STUDIES ON CAROTENOID METABOLISM

By

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PREFACE

Studies on various aspects of carotenoid metabolism have been carried out in the Biochemistry Department now for a number of years. A considerable fillip was given this work in 1954 with the return of Dr. W.A. McGillivray from study leave overseas spent at the National Institute for Research in Dairying at Shinfield, working in collaboration with Dr. S.K. Kon and Dr. S.Y. Thompson, two senior British workers in the field; and again in 1955 with the reciprocal visit of the latter worker to this laboratory for a period of some nine months.

The work reported in this thesis was carried out in the department between April 1955 and June 1957 during which time two separate investigations on different aspects of carotenoid metabolism were undertaken. The first of these was concerned with a study of the utilization of parenterally administered carotenoids, particularly in small animals, and the other with experiments on factors affecting the carotenoid and vitamin A contents of milk fat, in particular, on factors affecting the summer decline in the carotenoid and vitamin A contents of New Zealand milk fat. In order to facilitate the presentation of the results of these investigations, the work is reported in two separate sections.

The results discussed are for the most part the author's own. Certain of the work described, however, was carried out on a collaborative basis with Dr. McGillivray, under whose general supervision the author worked, and also with Dr. Thompson during part of the time for which he was present

in the department. Due to the nature of the work involved it is not possible to state precisely the exact contribution made by the author to these collaborative studies, nor the extent to which, in the course of the work, he availed himself of the assistance of the above workers. It is emphasised, however, that the author took an active part at all stages of the investigation and is familiar with all the experimental methods and techniques described.

It is with pleasure the author expresses his sincere thanks to Dr. McGillivray for his continued help and encouragement throughout the course of the investigation and for many helpful criticisms in the preparation of this manuscript. Also to Dr. Thompson for his assistance and interest during the early stages of the work and for numerous stimulating discussions.

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SECTION I

STUDIES ON THE UTILIZATION OF PARENTERALLY ADMINISTERED CAROTENOIDS

INTRODUCTION

Prior to 1929 it was generally recognised that vitamin A activity was shared by two distinct substances, the yellow pigment carotene, and the colourless vitamin A of fish liver oils, although at that time it was not known that a definite biochemical inter-relationship existed between them. It was Moore, working at Cambridge, who first demonstrated that carotene was a precursor of vitamin A and that it was converted into the active vitamin within the animal body. In a series of preliminary experiments (Moore, 1929 a, b), he showed that carotene extracted from carrot fat was effective in restoring growth and curing xerophthalmia in vitamin A deficient rats. In a later series of experiments (Moore, 1930), he confirmed these results and proved beyond doubt that "carotene possesses the physiological action of vitamin A". His conclusions were based on the recovery of vitamin A, identified by the antimony trichloride reaction and the formation of a typical absorption band at 328 mμ, from the livers of vitamin A depleted rats fed a supplement of highly purified carotene. Subsequent research by numerous other workers (reviewed, for example, by Kon & Thompson, 1951) has amply confirmed these findings and has established conversion to take place in a wide range of animal species.

Moore's work, demonstrating the <u>in vivo</u> conversion of carotene to vitamin A, naturally directed attention to the site of conversion and although Moore himself did not investigate the problem directly, he suggested, on the basis of his earlier findings, that:

> ".....conversion to the vitamin is not effected in the course of digestion but at some period subsequent to absorption into the blood stream. Since the liver is unique not only in its ability to hold large stores of vitamin A, but also in containing noteworthy amounts of unchanged pigment, it would seem unnecessary to look beyond this organ for the site of conversion of carotene". (Moore, 1931).

Thus it was that interest was first stimulated in the fate of carotene administered by the parenteral route since, quite clearly, if its activity under such conditions could be demonstrated it would go far in support of the hepatic theory of conversion. Although, as is now well known, subsequent work has proved Moore's original suggestion incorrect and has unequivocally demonstrated the wall of the intestine to be the main locus of transformation of carotene to vitamin A in the animal (Glover, Goodwin & Morton, 1947, 1948a; Mattson, Mehl & Deuel, 1947; Thompson, Ganguly & Ron, 1947, 1949), nevertheless for some fifteen years the hepatic theory found general acceptance in the literature and over this period numerous studies on the conversion of parenteral carotene were reported.

Early studies were concerned mainly with the value of carotene administered in oily solution or colloidal form and in general produced conflicting results. Using the intravenous route, Wolff, Overhoff & Eckelen (1930) noted an increase in the liver vitamin A levels of deficient rabbits injected with carotene in oil. The animals were first partially hepatectomized to obtain a control sample of liver which was assayed using antimony trichloride. Carotene was then injected and three days later the animals were slaughtered and the vitamin content of the residual liver tissue was determined and shown to be appreciably higher than the control sample. By essentially the same technique these findings were confirmed in the rabbit with colloidal carotene by Ahmad, Grewal & Malik (1934) using liver storage and, in addition, resumption of growth as criteria. On the other hand the latter authors were unable to repeat these results in the rat or dog, and negative results were also obtained by Drummond & MacWalter (1935) in the rat. Similarly, in perfusion experiments in the cat, Ahmad (1931),

Ahmad & Drummond (1931), Rea & Drummond (1932) and Drummond & MacWalter (1933) were unable to find any evidence of formation of vitamin A in this species from parenterally administered carotene.

Using both the subcutaneous and intramuscular routes, Rydbom (1930) and Euler & Rydbom (1930) in the rat, guinea pig and chick, demonstrated that carotene was effective in causing resumption of normal growth in vitamin A deficient animals. In agreement with this finding Greaves & Schmidt (1935, 1937) noted in the rat a progressive improvement in the appearance of vaginal smears from deficient animals injected with carotene, although the response was very much less than when carotene was administered orally. Likewise, Lease, Lease, Steenbock & Baumann (1942) obtained similar results using restoration of growth and cure of xerophthalmia as criteria. In marked contrast to these observations, however, Rokhlina, Balakhovski & Bodrova (1943) found subcutaneously injected carotene to be completely devoid of vitamin A activity and concluded parenterally administered carotene to be ineffective as a source of vitamin A. Similarly, Popper & Greenberg (1941) and Popper (1944), using the technique of fluorescence microscopy, were never able to detect the presence of vitamin A in the livers of deficient rats following parenteral administration of carotene.

In view of this mass of conflicting evidence a thorough re-examination of the problem was undertaken by Sexton, Mehl & Deuel (1946). Their results finally left no doubt as to the complete ineffectiveness of carotene when administered parenterally either in oily or colloidal form. In a series of carefully controlled experiments in rats, using liver storage and restoration of growth as criteria, they showed quite conclusively that such

preparations, irrespective of the route by which they were injected, were not metabolized to vitamin A but tended to accumulate largely unchanged in the liver.

As a result of these findings the question of the value of parenterally administered carotene appeared settled. A short while later, however, Tomarelli, Charney & Bernhart (1946) reported successful attempts to demonstrate parenteral conversion using carotene solubilised in water with a surface-active agent. In studies with rats these workers compared the growth promoting activity of carotene administered parenterally and orally to deficient animals both as an oily solution and as an aqueous dispersion prepared using the surface-active agent Tween 80 (polyoxyethylenesorbitan monooleate). From the results of these studies they showed carotene injected as a dispersion in Tween was very much more effective in promoting growth than carotene injected in oil, and even slightly more effective than carotene administered by the oral route, and on the basis of these findings they suggested its value as a precursor of vitamin A after injection was related to its state of dispersion.

Although the work of Tomarelli et al. (1946) appears at the time largely to have been overlooked, the results of more recent studies have amply confirmed their findings and have demonstrated conversion of aqueous carotene dispersions to take place in a number of different species. Bicri (1949), Bieri & Schultze (1951), Bieri & Sandman (1951) and Bieri & Pollard (1954) working with rats; Hentges, Grummer & Sorensen (1952) with pigs; Church, MacVicar, Bieri, Baker & Pope (1954) with sheep; Kon, McGillivray & Thompson (1955) with rats and rabbits; and Bieri (1955 a) with chicks and rabbits, have all shown beyond reasonable doubt that in

these species parenterally administered carotene, providing it is presented in a suitable physical state, may be converted to vitamin A. In all these experiments aqueous dispersions of carotene in water-miscible surface-active agents of the Tween type were used. Employing similar preparations Eaton, Matterson, Decker, Helmboldt & Jungherr (1951) also reported conversion in the calf, although subsequently, Church et al. (1954) and Kon et al. (1955) were unable to confirm this.

On the basis of the above results it was decided in 1955 to undertake a series of experiments in this department to study further the utilization of parenterally administered aqueous dispersions of carotene in Tween. Such studies, it was hoped, would not only assist in some measure in clarifying certain issues arising from the earlier work, but would also provide some information concerning the nature of the reaction which was involved. The details and results of these experiments are discussed in the following nine chapters comprising Section I of this thesis.

In Chapter I the results of a number of experiments are described on the effect of the thyroid gland on the conversion of parenterally administered carotene, while in Chapters II and III a series of studies on the site of conversion is reported. Chapter IV is concerned with a description of various attempts to demonstrate the in vitro conversion of carotene to vitamin A, while Chapter V is devoted to a consideration of the results of a number of studies of the effect on conversion of such factors as the route of administration, the level of dosing, the interval between dosing and slaughter and repeated dosing; also reported are the results of studies on other factors possibly affecting conversion, such as the effect of high vitamin A liver storage and the effect of simultaneous administration of xanthophyll,

tocopherol and benzoyl peroxide. In addition, the results of a series of studies on the metabolism of aqueous dispersions of several carotenoid pigments other than β-carotene are also considered.

In Chapter VI are reported further studies on the effect of tocopherol on conversion, while in Chapter VII are discussed the results of experiments on the utilization of parenterally administered aqueous dispersions of carotene in the guinea pig, and of carotene and vitamin A in the goat, two species which hitherto have not been investigated. A study of the utilization of parenterally administered carotene and vitamin A in emulsion form in rats and goats is described in Chapter VIII, and an integrating summary of the work as a whole is presented in Chapter IX.

EXPERIMENTAL

MATERIALS AND METHODS

In this section materials and methods which relate to the investigation as a whole are described; a number of specialized techniques which relate only to particular aspects of the work are not included here but are discussed later at appropriate places in the main body of the thesis.

Rats

General. The rats used in these experiments were albino animals of the Wistar strain bred from stock introduced into New Zealand and maintained as an inbred colony since 1932 by Dr. I.J. Cunningham, Superintendent of the Wallaceville Research Station, Wellington. The stock-colony rats were maintained on a basal diet prepared in pellet form by W. & R. Fletcher (N.Z.) Ltd., of the following approximate percentage composition: ground wheat 42, ground barley 10.5, ground oats 4, dried skim milk 34, wheat germ 8, $CaCO_3$ 1 and NaCl 0.5. The diet was supplemented occasionally with raw liver and a proprietary emulsion "Vetemul", containing both vitamins A and D (Nicholas Products Ltd., Wellington).

Preparation of Partially deficient rats. For work in which blood levels and liver storage of vitamin A were employed as criteria, the rats were prepared in the following manner. Pregnant does were given the basal diet supplemented only with vitamin D (Glaxo Laboratories (N.Z.) Ltd.) for a week before parturition and throughout lactation. After weaning, usually at 3 weeks of age, the young continued to receive this diet and were generally used when 200-300 g in weight. They normally grew well on this diet without any additional supplementation. At slaughter very low levels of vitamin A were present in the

blood and only traces were present in the liver. Typical levels for 6 individual animals are shown in Table 1.

Table 1

The vitamin A content of the blood plasma and liver of "partially deficient" rats.

Rat no.	Blood Plasma		Liver	
	Vitamin A alcohol (µg/100 ml.)	Vitamin A ester (µg/100 ml.)	Vitamin A alcohol (µg)	Vitamin A ester (µg)
1	10	6.5	0.7	0.7
2	11	8.0	0.4	0.6
3	15	9.0	0.4	0.5
4	12	7.0	0.8	0.8
5	10	5.5	0.6	0.8
6	10	6.5	0.7	0.9
Mean	11	7.1	0.6	0.7

These animals are referred to throughout the present work as being "partially deficient".

Preparation of completely deficient rats. The rats used in the experiments in which resumption of growth and cure of xerophthalmia were employed as criteria, were treated up to the time of weaning as described in the previous section. After weaning, however, they were placed on the U.S.P. vitamin A-free test diet (Hawk, Oser & Summerson, 1947) until such time as their weight remained steady (a period of approximately 3 weeks), or until xerophthalmia developed (a period of 4 to 6 weeks). These animals are subsequently referred to in the present work as being "completely deficient".

Preparation of aqueous dispersions

Aqueous dispersions of carotene (88% β - carotene, supplied by L. Light & Co. Ltd., Poyle) were prepared using a 20% (v/v) solution in water of Tween 40* (Atlas Powder Co., Wilmington, Delaware), essentially as described by Bieri (1951) and Bieri & Pollard (1954). The pigment was weighed out (usually 10 mg), dissolved in a minimum of chloroform and 2 ml. of Tween were added. The mixture was heated to 70°+ on a hotplate and the chloroform removed under vacuum. 8 ml. of distilled water heated to 70° were quickly added and the flask swirled to assist mixing. Further vacuum was applied to remove the last traces of chloroform and the dispersion (concentration approximately 1 mg/ml.) was then ready for use. Dispersions which were not perfectly clear were rejected since, as has been shown by Bieri & Pollard (1954), carotene administered as a dispersion exhibiting even only a very slight degree of turbidity was much more rapidly removed from the blood by the tissues than carotene administered as a clear dispersion. In view of the fact that the length of time for which carotene circulates in the plasma after injection appeared to be an important factor influencing the amount of vitamin A formed (Chapter V), considerable attention was paid to this matter at all times.

Injection of dispersions

Dispersions were normally injected intravenously. For this purpose a 1 ml. syringe fitted with a 26 G x ¾" hypodermic needle was found most convenient. In the earlier stages of

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* Polyoxyethylenesorbitan monopalmitate

\+ All temperatures are expressed on the Centigrade scale.

the work, injection was made into the vena cava exposed by ventral mid-line incision, as described by Kon et al. (1955). Later, however, injection was made into the jugular vein, exposed by neck incision, or into the iliac vein, exposed by ventral leg incision. These latter techniques were found very much quicker and more convenient and required less surgical interference with the animal. Attempts were also made to inject via the tail vein, as described by Bieri & Pollard (1954). This method of administration, however, was not always successful and proved to be very time consuming and for these reasons was not adopted as a routine procedure.

Anaesthetic

Diethyl-ether anaesthesia was used in all experiments in which a general anaesthetic was required.

Measurement of carotene and vitamin A

Carotene and vitamin A in individual blood and liver samples were estimated essentially as described by Thompson et al. (1949) and Kon et al. (1955). Livers were assayed for carotene, vitamin A alcohol and ester; bloods, however, were assayed for carotene and vitamin A alcohol only, since, as has been shown by Kon et al. (1955), and later confirmed in preliminary experiments in this laboratory (see Table 2), variations in blood vitamin A ester after injection of carotene are very slight. A brief outline of the procedure is as follows.

Table 2

Demonstrating the uniformity of vitamin A ester, relative to vitamin A alcohol, in the blood of partially deficient rats at various intervals after injection of 400 µg of carotene in Tween.

No. rats	Interval between dosing & slaughter (h)	Blood plasma		
		Vitamin A alcohol (µg/100 ml.)	Vitamin A ester (µg/100 ml.)	Carotene (µg/100 ml.)
6 *	-	11	7.1 +	0
1	1/12	57	7.7 +	4100
2	1	48	6.8 +	2200
2	2	43	10 +	920
8	24	14	8.7 +	Trace

* Control rats; data from Table 1.

+ It should be pointed out that these levels of vitamin A represent actual density readings on the absorptiometer (see p.16) of only about 0.02. Normally the blue colour resulting from the interaction of antimony trichloride and vitamin A cannot be identified visually below a density reading of 0.05 - 0.06. The vitamin A values present in the table must, therefore, merely be regarded as "blank" values to which no particular significance can be attached.

Extraction procedure.

Blood. Blood was obtained from the anaesthetized animal by cardiac puncture after obtaining access to the chest cavity by ventral incision. The sample (usually 6-8 ml.) was taken in a 10 ml. syringe fitted with a 16 or 18 G needle and transferred to a 10 ml. centrifuge tube containing one drop of heparin ‡ (Evans Medical Supplies Ltd., London) to prevent coagulation. The sample was centrifuged

‡ Prepared at a concentration of 3000 units/ml.

at 2000 r.p.m. for 30 min to separate the plasma (usually 3-4 ml.) which was drawn off by means of a syringe, transferred to a 100 ml. separating funnel and made up to 10 ml. with distilled water. 10 ml. of ethanol were added to denature the plasma, and the mixture was extracted with 25 ml. of petroleum ether by shaking in a mechanical shaker for 5 min. The lower layer was run off and re-extracted with a further 25 ml. of petroleum ether as described above. The petroleum extracts were then combined, centrifuged, adjusted to volume, and the carotene present estimated. The solution was then carefully evaporated on a hotplate at 70° and taken up in 2-3 ml. of petroleum ether for chromatography.

Liver. After blood sampling the liver was removed from the animal and extracted under nitrogen in a Waring Blendor for 2-3 min with 15 ml. of distilled water, 30 ml. of ethanol and 100 ml. of petroleum ether. The mixture was transferred to a 250 ml. separating funnel and allowed to settle for 10-15 min. The bottom layer was run off and re-extracted with a further 100 ml. of petroleum ether and the extracts were combined, centrifuged and made to volume. The carotene present was then estimated and the extract carefully evaporated on a hotplate at 70° and taken up in 2-3 ml. of petroleum ether for chromatography.

Chromatographic separation.

Reagents.

Alumina: Aluminium oxide for chromatography, (May & Baker Ltd., Dagenham), Brockmann grade III. *

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* Determined using the method described by Williams (1946).

Solvent:* Petroleum ether, boiling range 60-80°, (British Drug Houses Ltd., Poole).

Eluents: 1. 2% acetone (v/v) in petroleum ether.
2. 8% ethanol (v/v) in petroleum ether.

Preparation of adsorption column.

The columns were formed in tubes (13 x 1.3 cm) by pouring a suspension of alumina in 8% ethanol (eluent 2) to a height of 3-4 cm. This was most easily carried out using a 250 ml. polythene wash bottle filled about one-quarter with alumina and one-quarter with the eluent. Then the column had settled the top was strengthened with 1 ml. of petroleum ether, and was then ready for immediate use. In practice 6 columns were found a convenient number to handle at the one time.

Use of adsorption column.

The extract in petroleum ether was poured onto the column which adsorbed vitamin A alcohol and allowed carotene and vitamin A ester to pass through; any fat present also moved through with this fraction. The chromatogram was then developed with 10-20 ml. of 2% acetone (eluent 1) which quantitatively stripped carotene and vitamin A ester off the column. Further development with 20-25 ml. of 8% ethanol (eluent 2) removed vitamin A alcohol. Care was taken at all times to avoid unnecessary exposure of the top of the column to air as under these circumstances there is likely to be an undue destruction of both carotene and vitamin A (cf. Thompson et al. 1949).

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* During the course of the work, owing to supply problems in this country, n-hexane, as used by Thompson et al. (1949), was replaced for all chromatographic work by petroleum ether, comparative experiments having established that there was no difference in the behaviour of the two solvents.

Estimation of carotene and vitamin A.

Carotene. Carotene was estimated spectrophotometrically in a Beckman, model DU, photoelectric spectrophotometer at a wave length of 450 mμ ($\lambda_{max.}$), using $E^{1\%}_{1cm}$ = 2500 (Morton, 1942).

Vitamin A alcohol. After chromatography the vitamin A alcohol fraction was evaporated carefully on a hotplace at 70°, taken up in chloroform, and estimated colorimetrically with antimony trichloride using a Hilger "Biochem" Absorptiometer (model H 810) which had been standardized against pure vitamin A palmitate (L. Light & Co. Ltd., Poyle).

Vitamin A ester. Vitamin A ester was estimated colorimetrically as for vitamin A alcohol. Where fatty material or pigment (e.g. carotene) was present, however, the fraction was first saponified with 1 ml. of 60% KOH and 5 ml. of ethanol on a hotplate for 5 min. 16 ml. of distilled water were added and the mixture was transferred to a 100 ml. separating funnel and extracted with two 20 ml. lots of freshly distilled diethyl ether. The extracts were combined, washed with distilled water and dried over anhydrous sodium sulphate. The combined extracts were then evaporated on a hotplate at 70°. If no carotene was present the residue was taken up in chloroform and estimated for vitamin A in the normal way. Where carotene was present, however, the residue was taken up in 2-3 ml. of petroleum ether and re-chromatographed. After elution with 2% acetone to remove carotene, the original ester was recovered as vitamin A alcohol in the 8% ethanol fraction. This was then evaporated to dryness, taken up in chloroform, and the vitamin A present estimated in the normal way.

Although at the time the present investigations were commenced all of the techniques just described were in standard use in overseas laboratories, a number had not previously been employed in this laboratory. For this reason a considerable amount of time was spent at the outset in establishing these techniques, and in checking materials and methods, before employing them for routine investigations. Much time was also spent in the preparation of the animals and in the general organization and running of the small-animal colony which was just in the process of being formed when the present studies were commenced. During the course of this early work a number of factors which were suspected as being of importance in influencing conversion were investigated in a preliminary manner. These preliminary studies represented very largely an extension of the earlier work of Kon et al. (1955) and were of value not only in providing a suitable background for later work but also in indicating a number of possible future lines of investigation. As most of these earlier studies were later repeated in much greater detail, the results are not reported separately but have been intergrated with subsequent work and presented in the appropriate chapters of the thesis.

CHAPTER I

THE EFFECT OF THE THYROID ON THE CONVERSION OF INTRAVENOUSLY ADMINISTERED AQUEOUS DISPERSIONS OF CAROTENE TO VITAMIN A IN THE RAT

Introduction

Numerous reports have appeared concerning the effect of the thyroid on the conversion of carotene to vitamin A. The earlier work has been reviewed by Drill (1943) who concluded: "All the evidence to date indicates that in the absence of the thyroid gland carotene is not metabolised to vitamin A". The results of later work, however, appear somewhat confusing. Thus Crandall & Valdecanas (1947) reported that they were unable to prevent ocular symptoms in hypothroid rats following oral administration of carotene while Drill & Truant (1947) reported similar findings in thyroidectomised rats following subcutaneous administration of carotene in sesame oil. Remington, Harris & Smith (1942), on the other hand, noted that oral dosing with carotene was equally as effective as vitamin A in curing eye conditions in thyroidectomised rats.

Johnson & Baumann (1947) and Kelley & Day (1948), using liver storage of vitamin A as a criterion, found that, after the same oral dose of carotene, animals rendered hypothyroid stored less vitamin A than controls. In contrast, however, Wiese, Mehl & Deuel (1948), using growth as a criterion, showed that the same amount of carotene was required to elicit a fixed growth response in hypothyroid animals as in normal animals.

In an effort to collate these divergent findings it was thought desirable that a study should be made of the effect of the thyroid on the conversion of carotene administered by the parenteral route. Parenteral administration was chosen in preference to the oral route in order to overcome any possible effects that variations in absorption of carotene or its vehicle

might have on the outcome of the results (e.g. Cama & Goodwin, 1949). The present chapter describes the results of these studies using blood levels and liver storage of vitamin A, and in addition cure of xerophthalmia, as criteria.

Experimental

General. The materials and methods used were essentially as described earlier with the following additions:

Rats. The rats used in Exps. 1, 2 and 3 (weight range 150-300 g) were partially deficient in vitamin A and were maintained on the basal diet already described. The rats used in Exp. 4 (weight range 100-118 g) were completely deficient, as indicated by the presence of ocular symptoms, and were maintained on the U.S.P. vitamin A-free test diet (Hawk et al. 1947).

Rats were rendered hypothyroid either by surgical removal of the thyroids or by treatment with 2-thiouracil (May & Baker Ltd., Dagenham), or were rendered hyperthyroid by injections with the sodium salt of l-thyroxine (Burroughs Wellcome & Co., London), the dosage levels used (see Tables 3, 4 and 5) being those which reference to the literature suggested would give the desired degree of hypo- or hyperthyroidism (e.g. Johnson & Baumann, 1947; Kelley & Day, 1948; Wiese et al. 1948). All animals not thyroidectomized, including controls, were subjected to sham operation. As a measure of the effects of these treatments the oxygen consumption of the rats was determined by a method essentially as described by Morrison (1947). The determinations were made on groups of up to three rats in three consecutive 15 min periods.

All carotene injections were made into the vena cava exposed by ventral mid-line incision.

Results

The results of preliminary experiments on the effect of thyroid activity on the conversion of intravenously administered aqueous dispersions of carotene to vitamin A are presented in Table 3 (Exps. 1 and 2) from which it is apparent that, despite wide differences in thyroid activity, as indicated by oxygen consumption figures, the thyroid has little effect on conversion. Thus in the first study, 24 h after the injection of 400 µg of carotene in Tween, vitamin A in the liver of hyperthyroid and thyroidectomized animals increased from a control level of 0.7 µg/liver to 9.3 and 9.0 µg/liver, respectively, while blood levels increased from 10 µg/100 ml. plasma in controls to 14 µg/100 ml. plasma in both treated groups. Similarly, in the second study, in which a different Tween preparation of carotene was used, liver vitamin A levels of normal, hyperthyroid, hypothyroid and thyroidectomized animals increased from a control level of 0.7 µg/liver to 12, 13, 19 and 13 µg/liver, respectively, while blood levels increased from a control level of 10 µg to 25, 11, 23 and 18 µg/100 ml. plasma, respectively.

In later experiments (Table 4, Exp. 3), in which higher dosage rates of thyroxine and thiouracil were employed for a longer period prior to injection of carotene, the above findings were further confirmed. Thus, 24 h after injection of 400 µg of a Tween dispersion of carotene, liver vitamin A levels in normal, hyperthyroid, hypothyroid and thyroidectomized animals

Table 3. The effect of thyroid activity on the conversion of carotene to vitamin A 24 h after intravenous administration of carotene as an aqueous dispersion in Tween to rats partially deficient in vitamin A.

Exp.	Rats		Treatment	Oxygen consumption	Dose administered	Blood plasma		Liver			
	No. used	Mean weight (g)		(ml./kg/h)	(µg)	Vitamin A alcohol (µg/100 ml.)	Carotene (µg/100ml.)	Vitamin A Alcohol (µg)	Vitamin A Ester (µg)	Vitamin A Total (µg)	Carotene (µg)
1	2	200	None	-	-	10	0	0.6	0.1	0.7	0
	6	150	Sham operation, 10 µg thyroxine subcutaneously daily for 15 days before injection	1700	400	14	64	4.2	5.1	9.3	46
	6	165	Thyroidectomy 15 days before injection	1200	400	14	62	4.2	4.8	9.0	44
2	4	200	Sham operation 21 days before injection	1300	400	25	Trace	5.1	7.2	12	9
	4	190	Sham operation, 20 µg thyroxine subcutaneously daily for 21 days before injection	1800	400	11	Trace	5.3	7.3	13	10
	5	230	Sham operation 100 mg thiouracil orally daily for 21 days before injection	1000	400	23	Trace	7.7	11	19	10
	5	230	Thyroidectomy 21 days before injection	980	400	18	Trace	5.1	7.9	13	13

Table 4. The effect of thyroid activity on the conversion of carotene to vitamin A 24 h after intravenous administration of carotene as an aqueous dispersion in Tween to rats partially deficient in vitamin A.

Exp.	Rats		Treatment	Oxygen consumption	Dose administered	Blood plasma		Liver			
	No. used	Mean weight (g)		(ml/kg/h)	(µg)	Vitamin A alcohol (µg/100 ml.)	Carotene (µg/100ml.)	Vitamin A			Carotene
								Alcohol (µg)	Ester (µg)	Total (µg)	(µg)
3	2	230	Sham operated 28 days prior to injections	1460	-	11	0	0.5	0.6	1.1	Trace
	3	230	Sham operated 28 days prior to injection	1460	400	19	Trace	4.4	6.6	11	15
	5	200	Sham operated. 30 µg l-thyroxine subcutaneously daily for 28 days prior to injection	2180	400	17	Trace	5.0	5.6	11	11
	5	300	Sham operated. 250 mg thiouracil daily in feed for 28 days prior to injection	830	400	21	Trace	5.2	7.6	13	17
	5	300	Thyroidectomised. 100 mg thiouracil daily in feed for 28 days prior to injection	960	400	18	Trace	3.8	6.4	10	14

Table 5. The effect of the thyroid on the remission of xerophthalmia and on weight increases in rats following intravenous injection of carotene as an aqueous dispersion in Tween.

Exp.	No. rats used	Mean weight (g)	Treatment	Dose administered (µg)	Interval over which ocular symptoms disappeared in group (days)	Wt. increase after injection; 14 day period (g)
4	2	100	Sham operated at first sign of xerophthalmia. Injected 4 days later.	400	6 - 10	21
	5	108	Sham operated at first sign of xerophthalmia. 15 µg l-thyroxine daily for 4 days prior to injection.	400	5 - 10	24
	5	115	Sham operated at first sign of xerophthalmia. 150 mg thiouracil in feed daily for 4 days prior to injection.	400	5 - 11	14
	6	118	Thyroidectomised at first sign of xerophthalmia. Injected 4 days later.	400	7 - 9	19

increased from 1.1 µg/liver in controls to 11, 11, 13 and 10 µg/liver, respectively, while plasma vitamin A levels increased from 11 µg/100 ml. in controls to 19, 17, 21 and 18 µg/100 ml., respectively.

That the vitamin A appearing after injection was physiologically active and that its activity under the present experimental conditions was in no way affected by thyroid activity were confirmed by the results presented in Table 5 (Exp. 4). After injection of carotene ocular symptoms were relieved equally effectively in all four experimental groups, while weight increases in all groups, with the exception of that treated with thiouracil, were also approximately the same.

Discussion

The main aim of the present experiments was to determine whether the activity of the thyroid gland had any influence on the course of conversion of intravenously administered aqueous dispersions of carotene to vitamin A. Conflicting reports are still appearing in the literature concerning the effect of the thyroid on the conversion of carotene to vitamin A following oral administration, some workers claiming conversion to be impaired by hypothyroidism and enhanced by hyperthyroidism. Cama & Goodwin (1949) have suggested, however, that these differences may be associated more directly with the effect of the thyroid on intestinal absorption than with its effect on the mechanism of conversion. Parenteral administration of carotene, providing that the carotene is presented in a state suitable for utilization by the animal, offers a convenient

means of studying any direct effect which the thyroid may have on the conversion mechanism without the complicating effects of intestinal absorption.

From the results presented above it is clearly evident that thyroid activity has no direct effect on the conversion of intravenously administered carotene to vitamin A. This conclusion is based on increases in the vitamin A levels of the liver and blood after injection of a single dose of carotene into normal, hyperthyroid, hypothyroid and thyroidectomized rats, and on remission of specific symptoms of vitamin A deficiency after injection of carotene into animals similarly treated. The results confirm, therefore, the work carried out with orally administered carotene by Remington et al. (1942) and Wiese et al. (1948). They contrast, however, with that of Canadell & Valdecasas (1947), Johnson & Baumann (1947) and Kelley and Day (1948), due probably to differences associated with intestinal absorption. They contrast also with the results of Drill & Truant (1947) obtained with parenterally administered carotene but an explanation for this is difficult to offer.

Throughout the present experiments it is of interest to note the significantly higher liver vitamin A storage in the groups rendered hypothyroid with thiouracil (Tables 3 and 4), an irregularity which has also been reported in blood by Bieri (1949) and which would appear to be attributable to thiouracil per se rather than to the thyroid. The somewhat smaller weight increase in the hypothyroid group after injection (Table 5) would also appear to confirm the observations of Wiese et al. (1948), who have emphasized that when assessing

the effect of drugs such as thiouracil on carotene metabolism by biological assays involving weight increases, allowance must be made for the growth inhibiting action of the drugs themselves.

Summary

1. The effect of thyroid activity on the conversion of parenterally administered carotene to vitamin A has been studied in rats. Animals were rendered hyperthyroid by subcutaneous injection of l-thyroxine and hypothyroid by oral administration of thiouracil and by thyroidectomy. As a measure of the effectiveness of these treatments oxygen consumptions were estimated.

2. Following injection of carotene in Tween into normal, hyperthyroid, thiouracil-treated and thyroidectomised animals which were almost depleted of vitamin A, blood vitamin A levels increased uniformly in all four groups. Likewise, liver vitamin A levels increased uniformly with the exception of the thiouracil-treated groups which were invariably higher.

3. After injection of carotene in Tween into similarly treated groups which were completely deficient in vitamin A and suffering from xerophthalmia, eye symptoms disappeared at a comparable rate in all four groups and weight increases following injection, with the exception of the thiouracil-treated group which was lower, were approximately equal.

4. From these results it is concluded that the thyroid has no effect on the conversion of intravenously administered carotene to vitamin A, and that any apparent effect following oral administration is more likely to be associated with the absorption of carotene, or its vehicle, rather than with the mechanism of conversion.

CHAPTER II

STUDIES ON THE SITE OF CONVERSION OF INTRAVENOUSLY ADMINISTERED AQUEOUS DISPERSIONS OF CAROTENE TO VITAMIN A IN THE RAT AND RABBIT

I. The Effect of Complete Hepatectomy and Evisceration

Introduction

It is now well established that carotene, administered intravenously as an aqueous dispersion in Tween, can be converted to vitamin A in the rat, rabbit, pig, sheep and chick (e.g. Kon et al. 1955). The site at which conversion occurs, however, has not as yet been determined. It was originally suggested by Mattson et al. (1947) that parenterally administered carotene might be utilized after being secreted by way of the bile into the intestine, the normal site of conversion after oral administration. Bieri & Pollard (1954), however, could find no decrease in conversion in rats in which the bile duct had been completely ligated, or in rats from which the small intestine had been removed, and concluded that rats can effectively convert injected carotene at a site other than the small intestine. In further experiments the same authors showed that in nephrectomized rats, and in rats from which up to 75% of the liver tissue had been removed, the amounts of vitamin A formed after intravenous injection of carotene dispersions were essentially similar to the amounts formed in intact controls. Though it appeared probable from these experiments that the utilization of carotene was independent of the liver, the possibility remained that sufficient functional tissue was present to effect conversion. For this reason it was felt desirable to repeat the experiments in the complete absence of the liver. A series of experiments was therefore undertaken using hepatectomized and hepatectomized-eviscerated rats, and hepatectomized rabbits. The results of these experiments are described in the present chapter.

Experimental

General. The materials and methods were as described previously with the following additions:

Rats. The rats used in these experiments were maintained on the basal diet previously described and, unless otherwise stated, were all partially deficient in vitamin A.

