reference substance for experiments of this type. Chromium oxide had been used previously for determining the apparent digestibility of carotene but did not appear to mix uniformly with the contents, at least in the upper portions of the digestive tract. This apparent stratification has been referred to by Barnicoat (129). The method of Ellis, Matrone and Maynard (242) appeared to be the most satisfactory for lignin in herbage. It involves the following steps - 1. Fat extraction of the finely ground material with an ethanol-benzene mixture; 2. Incubation with pepsin and N/10 hydrochloric acid overnight; 3 Refluxing with 5% sulphuric acid for 1 hour; 4. Incubation with 72% sulphuric acid for 2 hours at 20°C; 5. Refluxing with 5% sulphuric acid for 2 hours; 6. Determination of lignin by loss of weight on ignition at 600°C.

The lignin estimated in this way appears to be completely non-digestible and the method has been

applied to digestibility trial work where results from lignin-ratio determinations agree closely with those obtained using the conventional "bag" method (242).

> Samples of the ingesta were collected from the four stomachs, from various points along the small intestine and from the caecum, colon and rectum of a pasture-fed sheep immediately following slaughter. Quantities equivalent to 1 to 2 grams of dry matter were ground thoroughly with a small quantity of acid-washed sand. The carotene content of these samples was determined using the petroleum ether-ethanol extraction procedure and the extracted pigments chromatographed on a column of Hyflo-Super-Cel as described in the previous chapter.
>
> The residue from the carotene estimations was used for the lignin determinations to reduce sampling errors. Since lignin methods are somewhat empirical, particular attention was paid to standardising the conditions of the assays and the experimental details given by Ellis, Matrone and Maynard (242) were strictly adhered to.
>
> A sample of freshly voided faeces and a sample, as representative as possible of the pasture which the animal had been grazing, were assayed for lignin and carotene.

The carotene to lignin ratios in mg. per gram were calculated for the various samples and are shown in Figure 27.

The reproducibility of the ratios was investigated by estimating carotene and lignin in a well-mixed sample of dried grass. Six determinations were made. The carotene content of the sample was 335 ($\pm$15) ug/gm. and the lignin content 4.8 ($\pm$ 0.20) % giving a carotene to lignin ratio of 7.4 mg/gm. with a deviation of $\pm$ 0.4 mg/gm. or 5.5%. Differences of the same order were found between three determinations on the freshly voided faeces and duplicate determinations on ingesta samples. The average carotene to lignin ratio in the faeces was 22.5 mg/gm., the individual ratios being 21.8, 23.7 and 22.0 mg/gm. Difficulty was experienced in obtaining representative

grass samples, the ratios for typical samples collected being 24.9, 18.0 and 20.7 mg/gm. giving an average of 21.2 mg/gm. This deviation of over 15% must be attributed to sampling errors.

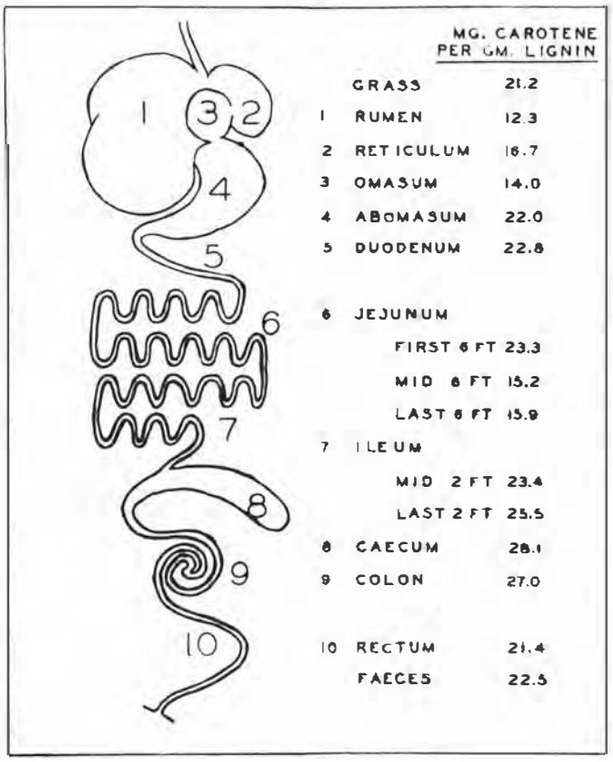


Fig.27 - Changes in carotene to lignin ratios through the digestive tract of a pasture-fed sheep.

From the results shown in Figure 27 the carotene ratio appears to decrease to a minimum in the jejunum and then increases reaching a maximum of 28.1 mg/gm. in the caecum followed by a small decrease through the colon and rectum. The ratios vary considerably through the four stomachs due possibly to the retention of more fibrous materials of low carotene content in the rumen and omasum. There is good agreement between the ratios in the

abomasum and in the grass.

The experiment was repeated with three other sheep, samples in one case being taken at more frequent intervals. The results (Table 17 and 18) show the same general trend.

TABLE 17

Carotene to Lignin ratios (expressed as mg/gm.) in a pasture-fed sheep.

Grass	16.7	Small Intestine 10th 6 ft.	22.2
Abomasum	14.9	11th 6 "	18.8
Duodenum	15.5	12th 6 "	17.6
Small intestine 1st 6 feet	11.7	Caecum	22.2
2nd 6 "	9.0	Colon 1st 2 ft.	21.8
3rd 6 "	9.9	2nd 2 "	21.0
4th 6 "	12.0	3rd 2 "	20.1
5th 6 "	13.9	4th 2 "	20.4
6th 6 "	13.4	5th 2 "	18.7
7th 6 "	16.0	6th 2 "	18.9
8th 6 "	13.8	Faeces	17.5
9th 6 "	16.6		

TABLE 18

Carotene to lignin ratios (expressed as mg/gm.) in two pasture-fed sheep.

	Sheep 1	Sheep 2
Grass	16.7	16.7
Abomasum	16.5	18.2
Mid jejunum	13.7	15.6
Caecum	17.3	20.0
Faeces	21.6	18.1

. . . . . .

The decrease through the upper portion of the small intestine could be attributed to absorption of the carotene and the decrease through the colon and rectum to oxidative decomposition, but the increase in carotene to lignin ratios in the ileum and caecum could only be explained by 1. a partial digestibility of the lignin or 2. the formation in the intestine of a pigment not separated from carotene on the chromatographic column or 3. a synthesis of carotene in the intestine.

Lignin was determined in samples of feed and faeces (from one sheep) which were available from a recently conducted digestibility trial and its recovery found to be 84.5%. This is lower than the figures reported for lignin recoveries by other workers (242) but this small apparent digestibility of lignin is insufficient to explain the increased carotene ratios.

A digestibility trial with pasture-fed sheep provided an opportunity for determining the recovery of lignin as estimated in grass by the Ellis, Matrone and Maynard method. At the same time, the excretion of carotene by sheep was further investigated by estimating carotene to lignin ratios in dung samples collected twice daily.

The pasture was relatively uniform and the feed intake was calculated by the usual method of cutting a representative portion of the pasture, weighing the yield and allowing the animals to graze the remainder to the same level. This digestibility trial was one of a number carried out at intervals by the Grasslands Division of the Department of

Scientific and Industrial Research. It is considered by Officers of the Division that the method used gives a reliable estimate of the total yield and hence the feed intake of the animals. The difficulties previously experienced with sampling were largely overcome by assaying a portion of the cut grass.

> Freshly voided dung samples (about 25 gm.) were collected twice daily from four pasture-fed sheep. These, together with representative grass samples, were assayed for lignin and carotene using the methods already described.

The average ratio of carotene to lignin in the pasture was 18.5 mg/gm and the ratios in the faeces are set out in Table 19.

TABLE 19

Carotene excretion expressed in milligrams carotene per gram lignin.

Day	Sheep 1	Sheep 2	Sheep 3	Sheep 4
1st day a.m.	15.7	22.4	17.0	16.0
p.m.	13.4	19.8	18.6	18.8
2nd day a.m.	12.2	21.8	18.2	21.1
p.m.	15.4	21.5	17.9	17.9
3rd day a.m.	15.4	20.3	16.5	16.3
Average	14.4	21.2	17.6	18.0
% Carotene excreted	78.0	114.5	95.2	97.4

. . . . . .

It appears that the carotene excretion is fairly uniform for each animal but varies somewhat between animals. It was realised later that this might be due to selective grazing. The differences in carotene content along leaf blades and the changes with maturity were estimated for a number of samples of rye-grass. From the results shown in Table 20 it is apparent that selective grazing could be one explanation for the differences in carotene excretion.

TABLE 20

Carotene content of rye-grass.

Sample	ug/gm fresh weight
1. Very young shoots	99
2. Immature grass	80
3. Young grass 2½ - 3"	125
4. Mature grass 8 - 9"	115
5. Top 3" mature grass blades (4)	200
6. 2nd 3" " " " (4)	120
7. 3rd 2 - 3" mature grass blades (4)	68
8. Mixed representative sample	124

The average lignin recovery was 96.0% indicating almost complete non-digestibility. The average carotene excretion calculated by the lignin ratio method was 96.3%.

That the increase in the carotene ratios in the caecum could not be due to lignin digestion was further demonstrated by an estimation of the nitrogen content of a number of lignin samples. Although no nitrogen is thought to be present in the lignin molecule, it has not so far been possible to isolate nitrogen-free lignin from succulent plant tissues. It is generally considered (243) that this nitrogen is due to the condensation of protein molecules with the lignin possibly during extraction. Any decrease in the amount of nitrogen associated with the lignin would results in low recoveries of lignin and an increase in the carotene to lignin ratios. Samples of lignin were therefore prepared from fresh grass, caecal contents and faeces, and assayed for nitrogen. No significant differences were found, all the lignin containing from 1.8 to 2.5% of nitrogen.

