CXVI. A DELICATE COLOUR REACTION FOR THE PRESENCE OF VITAMIN A.

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In a previous communication [1920] we stated that the well-known colour reaction of cod-liver oil with sulphuric acid is given not only by the liver oil of this fish but by the liver fats of all animals, and suggested a relationship of the chromogenic substance to vitamin A. The close parallel between the growth-promoting factor and the sulphuric acid test was afterwards emphasised by Drummond and Watson [1922] and further experience, extending over three years, has fully convinced us that this association is more than accidental. The transient nature of the reaction unfortunately prevents its use for a quantitative colorimetric comparison of different oils and all attempts to stabilise the reaction were unsuccessful.

In the course of work on the activation of cholesterol by ultra-violet light [Drummond, Rosenheim and Coward, 1925], we found that arsenic chloride (and various other reagents, see below) gives with cod-liver oil a brilliant ultramarine blue colour reaction. Further study of this reaction led us to the conclusion that, like the sulphuric acid test, it is characteristic for vitamin A. It has the advantage that the colour persists sufficiently long to allow a colorimetric comparison with a suitable standard.

The reaction is carried out by adding 1 cc. of pure arsenic chloride to one drop of cod-liver oil, and shaking the test-tube at once. The oil dissolves immediately to form a clear blue solution, which in the course of a few seconds assumes a purple tint and gradually fades. The reaction is characterised by a well-defined absorption band extending from λ 550–590. Under the above conditions the band persists for about 5 minutes.

Sensitiveness. The reaction is extremely sensitive. A 1 % solution in light petroleum of a highly growth-promoting Newfoundland cod-liver oil was still found to react intensely with 1 cc. $AsCl_3$ in amounts of 0·05 cc. = 0·5 mg. of oil. The limit was reached with 5 $mm.^3$ = 0·05 mg., measured with a Wright's capillary pipette. Compared with the ordinary sulphuric acid test in the same solution the sensitiveness was 20 times as great.

The oil was saponified and the unsaponifiable portion freed from cholesterol by means of digitonin. The cholesterol-free fraction now gave the reaction in a dilution of 1 : 2,000,000.

Relation to vitamin A. The fact that the chromogenic substance resists saponification and is concentrated in the cholesterol-free unsaponifiable fraction of cod-liver oil strongly suggests its close relationship to vitamin A, since it has previously been shown by Drummond and Coward [1922] that this fraction contains the fat-soluble vitamin A. This can be distilled with superheated steam in a nitrogen atmosphere, or in a high vacuum, without losing its activity, and still gives, as we found, the colour reaction with $AsCl_3$ in undiminished intensity. On the other hand, the chromogenic substance, and with it the growth-promoting power, is gradually destroyed by oxidation when a current of air is passed through the oil at 100°: after 30 minutes' aeration the reaction is diminished and is no longer given after 60 minutes.

We have further tested a series of over thirty oils and fats and have found complete agreement between the colour intensity and the growth-promoting activity, as tested by animal experiment. Amongst these samples was the butter made from the milk of a cow which had been fed with cod-liver oil. This butter gave the reaction most intensely as compared with ordinary butters, whilst vegetable oils free from vitamin A gave no reaction. Details of this work will be given in a later communication, together with the results of further tests still in progress.

It is interesting to note that this reaction makes it possible to differentiate the growth-promoting vitamin A from the anti-rachitic vitamin D. We have found that cholesterol which had become highly anti-rachitic by irradiation with ultra-violet light [Rosenheim and Webster, 1925] does not react in the way described above with arsenic chloride[1]. We intend to examine in this direction the oils from the marine diatom, Nitzschia, and of plankton, which were found not to respond to the sulphuric acid test by Drummond and Watson [1922], since in the light of more recent experience it is possible that such growth-promoting power as they exhibit may be due to their content of anti-rachitic vitamin and not to the true, growth-promoting, vitamin A.

Experiments which we made on the diffusibility of the fat-soluble vitamins make it highly probable that they both dialyse through a rubber membrane into light petroleum. The oil recovered from the dialysate gives the colour reaction intensely and is also anti-rachitic, as tested by the animal experiment (unpublished experiments by Rosenheim and Webster).

