CXXXIII. A STUDY OF SOME BIOCHEMICAL COLOUR TESTS.

III. COLOUR REACTIONS ASSOCIATED WITH VITAMIN A.

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THE familiar observation that liver oils give a transient purple colour on treatment with sulphuric acid acquired a new significance when Drummond and Watson [1922] demonstrated the close relationship between the presence of this reaction and the distribution of vitamin A. These observations have been confirmed by Poulsson and Weidemann [1923] and by Sjorslev [1924], although the specificity of the test has not yet been directly established.

The following work was undertaken to ascertain as far as possible the connection between the vitamin and the pigment; to see if the test would afford assistance in the isolation of the vitamin; and to obtain a colorimetric method for determining the quantitative distribution of the vitamin.

Since strong sulphuric acid under varying conditions produces a variety of colours with liver oils, and as only one of these, namely, the deep violet shade, appears to be associated with the vitamin, the test as performed and interpreted by the previous writers will be referred to in the present paper as the *Drummond* test.

THE NATURE OF THE PIGMENT PRODUCED IN THE DRUMMOND TEST.

The usual method of carrying out the test is to dissolve a drop or two of the liver oil in about 5 cc. of an anhydrous fat solvent. Addition of a drop of concentrated sulphuric acid followed by gentle agitation produces a deep violet colour if the test be positive. This colour soon fades, or is replaced by a red or brown shade. The violet colour is the essential part of the test.

That the actual pigment produced is independent of the nature of the solvent is shown by the similarity of the colour spectrum obtained in different solvents, or, in the absence of a solvent, by the direct addition of a dehydrating agent to the oil.

The intensity and rate of colour formation varies in different solvents approximately in the following order of decreasing response: xylene, toluene, benzene, light petroleum, "liquid paraffin," chloroform, carbon tetrachloride,

carbon disulphide, ethyl ether. This was found to be due chiefly, but not entirely, to the greater water content of the less effective solvents. The colour test, like many sterol reactions, is extremely sensitive towards water.

In order to obtain standard conditions for carrying out the test the various factors concerned were investigated in turn.

Selection of a Solvent.

Light petroleum, B.P. 40–60°, was eventually selected as a solvent, owing to cheapness and other advantages which will appear later.

The light petroleum was dried by being shaken thoroughly with one-third of its volume of pure concentrated sulphuric acid, remaining in contact with the acid for some days. This process was repeated three times. It was then stored in a capped bottle, which had previously been dried in an air oven. It is essential for the success of the work that the solvent be free from water.

Selection of a Condensing Agent.

Strong sulphuric acid being obviously unsuitable, owing to the difficulty of controlling its action, other agents were tried including dry hydrochloric acid gas, trichloroacetic acid, zinc chloride and phosphorus pentoxide. With the last of these it was observed that the pigment formed did not go into solution in the body of the solvent but was adsorbed by the pentoxide and gradually subsided from the mixture as a dark purple precipitate; this suggested a method for the separation of the pigment.

The Phosphorus Pentoxide Test.

1. About a gram of phosphorus pentoxide is added to 5 cc. of the oil to be tested, either undiluted or dissolved in dry light petroleum, employing a well-dried tube. On gentle shaking, a deep violet colour develops if the oil be positive to the Drummond test. The colour slowly changes to a muddy brown depending on the amount of moisture originally present in the oil.

2. A drop of the oil to be examined is let fall on to the centre of a small heap of phosphorus pentoxide on a dry filter paper. If the test be positive a purple develops in a minute, and persists until the pentoxide has begun to deliquesce. Oils negative to the Drummond test give only yellowish colorations. The spectrum of the pigment suspension in dry benzene shows a well-marked band in the orange, 650–700 μ .

Better results are obtained when the pentoxide is added directly to the oil and not to dilute solutions of the oil in an appropriate solvent. This appears to be due to the necessity of oxidation for the development of the pigment. Ordinary specimens of liver oil are able to effect this oxidation if present in sufficient amount. The peroxide value of liver oils is often high; some specimens can replace effectively the oxidised turpentine or hydrogen peroxide as a source of oxygen in the guaiacum test.

When the pentoxide test is applied to a weak solution of the oil, the development of the colour may be hastened by the addition of a suitable oxidising agent such as dry benzoyl peroxide. This difficulty does not arise in the original liver oil test as the sulphuric acid is able both to condense and to oxidise.

The Isolation of the Pigment in the Phosphorus Pentoxide Test.

It appears probable that the pigment obtained by the action of phosphorus pentoxide is the same as the transient violet compound formed in the Drummond test, and in the more recent tests introduced by Rosenheim and Drummond [1925]. The pigment formed in the pentoxide test can be easily separated from the oil by the following method: 50 g. of phosphorus pentoxide powder are rapidly ground up with 50 cc. of an active liver oil. The deep violet colour which develops reaches a maximum in about seven minutes. The mixture is then transferred to a set of *dry* centrifuge tubes and rapidly centrifuged. The pentoxide and adsorbed pigment separate, leaving a clear supernatant layer. This layer is poured off and the pigment precipitate washed while in the tube by means of dry light petroleum, and again centrifuged. This procedure is continued until the washings no longer give the Liebermann-Burchard test for sterols. The precipitates are then suspended in the light petroleum and poured into a litre flask three-quarters full of water, the tubes of the centrifuge being rinsed out with light petroleum.