The presence of a pigment other than carotene

seemed the most likely explanation for the increase in carotene to lignin ratios and led to an extensive rechecking of the method used for estimating the pro-vitamin. The presence in faeces and other materials of a yellow pigment which cannot be separated from carotene by normal phasic methods, has been mentioned in the previous chapter. It should be possible however, to effect a separation by the use of suitable adsorbants but repeated chromatography on columns of Hyflo Super-Cel or of Magnesium oxide and Hyflo Super-Cel, failed to reveal the presence of pigments other than the carotenes.

The identity of the pigment obtained from the caecal contents was further confirmed by a comparison of its absorption spectrum with that of a sample of carotene extracted from grass. As shown in Figure 28, the pigment from the intestine appears to be identical with the carotene in the grass, allowing for some isomerisation in the digestive tract.

Negative digestibilities for carotene have been reported (e.g. 236) but in all such cases phasic separation methods have been used for separating the carotenes from other pigments. These findings have not been reinvestigated since the results have been attributed to the estimation of other epiphasic pigments with the carotenes. The presence of these pigments, which form a yellow band immediately above the carotene on the Hyflo Super-Cel columns, has been noted in this investigation. The quantities however, have been small, representing less than 1% of the total epiphasic pigments present and it seems possible that carotene synthesis might also have contributed to the negative digestibilities previously reported.

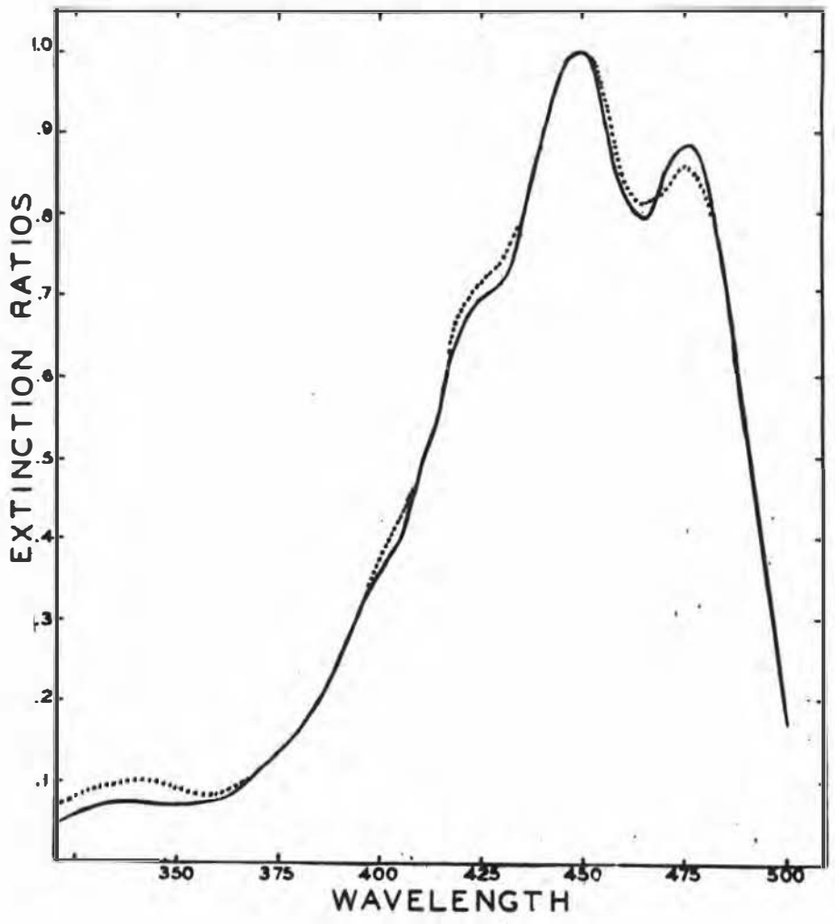


Fig.28 - Absorption spectrum of carotene isolated from grass ___ and from caecal contents .....

. . . . . .

A number of micro-organisms are capable of synthesising carotene (244) and the possibility of intestinal synthesis by these organisms has apparently been considered previously but in a recent report it was concluded that formation of carotene did not occur in the digestive tracts of humans (245).

If micro-organisms are responsible for the synthesis in sheep, it should be possible to show an increase in carotene content on incubating caecal or ileal contents. It has not been possible however, to demonstrate formation of carotene under these conditions but synthesis has occurred on an agar medium inoculated with caecal contents.

> The medium used contained per litre, tryptose 20 grams, dextrose 1 gram, sodium chloride 5 grams, and agar-agar 20 grams. The pH was adjusted to 7.2 and after auto-

claving, slants were inoculated with caecal contents diluted with sterile saline.

After incubating for 48 hours the carotene content of the cells was estimated by heating with alcoholic potash extracting the pigments into petroleum ether and chromatographing on a Hyflo Super-Cel column in the usual way.

Carotene, identified by its absorption spectrum, was formed equivalent to 1.2 to 1.8 ug/ml of medium. No attempt has been made to identify the micro-organisms responsible.

Apart from this synthesis of carotene, marked decomposition of the pro-vitamin appears to occur in the colon and rectum so that the apparent digestibility as calculated from the difference between the amounts ingested and excreted, gives no indication of actual absorption.

A point of immediate interest is whether this synthesised carotene can be utilised by the animal. No absorption of carotene or vitamin A occurs in the caecum or colon (246) but it is possible that some absorption occurs in the lower portions of the ileum.

This intestinal synthesis of carotene is being further investigated at the present time.

. . . . . .

SUMMARY

I. The estimation of vitamin A has been investigated particularly in the presence of substances which interfere in the usual antimony trichloride and spectrophotometric methods. It is considered that the recently introduced colorimetric reagent, glycerol dichlorohydrin, does not possess any advantages over the Carr-Price reagent but a spectrophotometric method is proposed based on a comparison of the absorption of test solution with that of vitamin A at three wavelengths in the region of maximum absorption. For vitamin A alcohol in ethanol or petroleum ether, readings are taken at 325, 310 and 340 mμ and the vitamin A estimated from these three absorptions by a simple calculation. The method is rapid and has proved satisfactory for the estimation of vitamin A in the presence of a number of interfering materials. The decomposition of vitamin A and carotene at different stages in extraction procedures is also discussed.

II. Chemical methods for the conversion of carotene to vitamin A have been investigated with particular reference to the reaction involving hydrogen peroxide catalysed with osmium tetroxide. It is considered that the product of the reaction is vitamin A and not, as seemed possible, a di-glycol formed by the addition of hydroxyl groups across the central double bond of β-carotene. A method for the synthesis of this di-glycol, which is a possible intermediate in the conversion of carotene to vitamin A in the animal body, is being investigated further.

III. It has been established that the conversion of carotene to vitamin A in the sheep occurs in the

wall of the intestine. This conclusion is based on surviving tissue experiments in which sections of intestine have been incubated with carotene and the product conclusively identified as vitamin A by colorimetric and spectrophotometric methods. It is further supported by the high vitamin A levels in intestinal as compared with non-intestinal lymph and similar observations with cattle suggest the intestine as the site of conversion in this species also. The intestinal lymph and portal blood plasma of sheep contains little or no carotene. In cattle a high level has been found in the intestinal lymph and the possibility of a secondary site of conversion in this species has been considered. It has not been possible however, to demonstrate the in vitro conversion of carotene to vitamin A in the livers of cattle. In sheep the vitamin A appears to be transported from the intestine by both the lymphatic and portal routes.

IV. The decomposition of carotene under accelerated conditions has been studied. Dilute solutions of carotene in liquid paraffin or hydrogenated coconut oil have been found to be extremely stable. The rate of decomposition of these solutions depends on the initial carotene concentrations and a linear relationship is shown to exist between the length of the induction period and the logarithmof the initial carotene concentration. In view of the stability of the carotene solutions, the use of hydrogenated coconut oil as a solvent in biological assay work is suggested.

V. Preliminary experiments have been carried out with sheep to determine the effect of the thyroid on the conversion of carotene to vitamin A. From a comparison of the plasma carotene and vitamin A levels

in normal, -hypo- and hyperthyroid animals, it is concluded that the thyroid has no direct action on either the enzyme system involved in the transformation or on the stability of the carotene in the intestinal tract and that any influence exerted must be on the rate of absorption of the provitamin.

VI It has not been possible to confirm the in vitro conversion of carotene to vitamin A using thyroprotein or thyroid extracts as claimed by Russian workers. It is suggested that incubation of carotene with thyroid extracts or iodinated casein results in the decomposition and isomerisation of the provitamin without the formation of vitamin A. The reasons for these changes have been investigated and it is concluded that thyroxine or thyroxine-like substances may influence the stability of the carotene solutions.

VII The carotene content of New Zealand pasture is discussed and reasons suggested for its apparent relative non-availability to ruminants during the summer months. A number of possible factors including the effect of high fibre and lo protein are under investigation. Experiments are also being carried out to determine seasonal changes in the tocopherol content of the pasture and the effects of these on the vitamin A potency of the butterfat.

VIII In view of the widely different procedures used in various laboratories, a survey has been made of the methods available for the estimation of carotene in plant materials. Errors inherent in a number of these are discussed and a method suggested based on a cold ethanol-petroleum ether extraction followed by chromatography on a column of Hyflo Super-Cel. This method, which introduces a number of modifications to

published procedures, is simple and rapid and is suitable for the routine assay of large numbers of samples. The method has been used for the estimation of carotene in fresh and dried herbage and faeces, and has given results in good agreement with those obtained by other commonly-used methods. The accuracy and reproducibility of the method has been extensively investigated.

IX. Carotene to lignin ratios have been determined at different points through the digestive tracts of a number of pasture-fed sheep. The ratios have been found to decrease through the upper portion of the small intestine, to increase through the ileum, reaching a maximum in the caecum, and to decrease slightly through the colon and rectum. It is shown that this increase in carotene to lignin ratio is not due to a partial digestibility of the lignin fraction of herbage or to the presence of a non-carotene pigment and it is suggested that there is a synthesis of carotene by the micro-organisms of the ileum and caecum. The synthesis of carotene by intestinal micro-organisms has been demonstrated on an agar medium inoculated with caecal contents.

