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THE QUANTITATIVE DETERMINATION OF VITAMIN A

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Recent research gives prominence to vitamin A among the chemical substances essential to nutrition and makes clear the importance of quantitative work in the determination of the relative concentrations of this substance. As in the determination of vitamins B[1] and C,[2] the general method is to feed different weighed quantities of the material to be tested, as the sole source of the vitamin, to a standard test animal on a basal diet fully adequate in all other respects, and thus to find the minimum amount of the material under examination which suffices to produce a standard result in or for a standard time.

As explained in a previous paper,[3] vitamin A can be stored in the body of the test animal to a relatively greater extent than either vitamin B or C, and special precautions are therefore required in order that comparisons shall not be vitiated (as many doubtless have been in the past) by the presence of significant and variable quantities of vitamin A in the bodies of the test animals at the beginning of the experimental period. This was first clearly emphasized by Drummond and Coward[4] and Zilva and Miura,[5] and it is mainly from their methods that ours have been developed.

The purpose of the present paper is to summarize the results of our investigations upon the basal diet, the test animals and the experimental procedure; and, through the application of these investigations, to systematize the method and the interpretation and mode of expression of the results, in the interest of increased accuracy and clearness in work involving quantitative comparisons of vitamin A.[6]

[1] Sherman and Edgeworth, THIS JOURNAL, **45**, 2712 (1923). Sherman and Spohn, *ibid.*, **45**, 2719 (1923). Sherman and Grose, *ibid.*, **45**, 2728 (1923).

[2] Sherman, LaMer and Campbell, *ibid.*, **44**, 165 (1922).

[3] Sherman and Kramer, *ibid.*, **46**, 1055 (1924).

[4] Drummond and Coward, *Biochem. J.*, **14**, 661 (1920).

[5] Zilva and Miura, *ibid.*, **15**, 654 (1921).

[6] While the experiments here described were in progress, Steenbock and his coworkers have shown that in animals from a certain type of stock diet, retardation of growth such as has previously been attributed simply to deficiency of vitamin A may be due in part at least to shortage of the antirachitic factor. Such confusion does not seem to occur under the experimental conditions recommended in this paper and in any case can be avoided by irradiation of the animals, or even, as has recently been shown, of their food. (Hess, paper presented to American Pediatric Society, June, 1924. Steenbock, *Science*, September 5, 1924 and paper presented to American Chemical Society, September, 1924. Steenbock and Black, *J. Biol. Chem.*, **61**, 405 (1924).)

The Basal Diet

Much attention has been given to the development of a basal diet which shall be free from vitamin A but fully adequate in all other respects and which can be duplicated with certainty.

As the protein component of our basal diets we have used both casein and meat residue with satisfactory results. In either case, our experience leads us to depend upon thorough extraction with hot alcohol for removal of vitamin A rather than upon the attempt to free the material completely from this vitamin by heating in the air. Alcoholic extraction of the meat residue has been discussed in a previous paper.[3] Casein is perhaps preferable to meat residue as a component of a standardized basal diet because it is more definite chemically. We conduct the extraction of the casein essentially as described by Osborne and Mendel. The following details of procedure in this extraction have been found convenient and effective.

Place 200 g. of fine-grained, air-dry, high grade casein in a 2-liter Pyrex flask with 500 cc. of 95% alcohol, boil for one hour on a steam-bath under a reflux condenser and then transfer the contents of the flask rapidly without cooling to a suction filter in a Büchner funnel of about 15 cm. diameter. After thorough removal of the hot alcohol, return the casein to the flask and add 500 cc. of fresh 95% alcohol, boil and filter as indicated above; then repeat the process again. In this way the casein receives three successive one-hour extractions with boiling alcohol. Finally it is washed in the Büchner funnel with 500 cc. of hot alcohol and then dried in the air at room temperature.

