CLXVIII. THE NATURE OF FEARON'S COLOUR REACTION AND ITS NON-SPECIFICITY FOR VITAMIN A.

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FEARON [1925] described a colour reaction which he considered to be associated with vitamin A and suitable for colorimetric work. Attracted by the permanence of the reaction, Willmott and Moore [1926], as well as Euler, Myrbäck and Karlsson [1926], have attempted to make it the basis of a method for the quantitative colorimetric estimation of vitamin A. The reaction consists essentially in the production of a stable bright rose colour by the addition of trichloroacetic acid to a mixture of cod-liver oil with a phenol (pyrogallol). The pigment formation is assumed to be due to the condensation of an aldehydic chromogenic constituent of the oil (= vitamin A) with the phenol, and is intensified by the addition of an oxidising agent (benzoyl peroxide).

It is surprising that neither the discoverer of the reaction nor his followers have applied the test to the unsaponifiable fraction of cod-liver oil and thereby obtained more definite evidence with regard to its specificity for vitamin A. It is now well established that this fraction contains the whole of the growth-promoting material present in the oil and that the soaps are free from vitamin A. Consequently the first condition to be satisfied by any such test must be a positive reaction when applied to the unsaponifiable fraction of an oil [Rosenheim and Drummond, 1925]. Since we found, when carrying out this crucial test [Rosenheim and Webster, 1926], that Fearon's colour reaction is not given by the unsaponifiable fraction of cod-liver oil, it is evident that this reaction has no relation to vitamin A, for the estimation of which it was suggested. Further, apart from the non-specificity of the reaction, it was found that trichloroacetic acid, intended to act as a condensing agent, itself gave rise to a reaction of an entirely different character and colour, in the absence of a phenol. Since the relative amounts of the two chromogens entering into the reaction vary in different oils, the resulting mixture of colours cannot be estimated by the ordinary colorimetric methods.

EXPERIMENTAL.

5 g. cod-liver oil, giving a strong Fearon's reaction, were saponified with alcoholic potash. The unsaponifiable portion, prepared in the usual way, gave an intense arsenious chloride reaction but was entirely negative to Fearon's

test. The same negative result was obtained with different liver oils, and also in the case of sardine oil, a fish body oil (see below). A preparation of the unsaponifiable fraction of cod-liver oil, freed from cholesterol by means of digitonin and kindly supplied to us by Prof. Drummond, gave identical results.

The nature of the chromogen. The substance responsible for Fearon's reaction is not destroyed by saponification, but can be recovered from the soap solution. After acidifying the vitamin-free soap solution, the fatty acids were extracted with pure ether and were found to give the reaction¹. The mixed fatty acids were separated by the well-known lead acetate process into two fractions. The unsaturated fatty acids liberated from the ether-soluble lead salts contained the chromogen, whilst the saturated fatty acids from the ether-insoluble lead salts gave a negative result. The chromogen is therefore associated with the unsaturated fatty acids. It can further be shown that it occurs in the original oil as an ester in combination either with glycerol or another alcohol (cholesterol?). This follows from the fact that the chromogen cannot be removed from the oil by shaking with 2 % KOH at room temperature and is only converted into a water-soluble salt by boiling with alcoholic potash.

We would suggest, as a working hypothesis, that the chromogen is an aldehydic oxidation product of a highly unsaturated fatty acid of the type of clupanodonic acid, $C_{22}H_{34}O_2$. This acid was first isolated by Tsujimoto from Japanese sardine oil (from *Clupanodon melanosticta*) and subsequently also from cod-liver oil, other fish oils and from green algae [Tsujimoto, 1922, 1925]. This suggestion is in agreement with the fact that all vegetable oils are negative in Fearon's test. In these oils clupanodonic acid cannot be detected by the "bromide" test, whilst we were able to isolate as much as 20 % of the decabromide of clupanodonic acid, $C_{22}H_{34}O_2Br_{10}$, from the fatty acids of an animal oil which gave an intense Fearon's reaction. It may further be pointed out that acids of similar type are easily oxidised to aldehydic fatty acids, which could act, owing to their aldehydic functions, as Fearon's postulated chromogen when condensed with phenols.

Comparative colour- and feeding-tests. When testing a large number of cod-liver oils and other liver oils, dissolved in light petroleum according to Fearon's directions, we found that only relatively few of them gave the bright rose colour, which was considered by him as characteristic for vitamin A. A large number gave a pea-green, others an intense emerald green and still others an equally intense greenish-blue colour. After addition of benzoyl peroxide the colours changed into carmine, reddish purple and various shades of magenta respectively. Similar erratic changes were recorded by Willimott and Moore [1926] and by Euler and his collaborators [1926]. The former observers ascribe the formation of two distinct colours to the varying proportions of phenol

¹ This fact agrees with and explains the recent observation of Moore and Willimott [1926], according to which Fearon's reaction persists in cod-liver oil after treatment with concentrated H_2SO_4 , whilst the $AsCl_3$ reaction disappears together with vitamin A, which is destroyed by the acid.

used and even suggest that in one stage two "vitamin" molecules combine per phenol molecule, whilst in the other stage only one such molecule combines.

We are able to offer a much simpler explanation, since we found that these green and blue colours are not connected with the chromogen of Fearon's reaction at all, but are given by certain oils when mixed with a solution of pure trichloroacetic acid itself. The test, therefore, does not fulfil the essential condition, laid down by Fearon, that the condensing agent should not produce any colour with the oil in the absence of a phenol. When these dark green or blue solutions are shaken with a small volume of water, the blue component descends into the watery phase, whilst the yellow pigment (lipochrome?) remains in the light petroleum. Further, the unsaponifiable portion of all active liver oils gives with pure trichloroacetic acid (in concentrated aqueous or light petroleum solution) a blue colour, identical in all respects with the blue colour in the AsCl_3 or SbCl_3 reactions, described by Rosenheim and Drummond [1925] and by Carr and Price [1926].

It is evident, therefore, that Fearon's reagents react in many oils with two entirely different chromogens, and this fact alone makes it impossible to use as a colorimetric standard a buffered solution of phenol red, as suggested by Fearon, or the mixture of dyes proposed by Willimott and Moore.

We overcame this difficulty by employing Lovibond's colorimeter, in which the amount of red colour present in a solution together with blue and yellow can be accurately measured and expressed in units of red standard tints. There is no need to describe in detail the arbitrarily selected conditions, under which we were able to obtain comparative colour values for a large number of liver oils and fish body oils. It is sufficient to mention that they varied in intensity from 0 to 80 red units in a 10 mm. layer, for 100 mg. of oil, contained in 5 cc. solvent. The results of parallel feeding tests on rats showed that the colour values bore no relationship whatever to the vitamin content of the oils tested. Two extreme cases only need be mentioned. The fat of a pig's liver gave no colour reaction with Fearon's reagents, whilst its growth promoting value in the animal test was found to be twice that of a standard cod-liver oil. A sardine oil gave as intense a Fearon's reaction as the standard cod-liver oil, without producing growth or having any effect on xerophthalmia¹.

SUMMARY.

1. Chemical and biological evidence is presented which shows that Fearon's reaction has no relation to vitamin A, for the estimation of which it was intended.

2. The suggestion is made that the chromogen of the reaction is associated with aldehydic oxidation products of unsaturated fatty acids, of the type of clupanodonic acid.

¹ We are informed by Prof. Drummond that he also found sardine oil devoid of vitamin A.

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