Surgical procedure in the rat. Owing to the difficulties of either cannulation* or the establishment of collateral circulation in the rat, a simple acute technique was developed for removal of the liver. After subcutaneous injection of 1 ml. of a saturated glucose solution the animals, maintained under diethyl-ether anaesthesia, were opened by ventral mid-line incision extended to the left laterally as near as possible to the ribs. The aorta was ligated as near as possible to the pleural cavity, the portal vein was ligated as near as possible to the liver, and the vena cava below and above the liver. The liver, including the section of the vena cava embedded in it, was then removed. The operated animals received the dose of carotene in Tween by the jugular vein and were slaughtered up to ½ h later, this being about the maximum period for which the animals could be kept alive. Blood samples were obtained in the normal way by cardiac puncture.

In experiments in which evisceration was performed the rats used were stock-colony does containing normal liver stores.

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* Attempts were made initially to cannulate the portal vein and divert its flow into the vena cava prior to removing the liver, but the technical difficulty associated with this operation were so great as to render the technique impracticable.

After removal of the liver, the viscera, comprising the stomach, small intestine, large intestine, kidneys, pancreas, adrenals and gonads were completely removed. The cavity was then lightly swabbed with 1 in 30,000 adrenaline solution prior to injection of carotene in Tween into the jugular vein. The hepatectomised-eviscerated animals were re-anaesthetized after ½ h and blood samples obtained by cardiac puncture.

Rabbits. The rabbits used were from an inbred smooth-coated albino strain supplied by the Ruakura Animal Research Station small-animal colony. They were maintained on a basal diet similar to that of the rats and received in addition a generous allowance of fresh grass daily. Their vitamin A blood levels and liver stores would, therefore, be expected to lie within the normal range.

Surgical procedure in the rabbit. The technique of liver removal was, in the main, similar to that described above in the rat. After subcutaneous injection under the foreleg of 3 ml. of saturated glucose solution, the anaesthetized animals were opened by ventral mid-line and left lateral incision. The aorta was ligated directly above the junction of the coeliac artery, the vena porta as closely as possible to the liver and the vena cava immediately below the liver. Next, the liver was tied off below the diaphragm, and the whole organ removed. A 10 ml. control blood sample was then taken from the lower vena cava. Carotene in Tween was immediately injected directly into the jugular vein and the animals allowed to recover from the anaesthetic; they were again anaesthetized 1-2 h later and bled by cardiac puncture. From animals of sufficient size an additional 10 ml. blood sample was also taken from the

jugular vein after half an hour.

Sampling and assay of blood. After removal of the liver by the method described above, the blood circulation of the animal was virtually restricted, apart from a small amount of collateral circulation through the body wall, to the anterior part of the body. In the rat this situation made it difficult to obtain sufficient blood from individual animals for assay purposes. For this reason blood was usually bulked from two or three rats, so as to yield not less than 3 ml. of plasma. In order to gain some idea of the extent of collateral circulation to the posterior part of the body after liver removal, blood samples were also taken from the vena cava of the hepatectomized rats at slaughter and similarly bulked for assay. This procedure was not applied to rabbits, however, because, as noted earlier, after removal of the liver and immediately before injection all blood in the vena cava was withdrawn to provide the predosing (control) sample.

Results

Conversion in hepatectomized rats. The results presented in Table 6 (Exps. 1 and 2) clearly demonstrate that removal of the liver has little effect on the formation of vitamin A from injected carotene, the amounts of vitamin A appearing after injection being similar to those in intact rats. Thus in Exp. 1, within 5 min and ½ h of injection of 400 µg of carotene in Tween into hepatectomized rats, plasma vitamin A alcohol increased from a control value of 10 µg/100 ml. to 57 and 50 µg/100 ml., respectively. Similarly in Exp. 2, in which a different carotene dispersion was used, within

10 min of injection of 320 µg of carotene plasma vitamin A alcohol rose from a value of 10 µg/100 ml. to a value of 49 µg/100 ml. while within ¼ h and ½ h of injection, respectively, values of 29 µg/100 ml. and 45 µg/100 ml. were recorded. Injection of intact animals with the same dispersion resulted in an increase in plasma vitamin A alcohol from a control level of 13 µg/100 ml. to 42 µg/100 ml.

It is interesting to note that carotene was present in blood from the posterior vena cava of hepatectomized rats at slaughter in concentrations ranging from 15-25% of that in the anterior blood obtained by heart puncture (Exp. 2). Although this finding confirms the presence of collateral circulation through the body wall to the lower part of the animal, the extent of the circulation and its bearing on the present studies would appear to be limited.

Conversion in hepatectomized-eviscerated rats. From the results presented in Table 6 (Exp. 3) it is clearly evident that hepatectomized-eviscerated rats are capable of converting intravenously injected carotene to vitamin A. Thus within ¼ h of injection of 320 µg of carotene in Tween, plasma vitamin A alcohol levels in treated animals were shown in the present experiments to increase from a control value of 10 µg/100 ml. to 40 µg/100 ml. i.e. to a value similar to that recorded for hepatectomized and intact animals.

Conversion in hepatectomized rabbits. In rabbits the overall picture was found to be essentially the same as in rats although increases in plasma vitamin A alcohol levels following injection of carotene in Tween into hepatectomized animals were not nearly

Table 6. Appearance of carotene and vitamin A alcohol in the blood of partially deficient hepatectomized and hepatectomized-eviscerated rats after intravenous injection of Tween dispersions of carotene.

Exp.	Rats		Treatment	Dose				Time between injection and slaughter (h)	Blood plasma		
				Vehicle		Substance			From heart		From vena cava
	No. used	Mean weight (g)		Nature	Amount (ml.)	Nature	Amount (µg)		Vitamin A alcohol (µg/100 ml.)	Carotene (µg/100 ml.)	Carotene (µg/100 ml.)
1	3	-	None	None	-	None	-	-	10	0	-
	3	-	Hepatectomy	Tween dispersion	0.4	Carotene	400	$\frac{1}{12}$	57	6500	-
	3	-	Hepatectomy	Tween dispersion	0.4	Carotene	400	$\frac{1}{2}$	50	3850	-
2	2	250	None	None	-	None	-	-	13	0	-
	2	240	None	Tween dispersion	0.4	Carotene	320	$\frac{1}{2}$	42	1400	-
	3	250	Hepatectomy	None	-	None	-	$\frac{1}{4}$*	10	0	0
	3	230	Hepatectomy	None	-	None	-	$\frac{1}{2}$*	10	0	0
	3	230	Hepatectomy	Tween dispersion	0.4	Carotene	320	1/6	49	3300	500
	3	250	Hepatectomy	Tween dispersion	c.0.4	Carotene	300+	$\frac{1}{4}$	29	4000	640
	12	230	Hepatectomy	Tween dispersion	0.4	Carotene	320	$\frac{1}{2}$	45	2600	650
3	2	370	Hepatectomy and eviscoration	None	-	None	-	$\frac{1}{4}$*	10	0	-
	4	370	Hepatectomy and eviscoration	Tween dispersion	0.4	Carotene	400	$\frac{1}{2}$	40	3300	-

* Interval between hepatectomy and slaughter

+ Short dose

Table 7. Appearance of carotene and vitamin A alcohol in the blood of hepatectomized rabbits after intravenous injection of Tween dispersions of carotene.

Rabbits		Dose				Time between injection and sampling (h)	Blood Plasma		
		Vehicle		Substance			Vitamin A alcohol		Carotene
No. used	Mean weight (kg)	Nature	Amount (ml.)	Nature	Amount (µg)		(µg/100 ml.)	Change (µg/100 ml.)	(µg/100 ml.)
2	2.0	None	-	None	-	-	73		0
						1	60	-13	
2	2.2	Distilled water	1.0	None	-	-	40		0
						2	24	-16	0
2	1.6	Tween dispersion	1.0	None	-	-	51		0
						1	31	-20	0
3	2.0	Tween dispersion	1.0	None	-	-	68		0
						2	41	-27	0
1	3.0	Tween dispersion	1.0	Carotene	920	-	52		0
						½	57	+ 5*	1330
						1	61	+ 9	1130
2	3.2	Tween dispersion	1.0	Carotene	920	-	46		0
						½	58	+12*	1420
						2	56	+10	1280
2	2.0	Tween dispersion	1.0	Carotene	920	-	63		0
						1	72	+ 9	2280
2	2.2	Tween dispersion	1.0	Carotene	920	-	58		0
						2	68	+10	1540

* Mean increase of 3 animals at ½ h = 9.7 µg/100 ml.

so great, an observation confirming that made previously by Kon et al. (1955) working with intact animals. Thus from Table 7 it will be seen that the increases recorded within ½ h, 1 h and 2 h of injection of 920 µg of carotene were 9.7, 9.0 and 10 µg/100 ml. plasma, respectively, above the normal level. On the other hand, however, values recorded in undosed animals or animals dosed with distilled water or Tween in the absence of carotene, showed a spectacular decline to values of from 13 to 27 µg/100 ml. plasma below the normal level within 1-2 h of liver removal. A consideration of these two opposing trends would appear, therefore, to leave little doubt that in the rabbit, as in the rat, complete hepatectomy has little effect on the course of conversion of intravenously administered aqueous dispersions of carotene into vitamin A.

Discussion

The main purpose of the present experiments was to confirm whether animals normally capable of converting intravenously administered aqueous dispersions of carotene to vitamin A could do so in the complete absence of the liver. In rats, the results obtained provide strikingly clear evidence, based on increases in plasma vitamin A alcohol levels after injection, that in the hepatectomized animal carotene in Tween is converted to vitamin A at an apparently normal rate. Similarly, in hepatectomized-eviscerated animals, formation of vitamin A appeared to proceed at an undiminished rate after injection of dispersions of carotene.

In rabbits the increases in plasma vitamin A alcohol levels noted after injection of carotene into hepatectomized animals were considerably smaller than in rats, owing possibly to the lower dose of carotene administered, on a weight basis, or to a species difference in the efficiency or rate of conversion (Kon et al. 1955). Since, however, in undosed animals and in animals dosed with distilled water or Tween alone, plasma alcohol levels showed a steady decrease after hepatectomy, and since carotene administration offset and reversed this trend, there would appear to be no doubt that the formation of vitamin A following injection of carotene dispersions into hepatectomized rabbits occurs independently of the liver.

After these downward trends in vitamin A plasma levels were observed in operated rabbits not dosed with carotene, efforts were made to check whether or not a similar situation existed in rats. This was made difficult, however, by the fact that the plasma vitamin A alcohol levels of our normal or deficient rats were not increased significantly by massive oral doses of vitamin A concentrate, so that efforts to check the possibility of any decline in the vitamin A in the blood of rats after hepatectomy met with no success.

The reason for the sharp drop in vitamin A in the blood of hepatectomized rabbits not dosed with carotene is at present a matter for conjecture. The decrease may possibly provide a measure of the rate at which vitamin A is metabolized by the tissues which are still functioning in the animal. It could also possibly be associated with and due to the shocked condition of the animal following surgery, although this is problematical since Kon et al. (1955) found marked fluctuations in the blood

of rabbits even after simple venepuncture and a frank decrease after venepuncture of rabbits injected with Tween. In any event the decrease would appear to be quite independent of any toxic or injurious effects of Tween since no significant difference in behaviour could be observed between those animals injected with Tween and those injected with distilled water.

It is significant to note that, as a result of the conditions under which these experiments were carried out, the formation of vitamin A after injection of carotene in Tween has been shown to be independent of not only the liver but also of many other organs and tissues. For example, as previously reported by Bieri & Pollard (1954), the appearance of vitamin A has been shown in the present series of experiments to be unaffected by ligation of the bile duct or by removal of the small intestine and kidneys. Similarly, the level of vitamin A circulating after injection was not diminished by extirpation of the stomach, large intestine, pancreas, kidneys, adrenals and gonads, or by complete ligation of all the major blood vessels supplying the lower part of the body.

Consideration of these results would appear to lend support to the conclusion of Bieri & Pollard (1954), with which Kon et al. (1955) concur, "that perhaps many tissues possess the ability to convert carotene". The further possibility must, however, also be considered that other tissues in the anterior part of the body may be specifically involved in conversion. Experiments were therefore planned to test this possibility, the results of which are presented in the following chapter.

Summary

1. The utilization of intravenously injected Tween dispersions of carotene has been studied in hepatectomized and hepatectomized-eviscerated rats and hepatectomized rabbits.

2. After injection of 300-320 µg of carotene into hepatectomized rats, plasma vitamin A alcohol increased markedly from the control level of 10 to as high as 57 µg/100 ml. at various intervals up to ½ h after injection.

3. In hepatectomized-eviscerated rats injection of 320 µg of carotene caused the level of vitamin A alcohol in the plasma to increase from a control value of 10 to 40 µg/100 ml. within ½ h.

4. Injection of 920 µg of carotene into hepatectomized rabbits caused an increase in vitamin A alcohol in the blood of up to 10 µg/100 ml. plasma within 2 h. In the absence of injected carotene, however, alcohol levels decreased in a spectacular fashion after hepatectomy, a maximum drop of 27 µg/100 ml. plasma being recorded after 2 h.

5. From these results it is concluded that the conversion of intravenously administered aqueous dispersions of carotene to vitamin A is not adversely affected by complete removal of the liver in rabbits, or by complete removal of the liver and viscera in rats.

CHAPTER III

FURTHER STUDIES ON THE SITE OF CONVERSION OF INTRAVENOUSLY ADMINISTERED AQUEOUS DISPERSIONS OF CAROTENE TO VITAMIN A IN THE RAT

II. The Effect of Removal of the Lungs and Decapitation

Introduction

In Chapter II it was demonstrated that in the rat the formation of vitamin A from carotene, administered intravenously as an aqueous dispersion in Tween, was unaffected by complete removal of the liver, stomach, small intestine, large intestine, pancreas, kidneys, adrenals and gonads. While these results were regarded as support for the conclusion of Dieri & Pollard (1954), with which Kon et al. (1955) concurred, "that perhaps many tissues possess the ability to convert carotene", the possibility still remained that perhaps other tissues in the anterior part of the body were specifically involved in conversion. From the evidence presented in Chapter I there appeared to be good grounds for rejecting the thyroid as the site of conversion. The possibility still remained, however, as suggested by Kon et al. (1955), that the lungs were specifically involved in the breakdown of injected carotene. It was thought important, therefore, that this possibility should be further investigated. The present chapter describes the results of this work together with the results of a further study concerning the effect of decapitation on the conversion of intravenously administered carotene into vitamin A.

Experimental

General. The materials and methods used were similar to those used earlier with the following additions:

Rats. The rats used were partially deficient in vitamin A and maintained on the basal diet previously described.

Surgical procedure in the rat.

Removal of the lungs. For anaesthesia a combination of

veterinary nembutal (Abbott Laboratories Ltd., London), injected subcutaneously, and diethyl ether was used. Nembutal was used to ensure prolonged anaesthesia since it was not possible to administer further ether once surgery had commenced. Firstly, the trachea was exposed and a cannula of nylex tubing (1 mm internal diameter) inserted and connected to a manually-operated "respirator"* containing pure oxygen. Next, the abdomen was opened by ventral mid-line and left and right lateral incisions as near as possible to the ribs, and the pleural cavity opened by two parallel incisions on either side of the sternum. At the same time, with the aid of an assistant, artificial respiration was applied by means of the respirator. Successive lobes of lung tissue were then quickly tied off and completely removed. Finally, 0.4 ml. of a solution of carotene in Tween plus 0.1 ml. of a 1 in 50,000 solution of adrenaline were injected directly into the vena cava and blood samples were taken by cardiac puncture approximately 5 min later (range 5 - 7 min), just as the action of the heart started to weaken.

Removal of the head. Although there was no convincing evidence to suggest that any of the organs and tissues of the head or neck was specifically involved in the breakdown of injected carotene it was thought advisable, for the sake of completeness, to check this point. A collar of fur was first cut from around the neck of the anaesthetized animal. The left carotid artery was then dissected out, tied and cut,

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* Illustrated in Plate 1.

followed by the left jugular vein. Next the right carotid artery was tied and cut followed by the right jugular vein. The whole head was then severed below the larynx and the stump swabbed liberally with a 1 in 30,000 solution of adrenaline. Two small arteries lying dorsally on either side of the spinal column were quickly clamped and the animal was opened and an injection given into the vena cava. Blood samples were obtained by cardiac puncture 5 min later.

In both series of experiments livers as well as bloods were taken for assay.

Results

The effect of removing the lungs.

From the results presented in Table 8 (xp. 1) it is clear that there was little apparent difference between the amount of vitamin A formed in intact animals after injection of carotene and the amount formed in animals from which all functional lung tissue was removed, plasma vitamin A alcohol levels of both groups increasing from the control level of 17 µg/100 ml. to 39 and 43 µg/100 ml., respectively, within 5 min. Liver levels of vitamin A of both treated groups, however, showed no increase over the control group due no doubt to the short time interval involved.

The effect of removing the head.

The results in Table 8 (Exp. 2) provide clear evidence that no part of the head or neck is in any way specifically involved in the conversion of injected carotene to vitamin A, blood levels of vitamin A alcohol within 5 min of injection

Table 8. Appearance of carotene and vitamin A in the blood and livers of pneumatectomized (Exp. 1) and decapitated (Exp. 2) rats 5 min after injection of carotene in Tween. (Mean weight 250 g).

Exp.	No. rats used	Treatment	Dose				Blood Plasma		Liver		
			Vehicle		Substance		Vitamin A Alcohol	Carotene	Vitamin A ester	Vitamin A alcohol	Carotene
			Nature	Amount (ml.)	Nature	Amount (µg)	(µg/100 ml.)	(µg/100 ml.)	(µg)	(µg)	(µg)
1	3	None	None	-	None	-	17	0	0.8	0.4	Trace
	3	None	Tween dispersion	0.4	Carotene	400	39	4500	0.7	0.5	Trace
	9	Lungs removed	Tween dispersion	0.4	Carotene	400	43	5000	1.1	0.7	Trace
2	2	None	None	-	None	-	19	0	0.8	0.6	Trace
	1	None	Tween dispersion	0.4	Carotene	400	46	6800	1.0	0.7	Trace
	5	Head removed	Tween dispersion	0.4	Carotene	400	40	7600	0.9	0.6	Trace

showing an increase from 19 µg/100 ml. plasma in controls to a mean level of 40 µg/100 ml. in operated animals i.e. to a level comparable with the treated groups in the previous experiment.

Discussion

In earlier experiments reported in Chapters I and II it was shown that removal of the thyroid, liver or viscera had no influence on the animal's ability to convert injected carotene to vitamin A. The aim of the present experiments was to determine what effect, if any, removal of the lungs or the head and neck had on this function. From the results it is evident that in neither case could any effect be demonstrated. It would appear probable, therefore, both from this evidence and from that submitted previously by Bieri & Pollard (1954) and Kon et al. (1955), that the ability to convert injected carotene is an attribute not of any one organ or tissue, but of many.

At the present time it is unfortunately not possible to say with any degree of certainty which tissues in particular are capable of converting injected carotene. It may well be that all tissues, or even perhaps all cells, are involved at least to some extent. The further possibility must also be considered that the blood may in some way be involved. With a view to investigating these possibilities more fully it was decided to carry out a series of experiments with tissues from the rat, guinea pig, and sheep, using various in vitro techniques. In this way it was hoped to obtain further information concerning not only which tissues were

capable of effecting conversion, but also the actual mechanism of conversion.

Summary

1. The effect of lung removal and decapitation on the conversion to vitamin A of intravenously administered aqueous dispersions of carotene has been studied in the rat.

2. Within 5 min of injection of 400 µg of carotene in Tween, the level of vitamin A circulating in the blood of animals without lungs was the same as the amount circulating in the blood of intact control animals.

3. Similarly, within 5 min of injection of carotene the level of vitamin A formed in decapitated animals was the same as that formed in normal animals.

4. From these results and from those presented previously, it would appear that the ability to convert injected carotene to vitamin A is not a function of any one organ or tissue, but of many. Possibly all tissues, or even all cells, are able to effect conversion.

CHAPTER IV

STUDIES ON THE IN VITRO CONVERSION OF CAROTENE TO VITAMIN A IN TISSUES FROM THE RAT, GUINEA PIG AND SHEEP

Introduction

Numerous attempts have been made to demonstrate the *in vitro* conversion of carotene to vitamin A but the results for the most part are conflicting. Thus, Olcott & McCann (1931), Pariente & Nalli (1932) and Euler & Klussmann (1932), in experiments in which minced liver tissue or liver extracts were incubated with colloidal carotene, obtained evidence for the formation of vitamin A by the presence of a typical absorption band at 328 mμ or by a positive antimony trichloride colour reaction. On the other hand, Rea & Drummond (1932), Euler (1932), and later, Ahmad (1934), were unable to confirm these results, and Drummond & MacWalter (1933) could not obtain vitamin A from carotene which had actually been taken up by the liver cells prior to mincing and incubation. In critically discussing these findings Woolf & Moore (1932) pointed out the difficulties inherent in such studies and the uncertainty of detecting vitamin A in the small amounts in which it was claimed to have been produced, and more recently, Glover *et al.* (1948a) have noted the difficulty of distinguishing the 335 mμ peak of carotene *cis*-isomerides from that of vitamin A at 325-328 mμ when other absorbing substances are present.

More recent studies have likewise produced equivocal results. Thus Wiese *et al.* (1947) and Rosenberg & Sobel (1953a, b) observed a positive increase in vitamin A after incubating the intestines of rats with carotene. A similar result was obtained also by Glover *et al.* (1948a) but the increase was considered by the authors to be insufficient to assert definitely that *in vitro* conversion had occurred. In

experiments with calf tissues Stallcup & Herman (1950) demonstrated conversion of colloidal carotene in minced liver preparations and in isolated intestines. Similarly, McGillivray (1951) obtained evidence of conversion in isolated sections of surviving sheep intestine. In contrast to these findings, however, De & Sundararajan (1951) in the rat, and Bieri & Pollard (1953) in the rat, rabbit and calf, were unable to demonstrate any increase in vitamin A after incubating whole intestine with carotene. Negative results were reported also by Kon & Thompson (1951) in the rat, working in collaboration with Fisher & Parsons using the perfusion technique described by the latter authors (Fisher & Parsons, 1949).

From the work presented in the preceding three chapters it seems clear that the conversion of carotene to vitamin A following intravenous administration is not restricted to any one particular site but is widespread throughout the body, and it has been suggested that all tissues, or perhaps even all cells, are capable of effecting conversion. With a view to examining this possibility in more detail it was decided that a series of <u>in vitro</u> experiments should be undertaken using carotene dispersed in Tween and in emulsion form* . Such studies it was hoped would provide information not only on the ability of different tissues to convert carotene to vitamin A <u>in vivo</u>, but ultimately, perhaps, also on the

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* For evidence concerning the conversion into vitamin A of carotene administered parenterally as an emulsion, see Chapter VIII.

actual mechanism of conversion. It was further hoped that the information obtained would also assist in some measure in collating the divergent findings of earlier workers in the *in vitro* field. Three different species were investigated (the rat, guinea pig and sheep) which were known normally to be capable of converting injected carotene*. Further, to help interpret the results obtained, *in vitro* studies on the metabolism of vitamin A and retinene by different tissues were also carried out.

Experimental

General. The materials and methods were as described earlier with the following additions:

Rats. Two different types of animals were used in the present work. The first were inbred Wistar animals similar to those used in the previous experiments. The second were an inbred strain of Hooded Norwegians obtained from the Dunedin Medical School small-animal colony. At the time the present experiments were undertaken the Massey colony was seriously depleted in numbers and the above animals were obtained to overcome this shortage. All animals when used were partially deficient in vitamin A and were maintained on the basal diet previously described. In every experiment a mixture of animals was used with roughly equal numbers of each type.

Guinea pigs. Guinea pigs (weight range 400-500 g) were obtained from the College Veterinary Department colony. They

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* Evidence confirming conversion of intravenously administered carotene to vitamin A in the guinea pig is presented in Chapter VII.

were inbred albino animals and were maintained throughout life on concentrate cubes* and fresh grass. Their vitamin A blood levels and body stores would be expected, therefore, to lie within the normal range.

Sheep. The two sheep from which blood was obtained were purebred male Romneys from the College flock and at the time of the experiments were pasturing on a typical clover/ryegrass association on the College farm.

Carotene. The carotene used in these experiments was 100% β-carotene as supplied by the Eastman Kodak Co. Ltd., New York.

Vitamin A. The vitamin A used was an oil concentrate of vitamin A ester obtained from Glaxo (N.Z.) Ltd. Vitamin A alcohol was obtained by saponification of the latter followed by chromatography on alumina as described on p.14.

Retinene. Retinene (vitamin A aldehyde) was prepared from vitamin A alcohol by oxidation with manganese dioxide using the method described by Ball, Goodwin & Morton (1948). The products were separated by chromatography on an alumina column prepared as described previously (see p.14). Retinene, characterized as a broad brown band which moved slowly down the column, was recovered in the 2% acetone fraction. Its identity was further confirmed from its absorption spectrum as published by Ball et al. (1948). The amount present was estimated spectrophotometrically in a Beckman, model DU, spectrophotometer at 370 mμ using a value for $E^{1\%}_{1cm}$ = 1250 (Ball et al. 1948).

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* These cubes were a special guinea pig ration, compounded for the Veterinary Department by W. & R. Fletcher (N.Z.) Ltd., of the following approximate composition: ground wheat 39, pollard 15, bran 13, ground maize 13, linseed meal 10, meat meal 5, dried blood 3, wheat germ 0.9, $CaCO_3$ 0.9, and NaCl 0.2%.

Preparation of aqueous dispersions of vitamin A and retinene. Aqueous dispersions of vitamin A and retinene were prepared with a 2% (v/v) solution in water of Tween exactly as described previously for carotene (see p.11).

Preparation of emulsions. Emulsions of carotene and vitamin A were prepared using the method of Shafiroff, Mulholland, Roth & Baron (1949). 50 g of glucose and 20 g of gelatin were dissolved separately in water and blended in a Waring Blendor with 100 g of arachis oil containing the appropriate quantities of either carotene or vitamin A alcohol or palmitate. The volume was adjusted to 1 l. with water and the suspension homogenized at 3500 lb./sq.in. in a single-stage, piston-type homogenizer to give an emulsion of average particle size about 0.6 μ and few particles larger than 1 μ diameter.

As the carotene and vitamin A were destroyed fairly rapidly, even when emulsions were stored under nitrogen at low temperatures, all emulsions were used as soon as possible after preparation.

Preparation of physiological salines. Krebs-Ringer-phosphate and Krebs-Ringer-bicarbonate salines were prepared by the standard methods as described by Umbreit, Burris & Stouffer (1945). The solutions were made up at approximately weekly intervals and maintained at 5°.

Perfusion experiments

Perfusion through the intact rat. In preliminary experiments with rats attempts were made to follow the build up of carotene and vitamin A in individual organs by perfusing the intact

animal with carotene and vitamin A in warm, well oxygenated (95% O_2; 5% CO_2) saline in the absence of blood. This was achieved by opening the chest cavity, rapidly cannulating the aorta and vena cava immediately above the diaphragm with 1 mm bore nylex tubing, pumping saline through the animal (at the rate of approximately 20 ml./min in the direction of normal blood flow) until the liquid leaving the vena cava was colourless, and then perfusing continuously with 200 ml. of Krebs-Ringer-phosphate or -bicarbonate at 37° containing carotene or vitamin A in Tween for periods varying from 5 min to 4 h. For this latter purpose a small perfusion apparatus with a variable delivery rate of from 20-50 ml. was constructed and set up as illustrated in Plate 2.

Perfusion through isolated rat organs. In other experiments, also with rats, attempts were made to perfuse various isolated organs (e.g. kidney, liver and lung) with carotene and vitamin A preparations, using the same apparatus as above, in an endeavour to determine the manner in which these preparations were metabolized by the different organs.

Perfusion through the lumen of the intestine. In a further series of experiments involving perfusion through the lumen of the intestine of the rat, both *in vivo* and *in vitro*, an apparatus similar in principle to that described by Fisher & Parsons (1949) was constructed. As pointed out by these authors the use of such an apparatus offered the advantage of allowing perfusion of oxygenated saline through the lumen of the intestine before the blood supply to the organ was severed. This was felt to be of importance since, as has been demonstrated by Fisher & Parsons (1949), even the briefest anoxia may produce

important changes in the intestinal mucosa which far outlast the period of anoxia and which may be a limiting factor to success.

The rats were lightly anaesthetized with ether and opened by ventral mid-line incision. The intestines were cannulated with fine glass cannulae of 3 mm internal diameter, one cannula being inserted in the region of the pyloric sphincter and the other in the region of the ileo-caecal valve. Circulation of 200 ml. of warm oxygenated saline through the lumen of the intestine was commenced and the organ was removed from the animal (care being taken to tie and ligate all blood vessels) and suspended in a flask of oxygenated saline held at 37°. At this point the dispersion or emulsion of carotene, vitamin A alcohol or retinene was introduced into the fluid passing through the lumen, and the apparatus left to run for 2-4 h. The circulating fluid was then collected and the intestine removed and assayed for carotene and vitamin A.

Incubation experiments

Preparation of tissues for incubation. Tissues were removed as rapidly as possible from animals (rats and guinea pigs), after a sharp head blow, and immediately placed in chilled Krebs-Ringer solution. They were then either sliced free hand to a thickness of approximately 1 mm or homogenised in a food blender for 5-10 sec with a small amount of saline. These preparations were weighed out in 2-5 g batches and incubated aerobically for 2-4 h at 37° with carotene, vitamin A or retinene in saline (1 g of tissue to 10 ml. of saline). Whole intestines, rapidly removed from the animal after suturing

t both ends and administering carotene or vitamin A by hypodermic syringe into the lumen, and finally chopped intestines, were also incubated for varying periods from 2-16 h.

Incubation of bloods. Blood samples from rats and guinea pigs were taken in the normal way by cardiac puncture. In the case of sheep, however, blood samples (normally 20-30 ml.) were taken into an heparinized container by means of a 15 G needle inserted into the jugular vein. All bloods were incubated immediately after collection with carotene or vitamin A for periods varying from 5 min to 16 h and were kept well oxygenated with a mixture of 95% O_2; 5% CO_2.

Measurement of carotene and vitamin A

Carotene and vitamin A alcohol and ester were estimated in body wall, intestine, kidney and lung exactly as described for liver on p.14.

Results

Perfusion experiments

Perfusion through the intact rat. In experiments in which the aorta and vena cava were cannulated, the blood washed out, and the body perfused with a Tween solution of carotene in saline (Table 9, Exp. 1), no build up of vitamin A in the different organs or tissues of the vitamin A deficient rat or in the circulating fluid could be demonstrated up to 4 h though carotene was taken up in considerable amounts by the kidney and liver. It was of interest to note the presence of considerable amounts of carotene oxidation products in the vitamin A alcohol fraction

on extraction of the circulating fluid after perfusion. That the reading obtained on this fraction was not true vitamin A was suspected from the atypical, non-fading, blue-grey colour formed on mixing with antimony trichloride reagent, and was confirmed by applying the three-point correction procedure of Cama, Collins & Morton (1951). When a Tween solution of vitamin A alcohol in saline was perfused through the animal (Table 9, Exp. 2), vitamin A was taken up by the carcase, kidney and liver in varying amounts depending on the perfusion interval, and it was noteworthy that the vitamin A appearing in the liver after perfusion was mainly in the ester form whereas that appearing in the kidneys and carcase was still in the form administered.

Perfusion through isolated rat organs. The results from these experiments are presented in Tables 10 and 11. From a consideration of the results presented in Table 10 it will be seen that there was no evidence to indicate any formation of vitamin A in the isolated kidney, liver or lung after perfusing with carotene, either as a dispersion in Tween or as an emulsion, although destruction of the circulating carotene was quite appreciable. In the perfused lung there was a relatively larger breakdown of carotene in Tween (as judged by total recovery) than in the liver or kidney although storage of carotene in the latter two greatly exceeded that in the lung. The greater breakdown of carotene in the lung was also accompanied by comparatively higher blue readings with antimony trichloride reagent. These, however, were non-fading atypical blues and when the three-point correction procedure was applied to the original solution there was no evidence of vitamin A being present. From a comparison

of the results obtained after perfusing carotene as a dispersion in Tween and as an emulsion it is apparent that the latter was very much more readily destroyed than the former, but whether this was due to a difference in particle size or to a difference between the two in their relative stability in saline is not known.

When vitamin A alcohol, as a Tween dispersion or as an emulsion, was perfused through the individual organs (Table 11) a build up of vitamin A was noted similar to that which occurred on perfusion of the intact animal. Further, as in the earlier experiment, the vitamin was observed to be present in the liver mainly as the ester and in the kidney mainly as the alcohol.

Perfusion through the lumen of the intestine. Using the technique of Fisher & Parsons (1949) no vitamin A could be demonstrated in either the intestinal wall or the perfusion fluid after circulating a Tween solution or an emulsion of carotene through the isolated intestine for periods from 2-4 h (Table 12, Exps. 1 and 2). On the other hand, perfusion of the intestine *in vivo* with carotene for 2-4 h resulted in accumulation in the wall of quite measurable quantities of vitamin A, in both the alcohol and ester forms. In the latter experiments animals were kept under diethyl-ether anaesthesia throughout the experimental period and, apart from insertion of the cannulae into the intestine and circulation of the perfusion fluid, were not interfered with in any other way. Perfusion of 100 μg vitamin A alcohol, either as a Tween dispersion or as an emulsion, through the isolated intestine using the Fisher & Parsons (1949) technique resulted

in a fairly large build up of vitamin A (mainly as the ester) in the intestine at 2 and 4 h (Table 12, Exps. 3 and 4). Furthermore, the level of blood vitamin A ester (the normal transport form from the intestine) and the levels of alcohol and ester in the liver were all significantly increased following perfusion in vivo (not, however, shown in Table). In a single experiment in which 100 µg of retinene in Tween was circulated through an isolated surviving intestine the amount and the form of the vitamin present after perfusion for 2 and 4 h was fairly similar to that after perfusion with an equivalent amount of vitamin A alcohol for the same time (Table 12, Exp. 5). This observation was felt to be of considerable significance since it quite clearly demonstrated that retinene, which has been postulated as a possible intermediate product in the conversion of carotene to vitamin A (Glover et al. 1948b), could be absorbed, metabolized to vitamin A alcohol and esterified in the surviving intestine, away from the animal body.

