THE ROLE OF CLOVER AS A FACTOR AFFECTING THE SUMMER DECLINE IN THE VITAMIN A POTENCY OF NEW ZEALAND BUTTERFAT.

by

NEIL ADRIAN WORKER.

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CHAPTER ONE.

A REVIEW OF LITERATURE.

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INTRODUCTION.

Prior to 1913 it was generally assumed that all fats had similar nutritive values and that their only function in the diet was to supply energy. In that year, however, McCollum and Davis (1) of Wisconsin, found in agreement with the earlier observations of Hopkins (2), that rats failed to grow on purified diets in which olive oil, almond oil and lard provided the sole source of fat, whereas normal growth resulted in the presence of milk fat, egg-yolk fat, or cod-liver oil.

Almost simultaneously Osborne and Mendel (3), working independently at Yale, observed the growth response of rats on a purified diet of "protein-free milk", protein, and starch was greatly enhanced when fat was supplied as butter-fat (or as whole milk powder) but not as lard. In a subsequent communication (4), Osborne and Mendel confirmed these results and called attention to the prevalence of an inflammation of the eyes of their rats restricted to the lard diet, a condition which they noted to be speedily alleviated by the introduction of butterfat into the diet. Shortly afterwards a similar eye condition, to which they gave the name xerophthalmia, was described by

McCollum and Simmonds (5) and likewise shown to be relieved by a supplement of butter or cod-liver oil.

These preliminary experiments indicated that certain fats contained a factor essential for normal health and growth, which was absent from others. McCollum designated the factor "fat-soluble A". At about the same time certain other accessory food factors were becoming recognised and the Vitamin Theory was gaining general acceptance. Subsequently "fat-soluble A" was shown to be a vitamin and on the suggestion of Drummond (6) was named vitamin A.

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THE ISOLATION AND INDENTIFICATION OF VITAMIN A.

In the years that followed, chemical isolation and indentification became the main aim of all vitamin A research. In 1914 McCollum and Davis (7) reported the vitamin to be stable to alkaline hydrolysis and demonstrated its growth-promoting activity to be concentrated in the unsaponifiable fraction of butterfat and cod-liver oil. These observations were subsequently confirmed by other workers and formed the basis of nearly all future attempts to isolate the vitamin.

During the initial stages,

however, progress was slow and results were confusing and extremely contradictory. In the first place, the chemical structure of vitamin A was unknown and the early investigators were forced to work with a substance whose identity could be described only in terms of certain specific deficiency symptoms. It was not, therefore, until after the pathology of vitamin A in the animal organism had been thoroughly investigated that rapid progress was possible in the accumulation of exact knowledge of the dietary factor involved.

In the second place, confusion arose due to the failure of the earlier workers to appreciate the multiple nature of the unsaponifiable fraction of certain fats, in particular butterfat and cod-liver oil. Mellanby (8) for example, in 1918 demonstrated the superiority of the unsaponifiable fraction of cod-liver oil in the treatment of experimental rickets in puppies, and from this inferred that rickets were caused by a deficiency of vitamin A, an observation which was subsequently confirmed by Korenchevsky (9) and Goldblatt (10). However Shipley, Park, McCollum, Simmonds and Parsons (11) questioned this explanation. In a series of studies involving many modifications of diet, they concluded that the content and ratios of calcium and phosphorus in the diet, together with the presence or absence of some organic factor contained in the cod-liver oil, constituted the

essential factors in the etiology of rickets in rats under experimental conditions. That this factor was not vitamin A was later demonstrated by McCollum, Simmonds, Becker and Shipley (12) when they showed that cod-liver oil, which had been treated with a stream of air bubbles to remove its vitamin A activity (13), was still potent in the prevention or cure of rickets. This removed any doubts that the antirachitic substance was not a definite chemical entity entirely distinct from vitamin A. Subsequently it was named vitamin D by McCollum.

In 1925, a Japanese team headed by Takahashi (14) reported the isolation of the active principal of vitamin A from the unsaponifiable fraction of cod-liver oil. This claim was, however, later discounted by the work of Drummond, Channon and Coward (15) and Drummond and Baker (16) who concluded that:

> "the active substance is present in cod-liver oil concentrates in amounts so minute that direct attempts at its isolation by ordinary chemical means are of little use".

More potent sources of the vitamin were sought, therefore, and in the search a large number of fish-liver oils was investigated. In 1929 Poulsen (17) reported halibut-liver oil to be an extremely potent source and fractionation methods were immediately applied to it in an attempt to isolate the active substance.

Despite repeated efforts it was not until 1930 that Karrer and his associates (18) in Switzerland, announced the isolation as an oil concentrate, of an active fraction which they identified as an unsaturated alcohol having the formula $C_{20}H_{30}O$. Its structure (fig.1A) was suggested by the same workers (19) and later confirmed by Heilbron, Morton and Webster (20) and subsequently by Karrer himself (21) by means of chemical synthesis of the hydrogenated derivative of the vitamin.

There followed numerous unsuccessful attempts to crystallize the vitamin, the main problem being to find a means of removing the last traces of impurity. It was 1937, seven years after its isolation, before Holmes and Corbet (22) were able to report the recovery of the first pure, pale yellow, needle-like crystals of vitamin A from the liver oil of the Atlantic Ocean Mackerel. In that same year the first quarter of a century of vitamin A research was successfully concluded when Fuson and Christ (23) and Kuhn and Morris (24) announced simultaneously the chemical synthesis of the vitamin by independent methods.

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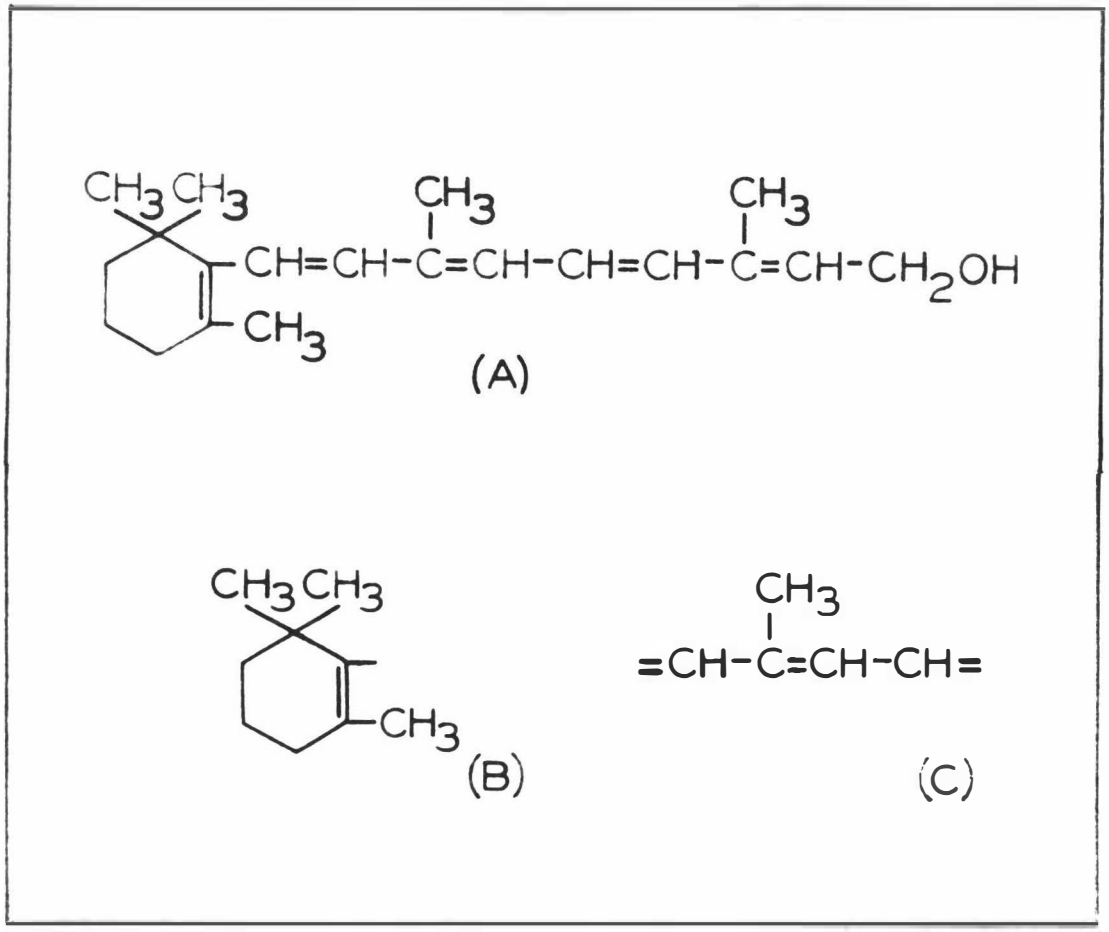

FIGURE 1.

(A) Structural formula for vitamin A.
(B) Beta-ionone ring structure.
(C) Isoprene residue.

PROVITAMIN ACTIVITY.

At about the time that Mellanby was carrying out his investigations into the cause of rickets, Steenbock and his colleagues (25,26,27) at Wisconsin first drew attention to the growth-promoting activity of certain plant and vegetable pigments. In feeding trials they demonstrated the superiority of yellow maize over white maize in maintaining growth in the vitamin A deficient rat, and concluded that this was due to the relatively higher carotenoid content of the former. To test this hypothesis they extracted carotene, the principal pigment of carrots, crystallized it and found the crystals to have vitamin A activity. A similar preparation of xanthophyll on the other hand was shown to be inactive. From these results they concluded that carotene, but not xanthophyll, afforded the same protection to rats as did butter-fat and cod-liver oil, and was the ultimate source of vitamin A in nature.

The results of other workers confirmed these views. For example Osborne and Mendel (28) noted the growth-promoting power of certain leaves to be correlated with their relative "greenness", while Coward and Drummond (29) noted that green leaves exhibited marked superiority over etiolated shoots which were apparently inactive. Hume (30) and Collinson et al (31) in guinea pigs, found white cabbage, in contrast to green, to be relatively inactive.

Although the weight of evidence pointing to some relationship between vitamin A and certain of the carotenoid plant pigments was considerable, such a relationship was not to be generally recognised for at least another ten years.

Many challenged the validity of the Wisconsin team's conclusions on the grounds that the carotene which they employed was not pure but contained, in addition, traces of vitamin A. This view was supported by Rosenheim and Drummond (32) and Drummond, Channon and Coward (15) who were unable to repeat the earlier work using carotene of varying degrees of purity and concluded that carotene is devoid of growth-promoting activity. Further, Stephenson (33) demonstrated the activity of butter to be unaffected by complete decolorization with charcoal. Others argued that since cod-liver oil, which was then the most potent source of vitamin A known, was only faintly coloured there could be no connection between pigmentation and growth-promoting activity. The matter seemed firmly settled when Palmer and Kempster (34) and Palmer and Kennedy (35) showed with fowls and rats respectively, that they could be reared successfully and later made to reproduce on a diet which was, to all intents and purposes, free from carotenoids.

The question was not raised again until 1928 when Euler, Euler and Hellstrom (36,37) prepared some very pure carotene crystals and tried feeding them to young rats on a diet otherwise devoid of vitamin A. They noted normal

weight gains in their experimental animals and established beyond all doubt the earlier hypothesis of Steenbock et al. Many other reports came forward confirming these conclusions (38,39) including a report from van Stolk, Guilbert, Penau and Simmonet (40) who showed that even after eleven successive crystallizations, carotene still retained its activity.

That the results of other workers were not in agreement with Steenbock and his associates must be attributed either to failure to supply vitamin D in the test diet, or more probably to insufficient precautions against oxidation of the pigment during administration of the test dose. Similarly, the contradictory results of Palmer and his team can be explained in the light of more recent knowledge by the presence of liver extracts in their basal diet. That their animals were able to grow and reproduce satisfactorily was no doubt due to the high vitamin A content of these extracts.

By 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. It remained for Moore at Cambridge to show that carotene was, in fact, provitamin A and was converted into the active vitamin within the animal body. In a series of preliminary experiments (41,42) he showed that carotene extracted from carrot fat was effective in restoring growth and

curing xerophthalmia in deficient rats. In a later series of experiments (43), he confirmed these results and proved beyond all doubt that "carotene possesses the physiological action of vitamin A". His conclusions were based on the recovery of vitamin A, identified by the Carr-Price reaction and the formation of a typical absorption band at 328mμ, from the livers of depleted rats fed a supplement of highly purified carotene. These results were confirmed by Capper at Belfast (44,45),working in collaboration with Moore and using identical techniques.

Subsequent research by numerous others (46,47,48) has established the conversion to take place in all animal species with the possible exception of the cat. Vitamin A is now generally recognised to be exclusively an animal product, the ultimate source in nature being certain of the carotenoid plant pigments.

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THE CONSTITUTION OF CAROTENE.

Moore's work demonstrating the *in vivo* conversion of carotene to vitamin A stimulated widespread research into the chemical structure and provitamin activity of the carotenoids. Amongst the foremost workers in this field were Kuhn and his collaborators in Germany. In a series

of experiments from 1931 onwards (49,50,51) they showed that 'carotene', which up to that time had always been considered a single entity, was a mixture of three naturally-occurring isomers which they designated alpha-carotene, beta-carotene and gamma-carotene respectively. By chromatographic adsorption and fractional precipitation they separated and purified these, determined their chemical structure (figs. 2A,B and C) and showed in feeding trials with rats that the β-isomer possessed approximately twice the growth-promoting activity of the other two. Karrer and his associates at Zurich working along similar lines on essentially the same problems, provided additional evidence in support of these conclusions (52,53).

It is not proposed to discuss in detail the chemistry of the isomeric carotenes except in so far as it bears on the vitamin A problem and it is considered sufficient merely to summarise the salient points. The Karrer School were the first to investigate the chemical criteria of provitamin activity and from their results concluded that the β-ionone ring structure (fig.1B) and the unsaturated polyene chain (fig.1C) were of major importance in this respect.

That the unsaturated, unoxidised, optically inactive ring structure was of importance was demonstrated by Karrer (54) when he showed that only those carotenoids having an ionone ring possessing these characteristics were converted to vitamin A. Thus β-carotene with two such rings (fig.2A)

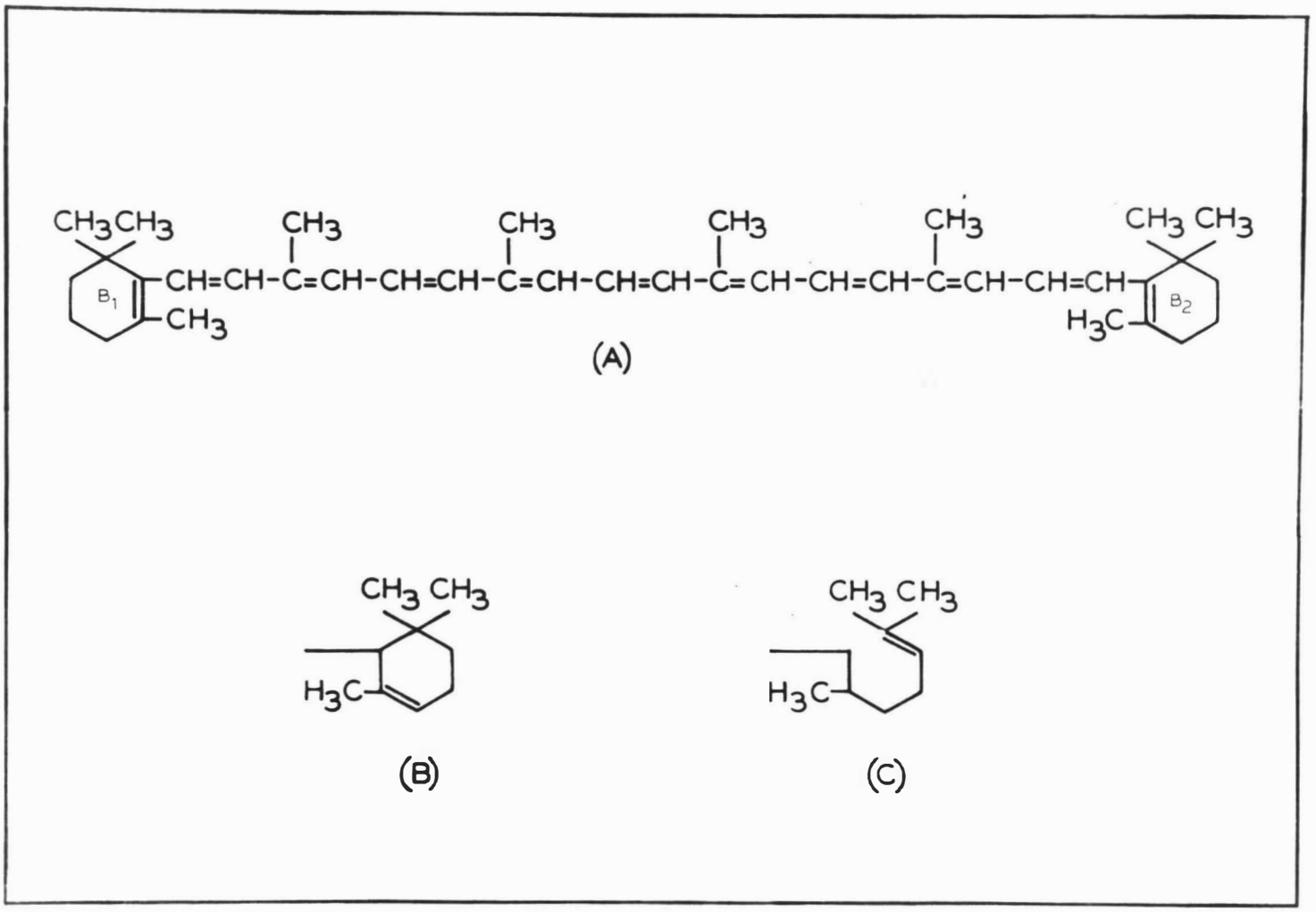

FIGURE 2.

(A) Structural formula, beta-carotene.
(B) B_2 ring structure of alpha-carotene.
(C) B_2 ring structure of gamma-carotene.

theoretically may be converted into two molecules of vitamin A, whereas alpha-carotene and gamma-carotene and cryptoxanthin (the only other carotenoid of importance exhibiting provitamin activity) can yield a maximum of only one molecule of vitamin A because they possess only one such ring. In practice a perfect 2:1 ratio is not achieved as the presence of a different type of ring structure in the B_2 position affects other properties such as absorption and stability. That a certain amount of unsaturation in the aliphatic side chain was required for provitamin activity was proved when Karrer et al (55) demonstrated β-dihydrocarotene and diiodocarotene to be active but perhydro-β-carotene to be inactive. Further, hydrogenation of fats and oils containing vitamin A or its precursors was shown to destroy vitamin activity due to saturation of the double bonds in the sidechain.

By 1935 the chemical basis of conversion of carotene to vitamin A was firmly established. Many other problems, however, remained to be solved. In the following section the more important of these will be discussed together with some of the miscellaneous aspects of vitamin A metabolism.

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VITAMIN A IN THE ANIMAL BODY.

The site of conversion is a

subject in which a great deal of interest has been shown, especially in recent years. The matter was first discussed by Moore (56) who concluded that:

> "A most consistent feature of the above results is the persistence of carotene, apparently unchanged, within the alimentary tracts of those animals which had received diets rich in carotene up to the time of killing. It is obvious, therefore, that conversion to the vitamin is not affected 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".

Since the work of Moore many other workers have attempted to confirm the liver as the main locus of transformation. In vitro experiments, in which minced liver tissue or extracts were incubated with colloidal carotene, produced conflicting results. Thus Ahmad (57), Olcott and McCann (58) and Euler and Klussmann (59) obtained evidence for the formation of vitamin A by the presence of a typical absorption band at 328mμ or by a positive Carr-Price colour reaction. On the other hand, Rea and Drummond (60), and later, Euler (61) and Ahmad (62) were unable to confirm these results. Furthermore, Drummond and MacWalter (63) could not obtain vitamin A from carotene which was actually taken up by the liver cells prior to mincing and incubating. In critically discussing these results, Woolf and Moore (64) pointed out the

the uncertainty of detecting vitamin A in the small amounts in which it was claimed to have been produced, and Glover, Goodwin and Morton (65) noted the difficulty of distinguishing the 335mμ peak of carotene cis-isomerides from that of vitamin A at 328mμ when other absorbing substances were present. For these reasons there appeared little justification in furthering the in vitro technique and workers turned their attention to studies on the living animal.

Most of the early in vivo work aimed at producing experimental proof of the value of carotene when given parenterally, since demonstration of its activity under such conditions would go far in support of the hepatic theory of conversion. Using the intravenous route Wolff, Overhof and van Eekelen (66) and Ahmad, Grewal and Malik (67) noted an increase in the liver vitamin A levels of rabbits after injection of colloidal carotene suspended in isotonic glucose; Ahmad et al could not, however, repeat these observations using rats and dogs. Similar experiments by Rea and Drummond (60), Drummond, Gilding and MacWalter (68) and Drummond and MacWalter (69) were also negative. Using the intramuscular route Chu and Coady (70), With and Wanscher (71) and Tomarelli, Charney and Bernhart (72) showed carotene to be effective in causing resumption of normal growth in depleted rats. Rokhlina, Balakhovski and Bardrova however, could not confirm these results and concluded parenterally administered carotene to be ineffective as a source of vitamin A (73).

In view of this mass of contradictory evidence the matter remained unsettled until 1946 when a thorough re-examination of the problem was undertaken by Sexton, Mehl and Deuel (74) working at California. In a series of carefully controlled experiments they proved conclusively that carotene, administered parenterally to depleted rats, was not converted to vitamin A but accumulated in the liver without relieving symptoms of deficiency. In discussing these results they concluded:

> "The possibility is suggested that the conversion of carotene to vitamin A may be an extra-hepatic function in the rat. The wall of the intestine is suggested as a possible site of such transformation".

Although this was the first firm suggestion that the intestine was the site of conversion the possibility had been noted previously by Verzar and McDougall (75) in man, Wagner and Vermeulen (76) in whales, and Popper (77) and Popper and Greenberg (78) in the rat. Using the technique of fluorescence microscopy the latter workers examined the various organs of a series of vitamin A depleted rats at intervals after the administration of carotene, orally and parenterally, in an attempt to find the organ in which fluorescence first appeared. Following parenteral administration they were never able to detect fluorescence due to vitamin A in the liver; following oral administration, however, fluorescence normally appeared first in the liver but was also occasionally noted first in the

intestine. From the observations they concluded that:

> "The first fluorescence after feeding of carotene was seen in the intestine or in the Kupffer cells and then in the adjacent parts of the liver cells or in the endothelial cells of the renal cortex...Probably carotene is converted into vitamin A in one or all of these locations".

Other evidence also pointed to the intestine as the site of conversion. In animals such as the sheep, goat, rat and pig the almost complete absence of blood carotenoids constituted a serious objection to the hepatic theory, since it failed to explain how in such species carotene taken up by the intestine could reach the liver. The possibility that it was transported via the portal circulation was investigated by Goodwin, Dewar and Gregory (79) and Goodwin and Gregory (80) working at Liverpool. In a series of experiments involving rabbits, sheep and goats they consistently failed to detect carotene in the systemic or portal blood following massive dosing with carotene, yet vitamin A similarly fed was shown to appear in the blood in large quantities. From these results they concluded that the older views on the transformation of carotene into vitamin A should be abandoned in favour of the view that the conversion took place in the intestinal wall. In later experiments on goats provided with thoracic cannulae they confirmed this conclusion by showing the vitamin A level of the lymph draining the gut

to be closely correlated with carotene intake of their experimental animals.

Additional evidence which directed attention to the intestine as the site of conversion was derived from the experiments of Ball, Glover, Goodwin and Morton (81) and Glover, Goodwin and Morton (82) showing the conversion of retinene$_1$ (vitamin A$_1$ aldehyde) to vitamin A in the rat. After oral administration conversion was shown to take place in the gut and after subcutaneous injection in the subcutaneous tissues. From these results it was suggested that:

> "The transformation of β-carotene into vitamin A$_1$ *in vivo* is more likely achieved by the oxidation of the former to retinene$_1$, which is then rapidly reduced to vitamin A, rather than by hydrolytic fission".

This work, which has since been confirmed (83), was noteworthy for two reasons. In the first place it gave an insight into the actual mechanism of conversion and, in the second, it formed a basis for future attempts to confirm the intestine as the site of conversion.

In 1947 further evidence supporting the intestinal theory of conversion was presented independently and almost simultaneously by Glover, Goodwin and Morton (84,65) at Liverpool, Wiese, Mehl and Deuel (85) and Mattson, Mehl and Deuel (86,87) at California, and Thompson, Ganguly and Kon (88,89)

at Reading. In each case this evidence was based on the recovery and identification of vitamin A from depleted animals fed a supplement of carotene under conditions making it clear that it originated from the small intestine.

The Liverpool workers for example, in a series of nine experiments followed vitamin A levels in the intestinal mucosa of depleted rats at regular intervals after dosing with β-carotene in oil. Their results showed that during the period of maximal absorption (6–8 hours after dosing), both carotene and vitamin A could be extracted from the gut wall, the latter in such quantities as to prove beyond all doubt its release at that site. The possibility that some conversion took place prior to absorption was also investigated. Such a possibility was, however, discounted by Glover et al when they consistently failed to detect even traces of vitamin A in the stomach and intestinal contents of rats actively absorbing carotene.

The approach of the Californian team was in many respects similar to that of the Liverpool workers. Using depleted rats as test animals they showed that, following oral administration of carotene, vitamin A appeared first in the intestinal wall and that for a period of at least four hours after dosing the levels present exceeded those in the liver. Furthermore, during the process of carotene absorption, no increase in the provitamin content of the liver could be demonstrated whereas that of the intestinal wall was

shown to increased markedly. The possibility that vitamin A present in the wall was derived from the liver was also investigated but was discounted when it was shown that following vitamin supplementation that intestine contained little vitamin while hepatic stores were high. Although these results left little room for doubting the intestine as the site of conversion a further series of experiments were undertaken in an attempt to demonstrate the conversion in vitro. A surviving-tissue technique was used in which the intestines of depleted rats were removed and incubated with colloidal carotene under anaerobic conditions in Ringer-Locke solution. Following incubation the presence of vitamin A was demonstrated in the intestinal mucosa in amounts sufficient to justify the assumption that:

> "In rats, and presumably in other species also, the wall of the intestine is the main site of conversion of carotene to vitamin A".

In their preliminary experiments, also in rats, the Reading group confirmed these conclusions and proceeded to investigate the site of conversion in the pig. Large White baconers were dosed with 600mg. of carotene in arachis oil and at slaughter (3-7 hours after dosing) samples of blood plasma, mesenteric lymph, and small intestine were taken for analysis. The results showed in every case that the vitamin A content of the samples taken from dosed animals was higher than similar samples from controls. In later experiments London

cannulae were established in the portal vein and mesenteric lymph duct. Following a meal of carotene no more than traces of pigment could be detected in the lymph or portal and systemic bloods whereas the content of vitamin A ester showed a marked increase. An increase was also noted in the alcohol some two hours later. When vitamin A ester was fed in place of carotene similar changes occurred, indicating the pattern of absorption and distribution to be identical irrespective of whether the vitamin was fed preformed or as carotene. On the basis of these results it was concluded that in the pig, as in the rat, the intestine was the main site of conversion of carotene to vitamin A.

Following publication of the work discussed above, numerous other reports appeared confirming the intestine as the site of conversion in the rat and pig (90,91,92). A further valuable contribution was made by Krause and Pierce (93) in which they demonstrated the intestinal conversion of carotene to vitamin A in rats in which the liver was tied off at the portal vein. The site of conversion in other species was also investigated and in chicks (90a,[redacted],94), sheep ([redacted],[redacted],95), and dairy cattle (96,97,98) results supporting the intestinal theory of conversion were obtained.

At present, the possibility of a secondary site of conversion is being actively investigated by groups at Texas and Reading.

Attention was first directed to this question in 1946 by Tomarelli, Charney and Bernhart (72) of the University of Pennsylvania who showed that parenteral carotene could serve as an effective source of vitamin A provided it was solubilized in water with a surface-active agent. This observation was subsequently confirmed by Eaton et al (99) at Connecticut, who demonstrated an increase in the plasma vitamin A levels of depleted calves following intravenous injection of an equeous suspension of carotene. Using a similar technique, a Belgian team under Kowalewski reported a similar increase in the plasma and liver vitamin A levels of dogs (100). At Texas, Bieri and Schultze (101) studied the availability of solubilized carotene injected intramuscularly into vitamin A-depleted rats and were able to demonstrate only small amounts of vitamin A in the serum and kidneys. Traces of carotene but no vitamin A could be detected in the liver and from these results it was concluded that the utilization of parenteral carotene was relatively inefficient. In a later experiment Bieri and Sandman (102) confirmed this point when they showed that for maximum growth young rats required almost five times as much carotene intramuscularly as orally. In a subsequent paper (103), Bieri and Pollard have demonstrated that under some conditions, carotene administered intravenously is as efficiently utilized as when given orally. Using vitamin A-depleted rats they noted that aqueous dispersions of carotene produced a similar growth response when adminstered by either route. When alpha-

tocopherol was incorporated in the supplement, however, the oral route was slightly more effective. In moderately or severly deficient rats, low doses of carotene with or without tocopherol were considerably less effective intravenously than orally, but a larger dose with tocopherol was utilized about equally by the two routes of administration. When repeated doses of carotene were given orally or intravenously to deficient rats, the resulting amounts of vitamin A in the serum, liver and kidneys were observed to be approximately similar.