A C K N O W L E D G M E N T S

The author wishes to express his appreciation of the co-operation received from various members of the staffs of Massey Agricultural College, the Dairy Research Institute, the Plant Chemistry Laboratory and the Grasslands Division of the Department of Scientific and Industrial Research. In particular it is desired to acknowledge the following -

Dr.C.N. Barnicoat for his interest and assistance throughout the investigation; other members of the Biochemistry Department - Mr.R.W. Cawley for help, particularly with the animal feeding work, and Mr.L. Jurd for advice on iodination experiments.

Messrs W.M. Webster, J.C. Newhook and B.A. Reynolds for veterinary assistance.

Mr.J.H. Tetley for use of an animal feeding shed and experimental animals.

Drs.F.H. McDowall and R.M. Dolby of the Dairy Research Institute for the use of a Beckman Spectrophotometer during the early stages of the investigation; Mr. A.K.M. McDowell, also of the Dairy Research Institute, for access to the Institute's figures for the vitamin A potency of butterfat.

Dr.J. Melville of the Plant Chemistry Laboratory for his interest and for the use of special equipment.

Mr.P.D. Sears of the Grasslands Division and his assistants for their willing co-operation at all times particularly in supplying grass samples, in providing pasture analysis figures and in allowing the use of experimental animals.

Finally Mr.H.D. Erlam and the College Library Staff for their assistance.

This work has been financed by University Research Grants and carried out during the tenure of a University Research Fellowship.

REFERENCES

1. Gridgeman N.T., "The Estimation of Vitamin A", Lever Bros. & Unilever Ltd., London, (1944).

2. Gridgeman N.T., "Recent Work on the Chemistry and Estimation of Vitamin A. Part II" Chem.& Ind. (1947), 574.

3. "Methods of Vitamin Assay" The Association of Vitamin Chemists Inc. Interscience Publishers Inc., New York (1947).

4. Hawk, P.B., Oser B.L. & Summerson W.H., "Practical Physiological Chemistry" The Blakiston Company, Toronto, 12th Edition (1947).

5. British Pharmacopoeia Addendum 1936. Pharmacopoeia of the United States 1942. Coward K.H. "Biological Standardisation of the Vitamins" Bailliere, Tindale & Cox., London, 2nd Edition (1947).

6. Oser B.L., Melnick D., Pader M., Roth R & Oser M. "Determination of Vitamin A. Correlation of Improved Spectrophotometric & Colorimetric Methods with Multiple-Level Bioassays." Ind.Eng.Chem. (Anal.Ed.) 17, 559, (1945).

7. Oser B.L., Melnick D. & Pader M. "Estimation of Vitamin A in Food Products". Ind.Eng.Chem.(Anal. Ed.) 15, 724, (1943).

8. Kobel A.E. & Herbin H. "Determination of Vitamin A in Fish Liver Oils with Activated Glycerol dichlorohydrin". Ind.Eng.Chem.(Anal.Ed.) 19, 107, (1947).

9. Ewing G.W. & Parsons T.Jr., "Intercomparison of Beckman Spectrophotometers". Anal.Chem. 20, 423, (1948).

10. "Handbook of Chem.& Physics" Chemical Rubber Publishing Co.,Cleveland, Ohio, 29th Edition. (page 2058)

11. Morton R.A. "Application of Absorption Spectra to the tudy of Vitamins, Hormones and Co-enzymes". Adam Hilger Ltd., London, 2nd Edition, (1942).

12. Kreider H.R., "2-Phenyl azo-p-cresol. A Photometric Standard for Vitamin A." Ind.Eng.Chem. (anal.Ed.) 17, 694, (1945).

13. Baxter J.G. & Robeson C.D. "Crystalline Vitamin A". J.Amer.Chem.Soc. 64, 2411, (1942).

14. Lehibated H. "New Test for Fat Aldehydes Resulting from Oxidation of Fats and Oils." Ind. Eng.Chem. (Anal.Ed.) 4, 204, (1932).

15. Morton R.A. & Stubbs A.L. "Studies in Vitamin A. 4. Spectrophotometric Determination of Vitamin A in Liver Oils. Correction for Irrelevant Absorption." Biochem.J. 42, 195 (1948).

16. Metcalf W.S., "Spectrophotometric Determination of Vitamin A. in Fish Oils". Nature, 155, 575 (1945).

17. Rawlings H .W.& Wait O.H. "Reliability & Precision of the Spectroscopic Determination of Vitamin A". Oil & Soap, 33, 83 (1946).

18. Oser B.L., Melnick D.& Pader M. "Chemical & Physical Determination of Vitamin A in Fish Liver Oils". Ind.Eng.Chem. (Anal.Ed.) 15, 717, (1943).

19. Little R.W. "Destructive Irradiation Technique of Spectrophotometric Vitamin A Assay". Ind.Eng. Chem.(Anal.Ed.) 16, 288 (1944).

20. Zechmeister L. "Cis-trans Isomerism & Stereo-Chemistry of Carotenoids & diphenylpolyenes". Chem.Revs. 34, 267, (1944).

21. Oser B.L. "Simplified Form of Morton & Stubb Correction of Vitamin A Absorption Curves." Anal.Chem. 21, 529 (1949).

22. Drummond J.C. & Watson A.F. "The Sulphuric Acid Reaction for Liver Oils" Analyst, 47, 341, (1922).

23. Rosenheim O. & Drummond J.C. "A Delicate Colour Reaction for the Presence of Vitamin A". Biochem.J. 19, 753, (1925).

24. Carr F.H. & Price E.A. "Colour Reactions Attributed to Vitamin A." Biochem.J. 20, 497 (1926).

25. Dann W.J. & Evelyn K.A. "The Determination of Vitamin A. with the Photoelectric Colorimeter." Biochem.J. 32, 1008 (1938).

26. "Colour Tests for Vitamin A". Nut.Revs. 4, 107 (1946).

27. Benham G.H. "The Antimony Trichloride Method for the Determination of Vitamin A." Canad.J. Res. 22B, 21 (1944).

28. Robin E.D. "Reaction of Vitamin A with Liebermann-Buchard Reagent". Science 102, 17, (1945).

29. Hogrady G. "Micro Determination of vitamin A by means of trichloro-acetic acid reagent." Hagy. Orv. Arch. 45, 135, (1944). (Brit.Abs.(1948) C.153)

30. Johnson R.W. & Baumann C.A. "Studies on the Reaction of certain Carotenoids with antimony trichloride". J.Biol. Chem. 169, 83 (1947).

31. Sobel A.E. & Werbin H. "Spectrophotometric Study of a new Colorimetric Reagent for Vitamin A." J.Biol. Chem. 159, 681 (1945).

32. Sobel A.E. & Werbin H. "Activated Glycerol dichloro-hydrin. A new colorimetric reagent for Vitamin A." Ind.Eng.Chem. (anal.Ed.) 18, 570, (1946).

33. Penketh D.S. "Glycerol dichlorohydrin and vitamin A". Nature, 161, 893, (1948).

34. Sobel A.E. & Snow S.D. "Estimation of Serum vitamin A with activated glycerol dichlorohydrin." J.Biol.Chem. 171, 617, (1947).

35. Wall. M.E. & Kelley E.G. "Determination of Vitamin A Meter in fortified poultry mashes with activated glycerol dichlorohydrin". Anal.Chem., 20, 757 (1948).

36. Squibb R.L., Cannon C.Y. & Allen R.S. "Effect of raw soya beans and so e bean oil on plasma carotene and on vitamin A as measured by activated glycerol dichlorohydrin." J.Dairy Sci. 31, 421, (1948).

37. McGillivray W.A., "A laboratory-built photoelectric colorimeter and fluorimeter." N.Z. J.Sci.& Techn. 29, 317, (1948).

38. Jones G.I., Sanford F.B., McKee L.G. & Miyauchi D.T. "Adaption of the Beckman Quartz Spectrophotometer for measurement of vitamin A by the Carr-Price reagent." Anal. Chem. 19, 142 (1947)

39. McGillivray W.A., "The vitamin content of New Zealand meat II, Mutton and Lamb" N.Z.J. Sci.& Tech. (in the press)

40. Antonaini C., Federico L. & Arton A. "Use of glycerol dichlorohydrin in the determination of vitamin A in oil concentrates". Olearia (1948) 405, (C.A. 42, 8244 (1948).

41. Lowman A. "A new reagent for vitamin A." Science 101, 183, (1945).

42. Kreider M.R. "Reaction of vitamin A with "super-filtrol" ". Science, 101, 377 (1945).

43. Meunier P. "Compes Rendus de l' Acad. France 215, 470 (1942) as quoted by Zechmeister L. & Sandoval A. "The coloration given by vitamin A and other polyenes on acid earths." Science, 101, 585 (1945).

44. Vecher A.S. "Colorimetric determ nation of vitamin A by adsorption on bentonite." Biokhimiya 13, 501, (1948). (C.A. 43, 3056, (1949)).

45. Shantz E.M., Lawley J.R. & Embee M.D. "Anhydro ("cyclized") vitamin A". J.Amer.Chem.Soc., 65, 901, (1943).

46. Gallup W.D. & Hoeffer J.A. "Determination of vitamin A in liver" Ind.Eng.Chem.(Anal.Ed.) 18, 288, (1946).

47. Davies A.W. "The colorimetric determination of vitamin A by the alkali digestion method." Biochem.J. 27, 1770, (1933).

48. Kimble M.S. "The photocolorimetric determination of vitamin A and carotene in human plasma." J.Lab.Clin.Med. 24, 1055, (1939).

49. Yudkin S. "The estimation of vitamin A and carotene in human blood". Biochem.J. 35, 551, (1941).

50. Fisher H .J. & Bailey E.M. "Vitamin A color reactions. A review of literature." J.Assoc.Offic.Agr.Chem. 13, 352, (1930).

