87. VITAMIN A IN BLOOD AND ITS RELATION TO BODY RESERVES

By P. C. LEONG

*From the Biochemical Department, King Edward VII College
of Medicine, Singapore*

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AN important line of vitamin research during recent years has been the establishment of chemical tests for the detection of vitamin deficiencies in human beings. While the presence of clinical signs will indicate the existence of a gross deficiency, it is recognized that the criteria for the detection of a partial deficiency are difficult to define.

A number of studies have been made with the object of correlating the level of vitamin A in blood with the state of vitamin A nutrition. Some evidence has been obtained that the level of vitamin A in blood is in general related to the economic status of the subject [Menken, 1932, 1934; Wolff, 1936; Wolff *et al.* 1936; de Haas & Meulemans, 1938, 1]. The subjects from the low-income groups were presumed to possess inadequate stores of the vitamin, which in turn were supposed to be reflected in the 'low' vitamin A values in blood. Arbitrary standards were suggested, describing certain levels of blood values as 'satisfactory' and others as 'insufficient'.

Also, it was found that generally the blood values of subjects with xerophthalmia contained either little or no vitamin A [Sie, 1937; de Haas & Meulemans, 1938, 2]. This finding appeared to fit in with the theory that the vitamin A level in blood is an index of the state of vitamin A nutrition. De Haas & Meulemans [1938, 2], however, also observed that at least 4 out of the 27 subjects with xerophthalmia had what the authors regarded as 'relatively high' vitamin A values in their blood and no explanation for this finding could be suggested.

Studies carried out by van Veen [1937] have shown that very often a direct relation between vitamin A deficiency and the vitamin A level in the blood appeared to be absent. What were regarded as 'high' or 'normal' values were sometimes found in patients with clinical symptoms of vitamin A deficiency, while 'low' values were observed in apparently healthy individuals. It appeared to us that such apparent anomalies can only be explained by the assumption that the level of blood vitamin A is not related to the body stores.

In all past work on this subject, no details of the previous dietary intake of the vitamin appear to have been taken into consideration. Without such data, the state of vitamin A nutrition of a subject is not definitely known. Thus it may happen that the diet of a subject at the time of the examination is deficient in some food factor and yet his previous diet may have provided him with ample reserves of that factor.

It appeared that the relation of the level of vitamin A in blood to the amount in the body reserves has not been defined and the present study was planned to throw more light on it. It was felt that the first step was to work on experimental animals whose whole dietary history was known and whose vitamin A intake could be easily kept under control.

The rat was found to be unsuitable for such studies because its blood level of vitamin A is normally too low to permit a reliable estimation to be made by the SbCl_3 reaction. Subsequently dogs were chosen for this study. After this work was started there appeared a paper by Steininger *et al.* [1939] on the blood vitamin A level of human beings whose vitamin A intake was controlled for a period of about 5 months. Although the amount of vitamin A in their fasting blood was, on the whole, found to be dependent on the amount in the diet, the evidence as to whether or not such determinations are of value in judging the nutritional status was considered by the authors to be contradictory.

EXPERIMENTAL

Study limited to vitamin A. Vitamin A metabolism in the animal body is intimately connected with the utilization of the various isomers of carotene. At present little is known of the quantitative aspects of carotene metabolism. It is very probable that the extent of its absorption, storage and transformation into vitamin A *in vivo* is governed by many factors which are as yet unknown. In order to reduce the problem to its simplest form it was decided that the experiment should be first carried out with reference to vitamin A alone. In this way the interpretation of the results would be simplified, since the evaluation of the significance of the carotenoid level in blood would not arise. With this consideration in view, the dogs were kept on a basal diet which was practically free from carotenoid pigments and a vitamin A concentrate (B.D.H.) was used as a supplement.

Plan of the experiment. The plan of this study was to rear young puppies of about 6 weeks on a diet of known vitamin A content. When about 6 months old, they were transferred to a vitamin A-deficient diet which was supplemented with graded doses of vitamin A. Their relative, though not absolute, reserves of vitamin A are thus known. The levels of vitamin A in their blood were determined at regular intervals to see if they were related to the vitamin A reserves. The object was to discover whether the vitamin A level in the blood can be used as a means of detecting inadequate vitamin A reserves.