Subsequently, attempts were made by Bieri and his colleagues to locate the site at which parenterally administered carotene was converted. The possibility that it was excreted into the intestine, reabsorbed, and converted in the intestinal wall was ruled out when it was shown, following removal of the small intestine, that conversion of the circulating carotene occurred essentially undiminished. Similarly, removal of the kidneys could not be shown to have any significant effect on conversion. In experiments in which varying proportions of the liver were removed before injection, no direct relationship could be established between the amount of tissue remaining and the amount of vitamin A formed, and even when the liver was reduced to one-fourth its normal size formation of vitamin A still occurred at a rapid rate. Although the ability of the liver to convert carotene was not directly discounted by these

observations it was concluded that:

> "If the liver is the site of conversion.....appreciable formation of vitamin A can occur even when the amount of liver tissue is considerably reduced".

Thus it is quite conceivable that many tissues may possess the ability to convert injected carotene; furthermore, the possibility must not be overlooked that the formation of vitamin A from carotene may not be a specific reaction, but in the breakdown and detoxication of carotene vitamin A, or a vitamin A-active grouping, might just be one of the many endproducts.

The more recent observations of the Reading group (104) confirm many of the points discussed above. Working with depleted rats, and later normal rabbits, they noted that carotene, which had been solubilized with the surface active agent "Tween 40" and injected intravenously, was converted to vitamin A, the appearance of the vitamin in the blood in the alcohol form as distinct from the ester being most significant. It is noteworthy that only Tween solubilized carotene was effective, as no vitamin A could be detected following the injection of oily or colloidal carotene. The results of similar experiments on calves were in sharp contrast to those on rats and rabbits and to those of Eaton et al mentioned above. In no case could the conversion of solubilized carotene be demonstrated

following injection, the carotene being rapidly metabolized and apparently destroyed, Unfortunately, the importance which can be attached to these observations (and to those of the Texas team) is limited by the unphysiological nature of the Tweens. Even in quite low concentration these agents cause various pathological changes in the tissues and blood which are often fatal. Furthermore, the possibility that the uptake and utilization of vitamin A might be affected must also be considered since the Reading group have shown that vitamin A, solubilized in Tween and injected in the same way as carotene, cannot be utilized by deficient animals. Despite these objections, there would appear to be sufficient evidence from the Reading and Texas work to justify the assumption that between those species which normally circulate carotene in their blood and those which do not, marked differences in carotene metabolism occur. It seems reasonable to assume that carotene is present in the former because they do not possess a secondary site for its conversion, whereas in the latter a secondary site is present and carotene is transformed immediately, following its uptake from the intestine. Although there is, as yet, no evidence as to the location of the secondary site of conversion, the work to date makes it clear that it is almost certainly not located in any one particular organ. One may, therefore, agree with Bieri and Pollard (103) that:

> "The possibility must be considered that perhaps many tissues have the ability to convert carotene".

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FUNCTIONS OF VITAMIN A AND SYMPTOMS OF ITS DEFICIENCY IN THE ANIMAL BODY.

Function and deficiency are two aspects of the same thing and for this reason, and for the sake of convenience, they will be considered together in this section.

(a) Functions of Vitamin A.

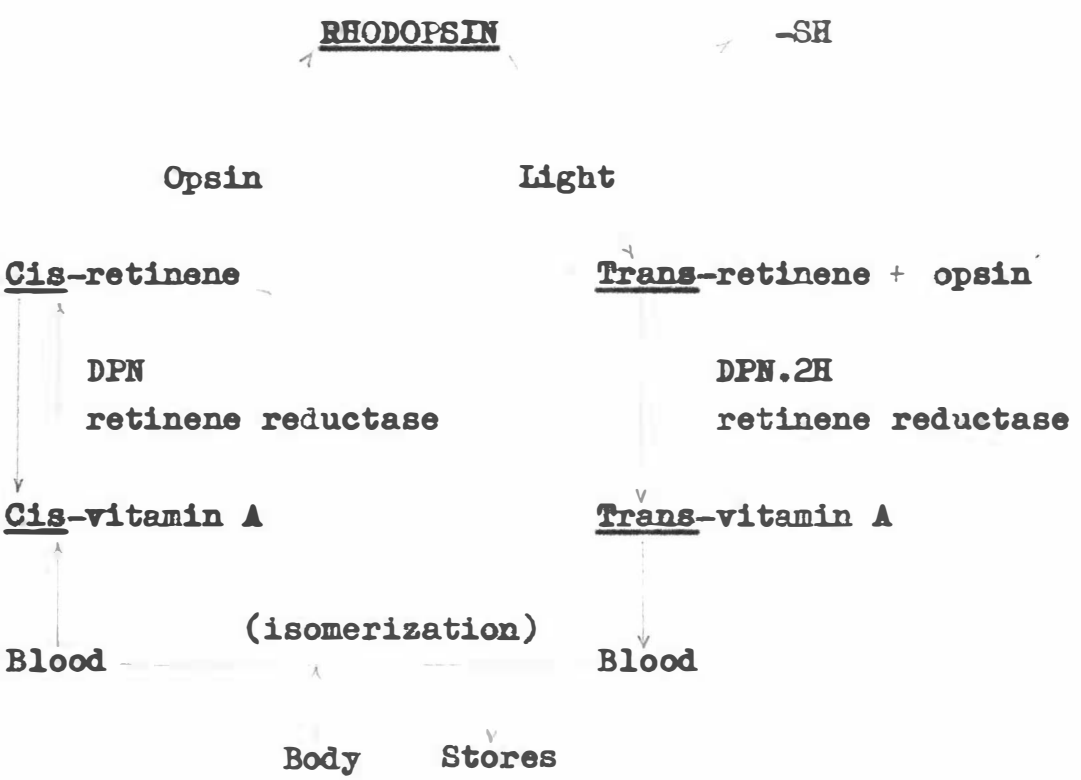
Although it is now over 40 years since the discovery of vitamin A, little is known of its mode of action or of its fundamental role in the physiology of the animal body.

Mention has already been made of its growth-promoting activity but it is not yet clear just how this effect is brought about. There is no evidence that this or any other vitamin actually accelerates the metabolic processes or modifies their efficiency and it is probably that the growth-promoting effect of vitamin A is exerted indirectly through its ability to maintain the integrity of the epithelial tissues (105). The further possibility that vitamin A may be concerned in oxidation-reduction reactions must also be considered. Although there is no direct evidence to support this hypothesis, the unsaturated nature of the polyene side-chain would suggest the vitamin to be both a hydrogen acceptor and an oxidation promoter, the latter especially when actively absorbing oxygen.

The central role of vitamin A in the photochemical processe associated with vision is one aspect of vitamin A metabolism which is particularly well understood. It is generally believed that there are two visual mechanisms, the first operating in dim light and mediated by the retinal rods, and the second in bright light, by the retinal cones. Each of these organs contains a photosensitive pigment which bleaches on exposure to light. This transformation leads to nervous excitation which, transmitted from neurone to neurone along the optic pathways to the brain, ends in producing visual sensations.

Three photosensitive pigments are known; (i) rhodopsin, which is contained in the retinal rods of all higher animals and marine vertebrates and is responsible for vision in dim light; (ii) porphyropsin, which is the counterpart of rhodosin in the retinal rods of freshwater fish; and, (iii) iodopsin, which is present in the retinal cones of certain of the higher animals, and is responsible for vision in bright light. All three pigments are known to be proteins, containing vitamin A prosthetic groups to which they owe their colour and sensitivity to light. Since a detailed consideration of each of these pigments and of the visual mechanisms with which they are associated is obviously beyond the scope of this review, the following discussion of the relation of vitamin A to the visual system in man and higher animals, will be limited to the rhodopsin cycle.

The simple cycle which was first proposed by Wald (106) in 1937, has undergone substantial elaboration and may now be summarized as follows:



(After Baumann,107).

On illumination with white light, rhodopsin is bleached to all-trans retinene (vitamin A aldehyde) which may be further reduced in the rod elements to all-trans vitamin A. In the initial stages of the bleaching process, free sulfhydryl groups are liberated (two for every molecule of retinene appearing), and it is thought

that it is by this means that electrical energy is released from the rods to the nervous system (108). In the normal individual the regeneration of rhodopsin occurs spontaneously providing opsin, cozymase, retinene reductase, and the correct stereoisomer of vitamin A are all present (109,110). In the deficient individual, however, this process is delayed because the amount of vitamin A reaching the retina from the bloodstream is insufficient to replace that which diffuses out during the operation of the cycle. As the rhodopsin level in the retinal rods decreases, dark adaption is impaired, and night blindness results. Night blindness is thus a manifestation of vitamin A deficiency, evidenced by poor ability to regenerate rhodopsin, and in the clinical diagnosis of vitamin A use is made of this relationship in the so-called "dark adaption" test.

The porphyrin cycle in fresh-water vertebrates is essentially similar to that described above, the only difference being that in the bleaching of porphyrin, a retinene related to vitamin A_2 is produced. However, the reactions which connect vitamin A_2, retinene$_2$, and porphyrin appear to be exact counterparts of those of the rhodopsin system (111).

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(b) <u>Symptoms of deficiency</u>.

A variety of symptoms follows

the depletion of vitamin A in the animal organism. They have been conveniently summarized by Harris (112), in their approximate order of appea ance, as follows:

TABLE 1.

Effects of Vitamin A Deficiency in Experimental Animals.

1. Night blindness (1,3).
2. Failure of growth (113).
3. Metapla ia (xerosis) of epithelial tissues (114.115).
4. Multiple secondary infections (116).
5. Xerophthalmia and keratomalacia (3,117).
6. Renal calculi (117a).
7. Periodontal hyperplasia (118).
8. Reproductive failure (119).
9. Degenerative changes in central or peripheral nervous system (120).
10. Bony overgrowth (121).

It is not intended to discuss these symptoms in detail in this section. The subject of vitamin A deficiency is considered to be somewhat beyond the scope of this thesis and has been dealt with adequately elsewhere (115,122, 123,124,125). Furthermore, the literature relating to it is now so vast that it does not lend itself easily to brief summarization. The above table has been included mainly for reference purposes, and in this capacity serves also as a convenient introduction to the subject of vitamin A requirements.

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VITAMIN A REQUIREMENTS.

The following daily allowances of vitamin A, for the different classes of stock listed, have been recommended by the Food and Nutrition Board of the National Research Council (126).

TABLE 2.

Recommended Daily Vitamin A or Provitamin A Allowances in the Diet.

	Growth.	Pregnancy.	Maintenance.
Humans	3500 I.U.***	6000 I.U.	5000 I.U.
Dairy Cattle	25mg.**	90mg.*	60mg.*
Beef Cattle	25mg.**	55mg.*	55mg.*
Swine	4mg.π	20mg.π	20mg.π
Sheep	3.3mg.τ	8mg.$\pi\pi$	8.2mg.$\pi\pi$
Turkeys	2500 I.U."	4000 I.U."	
Chickens	1800 I.U."	3300 I.U."	

***For a 55lb. child.
** For a 400lb. animal.
* For a 1000lb. animal.
π For a 100 lb animal.
τ For a 60 lb. animal.
$\pi\pi$ For a 150lb. animal.
" Units of either vitamin A or carotene/lb.feed.

The values for farm animals are given in terms of carotene alone.

(After Gortner,105).

The term "allowance" has been used by the board in its report (126) in preference to the term "requirements", since the latter term

implies the actual amount needed to promote normal health, and does not allow for a margin of safety to provide for variations in availability, conversion, storage and utilization of vitamin A and carotenoids between individuals.

A "recommended allowance" is intended to provide an intake of vitamin A which is sufficient to ensure adequate nutrition for all normal individuals under practical conditions. It is estimated by multiplying the "average minimum requirement" by a factor of five or ten (127). An average minimum requirement is the amount of vitamin A necessary to just prevent the onset of obvious symptoms of deficiency; it is determined experimentally by the prophylactic technique using either night blindness or failure of growth, i.e., early appearing symptoms (table 1.), as the criterion of response. In Cattle, sheep and swine, the average daily minimum requirement of vitamin A and carotene has been shown by Hart and his co-workers at California, to lie between 6-8µg/kilo L.W. and 25-30µg/kilo L.W. respectively (127,128,129,130). Considerably higher values than these have been reported for chickens and turkeys (131).

In practice, minimum levels are increased by at least five or ten times, for a number of reasons. Firstly large variations have been shown to exist between individuals in their ability to utilize vitamin A and carotene from similar sources (122). Secondly, the amount of vitamin A just necessary to prevent overt symptoms

of deficiency has been shown to be less than that required to maintain apparently normal health (131); furthermore, this quantity is probably again less than the amount necessary for "buoyant", "positive", or "optimum" health (132). Thirdly, Sherman and his colleagues (133,134) have demonstrated how much greater must be the intake of vitamin A if reserve stores are to accumulate in the liver than if normal increases of body weight are regarded as criteria of adequate vitamin A intake. Finally, vitamin A concentrates are freely available and comparatively inexpensive, so that from the cost angle there is little justification for not providing a liberal allowance in human and animal diets.

Little information is available regarding the intake of carotene by free-grazing species in New Zealand, However, Cawley (135) has shown that the average carotene content of typical New Zealand pastures varies from 550µg/g. dry matter ($\pm$ 100) during Autumn, Winter and Spring, to approximately 200µg./g. dry matter during Summer. Assuming an intake of 10kg. dry matter daily for cows on pasture, this would give a daily carotene intake of at least 2g., or nearly 80 times the recommended daily allowance. Similarly for sheep, assuming an intake of 1.5kg. dry matter daily, the carotene intake would be at least 0.3g. or nearly 100 times the recommended daily allowance. The digestibility of carotene from New Zealand pastures has not been reported, but it is probable that under normal conditions, it lies somewhere within the

range of 1-3% reported by overseas investigators (136). In any event there would appear to be little reason for suspecting that the carotene intake of free-grazing species in New Zealand is other than adequate.

In so far as human requirements are concerned no information is available as to how these are met by the average New Zealand diet. Dairy products, however, are known to make a substantial contribution. For example, the average daily intake of whole milk and butter can be shown to contribute 64.8% of the daily recommended allowance (appendix 1). Also cheese, cream, ice-cream and processed milk make a further small but significant contribution (136a). Similarly in Australia, Sweden, Canada and the United States of America, whole milk and butter can be shown to account for 44.8, 40.5, 32.6, and 25.0 percent, respectively, of the daily recommended allowance. Even in the United Kingdom during rationing, whole milk and butter supplied some 23.2% of the vitamin A requirements of the nation. Unfortunately there is no means of checking how the balance of requirements is made up but there can be little doubt that it is adequately supplied in eggs and green vegetables, and in other miscellaneous foods. The figures quoted above and in appendix 1 emphasise the importance of dairy products, and in particular butter, as sources of vitamin A in the human diet and in countries where the intake of these commodities is high this contention is borne out by the virtual absence of deficiency symptoms in the population.

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THE ESTIMATION OF VITAMIN A AND ITS PRECURSORS.

Vitamin A and its precursors may be estimated biologically, by means of feeding trials; chemically, by colour reactions; and physically, by absorption spectra estimations.

(a) The Biological Method:

Since vitamin A was recognised as such long before its chemical structure and properties were known, biological assays became firmly established amongst the early workers and the International Unit (I.U.) was defined in terms of these assays. Thus in 1934, the Permanent Commission on Biological Standardization of the League of Nations defined the International Unit of vitamin A as the biological activity of 0.6µg of standard β-carotene.

Essentially the method entails a comparison of the biological responses of two groups of rats (or other test animals), matched as to sex, age, weight and litter, one group being fed the substance under investigation and the other a standard vitamin A preparation.

Biological response may be measured by the prophylactic method or the curative method; that is, the potency of the substance being tested is estimated either by its capacity to prevent the onset of symptoms of vitamin A

deficiency, or to cure the symptoms once they are established. The curative method is generally considered to be the more satisfactory although it has been criticised on the grounds, firstly, that the ill-health of animals suffering deficiency symptoms causes large variations in their response to vitamin A treatment; secondly, that the depletion period necessary to produce deficiency symptoms increases the length of the feeding trial unnecessarily; and thirdly, that the subjective estimation of two "end-points" in the curative method introduces a larger error than in the prophylactic method where only one such "end-point" need be estimated.

In countering these criticisms Coward (137) has shown that depleted animals show no greater variation than do normal animals in their response to vitamin A; moreover, she has pointed out that in the prophylactic method, large individual variations in liver stores at the beginning of the test, may introduce far greater complications which can be overcome only by the use of extremely large numbers of animals.

In the curative method various criteria have been adopted to measure response:-

(i) Change in Weight or Growth - The chief objection to the use of growth as a criterion of biological response is that it is not, in itself, a specific effect of vitamin A deficiency (even though vitamin A may have a specific effect on growth), since other factors such as the "B-complex" vitamins, protein quality, etc. may also have a

profound effect on growth. The advances of nutritional knowledge have, however, largely overcome this objection and test diets can now be made "nutritionally adequate in all other respects". Change in weight, or growth, has the advantage that it is easy to measure and is objective. Thus in practice it finds wide application.

(ii) Xerophthalmia - A specific effect of vitamin A deficiency, is a useful criterion and one which is commonly used. It suffers from the disadvantage, however, that recognition of the appearance of symptoms and their subsequent disappearance is subjective and therefore variable depending on the standards adopted by individual workers.

(iii) Changes in Vaginal Epithelium - Remarks as for (ii) above; the method suffers, however, from a further disadvantage that the effects of oestrus often cannot be separated and distinguished from those of vitamin A deficiency. This may be overcome by spaying but since this is a rather delicate operation the method does not find general acceptance.

(iv) Degeneration of Medullary Nervous Tracts - In 1940, Irving and Richards (138) noted that very small differences in intake of vitamin A were required to protect against or induce medullary degeneration, and on the basis of this observation they designed an extremely critical bioassay for the vitamin. The technique is an histological one and is also subjective in nature and for these reasons it has not been found general favour.

All biological assays are

relative measures and are subject to considerable variation. Results vary, for example, between different laboratories, between colonies, and between individual workers. In addition many factors may affect the absorption, utilization and stability of both vitamin and provitamin. The sparing action of tocopherol, for example, in protecting them against destruction in the gastro-intestinal tract and bloodstream has been adequately demonstrated (139,140,141). Large differences in the availability of carotene from different sources have also been noted (142,143). In addition there is a well known "level of feeding" effect; small amounts being well utilized, but as the level increases, the percentage absorbed decreases until at high levels of intake only one to two percent of the carotene ingested is converted to vitamin A. Thus a substance with a certain relative bioassay value at the very low levels of intake used in say the growth method, will not always have the same value at higher levels of feeding; in fact it will invariably be lower. To overcome this difficulty the Liver Storage Method has been introduced. Using this method it is possible to measure "availability" at any level and under any conditions. Therefore, by working at higher, more normal, levels of intake a truer bioassay value can be obtained.

It is not intended to discuss in detail the methods underlying each of the bioassays mentioned above. Since in practice the rat-growth bioassay is the only one which has

found general acceptance it will now be considered more fully:

Rat-growth Bioassay: The method.

Young rats used for the estimation are first given a deficient diet until they cease growing or lose weight. This is called the depletion period and it may be reduced by feeding suckling mothers a deficient diet. The ideal is to have young rats stop growing or "plateau" at between 70–90g. At this stage the depleted specimens are divided equally into two groups with respect to age, sex, weight and litter. Subsequently, one group is fed the test diet under investigation and the other a standard diet. By comparing the weight gains of the group on the test diet with those of the group on the standard diet the potency of the substance under investigation can be estimated.

Interpretation: (After Coward, 137).

Mean gain of 6 rats on 0.5mg C.L.O. =2g in 3 wks.
" " " " " " 1.0mg " =10g" "
" " " " " " 2.0mg " =20g" "
" " " " " " 0.5mg Std. =7g " "
" " " " " " 1.0mg std. =12g " "
" " " " " " 2.0mg std. =28g" "

Let 1 unit of standard preparation
= 1000 I.U. (diluted x 1000).

Now, 0.5unit std. prepn. > 0.5mg. C.L.O.
1.0 " " " > 1.0mg. C. L.O.
2.0 " " " > 2.0mg. C.L.O.
∴ potency of C.L.O. < 1000 I.U./g.
1.0mg C.L.O. > 0.5unit std. prepn.
and ∴ > 500 I.U./g.
and 2.0mg C.L.O. > 1.0unit std. prepn.
∴ potency of C.L.O. is approx. 800 I.U./g.

From this example it will be seen that when test substance and standard are compared at only

three levels a very rough approximation of the potency of the test substance only can be made. When a more critical estimate is required it is important, therefore, to compare the test substance and the standard at a sufficient number of levels to provide data of satisfactory accuracy.

To overcome the necessity of always using a "standard" group, Coward (137) has drawn up a standard curve of rat growth (fig.3) in which average weight increment is plotted against a standard dose of cod liver oil (mg.daily), and a regression:

$$Y = 6.13 + 29.71(\text{Log } X)$$

fitted. Under normal conditions therefore, given weight gain (on basal diet plus a known amount of test substance), the potency of the test substance can be calculated approximately from this regression. It is of interest to note that the curve which Coward has obtained is curvilinear and that doubling the intake of cod liver oil (i.e. intake of vitamin A) does not necessarily double growth rate (as measured by weight increase/3 weeks), except at low levels:

E.g. 1.5mg. C.L.O. results in 9.8g weight increase.
3.0mg. " " "20.0g " "
4.0mg " " "23.0g " "
8.0mg " " "31.0g " "

Conversly, while 2.0mg C.L.O. results in 15g increase/group/3 weeks, it takes 7.5mg C.L.O. to double this increase to 30g.

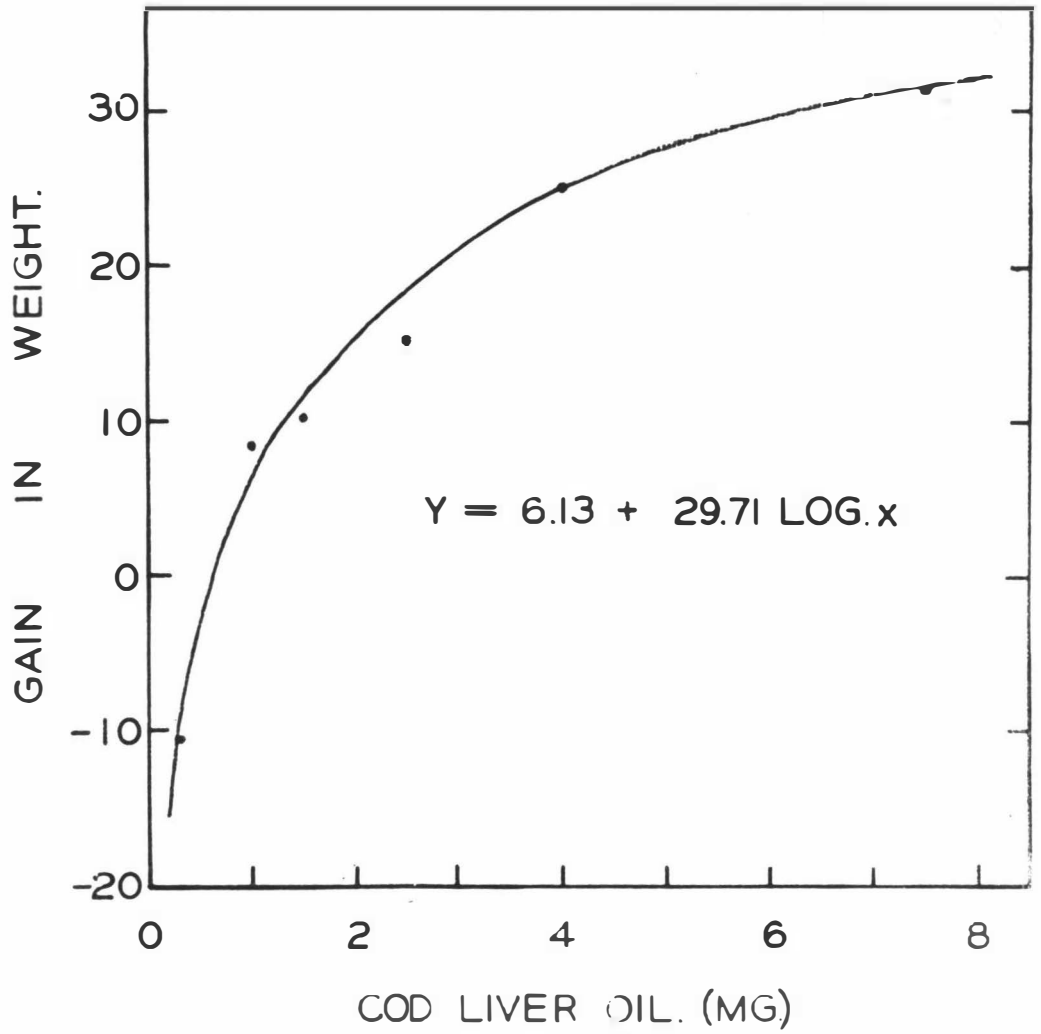

FIGURE 3.

Growth of Rats on Varying Daily Intakes of Cod Liver Oil. (After Coward, 137).

In her book Coward points out the importance of insuring that the basal diet used in all trials is identical, and that it is nutritionally adequate in respect to all dietary essentials, save vitamin A. The importance of removing all traces of vitamin A and carotene from the basal diet is also emphasised.

It should be noted that the curative rat-growth assay gives an estimate of the biological availability of certain active chemical groups. It does not, however, give any indication of the distribution of activity between these groups, and is therefore unsuitable for work requiring any fine degree of discrimination. Thus in a diet containing a mixture of vitamin A and isomeric carotenes, it is possible to measure the overall potency of the mixture but not the contribution of each to the growth-promoting activity.

Biological methods in general are tedious, time-consuming, and expensive, and require skillful attention throughout their 6--8 week duration and special care in calculations and interpretation of data. For these reasons they are not adaptable to routine assays, but must be reserved for occasional check runs and for standardization of the more rapid and precise physico-chemical methods.

.....

(b) Physical and Chemical Methods:

These methods depend on the measurement of a characteristic property of the vitamin, e.g. absorption of visible light by carotene and ultra violet light by vitamin A, or on the measurement of colour formation following the interaction of vitamin A with some other substance, e.g. antimony trichloride. The former methods, which involve a direct reading of light absorption, are usually referred to as physical methods, while the latter, which involve a prior chemical reaction, are usually referred to as chemical methods; the distinction, however, is not rigid.

Both methods presuppose:-

(i) The use of an instrument capable of measuring transmission at various wave lengths such as the Beckman Spectrophotometer.

(ii) The presence of a pure source of the vitamin, as a standard.

(iii) The removal of interfering materials, and substances inhibiting colour development, especially when working at short wave lengths.

Physical methods are based on the measurement of light absorption of vitamin A or carotene in solution. Measurement is made at $\lambda_{max.}$ (e.g. 325mμ for vitamin A alcohol, and 450mμ for β-carotene), since at this wavelength Beer's Law will be obeyed and the relative effect of interfering substances will be reduced to a

minimum. Absorption spectrum estimations have been found to be reliable, giving results in harmony with biological estimations (144), so long as the estimation is preceded by saponification if the test substance contains less than 10,000 I.U./g. of vitamin A. By assaying the unsaponifiable fraction of such substances, irrelevant absorption due to interfering impurities is greatly reduced. Interfering substances may also be "diluted out", or for some samples, the specificity of the spectrophotometric procedure may be increased by the application of a blank reading obtained by destructive irradiation of vitamin A in solution (144). This latter technique is open to the criticism that substances other than vitamin A may be destroyed in the process, giving rise to a series of absorbing by-products resulting in a blank reading which is too high. Chromatography may also be used, either for purification of vitamin A in solution, for preparation of the vitamin A-free blank, or for the separation and purification of the isomeric carotenes.