51. Bacharach A.L. & Smith E.L. "The Chemical evaluation of the vitamins" Analyst, 59, 70 (1934).

52. Allen R.S., Rwise O.H. & Jacobson N .L. "Occurrence in blood plasma from certain dairy calves of factors that interfere with the blue colour reactions of activated glycerol dichlorohydrin with vitamin A and carotenoids." J.Dairy Sci. 32, 688, (1949).

53. Parrish D.B., Wise G.H. & Hughes J.S. "Vitamin A and carotenoids in the blood serum of dairy cattle". Anal.Chem. 20, 230, (1948).

54. Gillam A.E. "A modified spectrophotometric method for the assay of carotene and vitamin A in butter." Biochem.J. 28, 73, (1934).

55. Baumann C.A., Steenbock H., Beeson W.M. & Rupel I.W. "Fat soluble vitamins. The influence of breed and diet of cows on the carotene and vitamin A content of butter." J.Biol.Chem. 105, 167 (1934).

56. McNicholas H.J. "The visible and ultra-violet absorption spectra of carotene and xanthophyll and the changes accompanying oxidation." Bureau of Standards Journal of Research, 7, 171 (1931).

57. Schrenk W.G., Silker R.W. & King H.H. "Effect of β carotene isomerisation on its absorption at 326 mμ". Ind.Eng.Chem.(Anal.Ed.) 16, 328 (1944).

58. Morton R.A. "Carotene and allied substances in foods and feeding stuffs." Analyst, 65, 263 (1940).

59. Hunter K.F. "Vitamin A and the biologically active carotenoids." Chem.& Ind. (1942) 89.

60. Euler H.v. Karrer P. & Kolmaeen U. "Homologe of vitamin A. and degradation product of α carotene, α apo-2-carotenal". Helv.Chim.Acta. 21, 211 (1938).

61. Hunter K.F. & Williams N.E. "The chemical conversion of carotene into vitamin A." J.Chem.Soc.1945, 554.

62. Hunter K.F. "The conversion of carotene into vitamin A." Nature 158, 257, (1946).

63. Lechmeister L., Le Rosen A.L., Schroder W.A., Polgar A. & Pauling L. "Spectral characteristics and configuration of stereo-isomeric carotenoids including prolycopene & pro- γ -carotene." J.Amer.Chem.Soc. 65, 1940 (1943).

64. Glover J., Goodwin T.W. & Morton R.A. "Studies in Vitamin A. 6. Conversion in vivo of vitamin A aldehyde to vitamin A." Biochem.J. 43, 109, (1948).

65. Goss C.C.L. & McFarlane W.D. "Oxidation of β carotene with osmium tetroxide." Science 106, 375 (1947).

66. Fredenhagen H. & Bonhoeffer K.F. "Cannizzaro reaction in heavy water." Z. phys.Chem. A181, 379 (1938).

67. Waters W.A. "Mechanisms of oxidation." Annual Reports Chemical Society, 42, 146, (1945).

68. Arens J.F. & van Dorp D.A. "Synthesis of some compounds possessing vitamin A activity" Nature, 157, 190 (1946).

69. Organic Reactions Vol.II, John Wiley & Sons Inc., New York, 1944.

70. Karrer P. & Co-workers. Helv.Chim.Acta. 13, 1084, (1930), 14, 1033, (1931).

71. Sutherland M.D. Private communication.

72. Hickinbottom W.J. "Reactions of organic compounds" Longmans, Green & Co., London 1942.

73. Karrer P., "Organic Chemistry" Nordeman Publishing Co. Inc. , New York.

74. Moore T. "A note on carotene and vitamin A." Lancet 1929 (i), 499.
"The relation of carotene to vitamin A." Lancet 1929 (ii), 380.
"The absence of the liver oil vitamin A from carotene. The conversion of carotene to vitamin A in vivo." Biochem.J. 4, 692, (1930).
"Vitamin A and carotene. The distribution of vitamin A and carotene in the body of the rat." Biochem.J. 25, 275, (1931).

75. Copper N.L. "The alleged contamination of carotene by vitamin A. The transformation of carotene into vitamin A as shown by a study of the absorption spectra of rat liver oils." Biochem.J. 24, 453 and 980 (1930).

76. Kapper K.L., McKibbin I.M.S. & Prentice J.H. "The conversion of carotene into vitamin A by fowl." Biochem.J. 25, 265, (1931).

77. Drummond J.C., Ahmad B. & Morton R.A. "Relation of carotene to vitamin A." J.Soc.Chem.Ind. 49, 291T (1930).

78. Rosenheim O. & Drummond J.C. "On the relation of the lipochrome pigments to the fat soluble accessory food factor." Lancet 1920 (i) 862.

79. Coward K.H. "The association of vitamin A with the lipochromes of plant tissues." Biochem.J. 17, 145 (1923).

80. Greaves J.D.& Schmidt C.L.A. On the absorption and utilisation of carotene and vitamin A in choledochocolonostomized vitamin A deficient rats. "The utilisation of carotene by jaundiced and phosphorus treated vitamin A deficient rats." Amer.J. Physiol. 111, 492, & 502, (1935).

81. Kith T.K. "Rate of conversion of carotene to vitamin A in the rat liver." Nord.Med. 5, 2901, (1939).

82. Popper H. "On the variations of the plasma vitamin A level after the administration of large doses of vitamin A in liver disease." J.Clin. Invest.22, 775 (1943).

83. Drummond J.C. & Macwalter R.J. "The fate of carotene injected into the circulation of the rat." J.Physiol. 83, 236 (1934).

84. Drummond J.C. "Observations on the absorption of carotene and vitamin A." Brit.Med.J. (1935) i, 1209.

85. Bicknell F.& Prescott F. "The vitamins in Medicine." William Heinemann, London, (1946).

86. Olcott,H.S. & McCann D.C. "Carotenase. The transformation of carotene to vitamin A in vitro." J.Biol.Chem. 94,185 (1931.

87. Parienti A.C. & Ralli E.P. "Presence of carotenase in the liver of the dog." Proc. Soc. Exp.Biol.Med. 29,1209 (1932)

88. Euler H.v. & Klussmann E. "Carotene and vitamin A." Svensk.Kim.Tidskr. 44,223 (1932)

89. Wilson H.A.C., Ahmad B. & Mazumdar D.K. "Transformation of carotene into vitamin A in liver autolysate." Indian J.Med.Res. 25, 85 (1937)

90. Ahmad B. "The fate of carotene after ingestion in the animal body." Biochem.J. 25, 1195 (1931)

91. Drummond J.C. & Rae J.L. Vitaminforsch. 1, 177 (1932)

92. Drummond J.C. & Macwalter R.J. "The biological relation between carotene and vitamin A." Biochem.J. 27, 1342 (1933)

93. Woolf B. & Moore T. "Carotene and Vitamin A." Lancet 123, (11), 13 (1932)

94. Kuhlmeister L. Ergeb.Physiol.biol.Chem.exp. Pharmakol. 39,117 (1937)

95. Wolff L.K., Overhoff J. & Kekulen M. "Carotene and vitamin A." Deut.med.Wochschr. 56, 1428 (1930)

96. Lease J.G., Lease E.J., Steenbock H. & Baumann C.A. "The comparative physiological value of injected carotene and vitamin A." J.Lab.Clin.Med. 27, 502 (1942)

97. Tomarelli R.M., Charney J. & Bernhart F.W. "Utilization of intramuscularly injected carotene." Proc.Soc.Exptl.Biol.Med. 63, 108 (1946)

98. Smith T.K. "Absorption, metabolism and storage of vitamin A and carotene." Oxford University Press (1941)

99. Vinet A., Plessier M. & Raoul Y. "Place and conditions of transformation of carotene to vitamin A in the animal organism." Bull.Soc.Chim.biol. Paris. 25, 87 (1943)

100. Kolhline W.L., Malakhovski S.C. & Rudnova A.A. "Mode of administering carotene." Biochimia 8, 168 (1943). (Chem.Abs. 38, 1608 (1944))

101. Sexton W.A., Kohl J.W. & Touel H.J.Jr. "Studies on carotenoid metabolism. 6. The relative provitamin A activity of carotene when introduced orally and parenterally." J.Nutrition 31, 299 (1946)

102. Kuhlmeister L. "Carotenoide." Julius Springer. Berlin. (1934)

103. Glover J. & Morton R.A. "The administration, storage and metabolism of vitamin A." Biochem.J. 43, xii. (1948)

104. Maximow A.A. & Bloom W. "Textbook of Histology." W.B. Saunders Co. Philadelphia. 2nd. Edition (1942) page 428.

105. Mattson F.H., Mehl J.W. and Daniel H.J. Jr. "Studies on carotenoid metabolism. 7. Site of conversion of carotene to vitamin A in the rat." Arch. Biochem. 15, 65 (1947)

106. Mason C.W., Mehl J.W. & Daniel H.J. Jr. "Studies on carotenoid metabolism. 8. The in vitro conversion of carotene to vitamin A in the intestine of the rat." Arch. Biochem. 15, 75 (1947)

107. Popper H. "Histological distribution of vitamin A in human organs under normal and under pathological conditions." Arch. Path. 31, 766 (1941)

108. Rimble W. . "The photocolorimetric determination of carotene in human plasma." J.Lab.Clin.Med. 24, 1055 (1939)

109. Mattson F.H. "The site of conversion of carotene to vitamin A." J.Biol.Chem. 176, 1467 (1948)

110. Glover J., Goodwin T.W. & Morton R.A. "Conversion of β carotene into vitamin A in the intestine of the rat." Biochem.J. 41, xlv, (1947)

111. Glover J., Goodwin T.W. & Morton R.A. "Studies in vitamin A. 8. Conversion of β carotene into vitamin A in the intestine of the rat." Biochem.J. 43, 512 (1948)

112. Thompson S.Y., Ganguly J. & Kon S.K. "The intestine as a possible site of conversion of carotene to vitamin A in the rat and the pig." Brit.J.Nutrition 1, v (1948)

113. Thompson S.Y., Arnaide M., Coulé A.M., Ganguly J. & Kon S.K. "The intestinal conversion of carotene to vitamin A." Biochem.J. 44, ix, (1949)

114. Wagner G.H. "Vitamin A and β carotene of the fin-, blue- and sperm-whales." Johann Ambrosius Barth. Leipzig. (1939)

115. Goodwin T.W. & Gregory M.E. "Studies in vitamin A. 7. Carotene metabolism in herbivores." Biochem.J. 43, 505 (1948)

116. "Conversion of carotene to vitamin A." Nut. Revs. 7, 125 (1949)

117. "The site of conversion of carotene to vitamin A." Nut. Revs. 6, 92 (1948)

118. Kon S. & Wellers H.C. "The absorption of vitamin A." Biochem. J. 42, xlix, (1948)

119. Frazer A.C., "Absorption of fat from the intestine." Chem. & Ind. (1947), 373.

120. Frazer A.C., Schulman J.H. & Stewart R.C. "Emulsification of fat in the intestine of the rat and its relationship to absorption." J. Physiol. 105, 506 (1944)

121. Umbreit W.W., Burris R.H. & Stauffer J.F. "Manometric techniques and related methods for the study of tissue metabolism." Burgess Publishing Co.

