SOME ASPECTS OF THE EFFECTS OF VITAMIN A DEFICIENCY ON THE RAT INCISOR, WITH PARTICULAR REFERENCE TO ERUPTION OF THE INCISOR.

BARRY J. SESSLE B.D.S., B.Sc.

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INTRODUCTION

Because of its property of continuous eruption, the rat incisor has been especially used for studies on the mechanism of tooth eruption, and for studies of the effects of various experimental procedures on this mechanism. Most of the studies, particularly those of the effects of vitamin A deficiency on tooth eruption, have been carried out on functionally occluding incisors. However, such a procedure does not distinguish between factors acting on attrition and those on the eruptive mechanism itself, and this may account for some of the controversy concerning the phenomenon of tooth eruption. Factors acting solely on the eruptive mechanism could be investigated by removing one of the incisors from occlusion, thus allowing a study on the unimpeded eruption of this tooth, free from the inhibitory influence of the force of functional occlusion.

Bryer (1957), who introduced the concept of unimpeded eruption, investigated the influence of various experimental procedures on the unimpeded eruption rate of the lower incisor. Included amongst these was a study on the unimpeded eruption rate of vitamin A deficient rats that had been placed on a vitamin A-free diet at weaning. However, this study was carried out over a period of only four weeks in clinically deficient rats. Consequently, it was decided to investigate more extensively the effects of vitamin A deficiency on the rat incisor, using both delayed deficient and rapidly

deficient rats. Particular emphasis was placed on the unimpeded eruption rate of the incisor, and observations were also made on the effect of vitamin A replacement therapy. Moreover, it was also decided to examine the attrition and eruption rates of functionally occluding lower incisors in normal rats. Such a study, it was thought, would act not only as a basis for comparison with the vitamin A deficient rats and the control animals, but also might provide information which would help to clarify our knowledge of the mechanism of tooth eruption.

CONTENTS

1. ERUPTION RATE OF THE RAT INCISOR

1.1 The Rat Incisor

"In relatively recent years man himself has learned to conduct experiments in order to elucidate further facts regarding the biologic mechanisms involved in structure and function.

A considerable impetus to our knowledge of normal oral histology and embryology has come about through experimentation. This approach permits an exaggeration of the normal processes so that normal structure and function can be more easily understood. Particularly for a better understanding of the processes in tooth development, the continuously growing rat incisor is significant and affords decided advantages in experimental studies." (Schour, 1960).

Because of the property of continuous eruption, the rat incisor is also an invaluable asset in the study of processes subsequent to tooth development, that is to say, in the study of tooth eruption.

The rat incisor develops primarily from an epithelial base, which differs both functionally and structurally on its labial and lingual aspects. This base, on the labial aspect, resembles the enamel organ of the human tooth while on the greater part of the lateral surfaces and on the lingual aspect, it resembles Hertwig's epithelial sheath. The term "odontogenic epithelium" has been applied to this germinal tissue. The odontogenic epithelium gives

rise to the enamel organ, but Schour pointed out that the latter is not transitory as in man. It persists throughout the life of the rat, but only on the tooth's convex surface up to the gingival margin, for it is rudimentary at the base of the concave surface of the mature incisor. At the basal end of the incisor, where there is constant renewal and growth of cells, the enamel organ has basically the same four layers as in man, that is, inner layer of ameloblasts, outer enamel epithelium, stratum intermedium and stellate reticulum. The enamel organ encloses the dental papilla, which is derived from mesenchyme, and from the peripheral cells of the dental papilla, odontoblasts develop, under the influence of the ameloblasts. These dentine-forming cells migrate toward the incisal end of the incisor and at the same time recede centrally toward the pulp.

The pulpal recession is in direct proportion to the amount of dentine laid down. Experimental studies (Schour and Steadman, 1935; Schour and Hoffman, 1937, 1939) revealed that dentine is laid down at the rate of 16 microns per day. The forward movement of the odontoblasts towards the incisal end of the incisor is directly proportional to the eruption rate of the tooth. Such incisal and pulpal movements result in an increasing approximation of the pupal surfaces of the dentine in the incisal part of the incisor. Thus, the incisal end tends to become solid.

In a rather similar manner to that of the odontoblasts, the ameloblasts move forward at a rate equal to the eruption rate, and recede labially with the apposition of the organic enamel matrix which proceeds, as does the dentine matrix, at the rate of 16 microns per day (Schour et al., 1935, 1937, 1939). However, this growth pattern is of more limited extent than that of the dentine.

Mention must be made at this point of the enamel pigment, since a loss of this pigment is a characteristic feature of vitamin A deficiency. This yellow or orange pigment is found in the outer surface of the enamel. It can be easily scraped off newly-formed enamel, and may also be seen within the enamel - forming cells, the ameloblasts. Its significance in vitamin A deficiency will be discussed more fully in subsequent sections.

Prior to eruption of the rat incisor, the basal end grows backward into the bone which resorbs before its advance. According to Addison and Appleton (1915), actual eruption begins in a forward direction at eight days of age, the tooth being extruded into the oral cavity by a force acting at its basal end. Eruption of the incisor is considered more fully inthe following section (I.2).

It must be realized that the rat incisor has no root, but consists of a powerful crown which has a labial convex part covered with enamel, and a lingual and lateral aspect covered with cementum. The characteristic convexity of the rat incisor is the result of greater cellular activity on its ~~labial surface than on its~~

labial surface than on its other surface. Because of its shape, the rat incisor is specially adapted for use as a chisel for cutting hard substances. Furthermore, gnawing produces more rapid wearing of the relatively soft cementum and dentine on the lingual than of the hard labial enamel. Thus is maintained a sharp, chisel-shaped edge, and since the lower incisors can be closed either labially or lingually to the upper incisors, the upper and lower incisors sharpen each other through their constant use. It is by this functional activity that the rat incisors wear away, and in order to continue their function, these teeth must continue to erupt throughout the life of the animal. From the time the upper and lower incisors come into functional occlusion, it has been stated (Schour, 1960) that "the rate of wear equals the rate of eruption, consequently the tooth remains of constant size. The failure of an incisor to occlude normally with its antagonist results in an elongation unchecked by wearing which may kill the animal by starvation". The latter statement has been confirmed by the author's own observations on the rat, but that "the tooth remains of constant size" is rather doubtful both from the author's experience, and the observations of Sicher and Weinmann (1944) who showed that continuous eruption of the incisor not only compensates for loss of tooth substance but also leads to an actual increase in length of the incisor.

Thus, it has been shown that the rat incisor "may be regarded as one of the special gifts of nature to dental research. It is a tooth of persistent growth, and permits a study, in a single adult of any age, of the structural changes which the dental cells and tissues undergo from their early development to their maturity and final state." (Schour, 1960).

1.2 Eruption Rates

At the present time, there is a good knowledge of the clinical and anatomical aspects of tooth eruption, but, as Bryer (1957) pointed out "much controversy still exists regarding the nature and interrelationships of the physiological forces concerned in the active movement of the teeth through the jaws to take up their functional position intra-orally. Many theories have been postulated in attempts to explain this mechanism, but as yet no single factor can account for all the clinical, anatomical and experimental findings."

No one theory of tooth eruption can explain all the factors involved in the eruptive process but, because each may contribute in some way towards a better understanding of the process, these theories will be considered briefly here.

There seems no doubt that eruption depends to a large extent on the active movements of the tooth incident to jaw growth, but experimental studies (Brash, 1928; Orban, 1928; Brodie, 1934;

Massler and Schour, 1941) indicate that the tooth also moves by its own growth. Furthermore, Sicher and Weinmann (1944) have found in the rat a close correlation between the growth of the teeth and of the jaws. As well as the position of the molars and incisors remaining practically constant, these workers observed that the growth of the rat incisor is proportional to jaw growth.

Schour (1960), in Noyes' textbook, considered the theories of eruption under two headings:

(a) Pressure from the Elongation of the Roots: Whereby the growing tooth root, pushing against the floor of the bony crypt, results in tooth movement towards the oral cavity. This theory breaks down on many points, which include the observations that some teeth erupt in the absence of root formation, some show delayed eruption despite root formation, while often the distance travelled by a tooth crown is considerably greater than the amount of root added.

(b) Pulp Pressure: Since the vascular supply of the pulp and the tissues beneath the tooth is greater than the supply above it, blood pressure brings about eruption. Schour (1960) quoted Noyes as saying "the force exerted by the growing tooth is the result of the multiplication of cells in the tooth germ, ------ It apparently is related to osmosis and has direct relations to blood-pressure. It is certainly a very complicated matter, with chemical affinities at the bottom of it."

As with the first theory, there are objections to this one. For example, the movement of the erupting tooth may not be in the direction of its longitudinal axis, as the pressure theory would have it.

This uncertainty of the mechanism of tooth eruption also results partly from the complication of endocrine factors. Schour (1934) has shown that the pituitary gland has a significant effect on tooth eruption. Additionally, this uncertainty may have been complicated further by the method, rationale and interpretations of previous experiments, especially those carried out on the continuously erupting rat incisor. As Bryer (1957) pointed out, "effects on the eruptive mechanism or eruptive force per se have been evaluated from changes in the eruption rate of functionally occluding incisors. This rate has been determined by making a mark with a fine file on the enamel surface close to the gingiva and measuring the distance from the mark to the gingiva at weekly intervals. The changes in this rate produced by various experimental conditions have been repeatedly interpreted as being the result of direct changes on the eruptive mechanism, yet in reality it does not distinguish between influences directly on the eruptive mechanism or on the attrition rate."

Attrition, as discussed above, is compensated for by continuous eruption of the rat incisors, and its rate can be altered by a change in attritional activity, in the structure and quality of

the incisors, or in the eruptive force itself. Studies by Wetzel (1927), and Downs (1931) showed that the rate of incisor attrition could be varied by a change in diet, while Taylor and Butcher (1951) reported a similar type of result when they demonstrated that the rate of attrition is affected by the use of the incisors in obtaining food. These results in no way support the findings of Addi son and Appleton (1915) that, irrespective of the consistency of the diet and the ability to gnaw on hard objects, the attrition rates remain constant.

Addison and Appleton (1915) had been the first to show that the "rates of growth" of rat incisors are 2.2 mm. and 2.8 mm. per week for the upper and lower incisors respectively. However, when Hoffman (1939), Schour and Medak (1951), and Taylor and Butcher (1951) cut down an incisor and thus removed it from occlusion, the full expression of the eruptive force of the shortened tooth was allowed to occur. This unimpeded eruption rate was found to be 100-200 per cent more than the attrition-eruption rate. The results of Taylor and Butcher are of further interest, since after cutting down one of the incisors, this tooth showed a greatly increased (unimpeded) eruption rate, but the contra-lateral incisor, then bearing all the occlusal load of the lower jaw, consequently showed an increased rate of attrition.

As might be expected, it has been shown (Bryer, 1957) that the unimpeded eruption rate is independent of attrition, since there

is no significant difference between rats on a hard or soft diet.

Consequently it can be said that the attrition-eruption rate of functionally occluding incisors is not really a full reflection of the eruptive force itself, but is the resultant expression of two antagonistic forces - the eruptive force on the one hand, and the impeding force of occlusion on the other. As Bryer said "it is therefore quite probable that in previous experiments a change in this eruption-attrition rate may reflect, to some extent at least, a change in the attrition rate and not in the eruptive force."

Because of the rationale of the use of the concept of unimpeded eruption rate in experiments on eruption, the author has adopted this concept for an investigation of the effect of vitamin A deficiency on incisor eruption.

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2. HISTORY OF VITAMIN A

The delay in the acceptance of the concept of the vitamins, including vitamin A, as essential food factors, was largely due to the minute amounts of each vitamin required by the body, and to the inability of investigators to appreciate that disease could be the result of a nutritional deficiency. Such a delay persisted well into this century, despite the fact that the curative effects of vitamin A - rich foods in what are now known to be deficiency states, had been recognized for thousands of years. As far back as 3,500 years ago, the Egyptians recognized the importance of liver as a cure for night blindness, a vitamin A deficiency symptom, while Hippocrates, of classical Greek times, similarly used liver for the treatment of night blindness.

However, it was not until the last century that the importance of nutrition to health and disease began to be appreciated. Bishop Heber (1846) related how he noted night blindness as a common affliction of the lower classes in India; the Sepoys, interestingly enough, actually attributed it to a nutritional deficiency. Dumas (1871), in a study of undernourished humans, almost grasped the truth when he said that "the smallest and most insignificant traces of matters may prove to be not only efficacious but even indispensable."