Morton and Stubbs (145,145a) have introduced a geometrical correction proceedure to allow for interfering materials in which the absorption curve as a whole is considered,(Fig. 4). It is based on the assumption that absorption by interfering material follows a regular mathematical shape with wavelength, which is a reasonable assumption if the curve is well away from its maximum. By taking readings at a series of wavelengths, the absorption at each can be shown to be

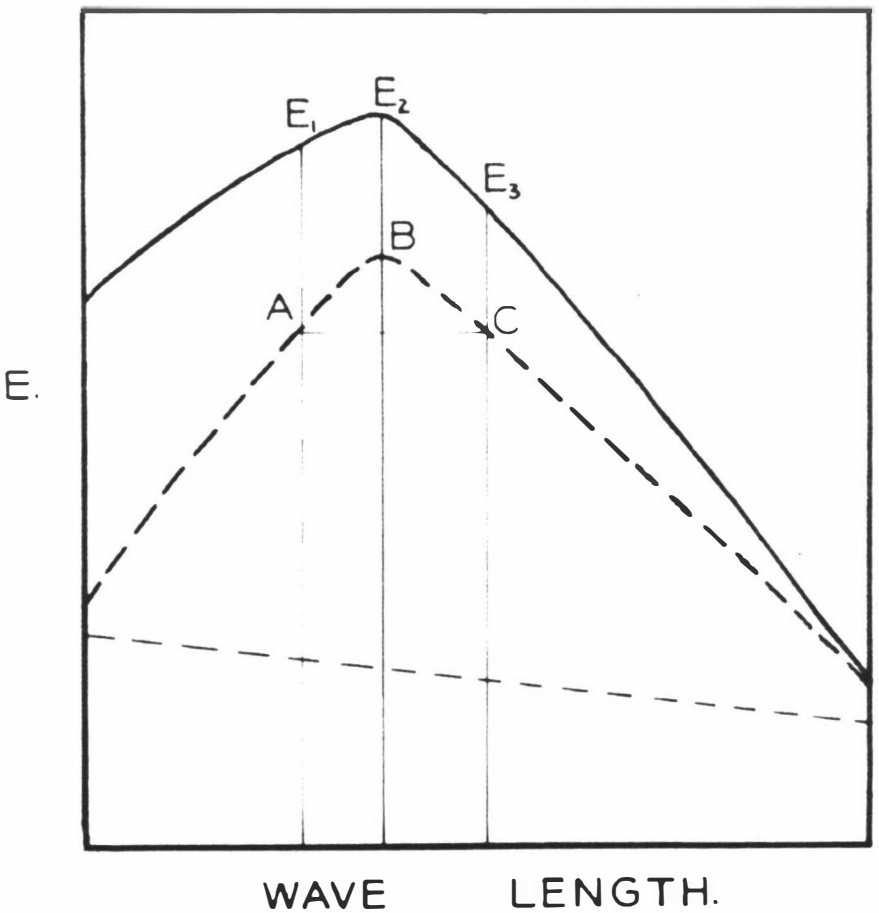

FIGURE 4.

Morton and Stubbs' correction procedure for irrelevent absorption is based on (a) accurate measurements on the pure substance under study at three wave-lengths λ_1, λ_2 and λ_3 not very far apart and (b) similar measurements on the sample at the same wave-lengths. The assumption is made that the irrelevant absorption is linear over the narrow wave-lengths chosen (broken straight line). Geometrical considerations lead to the formula:

$$E \text{ (corrected)} = AE_1 - BE_2 - CE_3$$

Where E_1, E_2 and E_3 are the readings at λ_1, λ_2 and λ_3 and A, B and C are constants calculated from the curve of the pure substance (broken curve).

partly due to pure vitamin A (and the curve for pure vitamin A is known), and partly due to interfering materials (which are assumed to follow a regular shape.) Thus for a sufficient number of readings, the contribution of vitamin A at 325mμ or carotene at 450mμ, can be calculated. If the interference is assumed linear, which is a reasonable assumption over a restricted wavelength, three readings (or "fix-points") only are required to enable absorption due to true vitamin A to be calculated (146). By subtracting the corrected absorption from the gross, the irrelevant absorption or absorption due to substances other than vitamin A can be obtained.

Of the chemical methods, the most widely used is the Carr-Price reaction based on measurement of the unstable blue colour formed by the interaction of antimony trichloride and vitamin A. The optical density of this blue solution at 620mμ is, within certain limits, a linear function of vitamin A concentration (144). Other inorganic chlorides and several other types of reagents have been used to produce colour reactions (147,148), but antimony trichloride gives the closest correlation between depth of colour and concentration of the vitamin (144) and is the reagent most widely used at present. The method will be described in more detail in a succeeding section. Though less precise than the ultra violet absorption method, blue colour assays are more specific for vitamin A and more sensitive, and for these reasons have wider applicability (144).

Further, the method does not require the use of such expensive equipment. On the other hand, there are numerous difficulties and disadvantages to be overcome. Firstly, the rapid fading of the blue colour necessitates speed and experience in determination of the point of maximum intensity; secondly, the extreme sensitivity of $SbCl_3$ reagent to traces of moisture and interfering substances such as carotenoids and sterols, results in inhibition of colour development or the production of atypical coloure; and thirdly, the corrosive nature of the reagent necessitates extreme care in handling. To overcome these disadvantages, efforts have been made to find a more suitable reagent. For example. glyeerol-dichloro-hydrin (G.D.H.) with vitamin A produces a more stable colour reaction than antimony trichloride but unfortunately the colour is less intense, and the reagent varies between successive samples. "Adsorption" methods using activated earths have also been tried and, although they show some promise, there is little to indicate that they will be extensively employed in routine assay work.

Physical and chemical methods of vitamin A assay, where applicable, are rapid and precise, are easy to carry out, and have a repeatability of 2-3%. They are concerned with determining accurately the amount of a particular chemical present and are particularly useful for standardizing drugs and foods, and for routine assay and comparative work. It should be pointed out that the accuracy of these methods has, in the

past, been seriously limited by the bioassay since the former were based on the latter as a standard. With the availability of pure vitamin A this difficulty has been largely overcome and the accuracy of physical and chemical methods has continually improved. It is at present not difficult to determine accurately the percentage of vitamin A in a preparation expressed in say, mg. or µg. of pure vitamin A. A problem arises, however, when an attempt is made to convert these percentage figures to "vitamin A potencies" expressed, for example, in International Units, as the conversion factors in use (bioassay/$E^{1\%}_{cm}{}_{\lambda max}$) vary widely. Since these variations can be attributed mainly to a lack of precision in the bioassays (which at best have a repeatability of only 0.8 - 0.9), it appears that much of the confusion which exists at present would be removed were we, as Gridgeman (149) suggests, to regard the bioassay as less absolute and ascribe greater analytical finality to physico-chemical methods. If this suggestion were adopted, the accuracy of non-biological methods would not then be restricted to within the limits of error of the less precise bioassay technique.

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THE EFFECT OF THYROID FUNCTION ON CAROTENE AND VITAMIN A METABOLISM.

Over the past 30 years, considerable literature has accumulated on the interrelationships between the function of the thyroid gland and vitamin A metabolism. In addition, numerous reports have appeared concerning the effect of the thyroid gland on the absorption and conversion of carotene. Most of these studies are experimental, although many observations of a clinical nature have also been reported (150,151, 152,153,154). It is not the intention to consider these latter papers in detail, as they, along with other clinical evidence have been adequately reviewed elsewhere by Drill (155), who concluded:

(a) "A deficiency of vitamin A produces thyroid hypertrophy, although a sex difference in response is present. An excess of vitamin A seems to decrease the amount of colloid in the thyroid gland, although this result is still controversial, as is the effect of thyroxine on hypervitaminosis A."

(b) "Hyperthyroidism decreases the requirement for vitamin A, also lowering serum vitamin A and serum carotene."

(c) "All the evidence to date indicates that in the absence of the thyroid gland carotene is not metabolised to vitamin A".

Laboratory studies have likewise pointed to the conclusion that a normally functioning thyroid gland is necessary for the

conversion of carotene to vitamin A. Thus Kunde (156) in 1926 noted the appearance of xerophthalmia in rabbits maintained for 8-12 months following thyroidectomy on diets rich in carotene, and somewhat later in 1932-3 Fellenberger and Grueter (157), and Fasold and Heidemann (158) observed that milk from thyroidectomised goats contained more carotene and less vitamin A than that from normal goats. Similarly, Abelin (159) reported that livers from thyroidectomised guinea pigs contained more carotene and less vitamin A than those from normal guinea pigs and Smith and Perman (160) noted that thyrotoxic cats, in contrast to untreated cats, could effect the conversion of carotene to vitamin A.

More recently, Canadell and Valdecasas (161) have shown carotene to be ineffective in relieving xerophthalmia when administered orally to thiouracil-treated rats, and Drill and Truant (162) have obtained similar results by injecting carotene into thyroidectomised rats. The effect of thiouracil on the metabolism of carotene in sheep and lambs has been studied by Bolin and Bolin (163) and Barrick, Andrews, Beeson and Harper (164) respectively, who have shown using plasma vitamin A levels as criteria, that very high doses of thiouracil inhibit the conversion of carotene to vitamin A. Similarly, in rats Johnson and Baumann (165), using the liver storage of vitamin A criterion, showed that the same dose of carotene produced less vitamin A when fed along with thiouracil than when fed alone. In a later paper (166) the same authors, using a similar technique, showed that thiouracil feeding

retarded the rate of depletion of vitamin A from the liver and on the basis of these results, together with those from the previous experiment, they concluded that the amount of vitamin A present in the liver at any one time was a resultant of two opposing effects: (a) the rate of conversion and deposition within the liver, and (b) the rate of utilization. On the basis of this conclusion thiouracil-fed animals analysed a short time after receiving a carotene supplement would be expencted to have less vitamin A in their livers than normal controls. On the other hand, however, animals given thiouracil for many days after beginning the carotene supplements might be expected to retain more of the vitamin A formed from the carotene and over a long term this amount might be great enough to equal or to possibly exceed any difference due to the lowered rate of conversion.

Goodwin (167), and Cama and Goodwin (168,169) were the first to discuss the possible mode of action of the thyroid on carotene metabolism. They considered that three possibilities existed:

(a) that the enzyme "carotenase" is inhibited;
(b) that the stability of carotene in the intestine is reduced, and
(c) that the absorption of carotene from the intestine is reduced.

The first possibility was ruled out when they consistently failed to detect carotene in the systemic blood of rabbits fed a carotene-rich diet along with large doese of thiouracil, and

there was good reason to reject the second possibility also when Cama and Goodwin (170) showed that thiouracil had no effect on the stability of carotene *in vitro*. That the thyroid exerts its main effect by reducing the absorption of carotene from the intestine is highly probable from the results of Cama and Goodwin (168), which showed that rats dosed with thiouracil excrete a greater proportion of a given supplement of carotene than do control animals, which in turn secrete more than rats dosed with desiccated thyroid. These results have also been confirmed in cows and goats by Owen and his colleagues at the Hannah Research Institute (171,172), who found in additinn that the milk ratio of carotene/vitamin A was decreased by thyroxine and increased by thiouracil.

It is of interest to note at this stage that subsequent to publication of the Liverpool work, the presence of a secondary site of conversion in rabbits was established by the Reading group (104). The possibility must be considered, therefore, that the failure of the Liverpool workers to identify carotene in the systemic circulation of their experimental animals may have been due to the fact that it was converted rapidly at a secondary site prior to entering the general circulation. If this were the case then there is nothing in the Liverpool work to indicate that intestinal "carotenase" is not inhibited in its action by thiouracil. To clarify this point it is highly desirable that this work should be repeated with the aid of cannulae established in

the portal vein and mesenteric lymph duct, and in combination with a degestibility trial, so that the fate of carotene following its ingestion can be traced and any losses accurately determined.

In contrast to the results discussed above there is considerable evidence to indicate that the thyroid has little or no effect on the metabolism of carotene in the animal body. Thus Remington, Harris and Smith (173) have shown carotene to be equally as effective as vitamin A in curing ocular symptoms in thyroidectomised rats. Similarly, Di Bella (174,175) found carotene to be effective but with reduced efficiency. Wiese, Mehl and Deuel (176) working at California demonstrated that the same amount of carotene was required to elicit a fixed growth response in hypothyroid animals as in controls, and in hypothyroid animals carotene was shown to be as effective as vitamin A at low levels of dosing in alleviating ocular symptoms and promoting growth. Attempts by Kon and his associates in England (177) and Smith, Niedermeier and Schultz in America (178) to repeat the earlier work of Fellenberger and Grueter (157) and Fasold and Heidemann (158) were unsuccessful, in neither case could the presence of carotene be detected in the milk or blood of goats following thyroidectomy. Furthermore, Smith et al showed heavy dosing with thiouracil to have little effect on the plasma vitamin A levels of goats fed diets rich in carotene. Similarly, Campell and McDowell (179) have noted that the feeding of large quantities of thiourea and thiouracil to dairy cows at pasture has little effect on either the carotene or vitamin A content of the milk.

These latter observations are contrary to the recent results of Owen and his co-workers at the Hannah who have shown in a series of experiments involving both goats and cows that the thyroid gland has a profound effect on the absorption and utilization of carotene, and on the levels of carotene and vitamin A present in the milk. In preliminary experiments (180,181), the effects of thyroxine and thiouracil injections on the digestibility of carotene were studied, and it was found in both species to be markedly increased by thyroxine and decreased by thiouracil. Furthermore, when on a carotene-free diet, faecal carotene reappeared more quickly following dosing with carotene in the thiouracil-treated group than in the thyroxine-treated group. In a later experiment (182) these results were confirmed and the effects of thyroxine and thiouracil on the vitamin A alcohol/ester ratio and on the carotene content of milk were investigated. When lactating cows were given a diet devoid of carotene, milk yields were not affected by the concentrations of carotene and vitamin A ester decreased. At the same time the vitamin A alcohol content of the milk increased to a level which was higher in the thyroxine-treated group than in the normal group. On resumption of a normal carotene diet the concentrations of both carotene and vitamin A ester were noted to increase, at a rate which was enhanced by thyroxine and retarded by thiouracil.

In their most recent studies (183), the Hannah group have repeated these experiments in both lactating goats and cows, using

thyrotrophin in the place of thyroxine. Blood levels of carotene and vitamin A were followed along with those of the milk, and changes in one were shown to closely parallel changes in the other. On feeding a diet free from carotene the blood and milk changes in composition in both species were identical to those found in previous experiments with the exception that no carotene or vitamin A alcohol could be detected in the blood or milk of either treated or untreated goats. One further paper of particular interest, is that by Chanda (184) concerning the partition of carotene and vitamin A in the milk of cows and goats throughout lactation. Using chromatographic and spectrophotometric methods, the partitions of carotenoids and vitamin A in the milk of three cows from the 2nd. to the 40th. week of lactation and from two goats from the 2nd. to the 30th. week of lactation were determined. All the animals were receiving winter rations. The vitamin A content of cow milk fat decreased gradually up to the 30th. week of lactation, and thereafter, when the milk yield was decreasing rapidly, both the percentage of fat in the milk and the percentage of vitamin A in the fat showed small but definite increases. When a quadratic curve was fitted to the data (fig. 1) the minimum concentration of vitamin A in the fat was shown to occur at 25 weeks post partum. In goat milk fat a similar trend was observed with a minimum occurring at 20 weeks post partum. The carotene content of cow milk fat showed no variation as lactation advanced, the amount present varying from 4.3 to 6.0 µg./g. with a mean of 5.1µg./g. At no stage could carotene be

detected in the milk fat of goats though traces were shown to be present in the liver and in the colostrum. Throughout lactation the vitamin A potency of goat milk fat persisted above that of cow milk fat, a fact which can perhaps be related to the greater activity of the thyroid gland of the goat which facilitates absorption of carotene and its conversion to vitamin A, thus making more vitamin available for secretion into the milk.

From the work discussed above it is apparent that considerable evidence has accumulated on the interrelationships between the function of the thyroid gland and carotene and vitamin A metabolism. Much of this evidence is based on clinical observations and much of it on the results of unconfirmed laboratory studies and is, therefore, of only limited value. Where work has been repeated in a number of different centres results have ofter been conflicting and difficult to interpret, especially when small numbers of animals and insufficient control groups have been used. However, there would appear to be little doubt that hypo function seriously impairs the absorption of carotene and the deposition of vitamin A in the liver. Furthermore, it would appear a reasonable assumption that in the absence of the thyroid gland carotene is not converted to vitamin A, although the evidence concerning this is far from satisfactory. The effect of the thyroid on vitamin A requirements is likewise open to question, although there is considerable evidence to suggest that in the hypothyroid state they are considerably reduced (155,185). Whether this is

due to a decreased maintenance requirement associated with a lower metabolic rate, or whether it is due to an increase in the efficiency of utilization of vitamin A, is not at present known. It is to be hoped, however, that when the results of the further work planned in this laboratory are forthcoming, additional light will be shed on this problem and on those other aspects of the thyroid-vitamin A relationship discussed above.

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CHAPTER TWO.

THE VITAMIN A CONTENT OF BUTTER.

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I. REVIEW OF LITERATURE.

Attention has already been drawn to the importance of butter as a source of vitamin A in the human diet and from the foregoing discussion it is obvious that a high potency* is desirable. Over the past 25 years much work has been carried out on the vitamin A content of butter, originally in order to estimate its potency relative to that of other dairy products, but more recently in order to trace the causes of the pronounced seasonal variations which have been shown to occur.

In England Booth, Kon, Dann and Moore (186) have shown that fat produced from

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* "The total vitamin A potency" of a butter refers to its total vitamin A activity. It is calculated from the formula:
(β-carotene µg./g. x 1.67) + (vitamin Aµg./g.x 3.33)
which allows for the activity of both vitamin and provitamin. The factor 1.67 is used for carotene since by definition the I.U. is defined as the biological activity of 0.6µg. of pure β-carotene, so that β-carotene possesses an activity of 1.67 I.U./g. Similarly, the factor 3.33 is used for vitamin A as the most reliable figures indicate 0.3µg. of pure vitamin A to be equivalent to one I.U. Thus 1.0µg. of vitamin A is equivalent to 3.33 I.U. although values ranging from 3.0 - 4.0 are commonly found in the current literature.

Shorthorn cows during the winter contained less carotene and vitamin A than that produced during the summer. Later, Kon (187) 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 and Pritchard (188) for mixed European butters showed a minimum in late winter and a maximum in late spring - early summer. In a more recent paper Lord (189) has confirmed these general trends and has correlated variations in the carotene and vitamin A contents of the milk with similar changes in the carotene and vitamin A levels of the blood; fig.5.

Seasonal variations in the vitamin A potency of American butter were first reported by Baumann and Steenbock (190) at Wisconsin, who observed wide differences between the carotene and vitamin A contents of milk fat produced under winter and spring conditions. These observations have been confirmed more recently by the results of the Nation-wide survey conducted by the Committee on Food and Nutrition of the National Research Council (191,192) which showed that:

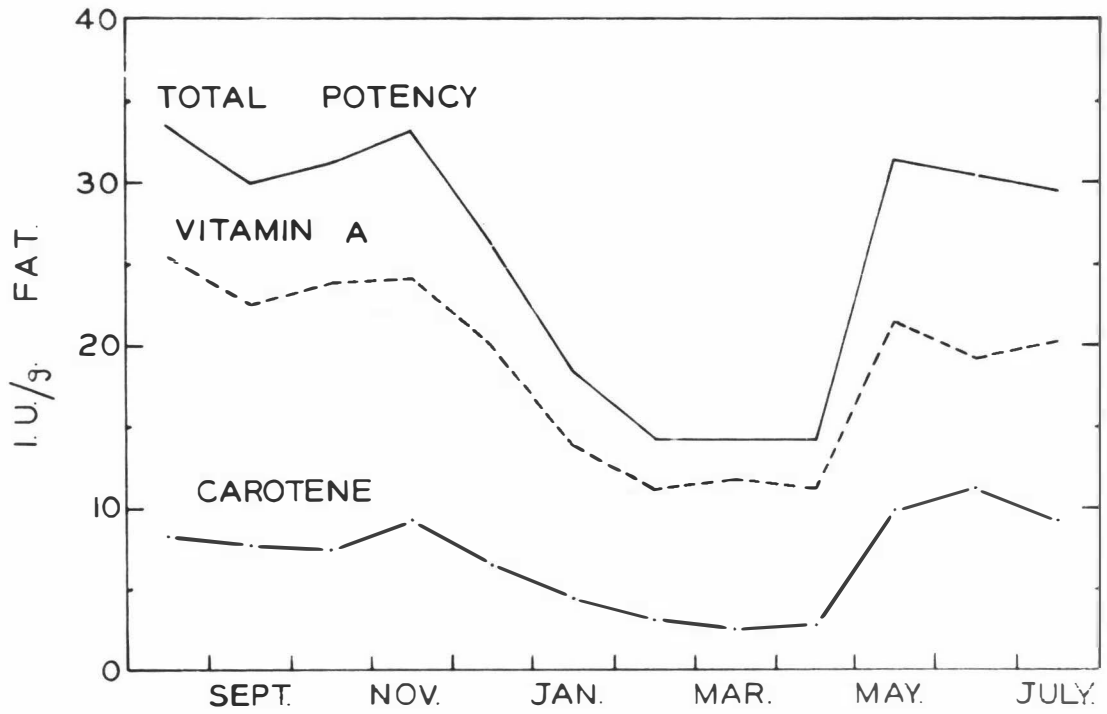

FIGURE 5.

SOURCE OF DATA: Table 3, p.55b.

TABLE 3.

SEASONAL VARIATION IN THE VITAMIN A POTENCY OF ENGLISH BUTTERFAT.

Mean monthly figures from a herd of 70 pure-bred Ayreshire cattle (189).

	CAROTENE I.U./gm.fat	VITAMIN A I.U./gm.fat	TOTAL POTENCY I.U./gm.fat	% CAROTENE of T.P. value
Aug.	8.2	25.5	33.7	24.3
Sept.	7.7	22.4	30.1	25.6
Oct.	7.5	23.8	31.2	24.0
Nov.	9.2	24.1	33.3	27.6
Dec.	6.4	20.1	26.3	24.3
Jan.	4.6	13.9	18.5	24.9
Feb.	3.3	11.1	14.4	22.9
Mar.	2.5	11.9	14.4	17.4
April	2.7	11.2	13.9	19.4
May	9.9	21.5	31.4	31.6
June	11.2	19.2	30.4	31.3
July	9.2	20.2	29.4	31.3

> "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 recorded 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 (193,194). The level of carotene in the diet is now known to be by far the most important factor influencing these variations (191,192,195-205). For example, Gillam, Heilbron, Morton, Bishop and Drummond (204,205), 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 dropped to their original levels. These observations have been confirmed by the results of work of a similar

nature at Beltsville (191,192) in which butter of maximum potency was produced throughout the winter by supplementing the normal concentrate ration with good quality alfalfa and corn silage.

In a study of milk from different breeds, Baumann, Steenbock, Beeson and Rupel (136) noted milk from Guernsey to be much richer in carotene than that from Friesians kept under the same conditions, but lower in vitamin A. Breed differences in total activity, however, appeared to be small. On the other hand, individual variations in the carotene and vitamin A contents of milk from different cows of the same breed were shown to amount to as much as 100%. Results of a similar nature have been reported by Gillam, Heilbron, Ferguson and watson (193) who, in a study of the carotene and vitamin A values of milk fats from four typical English breeds kept under the same conditions of feeding and management, concluded that:

> "individual variations among cows of the same breed are large, but apart from the abnormally high values of colostrum, the carotene and vitamin A values of the butters are much more dependent on diet than on stage of lactation.....Comparison of the summation of carotene plus vitamin A values places the breeds, in order of vitamin A activity of the butters, Guernsey > Friesian > Ayrshire > Shorthorn. The results of this and previous work indicate, however, that the differences between butters of the last three breeds are scarcely significant."

Similarly, Crawford, Perry, and Zilva (206) concluded that:

> "the breed of the dairy herd seems to have no significant influence on the vitamin content of the butters."

Numerous studies have been made of the carotene and vitamin A contents of colostral fat (207,208,209,210) and a comprehensive review of the subject has been published by Parrish, Wise, Atkeson and Hughes (211). It has been observed that in general the contents are highest in initial colostrum with a rapid decline to more normal values by the 4th. or 5th. day, unless prepartal diets rich in carotene (212) or supplemented with vitamin A per se are fed (213), when high levels may persist for 5 - 8 days.

Summarizing these results there appear to be three principal factors affecting the levels of carotene and vitamin A present in butterfat overseas:

(1) INDIVIDUALITY.
(2) BREED.
(3) SEASONAL CHANGES.

Of these the latter has been shown to be the most important. Seasonal changes were first thought to be due to purely "lactational effects" but are now known to be due mainly to diet. Under overseas stall-feeding conditions it is well recognised that variations in potency are primarily a function of the carotene content of the cow's ration, unless additional supplements

containing vitamin A *per se* are fed (214,215,216). Thus the decline in the activity of winter butter is explained by 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. Barnicoat (194), however, has reported results of an investigation carried out during the 1935-6 season into the carotene and vitamin A contents of butterfat from two of the principal butter-making districts of New Zealand, from which he concluded:

> "There were seasonal variations apparently due to nutritional rather than physiological causes. The minimum values (33-37 I.U./g. butterfat) for total vitamin A were found in late summer (February) at the time when the pasture normally tends to dry up, while peak values (42-53 I.U./g. butterfat) occurred in late winter and spring (July-October). The variations in vitamin A potency with season were in the opposite direction to the variations recorded in the literature for Europe and America."

Subsequently, these trends have been confirmed by other New Zealand workers (217,218) and by an

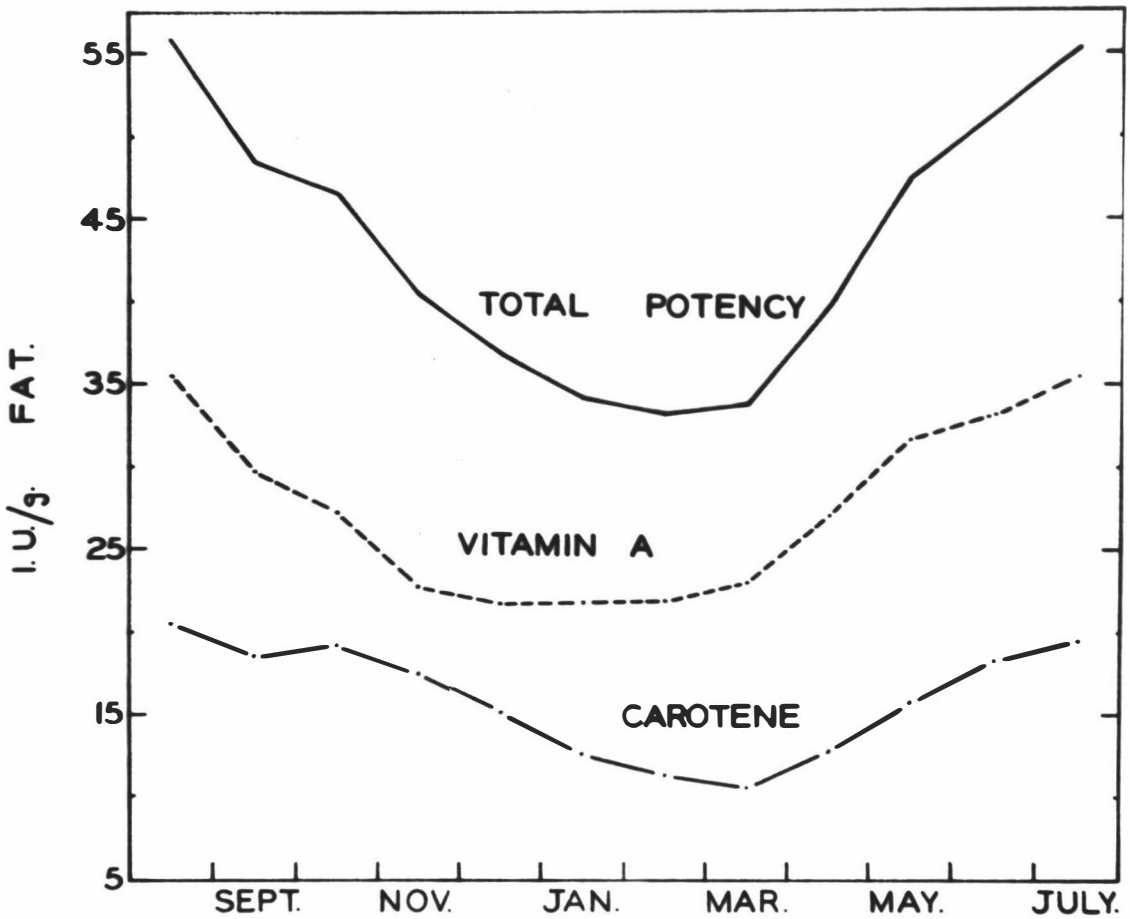

FIGURE 6.

SOURCE OF DATA: Table 4, p.59c.

