LX. RESEARCHES ON THE FAT-SOLUBLE ACCESSORY SUBSTANCE. III: TECHNIQUE FOR CARRYING OUT FEEDING TESTS FOR VITAMIN A (FAT-SOLUBLE A).

By JACK CECIL DRUMMOND and KATHARINE HOPE COWARD.

From the Institute of Physiology, University College, London.

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In a recent communication Osborne and Mendel [1920] referred to the experiments of Steenbock, Boutwell and Kent [1918], and of Drummond [1919, 1] on the destruction of the fat-soluble accessory factor by heat. They were unable to confirm these observations, and in other respects there were also discrepancies for which the reason was not apparent. We have made a careful examination of these discrepancies, and believe that satisfactory explanations are available. Of the difference between the opinions on the effect of heat on the nutritive value of butter we have little to say in this paper, because a later communication will be devoted entirely to that subject; it may, however, be said in passing that we have evidence that the destruction of fat-soluble A is an oxidation process, which confirms what has already been stated by Hopkins [1920]. It is discrepancies of another type which we wish to discuss in this paper, such for instance as the considerable differences noted between the amounts of butter fat required to supply the fat-soluble vitamin for growing rats, to which attention was drawn by Osborne and Mendel [1920]. At first our tendency was to ascribe such differences to variations in the food value of the samples of butter used by the separate investigators, but after giving the matter more careful attention, we have formed the opinion that this undoubted source of disagreement is frequently accompanied by one of a more serious nature, and one, fortunately, that can be readily eliminated. We refer to the very different types of basal diet used in various laboratories. In a previous paper of this series [Drummond, 1919, 2], an experimental method was described by which substances may be tested for the presence of the fat-soluble factor. This method was used for some time with success, but we were led to introduce certain improvements which are described here, and which may be helpful to other investigators who encounter the many difficulties which surround this type of research.

There appears to be little or no need to emphasise the importance of employing carefully selected animals for feeding tests of this nature, for one gathers from the published results that the majority of investigators fully

appreciate this point. In our opinion, however, it is frequently the composition of the basal dietary which is responsible for many of the misleading and contradictory statements which tend to confuse the literature on the vitamins at the present time.

Evidence has been produced which tends to show that the requirements of the growing rat for vitamin A become less as the animal approaches maturity [Drummond, 1919, 2], and our experience leads us to believe that the amount of vitamin which must be supplied to a rat in order to restore growth which has been inhibited by feeding on the deficient basal diet, will be inversely proportional to the weight of the animal.

Should this be confirmed, we think many of the discrepancies in the literature will be accounted for.

Preparation of Purified Basal Diets.

i. *Preparation of Pure Protein.*

Up to the present we have made a practice of using only highly purified caseinogen as a source of purified protein in our basal dietaries. We have done this following the general scheme used by many other investigators, but we are now considering whether it would not be advisable to employ another protein of equal or superior tissue building value, and one less prone to be contaminated with the fat-soluble factor. A search for such a protein is being made. Commercial caseinogen contains relatively large amounts of the fat-soluble vitamin, and should never be used for experiments relating to that factor without having been carefully purified. We have encountered numerous cases in which prolonged growth of young animals was observed in young rats which were fed upon a diet supposedly free from fat-soluble A, but which were in reality obtaining considerable supplies of the vitamin from the insufficiently purified caseinogen.

We heat our caseinogen for 24 hours or more in shallow dishes to a temperature of 102° C., after which it is subjected to a prolonged and continuous extraction with alcohol and ether.

ii. *Purity of Carbohydrate.*

In the past we have gone to the expense and trouble of preparing a highly extracted form of wheat starch to use as a source of carbohydrate in the basal diets. Later experiments have shown us however that rice starch in the crude form is almost entirely devoid of fat-soluble A, and may be employed without any lengthy and costly preliminary extraction.