122. Krause R.F. & Pierce E.E. "The extra-hepatic conversion of carotene to vitamin A." Arch. Biochem. 19, 147, (1948)

123. Karrer P., Helfenstein A. & Wehrli H. "Plant pigments. 18. Further contributions to the constitution of the carotenoids." Helv. Chim. Acta. 13, 1084 (1930)

124. Holmes H.N. & Corbet R.E. "Crystalline vitamin A concentrate." Science 85, 193 (1937) and "Isolation of vitamin A." J. Amer. Chem. Soc. 59, 2042 (1937)

125. Ward T.H., Underhill E.W. & Coward K.H. "Crystalline esters of vitamin A. I. Preparation and properties. II. Biological potency." Biochem. J. 33, 589 (1939)

126. Baxter J.G. & Robeson C.D. "Crystalline vitamin A palmitate and vitamin A alcohol." Science 92, 203 (1940)

127. Mickelsen O.C.L. "Fat soluble vitamins." Ann. Rev. Biochem. 12, 393 (1943)

128. Rodin G.F. "Relative activity of β carotene and vitamin A." Arch. Biochem. 17, 337 (1948)

129. Barnicoat C.R. "Estimation of apparent digestibility coefficients by means of an inert reference-substance." N.Z. J. Sci.& Tech. 27, 202 (1945)

130. Holmes E.A. "Oxidative decomposition of vitamin A. 1. Stability of vitamin A towards oxidation and irradiation." J.Biol.Chem. 169, 323 (1947)

131. Schultze A.B. & Turner C.W. "The determination of the rate of thyroid secretion by certain domestic animals." University of Missouri Research Bulletin No. 392 (1945)

132. King A.F., Roschen H.C. & Irwin W.H. "An accelerated stability test with the peroxide value as an index." Oil & Soap 10, 105 (1933)

133. Baumann C.A. & Steenbock H. "Fat soluble vitamins. The stability of carotene solutions." J.Biol.Chem. 101, 561 (1933)

134. Curtis A.C. & Dellaher L.J. "The prevention of carotene absorption by liquid petrolatum." J.A.M.A. 113, 11, 1785 (1939)

135. Noorden C.v. "Die Zuckerkrankheit." 4th Edition. Berlin. (1907) as quoted by Brill (143)

136. Anderson R.K. & Boley M.K. "Effects of carotenemia on the function of the thyroid and the liver." Am. J. Med. Sci. 195, 313 (1938)

137. Savy J., Vachon A. & Vincent D. J.de med. de Lyon 19, 245 (1938)

138. Macmillan E.F. "Carotenemia in myxedema - explanation of the typical slightly icteric tint." J. Clin. Endocrinol. 2, 33 (1942)

139. Wendt H. Munchen. Med. Wchnschr. 82, 1679 (1935)

140. Kohl M.C. & Feldman J.D. "Vitamin A deficiency in disease of the thyroid: its detection by dark adaption." Endocrinol. 24, 389 (1939)

142. Moore T. "The vitamin A reserves of the adult human being in health and disease." Biochem. J. 31, 155 (1937)

143. Drill V.A. "Interrelations between thyroid function and vitamin metabolism." Physiol. Revs. 23, 355 (1943)

144. Munds M.B. "Nutritional disturbances, pellagra and xerophthalmia." Proc. Soc. Exp. Biol. Med. 23, 312 (1926)

145. Smith D.C. & Perman J.M. "Effect of thyroxine combined with carotene on the oxygen consumption of rats." Endocrinol. 27, 110 (1940)

146. Schneider R. & Widmann E. Klin. Wchnschr. 14, 1786 (1935)

147. Pellenberger T.V. & Grutor F. "Effect on lactation in the goat of thyroidectomy alone or of thyroidectomy preceded by the administration of extract of placenta and macerated corpus luteum or followed by the administration of anterior pituitary extract." Biochem. J. 253, 42 (1932)

148. Parold H. & Heidemann E. Ztschr. f.d. ges. exper. Med. 92, 53 (1933)

149. Drill V.A. & Truant A.P. "Effect of thyroidectomy on the conversion of carotene to vitamin A." Endocrinol. 40, 249 (1947)

150. Comadell J.M. & Valdecenas F.M. "Inhibitory action of thiouracil on carotene." Experientia 3, 35 (1947)

151. Remington R.E., Harris P.L. & Smith C.A. "Relationship between vitamin A and iodine metabolism in the rat." J. Nutrition 24, 597 (1942)

152. Wiese H., Neuel H.G. Jr. & Mehl J.W. "Thiouracil and conversion of carotene to vitamin A measured by liver storage in the rat." Proc. Soc. Exp. Biol. Med. 66, 213 (1947)

153. Johnson B. C. & Baumann C. A. "The effect of thyroid on the conversion of carotene to vitamin A." J. Biol. Chem. 171, 513 (1947)

154. Kelley B. & Day H.G. "Thiouracil and the conversion of carotene to vitamin A in the rat." J. Biol. Chem. 175, 863 (1948)

155. Johnson B.C. & Baumann C.A. "Relative importance of growth and metabolic rate on the utilization of vitamin A by the rat." Federation Proc. 7, 290 (1948)

156. Wiese H., Mehl J.W. & Neuel H.G. Jr. "Studies in carotenoid metabolism. 9. Conversion of carotene to vitamin A in the hypothyroid rat." J. Biol. Chem. 175, 21 (1948)

157. Campbell I.L. & McDowell A.K.M. Dairy Research Institute (N.Z.) Physiological Project 32. (Unpublished results.)

158. Peirce A.W. "Changes in the concentrations of carotene and vitamin A in the blood of sheep grazing on natural pasture in N. Australia." Aust. J. Exptl. Biol. Med. Sci. 24, 231 (1946)

159. Moore L.A. "Determination of carotene in plant material." Ind. Eng. Chem. (Anal.Ed.) 12,726 (1940)

160. Goodwin T. W. "The thyroid gland and carotene and vitamin A metabolism." Biochem.J. 43, xliii (1948)

161. Allen N.N., Wise G.H. & Jacobson W.C. "Effect of thiouracil and thyroprotein on the concentrations of carotene and vitamin A in the blood of dairy cows." J. An. Sci. 7, 538 (1948)

162. Barrick E.L., Andrews F.N., Beeson W.M. & Harper C. "Effects of thiouracil and similar compounds on feeder lambs." J. An. Sci.7, 539 (1948)

163. Turhoff B.H. & Meuel H.J. Jr. "The effect of growth hormone on the vitamin-deficient rat." Endocrinol. 36, 280 (1945)

164. Althausen T.L. & Stockholm M. "Influence of the thyroid gland on absorption in the digestive tract." Am. J. Physiol. 123, 577 (1938)

165. Kalabe T.A. "Influence of thyroglobulin on vitamin A formation from carotene." J. Physiol. U.S.S.R. 29, 318 (1940) Chem.Abs. 36, 5519 (1942)

166. Koplenaky G. & Kalabe T. "Transformation of carotene into vitamin A under the action of iodinated casein." Biokhimiya 11, 327 (1946)

167. Harington C.R. "The thyroid gland. Its chemistry and physiology." Oxford University Press. London. (1933)

168. Heineke E.W. & Turner C.W. "The effect of manganese compounds and certain other factors on the formation of thyroxine in iodinated casein." J.Biol.Chem. 161, 613 (1945)

169. Heineke E.W. & Turner C.W. "Preparation of highly active thyroproteins, their biological assay, and practical use." University of Missouri Research Bulletin No. 355. (1942)

170. Cama H.R. & Goodwin T.W. "The role of thyroglobulin and iodinated casein in the in vitro conversion of carotene into vitamin A." Biochem. J. 43, xliv (1948)

171. Di Bella L., Caima G. & Boilini Comurri M.L. "Relations in vitro between β carotene and thyroxine." Arch. Sci. biol. Napoli 32, 321 (1948)

172. Barnicoat C.R. "Variations in the carotene and vitamin A contents of certain New Zealand butterfats." J. Dairy Res. 15, 80 (1947)

173. Dairy Research Institute (N.Z.) Unpublished results.

174. Ward A.H. "District and seasonal influence on lactation curves in New Zealand." Proc. Soc. Animal Production (1942), 30

175. Hauge S.M., Westfall R.J., Wilbur W.J. & Hilton J.H. "The vitamin A requirements of dairy cows for production of butterfat of high vitamin A value. III. Availability of carotene in dehydrated alfalfa hay as compared with carotene in oil." J.Dairy Sci. 27, 63 (1944).