The pentoxide with the adsorbed pigment is immediately hydrolysed, and the components of the pigment can be extracted from the solution by means of light petroleum by filling up the flask so that the solvent layer rises into the neck, where it separates on standing for a couple of days.

Notes on the Preparation. 1. Since water immediately decomposes the pigment great care must be taken throughout the preparation to exclude moisture. All the apparatus in contact with the pigment must be dried beforehand in an air-oven and stored in a desiccator. The presence of moisture is shown by the change in colour of the precipitate from purple to muddy brown.

2. Whitby's delicate test for sterols [1923] cannot be used in testing the washings, as the formaldehyde interacts with some unsaturated constituent of the light petroleum and obscures the test.

3. Unless the reagents are free from moisture it is not possible to wash the pigment precipitate free from sterol, since a moist solvent is able to decompose the pigment with the liberation of a substance capable of giving the general sterol reactions.

4. With a rapid centrifuge it does not appear necessary to seal the tubes, provided that the necks be kept quite free from any splashes of pentoxide.

5. Light petroleum is a particularly convenient solvent for washing the pigment precipitates since it can be separated very rapidly from the heavy

precipitate, and since it does not tend to form stable emulsions with the pigment components when added to water, as happens with the aromatic solvents, notably benzene.

The Character of the Pigment Constituents after Hydrolysis.

After evaporation of the light petroleum solvent the pigment constituents are left as a pale yellow oil. This oil gives the general sterol colour tests but does not give the Drummond test, nor the colour reaction with phosphorus pentoxide, nor the various polyphenol colour reactions described later in the paper.

The oil showed no signs of crystal formation on being kept for some weeks *in vacuo* at low temperature.

Before attempting any detailed chemical investigation of the residue, its growth-promoting effect was examined according to the method of Zilva and Miura [1921], the actual dietary having been previously tested in connection with other work in this laboratory [Pringle, 1925].

Six rats of comparable age and weight (25–55 g.) were employed (Fig. 1). The three controls were fed on the standard deficient dietary, with 20 mg. of cod-liver oil per rat per diem supplied by hand as the source of vitamin A. The second set of rats was fed on the same diet except that the pigment extract from an equivalent amount of oil was given instead of the oil. These rats had been on the deficient diet for three weeks previous to the experiment. Apart from an initial rise, the pigment extract showed no growth-promoting properties during a period of 20 days, after which the daily dose was increased to 0.1 g. with no appreciable result. This indicates either that the pigment is not intimately associated with the vitamin or that the process of condensation and subsequent hydrolysis changes the biochemical character of the material.

The latter alternative is supported by the fact that the final preparations no longer give the characteristic colour reactions.

THE EFFECT OF THE REMOVAL OF THE PIGMENT ON THE GROWTH-PROMOTING PROPERTIES OF COD-LIVER OIL.

Samples of cod-liver oil were treated with phosphorus pentoxide and centrifuged in the manner described until they no longer gave any colour with the reagent. The oils were then freed from excess of pentoxide by sedimentation during a period of one week.

These depigmented oils gave neither the Drummond test nor any of the colour reactions described in the present paper. The depigmented oil was administered in quantities of 20 mg. per rat per diem to each of the three rats which had been on the diet deficient in vitamin A supplemented by the pigment extract in the previous experiment, the pigment extract being discontinued during the administration of the depigmented oil.

No growth-promoting effect could be observed during the period when the depigmented oil was given, which supports the conclusion that the vitamin is a constituent of the pigment precursor. The potency of the oil used was shown by the control experiments.

Fig 1.

A—B = Pigment equivalent of 20 mg. oil per rat p.d.
 B—C = " " " 100
 C—D = De-pigmented oil, 20 mg. per rat p.d.

COLOUR REACTIONS SUITABLE FOR THE COLORIMETRIC ESTIMATION OF VITAMIN A.

If the pigment occurring in the Drummond and the phosphorus pentoxide tests be produced by a condensation between the vitamin and a sterol already present in the oil it should be possible to modify the test by replacing either component.

This has been done by Harden and Robison [1923], who have shown that small quantities of furfuraldehyde added to solutions of cholesterol, or to inactive oils containing cholesterol, react on treatment with strong sulphuric acid in a manner resembling that of liver oils.

The reaction appears to be similar in character to that devised by Whitby [1923] for the detection of sterols.

It has been suggested that the vitamin responsible for the colour test may itself be an aldehyde, but Drummond has pointed out that this is unlikely on account of the stability of the vitamin to the process of saponification, although it must be remembered that the vitamin may resemble the non-reducing disaccharides in being relatively stable in alkaline solution but readily undergoing decomposition in acid solution with the production of aldehydes.