iii. *Purity of Fat.*

In the selection of a fat to include in the basal fat-soluble-free diet the greatest care must be employed. As will be shown in the following paper the natural oils and fats which are usually supposed to be free from vitamin A,

are not always so, and disconcerting variations in a single oil may be encountered. As a consequence of many such conflicting results we have carefully reinvestigated this question, and have ascertained that no hard and fast line can be drawn between the animal and vegetable oils and fats, when their growth-promoting powers are being considered. We therefore think it advisable that no natural oil or fat should be used as a source of fat in a diet intended to be free from fat-soluble A. To overcome this difficulty we employ a fully hardened (hydrogenated) and refined vegetable oil, usually cotton seed oil. Such oils consist very largely of tristearin, and are, so far as we can ascertain from carefully controlled feeding tests, entirely devoid of vitamin A. It is possible that even greater security might be obtained by excluding fat entirely from the basal diet, in view of the fact that rats appear to be able to dispense with the presence of pure fats in their diet [Drummond, 1919, 2; 1920].

iv. *Purity of other Constituents of Basal Diet.*

The orange juice and salt mixture which we include in the basal ration have been proved by direct tests to be devoid of vitamin A. The yeast extract is also thought to be equally inactive in that respect, although definite results have not yet been obtained to make quite sure of this point.

The composition of the purified basal ration which we employ in all our routine tests is given below:

Purified caseinogen...	18 parts
Purified rice starch...	52 ,,
Refined hydrogenated vegetable oil	15 ,,
Yeast extract	5 ,,
Orange juice	5 ,,
Salt mixture	5 ,,

Rats fed upon this diet behave in somewhat different manner according to their age [Drummond, 1919, 2]. Small rats of 50–70 g. (4–5 weeks old) should show very little growth at all, and should remain stable for a week or two after the slight initial growth. Any considerable increase of body weight in rats of this age when fed upon a purified diet of this composition is interpreted by us as an indication that the basal ration is insufficiently purified. Animals considerably over 100 g. are in our opinion unsatisfactory for testing for vitamin A, and as far as possible we attempt to test all fractions on rats the growth of which has been suspended for 10–14 days, and which are not heavier than 80–120 g.

Testing Substances for Vitamin A.

As far as possible we make it a routine in this laboratory to test all substances for the presence of vitamins by administering a definite weight of the substance directly to the animal before the day's ration of the basal food is

given. In the large majority of cases this method, which is the only one permitting of an accurate quantitative measure of intake, is practicable. In cases where it is found to be difficult to carry this out, the supplement of foodstuff or fraction must be incorporated in the diet, and the intake judged by records of the total consumption of food.

Results.

Even with the employment of the greatest care, this method occasionally gives results of doubtful significance. The enormous amount of routine work which is entailed by feeding large numbers of experimental animals by such a process necessitates in an average laboratory some restriction in the size of the experimental groups, a fact which tends to increase the error due to individual variation. Such errors are frequently encountered, and are sometimes disconcerting, but one usually obtains a definite result from the majority of the animals in a particular group.

As we have previously remarked, we are of the opinion that insufficient purification of the basal diet is responsible for many misleading results.

In the curves given by Osborne and Mendel [1920], it will be observed that some of the young rats grew for a considerable time, and attained a fairly heavy weight before they showed the typical decline due to deficiency of vitamin A. This means that when the rats are fit to use for feeding tests they are often considerably over 100 g. in weight. Such animals would presumably recover health and recommence growing on receiving a much smaller amount of the missing vitamin than would be necessary to restore a declining rat of 60–90 g. Further, the former animal, although it had ultimately declined on a deficiency of fat-soluble A, would still be obtaining some fat-soluble factor in the impure basal diet in addition to that contained in the supplement. The observed result might therefore lead one to ascribe a higher food value to the supplement than was justifiable.

Our own experience has given us many examples of how the presence of very small amounts of the factor A in an insufficiently purified basal diet may confuse the issue of the experiment.

Summary.

In testing foodstuffs for the presence of the fat-soluble vitamin the greatest care should be devoted to ensuring that the basal dietary is rendered as free from that vitamin as possible. Details for the preparation of a highly purified ration are given. Failure to work with a sufficiently pure diet may lead to conflicting and misleading results.

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