176. "Feeding for maximum vitamin A in butter." Nutr. Revs. 2, 203 (1944)

177. Moon H.K. "The composition of grass at various stages of maturity and the changes occurring during hay making with particular reference to carotene content." Empire J. Exptl. Agric. 7, 225 (1939)

178. Moon H.K. "The carotene content of some grass and clover species with a note on pasture weeds." Empire J. Exptl. Agric. 7, 235 (1939)

179. Thomas M. & Moon H.K. "A preliminary study of the effects of manurial treatment and of age on the carotene content of grass." Empire J. Exptl. Agric. 6, 235 (1938)

180. Smith A.M. & Wang T. "Carotene content of certain species of grassland herbage." J. Agr. Sci. 31, 370 (1941)

181. Cawley R. . Biochemistry Department, Massey Agricultural College. Unpublished results.

182. Thorlund T.B. "Leaf lipids of forage grasses and clovers." Nature 153, 168 (1944)

183. Krupe G. . "Effect of bulk, casein and fat in the ration on the utilization of carotene by white rats." Arch. Biochem. 10, 485 (1946)

184. Harris P.L., Swanson W.J. & Nickson K.C.N. "Co-vitamin studies. Effect of tocopherol supplementation on the output of vitamin A, carotene and fat by dairy cows." J. Nutrition 33, 411 (1947)

185. Whiting F., Loosli J.K., Krukovsky V.N. & Turk K.L. "The influence of tocopherols and cod-liver oil on milk and fat production." J. Dairy Sci. 32, 139 (1949)

186. Hall R.J. & Kelley E.G. "Determination of tocopherol in plant tissues." Ind. Eng. Chem. (Anal.Ed.) 16, 198 (1944)

187. Ja N.N. "The assimilation of vitamin A and carotene by rats from some common foods with a note on the conversion factor I.U./γ proposed by the International Vitamin Conference." Indian J. Med. Res. 24, 751 (1937)

188. Guggenheim K. "The biological value of carotene from various sources and the effect of vitamin E on the utilization of carotene and vitamin A." Biochem. J. 38, 160 (1944)

189. Nickson K.C.N., Harris P.L. & Woodside W.M. "Interrelationship of vitamins A and E." Nature 150, 91 (1942)

190. Nickson K.C.N., Raley M. . & Harris P.L. "Co-vitamin studies. The sparing equivalence of tocopherols and mode of action." J.Biol.Chem. 152, 321 (1944)

191. Mac ...L. "Utilization of carotene from oils. Influence of vitamin E on the utilization of carotene from oils." Nature 156, 234 & 449 (1945)

192. Sherman H.C. "The effects of certain fats and unsaturated fatty acids upon the utilization of carotene." J. Nutrition 22, 153 (1941)

193. Nelson E.M. "Statement on carotene." J. Assoc. Off. Agr. Chem. 28, 544 (1945)

194. Coward K.H. "Some observations on the extraction and estimation of lipochromes from animal and plant tissues." Biochem. J. 18, 1114 (1924)

195. Ferguson W. . & Bishop L. "The estimation of carotene in agricultural products." Analyst 61, 725 (1938)

196. Bolton W. & Common R.H. "Modification of Wood's method for the estimation of carotene in silage." J. ...I. 61, 50 (1942)

197. Charkey H.... & Wilgus H. . Jr. "Determination of carotene in alfalfa." Ind Eng. Chem. (Anal. Ed.) 16, 184 (1944)

198. Willstatter R. & Stoll A. "Untersuchungen Uber Chlorophyll. Methoden und Ergebnisse." Julius Springer, Berlin (1913)

199. Russell W.C., Taylor M.W. & Chichester D.F. "Determination of carotene in plant tissues." Plant Physiol. 10, 325 (1935)

200. Wilker H.W., Schrenk W.G. & King H.H. "Determination of carotene in dehydrated alfalfa." Ind. Eng. Chem. (Anal. Ed.) 16, 513 (1944)

201. Petering H.G., Wolman W. & Hubbard R.P. "Determination of chlorophyll and carotene in plant tissue." Ind. Eng. Chem. (Anal.Ed.) 12, 148 (1940)

202. Seaber E.M. "The commercial determination of carotene and allied pigments with special reference to dried grass and other leafy material." Analyst 65, 266 (1940)

203. Hall M.H. & Kelley E.G. "Determination of pure carotene in plant tissue. A rapid chromatographic method." Ind. Eng. Chem. (Anal.Ed.) 15, 18 (1943)

204. "Official and tentative methods of analysis of the Assoc. of Official Agricultural Chemists." A.O.A.C. Washington. (1945)

205. Derby J.V. & Le Fitt J.E. "Comparison of methods for determination of carotene." J. Assoc. Off. Agr. Chem. 31, 704 (1948)

206. Kuhn R. & Brockmann H. "Determination of carotenoids." Z. physiol. Chem. 206, 41 (1932)

207. Zimmermann W.J., Treseler D.K. & Maynard L.A. "Determination of carotene in fresh and frozen vegetables. 1. Carotene content of green snap beans and sweet corn." Food Res. 6, 57 (1940)

208. Beadle B.W. & Zscheile F.P. "Studies on carotenoids. II The isomerization of β carotene and its relation to carotene analysis." J. Biol. Chem. 144, 21 (1942)

209. Binnington D.S., Libbitt L.S. & Geddes W.F. "Determination of wheat and flour pigments." Cereal Chem. 15, 119 (1938)

210. Fujita A., Morita A. & Ajisaka M. "Estimation of provitamin A. 1. Estimation of carotene." Biochem. Z. 308, 420 (1941)

211. Hyke M. "A rapid method for the determination of carotene, xanthophyll and chlorophyll in artificially dried grass meals." Chem.& Ind. 55, 197T (1936)

212. Smith L.I. & Smith C. "Light and the carotenoid content of certain fruits and vegetables." Plant Physiol. 6, 265 (1931)

213. Warn T.B. "The separation of vitamin A from xanthophylls in the presence of egg yolk sterols." Analyst 68, 233 (1943)

214. Wolin D. & Khalafur A.M. "A precise method for the determination of carotene in forage." Ind. Eng. Chem. (Anal.Ed.) 10, 417 (1938)

215. Wegstad D.M., Porter J.W. & Peterson W.H. "Determination of carotene in silage. An improved method." Ind. Eng. Chem. (Anal.Ed.) 11, 256 (1939)

216. Moore L.A. & Ely R. "Extraction of carotene from plant materials. A rapid quantitative method." Ind. Eng.Chem. (Anal.Ed.) 13, 600 (1941)

217. Ferrari C.G. "Colorimetric determination of carotene in plant tissue." Cereal Chem. 10, 277 (1933)

218. Miller E.C. "Photoelectric spectrophotometry applied to the quantitative analysis of carotenoid and chlorophyll pigments in ternary and quaternary systems." Cereal Chem. 15, 310 (1938)

219. Borodin J. Bull. Acad. Imp. Sci. St. Petersburg 9, 512. Bot. Ztg. 4, 577.

220. Haagen-Smit A.J., Jeffreys C.E.W. & Kirchner J.G. "Separation of carotenes from xanthophylls." Ind. Eng. Chem. (Anal.Ed.) 15, 179 (1943)

221. White J.W. Jr. & Zscheile F.P. "The carotenoids. 3. Distribution of pure pigments between immiscible solvents." J.Am.Chem. Soc. 64, 1440 (1942)

222. Bell M.F., Kelley E.G. & Williamson J.J. "Carotene concentrates from vegetable leaf wastes." Ind. Eng. Chem. 36, 1057 (1944)

223. Murri I. "A new method for extracting carotene from green plant materials." Lokl. Red. Acad. Agric. Sci. 4, 37 (1943) Nut. Revs.& Abs. 14, 64A (1945)

224. Sreenivasan A. & Vaidya R.H. "Determination of carotene in plant materials." Anal. Chem. 20, 720 (1948)

225. Fraps G.S. & Kemmerer A.R. "Improved method for estimating carotene in feeds." J. Assoc. Off. Agr. Chem. 22, 190 (1939)

Fraps G.S., Kemmerer A.R. & Greenberg L.M. "Factors affecting absorptive power of magnesia for carotene." Ind. Eng. Chem. (Anal.Ed.) 12, 16 (1940)

Fraps.G.S., Kemmerer A.R. & Greenberg L.M. "Determination of carotene in the presence of lycopene." J. Assoc Off. Agr. Chem. 23, 422 (1940)

Fraps G.S., Kemmerer A.R. & Greenberg L.M. "An adsorption method for the determination of pure carotene." J. Assoc Off. Agr. Chem. 23, 659 (1940)

226. Kernohan G. "Determination of carotene." Science 90, 623 (1939)

Cooley W.L., Christiansen J.L. & Schroeder C.H. "Chromatographic estimation of vitamin A in mixed feeds." Ind. Eng. Chem. (Anal.Ed.) 17 689 (1945)

227. Wilkes J.B. "High-speed filter aid for chromatographic analysis." "Chromatographic determination of carotene. Kieselguhr as chromatographic adsorbent." Ind. Eng. Chem. (Anal.Ed.) 18, 329 & 702 (1946)

228. Booth V.H. "Extraction and estimation of carotene from foodstuffs." J. Soc. Chem. Ind. 64, 162 (1945)

229. Moore L.A. "Activation of dicalcium phosphate for the chromatographic determination of carotene." Ind. Eng. Chem. (Anal.Ed.) 14, 707 (1942)

230. Kuhnmeister L. & Polgar A. "Cis-trans isomerization and spectral characteristics of carotenoids and some related compounds." J. Am. Chem. Soc. 64, 1856 (1942)

Kuhnmeister L. & Tuason R. "Isomerisation of the carotenoids." Biochem. J. 32, 1905 (1938)