Incubation experiments

Incubation of rat tissues. Table 13 presents the results obtained on incubating tissue slices and tissue homogenates from rats with carotene, vitamin A and retinene in Tween, and carotene and vitamin A in emulsion form. From the results of Exp. 1 it is quite clear that no vitamin A was formed on incubating carotene, either as an aqueous dispersion or as an emulsion, with tissue slices or homogenates of abdominal wall, intestine, kidney, liver and lung. In nearly every case, however, allowances had to be made for the presence of

carotene oxidation products which gave stable atypical blue colours with antimony trichloride. This was especially true after incubating carotene as an emulsion when much of the carotene present was invariably destroyed. Incubation of 80 µg of vitamin A alcohol in Tween and in emulsified form with rat tissue homogenates (Exp. 2) yielded interesting results. Under the conditions under which these experiments were carried out abdominal wall, kidney and lung were unable to esterfy the free vitamin either dispersed in Tween or emulsified. Homogenates of intestine and liver tissue, on the other hand, were each capable of esterification *in vitro* but the reaction in the latter case was very much slower than in the former. In a further experiment (Exp. 3) in which 100 µg of retinene in Tween was incubated with homogenates of intestine, kidney and liver, approximately one-third of the dose was recoverable as vitamin A after 2 h incubation and almost one-quarter after 4 h incubation. No free retinene could be identified after incubation, however, which is perhaps to be expected in view of its somewhat reactive nature.

Using whole and chopped intestine (approximately 1 cm in length), numerous attempts were made to demonstrate the *in vitro* conversion of carotene to vitamin A. A sample of these results is presented in Table 14 (Exp. 1). Many variations in the incubation interval, in the form and concentration of carotene and in the type of saline were tried and several different techniques published by workers who claim to have effected *in vitro* conversion were repeated (e.g. Wiese *et al.* 1947; Rosenberg & Sobel, 1953a, b), but without any apparent success. On the other hand, however, vitamin A alcohol was rapidly

esterified by these preparations (Table 19, Exp. 2), being virtually complete at 3 h. In the light of this latter result, it is interesting to note that if any of the intestinal preparations used had been able to convert carotene to vitamin A in vitro, the main increase in vitamin A should have been in the ester form. This, however, was quite certainly not the case, the only increase noted being in the vitamin A alcohol fraction, and after correction this was shown clearly to be due not to vitamin A but to oxidation products of carotene.

Incubation of guinea pig tissue. When carotene, either as a dispersion or as an emulsion, was incubated with guinea pig tissue slices, or homogenates, of abdominal wall, intestine, kidney, liver and lung there was no evidence to suggest formation of vitamin A by any tissue (Table 15, Exp. 1). The results obtained and the conclusions drawn, generally, were the same as those for the rat and will not, therefore, be discussed further here.

In contrast, however, the results obtained after incubating guinea pig tissue preparations with vitamin A alcohol were markedly different from the rat (Table 15, Exp. 2). Whereas in the rat approximately half of the vitamin A was recovered after incubation, mainly in the ester form in the case of the intestine and the liver, in the guinea pig some tissues completely destroyed vitamin A during incubation. After incubation vitamin A was recovered only from the lung preparation (as the alcohol) and from the liver preparation (mainly as the ester). Since the intestinal preparation was so effective in destroying over 90% of the vitamin A in the dose one wonders whether in the living animal a similar state of affairs exists and is not the

explanation for the extremely poor vitamin A economy of this species.

Incubation of rat, guinea pig and sheep blood. When blood from rats, guinea pigs and sheep were incubated for varying intervals with a dispersion of carotene in Tween or an emulsion of carotene (Table 16), there was a progressive increase with time in the antimony trichloride blue colour of the vitamin A alcohol fraction (but not the ester fraction)of the plasma. The blue colours obtained were typical and were observed to fade normally. When, however, a correction was carried out using the three-point procedure of Cama et al. (1951) no differences could be seen between the initial levels of vitamin A in the blood and the levels after incubation with carotene. These observations were felt to be of considerable significance since they demonstrate how easy it is to attribute a small increase in blue colour to vitamin A, when in fact it may be entirely due to oxidation products of carotene. This increase is normally comparatively easy to determine visually when vitamin A is absent in the original preparation but in the presence of vitamin A the atypical non-fading blue due to oxidation products may be easily masked by the typical fading blue due to vitamin A.

However, in order to further check that the increase in antimony trichloride blue colour observed on incubating carotene was actually due to oxidation products of carotene and not to the presence of true vitamin A, an experiment was designed in which a sample of rat blood was divided into 4 treatment groups as follows:

Table 9. Perfusion of the intact partially deficient rat with Tween dispersions of carotene and vitamin A alcohol in 200 ml. physiological saline for varying periods up to 4 h.

Exp.	No. rats used	Substance		Perfusion time	Carcass			Kidneys			Liver			Perfusion fluid		
		Nature	Amount		Vitamin A alcohol	Vitamin A ester	Carotene	Vitamin A alcohol	Vitamin A ester	Carotene	Vitamin A alcohol	Vitamin A ester	Carotene	Vitamin A alcohol. Total present	Vitamin A ester. Total present	Carotene. Total recovered
			(µg)	(h)	(µg/10g*)	(µg/10g*)	(µg/10g*)	(µg)	(µg)	(µg)	(µg)	(µg)	(µg)	(µg)	(µg)	(µg)
1	2	None	-	0	0	0	0	0.6	0.7	0	0.8	0.9	0	-	-	-
	1	Carotene	400	1/12	0	0	Trace	0.5	0.7	4	1.0	1.1	50	3.0+	-	260
	3	Carotene	400	1	0	0	Trace	0.7	1.1	7	0.7	1.2	86	4.9+	-	140
	2	Carotene	400	2	0	0	Trace	0.4	0.3	8	0.9	0.8	105	2.1+	-	100
	4	Carotene	400	4	0	0	Trace	0.6	0.5	11	0.6	0.8	140	6.7+	-	40
2	1	Vitamin A alcohol	100	1/12	1.4	0	-	1.0	0.8	-	4.6	1.2	-	60	0.2	-
	2	Vitamin A alcohol	100	1	1.8	0	-	7.6	0.6	-	6.1	7.1	-	43	0.9	-
	2	Vitamin A alcohol	100	2	2.4	0	-	5.4	0.7	-	7.8	8.9	-	41	0.7	-
	2	Vitamin A alcohol	100	4	2.2	0	-	8.1	1.4	-	6.8	10	-	24	1.2	-

*10 g being a convenient weight for assay purposes

+ Atypical non-fading blue colours with antimony trichloride reagent indicating the presence of carotene oxidation products (see text).

Table 10. Perfusion of isolated organs from partially deficient rats with Tween dispersions or emulsions of carotene in 15 ml. physiological saline for varying periods up to 4 h.

No. rats used	Vehicle		Substance		Perfusion time	Kidneys				Liver				Lungs			
	Nature	Amount (ml.)	Nature	Amount (µg)	(h)	Vitamin A alcohol (µg)	Vitamin A ester (µg)	Carotene (µg)	Perfusion fluid Carotene recovered (µg)	Vitamin A alcohol (µg)	Vitamin A ester (µg)	Carotene (µg)	Perfusion fluid Carotene recovered (µg)	Vitamin A alcohol* (µg)	Vitamin A ester (µg)	Carotene (µg)	Perfusion fluid. Carotene recovered (µg)
2	None	-	None	-	0	0.6	0.7	0	-	0.8	0.9	0	-	0.2	0.2	9	-
2	Tween dispersion	0.4	Carotene	400	1/12	0.6	0.9	Trace	350	1.1	0.9	30	320	0.4	0.3	20	140
2	Tween dispersion	0.4	Carotene	400	1	0.9	1.2	18	300	0.9	0.7	45	220	0.4	0.4	23	108
2	Tween dispersion	0.4	Carotene	400	2	0.8	1.0	42	120	0.7	0.6	90	78	0.2	0.3	35	45
2	Tween dispersion	0.4	Carotene	400	4	1.0	0.8	44	88	0.8	0.9	120	32	0.2	0.3	25	32
1	Emulsion	4.0	Carotene	400	1/12	0.4	0.7	Trace	280	0.6	0.8	11	300	0	0	18	140
1	Emulsion	4.0	Carotene	400	1	0.7	0.7	Trace	90	0.7	0.9	17	80	0	0	12	30
1	Emulsion	4.0	Carotene	400	2	0.6	0.3	17	18	1.0	1.2	29	Trace	0	0	Trace	Trace
2	Emulsion	4.0	Carotene	400	4	0.4	0.9	16	Trace	0.9	1.1	30	Trace	0	0	Trace	Trace

* Values corrected for presence of carotene oxidation products by the method of Cama et al. (1951)

Table 11. Perfusion of isolated organs from partially deficient rats with Tween dispersions or emulsions of vitamin A alcohol in 15 ml. physiological saline for varying periods up to 4 h.

No. rats used	Dose				Perfusion time	Kidneys			Liver			Lung		
	Vehicle		Substance			Vitamin A alcohol	Vitamin A ester	Perfusion fluid. Vitamin A alcohol recovered	Vitamin A alcohol	Vitamin A ester	Perfusion fluid. Vitamin A alcohol recovered	Vitamin A alcohol	Vitamin A ester	Perfusion fluid. Vitamin A alcohol recovered
	Nature	Amount (ml.)	Nature	Amount (µg.)	(h)	(µg.)	(µg.)	(µg.)	(µg.)	(µg.)	(µg.)	(µg.)	(µg.)	(µg.)
2	None	-	None	-	0	0.6	0.7	-	0.8	0.9	-	0.2	0.2	-
1	Tween dispersion	0.15	Vitamin A alcohol	100	1/12	4.1	0.9	70	7.9	3.7	76	6.8	0	64
1	Tween dispersion	0.15	Vitamin A alcohol	100	1	3.7	0.8	64	12	13	48	4.1	-	56
1	Tween dispersion	0.15	Vitamin A alcohol	100	2	5.4	0.6	31	10	30	32	7.3	-	27
1	Tween dispersion	0.15	Vitamin A alcohol	100	4	7.9	1.1	20	15	27	17	4.3	-	14
1	Emulsion	0.7	Vitamin A alcohol	100	1/12	1.2	0.7	59	4.3	2.1	65	11	0	48
1	Emulsion	0.7	Vitamin A alcohol	100	1	3.8	0.7	43	3.0	4.8	52	7.9	0	30
1	Emulsion	0.7	Vitamin A alcohol	100	2	3.1	1.2	18	5.1	6.4	27	8.9	-	17
1	Emulsion	0.7	Vitamin A alcohol	100	4	5.6	0.8	11	7.3	14	7	6.0	-	Trace

Table 12. Perfusion through the lumen of the intestine of the partially deficient rat both *in vivo* and *in vitro* with Tween dispersions of carotene, vitamin A alcohol and retinene in saline, or with emulsions of carotene and vitamin A alcohol in saline. Perfusion times 2-4 h.

Exp.	No. rats used	Dose: Vehicle Nature	Dose: Vehicle Amount (ml.)	Dose: Substance Nature	Dose: Substance Amount (µg)	Treatment	Perfusion time (h)	Intestinal wall: Vitamin A ester (µg)	Intestinal wall: Vitamin A alcohol (µg)	Intestinal wall: Carotene (µg)	Perfusion fluid: Total Vitamin A present (µg)
1	2	None	-	None	-	None	0	0	0	-	-
	3	Tween dispersion	4.0	Carotene	4000	Intestine cannulated and perfused in intact animal	2	3.2	2.8	42	0*
	3	Tween dispersion	4.0	Carotene	4000	Intestine cannulated and perfused in intact animal	4	6.4	2.2	44	0*
	2	Tween dispersion	4.0	Carotene	4000	Intestine cannulated, perfused *in vitro* using Fisher and Parson's technique	2	0.4	0.3	50	0*
	2	Tween dispersion	4.0	Carotene	4000	Intestine cannulated, perfused *in vitro* using Fisher and Parson's technique	4	0.8	0.4	67	0*
2	1	Emulsion	10	Carotene	1000	Intestine cannulated and perfused in intact animal	2	3.5	2.9	13	0*
	1	Emulsion	10	Carotene	1000	Intestine cannulated and perfused in intact animal	4	4.4	4.1	17	0*
	2	Emulsion	10	Carotene	1000	Intestine cannulated, perfused *in vitro* using Fisher and Parson's technique	2	0.1	0.4	15	0*
	2	Emulsion	10	Carotene	1000	Intestine cannulated, perfused *in vitro* using Fisher and Parson's technique	4	0.5	0.7	18	0*
3	1	Tween dispersion	0.15	Vitamin A alcohol	100	Intestine cannulated, perfused *in vitro* using Fisher and Parson's technique	2	29	5.2	-	36
	1	Tween dispersion	0.15	Vitamin A alcohol	100	Intestine cannulated, perfused *in vitro* using Fisher and Parson's technique	4	42	2.6	-	22
4	1	Emulsion	0.7	Vitamin A alcohol	100	Intestine cannulated, perfused *in vitro* using Fisher and Parson's technique	2	19	2.4	-	29
	1	Emulsion	0.7	Vitamin A alcohol	100	Intestine cannulated, perfused *in vitro* using Fisher and Parson's technique	4	26	3.0	-	13
5	1	Tween dispersion	0.22	Retinene	100	Intestine cannulated, perfused *in vitro* using Fisher and Parson's technique	2	23	4.0	-	-
	1	Tween dispersion	0.22	Retinene	100	Intestine cannulated, perfused *in vitro* using Fisher and Parson's technique	4	33	4.6	-	-

*Corrected for irrelevant absorption by the method of Cama *et al.* (1951)

Table 13. Incubation of tissue slices* and tissue homogenates* from partially deficient rats with carotene, vitamin A alcohol and retinene in Tween, and with carotene and vitamin A alcohol in emulsion form for periods of from 2-4 h.

Exp.	No. rats used	Dose				Preparation	Incubation time	Abdominal Wall+			Intestine+			Kidney+			Liver+			Lung+		
		Vehicle		Substance				Vitamin A alcohol‡	Vitamin A ester	Carotene	Vitamin A alcohol‡	Vitamin A ester	Carotene	Vitamin A alcohol‡	Vitamin A ester	Carotene	Vitamin A alcohol‡	Vitamin A ester	Carotene	Vitamin A alcohol‡	Vitamin A ester	Carotene
		Nature	Amount (ml.)	Nature	Amount (μg)		(h)	(μg/5 g)	(μg/5 g)	(μg/5 g)	(μg/5 g)	(μg/5 g)	(μg/5 g)	(μg/5 g)	(μg/5 g)	(μg/5 g)	(μg/5 g)	(μg/5 g)	(μg/5 g)	(μg/5 g)	(μg/5 g)	(μg/5 g)
1	2	None	-	None	-	-	0	0	0	0	0.1	0.1	0	0.6	0.7	0	0.8	0.9	0	0.2	0.2	0
	3	Tween dispersion	0.4	Carotene	440	Slices	3	0.4	0.1	350	0.2	0.3	320	0.7	0.7	280	1.0	0.8	360	0.3	0.4	260
	3	Tween dispersion	0.4	Carotene	440	Homogenate	2	0.1	0.2	360	0.2	0.1	300	0.8	0.7	300	0.9	1.0	320	0.4	0.1	270
	3	Tween dispersion	0.4	Carotene	440	Homogenate	4	0.2	0.1	360	0.1	0.2	360	0.6	0.8	320	0.9	1.2	350	0.4	0.3	220
	4	Emulsion	3.8	Carotene	400	Slices	2	0.1	0.1	320	0.3	0.2	48	0.6	0.5	50	0.7	0.7	270	0.1	0.1	100
	4	Emulsion	3.8	Carotene	400	Homogenate	4	0.3	0.1	300	0.2	0.4	35	0.8	0.3	43	0.6	1.0	260	0.4	0.2	85
2	3	Tween dispersion	0.2	Vitamin A alcohol	80	Homogenate	3	58	1.0	-	5.2	21	-	44	0.7	-	38	10	-	55	0.8	-
	3	Emulsion	0.6	Vitamin A alcohol	80	Hom		48	0.3	-	6.0	15	-	36	0.3	-	27	9.0	-	44	0.3	-
3	1	Tween dispersion	0.22	Retinene	100	Homogenate	2	40	0	-	4.0	14	-	41	0.8	-	33	7.2	-	25	0.2	-
	2	Tween dispersion	0.22	Retinene	100	Homogenate	4	23	0.8	-	1.0	11	-	35	0.2	-	22	3.1	-	29	0.1	-

* Lots of 5 g of tissue incubated with 50 ml. of saline.

\+ Tissue plus saline extracted after incubation.

‡ All vitamin A alcohol values corrected where necessary in Exp. 1 according to Cama et al. (1951).

Table 14. Incubation of whole and chopped rat intestines in 50 ml. physiological saline with carotene and vitamin A alcohol as emulsions and as Tween dispersions for various intervals up to 16 h.

Exp.	No. rats used	Dose				Preparation	Incubation time (h)	Intestine		
		Vehicle		Substance				Vitamin A alcohol (µg)	Vitamin A ester (µg)	Carotene (µg)
		Nature	Amount (ml.)	Nature	Amount (µg)					
1	2	None	-	None	-	Whole intestine	0	0.3	0.2	0
	2	Tween dispersion	0.4	Carotene	400	Whole intestine	2	0.3	0.5	210
	2	Tween dispersion	0.4	Carotene	400	Whole intestine	16	0.4	0.4	180
	2	Tween dispersion	2.0	Carotene	2000	Whole intestine	4	0*	0.6	2700
	2	Tween dispersion	2.0	Carotene	2000	Whole intestine	16	0*	0.3	1200
	2	Emulsion	4.0	Carotene	400	Whole intestine	4	0.6*	0.5	80
	1	Emulsion	10	Carotene	1000	Whole intestine	8	0.8*	0.3	130
	2	Tween dispersion	1.0	Carotene	1000	Chopped intestine	4	0.7*	0.2	390
	2	Emulsion	10	Carotene	1000	Chopped intestine	4	0.9*	0.1	170
2	2	Tween dispersion	0.15	Vitamin A alcohol	100	Whole intestine	3	6	26	-
	2	Tween dispersion	0.15	Vitamin A alcohol	100	Chopped intestine	3	3	31	-
	2	Emulsion	0.7	Vitamin A alcohol	100	Whole intestine	3	7	16	-
	2	Emulsion	0.7	Vitamin A alcohol	100	Chopped intestine	3	11	25	-

* Values corrected for the presence of carotene oxidation products by the method of Cama et al. (1951).

Table 15. Incubation of tissue slices* and tissue homogenates* from normal guinea pigs with Tween dispersions and emulsions of carotene and vitamin A alcohol in physiological saline for varying periods up to 24 h.

Exp.	No guinea pigs	Dose				Preparation	Incubation time	Abdominal Wall+			Intestine+			Kidney+			Liver+			Lung+		
		Vehicle		Substance				Vitamin A alcohol ‡	Vitamin A ester	Carotene	Vitamin A alcohol ‡	Vitamin A ester	Carotene	Vitamin A alcohol ‡	Vitamin A ester	Carotene	Vitamin A alcohol ‡	Vitamin A ester	Carotene	Vitamin A alcohol ‡	Vitamin A ester	Carotene
		Nature	Amount (ml.)	Nature	Amount (μg)		(h)	(μg/5 g)	(μg/5 g)	(μg/5 g)	(μg/5 g)	(μg/5 g)	(μg/5 g)	(μg/5 g)	(μg/5 g)	(μg/5 g)	(μg/5 g)	(μg/5 g)	(μg/5 g)	(μg/5 g)	(μg/5 g)	(μg/5 g)
1	2	None	-	None	-	-	0	1.0	1.0	Trace	0.2	0.3	Trace	0.8	1.0	0	4.0	23	Trace	0.8	1.1	0
	3	Tween dispersion	0.8	Carotene	800	Slices	24	0.8	0.4	420	0.3	0.5	400	1.0	1.3	470	0.5	19	430	0.3	0.2	320
	3	Tween dispersion	0.8	Carotene	800	Homogenate	24	0.7	0.9	370	0.6	0.2	420	0.9	1.0	390	0.9	17	410	0.7	0.9	320
	4	Emulsion	8.0	Carotene	800	Homogenate	12	0.8	0.6	140	0.5	0.7	130	0.7	0.8	140	2.9	16	220	0.8	1.2	100
2	2	Tween dispersion	0.3	Vitamin A alcohol	40	Homogenate	4	-	-	-	0.1	3.0	-	0.2	0.4	-	2.0	28	-	24	0.3	-
	2	Emulsion	0.7	Vitamin A alcohol	100	Homogenate	6	-	-	-	0.2	6.2	-	0.8	0.1	-	5.0	52	-	17	0.7	-

* 5 g tissue incubated with 50 ml. saline.

\+ Tissue plus saline extracted after incubation.

‡ All vitamin A alcohol values corrected where necessary in Exp. 1 according to Cama et al. (1951).

Table 16. Incubation of blood from the rat, guinea pig and sheep with various preparations of carotene and vitamin A alcohol for periods up to 16 h.

No. specimens	Dose: Vehicle: Nature	Dose: Vehicle: Amount (ml.)	Dose: Substance: Nature	Dose: Substance: Amount (µg)	Species	Incubation time (h)	Blood plasma: Vitamin A alcohol (µg/100 ml.)	Blood plasma: Vitamin A ester (µg/100 ml.)	Carotene recovered (µg)
6	None	-	None	-	Rat	0	16	-	0
4	Tween dispersion	0.4	Carotene	400	Rat	1/12	18*	-	320
2	Tween dispersion	0.4	Carotene	400	Rat	4	17*	-	350
2	Tween dispersion	0.8	Carotene	800	Rat	16	19*	-	480
2	Tween dispersion	0.15	Vitamin A alcohol	100	Rat	3	41+	1.8+	-
1	Emulsion	0.7	Vitamin A alcohol	100	Rat	3	23+	0.7+	-
3	None	-	None	-	Guinea pig	0	15	2.0	Trace
5	Tween dispersion	0.4	Carotene	400	Guinea pig	4	14*	3.1	120
2	Tween dispersion	0.8	Carotene	800	Guinea pig	16	17*	1.0	590
4	None	-	None	-	Sheep	0	18	-	0
2	Tween dispersion	0.4	Carotene	400	Sheep	4	19*	-	270
2	Tween dispersion	0.8	Carotene	800	Sheep	16	16*	-	500
1	Emulsion	4.0	Carotene	440	Sheep	3	20*	-	0

* Values corrected for the presence of carotene oxidation products by the method of Cama et al. (1951).

\+ Represent actual amounts of vitamin A recovered.

Table 17. Incubation of blood, separately and in combination, with carotene and with intestinal homogenate, to demonstrate esterification of true vitamin A alcohol and its chromatographic separation from oxidation products of carotene which interfere with the antimony trichloride reaction. Incubation time 1 h.

Blood (ml.)	Treatment	Blood Plasma	
		Vitamin A alcohol (µg/100 ml.)	Vitamin A ester (µg/100 ml.)
10	(1) Whole blood incubated alone (control)	14*	1.0
10	(2) Whole blood incubated with 2 g intestinal homogenate	0	10*
10	(3) Whole blood incubated with 400 µg carotene in Tween	25*	1.3
10	(4) Whole blood incubated with 400 µg carotene in Tween together with 2 g intestinal homogenate	10+	12*

* Normal fading blue with antimony trichloride reagent.

+ Atypical non-fading blue with antimony trichloride reagent.

(1) blood incubated alone,

(2) blood incubated with intestinal homogenate,

(3) blood incubated with carotene in Tween,

(4) blood incubated with carotene in Tween plus intestinal homogenate.

The results of this experiment are presented in Table 17. From these it can be seen that of the vitamin A alcohol originally present in the control blood (Treatment 1), all was capable of esterification in the presence of intestinal homogenate and was nearly completely recoverable in the ester fraction after chromatography (Treatment 2). Of the "vitamin A alcohol" present in the blood after incubation with carotene (Treatment 3), however, only about one-half (an amount equivalent to that in the control blood) was esterified in the presence of intestinal homogenate and recovered as the ester after chromatography (Treatment 4). The balance remained still with the alcohol fraction and in the presence of antimony trichloride gave a non-fading, grey-blue colour which was quite atypical of true vitamin A.

Discussion

The main objects of the present experiments were to determine by means of various incubation and perfusion techniques which tissues were capable of converting carotene to vitamin A *in vitro*, and, as an aid in interpreting these findings, to determine by means of the same techniques the fate of vitamin A and retinene under similar conditions. For this purpose tissue slices and homogenates were incubated with aqueous dispersions of carotene, vitamin A alcohol and

retinene and with emulsions of carotene and vitamin A alcohol, and intact animals and isolated organs were perfused with similar preparations. The results of such studies it was hoped would give an indication as to which tissues were potentially capable of converting carotene to vitamin A *in vivo* and also provide some insight into the nature of the reaction involved.

It is well recognised that a negative result obtained *in vitro* is a difficult premise from which to argue. For this reason, throughout the present work, considerable attention was paid to detail and extreme care was taken at all times to ensure that the results obtained were not limited in any way by poor technique. Carotene was prepared in forms which are known to be utilized quite effectively *in vivo*, the incubation and perfusion procedures were standard ones which have been used successfully by numerous other workers studying a variety of different problems, and the extraction, measurement and correction procedures used to estimate vitamin A were ones which have been employed routinely in this laboratory and elsewhere with perfectly satisfactory results. Every endeavour was thus made to avoid factors which were likely to have any possible adverse effect on the outcome of the results. Despite these precautions, however, it was not possible to demonstrate conversion of carotene to vitamin A *in vitro* either in the perfused intact animal, or in tissue slices and homogenates of body wall, intestine, kidney, liver and lung, or in isolated perfused organs (intestine, kidney, liver and lung) of the rat or guinea pig. Further it was not possible to demonstrate conversion in the blood of the rat, guinea pig or sheep.

While these results clearly confirm the findings of De & Sundararajan (1951), Kon & Thompson (1951) and Bieri & Pollard (1953) and contrast with those of Wiese *et al.* (1947), Rosenberg & Sobel (1953a, b), Stallcup & Herman (1950) and McGillivray (1951), it is difficult at the present time to offer a completely satisfactory explanation for these conflicting viewpoints. It should be pointed out, however, that in the work of Wiese *et al.* (1947) and Stallcup & Herman (1950) vitamin A was estimated solely by the antimony trichloride reaction, the validity of which, under such conditions, would appear very much open to question (e.g. see p.61). Furthermore, in the work of Rosenberg & Sobel (1953a, b) vitamin A was estimated in intestine by the difference in absorption at 325 mμ before and after ultraviolet irradiation, a procedure which although shown reliable for serum by Beasley, Lowry, Brock & Lopez (1946), has since been shown to be of little use for intestine by Bieri & Pollard (1953). For these various reasons it is felt that the findings of these workers cannot be accepted completely as demonstrating conclusively a significant degree of conversion of carotene to vitamin A *in vitro*. In a somewhat different category is the work of McGillivray (1951) in which significant increases in vitamin A, identified both colorimetrically and spectrophotometrically, were found in the isolated intestine of the sheep after incubation with carotene. In the light of the results of the present studies it is not easy to offer an explanation for this finding unless it is assumed to be due, at least in some measure, to a species difference in the rate or efficiency of conversion.

The results obtained on perfusing or incubating tissues with retinene and vitamin A alcohol support observations made by other workers *in vivo*. For example, Glover *et al.* (1948b) have shown that after oral administration retinene is converted very efficiently to vitamin A in the gut wall, and after subcutaneous administration in the subcutaneous tissues. In the present experiments retinene was completely absorbed and efficiently converted to vitamin A (mainly vitamin A ester) after perfusion through the isolated surviving intestine for 4 h. Furthermore, after incubation for 2-4 h with homogenates of abdominal wall, intestine, kidney, liver and lung retinene was completely metabolized, approximately one-third of the dose being recovered as vitamin A, as the alcohol in the case of abdominal wall, kidney and lung, and as the ester in the case of the liver and intestine. It would appear, therefore, as Glover *et al.* (1948b) have observed, that the conversion of retinene to vitamin A is a simple reduction reaction, the enzymes responsible being widespread throughout the body tissues.

In the case of vitamin A alcohol, esterification in the rat, under the present experimental conditions, was limited to the intestine and liver, being complete in the former and incomplete in the latter. These observations held good whether the intestine was perfused whole or incubated as slices or homogenates, and whether vitamin A was present as a Tween dispersion or as an emulsion. In the guinea pig, on the other hand, the position was somewhat different. After incubation of homogenates of intestine, kidney, liver and lung with vitamin A alcohol in Tween for varying periods, most

of the vitamin A present was destroyed in the case of the intestine and kidney. In the case of the lung preparation one-half of the vitamin A originally present was recovered (still as the alcohol), and in the case of the liver preparation somewhat less than one-half that originally present was recovered (as the ester). That the guinea pig intestine, incubated both whole and as an homogenate, was effective in destroying nearly all the vitamin A added is interesting and, as has been pointed out previously, is one possible explanation for the poor rate of carotene utilization and the low vitamin A liver reserves in this species.

The attempts which have been made using various tissue preparations to demonstrate the conversion of carotene to vitamin A *in vitro* have unfortunately not been successful and for this reason it has not been possible to obtain any further information concerning which tissues are capable of effecting the conversion of parenterally administered carotene to vitamin A in the intact animal. From a consideration of the results it would appear that the reaction is an exceedingly difficult one to demonstrate *in vitro* although the reasons behind this are not at all clear. It has been postulated by Glover and his associates (see Glover & Redfearn, 1954; Fazakerley & Glover, 1957; Fishwick & Glover, 1957) that the initial reaction in the breakdown of orally administered carotene to vitamin A involves an oxidative fission of the terminal double bond in the side-chain to form carotenals, which are then oxidised by stepwise oxidation to retinene, which is in turn reduced to vitamin A. From the results of the present studies with retinene, and from those obtained *in vivo* by Glover *et al.* (1948b), it would appear that it is the early stages of the

reaction (i.e. carotene → carotenals → retinene) that are inhibited *in vitro*. Of the enzyme systems involved in these changes little is known. It would appear, however, on the basis of the results of the present experiments, that either the enzymes involved must be extremely sensitive to nonphysiological conditions, or the necessary co-factors, perhaps supplied by the blood, are not present in isolated tissues in sufficient concentration to effect conversion.

Summary

1. Attempts have been made to demonstrate the *in vitro* conversion of carotene to vitamin A in the rat, guinea pig and sheep in an endeavour to locate directly the site at which conversion of injected carotene to vitamin A occurs in the normal animal.

2. For this purpose a series of perfusion and incubation experiments was carried out using different forms of carotene and a variety of tissue preparations.

3. Concurrently, a parallel series of experiments was carried out using retinene and vitamin A alcohol, in an attempt to determine the behaviour of these substances with similar tissue preparations.

4. Despite numerous precautions and the use of several refined techniques there was no evidence to indicate formation of vitamin A from carotene in any of the organs or tissues examined.

5. On the other hand, retinene and vitamin A alcohol were metabolized by a number of tissues, their behaviour *in vitro* being generally similar to that reported independently by other investigators working *in vivo*.

6. Possible reasons for failure to effect the *in vitro* conversion of carotene to vitamin A are discussed and attention is drawn to the fact that it is probably the initial stages of the reaction which are inhibited under *in vitro* conditions.

CHAPTER V

A STUDY OF SOME FACTORS AFFECTING THE UTILIZATION OF PARENTERALLY ADMINISTERED AQUEOUS DISPERSIONS OF CAROTENE TO VITAMIN A

Introduction

In earlier studies reported in Chapters I to III evidence was obtained suggesting that the ability to convert injected carotene to vitamin A was not an attribute of any one organ or tissue but of many, and it was concluded on the basis of these results that possibly all tissues, or even perhaps all cells, were capable of effecting conversion. Attempts, however, to check this possibility using different *in vitro* techniques were inconclusive (Chapter IV).

If, as appears to be the case, no one organ is specifically involved and conversion can occur in any tissue, some conversion into vitamin A of aqueous carotene dispersions administered by parenteral routes other than the intravenous might be expected. Various workers have in fact claimed restoration of growth and disappearance of other vitamin A deficiency symtoms after intramuscular, intraperitoneal or subcutaneous injection of various carotene preparations (e.g. Lease *et al.* 1942; Tomarelli *et al.* 1946; Biori & Schultze, 1951; Biori & Sandman, 1951). It therefore seemed that a comparison, as indicated by hepatic vitamin A stores, of the efficiency of conversion into vitamin A of aqueous carotene disperaions administered by various routes, and an investigation of some further factors influencing the utilization of intravenously administered carotene disperaions, might provide additional information on the ability of different tissues to convert carotene and on the nature of the processes involved. The results of these studies are reported in the present chapter. In addition the results of a series of studies on the metabolism of intravenously administered aqueous

dispersions of several carotenoid pigments other than β-carotene are also reported. These latter studies were undertaken in an attempt to investigate the breakdown of the various pigments after parenteral administration and to compare and contrast their provitamin A activity after parenteral administration with that after oral administration.

Experimental

The materials and methods were as described previously with the following additions:

Rats

General. The rats used in this work were all inbred albino animals of the Wistar strain.

In the experiments in which the effect of the vitamin A status of the animal on the conversion of injected carotene was studied, high liver storage was achieved by supplementing the basal diet with "Vetamul" a few days prior to the experiments.

The rats used in the experiments in which the utilization of the various carotenoid pigments was studied, using growth as a criteria, were completely deficient animals prepared as described previously (p.10). They were injected immediately their liveweight "plateaued" after which they were maintained for a further 14 days on the U.S.P. vitamin A-free test diet, during which time they were weighed every second day.

The rats used in all other experiments were partially deficient animals prepared as described on p.9.

Dosing. In experiments in which the effect of the route of administration was studied, oral administration was made by stomach tube consisting of approximately 8 cm of nylex tubing (1 mm bore) connected to an 18 G needle and a 2 ml. syringe; parenteral administration was by intravenous injection into the jugular vein exposed by ventral neck incision, by intramuscular injection into the gluteus maximus muscle, by intraperitoneal injection directly into the peritoneal cavity through the abdominal wall, and by subcutaneous injection into a fold of skin behind the foreleg.

In all other experiments in which carotene and other pigments were injected intravenously, injection was made directly into the vena cava exposed by ventral mid-line incision or into the jugular vein exposed by ventral neck incision.

Preparation of Carotenoid Pigments

General. Where pigments had to be prepared from natural sources the methods chosen in all cases were well recognised ones and for this reason their preparation is not described here in any great detail.

Carotene. Crystalline carotene (containing approximately 88% β-carotene) supplied by L. Light & Co. Ltd., Poyle, was used in the present experiments. For comparing the activity of α- and β-carotene this material was separated chromatographically on Magnesia (Hopkins & Williams Ltd., Sydney; heavy grade, No. 5368) into α and β fractions by a method similar to that of Strain (1934).

Xanthophylls. Fresh grass was extracted with acetone in the

cold, the extract was saponified (see p.16) and xanthophylls were separated from carotene by chromatography on alumina. The crude mixture of xanthophylls was used without further purification. Hereafter, for the sake of convenience, this fraction will be referred to simply as "xanthophyll".

