A HISTOLOGICAL METHOD FOR THE BIOLOGICAL ESTIMATION OF VITAMIN A.

THESIS

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by

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PART I.

REVIEW OF LITERATURE.

Introduction.

There are considerable differences between physical-chemical and biological results of Vitamin A estimations, owing to the fact that "the analyst determines the proportion of a substance present, whilst the bioassayist determines an effect" (202).

The biological vitamin A value of food or pharmaceutical products is the sum of the values of all substances present having vitamin A activity, such as preformed vitamin A, either as the free alcohol or esters, and various carotenoid pigments. The response of the experimental animal to a given dose of test substance is dependent upon the absorption and utilization of vitamin A and its precursors and is influenced by the digestibility of the food and the absence or presence of antioxidants. Physical-chemical estimations of the vitamin A content of a product, on the other hand, are dependent on the nature and number of compounds present revealing absorption in that region of the ultra-violet spectrum where vitamin A shows its maximal light absorption and the relative biological activities of these substances.

To convert extinction coefficients, as determined by physical-chemical means, to biological units a conversion factor was adopted. This factor, however, is chiefly based on the results of the curative "growth" method which is non-specific for vitamin A, but which is more accurate than the vaginal content and xerophthalmia method. The work presented in this thesis was undertaken as an attempt to work out a biological method for
the estimation of vitamin A based on the degeneration of the central nervous system, which was found to be a specific deficiency system for this vitamin (131,139).

The vitamin A potencies of the following oils were determined:
1. Vaalhaisi (Galeorhinus capensis) liver oil;
2. Vitamin A acetate in arachis oil;
3. Vitamin A distillate in arachis oil;
4. Seabuck (Thyrsoites atum) liver oil;
5. Mackerel (Merluccius capensis) liver oil;
6. Tuna (Germa albacora) liver oil;
7. Dogfish (Squalus species) liver oil;
8. Stonebaas (Polyprion americanus) liver oil.

The results were analysed statistically to obtain the limits of error for this histological method and corresponding conversion factors were calculated to test out the reliability of the present conversion factors.

**REVIEW OF THE CHEMISTRY AND PHYSIOLOGY OF VITAMIN A.**

**Chemical Properties.**

The history of vitamin A dates back to 1913 when McCollum and Davids (164) recognised the existence of a fat-soluble factor essential for growth. Five years later Mellanby (170, 171, 172) was able to produce experimental rickets in puppies by withholding from their diet a substance (the anti-rachitic vitamin), which he was inclined to identify with the fat-soluble factor of McCollum and Davis owing to the similarity of its distribution and properties.
Observations gradually accumulated which indicated that two fat-soluble accessory food factors were present. McCollum, et al. (167) and Steenbeck and Nelson (248) were unable to demonstrate the antixerophthalmic action of cod liver oil after oxidation for 12 to 20 hours at 100°C, whereas its antirachitic action was still present. Similarly, Goldblatt and Zilva (79) found a marked difference in the rate of destruction by heat and oxidation of these two factors in cod liver oil. A more prolonged and drastic treatment was necessary to decompose vitamin D than would affect the destruction of vitamin A.

McCollum and his co-workers also found that vegetable fats were lacking in fat-soluble vitamin A but contained a substance which stimulates the deposition of calcium salts in rickets in a manner similar to cod liver oil.

Another most important discovery was made in 1919 when Steenbeck (247) pointed out the relationship between the plant carotenoids and their vitamin A activity. However, it was in 1930 that his findings were confirmed for the first time by von Euler and his associates (261, 262, 283), who demonstrated the vitamin A activity of crystalline carotene. The real nature of this relationship was cleared up in the following year when Moore (192, 193) showed that carotene acts as a provitamin and is converted into vitamin A in the animal body. The final solution to this problem came when Karrer and his co-workers (140, 141) and Kuhn and Brockmann (146, 146, 147, 148) determined the chemical structure of both vitamin A and the members of the carotenoid group and showed the vitamin A activity of the latter.
Owing to the close relationship between vitamin A and its precursors it is impossible to discuss only the chemistry and physiology of the former without mentioning that of the latter compounds.

Attempts to isolate vitamin A or to prepare its crystalline derivatives failed until 1937, when Holmes and Gerret (116) obtained crystalline vitamin A by recrystallization of the unsaponifiable matter of fish liver oil from methyl alcohol at low temperatures. These crystals melted at 7.5 - 8°C., but contained solvent of crystallization according to Baxter and Robeson (12). These workers prepared crystalline vitamin A, melting at 63 - 66°C., by using ethyl formate as solvent instead of methyl alcohol. Several vitamin A esters, including the acetate, palmitate and divitamin A succinate, have also been prepared by them. Before this time Head, et al. (168), however, prepared crystalline esters, namely, vitamin A anthraquinone-2-carboxylate and vitamin A-naphthoate.

Vitamin A and the carotenoids possess two outstanding physico-chemical properties. The first is the brilliant blue colour reaction with a saturated solution of antimony trichloride in chloroform, varying in intensity with the vitamin A and/or carotene content on which the well-known Carr-Price test is based (32). The second is their ability to absorb ultra-violet rays: this forms the basis of the spectrophotometric test (303). Besides providing two rapid and convenient methods for estimation, these two characteristic properties were used successfully to investigate the purity of crystalline vitamin A and its esters, as well as to advance our knowledge regarding the distribution
of this vitamin and related compounds in natural products.

Examining the blue colour of the Carr-Price reaction spectrophotometrically, vitamin A and its esters show two absorption bands with maxima at 620 m\(\mu\) and at approximately 683 m\(\mu\) (203, 108, 109). In the case of low-potency oils with a high amount of irrelevant absorbing substances, these maxima are usually displaced to 603 - 606 m\(\mu\) and 572 m\(\mu\) respectively. Saponification of these oils removes most of these interfering substances and for this reason it is preferable to use the unsaponifiable matter when the estimation is carried out with the colour test.

Two other bands that are of considerable importance show maxima at 650 m\(\mu\) and 693 m\(\mu\) and derive from vitamin A\(_3\). It is present only in traces in mammalian liver oils; varies in amount in different fish liver oils and predominates in liver oils from fresh water fishes. The influence of irrelevant absorbing substances plays an important role in the accurate determination of vitamin A (150, 62) and will be dealt with later.

Horton and Heilbron, (198) showed in 1928 that vitamin A exhibits a broad absorption band in the ultraviolet spectrum with a maximum at 328 m\(\mu\) for vitamin A esters and at 225 m\(\mu\) for vitamin A alcohol. This and later work (34, 285, 215) showed it to be a characteristic property of vitamin A and resulted in the spectrographic method of estimation.

The chemical relationship between vitamin A and the carotenoids is clear when we look at their structures. Vitamin A is a poly-ene alcohol, C\(_{20}\) H\(_{29}\) OH.
All the carotenoids possess structures which include one-half of the symmetrical \( \beta \)-carotene molecule, \( \text{C}_{40} \text{H}_{56} \).

The most important biologically-active carotenoids are \( \alpha \)-, \( \beta \)- and \( \gamma \)-carotene, while echinonene, lepretene, myxoxanthin, aphanin, aphanicin and kryptoxanthin are also known. Literature on the isolation and structure of these compounds is voluminous, but reference may be made to the work of Kuhn and Brockmann (145, 146, 147) and the reviews written by Morton (200, 203) and Rosenberg (236).

These carotenoids are all biologically active and in nearly every case their potencies are one-half that of the \( \beta \) isomeride (148, 261). At first it was generally assumed that the fission of \( \beta \)-carotene achieved within the animal body consisted of adding the elements of water at the central double bond. Thus, the equations for the conversion of \( \beta \)-carotene and the other provitamins A, respectively, would have been:

\[
\begin{align*}
\text{C}_{40} \text{H}_{56} & \quad \text{IN VIVO} \quad \text{H}_{2} \text{O} \quad \text{OH} \\
(\beta \text{-carotene}) & \quad \text{H}_{2} \text{O} \quad ^{1} \text{Vitamin A alcohol}.
\end{align*}
\]


\[
\begin{align*}
\text{C}_{30} \text{H}_{28} \times & \quad \text{IN VIVO} \quad \text{C}_{20} \text{H}_{29} \text{OH} + \text{XOH} \\
(\text{Other provitamin A}) & \quad \text{H}_{2} \text{O} \quad ^{1} \text{Vitamin A alcohol}.
\end{align*}
\]

It is important, however, to note here that the experimental animal suffering from avitaminosis A will convert the provitamin A with higher efficiency than the well-nourished animal receiving carotene in excess of the minimum requirement.

The \text{IN VITRO} conversion of carotene to vitamin A was demonstrated recently, but will be discussed simultaneously with the site of conversion in the animal body.

This theory about the \text{IN VIVO} conversion of carotenoids to vitamin A was evidenced by the above-
mentioned fact of their biological activities. The biological assay of pure crystalline vitamin A and its esters against \( \beta \)-carotene standard in recent years, however, proves the incorrectness of these equations.

Pure \( \beta \)-carotene has, by definition, a potency of \( 1.67 \times 10^6 \) I.U. per gram and with a quantitative conversion into vitamin A according to the assumed equation, the expected potency of pure vitamin A alcohol would have been \( 1.56 \times 10^6 \) I.U./gm. Most recent data (10, 11, 12, 16, 33, 116, 168), however, shows the potency of pure vitamin A alcohol to be nearly double this figure, namely, about \( 3.0 - 3.5 \times 10^6 \) I.U./gm. These figures indicate that the experimental animal can form only one molecule of \( \beta \)-carotene, under the most favourable conditions. Thus, there is no reason to believe that the fission of the \( \beta \)-carotene molecule takes place symmetrically (201, 202, 203) and the mechanism of this reaction still remains obscure (125, 126, 200).

The Physiology of Vitamin A.

1. Daily requirements:

Vitamin A occurs exclusively in the animal world, where it is especially found in the livers of fishes, birds and mammals. It is absent from the plant world where its precursors, the yellow and red carotencoid pigments, are widespread. These pigments are distributed from bacteria (127) to garden fruit and vegetables (203) and are the origin of the vitamin A found in the animal bodies (53, 134, 168).

Vitamin A is fairly heat-stable but is destroyed by oxidation. Cooked and canned foods retain their potency, as well as foods stored in a frozen state; dried and dehydrated foods, however, show considerable
Because of the importance of vitamin A in nutrition it is interesting to have an idea of the approximate optimal human requirements. In a general paper on Vitamin A, Butt (27) gave the following figures:

For the average man and woman of 70 Kgm. and 56 Kgm. respectively the daily allowance is 5,000 I.U. In the latter half of pregnancy, 6,000 I.U. are required, and during lactation 8,000 I.U. It may be mentioned here that these figures correspond with the figures for rats and cattle, where the daily minimum requirements are approximately 18 to 30 I.U./Kgm. bodyweight (29, 82, 105, 151). The optimal requirement is about four times that of the minimum (217). During lactation a higher allowance is necessary owing to the high excretion of vitamin A in the colostrum and milk. Seeing that the requirements of vitamin A are a function of the bodyweight, children need less vitamin A than adults. For children under one year of age, 1,500 I.U. are necessary and the allowance is increased with age, till those aged from 7 to 9 years need 3,500 I.U. and those from 10 to 12 years, 4,500 units.

Many workers, using different techniques, determined the minimum daily vitamin A requirements of animals ranging from the rat to the cow. Their figures show good agreement for mammals, with an indication that the requirements for poultry are higher (105). One lot of workers expressed their results in terms of the amount of vitamin A per weight of air-dried diet, while the others calculated their figures per Kgm. of bodyweight.

In the interpretation of the results one must clearly distinguish between those expressed as International
The data in Table I. confirm the statement of Guibert and Hart (98) made in 1935 that the requirements of vitamin A are a function of the bodyweight.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Species</th>
<th>Min. daily requirement/ bodyweight/kgm.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Goss &amp; Guibert(82)</td>
<td>Rat</td>
<td>18-22 I.U. Vit.A.</td>
</tr>
<tr>
<td>Lewis, et al. (103)</td>
<td>*</td>
<td>20 *</td>
</tr>
<tr>
<td>Callison &amp; Knowles</td>
<td>*</td>
<td>20 *</td>
</tr>
<tr>
<td>Paul &amp; Paul (217)</td>
<td>*</td>
<td>20 U.S.P. units Vit.A.</td>
</tr>
<tr>
<td>Hart (105)</td>
<td>*</td>
<td>18-22 *</td>
</tr>
<tr>
<td></td>
<td>*</td>
<td>Sheep 17-26 I.U.</td>
</tr>
<tr>
<td></td>
<td>*</td>
<td>Swine 18-24 *</td>
</tr>
<tr>
<td></td>
<td>*</td>
<td>Horse 17-22 *</td>
</tr>
<tr>
<td></td>
<td>*</td>
<td>Cattle 21-27 *</td>
</tr>
<tr>
<td>Lewis &amp; Wilson(154)</td>
<td>Calves</td>
<td>32 U.S.P. units *</td>
</tr>
<tr>
<td>Butt (37)</td>
<td>Rumah</td>
<td>About 20 IU.</td>
</tr>
</tbody>
</table>

These authors also agree that the optimal requirement of vitamin A is about four times that of the minimum.

Another important factor that remains to be considered here, is the influence of age on the daily...
requirement of vitamin A. Paul and Paul (217) studied the relation of vitamin A intake to length of life, growth, tooth structure and eye condition and found that the amount of vitamin A necessary to keep the rat in a well-nourished condition increased with age. In this experiment the effect of increasing weights has been excluded by the adjustment of the size of the dose to the corresponding weight.

The maximal need for vitamin A in the rat seems to be at an age of about 60 - 90 days (29, 31). These results support the view of Irving and Richards (130), who came to the conclusion that the requirement of the animal for vitamin A increases with age, not only for tooth formation, but also to avert the other pathological changes associated with vitamin A deficiency. This is in somewhat surprising contrast with the requirements of other dietary factors, such as calcium and phosphorus, which are not supposed to increase in this manner.

2. HyperVitaminosis A.

Literature on the toxicity of high doses of vitamin to man is rare. The fact that ingestion of polar-bear liver causes severe illness in man and dogs has long been known among Eskimos and Arctic travellers. Redahl and Moore (235) found that the livers of these polar bears contain about 18,000 I.U. vitamin A per gram of wet material and ascribed the cause of this illness to the toxicity of too high doses of this vitamin to the human being. Butt (27), however, found that the administration of 50,000 to 300,000 or more U.S.P. units of vitamin A daily for as long as two to six months was not followed by any harmful effects in man.
Hypervitaminosis A can easily be demonstrated in rats. Moore and Wang (196) dosed young rats with 25,000 to 50,000 I.U. of vitamin A daily and found that it invariably caused pathological fractures of the bones after a period of 8 to 20 days. The same dose given to adult pregnant females gave rise to fatal uterine haemorrhage. The same authors published a review on hypervitaminosis A (197) in 1945, when they produced it with crystalline vitamin A.

3. Deficiency symptoms.

Vitamin A deficiency causes various symptoms in man and animal. Most investigators agree that severe deficiency of this, or of any other, vitamin, is accompanied by a lowered resistance of the body to infection. It is, however, not justifiable to describe vitamin A as the "anti-infective" (69) vitamin, owing to the fact that many other factors of even greater influence on infection than vitamin A exist. Thus, it seems wiser, in the words of Mendel (182), "to stress the indefinite function of the vitamin in preserving health and vigour, rather than to herald any specific action against definite microbiotic enemies."

In the human being the earliest cutaneous manifestation of deficiency is dryness and scaling of the skin. The most common type of cutaneous lesion is the appearance of small hard and deeply-pigmented pustules around the hair follicles, which vary in diameter up to 5 mm. Keratosis pilaris, ichthyosis follicularis are other synonyms (27). The response of these lesions to daily doses of vitamin A is slow and takes at least 2 to 3 months, depending on structural repair (108).

The methods used to detect early vitamin A
deficiency in man are based on dark adaptation of the retina (21, 100, 106, 218, 255).

The general vitamin A deficiency symptoms in animals, especially in the rat, are described in full detail in the literature and need not be discussed here. The epithelial structures of the organs are first to be involved (89, 251, 272, 273, 274), and uncomplicated pregnancies have also been observed as an effect of vitamin A deficiency on pregnant females (210). The occurrence of diverse pathological conditions such as inflammation and ulceration of the stomach and cecum, keratosis of the stomach and bladder, tongue abscess and pyelitis, are also common manifestations (250). Inco-ordination, paralysis and degeneration of the central nervous system are characteristic and will be discussed in detail later.


A specific symptom of vitamin A deficiency, especially in experimental animals, is xerophthalmia. It occurs most commonly in young animals and as early as 1916 McCollum and Simmonds (166) described that "the animals become emaciated and suffer from oedema of the eyes. Blindness results if the animals are permitted to go without this dietary essential (fat-soluble A) or with an inadequate supply for a sufficient time." Adults, on the other hand, develop night blindness as one of the earliest deficiency symptoms.

Fridericia and Holm (75) were the first to study the relationship between night blindness and malnutrition. These workers observed that in vitamin A-deficient rats, the regeneration of visual purple after bleaching by a bright light, was much slower than in
rats having an adequate supply of vitamin A. Their work was confirmed by Tansley (253), who followed the course of regeneration of the visual purple by extracting the latter with digitonin.

Blindness in calves is common when they are fed on a ration containing poor quality roughage. This blindness is connected with atrophy of the optic nerve, where it passes through the optic foramen, apparently due to bone overgrowth which causes pressure on the nerve (186, 187, 188). Blindness due to constriction of the optic nerve does not occur in mature cows. The relation between vitamin A and vision was studied by Wald (265, 266), whose diagrammatic illustration of the visual purple system is as follows:

\[
\begin{align*}
\text{Visual purple (rhodopsin)} & \quad \text{light} \\
\text{Vitamin A + protein} & \quad \text{dark} \\
\text{Retinene + protein} & \quad \text{visual white} \\
\text{("visual white")} & \quad \text{"visual yellow"}. \\
\end{align*}
\]

Vitamin A is the precursor of visual purple (rhodopsin) as well as the product of its decomposition. It unites in the retina with a protein to form visual purple (107). This takes place continuously and depends on a sufficient supply of vitamin A. Exposure of the retina to light leads to a reversible chemical change during which visual purple is bleached to visual yellow and retinene is liberated. This step may either be reversed to visual purple or retinene may decompose to vitamin A. The latter, in its turn, may partially be resynthesised into visual purple.

This visual purple system is found in all animals except in fresh water fish, where the visual pigment is porphyropsin in which vitamin A is replaced by
It is interesting to note here that the optimal retinal concentration of vitamin A (about 2 I.U. daily for the rat) is much lower than the daily vitamin A requirement for normal body functions (153); the optimal retinal concentration being approximately the same as the minimal body requirement. Severe vitamin A deficiency, however, may result in structural breakdown of the retina itself (157).

Although the essential function of vitamin A is still obscure, much light was thrown on its distribution in the tissues by means of fluorescent microscopy (90, 112, 214, 221, 284). The technique is as follows:

The whole organ or a slice of tissue is fixed for about 12 hours in formaldehyde solution. Frozen sections are mounted in water and then illuminated by a source of ultraviolet light from which all visible rays have been screened. If vitamin A is present, it glows with a bright green fluorescence, which is quickly destroyed by the ultraviolet light. Through the microscope the vitamin A is seen to occur in oil droplets in the organs. Tissues containing vitamin A2 show droplets with a reddish glow.

In the retina of a dark adapted eye no vitamin A fluorescence is found, while in the light adapted eye the fluorescence is found in a high degree in the pigment coat and to a lesser degree in the rod and cone layer. Even in severe deficiency vitamin A does not completely disappear from the retina (20, 76).

Thus, fluorescent microscopy reveals two types of vitamin A distributions in the eye, which confirm the validity of the Wald's cycle. The first type is found in the interstitial elements, such as the connective
tissue and the capillary endothelium, which is similar to that in the lungs, serous membranes, etc. Here the fluorescence reflects the nutritional status of the animal. The second type is found in the pigment coat and in the retina, where vitamin A has a specific distribution, depending upon the degree of light adaptation and reflects the functional state.

5. Formation and Storage.

Until recently it was generally assumed that a certain enzyme, carotenase, in the liver was responsible for effecting the transformation of carotene to vitamin A (14, 194). Nobody, however, was able to demonstrate this conversion IN VITRO with a liver extract (3, 56, 194, 238). In 1943 Nove (117) demonstrated the IN VITRO destruction of carotene by a water extract of minced rat stomach, which was a step forward towards the recognition of the intestine as the real seat of this chemical reaction. Popper (220), using fluorescent microscopy, was led to a somewhat similar conclusion and ascribed the site of conversion to either the intestine, kidney, lung, adrenal cortex or to all of these.

The next evidence came when Sexton, et al. (238), demonstrated that avitaminotic A rats, injected with large doses of carotene, died of vitamin A deficiency, in spite of the fact that their livers contained large amounts of carotene. These workers were also unable to detect any vitamin A in the livers of these animals. They suggested the possibility that carotene is converted to vitamin A in the wall of the intestine and that the carotene was stored in the Kupffer cells, in this case, as would any foreign body reaching the liver (165).
This proves the inability of the liver to convert carotene to vitamin A.

By following the vitamin A level after oral administration of carotene it was possible to demonstrate, further, that vitamin A appears first in the intestinal wall and only later in the liver (162, 254). Finally, direct proof to show the real seat of conversion, was presented when the IN VITRO transformation of carotene to vitamin A was demonstrated in the small intestine of vitamin A-deficient rats (78, 269).

The behaviour of vitamin A esters in the intestinal tract are similar to other esters of fatty acids. They are hydrolysed by the enzyme present and transferred through the gut wall as the free alcohol (80). In the case of abnormally high doses re-esterification takes place with the original (86), or with acids from the body fat under normal conditions (87). Absorption of vitamin A is facilitated by bile and the simultaneous absorption of fat (22, 88, 229, 239), while anti-oxidants (226) and bile salts (232) protect it against destruction.

Transport of vitamin A esters - and carotene in cattle (81, 74) - to the liver, are both by means of the lymphatic and blood systems (112, 216). Owing to the fact that these esters are not water-soluble, a carrier complex must be involved and the assumption is that vitamin A esters are linked to an albumen in the blood stream (61).

Vitamin A is stored in the liver, chiefly in the form of its esters (67, 110), and the palmitate is one of the natural occurring esters (10, 255). The liver contains about .8% of the bodily storage of vitamin A and there exists no parallelism between the liver storage and
blood vitamin A level. The only interpretation that may be made from a high blood vitamin A level, is that the subject has at least an average liver storage (100, 184).

The distribution of vitamin A in the liver was studied by Popper and Brenner (221) with the fluorescent technique. They made the following important observations:

In the hypervitaminotic state the excess of vitamin A is stored in the Kupffer cells, where it is destroyed and this explains the rapid loss of vitamin A in the first period of depletion. As the total amount of vitamin A drops, the fluorescence in the Kupffer cells decreases and gradually disappears, while the fluorescence in the liver cells remains unchanged. The amount of vitamin A in the liver corresponds at this stage with that found in the liver of the average adult stock rat and represents the physiological storage.

The liver reserves of vitamin A in new-born animals are low (20, 108), and several authors made the statement that it is independent of the mother’s diet during gestation (27, 50). By using Brucella abortus on cows, Braun and Carle (19), however, showed that although the vitamin A content of the fetal liver is low, it is in direct relationship to the mother’s diet.

The liver storage of the young rat increases 2 to 3-fold during suckling (50). This increase may be greater when the mother receives a diet rich in vitamin A, owing to the fact that milk with a high vitamin A content can be produced by supplementary administration of vitamin A to the mother (56). These facts also show that the most important channel for the excretion
of vitamin A is the milk, while large amounts are also excreted in the faeces (280).