Numerous feeding experiments showed that the casein thus extracted was free from any trace of vitamin A detectable by present methods and was indistinguishable in this respect from other portions of casein which had been subjected to still longer extraction with hot alcohol.

Corn starch and patent flour have been used alternatively as the chief source of energy in our basal diets, numerous experiments with the patent flour obtained from a local grocery having indicated that this contained no detectable amount of vitamin A. We have been told, however, of experiments in another laboratory in which patent flour, obtained directly from an experimental mill and known to have been unbleached, seemed to carry traces of vitamin A. In view of this possibility and the theoretical advantage of composing the basal diet as far as practicable of materials which are individual chemical substances, we now use starch instead of flour.

Dried brewery yeast was used as the source of vitamin B in the basal diet. Since Osborne and Mendel have shown[7] that such yeast, even when fed to the extent of 42.5% of the total food consumed, does not furnish any detectable amount of vitamin A, it seemed safe to assume that in feeding about one-eighth of the quantities fed by them no appreciable trace of vitamin A would be introduced into the basal diet. This has been con-

[7] Osborne and Mendel, *J. Biol. Chem.*, **45**, 277 (1921).

firmed by our three years' experience with the use of basal diets of the type here described.

Mineral elements were supplied by the use of the complete salt mixture prepared according to the directions of Osborne and Mendel.[8] Additional sodium chloride to the extent of 1% of the dry weight of the food mixture was also included in the basal diet, as this seemed to result in the diet being somewhat more uniformly well-relished by the experimental animals of our colony.

The composition of our final basal diet (Diet 380) was as follows: 20% of casein (extracted as described above), 70% of starch, 5% of dried brewery yeast, 4% of Osborne and Mendel salt mixture,[8] 1% of sodium chloride; total, 100%.

Fat was not included in the basal diet, as our animals seem to thrive as well without it and its omission rids us of any possible uncertainty as to its freedom from vitamin A or other fat-soluble vitamin.

Standardization of Test Animals

Albino rats have been used as test animals in many previous experiments upon vitamin A; but the lack of exact and comparable methods or criteria for the choice and use of rats for this purpose has undoubtedly been one of the greatest sources of error.

The present discussion relates to young albino rats which, unless otherwise stated, were from families fed upon a diet of one-third dried whole milk and two-thirds ground whole wheat, with sodium chloride in the proportion of 2% of the weight of the wheat. In some cases the mother had received 60 g. per week of fresh lean beef in addition to the wheat-and-milk mixture just described. This addition of lean beef was found to have no appreciable effect upon the amount of vitamin A stored in the body of the young rat as judged from the length of time it was able to survive when placed upon vitamin-A-free diet.

In all cases our experiments are begun by placing the young rats upon the vitamin-A-free diet immediately upon separation from their mothers at 28 to 29 days of age. Males and females are used interchangeably since, when experiments are started at four weeks of age, sex is found to have no appreciable influence upon the response of the animal to the vitamin-A-free food.

When rats having the same previous dietary history were fed the vitamin-A-free food from the age of 28 to 29 days until death, the survival period was nearly independent of the initial weight of the animal, within rather wide limits; but in order to avoid appreciable error from this source it is well to specify the use of rats whose initial weight is not less than 35 nor more than 55 g.

[8] Osborne and Mendel, THIS JOURNAL, **37**, 572 (1919).