Lycopene. Lycopene was extracted from fresh ripe tomatoes by means of acetone in the cold and purified by chromatography on alumina using the method of Strain (1942).

Zeaxanthin and Cryptoxanthin. These pigments were prepared from the calyx of Physalis franchetii essentially as described by Braude, Foot, Henry, Kon, Thompson & Mead (1941).

Retinene. Retinene was prepared as described previously on p.51.

Identification. The identity of all pigments was confirmed from their absorption spectra as published by Morton (1942), Karrer & Jucker (1950) and Goodwin (1952).

Preparation of aqueous dispersions of pigments. Aqueous dispersions of the various pigments were prepared exactly as described earlier for carotene on p.12. Dispersions of tocopherol* were similarly prepared.

Estimation of pigments

General. The various pigments were estimated as for carotene in a Beckman, model DU, photoelectric spectrophotometer using values for $\lambda_{max.}$ and $E^{1\%}_{1cm}$ quoted by Morton (1942) and

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* In the form of α-tocopheryl acetate supplied by L. Light & Co. Ltd., Poyle.

Goodwin (1952).

Positioning of pigments during chromatography. During the chromatographic separation of tissue extracts into vitamin A alcohol and ester, the positioning of the different pigments on the alumina column varied. Xanthophyll and zeaxanthin, for example, were strongly adsorbed and were eluted with the vitamin A alcohol fraction by 8% ethanol; cryptoxanthin and lycopene, on the other hand, were very much less strongly held and were eluted partly in the ester fraction with 2% acetone and partly in the alcohol fraction. Where much pigment was present in the ester fraction it was saponified and re-chromatographed as described on p.16 and the ester recovered as vitamin A alcohol for estimation. Where much pigment was present in the alcohol fraction, however, vitamin A could not be estimated. In cases where only small amounts of pigment were present in either fraction, a correction was applied to the blue reading with antimony trichloride equivalent to 0.06 µg vitamin A/ 1.0 µg of pigment present (McGillivray, Unpublished).

Results

Utilization of carotene dispersions administered by different routes.

The results of experiments on the utilization of carotene dispersions administered intravenously, intraperitoneally, intramuscularly, subcutaneously and orally are shown in Table 18. The rats were killed 4 days after dosing. With a dosage level of 400 µg/rat the carotene was equally well utilized, as indicated by hepatic vitamin A levels, whether injected intra-

venously or given orally and was almost as well utilized when injected intraperitoneally. Intramuscular injections of carotene were about one-third as well utilized as intravenous, but subcutaneous injections did not increase hepatic levels of vitamin A. At slaughter large quantities of carotene remained at the site of intramuscular and subcutaneous injection, that at the latter representing the bulk of the dose.

Effect of dosage level, interval between dosing and slaughter and repeated dosing on the utilization of carotene dispersions administered intravenously.

The effects of these factors are shown in Table 19 (Exps. 1-4). It is apparent from Exps. 1 and 2 that measurable amounts of vitamin A were stored in the liver after the intravenous injection of as little as 10 µg of carotene and that the hepatic stores of both carotene and vitamin A increased more or less directly with dosage level up to 800 µg of carotene, the highest level investigated.

From a consideration of Exps. 1 to 4 it is apparent that there is considerable variation in the behaviour of different Tween dispersions of carotene after injection. Exps. 2 and 3 were carried out with the same batch of carotene dispersion while in Exps. 1 and 4 two different dispersions were used. All dispersions were prepared in the same way, the only difference being that the dispersion used in Exp. 1 was prepared from a sample of carotene that had been in use for some time (although always stored at 0° under nitrogen when not in use, and showing no obvious signs of decomposition), whereas the other dispersions were prepared from a freshly opened ampoule of carotene. It is

apparent from the table that the dispersions differed in the total amount of vitamin A formed, in the ratio of vitamin A alcohol to ester in the liver and in the amount of carotene in the liver. While at the present time it is difficult to offer a satisfactory explanation for these findings, it is thought quite possible that part at least of the variations may be due to differences in the degree of dispersion of the carotene. They cannot be attributed to individual differences between the rats since the levels of carotene and vitamin A in the blood and livers of all animals injected with samples of the same dispersion were always remarkably constant. Throughout the present work, care has been taken to see that the same dispersion was used whenever comparisons were made between various treatments.

From the results of Exp. 3 it can be seen that liver vitamin A levels showed a steady increase with time up to 24 h after injection, but maximum carotene storage was achieved after 3 h. From the results of Exp. 4, if allowance is made for the fact that with the dispersion used in this experiment only 22 µg of vitamin A were stored in the liver from a dose of 400 µg of carotene as compared with 37 µg from the same dose level of another dispersion in Exps. 2 and 3, it appears that there is little difference in the utilization of the carotene whether it is given as a single injection or as smaller injections at 24 h intervals.

Effect of the vitamin A status of the animal on the conversion of injected carotene.

From the results of this experiment (Table 19, Exp. 5) it can be seen that 2 h after injection of 250 µg of carotene into rats with high vitamin A liver stores, blood levels of

vitamin A alcohol increased by approximately 50% which confirms the previous observation of Kon et al. (1955) in which a 20% increase was noted in non-deficient stock-colony rats injected with carotene. After injection of only 35 µg of carotene, however, no evidence of any increase in vitamin A blood levels could be detected. As the liver reserves were high, it is obvious that any increase in vitamin A in the liver due to injected carotene would have been masked by the normal variation in these reserves, and only mean values are given in order to indicate the vitamin A status of the animals before injection.

Effect of xanthophyll, tocopherol and benzoyl peroxide on the utilization of carotene dispersions administered intravenously.

That the simultaneous administration of xanthophyll or tocopherol inhibited the conversion of carotene dispersion into vitamin A is shown in Table 20. Since an anti-oxidant such as tocopherol exerts an inhibitory effect, it seemed possible that a pro-oxidant such as benzoyl peroxide might have the opposite effect. However, this was not so, the addition of benzoyl peroxide also markedly reducing the amount of vitamin A stored in the livers.

Conversion of carotenoids other than β-carotene.

Table 21 shows the results of injecting Tween dispersions of various carotenoid pigments other than β-carotene into rats. Of the pigments investigated, lycopene, xanthophyll and zeaxanthin showed no activity, although some blood and liver samples, particularly at 2 h after dosing, presented difficulties in interpretation of the antimony trichloride reaction owing to the presence of carotenoids, or their

Table 18. Utilization of aqueous carotene dispersions in Tween (400 µg carotene/0.4 ml. dispersion) administered by various routes to partially deficient rats of mean weight 240 g, killed 4 days after dosing.

No. rats used	Mode of administration	Liver			
		Vitamin A			Carotene (µg)
		Alcohol (µg)	Ester (µg)	Total (µg)	
3	-	0.4	0.7	1.1	0
6	Intravenous	2.5	11	14	36
3	Intraperitoneal	1.0	9.7	11	18
3	Intramuscular	0.5	2.8	3.3	14
3	Subcutaneous	0.4	0.9	1.3	4.2
3	Oral	3.3	10	13	5.0

Table 19. Effect of dose level (Exps. 1 and 2), interval between dosing and slaughter (Exp. 3), repeated dosing (Exp. 4) and vitamin A status (Exp. 5) on the utilization of aqueous carotene disperaions in Tween administered intravenously to rats of mean weight 250 g.

Exp.	No. rats used	Quantity carotene injected (µg)	Time between injection and slaughter (h)	Blood plasma		Liver			
				Vitamin A alcohol (µg/100 ml.)	Carotene (µg/100 ml.)	Vitamin A Alcohol (µg)	Vitamin A Ester (µg)	Vitamin A Total (µg)	Carotene (µg)
Controls	6	-	-	12	0	0.3	0.6	0.9	0
1	2	10	24	13	Not detectable	1.0	1.2	2.2	4
	2	20	24	12	Not detectable	0.8	1.7	2.5	6
	2	40	24	13	Trace	1.0	2.4	3.4	14
	2	75	24	14	Trace	1.1	5.0	6.1	27
	2	150	24	13	Trace	1.2	13.0	14	52
2	3	25	24	13	Not detectable	0.2	3.5	3.7	4
	3	50	24	12	Not detectable	0.3	5.7	6.0	19
	3	100	24	14	Trace	0.5	6.0	6.5	32
	3	200	24	13	Trace	2.3	12	14	33
	3	400	24	12	c. 25	4.7	32	37	64
	3	800	24	12	c. 50	4.2	40	44	160
3	3	400	3	67	3750	1.3	12	13	63
	3	400	6	23	380	1.0	13	14	60
	3	400	12	19	180	2.7	20	23	61
	3	400	18	16	35	2.0	24	26	60
	3	400	24	12	25	4.7	32	37	64
	3	400	48	13	Trace	3.5	33	37	62
	3	400	96	10	Trace	1.1	43	44	65
4	3	400	24	14	Trace	8.1	14	22	70
	3	400 + 400 24 h later	48 from first injection	16	Trace	10	22	32	110
	3	200, 200 + 400* at 24 h intervals	72 from first injection	15	Trace	4.0	23	27	66
5	5	-	-	21	0				0
	3	35	2	22	110	80	2000	2080	12
	6	250	2	33	1800				34

* Injected into the vena cava.

Table 20. Effect of xanthophyll, tocopheryl and benzoyl peroxide on the utilization of aqueous carotene dispersions in Tween administered intravenously to rats, of mean weight 300 g, killed 24 h after injection.

Exp.	No. rats used	Dose	Blood plasma		Liver			
			Vitamin A alcohol (µg/100 ml.)	Carotene (µg/100 ml.)	Vitamin A			Carotene (µg.)
					Alcohol (µg.)	Ester (µg.)	Total (µg.)	
1	6	-	12	0	0.3	0.6	0.9	0
	3	200 µg carotene	17	Trace	4.8	10	15	25
	3	+ 200 µg xanthophyll	12	Trace	2.3	3.3	5.6	21
	3	+ 400 µg xanthophyll	17	Trace	2.1	1.9	4.0	28
	3	+ 800 µg xanthophyll	15	Trace	2.3	1.7	4.0	28
	3	400 µg carotene	14	Trace	8.1	14	22	70
	3	+ 400 µg xanthophyll	14	Trace	3.0	4.3	7.3	87
2	3	400 µg carotene	12	c. 25	4.7	32	37	64
	3	+ 2 mg tocopheryl acetate	15	c. 15	1.0	25	26	98
	3	+ 4 mg tocopheryl acetate	14	c. 15	1.0	10	11	100
	3	+ 6 mg tocopheryl acetate	16	c. 15	0.7	10	11	100
3	3	400 µg carotene	14	Trace	8.1	14	22	70
	3	+ 1 mg tocopheryl acetate	15	Trace	6.8	9.0	16	63
	3	+ 2 mg tocopheryl acetate	12	Trace	5.8	7.5	13	55
	3	+ 3 mg tocopheryl acetate	10	Trace	5.1	6.0	11	60
	3	+ 4 mg tocopheryl acetate	18	Trace	3.4	4.3	7.7	61
	3	+ 20 mg tocopheryl acetate	8	Trace	2.5*	3.5*	6.0	65
4	3	200 µg carotene	17	Trace	4.8	10	15	25
	3	+ 100 mg tocopheryl acetate	12	Trace	1.3*	2.4*	3.7	42
	3	400 µg carotene	22	Trace	5.2	12	17	68
	3	+ 100 mg tocopheryl acetate	17	Trace	1.3*	2.8*	4.1	84
	3	+ 100 µg benzoyl peroxide	21	Trace	4.8	7.0	12	58
	3	+ 400 µg benzoyl peroxide	17	Trace	2.9	2.0	4.9	38

* Colour developed with antimony trichloride somewhat atypical.

Table 21. Appearance of carotenoid pigments and vitamin A in the blood and liver of rats partially deficient in vitamin A 24 h after the intravenous injection of the pigments as Tween dispersions.

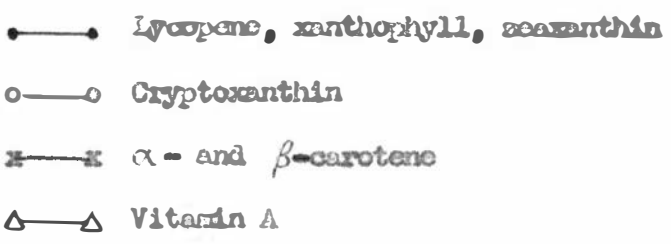
Rats		Dose				Time between injection and killing (h)	Blood plasma		Total liver content		
		Vehicle		Substance			Vitamin A alcohol	Carotenoid pigment	Vitamin A		Carotenoid pigment
No. used	Mean weight (g)	Nature	Amount (ml.)	Nature	Amount (µg)		(µg/100 ml.)	(µg/100 ml.)	Alcohol (µg)	Ester (µg)	(µg)
7	210	None	-	None	-	-	15	0	0.6	0.4	0
3	190	Tween dispersion	0.3	α-carotene	300	24	20	Trace	7.6	8.2	53
3	190		0.3	β-carotene	300	24	29	Trace	8.8	17	24
5	185		0.3	Lycopene	175	2	*	230	1.2+	0.7	34
3	190		0.3	"	180	24	14	Trace	0.6	0.8	32
3	165		0.4	Xanthophyll	230	2	*	260	0.2	0.9	34
3	230		0.4	"	250	24	16	Trace	0.6	0.5	54
4	200		0.2	Zeaxanthin	170	24	12	Trace	3.0+	0.7	88
6	200		0.3	Cryptoxanthin	220	24	16	20	2.0	1.9	34
4	225		0.4	Retinene	400	½	27	0	15	94	0
4	230		0.4	"	400	24	17	0	21	140	0

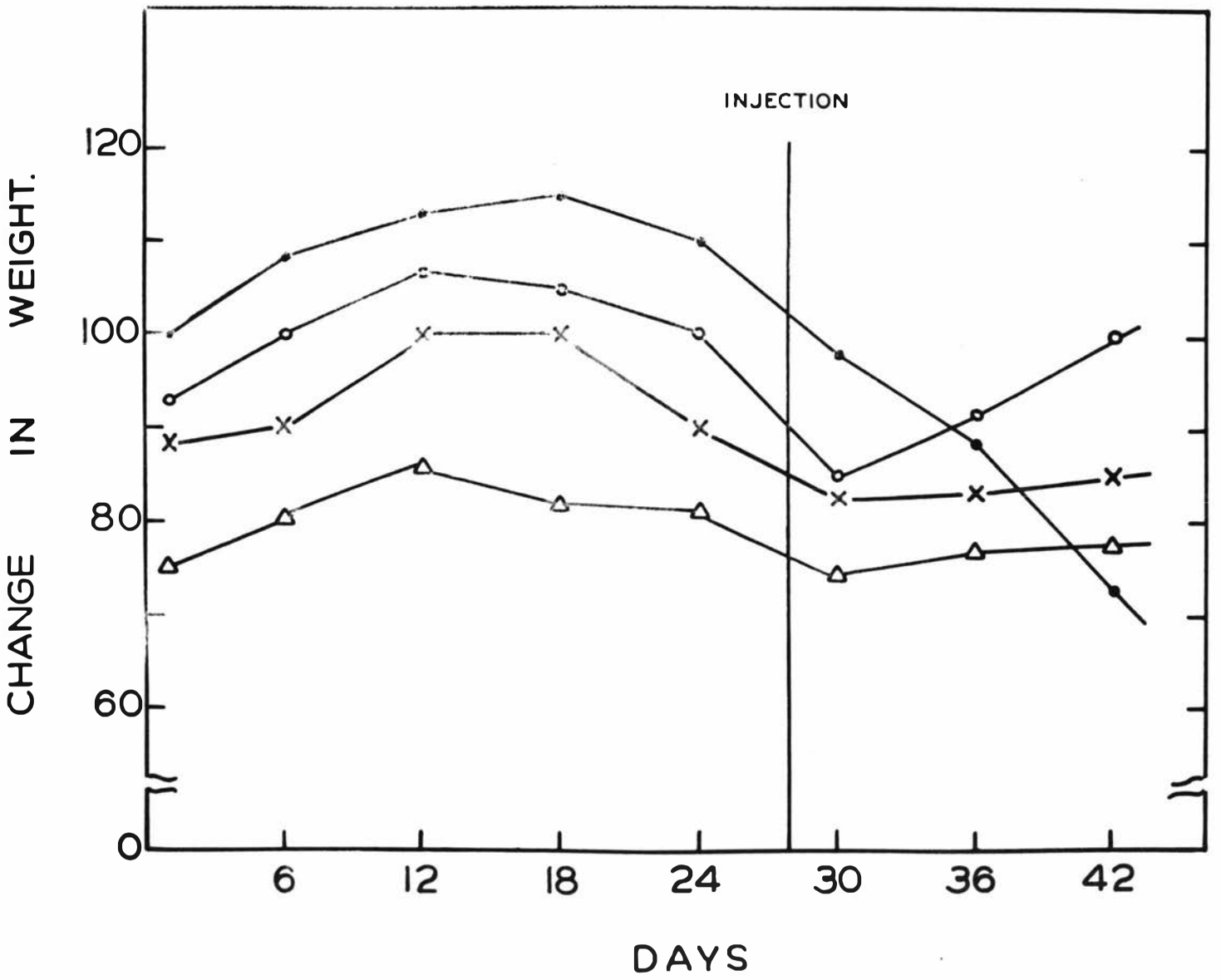
* Vitamin A could not be estimated accurately owing to presence of pigments.

\+ In these cases the colour developed with antimony trichloride was atypical of vitamin A due to the presence of pigments for which no satisfactory allowance could be made.

Figure 1.

Growth of completely deficient rats after injection of Tween dispersions of various carotenoids. 400 μg dose in each case




INJECTION
120
100
80
60
0
CHANGE IN WEIGHT.
6
12
18
24
30
36
42
DAYS

decomposition products, that could not be separated entirely from the vitamin A alcohol or ester. β-carotene was better utilized than α-carotene, as judged both by blood vitamin A alcohol and by total liver storage. Cryptoxanthin presents an anomaly, since there was little evidence of conversion to vitamin A after injection, although the same sample when administered orally showed activity, 1.2 mg giving rise in a deficient rat to 33 µg of vitamin A in the liver. Dispersions of retinene were well and rapidly utilized, since half an hour after the injection, the shortest time-interval studied, vitamin A mainly as the ester appeared in the liver and no retinene could be detected in either the blood or liver.

Similar results were obtained also in a further experiment using restoration of growth in young deficient rats as the criterion of activity. These results are summarized in Fig. 1. As expected, lycopene, xanthophyll and zeaxanthin showed no activity. On the other hand α-carotene, β-carotene and vitamin A were all active in causing a resumption of growth, as was also cryptoxanthin, which, rather surprisingly, was even more active than vitamin A.

Discussion

From the results presented in Table 18 it is clear that carotene in aqueous dispersion was converted into vitamin A by rats when injected intravenously, intraperitoneally or intramuscularly. There was, however, no evidence of any increase in liver vitamin A after subcutaneous injection. At slaughter, 4 days after dosing, no measureable amounts of carotene were present in any of the blood plasma samples and it is probable,

from the low level present in the liver, that measurable concentrations of carotene were not attained in the blood at any stage after subcutaneous injection. This is of considerable interest since it would appear from Table 19 (Exp. 3) that liver levels of vitamin A continued to increase only as long as reasonable levels of carotene remained in the blood plasma. These results also further suggest that, as long as a reasonably high level of carotene remains in the plasma, vitamin A is formed at some extrahepatic site and transported to the liver via the blood as vitamin A alcohol (see also Kon et al. 1955). There was no significant increase in liver vitamin A after plasma vitamin A levels had returned to normal, although the liver still contained large quantities of carotene. Further, the high blood plasma vitamin A alcohol levels attained in as little as 5 min after injection of carotene dispersions (e.g. Kon et al. 1955; also Tables 6 and 8) indicate an extremely rapid conversion. In contrast, the relatively slow increase in liver vitamin A (as shown, for example, in Table 19, Exp. 3) would suggest that there is no similar rapid reaction in the liver. It has already been shown (e.g. Chapter II) that hepatectomized animals are capable of converting intravenously administered carotene to vitamin A, and it would seem from the foregoing that in the intact animal also the liver is an unimportant site of conversion.

From the results presented in Table 19 (Exps. 1-3), it is apparent that the amount of vitamin A appearing in the liver after injection is more or less directly related to dosage level, within the range studied, and is also influenced by the interval between dosing and slaughter. However, except for dosage level, the various factors investigated appeared to have little influence on the amount of carotene taken up by the liver.

The level of carotene bore a fairly definite relationship to the dose injected (Table 19, Exps. 1 and 2), but showed surprisingly little change from 3 to 96 h after injection (Exp. 3) and was not markedly influenced by either xanthophyll or tocopherol (Table 20, Exps. 1-4). It is difficult to advance any simple explanation for the levels of carotene found in the livers in, for example, Exps. 2 and 3 (Table 19) in which the same dispersion was used. No simple equilibrium between plasma carotene and liver carotene, whether or not followed by a change in the physical nature of the liver carotene that prevents its subsequent release into the plasma as the level there decreases, seems compatible with the results obtained under the conditions of decreasing plasma carotene levels after the various initial levels injected. It has already been shown, however (Kon et al. 1955), that larger particles, such as are present in colloidal dispersions, are rapidly taken up by the liver and are not subsequently released into the plasma. Tween dispersions of carotene probably contain particles of a wide range of size, and a possible explanation for the liver carotene levels obtained is that all the carotene present as larger particles is removed rapidly, and retained, by the liver and possibly by other tissues. Thus differences in liver carotene levels obtained with the various dispersions used would, as suggested earlier, represent genuine differences in the physical state of these dispersions.

In the experiment with non-deficient rats (Table 19, Exp. 5), the increases of almost 50% in blood vitamin A after injection of 250 µg of carotene confirm those observed previously by Kon et al. (1955) with stock-colony rats. Although the increases were less on a percentage basis than in partially deficient rats,

it is clear that the conversion of intravenously administered carotene also occurs in normal rats and is not merely an alternative mechanism in animals with low vitamin A reserves. These findings also confirm that the differences in the efficiency of conversion of intravenously administered carotene between partially deficient rats and normal rabbits observed by Kon et al. (1955), and in the present studies in Chapter II, were not due to variations in the vitamin A status of the animals but to genuine species differences in conversion.

Kelley & Day (1950) have shown that rats given xanthophyll by mouth along with carotene stored less vitamin A in their livers than those given carotene alone. Exp. 1 (Table 20) shows that the simultaneous intravenous administration of xanthophyll and carotene produced a similar decrease in hepatic stores. An explanation for this finding is not easy to offer, but it would suggest that in oral administration the effect of xanthophyll may be more intimately associated with the mechanism of conversion than with the initial absorption of the carotene.

The sparing effect of tocopherol on carotene administered orally has been demonstrated by a number of workers (e.g. Hickman, Kaley & Harris, 1944a, b; Harris, Kaley & Hickman, 1944; see also review by Hobart & Morgan, 1953), but it is also established that higher levels of tocopherol (of the order of 5 mg/rat daily) depress the utilization of carotene as measured both by growth rate and liver storage of vitamin A (Johnson & Baumann, 1948). A similar decreased utilization of intravenously administered carotene associated with the simultaneous injection of tocopherol is clearly apparent from Table 20 (Exps. 2-4). Even as little as 1 mg of tocopheryl acetate had a marked effect on liver

vitamin A, decreasing the amounts of both alcohol and ester forms. The anti-oxidant properties of tocopherol would explain this inhibiting effect if the mechanism of conversion of carotene into vitamin A involves oxidation (cf. Glover & Redfearn, 1954; Fazakerley & Glover, 1957; Fishwick & Glover, 1957). The effect of benzoyl peroxide in also reducing liver storage of vitamin A could be due to a different type of oxidation.

It is apparent from the results in Table 21 that in general the activity of the various carotenoid pigments after intravenous injection is similar, as indicated by liver vitamin A levels, to that after oral administration. Carotenoids not containing the β-ionone ring structure showed, as might be expected, no vitamin A activity, although they were actively metabolized, as judged by their rapid disappearance, only a small fraction of the initial dose remaining in the blood and liver after 24 h. As when given orally, β-carotene was about twice as well utilized as α-carotene.

Bicri (1955a), working with chickens, has also reported inefficient conversion of injected cryptoxanthin to vitamin A as indicated by liver storage. Likewise, as in the present experiments (Fig. 1), he also demonstrated in growth studies that after injection some conversion does in fact take place. By way of an explanation for this apparent discrepancy, he concluded that after injection of cryptoxanthin just sufficient vitamin A was formed to permit normal growth but not sufficient to achieve appreciable liver storage.

In the experiments reported here it is clear that the behaviour of retinene differed both from that of the other

carotenoids, and from the vitamin A acetate dispersions used in the work of Kon et al. (1955). It was more rapidly converted to vitamin A than the other carotenoids, no retinene remaining in the blood or liver after half an hour, and it appeared to be somewhat more stable than vitamin A acetate similarly injected. Furthermore, the initial increase in liver vitamin A storage after half an hour was in the ester form whereas in all other cases the alcohol predominated. These observations are in keeping with those of Glover et al. (1948b) following oral and parenteral administration and with the results obtained in vitro in Chapter IV.

Whatever the processes involved in conversion, there seem to be marked similarities between the mechanism of breakdown after oral or intravenous administration of carotene. This similarity is evident from the utilization of the various carotenoid pigments (Table 21), and it is significant also that substances such as xanthophyll and tocopherol, which are known to affect the utilization of orally administered carotene, should be found to exert a similar effect on intravenous administration.

If, as has been suggested previously in Chapter III, all tissues are capable of effecting the conversion of parenterally administered carotene to vitamin A, the failure to detect some conversion after subcutaneous administration is somewhat surprising. The possibility cannot be overlooked, however, that conversion occurs, or is at least initiated, in the blood rather than the tissues themselves, a random oxidation resulting in a fragment, such as, for example, a carotenal, that can be readily converted into

vitamin A. The similar rates of removal of the different carotenoid pigments, including those not converted to vitamin A (Table 21), and of carotene from the blood of rats, rabbits and calves (although as discussed by Kon et al. (1955) the last do not form vitamin A) would support the idea of a random oxidation rather than a specific reaction producing vitamin A (see also Bieri & Pollard, 1953, 1954). With a view to investigating this possibility further and obtaining more precise information on the nature of the processes involved, it was decided to undertake a more detailed study of the effect of tocopherol on the conversion of intravenously administered carotene. Since, quite clearly, the function of the blood in conversion could not be studied by direct surgical procedures such as were used in the case of the liver and lung, it was thought that an investigation of some of the quantitative aspects of the inhibiting action of tocopherol might provide another means of approach to the problem. This work accordingly forms the subject of the next chapter.

Summary

1. The utilization of aqueous dispersions of β-carotene and other carotenoid pigments in Tween has been studied in rats. A number of factors affecting utilization have also been investigated.

2. Hepatic vitamin A stores were increased by the intravenous, intraperitoneal, intramuscular or oral administration of carotene dispersions, but not by subcutaneous injection.

3. There was a fairly direct relationship between the level of carotene and vitamin A in the liver and the amount of carotene injected. Liver stores of vitamin A continued to increase as long as measurable amounts of carotene remained in the blood plasma, but liver levels of carotene remained constant after 3 h.

4. It is suggested that the carotene present in the liver after intravenous administration merely represents the fraction of the dose present as larger particles.

5. Conversion was observed to occur in both deficient and non-deficient animals.

6. Lower hepatic stores of vitamin A resulted from the simultaneous intravenous administration of xanthophyll or of relatively large amounts of tocopherol with the carotene, and attention is drawn to the apparent similarity in the mechanism of breakdown of carotene after oral or intravenous administration.

7. Using vitamin A storage as a measure of activity, α-carotene was shown to be about one-half as active as β-carotene similarly injected. Lycopene, xanthophyll and zeaxanthin were completely inactive, as judged both by liver storage of vitamin A and growth studies. Cryptoxanthin produced little liver storage but was shown to be capable of supporting normal growth. Retinene disappeared rapidly from the blood after injection and was efficiently converted to vitamin A ester.

8. It is suggested that the conversion of intravenously administered carotene dispersions into vitamin A results primarily from a random oxidation of the carotene and that at least the initial stages of the oxidation may occur in the blood.

CHAPTER VI

FURTHER STUDIES ON THE EFFECT OF TOCOPHEROL ON THE UTILIZATION OF INTRAVENOUSLY ADMINISTERED AQUEOUS DISPERSIONS OF CAROTENE

Introduction

In the previous chapter evidence was presented suggesting that the blood may play a much more specific role in the conversion of intravenously administered aqueous dispersions of carotene to vitamin A than had hitherto been suspected. Evidence was also presented showing that the simultaneous intravenous administration of tocopherol inhibited the conversion of injected carotene and that the degree of inhibition was more or less directly related to the level of tocopherol injected (Table 20, Expts. 2-4). At about the time that this work was carried out there came to the author's notice a publication by Bieri (1955b) in which this worker reported that in experiments in which tocopherol had been administered orally to rats to build up reasonably high tocopherol levels in the tissues, no effect on the efficiency of conversion of injected carotene could be detected either by growth or tissue vitamin A studies. From a consideration of this evidence, along with that obtained in the last chapter, it seemed that further experiments, more detailed than the last, in which the fate of injected tocopherol was followed, and in which carotene was injected at various time intervals before and after tocopherol injections, might throw further light on the role played by the blood, and perhaps other tissues, in the conversion of intravenously administered carotene to vitamin A in the intact animal.

Experimental

The materials and methods used in the present experiments were as described previously with the following additions:

Rats. The rats used were albino of the Wistar strain, partially deficient in vitamin A, and maintained on the basal diet previously described. Their mean weight at the time of use was 250 g.

Injections. All injections were into the iliac vein exposed by ventral leg incision.

Estimation of tocopherol in tissues. Total tocopherol in different tissues was estimated as described on p. 166 et seq.

Estimation of vitamin A in carcass. In order to follow the build up of vitamin A in the rat at various intervals after injection of carotene, the whole carcass was assayed. After a sharp head blow the animal was dropped into a mixture of 20 ml. of 60% KOH and 100 ml. of ethanol at 70° and saponified for 10-20 min until the carcass was digested. 100 ml. of distilled water were added and the mixture was extracted with two successive lots of freshly distilled diethyl ether. The combined ether extracts were washed thoroughly with distilled water, dried over anhydrous sodium sulphate and evaporated to dryness on a hotplate at 70°. The residue was then taken up in 2-3 ml. of petroleum ether and chromatographed (see p.14). Vitamin A (as the alcohol) was recovered in the 8% ethanol fraction and estimated with antimony trichloride.

Results

Injection of tocopherol alone. The results presented in

Table 22 show that, after the intravenous injection of an aqueous dispersion of 20 mg of α-tocopheryl acetate, there was a rapid disappearance of this substance from the blood. After 5 min approximately 75% of the dose was recovered from the blood but after 1 h only about 5% remained in the blood and at 24 h the level had returned to normal. The levels in the lungs and kidneys were also highest immediately after injection and showed decreases with time similar to that in the blood. At 24 h, however, the level in the lungs was still about ten times normal and in the kidneys almost twice normal. On the other hand levels of tocopherol in the liver increased steadily during the first hour after injection, reaching a maximum between 1 and 2 h, and then decreasing steadily to levels 15-20 times normal at 24 h.

In the tissues examined, the total recovery of tocopherol was about 90% at 5 min and this level gradually decreased with time to 10% at 24 h.

Injection of tocopherol at various time intervals before and after injection of carotene.

Table 23 (Exps. 2 and 3) shows the effect on conversion of injecting an aqueous dispersion of 20 mg of α-tocopherol at various intervals before and after injection of 400 μg of carotene as an aqueous dispersion, while Table 23 (Exp. 1) shows the effect on conversion of administering 20 mg of α-tocopherol in aqueous dispersion simultaneously with 400 μg of carotene in aqueous dispersion. From the results presented it is apparent that the efficiency with which the rat is capable of utilizing intravenously administered carotene

Table 22. Levels of tocopherol in rat tissues at various time intervals after the intravenous injection of aqueous dispersions of α-tocopheryl acetate. (20 mg dose in 0.4 ml. Tween).

No. rats used	Time after injection (h)	Blood* (mg)	Liver (mg)	Lungs (µg)	Kidneys (µg)	Total recovery (mg)
3	Control	0.13	0.11	3.5	9	0.2
4	1/12	14.5	1.8	1200	145	18
4	1/4	10.0	2.9	670	105	14
4	1/2	4.2	6.0	335	110	11
4	3/4	1.6	8.0	125	37	10
4	1	1.1	11.2	135	41	12
4	2	0.53	8.0	115	35	8.6
4	4	0.33	2.9	60	22	3.2
4	24	0.11	1.9	32	14	2.0

* Total tocopherol present/rat.

Table 23. The effect of preliminary, simultaneous or subsequent intravenous injections of an aqueous dispersion of 20 mg ∝-tocopheryl acetate in 0.4 ml. Tween on the liver and blood levels of vitamin A formed from 400 µg to carotene similarly administered. All animals killed 24 h after carotene injection.

Exp.	No. rats used	Treatment	Interval between injections (h)	Blood vitamin A alcohol (µg/100 ml.)	Liver vitamin A		
					Alcohol (µg)	Ester (µg)	Total (µg)
1	2	None	-	8	0.3	0.6	0.9
	6	Carotene alone	-	14	2.8	28	31
	2	Carotene and tocopherol	Simultaneous	8	1.6*	4.0*	5.6
2	4	Carotene after tocopherol	1/12	9	2.2*	5.8*	8.0
	2		1/4	11	2.0	11	13
	4		1/2	11	2.0	17	19
	4		1	12	2.7	22	25
	2		2	14	4.9	21	26
	2		4	13	1.9	24	26
	4		24	13	2.0	28	30
3	3	Tocopherol after carotene	1/12	14	2.3	9.0	11
	2		1/4	14	4.9	25	30
	3		1/2	16	3.0	31	34
	3		1	15	2.1	31	33
	3		2	14	1.8	35	37
	3		4	13	2.0	30	32

* Colour developed with antimony trichloride somewhat atypical.

Table 24. Appearance of carotene and vitamin A in whole rat carcasses at various time intervals after the intravenous injection of an aqueous dispersion of 400 µg carotene in 0.4 ml. Tween (one rat at each time interval).