6. Mobilization of Vitamin A from its Stores:

The mechanism which causes the liberation of vitamin A from its stores in the body is not yet clear, although interesting facts have been observed. Young and Wald (278) found an increase in the vitamin A concentration of the blood in the rabbit by electric stimulation of the splanchnic nerve, injection of adrenaline and extirpation of one lobe of the liver. These workers came to the conclusion that "vitamin A is mobilized from its reserves in the liver by sympathetic-adrenal stimulation comparable with that which mobilizes sugar and certain plasma proteins."

The rate of disappearance of this vitamin is not accelerated by thyroid extract (280), but growth hormone of the anterior pituitary may play an important rôle (68). Pett (219) measured the rate of visual adaptation of man to dim light and found that this rate was unusually rapid on the day following the ingestion of alcohol. He ascribed this phenomenon to the liberation of vitamin A from its stores. Results of experimental work on rats and dogs (34, 35, 36, 136) confirmed the validity of Pett's observation. These data show that administration of ethyl alcohol is always followed by an increase in the blood vitamin A concentration.

Matot and Matot (161) were able to shorten the survival period of rats on a vitamin A-deficient diet by including 2% activated charcoal. The activated charcoal, however, has no effect on the liver reserves but destroyed small amounts of dietary vitamin A, probably by absorption in the intestinal tract.
These facts may find useful practical application in the biological assay of vitamin A. Ethyl alcohol and/or activated charcoal may perhaps be used to deplete rats more quickly of their vitamin A reserves and to start the bio-assay with rats without any pathological conditions. The weights of these depleted rats will be much smaller and they will, therefore, be much more sensitive to administration of vitamin A.
DEGENERATION DUE TO VITAMIN A DEFICIENCY.

In the demonstration of degeneration in the nervous system, most workers prefer to use the Barshi method, while Kulcheski's stain and polarized light (101, 237) are less often applied.

Degeneration of the central nervous system in young animals, as a specific symptom of vitamin A deficiency, was described as early as 1914 by Hart and McCollum (103, 104). Young swine were kept on an artificial ration containing all necessary food factors known at that time and developed inco-ordination and nerve degeneration. This ration contained a high amount of cereals and it was believed that cereal toxicity was the cause of nervous degeneration. An unknown factor (vitamin A) "associated with butter fat and certain other fats and found... in considerable higher concentration in the leaves... of alfalfa" (loc. cit.) had a protective action against nervous lesions.

In 1925 Welbach and Howe (272) pointed out the importance of age in the demonstration of nervous lesions, seeing "that a much longer period of deficient diet is required to produce symptoms in fullgrown adult rats than with young, partially-grown rats."

Hellamby, 1926, (173) reported severe degeneration of the nervous system in puppies and laid stress on the toxicity of a high cereal diet in the absence of fat-soluble vitamins. He suggested the name "toxamins" for the toxic substance and ascribed the toxicity to its interference with the calcification of bones. This assumption was based on the fact that when puppies received a diet deficient in vitamin D, the calcification of bones became "worse". Fat-soluble vitamins and
Calcium carbonate acted as protective substances. Differences in the degree of toxicity were also found among cereals themselves.

The belief in the toxicity of cereals as the cause of degeneration, was held by this author through the years (174, 175, 176). Working on the experimental production of deafness in puppies, fed on diets of natural foodstuffs but deficient in vitamin A and rich in cereals, he still concluded: "Substitution of potato for the cereal element of these vitamin A deficient diets greatly reduced the abnormal changes in the labyrinth,...".

A possible explanation for Mellanby's continual belief in the toxicity of cereals and his conflicting results, may, perhaps, be offered. The composition of his vitamin A-free diet is as follows (180).

Oats and bran in proportion 4:1 ... 30-60 gm.
Alfalfa leaf, heated and oxygenated to destroy carotene ... 10 gm.
Baker's yeast ... 5% of oats & bran.
Ascorbic acid ... 0.5 mgm.
Vitamin D₂ ... 200 I.U.

Comparing the composition of this diet with that of the vitamin A-free diet generally used (4,5,45), the difference in the contents of yeast is striking. The amount of vitamin B complex in Baker's and Brewer's yeast is approximately the same (80). Mellanby's diet contains about 5% yeast, whereas the diets of Coward and Bacharach contain at least 17%. This fact points to the possibility that Mellanby actually described a combined vitamin A and vitamin B deficiency degeneration.

Further support for this possibility is that
The posterior columns of Goll and Burdach were most affected (180), which is typical of vitamin B deficiency degeneration (250). That substitution of potato for cereal starch reduced the intensity of vitamin A deficiency signs can be expected, owing to the fact that potatoes contain small amounts of carotene (102).

The first partial evidence against the idea of cereal toxicity was given by Hughes, et al. (119) in 1929, who described nerve degeneration in pigs resulting from avitaminosis A: "This paper seems to be the first published data on nervous degeneration resulting from avitaminosis A as far as the authors are aware. Zimmerman (280) produced lesions in the nervous system with a diet containing no cereals. Finally, Irving and Richards (129) replaced the cereal element in the diet by a mixture of tapioca starch and pure glucose (2:1) and proved that the belief in the toxicity of cereals was unjustified.

The influence of age as an essential factor in the production of nerve lesions in vitamin A deficiency is very important. The main factors are, namely, the amount of vitamin A stored in the animal body at the beginning of the experiment and the early period of rapid growth.

As already described, the amount of vitamin A in the new-born animal, although small at birth, depends on the mother's diet during gestation, but increases 2 to 3 times during lactation. Comparison of the results obtained with rats on diets containing full- and restricted amounts of vitamin A before weaning, shows a difference of a week or more in the development of nerve lesions (129). This delay, in the case of rats which received a full pre-weaning diet, corresponds roughly to the increase in the length of their survival period, which was observed
by the same authors under similar conditions (131).

Suzman and his co-workers (352) failed in their
attempt to produce spinal cord degeneration in adult
dogs on a vitamin A-deficient diet, although the other
typical deficiency symptoms were present. They over-
looked the importance of age and assumed that "the basal
diet was deficient in some other essential factor, which
is considered to be probably some portion of the B-complex
other than vitamin B2". The possibility of failure of
absorption or utilization of iron was also considered.

Ginker and Kandel (94), on the other hand, reported
failure to demonstrate characteristic symptoms of involve-
ment of the cord in monkeys and advised that "the work of
Nellanby with the same type of vitamin deficiency in dogs
requires checking by other histological methods."

The four swine in Eveleth and Bister's (71) experi-
ment, which were fed on skimmed milk until twelve months
of age, did not show any degeneration after two months
on a deficient diet, probably owing to the fact that they
were past their early period of rapid growth and their
livers apparently contained large amounts of vitamin A when
the experiment started (234). The same explanation holds
good for the failure of Wolbach and Bessey (276) to pro-
duce nerve lesions in rats on a vitamin A-deficient diet,
after they reached an age of 10 - 12 weeks.

Unlike degeneration due to vitamin A deficiency,
age seems to have no influence on the production of
degeneration in vitamin B2 deficiency. This is clearly
demonstrated by the work of Zimmerman and Burack (281),
who started their experiments with adult dogs on a diet
adequate in all dietary essentials except for vitamin B2.
After a sufficient time these dogs developed character-
istic deficiency symptoms, such as loss of weight, persistent vomiting, diarrhoea and muscular weakness, which ended fatally after 200 - 300 days. The observed nervous lesions were marked degeneration of the peripheral nerves and posterior columns in the cord. The authors also pointed out that the lesions in the central nervous system were similar to or identical with those described in pellagra in man.

It would appear from these results that these workers were possibly observing a thiamin deficiency, but in spite of this it is evident that the neurological changes are quite different from that caused by vitamin A deficiency.

That loss of weight alone does not account for paralysis or degeneration in the cord, was shown by several workers (1, 275, 277). Rats received a diet adequate in vitamin A but restricted in amount, so that their weights paralleled those of littermates on a vitamin A-deficient diet. All the rats died after approximately the same period, but, in contrast with the controls, those receiving vitamin A or carotene showed no paralysis or degeneration of the cord. It was also demonstrated that neither tocopherol (277) nor unsaturated fatty acids (280, 282) play a role in the development of nervous lesions.

Nerve degeneration due to vitamin B deficiency is well-known. Authors agree that, amongst other nerves involved, it is characterized by myelin degeneration of the peripheral nerves and the posterior columns of the spinal cord, becoming more extensive with the length of the period on the deficient diet" (280). Addition of 'crystalline' riboflavin to this diet prevents the
occurrence of these lesions. The existence of a certain relationship between the period on the vitamin B-deficient diet and the severity of the resulting degeneration is confirmed by the work of Zimmerman and Burack (279) and Prickett (223). This fact indicates a possible relationship between dosage level of vitamin B1 and the severity of the degeneration, which may in future, perhaps, be used as the basis for a new biological assay of this vitamin.

Wolkoff and Bessey (276, 277) observed another very important fact with respect to vitamin B deficiency, namely, that "rats whose growth is stunted...because of certain vitamin deficiencies (riboflavin and pyridoxine) show a normal relation between the central nervous system and its bony investment." This is not valid in the case of vitamin A deficiency and is due to the fundamental differences between the deficiency manifestations of these two vitamins.

Several of the cranial nerves are affected in vitamin A deficiency. It has been demonstrated that in nystagmus (176), deafness (177, 178) and in dental nerve degeneration (145, 144), which result as typical symptoms of this deficiency, the corresponding nerves always show destructive changes.

To obtain an idea of the general picture of vitamin A deficiency degeneration in the central nervous system, several papers by Irving and Richards contain valuable information. The maximal degeneration was usually found to be at the level of, or just below, the pyramidal decussation. The degeneration gradually becomes less and more diffusely spread at lower levels in the cervical cord (179). Microscopic examination of a cross-section reveals the site of maximal degeneration
in the anterior columns, in contrast with the findings in vitamin B deficiency.

As the severity of the lesions increases, the posterior columns, and especially the column of Burdach and the spino-cerebellar tracts, become gradually more affected, though the degeneration never reaches the same intensity as in the funiculus praedorsalis. It may just be mentioned here that these observations correspond with the experimental work presented in this thesis. In the spinal cord the degeneration is much less uniform than in the medulla and differs in intensity from segment to segment.

The uniformity with which the condition described occurs in every one of the rats examined is in striking contrast with the variability in pathological condition, and in the occurrence of epithelial metaplasia found in animals in advancedavitaminosis.

The fact that this degeneration in the medulla occurs with such uniformity before the weight curve is affected to any extent, suggests that it is one of the fundamental lesions of vitamin A deficiency* (129).

The results of graded doses of vitamin A upon the pathological changes in the central nervous system of the rat, led Irving and Richards (131) to suggest that this procedure might be used to form the basis of a prophylactic assay of vitamin A. At weaning rats were placed on a vitamin A-deficient diet and graded doses of this vitamin were administered. When they were killed after seven weeks, degeneration was found in those receiving 1 I.U. or less daily, but not in those receiving 1.5 I.U. or more, and the demarcation was extremely sharp.
The Mechanism of the Degeneration.

Several investigators made efforts to explain the mechanism of vitamin A deficiency degeneration, but no one succeeded in producing a theory to account for all the accompanying complications. That vitamin A plays an important role in the calcification of bones was pointed out by Copp and Greenberg (37). They found that the fracture callus is much smaller in the deficient rat than in the normal animal and the calcification is less active. In rats receiving large doses of this vitamin the healing of fractured bones is comparable to that in normal animals, whereas the untreated rats show a significant delay.

Hollanby (178, 179, 181) postulated that bone growth, as a result of vitamin A deficiency, is responsible for the degeneration of the nervous system. Administration of this vitamin results in a correction of this malformation. According to this author, vitamin A deficiency upsets the regulation of skeletal growth by means of interference with the activity of the osteoblasts and osteoclasts. On a surface where osteoclasts are normally active, their activity can be suppressed and that of the osteoblasts may predominate and vice versa.

The addition of vitamin A to the diet restores the normal relationship and starts the reconstruction of bones to normality. It was concluded that "bone surfaces usually possess both osteoclasts and osteoblasts, though the one or other type of cell may be flattened and inactive but capable of having its activity altered by a change in the supply of vitamin A. In some instances migration of osteoclasts seemed the only explanation of their sudden appearance in large numbers, unless they were formed on the spot from some other type of cell" (181). This overgrowth of the bones causes pressure on the nerves by
squeezing and stretching and results in degeneration due
to mechanical injuries.

Welbach and Bessey (275, 276, 277) and Moore and
Sykes (190) agree with Hellankby that degeneration is
caused by mechanical injuries due to pressure. They,
however, ascribed causes other than bone-overgrowth.
The bones cease to grow with the establishment of vitamin
A deficiency, which causes a disproportionate growth of
the central nervous system in relation to its bony
enclosure. "There is overcrowding of the cranial

cavity, resulting in distortion of the brain, disloca-
tion towards the foramen magnum, with herniation of the
cerebellum therein and multiple herniation of the cerebrum
and cerebellum into the venous sinuses of the dura at
sites of arachnoidal drainage structures. There is over-
crowding of the spinal canal with distortion of the spinal
cord and herniation of nerve roots into inter-vertebral
foramina and into bodies of vertebrae" (277). Attention
was also drawn to the possibility that vitamin A deficiency
accelerates the growth of the central nervous system (275).

Another factor that needs to be considered here is
the increase in spinal fluid pressure that accompanies
vitamin A deficiency. This manifestation was demonstra-
ted by Moore and Sykes (189, 190) in bovines.
Cerebro-spinal fluid pressures were obtained by a
puncture in the sub-arachnoid space and the insertion was
made through the dorsal opening in the atlanto-occipital
articulation, with the animals in a standing position.

The normal pressure in bovine is about 110 mm. of
saline and increases gradually with the severity of the
deficiency, until it reaches a terminal value of 400 -
600 mm. Care must be taken not to excite the animals,
owing to the fact that excitement increases the pressure.
On return to a normal ration with adequate vitamin A, the pressure slowly returns to normal. It is suggested by these workers that inco-ordination and nervous lesions may be related to changes in the cerebro-spinal fluid pressure. The latter is due to an increased volume of the cranial contents, caused by either a relative overgrowth as indicated by Walbach and Bessey, or to bone overgrowth as postulated by Mallanby.

Bayer and his co-workers (17) showed that the increase in intra-cranial pressure in avitaminotic A calves is paralleled by a marked increase in the vitamin C content of this fluid. The vitamin C content in the cerebro-spinal fluid of cows is normally 5-10 times that of the blood plasma. Injection of vitamin C into the vitamin A-deficient calves was accompanied by a rise in the content of this vitamin in the cerebro-spinal fluid and a reduction in the pressure of the latter in three out of five calves. By virtue of these results, the authors suggested that the increased spinal fluid pressure observed in vitamin A deficiency, was associated with a decreased synthesis of ascorbic acid. This was partially confirmed by Johnson, et al. (139), who demonstrated scorbutic changes in the teeth and other organs of the rat as secondary symptoms of vitamin A deficiency, due to the inability of these animals to synthesise or to utilize vitamin C.

However, when Moore (191) maintained normal ascorbic acid values in the blood and spinal fluid of avitaminotic bovines, by feeding of chlorobutanol to stimulate the synthesis of vitamin C, or by subcutaneous injections of the vitamin, there was still an increase in the spinal fluid pressure. He concluded that "the disturbance in the synthesis of ascorbic acid in the vitamin A-deficient
calves plays no part in the mechanism of the increased spinal fluid pressure in calves." Boyer, et al, injection vitamin C at a time when calves naturally show a drop in spinal fluid pressure, because of their moribund condition.

These data indicate that, whatever the mechanism of vitamin A deficiency degeneration may be, it is still obscure. As far as the hypothesis of cessation of bone growth is concerned, the work of Orr and Richards (211) disproves it. Bone-overgrowth and increased intracranial pressure are definitely demonstrated. If, however, degeneration is due to any kind of pressure effect, an even distribution of demyelated fibres is to be expected in a cross-section of the cord and not a well-defined degeneration that always occurs in the same tracts (see photos).
The estimation of vitamin A by means of the physical-chemical methods has been described by several authors. It is unnecessary, therefore, to deal with it in detail here.

The disadvantage of the estimation of vitamin A (51) by means of the Carr-Price reaction, is the rapid fading of the blue colour. Many attempts were made to eliminate this drawback. Hoch (115) advised that the tube containing the reaction mixture should be photographed immediately, together with a series of standard tubes of equal dimensions. The photographic film is analysed afterwards by means of photo-electric photometry. This micro-method gives good results but is unsuitable for routine analysis. The Carr-Price reaction was also used by Urban, et al. (258) to analyse an oil sample simultaneously for carotene and vitamin A. A modified photo-electric colorimeter is used where a beam of light, after it has traversed the reaction tube, is split into two beams which pass through a 620 m $\mu$ and a 589 m $\mu$ filter, respectively, before reaching the two recording photocells. The disadvantage of this method is, however, that it can only be used in a cold room at 0°C, where the colour is stable for about two minutes. At room temperature it can only be used for vitamin A estimations and the fading is as rapid as usual.

The physical methods generally used are the spectrographic (93, 203) and the spectrophotometric (228). These methods are accurate and convenient to use, but the latter is preferable, seeing that it excludes the photographic part of the former.
BIOLOGICAL METHODS:

(a) Curative method.

Present official methods for the biological estimation of vitamin A are not completely satisfactory with respect to both the standards and the procedures. The generally-used curative "rat-growth" assay, although simple in its description, is one of the most difficult of all bio-assays.

In 1932 the Medical Research Council (169) summarized the curative method as follows: - "In the curative test the animals are allowed to exhaust their reserves of vitamin A, as indicated by cessation of growth, after which doses of the test substance are administered for about five weeks.... Comparisons are drawn between doses which give equal average increments in weight."

Reference to the literature makes it clear that results obtained in practice are far from satisfactory. There is abundant evidence of the loss of weight which occurs in the absence of vitamin A, but Orr and Richards (211) proved that this loss of weight does not indicate a cessation of growth. The weight of any animal is dependent on the weight of its skeleton, organs and the amount of muscle and fat which cover the skeletal framework. By measuring body-length these two workers demonstrated that an animal continues to grow after it ceases to increase in weight. It is, therefore, wrong to describe vitamin A as the "growth-promoting" vitamin, for "the most important role of vitamin A in nutrition lies in its power of preventing the onset of pathological conditions, which are the cause of the loss of weight that occurs in vitamin A deficiency" (211).

To get a clear understanding of all the difficulties encountered in the curative assay of vitamin A, it is
best to discuss it under different headings:

1. Reserve stores in the young rat:

   The practice of breeding so as to reduce the reserve stores of the young experimental animal to a minimum, requires further investigation. There seems to be a serious danger that restriction of the mother’s intake of vitamin A, may result in the production of rats with a low reserve of vitamin A, but also low in health and vitality. The health of the mother may also be affected badly. The only alternative seems to work out a diet with a low but fixed amount of vitamin A on which to keep the breeding stock for vitamin A work (95). Such a diet must contain just enough vitamin A to keep the mother in a well-nourished condition and to produce young rats with a store of this vitamin just enough for good health and vitality.

2. Composition of diet.

   The course of the weight curve during the depletion period is influenced to a great extent both by the composition of the basal diet and the type of experimental animal used. The weight level at which the curve reaches a plateau will be higher for a vigorous animal than for a weaker type on a given diet. The use of a more complete or more palatable diet, on the other hand, will tend to raise the weight level of all the rats.

   Coward, et al. (40) laid stress on the type of caseinogen used in the basal diet. They found that it was possible to obtain greater uniformity in the results by substitution of extracted "light white casein" for the "vitamin-free casein" previously used. However, Bacharach’s results (4) “in their bearing on the source of protein in the vitamin A-free diets, do not confirm those reported by Coward.” Culhane (49), on the other hand, came to the conclusion
that Coward's "light white casein" contained some vitamin A.

Another paper by Coward and her co-workers (42) is of even greater significance. These workers reported that they found great variations in the vitamin A value (0.73 : 3.43) of the same sample of cod liver oil when tested in the same laboratory at different times. After considering all the possible causes of these variations in response, such as changes in the basal diet, seasonal variations and differences in the initial weights of the rats, they concluded that the variations must be due to "some influence not yet determined."

3. Type of experimental animal.

There exist great individual variations, even among rats of the same litter. More vigorous rats maintain appetite and weight-increase after their weaker litter-mates decrease in weight. By rejecting the former rats from the experiment as "unsuitable" (469), the bio-assay determines the minimum effective dose of vitamin A for a rat of poor growth. This dose will not necessarily be effective for more vigorous animals and "a biological method cannot be regarded as satisfactory if its results are applicable only to a limited class of animals of a subnormal standard" (230).

It was also demonstrated by Cross (46) that there is no correlation between the initial weight of the vitamin A deficient rat and the increase in weight after administration of this vitamin.

4. Estimation of the end of the depletion period.

In the curative test, dosing with vitamin A is begun when weight increase has ceased. To decide when to start the administration is one of the main difficulties in this assay. The criterion adopted in the Medical Research
Council’s Report is “cessation of growth”. Owing to the fact that the weight curve may stop suddenly, followed by a rapid decline, or may fluctuate for a few days before its decline, every worker usually uses his own discretion as to when to start his experiment.

In the Sherman technique, which is generally used in America, the instructions are to “begin the test period a few days after growth has ceased on the basal diet, and about one day before the appearance of distinct sore eyes... Have the animals thoroughly depleted of surplus vitamin A but not in a seriously pathological condition when the experimental period is begun” (241).

It is obvious that the determination of the end of the depletion period will influence the response of these animals to vitamin A administration very much, seeing that the severity of the pathological conditions increases with the length on the vitamin A-free diet. Weight curves of two series of rats to illustrate these variations were given by Orr and Richards (230). When dosing started after 3½ to 4½ weeks on the basal diet, the weight response was satisfactory and no macroscopic lesions were found on post mortem examination at the end of the test period. However, when they started dosing after 34 - 42 days, the weight response was much poorer and, in most cases, post mortem examination revealed bad pathological conditions after five weeks of dosing with vitamin A. These data point out a close relationship between the weight response of animals and their pathological conditions.

Butcher and his co-workers (28) reported weekly gains of 8 - 9 gm. of rats on 3 ml. of cow's milk, while Macy, et al. (159) found weekly gains of 12 - 15gms.
on the same volume of milk. These workers suggested that the diets of the cows might account for this difference. A study of the length of their preliminary periods, however, indicates that Macy's dosing started 7 - 9 days earlier than that of Butcher.

5. Weight curve during the test period.

Coward (43) recommended that the duration of the test period must be at least three weeks to obtain a reasonable degree of accuracy. With a test period of 4 or 5 weeks, the increase in accuracy is too slight to justify the extra time and labour involved. To ensure an accurate weight at the end of the test period it is best to weigh the rat on three successive days, namely, the 20th, 21st and 22nd of the test and then to take the average as the final weight (45).

Gridgeman (92) demonstrated that inter-litter variations in response are large enough to justify the use of isogenic pairs. He also showed that variations in the slope (b) of the log-dosage-response curve occur over comparatively short periods of time. It is, therefore, necessary to estimate a standard curve simultaneously with every biological vitamin A assay.

In spite of all these precautions serious discrepancies occur among results obtained by workers in different laboratories, as well as among those obtained in one laboratory by the same workers using carefully standardized procedures. Drummond and Norton (54) reported the case of two litter-mates on the same dose of cod-liver oil, of which one lost 24 gm. in 4 weeks, while the other gained 34 gm. in 5 weeks. Such variations occur frequently and are by no means exceptions to the rule.