Experimental Procedure

When rats at four weeks of age are placed upon a diet well adapted to their needs in other respects but devoid of vitamin A, such as the basal diet described above (Diet 380), growth continues for a longer or shorter time, depending mainly upon the store of vitamin A which the young rat had in its body at the beginning of the experiment which, in turn, depends mainly upon the previous dietary history of the young rat and its mother. When the family dietary has furnished this vitamin in about the proportion in which it is furnished by the wheat-and-milk mixture described above, growth usually continues for from four to five weeks. Around the time that cessation of growth is anticipated the animals should be weighed daily or every second day. When the weight has been approximately stationary (or declining) for about one week, and the general appearance of the animal indicates that it has definitely ceased to grow, the "fore-period" is ended and the "test period" is begun. Under conditions such as have been described above, the test period, or period of feeding the material to be tested, usually begins during the sixth week of the fore-period or the tenth week of the life of the rat. Having decided upon a definite and uniform diet for the families from which the young are to be drawn, and a uniform age (preferably within the range of 25 to 29 days) at which to place the young upon the vitamin-A-free diet, one can learn by experience about when to expect cessation of growth, and will also be guided by the general appearance of the animals in determining when the fore-period should end and the test period begin. This should be after the animals have certainly used up their previous reserve store of vitamin A but before they have been permanently injured by the vitamin-A deficiency. Sometimes, but not always, the appearance of the earliest symptoms of the eye trouble may aid the observer in reaching a decision. Throughout the test period each rat is kept in a separate cage. So far as possible, rats of the same litter should be used for those tests the results of which are to be most directly compared with one another, and at least one representative rat from each litter should be continued on the vitamin-A-free diet only, as a "negative control." Careful daily observation of these latter will aid in acquiring the experience that is so helpful in determining when the fore-period should end and the test period begin. From the beginning of the test period each rat (except the "negative controls") is fed daily, or at other suitable intervals, a weighed amount of the food to be tested; this, of course, the experimenter must ascertain with certainty to be entirely consumed. All of the rats have the basal diet always available. They are kept in individual round cages, 23 cm. in diameter and 23 cm. high, without bedding and with raised bottoms of wire screen to prevent access to excreta. Our average negative controls live about three weeks beyond the end of the fore-period, usually losing weight through-

out this time. Any advantage in survival period or maintenance or gain in weight shown by a test animal over its "negative control" must be attributed to the vitamin A received from the weighed portions of the food or other material which is being tested, provided the experiments are conducted properly and in sufficient numbers to avoid vitiation of results through individual variability of the animals. Hence, animals making equal gains in weight during the experimental period may be regarded as receiving equal amounts of vitamin A, and the richness of different foods in vitamin A may be taken as inversely proportional to the amounts of the foods required to furnish that fixed amount of the vitamin which will produce a given result in a standard experimental animal, for example, a given rate of gain in weight during the experimental period in a rat that has been prepared for the purpose in the manner described above. As was pointed out by Drummond, Coward and Zilva, a definite small gain in weight furnishes the best basis for quantitative comparison. We recommend, as a suitable standard for this purpose, an average gain of 3 g. per week during an experimental period of eight weeks' duration. In all of the work here described we have chloroformed and autopsied all surviving animals at the end of the eight-weeks' test period.

The two main purposes of these autopsies are, first, to detect any possible cases of abnormality due to causes unrelated to the vitamin deficiency; and, second, to confirm the conclusions drawn from the weight curves as to the extent to which the vitamin deficiency, whether absolute or relative, has affected the life and growth of the experimental animal. This involves the use of the "negative controls" as a basis of comparison. Among these animals, which from the age of 28 or 29 days had received a diet adequate in other respects but devoid of vitamin A, 85% developed the ophthalmia first noted by Osborne and Mendel as characteristic of the results of this dietary deficiency.[9] Pus in one or more of the glands near the base of the tongue was found in 76% of the "negative controls" examined for this sign, which was, therefore, in this series, almost as characteristic of the vitamin A deficiency as was the ophthalmia. These animals, placed at an early age upon a diet entirely devoid of vitamin A and dying usually within about two months thereafter, did not show the frequency of lung trouble stated by Steenbock and Nelson[10] to be one of the characteristic effects of vitamin A deficiency. There is, however, probably no real discrepancy between the work of the two laboratories in this respect. It is probably because of the complete deprivation and consequent early death of our animals in this series that the lung trouble did not show itself in them. Where the diet is deficient in, rather than devoid of, this

[9] Osborne and Mendel, *J. Biol. Chem.*, **15**, 311 (1913).