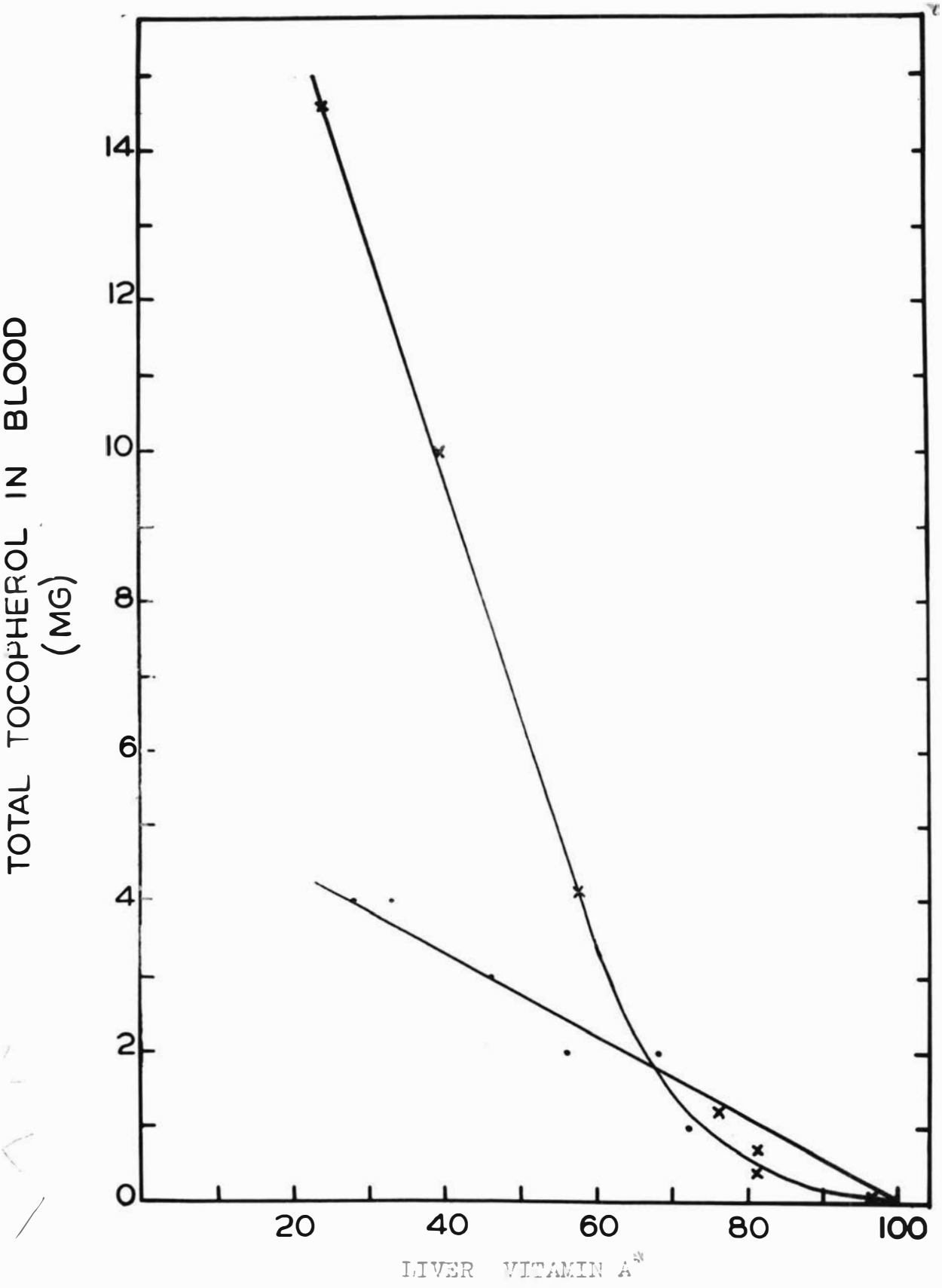
Interval between injection and slaughter (h)	Carotene (µg)	Vitamin A (µg)
Control (no injection)	0	1.8
$\frac{1}{12}$	400	7.2
$\frac{1}{4}$	380	7.8
$\frac{1}{2}$	340	7.2
1	350	13
2	320	14
4	300	21
24	230	26

Figure 2.

Relationship between blood levels of tocopherol at the time of carotene injection and the quantity of vitamin A appearing in the liver of the rat 24 h later.

●——● Tocopherol resulting from the injection of varying amounts of tocopheryl acetate simultaneously with the carotene.

×——× Tocopherol resulting from a preliminary injection of tocopheryl acetate.



* To allow for slight differences in the carotene dispersion vitamin A is expressed as a percentage of that appearing in the liver 24 h after injection of carotene alone.

is depressed by a simultaneous or preliminary injection of tocopherol. The effect of the tocopherol decreased with time, however, and conversion appeared to be normal when the injections were separated by 24 h.

From the results of Exp. 3 it is apparent that tocopheryl acetate injected 5 min after the carotene depressed conversion to about one-third, but when injections were separated by 15 min or longer, no effect was apparent.

Total vitamin A formed at various time intervals after injection of carotene.

The total amount of vitamin A present in the rat carcass at various time intervals after injection of carotene is shown in Table 24. Particularly at the shorter time intervals difficulties were encountered in estimating vitamin A due to the presence of an artifact which could not be separated from vitamin A alcohol and which produced, with antimony trichloride, an atypical non-fading colour. The vitamin A levels must therefore be regarded as approximate only but, whatever the absolute level, it is apparent that there was a steady increase in vitamin A in the whole carcass up to 24 h after carotene injection.

Discussion

The present experiments were undertaken for the purpose of investigating in more detail the effect of tocopherol on the utilization of intravenously administered aqueous dispersions of carotene, and in the hope of obtaining further information on the role played by the blood in this regard.

From the results presented in Table 23 it is clear that, not only is the utilization of intravenously administered carotene dispersions markedly reduced by the simultaneous injection of tocopherol, but that the effect of tocopherol persists, to a steadily decreasing extent, for some hours. Thus with the dosage levels used in the present investigation (400 µg β-carotene and 20 mg α-tocopheryl acetate), conversion of carotene, as indicated by liver stores of vitamin A 24 h after injection, was about one-fifth normal when carotene and tocopherol were administered simultaneously, about four-fifths normal when the carotene was injected 1 h after the tocopherol and was normal again when the interval between injections was increased to 24 h.

In earlier studies (e.g. Chapter V, Table 19; see also Kon et al. 1955) it was shown that the level of vitamin A in the liver increases steadily during the first 24 h after injection. Similarly in the present investigation, although some difficulties were encountered, due to the presence of interfering artifacts, in measuring the total amount of vitamin A in the whole carcass, the results obtained also indicate a steady formation of vitamin A over this period (Table 24). In view of this apparent formation of vitamin A over 24 h, it was surprising to find that when tocopherol was injected after the carotene, liver storage of vitamin A was depressed only at the 5 min interval and that conversion was normal when the injection was separated by quarter of an hour or longer. The amount of vitamin A in the carcass quarter of an hour after injection was only about one-third that present at 24 h (Table 24) and it would have been anticipated that the injection at that time of tocopherol would have depressed the formation of further

vitamin A. However, no such effect was apparent and the most probable explanation for these observations would appear to be that there is an initial rapid breakdown of the carotene to an intermediate which is then more slowly converted to vitamin A. It is probable that it is the formation of this intermediate which is inhibited by tocopherol (cf. also p.97, para. 3) and that the formation of vitamin A during the 24 h following injection is dependent on the further breakdown of this intermediate rather than on the continued level of carotene in the blood (cf. p.93, para. 1). Indeed as has been suggested for the pigment present in the liver (Chapter V), carotene in the blood and other tissues may, in the course of quarter of an hour or less, have been converted to a physical state from which it is no longer available to the animal for conversion to vitamin A. Kon et al. (1955) have already shown that the utilization of intravenously administered carotene is intimately related to its physical state and it is significant that although the initial appearance of vitamin A in the blood is extremely rapid, the vitamin A which finally appears in the liver represents only a very small proportion of the injected carotene (about 10% of a 400 µg dose). Although no information is available regarding the fate of intravenously administered Tween 40, it is possible that it could be metabolized sufficiently rapidly to explain a change in the physical state of the carotene.

In the experiments reported in Chapters I to III it was shown that conversion of injected carotene to vitamin A was little affected by removal of the liver, stomach, small intestine, large intestine, pancreas, kidneys, adrenals, gonads,

head or neck and on the basis of these results it was concluded that none of these organs or tissues was specifically involved in the conversion of injected carotene and that probably many tissues were capable of effecting the transformation (cf. Bieri & Pollard, 1954, Kon et al. 1955). Experiments of this type, however, tend to give qualitative rather than quantitative results due to the differences in blood volumes involved and the short time during which the animal can survive following acute surgery. Thus conversion may have been considerably reduced by the removal of some of these organs and possibly in the intact animal one or more of these organs may in fact be a main site of conversion. From the evidence presented by Kon et al. (1955), however, there would appear to be good grounds for rejecting the intestine as a main site of conversion in the intact animal and from the results presented in Chapter V it would appear likewise that the liver is not involved to any significant extent.

Further evidence regarding possible sites of carotene conversion can be obtained from the known effect of tocopherol and the rate at which it appears and disappears from tissues after intravenous injection. Since the reaction which tocopherol inhibits is an extremely rapid one, the levels of tocopherol in the tissues during and immediately following carotene injection are clearly of greatest interest. The tissue levels of tocopherol shown in Table 22 therefore afford further evidence for rejecting the liver as the main site of conversion in the intact animal. The levels of tocopherol in the liver bear no relationship to the utilization of carotene injected at increasing time intervals after tocopherol,

the level of tocopherol being low immediately after injection when the inhibition is greatest and highest some 2 h after injection by which time conversion is returning to normal. In the blood, kidneys and lungs, however, levels of tocopherol are highest immediately following injection and show decreases with time similar to the decreasing effect of tocopherol on conversion. Although this relationship is closest in the case of blood tocopherol levels, the experiment in itself hardly affords evidence for rejecting tissues such as the lungs and kidneys in favour of the blood as the site of conversion. It is significant, however, that, on the basis of other evidence presented in Chapter V, attention has already been drawn to the blood as the site where conversion could at least be initiated ".....a random oxidation resulting in a fragment, such as, for example, a carotenal, that can readily be converted into vitamin A".

Obviously further work is required to corroborate the present findings but all the evidence presented here is entirely compatible with the suggestion of the breakdown being initiated in the blood, with the rapid formation of an intermediate such a carotenal, which is then more slowly converted to vitamin A. All attempts to effect the conversion of carotene to vitamin A by incubation in vitro with blood (Chapter IV) unfortunately have failed, nor have any likely intermediates been identified in the blood of the intact animal after carotene injection or in the incubated material. The intermediate could, however, be a labile substance which rapidly undergoes further oxidation unless removal from the blood by the tissues where its conversion into vitamin A could occur.

The close negative relationship between blood tocopherol levels at the time of carotene injection and liver levels of vitamin A 24 h later is shown in Fig. 2. Data for this figure were obtained from Tables 22 and 23. Also plotted on the same figure is the effect of the injection, simultaneously with carotene, of comparable levels of tocopherol. These data were obtained from Tables 20 (exps. 2-4). If it is only the level of tocopherol in the blood which influences conversion, it would be anticipated that a given level of tocopherol, whether injected with carotene or resulting from a previous injection of tocopherol, would have produced the same depression in conversion. It would appear, however, that tocopherol injected simultaneously, at least at the higher levels, is considerably more effective in reducing conversion. It is difficult to explain this difference unless it is again the physical state of the tocopherol which is involved.

Summary

1. The effect of tocopherol on the conversion of intravenously administered aqueous dispersions of carotene has been further investigated.

2. After intravenous injection of an aqueous dispersion of α-tocopheryl acetate there was a rapid disappearance of this substance from the blood; at 5 min approximately 75% of the dose was recovered but at 1 h only 5% remained and at 24 h the level had returned to normal. The levels in the lungs and kidneys were also highest immediately after

injection and showed decreases with time similar to the blood. At 24 h, however, the level in the lungs was still about ten times normal and in the kidneys almost twice normal. In contrast, levels in the liver increased steadily during the first hour after injection, reaching a maximum between 1-2 h and then decreasing slowly to levels 15-20 times normal at 24 h.

3. Conversion of carotene into vitamin A was reduced by simultaneous or preliminary injections of tocopherol, the effect of tocopherol in the latter case decreasing with time up to 24 h when conversion was again normal. When tocopherol was injected after carotene, conversion was reduced at the 5 min interval but when injections were separated by 15 min or longer no effect was apparent.

4. It is concluded that intravenously administered carotene undergoes a rapid breakdown, probably in the blood, to an intermediate which is then more slowly converted to vitamin A.

CHAPTER VII

THE UTILIZATION OF AQUEOUS DISPERSIONS OF CAROTENE BY GUINEA PIGS AND CAROTENE AND VITAMIN A BY LACTATING GOATS

Introduction

Although it is now well established that parenterally administered carotene can be converted to vitamin A in the rat, rabbit, pig, sheep and chick (e.g. Kon et al. 1955), no evidence has appeared to date concerning conversion in the guinea pig or goat. This is somewhat surprising in view of the fact that both species are extensively used for experimental purposes. It was thought desirable, therefore, that the matter should be investigated. At the same time it was decided to undertake a study of the effect of aqueous dispersions of vitamin A alcohol and ester injected intravenously into lactating goats, following the claim by Sobel, Rosenberg & Engel (1952) that intravenous administration of an aqueous dispersion of vitamin A to lactating cows resulted in a marked increase in the vitamin A level of their milk. A study was also carried out of the effect on milk levels of carotene and vitamin A of injecting carotene and vitamin A dispersions directly into the tissue of the mammary gland. It is the purpose of the present chapter to report the results of these experiments.

Experimental

General. The materials and methods were essentially as described previously with the following additions:

Guinea pigs. The guinea pigs used in these experiments were suckling animals approximately one week old obtained from the College Veterinary Department colony. They were offspring of inbred albino stock-colony does which had been maintained throughout the later stages of gestation on a

diet fairly low in vitamin A consisting of a basal ration of concentrate cubes* plus hay. Their vitamin A stores at the time of use were, therefore, very low. All injections were into the jugular vein exposed by ventral neck incision.

Goats. The goats used were of a mixed breed, predominantly Saanen, and were tethered on pasture throughout the experimental period. Unless otherwise stated they were milked only once daily. All intravenous injections were made into, and blood samples were withdrawn from, the jugular vein, using an 18 G hypodermic needle. Intramammary injections were made into the tissue of the mammary gland, using a 24 G needle, the needle being withdrawn slowly during the injection to distribute the dispersion through the tissue; each dose was given as two equal injections.

Assay of milk. Fat was extracted from the milk samples by an adaption of the method of Olson, Hegsted & Peterson (1939) in general use in this laboratory. 50 ml. of milk (or 25 ml. of milk and 25 ml. of water if milk of high fat test) was denatured with 7.5 ml. of conc. NH_4OH and 50 ml. of alcohol and extracted by shaking with 60 ml. of diethyl ether and 30 ml. of petroleum ether. The mixture was allowed to settle and the bottom layer run off and re-extracted with 40 ml. of diethyl ether and 20 ml. of petroleum ether. The ether extracts were combined, centrifuged, washed twice with 25 ml. of warm distilled water and dryed over anhydrous sodium sulphate. The ether was next evaporated on a hotplate at 70° and the weight of the remaining fat determined. The fat was then

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* See p.51.

taken up in 2-3 ml. of petroleum ether, chromatographed on alumina, and the contents of carotene, vitamin A alcohol and ester estimated as described on pp. 14-16. (See also pp. 169, 170).

Results

The results presented in Table 25 show clearly that the guinea pig is capable of effectively utilizing parenterally administered carotene in Tween as a source of vitamin A. This is indicated by significant increases in the vitamin A levels of the blood and liver at intervals following injection of carotene into young animals with lower-than-normal vitamin A reserves. From the results of Exp. 1 it can be seen that, as in the rat, there was a rapid increase in the vitamin A alcohol content of the blood after injection, followed by a slower build up in the liver, initially as the alcohol but later mainly as the ester. It is of interest that the amount of vitamin A formed after injection of comparable doses of carotene was approximately the same as in the rat and that the amount of carotene taken up by the liver reached a maximum 3 h after injection and thereafter remained constant (cf. Table 19). From the results of Exp. 2 (Table 25) it is clear also that, over the range studied, the amount of vitamin A appearing after injection and the amount of carotene stored in the liver were both influenced by the level of carotene administered.

From the results presented in Table 26 (Exp. 1) it can be seen that although intravenous administration of carotene in Tween to goats resulted in comparatively high blood levels of carotene for some time after injection, there was no evidence

whatsoever to suggest any formation of vitamin A from this circulating carotene. While these results are admittedly limited in extent and obtained from only a small number of animals, they nevertheless provide an indication that conversion in the goat, if it occurs at all, is extremely inefficient when compared with that in some other species. The results presented also make it clear that no carotene was transferred to the milk after intravenous administration of carotene dispersion, nor was the level of vitamin A alcohol or ester in the milk measurably increased by any vitamin that may have been formed from the carotene. Similarly, there was little indication of the transfer to the milk of intravenously administered vitamin A alcohol or palmitate (Exps. 2-4). In Exps. 2 and, particularly, 3 there was some increase in vitamin A alcohol but, considering the level of vitamin A alcohol injected, the effect was very slight.

Blood plasma alcohol and ester levels recorded in Exp. 5 indicate that, as has been shown previously for Tween dispersions of vitamin A acetate injected into rats, rabbits and calves by Kon et al. (1955), vitamin A palmitate was rapidly hydrolysed in goats. It was also rapidly destroyed in or removed from the blood plasma, both alcohol and ester levels having returned to normal within 2 h. The equally rapid esterification of vitamin A alcohol, as shown by plasma levels in Exps. 3 and 4, indicates an equilibrium reaction.

In contrast to intravenous administration, injection of carotene dispersions directly into the tissue of the mammary gland gave some indication (Table 27, Exps. 1-3) of conversion into vitamin A, the alcohol level in the milk always

Table 25. Appearance of carotene and vitamin A in the blood and liver of young guinea pigs after intravenous administration of aqueous dispersions of carotene in Tween.

Exp.	No. animals used	Amount carotene injected (µg)	Time from dosing to slaughter (h)	Blood Plasma		Liver		
				Vitamin A alcohol (µg/100 ml.)	Carotene (µg/100 ml.)	Vitamin A alcohol (µg)	Vitamin A ester (µg)	Carotene (µg)
1	2	-	-	12	0	2.6	3.8	Trace
	1	400	1/12	60	4400	2.5	3.0	Trace
	1	400	1	56	1900	14	4.1	29
	2	400	3	40	900	8.8	8.8	70
	1	400	12	26	480	6.4	17	66
	2	400	24	18	Trace	6.6	31	72
2	2	200	24	14	Trace	3.4	23	49
	2	400	24	18	Trace	6.6	31	72
	2	800	24	15	Trace	5.8	40	90

Table 26. Utilization of aqueous dispersions of carotene and of vitamin A alcohol and palmitate injected intravenously into lactating goats.

Exp.	No. goats used	Dose of dispersion (ml.)	Substance		Time after injection (h)	Blood Plasma			Milk Fat		
						Vitamin A			Vitamin A		
			Nature	Amount (mg)		Alcohol (µg/100 ml.)	Ester (µg/100 ml.)	Carotene (µg/100 ml.)	Alcohol (µg/g)	Ester (µg/g)	Carotene (µg/g)
1	3*	0+	None	-	-	41	-	0	0.2	15	0
		20	Carotene	20	1/12	44	-	700	0.2	14	0
					2	42	-	140	0.3	16	0
					4	40	-	80	0.2	16	0
					18	40	-	55	0.4	18	0
					24	42	-	30	0.3	15	0
					48	41	-	21	0.2	17	0
					96	40	-	12	0.3	16	0
					120	43	-	0	0.2	15	0
2	1	0+	None	-	-	47	5.0	-	0.4	14	0
		20	Vitamin A alcohol	20	1/12	350	175	-	-	-	-
					4	44	4.5	-	0.5	17	0
					24	42	3.7	-	0.3	14	0
					48	46	4.1	-	0.3	15	0
3	1	0+	None	-	-	51	3.0	-	0.3	19	0
		10	Vitamin A alcohol	10	1/12	165	41	-	-	-	-
					1/2	68	8.7	-	-	-	-
					1	52	5.1	-	-	-	-
					2	49	2.9	-	0.9	18	0
					4	49	3.4	-	0.5	18	0
4	1	0+	None	-	-	44	8.2	-	0.3	18	0
		10	Vitamin A palmitate	10	1/12	59	280	-	-	-	-
					1/2	52	79	-	-	-	-
					1	44	24	-	-	-	-
					2	42	9.3	-	0.5	19	0
					4	42	8.5	-	0.5	17	0

* One animal non-lactating.

+ Values before injection.

Table 27. Utilization of aqueous dispersions of carotene and of vitamin A alcohol and palmitate injected directly into the tissue of mammary gland of lactating goats. (Dose: 5 mg of substance in 5 ml. of dispersion)

Exp.	No. goats used	Substance	Time after injection (h)	Milk fat				
				Control Gland*		Treated Gland		
				Vitamin A		Vitamin A		Carotene
				Alcohol (µg/g)	Ester (µg/g)	Alcohol (µg/g)	Ester (µg/g)	(µg/g)
1	1	None +	-	0.3	17	0.5	17	0
		Carotene	24	0.6	16	2.2	16	420
			48	0.2	17	1.2	18	170
2	1	None +	-	0.4	18	0.4	16	0
		Carotene	4	-	-	-	-	380
		Carotene ‡	24	0.3	14	1.0	17	160
			48	0.4	16	1.6	16	180
			72	0.5	16	1.3	16	110
3	1	None +	-	0.3	14	0.4	14	0
		Carotene	6	0.2	14	1.4	15	340
			24	0.2	13	1.0	15	110
			48	0.3	14	0.8	12	21
			72	0.3	15	0.4	16	7
4	1	None +	-	-	-	0.2	16	0
		Vitamin A alcohol	24	-	-	5.0	21	0
			48	-	-	4.0	20	0
			96	-	-	0.3	18	0
5	1	None +	-	-	-	0.2	16	0
		Vitamin A	18	-	-	22	780	0
		palmitate	42	-	-	8.4	280	0
			66	-	-	4.5	140	0
			168	-	-	0.2	18	0

* Injected with 5 ml. of 20% Tween solution (see p. 124).
\+ Values before injection.
‡ 2nd injection 24 h after first.

increasing. In Exp. 3, 5 ml. of a 20% Tween solution were injected into the control gland at the same time as the injection of carotene dispersion into the treated gland. The results indicate that the increases in milk vitamin A alcohol were associated with the carotene and did not result from an infiltration of blood plasma vitamin A alcohol due to tissue damage after injection of Tween.

From the results presented in Table 27 (Exps. 4 and 5, respectively) it is apparent that some esterification of vitamin A alcohol and some hydrolysis of vitamin A palmitate occurred after direct injection into the gland. The appearance in the milk, and the continued secretion of carotene and vitamin A, in Exps. 3-5 is not surprising and is not contradictory to the results obtained after intravenous administration. By the former route some of the dose would be injected directly into the alveoli and ducts, where, owing to local blockage, it could be retained for some time before release. The permeability of the membranes to aqueous dispersions would, therefore, not be involved.

Discussion

The main aim of the present experiments was to obtain evidence on the utilization of aqueous dispersions of carotene administered by vein to guinea pigs and of carotene, vitamin A alcohol and ester administered by vein to goats. In addition the utilization of similar preparations of carotene, vitamin A alcohol and ester administered directly into the mammary gland of goats was also studied.

From the results obtained with guinea pigs (Table 25), it

is apparent that this species is capable of converting intravenously administed aqueous dispersions of carotene to vitamin A about as effectively as the rat. The build up of vitamin A alcohol and ester in the liver after injection simulated that in the rat and the levels of vitamin A appearing 24 h after injection of varying amounts of carotene were also similar (cf. Table 19). It was of interest to note also that, as in the rat, the level of carotene stored in the liver reached a maximum at 3 h after injection and thereafter remained constant*.

After intravenous injection of carotene into goats there was no evidence of any increase in the vitamin A alcohol content of the blood either in lactating or non-lactating animals, and, on the basis of this finding, it must be concluded that in the goat, as in the calf (e.g. Church et al. 1954; Kon et al. 1955), injected carotene is converted to vitamin A very inefficiently, if at all. Why certain species should be unable to convert injected carotene is not known. Church et al. (1954) have suggested that the failure of the calf in this respect may be associated with the fact that in this species carotene normally circulates in the blood, but clearly,

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* This similarity in behaviour between the rat and the guinea pig was further borne out by the results of two other experiments; the first, in which the livers were removed from two guinea pigs (using the method described previously on p.30), and the second, in which the lungs were removed from two animals (using the method described on p.41). After injection of 400 ug of carotene in Tween into the hepatectomized animals, plasma vitamin A alcohol levels increased from a control level (one animal) of 15 µg/100 ml. to 53 µg/100 ml. in ¼ h, while after injection into the pneumonectomized animals, plasma vitamin A alcohol levels increased to 47 µg/100 ml. in 7 min. These levels resembled very closely those observed in rats similarly treated (cf. Tables 6 and 8).

such reasoning cannot be applied to the goat in which carotene does not circulate to any appreciable extent. A more likely reason, therefore, as has been suggested by Kon et al. (1955), would appear to be a difference in the katabolic pathway of carotene between those species normally capable of effecting conversion and those not. In this respect it is of interest that the rate of katabolism of carotene, as measured by its rate of disappearance from the blood, appears to be greater in the former group than the latter. For example Church et al. (1954) have shown the rate of disappearance of injected carotene from the blood to be greater in sheep than in calves while in the present work it has been shown to be greater in rats and guinea pigs (Tables 19 and 25) than in goats (Table 26). Similarly, Kon et al. (1955) have shown that although the initial rate of disappearance of carotene (i.e. over the first 2 h after injection) is the same in the rat, rabbit and calf, carotene persists in the blood of the calf for a much longer period following injection. Quite possibly these observations may have an important bearing on the problem although their exact significance at the moment is not easy to see.

Neither carotene, vitamin A alcohol nor ester injected intravenously in aqueous dispersion into goats had any significant effect on the levels of these substances appearing in the milk fat. This result is somewhat surprising in view of the findings of Sobel et al. (1952) in cows, which showed that intravenous administration of aqueous dispersions of vitamin A caused a marked increase in the vitamin A content of the milk fat. This discrepancy may represent a species difference associated perhaps with the fact that after injection vitamin A

was removed much more rapidly from the blood of goats than cows, but appears more likely to be attributable to a difference in the type of dispersion used*. That no carotene appeared in the milk, even though considerable amounts continued to circulate in the blood several days after injection by which time presumably it was no longer present as a Tween dispersion but in some more normal physiological form, is curious and can be explained only by assuming the mammary-gland membrane to be completely impermeable to carotene.

The fact that carotene in Tween injected directly into the tissue of the mammary gland, in contrast to that injected by vein, is converted to vitamin A in quite appreciable amounts is surprising and is difficult to explain. It is a matter which requires further study but unfortunately this was not possible in the present instance. Goats are not very readily obtainable for experimental work in this country and the ones used in the present series of experiments and in those of the following chapter were wild animals obtained from hill-country sheep stations. The animals were nervous and fairly difficult to handle and the number which could be kept at any one time was limited. For these reasons the amount of work which could be carried out with them was restricted. The experiments in which carotene was injected directly into the mammary gland were the very last to be performed in goats and were carried out in animals which were rapidly nearing the end of lactation, and, on this account, could not be extended.

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* This is further borne out by the fact that in an isolated experiment in which vitamin A alcohol as an aqueous dispersion in Tween was injected intravenously into a single cow (McGillivray, 1956, unpublished), no change could be shown in the vitamin A content of the milk fat.

Summary

1. The utilization of aqueous dispersions of carotene administered by vein has been studied in guinea pigs and the utilization of similar preparations of carotene, vitamin A alcohol and ester administered by vein and directly into the mammary gland has been studied in goats.

2. Young guinea pigs were able to utilize injected carotene as evidenced by marked increases in the levels of vitamin A alcohol in the blood and vitamin A alcohol and ester in the liver at various intervals after injection. The total level of vitamin A alcohol present in the liver increased steadily up to 24 h after injection; the level of carotene present, on the other hand, remained constant after 3 h. The total amount of vitamin A finally appearing in the liver was shown to be influenced by the level of carotene administered.

3. There was no evidence of any increase in the vitamin A alcohol content of the plasma of goats up to 120 h after injection of 20 mg of carotene in Tween. From this it was concluded that goats do not convert injected carotene to any appreciable extent.

4. After intravenous injection of carotene, vitamin A alcohol and ester there was no indication of any significant increase in the levels of these substances in the milk fat. Vitamin A alcohol injected by vein was rapidly esterified and removed from (or destroyed in) the blood. Likewise,

after injection vitamin A palmitate was rapidly hydrolysed and removed from (or destoyed in) the blood. Following injection of either alcohol or ester, blood levels returned to normal within 2 h. After injection of carotene, however, traces of pigment were still present in the blood 4 days later.

5. After injection of carotene directly into the mammary gland there was evidence of an increase in the vitamin A alcohol content of the milk from which it was concluded that some conversion of carotene had occurred. After injection of vitamin A palmitate some hydrolysis was again apparent as was also some esterification of injected alcohol. Injection of carotene, alcohol and ester into the gland results in increases in all these substances in the milk.

CHAPTER VIII

THE UTILIZATION OF INTRAVENOUSLY ADMINISTERED CAROTENE AND VITAMIN A EMULSIONS BY RATS AND GOATS

Introduction

It is now well established, both from earlier work (e.g. Kon et al. 1955) and from the results discussed in the preceding chapters, that intravenously administered aqueous dispersions of carotene in Tween can be converted to vitamin A in a wide range of animal species. So far, these Tween preparations of carotene are the only forms of the pigment which have been unequivocally demonstrated to be converted to vitamin A to any appreciable extent when administered by routes other than the oral. Colloidal and oily forms of the pigment, for example, when administered intravenously appear to be quite inactive (e.g. Sexton et al. 1946; Kon et al. 1955). Recently, however, Greenberg, Levenson & Rose (1955) reported that carotene administered intravenously to vitamin A-depleted rats as an emulsion* could be effectively utilized as a source of vitamin A, approximately 20% of the dose administered being recovered from the liver as vitamin A forty five minutes after dosing. In view of the fact that colloidal and oily preparations of carotene are inactive and Tween dispersions at best, even 24 h after administration, yield only about 10% of vitamin A (see Chapter VI), it was thought desirable that the utilization of carotene from an emulsion should be further investigated and an attempt made to confirm the work of Greenberg and his colleagues. It was thought of interest also to investigate the utilization of vitamin A

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* Prepared by Emuo Products Inc., New York, and containing 10% carrot oil and 7.2% gelatin (R. Greenberg, private communication).

alcohol and palmitate emulsions similarly administered. The results of those studies are discussed in the present chapter together with the results of some experiments in goats with similar preparations.

Experimental

General. All methods and materials were as described earlier.

Rats. All rats were inbred albino animals of the Wistar strain partially deficient in vitamin A.

Goats. The goats were predominantly Saanen, in full lactation, with normal vitamin A reserves. They were tethered on pasture throughout the experimental period. All injections were into, and blood samples were withdrawn from, the jugular vein.

Preparation of emulsions. Emulsions of carotene and of vitamin A alcohol and palmitate (all supplied by L. Light & Co. Ltd., Poyle) were prepared essentially as described previously in Chapter IV. In the case of the carotene emulsions, tocopherol was added during the preparation of the emulsion to stabilize the pigment present and prevent its too rapid destruction (see Table 28).

Results

Intravenous administration of carotene and vitamin A emulsions to rats partially deficient in vitamin A.

Blood plasma and liver levels attained at intervals after the intravenous administration of carotene or vitamin A

alcohol or palmitate emulsions to rats are shown in Table 28. From the results obtained in Exp. 1 it is apparent that vitamin A palmitate administered in this way is well utilized, over one half of the dose being accounted for in the plasma and liver at all time intervals studied. The increased vitamin A alcohol levels in both plasma and liver at the shorter time intervals indicates some hydrolysis of the palmitate, but at 4 h most of the vitamin A in the liver was in the esterified form and at 24 h re-esterification was complete. From the results of Exp. 2 it is clear that vitamin A alcohol was not as well utilized as the palmitate, slightly less than one third of the dose being recovered from the plasma and liver after injection at the various intervals studied. Most of the vitamin A in the liver immediately after injection was present in the alcohol form but as the interval after injection increased this situation was gradually reversed until after 24 h the ester form predominated.

It can be seen from Exps. 3 and 4 that there was some indication of conversion of the emulsified carotene into vitamin A. At the shorter time intervals there was an increased plasma vitamin A alcohol level succeeded by an increase in liver vitamin A alcohol and finally esterification of this liver vitamin A. It is interesting that after injection of 400 µg. of carotene (Exp. 4A), the level of carotene in the liver showed no significant change from 5 min to 24 h, although the level in the plasma fell from about 100 µg/100 ml. to just a trace

A feature of the work was the rapidity with which the

Table 28. Effect of intravenous administration of emulsions of vitamin A palmitate, vitamin A alcohol and carotene to rats partially deficient in vitamin A (mean weight 300 g).

Exp.	No. rats used	Dose: Vol. (ml.)	Dose: Substance: Nature	Dose: Substance: Amount (µg)	Time between injection and killing (h)	Blood Plasma: Carotene (µg/100ml.)	Blood Plasma: Vitamin A: Alcohol (µg/100ml.)	Blood Plasma: Vitamin A: Ester (µg/100ml.)	Blood Plasma: Vitamin A: Total per rat * (µg)	Liver: Carotene (µg)	Liver: Vitamin A: Alcohol (µg)	Liver: Vitamin A: Ester (µg)	Liver: Vitamin A: Total (µg)	Total vitamin A per rat (plasma + liver) (µg)
1	2	-	None	-	-	0	7	5	1	0	4	7	11	12
	3	2.0	Vitamin A palmitate	Equivalent to 100 µg vitamin A	1/12	0	28	157	29	0	9	24	33	62
	3				1/2	0	25	100	18	0	12	42	54	72
	3				2	0	29	53	9	0	10	47	57	66
	3				4	0	34	9	4	0	9	65	74	78
	3				24	0	12	7	2	0	6	62	68	70
2	2	-	None	-	-	0	20	4	2	0	1.0	2.5	3.5	5.5
	1	2.0	Vitamin A alcohol	Equivalent to 100 µg vitamin A	1/12	0	65	11	8	0	20	10	30	38
	1				1/2	0	49	13	6	0	13	17	30	36
	1				2	0	39	8	6	0	6.5	19	26	32
	1				4	0	28	9	4	0	5.3	27	32	36
	1				24	0	19	5	2	0	2.0	29	31	33
3	3	-	None	-	-	0	10	-	1.0	0	0.4	0.6	1.0	2.0
	3	2.0	Carotene	400	24	Trace	14	-	1.3	c.15	0.5	1.7	2.2	3.5
4A	6	-	None	-	-	0	13	-	1.2	0	0.6	0.8	1.4	2.6
	3	2.0	Carotene	400	1/12	c.1000	16	-	1.5	26	1.6	0.8	2.4	3.9
	3		α-tocopheryl acetate ‡	1000	1/2	c.500	23	-	2.0	29	2.2	0.8	3.0	5.0
	3				2	c.200	21	-	1.9	25	1.9	1.6	3.5	5.4
	3				4	c.200	21	-	1.8	31	2.0	3.2	5.2	7.0
	6				24	Trace	20	-	1.8	33	1.3	2.6	3.9	5.7
B	4	2.0	Carotene 200 α-tocopheryl acetate ‡ 1000		24	Trace	14	-	1.4	11	0.8	1.7	2.5	3.9
C	3	1.0	Carotene 100 α-tocopheryl acetate ‡ 500		24	Trace	13	-	1.2	9	1.0 +	2.5 +	3.5	4.7

* These figures show the total amount of vitamin A in the blood in µg calculated on the assumption that the rat contains 6.7 ml. blood/100 g (Cartland & Koch, 1928) of which plasma constitutes 50%.

