LXVII. A NOTE ON THE DIFFERENTIATION OF THE YELLOW PLANT PIGMENTS FROM THE FAT-SOLUBLE VITAMINE.

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THE identification of the yellow plant pigments—carotene and xanthophyll—with certain lipochromes of the animal body has been established by Palmer and his co-workers and has formed the substance of an interesting series of investigations.

Palmer and Eckles [1914, 1] first identified the yellow colouring matter of cow's milk with carotene accompanied by insignificant traces of xanthophyll. They showed that cows fed on a carotene-free diet yielded, after some weeks, a completely colourless milk, and further that, on the restoration of green food to the diet, the carotene was immediately diverted to the milk fat. The same writers also showed that carotene—with traces of xanthophyll—forms the yellow colouring matter of the body fat, corpus luteum and skin secretions of the cow and made the further observation that the same pigments form the colouring matter of the serum albumin from which they can be separated by drying with plaster of Paris, and treating the dried mass with alcohol, followed by extraction with light petroleum [Palmer and Eckles, 1914, 2, 3].

On extending these observations to other animals Palmer could discover no trace of carotene in the blood serum of swine on grass feed, traces only were found in the blood serum of the sheep and none could with certainty be isolated from ewe's milk. Goat's milk showed possible faint traces of carotene. Horse serum, on the contrary, showed a content of lipochromes comparable with that of the cow [Palmer, 1916].

Examination of the lipochrome content of the fowl [Palmer, 1915] brought to light interesting differences between the chemical habits of birds and mammals in this respect. It appears that, whilst in mammals the predominant plant pigment stored in the body is carotene, yet in the fowl the pigment stored in the egg-yolk body fat and blood serum is principally xanthophyll. Another striking point of difference is the manner of the association of this pigment in the serum albumin. In the case of the mammal the carotene could not be separated from the blood serum by merely shaking with light petroleum,

in which this pigment is soluble; a preliminary breakdown of the albumin-carotene complex by treatment of the dried mass with alcohol was necessary. In the case of the xanthophyll-albumin complex of the bird mere shaking with light petroleum sufficed to remove the pigment.

The distribution of these yellow plant pigments in animal tissues, and particularly their concentration in the milk of the mammal and the egg yolk of the fowl, has naturally led to considerable speculation as to their function and has tempted several workers to seek for their association or identity with the fat-soluble vitamine. The absence of these pigments from the body fat of the pig—a fat which has been shown to be low in vitamine content—also formed a finger-post pointing in the same direction.

Drummond [1919] fed crude carotene in the proportion of 0.003 % of the total diet and obtained evidence of traces of the fat-soluble vitamine. When, however, pure recrystallised carotene was substituted for the crude product a negative result was obtained. Zilva [1920] found that an alcoholic extract of carrot—which must have contained small quantities of the pigment—carried the fat-soluble factor and when fed in quantities corresponding to 25 g. of fresh carrot per rat per day sufficed to restore normal growth.

Palmer and Kempster [1919] working on the growth and fecundity of fowls obtained interesting results negating the importance of yellow plant pigments in the animal. These workers experimented first with a ration which they stated to be “carotinoid free”; as however they mention that this ration contained bone meal *ad lib.* it would have been more satisfactory had they furnished evidence that this was fat-free, since from freshly ground beef bones it is possible to extract a bright yellow fat which in view of Palmer’s earlier work probably contains carotene. In the course of their investigations, however, they resort to a special carotinoid-free diet in which bone meal was replaced by ground limestone. Hens on this ration laid eggs which were almost but not quite pigmentless; the eggs showed normal fertility, but were very slightly below the normal in percentage hatched. Considerable trouble was taken to prove the nature of the traces of pigment in these eggs. Although this was not actually identified, it was shown that it was neither xanthophyll nor carotene. These experiments showed fairly conclusively that the absence of yellow plant pigments does not affect prejudicially the health or fecundity of hens, and as a consequence renders it highly improbable that the fat-soluble vitamine can be identified with these pigments. •

The experiments described in the present paper were begun in June, 1919; while they were in progress, work bearing on the same subject has been published both by Palmer and Kempster [1919] and by Zilva [1920]. The experiments about to be recorded are therefore chiefly of interest as independent corroboration of their results. As, however, our knowledge of the fat-soluble vitamine is at present mainly derived from feeding experiments for which the widest possible statistical evidence is desirable, it has been decided to publish them.

EXPERIMENT 1. *The effect of an alcohol-light-petroleum extract of dried*

carrot on the growth of young rats. In order to determine whether carotene is the substance responsible for the growth-promoting properties of butter fat a preliminary trial was made with a crude extract of dried carrot containing the pigment. The fresh carrots were passed through a mincing machine, dried by means of an electric fan between 30° and 35° and the dried substance reduced to powder in a mill. The powder was placed in screw-stoppered bottling jars and covered with 98 % alcohol for 24 hours. The alcohol was then filtered off on a Buchner funnel and the residue covered with light petroleum (B.P. 40°–60°) for 24 hours, again filtered and the light petroleum extraction repeated three times. The yellow alcoholic filtrate was reduced in vacuo to a syrupy residue and the deep red petroleum extracts from the combined washings were concentrated until the volume of the solution was about 0.1 of the original combined extracts. The alcoholic and light petroleum extracts were then mixed, and a portion of the extract corresponding to 250 g. of fresh carrot was evaporated to a syrup on a water-bath and mixed with 250 g. of a palm kernel oil, known by repeated previous experiments to be deficient in the fat-soluble vitamine. Ten young rats (43–52 g. initial weight) were put on the usual synthetic diet employed in this laboratory, consisting of a mixture of starch, sugar, and purified caseinogen to which was added McCollum's salt mixture [McCollum, Simmonds and Pitz, 1916]. The water-soluble vitamine was supplied by a fat-free alcoholic extract of yeast and the anti-scurvy factor by 0.5 cc. lemon juice per rat per day. The fat given was palm kernel oil mixed with the crude extract of carrot as above described. By this means, extract of carrot from 2–3 g. fresh carrot per rat per day was consumed. The growth of these rats is shown on Curve 1. Ten similar rats serving as a control were given the same synthetic diet with palm kernel oil alone. The growth of these rats is shown on Curve 2. After the 36th day the diet of the control set was changed with a view to the study of keratomalacia. No symptoms of eye disease were shown by the rats receiving the extract of carrot, whilst six out of the ten control animals had the disease unmistakably. This, together with the striking dissimilarity in the growth curve, is strong evidence for the presence of the growth promoting factor in the extract of carrot.