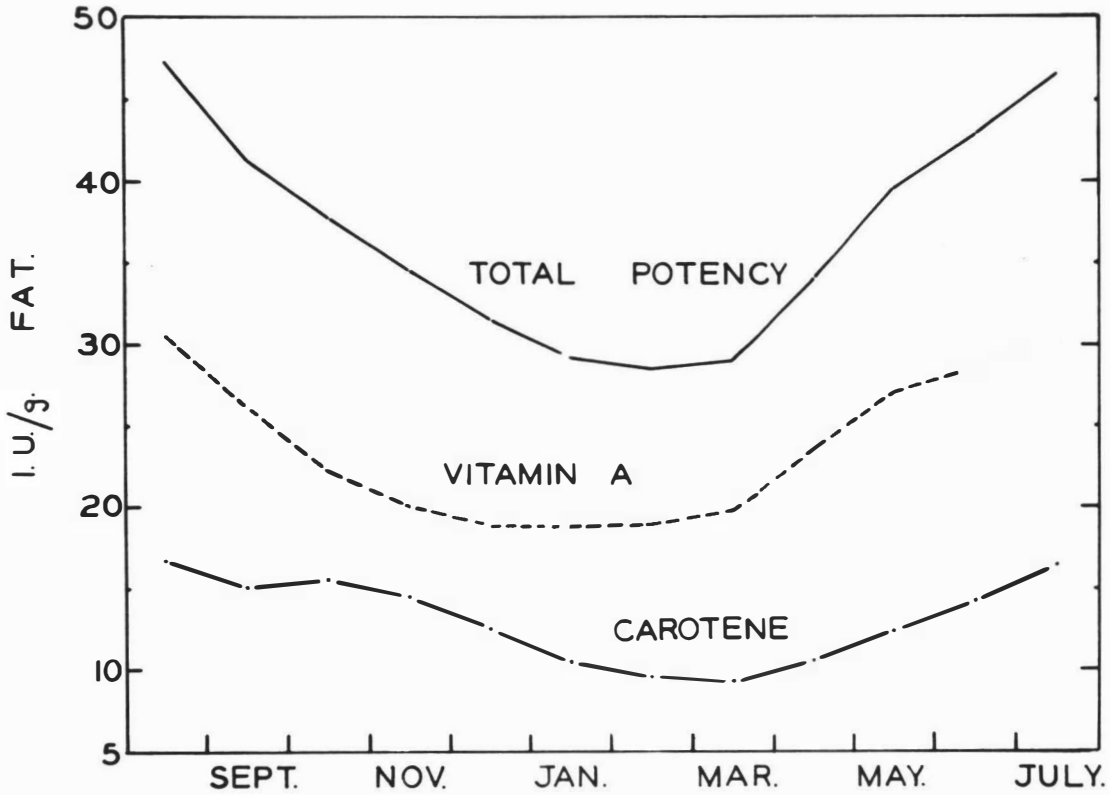

FIGURE 7.

SOURCE OF DATA: Table 5, p.59d.

TABLE 4.

SEASONAL VARIATION IN THE VITAMIN A POTENCY OF NEW ZEALAND BUTTERFAT.

Average unweighted monthly values obtained over the 1946-7-8 season from factories throughout the North Island (218).

	CAROTENE I.U./gm fat	VITAMIN A I.U./gm fat	TOTAL POTENCY I.U./gm fat	% CAROTENE of T.P. due to ~~I.U./gm fat~~
Aug.	20.64	35.46	56.10	36.8
Sept.	18.65	29.79	48.44	38.5
Oct.	19.37	27.23	46.60	41.6
Nov.	17.67	22.85	40.52	43.6
Dec.	15.11	21.89	37.00	40.8
Jan.	12.56	21.82	34.38	36.5
Feb.	11.29	22.02	33.31	33.9
Mar.	10.79	23.04	33.83	31.9
April	12.76	27.01	39.77	32.1
May	15.72	31.81	47.53	33.1
June	18.34	33.34	51.68	35.5
July	19.62	35.55	55.17	35.6

TABLE 5.

SEASONAL VARIATION IN THE VITAMIN A POTENCY OF NEW ZEALAND BUTTERFAT.

Average monthly values, weighted for production, obtained over the 1946-7-8 season from factories throughout the North Island (218).

	CAROTENE IU./gm fat	VITAMIN A I.U./gm Fat	TOTAL POTENCY.
Aug.	16.7	30.6	47.3
Sep.	15.1	26.2	41.3
Oct.	15.5	22.3	37.8
Nov.	14.4	20.0	34.4
Dec.	12.5	18.9	31.4
Jan.	10.4	18.8	29.2
Feb.	9.5	19.0	28.5
Mar.	9.2	19.8	29.0
April	10.6	23.5	34.1
May	12.4	27.0	39.4
June	14.2	28.7	42.9
July	16.5	30.1	46.5

Australian group (219) working with butterfat from a number of districts in Victoria where dairying conditions are comparable to those existing in New Zealand.

Several aspects of the problem have already been investigated. Barnicoat (194), for example, using spring- and autumn-calving cows, showed that there was no relationship between Vitamin A potency and "stage of lactation". He concluded from this observation that none of the variation was due to physiological causes per se, and that the variation must, therefore, be attributed mainly to factors of nutritional origin. Numerous estimates have been made of the intake of carotene required for the production of fat of maximum vitamin A potency. Russell et al (220), Atkeson et al (221), and Fraps (222) have recorded intakes ranging from 400mg. to 600mg. of carotene per day (assuming that 1 Sherman-Munsell Unit is equivalent to 1.4I.U. (223), that 1 I.U. is equivalent to 1.8 U.S.P.X.1934 Units (22), and assuming butter to contain 80% fat). Similarly, Wilbur, Hilton and Hauge (224) concluded that cows receiving approximately 460mg. of carotene per day produced fat of a maximum potency containing about 53 I.U./g. butterfat, and Hauge, Westfall, Wilbur and Hilton (225) obtained fat of approximately 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. The carotene intake of cows at pasture in New Zealand has already been discussed and throughout the year has been shown to vary between comparatively narrow limits.

Furthermore, at any one time at appears to be well above the level required for the production of fat of maximum vitamin A potency (p.31). The decline in the potency of butterfat from cows grazing summer pastures cannot, therefore, be ascribed to a low carotene intake, but must be assumed to be due to a lowered availability of the carotene from these pastures (217).

Several factors have been shown to affect the availability of carotene. Fraps (226), for example, has reported the apparent digestibility of carotene to be reduced by diets rich in fibre, and Graves (142), and van Eekelen and Pannevis (143)have demonstrated large variations in the availability of carotene from different sources. While other factors also are undoubtedly involved it is well recognised that adequate tocopherol plays an important part in "sparing" carotene and in protecting it against destruction in the gastro-intestinal tract and blood-stream (139,140,141). Working on the assumption, therefore, that low summer vitamin A potencies were due to low tocopherol rather than low carotene levels in the pasture, McGillivray (217) 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 one gram of alpha-tocopherol per day he was able to raise the potency of their fat from a typical mid-summer 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 3g. per day or when 300mg. of readily available carotene in oil was fed in addition to the 3g. 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 300mg. 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 the 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 at Grasslands Division, Palmerston North (227,228), for example, with clover/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 (217).

Summarizing these results, it appears that the low vitamin A potency of butterfat produced by cows grazing summer pastures in New Zealand is due to a decrease in the availability of the carotene from these pastures. This is suggested by the fact that the level of carotene present in the pasture at any one time is well above that required for the production of fat of maximum vitamin A potency. It appears probable that this decrease is associated with a low level of tocopherol in the pasture since by supplementing cows grazing summer pasture with tocopherol it is possible to raise the potency of their fat to a normal winter maximum. It has been suggested that the low vitamin A potency of summer fat may be associated with a high percentage of clover in the pasture since the emergence of clover as the dominant summer pasture species has been shown to coincide with the production of fat of lowest potency. Furthermore, preliminary work has established the tocopherol content of clover to be considerably less than that of ryegrass and has shown the tocopherol content of pasture to vary throughout the year and to reach a minimum in late summer.

During the summer of 1953 a series of experiments were undertaken using animals from the Dairy Research Institute farm, Palmerston North, in an endeavour to establish a relationship between the level of clover in a pasture and the vitamin A potency of fat produced from it. The

following section describes in detail the materials used and the experimental methods and procedures adopted in an attempt to elucidate this problem.

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II. EXPERIMENTAL.

INTRODUCTION.

The experimental work described below falls into two broad sections. The first section, which will hereafter be referred to as Section I, is devoted to a study of the effect of day to day variations in clover intake on the vitamin A potency of bulked herd butterfat. For this purpose bulked milk samples were collected twice weekly from the Dairy Research Institute herd for a period of approximately two months, extending from the 21st January to the 21st March 1953. Following separation of the milk by gravity creaming and preparation of the fat for assay, chemical determinations were made for:-

(a) Total Vitamin A. (Ester + alcohol).
(b) Carotene.
(c) Tocopherol.
(d) Iodine Number.
(e) Oleic Acid.
(f) Vitamin A alcohol.

at the same time the levels of clover in the pastures grazed were followed and attempts were made to establish a relationship between clover intake and vitamin A potency of fat produced. Further, attempts were made to establish a relationship between clover intake and the tocopherol content and iodine number of the fat. A check was also maintained on the carotene and fibre contents of the pastures in order to determine any possible effect which they might have on fat potency.

The second section, which will hereafter be referred to as section II, is devoted to a study of the effects of high and low clover diets on the vitamin A potency, the tocopherol and vitamin A alcohol (and ester) contents, and iodine numbers of the milk fat of monozygous twins. For this purpose three sets of twins (designated 5,6; 49,50; and 59,80 respectively) were used. Following a 4-day pre-treatment period to establish within-twin variations, during which time they were grazing a pasture containing approximately 36% clover, the even-numbered animals of each pair were placed on high clover pastures (containing approximately 70% clover) and the odd-numbered animals were placed on low clover pastures (containing approximately 18% clover), for a period of 5 days (period 1). For the following 7 days (period 2), the even-numbered animals were brought in from pasture and "stall" fed with clover cut from a pure stand at the Grasslands Division, while the odd - numbered animals remained on low clover pastures. Throughout the experiment (18th March-2nd April), milk samples were collected daily during the evening milking.

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NOTE ON THE MANAGEMENT OF THE DAIRY RESEARCH INSTITUTE HERD.

The herd, comprising a total of 38 mixed age cows, is a grade herd containing a predominance of Jersey blood. Calving normally takes place from mid-July onwards so that at the time of

the experiment the majority of the herd were in their 7th month of lactation. Throughout the experimental period they were rotationally grazing mixed pastures of the ryegrass, cocksfoot, white clover association typical of the Manawatu. Under the system of grazing practised the cows were offered a fresh break of approximately half an acre of pasture after each milking, the area varying according to the quality and quantity of pasture present. Since each paddock on the farm was approximately an acre in area the herd was shifted into a fresh paddock, on the average, after every second milking.

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METHODS AND MATERIALS.

(a) Pasture Analysis. From the outset it was obvious that the success of the project depended to a very large extent on the choice of a suitable pasture sampling technique. The choice was made difficult since studies of this particular type had not previously been undertaken at the college, at the Grasslands Division, or at the Dairy Research Institute. It was found necessary, therefore, to spend some little time on this problem.

Under the system of rotational grazing practised on the farm, at least two and sometimes four pasture analyses were required each day. Thus it was necessary to have a method that was both rapid and accurate. Point analysis was considered impracticable due to the rankness of the

pastures, and it became obvious that samples would have to be cut and sorted by hand. The immediate problem was to determine (1) the minimum size, and (2) the minimum number of samples necessary to give an accurate estimate of clover percentage.

For this purpose a special trial was conducted to determine the variation between successive samples. From a statistical analysis of the results (appendix 2) it was shown that 24 bulked random samples per paddock, each 6" x 4", could be relied upon to give an accurate estimate of the clover percentage present before grazing. The technique adopted for estimating the precentage of clover eaten was to choose 24 plots at random, each 6" x 8", the plots being pegged and numbered for identification purposes and placed at a distance of approximately 3 feet from the plots so as to overcome any effect which their presence might have on normal grazing behaviour, one half of each plot (6" x 4") being cut before grazing, the other half after grazing. The percentage of clover eaten from each paddock was then calculated from the equation:

$$\% \text{ clover eaten} = \frac{C_B - C_A}{T_B - T_A} \cdot 100$$

Where C_B = Wet weight of clover in sample before grazing.
C_A = " " " " " " after "
T_B = Total wet weight of sample before grazing.
T_A = " " " " " after "

The before- and after-grazing samples were cut from adjacent areas:
(i) to increase the accuracy of analysis, and
(ii) as a precautionary measure, to provide a check on selective grazing. This aspect is discussed more fully in a later chapter.

A large amount of work was involved in cutting, pegging, collecting and sorting the above samples. The samples were cut from within a wire frame (6" x 4") as close to the ground as possible using grass shears. The average wet weight before grazing was in the vicinity of 260g. and the average wet weight after grazing, 135g. Cutting, pegging and collecting took approximately an hour for both before- and after-grazing samples, and the sorting of both into clover and grass took, on the average, a further 30 minutes. Thus sometimes up to 3 hours per day were required for pasture sampling.

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(b) <u>The Determination of Carotene and Vitamin A in Butterfat.</u>

A photoelectric method was chosen for the estimation of both carotene and the blue colour developed by the interaction of vitamin A and antimony trichloride (Carr-Price reagent), as being more accurate than the older visual methods. The instrument used was an Eel colorimeter based on the Evelyn design, readings being recorded by means of a mirror galvonometer and projection scale.

Special matched colorimeter test tubes were used, the equivalent light path of these being 1.3cms.

Standardization:

(i) β-carotene. The instrument was standardized against a solution of pure carotene (British Chlorophyll Co.) in petrol ether.

0.1g. of pure β-carotene was carefully weighed and transferred to a standard litre flask. The carotene was dissolved in a small amount of petrol ether (boiling point 40-60°C.) and the solution made up to the mark. 10ml. of this solution (concentration 0.0001 g./ml.) were taken and made up to 100ml. in a standard flask. The percentage transmission of this solution (concentration 10µg./ml.) was then determined using a tri-colour blue filter giving maximum transmission at 440mµ. Solutions containing 9,8.....1µg./ml. respectively, were next made up and a standard curve for carotene (fig. 8) was determined.

(ii) Vitamin A. The instrument was standardized for vitamin A against a standard vitamin A concentrate (containing 51,760.0 µg. vitamin A per ml.) supplied by Glaxo Laboratories (N.Z.) Ltd. 1ml. of this concentrate was made up to 200ml. with chloroform in a standard flask. 10ml. of this solution were then transferred to a 100ml. standard flask and made up to the mark with chloroform. 1ml. of this solution (containing 25.88µg./ml.) was then transferred to a colorimeter tube and 3 drops of acetic anhydride and 6ml. of Carr-Price reagent added and the percentage transmission of the resulting

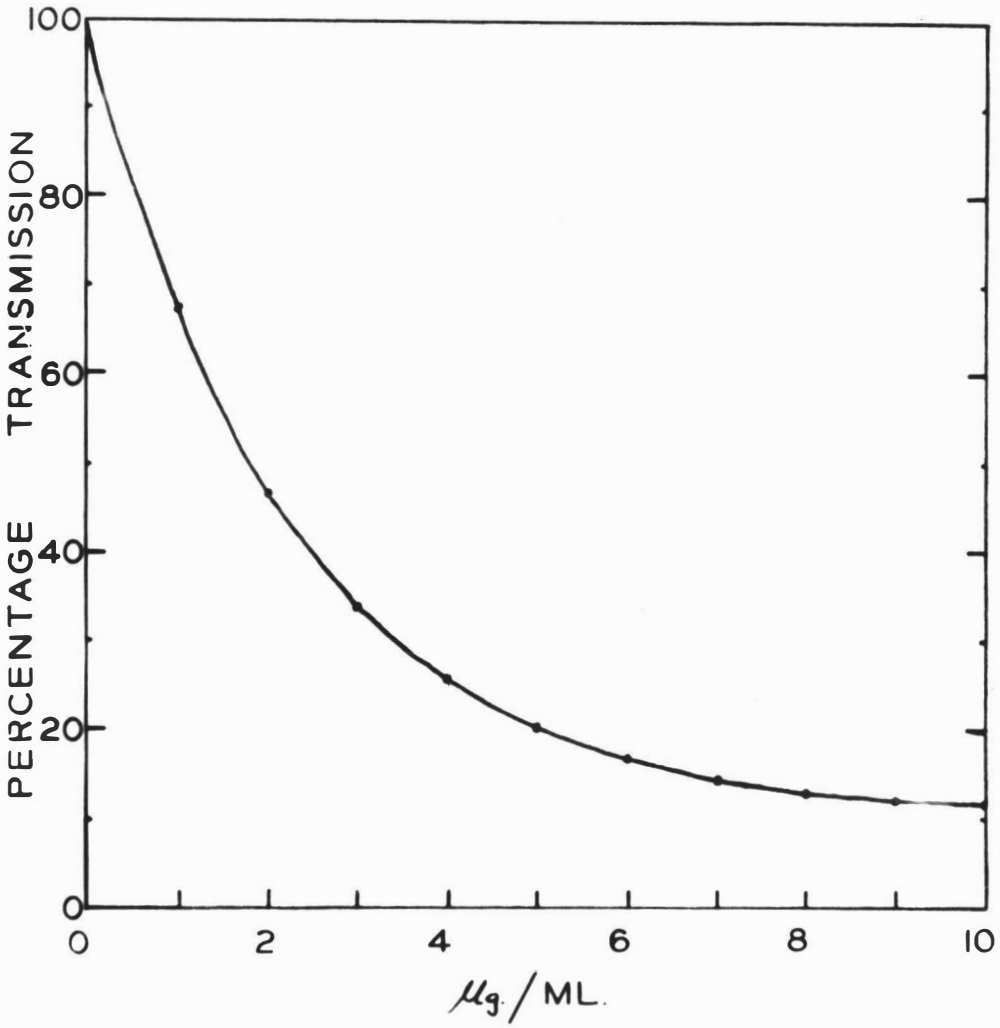

FIGURE 8.

(Standard Curve for Carotene).

blue colour measured (using a tri-colour red filter giving maximum transmission at 610mμ*) in the interval before the solution began to fade, which was, on the average eight seconds. Further suitable dilutions were made, as for carotene, and a standard curve for vitamin A (fig.9) was determined. These procedures, both for carotene and vitamin A, were carried out at least in duplicate.

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Preparation of Samples for Analysis. After collection the milk samples were stored at 0°C. for 12 hours. The cream was drawn off and churned and the butter obtained was melted and filtered at 60°C. The pure butterfat was then stored at 0°C. and assayed shortly afterwards.

The possibility of a small error being introduced by this method of separation, due to incomplete recovery of the small fat globules, must be considered since Kon, Mawson and Thompson (229) have shown the proportion of carotene to be greater per unit volume in small fat globules than in large. Such error as is introduced by the method, has, however, been shown in this laboratory to be so small that it may be disregarded (230).

Method of Analysis. 10g. of butterfat were saponified with 10ml. 50% NaOH and 5ml. ethanol for 15 minutes; before cooling, 40ml. distilled water were added. When the mixture was cool it was transferred to a 200ml. separat-

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* The only one available at the time.

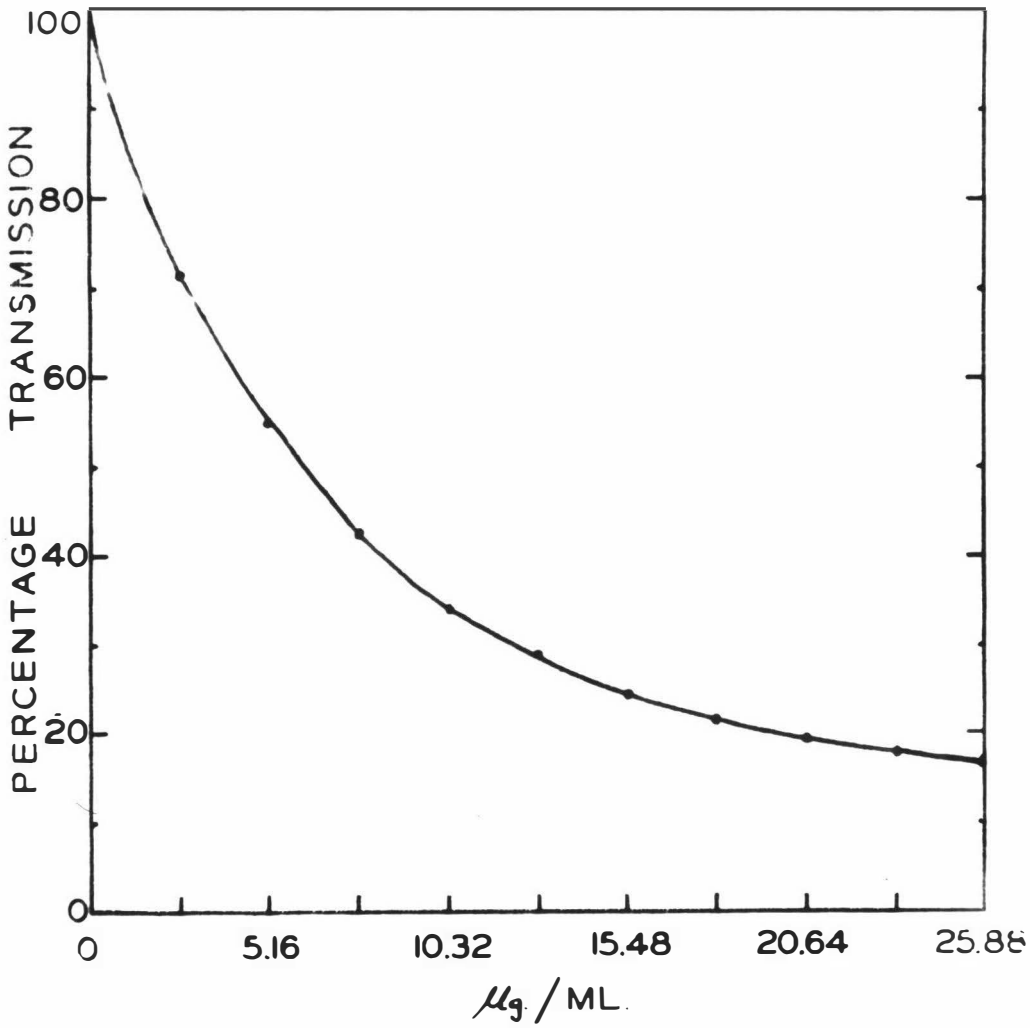

FIGURE 9.

(Standard Curve for Vitamin A).

ing funnel and extracted three times with 30ml. of freshly distilled and dried, peroxide-free ethyl ether. The combined ether extracts were given three successive washings with 100ml. distilled water to remove all traces of NaOH and then dried over Na_2SO_4 and made up to 100ml.

Carotene. 40ml. of the extract were evaporated to dryness in a 200ml. round-bottom flask under reduced pressure over a water-bath. The flask was immediately cooled and the residue taken up in 20ml. petrol ether (boiling point 40-60°C.). The solution was then extracted twice with 90% methyl alcohol to remove non-carotene pigments. The intensity of the yellow colour due to carotene was then estimated as for the standard solution. No correction was made for possible oxidative degradation products (231), or for isomeric forms of carotene (232).

The carotene content of the butterfat sample in µg./g. was then calculated using the formula:

$$\left(\frac{a}{b} \times \frac{20}{40} \times \frac{100}{1} \times \frac{0.77}{1} \right)$$

Where a = concentration of carotene, µg./ml. from standard curve.

b = weight of fat taken.

0.77 = factor $(\frac{15.4}{20.0})$ allowing for volume change after washing with methyl alcohol.

Vitamin A. A further 40ml. of the extract were extracted as before and the flask immediately cooled. The residue was then taken up in 5ml. of chloroform. 1ml. of this solution was transferred to a colorimeter tube

and three drops of acetic anhydride and 6ml. of Carr-Price reagent were added. The resulting maximum blue colour was recorded as for the standard solution and the vitamin A content of the sample in µg./g. was then calculated from the formula:

$$\left(\frac{m}{n} \times \frac{5}{40} \times \frac{100}{1} \right)$$

Where m = concentration of vitamin A, µg./ml., from standard curve.

n = weight of fat taken.

To prevent losses of carotene and vitamin A during the assays, all chemical work was carried out in subdued light and all determinations were carried out as soon as possible after the collection of the samples.

In order that the total vitamin A potencies of the fats might be compared, the carotene and vitamin A figures obtained were converted to International Units using the factors 1.67 (by definition), and 3.2 respectively. The factor 3.2 was used for vitamin A to keep values in line with those previously published from this laboratory by Barnicoat (194) and McGillivray (217).

.....

(c) <u>The Determination of Tocopherol in Butterfat</u>.*

The method in use in this

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* To expidite the chemical work and to decrease the time between collection and assay, these estimations were carried out by Dr. McGillivray.

laboratory is essentially similar to that previously described by Quaife (233).

.....

(d) The Determination of the Iodine Value of Butterfat.

Iodine values of the herd and twin fats were estimated by an adapted standard method (234) in general use in this laboratory:

> 0.2 - 0.3g. of butterfat was dissolved in 5ml. chloroform in a glass-stoppered bottle. 10ml. Wig's solution were added from a burette and the glass stopper was moistened with 10% potassium iodide solution. The mixture was then set aside in the dark for 30 minutes. Next 10ml. KI were added along with 100ml. distilled water and the whole was titrated against 0.1N $Na_2S_2O_3$. A blank determination was also run. The iodine value of the fat was then calculated from the equation:

$$I = 1.27 \times \frac{\text{ml.0.1N } Na_2S_2O_3\text{blank} - \text{ml.0.1N } Na_2S_2O_3\text{sample.}}{\text{weight of fat.}}$$

.....

(e) The Determination of Unsaturated Acid Constituents of Butterfat*

These determinations were carried out according to the methods and calculations described by Brice and Swain (235,236). These methods were preferred to those of Hilditch, Moore and Riley (237), in which dienoic and trienoic acids are estimated on separate portions of the same sample, because the estimation of all polyethylenic non-conjugated acids can be carried out on the same isomerized solution.

.....

(f) The Determination of Vitamin A Alcohol in Butterfat*

These assays were carried out, using the solvent extraction procedure recommended by Olsen, Hegsted and Peterson (238). Vitamin A alcohol was then separated from the ester and from carotenoids by the chromatographic method described by Thompson, Ganguly and Kon (89), and estimated spectrophotometrically using a Beckman Spectrophotometer. The extraction and separation procedure is that generally used and recommended by Thompson and Kon (239) at Reading.

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* Refer to footnote Page. 73.

(g) The Determination of Carotene in Pasture.

At various intervals throughout section I of the experimental period determinations were carried out on the carotene content of pasture using a modification (240) of Moore and Ely's (241) extraction method using a "foaming mixture" of petroleum ether and ethanol. The intensity of the yellow colour due to carotene was then estimated as for the standard solution.

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CHAPTER THREE.

RESULTS AND INTERPRETATION.

.....

I. RESULTS.

(a) Section I.

The results obtained during section I of the experimental work are presented in tables 6 and 7 and figures 10 and 11. The total vitamin A potency of the butterfat samples varied irregularly throughout the experiment from a minimum value of 26.08 I.U./g. fat to a maximum of 35.63 I.U./g. fat. The vitamin A content varied likewise from a minimum value of 15.07 I.U./g. fat to a maximum of 23.92 I.U./g. fat. The carotene content, on the other hand, showed much less variation (10.20 I.U./g. fat - 14.41 I.U./g. fat) and throughout the experiment a regular increase in values occurred.

Large variations were also observed in the tocopherol content (44µg./g. fat - 59µg./g. fat), the oleic acid content (33.3% - 36.1%), and the iodine number (35.1 - 39.6) of the fats. In contrast, little variation was observed in either the conjugated or the non-conjugated di-, tri-, and tetraenoic fatty acid contents of the fats, or in the vitamin A alcohol content which tended at all times to be extremely low compared with overseas values reported by Chanda et al (182).