The curative method is based on the assumption that increase in weight during the test period is an
indication of the presence of vitamin A in the tested substance. That this assumption is not always valid was proved by Richards and Orr (230) in the following experiment:

It was decided to carry out a biological assay on the vitamin A content of "fortified milk", i.e., dried, separated milk to which was added a vitamin A concentrate before evaporation. Although a blue test on the ether extract showed that only a small amount of the vitamin had been retained after evaporation, the weight curves indicated the presence of a considerable amount of vitamin A. Twenty out of the thirty rats used on three doses (0.025, 0.05 and 0.075 gm. of milk daily) survived the test period of 5 weeks. Owing to the obvious poor condition of the rats, it was decided that the increases in weight and the prolonged survival periods were to be ascribed to the food value of the milk and to its stimulating effect on the appetite, rather than to the presence of vitamin A.

To exclude the effect of this extremely palatable supplement, the assay was repeated with a slightly modified technique. During the depletion period each rat received a dose of "unfortified milk", i.e., dried separated milk, equal in volume to the dose of "fortified milk" it was going to receive during the test period. When the test period started the "unfortified milk" was replaced by "fortified milk". As a result of this change in technique only 2 out of 35 rats survived the test period of 5 weeks and they were found to be in a very bad pathological state.

"The marked contrast between the results obtained in these two assays served to bring into prominence the mistaken assumptions on which the curative technique is based" (230).
These data suggest that the administration of any dosing solution may have a marked influence on the response of animals. A difference in results is also to be expected when dosing takes place before or after feeding.

6. Pathological conditions.

Until recently a fundamental source of error in the technique of the curative assay has been overlooked, namely, the significance of the pathological conditions that occur in the absence of vitamin A from the diet. Obvious signs of disease such as xerophthalmia and lung trouble have received attention, while other more dangerous but less apparent symptoms have been almost ignored. These who have been interested in the relationship of vitamin A to disease have not, as a rule, been concerned with the quantitative estimation of the vitamin, while the assay workers have not recognised fully the extent to which their results can be affected by the varied nature of the symptoms arising in the absence of vitamin A (230).

In a biological assay it is essential that the experimental animals should be as nearly alike as possible in all respects. The real beginning of the curative test is the end of the depletion period. Post mortem examination of rats at this stage reveals pathological conditions in varying degrees of severity, which are responsible for corresponding variations in the weight response of individual animals to the same dose of vitamin A.

Appreciable weight increase at first followed by a sudden decline (2) may occur, or rats may die "rather suddenly without any obvious cause" (49). These cases are due to the presence of incurable pathological conditions which have increased in severity, while healing
of other less serious conditions has resulted in a temporary increase in weight.

Different types of subnormal growth responses are also well-known and can only be explained by the persistence of pathological conditions during the experimental period. There are, for example, "recovery with subnormal or normal growth for a time, followed by premature flattening" (120), "continuous subnormal response" (125) and "frequent failure of the animals... to respond to even relatively large doses of the vitamin" (4).

On post-mortem examination of 37 rats after 3 - 4 weeks on a vitamin A-free diet, i.e., before they should be considered to be "depleted"; only 9 revealed no macroscopic lesions (230). This evidence shows that any assay in which administration of vitamin A is started after 3 weeks on the deficient diet, belongs to the curative type.

(b) Prophylactic method:

To carry out the prophylactic, or "preventive" assay of vitamin A the Medical Research Council's Report (169) gave the following instructions: "Doses of the material to be tested are given either from the beginning of the feeding with the experimental diet or from about three weeks after, when the rats' reserves of vitamin A have been partially exhausted. After considering the serious disadvantages of the curative method it is clear that any biological assay of vitamin A to be satisfactory must be of the prophylactic type. It is important, however, not to start the dosing too late, owing to the fact that the animals may then have developed pathological conditions.

In the prophylactic assay the test period is continued until the control rats receiving no supplement have died and until distinct differences are present in
the weight curves of the different dosage groups. At
the end of the test period a "composite curve of growth"
(45), is constructed for each group of rats. This is done
by plotting weekly average weights against the time.
In calculating the results, the slopes of these curves
are compared.

(c) Xerophthalmia method.

The discrepancy in the vitamin A determination with
the two general methods is that they are based on weight
changes which are not specific for vitamin A. Methods
based on the occurrence of xerophthalmia and changes in
the vaginal contents of the rat in vitamin A deficiency
are less often used and their results are not very
satisfactory either.

When the occurrence of xerophthalmia is used as
the criterion for the determination of vitamin A in the
curative sense, the average number of days to cure every
group of rats is compared. Using the method prophyl-
actically, the average number of days which elapse
before the development of the desired stage of xeroph-
thalmia is taken as the criterion for calculations (45).

(d) The vaginal contents method.

Evans and Bishop, 1922, (60) were the first to show
that vitamin A deficiency upsets the cyclic changes in
the vagina of the rat. The continual appearance of
"cornified cells" (keratinised epithelial cells) day
after day in the vagina is characteristic. Administra-
tion of vitamin A restores the regular cycles. During
this abnormal period of vitamin A deficiency the
oestrous cycles occur naturally, but the usual signs given
by the vagina are masked (70).

Coward (39) was the first who made an attempt to
use this specific symptom of vitamin A deficiency as a
criterion for vitamin A determination. Her attempt
failed owing to the fact that her females were too young
and many of them became constant in weight before the
vagina opened. In 1932 Baumann and Steenbock (9)
suggested that mature females should be used and Coward,
Cambden and Lee (44) worked out the procedures for the
present method.

Female rats are reared on a diet containing only
moderate amounts of vitamin A. When they reach a
weight of about 140 gm. their vaginal contents are ex-
amined daily by means of Evans' method. A little of the
material is removed from the inside of the vagina with a
small spatula and examined at once in a small drop of
tap water under the low power of the microscope. If the
first smear proves unsatisfactory another can immedi-
ately be made. When it is ascertained that the cycles
occur regularly, the rats are placed on a vitamin A-free
diet until the vaginal smears show keratinised epithelial
cells for ten successive days. The rats are then con-
sidered ready for the test.

When this stage is reached, the rats are distri-
buted in different dosing groups and receive a single
dose of the vitamin A-containing substance. Vaginal
smears are examined daily and the number of days elapsing
before the disappearance of the keratinised cells from
the vagina is taken as the measurement of response.

Coward found that rats which do not respond by
ten days will never respond and every rat which does not
respond within this period of ten days, receives the value
10. Using this criterion as the basis for the calcula-
ting of the results, a logarithmic curve of response is
obtained.
When rats remain on a vitamin A-free diet after the assay is finished, without any further vitamin A supplement until they reach the stage to be considered as ready for the test, they can again be used. They appear, however, to be less sensitive to doses of vitamin A and can only be used for comparison with other rats which are also used for the second time.

As far as the accuracy of this method is concerned, it can just be mentioned that it was found by Coward (45) to be less accurate than the 3 weeks’ “increase in weight” method.

Four years ago this method was improved by the use of a modified technique, when Fuglesey, Willis and Crandall (225) demonstrated the use of younger ovariectomised females.

The animals are fed on a maintenance diet, low in vitamin A content, from 21 days of age until vaginal introitus occurs in the whole group at about 45 days of age. At this time the whole group is ovariectomised and vaginal smears are taken for 2 or 3 days after the operation. Some rats remain on the maintenance diet, while others are fed vitamin A-free diet, depending upon the dominant type of cell in the vaginal content, until the whole group shows the same evidence of depletion. The whole group is now placed on the vitamin A-free diet and vaginal smears are examined daily to determine the end of the depletion period, when they are distributed in dosing groups according to equal average bodyweight.

Doses of vitamin A are administered orally morning and night for two or more successive days. As the criterion for response, the number of days is taken from the first day of dosing until the depleted state is again reached (reappearance of keratinised cells). During this
period a temporary curative stage occurs.

At the end of the test period the rats go back to the maintenance diet and can be used repeatedly for eight to ten assays in the same way, or until the groups become too small to use. Fugley, et al. did not mention whether these rats became less sensitive to vitamin A dosing. The authors concluded that this modified method "shows considerable advantage over the increase in weight method with respect to precision and economy of time, material and effort."

(e) The liver storage method.

Another promising new method was also described four years ago. Guggenheim and Koch (96) used the amount of vitamin A stored in the livers of previously depleted young rats, when the material under test is fed on two successive days, as the basis for their assay. The results obtained with this method depend upon the utilisation of vitamin A by the organism and give a true index of the physiological value of this vitamin in any tested substance. The duration of the test period is only four days, the experimental procedure is much simpler than that of any other biological method in use at present and it is to be hoped that investigators in this field will develop it to its maximal efficiency.

Rats from 35 to 50 gm. in weight are used and each group contains equal numbers of males and females. The mothers' diet is regulated so as to reduce the liver storage of the young to about 3 to 10 I.U. per liver at weaning. This amount of stored vitamin A disappears completely within two to six days when the rats are given a vitamin A-free diet. This treatment produced healthy rats with no signs of vitamin A deficiency.
The vitamin A-containing substance is administered orally on the first and second days in quantities calculated per ten gm. of bodyweight. On the fourth day, the rats are killed, the livers prepared and their vitamin A contents determined by means of physico-chemical methods. Two livers from rats belonging to the same dosage group and sex are pooled for the vitamin determination. This method was found to be as accurate in its results as the 3-weeks' curative test.
It was found by Moore (195) in 1940 that the presence of vitamin E increases the storage of vitamin A in the liver of the rat, delays the onset of dental changes due to vitamin A deficiency and prolongs the time to deplete the animals of this vitamin. This work was confirmed by Bacharach (1) as far as the massive-dosage levels are concerned and by Davies and Moore (52) in all respects. Hickman (112) ascribes the incomplete confirmation of Bacharach "to the vitamin E being fed as the acetate of the synthetic 6,1-form which, though active as a preservative in the liver and tissues after hydrolysis and absorption, is relatively inactive in the intestines."

That the major seat of the syn-anti-effects is in the stomach and intestine was proved by Quackenbush and his associates (226), who examined the action of soybean distillates and other substances on carotene. They found that daily supplements of 5 μg. carotene in ethyl linolate failed to produce growth in young vitamin A-deficient rats, unless some of the soybean distillate was given simultaneously. The latter was also found to be highly effective in preventing antioxidation of carotene IN VITRO. The protective factor was α-tocopherol, since (a) both the protective substance of soybean oil and α-tocopherol distilled under similar conditions, and (b) small amounts of α-tocopherol showed approximately the same potency as an equal amount of soybean distillate in both the biological and the IN VITRO tests.

"The evidence indicates that in promoting a biological response to carotene the tocopherol functions as an antioxidant in the gastro-intestinal tract rather
than as a vitamin regulating some phase of metabolism in the tissues" (loc. cit.).

Hickman and his colleagues (101, 111, 113, 114) published results of experiments on the influence of vitamin E on the response of young rats depleted in vitamin A and receiving doses of \(\alpha\)-carotene, pure vitamin A (as alcohol or acetate) or \(\beta\)-carotene. Reference oil. The evidence of these workers for the intestinal action of vitamin E, is the change in result when vitamin A and tocopherol are given at different times or by different routes. Should the effect be physiological in nature, e.g., by the stimulation of a nerve or endocrine mechanism, changes in the time and route of administration are expected to be relatively unimportant. If a chemical action is concerned the simultaneous arrival of the two vitamins at the site of action will be most important. Data presented in these publications support the latter alternative.

Administration of the free vitamins on alternate days diminishes the synergy, while intramuscular injection of vitamin E proves to be inefficient, no matter how vitamin A is introduced. An interesting fact is that a maximal synergy is acquired by giving the coenzyme orally and the vitamin A parenterally. This is explained by the assumption that vitamin E diffuses inwards through the intestinal mucosa and protects vitamin A even when it is not present in the intestine.

"It may be assumed as a working hypothesis that that portion of the body reserves of these vitamins which is at any moment circulating in the blood stream will be in danger of destruction each time it passes through the muscular system connected with the intestinal wall. The enzymes and oxidizing agents which ordinarily endanger
the vitamin A during digestive absorption will again be dangerous when the vitamin approaches the same wall from the other side" (113). It is here where the covitamin exerts its protecting action, although vitamin A may not be present in the intestine.

It was found that approximately 0.5 mgm. of natural mixed tocopherols is the optimal daily dose to demonstrate the sparing action of vitamin E.

That the function of tocopherol in the vitamin A-E synergism is due to its antioxidant activity can readily be assumed. Sherman (242) has demonstrated that the action of carotene, administered to vitamin A-deficient rats, is inactivated by the simultaneous feeding of methyl linolate. The destructive agent of the latter can, on the other hand, be prevented by the addition of small amounts of tocopherol. This author suggested that the variations in growth response of vitamin A-deficient rats to carotene dissolved in different oils, can partially be attributed to the difference in the linoleic acid contents of these oils.

By equalizing the intake of linoleic acid, Rac (227) disproved this hypothesis and came to the conclusion that the utilization of carotene dissolved in different oils "appears to depend on the tocopherol content" of these oils. This was confirmed by Guggenheim (97), who showed that "the utilization of carotene from various plant sources, or of carotene dissolved in different oils, varies according to the vitamin E content of these materials.

Tocopherol performs its function by protecting carotene and vitamin A against oxidative destruction in the intestinal tract, as a result of which the absorption of vitamin A is increased.
A further proof for the mode of action of vitamin E is given by the work of Tomarelli and György, who demonstrated with IN VITRO experiments that "rice bran extract acts synergistically with mixed tocopherols in retarding oxidation of linoleic acid and consequently preserves carotene" (256). Being an antioxidant vitamin E also exerts a sparing action on essential unsaturated fatty acids in rats (118).

That the sparing action of vitamin E is the same for vitamin A and its provitamin \( \beta \)-carotene cannot be taken for granted, and the work of Brunius (23) gives an indication that it may not be the case. Determining vitamin A in five cod liver oils and thirteen high-potency oils (with arachis oil in the vitamin A-free diet) he found a mean conversion factor of about 1500 with a range of 1010 to 1660. Owing to the fact that arachis oil contains a high amount of vitamin E, the administration of extra tocopherol had no effect on the conversion factor. When the arachis oil in this diet was replaced by aerated lard and no tocopherol was given, conversion factors ranging from 1,850 to 2,430 were obtained for the same oils. However, when each rat received 0.6 mgm. of \( \alpha \)-tocopherol daily, conversion factors ranging from 1,310 to 1,550 were obtained for these oils.

The author's conclusion was: "These results are interpreted as indicating that the oxidised fat in the diet destroyed the carotene used as standard of reference more rapidly than the vitamin A under test" (loc. cit.).

After having discussed tocopherol and its sparing action on vitamin A, it is interesting to consider substances with a destructive action on vitamin A and its precursors. That linolates and linolemates are antagonistic to moderate amounts of carotene has already
been mentioned (242). Fatty peroxides cause rapid destruction of vitamin A which increases with a rise in temperature. From a practical point of view it is wise not to consider the peroxide value of oil solutions only as an index of potential rancidity, but also as an index of the stability of vitamin A present in the oil.

Free vitamin A alcohol is more susceptible to destructive changes due to fatty peroxides than the esterified form and rancid butter can depress carotene activity to one-third of the original potency (243). Owing to the fact that unsaturated glycerides contained in linseed and fish liver oils readily form peroxides destructive to vitamin A in the intestinal tract, they occupy a special position in this picture because they are essential for growth (25, 26).
THE INFLUENCE OF THE DIET ON

VITAMIN A UTILIZATION.

Another important factor in the utilization of vitamin A and carotene by the experimental animal, irrespective of vitamin E, is the composition of the assay diet. In an attempt to obtain a standard factor for converting extinction coefficients of vitamin A preparations into biological units, Hickman (110) stated that an important factor which influences the variability of the conversion factor, is the kind of basal diet on which the animals are depleted. Since then several authors have laid stress on this fact.

Dyer, et al. (60) observed that the biological activity of carotene or cod liver oils was affected by the oil used as a diluent for feeding. The influence of fats and oils in the diet, however, is chiefly due to their contents of vitamin E and other antioxidants, as well as of substances with a destructive action on vitamin A, such as fatty peroxides. The nature of the fat being absorbed is also of importance (22), although to a lesser degree.

The utilization of vitamin A is affected to a large degree by the medium in which it is dissolved or dispersed and the route by which it is administered. Barlow and Kocher (7) found that when vitamin A is injected intramuscularly in oil to a vitamin A-deficient rat, it is approximately 10 to 15% as effective as the same material given orally, as judged by the weight increase and the condition of the eyes. This may be due to the fact that a large amount of the oil and the vitamin A dissolved in it remains at the site of injection. Vitamin A is destroyed here and poor absorption takes place. On the other hand, when vitamin A is dissolved in propylene glycol it is equally effective by both routes.
Sobel, et al. (246) demonstrated that the liver storage of young rats was three times higher when they received the unsaponifiable fraction of fish liver oils dispersed in water, as compared with the group where the same fraction was administered as a solution in maize oil. Distilled esters gave a 2.2 times higher deposit when administered in an aqueous medium. This higher efficiency of vitamin A when dispersed in water, indicates the importance of considering the nature of the diluent in the biological evaluation of vitamin A. The authors suggested that the size of the individual vitamin A-containing particles is in itself a most important factor in the absorption of vitamin A.

To exclude the possibility that the casein in the general vitamin A-free diet may contain traces of this vitamin, Bridgeman, et al. (91) replaced all the casein and part of the starch by 30% coconut-cake meal and 10% defatted meat meal. A sharper depletion and greater sensitivity to vitamin A was obtained. It was also found that rats on this diet excreted no carotenoids when they received 40 I.U. \( \beta \)-carotene daily for three weeks, in contrast to the group on the general diet which excreted significant quantities. The carotene:vitamin response ratio was higher on the "coconut" diet and it was concluded that "coconut-cake meal contains some factor governing the proper conversion of \( \beta \)-carotene into vitamin A and utilization of the vitamin A so formed". These effects, however, can be attributed to impurities in the casein available at that time (93).

Determining the effect of the level of protein in the diet on the utilization of vitamin A in 1945 it was found that "the level of vitamin A intake was directly related to rate of gain up to a level of 3 I.U./day"
regardless of the level of protein in the diet (59).

Yeast present in the diet was found to play a significant rôle in the increase of the liver vitamin A storage (257). Cutaneous signs different from those previously accepted as typical for vitamin A deficiency, were demonstrated by Sullivan and Evans (251) in young rats on a vitamin A-free diet, insofar as it is possible to produce such a ration. "Complicating deficiencies of vitamin B-complex, fat and essential fatty acids were responsible for cutaneous alterations which resulted in the misrepresentation of the vitamin A deficiency syndrome."

The authors suggested that the composition of the vitamin A-free diet should be revised if an uncomplicated deficiency is to be obtained.

Russel (256), on the other hand, thought it wise to be careful in this respect and pointed out that "the plateauing of growth and the appearance of deficiency symptoms occurred later in the more complete diets ... Thus the animals would be heavier at the beginning of the assay period and, therefore, possibly might not be as sensitive to the differences in the vitamin A potency of dosage levels as animals of lower weight."

Whatever the case may be, these data indicate that the present vitamin A-free diet is not the "ideal" one and needs standardization.
THE CONVERSION FACTOR

Many attempts were made to establish a true conversion factor for converting spectral extinction coefficients of vitamin A-containing substances into biological units. To get a clear understanding of the difficulties involved in estimating such a conversion factor, reference must be made to the review of Hickman (112).

The biological assay gives a true measurement of the molecular content of the vitamin A in food, multiplied by a utility coefficient ($U$) which is generally less than one. This coefficient $U$ is dependent on a large number of factors, e.g., the chemical form of vitamin A, the presence or absence of protective and destructive agents and all the other factors, known and unknown, which are concerned with the absorption and utilization of vitamin A by the experimental animal. All the experimental animals are not alike and their response will further be influenced by their individual health and vitality, age, species and the kind of diet employed. In addition to vitamin A, the bio-assay measures the active carotenoids and all the substances curative to the vitamin A-deficient rat, which may be present in traces in the experimental diet.

The spectral absorption, on the other hand, does not give a measurement of the true quantity of vitamin A. The extinction coefficient, $\varepsilon_{260}$ $\mu$, is influenced by several variables. It varies with the nature ($n$) of the vitamin A moieties, i.e., whether it is present as the alcohol or ester and whether it is present as an equilibrium mixture of different isomers of vitamin A.

The spectral purity ($p$) depends on the presence or absence of irrelevant absorbing substances with little or no vitamin A activity, such as anhydro-vitamin A, vitamin A2
oxidised vitamin A products, etc. The influence of the solvent being used in spectrophotometric (226) and spectrographic analysis (227) may also be included here, as well as all the factors influencing the accuracy of such determinations.

The position as regards the conversion factor may thus be written:

\[
\text{Biological units} \times \text{U} = \text{X} \times \text{cm. } 395 = \mu \times \text{X conversion factor} \times \text{X cm. p.}
\]

Conversion factor = biological units/extinction coefficient \( \times \mu \times \text{cm. p.} \)

From this equation it is clear that the factor \( \mu \times \text{cm. p.} \) causes all the trouble in the establishment of a true conversion factor. The spectral contaminants (p) cause less trouble in high-potency than in low-potency oils and may be corrected for (228) to a certain degree. However, much research work remains to be done concerning the dependence of \( \mu \) on the state of \( \text{p} \) of vitamin A.

A good example of the different conversion factors found in different laboratories, is the case of the re-examination of the value of 1,200 for the conversion factor. This task was undertaken by the Vitamin A Sub-committee soon after the Second International Conference on Vitamin Standardisation allotted this figure in 1934. A short report on the results of the first experiment was published by the secretary in 1937 (122).

Unpublished data of biological and spectroscopic tests for vitamin A in various materials was collected. These data contained series of biological tests of concentrates which showed conversion factors of about 1,000 to 1,200, whereas some cod and halibut liver oils showed figures nearer to 2,000. A series of co-operative assays were, therefore, started so as to reduce discrepancies due
to variations in technique to a minimum.

"It was obvious that with the biological tests the most trustworthy results would be obtained by confining attention to a small number of materials and using as many experimental animals as possible."

Nine different laboratories determined the vitamin A content of a sample of halibut liver oil and a concentrate prepared from the same oil. They made simultaneous biological tests on these two oils and the International ß-carotene standard. Spectro-photographic determinations of the oil and the concentrate were made on the original material and on the remainder of the samples at the end of each worker's experiment.

A conversion factor of 1.470 was obtained for the halibut liver oil, but a recalculation by Irwin (132) indicated a value of 1.570. The concentrate showed a lower figure, but the spectrographic values of the residues were found to have fallen seriously and were, therefore, not taken into consideration.

Ten laboratories took part in a second collaborative biological assay of the U.S.P. Reference cod liver oil against the International standard (123). Conversion factors ranging from 930 to 2,210 were obtained. Statistical analysis, however, showed that six out of ten values gave a weighted mean of 1,820. It was found that the odds against the possibility of the difference between this factor and 1,570 being due to chance were approximately 30 to 1 (133).

A third experiment was, therefore, arranged (124). This time a crystalline ester was used, namely, vitamin A-ß-ß-naphthoate dissolved in arachis oil. Nine laboratories sent in results of biological tests. They ranged from the lowest conversion factor of 1,050 to the
highest of 2,770. The weighted mean of these conversion factors, which were obtained in all cases from five weeks' curative tests, was 1,770.

This value lies between the figures 1,870 and 1,920 obtained in the two previous experiments. By considering the logarithms of these three results and their standard errors, it was decided that they did not differ significantly, and by pooling the results a conversion factor of 1,740 was obtained.

Hume stated, however: "Practical application of such a conversion factor to commercial oils and concentrates is, of course, conditional on the absence or elimination of irrelevant absorption at 328 mμ... It would appear that a single conversion factor is still justified for all spectrophotometrically normal vitamin A preparations, oils or concentrates" (124).