Unfortunately, however, this belief was not appreciated by his contemporaries, and it was not until the introduction of

experimental nutritional studies at the close of the nineteenth century that advancement in this field actually occurred, largely due to the pioneer studies of Lunin (1881), Socin (1891), Pekelharing, Grijns, and Eijkman (1897). These workers were the first to realize the importance of minute amounts of essential nutrients in the well-being of animals, and the concept of these "accessory food factors" was further extended in the early part of this century by such investigators as Hopkins (1906), Stepp (1909, 1912), Osborne and Mendel (1911), and Funk (1912). Funk's contribution to this field is especially significant, since, as well as introducing the concept of deficiency diseases, he also coined the name "vitamines" for these newly discovered "accessory food factors" which were effective in the treatment of deficiency states. Since proof was lacking that these factors were, in fact, amines, Drummond (1920) proposed the word "vitamin".

With regard to vitamin A, the above contributions enabled McCollum and Davis (1913, 1914, 1915) to extend the observations to show that "there are necessary for normal nutrition during growth two classes of unknown accessory substances, one soluble in fats and accompanying these in the process of isolation of fats from certain foodstuffs, and the other soluble in water, but apparently not in fats." Thus, the fat-soluble vitamins, of which vitamin A is an example, were discovered.

Confusion and controversy still existed, however, regarding the components of this fat-soluble fraction. Such confusion was probably due to the presence of vitamin D along with vitamin A in the fat-soluble portion used in many subsequent studies, and to the poorly understood relationship of the plant carotenoids to this fraction. It was not until the chemistry, isolation and synthesis of vitamin A were accomplished by workers such as Karrer et al. (1930, 1931, 1933), Heilbron et al.(1932), and Holmes and Corbet (1937) that such confusion began to subside.

Accompanying this elucidation of the chemistry and physical properties of vitamin A were the introduction and gradual development of International Standards and Units for this vitamin. The initial standardization of vitamin A in 1931 was introduced at a time when vitamin A was not sufficiently well defined chemically and physically to permit a quantitative chemical or physical test, although the methods for the colorimetric determination of vitamin A, as introduced by Rosenheim and Drummond (1925) and improved upon by Carr and Price (1926), were beginning to be appreciated for their true worth. These supplied more accurate estimations of vitamin A than the more laborious biological tests on experimental animals so frequently used at that time. Thus, since carotene is converted to vitamin A in vivo and was thought to be well defined chemically, the international unit (i.u.) of vitamin A was expressed in terms of the biological activity of carotene.

However, it was soon learned that the standard sample of carotene was actually a mixture of carotenes with widely different biological activities, and so a more clearly defined, reproducible chemical substance was required.

In 1934, the Second International Conference on Vitamin Standardization recommended that pure beta-carotene be used as the international unit for vitamin A, and the international unit was defined as the vitamin A activity of 0.6 microgram of the standard preparation of beta-carotene.

However, by 1939, several esters of vitamin A had been isolated, and after the Second World War, in 1949, the newly formed World Health Organization recommended, the adoption of pure crystalline vitamin A acetate as the standard preparation. "The unit of activity of vitamin A was defined as being that of 0.344 microgram of the standard preparation of crystalline vitamin A acetate equivalent to 0.3 microgram of vitamin A alcohol." The earlier beta-carotene standard was renamed as the standard of provitamin A.

Since 1949, although the role of vitamin A in the body has, on the whole, been dealt with successfully, vitamin A biochemistry has proved the stumbling block of vitamin A research. Despite the brilliant investigations by Wald (1951) on the biochemical role of vitamin A in vision, very little knowledge of its general biochemistry has been forthcoming.

Consequently, it can be said that the history of vitamin A reveals a very long period of inactivity in scientific investigation until towards the end of the last century, after which much of the present day knowledge of vitamin A was gained. However, at the present time, a "stalemate" seems to be in existence, at least in vitamin A biochemistry, and concentrated efforts in this direction are necessary before we can understand, even in superficial details, the biological processes by which vitamin A acts.

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3. ASPECTS OF VITAMIN A AND NUTRITION

3.1 Vitamin A and Carotene

Plant tissues, as early as 1915, were recognized as possessing activity which resembled that nowadays attributed to vitamin A (McCollum and Davis, 1915). This activity was, in fact, due to the carotenoid pigments which are now known to act as provitamins A.

Palmer and Eckles (1914) had already shown that the fat of cow's milk owes its natural yellow colour to the possession of the carotenoid pigments; such pigments are not synthesized by the animal but, as Palmer and Eckles concluded, "are merely taken up from the food and subsequently secreted in the milk fat."

Steenbock (1919) contributed further knowledge when he observed that a relationship exists between the yellow colour of certain foods and their vitamin A activity and he suggested that the vitamin is either a pigment itself or certainly a closely related compound. That vitamin A is in fact a chemical substance separate from carotene was not fully accepted until ten years later, even though Palmer and Kempster (1919) had indicated that vitamin A is not identical with any of the carotenoid pigments, and their work had been supported by the observations of others (Drummond 1919; Stephenson, 1920).

Such a delay in the acceptance of vitamin A as a separate

chemical compound was probably due to a large extent, as von Euler et al.(1928) pointed out, to the fact that earlier research had been carried out when the separate existence of vitamins A and D was not realized. Thus, the apparent contradictions between various experiments on the relation of vitamin A to the carotenoid pigments may well have arisen from the influence of vitamin D effects.

Thus, the stage was set for Moore to show conclusively the relation between vitamin A and carotene. Moore from his 1929 studies, recognized the "possibility that the pigment may not independently exert the same action as the vitamin, but that it may act as a precursor capable of giving rise to the vitamin in vivo." He realized, in 1930, that the two substances must have different structures on the basis of different spectroscopic absorption features and of differences in their solubility in different solvents. From further experiments he concluded that carotene acts as a precursor of vitamin A in vivo, which accounts for the fact that when carotene is readily available in the diet of animals, such animals become useless for studies requiring depletion of vitamin A. Furthermore, he deduced that conversion of carotene to vitamin A occurs in the liver, which not only stores the vitamin but regulates its uptake and release (Moore, 1930, 1931). It was these classic investigations by Moore that brought previous speculation and confusion on this aspect of the vitamin A story to an abrupt conclusion.

3.2 Occurrence and Nutritional Requirements of Vitamin A

Vitamin A as such is found in many animal tissues and products; these include eggs, milk, cheese, butter and especially liver, cod-liver oil and halibut-liver oil. There is very little of the vitamin present in meat and meat fats, and it is absent from green vegetables, carrots and purely vegetable fats such as linseed oil and olive oil. However, green vegetables and carrots contain variable amounts of carotene and other provitamins. Thus, indirectly the latter are important sources of vitamin A, although, nutritionally, they are less satisfactory than vitamin A itself.

Most animals have a remarkable capacity for the storage of vitamin A. Moore (1957) pointed out that "the rat, with a life span of some three years, can accumulate enough vitamin in its liver to last, if economically used, for a century. Information is lacking, however, on the particular properties of either the vitamin or the liver tissues, which make this vast storage possible." "This vast storage" is exemplified further by the fact that a rat may, even in a few days, store enough vitamin A to supply its nutritional needs for many months; in times of low dietary intake of the vitamin, this store is used to supply the physiological needs of the animal.

Although the liver is the main storehouse of vitamin A, the kidneys also accumulate appreciable amounts of the vitamin, while detectable quantities occur in the lungs, adrenal glands, body fat

and blood. The vitamin A level in the latter depends to a large extent on the capacity of the liver to absorb or make available the vitamin as required.

Lewis and his colleagues (1942) had found that, in the rat, the plasma vitamin A level increases with increasing doses of vitamin A intake; at an intake of 50 i.u. per day, the optimum plasma level occurs, being 100 i.u. per 100 ml. of plasma. From his own wide experience, and from reviewing the results of other workers, Moore (1957) placed the "normal" plasma level at approximately 80 i.u. per 100 ml., although, of course, as inferred above, the rat's intake would influence this "normal" figure.

It is interesting to note that a sex difference has been repeatedly observed in the liver and plasma vitamin A levels. Brenner et al.(1942) and Moore, Sharman and Ward (1951) generally observed higher vitamin A levels in blood from male rats than that from female rats. Moreover, workers such as Kimble (1939), Abels et al.(1941) and Campbell and Tonks (1949), reported similar findings in humans. With regard to the liver, however, Brenner et al.(1942) and Esh and Sutton (1948), found that female rats have higher liver levels of vitamin A than male rats.

Lewis et al.(1942) also observed that liver storage of vitamin A in the rat only becomes perceptible at daily intakes of 25 i.u., but 50 i.u. per day are required for appreciable storage to take place. Furthermore, these workers noted that at a daily intake of

two i.u., at which dosage there is no liver storage and only a very low plasma vitamin A level, the vitamin A content in the retina of the eye is still optimal for retinal function. Such an observation appears related to the study of Dowling and Wald (1958), which showed that in vitamin A deficiency, the liver level has fallen practically to zero by the fifth week of deficiency; then, about a week later, the blood level falls rapidly to zero and results in a steady fall in retinal vitamin A. The rat's weight becomes steady during the fifth to seventh weeks, and then falls rapidly; by the eighth week, deficiency symptoms begin to appear. Obviously, the specific times mentioned by Dowling and Wald will not apply to all studies of vitamin A deficiency, since the time course of the deficiency depends on the amount of vitamin A stored by the animal in the pre-deficient period. However, this study does demonstrate the chronological relationship of the effects of the deficiency state on liver storage, blood level, retinal level, weight and clinical symptoms. Depletion of retinal vitamin A does not commence until after the disappearance of the vitamin from the liver and subsequently from the blood, and the fall in body weight. Such a time sequence explains why the occurrence of eye symptoms is a late manifestation of vitamin A deficiency.

Moore (1957) summarized the findings of Lewis et al.(1942), Sherman and Trupp (1949), Fraps (1947), and Paul and Paul (1946) on the vitamin A requirements of the rat by concluding that, to

allow for storage of the vitamin, daily doses of 20-40 i.u. are required. However, to allow for full longevity and a "natural" storage of 113 i.u. per gram of liver, Moore advocated a daily dose of 100 i.u. Such a dose, according to Lewis et al.(1942), results in a plasma level of 112 i.u. per 100 ml. Moore considered that the average human adult has a daily vitamin A intake of 2500 i.u., and on a metabolic basis, this would correspond to a daily intake of 63 i.u. in the rat. Such intakes, he said, would be "adequate for a long life and for the accumulation of substantial stores of vitamin."

3.3 Aspects of Vitamin A and Nutrition - References

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4. BIOCHEMISTRY OF VITAMIN A

4.1 General Biochemistry

Vitamin A is one of the fat-soluble vitamins, and its property of instability to acids, oxygen, oxidizing agents and light demands that cautious handling of the vitamin be observed at all times.

Knowledge of the chemistry and synthesis of vitamin A and its isolation were largely the result of the efforts of such workers as Karrer et al.(1930, 1931, 1933), Heilbron et al.(1932) and Holmes and Corbet (1937). The property of cis-trans isomerism is an important feature of vitamin A especially in its relationship with vision; its chemical structures with regard to this isomerism are shown in figure 1.

Vitamin A as such is absorbed from the intestine much more efficiently than its provitamins. Studies of Vitamin A absorption, for example, the study of Gray et al.(1940), seem to indicate that vitamin A is absorbed mainly in the form of its alcohol, but the picture is still by no means clear. Wald (1954) has suggested that the absorption of vitamin A and its related carotenoids may be due to "the existence in the blood plasma of more or less specific proteins, which permit the absorption of these carotenoids with which they can combine, ---". Furthermore, in the visual cycle, as will be seen below, interconversion occurs between vitamin A and retinene, the prosthetic group of the visual pigment rhodopsin,

and Wald has proposed that a similar reaction accounts, to some extent at least, for the utilization of vitamin A by the body. Wald carried his postulate even further by suggesting that even carotene is involved in this reaction. He feels that carotene, perhaps as a protein complex, is first oxidized to retinene. "This is reduced by the alcohol dehydrogenase system to vitamin A. Some of the vitamin A is converted in the tissues to esters, which are in turn hydrolyzed to the free alcohol for transport and to perform certain metabolic functions."

Only time will tell whether or not such postulates of the very fundamentals of vitamin A biochemistry are actually statements of the facts.