This study can be conveniently divided into 3 parts: (1) a preliminary period of about 4 months during which an adequate diet of known vitamin A content was given to the puppies; (2) a dosing period of about $2\frac{1}{2}$ months during which the animals received graded doses of vitamin A daily and the vitamin A in their fasting blood was determined at regular intervals; (3) a depletion period of about 16 months on a vitamin A-free diet. In the first 6 months the blood of these dogs was also assayed regularly for vitamin A. The dogs were weighed periodically and signs of vitamin A deficiency were watched for during the depletion period.

Diet during the preliminary period. The puppies were placed on the following diet when about 6 weeks old: raw (slightly polished) rice 500 parts, skimmed milk powder 100 parts, marmite 10 parts and coconut oil 20 parts. The rice was cooked before the other ingredients were added and 2 meals (morning and evening) were given daily. The skimmed milk powder was found by chemical tests to contain about 1.0 I.U. of vitamin A per g. Each dog in addition received 2 ml. of cod liver oil (180 I.U. vitamin A) daily and 50 I.U. vitamin D daily in the form of a diluted solution of Ostelin (Glaxo).

Diet during the dosing period. This was similar to that given above with the exception that the skimmed milk powder was extracted twice with hot 95% alcohol. The skimmed milk powder (500 g.) was heated in shallow dishes in an air oven for 12 hr. at 110° and was then extracted with 2 l. EtOH under a reflux

condenser for 8 hr. After filtration by suction the milk powder was extracted again in a similar manner, after which it was filtered off and dried. That this basal diet is reasonably free from vitamin A is shown by the observation that rats kept on it developed xerophthalmia in about 6 weeks.

Vitamin A supplements during the dosing period. At first 4 dogs were given the following amounts of vitamin A: 0, 100, 400 and 2000 I.U./kg. body-weight per day. Unfortunately the negative control animal died under anaesthesia soon after the experiment was started. The vitamin A supplement was given in the form of a concentrate (B.D.H.) diluted in olive oil, and its potency was checked periodically by means of the SbCl_3 reaction. The required amount of vitamin A solution was mixed with the morning meal and precautions were taken to ensure that the supplement was consumed. It was observed that the dogs invariably ate their morning meal, with the exception of the dog on the highest level of dosage (2000 I.U./kg. body-weight per day) which began to lose its appetite about 8 weeks after the dosing started. This loss of appetite was accompanied by a loss of weight and was presumably due to the excessive intake of the vitamin since the appetite returned and the weight again increased when the supplement was discontinued.

Diet during the depletion period. During this period of about 16 months the vitamin A supplements were stopped and the dogs were kept on the vitamin A-free diet mentioned above.

Total intake of vitamin A by the dogs. This was calculated by taking into consideration the vitamin A administered in the form of cod liver oil during the preliminary period and in the form of the concentrate during the dosing period. The following table summarizes the figures.

Table 1. *Total intake of vitamin A by the dogs*

	Preliminary period I.U.	Dosing period I.U.	Total I.U.
Dog A	26,000	56,000	82,000
Dog B	26,000	198,000	224,000
Dog C	26,000	1,064,000	1,090,000

As these figures represent almost all the vitamin A received by the dogs (with the exception of that obtained during their first 6 weeks of life), it is reasonable to conclude that their relative stores of vitamin A were of the same order of magnitude as their vitamin A intake.

Determination of vitamin A. Blood was drawn from the dogs once in about 3 weeks during the dosing period. The dogs were anaesthetized with a mixture of equal parts of ether and CHCl_3 and 15 to 30 ml. were taken from a vein in the outer part of the hind leg. Blood was drawn in the morning at least 24 hr. after the previous dose of vitamin A had been consumed. The vitamin was estimated as follows. The blood was diluted with an equal volume of water in a flask and 10 ml. each of 95% EtOH and 50% aqueous KOH added. The mixture was heated in an air oven at 100° for 45 min. and then extracted with 3 successive lots of ether (100, 100 and 50 ml. respectively). The combined ether extracts were washed with water until free from alkali and evaporated to dryness *in vacuo*. The residue was then taken up in a known volume of chloroform and the vitamin A estimated by the SbCl_3 reaction in a Lovibond Tintometer. The blue units (Moore) were converted into I.U. by multiplying by the factor 0.6 [Moore, 1937], but in the interpretation of results these values should not be regarded as absolute but as relative only.