The following Table contains some observed conversion factors, which will illustrate their variability and give an indication of the accuracy of the biological method. This Table will also be useful in the evaluation of work reported in this thesis.
<table>
<thead>
<tr>
<th>Reference</th>
<th>Material Standard</th>
<th>Mean O.P.</th>
<th>Approx. upper limit of error ($\pm 0.95%$)</th>
</tr>
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<tr>
<td>12 laboratories</td>
<td>1 U.S.P. cod 1931 Int. l.o. l. Std.</td>
<td>9080</td>
<td>137</td>
</tr>
<tr>
<td>10 ditto</td>
<td>1 cod l.o. *</td>
<td>9080</td>
<td>165</td>
</tr>
<tr>
<td>4 ditto</td>
<td>1 fish l.o. cons. *</td>
<td>1360</td>
<td>247</td>
</tr>
<tr>
<td>Grigeman (93)</td>
<td>1 fish l.o. oils *</td>
<td>1300</td>
<td>135</td>
</tr>
<tr>
<td></td>
<td>5 cod l.o. cons. *</td>
<td>880</td>
<td>134</td>
</tr>
<tr>
<td></td>
<td>7 whale l.o. oils *</td>
<td>900</td>
<td>127</td>
</tr>
<tr>
<td>9 lbs. (122, 125, 133)</td>
<td>1 halibut 1934 Int. o. Std.</td>
<td>1570</td>
<td>140</td>
</tr>
<tr>
<td>Robinson (252)</td>
<td>10 fish l.o. cons. *</td>
<td>1000</td>
<td>135</td>
</tr>
<tr>
<td></td>
<td>4 fish l.o. whale 1 o. cons. *</td>
<td>1000</td>
<td>132</td>
</tr>
<tr>
<td>Head, et al. (168)</td>
<td>2 cryst. Vit. A esters *</td>
<td>2000</td>
<td>160</td>
</tr>
<tr>
<td>Grab (83)</td>
<td>1 &quot;Vegan&quot; *</td>
<td>3400</td>
<td>135</td>
</tr>
<tr>
<td></td>
<td>1 &quot;Vegan&quot; unsap. matter *</td>
<td>1800</td>
<td>144</td>
</tr>
<tr>
<td>Roll and Reid (188)</td>
<td>1 &quot;Vegan&quot; unsap. matter *</td>
<td>3800</td>
<td>144</td>
</tr>
<tr>
<td></td>
<td>10 lbs. (125, 133)</td>
<td>1820</td>
<td>129</td>
</tr>
<tr>
<td>14 (259)</td>
<td>1 U.S.P. Ref. cod 1931 l.o. I. *</td>
<td>2140</td>
<td>139</td>
</tr>
<tr>
<td>9 (124, 133)</td>
<td>1 Vit.A. naph theose *</td>
<td>1770</td>
<td>139</td>
</tr>
<tr>
<td>Grigeman (93)</td>
<td>27 fish l.oils *</td>
<td>1900</td>
<td>128</td>
</tr>
<tr>
<td></td>
<td>15 whale *</td>
<td>960</td>
<td>125</td>
</tr>
<tr>
<td></td>
<td>14 refined products *</td>
<td>1310</td>
<td>128</td>
</tr>
<tr>
<td></td>
<td>1 cryst. Vit. A.ac. *</td>
<td>1660</td>
<td>114</td>
</tr>
<tr>
<td></td>
<td>1 uncryst. Vit.A.ac. *</td>
<td>1040</td>
<td>125</td>
</tr>
<tr>
<td>Braude, et al. (16)</td>
<td>1 Vit.A.ac. crys - tene *</td>
<td>1540</td>
<td>153</td>
</tr>
<tr>
<td></td>
<td>1 Vit.A. ester *</td>
<td>1370</td>
<td>156</td>
</tr>
<tr>
<td>Jone &amp; Christiansen (130)</td>
<td>11 cod l.oils U.S.P. Ref. I.</td>
<td>1900</td>
<td>147</td>
</tr>
<tr>
<td>Reference</td>
<td>Material</td>
<td>Standard</td>
<td>Mean C.F.</td>
</tr>
<tr>
<td>-----------</td>
<td>---------</td>
<td>----------</td>
<td>----------</td>
</tr>
<tr>
<td>Barthen &amp; Leonard (8)</td>
<td>17 cod l.oils</td>
<td>U.S.P. Ref. I.</td>
<td>2270</td>
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<td>=</td>
<td>2285</td>
</tr>
<tr>
<td>Black, et al. (15)</td>
<td>11 fish l.c. conc.</td>
<td>=</td>
<td>2150</td>
</tr>
<tr>
<td>Black, et al. (15)</td>
<td>41 fish l. conc.</td>
<td>=</td>
<td>2050</td>
</tr>
<tr>
<td>Black, et al. (15)</td>
<td>21 l. conc.</td>
<td>=</td>
<td>1920</td>
</tr>
<tr>
<td>Ewing, et al. (22)</td>
<td>6 fish l.</td>
<td>=</td>
<td>2130</td>
</tr>
<tr>
<td>Parker &amp; Omer (22)</td>
<td>11 fish l.</td>
<td>=</td>
<td>2050</td>
</tr>
<tr>
<td>Coy, et al. (46)</td>
<td>22 cod l.</td>
<td>=</td>
<td>2650</td>
</tr>
<tr>
<td>Coy, et al. (46)</td>
<td>30 fish l.</td>
<td>=</td>
<td>2250</td>
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<td>Makke &amp; Robeson (12)</td>
<td>1 cryst.Vit. A.ale.</td>
<td>= Ref. 1/3</td>
<td>2400</td>
</tr>
<tr>
<td>Makke &amp; Robeson (12)</td>
<td>1 cryst.Vit. A.maphthoene</td>
<td>=</td>
<td>3160</td>
</tr>
<tr>
<td>Makke &amp; Robeson (12)</td>
<td>1 cryst.Vit. A.ace.</td>
<td>=</td>
<td>2350</td>
</tr>
<tr>
<td>Makke &amp; Robeson (12)</td>
<td>1 cryst.Vit. A.palmitate</td>
<td>=</td>
<td>2520</td>
</tr>
<tr>
<td>Vahlteich &amp; Neal (260)</td>
<td>12 &quot;Vit.A&quot; oils</td>
<td>=</td>
<td>1740</td>
</tr>
<tr>
<td>Zecheila &amp; Henry (286)</td>
<td>8 mix. fish l. oils</td>
<td>=</td>
<td>2090</td>
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<td>Coy, et al. (47)</td>
<td>32 cod l.oils</td>
<td>= Ref. 2</td>
<td>2170</td>
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<tr>
<td>Coy, et al. (47)</td>
<td>31 fish l.</td>
<td>=</td>
<td>1950</td>
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<td>Coy, et al. (47)</td>
<td>7 Vit.A. conc.</td>
<td>=</td>
<td>1880</td>
</tr>
<tr>
<td>Coy, et al. (47)</td>
<td>7 &quot;esters&quot;</td>
<td>=</td>
<td>1970</td>
</tr>
<tr>
<td>Coy, et al. (47)</td>
<td>16 mix. products</td>
<td>=</td>
<td>2000</td>
</tr>
</tbody>
</table>

* In drawing up this Table use was made of data given by Gridgeman (93).
FACTORs affecting the variability of the conversion factor:

1. Saponification:

The general belief of the spectroscopists is that a unit of absorption at the peak of a characteristic band indicates a fixed number of vitamin A molecules which have a fixed curative value (198). This assumption is wrong. Crystalline vitamin A alcohol has a higher potency per molecule than the vitamin in the parent fish liver oil. Commercial concentrates prepared by saponification, on the other hand, reveal conversion factors ranging from 50 to 85% of the factor for the natural oil (46, 224). This is, however, not the case with vitamin A concentrates prepared by molecular distillation, for there the potency per molecule is about the same as in the parent fish liver oil (18).

Since vitamin A in the natural fish liver oil is in the esterified form, while that in the concentrates prepared by saponification is present as the free alcohol, the question arises as to whether the alcohol has a lower conversion factor than the esters (110). The work of Emmett and Bird (67) seems to lend some confirmation to this view. Different amounts of caustic soda were used to prepare three concentrates, which were saponified to different degrees. These were, namely, (1) partially-saponified, where a small amount of oil was left unsaponified; (2) strongly saponified, where the theoretical amount of caustic soda did not give complete saponification of all the different esters present in the oil; (3) completely saponified. The conversion factor found for the natural oil was 1975, while the figures 1970, 1420 and 905 were obtained for the three concentrates respectively.
Thus, "in the case of concentrates prepared from oils high in vitamin A, the conversion factor seems to vary with the amount of caustic employed in the saponification" (loc. cit.) Similarly Noll and Reid (185) pointed out that the conversion factor is greater for natural oils than for the concentrates prepared from them by saponification. They suggested that this is due to the higher biological efficiency of the natural vitamin A esters.

As soon as pure crystalline products became available, Baxter and Robeson (12) were able to demonstrate that there exists no difference in biological potencies of vitamin A alcohol and its esters. These authors suggested that the difference found by the previous workers was due to the presence of impurities in the oil absorbing at 328 mμ, but which had little or no biological vitamin A activity.

Hitherto it was generally assumed that irrelevant absorption is negligible if the observed value of the extinction coefficient of an oil is greater than 4. For low-potency oils with an E-value lower than 4 the physico-chemical estimations are made on the unsaponifiable fraction. This procedure excludes unsaturated fatty acids, which are the chief cause of irrelevant absorption in such oils (206).

Oser, et al (212) emphasized the importance of conducting any determination on the unsaponifiable matter of oils, regardless of the potency. Experimental results show that even in high potency oils a lower vitamin A value is obtained after saponification. This is due not only to the removal of unsaturated fatty acids but partially to the removal of oxidation products of vitamin A.
Chromatographic absorption is also used with success in the purification of oils from irrelevant absorbing substances (270).

2. Oxidation Products:

For the demonstration of the oxidative destruction of vitamin A different methods can be used (158, 160, 271). The following simple method was used by Robinson (232):

An accurately weighed amount of vitamin A concentrate was spread on strips of filter paper, which were dropped into a flask connected with a Hg manometer. The entire apparatus was filled with oxygen, the flask heated to 100°C, and the amount of oxygen absorbed was measured at intervals for 5½ hours.

During this period the vitamin A was found to have taken up 6% of its weight of oxygen and the extinction coefficient had fallen from 700 to 100. That heat only plays a secondary role by accelerating the reaction, was demonstrated when the experiment was repeated in the absence of oxygen at 12°C, and the E-value had fallen from 700 to 530 only.

After treatment with oxygen the ultra-violet absorption had thus fallen to a low value and the biological activity was small as compared with that of the original concentrate. The decrease in the biological activity as measured by the bioassay, however, was more rapid than was indicated by the physical estimations. This fact indicates that oxidised products of vitamin A, although biologically inactive, reveal considerable absorption at the 328 mμ band. The presence of these substances in stale fish liver oils and concentrates, is believed to be responsible for their low conversion factors.
Robinacm found further that after the treatment with oxygen there was a decrease in the iodine and acetyl values, a small amount of aldehyde was present, but no peroxides were found. The product also showed an increase in the saponification value, molecular weight and ketone content.

From these data it seems likely that the following chemical reaction takes place during the oxidative destruction of vitamin A: the hydroxyl group of the vitamin A alcohol is oxidised to an aldehyde. That this is the first oxidation step is proved by the fact that the ester form is less sensitive to destruction by oxygen (243). The unsaturated linkages are next to be oxidised; first to an intermediate peroxide stage which is immediately destroyed by disruption to aldehydes and ketones and by the reaction with unsaturated linkages to form polymers. The immediate destruction of the peroxide stage will explain the fact that Robinson found no peroxide in the final mixture. The occurrence of different stages in the oxidation of vitamin A is confirmed by the work of Oser, et al (212).

3. Isomers of vitamin A and closely-related compounds:

As early as 1928 Morton and Heilbron (196) observed that irradiation causes destruction of vitamin A. This was confirmed by Smith, et al (244), who demonstrated that irradiation with ultra-violet light of wave-lengths exceeding 300 µm resulted in a progressive drop in the extinction coefficients of vitamin A concentrates and oils. This fall increases with the intensity of irradiation but even diffuse daylight has some effect. When these solutions are put in the dark after irradiation the Φ-values increase towards the original figures, but the further it was decreased the less complete is the recovery in the dark.
These facts led the authors to suggest that the vitamin A in any oily solution exists as a mixture of geometrical isomers. On irradiation energy is absorbed which causes a change in the proportion of the original equilibrium mixture. It is likely that this change is responsible for the decrease in the extinction coefficient of the mixture during irradiation.

On standing in the dark the reaction is reversed to the original equilibrium mixture. However, seeing that this recovery is never complete, it is evident that a simultaneous irreversible photo-chemical change has taken place, with the formation of some other substance or substances with little or no absorption at 326 m/.μ.

It is interesting to know that not only vitamin A, but also β-carotene (143) reveals stereo-isomeric forms. According to these workers, carotene extracts of plants contain considerable quantities of two stereo-isomers of β-carotene, namely, neo-β-carotene B and neo-β-carotene U. The former has about one-half of the biological activity of β-carotene, while the latter possesses approximately 26%. It was determined by passage analysis that the activity of these substances are due to steric rearrangement in the digestive tract of the animal.

It is possible that these isomers of vitamin A which have different biological activities are concentrated in the "soluble A" fraction of Baxter and his co-workers (11, 231). Ling cod (Ophiodon elongatus) liver oil distillate containing about 23% vitamin A esters was saponified. Free vitamin A alcohol in the resulting concentrate was crystallized from ethyl formate. The remaining mother liquor was evaporated under reduced pressure and redistilled in order to carry the vitamin A crystallization as far as possible, but only a small amount of crystalline vitamin A could be gained from ethyl formate.
The resulting mother liquor was, therefore, evaporated and the residue examined. It was an orange-red oil which contained about 46% of the original vitamin A. The absorption curve of this oil was slightly broader than that of crystalline vitamin A and biological assays showed conversion factors of 1,500 for this un-crystallisable residue and 2,600 for the crystalline product.

Thus, these workers succeeded in preparing two vitamin A preparations from the same fish liver oil, with more or less similar ultra-violet absorption spectra, but different conversion factors and different crystallisation abilities.

"Natural oils which have been reported as having significantly different conversion factors may have possessed different proportions of these vitamin A's or inactive material absorbing sharply at 328 mλ. The preparation of concentrates, by distillation or extraction, which showed conversion factors different from those of the original oils from which they were prepared, may have concentrated the two vitamin A's differently or destroyed one form preferentially" (11). This vitamin A isomer was later obtained in crystalline form by Dexter and Robeson (12) and they suggested the name vitamin A₁ for it.

More recent investigations by the same authors (231) showed the existence of another compound, which occurs in high concentrations in fish liver oils and is responsible for approximately one-third of the total vitamin A potency in the liver oils of some fish species. It is suggested that it may be a geometrical isomer of vitamin A₁, which differs in the cis-trans configuration at the double bond nearest to the hydroxyl group.
and is called neo-vitamin A. Pure vitamin A has an absorption maximum at 326 m\(\mu\) with an \(E_{1%}\) equal to 1,760, whereas neo-vitamin A has its maximum absorption at 326 m\(\mu\) with an \(E_{1%}\) of 1,675. Neo-vitamin A has the same biological activity as vitamin A.

Gray and Cowley (64) said the following about the structure of vitamin A2: "Our observations are in accord with the view that vitamin A2 contains the same number of carbon atoms as vitamin A but differs in that it has one additional conjugated double bond."

Gillam (77) made a very interesting study with reference to the distribution of vitamin A and its isomers in the livers of various animals. Generally mammalian and bird livers contain vitamin A2 but no vitamin A2, except for animals such as the seal and the other which feed on fish. The livers of fresh water fish, on the other hand, are rich in vitamin A2 (186, 187).

By feeding rats with a concentrate prepared from fresh water fish livers, Gillam was able to obtain a mammalian liver high in vitamin A2. This proves that mammals are able to pass vitamin A2 into their livers if they receive it in their diet.

Jensen, et al (133) prepared a vitamin A2 concentrate from the livers of freshly-caught Northern pike (Esox lucius) and found a "conversion factor" of 970 for it. They found that this vitamin was toxic in very high doses and was present in large quantities in the livers of their experimental rats.

It is well-known that perch and dace thrive for a considerable period on a diet of blow-fly larvae. By enriching this diet with carotene for a few weeks Morton and Creed (199) were able to increase the liver storage of both vitamin A and vitamin A2 in these fresh-water fish. From this fact it is evident that carotene acts as the provitamin for the IN VIVO formation of both
vitamins.

"Sub-vitamin A" is described by Embree and Schantz (64) as a possible new member of the vitamins A₁ and A₂ group. It occurs in traces in the livers of several fish and possesses properties related to those of vitamins A₁ and A₂.

Another substance to take into consideration here is cyclised vitamin A. This material is formed as an artefact by the treatment of vitamin A with alcohol and HCl, but it has also been found to occur naturally in tuna and other fish liver oils (63, 87). Cyclised vitamin A gives practically the same colour reaction with antimony-trichloride as vitamin A and reveals considerable ultra-violet absorption at 328 mμ although it has no biological activity.

Because their diet consists chiefly of marine animals, the vitamin A concentration of whale and other sea mammal livers is much higher in comparison than that of the land mammals. In addition to vitamin A, whale liver oil contains kitol (204), which appears to be a divitamin A containing two hydroxyl groups, although only one molecule of vitamin A can be produced on heating in the laboratory. It is a viscous, pale yellow oil at higher temperatures and a solid, glassy substance at room temperature, with no biological activity.

It is suggested that a mammal such as the whale, which is confined to a marine diet, "may well be embarrassed with too much vitamin A which it might detoxify by converting two molecules to one of inactive kitol. However, kitol has been found in the livers of oxen and sheep which are not overburdened with supplies of vitamin A. Etiologically it would seem that kitol is a post-rather than a pre-vitamin" (112).
Correction for irrelevant absorption.

The above-mentioned facts with regard to the presence of irrelevant absorbing substances in all natural fish liver oils and commercial concentrates, afford evidence that the physico-chemical estimations of vitamin A must be regarded with some suspicion. The biological assay, on the other hand, gives an index of the physiological value of vitamin A-containing substances but is subjected to the influence of numerous known and unknown factors that affect its reliability.

As far as the interference of irrelevant absorption is concerned, it can be excluded to a certain degree by previous saponification and chromatographic purification, but both of these methods have serious disadvantages as was pointed out. The failure of these methods and the development of an improved spectrophotometric method led to recent investigations, which try to eliminate irrelevant absorption by the use of a certain correction formula. This idea is based on the knowledge of the whole absorption curve for pure vitamin A alcohol and its esters, which was constructed after these products became available.

Data presented by several authors show that this method improves the accuracy of the spectrophotometric estimations. The only trouble seems to be the presence of substances such as vitamin Al and Ag, with considerable absorption at 328 m, but with biological activities lower than that of vitamin A. It is necessary to mention the names of Norton and Stubbs (205, 206, 207, 208) and Chevallier (33) in this connection and this method of correction will be discussed in detail when applying it to the experimental work reported later in this thesis.
The Present Biological Standards.

The International Standard preparation of \( \beta \)-carotene is used as biological standard in England, while the United States Pharmacopoeia Reference cod liver oil 1 and 2 have been more generally used in the United States. These standards are unsatisfactory for vitamin A work owing to their unsuitability as physico-chemical standards and also to the fact that serious deterioration has taken place in the two reference cod liver oils. It is, however, difficult to insist on the adoption of a new standard which will suit the purpose of both physical and biological standards, owing to the danger involved that it may upset previously determined relationships.

In 1934 the U.S.P. reference cod liver oil 1 was assayed against the 1931 International Standard of mixed carotene by nine American and three British laboratories and a potency of 3,000 I.U. per gram was assigned to it (15). Four years later one American and nine British laboratories carried out a co-operative test on the same oil against the 1934 pure \( \beta \) -carotene International standard and a value of 2,019 I.U. per gram was found (123).

It is considered (124) that this value explains the difference between the value of 2,000 for the conversion factor which is preferred in the United States and that of 1,740 found by the Vitamin A Sub-Committee.

The U.S.P. reference oil 1 had deteriorated and "the simplest solution would, therefore, appear to be to recognise that the U.S.P. and International units have not, after all, the same magnitude, but that the former is only 2,619/3,000 or about 7/8 of the latter and that the respective conversion factors are about 2,000 and about 1,740". (loc. cit.).
Similar results were found for the U.S.P. reference cod liver oil 2 by Callison and Orent-Keiles (30): "A result of this discrepancy, assays of vitamin A value performed by bioassay using U.S.P. reference cod liver oil 2 as standard and expressed in terms of international units may be from 30 to 44% higher than the actual value.... Pure \( \beta \)-carotene may be used as a standard for the biological assay of vitamin A until a more stable and satisfactory standard is developed."

During a comparative study between the two U.S.P. reference cod liver oils, Gey, et al. (47) came to the conclusion that there exists a difference between the stability of these two standards resulting in different conversion factors for the same oil. Comparison of the absorption curve of pure vitamin A with that of the unsaponifiable fraction of a fresh sample of reference oil 2 shows that this standard of reference contains 'appreciable quantities of irrelevant light-absorbing material not entirely removed by saponification; it is unstable even when stored under nitrogen in the refrigerator for a period of only one month; and there is a marked discrepancy in its vitamin A content estimated by the spectrophotometric and colorimetric methods on either the whole sample or the unsaponifiable fraction' (212).

Selecting \( \beta \)-carotene as the best biological standard for vitamin A at present, does not mean the necessary assumption of complete utilization of carotene by experimental rats, but an assumption of constancy of utilization at any dosage level and under any experimental condition. This assumption is not justified if all the factors affecting absorption and utilization of vitamin A and carotene are taken into consideration as well as the possibility that the action of both substances may not be the same.
Guilbert, Newell and Hart (99) demonstrated that the ratio of the relative efficiencies of vitamin A and carotene widens with increasing levels of intake and they suggested: "Double standards for requirements must be recognized, one for carotene and one for vitamin A, and both must be considered in evaluating the status of a dietary furnishing both sources."

Huller and Reinert (209) obtained somewhat similar results. They found that the growth-promoting activities of \( \beta \)-carotene or equivalent quantities of vitamin A are subject to great variations, depending on the diet used, the amount of carotene or vitamin A administered per dose, the type of animals and the season of the year.

Studying the influence of choline and trypan blue on the utilization of carotene and vitamin A for liver storage, Bentley and Morgan (13) came to the conclusion "that the mechanisms of utilization and storage of vitamin A and carotene are probably affected by different conditions and that the composition of the accompanying vitamin A-deficient basal diets is an important factor in determining their efficiency."

Considering all these data it is evident that neither \( \beta \)-carotene nor any of U.S.P. reference cod liver oils can be regarded as an ideal biological standard for vitamin A.

With the recent production of pure vitamin A alcohol and its esters, the obvious solution for this problem of unsatisfactory standards, seems to be the adoption of a new vitamin A standard. Several recommendations have been made in this connection (65, 66). According to these recommendations the new standard will probably be a solution of pure vitamin A-acetate in refined deodorized cottonseed oil, slightly fortified with
tocopherol and having a potency of approximately 3,000 µgm per gram. Such a standard came into use in America at the beginning of this year. The stability will be excellent as compared with that of the present standards and it will show little, if any, interference with physico-chemical tests.

It may just be mentioned here that a solution of vitamin A acetate in a good-quality arachis oil without the use of any added antioxidants, proved to be a very satisfactory biological standard for vitamin A work during this work. It has been used since the beginning of 1947 and showed no deterioration after more than a year – owing to the high tocopherol content of the arachis oil. The preparation was stored in the refrigerator, without the use of carbon-dioxide or nitrogen and the bottle was opened several times during this period when dosing solutions and spectrophotometric estimations were made. These facts thus support the recommendations mentioned above.