The converse of the test does not yet appear to have been investigated; that is to say, the effect of the addition of sterols and polyphenols to solutions containing vitamin A before treatment with the condensing agent.

No difference was observed when excess of cholesterol was dissolved in a liver oil preparatory to carrying out the vitamin colour tests, presumably because the quantity of sterol naturally present was sufficient to give the maximum colour.

The effect of various polyphenols was then examined in the hope that they might interact with the vitamin to produce soluble pigments similar to those obtained by condensing pyrogallol with various aldehydes [Fearon, 1919].

The Condensing Agent.

The condensing agent must be of sufficient strength to bring about the reaction, without being in such concentration that it is able to produce a colour with the oil in the absence of the phenol. The condensing agent must also be able to hold the pigment in solution.

An entirely satisfactory result was obtained by employing a 12 % solution of dry trichloroacetic acid in dry light petroleum (B.P. 40–60°). This can conveniently be prepared by diluting a saturated solution of dry trichloroacetic acid in dry light petroleum (approximately 49 % at laboratory temperature) with three times its volume of light petroleum. Both the stock solution and the reagent should remain perfectly clear. They appear to keep indefinitely in the absence of moisture.

Pyrogallol, phloroglucinol, α -naphthol, and substituted phenols, such as vanillin and amidol, all give definite colours with liver oils in presence of the condensing agent.

Pyrogallol is probably the most suitable. It gives a bright rose colour with active oils. The colour is stable for a long period, and can be diluted with light petroleum for colorimetric comparison. It can be closely matched by a buffer solution containing phenol red.

The Pyrogallol Test for Vitamin A.

One or two drops of the oil, or dry ethereal solution of the substance to be examined, are placed in a dry 10 or 25 cc. flask. A few dry crystals of pyrogallol are added, and then 5 cc. of the condensing agent (12 % trichloroacetic acid in dry light petroleum). The contents are well shaken. A bluish pink colour slowly develops if the test be positive, changing to deep rose, and reaching its maximum intensity in 15 to 20 minutes.

Notes on the Test. 1. The rate of the development of the colour may be hastened greatly by warming the flask for a few seconds in a boiling water-bath. When the light petroleum begins to boil vigorously the flask should be removed, otherwise the liquid is apt to become cloudy.

2. Oxidation is necessary for the production of the colour. This is brought about partly by shaking the flask, and partly by peroxides already present in the oil. Some oils are very slow in producing a colour. This can be corrected by the addition of a very small quantity of dry benzoyl peroxide before

warming the solution. Excess of the peroxide must be avoided in colorimetric work, as it gives a yellow shade with the reagent on standing for some time.

3. As it is difficult to find a stable anhydrous solvent for pyrogallol which will not interact with the other reagents the pyrogallol is added in the solid form. Since it is only sparingly soluble in the acid light petroleum, the mixture must be well shaken at intervals.

4. Once formed, the colour is stable and will remain for days if the flask be properly closed. It can be diluted with dry light petroleum or with the condensing agent.

5. When the colour has developed, the contents of the flask should be perfectly clear, except for the undissolved crystals of pyrogallol at the bottom.

If the liquid is opalescent it indicates moisture. This sometimes may be removed by adding more of the condensing agent, shaking the flask and allowing it to stand for some time.

The Specificity of the Test.

In distribution and intensity, the pyrogallol test closely follows the Drummond and the phosphorus pentoxide tests. Oils inactivated by aeration, inactive vegetable oils, and hardened fats give no colour on standing in contact with the reagents for days, or, at most, a muddy yellow colour if an excess of the oil be taken.

It has not been found possible to reproduce the reaction by the addition of the commoner aldehydes and ketones, with and without cholesterol, to (1) medicinal petroleum, (2) aerated liver oil, (3) vegetable oil. The reaction with benzaldehyde most nearly resembles that of an oil containing vitamin A. The colour, however, is orange-red and soon fades.

Owing to the lack of success in the attempt to obtain a biologically active preparation from the pigment separated by the phosphorus pentoxide method no effort was made to isolate the pigment formed in the pyrogallol test.

It is of interest to note that the pyrogallol test is not given by "ostelin," "oxy-cholesterol," radiated cholesterol, or radiated hardened fat, which may be of significance in the differentiation between the fat-soluble vitamins.

CONCLUSIONS.

1. Phosphorus pentoxide forms a deep violet colour on addition to oils containing vitamin A.

2. The pigment may be completely separated from the oil leaving a residue deficient in growth-promoting properties.

3. The vitamin enters into the formation of the pigment, probably by condensation with a sterol.

4. It has not been found possible to recover the vitamin from the products of hydrolysis of the pigment.

5. Using a 12 % solution of trichloroacetic acid in dry light petroleum as a condensing agent, pyrogallol and other polyphenols interact with oils containing vitamin A to give stable pigments which are suitable for colorimetry.

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