Results

Vitamin A in blood during the period of dosing. The results are shown in Fig. 1. It will be noted that the levels of vitamin A in the blood rose gradually throughout the period of dosing and were proportional to the vitamin A intake of the dogs. If it be assumed that their body stores were in proportion to their intakes, it can be said that the blood vitamin A level was proportional to the

Fig. 1. Vitamin A in blood during the period of dosing. Arrow indicates commencement of dosing.

extent of the body reserves. This conclusion is valid only under certain conditions, viz. when appreciable amounts of the vitamin are present in the blood. Why this conclusion is sometimes not valid is explained below.

Vitamin A in blood during the depletion period. As soon as the vitamin A supplements were stopped the level of vitamin A in the blood began to fall (see Fig. 2). After about 6 months on the vitamin A-free diet, no vitamin A could be detected in as much as 30 ml. of blood by the SbCl_3 reaction (i.e. there was less than 5 I.U./100 ml. blood). There was no great difference in the time taken for the blood vitamin A of the 3 dogs to fall to 'zero' level, the respective times for dogs A, B and C being 154, 138 and 168 days.

After the blood vitamin A had fallen to this low level, the experiment was continued for another 10 months. During this period the animals were watched for signs of vitamin A deficiency but none were observed. Throughout the depletion period the dogs maintained their weights, and their general health and condition were excellent.

The female dog gave birth to a live litter of 5 young on the 276th day of the depletion period. The puppies appeared to be growing normally for about 3 weeks after which the mother's milk began to fail. The puppies quickly lost weight and 3 died while the remaining 2 were killed by coal gas. Their livers were examined for vitamin A but no appreciable amount could be detected by the SbCl_3 reaction.

After about 400 days on the depletion diet blood was again drawn from the dogs and examined for vitamin A, this time by the spectrophotometric method. Again no vitamin A could be detected in 30 ml. samples.

As has been mentioned above, the blood vitamin A level dropped to values of less than 5 I.U./100 ml. blood about 6 months after the vitamin A supplements were stopped. This finding, by itself, might be wrongly taken to mean that a state of deficiency existed. The important point to note is that these dogs were in a healthy condition and were free from any sign of vitamin A deficiency for a further period of 10 months.

Fig. 2. Vitamin A in blood during the period of depletion.

Dog A received 100 I.U. vitamin A/kg./day for 72 days.
 Dog B received 400 I.U. vitamin A/kg./day for 72 days.
 Dog C received 2000 I.U. vitamin A/kg./day for 76 days.

At present the metabolic requirement of vitamin A by the animal organism is not known with any degree of certainty. According to the findings of Guilbert *et al.* [1940] the daily minimum requirement of many mammalian species is about 20 I.U. of vitamin A per kg. body-weight per day. Taking this figure as a rough guide, it can be calculated that the minimum amount of vitamin A utilized by each dog during the last 10 months of the experiment would be about 54,000 I.U. At least this amount of vitamin A was presumably stored in the dog's tissues when the level of vitamin A in its blood was practically zero. On the basis of this observation it seems that a 'low' or 'negative' vitamin A value in dog's blood (as determined by the SbCl_3 reaction) cannot be regarded as a sign that the vitamin A reserve is seriously depleted.

Dogs A, B and C were killed after 470, 470 and 452 days respectively on the depletion diet. No abnormalities were observed on post-mortem examination. It has been shown that distinct lesions appear in certain nervous tissues of animals suffering from vitamin A deficiency [Mellanby, 1935]. Tissues from dogs B and C were examined for histological changes by Helen F. Burn at the Department of Pathology, Singapore. The results are given in the appendix at the end of this paper. The total liver reserves of vitamin A were 70, 50 and 1370 I.U. in dogs A, B and C respectively. It appears from these figures that dogs A and B were almost completely depleted of vitamin A after about 16 months on the deficient diet.