At present manufacturers who handle vitamin A are dependent on the extinction coefficient of this substance estimated by means of physico-chemical methods, as a linear function of vitamin A activity. A conversion factor has been adopted to relate the extinction coefficient with biological units. Unfortunately, differences in the biological vitamin A standards resulted in two conversion factors of different magnitudes.

The opinion of Bridgeman (93) is that this problem ought to be solved by the adoption of a new unit for vitamin A, which is based on the spectrophotometric assay only. He recommended a provisional skeleton definition such as “the unit of vitamin A is that quantity which, when made up to 100 ml. with a specified organic solvent,
gives a solution having an extinction coefficient at 328 m \mu of 0.0005. It is added that "the quest for a new, easily measurable, physiological criterion of response sharply graded to increasing dosage, prophylactic or therapeutic, of vitamin A, can itself be regarded as a fruitful research problem."

Netseilf (183) supported the view of Gridgeman and recommending that the vitamin A content of a substance should be expressed in mgm. of vitamin A per gram. Elom 328 m \mu should also be stated but just for the convenience of the chemist. The author "had experience of the confusion that is rife in commercial transactions and investigational surveys when the results of the spectroscopic estimations of vitamin A in fish oils are expressed in International Units per gram, and.... seek the co-operation of chemists in removal of this source of confusion."

Considering the influence of irrelevant absorption, it is, however, not justifiable to take the extinction coefficient as proportional to the concentration of vitamin A; neither can it be assumed that equal quantities of vitamin A (as determined by physico-chemical assays) from different sources have the same physiological value. Irrespective of the fact that it is a practical impossibility to persuade a world industry to accept a new unit as suggested by Gridgeman and Netseilf, the fact remains that the consumers of vitamin A are biological subjects and the physiological value of vitamin A can, therefore, not be excluded from its estimations.

A more satisfactory biological vitamin A standard is necessary. It seems desirable that such a standard ought to be standardized by large-scale collaborative experiments, using carefully-standardized diets and including all the present biological and physico-chemical methods.
PART II.

EXPERIMENTAL.

MATERIAL AND TECHNIQUE.

Although South Africa has a flourishing vitamin A industry, and extensive chemical research has been done in this field, the biological side has hitherto been neglected. The work presented in this thesis was undertaken on a decision of the Department of Physiology and Pharmacology of this University to do something in this direction and found its basis in a suggestion made by Irving and Richards (loc. cit.). When the work started, facilities for biological vitamin A assays were not available and had to be built up from the beginning.

1. Housing of rats:

Albino rats of the Wistar Institute strain were used. For convenience, and to exclude the danger of mixing up the diets of the stock and experimental rats, different rooms were used for breeding and assay work. Both rooms were kept at a fairly constant temperature of about 65 - 70°F and provision was made for ample ventilation. Owing to the fact that the windows face east, Venetian blinds were used to eliminate the direct rays of the sun during summer.

The rooms were swept daily and once a week the cement floors were washed with a dilute solution of lysol in water. Rats were kept in loose-bottomed wire cages and the bedding consisted of sawdust and Canadian pine shavings, which were changed once a week.

Ordinary bottles were used to supply the water by means of a straight delivery tube. The one end of this tube fitted into the neck of the bottle with a piece of
rubber tubing, while the other end was inverted between the wires of the upper part of the cage. Food-pots and water bottles were cleaned and refilled every morning.

2. Diets.

The rats used for breeding were kept on the normal departmental stock diet, which has the following composition:

- Maize meal: 32%
- Sussex ground oats: 32%
- Lucerne meal: 6%
- Wheaten bran: 6%
- Peanut meal: 5%
- Linseed meal: 5%
- Fish meal: 10%
- Oat germ meal: 2%
- Bone meal: 1%
- Cod Liver Oil: 2%

(Ca = 1.25%, P = 0.24%, Ca:P ratio = 1.46:1).

In addition to this diet, the rats received fresh carrots and green-leaved vegetables every day.

As this diet contains 2% of cod liver oil and the rats received carrots in addition, the young rats probably received large quantities of vitamin A during suckling. This, however, was allowed in order to protect the mother's health.

The composition of the vitamin A-free diet was as follows:

- Casein: 17%
- Potato starch: 47%
- Arachis oil: 14.1%
- Dried Brewer's Yeast: 17.2%
- Salt mixture: 4.7%
It is said that rats require no ascorbic acid but to be on the safe side one ground tablet, equal to 50 mgm. vitamin C, was included in every two kgm. of diet.

The following salt mixture — 165 — of McCollum, Simmonds and Pitz (165) was used:

<table>
<thead>
<tr>
<th>Salt</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>87 gm.</td>
</tr>
<tr>
<td>MgSO4 (anhydrous)</td>
<td>133 gm.</td>
</tr>
<tr>
<td>NaH2PO4·H2O</td>
<td>173 gm.</td>
</tr>
<tr>
<td>K2HPO4</td>
<td>477 gm.</td>
</tr>
<tr>
<td>CaH4(PO4)2·H2O</td>
<td>270 gm.</td>
</tr>
<tr>
<td>Ca lactate</td>
<td>650 gm.</td>
</tr>
<tr>
<td>Fe citrate</td>
<td>150 gm.</td>
</tr>
</tbody>
</table>

During this work it was always felt that the vitamin A-free diet was not a very satisfactory one. Owing to post-war restrictions it was impossible to obtain corn-starch. Potato-starch, which was not always of a very good quality, was used instead. The arachis oil was obtained in one-gallon tins and was of a good quality. The colour varied from light straw-colour to light brown and no vitamin A or carotenoids could be detected in it by the antimony trichloride test. Before using a new tin it was always fortified with calciferol so as to obtain approximately 100 I.U. vitamin D per kgm. of mixed diet.

The casein was bought as "fat and vitamin A-free" but was always extracted with 95% alcohol for about 7 - 8 hours before using. Care was taken not to overheat the casein during this treatment, to prevent diarrhoea in the experimental animals. After the extraction, which was done in batches of 15 - 20 lbs., the casein was dried at approximately 40° C. This process was facilitated by the use of an electric fan.
The vitamin A-free food mixture (without the arachis oil) was mixed in quantities of 5 kgm. at a time. Each day the necessary amount of this dry food mixture was weighed out and the corresponding amount of KJ-solution (0.64 gm. per litre) added. The oil was rubbed in as in baking and afterwards enough water was added to make a stiff paste, which was crumbled up and put into the food-pots.

For convenience in making up different quantities of diet a table was drawn up to show the amounts of arachis oil and KJ-solution for different quantities of dry food mixture and was found very useful. One unit in this table was: 71 gm. dry food mixture, 12 gm. of arachis oil and 0.5 ml. KJ-solution.

To economise the food, residues remaining in the pots were collected every morning and after any pine-shavings were removed, it was rubbed into the fresh food. Every third or fourth morning, however, all the residues were discarded and the pots rinsed with soap and hot water.

3. The Marchi Technique:

If properly used, the Marchi method is sufficiently accurate to follow degenerative changes in the myelin substance of the central nervous system. Duncan (57) demonstrated that the number, size and general appearance of the black areas vary with the fixing fluid used, the strength of the osmic acid and the time that the tissues are exposed to its action. Irving and Richards (129), on the other hand, stated that "great care must be taken in dissection to avoid any straining or distortion of the tissues which may give rise to artefacts" and formal fixation must be avoided, since this gives very unsatisfactory results.
Thus, it is evident that the Marchi technique for quantitative work depends on the constancy with which it is performed and it is essential that the inexperienced worker must do a lot of preliminary work to gain experience with this apparently simple method before reliable results can be expected.

For this work the general procedure of the Marchi method was modified in some respects in order to obtain a technique suitable for routine work and more than once during the actual experimental work as many as 80 medullas were embedded on the same day.

Rats were killed with coal gas in groups of about ten each. Part of the skin and the muscles on the back were removed to expose the skull and upper cervical vertebrae but without removing the ears. The cranium and spinal canal were opened with great care to avoid injuring the medulla. Having finished that operation, the skull with the central nervous system inside was cut off from the rest of the body. Owing to the tension of the ligaments the cervical vertebrae were then pulled at an angle to the skull. To avoid fixing of the medulla in that right-angled position, the axis of the skull was aligned to that of the spinal cord by inserting the sharpened end of a match through the trachea into the mouth.

The skulls of the rats belonging to different dosage groups were placed in bottles containing 2.5% aqueous potassium bichromate solutions with corresponding labels. After five days the dissection was completed by removing the lower medulla and upper cervical cord (afterwards referred to as the medulla), which was transferred to the same strength of potassium bichromate solution in a cork-stoppered flat bottom specimen tube where it stayed for a further five days. The nervous
tissue was very soft at that stage and it was necessary to handle it with the utmost care to avoid squashing it between the fingers.

The 2.5% bichromate solution was next poured out and about 3 cc. of a mixture consisting of one part of a 1% acetic acid solution in two parts of a 5.3% solution of potassium bichromate, thoroughly mixed in a glass-stoppered measuring cylinder, was put into the tube containing the specimen. That solution was changed for a similar one after five days for the same period.

Before washing, the medullas were put into separate pieces of cheesecloth, which were tied up with cotton and/or wool threads of different colours according to a previously drawn-up list which identified the medullas with their corresponding threads. To remove all the chrome salts washing was continued in running water for 24 to 48 hours. Dehydration was started in the afternoon and by using the following time-table it was possible to finish the embedding without interfering with the other part of the work.

<table>
<thead>
<tr>
<th>Time</th>
<th>Solutions</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.30 p.m.-6 p.m.</td>
<td>50% alcohol</td>
</tr>
<tr>
<td>6</td>
<td>70%</td>
</tr>
<tr>
<td>7.30 a.m.-8.30 a.m.</td>
<td>90%</td>
</tr>
<tr>
<td>8.30</td>
<td>95%</td>
</tr>
<tr>
<td>9.30</td>
<td>100%</td>
</tr>
<tr>
<td>10.30  11.30</td>
<td>100% + chloroform(1:1)</td>
</tr>
<tr>
<td>11.30-12.30</td>
<td>Chloroform</td>
</tr>
<tr>
<td>12.30  2 p.m.</td>
<td>Paraffin bath</td>
</tr>
<tr>
<td>2 p.m.</td>
<td>4.30 p.m.-6 p.m.</td>
</tr>
</tbody>
</table>

By the use of match boxes and square pieces of paper with details written on them, it was possible to embed a large number of medullas per day without the danger of making a mistake.
The sections (10µ) were cut serially starting from the cord end. Two out of every ten were taken and arranged in sequence on the microscopic slide in two parallel rows of ten each. Cover-glasses of two inches in length were used and every slide received a label with the same details as were written on the corresponding match box.

As a general rule, Marchi stained nervous tissues are very brittle, but the use of the following precautions seemed to improve the method and sections without any cracks were obtained. Care was taken to perform the transference of the medullas from the cold alcohol to the wax baths as quickly as possible, lest the tissues might get dry, because it was experienced that should this happen cracks were formed by shrinkage. A vacuum oven was used for the embedding and it was found important to decrease the pressure very gradually for a rapid decrease also resulted in cracks.

For the classification of the slides, two microscopes with mechanical stages were used. Slides belonging to the same assay were first classified roughly with one microscope in different groups according to their "stage of protection". A standard slide, say of stage 4, was next put under one microscope and slides which were expected to show approximately the same protection were examined individually under the other microscope. By finding first the section in the two rows revealing the maximal degeneration and, secondly, by comparing the number and size of the black areas in this section with that of the standard under the first microscope, it was easy to recognise the actual stage of protection. If the black spots showed a similar intensity to that of the standard, that specific slide was taken as stage 4.
while a little more degeneration denoted stage 3.5 and a little less stage 4.5. This procedure was repeated for every group.

With a little experience it was found that classification of the same slides after a few weeks showed no significant difference in the results. Owing to the strain on the eyes it is best to classify only one set of slides per day and not on two successive days.

Irving and Richards (123) suggested that the short Marchi technique described by Stewart (249) might be of value for hastening the results, but it was not tried out for the following reasons:

It is doubted if the consistency of the results would be the same as that obtained with the ordinary Marchi method. The chief objections to the latter method are the length of time necessary to complete the sections and that artefacts are fairly common. The short Marchi method, on the other hand, is rapid and cheaper since less osmic acid is required.

To get a clear understanding of the difficulties involved in this short method the following summary of its technique was made:

(1) Fix the tissues (2 - 3 mm. thick) in 10% formal-saline for at least two, but preferably for 8 to 10 days. Change the formal-saline after the first 24 hours.

(2) Cut frozen sections 30 µ in thickness.

(3) To prevent the formation of precipitates all the traces of formalin must be removed. Thus, wash for 1½ hours in as many changes of water as possible. Use distilled water for the last four washings.

(4) Place in 2.5% potassium bichromate solution for 24 hours at 21°C.

(5) Wash in distilled water until the deep yellow
It is essential to cease washing while the sections are still yellow lest the normal myelin should be rather dark.

(6). Transfer the sections to 1% osmic acid and keep in a dark place for 16 to 36 hours at 21°C. This step causes much trouble and will require much skill and experience if the method should be used for routine quantitative work.

The bottles containing the sections are usually placed in a tin having a light-tight cover and left overnight in a warm room. The sections are examined the next morning and periodically until they are dark enough for mounting; this can only be judged by experience.

Normally the sections are ready for mounting after 16 hours. The higher temperature appears to assist in the penetration of the fat globules by osmic acid and the sections do not, therefore, fade so easily. If sections in the osmic acid are exposed to light precipitates will be found to occur more often (loc. cit.).

(7) This stage is only required when the normal myelin is too dark and must be repeated as often as necessary.

(a) Wash in two changes of tap water for five minutes;

(b) Put in 0.05% potassium permanganate solution for 30 seconds;

(c) Rinse in tap water;

(d) Place in Pal’s Solution (equal parts of 1% osmic acid and 1% potassium sulphite) until all the brown deposit is removed.

(8) Wash in many changes of tap water to remove all acidity and to prevent fading.

(9) Dehydrate rapidly and mount the sections.

It is obvious that this method requires too much manipulation of the sections to be suitable for routine work and it is doubted if constant results will be obtained.
4. Preliminary Experiments.

Owing to the fact that the young rats start to eat their mother's diet when they are about 16 days of age, the mother with her litter was placed on the vitamin A-free diet 17 days after the birth of the litter. Litters were weaned at 22 days of age and, without exception, the mothers developed diarrhoea to a greater or lesser degree during the five days on the vitamin A-free diet. This symptom disappeared soon after she went back on to the stock diet. However, diarrhoea did not develop in the experimental animals.

One experiment was started with the intention of using an eight-day period of vitamin A restriction before weaning, but was stopped because the mothers developed very bad diarrhoea during that time. This condition affected the mother's milk production for the litters were in a very poor condition of health at weaning.

However, owing to the fact that the period before vitamin A deficiency degeneration started was found to be delayed by about one week when the rats received no vitamin A-free diet before weaning, it was decided to adhere to the practice of a five-day vitamin A-restricted period before weaning. More divergent results were also found with rats which remained on the stock diet until weaning.

The objects of the preliminary experiments were the following:

(a) To confirm the work of Irving and Richards on which the new method for the biological estimation of vitamin A is based (131);

(b) To determine (i) the effect of extra vitamin E on the degeneration in the spinal cord;
(ii) the best dosage range to use, and
(iii) the length of the test period so as to kill the rats when a maximal spread in degeneration occurs for the chosen dosage levels.

Rats were placed on the vitamin A-free diet at 17 days of age and weaned after five days when the test
period started. One group of rats received a diet which was fortified with an extra amount of vitamin E. In another group vitamin A was administered daily while the rest received their vitamin A every three days. Graded doses of vitamin A (0.25, 0.5, and 1 I.U./day) were administered orally and after 28 days the experimental rats were killed at intervals and their medullas examined by the Marchi method.

The maximal degeneration was found in the region about 3 mm. above and below the pyramidal decussation. A marked degeneration was found in the control animals receiving no vitamin A after 28 days, while those receiving 0.25 I.U./day showed little degeneration at that time.

The control rats showed their maximal degeneration after 35 days, when the group receiving 0.25 I.U./day were affected very slightly. After 42 days nearly the whole group receiving the lowest dose of vitamin A were in a moribund condition and sections of their spinal cords showed maximal degeneration, whereas the 1 I.U. group were slightly affected.

No significant difference was found between the degeneration of the group which had an extra amount of tocopherol in their diet and those who were on the usual vitamin A-free diet. The results were found to be the same whether vitamin A was administered daily or every three days.

The following conclusions were drawn from the data obtained from the preliminary experiments:

(a) The best length for the test period is between 35 and 42 days;

(b) The most satisfactory dosage range for this work is probably 0.25, 0.4 and 0.8 I.U. vitamin A/day. These three levels were selected owing to the fact that the dosage response curve was expected to be a straight line when the response was plotted against the logarithm (to the base 10) of the doses;
(a) To economise time and labour vitamin A can be administered every third day;

(b) The arachis oil in the vitamin A-free diet contains enough tocopherol to give a maximum vitamin A efficiency;

(c) Owing to the fact that rats received enough vitamin A show no degeneration in the spinal cord, it is obvious that this symptom is not due to the presence of any toxic substance in the experimental diet and is, therefore, specific for vitamin A deficiency;

(d) These results confirm the work of Irving and Richards. The constancy with which the degeneration occurs at various dosage levels of vitamin A, makes it suitable for use as the basis of a new prophylactic biological assay for this vitamin;

(e) The Marchi technique is reliable for the demonstration of the nervous degeneration and can be used for routine work with slight modifications as far as the histological procedure is concerned;

(f) There exists a significant difference between the response of male and female albino rats to the same dose of vitamin A;

(g) The condition of the eyes and the weights can be used as an indication of when the test period should cease;

(h) These first results indicated further that the response to a certain dose of International \( \beta \)-carotene standard (1934) will be similar to that of an equal amount of vitamin A.

The main objection to the use of a prophylactic method is that it takes longer to perform than the curative test. Time, labour and money, however, are saved at the feeding stage of this experiment, seeing that the test period is not longer than six weeks, whereas the depletion period of the curative method takes about five weeks and the test period is at least three weeks in addition. Thus, the experimental periods for this prophylactic method and the curative test are six, and at least eight weeks, respectively.

The curative method has the further disadvantage that frequently rats have to be rejected at the end of the preliminary period, because their weight curves render
them "unsuitable" for the test. A further reduction in the number of available animals may result owing to losses by death during the depletion period. The curative method is also carried out on animals which may differ widely in pathological condition, and this main source of error is avoided by using the prophylactic method. In this bioassay no animals need to be discarded.

In comparing the different biological methods for vitamin A estimation Coward (45 - p.51) came to the following conclusion:

"The "increase in weight" method has the distinct advantage over the other methods in having a criterion that is easily measured, though even this is not quite as accurate as one might at first imagine it to be, for a rat's weight fluctuates during 24 hours - it does not increase or decrease at a steady rate. The criterion is, however, much more accurately measured than either of the other criteria used for the determination of vitamin A, the error due to the uncertainty of whether xerophthalmia may be considered cured or not on any particular day being much greater than the error due to the fluctuation in weight of a rat during the day.

On the other hand, both the occurrence of xerophthalmia and the changes in the vaginal contents are thought to be due specifically to vitamin A deficiency and many people hold the opinion that a determination based on a reaction which is specific for the factor being measured is far better than a more accurate method based on a reaction such as the increase in weight which is not specific for any one substance."

5. Weight and Degeneration:

To prove the statement that the weight can be used in
this method as an indication as to when to stop the test period, the following calculations were made:

Rats used for the different determinations were weighed once a week for the first four weeks and more frequently after that time as judged by their general condition.

The average weight of every group at a given time was obtained by dividing the sum of the average weights of the males and the females in this specific group by two.

These average weights were plotted against the time of the test period and the slope of this curve was estimated by the use of the formula for a straight line, namely:

$$y = \bar{y} + b (x - \bar{x})$$

Where:
- $y$ = average weight of group
- $x$ = time
- $\bar{y}$ and $\bar{x}$ = averages
- $b$ = slope of curve.

Table III. was obtained from these results.

It is evident from these figures that there exist distinct differences between the weights of the different groups of rats at the end of the test period and these can be used as an indication as to when to stop the experiment. These distinctions are, however, not large enough at this stage to justify calculations for the prophylactic "growth" test. Therefore, such calculations were not carried out, for the results would not represent a true index for the evaluation of this method. A longer test period is probably required for the latter method.

Thus it was justifiable to kill the rats when the lowest dosage group became more or less constant in weight. At that stage the eyes of this group were also markedly affected, while the other groups were still normal with respect to eyes and weight.
TABLE III

<table>
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<tr>
<th>Section</th>
<th>Gill Sample</th>
<th>Slopes (B) for different dosage groups</th>
<th>Length of Test Period</th>
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<td>G.4 10/ day</td>
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<td>1.639</td>
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</tbody>
</table>

**Average:** 1.648 1.756 1.939

*This assay must be regarded with some suspicion. The rats behaved abnormally with respect to weight and eye conditions and, although the test period was much longer than in the other assays, the average degeneration was much lower. As will be seen later, the limits of error for this assay were very wide in comparison with that of other assays. The same diet was used for all the experiments and special care was taken that the stock and vitamin A-free diets did not get mixed up. No explanation can be offered for the abnormal behaviour of the rats in this assay.*
6. Technique of the Bioassay.

Except for the vitamin A acetate and distillate natural fish liver oils were used, where the vitamin A exists chiefly in the form of its esters (149). Estimations of the extinction coefficients were made with the Beckman spectrophotometer using cyclohexane as solvent and only in the case of the dogfish liver oil was the estimation carried out on the unsaponifiable fraction. The vitamin A acetate and distillate were dissolved in arachis oil to an E-value of about 7 and the spectrophotometric estimations were carried out on these solutions.

To obtain the final dosing solutions, the oils were diluted in steps with arachis oil to a potency of about 60 I.U. per gram. Fresh dosing solutions were made up every two weeks from a solution of intermediate strength. All the oils were stored in the refrigerator in glass-stoppered bottles. International β-carotene standard and vitamin A acetate were used as biological standards and each experiment was a comparison between standard and test oil responses which were obtained simultaneously.

Rats were distributed between the different dosage groups according to litter and sex, when they were 22 days of age and after they have been on the vitamin A-free diet for five days.

Dosing started at the beginning of the experiment and vitamin A was administered orally every three days by means of a tuberculin syringe with a micro-meter-screw attachment, which released a standard drop of 0.01 gm. (= 0.6 I.U. vitamin A) per turn. The different dosage groups received, therefore, 1, 2 and 4 turns per rat every third day, which was equal to approximately 0.2, 0.4 and 0.8 I.U. of this vitamin per day. Owing to the possibility that the time of dosing might have an influence on the absorption and utilization of vitamin A and
β-carotene, dosing was always carried out before feeding.

The test period lasted for 35 to 42 days and the weights and eye conditions were taken as the criterion as to when to stop the experiment. Rats were killed with carbon dioxide, the medullas stained with the Marchi technique and sections examined under the microscope to determine the degree of protection against degeneration. These results were used as the basis to calculate the biological vitamin A potency of the test oils and to work out corresponding conversion factors for them.
Irving and Richards (129, 131) indicated the degree of severity of the nervous lesions by numbers according to the following scheme:

Stage 1: a very few fibres degenerated.
Stage 2: a few scattered fibres degenerated.
Stage 3: well-marked degeneration.
Stage 4: very marked degeneration.
Stage 5: heavy degeneration.