Nature of chromogenic substance. In searching for an explanation of the chemical nature of the reaction, we found that cod-liver oil (and other liver fats) reacted not only with arsenic chloride, but yielded precisely similar blue colour reactions with a number of heterogeneous reagents, which are known to share with arsenic chloride the property of giving a red colour with chole-

[1] The colour reactions of irradiated cholesterol will be described together with its anti-rachitic power in another communication.

sterol. These reagents are dimethyl sulphate, trichloroacetic acid, acetyl chloride and benzoyl chloride (the last two only in the presence of zinc chloride). Whilst these substances react with cholesterol only when heated, and yield a permanent red colour (absorption band in green), they react with cod-liver oil at room temperature, the blue colour produced (absorption band in yellow) fading within 5–10 minutes.

The reaction with trichloroacetic acid is particularly striking and may be carried out either by allowing a drop of cod-liver oil to fall on a few crystals of the acid or by adding 1 cc. of a saturated chloroform solution of trichloroacetic acid to one drop of the oil[1]. The colour produced is slightly more purple than that with $AsCl_3$ and the absorption band is correspondingly shifted to the red. The colour is discharged by alcohol, ether, ethyl acetate, acetic anhydride, glacial acetic acid and 90 % formic acid, but not by benzene, toluene, light petroleum and chloroform. Pure mono- and dichloroacetic acids do not react.

It would be premature to assume from the analogy of these reactions to those of cholesterol that vitamin A is a sterol derivative. The interesting suggestion of Harden and Robison [1923] with regard to the mechanism of the sulphuric acid test and the observations of Whitby [1923] on cholesterol reactions in general, would tempt one to assume as an explanation of the new reactions that they are due to the presence of an aldehydic coupling substance or to a substance allied to cholesterol, possessing an aldehydic group in its molecule. It has indeed been stated by Takahashi [1922] that a fraction of the unsaponifiable matter of cod-liver oil, claimed by him to be vitamin A, possesses aldehydic character, as evidenced by the reduction of Fehling's solution and ammoniacal silver nitrate. We also have found clear evidence for the presence of a reducing substance in cod-liver oil, in so far as it gives Schiff's reaction and reduces phosphomolybdic acid. The reducing substance is destroyed by aeration, but withstands saponification to a considerable extent, and is therefore not likely to be aldehydic. A colorimetric comparison by means of Welmans' [1892] phosphomolybdic acid test moreover convinced us that the amount of the reducing substance in various oils does not run parallel to their vitamin A content, as measured by the animal experiment and by the arsenic chloride reaction. The nature of the reducing substance remains to be investigated.

Another observation we made links the chromogenic substance to the lipochromes, of which carotene especially yields, in light petroleum or chloroform solution, a slate-blue colour with arsenic chloride (and the other reagents). This reaction is distinguished from that of cod-liver oil by being permanent.

In view of the possible relationship between sterols and lipochromes and their general association with vitamin A in plant tissues [Coward, 1923], it is suggested that the arsenic chloride reaction is concerned with a substance

[1] The chloroform solution should be freshly prepared, as it slowly generates phosgene on keeping in light. A light petroleum solution may be used.

derived from these types of synthetic plant products under the influence of sunlight.

Colorimetric estimation of vitamin A. The application of the colour reaction to the evaluation of the vitamin A content of cod-liver oil is still under investigation. Should further work confirm the results, which show, so far in every case examined, that the colour intensity is proportional to the growth-promoting power, it will be feasible to replace the animal experiment to a large extent by a chemical test. Preliminary experiments indicate that even a rough colorimetric comparison with a suitable standard yields results of a higher degree of accuracy than those obtainable by a laborious animal experiment. The presence of large amounts of yellow pigment naturally influences the tint of the reaction, but since modern medicinal oils, the main source of vitamin A, are usually only slightly pigmented there is no difficulty in this respect.

We have so far made use of the arsenic chloride and the trichloroacetic acid reaction, and have taken as our standard of vitamin A a highly active Newfoundland oil. A suitable colour standard for the arsenic chloride reaction is a mixture of 100 cc. crystal violet solution (1 : 10,000) with 50 cc. methylene blue solution of the same strength (both in alcohol). Under the conditions chosen we found that the colour produced by 20 mg. of the oil (= 1 drop from a pointed glass rod of 3 mm. diameter) + 1 cc. $AsCl_3$ matches the standard dye solution diluted in the proportion 3 : 2.

It is advisable to add the reagent by means of a teat-pipette, holding 1 cc., and to make the comparison rapidly within a few seconds after the addition of the reagent. After preparing a set of suitably diluted standard solutions in test-tubes of uniform diameter, we found, after a little experience, no difficulty in ranging all the oils so far examined in a series which agreed with their growth-promoting activity.

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