231. Clausen S.W. & McCord A.B. "The determination of carotene and xanthophyll by a single distribution between liquid phases." J.Biol.Chem. 113, 89 (1936)

Hoch H. "Chromatographic investigation of the carotene content of serum of the newborn infant." Biochem. J. 38, 304 (1944)

232. Lease E.J. & Mitchell J.H. "Effect of certain carbohydrates on the determination of carotene." Ind. Eng. Chem. (Anal.Ed.) 12, 337 (1940)

233. Fraps.G.S., Heinke E.W. & Kemmerer A.R. "Determination of pure carotene in green vegetables and green feeds." J. Assoc. Off. Agr. Chem. 24, 739 (1941)

234. Miller E.C. "A rapid and accurate quantitative method for the determination of the common carotenoids." J. Am. Chem. Soc. 57, 347 (1935)

235. Rosenkrans C.B. & Rupkin D.K. "Absorption of minimal doses of β carotene by vitamin-deficient rats." Nature 149, 611 (1942)

236. Whitnah C.H., Peterson W.E., Atkeson F.W. & Cave H.W. "Carotene balance and blood carotene levels in heifers and lactating dairy cows and their relation to the production of off-flavours in milk." J. Agr. Res. 58, 343 (1939)

237. Quackenbush F.W., Steenbock H. & Peterson W.H. "The effects of acids on carotenoids." J. Am. Chem. Soc. 60, 2937 (1938)

238. Hartman A.M., Kane E.A. & Dhinn L.A. "Does the carotene in alfalfa hay account completely for its vitamin A activity." J.Biol.Chem. 105, xxxvi. (1934)

239. Bacsern H.W., Kane E.A., Dhinn L.A. & Cary C.A. "The carotene content of market hays and corn silage." J. Agr. Res. 57, 635 (1938)

240. Bathurst N.E. Plant Chemistry Laboratory, D.S.I.R. Private communication.

241. Moore L.A. "Determination of carotene in plant material. Dicalcium phosphate as adsorbent." Ind. Eng. Chem. (Anal.Ed.) 12, 726 (1940)

242. Willis C.F., Pentrone A. & Maynard L.A. "A 72% sulphuric acid method for the determination of lignin and its use in animal nutrition studies." J. Animal Sci. 5, 285 (1946)

243. MacDougall D. & Fleming W.A. "A study of methods for the extraction of nitrogenous materials from plants with particular reference to subsequent determination of lignin content." "A study of the effect of reducing conditions on the amount and nature of lignin isolable from plants." "A method for the determination of lignin content of fresh plant tissue without preliminary drying." Canad. J. Res. 26, 457 & 464 & 468 (Section B) (1948)

244. Leonhester L. & Cholnoky L. "Principles and practice of chromatography" Chapman & Hall Ltd. London (1945) pages 126 & 127.

245. Hume E.M. & Krebs H.A. "An experimental study of vitamin A deprivation in man." Medical Research Council. Special Report Series No. 264. London. (1949)

246. Merrick J.A., Andrews F.N., Peterson W.H. & Harper C. "The absorption of carotene and vitamin A from various levels of the gastrointestinal tract of sheep." J. Animal Sci. 7, 539 (1948)

A P P E N D I X

Vitamin Content of New Zealand Meats

As already mentioned a short investigation into the vitamin content of New Zealand mutton and lamb was undertaken during the course of the preliminary work associated with the main research.

The vitamin content of meat has been studied extensively particularly in America but little work has been carried out on New Zealand meats. Most of the workers have been interested in the vitamin content of average commercial cuts as supplied to the customer rather than in the amounts present in the tissues at slaughter and the relation of this to the past history of the animal. The present investigation has however, been restricted to organs and individual muscles taken from a number of lambs and one sheep raised on the Massey College farms for projects under investigation in other Departments. One group of lambs and the sheep were raised under normal conditions. The other group of lambs had been raised under conditions of indoor feeding not normal in New Zealand (i.e. hay and concentrates) and was included to give an indication of the effect of such widely different treatments on the vitamin content of the tissues. Thiamin, riboflavin and niacin, and in a few cases, pantothenic acid and biotin, were estimated in the tissues.

With the exception of riboflavin, microbiological methods were used for all assays. The microorganisms, methods of assay and media used were those recommended by Barton-Wright (1). These microbiological methods lend themselves readily to the assay of large numbers of samples such as were handled in this investigation and once their technique is mastered are rapid and relatively simple to carry out. While admitting the general

superiority of these microbiological methods over other methods, it is considered that a chemical method based on a readily measureable natural physical property of the vitamin such as the fluorescence of riboflavin, is likely to be at least as simple and reliable as a microbiological method. For this reason, particular attention had previously been devoted to the chemical assay of riboflavin and a suitable method suggested (2). This method which is a modification of that proposed by McLaren, Cover and Pearson (3) was used for all riboflavin assays.

Description of the Animals

The group of lambs, eight ewes and four wethers, raised under normal conditions, consisted of twelve Romney-Southdown lambs killed at as near to constant weight as possible. The mean hot carcase weight was 33.7 lbs. (S.D.0.9 lbs.) and the average age, 107 days. The second group, seven ewes and five wethers, consisted of twelve Romney lambs reared indoors from birth until 21 days before slaughter when they were turned out to normal pasture. During the indoor period after weaning the lambs were fed a mixed feed consisting of, lucerne hay 11 parts, bran 9 parts, linseed meal 13 parts, crushed oats 18 parts and peas 13 parts. The lambs did not thrive well under the conditions of the experiment only a few gaining weight regularly, and as most received a further setback on being put on to pasture they were in a poor condition when slaughtered. This change from indoor to outdoor feeding was part of another experiment out of our control. The mean hot carcase weight was 22.5 lbs. In view of the results obtained from the indoor fed lambs, one older lamb which had been fed indoors from birth until slaughter was assayed. This lamb, a wether, had gained weight consistently and was

one of the best out of over fifty lambs in the indoor-feeding experiment. When slaughtered it was in good condition- hot carcase weight 35.5 lbs, age 165 days.

Sampling

Organs required for assay were removed immediately after slaughter. It was not possible to obtain all muscle samples at once so a standard procedure was adopted of taking all such samples after the carcase had hung for 48 hours in the cooler. Whether for assay by chemical or microbiological methods, the preparation of samples was the same being based on the procedure recommended by Bathurst (4). After the removal of all visible fat, each sample was minced and well mixed. A large sample (usually 25 to 50 grams) was agitated on a Waring Blendor with distilled water. The resulting suspension was made up to a definite volume (depending on the approximate vitamin content), the volume occupied by the solid material in the sample being allowed for where necessary, and aliquots withdrawn and mixed with hydrochloric acid of sufficient strength to bring the total sample to the appropriate normality (0.25N in the case of thiamin and riboflavin and N for niacin). The tubes were plugged and after autoclaving at 15 lbs. pressure for 15 minutes, stored in a cool place protected from light until assayed. Immediately prior to assay thiamin samples were adjusted to pH 4.5, 0.2 grams of clarase added and incubated for 24 hours at 37°C with a few drops of toluene.

In all cases absorption techniques with subsequent elution were avoided owing to the difficulties in completely recovering the vitamin (5); likewise the Waring Blendor was used in preference to procedures such as grinding with sand (as recommended by several

workers) owing to the possibility of vitamin being adsorbed on the sand (6).

Results

The results for the various tissues assayed are set out in Tables 1 to 4 and in Figure 1.

TABLE 1

Vitamin content of tissues (fresh weight) from group of 12 lambs raised outdoors on pasture.

Tissue	Thiamin ug/gm	S.D.	Riboflavin ug/gm	S.D.	Niacin ug/gm	S.D.
Liver	4.6	0.4	36.4	5.5	180	12
Heart	6.2	0.7	6.8	0.2	50.0	3.3
Neck Muscle[1]	2.3	0.3	2.7	0.3	53.2	5.8
Long.Dorsi[2 3]	2.9	0.3	3.7	0.5	61.5	5.4
Psoas Major[2]	2.5	0.3	3.2	0.3	60.0	4.5
Diaphragm[2]	3.4	0.2	6.0	0.4	66.5	5.3

1 - Neck muscle from the region of the atlas.

2 - Assays on six animals only.

3 - Taken at the junction of the last thoracii and first lumbar.

. . . . . .

TABLE 2

Vitamin content of tissues (fresh weight) from group of 12 lambs raised indoors on hay and concentrates.

Tissue	Thiamin ug/gm	S.D.	Riboflavin ug/gm	S.D.	Niacin ug/gm	S.D.
Liver	4.5	0.6	49.0	5.5	171	13.6
Heart	6.7	0.8	6.9	0.3	49.0	9.8
Neck Muscle	2.4	0.3	3.9	0.5	57.5	7.4
Long.Dorsi[1]	3.0	-	4.1	-	62.0	-

1 - Assay on one animal only.

. . . . . .

TABLE 3

Vitamin content in ug/gm. fresh weight of tissues from mature ewe raised outdoors on pasture.

Tissue	Thiamin	Riboflavin	Niacin	Pantothenic Acid	Biotin
Liver	4.5	30.0	167	65	0.88
Heart	5.0	6.5	60.0	24	0.07
Kidney	4.6	20.5	85.0	-	-
Neck Muscle	1.3	2.9	44.5	3.8	0.02
Mean for eight representative muscles.					
	2.0	3.1	49.0	4.1	0.023
S.D.	0.6	1.3	9.9	0.8	0.01

. . . . . .

TABLE 4

Vitamin content in ug/gm. fresh weight of tissues from wether fed indoors on hay and concentrates.