These workers added that the degree of degeneration represented by the numbers 1 and 1 is of no significance since the few scattered fibres found here are present equally in positive controls and stock rats and resulted as an artefact of the Marchi technique. The present work proved the contrary. No degenerated fibres could be detected under the low power of the microscope in stock rats and positive controls, and the slightest degeneration found in the experimental rats was, therefore, taken as an indication of the first vitamin A deficiency symptom.

The system of classification used by Irving and Richards was found to be impractical for assay purposes. In the first instance, the resulting curve of response is negative and, secondly, it was found that with a little experience the degeneration can be divided into six different stages, which increase the accuracy of the estimation.

With the object of obtaining a positive curve of response it was decided to use the degree of protection against degeneration as the criterion of response. The value 0 was assigned to a section showing maximal degeneration to indicate that the degree of protection against degeneration was 0 in this case. A section with no degenerated fibres received the value 5 indicating full protection.
Much trouble was experienced in obtaining low power photomicrographs of the whole cross sections, but it was felt that this was the only way to give a true picture of the entire degeneration phenomenon. Marchi stained sections of the cord do not lend themselves readily to photography, especially at lower magnifications. The contrast on the normal photomicrograph proved to be insufficient, especially for comparisons of the lower stages of degeneration. To overcome this trouble and to produce a greater degree of contrast, it was necessary to transpose the negatives, thereby losing the detail of the undegenerated structures and increasing that of the degenerated tracts. In photographing the section with the lowest degeneration (stage 4 of protection) the required contrast was so high that the fibres crossing each other in the ground structures stood out as little black spots on this particular photomicrograph.

Owing to the fact that the classification was based chiefly on degeneration in the anterior half of the sections, higher power photomicrographs were taken from the antero-lateral columns of the same sections. These photomicrographs are normal reproductions.

The photomicrographs on the following pages represent the six stages of protection against degeneration on which the classification of the slides are based. The low power photos have a 14x magnification, while the higher power photos have a 45x magnification. For convenience in comparing the different stages of protection, the latter photomicrographs are placed right opposite the corresponding lower power photos.

It is evident from these photomicrographs that the first degeneration in vitamin A deficiency occurs in the funiculus praedorsalis and develops gradually
in the columns of Goll and Burdach. Even in the second stage of protection the posterior columns are affected to only a very slight degree in comparison with the degeneration in the antero-lateral columns. However, the severity of degeneration in the posterior columns increases rapidly in the later stages and reaches the same degree as in the anterior columns when there is no protection.
In 1952, Coward (41) described the difference in response to vitamin A dosage of male and female rats. She found that males gave results with a greater standard deviation than that given by female rats. However, the steeper slope of the curve of response of the former caused them to give results of greater accuracy than those obtained with female rats.

Brenner, Brookes and Roberts (20) observed a significant sex difference in the liver storage of vitamin A in rats. "The females stored and retained more vitamin A in the liver than the males, while the males had higher blood values." Using the fluorescent technique to demonstrate vitamin A in the liver, Popper and Brenner (221) came to the conclusion that "during depletion the livers of male rats lose vitamin A faster than those of females. There is, however, no sex difference with respect to method of utilization as judged from the histological picture."

These observations are in accord with results obtained with male and female rats as demonstrated in tables IV and V.

The dosing solutions were made up so as to contain approximately 0.6 I.U. of vitamin A per 10 mgm. When dosing every three days the 0.2 I.U./day group of rats received 10 mgm. of this dosing oil, while the 0.4 and 0.8 I.U./day groups were given 20 and 40 mgm. respectively.

By plotting the average results obtained in Table IV, against the logarithms (to the base 10) of the
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<tr>
<th>Section</th>
<th>Oil Sample</th>
<th>No. Males</th>
<th>C.2 m/day</th>
<th>No. Males</th>
<th>U.4 m/day</th>
<th>No. Males</th>
<th>U.8 m/day</th>
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<td>4</td>
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**TOTALS:**

111 122.0 120 208.0 121 357.5

**WEIGHTED MEANS:**

1.108 1.708 2.954
**TABLE V.**

PROTECTION OF FEMALE RATS ON DIFFERENT DOSES OF VITAMIN A.

<table>
<thead>
<tr>
<th>Section</th>
<th>Oil Sample</th>
<th>No.</th>
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<th>0.4 IU/day</th>
<th>No.</th>
<th>0.6 IU/day</th>
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<td>5</td>
<td>20.5</td>
</tr>
<tr>
<td>6.</td>
<td>Snek l.o.(b)</td>
<td>4</td>
<td>3.5</td>
<td>4</td>
<td>9.5</td>
<td>5</td>
<td>20.0</td>
</tr>
<tr>
<td></td>
<td>Vit.A.ac.Std.</td>
<td>4</td>
<td>6.0</td>
<td>5</td>
<td>15.5</td>
<td>5</td>
<td>22.0</td>
</tr>
<tr>
<td></td>
<td>Hake l.o.(b)</td>
<td>4</td>
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<td>4</td>
<td>11.0</td>
<td>5</td>
<td>23.5</td>
</tr>
<tr>
<td>7.</td>
<td>Tuna l.o.(a)</td>
<td>5</td>
<td>12.0</td>
<td>5</td>
<td>13.5</td>
<td>5</td>
<td>22.5</td>
</tr>
<tr>
<td></td>
<td>Intern.Std.(a)</td>
<td>5</td>
<td>14.0</td>
<td>5</td>
<td>17.5</td>
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<td>21.5</td>
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<tr>
<td></td>
<td>Dogfish l.o.(a)</td>
<td>5</td>
<td>8.5</td>
<td>5</td>
<td>19.5</td>
<td>4</td>
<td>17.5</td>
</tr>
<tr>
<td>8.</td>
<td>Tuna l.o.(b)</td>
<td>5</td>
<td>7.5</td>
<td>5</td>
<td>13.5</td>
<td>5</td>
<td>22.5</td>
</tr>
<tr>
<td></td>
<td>Intern.Std.(b)</td>
<td>5</td>
<td>7.5</td>
<td>5</td>
<td>15.5</td>
<td>5</td>
<td>23.5</td>
</tr>
<tr>
<td></td>
<td>Dogfish l.o.(b)</td>
<td>5</td>
<td>5.0</td>
<td>5</td>
<td>11.5</td>
<td>5</td>
<td>22.5</td>
</tr>
<tr>
<td>9.</td>
<td>Stonesbass l.o.</td>
<td>5</td>
<td>2.0</td>
<td>6</td>
<td>17.5</td>
<td>5</td>
<td>20.5</td>
</tr>
<tr>
<td></td>
<td>Vit.A.ac.Std.</td>
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<td>6</td>
<td>25.5</td>
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<td>Stonesbass l.o.</td>
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<td>6</td>
<td>26.5</td>
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<tr>
<td></td>
<td>Vaalhaal l.o.</td>
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<td>9.0</td>
<td>6</td>
<td>15.0</td>
<td>4</td>
<td>13.0</td>
</tr>
</tbody>
</table>

**TOTALS:** 144 276.5 133 280.0 131 533.0

**WEIGHTED MEAN:** 1.920 2.657 4.069
doses the curve of response proves to be a straight line

(See Fig. I).

This curve of response is calculated as follows:

<table>
<thead>
<tr>
<th>Average Protection given in mgm.</th>
<th>Dose of Dose</th>
<th>Deviation of y into mean X = X</th>
<th>Product of y(x-X)</th>
<th>Square of deviation from mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.108</td>
<td>10</td>
<td>1.000</td>
<td>-0.301</td>
<td>-0.33327</td>
</tr>
<tr>
<td>1.708</td>
<td>20</td>
<td>1.301</td>
<td>0.000</td>
<td>0.000</td>
</tr>
<tr>
<td>2.854</td>
<td>40</td>
<td>1.602</td>
<td>0.301</td>
<td>0.8892</td>
</tr>
<tr>
<td>3.870</td>
<td>50</td>
<td>1.903</td>
<td>0.606</td>
<td>0.5565</td>
</tr>
<tr>
<td>= 8</td>
<td>= X</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\[
b (\text{the slope of the curve}) = \frac{\sum y(x - \bar{X})}{\sum (x - \bar{X})^2}
\]

\[= \frac{0.8892}{0.1812} = 5.071\]

The straight line is represented by the equation

\[y = \bar{Y} + b (x - \bar{X})\]

\[= 1.923 + 5.071 (x - 1.301)\]

(See Fig. I.).

Using the average figures of Table V in a similar calculation the log-dose-response curve for the females also proves to be a straight line, with almost the same slope, namely,

\[b = \frac{0.6470}{0.1812} = 3.571\]

The straight line here is represented by the equation

\[y = 2.949 + 3.571 (x - 1.301)\] (See Fig. I).
The average protection of females against degeneration is, however, 2.949/1.923 = 1.533 times higher than that of the males and it is, therefore, justifiable to assume that the minimum vitamin A requirement of male rats (about 9 weeks of age) is about 1.5 times higher than that of females of the same age. Several workers showed that the vitamin A requirement is a function of the bodyweight. However, the latter alone cannot account for the sex difference in the daily requirement of this vitamin, or vice versa for the sex difference of protection against degeneration, as illustrated in Table VI.
### TABLE VI. AVERAGE WEIGHTS OF RATS AT THE END OF THE TEST PERIOD IN GMs.

<table>
<thead>
<tr>
<th>Oil Sample</th>
<th>0.2 IU/day Males</th>
<th>0.2 IU/day Females</th>
<th>0.4 IU/day Males</th>
<th>0.4 IU/day Females</th>
<th>0.8 IU/day Males</th>
<th>0.8 IU/day Females</th>
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</thead>
<tbody>
<tr>
<td>Internat. Std. (a)</td>
<td>99</td>
<td>85</td>
<td>112</td>
<td>89</td>
<td>108</td>
<td>90</td>
</tr>
<tr>
<td>Vaalhaai l.o. (a)</td>
<td>101</td>
<td>84</td>
<td>111</td>
<td>87</td>
<td>102</td>
<td>93</td>
</tr>
<tr>
<td>Intern. Std. (b)</td>
<td>86</td>
<td>83</td>
<td>92</td>
<td>87</td>
<td>109</td>
<td>89</td>
</tr>
<tr>
<td>Vaalhaai l.o. (b)</td>
<td>84</td>
<td>85</td>
<td>97</td>
<td>84</td>
<td>113</td>
<td>87</td>
</tr>
<tr>
<td>Vit. A.ac. (a)</td>
<td>135</td>
<td>105</td>
<td>140</td>
<td>99</td>
<td>151</td>
<td>116</td>
</tr>
<tr>
<td>Intern. Std. (a)</td>
<td>135</td>
<td>108</td>
<td>145</td>
<td>104</td>
<td>145</td>
<td>110</td>
</tr>
<tr>
<td>Vit. A.ac. (b)</td>
<td>155</td>
<td>107</td>
<td>142</td>
<td>108</td>
<td>155</td>
<td>109</td>
</tr>
<tr>
<td>Vit. A.ac. (b)</td>
<td>74</td>
<td>74</td>
<td>79</td>
<td>73</td>
<td>91</td>
<td>74</td>
</tr>
<tr>
<td>Intern. Std. (b)</td>
<td>70</td>
<td>73</td>
<td>80</td>
<td>70</td>
<td>94</td>
<td>82</td>
</tr>
<tr>
<td>Vit. A.dist. (b)</td>
<td>72</td>
<td>76</td>
<td>75</td>
<td>77</td>
<td>98</td>
<td>74</td>
</tr>
<tr>
<td>Snoek l.o. (a)</td>
<td>81</td>
<td>86</td>
<td>100</td>
<td>83</td>
<td>113</td>
<td>85</td>
</tr>
<tr>
<td>Vit. A.ac. Std. (a)</td>
<td>75</td>
<td>85</td>
<td>102</td>
<td>79</td>
<td>91</td>
<td>103</td>
</tr>
<tr>
<td>Make l.o. (a)</td>
<td>85</td>
<td>82</td>
<td>95</td>
<td>86</td>
<td>103</td>
<td>88</td>
</tr>
<tr>
<td>Snoek l.o. (b)</td>
<td>88</td>
<td>69</td>
<td>94</td>
<td>83</td>
<td>109</td>
<td>90</td>
</tr>
<tr>
<td>Vit. A.ac. (b)</td>
<td>91</td>
<td>81</td>
<td>100</td>
<td>90</td>
<td>112</td>
<td>94</td>
</tr>
<tr>
<td>Make l.o. (b)</td>
<td>93</td>
<td>79</td>
<td>91</td>
<td>84</td>
<td>105</td>
<td>87</td>
</tr>
<tr>
<td>Tuna l.o. (a)</td>
<td>100</td>
<td>80</td>
<td>102</td>
<td>85</td>
<td>111</td>
<td>88</td>
</tr>
<tr>
<td>Intern. Std. (a)</td>
<td>96</td>
<td>84</td>
<td>100</td>
<td>92</td>
<td>112</td>
<td>88</td>
</tr>
<tr>
<td>Dogfish l.o. (a)</td>
<td>99</td>
<td>81</td>
<td>109</td>
<td>89</td>
<td>112</td>
<td>88</td>
</tr>
<tr>
<td>Tuna l.o. (b)</td>
<td>91</td>
<td>81</td>
<td>99</td>
<td>84</td>
<td>106</td>
<td>94</td>
</tr>
<tr>
<td>Intern. Std. (b)</td>
<td>100</td>
<td>86</td>
<td>95</td>
<td>95</td>
<td>107</td>
<td>95</td>
</tr>
<tr>
<td>Dogfish l.o. (b)</td>
<td>93</td>
<td>81</td>
<td>105</td>
<td>89</td>
<td>111</td>
<td>99</td>
</tr>
<tr>
<td>Stonebass l.o. (a)</td>
<td>75</td>
<td>84</td>
<td>91</td>
<td>89</td>
<td>106</td>
<td>90</td>
</tr>
<tr>
<td>Vit. A.ac. Std.</td>
<td>90</td>
<td>81</td>
<td>95</td>
<td>85</td>
<td>113</td>
<td>96</td>
</tr>
<tr>
<td>Stonebass l.o. (b)</td>
<td>85</td>
<td>82</td>
<td>84</td>
<td>89</td>
<td>114</td>
<td>81</td>
</tr>
<tr>
<td>Vaalhaai l.o. (c)</td>
<td>85</td>
<td>80</td>
<td>86</td>
<td>91</td>
<td>121</td>
<td>93</td>
</tr>
</tbody>
</table>

**TOTALS:** 2441 2179 2526 2271 2910 2360

**AVERAGES:** 94 84 101 87 112 91

**MALES/FEMALES** 1.12 1.16 1.22
It is evident from these figures that the difference in the body-weights of male and female rats can only partially be responsible for the sex difference in protection and the daily vitamin A requirements of rats. Other factors must be concluded to account for the rest of this difference.

Taking the average weight of these experimental rats at approximately 100 gm., Fig.1. shows that the minimum daily vitamin A requirement is about 20 - 30 I.U. per kgm. bodyweight for rats up to nine weeks of age.
CURVE OF RESPONSE.

The above-mentioned sex difference in the response of rats to the same dose of vitamin A created a difficult situation. It was impossible to use just one sex owing to the limitations of the breeding facilities. Two alternatives to solve the problem remained, namely, to keep the females longer on experiment until they reached a degeneration stage equal to that of the males, or to use equal numbers of males and females in all the groups. The former possibility was considered impracticable owing to the fact that it took more than three weeks to determine the stage of degeneration. With limited numbers of rats the latter alternative could not be followed.

It was observed in this department that, as a general rule, the litters contained more females than males. To spare time and material, two different oils were tested simultaneously against the same standard and with three groups for every oil solution, each assay consisted of nine dosage groups. Litters containing more than nine young at birth were always reduced to nine so as to ensure healthy rats at weaning. With a limited number of litters, which consisted of a maximum of nine rats per litter, it was not always possible to start such an experiment with equal numbers of males and females (5,5) in each group. The distribution was, therefore, always carried out with the main object of starting an experiment with ten rats in each group which consisted as far as possible of five males and five females and to minimise the influence of litter variations.

To overcome this trouble of sex difference and to obtain results which might lend themselves readily to statistical analysis, Professor Pretorius (222) suggested that an average factor should be calculated for each
experiment by which to multiply the response of every individual female in that assay, so as to obtain a response corresponding to that of a male or vice versa. Such a factor represents the ratio of average response of males: average response of females in the present experiments.

This suggestion was tested out and gave satisfactory results as may be seen from tables VII and VIII.

The assay given in table VII (Section I) gave an average factor of 0.513 while the duplicate of this assay (Section 2) revealed an average factor of 0.400. By multiplying the individual responses of females with the factors 0.513 and 0.400 for Sections 1 and 2 respectively, table VIII was obtained. Such transformed results were used in calculating the vitamin A potencies of the different oils.

TABLE VII.

PROTOCOLS OF AN ASSAY OF VAALMAAL LIVER OIL

SECTION I.

<table>
<thead>
<tr>
<th>Daily doses of Intern. α - car. Standard</th>
<th>0.2 I.U.</th>
<th>0.4 I.U.</th>
<th>0.6 I.U.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>M</td>
<td>F</td>
<td>M</td>
</tr>
<tr>
<td>0.5</td>
<td>0.5</td>
<td>2.0</td>
<td>5.0</td>
</tr>
<tr>
<td>1.0</td>
<td>1.5</td>
<td>0.0</td>
<td>4.8</td>
</tr>
<tr>
<td>2.0</td>
<td>2.0</td>
<td>1.5</td>
<td>2.0</td>
</tr>
<tr>
<td>0.5</td>
<td>1.0</td>
<td>4.0</td>
<td>1.5</td>
</tr>
<tr>
<td>1.5</td>
<td>2.0</td>
<td>4.5</td>
<td></td>
</tr>
</tbody>
</table>

TOTALES: 1.5 6.0 5.0 17.0 10.0 21.0

AVERAGE: 0.5 1.0 1.25 3.4 2.5 4.2

MALES/FEMALES: 0.500 0.348 0.595
### TABLE VII. (Contd).

**Daily doses of Vaalhais Liver Oil**

<table>
<thead>
<tr>
<th></th>
<th>GM.</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1/63,400</td>
<td></td>
<td>2/63,400</td>
<td></td>
<td>4/63,400</td>
<td></td>
<td></td>
</tr>
<tr>
<td>M</td>
<td>F</td>
<td>M</td>
<td>F</td>
<td>M</td>
<td>F</td>
<td>M</td>
</tr>
<tr>
<td>1.0</td>
<td>2.0</td>
<td>2.0</td>
<td>2.0</td>
<td>2.0</td>
<td>3.5</td>
<td></td>
</tr>
<tr>
<td>1.0</td>
<td>3.0</td>
<td>1.0</td>
<td>2.0</td>
<td>4.5</td>
<td>4.5</td>
<td></td>
</tr>
<tr>
<td>0.0</td>
<td>2.0</td>
<td>1.5</td>
<td>1.5</td>
<td>1.5</td>
<td>5.0</td>
<td></td>
</tr>
<tr>
<td>2.0</td>
<td>3.0</td>
<td>1.5</td>
<td>3.0</td>
<td>3.0</td>
<td>5.0</td>
<td></td>
</tr>
<tr>
<td>3.0</td>
<td>2.0</td>
<td>5.0</td>
<td></td>
<td></td>
<td></td>
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</tr>
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<td>0.0</td>
<td></td>
<td>2.0</td>
<td></td>
<td>31.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>TOTALS:</strong></td>
<td>2.0</td>
<td>12.0</td>
<td>4.5</td>
<td>10.5</td>
<td>9.5</td>
<td>21.0</td>
</tr>
<tr>
<td><strong>AVERAGES:</strong></td>
<td>0.667</td>
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<td>1.5</td>
<td>2.1</td>
<td>2.378</td>
<td>4.2</td>
</tr>
<tr>
<td><strong>MALES/FEMALES:</strong></td>
<td>0.533</td>
<td>0.714</td>
<td>0.566</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>GROUP AVERAGES:</strong></td>
<td>0.417</td>
<td>0.541</td>
<td>0.680</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Average factor for section I**

\[
= 0.813.
\]

### TABLE VIII.

**Protocols of two assays of Vaalhais Liver Oil.**

**Section I.**

<p>| | | | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Daily Doses of Standard</td>
<td>Daily Doses of Vaalhais L.O.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5</td>
<td>2.0</td>
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<tr>
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<td>0.0</td>
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<td>1.0</td>
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<td>0.0</td>
<td>1.5</td>
<td>1.5</td>
</tr>
<tr>
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<td>1.04</td>
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<td>2.33</td>
<td>1.55</td>
<td>1.04</td>
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<td>2.33</td>
<td>1.55</td>
<td>1.04</td>
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<td>2.33</td>
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<tr>
<td><strong>TOTALS:</strong></td>
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<td>20.67</td>
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<td>20.37</td>
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<td><strong>AVERAGE:</strong></td>
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<td>1.534</td>
<td>2.519</td>
<td>0.9133</td>
<td>1.234</td>
</tr>
<tr>
<td>Daily Doses of Standard</td>
<td>Daily doses of Vaalhais L/O</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>------------------------</td>
<td>-----------------------------</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.2 IU</td>
<td>0.4 IU</td>
<td>0.8 IU</td>
<td>1/23,400 PM</td>
<td>2/23,400 PM</td>
<td>4/23,400 PM</td>
</tr>
<tr>
<td>0.5</td>
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<td>1.4</td>
</tr>
<tr>
<td>0.0</td>
<td>0.2</td>
<td>1.0</td>
<td>0.4</td>
<td>0.4</td>
<td>0.6</td>
</tr>
<tr>
<td>0.4</td>
<td>1.0</td>
<td>1.8</td>
<td>0.6</td>
<td>0.6</td>
<td>0.8</td>
</tr>
<tr>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>0.6</td>
<td>0.6</td>
<td>0.6</td>
</tr>
<tr>
<td>TOTAL:</td>
<td>2.1</td>
<td>5.4</td>
<td>10.7</td>
<td>2.7</td>
<td>4.3</td>
</tr>
<tr>
<td>AVERAGE:</td>
<td>0.3</td>
<td>0.675</td>
<td>1.3375</td>
<td>0.3687</td>
<td>0.5575</td>
</tr>
</tbody>
</table>

The following average factors were calculated for the different assays:

Section 1: (Vaalhais L.O. (a) and Std.) 0.313
Section 2: (Vaalhais L.O. (a) and Std.) 0.400
Section 3: (Vit. A. and Std.) 0.847
Section 4: (Vit. A. and Std.) 0.724
Section 5: (Vit. A. and Std.) 0.569
Section 6: (Vit. A. and Std.) 0.621
Section 7: (Vit. A. and Std.) 0.678
Section 8: (Vit. A. and Std.) 0.630
Section 9: (Vit. A. and Std.) 0.537

The log-dosage-response curve with this method is a straight line both for carotene Standard (Table IX) and vitamin A-containing oils (Table X). The data of these two tables were used to plot the graphs in Figure II.
FIGURE II.

TABLE IX.

PROTOCOLS OF TWO ASSAYS OF TUNA AND DOGFISH LIVER OILS.