Our results indicate that it is not possible to lay down a 'normal' vitamin A value for dog's blood. On theoretical grounds it seems reasonable to believe that there is a critical level of blood vitamin A below which signs of deficiency will appear. This level may be defined as the least amount of vitamin A in blood that is sufficient to meet the normal requirement of the tissue cells. Amounts below this level will correspond to a state of deficiency of the vitamin and the prolonged effect of this condition will result in the appearance of anatomical changes characteristic of the deficiency. It appears that the SbCl_3 reaction is not sufficiently sensitive to detect the critical level of vitamin A in dog's blood. It can also be concluded that there is no danger of avitaminosis-A in dogs so long as there are even traces of vitamin A in the fasting blood.

There is evidence that the critical levels of blood vitamin A in various species are different. Thus, the recent observations of Davis & Madsen [1941] indicate that the critical level of carotene in cattle plasma is comparatively high, viz. 25 $\mu\text{g.}/100\text{ ml.}$ and for vitamin A in the same sample about 16 $\mu\text{g.}/100\text{ ml.}$ When the critical level in a species is comparatively high, its estimation by the ordinary methods of vitamin A analysis will be possible and the detection of depleted vitamin A reserves can be made by blood analysis.

In the living body different levels of blood vitamin A may be found, depending on factors such as (1) the extent of the vitamin A intake and (2) whether or not the blood was drawn during the process of intestinal absorption. When the body reserves are totally depleted, it is to be expected that the blood will not contain any detectable amounts of vitamin A. On the other hand, the results of our experiments with dogs indicate that a negative SbCl_3 reaction does not necessarily mean that the body reserves are depleted.

APPENDIX

BY HELEN F. BURN

Immediately after death, the following tissues were removed from each dog: (1) fundus of the stomach, tongue and striated muscle and (2) peripheral nerve, optic nerve, spinal cord (cervical, thoracic and lumbar levels), trigeminal nerve, frontal cortex, motor cortex, basal and caudate nuclei, medulla, pons and cerebellum.

The stomach, tongue and muscle were stained with (1) haematoxylin and eosin and (2) haematoxylin and Van Gieson. They were found normal in both dogs.

The peripheral nerve, optic nerve and trigeminal nerve were studied by the Marchi and Weigert-Pal methods. The peripheral and trigeminal nerves were normal. The optic nerve from dog B showed slight degeneration in the Marchi sections.

The spinal cord and brain sections were studied by the Weigert-Pal method and also by the routine stains of (1) haematoxylin and eosin and (2) haematoxylin and Van Gieson. No change was noted except for a very slight degeneration of the lumbar cord in the posterior column in dog B. This change was probably not pathologically significant.

SUMMARY

A study has been made of the level of vitamin A in the blood of dogs receiving graded doses of the vitamin. The experiment included a period of dosing with vitamin A and a depletion period on a vitamin A-free diet.

During the period of dosing, the level of vitamin A in the blood was proportional to the intake of the vitamin.

During the depletion period, the blood vitamin A gradually fell to a very low level (not detectable by the SbCl_3 reaction in 30 ml. blood).

A 'low' blood vitamin level, however, cannot be considered as a definite sign of depleted reserves of the vitamin.

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REFERENCES

- Davis & Madsen (1941). *J. Nutrit.* **21**, 135.
de Haas & Meulemans (1938, 1). *Meded. Dienst Volksgezondh. Ned.-Ind.* **27**, 492.
—— (1938, 2). *Lancet*, **1**, 1110.
Guilbert, Howell & Hart (1940). *J. Nutrit.* **19**, 91.
Mellanby (1935). *Brain*, **58**, 141.
Menken (1932). *Dtsch. med. Wschr.* **58**, 1484.
—— (1934). *Maandschr. Kindergeneesk.* **4**, 22; quoted from *Nutr. Abstr. Rev.* (1935), **5**, 57.
Moore (1937). *Biochem. J.* **31**, 155.
Sie (1937). *Arch. Augenheilk.* **110**, 610.
Steininger, Roberts & Brenner (1939). *J. Amer. med. Ass.* **113**, 2381.
van Veen (1937). *Meded. Dienst Volksgezondh. Ned.-Ind.* **26**, 300.
Wolff (1936). *Schweiz. med. Wschr.* **66**, 979.
—— Banning & van Eekelen (1936). *Quart. Bull. Hlth Org. L.o.N.* **5**, 566.