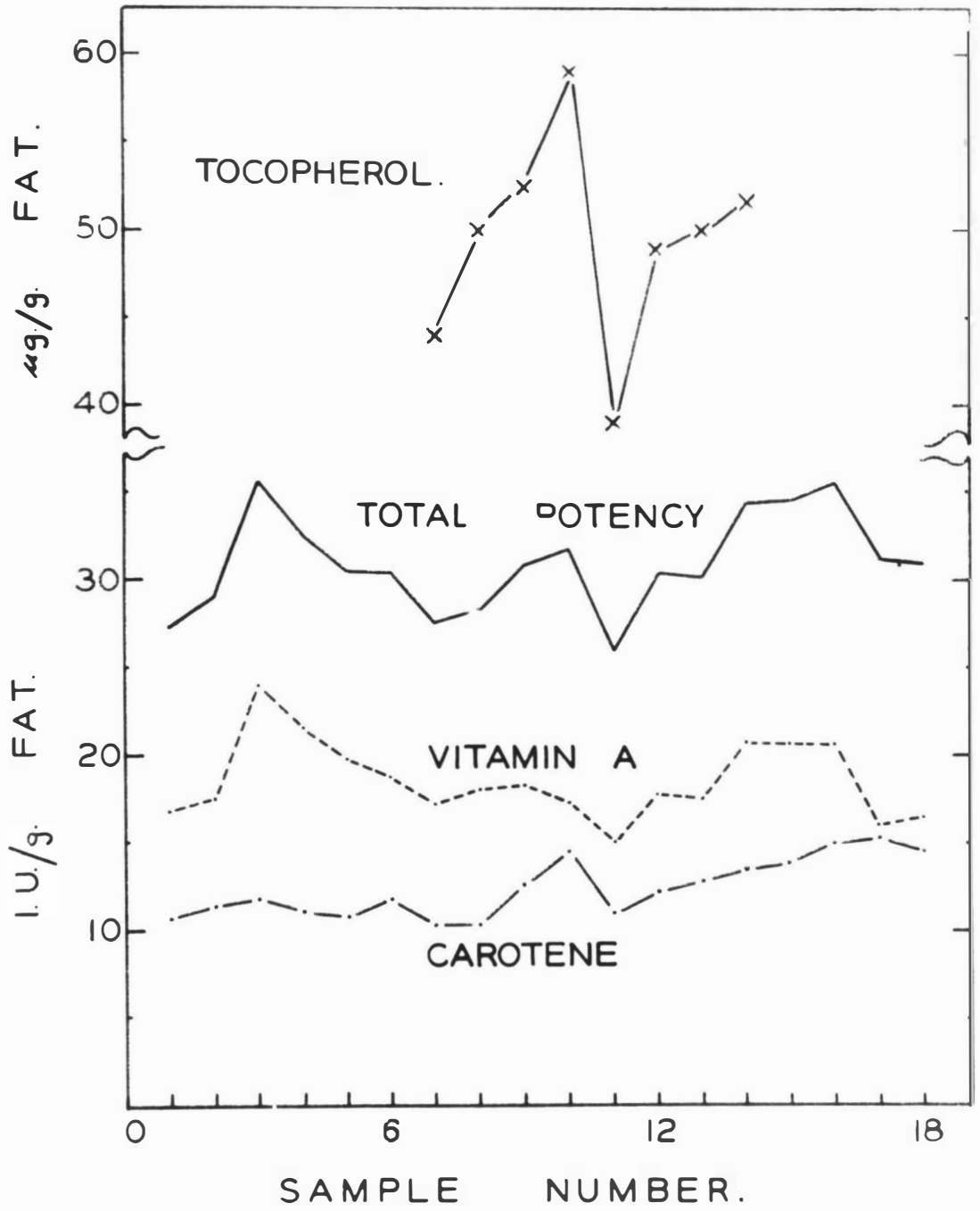

FIGURE 10.

SOURCE OF DATA: Table 6, p.77c.

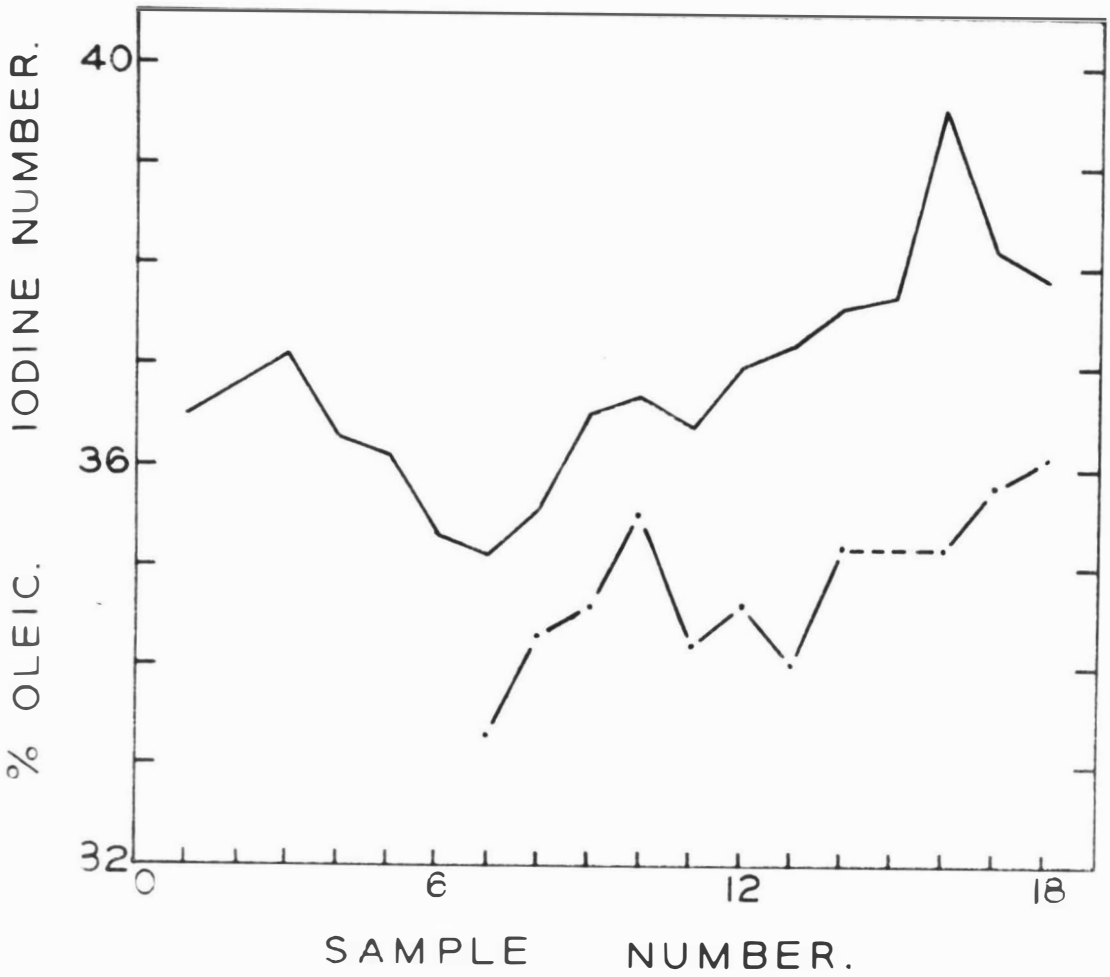

FIGURE 11.

SOURCE OF DATA: Table 6, p.77c.

HERD VALUES.

TABLE 6.

	VITAMIN A		CAROTENE		TOTAL POTENCY.	IODINE NUMBER.	OLEIC ACID %	TOCOPHEROL	VITAMIN A ALCOHOL.	PERCENTAGE CAROTENE.
	ug./g. fat.	I.U./g. fat.	ug./g. fat.	I.U./g. fat.	I.U./g. fat.			ug./g. fat.		
1.	5.225	16.72	6.385	10.66	27.38	36.5				38.9
2.	5.485	17.55	6.805	11.36	28.91	36.8				39.3
3.	7.475	23.92	7.010	11.71	35.63	37.1				32.9
4.	6.755	21.62	6.550	10.94	32.56	36.3				33.6
5.	6.157	19.70	6.475	10.81	30.51	36.1				35.4
6.	5.850	18.72	7.030	11.74	30.46	35.3				38.5
7.	5.415	17.33	6.105	10.20	27.53	35.1	33.3	44.0		37.1
8.	5.635	18.03	6.170	10.30	28.33	35.6	34.3	50.0		36.4
9.	5.710	18.27	7.500	12.53	30.80	36.5	34.6	52.5	0.13	40.7
10.	5.405	17.30	8.630	14.41	31.71	36.7	35.5	59.0	0.12	45.4
11.	4.710	15.07	6.590	11.01	26.08	36.4	34.2	39.0	0.12	42.2
12.	5.570	17.82	7.410	12.38	30.20	37.0	34.6	49.0	0.14	41.0
13.	5.440	17.41	7.600	12.69	30.10	37.2	34.0	50.0	0.14	42.2
14.	6.500	20.80	8.110	13.54	34.34	37.6	35.2	51.5	0.15	39.4
15.	6.480	20.74	8.290	13.84	34.58	37.7			0.14	40.0
16.	6.470	20.70	8.920	14.90	35.60	39.6	35.2		0.13	41.9
17.	5.030	16.10	9.100	15.20	31.30	38.2	35.8		0.13	48.6
18.	5.130	16.42	8.720	14.56	30.98	37.9	36.1		0.12	47.0

Table 7

HERD VALUES.

TABLE 7.

PERCENTAGE FATTY ACID COMPOSITION OF BUTTERFATS.

SAMPLE NUMBER	DIENOIC		TRIENOIC		TETRAENOIC	
	C.	N.C.	C.	N.C.	C.	N.C.
7.	1.1	0.2	0.03	0.6	Nil	0.4
8.	1.0	0.2	0.02	0.6	Nil	0.3
9.	1.1	0.2	0.03	0.7	Nil	0.3
10.	1.1	0.2	0.04	0.5	Nil	0.3
11.	1.2	0.3	0.03	0.6	Nil	0.4
12.	1.4	0.5	0.03	0.5	Nil	0.3
13.	1.4	0.4	0.04	0.7	Nil	0.4
14.	1.4	0.4	0.04	0.6	Nil	0.3
15.	1.3	0.3	0.03	0.7	Nil	0.3
16.	1.3	0.4	0.03	0.7	Nil	0.3
17.	1.3	0.3	0.04	0.6	Nil	0.4
18.	1.4	0.4	0.03	0.7	Nil	0.3

These results were subjected to statistical analysis and the following correlations were established using the formula:

$$r_{xy} = \frac{SSx.SSy}{(Sx)^2(Sy)^2}$$ for (n-2) degrees of freedom (240).

Total Potency with:

(i) vitamin A content, $r_{TP.VA}$ = 0.773**(16d.f.)
(ii) Carotene content, $r_{TP.CC}$ = 0.624** "
(iii)tocopherol content, $r_{TP.TC}$ = 0.760* (6 d.f.)
(iv) iodine number, $r_{TP.IN}$ = 0.650**(16d.f.)
(v) oleic acid, $r_{TP.OA}$ = 0.743**(9d.f.)

Tocopherol content with:

(vi) vitamin A content, $r_{TC.VA}$ = 0.519N.S.(6d.f.)
(vii)carotene content, $r_{TC.CC}$ = 0.756* "
(viii)iodine number, $r_{TC.IN}$ = 0.366N.S. "
(ix) oleic acid %, $r_{TC,OA}$ = 0.720* "

Iodine Number with:

(x) oleic acid %, $r_{IN.OA}$ = 0.693* (9d.f.)

Carotene Content with:

(xi) vitamin A content, $r_{CC.VA}$ = 0.008N.S.(16d.f.)

The carotene and crude fibre contents of several pasture samples, corrected for moisture and excess ash* are shown in table 8.

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* On analysis many of the pasture samples gave ash figures in the vicinity of 25%. This was assumed to be due to contamination of the pasture with road dust and river silt (following serious flooding of the Manawatu River) and an average ash figure of 15.0% taken for all samples. Accordingly, adjustments were made to the other constituents on this basis.

TABLE 8.

CAROTENE AND CRUDE FIBRE FIGURES
CORRECTED FOR MOISTURE AND EXCESS ASH.

SAMPLE NUMBER	PADDOCKS	CRUDE FIBRE PERCENTAGE.	CAROTENE ug./gm.
I	17	27.7	199
II	25,26,27	21.8	249
III	20,21,17,14,13	23.1	296
IV	2	26.6	245
V	11	25.1	299
VI	3	27.1	260
VII	5,4	21.0	379
VIII	5,6,7,8,9	27.5	276
IX	16	29.0	321
X	10,23	24.7	263
XI	12,13,15,25,27	29.5	208
XII	8,7	23.1	319
XIII	46,18	25.2	268
XIV	27	20.5	294
XV	12,18,19	22.2	263
XVI	24,26,21	23.5	195

Clover fed cut to twins 6,50 and 80 (period 2), carotene = 403 ug./gm.
Low clover pasture - grab samples, carotene = 273 ug./gm.
High clover pastures - grab samples, carotene = 248 ug./gm.

The samples were obtained from a series of paddocks grazed in succession over a period of approximately six weeks (8th February - 21st March) and were grouped according to the percentage of clover present in the pasture. Variations in fibre contents were low (20.5% - 29.5%) and were too uniform to have any marked effect on the milk fat. The carotene content of the pastures, on the other hand, varied from 195µg./g. D.M. to 379µg./g. D.M. with an average of 270µg./g. D.M. but at all times it appeared to be well above the level required for the production of fat of maximum vitamin A potency (217). This was further borne out by the fact that no relationship could be demonstrated between the carotene content of the pastures and the vitamin A potency of the fats produced from them.

The percentage of clover eaten throughout the experiment varied greatly (table 9) from a minimum of 5.5% to a maximum of 70.4%, Attempts were made to establish a relationship between the vitamin A potency of butterfat (Y) and the percentage of clover eaten 12 hours (x_1), 24 hours (x_2), 36 hours (x_3)..... and 120 hours (x_{10}) prior to milking, with the following results:

r_{Yx_1} = + 0.046N.S.(10 d.f.)
r_{Yx_2} = - 0.087N.S.("
r_{Yx_3} = + 0.356N.S. "
r_{Yx_4} = + 0.337N.S. "
r_{Yx_5} = + 0.238N.S. "
r_{Yx_6} = + 0.053N.S. "
r_{Yx_7} = + 0.055N.S. "
r_{Yx_8} = + 0.634* (10 d.f.)
r_{Yx_9} = + 0.610* "
$r_{Yx_{10}}$ = + 0.000N.S. "

TABLE 9.

SAMPLE NUMBER	TOTAL POTENCY I.U./g.fat	PERCENTAGE CLOVER EATEN.									
		X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	X_9	X_{10}
7,	27.53	70.4	36.9	36.9	18.4	18.4	19.5	19.5	20.0	20.0	
8.	28.33	5.5.	40.0	36.2	9.2	70.4	70.4	70.4	36.9	36.9	18.4
9.	30.80	25.3	25.3	29.4	29.4	33.7	39.1	39.1	37.7	5.5	40.0
10.	31.71	25.6	16.2	34.8	35.6	8.8	8.8	25.3	25.3	29.4	29.4
11.	26.08	39.1	30.9	16.1	33.7	15.3	16.6	37.9	32.6	25.6	16.2
12.	30.20	38.4	32.6	25.6	39.1	39.1	39.1	39.1	30.9	16.1	33.7
13.	30.10	40.2	40.2	47.4	15.2	49.4	15.6	15.6	15.6	38.4	32.6
14.	34.34	39.1	13.7	31.8	31.8	40.2	40.2	40.2	40.2	47.4	15.2
15.	34.58	63.3	63.3	63.3	31.4	74.2	33.7	16.1	43.2	39.1	13.7
16.	35.60	32.6	24.3	29.8	29.8	29.8	29.8	63.3	63.3	63.3	31.4
17.	31.30	26.9	25.7	25.7	25.7	25.7	21.8	30.0	38.4	32.6	24.3
18.	30.98	18.6	17.9	17.9	33.2	57.1	24.9	26.9	25.7	25.7	25.7

The percentage of clover in the pasture before grazing was found to be highly correlated (0.264*,55d.f.) with the percentage of clover eaten. Furthermore, the difference between the mean percentage of clover in the pasture before and after grazing was found to be small (1.91%) and not significant and on the basis of these results it was concluded that the herd was not selecting either for or against clover throughout the duration of the experiment.

(b) Section II.

(i) Pre-treatment Period.

The results obtained during this period established within-twin cariations in total vitamin A potency, in vitamin A, carotene, tocopherol and oleic acid contents, and in iodine number to be small, although large cariations between pairs (due presumably to breed and individual differences), were noted to exist. In the one or two cases where the within-twin variation was greater than 5%, allowance was made for this difference in all statistical procedures.

Over this period the average vitamin A potency of the six animals was 36.0I.U./g. butterfat. This figure is slightly above the minimum levels reported previously by Barnicoat (194) and McGillivray (217) owing to the fact that the average vitamin A potency of twins 59,80 (41.5 I.U./g. fat) was considerably higher than that of the other two pairs 5,6;49,50, which were 34.9 I.U./g. and 30.5 I.U./g.,respectively.

(ii) Period 1.

When the twins were divided and moved from "average" pasture (containing approximately 36% clover) onto high and low clover pastures (containing approximately 70% and 18% clover, respectively) an immediate response was apparent. For example, the vitamin A potency of fat from the even-numbered animals on high clover pastures decreased, on the average, 8.2 I.U./g. fat in the first 24 hours and the vitamin A potency of fat from the odd-numbered animals on low clover pastures increased, on the average, 4.1 I.U./g. fat (figs.12, 13,14). It is apparent (figs.15-20) that these changes were due more to variations in the vitamin A content of the fat than to changes in the carotene content.

Variations in the iodine number (figs.21,22,23) and the tocopherol content (fig.24) were closely related to changes in total potency but variations in the oleic acid (fig.25) and vitamin A alcohol (fig.26) contents were less clear-cut due presumably, to the paucity of data relating to these constituents. No changes were observed in the di-, tri-, and tetraenoic fatty acid contents of the fats (tables 10,11). Variations in percentage carotene (i.e. percentage contribution of carotene to total potency) were noted to occur, however, but in the opposite direction from those recorded for total potency (figs.27,28,29). Throughout the period milk production figures remained unaffected by treatment.

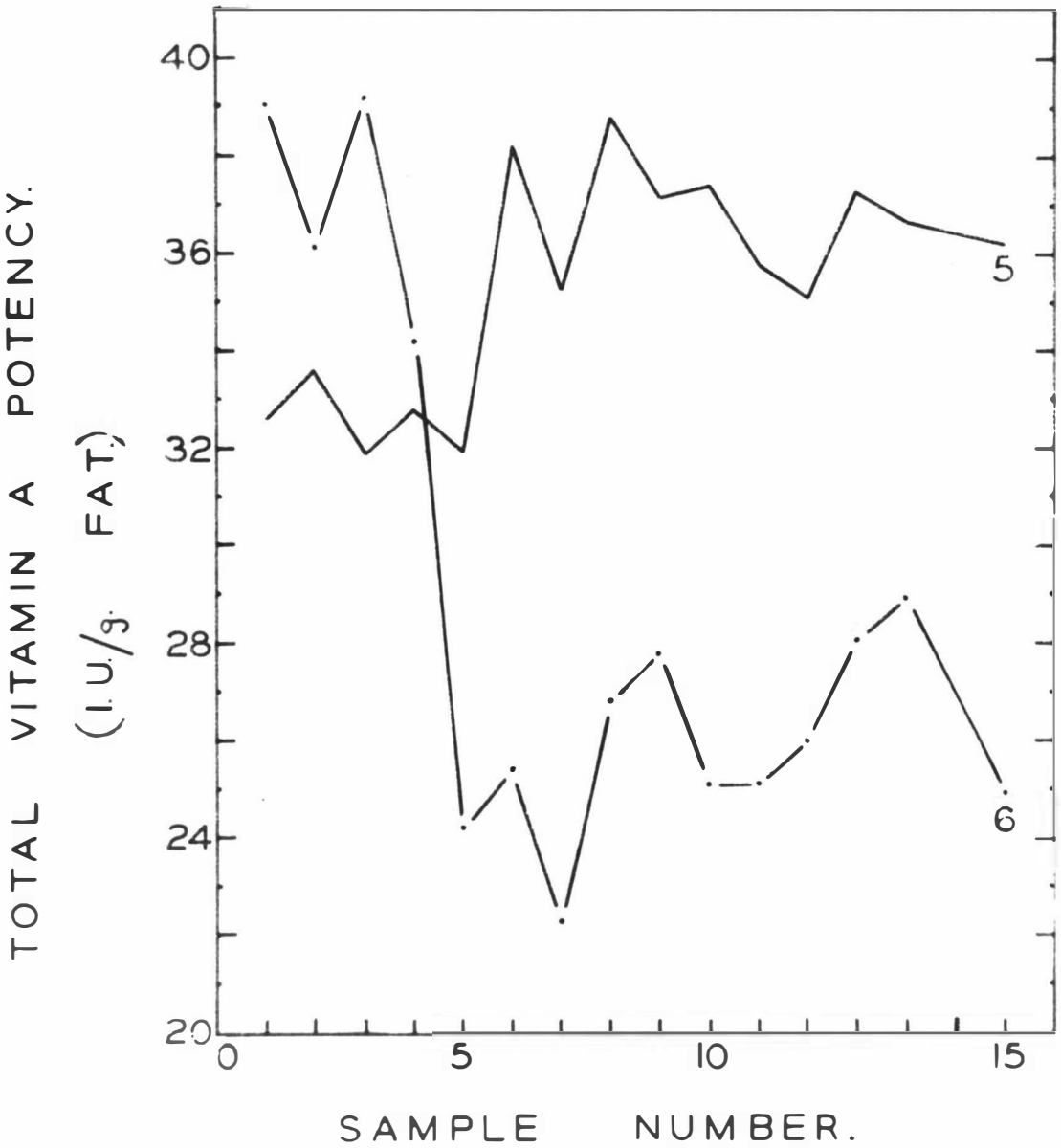

FIGURE 12.

SOURCE OF DATA: Tables 10,11, pp. 81s,81t.

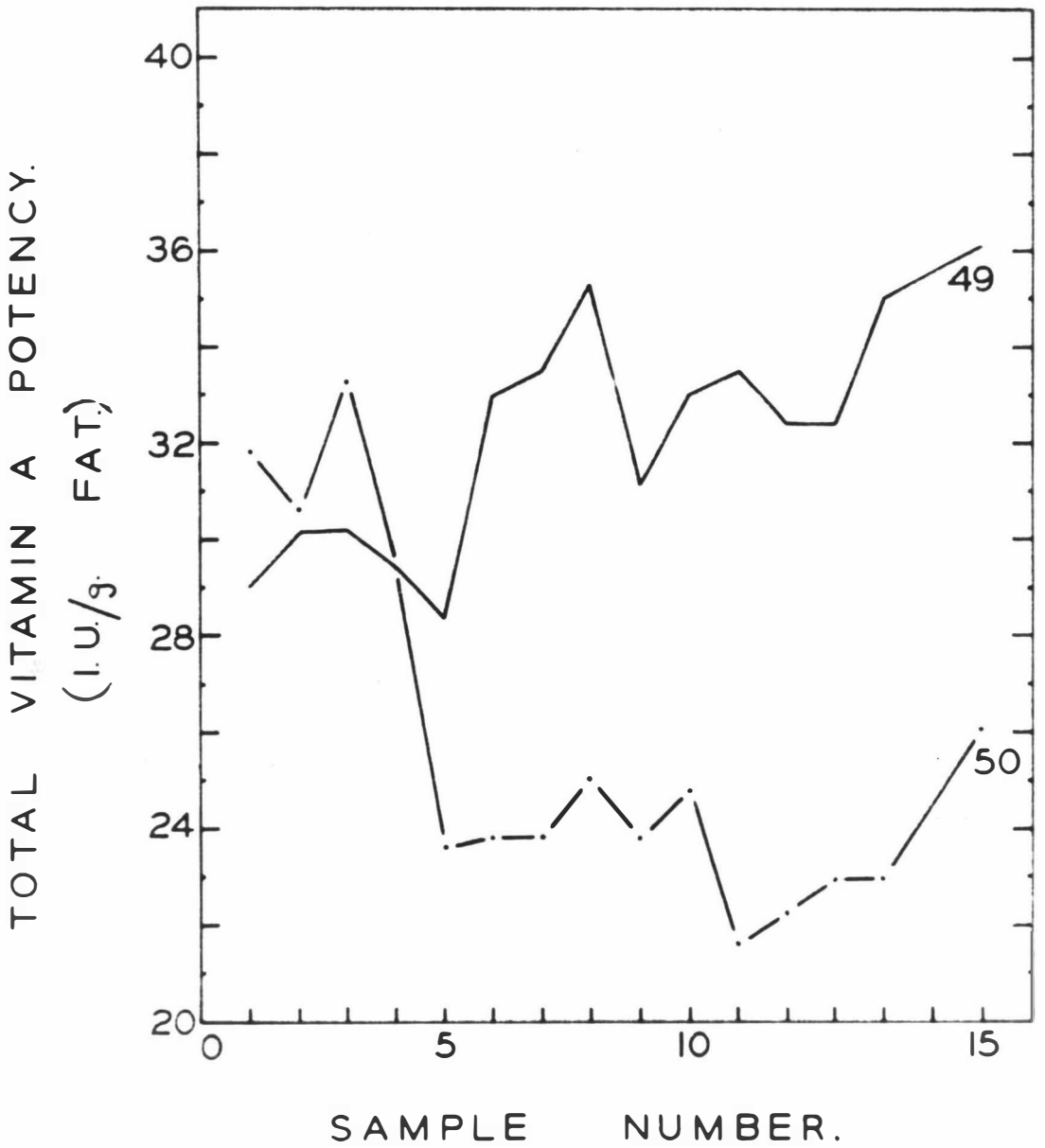

FIGURE 13.

SOURCE OF DATA: Tables 12,13, pp. 81u,81v.

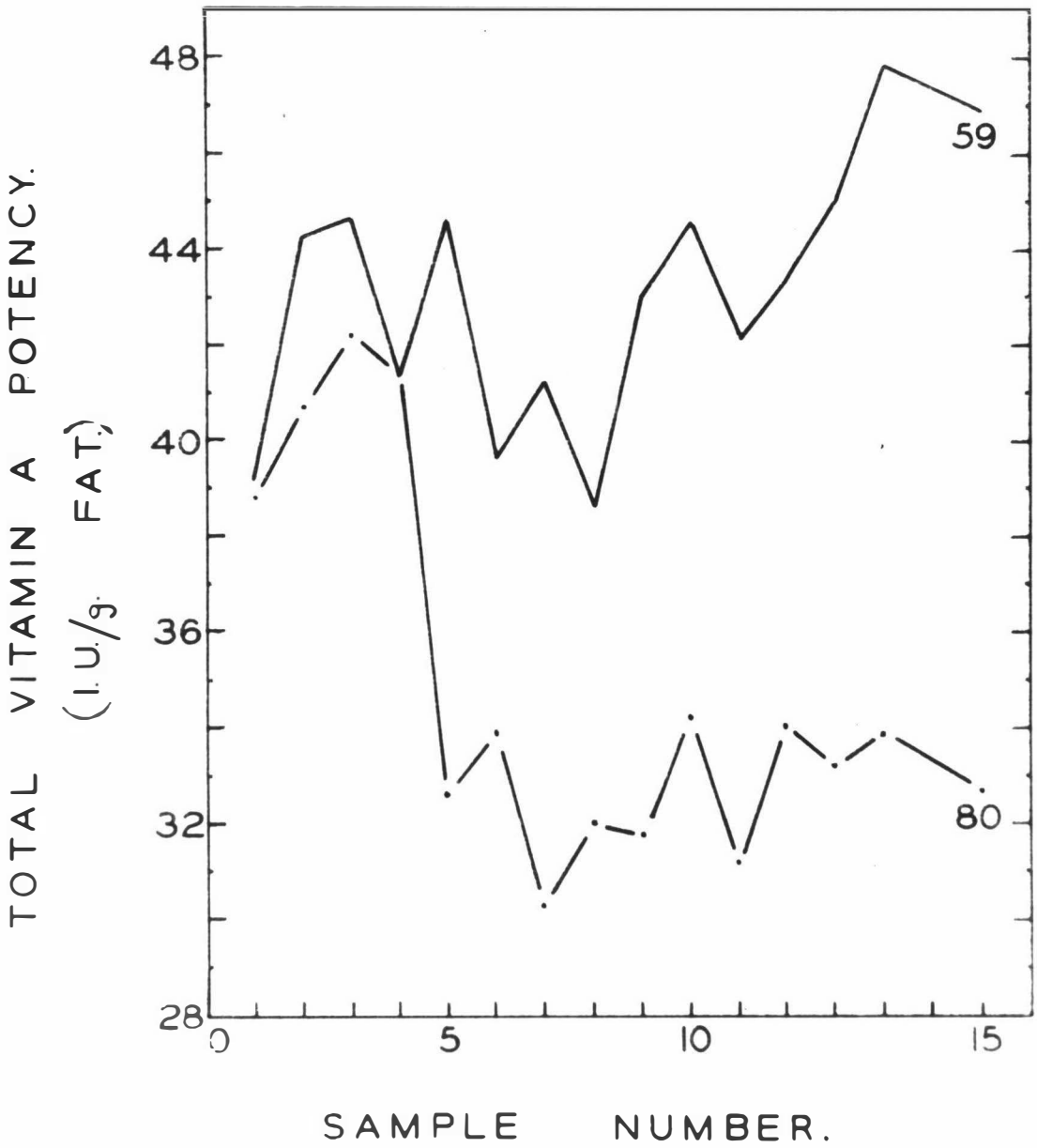

FIGURE 14.

SOURCE OF DATA: Tables 14,15, pp. 81w,81x.