4.2 Specific Biochemical Relationships

4.21 Vision

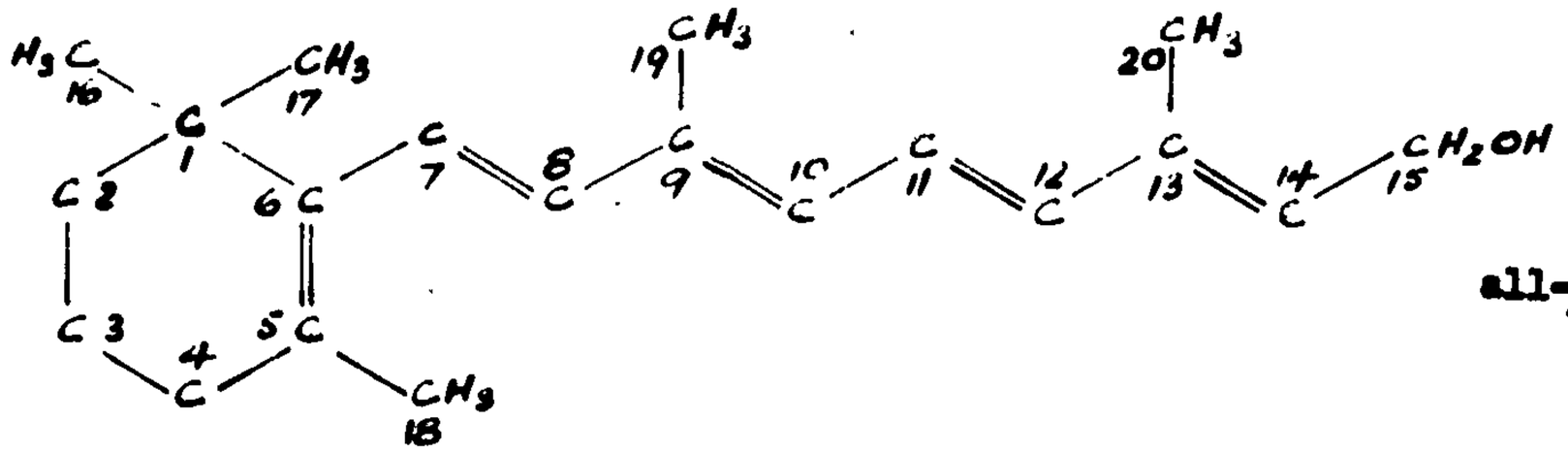
Despite the fact that vitamin A has biochemical functions throughout the body, it is only the visual role of vitamin A that is clearly understood, but, as inferred above, it would appear that certain aspects of this relationship between vitamin A and vision find application in other body processes.

Vitamin A is necessary for the synthesis of rhodopsin ("visual purple") which is the light-sensitive pigment found in the retinal rods in land vertebrates; these rods, together with retinal cones, comprise the photoreceptors of the eye.

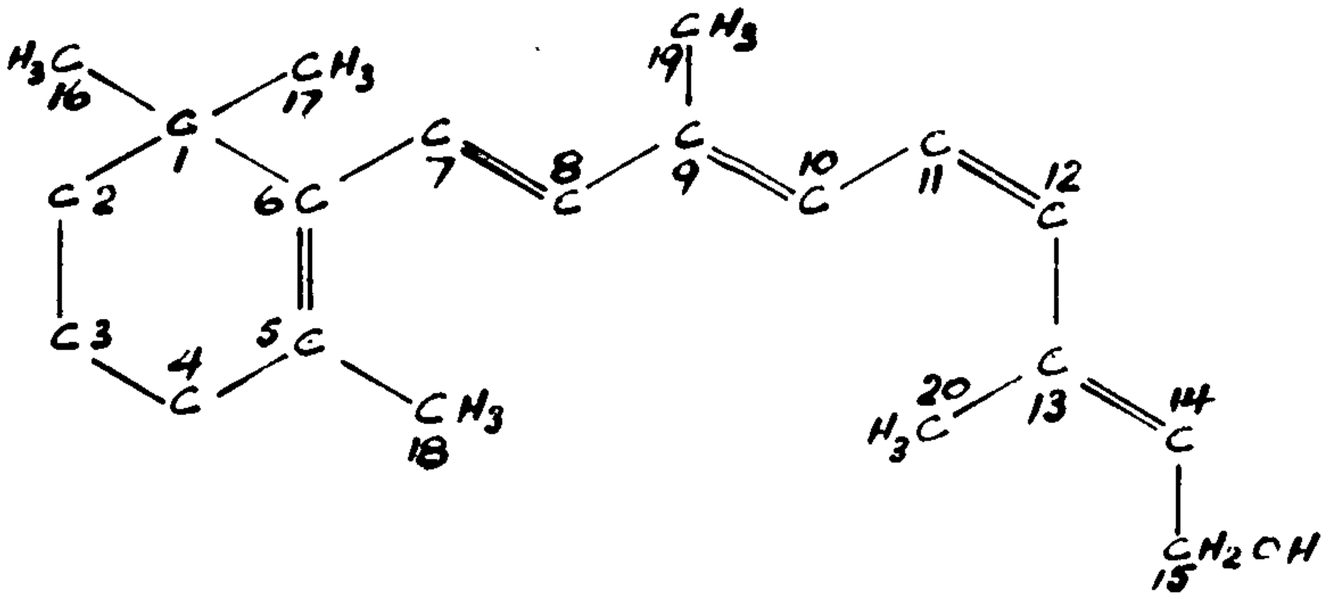
The concept of the rhodopsin - vitamin A cycle was largely the result of the classical studies of Wald (1951), and Hubbard and Wald (1952). Such a cycle was shown by these investigators to be dependent on the "geometrical configuration of vitamin A", since the isomerization is the characteristic feature of the cycle (see figures 1 and 2).

The initial step in the cycle is taken to be the oxidation of vitamin A to the yellow carotenoid, retinene, which spontaneously combines with the protein opsin to form rhodopsin. Bleaching of rhodopsin by light results in the formation of all-trans retinene, which must be isomerized via 11-cis retinene before rhodopsin can be resynthesized. Such a "fast" conversion is nowadays believed to occur to some extent, since Wald demonstrated that the eye contains an enzyme, retinene isomerase, catalyzing this inter-conversion of all-trans retinene to 11-cis retinene. However, it is thought that a "slow" pathway is the important process for the reconversion of all-trans retinene : the latter is reduced to all-trans vitamin A which only after isomerization to 11-cis vitamin A regenerates 11-cis retinene; this, with opsin, immediately forms rhodopsin (see figure 2).

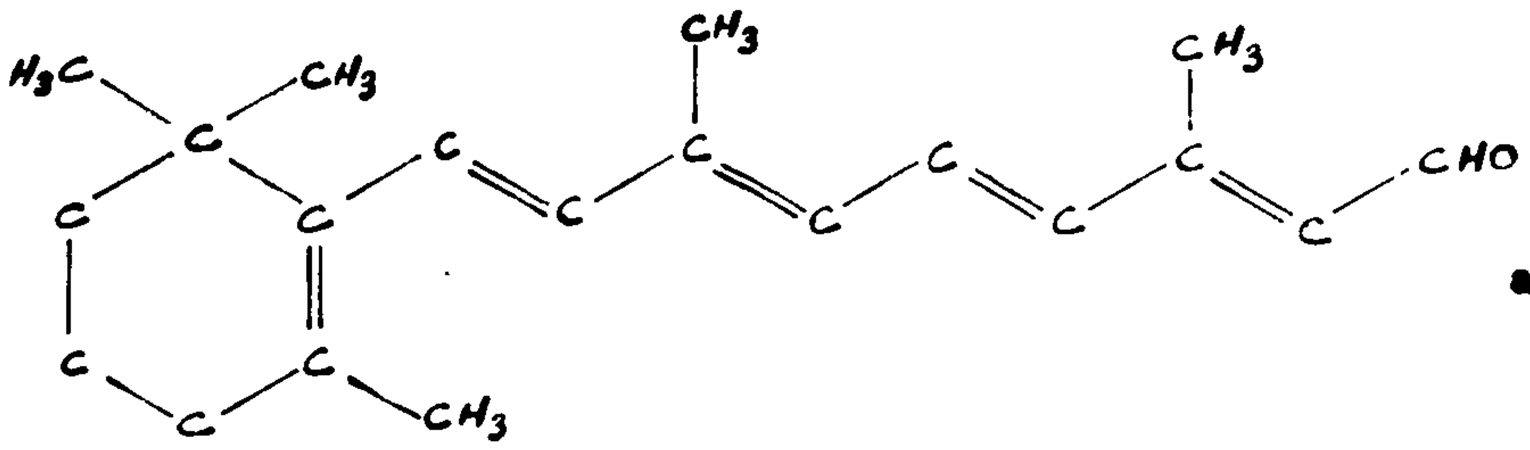
The importance of vitamin A in this system is obvious while, as Wald (1960) put it "the only thing that light does in vision is to isomerize retinine." This suggests that Wald did not take into account that only one photon of light acting on one rhodopsin molecule



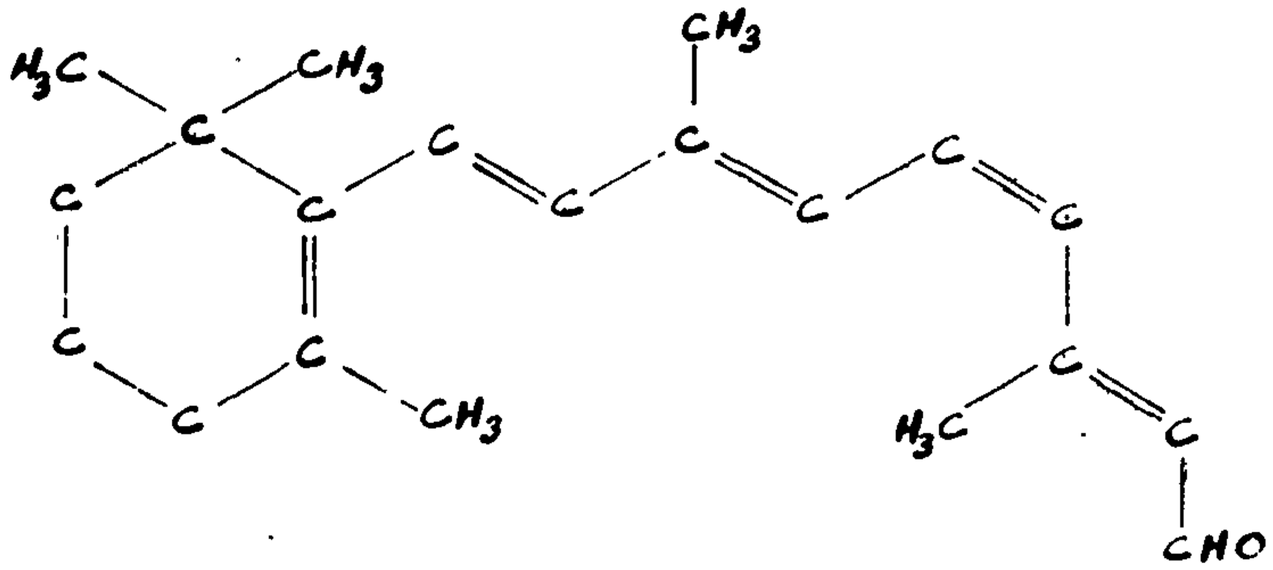
all-trans vitamin A



11 - cis vitamin A



all-trans retinene



11-cis retinene

Figure 1. Structural formulae for vitamin A alcohol and the corresponding aldehyde retinene.

* The numbering system is that proposed by Karrer and recommended by the International Union of Pure and Applied Chemistry (1947) for the carotenoids.