[10] Steenbock and Nelson, *ibid.*, **56**, 355 (1923).

vitamin, the experience of this Laboratory[11] fully confirms the view of Steenbock and Nelson that vitamin A deficiency leads to marked increase in the incidence of lung disease.

Typical Experiments upon the Vitamin-A Content of a Food

Young rats standardized as explained above and receiving the basal diet already described (Diet 380) were given, daily except Sundays, uniform amounts varied in different cases, from 0.05 g. to 2.0 g. of tomato as sole source of vitamin A, or were kept on the basal diet only as controls as described above. The average gain curve for each different amount of tomato fed is shown in Fig. 1. It will be seen that the animals receiving 0.2 g. of tomato six times per week (an actual daily allowance of 0.17 g.), showed almost precisely the desired average gain of 3 g. per week for the eight weeks of the experimental period.

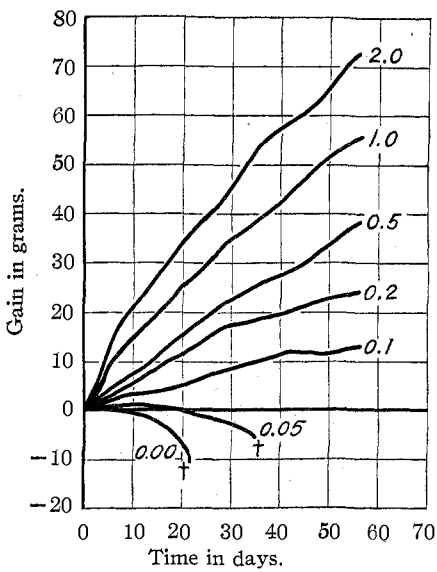

Fig. 1.—Curves showing gains in weight, during test period, made by groups of rats fed graded portions of tomato as sole source of vitamin A. The amount in grams of tomato fed to each rat six times weekly is shown at the termination of each curve. All received the vitamin-A-free basal diet (Diet 380) *ad libitum*. Each curve expresses the average result obtained from several tests.

With larger feedings the results were about equally regular but the gains increased in much less than arithmetical proportion to the amount of vitamin A received. On the other hand, with feedings so small as to permit no gain in weight whatever, there is danger that the test animal may fail to survive the experimental period.

From our experience in these and other cases we conclude that the most desirable unit in terms of which to measure vitamin-A content is that amount which when fed daily suffices to induce in a standard test animal, prepared as described above, a gain in weight of 3 g. per week during the experimental period. If such a unit be adopted, then the relative vitamin-A contents of foods may be expressed as units of vitamin A per gram, per ounce, per pound or per 100-Calorie portion of the food.

In this paper we have used the term vitamin A in essentially the sense in which it has been understood during the years in which the experiments

[11] Sherman and MacLeod, THIS JOURNAL, **47**, 1658 (1925).

here described were performed (1921–1924), namely, as signifying fat-soluble vitamin essential to growth. During this time it has been made clear that the antirachitic is to be differentiated from the antiophthalmic factor, and it has been suggested that the term vitamin A be confined to the latter. Steenbock and his associates have recently shown that under certain conditions the general growth may be retarded for lack of the antirachitic factor. The question therefore arises whether shortage of antirachitic vitamin is ever the factor limiting growth in experiments for the measurement of vitamin A by the method here described. A final answer to this question must await the development of methods for the more strictly quantitative differentiation of these factors. Some indication is, however, afforded by a comparison of the weight records and autopsy findings of our experiments. Some of our autopsies, particularly of animals in which higher gains in weight had been sought, revealed bone fractures and so-called beading of the ribs, suggestive of shortage of the antirachitic vitamin (as this term has been used by Steenbock); and in the cases in which this was most pronounced there was a tendency for the rate of gain to reach a maximum at 4 to 7 g. per week, whereas with animals that had received foods known to furnish the antirachitic factor, such as egg-yolk and whole milk, the rate of gain increased progressively with the food increments up to 10, 12, or even 15 g. per week with little if any appearance of bone defect. From these observations it would appear probable that the shortage of antirachitic substance in certain foods may become the factor limiting growth when considerable growth is sought or when the previous diet has been such as described by Steenbock; but that when animals from previous diets such as ours are used and the gain in weight is limited to 3 g. per week, the weight curve is determined not by any antirachitic factor but by vitamin A in the usual acceptation of the term. In any case it now appears that the method can easily be so applied as to avoid the possibility of retardation of growth by shortage of antirachitic factor, since as noted above this may be supplied by irradiation either of the test animal or of the food.