EXPERIMENT 2. *The effect of pure carotene on the growth of young rats.* The previous experiment having shown the presence of the fat-soluble vitamine in a crude alcohol-light-petroleum extract of dried carrot, a second experiment was undertaken to determine whether the substance responsible for the activity of the extract was carotene. This was prepared as follows:

5000 g. of fresh carrot were dried as described in Exp. 1, ground and extracted with light petroleum (without preliminary treatment with alcohol) at room temperature; the deep red extract (about eight litres) was concentrated on an electric heater to about 600 cc. and then in vacuo to about 5 cc. The residue was taken up with chloroform (10–20 cc.) and precipitated with methyl alcohol; the crude carotene rapidly crystallised out and was filtered

off under slight pressure. Yield from first crystallisation 0.1678 g. or 0.0033 % of the fresh carrot. The crude product was recrystallised from chloroform and methyl alcohol four times—that is until the m.p. was constant at 172–173° (uncorrected). The final yield was 0.0675 g., i.e. 0.0013 % of the fresh carrot, 0.004 g. of the recrystallised carotene corresponding to about 100 g. of fresh carrot was dissolved in 100 g. of palm kernel oil, which thus contained the same concentration of carotene as the palm kernel oil and crude extract of carrot fed in the preceding experiment. Five young rats were fed in the usual way on the standard synthetic diet with palm kernel oil and pure carotene as their source of fat (see Curve 3); five other rats, to serve as a control, were fed

Fat supplied to rats in

- Curve 1 (10 rats). Palm kernel oil + crude extract of carrot.
- Curve 2 (10 rats). Palm kernel oil.
- Curve 3 (5 rats). Palm kernel oil + recrystallized carotene.
- Curve 4 (5 rats). A—C. Palm kernel oil.
C—D. Palm kernel oil + crude extract of carrot.
- Curve 5 (8 rats). Butter fat decolorised by charcoal.
- Curve 6 (8 rats). Untreated butter fat.

with palm kernel oil alone as their fat supply (see Curve 4 A—C). It is seen from these curves that the growth of the rats receiving carotene showed no superiority over that of the control animals and three out of the five suffered from eye disease. The period represented by Curves 3 and 4 covers 82 days. On the 82nd day one rat was killed and the diet of the remaining four

was changed by the substitution of crude extract of carrot for pure carotene; all symptoms of eye disease cleared up in the three rats suffering from keratomalacia. Three of the four, however, failed to survive and died, two on the 14th and one on the 46th day after the change of diet. From this experiment it is clear that the carotene is not the substance responsible for the growth-promoting properties of the crude light petroleum extract of carrot. (On the 54th day of experiment (Curve 4 C) crude extract of carrot was given to the control animals and resulted in the improved growth (Curve 4 C-D).)

EXPERIMENT 3. *The removal of the pigment from butter fat and the effect on its growth-promoting properties.* The decolorisation of butter was carried out as follows: 300 g. of filtered butter fat were dissolved in one litre of light petroleum and about 4 g. of fine birch charcoal added: the mixture was placed in a Winchester quart bottle and shaken for two hours on a shaking machine. At the end of that time the light petroleum extract was filtered free from charcoal and the solution found to be only very slightly tinted; a second treatment with charcoal and subsequent filtration resulted in the complete decolorisation of the butter fat. The light petroleum was then distilled off in vacuo final traces being removed at 60°. The fat thus treated was perfectly white and resembled lard in appearance; it was not ascertained whether this effect was due to the adsorption of the carotene by the charcoal or to oxidation on its surface. It may also be mentioned that only one out of three samples of birch charcoal (all of which bore the same description on the label) had the effect described. An explanation of this anomaly is probably to be found in the work of Philip, Dunnill and Workman [1920] on the activation of wood charcoal by heat.

Two sets of eight young rats were put on experimental diet, the fat in the one case being the decolorised butter fat (see Curve 5), that in the other case being the same sample of butter fat untreated (see Curve 6). One rat on the decolorised butter died on the 14th day of experiment without previous loss of weight and obviously from accidental causes. Perfect growth was maintained in every other individual of both sets for eight weeks after which the experiment was discontinued.

This experiment shows clearly that the removal (or destruction) of the colouring matter of butter does not affect its vitamine content.

SUMMARY.

From these three experiments we may conclude:

1. That a crude alcohol-light-petroleum extract of dried carrot when added to a fat not containing vitamine confers upon it the growth-promoting property and the power of protecting the animal from, or curing it of, keratomalacia.

2. That the substance or substances in the above extract responsible for these properties is not carotene.

3. That the colouring matter of butter fat may be completely removed or destroyed by filtration through charcoal without in the least affecting the vitamine content of the butter.

I wish to take this opportunity of thanking Professor Hopkins for the benefit I have derived from his wide experience in feeding experiments.

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