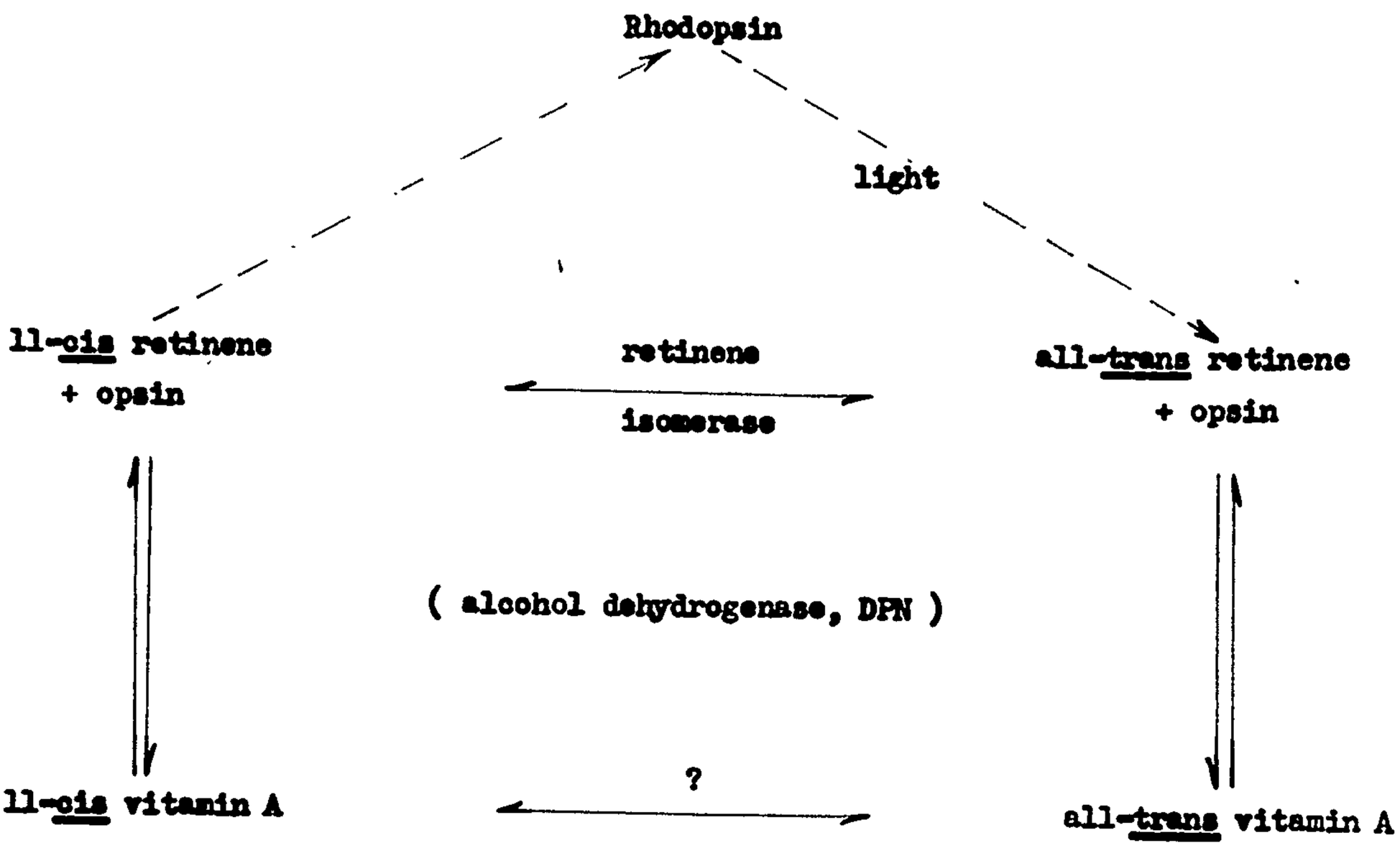


Figure 2: The Rhodopsin cycle.

will depolarize a rod. Consequently, although light may not be important in the actual resynthesis of rhodopsin, it is of extreme importance in vision by virtue of the fact that it is the all-important initiating excitation for the eventual appreciation of the visual stimulus by the animal. Compare this with vitamin A which is required for what amounts to the replenishment of retinene and subsequently rhodopsin. It may be that the low vitamin A requirements of the retina, as shown by Dowling and Wald (1958), can be accounted for by the "fast" interconversion of the retinenes being the major pathway of the visual cycle, thus dispensing with the large amounts of vitamin A as required by the rest of the body.

4.22 Adrenocorticoid Activity and Carbohydrate Metabolism

Vitamin A has been found essential for the biosynthesis of glycogen from acetate, lactate and glycerol; such a finding by Wolf, Lane and Johnson (1957) was supplemented by further research by these workers when they noted that vitamin A is involved in the reversal of glycolysis between the triose and glucose stages of carbohydrate metabolism, but is not necessary for glycogen synthesis from glucose. As stated so aptly by Wolf and his co-workers, such findings "would account for the extremely low level of liver glycogen in the vitamin A deficient animal. Though fairly capable of synthesizing glycogen from glucose, the deficient animal suffers from the effects of inanition; its glycogen stores are rapidly depleted

and cannot be replenished by gluconeogenesis, because of the blocked reaction."

The administration of cortisone to vitamin A deficient animals has been found to restore glycogen biosynthesis from acetate to a normal level (Wolf et al.1958). Since Lowe, Morton and Harrison (1953) had suggested that there is "some disorder in the metabolism of adrenal cortical cells in vitamin A deficiency with consequent alteration of their secretory activity," Wolf et al. (1958) tested the hypothesis that vitamin A is essential for the formation of a glucocorticoid hormone from the zona fasciculata of the adrenal cortex. Adrenalectomized, vitamin A deficient rats were found to be still unable to utilize labelled acetate for glycogen synthesis, even after supplementation with vitamin A. When cortisone alone is given, however, to these rats, glycogen can be produced normally.

This block in glycogen synthesis from acetate due to a deficiency in vitamin A is not corrected by the administration of adrenocorticotrophic hormone (Johnson and Wolf., 1960). Thus it would seem that vitamin A deficiency produces a "chemical adrenalectomy" with regard to glucocorticoid biosynthesis, or as Lowe, Morton and Harrison (1953) had stated with more reserve, seven years previous to Johnson's and Wolf's results, "it would appear that there is some abnormality in the glucocorticoid

activity of adrenal cortical secretion, while the secretion of mineralocorticoids (largely secreted by the zona glomerulosa of the adrenal cortex*) is unaffected or even enhanced."

Wolf et al.(1958) probably put the relation between vitamin A deficiency and glucocorticoid activity in its proper perspective when they concluded "that vitamin A is required for glucocorticoid synthesis in a way which is independent of its function in the rest of the organism. However, it is more rational to conclude that vitamin A does not, in fact, function through its influence on cortical hormone synthesis at all, but rather the vitamin A deficiency leads to a degeneration of certain cells and tissues, including those of the adrenal cortex, and hence to a deficiency in glucocorticoid hormones." Wolf and his co-workers had tested this hypothesis by showing histologically that as a state of vitamin A deficiency approaches, there is a disturbance in the glucocorticoid - producing cells of the adrenal cortex, even before depression of glyconeogenesis, the latter condition being caused by the impaired glucocorticoid production. Such disturbances in glyconeogenesis occur very early in vitamin A deficiency, preceding all other symptoms except weight loss.

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* Words in brackets - mine

Wolf and his colleagues (1959) found that vitamin A deficiency causes a reduction in adrenosteroid production in the rat and such a reduction could be reversed by injection of vitamin A four hours prior to sacrifice of the vitamin A deficient rat. Further research in this direction by these workers (Van Dyke, Johnson and Wolf, 1960) brought them to the conclusion that "the ability to restore, at least partially, the synthesis of corticosterone in an in vitro system by the addition of vitamin A alcohol and acid may indicate the possibility that some form of vitamin A may be functioning as a cofactor or coenzyme for one of the enzymes necessary for the transformation of cholesterol to corticosterone."

To conclude this section, mention must be made of investigations which indicate that the degree of vitamin A deficiency may be the factor determining whether or not steroid synthesis, and consequently carbohydrate metabolism, is affected by the deficiency. Johnson and Wolf (1960) found that in severe deficiency several steps in steroid synthesis are inhibited, including cholesterol biosynthesis. Mild vitamin A deficiency results in no inhibition of cholesterol biosynthesis, and there is no effect on the biosynthesis of desoxycorticosterone and progesterone; however, the conversion of desoxycorticosterone to corticosterone is suppressed. Apparently, the participation of vitamin A in the biosynthesis of corticosterone is a direct one since vitamin A is effective, in correcting the conversion, by direct addition to adrenal homogenate in vitro (Johnson and Wolf, 1960; Wolf, 1961).

4.23 Mucopolysaccharides

From their knowledge of vitamin A deficiency, Wolf and Johnson (1960) considered that the effect of vitamin A deficiency on adrenocorticoid hormones is only one of many disturbances caused by the deficiency state. In particular, there seems to be an effect on mucus formation and on mucus-secreting epithelium. Moore (1957) had expressed as one of the functions of vitamin A "the formation of large molecules containing glucosamine." Mucopolysaccharides (MPS) are large molecules containing hexosamines and much research has consequently been carried out in an effort to establish a relationship between vitamin A and mucopolysaccharide biosynthesis.

At the present moment, from the studies of Wolf and Varandani (1960), vitamin A appears to be necessary for the biosynthesis of mucopolysaccharide of the colon mucosa, at least in the rat and pig. Also, vitamin A has been found to act directly on the chondrocytes in the metabolism of the ground substance of cartilage (Fell and Mellanby, 1952). As yet, however, the results on this tissue are somewhat conflicting, and further research is needed to determine the exact mechanism of this action. A relation definitely exists between vitamin A intake and chondroitin sulphate synthesis (Fell and Mellanby, 1952; Dziewiatkowski, 1954; Frape et al.,1959) and studies such as those by Thomas and his

colleagues (1960) indicated that excess vitamin A causes a loss of chondroitin sulphate from the ground substance of cartilage.

An interesting point that has arisen from the studies of Fell and her colleagues (1952, 1953, 1954) and Frape et al.(1959) is that vitamin A has an effect on the mucopolysaccharide of mucous epithelium different from that on the mucopolysaccharide of cartilage. Excess vitamin A causes sulphate uptake into the mucopolysaccharide of mucous epithelium but results in dissolution of chondroitin sulphate of the ground substance of cartilage.

Although, as stated above, the precise mechanism of the action of vitamin A on the mucopolysaccharide of cartilage matrix remains somewhat obscure, its action on the mucosa of the colon is much more certain, as seen in the words of Wolf and his colleagues (1960) "the block in MPS synthesis due to vitamin A deficiency is at the sulfate activation step, that is, in the synthesis of 3' - phosphoadenosine - 5' - phosphosulfate (PAPS) and that this defect can be corrected by the in vitro addition of vitamin A."

The role of vitamin A in mucopolysaccharide metabolism can be summarized in Jolly's (1964) quotation from Wolf (1961) "We are led to the hypothesis that the activation of sulfate is the rate limiting step in MPS synthesis. Sufficient vitamin A leads to a maximum rate of MPS synthesis. Large amounts of vitamin A in some unexplained way, cause dissolution of the MPS - protein bound in cartilage and the increased formation of MPS bound in

mucus. The concentration of MPS itself may be the regulatory factor determining whether it is to be bound in cartilage or in mucus. Future research will show whether or not these hypotheses are borne out in fact."

4.24 Ubiquinones

The ubiquinones are normal components of mitochondria from a wide range of animal, plant and microbial tissues. Ubichromenol is a substance closely related to ubiquinone and it is likely that interconversion occurs between them. Green and Lester (1959), and Ziegler (1961) have put forward much evidence to show that ubiquinone has an important role in the mitochondrial electron transport system. Slater (1961) believed that it acts between the flavoproteins and cytochrome elements of the respiratory chain. King (1962) preferred to place ubiquinone "as a 'reservoir' or as an alternative to some stage which is not clearly defined", within the electron transport pathway.

Workers such as Heaton et al. (1955, 1957) and Wiss et al. (1961) have shown a relationship between vitamin A and the concentrations of ubiquinone and ubichromenol - in vitamin A deficiency, the concentrations of these two substances have been found to increase in the liver and other tissues of certain animals. Moreover, Wiss and his colleagues maintained that such changes take place very early in vitamin A deficient rats, and could be

reversed by vitamin A administration in either mild or severe deficiency.

However, other workers such as Moore and Sharman (1960) and Edwin et al.(1961, 1962) have cast much doubt on these concentration changes. They maintained that such changes could be due to three factors:

(a) the increases in ubiquinone concentrations may not be increases in absolute amounts of ubiquinone but could be due to reduced size of the various organs in the deficiency state.

(b) vitamin E influences ubiquinone and ubichromenol concentrations, and since vitamin A deficiency is often accompanied by increased vitamin E concentrations, such changes may be explained by simultaneous variations in vitamin E levels.

(c) gross disturbances in lipid metabolism occur in severe vitamin A deficiency and this may explain the increased concentrations of ubiquinone and ubichromenol in heart and liver.

Thus, as Moore and Sharman concluded "the specificity of the relationship between avitaminosis A and increased hepatic ubiquinone remains open to question." This statement can be applied not only to the liver, but also to other tissues.

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5. EFFECTS OF VITAMIN A DEFICIENCY

5.11 General

Two of the earliest studies on vitamin A deficiency were those carried out by Mori (1922), and Yudkin and Lambert (1923). Mori, however, did not give any details of the diet fed to his rats nor of the weights and age of these rats, while Yudkin and Lambert, although giving a good description of eye changes in vitamin A deficiency, placed too much emphasis on the importance of infection in the deficiency state.