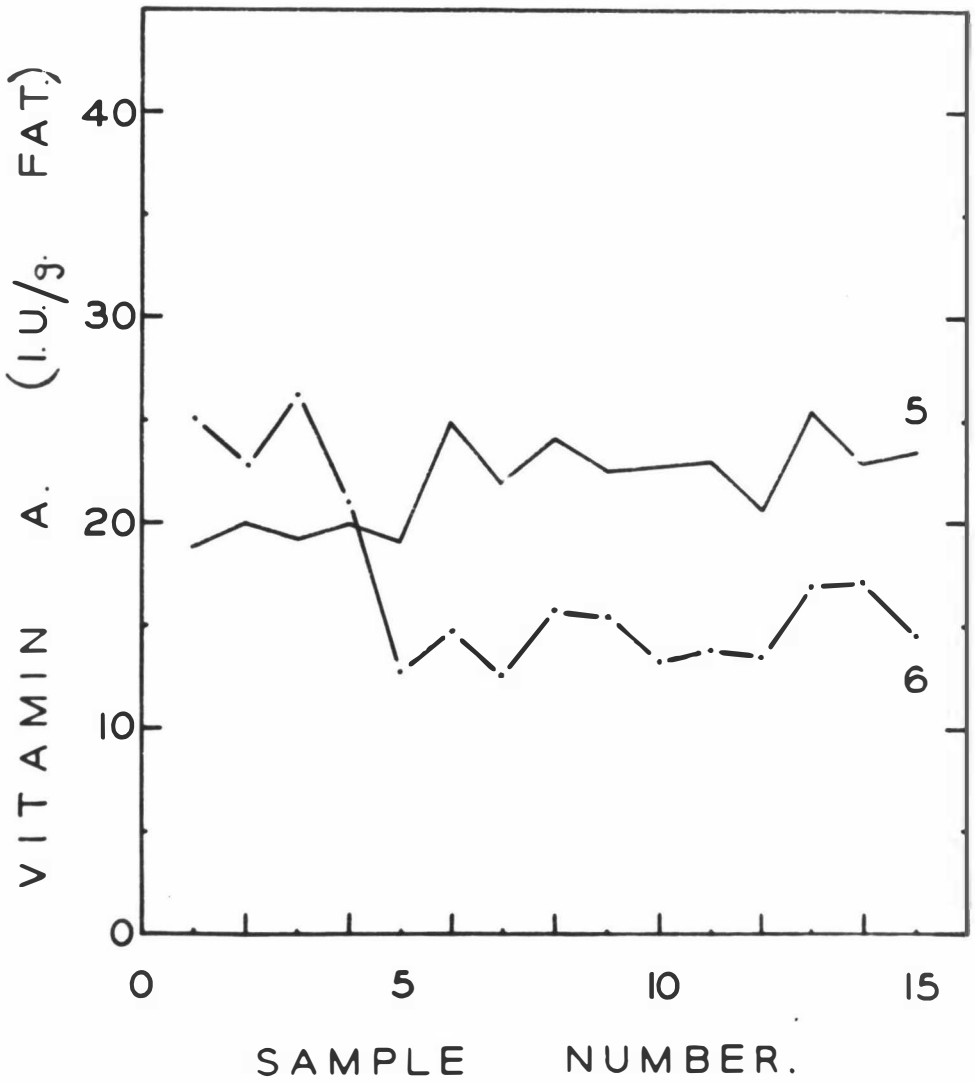

FIGURE 15.

SOURCE OF DATA: Tables 10,11, pp. 81s,81t.

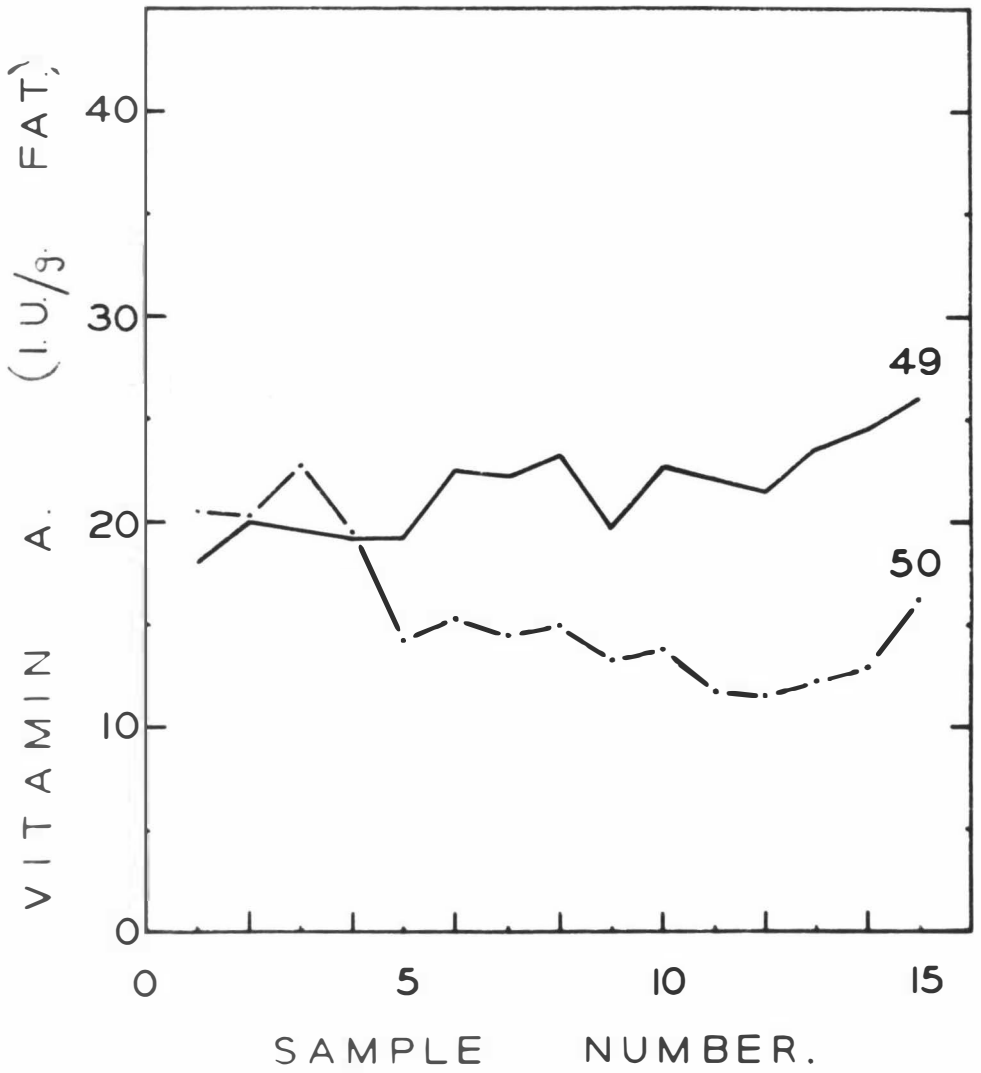

FIGURE 16.

SOURCE OF DATA: Tables 12,13, pp. 81u,81v.

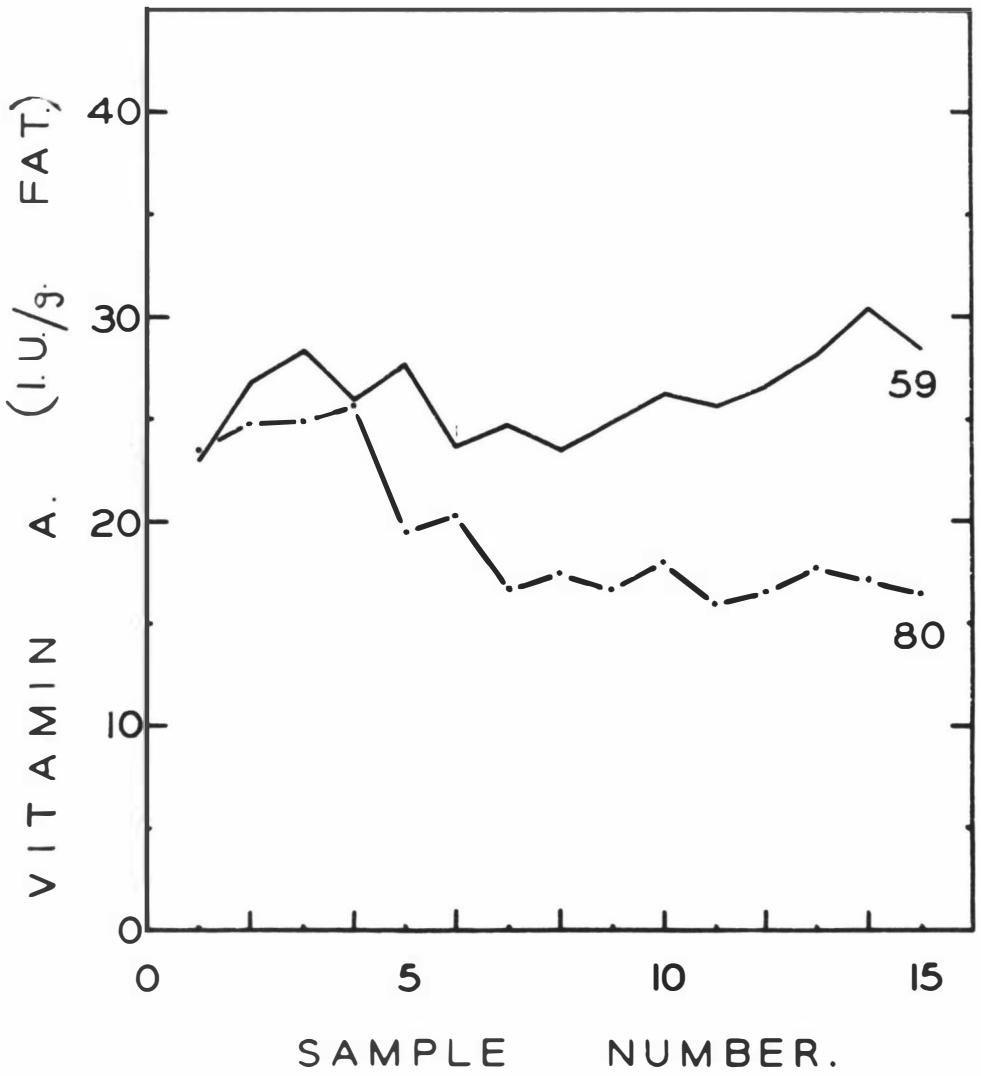

FIGURE 17.

SOURCE OF DATA: Tables 14,15, pp.81w,81x.

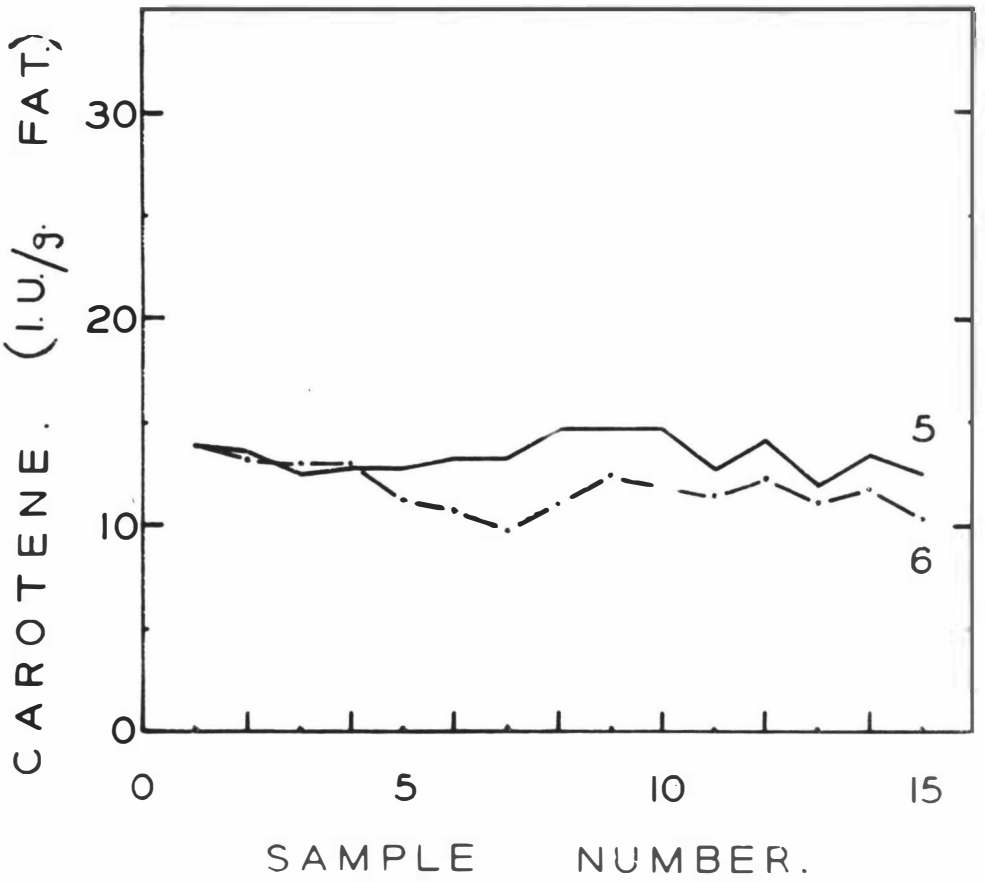

FIGURE 18.

SOURCE OF DATA: Tables 10,11, pp.81s,81t.

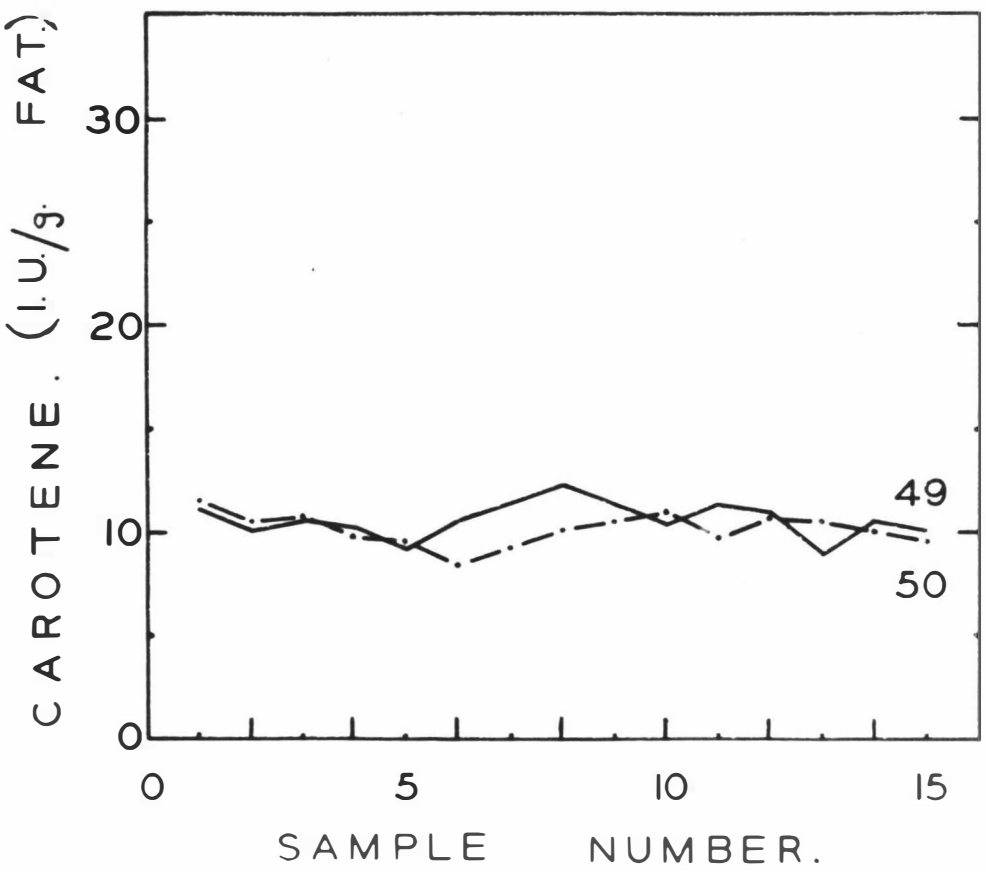

FIGURE 19.

SOURCE OF DATA: Tables 12,13, pp.81u,81v.

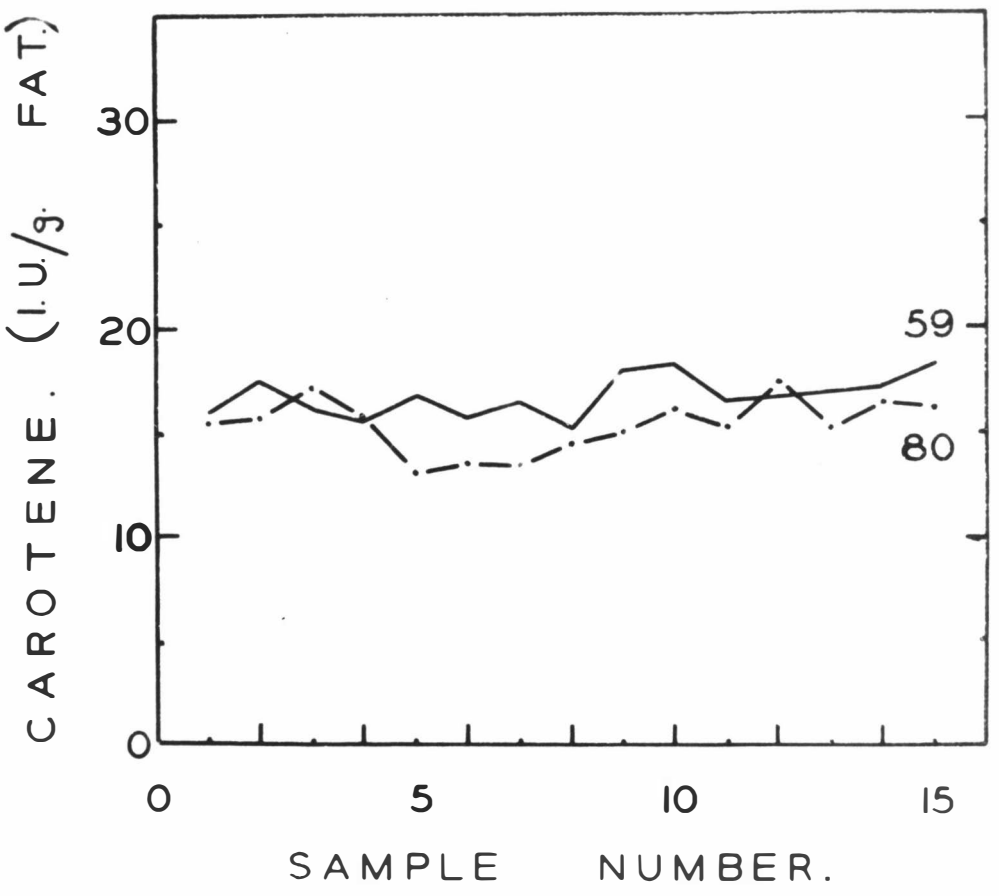

FIGURE 20.

SOURCE OF DATA: Tables 14,15, pp. 81w,81x.

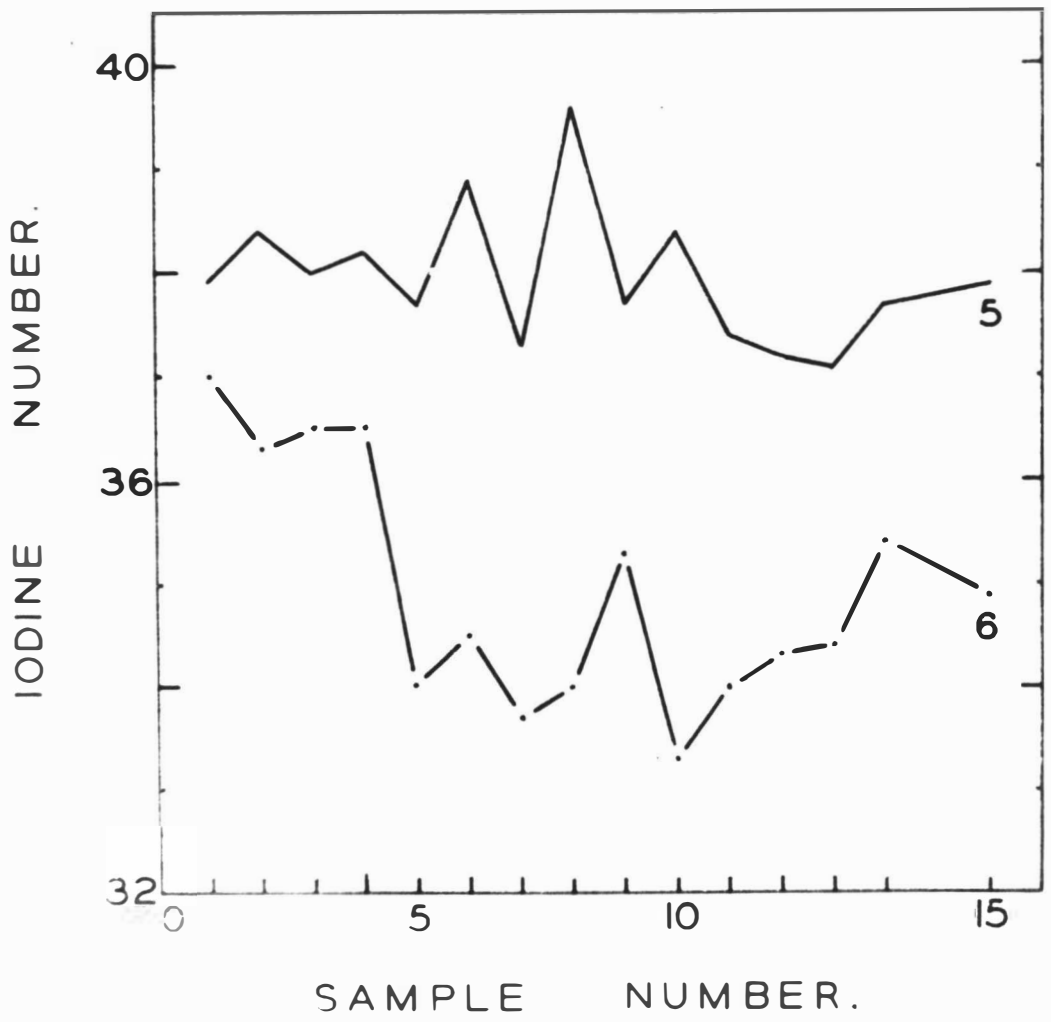

FIGURE 21.

SOURCE OF DATA: Tables 10,11, pp.81s,81t.

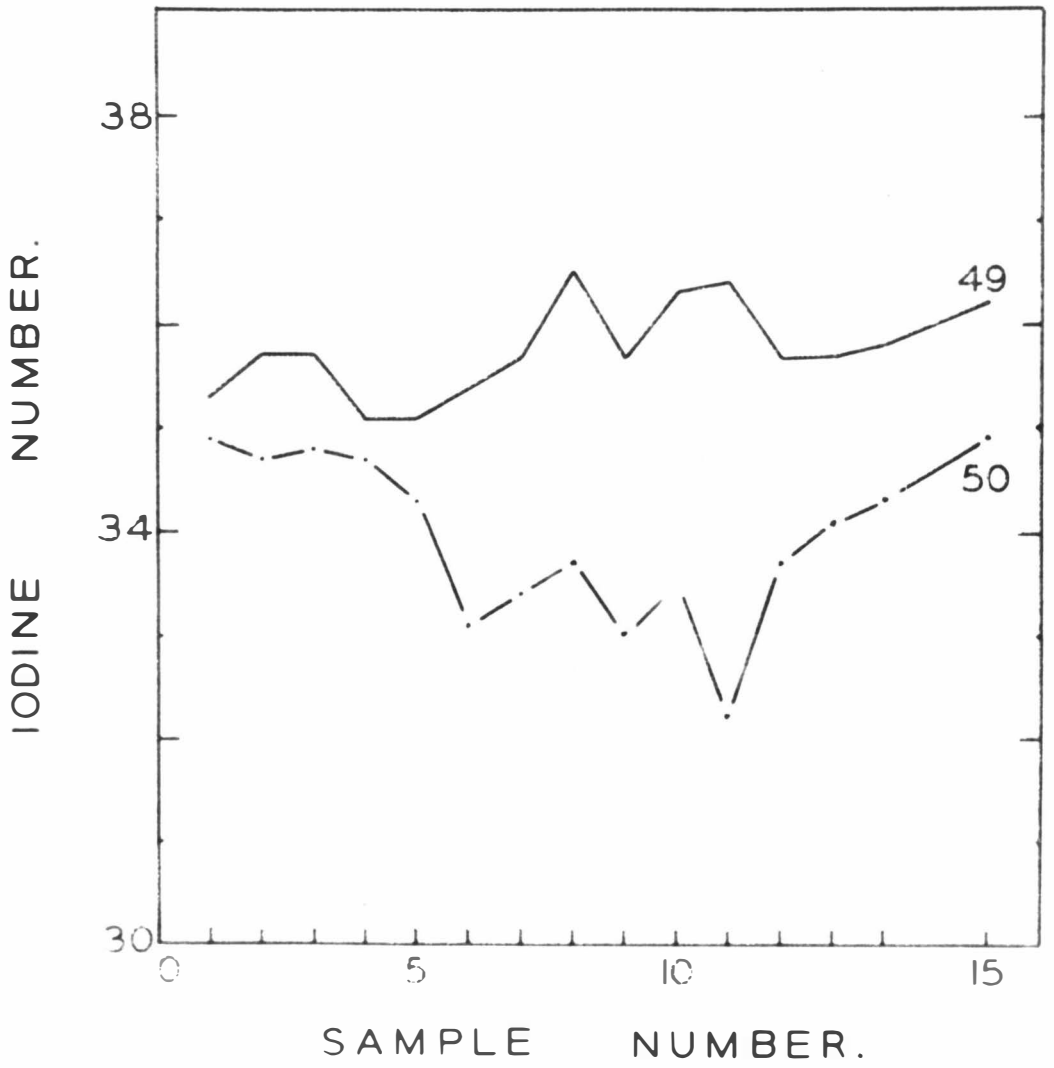

FIGURE 22.

SOURCE OF DATA: Tables 12,13, pp.81u,81v.

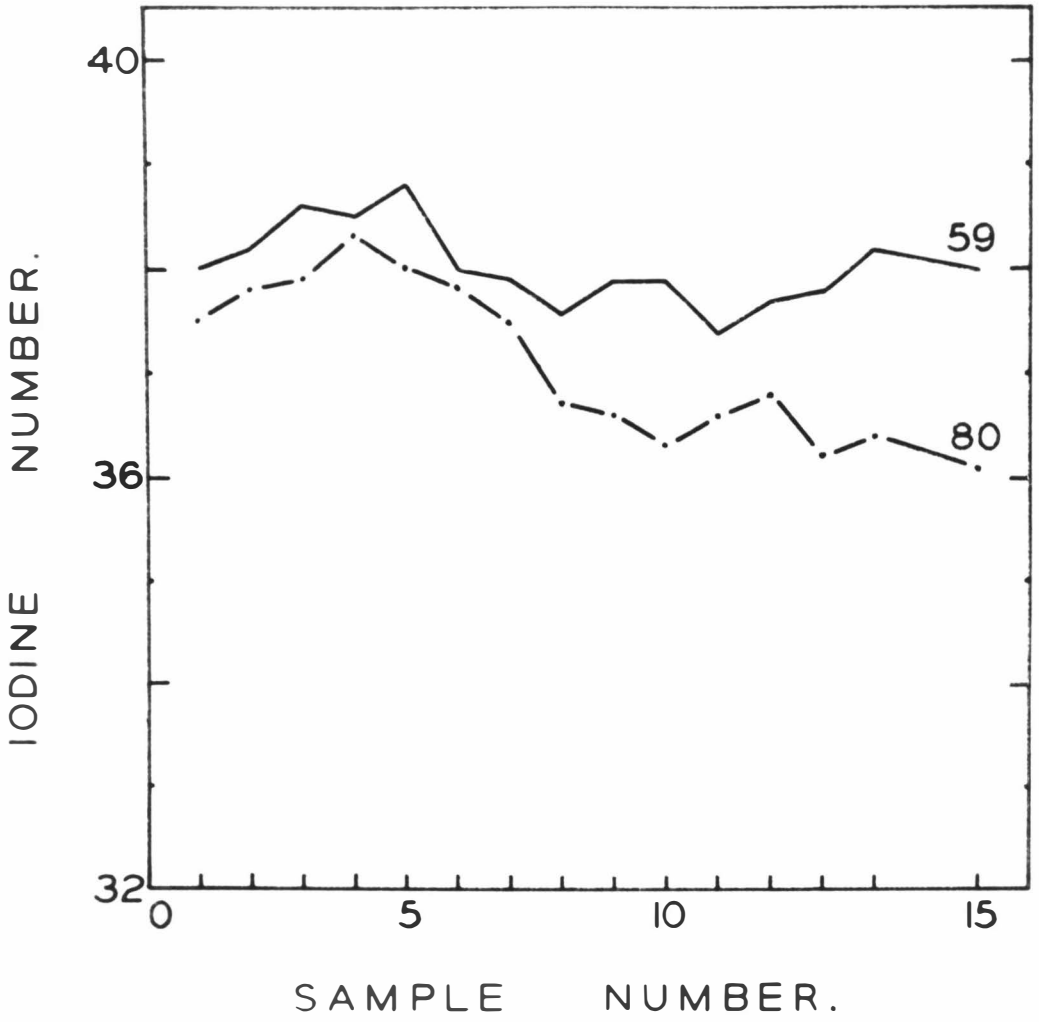

FIGURE 23.