Tissue	Thiamin	Riboflavin	Niacin
Liver	5.5	46.0	198
Heart	5.9	-	63.5
Kidney	4.8	27.2	78.0
Spleen	2.2	5.8	61.0
Brain	2.6	5.0	51.5
Lung	1.6	4.5	59.5
Rumen Wall	1.2	3.0	47.0
Tongue	1.7	3.4	66.5
Neck Muscle	1.2	2.6	55.5
Mean for nine representative muscles.			
	2.0	3.9	67.5
S.D.	0.4	0.9	11.0

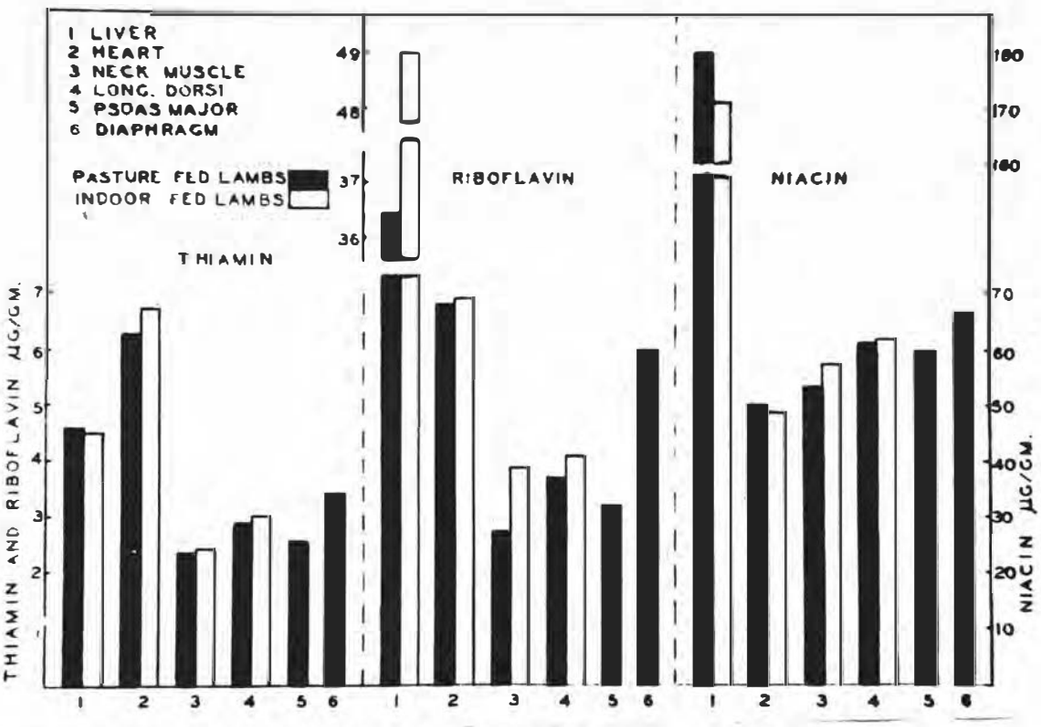


Fig.1 - Thiamin, riboflavin and niacin content of tissues from indoor-fed and pasture-fed lambs.

. . . . . .

In the carrying out of these assays a considerable saving of the time normally involved in plugging and handling test tubes may be affected by the use of small bottles such as 20 ml penicillin bottles. The normal rubber seal is removed and the bottles freed from any traces of penicillin by boiling with alkali. After running in the required amount of media and test solution the bottles are protected by their loose fitting aluminium caps only and placed in trays for autoclaving. Protected in this way the solutions remain sterile for considerably longer than the 72 hours required for carrying out most of the assays.

Discussion of Results

(a) Normal Outdoor-fed Animals

The animals of this group are of more interest since they are representative of normal New Zealand lamb and mutton. The tables show that although there is a fairly large variation between animals raised under

apparently identical conditions and between different muscles from the same carcase, the average values are of the same order as those reported overseas. The values for the mature ewe are slightly lower than the average for the lambs. Sufficient data is available from Tables 1 and 3 to enable correlations between the various vitamins to be calculated. These correlations are set out in Table 5.

TABLE 5

Correlation between vitamin content of various tissues.

	Correlation
Considering group of 12 lambs -	
Thiamin, riboflavin & niacin content of livers	N.S.
-do- hearts	N.S.
-do- neck muscles	N.S.
-do- other muscles	S.
B Group content of livers, hearts & muscles	N.S.
Considering individual animals -	
Thiamin, riboflavin & niacin content of muscles	S.

H.S. Significant at 1% level.
S. Significant at 5% level.
N.S. Not significant at 5% level.

. . . . . .

The lack of correlation between the vitamin content of hearts and other muscles is probably explained by the small variations found in the former which were in many cases only of the same order as the experimental error. As might be expected a significant (and in some cases highly significant) correlation exists between the amounts of the three vitamins in the muscles whether different muscles from the same carcase or corresponding muscles from different animals are considered. The variations between muscles from the one carcase are considerably greater than those between corresponding muscles from different animals.

These variations were much greater than could be explained by the small variations found in the fat and moisture content of the muscles and when calculated on a fat-free, moisture-free basis, variations in vitamin content remained of the same order. Wide variations have been reported in the amounts of connective tissue in different muscles (7) and it was thought that a negative correlation might exist between vitamin content and connective tissue. Using the enzyme digestion method of Mitchell and others, (7) the connective tissue was determined in the muscles samples taken from the mature ewe. No significant correlation was found but when calculated on a moisture, fat and connective tissue free basis, the variations in vitamin content were somewhat smaller.

The muscle samples assayed from these normal out-door fed lambs are regarded as representative of the whole carcase. Since the correlation already mentioned exists between the amounts of the three B vitamins in the various muscles, it is possible to assess the approximate average vitamin content of all muscles from a carcase by assays on one muscle only. Considering only the six lambs and the ewe where more than one muscle was assayed, the muscle with vitamin content most nearly equal to the average for all the muscles for each individual animal was the Longissimus Dorsi which is 105% of the mean with a standard deviation of 12%. From the point of view of accessibility however, a more suitable muscle to assay is the neck muscle which contains on the average 77% of the mean for each animal with the same standard deviation. The calculation can be carried a step further and an indication of the amounts of all three vitamins present obtained by the assay of one only. Probably the easiest vitamin to assay is niacin and from

this thiamin and riboflavin figures may be obtained from the curves given in Fig.2. These curves are drawn from the calculated equations :

Thiamin = 0.55 + 0.0325 Niacin

and Riboflavin = 0.23 + 0.047 Niacin

all expressed in ug/gram fresh weight.

It is probable that the calculation could be extended to other members of the B group.

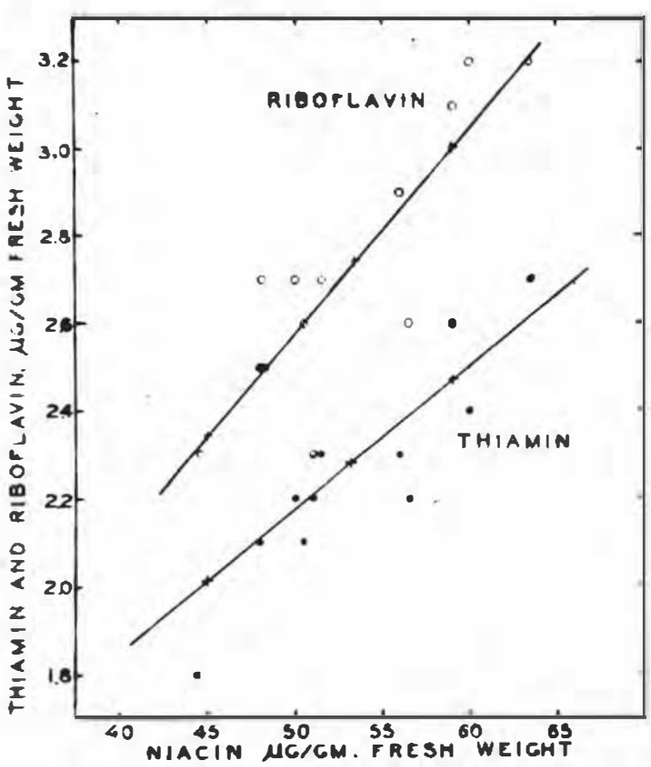


Fig.2 - Correlation between thiamin, riboflavin and niacin content of muscles.

(b) Indoor Fed Animals

The group of indoor fed lambs was much less uniform than the outdoor fed animals and a greater variation in vitamin content was shown in most cases. No significant correlations were found between vitamin content of the different tissues except in the case of the muscle samples from the single wether (Table 4). Tables 1 and 2 show that there is little difference in the mean thiamin and niacin content of the tissues from the two groups. There is however, a highly

significant difference in the riboflavin content of the livers and the muscles from the two groups indicating that the vitamin content of these tissues may be affected by the conditions under which the animals are raised. No conclusion can be drawn however, because of the lack of the correlation and the somewhat conflicting results from the wether listed in Table 4. The high riboflavin content may be due to the indoor diet favouring increased synthesis by the microflora of the rumen. It is more probable that the differences would be explained by breed or by factors associated with the indoor feeding, such as lack of exercise resulting in different degrees of development of the various muscles together with the poor condition of the lambs as compared with the outdoor fed group. No significant sex differences were found in either group.

Considering the extremely different conditions under which the two groups of animals were raised and the differences in condition at the time of slaughter, the differences in vitamin content of the tissues are surprisingly small. Unless there is a large seasonal or age variation it is not likely that lambs of average quality raised under the reasonably similar conditions existing throughout New Zealand would differ markedly from the figures shown in Table 1.

References

(1) Barton-Wright E.C.(1946): "Practical Methods for the Microbiological Assay of the Vitamin B Complex and Essential Amino Acids", Ashe Laboratories Ltd., London.

(2) McGillivray W.A., N.Z.J. Sci.& Tech. (in the press)

(3) McLaren B.A., Cover S., & Pearson P.B. (1944): Arch.Biochem.4, 1.

(4) Bathurst N.O., (1945): N.Z.J.Sci.& Tech., 27, 231.

(5) Harris L.J. & Wang Y.L. (1941): Biochem.J. 35, 1063.

(6) Ridyard H.N. (1946): J.Soc.Chem.Ind. 65, 92.