The classical studies of Wolbach and Howe (1925) proved the outstanding contribution in providing a clear and accurate description of tissue changes which occur in vitamin A deficiency. These workers noted microscopic changes which included reduction in size of the liver and spleen, "atrophy of the following glands : submaxillary, parotid, lacrimal, Harderian, extraorbital, pancreas, thyroid, pituitary, thymus, and testes" (such "atrophy" is really due to changes in the component cells of the atrophic organs), disappearance of fat from adipose tissue, and cyst formation in the submaxillary glands and accessory salivary glands at the base of the tongue.

Histopathologically, Wolbach and Howe noted primarily a replacement of various epithelia by a stratified squamous keratinizing epithelium: "this effect is noted in cells having presumably widely different chemical (secretory) functions, and

terminates in the complete loss of specific function and transformation into a common type of chemically inactive (non-secretory) epithelium." This keratinizing epithelium is found in such locations as the respiratory, genital, urinary and alimentary systems, and the salivary glands. Orbital glands are also affected, but later and to a lesser degree than the salivary glands. Although chemical functions are suppressed, Wolbach (1937) emphasized that "proliferative powers are not inhibited; neither are the potentialities of cells lost, as is shown by the return to normal physiologic function when vitamin A is restored to the animal."

Furthermore, Wolbach in his 1937 review of the pathology of vitamin A deficiency, listed the order of response of various tissues to this condition as firstly the salivary glands - submaxillary, parotid and accessory glands, followed by the respiratory tract, then the genitourinary tract and finally the eye and parocular glands.

He classified the secondary effects as being -

(a) Loss of weight.

(b) Anaemia.

(c) Cessation of growth.

(d) Lymphoid hypoplasia of the spleen.

(e) Degeneration of skeletal muscle.

Wolbach and Howe stressed the point that infection plays only a minor, secondary role in the epithelial changes of vitamin A

deficiency. Still, Green and Mellanby (1928) called vitamin A the "anti-infective vitamin" since infection is such a common finding in vitamin A deficient rats. However, in regard to this finding, they thought that it may be the result of obstruction by desquamated cells acting as a favourable medium for bacterial growth, or "possibly it is a combination of the keratinizing process and a diminished resistance which makes animals with vitamin A deficiency so prone to infection." Vitamin A became advocated for use in the treatment of a wide variety of infections, and it was not until Harris and his colleagues (1932) refuted such claims, by a series of experiments, that the relationship between vitamin A and infection was placed in its proper perspective, that is, as being only of secondary importance.

The studies of Wolbach and Howe (1925) on vitamin A deficiency have proven to be the outstanding contribution to this aspect of nutrition research, and provided the basis upon which a good deal of later research, in this field, has been established. However, it is quite probable that their vitamin A deficiency studies were also influenced by a concomitant partial deficiency in the other fat-soluble vitamins, D, E and K since, although Wolbach and Howe recognized the possibility that other vitamins were missing from their deficiency diet, they substituted, into the diet of their control rats, butter fat for lard. However, butter fat and lard differ in their relative compositions of the fat-soluble vitamins A, D, E and K

and so the control and experimental rats were receiving different proportions of these vitamins. Lard is a poor source of vitamins D, E and K but may possess some vitamin A activity (Kaunitz and Slanetz, 1950, 1951), so it is quite likely that the experimental rats were being deprived, to some extent at least, of the other fat-soluble vitamins, and not completely of vitamin A.

As Jolly (1964) pointed out "the rats used in the investigations of Wolbach and Howe received a diet which by way of its lard content alone, provided them with a possible 0.5 to 2.5 i.u. of vitamin A per day whilst the daily ration of vitamin E was in the vicinity of only 0.006 to 0.035 mg. The effect of a superimposed dietary deficiency of vitamins D and K remains a matter of conjecture. It would seem then, in the light of nutritional data available to-day, that the experimental rats studied by Wolbach and Howe were subjected not only to the total deprivation of one vitamin but rather to a partial deficiency of at least two of the fat-soluble vitamins."

Still, it must be realized that the effects of the deficiency state produced by Wolbach and Howe were confirmed by other investigations on vitamin A deficiency. Consequently, as Jolly (1964) concluded "the pathological changes reported are undoubtedly those of vitamin A deficiency."

Finally, it must be emphasized that regardless of the original morphology and function of a certain epithelium, in vitamin A deficiency it is replaced by a stratified keratinizing epithelium.

Wolbach and Bessey (1942) pointed out that vitamin A deficiency generally affects those epithelia "which have a secreting (chemical) function in addition to the role of a covering layer and whose functioning cells are without power to divide." On the other hand, "epithelial cells with chemical roles, as liver and renal tubules, which do have the power of dividing, do not exhibit marked degrees of atrophy nor are they replaced by keratinizing epithelium". Thus the role of vitamin A is to maintain the functional integrity of certain epithelial structures.

5.12 Skin

Because of the cutaneous changes, and the observation that many epithelial tissues change into a structure similar to skin, the effects of vitamin A deficiency on skin have received considerable attention.

The early work of Frazier and Hu (1931) revealed that the main effect on the skin in humans is excessive epithelial keratinization; this they said, results in the occlusion of hair follicles and sweat ducts and secondary degeneration of sebaceous and sweat glands.

In their study of vitamin deficient patients, Sweet and K'ang (1935) noted dryness of the skin, but the most specific cutaneous lesion is follicular keratosis. Frazier and Hu, in a later study (1936), also observed follicular keratosis in deficient humans.

Histologically, such a condition shows "hyperkeratinization of the lining epithelium of the hair follicles" which are obstructed by a mass of cornified cells. There is also hyperplasia of epidermal cells adjacent to the hair follicles, and some hyperkeratinization of the surface epithelium.

Both Frazier and Hu (1936), and Sweet and K'ang (1935) reported that the cutaneous lesions usually precede the ocular symptoms of vitamin A deficiency, but that, under dietary treatment, the cutaneous lesions subside more slowly than the ocular lesions.

Animal experiments, however, have failed to give definite proof to the concept of vitamin A deficiency as being the cause of follicular hyperkeratosis. Such negative evidence stresses the fallacy of placing too much emphasis on results from undernourished patients who are, in all probability, deficient in other vitamins as well. As Jolly (1964) so aptly stated "in a deficient population, the problem of determining which particular deficiency is responsible for a particular sign or symptom, in this case a skin lesion, is exceedingly difficult."

5.13 Alimentary Tract

Research on the alimentary tract has revealed that the susceptibility of the gut to vitamin A deficiency is only slight. Wolbach and Howe (1925) demonstrated very little change in the oesophagus, stomach and intestines of vitamin A deficient rats. A

later study by Wolbach and Howe (1933) however, showed the presence of focal areas of hyperkeratosis in the first portion of the rat stomach.

Diarrhoea, colitis and ulceration of the colon have been observed by most workers in vitamin A deficient rats but such effects could be due to secondary effects of the deficiency state, such as a greater susceptibility to infection.

In undernourished humans, Sweet and K'ang (1935) noted marked hyperkeratinization of the oesophagus. In both animals and humans, however, very little research has been carried out on vitamin A deficiency effects on the oral tissues. Such a situation prompted Jolly (1964) to investigate this rather neglected phase of vitamin A research. His findings are included below in the separate section (5.22) on vitamin A deficiency effects on oral tissues.

5.14 Respiratory Tract

Wolbach and Howe (1925) had noted, in vitamin A deficiency, a keratinizing metaplasia of the respiratory mucosa of the nares, accessory sinuses, larynx, trachea and bronchi. Bronchiectasis is a common finding, being the result of bronchial occlusion by masses of desquamated keratinized cells, and secondary infection is by no means infrequent. In the author's study, respiratory distress was something noted in vitamin A deficient rats; such distress was probably due to bacterial influences, and not simply

to bronchial occlusion, since antibiotic therapy was rapidly effective in overcoming the condition.

Keratinizing metaplasia in the respiratory system occurs quite early in the deficiency state. In fact, Blackfan and Wolbach (1933) noted that in infants thought to be suffering from vitamin A deficiency, the earliest appearance of keratinizing metaplasia occurs in the trachea and bronchi.

5.15 Genital System

The epithelia of the testes and vagina were shown, in the classical study of Wolbach and Howe (1925), to be highly susceptible to vitamin A deficiency. In the vagina, keratinizing metaplasia occurs, yet on the other hand, the seminiferous tubules atrophy but do not keratinize.

The testes of male rats undergo transient oedema (Wolbach and Howe, 1925), while histological studies by Sampson and Korenchevsky (1932) revealed initially swelling of the spermatozoa, followed by lysis, then swelling of the seminiferous cells and degenerative changes in the spermatids which eventually form a syncitial mass.

Also, in male rats, the epididymis becomes atrophic, while the vasa efferentia, prostate gland, coagulating gland, and seminal vesicles display a sequence of atrophy, keratinization and fibrosis.

Vaginal keratinizing metaplasia in female rats is so characteristic of vitamin A deficiency that vaginal smears are often used for biological tests and assays of vitamin A. This keratinizing metaplasia occurs very early in vitamin A deficiency. It also occurs in the uterus and oviducts but Wolbach and Howe noted no changes in the ovaries.

It must be realized that one of the actions of oestrogen is to promote cornification of the vaginal epithelium. Such an effect displays cornified cells in vaginal smears taken at the first, and especially the second, stages of oestrus. These cornified cells occur constantly in vitamin A deficient rats and Evans and Bishop (1922) believed that this effect is independent of ovarian influences because of the exhibition of continuous cornification even after double ovariectomy.

At first glance it would appear that the vaginal effects of vitamin A and oestrogen are mutually opposed. Moore (1957) however believed that continuous vaginal cornification of vitamin A deficiency is not due to the unimpeded action of oestrogen. He suggested that this epithelium, even in the absence of oestrogen, tends to cornify unless stimulated by vitamin A to produce mucus. Thus, when oestrogen levels rise during the oestrus cycle of normal rats, the action of vitamin A is inhibited by these increased oestrogen levels, and cornification of the vagina occurs. Such a suggestion seems reasonable, but more research is required in this

direction before the relationship between vitamin A and oestrogen can be placed on a firmer basis.

5.16 Urinary Tract

Again it was Wolbach and Howe (1925) who accurately described keratinizing metaplasia of the epithelium of the bladder, ureters and renal pelvis in vitamin A deficient rats. They noted the remarkable degree of proliferation of these keratinizing epithelia. In fact, they said that this behaviour of the epithelia is suggestive of neoplastic activity.

In rats, Wolbach (1954) observed that obstruction of the urinary system sometimes occurs and is due to masses of keratinized cells; this may be the immediate cause of death in deficient rats. He noted keratinizing metaplasia in the human bladder where desquamated keratinized cells could act as a nidus for urinary calculosis. As Wolbach pointed out, such calculus formation in vitamin A deficiency is only a secondary effect; vitamin A deficiency is thus one of many possible predisposing causes of this condition.

5.17 The Eyes

Involvement of the eyes is one of the later effects of vitamin A deficiency. The first change is the appearance of keratinizing metaplasia of the cornea and conjunctivae and vascularization of

the cornea. Atrophy of the lacrimal glands with metaplasia of their ducts and conjunctival changes result in loss of secretions. Xerophthalmia is a consequence of the accumulation of keratinizing cells in the conjunctival sac, while keratomalacia is the actual oedema and necrosis of the cornea. Infection of the cornea is favoured by the accumulation of keratinized cells and may lead to ulceration of the cornea and then hypopyon (Wolbach, 1954).

In the first stages of deficiency effects on the eye, Wolbach and Howe (1925) reported that desquamated keratinized cells are rapidly removed from the conjunctival sac but gradually accumulate and adhere to the lid margins. Later in deficiency, leucocytes accumulate in the lamina propria, and corneal ulceration may finally occur.

Parnell and Sherman (1962) have recently introduced a new method for studying vitamin A deficiency effects on the cornea of the rat. These workers examined eye washings from rats at various stages of deficiency and noted increasing numbers of non-nucleated cells as the deficiency progresses.

Histologically, the corneal epithelium, normally non-keratinizing stratified squamous, shows cells with indistinct outlines in the intermediate layers of the epithelium. Parnell and Sherman also noted that the free surface of the epithelium becomes irregular and accumulates masses of desquamated cells. When the deficiency has progressed even further, it was noted that the

basal cell layer is irregular, while the intermediate cell layers have by this time formed a "homogenous zone".

Few mitotic figures are seen, for, contrary to the studies of Wolbach and Howe (1933), Parnell and Sherman observed that epithelia of vitamin A deficient rats have a significantly lower mitotic index than those of normal rats. Furthermore, these workers showed that "vitamin A - deficient epithelia are more responsive to the action of the vitamin A than are normal epithelia" in regard to increased mitotic activity. Such utilization of vitamin by the corneal epithelium was shown to be a direct utilization.