\+ The colour developed with the antimony trichloride reagent was somewhat atypical but still indicative of some vitamin A.

‡ See p. 132.

Table 29. Levels of carotene and vitamin A in the blood plasma and milk fat after intravenous administration to a goat of 20 ml. of emulsion containing 4 mg of carotene.

Time after injection (h)	Blood Plasma*		Milk Fat*	
	Vitamin A alcohol (µg/100 ml.)	Carotene (µg/100 ml.)	Vitamin A ester (µg/g)	Carotene (µg/g)
Before dosing	30	0	13	0
½	32	13	-	-
½	28	6.0	-	-
1	27	4.0	-	-
2	28	4.0	12	0
4	30	3.0	11	0

* There was no measurable quantity of vitamin A ester in the blood plasma or of vitamin A alcohol in the milk fat.

carotene and vitamin A remaining in the plasma were destroyed after the blood was withdrawn from the animal. All assays were therefore carried out as soon as possible after slaughter of the animals. This destruction was particularly marked with plasma carotene after injection of carotene emulsion; despite the precautions taken extremely variable plasma carotene levels were recorded. The plasma carotene levels are shown in Table 28, therefore, as approximate means only.

Intravenous administration of carotene emulsions to lactating goats.

Carotene emulsions (10-20 ml.) were injected intravenously into three lactating goats. In none was carotene detected in the milk drawn 2-48 h after injection, nor was there any significant increase in the vitamin A ester or alcohol in the milk or blood plasma. Typical figures are given in Table 29 for one goat* injected with the same carotene emulsion as was used for the rats recorded in Table 28 (Exp. 4A).

Discussion

The conversion of intravenously administered carotene emulsion to vitamin A confirms the findings of Greenberg et al. (1955) who demonstrated the appearance of vitamin A in the rat following intravenous injections of a commercial carotene emulsion specially prepared from carrot oil. The

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* It should be pointed out that both the goats and rats invariably showed signs of distress after injection of the emulsion. As a result two of the goats and several of the rats died after injection due presumably, from the appearance of the animals, to some form of respiratory failure.

efficiency of conversion in the present experiments, however, was of a very much lower order than that reported by the latter authors, due, presumably, to differences in the physical state of the two emulsions, or, perhaps, to the presence of tocopherol which was added to stabilize the carotene present in the emulsion. It is obvious also that conversion was much less efficient than in the case of the aqueous carotene dispersions reported in preceding chapters with the same quantity of carotene. From the plasma vitamin A alcohol levels and the liver vitamin A alcohol and ester levels at the various intervals, however, it would appear to follow a similar course, the vitamin A appearing first in the blood plasma as the alcohol from which it was taken up by the liver and subsequently esterified.

From the results obtained after injection of emulsions of vitamin A alcohol and ester into rats it is apparent that the alcohol is less efficiently utilized than the ester but why this should be so is not clear unless, due to its somewhat more reactive nature, the free vitamin is more prone to destruction. The utilization of vitamin A palmitate from an emulsion is of particular interest since the absorbed vitamin A, following oral administration, is transported from the intestines to the storage organs in an esterified form (Thompson et al. 1949). If the utilization of this palmitate emulsion does to any extent simulate the uptake from the plasma of vitamin A ester in transport, it would appear to be a rapid and relatively efficient process, one-fifth of the dose being taken up

in the first 5 min and almost half the dose in ½ h.

In the goat, although the mammary gland is capable of utilizing dietary fats by a direct uptake of blood chylomicrons (e.g. review by Glascock, 1954), it would appear that the membrane is either impermeable to fat globules of the type or size present in the carotene emulsion or that the carotene is destroyed or removed from the fat during the process. Furthermore, from the plasma vitamin A alcohol levels recorded after injection there was little to indicate any conversion of the emulsion into vitamin A.

Summary

1. The utilization by rats and by lactating goats of intravenously administered emulsions of carotene and of vitamin A alcohol and palmitate has been investigated.

2. In the rat vitamin A palmitate was rapidly and relatively efficiently taken up by the liver, almost half the dose appearing in the liver in ½ - 2 h.

3. Less than one-third of the dose of vitamin A alcohol injected into rats was stored in the liver, mainly as the ester, after 24 h.

4. Rats converted carotene injected in an emulsified form into vitamin A. The conversion was effected in a similar manner, but not as efficiently, as when the carotene was injected as aqueous dispersions in earlier experiments.

5. No carotene ap eared in the milk and the vitamin A level was unchanged after the intravenous administration of carotene emulsions to lactating goats. Blood levels of vitamin A alcohol appeared to remain unaffected by injection of carotene emulsion.

CHAPTER IX

INTERGRATING SUMMARY

From the results of the work discussed in the preceding eight chapters the following points of general interest emerge:

1. Carotene administered parenterally as an aqueous dispersion in Tween can be effectively utilized as a source of vitamin A by the rat, rabbit and guinea pig but not by the goat. Roughly about 10% of the dose administered appeared in the livers of rats and guinea pigs 24 h after injection; rabbits, however, were very much less efficient converters than the latter species. Carotene administered intravenously as an emulsion was also converted to vitamin A in the rat although the reaction was of a very low order of efficiency. Carotene administered intravenously as an emulsion to goats was not converted to vitamin A nor was there any evidence of any increase in the carotene or vitamin A contents of the milk.

2. A number of factors have been shown to affect the efficiency of utilization of parenterally administered aqueous dispersions of carotene:

   (a) Carotene administered intravenously or intraperitoneally was more effectively utilized than carotene administered intramuscularly. Carotene administered subcutaneously was utilized very inefficiently, if at all.

   (b) As the dose of carotene increased, hepatic stores of both carotene and vitamin A also increased, the relationship over the dose range 10-300 μg being more or less linear.

(c) The level of vitamin A in the liver increased steadily up to 24 h after injection and thereafter remained steady. Maximum storage of carotene was achieved after 3 h.

(d) Simultaneous administration of xanthophyll, tocopherol and benzoyl peroxide decreased the efficiency of conversion of carotene into vitamin A.

3. Conversion of injected carotene into vitamin A has been demonstrated to take place in both deficient and non-deficient animals.

4. Carotenoids not possessing the β-ionone ring structure were shown to be completely lacking in vitamin A activity when administered intravenously although they were actively metabolized as indicated by their rapid disappearance from the tissues after injection. β-carotene was shown to be about twice as active as α-carotene as judged by liver storage of vitamin A. Cryptoxanthin produced little liver storage of vitamin A but was shown to be capable of supporting normal growth. Retinene disappeared rapidly from the blood after injection and was efficiently converted to vitamin A.

5. Evidence was obtained showing that the reaction involved in the conversion of carotene to vitamin A after parenteral administration of carotene was similar to the reaction involved after oral administration.

6. Thyroid activity was shown to be without any effect on the conversion of intravenously administered carotene into vitamin A, from which it was concluded that the effect of the

thyroid on the conversion of orally administered carotene must be by way of its effect on the intestinal absorption of carotene rather than on the mechanism of conversion into vitamin A.

7. A series of experiments is reported in which different tissues were removed prior to injection of carotene in an attempt to locate the site at which carotene is converted into vitamin A. Removal of the small intestine, large intestine, pancreas, kidneys, adrenals, gonads, lungs, head or neck had little apparent effect on the level of vitamin A alcohol appearing in the blood after injection, from which it was concluded that possibly many tissues are capable of effecting conversion. Attempts to check which tissues in particular were involved in conversion using various in vitro techniques unfortunately were not successful. It would appear that the reaction in vitro is exceedingly difficult to demonstrate although retinene and vitamin A alcohol were metabolized normally under these conditions.

8. In a further study of the inhibiting effect of tocopherol on conversion, it was shown that the reaction which tocopherol inhibits is a rapid one occurring within the first 15 min after injection at a time when tocopherol is highest in the blood, lung and kidney. While conversion possibly may occur at any one or all of these sites, it is suggested that the breakdown of carotene is most probably initiated in the blood, with the rapid formation of an intermediate such as a carotenal, which is then more slowly converted to vitamin A.

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SECTION II

A STUDY OF SOME FACTORS AFFECTING THE VITAMIN A POTENCY OF MILK FAT

With Particular Reference to Factors Affecting the Summer Decline in the Vitamin A Potency of New Zealand Milk Fat.

INTRODUCTION

The vitamin A potency of English, European and American butters has been investigated by a number of workers and clearly defined seasonal variations in potency have been shown to occur. Booth, Kon, Dann & Moore (1933), in England, showed that fat produced from Shorthorn cows during the winter contained less carotene and vitamin A than that produced during the summer. Later, Kon (1945) confirmed these results using bulked milk collected from sixteen districts throughout the country, and concluded:

> "A general seasonal trend is plainly visible for each locality.... In general summer milk in this country has a potency of 30 or 40 I.U./g. butterfat. In winter the value may fall to 10 or 20 units."

Similarly, values recorded by Morgan & Pritchard (1937) for mixed European butters showed a minimum in late winter and a maximum in late spring/early summer. In a more recent paper Lord (1945) has confirmed these general trends and has correlated variations in the carotene and vitamin A contents of the milk fat with similar changes in the carotene and vitamin A levels of the blood.

Seasonal variations in the vitamin A potency of American butter were first reported by Baumann & Steenbock (1933) at Wisconsin, who observed wide differences between the carotene and vitamin A contents of milk fat produced under winter and summer conditions. These observations have been confirmed more recently by the results of the nation-wide survey published by the United States Department of Agriculture (1945, 1947), which showed that:

> "In all but two states there was a distinct difference between the vitamin A potency of butter produced under winter-feeding conditions and that produced under summer-feeding conditions. About 35% of all creamery butter is "winter" butter and it has an average vitamin A potency of 10,500 or 11,200 I.U./lb.... About 65% of all creamery butter is "summer" butter (produced by cows on pasture) and it has an average vitamin A potency of 17,000 or 18,000 I.U./lb."

Values showed a sharp rise following the transition from stall-feeding to pasture-feeding conditions, after which they remained almost constant until the autumn, when they fell gradually reaching a minimum in late winter.

Early workers regarded these trends as part of the normal lactational cycle but this has since been proved incorrect (e.g. Gillam, Heilbron, Ferguson & Watson, 1936; Hibbs, Krause & Monroe, 1949). The level of carotene in the diet is now known to be the most important factor influencing these variations. For example, Heilbron & Gillam (1937), in experiments with stall-fed cows on winter rations, observed a rapid increase in the carotene and vitamin A contents of butter following supplementation with dried grass or A.I.V. silage, these higher levels being successfully maintained until all groups were again placed on the control diet, when they fell to their original levels. These observations were confirmed also by the results of similar work at Beltsville*, in which butter of maximum potency was produced throughout the winter by supplementing the normal concentrate ration with

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* See Misc. Publ. U.S. Dep. Agric. No. 636, 1947: Butter as a source of vitamin A in the diet of the people of the United States, pp. 30-31. Washington: U.S. Gov. Printing Office.

good quality alfalfa and corn silage. From these results it was concluded that under stall-feeding conditions variations in potency were primarily a function of the carotene content of the cow's ration, and that the decline in activity of normal winter milk fat was due to an absence from the diet of foods rich in carotene.

This relationship under New Zealand dairying conditions is somewhat different since cows are continuously at pasture and the complicating effects of stall-feeding are thus eliminated. Even during periods of relative pasture shortage, crops such as green maize, chou moellier, lucerne and grass silage, all of which are rich in carotene, are normally fed. Under such conditions the production of fat of uniformly high potency might be expected. That this was not the case was first reported by Barnicoat (1947), who found marked seasonal variations in butter representative of two North Island dairying districts (Waikato and Manawatu) during the 1935-36 season. Both carotene and vitamin A were shown to be affected but variations were in the opposite direction from those reported for overseas butters, maximum potencies (42-53 i.u./g fat) being recorded in late winter and spring and minimum potencies (33-37 i.u./g fat) in late summer. Subsequently, these trends were confirmed by McDowell & McDowall (1953) in a more extensive investigation covering other New Zealand dairying districts and extending over several seasons, and by Farrer, Balding, Warren & Miller (1949) for a number of districts in Australia where dairying conditions are comparable with those existing in New Zealand.

Several aspects of the problem have already been investigated.

The possible influence of the high vitamin A potency of colostrum on the potency of New Zealand factory-made butter has been discussed at some length by McDowall & McDowall (1953) who concluded that:

> "When account is taken of the possible proportion of early post-parturition fat in the butter..... it is obvious that the carotenoid contents of New Zealand butterfat in the early spring months cannot be due to the inclusion of post-colostral fat; the values should fall as the number of newly calved cows to the total number of cows decreases during July and August, whereas, on the contrary, the values continue to rise during August. Furthermore, it is clear.... (a) that the rises in carotene and vitamin A contents commence in the late autumn and continue throughout the winter period, and that, therefore, the high values for July and August butterfats are a continuation of a trend already in progress before the spring, i.e. the calving season; and (b) that in some years the high carotene values persist for such a long period that they could not possibly be attributed to the presence of post-colostral fat in the butter....."

The production of butterfat in New Zealand is highly seasonal, the majority of cows being calved down in late winter and early spring (July, August, September), with the result that the maximum production of fat is obtained in the late spring and early summer (November, December, January). It is possible, therefore, that the summer decline in potency is a "lactational" effect associated with the high production of butterfat over this period. McGillivray (1949, 1956) has shown, however, that total vitamin A secretion (i.e. total potency x weight of fat produced) follows the same general seasonal trend as total potency. Further, Barnicoat (1947) and McDowall (1956), using spring- and autumn-calvers, found that there was no relationship between vitamin A potency and stage of lactation and concluded that variations with season were associated mainly

with factors of nutritional origin (see also McGillivray, 1957a).

Numerous estimates have been made of the intake of carotene required for the production of fat of maximum vitamin A potency*. Russell, Taylor, Chichester & Wilson (1935), Atkeson, Hughes, Kunerth, Peterson & Kramer (1937) and Fraps, Copeland, Treichler & Kemmer (1937) have recorded intakes ranging from an equivalent of 400 to 600 mg of carotene per day. Similarly, Wilbur, Hilton & Hauge (1940) concluded that cows receiving approximately 460 mg of carotene per day produced fat of maximum potency containing 53 i.u./g butterfat, and Hauge, Westfall, Wilbur & Hilton (1944) obtained of the same potency with a carotene intake of 300 mg per day, using dehydrated alfalfa hay or carotene in oil as the source of provitamin. Wiseman, Sheppard & Cary (1949), on the other hand, concluded that an intake in the vicinity of 2500 mg was required to maintain maximum potency. Likewise, Hibbs, Krauss & Monroe (1949) found that after the initial rise following the change from stall- to pasture-feeding, milk carotenoid content did not appear to follow closely changes in pasture carotenoid content except when the pasture carotenoid level was below 260 µg/g dry matter (i.e. a total carotene intake in the region of 2600 mg/day), and they concluded that there was a maximum response at this level.

Only limited information is available concerning the carotene content of typical New Zealand dairy pastures but from the work of Shorland (1949), Cawley (1950) and McGillivray

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* The term "maximum vitamin A potency" refers to the maximum potency obtainable on a diet containing carotene as the only source of vitamin A.

(1952) it would appear to be fairly uniform throughout the winter, spring and early summer within the range 450-650 µg/g dry matter. There is no evidence of any general fall in carotene content of pasture from September to January which might account for the decline in the carotene and vitamin A contents of the milk fat over this period, and further, the amount of carotene in the pasture at any one time is considerably in excess of even the highest levels reported necessary for the production of fat of high vitamin A potency (e.g. Wiseman et al. 1949; Hibbs et al. 1949). Even under drought conditions in midsummer (February-March) when pasture carotene levels may fall to around 200 µg/g dry matter due to drying up of the pasture (McGillivray, 1952), carotene intake should still be sufficient to support a relatively high fat potency. Thus it would appear that the decline in the carotene and vitamin A contents of milk fat over the late spring - early summer period cannot be attributed to a lack of carotene in the diet, but must be assumed to be due to a lowered availability of the carotene in the pasture at this time (McDowell & McDowell, 1953; McDowell, 1956; McGillivray, 1956).

Several factors have been shown to affect the availability of carotene. Fraps (1946), for example, has reported the apparent digestibility of carotene to be reduced by diets rich in fibre, and Eckelen & Pannevis (1938) and Graves (1942) have demonstrated large variations in the availability of carotene from different sources. While other factors are also undoubtedly involved it is well recognised that adequate tocopherol plays an important part in sparing carotene and protecting it against destruction in the gastro-intestinal

tract and bloodstream (e.g. Hickman, Kaley & Harris, 1944a, b; Harris, Kaley & Hickman, 1944).

Working on the assumption that the low summer vitamin A potencies of New Zealand butterfats were due to low tocopherol rather than low carotene levels in the pasture, McGillivray (1942) investigated the effect of a tocopherol supplement on the vitamin A potency of butterfat from cows grazing typical New Zealand summer pastures. By supplementing one member of each of three pairs of monozygotic twins with 1 g of α-tocopherol per day he was able to raise the potency of their fat from a typical midsummer minimum (34 i.u./g butterfat) to a normal winter maximum (50 i.u./g butterfat) in a matter of 10 days. This maximum level remained unaffected when the tocopherol supplement was increased to 3 g per day or when 300 mg of readily available carotene in oil was fed in addition to the 3 g of tocopherol. Tocopherol supplements likewise increased the tocopherol content of the milk fat and a significant correlation was established between vitamin A potency and tocopherol content. When tocopherol supplementation ceased the potency of the milk fat fell to that of the untreated twin within 6 days. Administration of 300 mg of carotene in oil for the following 6 days, in the absence of additional tocopherol, was without effect on the potency of the milk fat, thus confirming the assumption that the carotene intake was already adequate.

From these results McGillivray concluded that the low potencies reported for New Zealand butterfats were probably due to inadequate tocopherol or other anti-oxidant in summer pasture and were presumably associated with the drying up of the pasture or with changes in its botanical composition. He

further suggested that clover might be a factor complicating the problem since its emergence as the dominant species of normal summer pasture coincided with the production of fat of minimum vitamin A potency. Work by Sears, Goodall & Newbold (1942, 1948) at the Grasslands Division, Palmerston North, for example, with clover and ryegrass associations, has shown that the percentage of clover in the sward increases from 10-15% in the winter to 50% or higher in the summer. Furthermore, it has been established that clover contains considerably less tocopherol than ryegrass sampled at the same time and that the tocopherol content of mixed pasture (predominantly clover and ryegrass) decreases throughout the spring to reach a minimum in late summer (McGillivray, 1952).

Recently, with a view to examining this relationship further, a series of experiments was undertaken by Worker & McGillivray (1957)* in an endeavour to establish a correlation between clover intake and milk fat vitamin A potency. In preliminary experiments in which the effect of day-to-day variations in the botanical composition of pasture on the vitamin A potency of bulked herd milk fat was studied, it was unfortunately not possible to establish any relationship between clover intake and fat potency, due most probably to the rapidity with which the level of clover intake varied under the system of rotational grazing practised.

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* The results reported in this publication formed a section of a thesis submitted by the author in 1955 in partial fulfilment of the requirements for the degree of M.Agr.Sc. of the University of New Zealand.

the effect of one level being completely masked by the effect of a widely differing level during the following grazing period. In later experiments, however, in which the effect of feeding separately white clover (Trifolium repens, N.Z. pedigree strain), red clover (T. pratense), and ryegrass (Lolium perenne) on the carotene and vitamin A contents of milk fat from individual cows was studied, interesting results were obtained. In one study carried out during the late summer of 1953 a comparison of the effects of white clover versus ryegrass feeding was carried out using three pairs of monozygotic twins. Following a 4-day pretreatment period to establish the magnitude of within-twin variation, during which time the six animals were grazing a mixed pasture containing approximately 36% clover, one animal of each pair was placed on a high clover pasture containing approximately 70% clover and the other on a low clover pasture containing less than 10% clover. After 5 days the animals on the high clover pasture were brought in and for a further 7 days were stall fed, ad lib, clover cut from a pure stand while the others remained on low clover pasture. The pasture clover, like that used for stall feeding, was almost entirely white clover with a cyanide content of approximately 0.03% on a dry matter basis (Doak, 1935), and was at a lush and rapidly growing stage. The milk fat of the clover fed twins showed an average decrease in vitamin A potency of 8 i.u./g over the first 24 h while their twin mates on the low clover pasture showed a corresponding increase of 4 i.u./g, these differences being due more to variations in the vitamin A content of the fat than to changes in the carotene content. Variations in iodine value also occurred and these were shown to be closely related to changes in total vitamin A potency. The

average iodine value prior to treatment was 36.8, decreasing to a mean value of 35.4 in the clover fed group and increasing to a mean value of 37.2 in the ryefed group within 24 h, and remaining thereafter relatively constant. Other milk fat components, investigated for only one set of twins, showed the following average levels for pretreatment, high clover, and low clover periods: tocopherol, 52, 44, 58 µg/g; oleic acid 34, 30, 35%; vitamin A alcohol, 0.15, 0.19, 0.17 µg/g. In a further study carried out during the summer of 1955 a similar comparison of the effects of red clover versus ryegrass feeding was carried out using two pairs of identical twins. In marked contrast, however, to the results obtained with white clover the feeding of red clover had no effects on the levels of carotene and vitamin A in the milk fat nor had it any effect on other fat components. Similarly also, blood levels were unaffected by treatment and remained relatively constant throughout.

At the present time the mechanism by which diets high in white clover depress carotene utilization from pasture and vitamin and provitamin secretion from the mammary gland, and the significance of these findings in relation to the summer decline in the vitamin A potency of New Zealand butterfat, are not known. There would, however, appear to be four possibilities worthy of consideration. In the first place, McGillivray (1952) has shown that the tocopherol content of clover is lower than that of grass sampled at the same time and that the level of tocopherol in pasture declines throughout the spring to reach a minimum in summer. These observations, together with the fact that he was able to raise the potency of summer milk fat by tocopherol supplementation would suggest

that a lower level of tocopherol (or other anti-oxidant) in the pasture was at least partly responsible for the summer decline in potency.

In the second place, it was noteworthy that the white clover used in the investigations of Worker & McGillivray (1957) was a high cyanide strain. It was thought probable, therefore, that cyanide, which is liberated from white clover by enzymic and microbial hydrolysis in the rumen, might be a further factor contributing to the problem. Following its absorption into the bloodstream, it may possibly have some direct physiological effect or it may act indirectly after detoxication to thiocyanate. There are several *a priori* grounds on which to base the latter assumption. First, a very large proportion of the white clovers present in high producing New Zealand dairy pastures are improved, high cyanide strains; moreover, it has been shown that the percentage of clover in typical pastures in this country follows a regular seasonal trend with maximum levels being present in the summer (Sears *et al.* 1942, 1948). Secondly, Coop & Blakely (1949a, b) have shown the release, the absorption and the detoxication of cyanide to thiocyanate in the liver to be a comparatively efficient process in the sheep. Thirdly, Barker (1936), Raben (1949) and Bourne & Kidder (1953) have shown thiocyanate to possess goitrogenic properties; and fourthly, Cama & Goodwin (1949) and Chanda, Clapham, McNaught & Owen (1952) have demonstrated a rapid decrease in the absorption of carotene in the rabbit, goat and cow following experimental induction of hypothyroidism with thiouracil. On these grounds it is possible, therefore,

that the decreased availability of carotene from summer pastures in New Zealand and the low levels of carotene and vitamin A in the butterfat at this time are associated with and are partly due to a lowered thyroid function resulting from higher than normal levels of blood thiocyanate.

In the third place, since carotene is a fat-soluble pigment it is reasonable to assume that its availability from pasture will depend to a certain extent on the amount and on the nature of the fat present in the pasture. Indeed, there is a considerable body of indirect evidence in the literature to support this hypothesis (e.g. Drummond, 1938; Dyer, Key & Coward, 1934; Smith & Spector, 1940; Krukovsky, 1942). For example, Krukovsky (1942) has presented evidence to show that the solubility of carotene in a fat is to a large degree dependent on its degree of unsaturation and from this concluded that "the efficiency of absorption of carotene from a feed might be influenced by the degree of unsaturation of the fat present in the feed". Thus a low proportion of unsaturated acids in the feed would result not only in a low iodine value but also in a less efficient absorption of carotene from the feed and in a low carotene and vitamin A content of the milk fat. Unfortunately, the nature of the fat present in New Zealand pastures has not been extensively investigated, and there is as yet no confirmation of any seasonal change in its degree of unsaturation. (Shorland (1944), however, has shown New Zealand pastures, like those overseas (e.g. Hilditch, 1956), to be characterized by a high content of C_{16} and C_{18} unsaturated acids of which one of the commonest is oleic. At the same time it has been demonstrated by Hansen & Shorland (1952) and

McDowell (1953), working independently with New Zealand butterfats, that oleic acid, which is derived directly from the diet and is known not to be synthesized to any appreciable extent within the mammary gland (e.g. Popjak, 1952), varies in the fat throughout the season, reaching minimum levels in November and January. If then, as would appear likely, this trend reflects similar changes in either the level or availability of oleic acid in the pasture and if, as has been suggested by Krukovsky (1942), carotene is associated with oleic and other unsaturated acids during absorption, the low availability of carotene from summer pastures may be explained in part by a decrease in the content of oleic acid (or other unsaturated acids) in the pasture resulting perhaps from the drying up or hardening off of the pasture at this time or from changes in its botanical composition. Variations in the degree of hydrogenation of the acids in the rumen prior to digestion may also possibly be a further factor contributing to the problem (e.g. Shorland, Weenink & Johns, 1955). Seasonal variations in the amounts and nature of the fats present in New Zealand pastures are at present being studied at the Plant Chemistry Laboratory of the Department of Scientific and Industrial Research, Palmerston North, and when the results of these investigations come to hand it is to be hoped that additional light will be shed on this particular aspect of the problem.

Finally, although in the experiments of Worker & McGillivray (1957) it was shown that white clover may have a marked effect on the vitamin A potency and on other components of the milk fat, the further possibility must be considered that the summer decline in potency may not be due to the effect of clover per se but may be caused by the accumulation of some specific substance (or group of substances) associated with the stage of development of the pasture as a whole at a time when clover happens to be the dominant species present. This is strongly suggested by

the fact that in the twin-cow experiments of Worker & McGillivray (1957), even though the twins on low clover pasture were grazing pasture containing approximately the same amount of clover as average winter pastures, the vitamin A potency of their fat was considerably lower (approximately 37 i.u./g) than that of winter fat, and remained so throughout the experimental period. This would seem to indicate that some factor in summer grass as well as in clover was interfering with the utilization of carotene. The finding that clover has a relatively greater effect on fat potency than grass might be merely due to the fact that it contains a somewhat greater concentration of the factor than grass.

With a view to investigating these possibilities in greater detail and obtaining further general information on certain other aspects of the problem of carotene utilization from pasture, it was decided in this department in August 1955 to extend the earlier studies on carotenoid metabolism in the cow and to undertake further studies also in the rat. The details of these experiments and the results obtained are described in the following three chapters which comprise Section II of this thesis.

In Chapter X the results of a seasonal survey are described in which various plasma and milk fat constituents were followed at fortnightly intervals in six pairs of monozygotic twin cows for the major part of one lactation; at the same time various pasture constituents were followed at similar intervals in an attempt to relate any changes occurring in the pasture with those occurring in the plasma or milk fat. In Chapter XI

two experiments with monozygotic twins are described on the effect of reduced carotene intake on the vitamin A potency of the milk fat, while in Chapter XII the results of a series of experiments in rats on the effect of various nutritional factors on the utilization of carotene are discussed. In Chapter XIII a short summary of the section as a whole is presented.

EXPERIMENTAL MATERIAL

AND METHODS

Measurement of carotene, xanthophyll, vitamin A alcohol and ester, and tocopherol in cow blood plasma.

Blood sampling. Blood samples were collected in 50 ml. oxalated centrifuge tubes by means of a 15 G hypodermic needle inserted into the jugular vein. Immediately after collection the samples were centrifuged at approximately 2000 r.p.m. for 30 min.

Extraction of plasma. After centrifugation, 20 ml. of plasma were drawn off into a syringe and transferred to a 150 ml. separating funnel. 20 ml. of ethanol were added to denature the plasma and the mixture was extracted with 50 ml. of petroleum ether by shaking in a mechanical shaker for 5 min. The bottom layer was run off and re-extracted with a further 50 ml. of petroleum ether. The extracts were combined, centrifuged, evaporated on a hotplate at 70° and then taken up in 2-3 ml. of petroleum ether for chromatography.

Chromatographic separation. In order to make it possible to measure all the substances under consideration in the one plasma extract, a slight modification of the original chromatographic procedure of Thompson et al. (1949) and Kon et al. (1955) was evolved. An examination of the behaviour of free tocopherol* (prepared by saponification of

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* The form of tocopherol in all natural sources (Tosic & Moore, 1948).

α-tocopheryl acetate*) on alumina during the normal separation of vitamin A alcohol and ester (see p.15) showed that, after development of the column, most of the tocopherol had passed into the 8% ethanol fraction and only a very small proportion (approximately 10%) was present in the 2% acetone fraction. By reducing the strength of the acetone eluent to 1% (v/v) in petroleum ether, however, it was found possible to recover all of the tocopherol in the 8% ethanol fraction and at the same time still effect a satisfactory separation of vitamin A alcohol and ester. An outline of the modified procedure is as follows.

Reagents.

Alumina: Aluminium oxide for chromatography, (May & Baker Ltd., Dagenham), Brockmann grade III.

Solvent: Petroleum ether, boiling range 60-80° (British Drug Houses Ltd., Poole).

Eluents: 1. 1% acetone (v/v) in petroleum ether.

2. 8% ethanol (v/v) in petroleum ether.

Preparation and use of adsorption column

The columns were prepared as described previously on p.15. The extract in petroleum ether was poured onto the column which adsorbed xanthophyll, vitamin A alcohol and tocopherol and allowed carotene and vitamin A ester to pass through. The column was then developed with 10 ml. of 1% acetone (eluent 1) which quantitatively removed carotene and vitamin A ester. On further development with 20-25 ml. of 8% ethanol (eluent 2), xanthophyll, vitamin A alcohol and tocopherol were recovered.

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*) Supplied by L. Light & Co. Ltd., Poyle.

Estimation of carotene and vitamin A ester. After chromatography the 1% acetone fraction was made to volume and the carotene present estimated spectrophotometrically as described on p.16. The solution was then evaporated to dryness on a hotplate at 70°, saponified, re-chromatographed, and the vitamin A ester originally present estimated as vitamin A alcohol (see p.16).

Estimation of xanthophyll, vitamin A alcohol and tocopherol. After chromatography the 8% ethanol fraction was made to volume and the xanthophyll was estimated spectrophotometrically as described previously (p.82). The solution was then divided into two equal parts. One half was evaporated at 70° on a hotplate, the residue taken up in chloroform, and the vitamin A alcohol present was estimated colorimetrically using antimony trichloride (see p.16), allowance being made for the presence of xanthophyll as described on p.83. The other half was also evaporated and the residue was taken up in ethanol. The tocopherol present was then estimated colorimetrically by the method of Emmerie & Engel (1938) involving the reduction of ferric ion to ferrous ion, which with α, α'-dipyridyl forms a red coloured complex exhibiting maximum absorption at 520 mμ. The intensity of the red colour was determined 15 sec after mixing using a Hilger "Biochem" Absorptiometer (model H 810) which had been standardized against free tocopherol obtained by saponification* of pure α-tocopheryl acetate. A correction for the small amount of xanthophyll in the sample was found necessary.

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* The method of saponification is described on p. 170.

The correction applied (determined by experiment) was equivalent to 1.7 µg tocopherol/1.0 µg xanthophyll present*. No correction was found necessary for the presence of vitamin A alcohol in the sample.

Measurement of thiocyanate in blood

The level of thiocyanate in the blood plasma was estimated colorimetrically by the standard method described by Fister (1950).

Measurement of carotene, xanthophyll, vitamin A alcohol and ester, and tocopherol in milk fat

Preparation of samples for analysis. After collection milk samples were stored at 5° for 12 h. The milk layer was drawn off and the cream was churned in a Waring Blendor running at a low speed. The butter was melted and filtered at 60° and the butterfat obtained was then stored at 5°.

Chromatographic separation and estimation of carotene, xanthophyll, vitamin A alcohol and ester. Approximately 2 g of melted butterfat were dissolved in about 5 ml. of petroleum ether and the mixture was chromatographed on

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* Under normal circumstances the level of xanthophyll present in the final sample was usually about 4-8 µg while the level of tocopherol present was in the region of 80-120 µg, so that the actual correction for xanthophyll was always small in comparison with the total level of tocopherol present. A similar situation obtained also in the case of butterfat (see following page).

alumina as described on p.15. Carotene, vitamin A alcohol and ester were assayed as described on p.16 and xanthophylls as described on p.82.

Chromatographic separation and estimation of tocopherol. Approximately 2 g of melted butterfat were saponified for 5 min at 70° with 1 ml. of 60% KOH and 5 ml. of ethanol, the latter containing 5% (w/v) of pyrogallol to prevent destruction of the tocopherol (Tosic & Moore, 1945). The mixture was extracted with diethyl ether, washed with water and dried over anhydrous sodium sulphate. The ether extract was then evaporated to dryness and the residue taken up in 2-3 ml. of petrol ether and chromatographed on alumina as described on p.167. The tocopherol present was estimated colorimetrically using the Emmerie-Engel reaction as outlined on p.168, the small amount of xanthophyll present being corrected for as described on p.169.

Estimation of carotene, xanthophyll, chlorophyll and tocopherol in pasture

Carotene, xanthophyll and chlorophyll.