Summary

For the quantitative determination of the relative vitamin-A content of foods, it is recommended that albino rats of known nutritional history be placed when 28 to 29 days of age upon a diet adequate in all other respects but free from vitamin A. After growth has ceased, the young rats are to be kept in individual cages and at least one of each litter should be continued on the basal vitamin-A-free diet until death, as a "negative control," while the others are to be fed graded portions of the food to be tested, as their sole source of vitamin A, daily or at other suitable intervals, during a test period of eight weeks. By sufficient repetitions, the mini-

mum allowance of food which will induce an average gain in weight of 3 g. per week during the test period is thus ascertained.

The unit recommended for numerical expression of results is that amount of vitamin A which when fed daily induces an average gain of 3 g. per week in a standard test animal under the conditions described.

The choice and control of the basal diet, of the test animals, and of the experimental procedure, and the interpretation of the findings, are discussed.

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QUANTITATIVE EXPERIMENTS UPON THE OCCURRENCE AND DISTRIBUTION OF VITAMIN A IN THE BODY, AND THE INFLUENCE OF THE FOOD

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A conspicuous feature of the experiments by means of which the existence of fat-soluble vitamin was demonstrated by McCollum and Davis[1] and Osborne and Mendel[2] was the fact that feeding tests of relatively long duration were required before the results of a lack of this substance in the food were made manifest in the condition of the test animal. This led to the general belief[3,4] that the animal body might be able to carry a store of this vitamin, and soon afterward it was shown[5] that animals kept under like conditions upon the same diet deficient in vitamin A survive for very different lengths of time, according as their previous diet has been rich or poor in this substance.

That the liver is especially concerned in this storage function was early suggested by Osborne and Mendel's[6] discovery of the high vitamin-A potency of cod-liver oil, and by the finding of liberal proportions of this vitamin in pig and beef livers.[7] In a paper appearing since the completion of most of the experiments here described, Steenbock has shown[8] that rat liver may be relatively rich or relatively poor in vitamin A, according to the nutritional history of the animal.

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[1] McCollum and Davis, *J. Biol. Chem.*, **15**, 167 (1913).

[2] Osborne and Mendel, *ibid.*, **15**, 311 (1913).

[3] Hopkins and others, *Nat. Health Insur. Med. Res. Com. (Great Britain), Special Rept.*, No. 38, **1919**.

[4] Osborne and Mendel, *J. Biol. Chem.*, **45**, 277 (1921).

[5] Sherman, MacLeod and Kramer, *Proc. Soc. Exptl. Biol. Med.*, **17**, 41 (1920). See also Sherman and Kramer, THIS JOURNAL, **46**, 1055 (1924).

[6] Osborne and Mendel, *ibid.*, **17**, 401 (1914).

[7] Osborne and Mendel, *ibid.*, **32**, 309 (1917); **34**, 17 (1918). McCollum, Simmonds and Parsons, *ibid.*, **47**, 111 (1921). Zilva and Drummond, *Lancet*, **1922**, I, 1243.

[8] Steenbock, Sell and Nelson, *J. Biol. Chem.*, **56**, 327 (1923).