CXXXI. THE REACTION OF FISH-LIVER OILS WITH ANTIMONY TRICHLORIDE. (PRELIMINARY COMMUNICATION.)

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It is a well-known fact that fish-liver oils and other products containing vitamin A give a blue colour reaction with antimony trichloride, the intensity of which, within certain limits and under definite conditions regarding the concentration of the solutions, runs parallel with the biological activity of the oil. As yet the question whether the substance reacting with SbCl_3 is the vitamin itself or an accompanying compound has not been definitely settled.

The purpose of the present communication is to report some experiments bearing on this problem.

It is difficult to obtain the blue product resulting from the interaction of antimony trichloride and liver oil in quantity and condition suitable for examination. As is well known, the blue colour is exceedingly unstable. It reaches its maximum intensity some few seconds after the mixing of the solution of oil in dry chloroform with the reagent (a saturated solution of antimony trichloride in chloroform). After about 60 seconds the colour has already begun to fade, and it disappears completely in the course of the next 15 minutes.

A number of other dry, inorganic salts¹ were tried with no evident advantage. With concentrates of vitamin A, a blue or bluish colour was always obtained, but the colour was no more stable and often fainter than that produced by the antimony trichloride. This happened especially with salts only slightly soluble in chloroform. As is well known, the addition of water makes the colour disappear almost instantaneously; the formation of the blue colour is also prevented by acetone, ether, acetaldehyde and various alcohols. The stability of the blue colour is largely influenced by even small temperature changes, an increase of 10° above the ordinary room temperature makes the colour fade more quickly, while a corresponding fall in temperature has the opposite effect. An intense cooling increased the stability of the blue colour for a considerable time. At -35° it persisted for at least some hours, and after subsequent cooling to -50° a heavy blue oil was precipitated on the bottom of the vessel.

¹ AlCl_3 , AlBr_3 , AsCl_3 , BiCl_3 , FeCl_3 , SbBr_3 , SbCl_5 , SnCl_4 , SnBr_4 , TeCl_4 , TiCl_4 , ZnCl_2 , ZnBr_2

It was therefore decided to work at a low temperature, and then to decompose the blue oil produced according to the following hypothetical equation.

1. Chromogenic substance (Vitamin A?) + SbCl_3 = Blue product.
2. Blue product + H_2O = Chromogenic fraction + SbOCl + 2HCl .

Liver oil of halibut was chosen as a convenient raw material. It contains 50–100 times more vitamin A than cod-liver oil, and at the same time it is relatively poor in vitamin D. The saponification was carried out in the usual way, the oil being boiled with an excess of 5 % alcoholic KOH. The saponification was repeated until a sample of the product was practically free from glycerides. The product was next dissolved in methyl alcohol, cooled down to about -5° and the crystalline cholesterol filtered off. After evaporation of the methyl alcohol the residue consisted of a reddish-yellow oil, which showed an antimony trichloride value of 13,500 blue units (Lovibond)¹, and when tested on rats proved active in daily doses of 0.002–0.003 mg. All experiments were carried out in an atmosphere of nitrogen or carbon dioxide. The distillations were likewise performed in indifferent gases or *in vacuo* and at a low temperature.

Preparation of the blue product.

15 g. of the unsaponifiable fraction were dissolved in 600 cc. of dry chloroform, and the solution was at once cooled down to -35° in a cooling mixture of carbon dioxide, alcohol and glycol. About 1300 cc. of a saturated solution of SbCl_3 in dry chloroform were added under constant stirring, care being taken that the temperature did not exceed -30° . On subsequent cooling to -55° a heavy blue oil was precipitated, from which the faintly coloured chloroform solution could be decanted. To this oil pieces of ice or an ice-cold mixture of water and 10 % KOH (for the neutralisation of the possibly harmful hydrochloric acid) were added under constant stirring until the blue colour disappeared. Then the mixture was extracted with benzene. The benzene solution was filtered, washed with water and subsequently dried with sodium sulphate and the benzene distilled off *in vacuo*. The residue weighed 11.25 g. It was a highly unsaturated reddish-yellow oil, which we may call "the chromogenic fraction," as it still reacts with antimony trichloride. The antimony trichloride value is however only 4000–5000 blue units, *i.e.* about 1/3 of the colour value given by the original concentrate. In doses of 0.01–1 mg. per day it showed no biological effect (23 rats). On the addition of ethyl alcohol to the oil an amorphous yellow powder was precipitated, the blue value (SbCl_3) of which was variable, *viz.* 1700–8700 Lovibond units. The reason apparently was that the powder, when dried and exposed to the air, underwent a rapid transformation and became almost colourless. The freshly prepared product had an unsharp melting-point between 160° – 170° . It was easily soluble in benzene,