Finally, mention must be made of the concept of a relation between vitamin A deficiency and Bitot's spots. These spots are visible to the naked eye on the temporal aspect of the bulbar conjunctivae. Their size and shape are variable but they usually occur bilaterally, as foamy spots or lustreless plaques. Bitot's spots occur in undernourished populations, and many noted workers such as Mason (1954) supported the concept that they are related specifically to vitamin A deficiency and, despite even a recent study (Darby et al.1960) which did not show such a relationship, the concept still appears to persist.

Jolly (1964) put forward a suggestion to explain the presence of Bitot's spots in vitamin A deficiency; such a suggestion was supported by his own experimental observations, and these have been confirmed by the writer's present study. "The explanation

may be that Bitot's spots are secondary or indirect manifestations of vitamin A deficiency and may depend directly on a diminished secretion of the lachrymal glands, and this may be brought about by more than one primary cause. Thus Bitot's spots may be merely accumulations of the more or less normal debris of the eye (epithelial, bacterial, glandular and foreign matter) which under normal conditions of lachrymal secretion is swept away." Thus, as well as vitamin A, any factor causing decreased lachrymal secretion or excessive loss of moisture from the eye may be, to some degree at least, the causative agent for the development of Bitot's spots.

5.2 Dental

5.21 Salivary Glands

The classical work of Wolbach and Howe (1925) again comes to the fore in this section, since it gives a very clear and even chronological description of the effects of vitamin A deficiency on salivary glands. The epithelium of the submaxillary, parotid and accessory salivary glands was seen to be replaced by a stratified keratinizing epithelium. The submaxillary glands are first affected, with slight atrophy of the gland acini of both serous and mucous types, and atrophy of the duct epithelium which is the first epithelium to undergo keratinizing metaplasia. Infection is not present at this stage. The nuclei of some cells degenerate and the cells become rounded

and vacuolated.

At a later stage in the deficiency, duct keratinization and atrophy of gland tissue are more prominent, and still later infection intervenes in most cases. Stromal connective tissue gradually increases, until the whole gland is markedly fibrosed and filled with cysts having a keratinizing lining.

Similar changes occur in the parotid glands, and in the accessory salivary glands of the pharynx and the base of the tongue, but at a later stage of deficiency. Infection, however, is not as common as with the submaxillary glands.

Wolbach and Howe (1933) reported that, despite the morphological change of the deficient epithelium, replacement therapy with vitamin A enables the epithelium to return to its original form. However, it only seems logical to assume that, if gross destruction of the gland's functional tissues has occurred, a return to normal is very unlikely.

Apart from rats, studies have been carried out on other animal species, including the ox (Jungherr et al.1950), hamster (Salley and Bryson, 1957) and man (Blackfan and Wolbach, 1933). Salley and Bryson noted striking changes in hamster salivary glands in vitamin A deficiency. In contrast to the rat (Wolbach and Howe, 1925), the serous portion of the submaxillary glands exhibits the earliest and most severe changes. Cyst formation is common, and Salley and Bryson believed that duct obstruction is a very important

causative agent of gland pathology. In human infants, Blackfan and Wolbach noted some degree of keratinizing metaplasia, especially of the duct epithelium.

However, while the effects of vitamin A deficiency on salivary gland morphology are generally accepted, its effects on salivary gland function are still controversial. May, Mellanby (1930) reported "tartar" in the gingival sulci of teeth examined for vitamin A deficiency effects on gingival tissues, but no explanation was offered for such "tartar". It could be explained as being the result of the deficiency state affecting the salivary glands and producing a decreased salivary flow. Thoma and Goldman (1960) believed that vitamin A deficiency may play a part in the production of xerostomia, but Stones (1962) did not recognize vitamin A deficiency as a cause of xerostomia. From his investigations, Jolly (1964) came to the conclusion that "vitamin A deficiency can produce such severe xerostomia in the rat as to preclude the normal clearing of food from the mouth. Food particles are left adhering to the oral mucous membrane for long periods after eating." 'At a later stage of the deficiency, calculus formation on the labial surfaces of the upper incisors is very common. Such findings were confirmed by the writer's own observations.

5.22 Oral Epithelium

Except for gingival and periodontal tissues, the effect on the oral epithelium is a rather neglected phase of research into vitamin A deficiency. This, to some extent at least, may be due to the lack of gross changes in the oral mucous membranes and to the influence of the early studies in vitamin A deficiency by Mori (1922), and Wolbach and Howe (1925) who found no oral epithelial changes.

Although changes in the gingival and periodontal tissues in vitamin A deficiency have been frequently reported, (Mellanby, 1930; Mellanby and King, 1934; Topping and Fraser, 1939; Tomlinson, 1939; King, 1940; Mellanby, 1941; Baume and Frandsen, 1953; Miglani, 1959; and Frandsen, 1963) there have also been investigations refuting such changes (Boyle, 1938, 1941; Boyle and Bessy, 1941; Glickman and Stoller, 1948).

Mellanby (1930), and Mellanby and King (1934) had shown that a diet deficient in vitamin A produces definite abnormalities of the gingival tissues of dogs. The gingival epithelium is hyperplastic, the crevicular epithelium is thickened and irregular, and the epithelial attachment extends onto cementum. King (1940), in the rat, demonstrated gingival hyperplasia, with thickening of the keratinous outer layer and the subgingival epithelium.

Despite Boyle's (1938, 1941), and Boyle's and Bessey's (1941) contradictions to the above findings, more recent findings give

convincing evidence of a relationship between vitamin A deficiency and periodontal disease. Baume and Frandsen (1953) found that the normally thick epithelium covering the gingival papilla is usually thinner in vitamin A deficient animals and the basement membrane between the epithelium and the connective tissue is no longer visible (such findings are very similar to Jolly's findings (1964) on the oral epithelium; these findings are discussed below). Furthermore, Frandsen (1963) reported decreased repair of interdental tissues in vitamin A deficient rats.

Consequently, a relationship between vitamin A deficiency and periodontal disease appears to have been established. The epithelial hyperplasia and cellular infiltration of the gingival tissues, the changes in the periodontal ligament, the atrophy of the alveolar bone, and the presence of soft deposits, calculus formation and obvious xerostomia as noted by Jolly (1964) and this writer, are all characteristic of the vitamin A deficiency state, and certainly indicate an increased susceptibility to periodontal disease in vitamin A deficiency.

Despite the research carried out on the gingival tissues, it is only recent research that has swung the pendulum in favour of a relationship between vitamin A deficiency and gingival disease. However, with regard to the oral epithelium, until 1964 there did not appear to be any investigations carried out on experimental vitamin A deficiency effects on the mucous membranes of the

tongue, palate and cheeks.

Abels et al.(1942) had shown that the incidence of dietary deficiency is high in patients with papillary atrophy of the tongue and oral leukoplakia. Thoma and Goldman (1960) believed vitamin A deficiency may predispose to oral leukoplakia, while Cheraskin and Langley (1956) had reported hyperkeratosis as the main finding in vitamin A deficiency. But Shafer et al.(1963) warned that "Current literature gives little clinical support, however, to the role of endocrine dysfunction and vitamin deficiency, and there has been a general lack of recent investigations of these factors making their significance in the etiology of leukoplakia difficult to evaluate."

It was this "general lack of recent investigations" in vitamin A deficiency which prompted Jolly (1964) to carry out such research. His findings on an epithelium already keratinized (the oral epithelium) showed a decrease in thickness of the epithelium of the tongue, palate and cheeks; such effects are very interesting for they contrast with the keratinizing metaplasia which occurs in other epithelia of the body in vitamin A deficient rats. Much of this reduced thickness is the result of atrophy in regions normally exhibiting "complete orthokeratinization" - hard palate and papillary regions of the tongue - or a combined effect of atrophy and metaplasia of regions normally showing "incomplete orthokeratinization" - soft palate, cheeks, interpapillary regions of the tongue. Jolly's study also revealed a reduced mitotic rate of the oral

epithelium, hyalinization of subepithelial tissue, disruption of the basal epithelial cells and an increased number of cells resembling melanocytes.

Such findings definitely indicate involvement of the oral epithelium in vitamin A deficiency. The importance of such findings can be appreciated from the fact that they may implicate vitamin A deficiency, per se or in association with other nutritional deficiencies, in many of the epithelial and perhaps subepithelial disturbances of oral tissues.

5.23 Teeth

Many investigations have been made of vitamin A deficiency effects on the rat incisor (Wolbach and Howe, 1925, 1933; Shibata, 1931; Smith and Lantz, 1933; Mellanby and King, 1934; Fridericia and Gudjonsson, 1936; King, 1936; Orten et al, 1937; Bessey and Wolbach, 1938; Schour et al, 1938; Mellanby, H, 1939; Burn et al., 1941; Schour et al, 1941; Irving, 1943; Bryer, 1957); most of these however, have contributed little to the knowledge already gained of this aspect of vitamin A deficiency from those classical studies of Wolbach and Howe (1925, 1933). For this reason, the following will be largely a review of these initial findings, with occasional reference to additional contributions from the above list of authors.

As a result of atrophy and metaplasia of the enamel-forming organ, the continuously erupting rat incisor is profoundly affected.

Atrophy and cessation or depression of the function of the odontoblasts follows atrophy of the enamel-forming organ. Consequently there is a decreased and defective formation of dentine, together with a cessation of enamel formation, and severe deformities of the tooth may result. Wolbach and Howe (1925) showed actual wearing down of some incisors, indicating cessation of growth. Their studies on the incisor of the guinea pig revealed similar effects to those shown by the rat incisor but the morphology and function of the odontoblasts were found to persist in the guinea pig incisor despite marked enamel organ atrophy.

The enamel organ finally consists of the atrophic remnants of the epithelial papillae, and of squamous cells replacing the ameloblasts and stratum intermedium. At first, only the anterior part of the enamel organ is affected but eventually the whole length is involved, including its basal (formative) end. Schour et al. (1941) agreed with Wolbach and Howe (1933) that the histodifferentiation of theodontogenic epithelium, particularly the lingual epithelium, is disturbed, such that its normal organizing influence, that causes the pulpal cells to differentiate into odontoblasts, is ineffective. They stated that "most of the other dental changes, such as the uninhibited proliferative growth of the odontogenic epithelium and the disturbances in appositional growth, may be regarded as secondary effects which are the resultants of a disturbance in histodifferentiation."

The resulting disturbance in the odontoblasts shows marked reduction in size of the cells, with loss of their normal epithelial arrangement. Odontoblastic atrophy is rapid (in the rat), but during the atrophy, the odontoblasts continue to form an irregular, poorly calcified dentine matrix; eventually they are replaced by cells indistinguishable from the other connective tissue cells of the pulp. Some odontoblasts may become incorporated into the dentine, which may also show vascular inclusions. Those odontoblasts on the labial side are affected last, and Wolbach and Howe (1933) believed that this accounts for "the dentine on the labial side of the tooth (being)* disproportionately thick compared to the lingual, lateral and mesial sides." Schour et al. (1941), however, suggested that the final cross-sectional shape may be due to "a compensatory thickening of the labial dentine which has to carry most of the functional stress in mastication." Probably as a result of this thickening, the pulp cavity is displaced lingually.

Furthermore, Schour and his colleagues described disturbances in dentine calcification and differences in the rate of dentine apposition - the enamel-covered dentine has an accelerated rate of apposition (nearly 20 microns per day) while the cementum-covered dentine has a decreased rate of apposition,

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* Word in brackets - mine.

being as low as 6 microns per day (normal apposition rate is 16 microns per day).

The findings of Wolbach and Howe also included denticle and cementicle formation, and deposits of osteoid tissue in the pulp. Concerning the latter, they observed "first, minute spherical globules of hyaline matrix appear between the pulp cells and simultaneously narrow zones of similar material form about occasional capillaries; these deposits increase and incorporate pulp cells which assume the morphology of osteoblasts, and finally cells in contact with the periphery acquire the morphology of osteoblasts and presumably function as such."

In passing, the similarity of the connective tissue hyalinization of oral epithelium in Jolly's study (1964) to this formation of hyaline matrix in the vitamin A deficient tooth pulp should be noted. Such findings seem to implicate vitamin A in the physiology and pathology of connective tissue. It could well be that, since vitamin A has never been demonstrated in epithelia, this vitamin acts on epithelial structures via the connective tissue. Future research on vitamin A physiology and pathology in this direction may well prove fruitful in determining the mode of action of vitamin A at a biochemical level.