SOURCE OF DATA: Tables 14,15, pp.81w,81x.

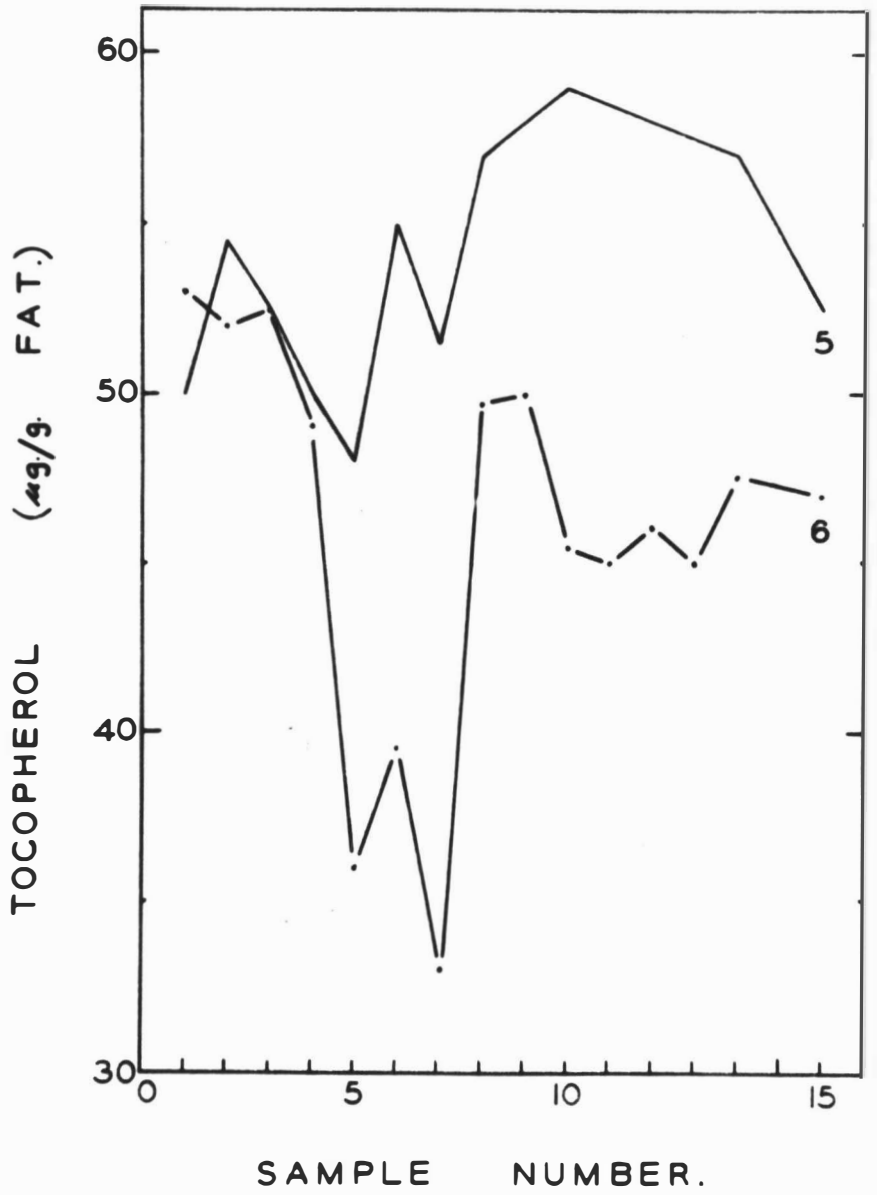

FIGURE 24.

SOURCE OF DATA: Tables 10,11, pp. 81s, 81t.

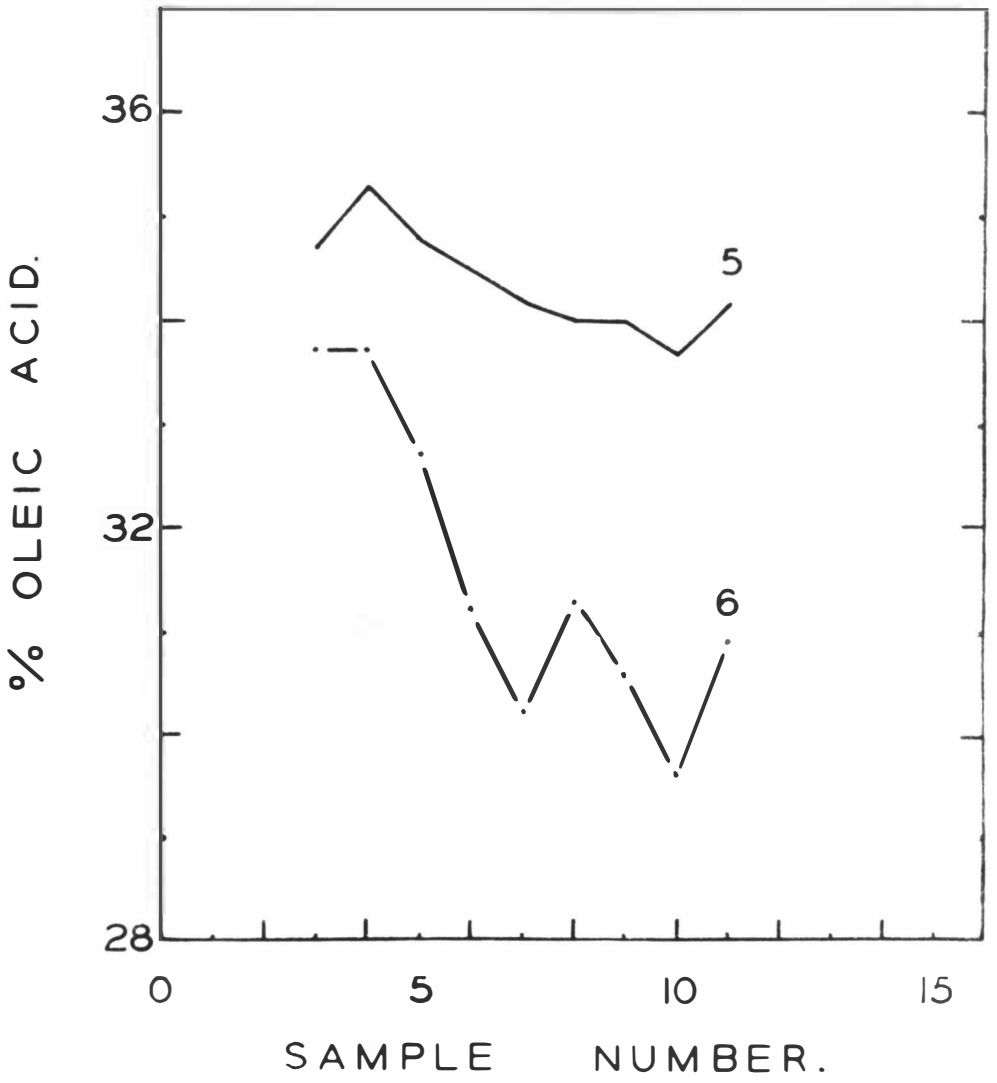

FIGURE 25.

SOURCE OF DATA: Tables 10,11, pp.81s,81t.

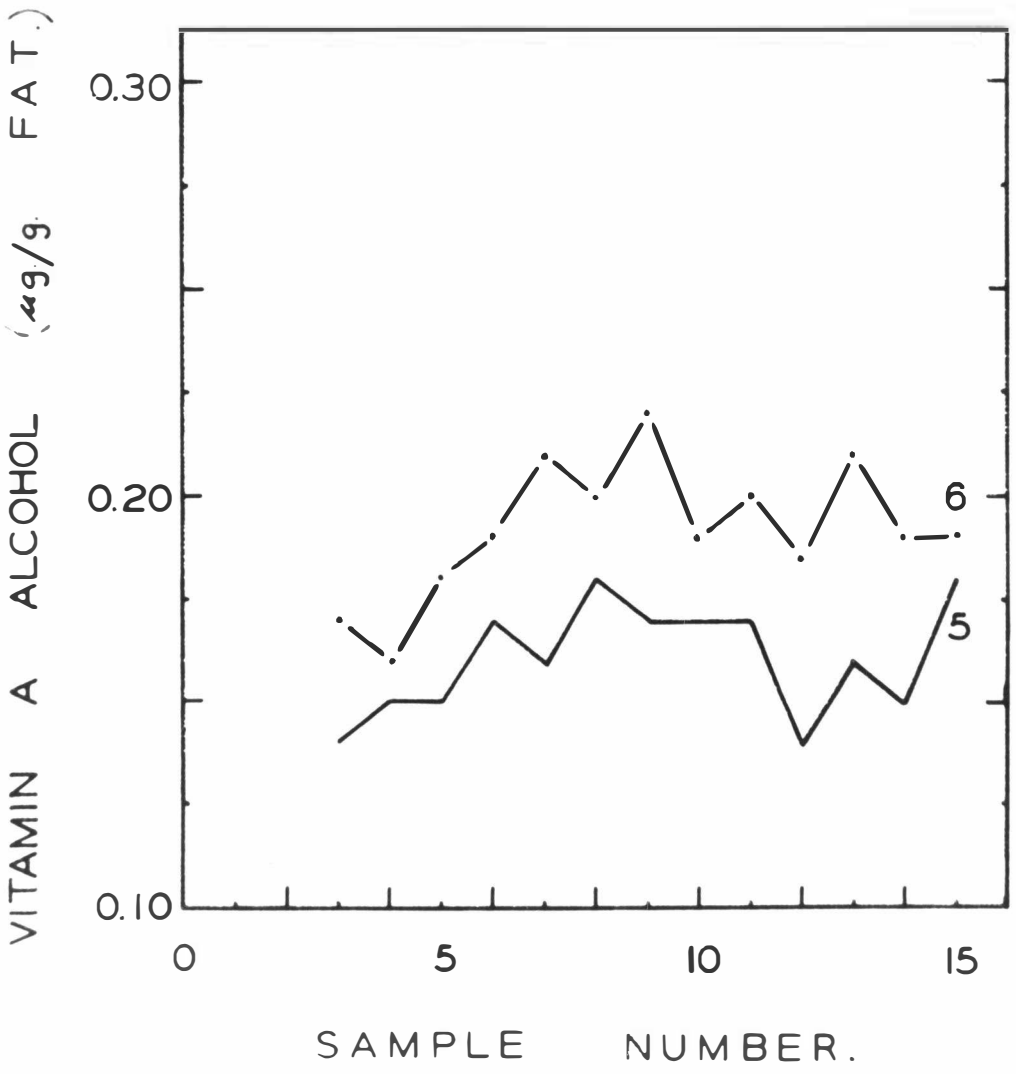

FIGURE 26.

SOURCE OF DATA: Tables 10,11, pp.81s,81t.

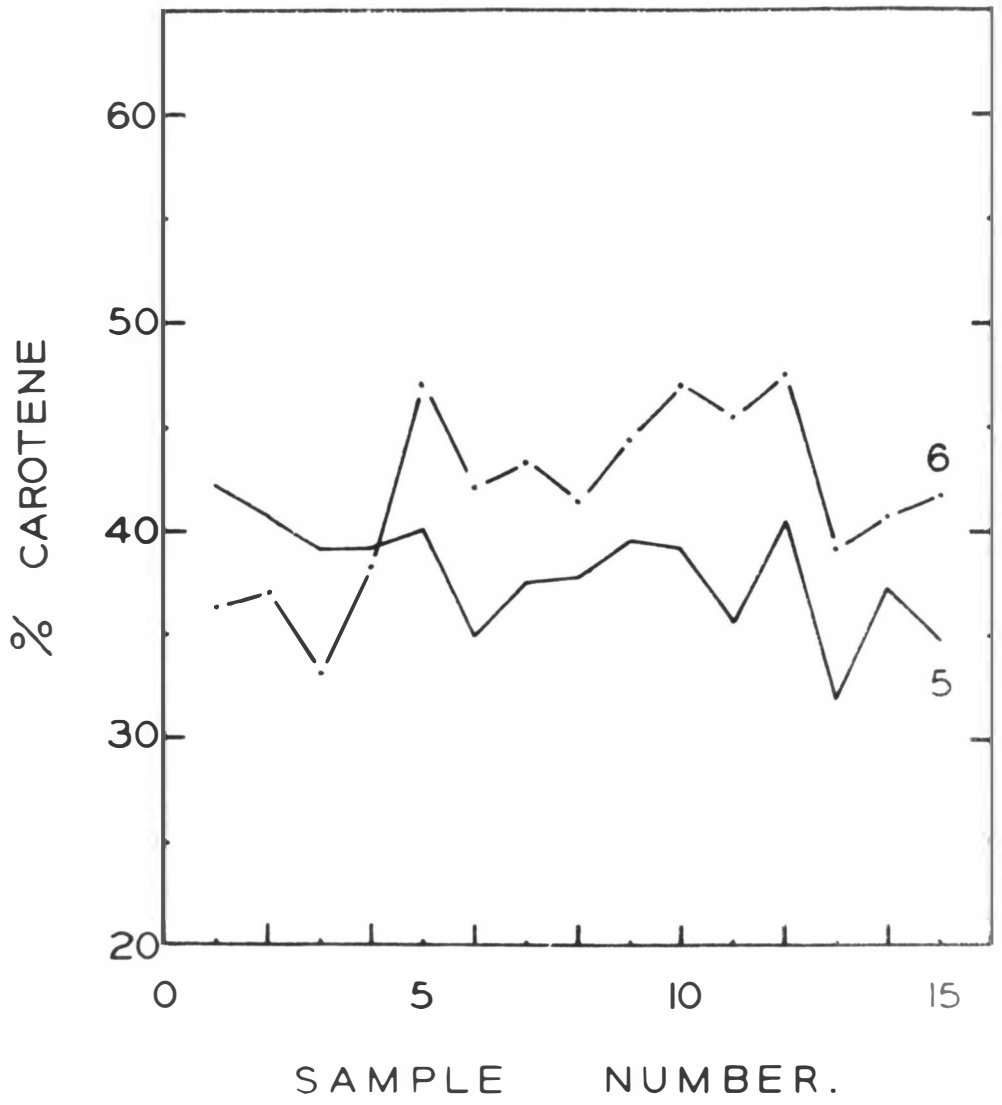

FIGURE 27.

SOURCE OF DATA: Tables 10,11, pp.81s,81t.

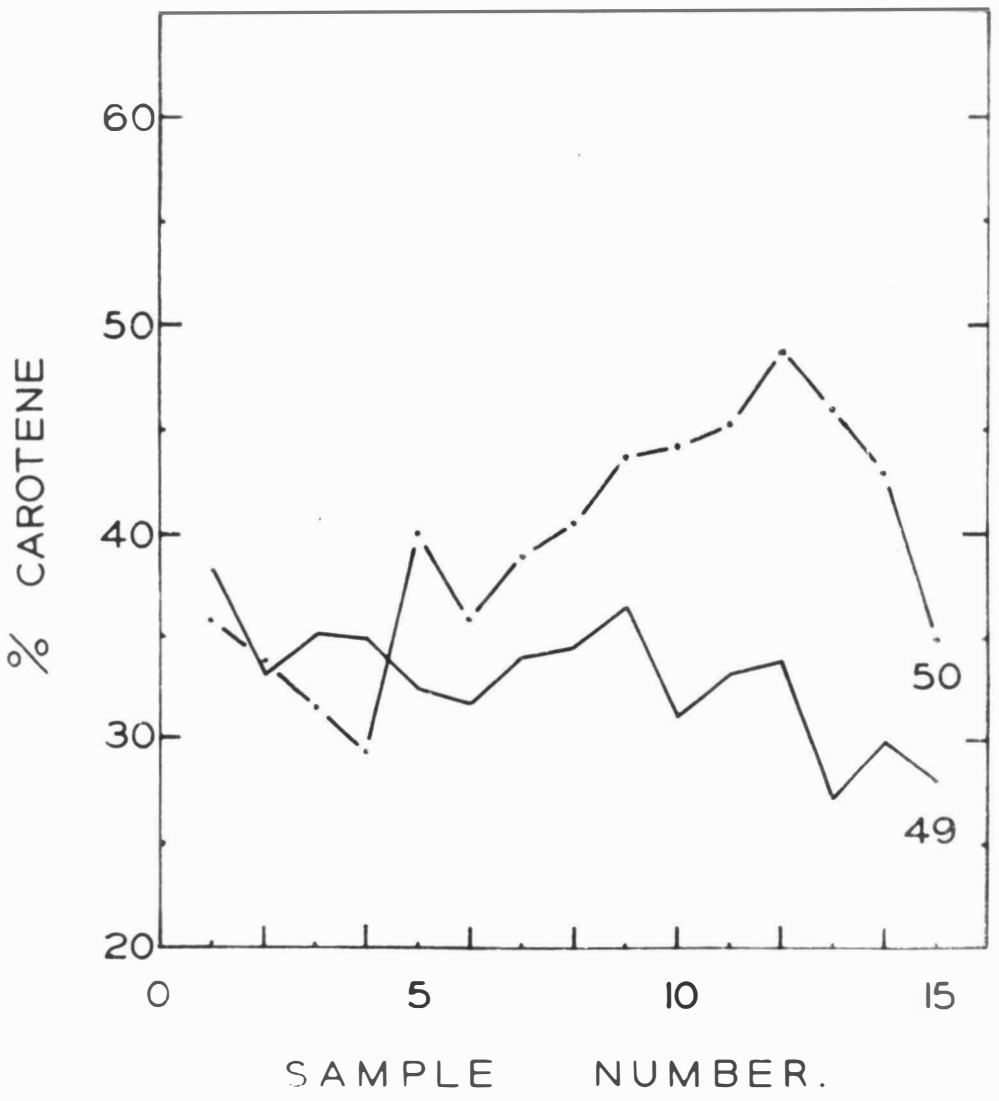

FIGURE 28.

SOURCE OF DATA: Tables 12,13, pp.81u,81v.

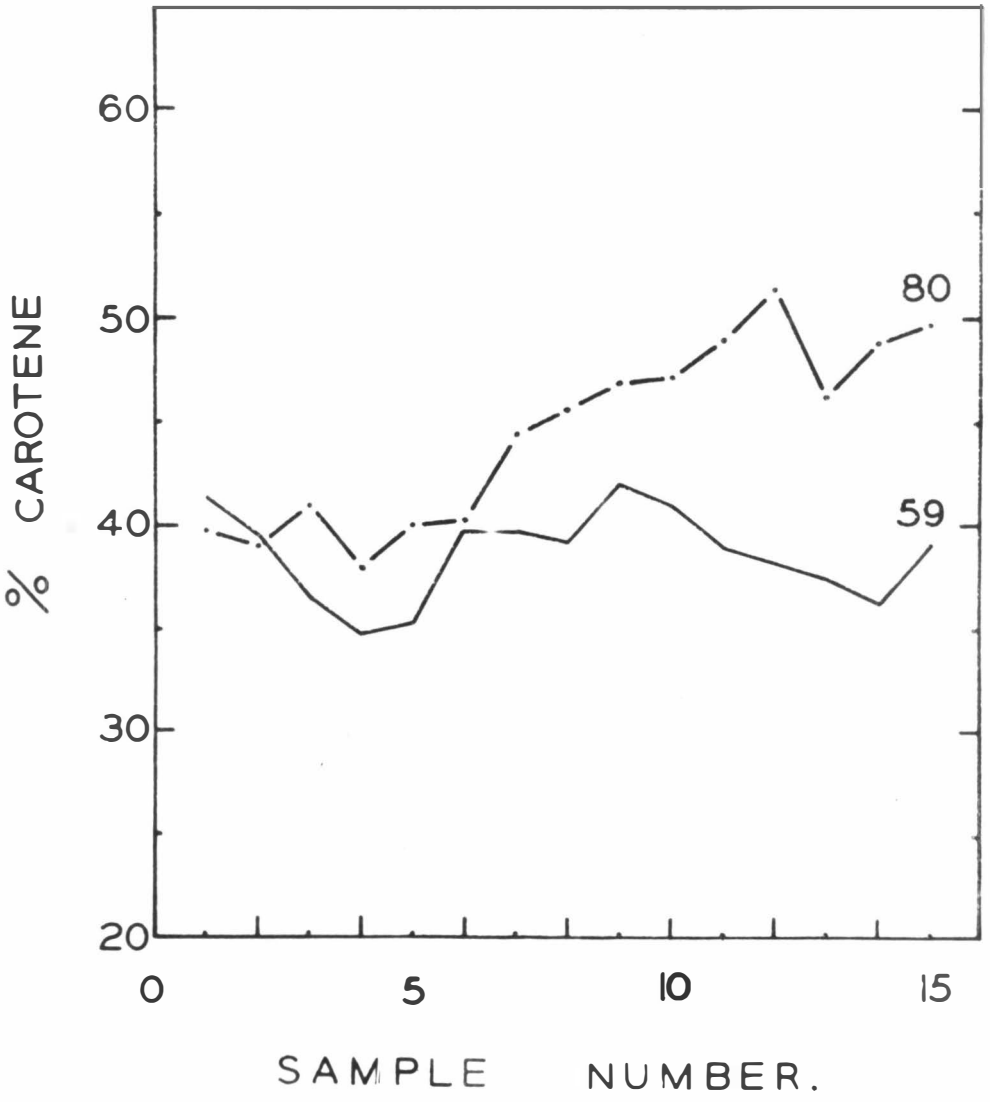

FIGURE 29.

SOURCE OF DATA: Tables 14,15, pp.81w,91x.

TWIN 5.

TABLE 10.

	VITAMIN A.		CAROTENE.		TOTAL POTENCY.	IODINE NUMBER.	OLEIC ACID %.	TOCOPHEROL.	VITAMIN A ALCOHOL.	PERCENTAGE CAROTENE.
	ug./g. fat.	I.U./g. fat.	ug./g. fat.	I.U./g. fat.	I.U./g. fat.			ug./g. fat.		
1.	5.88	18.82	8.25	13.79	32.61	37.9		50.0		42.3
2.	6.24	19.96	8.20	13.69	33.65	38.4		54.5		40.7
3.	6.05	19.36	7.52	12.56	31.92	38.0	34.7	52.5	0.14	39.3
4.	6.23	19.93	7.70	12.86	32.79	38.2	35.3	50.0	0.15	39.2
5.	5.98	19.14	7.67	12.80	31.94	37.7	34.8	48.0	0.15	40.1
6.	7.77	24.87	8.00	13.36	38.23	38.9	34.5	55.0	0.17	34.9
7.	6.90	22.09	7.93	13.24	35.33	37.3	34.2	51.5	0.16	37.5
8.	7.55	24.16	8.78	14.67	38.83	39.6	34.0	57.0	0.18	37.8
9.	7.02	22.46	8.81	14.72	37.18	37.7	34.0	58.0	0.17	39.6
10.	7.11	22.73	8.78	14.67	37.40	38.4	33.7	59.0	0.17	39.2
11.	7.19	23.00	7.67	12.81	35.81	37.4	34.2	58.5	0.17	35.8
12.	6.52	20.88	8.54	14.26	35.14	37.2		58.0	0.14	40.6
13.	7.93	25.38	7.15	11.95	37.33	37.1		57.5	0.16	32.0
14.	7.20	23.05	8.18	13.66	36.71	37.7		57.0	0.15	37.2
15.	7.36	23.55	7.57	12.65	36.20	37.9		52.5	0.18	34.7

TWIN 6.

TABLE 11

	VITAMIN A.		CAROTENE.		TOTAL POTENCY.	IODINE NUMBER	OLEIC ACID %.	TOCOPHEROL.	VITAMIN A ALCOHOL.	PERCENTAGE CAROTENE.
	ug./g. fat.	I.U./g. fat.	ug./g. fat.	I.U./g. fat.	I.U./g. fat.			ug./g. fat.		
1.	7.78	24.90	8.47	14.14	39.04	37.0		53.0		36.2
2.	7.11	22.75	8.01	13.38	36.13	36.3		52.0		37.0
3.	8.19	26.21	7.81	13.03	39.24	36.5	33.7	52.5	0.17	33.2
4.	6.60	21.12	7.80	13.04	34.16	36.5	33.7	49.0	0.16	38.2
5.	4.02	12.86	6.80	11.35	24.21	34.0	32.7	36.0	0.18	46.9
6.	4.60	14.71	6.42	10.72	25.43	34.5	31.2	39.5	0.19	42.2
7.	3.94	12.62	5.82	9.72	22.34	33.7	30.2	33.0	0.21	43.5
8.	4.91	15.72	6.64	11.09	26.81	34.0	31.3	49.5	0.20	41.4
9.	4.81	15.40	7.44	12.42	27.82	35.3	30.6	50.0	0.22	44.6
10.	4.13	13.21	7.12	11.89	25.10	33.3	29.6	45.5	0.19	47.4
11.	4.29	13.73	6.83	11.40	25.13	34.0	30.9	45.0	0.20	45.4
12.	4.25	13.59	7.41	12.37	25.96	34.3		46.0	0.17	47.7
13.	5.53	17.05	6.57	10.96	28.01	34.4		45.0	0.21	39.1
14.	5.35	17.12	7.06	11.79	28.91	35.4		47.5	0.19	40.8
15.	4.54	14.53	6.22	10.39	24.92	34.9		47.0	0.19	41.7

TWIN 49.

TABLE 12

	VITAMIN A.		CAROTENE.		TOTAL POTENCY.	IODINE NUMBER	PERCENTAGE CAROTENE.
	ug./g. fat.	I.U./g. fat.	ug./g. fat.	I.U./g. fat.	I.U./g. fat.		
1.	5.61	17.95	6.65	11.11	29.06	35.3	38.2
2.	6.28	20.09	6.01	10.03	30.12	35.7	33.3
3.	6.13	19.61	6.34	10.59	30.20	35.7	35.1
4.	5.98	19.13	6.16	10.28	29.41	35.1	35.0
5.	5.99	19.17	5.53	9.25	28.42	35.1	32.5
6.	7.05	22.56	6.27	10.46	33.02	35.4	31.7
7.	6.92	22.15	6.81	11.37	33.52	35.7	33.9
8.	7.23	23.13	7.29	12.17	35.30	36.5	34.5
9.	6.20	19.83	6.82	11.39	31.22	35.7	36.5
10.	7.09	22.68	6.20	10.35	33.03	36.3	31.3
11.	6.98	22.34	6.66	11.12	33.46	36.4	33.2
12.	6.70	21.45	6.53	10.91	32.36	35.7	33.7
13.	7.36	23.55	5.28	8.81	32.36	35.7	27.3
14.	7.69	24.60	6.26	10.45	35.05	35.8	29.8
15.	8.13	26.01	6.06	10.11	36.12	36.2	28.0

TWIN 50.

TABLE 13

	VITAMIN A.		CAROTENE.		TOTAL POTENCY.	IODINE NUMBER.	PERCENTAGE CAROTENE.
	ug./g. fat.	I.U./g. fat.	ug./g. fat.	I.U./g. fat.	I.U./g. fat.		
1.	6.38	20.42	6.81	11.38	31.80	34.9	35.8
2.	6.34	20.29	6.18	10.32	30.61	34.7	33.7
3.	7.13	22.82	6.25	10.44	33.26	34.8	31.4
4.	6.07	19.42	5.98	9.99	29.41	34.7	29.4
5.	4.43	14.18	5.64	9.43	23.61	34.3	40.0
6.	4.78	15.29	5.10	8.51	23.80	33.1	35.8
7.	4.54	14.52	5.57	9.30	23.82	33.4	39.0
8.	4.68	14.98	6.08	10.15	25.03	33.7	40.6
9.	4.18	13.37	6.26	10.44	23.81	33.0	43.9
10.	4.32	13.83	6.57	10.97	24.80	33.5	44.2
11.	3.70	11.84	5.88	9.82	21.66	32.2	45.3
12.	3.57	11.42	6.48	10.82	22.24	33.7	48.7
13.	3.86	12.34	6.33	10.56	22.91	34.1	46.1
14.	4.08	13.06	5.93	9.90	22.96	34.3	43.1
15.	5.10	16.33	5.84	9.75	26.08	34.9	37.4

TWIN 59.