¹ Measured with the Rosenheim-Schuster colorimeter according to the method described by Rosenheim [1927], the highly active preparations being taken into consideration and the readings being made half a minute after the addition of the SbCl_3 solution.

chloroform and ether with an intense yellow colour. In daily doses of 0.002–0.02 mg. it was biologically inactive (24 rats). When the chromogenic fraction was treated with SbCl_3 at low temperature and the reaction product decomposed with water a chromogenic substance was again formed.

The non-chromogenic fraction.

On addition of cold light petroleum (-45°) to the faintly coloured chloroform solution mentioned above, the last traces of the blue product were precipitated. To the separated solution which was almost colourless, ice or cold aqueous KOH was added under constant stirring. The aqueous layer was removed in a separating funnel, the chloroform-light petroleum solution was dried with sodium sulphate and evaporated *in vacuo* on a water-bath. The residue, a faintly yellow oil, only gave a slight coloration with antimony trichloride and in daily doses of 0.05 and 0.4 mg. the biological effect was nil (6 rats).

The results of the biological experiments seem to justify the conclusion that the antimony trichloride reaction is not specific for vitamin A. This is in agreement with Euler and Euler [1930]. It was shown by these authors that a series of carotenoids devoid of any biological effect (vitamin A), gave a blue colour with antimony trichloride. In addition to its biological action, vitamin A, according to several investigators, is characterised by a broad, selective absorption in the ultra-violet region of the spectrum, with a definite maximum at 328 $m\mu$. It was accordingly desirable to make a spectrographic comparison of the chromogenic fraction and the liver oil concentrates of *Hippoglossus hippoglossus*. It was found that the exceedingly active preparations of the halibut-liver oil showed a very distinct absorption band in the ultra-violet range at 326–327 $m\mu$. This band was not observed in the chromogenic fraction. The characteristics of the absorption spectrum of this fraction seem to be the following.

A strong absorption in the ultra-violet from 390 $m\mu$ towards shorter wavelengths, with bands at 370, 351 and 300–290 $m\mu$. The absorption curves obtained with some of the preparations are presented in Fig. 1 and Fig. 2.

The absorption spectra given by the two blue products (SbCl_3 + concentrate, or chromogenic fraction) were compared both spectrographically and spectrophotometrically. As the spectrophotometric measurements in the Albrecht apparatus gave only approximate values, the more exact spectrographic data are alone reported. The spectrogram of the blue product, obtained from the concentrates, showed two absorption bands, at 622.5 (621.1) and 590 $m\mu$ respectively. The first was very sharp and its position easy to place, the other was less distinct and the wave-length in this case could hardly be determined with any degree of accuracy. In some preparations of the blue product the second band was localised at 575 $m\mu$. In contrast with the concentrate the chromogenic fraction is partially insoluble in alcohol. The blue product obtained from the chromogenic fraction exhibits two absorption bands, differing from those

seen in the blue product from the concentrate. In the case of the chromogenic fraction the maximum absorption is at 590 (586.8) $m\mu$ and 550–522 $m\mu$. Concerning these bands it is likewise to be noted that the first is the most

Fig. 1.

Absorption curves for the concentrate (I) and the "chromogenic fraction" (II).

The positions of the points marked • were determined photometrically, the others visually.

$$\epsilon = 1/d \log I_0/I.$$

I_0 = intensity of the incident light.
 I = intensity of the transmitted light.
 d = thickness of the cell in cm.