The loss of the normal yellow or orange pigment of the enamel of the rat incisor is a later characteristic sign of vitamin A deficiency. Leicester (1949) believed that this "bleaching of the

enamel pigment produced in vitamin A deficiency is most probably a secondary effect. It may be due to the disorganization of the ameloblasts with consequent disturbance of enamel formation, but it more probably reflects an iron deficiency in the system, -- for anemia is one symptom of vitamin A deficiency." That it may be a reflection of a generalised iron deficiency is supported by the work of Moore and Mitchell (1955), who found a decreased iron content in the depigmented enamel of the incisors of vitamin A deficient rats. However, in regard to this loss of pigment and the structural changes in the vitamin A deficient rat incisor, Moore (1957) has pointed out "that the condition of the surfaces of the teeth which are visible during life do not reflect the current status of the animal in regard to vitamin A, but the status as it was several weeks previously. Time must elapse before injuries sustained beneath the gums can be brought into view by the growth of the tooth." Thus, as he concludes, "Inspection of only the exposed surfaces of the teeth therefore proves quite deceptive as a guide to the condition of the rat at the time of observation."

Mellanby (1939, 1941) made interesting studies in the offspring of vitamin A deficient rats. Pre-natal deficiency results in an extremely irregular and distorted enamel organ and in many cases, both ameloblasts and odontoblasts degenerate quite early so that only extremely malformed teeth are produced. Thus, from a review of the effects of prolonged or pre-natal deficiency states

on the rat incisor it could be said that the severity of such effects are proportional to the degree of vitamin A deficiency.

This section has demonstrated that vitamin A deficiency is capable of producing striking changes in the rat incisor, but pulp ossification, cementicle, denticle and defective enamel formation are all possible consequences of vitamin A deficiency in the human. However, as Wolbach and Howe (1933) have pointed out, such effects on human teeth are restricted to teeth in their formative stage, and particularly to those of the second dentition.

5.24 Eruption Rate of Teeth

The previous section has shown that much research has been carried out on vitamin A deficiency effects on the structure of the rat incisor. However, only a few references have been made as to the effect of vitamin A deficiency on its eruptive mechanism (Wolbach and Howe, 1925; Shibata, 1931; Smith and Lantz, 1933; Fridericia and Gudjonsson, 1936; Schour et al., 1941; Bryer, 1957; Jolly, 1964), and most of these were just observations, with no actual measurements of eruption rate. All of these studies, except that of Bryer, reported that vitamin A deficiency causes a retardation of the eruption rate of functionally occluding incisors, that is, of the attrition-eruption rate. However, as pointed out in the section on incisor eruption rate (1.2) this attrition-eruption rate is not a true reflection of the eruptive force, since the

inhibiting force of functional occlusion does not allow the full expression of the tooth's eruptive force. Thus, a true indication of the effects of vitamin A deficiency on eruption can only be obtained by measuring the unimpeded eruption rate.

Bryer measured unimpeded eruption by shortening the left mandibular incisor, thus removing it from functional occlusion and permitting the measurement of the eruption rate of this unopposed, uninhibited incisor, that is, of its unimpeded eruption rate. He then carried out a series of experiments to investigate the influence of various dietary, circulatory, surgical and endocrine factors on tooth eruption. One of these many investigations involved a short experiment on the effect of vitamin A deficiency on the unimpeded eruption rate of the rat incisor. Male weanling rats were placed on a vitamin A-free diet, and when clinically deficient, the unimpeded eruption rates of these clinically deficient rats, and their controls (receiving vitamin A supplements), were measured over a four week period. Bryer found that, in post-natal vitamin A deficiency, the unimpeded eruption rate in the eighth week of deficiency is 22 per cent lower than that of the control rats, and by the twelfth week, is 82 per cent lower than the control rate. Replacement therapy in two rats "showed an immediate progressive increase in the unimpeded eruption rate." Bryer checked the vitamin A status of his rats by

determination of the vitamin A liver levels; such determinations "showed little or none present in the deficient rats."

The importance of the concept of unimpeded eruption in experimental investigations on the eruptive process can be readily appreciated, and Bryer's findings in vitamin A deficiency over a restricted period of time prompted the author to carry out a more extensive study of the effects of vitamin A deficiency and replacement therapy on the eruptive process of the rat incisor, using especially the concept of unimpeded eruption. The author's methods, and its comparisons with those of Bryer, are described and discussed in the following sections.

5.3 Effects of Vitamin A Deficiency - References

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6. THE EXPERIMENTAL INVESTIGATION.

6.1 Materials and Methods

A total of seventy albino rats of the Sprague-Dawley strain were used for the experimental investigations. All rats were housed in wire cages in groups of not more than five animals of the same sex, and each rat was numbered by ear-punching.

Diet: The seventy rats were divided into four experimental groups and maintained on a vitamin A-free diet. This diet was similar to the one used by the Nutritional Laboratory, Cambridge (Moore, 1957), and had the following composition:-

Casein (vitamin free)	20 gm.
Sucrose	60 "
Arachis Oil	15 "
Brewer's yeast (dried)	10 "
Salt mixture	5 "

The composition of the salt mixture was

Calcium phosphate, $Ca_3(PO_4)_2$	43.5 gm.
Potassium chloride	27.2 "
Acid sodium phosphate (anhydrous)	11.4 "
Magnesium sulphate	8.7 "
Sodium chloride	5.4 "
Iron citrate	3.8 "
Potassium iodide	0.1 "
Manganese sulphate	0.22 "
Sodium fluoride	0.004 "

All rats received this diet and tap water ad libitum, and weekly oral doses of vitamins D, E and K dissolved in arachis oil, the proportions of these vitamins being:-

Vitamin D, as ergocalciferol	1.5 µg. per rat per week.
Vitamin E, as dl-alpha-tocopherol acetate	2 mg. per rat per week.
Vitamin K, as 2-methyl-1, 4-naphthoquinone	50 µg. per rat per week.

A total weekly supplement of 200 i.u. of vitamin A acetate in arachis oil was given orally to those rats not acting as experimentals (i.e. controls and "normals") over the period of study.

Grouping of Rats: The first group of rats consisted of ten "normal" rats, with equal numbers of males and females, which were taken from their mothers at six weeks of age and placed on the synthetic diet. These rats were called "normals" because both their lower incisors were allowed to remain in functional occlusion, and they also received the full supplement of vitamins (viz. A, D, E and K). Thus, the eruption rates of functionally occluding left and right lower incisors were able to be studied; such studies extended over a period of approximately 20 weeks.

The second group also consisted of five male and five female rats separated from their mothers at six weeks of age and placed on the vitamin A deficient diet. As with the first group, leaving these rats for so long in the presence of their mothers meant that they had already stored appreciable amounts of vitamin A, since they

had been allowed considerable time in which to partake of the standard stock diet of their mothers. However, unlike the first group of rats, these rats were not given a supplement of vitamin A, but comprised the delayed vitamin A deficient group in which the effects of a delayed vitamin A deficiency, especially on unimpeded eruption, were studied over a period of many months. Over this prolonged study period, four of the rats of this group died at different stages. Since at these stages their eruption rates and general condition were following the same time course and pattern of the other rats of this group, and since they had been only partially depleted of vitamin A, their results were not included in the experimental findings.

The third group was made up of five male and five female rats which acted as the control animals for the delayed deficient rats. The ten rats were therefore arranged to be of corresponding age and sex to the rats of the second group, and so were also separated from their mothers at six weeks and placed on the deficient diet, but unlike the rats of the second group, received vitamin A supplements. The incisor eruption rates, including the unimpeded eruption rate of the lower left incisor, of these control rats were then determined each week for a length of time similar to that of the rats of the second group.

The fourth and final group of rats consisted of forty rats with equal numbers of males and females. This number of rats was

required as it was realized that a number of them might not survive all the repeated anaesthesia necessary for each rat over the study period, or might survive these but succumb to the deficient state. As will be seen below, replacement therapy was given to these rats. Since only rats surviving the deficient period could be given such therapy, and 24 were required, the initial number of rats had to be sufficient to offset the losses during the pre-deficient and deficient periods.

Unlike the rats of the other three groups, these forty rats were separated from their mothers at approximately three and a half weeks of age. Thus, much smaller amounts of vitamin A had been stored by these rats, and a study of the effect of a rapid vitamin A deficiency on the eruption rates of the lower left incisor was possible. As with the other groups of rats, they were fed the vitamin A-free diet but were not supplemented each week with vitamin A. In addition to this deficiency study, these rats were used to study the effect of vitamin A replacement therapy on the eruption rates. When deficient (the criteria being the usual clinical deficiency signs, a plasma vitamin A level of zero and an unimpeded eruption rate equal to or less than 1 mm. per week), the rats were randomly placed into four sub-groups. All four of these sub-groups had equal numbers of rats (six) and within each sub-group, there were equal numbers of males and females. An oral vitamin A dose supplement was assigned to each of these sub-groups, such that rats

of sub-group I received a total of 10 i.u. of vitamin A each week;

" II " " " 160 " " " " " ";

" III " " " 40 " " " " " ";

" IV " " " 640 " " " " " ".

This vitamin A replacement therapy was instituted to study the effect of different dosages of vitamin A on the ability of the deficient rat's eruptive mechanism to recover from the vitamin A deficient state. This procedure involved a study period of some six weeks, until the unimpeded eruption rates of the rats in the different sub-groups regained their normal values or reached a steady level.

The weights and eruption rates of each rat of each group were noted twice weekly and recorded on individual graphs. Also at these times, each rat was examined for gross signs of vitamin A deficiency with particular emphasis on the condition of the animal's eyes and coat, the degree of incisor pigmentation, the presence of xerostomia, soft deposits, calculus and gingival and periodontal changes in the mouth, respiratory distress, general muscular weakness and the presence of a blood-stained nasal discharge, which was noted by the author as being of common occurrence in the vitamin A deficient rat.

At the end of the experimental period for each group of rats, each rat was killed by using excess ether, after final blood samples had been collected (as described below).

General Anaesthesia: Prior to the measurement of its eruption rates, each rat was anaesthetized with ether. It had been found necessary to use ether as the general anaesthetic because barbiturate (sodium pentobarbital), injected intra-peritoneally, had proven both disadvantageous and inconvenient. Although the amount of barbiturate injected was calculated on a dose per weight basis for each animal, the dose required for each rat was found to vary, not only between rats of similar weight but even in the same animal. Since the dose of barbiturate is quite critical, and since a large number of animals had to be anaesthetized twice a week, this individual variation in tolerance to the drug introduced the distinct possibility of a high mortality rate. Moreover, recovery from such barbiturate anaesthesia was found to be quite slow, and those rats recovering first often began to devour their cage-mates that were still under the effect of the anaesthetic. Consequently, this would have required housing each rat in individual cages until it eventually recovered.

For these reasons, anaesthetic ether (B.P.) was the general anaesthetic of choice. Ether displayed an extremely rapid recovery time, thus dispensing with the need for individual cages. There were no apparent after-effects of the anaesthetic, and in view of the fact that the anaesthetic was administered well over one hundred times each week, the effect of this general anaesthetic was even

the more remarkable since a mortality rate of less than one per cent was achieved!

The remarkably low mortality rate is further significant, since some rats (the twenty control and delayed deficient rats) were studied for nearly forty weeks. On the basis of two general anaesthetic procedures for each rat per week, this meant that each of these rats was subjected to a total of some seventy general anaesthetics during the study period, with no apparent ill-effects, and an extremely low mortality rate for the total number of rats over this long period of time. This further emphasizes the safety and practicability of the use of ether as the general anaesthetic for such procedures.

Because of the extremely low mortality rate of the general anaesthetic procedure, it will now be described, along with the signs of adequate general anaesthesia by which the author was guided and the hazards that may be encountered.

The rat, on removal from its cage, was placed into a large glass jar, at the bottom of which had been placed an ether-wetted pad of cotton wool covering the base. A large filter funnel was then turned upside down and positioned over the top of the glass jar; this not only prevented the animal from escaping, but also allowed into the jar a supply of air, through the stem of the funnel. A means of supplying air to the animal is very necessary, since

without it, the anaesthesia would be complicated by anoxia and undoubtedly result in respiratory failure, which even artificial respiration may find difficult to reverse.

A large glass jar was used so that the signs of the onset of general anaesthesia could be readily observed. General anaesthesia is usually divided into four stages, and each stage has characteristic signs which indicate the degree of anaesthesia. The first stage, usually referred to as the stage of induction, is that wherein consciousness is gradually altered until the second stage is reached, the stage of excitement. This is the period during which consciousness is lost, and it is the stage most critical for the animal's survival of the anaesthetic. Since the drug is now acting on the motor cortex, there is a general increase in muscle tone and activity. The passage from the second to the third stage, the stage of surgical anaesthesia, is characterized by a "smoothing out" of the respiration and constriction of the pupils of the eyes which were dilated in the second stage. The author regarded the animal to be adequately anaesthetized when it displayed a regular respiratory pattern, cessation of a nose twitching reflex which became apparent in the latter part of the second stage, and the loss of the conjunctival reflex (blinking) which seemed to be the last reflex to be lost as the stage of surgical anaesthesia was reached.