A search of the relevant literature indicated that while a number of excellent methods have been published for the estimation of carotene, xanthophyll and chlorophyll in pasture, none was entirely satisfactory for the present purposes. Many, for example, were not suitable for routine work involving large numbers of samples. Some involved saponification, a tedious and time-consuming step which it was wished to avoid if possible, and others required

the use of adsorbents which were known to allow only extremely slow passage of solvents. Also speed and simplicity had sometimes been sacrificed to obtain an absolute analytical precision which for comparative purposes is seldom required. To overcome these difficulties a chromatographic technique was developed on alumina which allows a rapid and quantitative separation of the pigments on one column of adsorbent. The method is simple, rapid and accurate and is thus ideally suited for the routine assay of large numbers of samples.

Reagents.

Alumina: Aluminium oxide for chromatography, (May & Baker, Ltd., Dagenham), Brockmann grade III.

Solvents: Petroleum ether, boiling range 60-80°, (British Drug Houses Ltd., Poole).

Acetone, A.R., (British Drug Houses Ltd., Poole).

Eluents: 1. 1% acetone (v/v) in petroleum ether.

2. 1% ethanol (v/v) in petroleum ether.

3. 8% ethanol (v/v) in petroleum ether.

Extraction procedure. 1 g of dried grass ground to pass through a 0.5 mm circular mesh sieve and thoroughly moistened with water prior to extraction, or 5 g of fresh grass, are convenient quantities to use. The sample is extracted under nitrogen in a Waring Blendor with 60 ml. of acetone in the cold for 5 min. The mixture is filtered through a Buchner funnel and the residue re-extracted under nitrogen for 5 min with an additional 60 ml. of acetone. The mixture is filtered as before and the residue washed with 25 ml. of acetone. The filtered extracts are then

combined and made up to 150 ml. An aliquot of 3 ml. is evaporated to dryness and taken up in 2-3 ml. of petroleum ether for chromatography.

Preparation and use of column. The column is prepared exactly as described previously for the separation of vitamin A alcohol and ester (see p.15). The solution of pigments in petroleum is poured onto the column which adsorbs the chlorophyll and xanthophyll in a narrow band at the top and allows the carotene to move through. (Overloading the column will cause undue destruction of all pigments). The chromatogram is then developed with 10 ml. of 1% acetone (eluent 1) which quantitatively removes the carotene fraction. Further development with 25 ml. of 1% ethanol (eluent 2) removes chlorophyll with less than 3% loss. Xanthophylls are quantitatively recovered with 25 ml. of 8% ethanol (eluent 3). The solutions are made to volume and are then ready for spectrophotometry. During the separation the tops of the columns must not be allowed to become dry but must be topped up just as the last of the previous batch of eluent is about to percolate through. In addition the general precautions outlined previously by Davidson (1954) must also be observed.

Estimation of pigments. Carotene and xanthophyll were estimated spectrophotometrically in a Beckman, model DU, photoelectric spectrophotometer as described earlier (see p.82). Because of the difficulty of choosing a suitable wave length at which to measure total chlorophyll, due to variation in $\lambda_{max.}$ with variations in the ratio of chlorophyll-a to chlorophyll-b, the latter were estimated separately and summed. Absorption

curves for the pure isomers were prepared from pure solutions obtained by chromatography on sucrose (Winterstein & Stein, 1934) of the mixed chlorophyll fraction obtained by chromatography on alumina. The maxima in the curves occurred at 664 mμ and 645 mμ for chlorophyll-a and -b respectively, but each had appreciable absorption at the $\lambda_{max.}$ of the other. Corrections (found by measurement) were therefore applied to the optical densities of 1-cm layers of solutions (D_a and D_b, respectively) by deducting $0.2065D_b$ in the case of chlorophyll-a and $0.2028D_a$ in the case of chlorophyll-b. That these corrections were valid was shown by tests on known mixtures of solutions of the two chlorophylls, when the difference between the absorption found and that calculated was not more than 1%. The E values at the $\lambda_{max.}$ used in the calculations were 1020 for chlorophyll-a and 568 for chlorophyll-b (computed from the results of Scheibe & Conar, 1941).

Recovery experiments. The results of recovery experiments in which known amounts of the different pigments were blended with acetone, transferred to petroleum ether, chromatographed on alumina and re-estimated are shown in Table 30. From these results it is apparent that over the range of concentrations usually encountered in the method, recoveries were well within the limits of normal experimental error.

Table 30

Recovery of pigments after blending with acetone and chromatographing on alumina.

	Carotene (µg)		Xanthophyll (µg)		Total chlorophyll (µg)	
Added	5.0		10.0		50.0	
Recovered	4.9		9.8		49.0	
% Recovered		98.0		98.0		98.0
Added	10.0		20.0		100.0	
Recovered	10.0		19.8		97.4	
% Recovered		100.0		99.0		97.4
Added	15.0*		30.0*		150.0*	
Recovered	14.8		29.6		145.5	
% Recovered		99.0		98.7		97.0
Added	20.0		40.0		200.0	
Recovered	19.7		39.5		193.8	
% Recovered		98.5		98.5		96.9
Added	50.0		100.0		500.0	
Recovered	48.6		96.9		468.0	
% Recovered		97.2		96.9		93.6

* Approximate amounts of pigments present on column during the course of a normal separation.

Tocopherol.

The most satisfactory method of estimating tocopherol is by the Emmerie-Engel reaction, a colorimetric procedure involving the reduction of ferric chloride by tocopherol (see p.168). There is the general difficulty, however, when determining tocopherol in natural products of overcoming the effects of other substances (e.g. chlorophyll, carotenoids, unsaturated fatty acids, etc.) which interfere with the reaction. Quaife & Harris (1944) and Quaife (1947), for estimating tocopherol in milk fat and blood plasma, have recommended hydrogenation but in the author's experience this is a time-consuming step, especially where large sample numbers are involved. Hall & Kelley (1946), for plant tissue, favour chromatography on magnesia to separate chlorophyll and xanthophylls followed by treatment with 85% (v/v) sulphuric acid to destroy carotene but in this laboratory it was not found possible to obtain a satisfactory recovery of tocopherol after the latter treatment. Attempts to apply a correction for the carotene present were also unsuccessful due to the high concentration present. Accordingly, for the present purposes, this step has been modified and the carotene and tocopherol separated chromatographically on alumina as described on p.167. A brief outline of the complete procedure is as follows.

Reagents. As in the previous section (p. 171), with the addition of magnesium oxide (Hopkins & Williams Ltd., Sydney; heavy grade, no. 5368).

Preparation of columns.

Alumina: As described previously (see p.15).

Magnesia: The magnesia, without any prior treatment, is suspended in 1% ethanol (eluent 2) and poured to form a column after settling of 8-9 cm. The column is then ready for immediate use.

30 ml. of acetone extract are evaporated on a hotplate and the residue taken up in 2-3 ml. of 1% ethanol (eluent 2). The solution is poured onto the magnesia column which adsorbs chlorophyll and xanthophylls in a band at the top and allows carotene and tocopherol to pass through. The latter are quantitatively recovered in the eluate after development with 40 ml. of eluent 2 (a slight vacuum applied at the foot of the column is helpful in speeding development). The eluate is evaporated and taken up in 2-3 ml. of petroleum ether and re-chromatographed on alumina to separate the carotene and tocopherol as described on p.167. Tocopherol is recovered quantitatively in the 8% ethanol fraction. The solution is then evaporated and taken up in ethanol for estimation (see p.168).

Recovery experiments. A series of recovery experiments was carried out in which measured amounts of tocopherol were added to pasture samples of known tocopherol content, blended with acetone, separated chromatographically and re-estimated. The results are shown in Table 31, from which it can be seen that, within the limits of normal experimental error, recoveries were satisfactory.

Table 31

Recovery of tocopherol after adding to dried grass, blending with acetone and chromatographing on magnesia and alumina.

Species	Tocopherol content of pasture sample (µg/g D.M.)	Tocopherol added (µg)	Added tocopherol recovered (µg)	% recovery
Ryegrass	220	100	97	97
	220	300	306	102
	220	1000	980	98
Ryegrass	370	100	96	96
	370	300	286	95
	370	1000	958	96
Ryegrass/ white clover mixture	320	100	101	101
	320	300	310	103
	320	1000	984	98

CHAPTER X

A SURVEY OF THE CHANGES OCCURRING IN THE LEVELS OF VARIOUS BLOOD PLASMA AND MILK FAT CONSTITUENTS IN COWS GRAZING NEW ZEALAND PASTURES OVER THE SPRING AND SUMMER

Introduction

Seasonal surveys of the vitamin A potency of New Zealand butterfat have been published by a number of workers. Barnicoat (1947), for example followed the contents of carotene and vitamin A in factory samples from the Waikato and Manawatu districts over the 1935-36 season and showed the levels to be highest in late winter and spring and lowest in late summer. Similar results were obtained also by McDowall & McDowall (1953) in a comprehensive survey of factory butters from a number of North Island dairying districts over the 1946-48 seasons. McGillivray (1956) likewise, over the 1952-53 season, confirmed these findings with butters from the Manawatu district, and in addition found the levels of xanthophyll and tocopherol in the fat showed a similar seasonal trend. More recently, McGillivray (1957a) followed the levels of carotene, xanthophyll, vitamin A alcohol and ester in the blood plasma and milk fat of six cows throughout lactation and from the results concluded that:

> "The seasonal trend in the vitamin A and carotenoid content of New Zealand milk fat is a reflection of the changing level of these substances in the blood plasma....The decreased level of carotene, xanthophyll and vitamin A ester in the blood plasma over the summer months is consistent with a decreased absorption or utilization of carotenoids (from the pasture) over this period."

With a view to confirming these results and investigating the relationship between blood plasma and milk fat levels of carotenoids and vitamin A in greater detail, it was decided to repeat this work with a larger number of animals. Furthermore, tocopherol levels in blood plasma and milk fat were investigated and thiocyanate levels in the plasma were

also followed. In addition, a seasonal survey of the carotene, xanthophyll, chlorophyll and tocopherol levels in pasture was undertaken in an attempt to follow the variations in these substances throughout the season and to determine any possible effects these variations might have on the different plasma and milk fat constituents.

Experimental

General. The materials and methods were as described earlier with the following additions:

Cows. Six pairs of identical twin cows, predominantly Jersey, from the herd of The Dairy Research Institute (N.Z.) were used. The cows were grade animals of varying ages. They were fed out-of-doors on pasture as normal for the Institute herd. The mating of the animals had been arranged so that individual animals within each pair calved, on the average, some 7 weeks apart*.

Sampling.

Blood. Individual blood samples were taken at fortnightly intervals and assayed separately immediately after collection.

Milk. Individual milk samples were obtained at fortnightly intervals, each sample being a composite of the morning and evening milks. Fat samples prepared from the milks were stored at 5° and assayed as soon as possible after collection.

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* This had been arranged independently by The Dairy Research Institute for purposes quite divorced from the present study.

Pasture. Pasture was sampled, on an average, at approximately 3-weekly intervals. The samples were taken from a plot (located at the Grasslands Division, Palmerston North) containing a clover/ryegrass association reasonably typical of an average New Zealand dairy pasture. Samples were taken when the pasture was approximately 4" in height, the grass being clipped back to within an inch or so of the ground with hand shears. After cutting the samples were freeze dried and then ground in a C & N junior laboratory mill to pass through a 0.5 mm mesh sieve.

Results

Milk fat constituents. The levels found for various milk fat constituents are shown in Fig. 3. Average values only are recorded, but all samples were assayed separately and all animals showed similar trends. From the results obtained it is apparent that from early spring (October) through to mid summer (February) there was a steady fall in the level of carotene, xanthophyll and tocopherol in the milk fat of both early- and late-calving cows. The levels of vitamin A alcohol and ester in the milk fat of the two groups on the other hand showed little seasonal change over this period, the variations observed being mainly of an irregular nature*. As might be expected, the total vitamin A potency of the milk fat, due to the decreasing level of carotene present, declined steadily throughout the duration of the experiment in both

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* Changes in vitamin A alcohol were so small that they were regarded as being of no significance (see, for example, McGillivray, 1957a). The levels shown in the figure are average levels for the early- and late-calving cows.

groups of animals.

Blood plasma constituents. Average values for the various plasma constituents followed are shown in Fig. 4. From a consideration of these results it is clear that throughout the experiment there was a steady fall in the levels of carotene, xanthophyll, vitamin A alcohol and tocopherol. There was also a similar decrease in vitamin A ester. The levels of thiocyanate found in the plasma are not shown in the figure. Contrary to expectation, the levels for all cows remained remarkably constant through the experiment at 2.0 ± 0.2 mg/100 ml. plasma.

Pasture constituents. Values found for carotene, xanthophyll, chlorophyll and tocopherol in the pasture over a 9-month period extending from August through to May are shown in Fig. 5. The values showed little regular variation throughout the year and no seasonal trend in any of the constituents was apparent. The average level of carotene present in the pasture over the whole period was 500 µg/g dry matter* with individual assays varying from 430 to 790 µg/g. Xanthophyll varied from 1170 to 1560 µg/g dry matter with a mean value of 1300 µg/g while total chlorophyll varied from 6.4 mg/g to 11.7 mg/g dry matter with a mean value of 8.7 mg/g. Values for tocopherol varied from 270 to 470 µg/g with a mean of 350 µg/g dry matter.

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* On an air-dried basis.

Figure 3.

Changes in the levels of various constituents in the milk fat of early- and late-calving, pasture-fed cows from early spring to late summer.

Solid lines: early-calving cows.

Broken lines: late-calving cows.

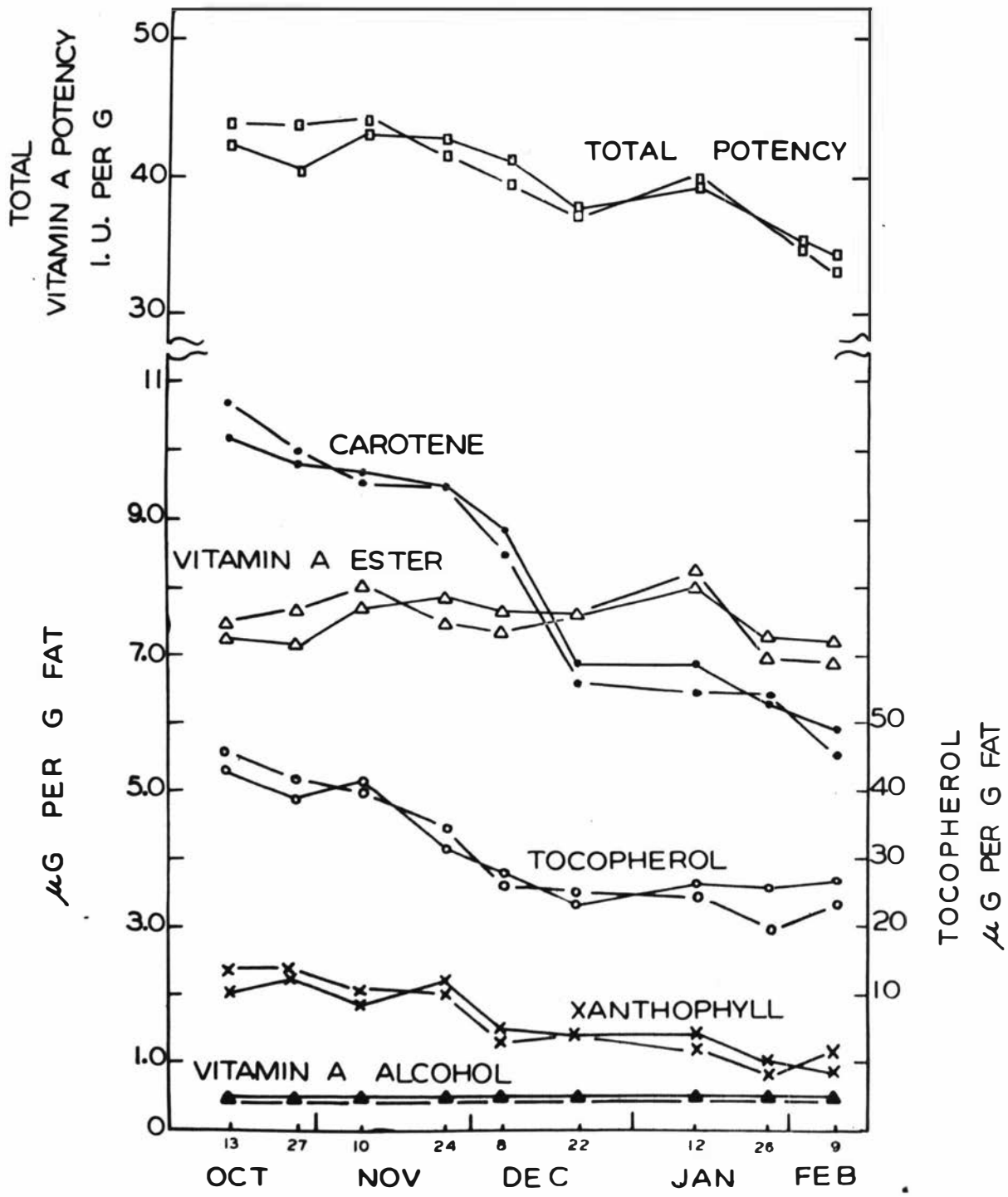

TOTAL VITAMIN A POTENCY I. U. PER G
50
40
30
TOTAL POTENCY
11
9.0
7.0
5.0
3.0
1.0
0
μG PER G FAT
CAROTENE
VITAMIN A ESTER
TOCOPHEROL
XANTHOPHYLL
VITAMIN A ALCOHOL
50
40
30
20
10
TOCOPHEROL μG PER G FAT
13
27
10
24
8
22
12
26
9
OCT
NOV
DEC
JAN
FEB

Figure 4.

Changes in the levels of various constituents in the blood plasma of early- and late-calving, pasture-fed cows from early spring to late summer.

Solid lines: early-calving cows.

Broken lines: late-calving cows.

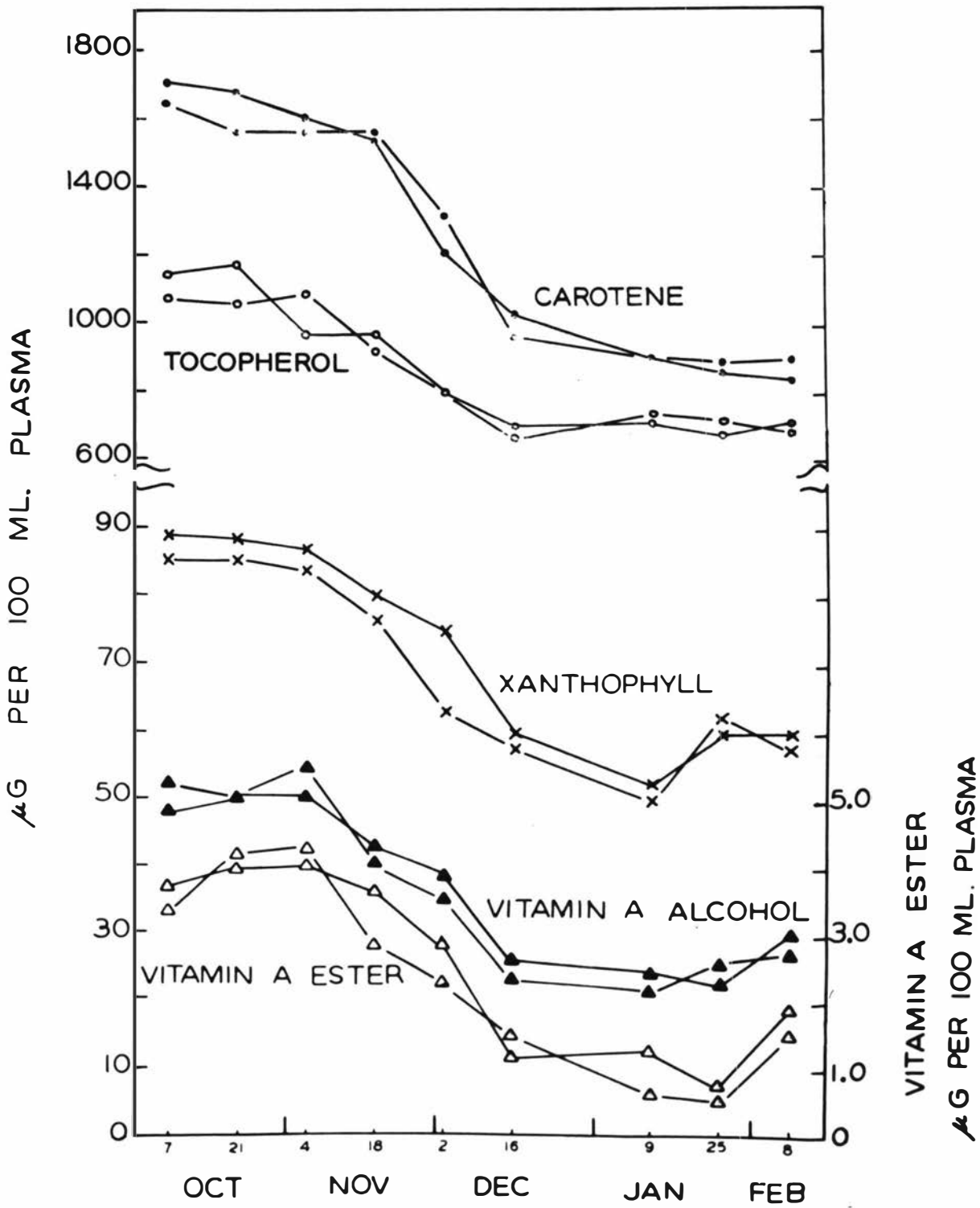
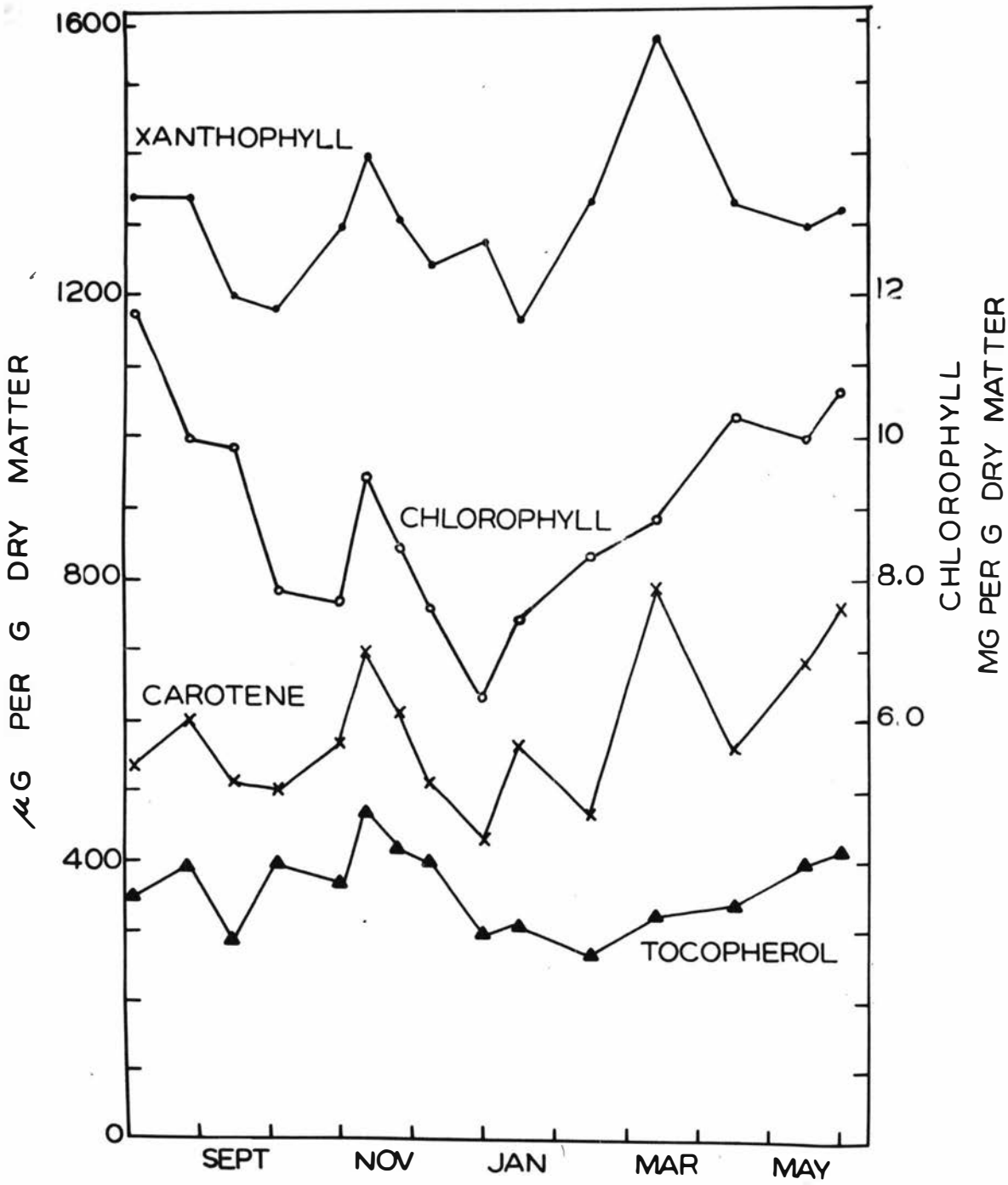

1800
1400
1000
600
90
70
50
30
10
0
CAROTENE
TOCOPHEROL
XANTHOPHYLL
VITAMIN A ALCOHOL
VITAMIN A ESTER
5.0
3.0
1.0
0
7
21
4
18
2
16
9
25
8
OCT
NOV
DEC
JAN
FEB
μG PER 100 ML. PLASMA
VITAMIN A ESTER
μG PER 100 ML. PLASMA

Figure 5.

Changes in the levels of various pasture constituents throughout the season.


1600
1200
800
400
0
XANTHOPHYLL
CHLOROPHYLL
CAROTENE
TOCOPHEROL
SEPT
NOV
JAN
MAR
MAY
μG PER G DRY MATTER
12
10
8.0
6.0
CHLOROPHYLL
MG PER G DRY MATTER

Discussion

The main aim of the present experiments was to investigate in more detail relationships between various constituents in the milk fat and blood plasma of cows, and in pasture, in an attempt to obtain further information on the nature of the summer decline in the vitamin A potency of New Zealand butterfat. The results presented in Fig. 3 indicate a steady decrease in the levels of carotene, xanthophyll and tocopherol, and in the total vitamin A potency of the milk fat from early October through to late January/early February in both early- and late-calving cows, thus confirming the observations reported previously by other workers (e.g. Barnicoat, 1947; McDowell & McDowell, 1953; McDowell, 1956; McGillivray, 1956). Vitamin A alcohol and ester, on the other hand, did not appear to be appreciably affected, the variations observed being, for the most part, irregular (cf. also McDowell, 1956).

From a comparison of the results presented in Fig. 3 with those in Fig. 4 it is clear that in general the gradual decline in the levels of the various milk fat constituents results primarily from a decrease in the level of these constituents in the blood plasma (cf. also McGillivray, 1957a). In the case of carotene, xanthophyll and tocopherol it would appear that this relationship is a fairly direct one but in the case of vitamin A the picture is somewhat different and is complicated by the fact that, while the free and esterified forms of vitamin A are present in both milk fat and blood plasma, in the former the esterified form predominates, while in the latter the reverse is normally true. If, as would

appear to be the case, plasma vitamin A ester is the main source of milk fat vitamin A (see McGillivray, 1957b), it is surprising that milk fat vitamin A levels should have remained almost constant throughout the survey despite the well defined fall in plasma vitamin A ester levels. It is possible that the animal is able to maintain the level of vitamin A in its milk fat, at the expense of liver stores, by way of plasma vitamin A alcohol and the decline in plasma vitamin A alcohol levels apparent in the survey would appear to bear this out. Further there is a considerable body of evidence published demonstrating that in times of need the animal may make considerable demands on liver stores for this purpose (see Chanda & Owen, 1952; Chanda, Clapham & Owen, 1954, 1955). Against this, however, McGillivray (1957b) has shown the extent to which cows can utilize plasma vitamin A alcohol to maintain milk fat levels is fairly limited and has suggested a level of utilization equivalent to about 2 µg/g fat. He considers that utilization of plasma vitamin A alcohol is, within normal limits, independent of the vitamin A status of the animal - either its immediate carotene intake or its liver storage of vitamin A - and suggests that utilization of plasma vitamin A alcohol depends on the uptake by the mammary gland of plasma protein with which the vitamin A alcohol is associated. The vitamin A alcohol is then apparently transferred largely to the fat phase in the milk and is fairly completely esterified. Under these circumstances the level of vitamin A in the milk would be related not only to the uptake of chylomicrons and the level of vitamin A ester in them, but also to the uptake of plasma

protein and in attempting to relate levels of vitamin A in the blood plasma and in the milk fat, milk and fat yields would need also to be taken into account. Clearly, further more detailed studies are required in order to settle this matter finally.

The finding that the level of thiocyanate in the blood of cows remains constant despite comparatively wide fluctuations in cyanide intake from white clover, would appear to rule out the possibility of thiocyanate being of any importance in connection with the present problem by virtue of its effect on thyroid function. Despite this fact, however, the low level of thiocyanate in the blood, in itself, is most interesting and merits further investigation. In sheep on high intakes of high cyanide white clover, plasma levels of over 17 mg/100 ml. plasma have been reported (Flux, Butler, Johnson, Glenday & Petersen, 1956). The fact that in the cow the level remains constant at about 2.0 mg/100 ml. plasma and appears independent of cyanide intake would seem to indicate that either urinary excretion of thiocyanate in the cow is much more efficient than in the sheep or in the cow an alternative mechanism is present for detoxicating ingested cyanide.

From a consideration of the results presented in Fig. 5 it is evident that, while there are sample-to-sample fluctuations in the levels of carotene, xanthophyll, chlorophyll and tocopherol in the pasture throughout the year, no clear-cut seasonal variations in the levels of these constituents are apparent. McGillivray (1952) has reported a decrease in the levels of tocopherol in mixed pastures in the Manawatu over the summer

months, but the findings in the present study, at least over this one particular season, would not seem to substantiate this. Since the levels of carotene, xanthophyll and tocopherol in pasture vary between comparatively narrow limits, and since in winter and early spring these are sufficient to maintain high blood plasma and milk fat values of these constituents, it would seem likely, in agreement with the findings of earlier workers (e.g. McGillivray, 1952; McDowell & McDowell, 1953; McGillivray, 1957a), that the lowered blood plasma and milk fat values recorded over the late spring/summer period are due to a decrease in the availability of these constituents in the pasture resulting in a different relationship between the level of intake of these substances and plasma and milk fat values in the summer than in the winter and spring. Little is known of the minimum levels of xanthophyll and tocopherol in the pasture necessary to maintain normal values of these constituents in the milk fat. A considerable amount of work has been carried out overseas, however, on the minimum levels of carotene necessary to maintain high values of carotene and vitamin A in the milk fat (discussed in the next chapter), on the basis of which it would seem that, insofar as the actual carotene content of the pasture is concerned, summer pastures in New Zealand should be able to support the production of milk fat of maximum vitamin A potency.

In view of the above findings it was decided to undertake further experiments with a view to investigating in greater detail the relationship between the level of carotene intake from summer pasture and the vitamin A potency of milk fat

produced therefrom. The results of these experiments will be considered in the next chapter.

Summary

1. The nature of the summer decline in the vitamin A potency of New Zealand milk fat has been investigated using 6 pairs of identical twin cows. Calving was arranged so that individual cows within each pair calved approximately 7 weeks apart.

2. There was a steady decline from early November to late January in the levels of carotene, xanthophyll and tocopherol in the milk fat, vitamin A alcohol and ester levels, however, remaining relatively steady. Due to the decreasing levels of carotene present there was a noticeable fall in vitamin A potency of the milk fat.

3. Plasma levels of carotene, xanthophyll, vitamin A alcohol and ester, and tocopherol all declined over the period from November to January indicating that changes in milk fat constituents are the result of the changing levels of these substances in the blood plasma.

4. No differences in the behaviour of early- and late-calving cows was observed confirming that the changing levels of the various constituents in the blood plasma and milk fat throughout the survey were independent of lactational influences.

5. Levels of carotene, xanthophyll, chlorophyll and

tocopherol in mixed pasture showed sample-to-sample fluctuations throughout the survey but no distinct seasonal trends were observed which might account for changes in the levels of the various milk fat and blood plasma constituents. Carotene in the pasture was shown at all times to be well above the level necessary, on the basis of overseas work, for the production of fat of maximum vitamin A potency. It is concluded that the low vitamin A potencies and the low levels of the various milk fat and blood plasma constituents over the summer period is due to a lowered availability of carotene and other fat-soluble constituents in the pasture.

CHAPTER XI

STUDIES ON THE UTILIZATION OF CAROTENE FROM SUMMER PASTURE AND FROM ARACHIS OIL BY THE COW

Introduction

The relationship between the daily carotene intake of cows and the levels of carotene and vitamin A in their milk fat has been investigated by a number of workers (e.g. Russell et al. 1935; Atkeson et al. 1937; Fraps et al. 1937; Wilbur et al. 1940; Hauge et al. 1944; Wiseman et al. 1949; Hibbs et al. 1949). Clear relationships have been established at lower levels of carotene intake but it is apparent that there are maximum levels in the milk fat which cannot be exceeded by feeding carotene as the sole source of vitamin A. Most workers find these levels equivalent to about 50-60 i.u./g fat. There is, however, less general agreement on the actual minimum carotene intake necessary to attain these maximum milk fat levels and figures ranging from 300 mg (Hauge et al. 1944) up to 2500-2600 mg (Wiseman et al. 1949; Hibbs et al. 1949) of carotene per day have been suggested.

Vitamin A potencies varying between 50-60 i.u./g fat have been reported for New Zealand milk fats produced during the winter and early spring but the potency of summer milk fat has been found to be much lower, in the region of 30-40 i.u./g fat (e.g. McDowall & McDowall, 1953; McGillivray, 1956). On the basis of overseas work these lower potencies might be expected to be associated with a lower carotene intake, but this is now known not to be the case since it has been shown that the carotene content of typical dairy pastures in New Zealand is relatively high throughout the year and that the daily carotene intake of cows on these pastures is in the region of 4-8 g/day (McGillivray, 1956; see also Chapter X).

This is considerably in excess of even the highest levels referred to above as being adequate for the production of milk fat of maximum vitamin A potency and is in the range where milk fat carotene and vitamin A would be expected to be independent of carotene intake.

It would appear, therefore, that the utilization of carotene from New Zealand summer pastures is in some way impaired, resulted possibly in a different relationship between carotene intake and milk fat potency from that reported overseas. Accordingly, it was decided to carry out experiments to determine more precisely the nature of this relationship and also to investigate the utilization of an alternative source of provitamin (carotene in arachis oil).