<table>
<thead>
<tr>
<th>Section</th>
<th>Daily Doses of Tuna I.C.</th>
<th>Daily doses of Intern. Std.</th>
<th>Daily doses of dogfish I.C.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.5</td>
<td>1.0</td>
<td>1.5</td>
<td>3.5</td>
</tr>
<tr>
<td>0.5</td>
<td>2.0</td>
<td>4.0</td>
<td>4.0</td>
</tr>
<tr>
<td>0.5</td>
<td>4.0</td>
<td>4.0</td>
<td>4.0</td>
</tr>
<tr>
<td>1.5</td>
<td>2.5</td>
<td>4.0</td>
<td>2.0</td>
</tr>
<tr>
<td>2.0</td>
<td>2.5</td>
<td>3.5</td>
<td>1.5</td>
</tr>
<tr>
<td>2.71</td>
<td>3.05</td>
<td>3.39</td>
<td>2.71</td>
</tr>
<tr>
<td>1.36</td>
<td>2.03</td>
<td>3.03</td>
<td>1.70</td>
</tr>
<tr>
<td>1.36</td>
<td>2.71</td>
<td>5.05</td>
<td>1.70</td>
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<tr>
<td>1.70</td>
<td>1.36</td>
<td>2.71</td>
<td>5.05</td>
</tr>
<tr>
<td>1.02</td>
<td>3.05</td>
<td>1.36</td>
<td>2.71</td>
</tr>
</tbody>
</table>
### Table IX. Contd.

#### Section B.

<table>
<thead>
<tr>
<th>Daily doses of Tuna l.c.</th>
<th>Daily doses of Intern. Std.</th>
<th>Daily doses of Dogfish l.c.</th>
</tr>
</thead>
<tbody>
<tr>
<td>% 0.2 I. U.</td>
<td>% 0.4 I. U.</td>
<td>% 0.8 I. U.</td>
</tr>
<tr>
<td>0.5</td>
<td>2.0</td>
<td>3.0</td>
</tr>
<tr>
<td>0.5</td>
<td>2.5</td>
<td>3.5</td>
</tr>
<tr>
<td>0.5</td>
<td>2.0</td>
<td>2.0</td>
</tr>
<tr>
<td>0.0</td>
<td>2.0</td>
<td>3.5</td>
</tr>
<tr>
<td>1.0</td>
<td>2.0</td>
<td>2.0</td>
</tr>
<tr>
<td>1.25</td>
<td>2.25</td>
<td>2.84</td>
</tr>
<tr>
<td>0.63</td>
<td>2.84</td>
<td>2.52</td>
</tr>
<tr>
<td>0.65</td>
<td>1.90</td>
<td>2.52</td>
</tr>
<tr>
<td>1.25</td>
<td>0.63</td>
<td>3.15</td>
</tr>
<tr>
<td>0.95</td>
<td>1.90</td>
<td>2.84</td>
</tr>
</tbody>
</table>

**TOTALES:**

27.24 41.67 60.74

**WEIGHTED MEANS:**

1.568 2.0835 3.057

The slope (b) of this curve for β-carotene is 2.782 and the straight line is represented by the equation \( y = 2.161 + 2.782 \) (\( x = 1.301 \)) - See Fig. II.

### Table X.

**PROTECTION WITH DIFFERENT DOSES OF VITAMIN A.**

<table>
<thead>
<tr>
<th>Oil Sample</th>
<th>Rats</th>
<th>0.2 Group</th>
<th>Rats</th>
<th>0.4 Group</th>
<th>Rats</th>
<th>0.8 Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Snook l.c.(a)</td>
<td>10</td>
<td>9.76</td>
<td>10</td>
<td>19.34</td>
<td>10</td>
<td>24.96</td>
</tr>
<tr>
<td>Vit. A. as. Std. (a)</td>
<td>10</td>
<td>6.05</td>
<td>10</td>
<td>19.61</td>
<td>9</td>
<td>21.95</td>
</tr>
<tr>
<td>Hake l.c.(a)</td>
<td>9</td>
<td>7.47</td>
<td>10</td>
<td>18.63</td>
<td>10</td>
<td>28.18</td>
</tr>
<tr>
<td>Snook l.c.(b)</td>
<td>10</td>
<td>6.17</td>
<td>10</td>
<td>15.89</td>
<td>10</td>
<td>22.93</td>
</tr>
<tr>
<td>Vit. A. as. Std. (b)</td>
<td>10</td>
<td>9.22</td>
<td>10</td>
<td>15.63</td>
<td>10</td>
<td>32.17</td>
</tr>
<tr>
<td>Hake l.c.(b)</td>
<td>10</td>
<td>12.58</td>
<td>10</td>
<td>19.62</td>
<td>10</td>
<td>29.62</td>
</tr>
<tr>
<td>Tuna l.c.(a)</td>
<td>10</td>
<td>14.15</td>
<td>9</td>
<td>21.15</td>
<td>10</td>
<td>35.25</td>
</tr>
<tr>
<td>Dogfish l.c.(a)</td>
<td>9</td>
<td>10.27</td>
<td>10</td>
<td>19.01</td>
<td>9</td>
<td>30.88</td>
</tr>
<tr>
<td>Tuna l.c.(b)</td>
<td>10</td>
<td>7.28</td>
<td>10</td>
<td>19.03</td>
<td>10</td>
<td>26.37</td>
</tr>
<tr>
<td>Dogfish l.c.(b)</td>
<td>10</td>
<td>9.05</td>
<td>9</td>
<td>11.75</td>
<td>10</td>
<td>22.67</td>
</tr>
<tr>
<td>Stonebass l.c.(a)</td>
<td>10</td>
<td>2.53</td>
<td>10</td>
<td>11.39</td>
<td>10</td>
<td>24.02</td>
</tr>
<tr>
<td>Vit. A. as. Std.</td>
<td>12</td>
<td>10.96</td>
<td>11</td>
<td>11.39</td>
<td>10</td>
<td>24.02</td>
</tr>
<tr>
<td>Stonebass l.c.(b)</td>
<td>10</td>
<td>7.09</td>
<td>10</td>
<td>15.89</td>
<td>10</td>
<td>21.71</td>
</tr>
<tr>
<td>Vaalhaa l.c.(a)</td>
<td>10</td>
<td>6.68</td>
<td>10</td>
<td>18.00</td>
<td>9</td>
<td>19.49</td>
</tr>
</tbody>
</table>
TAB.,....X. (Contd).  

<table>
<thead>
<tr>
<th></th>
<th>Rats Group</th>
<th>Rats Group</th>
<th>Rats Group</th>
<th>Rats Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>TOTALS:</td>
<td>140</td>
<td>119.44</td>
<td>139</td>
<td>220.86</td>
</tr>
<tr>
<td>WEIGHTED MEANS:</td>
<td>0.8531</td>
<td>1.5891</td>
<td>2.6934</td>
<td></td>
</tr>
</tbody>
</table>

b = 3.068  
y = 1.712 + 3.068 (x - 1.301) — See Fig. II.

Owing to the fact that the response of rats to a given dose of vitamin A is subject to fluctuations, "it is absolutely essential that each test should be a comparison between the response given to a dose of the test substance and the response given to a dose of the standard at the same time" (Coward 46 - p.2). Experimental rats must be distributed in equal numbers between the biological standard and the substances to be tested and the slope of the response curve must be obtained from every individual test itself.

The slope from a previously constructed dosage-response curve cannot be used, because "large changes of slope may occur with time.... These changes do not occur in a random fashion but exhibit a secular or quasi-periodic tendency. The use of a fixed slope, therefore, introduces an uncontrolled error for which it is impossible to allow. Even if regular records of slopes are kept, and their standard deviations over a considerable period of time calculated, this does not get over the difficulty, for the variations are not random, and so his standard deviation gives no clue to the error of the slope at any particular time" (Irwin 135).

This author demonstrated further that it is a faulty procedure to take the position and slope of the response curve given by the doses of vitamin A standard as correct and to use that for calculating the potency of the test substance. The correct procedure is to find the slope of the best-fitting straight line through all the
observed points; that is to say, it assumes that, apart from sampling error, the slopes provided by the standard and the test substance are the same. The horizontal distance between the parallel straight lines then provides an estimate of the potency ratio (loc. cit.).

A similar method of calculation was used in this work and Table XI illustrates the fluctuations which were found in the slopes of the response curves.

The slopes found for every section show very good agreement, but significant differences exist between slopes of different assays, e.g., section No. 3 and Section No. 6. It is evident from these data that "it is absolutely essential that each test should be a comparison between the response given to the dose of the test substance and the response given to the dose of the standard at the same time" (48).

**TABLE XI.**

**FLUCTUATIONS IN THE SLOPES (b) OF THE RESPONSE CURVES.**

<table>
<thead>
<tr>
<th>Section</th>
<th>Test Oil - Standard</th>
<th>Test Period</th>
<th>Slope (b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Vaalhaai l.o.(a)-Intern.Std</td>
<td>2/3/47 - 13/4/47</td>
<td>0.766</td>
</tr>
<tr>
<td>2.</td>
<td>Vaalhaai l.o.(b)</td>
<td>24/3/47 - 6/5/47</td>
<td>0.475</td>
</tr>
<tr>
<td>3.</td>
<td>Vit.A.ac.(a) - Intern.Std</td>
<td>27/4/47 - 29/6/47</td>
<td>0.563</td>
</tr>
<tr>
<td></td>
<td>Vit.A.dist.(a) - Intern.Std</td>
<td></td>
<td>0.547</td>
</tr>
<tr>
<td>4.</td>
<td>Vit.A.ac.(b) - Vit.A.ac.(a)</td>
<td>1/5/47 - 14/7/47</td>
<td>0.635</td>
</tr>
<tr>
<td></td>
<td>Vit.A.dist.(b) - Vit.A.ac.(b)</td>
<td></td>
<td>0.822</td>
</tr>
<tr>
<td>5.</td>
<td>Snoek l.o.(a)-Vit. A.ac.Std, Male l.o.(a)-Vit.A.ac.Std</td>
<td>21/6/47-11/8/47</td>
<td>0.756</td>
</tr>
<tr>
<td>6.</td>
<td>Snoek l.o.(b)Vit.A.ac.(Std)</td>
<td>21/7/47-9/9/47</td>
<td>0.993</td>
</tr>
<tr>
<td></td>
<td>Male l.o.(a) - Vit.A.ac.Std</td>
<td></td>
<td>1.000</td>
</tr>
<tr>
<td>7.</td>
<td>Tuna l.o.(a)-Intern.Std</td>
<td>12/5/47-28/9/47</td>
<td>0.954</td>
</tr>
<tr>
<td></td>
<td>Dogfish l.o.(a)-Intern.Std</td>
<td></td>
<td>1.007</td>
</tr>
<tr>
<td>8.</td>
<td>Tuna l.o.(b)-Intern.Std</td>
<td>14/6/47-23/10/47</td>
<td>0.914</td>
</tr>
<tr>
<td></td>
<td>Dogfish l.o.(b) Intern.Std</td>
<td></td>
<td>0.726</td>
</tr>
<tr>
<td>9.</td>
<td>Stonebass l.o.(a)-Vit.A.ac.Std</td>
<td>31/10/47-12/11/47</td>
<td>0.970</td>
</tr>
<tr>
<td></td>
<td>l.o.(b) - Vit.A.ac.Std</td>
<td></td>
<td>0.815</td>
</tr>
<tr>
<td></td>
<td>Vaalhaai l.o.(c)-Vit.A.ac.Std</td>
<td></td>
<td>0.819</td>
</tr>
</tbody>
</table>

The values of these slopes are not of the same magnitude as those previously used.
Several authors (16,24,128,133) described statistical methods to analyse results obtained with biological assays. In view of the unfamiliarity of some research workers and analysts with the use of statistical methods in the design and interpretation of experiments, it was decided that a worked out sample should be added...to the British Standards Institution Specification No.911 (24) in which a biological assay of vitamin D₃ by the chick method is described. Owing to the fact that the log-dosage-response curve is a straight line in both methods, it was decided to use the latter method to analyse the results.

The histological method for the biological estimation of vitamin A is based on the fact that if groups of young rats are kept under the same experimental conditions and receive graded doses of this vitamin over a suitable dosage range, equal proportional increases in the doses of this vitamin produce approximately equal average increases in the protection of these rats against degeneration in the central nervous system.

As far as the protective action is concerned, the test oil may be regarded as a dilution or concentration of the biological standard for vitamin A. If the observed average protections of the different groups of an assay are, therefore, plotted as ordinates against the logarithms of the corresponding doses as abscissae, then these two series of points will lie on approximately parallel straight lines, provided that, apart from sampling error, the slopes for the standard and test substances are the same. The horizontal distance between these two parallel straight lines then gives an estimate of the relative potencies of the two preparations.
**TABLE XII.**

### Section 5.

<table>
<thead>
<tr>
<th>Daily Dose of Shmck L.D.</th>
<th>Daily Dose of Vit. A.C. Std.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1/144,000 gm.</td>
<td>1/144,000 gm.</td>
</tr>
<tr>
<td>2.0</td>
<td>2.5</td>
</tr>
<tr>
<td>0.5</td>
<td>2.0</td>
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<td>1.0</td>
<td>1.5</td>
</tr>
<tr>
<td>0.0</td>
<td>1.5</td>
</tr>
<tr>
<td>1.14</td>
<td>0.28</td>
</tr>
<tr>
<td>1.14</td>
<td>1.14</td>
</tr>
<tr>
<td>0.57</td>
<td>1.14</td>
</tr>
<tr>
<td>1.14</td>
<td>2.28</td>
</tr>
<tr>
<td>2.42</td>
<td>2.00</td>
</tr>
<tr>
<td>0.85</td>
<td>2.00</td>
</tr>
<tr>
<td>0.976</td>
<td>1.654</td>
</tr>
</tbody>
</table>

**TABLE XIII.**

Calculations on responses to vitamin A acetate standard.

<table>
<thead>
<tr>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0</td>
<td>50</td>
<td>20</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>1.0</td>
</tr>
<tr>
<td>0.5</td>
<td>20</td>
<td>15</td>
<td>15</td>
<td>10</td>
<td>20</td>
<td>1.5</td>
</tr>
<tr>
<td>1.0</td>
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<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>2.0</td>
</tr>
<tr>
<td>2.0</td>
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<td>10</td>
<td>10</td>
<td>10</td>
<td>5</td>
</tr>
<tr>
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<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>1.0</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
</tr>
</tbody>
</table>

**TABLE XIV.**

Calculations on responses to tnt Shmck liver oil (a).

<table>
<thead>
<tr>
<th>1/144,000 gm.</th>
<th>5.8415</th>
<th>2/144,000 gm.</th>
<th>5.1427</th>
<th>4/144,000 gm.</th>
<th>5.4437</th>
</tr>
</thead>
<tbody>
<tr>
<td>1/144,000 gm.</td>
<td>0.976</td>
<td>1/144,000 gm.</td>
<td>1.654</td>
<td>1/144,000 gm.</td>
<td>2.495</td>
</tr>
<tr>
<td>2/144,000 gm.</td>
<td>0.760</td>
<td>30</td>
<td>10</td>
<td>24.95</td>
<td>10</td>
</tr>
<tr>
<td>4/144,000 gm.</td>
<td>0.630</td>
<td>30</td>
<td>10</td>
<td>24.95</td>
<td>10</td>
</tr>
<tr>
<td>Sums</td>
<td>30</td>
<td>30</td>
<td>15.2</td>
<td>69.53</td>
<td>111.66</td>
</tr>
</tbody>
</table>

**TABLE XV.**

Calculations on responses to tnt Shmck liver oil (b).

<table>
<thead>
<tr>
<th>1/144,000 gm.</th>
<th>5.8415</th>
<th>2/144,000 gm.</th>
<th>5.1427</th>
<th>4/144,000 gm.</th>
<th>5.4437</th>
</tr>
</thead>
<tbody>
<tr>
<td>1/144,000 gm.</td>
<td>0.976</td>
<td>1/144,000 gm.</td>
<td>1.654</td>
<td>1/144,000 gm.</td>
<td>2.495</td>
</tr>
<tr>
<td>2/144,000 gm.</td>
<td>0.760</td>
<td>30</td>
<td>10</td>
<td>24.95</td>
<td>10</td>
</tr>
<tr>
<td>4/144,000 gm.</td>
<td>0.630</td>
<td>30</td>
<td>10</td>
<td>24.95</td>
<td>10</td>
</tr>
<tr>
<td>Sums</td>
<td>30</td>
<td>30</td>
<td>15.2</td>
<td>69.53</td>
<td>111.66</td>
</tr>
</tbody>
</table>
To illustrate the different steps in the statistical analysis of the results of an experiment, the observed data for the snook liver oil (a) (Section 5 of Table XII) may be used.

The successive steps in the numerical analysis of an assay are:

1. To estimate the standard deviation of the protection of rats which received the same dose of vitamin A;

2. To check whether the points obtained for both the standard and the test oil lie within the limits of sampling deviations on two straight lines and if these two response-lines may be regarded as parallel;

3. Estimating the common slope of these two lines, the distance between them and then the potency of the test oil;

4. To find the fiducial limits for the potency of the test oil.

The most important part of the calculations is to use the experimental data to construct Tables XIII and XIV, where the responses obtained for the biological standard (vitamin A acetate in this case) and the test oil (snook liver oil (a)) are treated in parallel successive rows which correspond to the successive dosage groups.

(For the convenience of the reader an extra loose sheet containing these tables is included).
### Table XII

**Protocols of Two Assays of Sockeye & Hake Liver Oils.**

#### Section 5

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>2.0</td>
<td>2.5</td>
<td>2.0</td>
</tr>
<tr>
<td>0.5</td>
<td>2.0</td>
<td>2.5</td>
</tr>
<tr>
<td>1.0</td>
<td>2.5</td>
<td>2.0</td>
</tr>
<tr>
<td>0.0</td>
<td>2.0</td>
<td>2.5</td>
</tr>
<tr>
<td>1.44</td>
<td>0.28</td>
<td>1.0</td>
</tr>
<tr>
<td>1.14</td>
<td>1.14</td>
<td>1.14</td>
</tr>
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**TOTALS:**

| 0.976 | 1.634 | 2.496 | 0.606 | 1.661 | 2.43 |

**Section 6**

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### TABLE XIII.

**CALCULATIONS ON RESPONSES TO VITAMIN A ACETATE STANDARD**

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<th>No</th>
<th>1.U/day</th>
<th>Log dose</th>
<th>XL</th>
<th>YL</th>
<th>n1</th>
<th>n1^2</th>
<th>n1(x1)</th>
<th>n1(x1)^2</th>
<th>n1(y1)</th>
<th>n1(y1)^2</th>
<th>n1(y1^2)</th>
<th>n1(y1^2)-1</th>
<th>S1((y1^2))</th>
<th>(f1(y1^2))</th>
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<td>-6.05</td>
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<td>1.461</td>
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<td>32.35</td>
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**Sums:**

- \(\sum Y_1 = -1\)
- \(\sum Y_2 = 1.8856\)

**Correction Terms:**

- \(C_1 = -0.0345\)
- \(C_2 = 1.65\)

**Differences:**

- \(\bar{y}_1 = 0.8651\)
- \(\bar{y}_2 = 1.65\)

**Remainder:**

- \(1 \times A_1 = 0.14\)

### TABLE XIV.

**CALCULATIONS ON RESPONSES TO TEST SHEEP LIVER OIL (a)**

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<th>Sm/day</th>
<th>Log dose</th>
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<th>Y2</th>
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<th>n2(y2)</th>
<th>n2(y2)^2</th>
<th>n2(y2)^2)</th>
<th>S2((y2^2))</th>
<th>(f2(y2^2))</th>
<th>(f2)</th>
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<td>30.63</td>
<td>3.93</td>
<td>9</td>
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<td>4/144000</td>
<td>4.4437</td>
<td>1</td>
<td>2.496</td>
<td>10</td>
<td>10</td>
<td>24.96</td>
<td>24.96</td>
<td>25.30</td>
<td>30.84</td>
<td>6.54</td>
<td>9</td>
</tr>
</tbody>
</table>

**Sums:**

- \(\sum Y_2 = 55.29\)
- \(\sum Y_2 = 44.61\)

**Correction Terms:**

- \(D_1 = 0\)
- \(D_2 = 0\)

**Differences:**

- \(\bar{x}_2 = 0.86\)

**Remainder:**

- \(1 \times A_2 = 0.4019\)
In Tables XIII and XIV column (1) contains the daily dose of vitamin A and column (2) its logarithm to the base 10. Owing to the fact that the doses both of the vitamin A acetate standard and the test oil (snook liver oil(a)) were in geometrical progression, the entries in Col. (2) increase by steps of 0.301. This interval is, therefore, taken as the working unit of the abscissae and afterwards the much more convenient entries in Col. (3) are used instead of the entries in Col. (2). The zero entries in Col. (3) may be placed wherever seems most convenient. The first row of Table XIII is completed as follows:

Col. (5): \( n_1 \) = number of rats receiving 0.1959 I. U. = 10,
Col. (7): \( n_1Y_1 \) = the sum of their potencies
\[
1.0 + 0.0 + 0.3 + \ldots + 1.14 = 6.05
\]
Col. (4): \( Y_1 \) = their average protection
\[
(7)/(5) = 6.05/10 = 0.605
\]
Col. (6): Multiply (5) by (3): (+10) \( \times \) (-1)
\[= -10.\]
Col. (8): Multiply (6) by (3): (-10) \( \times \) (-1) \( = \) 10
Col. (9): Multiply (7) by (3): (6.05) \( \times \) (-1)
\[= -6.05\]
Col. (10): Multiply (7) by itself and divide by (5): (6.05 \( \times \) 6.05) / 10 \( = \) 3.66
Col. (11): \( n_1 [5]^2 \) = sum of the squares of the 10 protections:
\[
(1.0)^2 + (0.0)^2 + (0.3)^2 + \ldots + (1.14)^2 = 5.29
\]
Col. (12): Subtract (10) from (11):
\[
5.29 - 3.66 = 1.63
\]
Col. (13): Reduce entry in Col. (5) by one. The remaining rows in Table XIII are completed in the same way and columns (5) to (13) are totalled.

Enter the quantities \( \bar{n}_1 \) and \( \bar{Y}_1 \) under Columns (1) to (3) for later use.
These quantities are calculated as follows from the totals of columns (5), (6) and (7):

\[ \bar{y}_1 = \frac{-1}{29} = -0.0345. \]
\[ \bar{y}_2 = \frac{44.61}{29} \approx 1.5583. \]

The "correction terms" which are subtracted from the totals of columns (8), (9) and (10) are also calculated from the totals of columns (5), (6) and (7):

For Col. (8): Multiply (6) by itself and divide by (5):

\[ \frac{-1}{29} = 0.03 \]

For Col. (9): Multiply (6) by (7) and divide by (5):

\[ \frac{-1}{29} \approx -1.54. \]

For Col. (10): Multiply (7) by itself and divide by (5):

\[ \frac{44.61}{29} \approx 1.55. \]

For convenience the differences left in columns (8), (9) and (10) of Table XIII, after the subtraction of these correction terms, are denoted by \( P_1 \), \( Q_1 \) and \( P_2 \), respectively, thus:

\[ P_1 = 18.97; \quad Q_1 = 17.44; \quad P_2 = 16.17. \]

To complete Table XIII a further term, \( \frac{Q_1^2}{P_1} = 16.05 \), is subtracted from \( P_1 \). The remaining difference is 0.14 and is denoted by \( \Delta A_1 \). The numerical multiplier attached to \( A_1 \) is obtained by reducing the number of the separate dosage groups receiving the same oil preparation by two.

Table XIV is constructed in exactly the same way as Table XIII.

The corresponding values for the snook liver oil in Table XIV are:

\[ \bar{y}_2 = \frac{0}{30} = 0; \quad \bar{y}_3 = \frac{81.06}{30} \approx 1.7020. \]
\[ Y_2 = 30; \quad Y_3 = 15.20 \]
\[ Y_4 = 11.63; \quad \frac{Y_2^2}{Y_3} = 11.55 \]
\[ \text{Remainder} = 1 \times A_3 = 0.08. \]
From these final values of the two tables, the following are obtained:

\[ \bar{Y}_1 = \bar{Y}_2 = -0.3245 \quad \bar{Y}_1 - \bar{Y}_2 = -0.1637 \]

\[ p = \bar{Y}_1 + \bar{Y}_2 = 38.97 \quad q = \bar{Q}_1 + \bar{Q}_2 = 32.65 \]

\[ r = \bar{Y}_1 + \bar{Y}_2 = 27.80 \quad q^2/r = 27.35 \]

The successive steps (1) to (4) in the numerical analysis of the assay are now calculated.