The animal must be carefully observed to see that it does not pass through the third stage, which is the period required for

surgical procedures, to the fourth stage which marks medullary paralysis. At this terminal stage, the respiration becomes wholly abdominal, the pupils dilate, and cyanosis occurs, with eventual cessation of respiration and finally heart failure. Should the animal show signs of respiratory failure, artificial respiration should be instituted immediately. Even this may sometimes prove unsuccessful, although the heart may keep beating for some time before it finally stops. In the author's experience, death can be most often the result of using too much anaesthetic, thus causing the animal to pass rapidly through the stages of anaesthesia with medullary paralysis and death the consequences. Furthermore, one cannot emphasize too strongly the necessity of close observation of the animal's condition at all times, from the very beginning to the completion of the operation. Even the slightest distraction can result in death of the animal.

Finally, mention must be made of the property of hypothermia. This is a property of most general anaesthetics, including ether, and is the result of a general vasodilation. Although this was not a problem in the present study, since the operating procedure for each rat was completed within five to six minutes, a means for keeping the animal warm (e.g. use of an operating table warmed underneath by electric light bulbs) may be important in operations requiring each animal for a considerable period of time.

Once anaesthetized, the rat was set up for eruption rate measurement as described below, with a cup containing an ether-wetted wad of cotton wool positioned loosely above its nostrils.

Measurement of Eruption Rates: The anaesthetized rat was placed on its back with its jaws held apart by two wire clamps. These wires were so fashioned as to be able to engage the palatal and lingual surfaces of the upper and lower incisors respectively; when the clamps were tightened, the jaws were thus held widely and rigidly apart, with the longitudinal plane of the lower incisors always maintained perpendicular to the eye-pieces of a binocular microscope. The binocular microscope had incorporated into it a micrometer gauge and, under reflected light, measurements of the eruption rates of the lower incisors were possible.

The method for the measurement of the unimpeded eruption rate, as advocated and used by Bryer (1957), was the method of choice for use in the rats of groups two, three and four. Using a thin abrasive separating disc, the lower left incisor was cut out of occlusion, care being taken to avoid overheating of the tooth. It must be stressed that not only should the tooth be completely free of occlusion with the upper incisors but also it should be sufficiently short of contact with the animal's palate. The rat's lower incisors often contact the palate in function, and if the left incisor has not been shortened sufficiently, the palate could prevent the full expression of the tooth's eruptive force. The next step was to make

a fine mark with a scalpel on the labial enamel of the lower right incisor, well towards the gingival margin of the tooth (the latter was mainly for the author's convenience, since it avoided the necessity of placing a new mark on this tooth at each measurement time; if placed near the incisal edge, this mark would, because of the tooth's continual eruption, have worn away at the next measurement, and required placing a new mark).

In Bryer's method, two measurements were then made (see figure 3) - the distance from the mark on the right incisor to the incisal edge of that same tooth (x), and the distance from the mark to the cut edge of the left incisor (y). At the next measurement, these distances were measured (x' and y' respectively). Thus, the attrition rate of the right incisor over the period between measurements was equal to $x-x'$, while the unimpeded eruption rate of the left incisor was $(x-x') + (y' - y)$. Then, the above procedure was repeated, with the left incisor again being cut down and the new distances of x and y measured, and remeasured (x' and y') at the following measuring date. This procedure was carried out twice weekly, on the same days each week, so that weekly eruption rates could be noted.

However, as well as the measurements noted above, the distance from the mark on the right incisor to the gingival margin of the tooth, and the distance from the cut surface of the left incisor to the gingival margin of that tooth, were measured and recorded at the same times as the measurement and recording

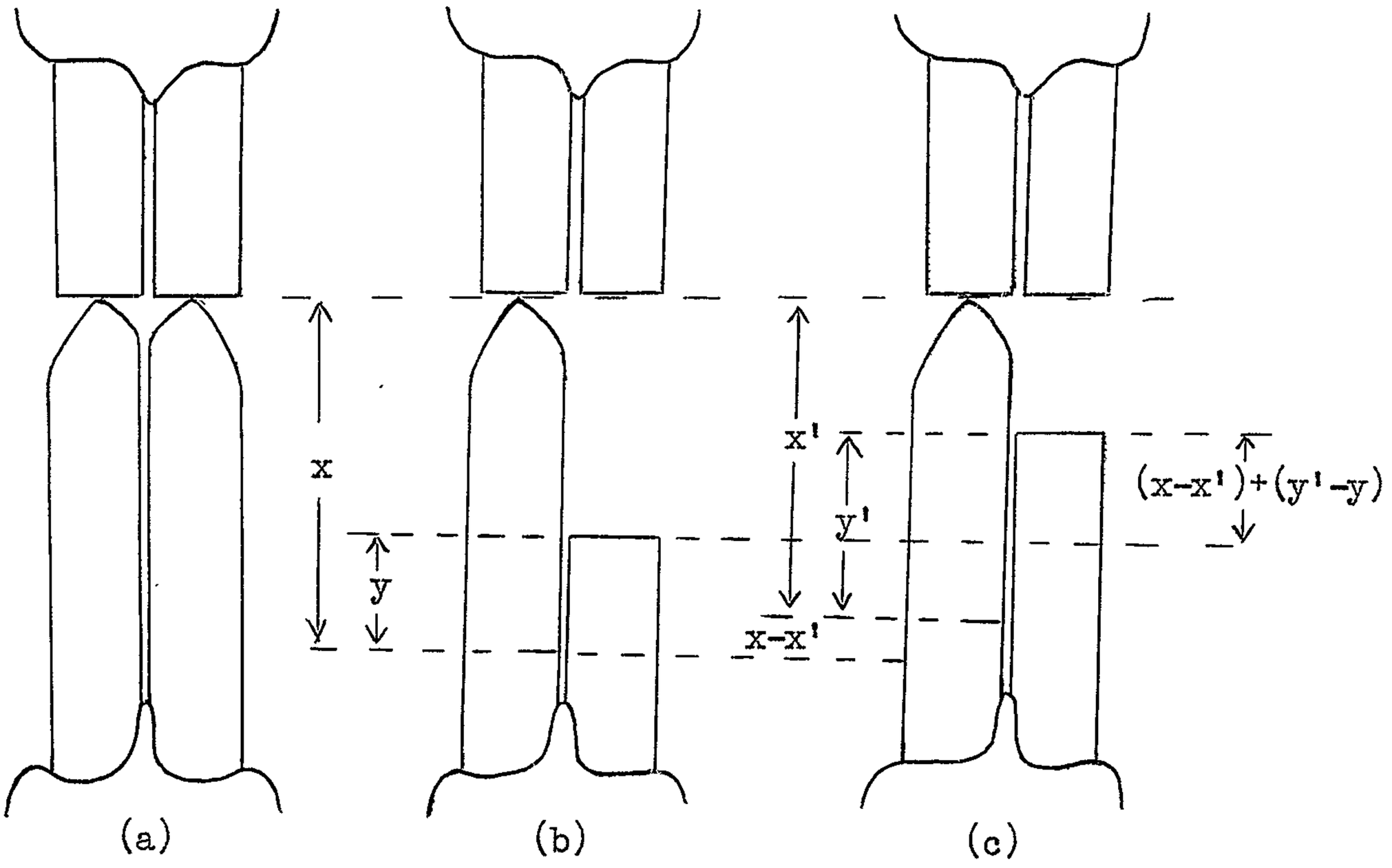

Figure 3 : Measurement of unimpeded eruption rate of rat's lower left incisor.

(a) : teeth still in functional occlusion.

(b) : lower left incisor cut out of occlusion - distances x and y measured.

(c) : 2-3 days later - x' and y' measured.

Unimpeded Eruption Rate = $(x-x')+(y'-y)$

of the distances mentioned in the previous paragraph. As discussed below, these gingival measurements were compared with the measurements of unimpeded eruption rate and attrition.

In the first group, however, which consisted solely of so-called "normal" rats in which all incisors were in functional occlusion, the above procedure was obviously not used. Instead, fine marks were placed on both the left and right lower incisors, and in each tooth the distances from the mark to the incisal edge, and also to the gingival margin, were recorded each week so as to give weekly attrition and eruption rates of incisors in functional occlusion.

Blood Samples and Analyses: Blood samples were collected from all groups of rats for analyses of their plasma vitamin A content. For all rats, no more than five ml. of blood were taken at the one time, and each rat was allowed at least four weeks to recover before a further sample was taken. Samples were obtained by cutting off the end of each rat's tail and allowing blood to drip into a dark bottle containing one drop of EDTA (to prevent clotting). Such samples were obtained from the group of normal rats at the time of their sacrifice to check that their plasma vitamin A levels were normal. In the deficient and control groups of rats (groups two, three and four), as well as the final blood sample, blood was also collected from rats of these groups before the unimpeded eruption rate of the deficient rats began to fall and also after it had

reached its deficient level. Thus, the plasma vitamin A level at which the eruptive mechanism was affected could be noted.

Each blood sample was analyzed by a method similar to that used by Bessey et al. (1946). Briefly, this involved centrifuging the blood sample (treated with EDTA) to obtain the plasma, then saponification of the plasma by the addition of an equal volume of alcoholic potassium hydroxide and heating this mixture at 60°C. for twenty minutes. After cooling, extraction of the vitamin A from the plasma was accomplished by adding a kerosene-xylol mixture (1:1) and agitating the tube now containing plasma, alcoholic potassium hydroxide and kerosene-xylol. After centrifuging at 3,000 r.p.m. for ten minutes, the kerosene-xylol layer containing the vitamin A content of the blood sample was then pipetted off and, using a Unicam SP 500 spectrophotometer, the optical density of this layer was read against a kerosene-xylol blank at 328 mμ. The amount of vitamin A present (if any) was then determined by comparison with standards of known concentration, the determinations being expressed in i.u. per 100 ml. of plasma.

Because of the instability of vitamin A to light, all procedures involving treatment of the plasma were carried out in the dark.

6.2. Findings

6.21 General Response to the Diet - Gross Signs of Vitamin A Deficiency

All rats of the four experimental groups ate similar amounts of the synthetic diet. However, whereas the normal and control animals (groups 1 and 3), receiving a full vitamin supplement each week, flourished on this diet, the delayed deficient and rapidly deficient rats (groups 2 and 4), receiving no vitamin A supplement, were markedly affected by the vitamin A deficient diet. A number of characteristic gross signs of the deficiency produced in these rats were observed; such signs were largely the result of the deficient state affecting the animals' weights, eyes, coats, respiratory tracts and oral cavities.

Weight Changes: One of the earliest and most striking effects in the vitamin A deficient rats was a rapid decrease in weight. On the other hand, the control and normal rats showed a steady rate of increase in weight, the male rats having a greater rate of increase than the female rats. However, in a few of the rapidly deficient rats, the weight did not fall but rather became steady. The younger the animal when placed on the vitamin A deficient diet, the more rapidly it became deficient and the more rapidly it showed weight losses. Thus, the rapidly deficient rats showed for a short time the normal weight pattern of an animal with vitamin A reserves, but once these became exhausted, the rats began to lose weight.

This weight loss began to occur at less than three months after the introduction to the vitamin A deficient diet. The delayed deficient rats however, displayed a much longer time during which the rates of weight increase were similar to their control animals. In contrast to the rapidly deficient rats, this group of rats did not show definite weight losses until five to six months after being placed on the deficient diet.

With the institution of vitamin A replacement therapy, the rapidly deficient rats showed generally a rapid increase in weight. It is of interest to note, however, that this weight increase did not differ markedly in rats receiving different dosages of the vitamin A supplement. Apparently, the higher dose supplements were more than enough to allow a return to normal "growth", and the lower doses were quite sufficient for this purpose. Furthermore, there could be found in replenished rats, no close correlation between improvement in the animals' weight and improvement in the other signs of deficiency of the animals. For example, although there may have been marked improvement in the weight of a replenished rat, the condition of its eyes and incisor eruption rate may have improved very little. Consequently, it must be emphasized that the weight should not be the only factor considered in establishing macroscopically the vitamin A status of an animal, but all the signs characteristic of the deficient state, or their absence (in animals receiving vitamin A supplements), must be collectively taken into account.