Fig. 2.

distinct. The positions of some of the absorption bands in the spectrograms mentioned above were determined both visually and photometrically. The values obtained with the photometer are given in parentheses.

The maxima of the most distinct bands can be localised with an accuracy of about $\pm 1\text{--}2\ m\mu$; as to the weaker bands the position cannot be determined with any great degree of accuracy owing to their lack of sharpness.

DISCUSSION.

The spectrographic results obtained with the vitamin A concentrates (of halibut-liver oil) are in close agreement with the findings of Morton, Heilbron and Thompson [1931], Heilbron, Gillam and Morton [1931], and Coward, Dyer, Morton and Gaddum [1931]. According to several investigators, the ultra-violet absorption with its maximum near 328 $m\mu$ has been observed in all the biologically active preparations examined, and seems to be characteristic for vitamin A. By treating the vitamin concentrates with SbCl_3 and adding water to the blue compound thus formed, the vitamin is rendered inactive.

The addition of water results in the formation of a yellow oil, which I have called the "chromogenic fraction." With antimony trichloride this fraction gives a strong blue colour, which cannot be attributed to vitamin A itself, but must be due to yet another chromogen. As mentioned by Heilbron, Gillam and Morton [1931] the concentrate of halibut-liver oil probably contains two chromogens.

One of these chromogens is supposed to cause the absorption at $328\text{ m}\mu$, and that at $583\text{ m}\mu$ of the blue product, while the other chromogen in the colour reaction is responsible for the absorption band at $620\text{ m}\mu$. The physico-chemical characteristics of vitamin A, according to these authors, are as follows: "a colourless or pale yellow substance exhibiting selective ultra-violet absorption free from fine structure and absorbing maximally at or near $328\text{ m}\mu$ and giving with antimony trichloride a coloured substance, characterised by a maximum in the yellow between 580 and $590\text{ m}\mu$, whilst the molecular extinction coefficients at 328 and 580 – $590\text{ m}\mu$ expressed in terms of vitamin concentration should be approximately equal."

Comparing my own results with the findings of Heilbron, Gillam and Morton [1931], it is striking that my highly active concentrates exhibit with antimony trichloride a decided absorption maximum at $622\text{ m}\mu$, while, on the other hand, the inactive preparation ("chromogenic fraction") in the colour test does not show this band, but exhibits a strong absorption in the neighbourhood of $590\text{ m}\mu$ and a fainter one at 551 in the green part of the spectrum.

While Heilbron, Gillam and Morton state that oxidation of certain liver oils, containing vitamin A, leads to an increase in the intensity of the absorption at 606 , or 620 for concentrates, I have observed that a destruction of vitamin A subsequent to treatment with antimony trichloride and water abolishes this band (622). On the other hand, the absorption at $590\text{ m}\mu$ is exceedingly pronounced. I must conclude therefore that an absorption band at $590\text{ m}\mu$ produced by the interaction of antimony trichloride and the "chromogenic fraction" cannot be attributed to vitamin A. It must however be taken into account that so powerful an influence on the inner structure of the substance, as a treatment with SbCl_3 and water must probably exert, may also show itself in a displacement of the absorption bands. From that point of view it is possible that the chromogenic nature of the concentrates after the treatment with SbCl_3 and water may be due to the decomposed vitamin.

SUMMARY.

A certain parallelism exists between the biological activity and the intensity of the blue colour given by liver oil concentrates of *Hippoglossus hippoglossus* with antimony trichloride.

At room temperature the blue colour thus formed is exceedingly evanescent, but at -40° the reaction-product is stable for hours.

Concentrates of halibut-liver oil probably contain two chromogens, of which one is possibly identical with vitamin A, and is destroyed by treatment with antimony trichloride and water, while the other, which presumably has no connection with growth in animals, still reacts with antimony trichloride forming a blue product from which the chromogen may be regenerated by the addition of water. The absorption bands of the liver oil concentrate and the "chromogenic fraction," together with the spectra of their reaction products with antimony trichloride, are described.

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