1. **Estimation of the Standard Deviation of Individual Responses:**

   The estimate \( s^2 \) of the variance (squared of the standard deviation) of the protection of rats receiving the same dose of vitamin A is calculated at the foot of Table XIV. It is obtained by dividing the sum of the totals of columns (12) in Tables XIII and XIV by the sum of the totals of columns (13).

   \[ s^2 = 0.4019 \text{ (53 Degrees of Freedom)} \]

   This value is used in the investigation of steps (2) and (4). In the calculations certain subsidiary constants, which are obtained from published tables (73, 245), are used. These constants depend on the difference between the total number of rats and the total number of dosage groups used in the assay. This difference is called "the number of degrees of freedom available for calculating \( s^2 \), and is the sum of the totals of columns (13) in Tables XIII and XIV.

2. **Can the Response Curves be regarded as Straight Lines and are they Parallel?**

   The answer to these questions may be obtained by a single test. The following calculation is necessary:

   Subtract the value found for \( q^2/p \) from that found for \( r \), and divide the difference by the total number of dosage groups minus 3. In this case the result is:

   \[ \frac{27.80 - 27.35}{(3+3) - 3} = 0.45/3 = 0.15. \]
This value is now divided by $s^2$:

$\frac{0.15}{0.4019^2} = 0.3733$.

Should this final figure be less than 2, as in this example, the two response curves may be regarded as rectilinear and parallel, provided that there are not more than ten dosage groups in the assay. In other cases this figure should be checked against a "Table of the 5 per cent Points of the Variance Ratio" (75, 245) which will generally result in the rejection of one or more of the extreme dosage groups. Such a rejection will, however, usually prove the fiducial limits for the potency to lie so far apart that it will be necessary to repeat the assay.

(3) Estimation of the Potency of the Sneek Liver Oil:

Still using the working unit of 0.301 the common slope of the two log-dosage-response curves is estimated as

$$b = \frac{a}{2} = \frac{32.68}{28.97} = 0.8376.$$

and the horizontal distance between them as

$$d = (\bar{x}_1 - \bar{x}_2) - (\bar{y}_1 - \bar{y}_2)/b$$

$$= -0.0345 - (-0.161/0.8376)$$

$$\approx -0.0345 + 0.1954.$$  

$$\approx 0.1609.$$

By multiplying this horizontal distance \(d\) between the two response lines by the working unit 0.301, the logarithm to the base 10 is obtained for the ratio:

\[ \text{Activity of } \frac{2}{144,000} \text{ gm. of Sneek Liver Oil} \]
\[ \text{Activity of } 0.3918 \text{ I.U. of Standard.} \]

The doses of the Standard and the test oil which appear here, are those doses to which zero values of \(x\) were assigned in Tables XIII and XIV. The resulting value for this ratio is

\[ (0.1609)(0.301) \approx 0.4864 = \log (1.118). \]
Thus, it is estimated that $2/144,000 = 1/72,000 \text{ gm.}$
of Snoek liver oil contains $(1.118)(0.3918)$ I.U. of
vitamin A. Therefore, 1 gm. contains:

$(1.118)(0.3918) (72,000) \text{ I.U. Vitamin A.}$

\[ \approx 31,538 \text{ I.U.} \]

The cm. 328 of this oil was estimated as 17.64
and the corresponding conversion factor is

\[ 31,538 / 17.64 \approx 1838. \]

(4) Calculation of the true fiducial limits for the
potency:

The limits of error for an assay are usually ex-
pressed as $P\leq 0.95$, which indicates that the result of the
test will be within the given limits 95 times out of
every 100 times that the test is made.

To obtain the fiducial limits for the potency the
range covered by the limits for the distance $d$ is first
calculated. These values are then multiplied by the
working unit and the antilogarithms taken. A subsidiary
constant is used here which is extracted from a “Table of
the Distribution of $t$” (73,245). The required value here
is $t = 2.060$ and is obtained from the column headed
“Probability 0.05” and the row corresponding to the number
of degrees of freedom (55) on which $\sigma^2$ is based.

The $P\leq 0.95$ limits for $d$ are

\[ d' - t s' \quad \text{and} \quad d' + t s' \]

where

\[ d' = (\bar{x}_1 - \bar{x}_2) - c (\bar{y}_1 - \bar{y}_2) / b. \]

$s'$ is calculated from the formula

\[ s'^2 = \frac{S^2}{\frac{N_1}{b^2} + \frac{1}{b^2}} \left( \frac{\bar{y}_1 - \bar{y}_2}{b} \right)^2 \]

where

$C$ = a corrective factor

$N_1$ = Total number of rats receiving doses of the Standard.

$N_2$ = Total number of rats receiving doses of the test oil.

The corrective factor $C$ is calculated from the
formula

\[ C = \frac{N_2}{N_1} \]
The following values have been found earlier in this analysis:

\[ \bar{x}_1 - \bar{x}_2 = -0.0345 ; \quad \bar{y}_1 - \bar{y}_2 = -0.1637. \]
\[ p = 26.97 ; \quad q = 32.65 \]
\[ s^2 = 0.4019 ; \quad q^2/p = 27.35. \]
\[ t = 2.006 ; \quad q^2/s^2 = 68.05 \]
\[ b = 0.6278 ; \quad (\bar{y}_1 - \bar{y}_2)/b = 0.1954. \]

\[ c = 68.05 \]
\[ = 68.05/64.03 \]
\[ = 1.063 \]
\[ d' = -0.0345 - (1.063)(-0.1954) \]
\[ = -0.0345 + 0.2077 \]
\[ = 0.1732. \]
\[ s' = \left[ \frac{(0.4019)}{(0.6278)^2} \left( \frac{1}{26.97} + \frac{1}{32.65} + (1.063)(0.1954)^2 \right) \right] \]
\[ = (1.063)(0.4104)(0.0688) \]
\[ = 0.04104. \]

Whence
\[ s' = 0.2046 \]
\[ t s' = 0.4104 \]
\[ d' = t s' = -0.2375 \]
\[ d' + t s' = 0.5836. \]

Multiply now by the working unit 0.301:
\[ (-0.2375)(0.301) = -0.0714 = 1.9286. \]
\[ = \log (0.8484). \]
\[ (0.5836)(0.301) = 0.1756 = \log (1.498). \]

The values for the 95% limits of potency of the test oil are now
\[ (0.8484)(0.3918)(72,000) \text{ I.U.} \]
\[ (1.498)(0.3918)(72,000) \text{ I.U./gm}. \]

These values give the lower and upper limits of error for the assay as
75.9% to 134.0% respectively.
RESULTS:
The protocols of the different assays given already are:

- Vaalbaai liver oil ........ Table VIII.
- Tuna and Dogfish Liver Oils.. Table IX.
- Snoek and Hake Liver Oils.... Table XII.

Before giving the results obtained for the different oils Tables XV and XVI may be added. These two tables contain the protocols for the remaining oils.

The statistical method as described was used to analyse the observed experimental data. The results are summarised in Table XVII. A comparison between the limits of error in this Table and that given in Table II for the three weeks' curative "growth" method, can be used in the evaluation of the accuracy of this histological method for Vitamin A estimations.

The accuracy of a biological assay is, however, also dependent upon the number of animals used. The disadvantage of Table II is that these numbers are unknown. Dr. Irwin (139) calculated the limits of error for the Pharmacopoeia for different vitamins with varying numbers of experimental animals and Table XVIII was extracted from his findings for vitamins A and B₁. The data for the latter vitamin was included to serve as a comparison between the accuracy of the bioassay of vitamin A and that of another vitamin.
**TABLE XV.**

PROTOCOLS OF THE ASSAYS OF VITAMIN A ACETATE AND VITAMIN A DICOT BomHIS OIL.

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<th>Daily doses of Int. std., 0.2 I. U.</th>
<th>Daily doses of Int. std., 0.4 I. U.</th>
<th>Daily doses of Int. std., 0.8 I. U.</th>
<th>Daily doses of Vit. A. dist.</th>
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<th>Daily doses of Int. std., 0.4 I. U.</th>
<th>Daily doses of Int. std., 0.8 I. U.</th>
<th>Daily doses of Vit. A. dist.</th>
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<td>2.90</td>
<td>3.26</td>
<td>2.90</td>
</tr>
<tr>
<td>0.72</td>
<td>2.17</td>
<td>2.17</td>
<td>1.61</td>
<td>2.17</td>
<td>2.90</td>
</tr>
</tbody>
</table>

| 17.96     | 23.18    | 32.43   | 28.00   | 23.52   | 30.94   | 24.44   | 27.03   | 34.33   |
| 1.796     | 2.318    | 3.243   | 2.000   | 2.352   | 3.094   | 2.444   | 2.705   | 5.435   |
### Section 9.

#### TABLE XVI.

**Protocols of an Assay of Stonebass and Vaalnai Liver Oils.**

<table>
<thead>
<tr>
<th>Daily doses of Stonebass L.C.</th>
<th>Daily Doses of Vit. A ac., etc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1/48,000 gm.</td>
<td>1/240,000 gm.</td>
</tr>
<tr>
<td>0.5</td>
<td>0.0</td>
</tr>
<tr>
<td>0.5</td>
<td>1.0</td>
</tr>
<tr>
<td>0.5</td>
<td>1.0</td>
</tr>
<tr>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>0.0</td>
<td>1.61</td>
</tr>
<tr>
<td>0.0</td>
<td>1.61</td>
</tr>
<tr>
<td>0.27</td>
<td>1.61</td>
</tr>
<tr>
<td>0.27</td>
<td>1.34</td>
</tr>
<tr>
<td>0.0</td>
<td>1.34</td>
</tr>
<tr>
<td>0.54</td>
<td>1.88</td>
</tr>
<tr>
<td>0.54</td>
<td>1.88</td>
</tr>
<tr>
<td>2.66</td>
<td>11.39</td>
</tr>
<tr>
<td>0.258</td>
<td>1.139</td>
</tr>
</tbody>
</table>

#### Similar daily doses of Stonebass Liver Oil

<table>
<thead>
<tr>
<th>Daily doses of Vaalnai Liver</th>
<th>1/81,000 gm.</th>
<th>2/81,000 gm.</th>
<th>4/81,000 gm.</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>2.0</td>
<td>2.0</td>
<td>0.0</td>
</tr>
<tr>
<td>0.5</td>
<td>2.5</td>
<td>2.5</td>
<td>1.0</td>
</tr>
<tr>
<td>0.5</td>
<td>2.0</td>
<td>2.0</td>
<td>0.0</td>
</tr>
<tr>
<td>2.0</td>
<td>2.0</td>
<td>1.5</td>
<td>1.0</td>
</tr>
<tr>
<td>1.08</td>
<td>0.0</td>
<td>2.42</td>
<td>0.54</td>
</tr>
<tr>
<td>0.54</td>
<td>2.15</td>
<td>2.15</td>
<td>0.54</td>
</tr>
<tr>
<td>0.54</td>
<td>0.54</td>
<td>2.15</td>
<td>1.08</td>
</tr>
<tr>
<td>0.54</td>
<td>1.08</td>
<td>2.15</td>
<td>0.81</td>
</tr>
<tr>
<td>1.08</td>
<td>0.54</td>
<td>2.42</td>
<td>0.81</td>
</tr>
<tr>
<td>0.81</td>
<td>1.08</td>
<td>2.42</td>
<td>1.08</td>
</tr>
<tr>
<td>7.09</td>
<td>13.39</td>
<td>21.71</td>
<td>6.88</td>
</tr>
<tr>
<td>0.709</td>
<td>1.369</td>
<td>2.171</td>
<td>0.686</td>
</tr>
</tbody>
</table>
### TABLE XVII.
**LIMITS OF ERROR \( (p=0.95) \) AND CONVERSION FACTORS ESTIMATED FOR DIFFERENT VITAMIN A PREPARATIONS.**

<table>
<thead>
<tr>
<th>Oil Sample</th>
<th>Conv. Fact.</th>
<th>Av. Conv. Fact.</th>
<th>Limit of Error (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vaalhai l.o. (a)</td>
<td>1805</td>
<td>1659</td>
<td>74.3 * 130.9</td>
</tr>
<tr>
<td>&quot; (b)</td>
<td>1391</td>
<td>1618</td>
<td></td>
</tr>
<tr>
<td>&quot; (c)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vit. A. asst. (a)</td>
<td>1918</td>
<td>1774</td>
<td>52.6 * 151.2</td>
</tr>
<tr>
<td>&quot; (b)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vit. A. distillate</td>
<td>1455</td>
<td>1957</td>
<td>67.9 * 292.2</td>
</tr>
<tr>
<td>Tuna l.o. (a)</td>
<td>1776</td>
<td>1984</td>
<td>78.4 * 188.8</td>
</tr>
<tr>
<td>&quot; (b)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dogfish l.o. (a)</td>
<td>1857</td>
<td>1411</td>
<td>76.4 * 128.3</td>
</tr>
<tr>
<td>&quot; (b)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sockey l.o. (a)</td>
<td>1738</td>
<td>1411</td>
<td>73.0 * 124.0</td>
</tr>
<tr>
<td>&quot; (b)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tuna l.o. (a)</td>
<td>1981</td>
<td>1484</td>
<td>72.7 * 122.0</td>
</tr>
<tr>
<td>&quot; (b)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stonebass l.o. (a)</td>
<td>1408</td>
<td>1475</td>
<td>70.9 * 120.0</td>
</tr>
<tr>
<td>&quot; (b)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

(The average number of rats used in an assay of one oil in this work was 30).

### TABLE XVIII.
**THE LIMITS OF ERROR \( (p=0.95) \) WITH VARYING NUMBERS OF ANIMALS.**

<table>
<thead>
<tr>
<th>VITAMIN A (CURATIVE).</th>
<th>3 Weeks</th>
<th>5 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td>20 rats</td>
<td>47 and 214</td>
<td>56 * 172</td>
</tr>
<tr>
<td>40 rats</td>
<td>52 * 193</td>
<td>56 * 172</td>
</tr>
<tr>
<td>80 rats</td>
<td>63 * 199</td>
<td>68 * 146</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>VITAMIN B1</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 pigeons</td>
</tr>
<tr>
<td>40 &quot;</td>
</tr>
<tr>
<td>80 &quot;</td>
</tr>
</tbody>
</table>
From a comparison of the limits of error in Table II, XVII and XVIII, this histological method for the bioassay of vitamin A seems to be as accurate as and possibly more accurate than the three weeks' curative "growth" test.

The given doses of both the vitamin A standard and the test oil were always in geometrical progression. This did not apply to the total vitamin A intake, owing to the fact that the rats received extra small equal amounts of carotene derived from the potato starch. Such an extra allowance, although small, should obviously tend to interfere with the logarithmic dosage-response relationship. Therefore, a more satisfactory vitamin A-free diet would probably increase the accuracy of this method.
CORRECTION FOR IRRLEVANT ABSORPTION.

Until recently it was believed that the full intensity of absorption at 328 mμ was due to the presence of vitamin A. This assumption was proved to be unjustified, owing to the presence of irrelevant absorbing substances, even in high potency oils and their unsaponifiable fractions.

To detect the presence of irrelevant absorbing substances in an oil Chevallier (35) stated that "oils with abnormal spectral absorption, in spite of possessing a clear maximum at 328 mμ, could be recognised by tracing the curve of absorption beyond 310 mμ and 340 mμ; if the value at 310 mμ lay higher than that at 340 mμ, the absorption was abnormal."

Norton and Stubbs (206) showed that for vitamin A preparations free from detectable irrelevant absorption 3313 mμ = 3365.5 μμ = 1.166. These workers demonstrated that these criteria were satisfied by the purest preparations of crystalline vitamin A acetate dissolved in cyclohexane and they worked out a formula for the correction of irrelevant absorption.

By applying this formula in the examination of the halibut liver oils, used in the large-scale co-operative assays of 1936 (122, 125, 135) it was shown (207) that the original K-values should have been subjected to some correction for absorption which was mainly due to the presence of anhydro-vitamin A. Using these new K-values instead of those originally observed, the estimate of 1.570 for the conversion factor was raised to 1.820. The validity of the correction procedure was further demonstrated by fractionation of the oils.

These data led Norton and Stubbs to conclude: "The principal significance of these results is that a single
conversion factor can be applied to spectrophotometric measurements on fish liver oils, on unsaponifiable fractions of fish liver oils and on crystalline vitamin A esters, provided that proper correction is made for irrelevant absorption. The potencies in I.U./gm. so obtained will then be in accord with the biological estimates, provided that the biological assays are carried out under strictly comparable conditions. Thus, the conversion factor of 1,800 suggested by the present work only applies to the particular condition of diet, dosage level, etc. under which the three cooperative assays were carried out; changes in these conditions might well lead to a conversion factor appreciably different from 1800° (loc. cit.).

The correction necessary for molecular distillates (average about 8%) is smaller than for commercial concentrates prepared by saponification (average about 11 to 15%) and variations between samples, even from a single species, exist. The conversion factor of 1,600 remains appropriate enough for uncorrected E-values, but 1,800 should be used for corrected E-values. The need for correction arises from the presence of anhydro-vitamin A and sometimes oxidation products* (205).

It has been the custom to use the unsaponifiable fraction of low-potency oils (E-values lower than 4) for the physico-chemical estimation of vitamin A. The absorption at 328 mμ is hereby significantly reduced and the shape of the whole absorption curve now resembles that of high-potency oils and concentrates. There still remains, however, a certain amount of irrelevant absorbing substances. The reason why irrelevant absorption occurs so frequently in high-
potency oils is that vitamin A decomposition products are formed either before or during the process of extracting livers. There may also be deterioration during transit or storage. Whatever the exact reason for their occurrence, the fact of contamination by such artefacts is undeniable. Most of the decomposition products remain in the unsaponifiable fraction (208).

Correction procedure:

The correction procedure described by Norton and Stubbs (206) is based on the assumption that the irrelevant absorption "curve" is linear over the approximate range of 310 to 340 m μ, which means that no impurity or artefact shows a maximum close to that of vitamin A.

"A good deal is now known concerning the spectra of vitamin A and oxidation products of vitamin A, and it can be said that over the range 310 - 340 m μ their absorption curves are least approximately linear. Nevertheless, slight departure from linearity would give rise to appreciable errors in the final result, and the final verdict on the validity of the assumption must rest on .... experience" (206).

Three chosen wave-lengths ("fixation points") for vitamin A alcohol or ester in a specified solvent are necessary to apply the correction formula, namely, E-maximum and two others at which the E-value is 6/7 of E-maximum. These three wave-lengths are 313, 326 and 338.5 m μ. The difference between the resulting corrected curve and the uncorrected one will represent the absorption of substances other than vitamin A, provided that the assumption of the linearity is valid.
The correction is made as follows:

**Vaalhaai liver oil in cyclohexane:**

<table>
<thead>
<tr>
<th>Wave length</th>
<th>m</th>
</tr>
</thead>
<tbody>
<tr>
<td>315 mμ</td>
<td>0.705</td>
</tr>
<tr>
<td>328 mμ</td>
<td>0.799</td>
</tr>
<tr>
<td>338.5 mμ</td>
<td>0.683</td>
</tr>
</tbody>
</table>

The intervals between the different wave-lengths are:

- 328 - 315 mμ = 15 mμ
- 338.5 - 328 mμ = 10.5 mμ
- 338.5 - 313 mμ = 25.5 mμ

From the observed values:

E315 mμ - E338.5 mμ = 0.705 - 0.683 = 0.022.

According to the formula the correction for the slope is:

\[
\frac{0.022 \times 10.5}{88.5} = 0.009.
\]

\[
0.799 - 0.009 = 0.790.
\]

The correction for the height of the general absorption:

\[
0.790 - \frac{x}{7} = \frac{0.683 - 0.790}{7}
\]

Whence \( x = 0.041 \)

\[
0.790 - 0.041 = 0.749.
\]

This corrected value for E-maximum changes the observed E-value for the Vaalhaai liver oil to 0.749/0.799 or 9.26, which was the original estimate. Thus, the application of the correction formula gives a reduction of 6% in this case.

The influence of this correction procedure on the conversion factors obtained in this work is illustrated in Table XIX.
It is evident from the data in Table XIX that a conversion factor of 1,600 is preferable for uncorrected E-values and that the conversion factor of 2,000, which is generally used in America, is definitely too high. A higher conversion factor should be used for corrected E-values and the value of 1,800 suggested by Horton and Stubb is supported by these data.

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Vaalhai 1.e</td>
<td>15,000</td>
<td>9226</td>
<td>8.68</td>
<td>1618</td>
<td>1727</td>
</tr>
<tr>
<td>Vit.A.ac.</td>
<td>12,300</td>
<td>6.85</td>
<td>6.80</td>
<td>1796</td>
<td>1810</td>
</tr>
<tr>
<td>Vit.A.dist</td>
<td>14,500</td>
<td>8.44</td>
<td>8.15</td>
<td>1927</td>
<td>2028</td>
</tr>
<tr>
<td>Tuna 1.e</td>
<td>129,900</td>
<td>75.10</td>
<td>67.50</td>
<td>1730</td>
<td>1928</td>
</tr>
<tr>
<td>Dogfish 1.e</td>
<td>8,710</td>
<td>4.03</td>
<td>3.60</td>
<td>1411</td>
<td>1586</td>
</tr>
<tr>
<td>Snook 1.e</td>
<td>26,700</td>
<td>17.44</td>
<td>16.56</td>
<td>1511</td>
<td>1610</td>
</tr>
<tr>
<td>Hake 1.e</td>
<td>13,900</td>
<td>7.08</td>
<td>7.25</td>
<td>1817</td>
<td>1924</td>
</tr>
<tr>
<td>Stonebass 1.e</td>
<td>78,600</td>
<td>53.37</td>
<td>46.75</td>
<td>1473</td>
<td>1682</td>
</tr>
</tbody>
</table>

AVERAGES: 1665 1786
SUMMARY AND CONCLUSIONS.

1. Degeneration in the central nervous system is a characteristic symptom of vitamin A deficiency in rats. The Marchi technique is sufficiently accurate to follow such degenerative changes in the myelin substance of the central nervous system, provided that it is properly used. This technique can be used for quantitative routine work with slight modifications as far as the histological procedure is concerned.

2. A histological method for the biological estimation of vitamin A is described. This method is based on the fact that if groups of young rats are kept under the same experimental conditions and receive graded doses of vitamin A over a suitable dosage range, equal proportional increases in the doses of this vitamin produce approximately equal average increases in the protection of these rats against degeneration in the central nervous system.

3. There exists a significant difference between the response (protection against degeneration) of male and female rats to the same dose of vitamin A. This difference indicates that the minimum vitamin A requirement of male rats is about 1.5 times higher than that of females of the same age. Although it is generally assumed that the vitamin A requirement is a function of the body-weight, the latter alone cannot account for this sex difference.

4. The vitamin A potencies of vitamin A acetate, vitamin A distillate and six natural fish liver oils were estimated by means of this histological method and corresponding conversion factors were calculated to test out the reliability of the present conversion factors.
5. The observed experimental data were analysed statistically and this method seems to be as accurate as and possibly more accurate than the 3-weeks' curative "growth" test.

6. The calculated conversion factors indicate that the value of 1,600 for this factor is preferable to that of 2,000 for uncorrected \( E \)-values. A higher value should be used for corrected \( E \)-values.
ACKNOWLEDGMENTS.

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OPGEDRA....

Hierdie werk word in dankbaarheid opgedra
aan my Ouers en Isabel.