TABLE 14

	VITAMIN A.		CAROTENE.		TOTAL POTENCY.	IODINE NUMBER.	PERCENTAGE CAROTENE.
	ug./g. fat.	I.U./g fat.	ug./g. fat.	I.U./g. fat.	I.U./g. fat.		
1.	7.20	23.05	9.68	16.17	39.22	38.0	41.2
2.	8.37	26.79	10.43	17.42	44.21	38.2	39.4
3.	8.85	28.32	9.77	16.32	44.64	38.6	36.6
4.	8.11	25.96	9.22	15.40	41.36	38.5	37.2
5.	8.68	27.79	10.07	16.82	44.61	38.8	37.7
6.	7.46	23.87	9.45	15.78	39.65	38.0	39.8
7.	7.75	24.80	9.82	16.40	41.20	37.9	39.8
8.	7.33	23.45	9.11	15.20	38.65	37.6	39.3
9.	7.79	24.93	10.82	18.07	43.00	37.9	42.0
10.	8.23	26.34	10.98	18.34	44.68	37.9	41.1
11.	8.05	25.76	9.87	16.48	42.24	37.4	39.0
12.	8.37	26.78	9.96	16.63	43.41	37.7	38.3
13.	8.80	28.16	10.12	16.88	45.04	37.8	37.5
14.	9.52	30.46	10.39	17.35	47.81	38.2	36.3
15.	8.92	28.55	10.99	18.35	46.90	38.0	39.1

TWIN 80.

TABLE 15.

	VITAMIN A.		CAROTENE.		TOTAL POTENCY.	IODINE NUMBER.	PERCENTAGE CAROTENE.
	ug./g. fat.	I.U./g. fat.	ug./g. fat.	I.U./g. fat.	I.U./g. fat.		
1.	7.31	23.39	9.24	15.43	38.82	37.5	39.7
2.	7.77	24.85	9.48	15.83	40.68	37.8	38.9
3.	7.78	24.90	10.38	17.33	42.23	37.9	41.0
4.	8.01	25.64	9.40	15.70	41.34	38.3	38.0
5.	6.11	19.55	7.81	13.05	32.60	38.0	40.1
6.	6.33	20.25	8.19	13.67	33.92	37.8	40.3
7.	5.25	16.80	8.06	13.47	30.27	37.5	44.5
8.	5.42	17.33	8.79	14.67	32.00	36.7	45.9
9.	5.27	16.87	8.94	14.93	31.80	36.6	46.9
10.	5.64	18.05	9.67	16.16	34.21	36.3	47.2
11.	4.98	15.94	9.14	15.27	31.21	36.6	48.9
12.	5.16	16.51	10.47	17.49	34.00	36.8	51.4
13.	5.58	17.86	9.19	15.35	33.21	36.2	46.2
14.	5.43	17.38	9.87	16.48	33.86	36.4	48.7
15.	5.13	16.41	9.74	16.26	33.67	36.1	49.8

TWIN 5.

TABLE 16.

PERCENTAGE FATTY ACID COMPOSITION OF BUTTERFATS.

SAMPLE NUMBER	DIENOIC		TRIENOIC		TETRAENOIC	
	C.	N.C.	C.	N.C.	C.	N.C.
3.	1.0	0.4	0.01	1.1	Nil	0.4
4.	0.9	0.3	0.01	1.2	Trace	0.3
5.	1.1	0.3	0.01	1.1	Nil	0.3
6.	1.2	0.4	0.02	1.1	Nil	0.3
7.	0.9	0.6	0.01	1.0	Trace	0.3
8.	1.2	0.5	Trace	1.0	Nil	0.4
9.	1.0	0.4	Trace	1.2	Nil	0.4
10.	1.2	0.4	0.01	1.1	Trace	0.4
11.	1.2	0.4	0.02	1.0	Trace	0.3

TWIN 6.

TABLE 19.

PERCENTAGE FATTY ACID COMPOSITION OF BUTTERFATS.

SAMPLE NUMBER	DIENOIC		TRIENOIC		TETRAENOIC	
	C.	N.C.	C.	N.C.	C.	N.C.
3.	1.1	0.3	0.01	1.0	Nil	0.3
4.	1.0	0.3	Trace	0.9	Nil	0.3
5.	1.2	0.5	Trace	0.9	Trace	0.2
6.	1.4	0.6	Trace	0.7	Trace	0.3
7.	1.5	0.6	Nil	1.0	Nil	0.3
8.	1.3	0.6	Trace	0.8	Trace	0.3
9.	1.4	0.6	Trace	1.0	Nil	0.3
10.	1.3	0.5	Nil	0.8	Nil	0.4
11.	1.5	0.5	Trace	0.7	Nil	0.2

(iii) Period 2.

During this period the even-numbered twins were fed cut clover (100% pure) while the odd-numbered twins remained on low clover pastures. The results show that, while the differences between high- and low-clover twins were maintained, they did not appear to be increased by the extra clover intake. These differences were analysed by means of the "t" test using the formula:

$$t = \frac{\bar{x}}{S_{\bar{x}}} \qquad \text{for } (n-1) \text{ degrees of freedom (240).}$$

and the following results were obtained:

(i) Total vitamin A potency,(10 d.f.):
Twins 5 and 6, t = 8.834**(11.3154±1.2809).
Twins 49 and 50,t =15.321**(9.3764±0.6120).
Twins 59 and 80,t =13.458**(10.6800±0.7936).

(ii) Vitamin A Content,(10 d.f.):
Twins 5 and 6, t = 3.120* (8.2445±2.6421).
Twins 49 and 50,t = 3.085* (8.7554±2.8378).
Twins 59 and 80,t = 3.042* (8.9036±2.9274).

(iii) Carotene Content,(10 d.f.):
Twins 5 and 6, t = 2.979* (2.2445±0.7534).
Twins 49 and 50,t = 1.486NS(0.6127±0.4122).
Twins 59 and 80,t = 2.673* (2.0082±0.7514).

(iv) Iodine Number,(10 d.f.):
Twins 5 and 6, t = 6.145**(2.0045±0.3262).
Twins 49 and 50,t = 5.471**(1.5340±0.2804).
Twins 59 and 80,t = 6.210**(1.0640±0.1713).

(v) Tocopherol Content, (10 d.f.):
Twins 5 and 6, $t = 10.239^{**}(11.5909 \pm 1.132)$.

(vi) Vitamin A Alcohol Content, (10 d.f.):
Twins 5 and 6, $t = 3.750^{**}(0.0136 \pm 0.0036)$.

(vii) Oleic Acid %. (6 d.f.):
Twins 5 and 6, $t = 7.458^{**}(1.9714 \pm 0.2643)$.

As for the previous period, milk production was unaffected by treatment except over the last three days (samples 14 and 15) when the production from the even-numbered twins fell considerably.

.....

II. INTERPRETATION.

The results from section I of the experiment confirm the low summer potencies and iodine values reported previously for New Zealand butterfats (194,217,218). At the same time they also draw attention to the relatively large variations present in the vitamin A potency and iodine number of bi-weekly summer butterfat samples. None of these variations could be shown, however, to be due to changes in clover intake. In planning the experiments it was thought that the variations in clover content noted between paddocks would be sufficiently large to be reflected by significant changes in fat potency, and by this means it was hoped to establish a reciprocal relationship between

the clover content of the pasture on the one hand and the vitamin A potency of the fat produced from it, on the other. Unfortunately it was not possible to establish this relationship and considerable doubt arose as to whether the effect of clover was a real one or not. That no relationship could be established was possibly due to the true effect of clover being masked either (1) by the rapidity with which the level of clover intake varied; (2) by the high carotene content of the clover relative to that of the grass (appendix 3); or (3) by some other uncontrolled extraneous factor. Throughout the experimental period, however, weather conditions were fine and warm, pasture was plentiful, and production remained relatively constant. Furthermore, the level of carotene present in the pasture was shown to be adequate for the production of fat of maximum potency. It would appear, therefore, on the basis of these observations, that the variations noted can be explained only by assuming the presence (or absence) of some factor in the pasture affecting availability.

The results from section II of the experiment, strongly indicate this factor to be closely associated with the clover content of the pasture since diets high in clover markedly decreased fat potency, iodine number, and the contents of oleic acid and tocopherol, whereas diets low in clover had the opposite effect. One further observation of significance was the rapidity with which changes in fat potency responded to variations in clover intake. For example, the

potency of the fat from the even-numbered twins during the first 24 hours on high clover pastures (period 1) fell, on an average, 8.2 I.U./g. fat while the potency of the fat from the odd-numbered twins on low clover pastures rose, on an average, 4.1 I.U./g. fat. These observations would suggest the availability of carotene to be affected by the presence of some water-soluble factor which is rapidly absorbed into the system, possibly via the ruminal wall, since the effect of any factor absorbed through the intestinal wall would not be expected to become apparent so soon. On this basis the low Summer potencies of New Zealand butterfat would appear to be due primarily not to a low intake of tocopherol (which is fat soluble and therefore absorbed through the intestinal wall in the normal course of digestion but to some other factor, present in clover, which has a much more immediate effect on the availability of carotene from the pasture.

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CHAPTER FOUR.

DISCUSSION AND INTERGRATING SUMMARY.

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Variations in the total vitamin A potency, in the carotene and vitamin A contents, and in the iodine number of New Zealand butterfat are of particular interest because of (i) the regularity of the seasonal changes which have been reported, (ii)the difference in the nature of the trend reported here and that reported overseas, and (iii) the fact that the carotene content of pastures throughout the year has been shown to be adequate for the production of fat of maximum potency.

Several factors may possibly be involved in causing these variations. The production of butterfat in New Zealand is highly seasonal, the majority of cows being calved down in early spring (July - August), 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 (241) has shown, however, that total vitamin A secretion (i.e. total potency x weight of fat produced) follows the same general trend as total potency (fig.30). Furthermore, Barnicoat (194) could find no difference between the carotene and vitamin A

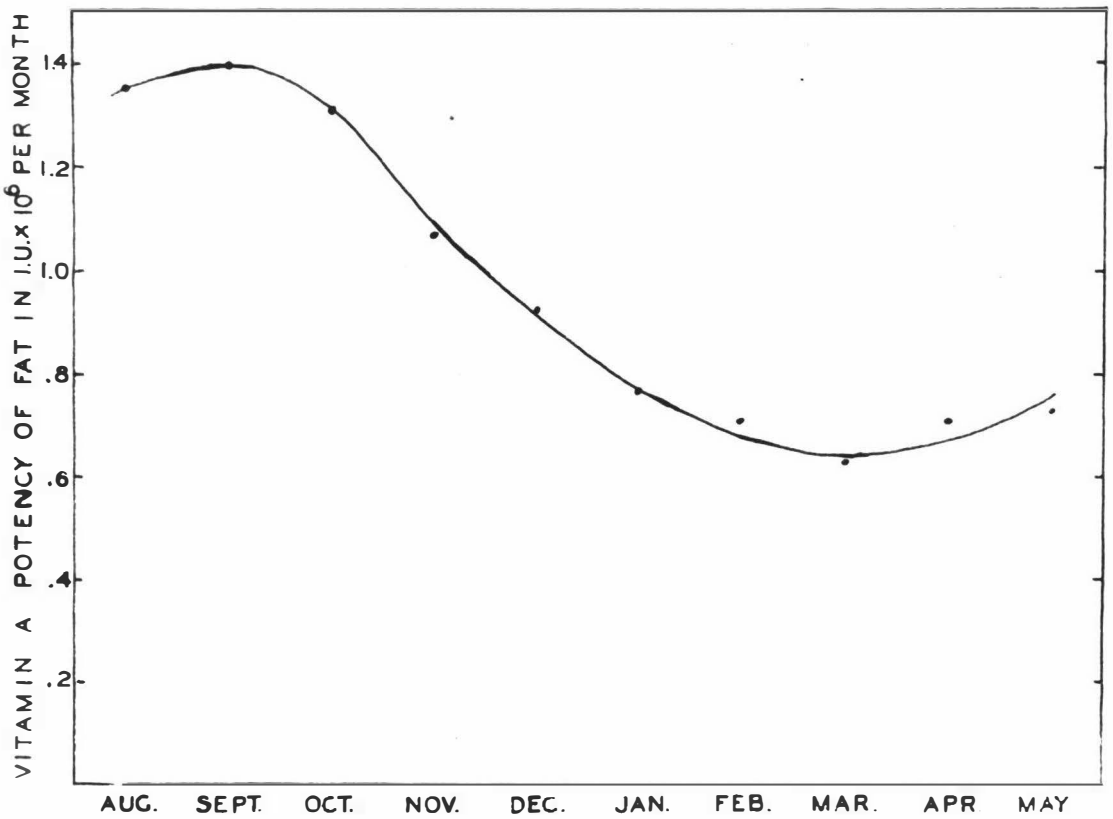

FIGURE 30.

(Seasonal Trend in Total Vitamin A Secretion).

contents of butterfats drawn simultaneously from spring- and autumn-calvers kept under the same feeding and management conditions.

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 McDowell and McDowall (218), who concluded that:

> "When account is taken of the possible proportion of early post-parturition fat in the butter.....it is obvious that the high 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 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. 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..... It may be concluded, therefore, that the peculiar seasonal changes in vitamin A potency of New Zealand butterfat are not caused solely by purely lactational influences".

The claim by Chanda (184) to have established a parabolic relationship between vitamin A content and stage of lactation has already

been discussed. In a preliminary abstract (242) the statement was made that "all animals were receiving winter rations". It has since been found, however, (243) that this was so only up until the 30th week of lactation after which the cows went out on to spring pastures which provided a carotene intake of 2000mg. to 4000mg. per day. It appears, therefore, that the curve published by Chanda (184) is a normal seasonal curve representing variations in the vitamin A content which are characteristic of Northern Hemisphere butters.

Barnicoat (194) has suggested that low summer potencies might be associated with the drying up of the pastures following the spring flush. If this were the case then a rapid rise in the total potency and in the carotene and vitamin A contents of butterfat would be expected soon after the first rains following a summer drought. No such rise was apparent, however, in figures published by McDowell and McDowall (218) for four North Auckland factories during the 1946-7 season when drought conditions prevailed.

McGillivray (217) has shown that a tocopherol supplement fed to cows in mid-summer is capable of raising the potency of their fat to a normal winter maximum. It is desirable, however, that this work should be repeated, preferably in the winter, to determine whether the effect which he obtained was a general "sparing" effect, or whether it was a specific effect due to a lack of tocopherol in the pasture. In his

discussion, McGillivray draws attention to the emergence of clover as the dominant summer pasture species and suggests that the low potencies of New Zealand summer butterfat may be associated with this trend.

In the present series of experiments the clover content of pasture has been clearly demonstrated to have a marked effect on the composition of the butterfat produced. Thus, the total vitamin A potency and the contents of both carotene and vitamin A have been shown to be significantly decreased by a high clover intake and significantly increased by a low clover intake. Similarly, the iodine number, the oleic acid and tocopherol contents, and to a lesser extent the vitamin A alcohol content, have likewise been shown to be affected. The most significant feature of these variations was the rapidity with which they occurred following changes in clover intake and, as has been previously pointed out, this indicated the presence of some water-soluble factor which was rapidly absorbed into the system possibly via the ruminal wall.

At the present time, the mechanism by which diets high in clover depress vitamin and provitamin secretion from the mammary gland is not clear. There would, however, appear to be three possibilities. In the first place, McGillivray (217) 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 the 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 fat by tocopherol supplementation, would suggest that a low tocopherol level in the pasture is perhaps partly responsible for the summer decline in potency. That tocopherol is not wholly responsible, however, is indicated by the fact that the effect of supplementation was not definitely apparent in McGillivray's results for some seven days following dosing, whereas in the present study the effect of a change of clover intake was clearly noticeable within 24 hours.

In the second place, it is noteworthy that a very high proportion of the clover in the pastures during section I and section II (period 1) of the experimental work, and of the clover fed during section II (period 2) was high-HCN white clover. It is tentatively suggested, therefore, that HCN, which is liberated from white clover by enzymic and microbial hydrolysis in the rumen, may possibly be a further factor contributing to the decreased availability of carotene over the summer period. There are several <u>a priori</u> grounds on which to base this assumption. Firstly, Coop and Blakely (244,245) have shown the release, the absorption (directly via the rumen), and the detoxication of HCN to thiocyanate in the liver to be a comparatively rapid process, in ruminants. Secondly, Barker (246) and others (247, 248) have shown thiocyanate to possess anti-thyroid properties; and, thirdly, as has already been discussed, Morton and his team at Liverpool (168) and Chanda et al at the Hannah Research Institute (171,172) 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, therefore, possible that the decreased availability of carotene from summer pastures is associated with, and due in part to, a lowered thyroid function resulting from higher-than-normal levels of blood thiocyanate. It is hoped that when the results of experiments at present being planned in this laboratory to test this hypothesis come to hand, this particular aspect of the problem will be more completely understood.

In the third place, since carotene is a fat-soluble pigment it is reasonable to assume that its availability from a pasture depends to a certain extent on the fat content of the pasture. The possibility must be considered, therefore, that the low availability of carotene from summer pasture may also be associated with a decrease in the fat content of the pasture at this time. Unfortunately, little information is available concerning the seasonal variation in the fat content of New Zealand pastures. It is well established, however, that the higher fatty acids of milk fat (i.e. C_{16}-C_{18}), of which the most important is oleic acid, are derived directly from the cow's ration, while the lower fatty acids are synthesised within the gland from "active" C_2 units (249,250,251). In the present study the oleic acid content of butterfat was shown to be highly correlated with total potency. Total potency, however,

has been shown elsewhere* to be highly correlated with carotene intake so that is is logical to assume that carotene will be associated with oleic acid during absorption. The oleic acid content of butterfat would appear, therefore, to be a useful relative measure of the availability of carotene in the ration. Hansen and Shorland (252), and McDowell (253) working independently with New Zealand butters, have shown the oleic acid content throughout the season to be lowest in November and January; i.e. when the clover content of pasture is highest. Similarly, in section II of the present experiments the oleic acid content of the fat from the even-numbered twin (6) on high clover pasture was noted to be significantly lower than that from the odd-numbered twin (5) on low clover pasture (fig.25). It is suggested on the basis of these observations that the low availability of carotene from summer pastures is possibly due, at least in part, to a decrease in the content of oleic (or other C_{16-18}) acid in the pasture at this time. Whether this is the result of a general decrease in the oleic acid content of the pasture as a whole (associated, perhaps, with the increase in clover content), or whether it is the result of a decrease in the availability of oleic acid from the pasture

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* McGillivray (254), for example, has recently shown that for the production of butterfat of high vitamin A potency cows are mainly dependent on a reasonable daily intake of carotene (or vitamin A), rather than on high liver stores, which appear to be relatively unavailable for this purpose. Thus most of the carotene and all of the vitamin A ester (which represents the bulk of the vitamin A) in the milk fat can be assumed to be derived directly from the cow's ration.

(associated, perhaps, with a lowered thyroid activity and a decrease in absorption), is not, however, known.

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CONCLUSION.

In the present series of experiments it has been demonstrated that the clover content of summer pasture may have a marked effect on the composition of the butterfat produced. It is not clear, however, whether this effect is due, to a low tocopherol content in the clover, to a low oleic acid content, to a high HCN content, or, whether indeed it is due specifically to clover at all, for the further possibility must be considered that it may be caused by an accumulation of some 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, even though the odd-numbered twins during section II of the experiment were grazing pasture containing approximately the same amount of clover as average winter pasture (227,228), the vitamin A potency of their fat was considerably lower than that of winter fat, which would seem to indicate that some factor in the grass rather than in the clover was interfering with the normal utilization of carotene.It is also not clear whether an effect similar

to that obtained in the present study may be produced by high intakes of other clover species(such as, for example, red clover, low-HCN white clover, ladino clover), or whether the effect is specific for high-HCN white clover only. It is of interest to note, however, that in a recent experiment in which the availability of carotene from different hays was compared, Loosli, Krukovsky, Lofgreen and Musgrave (257) have shown, that although the carotene content of ladino clover hay was higher than that of the other hays investigated, the plasma vitamin A and milk fat levels of cows were lowest when ladino clover hay was fed.

Further experiments are at present being planned in this laboratory to investigate more fully the relationship between clover intake and the vitamin A potency of butterfat, and in particular the mechanism by which diets high in clover depress fat potency. It is to be hoped that when the results of these experiments are forthcoming, additional light will be shed on the problem of the summer decline in the vitamin A potency of New Zealand butterfat.

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SUMMARY OF CHAPTERS
THREE AND FOUR.

(1) A brief review of literature relating to factors affecting the vitamin A potency of butterfat in general, and the summer decline in the vitamin A potency of New Zealand butterfat in particular, has been made, and the significance of clover as the dominant species of New Zealand summer pastures has been noted.

(2) The results of experiments designed to establish some relationship between the level of clover intake and the composition of the butterfat produced are presented. Diets high in clover were shown to depress carotene, vitamin A, total potency, iodine number, oleic acid, and to a lesser extent vitamin A alcohol, whereas diets low in clover were shown to have the opposite effects. The level of clover in the diet appeared to have no effect on the contents of di-, tri-, and tetraenoic fatty acids in the butterfat. Significant correlations were established between; (a) total potency, and, vitamin A, carotene, tocopherol, oleic acid, and iodine number; (b) tocopherol content, and, carotene and oleic acid; (c) iodine number and oleic acid.

(3) The mechanism by which diets high in clover depress fat potency, iodine number and oleic acid content is not clear. It is suggested, however, that it may be associated with (i) the low tocopherol content of clover, (ii) the possibly lower

oleic acid contents, and (iii) the high HCN content of certainspecies. The possibility is also considered that it may be associated with the stage of development of the pasture as a whole, in which clover is the dominant species present.

(4) Further work is being planned with a view to investigating these possibilities more fully.

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APPENDIX 1.

BUTTER AND MILK AS SOURCES OF VITAMIN A IN THE HUMAN DIET.

NEW ZEALAND.

Annual average consumption butter	= 44 lbs./year(256)
	= 55 grams/day
Assuming butter to contain 80% fat.	= 44 " fat/day
and assuming potency of fat to be 40 I.U./g.	= 1760 I.U./day.
Annual average consumption whole milk	= 270 kg. (256)
Assuming milk to contain 5% fat (40 I.U./g.fat)	= 1480 I.U./day.
Total	= 3240 I.U.
or	64.8%.

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AUSTRALIA.

Annual average consumption butter	= 30 lbs. (256).
Assuming butter to contain 80% fat.	= 30 grams fat/day
and assuming potency of fat to be 40 I.U./g.	= 1200 I.U./day.
Annual average consumption whole milk	= 190 kg. (256)
Assuming milk to contain 5% fat (40 I.U./g.fat)	= 1040 I.U./day
Total	= 2240 I.U./day
or	44.8%.

SWEDEN.

Annual average consumption butter	= 25 lbs./year (256)
Assuming butter to contain 80% fat.	= 25 grams/day.
and assuming potency of fat to be 34 I.U./g.	= 850 I.U./day
Annual average consumption whole milk	315 kg./year
Assuming milk to contain 4% fat (34 I.U./g.fat)	= 1175 I.U./day
Total	= 2025 I.U./day
or	40.5%

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CANADA.

Annual average consumption butter	= 22 lbs. (256)
Assuming butter to contain 80% fat	= 22 grams/day
and assuming potency of fat to be 34. I.U./g.	= 748 I.U./day
Annual average consumption whole milk	= 237 kg (256)
Assuming milk to contain 4% fat (34 I.U./g.fat)	= 882 I.U./day
Total	= 1630 I.U./day
or	32.6%

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UNITED STATES OF AMERICA.

Annual average consumption butter	= 9 lbs./year(256)
Assuming butter to contain 80% fat	= 9 grams fat/day
and assuming potency of fat to be 34 I.U./g.	= 306 I.U./day
Annual average consumption whole milk	= 253 kg (256)
Assuming milk to contain 4% fat (34 I.U./g.fat)	= 944 I.U./day
Total	= 1250 I.U./day
or	25.0%

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UNITED KINGDOM.

Annual average consumption butter	= 10 lbs./year (256)
Assuming butter to contain 80% fat	= 10 g./day
and assuming potency of fat to be 34 I.U./g.	= 340 I.U./day
Annual average consumption whole milk	= 220 kg. (256)
Assuming milk to contain 4% fat (34 I.U./g.fat)	= 820 I.U./day
Total	= 1160 I.U./day
or	23.2%

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APPENDIX 2.

The analysis of variance to determine the minimum size and minimum number of samples necessary to give an accurate estimate of clover percentage was carried out by Mr. A.C.Glenday, biometrician, of the Grasslands Division. A 24 " sample was arbitrarily decided on as a convenient area to sample and the first problem was to decide what shape the plot should be, e.g.,12" x 2", 8" x 3", or 6" x 4". In a pilot trial the standard errors of 100 samples (bagged and sorted independently) per paddock, cut from within three wire frames of the above shape were shown to be 5.8, 4.1, and 1.4, respectively. Thus a 6" x 4" frame was chosen for sampling purposes.

The number of samples necessary per paddock required to give an estimate of the clover percentage (within 5%) was calculated from the equation:-

$$n = \frac{t^2 s^2}{\bar{x}^2} \qquad (240)$$

Where n = number of replications.
$\bar{x}$ = mean of samples.
s^2 = variance.
t = specified probability of "t".

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APPENDIX 3.

At odd times throughout the course of the experimental work determinations were carried out on pure ryegrass and pure clover (sampled simultaneously from adjacent areas), with a view to comparing their carotene contents. The following results were obtained.

TABLE 18.

CLOVER. µg./g.	GRASS. µg./g.
250.5	237.0
381.0	361.5
376.5	349.0
403.5	351.0
280.0	299.5
297.5	267.5
466.5	312.0
323.5	331.5
440.0	410.5
480.0	437.5
403.5	398.5
211.0	256.5
476.0	401.5
370.0	319.5
335.5	287.0
358.0	321.0

With the exception of one or two odd samples, the carotene content of the clover in the pastures throughout the experimental period appeared to be somewhat higher than ryegrass.

APPENDIX 4.

During the winter (May) of the 1954-5 season a pilot experiment was conducted using two cows from the Massey College herd to determine the effect of a high clover diet on the carotene and vitamin A contents of the blood and milk fat. The cows were grade Jerseys and were nearing the end of their lactation. Daily variations were established over a four day pre-treatment period during which blood samples were obtained after morning milking and milk samples after the morning and evening milkings. Following the pre-treatment period high-HCN white clover (with some red clover) was cut and fed once a day for four days during which time milk and blood samples were obtained as above.

RESULTS.

The results, presented in tables 19 and 20, were analysed statistically and the following differences were obtained:

	COW 2.	COW 34.
(a) Blood, 4 d.f.		
Vitamin A alcohol	1.354 NS	4.375*
" " ester	1.883 NS	2.003NS
Carotene	1.401 NS	4.359*
(b) Milk Fat, 14 d.f.		
Carotene	3.885**	5.090**
Vitamin A	- N.S	4.530**

The results of the trial generally speaking were inconclusive and it is obvious that a larger trial involving more cows for a longer period, preferably grazing clover *in situ*, is required. It is of interest, however, to note the very highly significant drop in the carotene and vitamin A content of the milk fat even though the yield of milk throughout the trial declined rapidly, and even though the levels of the vitamin A present were very low.

If differences of a similar magnitude could be established over a longer period during the winter using more animals, it would suggest that the depressant effect of clover is a permanent property of the plant and not one that is necessarily associated with any particular season of the year or with any particular stage of development.

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COW 2. TABLE 19.

BLOOD LEVELS	VITAMIN A ALCOHOL . I.U./100ml.	VITAMIN A ESTER. I.U./100ml.	CAROTENE. µg./100ml.
(a) Pre-treatment.			
	107	20	1380
	112	22	1380
	113	20	1400
(b) Treatment.			
	106	21	1080
	102	9	1340
	111	7	1360

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COW 34.

(a) Pre-treatment.			
	105	11	1550
	109	12	1660
	108	13	1620
(b) Treatment.			
	94	11	1400
	89	10	1260
	79	7	1410

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COW 2. TABLE 20.

MILK FAT LEVELS.	CAROTENE µg./g.	VITAMIN A. µg./g.
(a) Pre-treatment.		
	10.1	4.5
	10.8	4.5
	10.0	4.9
	10.3	4.9
	11.5	5.7
	10.2	5.1
	10.2	5.3
	11.2	5.2
(b) Treatment.		
	8.9	4.6
	9.8	4.3
	9.5	5.0
	9.0	4.9
	10.0	5.0
	9.8	4.8
	-	-
	9.7	4.7

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COW 34.

(a) Pre-treatment.		
	10.6	4.3
	11.6	4.0
	11.0	4.5
	11.9	4.3
	11.7	4.8
	11.2	4.4
	10.8	4.6
	11.2	4.4
(b) Treatment.		
	9.7	3.8
	10.3	3.8
	8.0	3.7
	10.0	4.1
	8.1	3.8
	10.3	3.9
	8.2	4.2
	10.4	3.7

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