Experimental

General. The materials and methods were as described previously with the following additions:

Cows.

Exp. 1. Four pairs of monozygotic twins, predominantly Jersey, from the herd of The Dairy Research Institute (N.Z.) were used in the investigation which was commenced in January 1956, when the levels of carotene and vitamin A in the milk fat were at a minimum. The animals were grazed out-of-doors on typical dairy pastures which were at the time predominantly white clover with a small proportion of ryegrass and cocksfoot. During the 7-day pretreatment period (Period 1) all animals were on pasture and were permitted to graze to appetite each

day on an area which was limited by means of the electric fence. For the duration of the experiment 4 cows were offered an area of pasture equivalent to this each day. Their twin mates were then restricted for 8 days to one-half this area per day (Period 2), for 8 days to one-quarter of this area per day (Period 3), and finally, for 8 days they were deprived of pasture almost completely (Period 4). The eight animals then ran together on pasture for a further 15 days (Period 5). During the periods of restricted pasture intake, the balance of the requirements for maintenance and lactation (calculated from Woodman, 1948) was provided by poor quality hay and a meal consisting of equal parts of linseed meal and crushed barley offered in the field simultaneously with the pasture which was rationed throughout the day to ensure a relatively uniform intake of pasture and meal.

Exp. 2. To investigate the utilization of an alternative source of carotene, two of the four pairs of monozygotic twins employed in the first experiment were used. Two animals remained on pasture throughout the experiment while their twin mates were fed out-of-doors on concentrates and hay for 18 days (Period 7). During the last 9 days of this period (Period 7b) the meal-fed animals were both drenched twice daily, after the morning and evening milking, with a solution of carotene (pure synthetic β-carotene as supplied by L. Light & Co. Ltd., Poyle) in arachis oil to give a daily intake of 750 mg of carotene in 150 ml. of oil. The four animals were then maintained on pasture for a further 12 days (Period 8).

Sampling.

Milk fat samples. All cows were milked normally twice a

day, milk samples being collected at the evening milking only. Fat samples were prepared for assay and estimated individually for carotenoids and vitamin A as described on pp. 169, 170.

Pasture and feed samples. Pasture and feed samples were taken at intervals of approximately 7 days and assayed for carotene using the method described on p. 170 et seq.

Results

Exp. 1. Throughout the experimental periods the level of carotene in the pasture remained relatively constant at 500-550 µg/g dry matter. Assuming a dry matter feed intake of approximately 2½% body weight (Woodman, 1948), this gave a daily carotene intake of about 5000 mg when the animals were on full pasture (Periods 1 and 5). Allowing for the carotene content of the dry feed, the daily carotene intake was approximately 2500 mg during Period 2, 1300 mg during Period 3 and 100 mg during Period 4 when pasture contributed only about 1% of the total feed intake.

Average milk fat vitamin A and carotenoid levels for the 4 pairs of animals are shown in Fig. 6. All animals showed similar trends and similar sample-to-sample fluctuations which in many cases exceeded treatment differences except during Period 4. However, it seems apparent that a reduction in carotene intake to one-half or one-quarter normal has little effect on the levels of vitamin A ester or alcohol. When the carotene intake was further reduced to 100 mg (Period 4), vitamin A ester was depressed on the average by about 1.5 µg/g but vitamin A alcohol was not appreciably affected. During

Period 2 the average level of carotene in the milk fat was reduced by about 1.0 μg/g fat and during Periods 3 and 4 by about 2.0 μg/g fat. Treatment had little effect on xanthophyll levels until Period 4 when a depression of about 1.0 μg/g fat occurred.

Milk fat vitamin A and carotenoid levels returned slowly to normal during the post-experimental period (Period 5), vitamin A ester being somewhat higher for several days in the milk fat of animals whose carotene intake had previously been restricted.

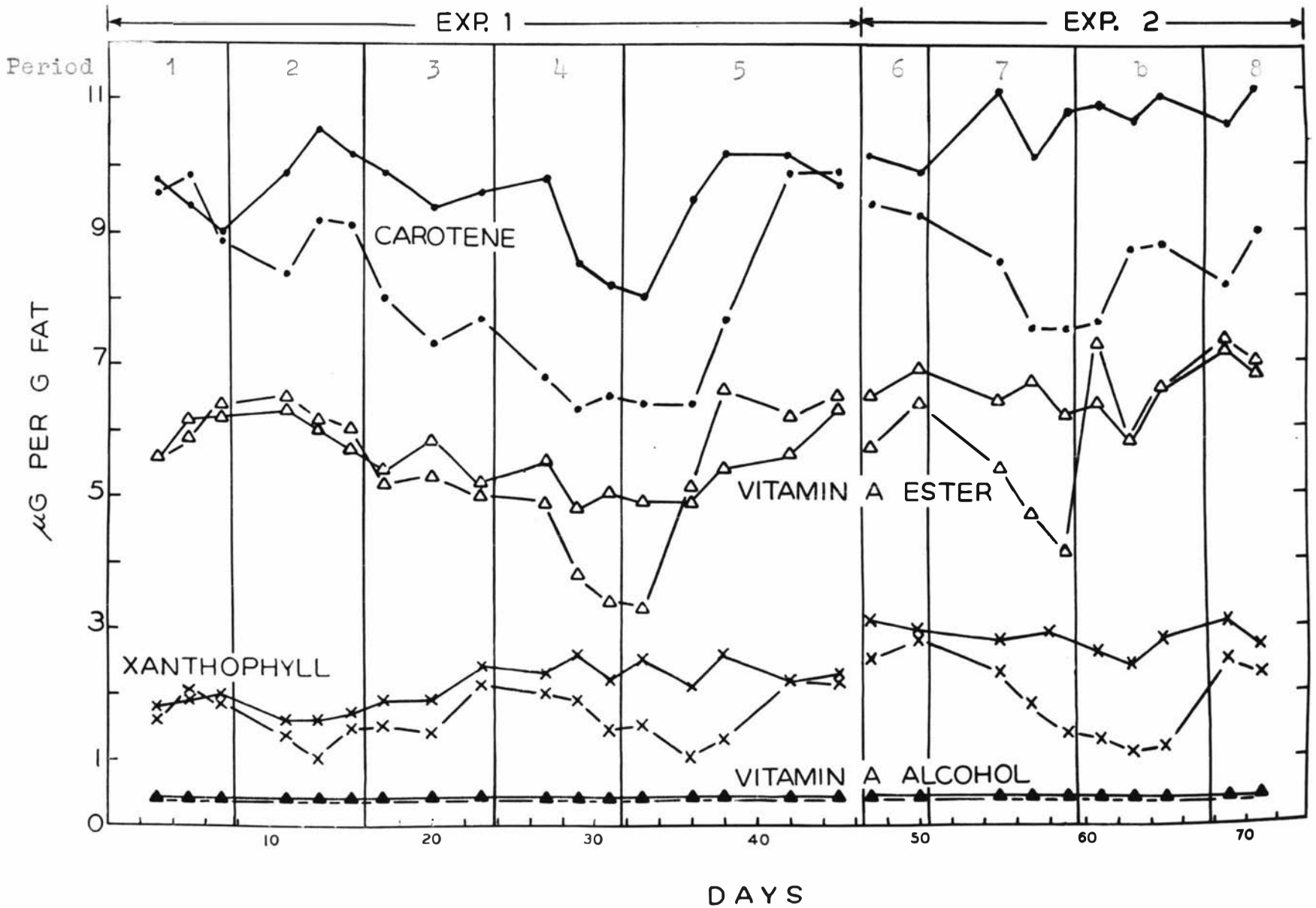
Exp. 2. The results of the experiment in which 2 animals were dosed with carotene in oil as an alternative source of carotene are shown also in Fig. 6. During Period 7 the treated animals received the same "low-carotene" feed as was used during Period 4 (Exp. 1) and their daily carotene intake was again about 100 mg. As in the previous experiment, the restricted carotene intake caused a decrease in milk fat vitamin A ester, carotene and xanthophyll levels. When, however, the feed was supplemented during Period 7b with 750 mg of carotene in oil to give a total carotene intake of approximately 850 mg per day, milk fat vitamin A ester and carotene values rose rapidly, the vitamin A ester levels for a short period exceeding those in the milk fat of the control cows. By the end of Period 7b vitamin A ester levels were identical in the control and treated cows and no significant changes occurred on return to full pasture during the post-experimental period. Carotene, on the other hand, was still slightly lower in the milk fat of the treated cows than of the controls at the end of Period 7b and a slight

Figure 6.

Effect of reduced carotene intake (Exp. 1) and of supplementing with carotene in oil (Exp. 2) on the levels of carotene, xanthophyll, vitamin A alcohol and ester in milk fat.

Solid lines: control cows.

Broken lines: treated cows.


EXP. 1
EXP. 2
Period
1
2
3
4
5
6
7
8
μG PER G FAT
11
9
7
5
3
1
0
CAROTENE
VITAMIN A ESTER
XANTHOPHYLL
VITAMIN A ALCOHOL
10
20
30
40
50
60
70
DAYS

increase occurred on return to full pasture. Xanthophyll levels returned rapidly to normal during the post-experimental period.

Milk and fat yields remained relatively constant throughout the two experiments and no significant differences were found between treated and control cows.

Discussion

The present experiments were undertaken in an attempt to obtain further information on the utilization of carotene by cows grazing summer pasture in New Zealand and to compare the availability of carotene from such pasture with that from an alternative source (viz. arachis oil).

From the results presented in Fig. 6 it is apparent that although the carotene intake of the control animals was relatively constant throughout the experiment at about 5000 mg per day, considerable fluctuations occurred in the levels of carotene and vitamin A in the milk fat. For example, during Exp. 1 the milk fat carotene varied from a maximum of 10.4 to a minimum of 8.0 μg/g fat, and vitamin A ester from 6.3 to 4.9 μg/g fat, giving a variation in total potency from approximately 40 to 30 i.u./g fat. That these sample-to-sample differences were of the same order as the differences between control and treated animals during Period 4 would seem to indicate that some factor other than carotene intake was capable of producing as great a change in milk fat carotene and vitamin A as a 50-fold change in carotene intake. At the

present time the nature of the factor involved is not at all clear. It is of interest to note, however, throughout both experiments, the close correlation between the sample-to-sample fluctuations in the milk fats of control and treated animals suggesting that variations possibly in the external environment of the animal may have a marked effect on carotene utilization. Although these fluctuations tended to mask the effect of variations in carotene intake, it appears quite clear from the results of Exp. 1 that a relationship does exist between carotene intake and total fat potency even up to relatively high levels of carotene intake. For example, there was some depression of milk fat vitamin A ester levels when the carotene intake was reduced to 1500 mg per day (Period 3) and milk fat carotene levels were affected to some extent when carotene intake was reduced to 2500 mg per day (Period 2). The latter effect was slight, however, so that generally speaking with intakes from pasture in excess of 2500 mg per day (equivalent to a level in the pasture of approximately 250 µg/g dry matter), it seems that the total vitamin A potency of the milk fat may be regarded as virtually independent of carotene intake. This is in agreement with the earlier findings of Wiseman et al. (1949) and Hibbs et al. (1949), although in the present investigation the maximum total vitamin A potency of the milk fat produced at this level of carotene intake was lower than that reported by these workers.

Throughout the present experiments little relationship could be observed between carotene intake and the level of vitamin A alcohol in the milk fat although there did appear

to be a very slight tendency for the alcohol level in the milk fat to increase as the carotene intake decreased (cf. Chanda & Owen, 1952; Chanda et al. 1954, 1955). However, the levels present were so low and the differences noted so consistently small, that no significance can be attached to them (see also McGillivray, 1957a, b).

In comparing the results obtained in Exp. 2 in which 750 mg of carotene in arachis oil was used as almost the sole source of carotene in the diet, with those obtained in Exp. 1 it is noteworthy that this level of carotene in oil was more effective than 1300 mg of carotene from pasture in maintaining milk fat vitamin A potency, and only slightly less effective than 2500 mg of pasture carotene. It was also of interest that on transferring from the "meal-plus-carotene-in-oil" rations used in Exp. 2 (Period 7b) to full pasture, no change occurred in milk fat vitamin A ester levels and milk fat carotene levels increased only slightly. Carotene in oil would appear, therefore, to be relatively better utilized than carotene in pasture grown under New Zealand summer conditions. In view of the fact, however, that dosing with carotene in oil did not increase the total potency of the fat above normal summer levels (i.e. 30-35 i.u/g fat), it would appear that factors limiting the utilization of carotene from summer pasture exert a similar overall effect on carotene administered in oil.

From a consideration of the results of both experiments it seems apparent that under New Zealand conditions a relationship similar to that reported by overseas workers exists between the carotene intake from pasture and the levels of

carotene and vitamin A in the milk fat. During winter and early spring the total vitamin A potency of the milk fat is of the same order as would be predicted from the known carotene intake of the animals but in midsummer certain factors prevent these maximum levels being attained. While part at least of this effect may be due to environmental influences (p. 200) it would seem likely that nutritional factors also are involved to a certain extent. It was thought desirable, therefore, that this latter possibility should be further investigated. Accordingly, a series of experiments was undertaken for this purpose in rats. The results of these studies form the subject of the next chapter.

Summary

1. The results are discussed of an investigation into the efficiency of utilization by dairy cows of carotene from New Zealand summer pasture and from arachis oil.

2. Above an intake of 2500 mg of carotene/day from pasture (corresponding to a level in the pasture of approximately 250 µg/g dry matter), it would appear that milk fat vitamin A potency is virtually independent of the level of carotene in the diet.

3. A 50-fold decrease in the normal intake of carotene from summer pasture over a short period was shown to have relatively little effect on the vitamin A potency of the milk fat, carotene content being reduced by approximately 2.0 µg/g fat and vitamin A ester by approximately 1.5 µg/g fat. Little change was observed in vitamin A alcohol

content.

4. Sample-to-sample variations in both control and treated animals were observed in many cases to exceed the differences due to treatment except during the period of extreme carotene deprivation. Sample-to-sample variations of control and treated animals were also observed to be highly correlated which would suggest that perhaps variations in the external environment of the animal may have some effect on carotene utilization.

5. Carotene in arachis oil was shown to be relatively more effective in maintaining the vitamin A potency of milk fat than summer pasture but the total potency of the fat obtained after dosing with carotene in oil was of the same order as that produced from summer pasture.

CHAPTER XII

THE INFLUENCE OF VARIOUS NUTRITIONAL FACTORS ON THE UTILIZATION OF CAROTENE ADMINISTERED ORALLY TO RATS

Introduction

Nutritional factors influencing carotene utilization have been investigated by a number of workers but in general the level of carotene intake has been relatively low and it cannot be assumed that the same effects would be apparent at the very considerably higher relative levels of carotene intake obtaining with cows on pasture in this country (see p. 193) Accordingly, using rats as experimental animals and comparatively high carotene intakes, the effects on carotene utilization, as indicated by blood levels and liver stores, of a number of possible changes in pasture composition were investigated in the hope of obtaining information applicable to the general problem of carotene utilization from pasture by cows. In addition, since the seasonal changes noted previously in Chapter X, particularly in plasma vitamin A alcohol levels, could perhaps be hormonal, the effects of adrenaline and insulin which other workers have claimed could influence vitamin A metabolism (see, for example, Young & Wald, 1940; Bauereisen, 1939) were also investigated.

Experimental

General. The materials and methods were as described previously with the following additions:

Rats. The rats used were albino of the Wistar strain, partially deficient in vitamin A and maintained on the basal diet described earlier.

Dosing of rats. Carotene (approximately 90% β) and

α-tocopheryl acetate were supplied by the Eastman Kodak Co. Ltd., New York. Chlorophyll and xanthophyll were extracted from fresh grass and separated chromatographically as described on p.170. Unless otherwise stated the carotene and other fat-soluble substances were dissolved in arachis oil and the animals dosed by stomach tube (see p.61) and killed 48 h later.

Factors studied. As has been noted earlier, little information is available regarding variations in the general composition of typical New Zealand pastures. It is well known, however, that there are seasonal changes in the composition of the pasture, particularly in the proportion of ryegrass (*Lolium perenne*) and red and white clover (*Trifolium pratense* and *T. repens*)*, and also in the stage of maturity. A number of variations in known pasture constituents which might be expected to affect carotene utilization were therefore investigated as follows:

(a) effect of level of carotene

(b) effect of concentration of carotene in oil used as vehicle

(c) effect of degree of unsaturation of the vehicle

(d) effect of non-digestible material in the oil

(e) effect of tocopherol

(f) effect of chlorophyll and xanthophyll

(g) effect of botanical composition of the feed

(h) effect of cyanide

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* See Sears *et al.* (1942, 1948).

Results

The results presented in Table 32 indicate that a number of possible variations in pasture composition might be expected to influence quite markedly carotene utilization despite the relatively high level fed. Thus, from Exp. 1 it is clear that a fairly direct relationship exists between liver storage of vitamin A and the level of carotene fed over the range 0.25-4 mg/rat*. The concentration of carotene in the oil is also important (Exp. 2), too low or too high a concentration tending to reduce liver storage, the latter due probably to an overloading of the digestive system with lipid material. From the results of Exp. 3 it is apparent that the nature of the vehicle may have a profound effect on carotene utilization, the level of vitamin A in the liver decreasing sharply as the proportion of triolein in the oil used as vehicle was decreased below about 70%. The incorporation of a small amount of beeswax had a much greater depressant effect than the equivalent amount of tristearin, the mixture of 60% triolein, 25% tristearin and 15% beeswax resulting in a liver storage of only 8.3 µg of vitamin A whereas a mixture of 60% triolein and 40% tristearin alone would have been expected to give a liver storage of the order of 40-45 ug of vitamin A. Varying the level of tocopherol from 0 to 8 mg/rat had no effect on the utilization of a 4 mg dose of carotene (Exp. 4). Similarly, 40 mg of chlorophyll dosed with the carotene had no effect (Exp. 5), while 3 mg of xanthophyll had a slight

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* Equivalent on a weight basis to approximately 500-8000 mg/cow.

but probably insignificant depressant effect on liver vitamin A stores. In general liver carotene levels, although low, followed the liver vitamin A levels. Plasma vitamin A alcohol levels varied from 13 to 31 µg/100 ml. plasma, and, as would be expected, bore no apparent relationship to treatment. On the other hand, plasma ester values, which ranged from 1.1 to 8.6 µg/100 ml., bore a fairly direct relationship to liver vitamin A levels indicating that absorption of vitamin A was still proceeding and at a rate proportional to liver stores.

From the results presented in Table 33 it would appear that there was some difference in the utilization of carotene by rats from the 3 pasture species investigated but the addition of cyanide in quantities equivalent to those likely to be derived from a high cyanide white clover had no marked effect on the utilization of carotene either from arachis oil or from a mixed pasture. In Exp. 6, the lowest liver storage of vitamin A resulted from feeding high cyanide clover. This was associated with a plasma vitamin A alcohol level of 34 µg/100 ml. as compared with 20 and 26 µg/100 ml. for ryegrass and low cyanide white clover, respectively. The same effect is apparent in Exp. 7 where the addition of cyanide to the drinking water resulted in lower liver storage of vitamin A again associated with slightly higher blood plasma alcohol levels but these differences were not significant. Similarly no significant treatment differences were apparent with the higher dosage rate of carotene in oil used in Exp. 8.

To investigate the effect of adrenaline and insulin on plasma vitamin A levels, 6 female stock-colony rats with normal

Table 32. The effect of various factors on the appearance of carotene and vitamin A in the blood plasma and livers of rats (mean weight 250 g) partially deficient in vitamin A dosed orally with carotene. All rats were slaughtered 48 h after dosing.

Exp.	No. Rats Used	Dose: Vehicle Nature	Dose: Vehicle Amount (g)	Dose: Amount Carotene (mg)	Dose: Other Substances Nature	Dose: Other Substances Amount (mg)	Blood plasma: Vitamin A Alcohol (µg/100 ml.)	Blood plasma: Vitamin A Ester (µg/100ml.)	Liver: Vitamin A Alcohol (µg)	Liver: Vitamin A Ester (µg)	Liver: Vitamin A Total (µg)	Liver: Carotene (µg)
1	6	-	-	-	-	-	13	-	0.5	0.7	1.2	-
	3	Arachis oil	1	0.25	-	-	18	1.1	1.0	2.3	3.3	1.8
	3		1	0.5	-	-	16	1.5	2.2	4.9	7.1	1.9
	3		1	1.0	-	-	19	3.3	3.8	8.2	12	2.3
	3		1	2.0	-	-	20	4.0	9.1	19	28	5.3
	3		1	4.0	-	-	24	7.0	10	38	48	8.7
2	3	Arachis oil	0.5	4.0	-	-	25	5.2	14	24	38	6.7
	3		1.0	4.0	-	-	24	7.0	10	38	48	8.7
	3		2.0	2.0	-	-	31	3.3	10	16	26	4.8
3	3	(30% triolein (70% tristearin	1	4.0	-	-	19	2.8	2.1	3.7	5.8	5.4
	3	(40% triolein (60% tristearin	1	4.0	-	-	19	3.7	6.3	19	25	8.2
	3	(50% triolein (50% tristearin	1	4.0	-	-	20	3.9	9.8	26	36	13
	3	(70% triolein (30% tristearin	1	4.0	-	-	16	5.7	11	38	49	10
	3	triolein	1	4.0	-	-	18	5.2	12	40	52	11
	3	(60% triolein (25% tristearin (15% beeswax	1	4.0	-	-	17	3.1	2.5	5.8	8.3	6.3
4	3	Arachis oil	1	4.0	α-tocopheryl acetate	-	24	7.0	10	38	48	8.7
	3		1	4.0		0.5	25	4.2	11	30	41	8.1
	3		1	4.0		1.0	20	5.0	11	38	49	8.3
	3		1	4.0		2.0	21	8.6	11	38	49	11
	3		1	4.0		4.0	25	2.9	10	32	42	5.6
	3		1	4.0		8.0	17	2.2	11	33	44	4.8
5	3	Arachis oil	1	4.0	-	-	24	7.0	10	38	48	8.7
	3		1	4.0	Chlorophyll	40	25	2.3	14	33	47	4.1
	3		1	4.0	Xanthophyll	3	25	3.0	12	26	38	4.6
	3		1	4.0	(Chlorophyll (Xanthophyll	40) 3)	25	3.4	13	30	43	4.7

Table 33. The effect of feeding separately ryegrass, low cyanide and high cyanide white clover and of feeding cyanide on the appearance of carotene and vitamin A in the blood plasma and liver of rats (mean weight 170 g) partially deficient in vitamin A.

Exp.	No. rats used	Treatment: Diet	Duration (days)	Total Carotene intake per rat (mg)	Blood Plasma: Vitamin A Alcohol (µg/100 ml)	Blood Plasma: Vitamin A Ester (µg/100 ml)	Liver: Vitamin A Alcohol (µg)	Liver: Vitamin A Ester (µg)	Liver: Vitamin A Total (µg)	Liver: Carotenoids Carotene (µg)	Liver: Carotenoids Xanthophyll (µg)
6	6	Normal basal diet (controls).	-	-	14	-	0.5	0.4	0.9	0	0
	6	Skim milk powder + 28% freeze dried ryegrass.	6	7.1	20	7.6	24	82	106	2.8	5.3
	6	Skim milk powder + 34% freeze dried low cyanide white clover.	6	7.1	26	8.2	21	73	94	2.6	2.6
	6	Skim milk powder + 50% freeze dried high cyanide white clover.	6	7.1	34	8.8	20	60	80	3.2	3.0
7	4	Skim milk powder + 30% freeze dried pasture (approx. 50% ryegrass 50% low cyanide clover on D.M. basis).	5	6.6	27	-	15	113	128	6.2	4.3
	4	- ditto - + KCN equivalent to 0.05% HCN in drinking water.	5	6.6	33	-	20	91	111	6.9	3.5
8	3	Normal basal diet + 4 mg carotene in 400 mg arachis oil/rat/day.	6	24	22	-	23	310	333	34	-
	3	- ditto - + KCN equivalent to 25 mg HCN/rat/day mixed with feed.	6	24	22	-	28	334	362	34	-

Table 34. The effect of subcutaneously administered adrenaline and insulin on the plasma vitamin A alcohol levels of stock-colony rats. Slaughtered $\frac{1}{2}$ h after injection. Mean weight 300 g.

Exp.	No. rats used	Liver reserves of vitamin A	Dose		Blood plasma	
			Nature	Amount (ml.)	Vitamin A alcohol (µg/100 ml.)	Vitamin A ester (µg/100 ml.)
9	6	Normal	Distilled water	0.5	15.1	4.0
	6	Normal	1:1000 adrenaline	0.5	11.3	5.2
10	6	High	Distilled water	0.5	14.6	9.2
	6	High	1:1000 adrenaline	0.5	14.4	8.7
	6	High	Insulin (= 40 units)	0.5	13.2	9.6

vitamin A reserves were injected subcutaneously with 0.5 ml. of 1:1000 adrenaline and killed ½ h later. 6 similar rats but injected with 0.5 ml. of distilled water were used as controls. From the results (Table 34, Exp. 9) it can be seen that the plasma vitamin A alcohol levels found for treated animals (11.3 µg/100 ml.) were lower than those found for the control animals (15.1 µg/100 ml.), these differences being, however, statistically not significant. When this experiment was repeated using animals with considerably higher liver vitamin A reserves (Exp. 10), similar injections produced no significant changes in plasma vitamin A alcohol, 6 treated animals averaging 14.4 µg/100 ml. and 6 control animals 14.6 µg/100 ml. 6 similar animals injected subcutaneously with 0.5 ml. of insulin (equivalent to 40 units) and killed ½ h later showed an average plasma alcohol level of 13.2 µg/100 ml. which was again not significantly different from the controls. Treatment with either adrenaline or insulin had no effect on plasma vitamin A ester levels.

Discussion

Factors influencing carotene utilization at low levels of intake have been extensively reviewed elsewhere by a number of workers (see, for example, Goodwin, 1952) and will not therefore be dealt with further here. The main purpose underlying the present experiments was to determine whether similar relationships could be established at considerably higher levels of carotene intake as a means of explaining certain irregularities in the utilization of carotene from pasture by dairy cows grazing under New Zealand summer conditions.

For this purpose rats have been used as experimental animals. All dosage levels were adjusted, on a body weight basis, so as to simulate as closely as possible conditions in the field.

From the results obtained it is apparent that certain factors may have a profound influence on the vitamin A economy of the animal even at these relatively high levels of carotene intake. For example, it is clear from the present results, that over a wide range of carotene intake there is an almost linear relationship between hepatic storage of vitamin A and the level of intake, although these observations, insofar as they apply to animals in the field, are probably only of fairly limited import (see, for example, p. 196). Further, there would appear to be a relationship between the level of fat in the diet and the efficiency of carotene utilization providing at least that the digestive system does not become unduly overloaded with lipid material when utilization may become seriously impaired. In contrast to these results, however, it would appear that the utilization of carotene when fed at high levels in the diet is but little affected by addition of either tocopherol, chlorophyll or xanthophyll. From a consideration of the results obtained in Exp. 3 (Table 32), however, it is apparent that the degree of unsaturation of the vehicle may be the most important single factor determining the efficiency of utilization of carotene at high intake levels. In practice in the field the degree of unsaturation of the pasture fat could affect the utilization of carotene in at least two ways; directly, due to the proportion of unsaturated fatty acids present in the fat (e.g. Krukovsky, 1942), and indirectly, depending on the extent to which the unsaturated

acids present are hydrogenated due to microbial activity during their passage through the rumen. Shorland et al. (1955) have demonstrated that unsaturated acids of pasture fat are readily hydrogenated in the rumen, but there is to date no indication of any seasonal variation in this function which might account for possible variations in carotene absorption. Oleic acid, however, which is the major unsaturated acid of milk fat, is known to vary in New Zealand milk fat in much the same way throughout the season as carotene, xanthophyll and tocopherol (e.g. results of Hansen & Shorland, 1952; McDowall, 1953; Worker & McGillivray, 1957; see also pp. 159, 161), and since oleic acid is not synthesized to any appreciable extent in the mammary gland (Popjak, 1952) it might be assumed, on the basis of the results obtained in Chapter X, that this trend reflects similar changes in the oleic acid content of the blood plasma. If in turn the level of oleic acid in the plasma reflects hydrogenation activity in the rumen, as it appears very well it might from the results of Shorland et al. (1955), then the latter function may quite well be a fundamental contributing factor to the low availability of carotene from New Zealand summer pastures. Clearly, however, much further work will be required in order to elucidate these relationships in more detail.

From the results of the experiments, in which the effect of feeding separately ryegrass, low cyanide and high cyanide white clover on plasma levels and hepatic stores of vitamin A were studied (Table 33), a difference was observed in the utilization of carotene from the three species in favour of the ryegrass and low cyanide clover (Exp. 6). This species

difference was, however, probably due to some factor other than the cyanide content, since incorporation of cyanide in the diet in amounts calculated to be equivalent to those derived from high cyanide clover did not appear to appreciably affect the efficiency of utilization of carotene either from a mixed pasture (Exp. 7) or from arachis oil (Exp. 8).

The results presented in Table 34 are in sharp contrast with the findings of Young & Wald (1940) and Bauereisen (1939). From a consideration of the results it is apparent that under the present experimental conditions neither adrenaline nor insulin had any appreciable effect on the levels of vitamin A alcohol or ester in the blood plasma of rats. From this evidence and from that presented by Thompson & McGillivray (1957) it would appear that, under normal conditions in the field, hormonal influences are probably unimportant insofar as blood plasma and milk fat levels of carotene and vitamin A are concerned.

Summary

1. The influence of a number of factors on the utilization of carotene by rats has been investigated. A high dosage level equivalent to that derived from pasture by cows has been employed and plasma levels and liver storage of vitamin A has been used as a measure of the efficiency of carotene utilization.

2. Utilization was influenced by the level of carotene intake, by the concentration of carotene in the oil used as vehicle, by the degree of unsaturation of the vehicle and by the presence of non-digestible wax.

3. Carotene was better utilized from ryegrass and low cyanide white clover than from high cyanide clover but added cyanide had little or no effect on the uptake of carotene from cyanide-free pasture or from a solution in oil.

4. Insofar as the results of these investigations can be applied to cows grazing typical New Zealand dairy pasture, it seems that factors which may throw some light on the poor utilization of carotene from the pasture over the summer months are (a) variations in the amount, nature and composition of the fat present in the pasture, and (b) variations in the degree of hydrogenation of fat in the rumen.

5. Adrenaline and insulin appeared to have no effect on the levels of vitamin A alcohol or ester in the blood plasma of rats. From these results and those obtained by other authors it would seem unlikely that hormonal effects are important in influencing carotene and vitamin A levels of the blood plasma or milk fat.

CHAPTER XIII

INTEGRATING SUMMARY

Certain aspects of the problem of the decline in the vitamin A potency of milk fat from cows grazing summer pasture in New Zealand have been investigated. In the course of these studies the following points of general interest emerged:

1. In a survey in which the levels of carotene, xanthophyll, vitamin A alcohol, vitamin A ester and tocopherol were followed at fortnightly intervals in the milk fat and blood plasma of 6 pairs of monozygotic twin cows from early October (spring) through to early February (midsummer), variations were observed in all constituents (with the exception of vitamin A alcohol and ester in milk fat which was relatively constant) similar to those reported by earlier workers, a steady decline in levels being recorded from early November through to late January. No differences were observed between early- and late-calving cows which confirms that the trends noted were quite independent of any lactational effect. From a comparison of the blood picture with that obtained in milk fat it is clear that the variations observed in milk fat constituents are due primarily to the changing levels of these substances in the blood plasma. The lower plasma levels of carotene, xanthophyll and tocopherol over the summer period would seem to suggest, in agreement with the findings of other workers, that there is a decrease in the availability of these substances from the pasture at this time, since pasture levels of each were shown throughout the survey to vary but little, and the level of carotene in the pasture was found at all times to be well above the level suspected as adequate for the production of fat of maximum vitamin A potency.

2. Factors likely to affect the availability of carotene

from pasture have been investigated in some detail.

(i) Evidence is presented suggesting that, in the cow environmental factors may have quite an important effect in influencing carotene utilization.

(ii) The possibility that a different relationship than that indicated from overseas work existed between carotene intake and milk fat potency over the summer months was examined using 4 pairs of monozygous twins, from which it was shown that above an intake of 2500 mg of carotene per day (equivalent to a level of approximately 250 µg/g dry matter in the pasture) milk fat potency was virtually independent of carotene intake.

(iii) In a further study using 2 pairs of twin cows, in which the availability of carotene from arachis oil was compared with that from summer pasture, carotene from the former source was shown to be relatively better utilized than from the latter. Drenching with carotene in oil, however, did not increase milk fat potency above normal summer levels indicating that factors affecting the utilization of carotene from summer pasture exert a similar overall effect on carotene from other sources.

(iv) In experiments with partially deficient rats, in which the influence of a number of factors on carotene utilization was investigated using blood levels and liver storage of vitamin A as criteria and high dosage levels of carotene equivalent to that derived by cows from pasture, utilization was shown to be influenced by the level of carotene intake, by the concentration of carotene in the oil used as vehicle, by the degree

of unsaturation of the vehicle and by the presence of non-digestible wax. On the other hand tocopherol, chlorophyll and xanthophyll appeared to be without effect on carotene utilization.

(v) Carotene was better utilized from ryegrass and low cyanide white clover by rats then from high cyanide clover but incorporation of cyanide in the diet in amounts calculated to be equivalent to those derived from high cyanide clover did not appear to appreciably affect the uptake of carotene either from cyanide-free pasture or from oil.

(vi) Adrenaline and insulin appeared to be without effect on blood vitamin A alcohol or ester levels in rats.

5. From the results obtained in the present series of experiments the following general conclusions may be drawn:

(a) The level of carotene in New Zealand pasture varies for the most part between comparatively narrow limits and throughout the year would appear to be well above the level required for the production of fat of maximum vitamin A potency. Similarly, xanthophyll and tocopherol do not appear to vary greatly in the pasture and the levels present at all times should be sufficient to maintain continuously high levels of these substances in the milk fat.

(b) The fact that lower milk fat vitamin A potencies and lower levels of xanthophyll and tocopherol are found in milk fat produced over the summer period is due primarily to lower levels of various plasma constit-

unts over this period apparently associated with their lowered availability from the pasture.

(c) A number of factors may be of importance in contributing to the lowered availability of fat-soluble pasture constituents over the summer. Environmental factors are suspected as perhaps being partly responsible. Insofar as results obtained with rats may be applied to the problem, however, it would appear that by far the most important factor contributing to the problem is a possible variation in either the amount, nature or composition of the fat present in the pasture throughout the year. Variations in the degree of hydrogenation of this fat in the rumen may also be a factor complicating the problem.

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