CLXXIV. A NEW COLORIMETER BASED ON THE LOVIBOND COLOUR SYSTEM, AND ITS APPLICATION TO THE TESTING OF COD-LIVER OIL, AND OTHER PURPOSES.

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THE need for the construction of an apparatus of the type described in this communication¹ arose during the course of work on the colorimetric estimation, by means of the arsenic—or antimony—trichloride reaction, of the chromogen contained in cod-liver oil and other fats, which is assumed to be identical with vitamin A [Rosenheim and Drummond, 1925; Carr and Price, 1926]. The difficulties which had to be overcome before this test could be applied quantitatively were twofold and were due (1) to the characteristic instability and transient nature of the brilliant blue colour developed in the test, and (2) to the variable tints of blue (*i.e.* greenish or reddish) produced and determined by the uncontrollable and variable admixture of yellow or red pigments in the oils to be tested. The formation of these adventitious colours excluded the employment of any one fixed standard for comparison, as well as the use of a colorimeter of the usual Duboscq type. Lovibond's Tintometer, on the other hand, enables a perfect matching of any mixture of colours to be made, but the apparatus in its present form is hardly suitable for the extremely rapid work essential in the above test. Considerable experience is required in the expeditious selection of the suitable standard glasses, which have to be inserted into the apparatus singly and exchanged by hand if unsuitable. This drawback has been overcome in the model illustrated (see Fig. 1) by arranging the standard glasses in frames containing ten each of the red, yellow and blue units, in such a way that they can be rapidly moved horizontally behind a window and below the cell (or test-tube) containing the coloured solution to be matched. A mounting of the standard glasses on discs was considered in the first instance. The horizontal arrangement, in preference to a vertical one, was chosen for technical reasons in discussion with Dr W. V. Thorpe, to whom I am also indebted for the construction of a small working model. The apparatus now described, which is entirely different in general arrangement and mechanical details from Dr Thorpe's model, was designed and constructed by Dr E. Schuster, who has kindly furnished the description of it given in this paper.

¹ The Tintometer, Ltd., Salisbury, have undertaken the manufacture of the apparatus, all rights in it having been put freely at their disposal.

The apparatus has proved itself not only eminently suitable for the purpose for which it was designed, but possesses certain advantages, which seem to justify a recommendation for its use in colorimetric work generally and in biochemistry in particular. Estimations made with it are free from the error inherent in those obtained by the use of Duboscq's colorimeter, or any of its modifications, in which two coloured solutions of different concentration are

Fig. 1.

compared in layers of different thicknesses. This error, which may in extreme cases amount to as much as 25 %, is discussed fully by Stanford [1913, 1923]. Further, a well-known difficulty in biochemical colorimetric work, which cannot be overcome in the usual type of colorimeter, is due to the difference in tint between the colours given by the "unknown" and by the standard prepared from a pure substance respectively. The formation of adventitious

colours due to constituents, other than those to be determined, occurring in physiological fluids or extracts of organs is unavoidable. In the new apparatus the variable accidental colour admixtures can be easily matched, but only the essential colour value of the reaction itself is taken for the purpose of calculation (see example later). As a final advantage may be claimed the saving in time in preparing, as is usually necessary, a fresh standard solution for comparison. This is replaced by Lovibond's colour units, which are reproducible and permanent. The practical advantages of this colour system are recognised in industrial chemistry, where it has been used extensively since its introduction forty years ago [Lovibond, 1887], but references to its application are scanty in biochemical literature [Palmer and Kempster, 1919; Calvert, 1923; Baker and Marrian, 1927]. The accuracy of the colour units may be controlled spectrophotometrically [Gibson, Harris and Priest, 1927].

Although all the evidence so far available [Rosenheim and Webster, 1926, 1927; Willimott and Wokes, 1927; Accessory Food Factors Committee, 1927] is in favour of the specificity for vitamin A of the AsCl_3 (or SbCl_3) colour reaction, it seems advisable to await still further confirmation before recommending the colorimetric test for general use as a reliable substitute for the animal experiment. This caution seems to be the more necessary, in that the ease with which estimations can be carried out with the new colorimeter might encourage manufacturers, who have no facilities for controlling the results biologically, to rely on the colorimetric test exclusively.

In order to correlate the colorimetric with the biological test, it was decided after considerable experimental work to take 20 mg. of cod-liver oil as the standard amount for analysis. It was found that the daily administration of this quantity of a good average cod-liver oil, which served as a standard, was the minimum amount required to restore normal growth, *i.e.* to induce a weekly increase of 8–10 g. in rats kept on a diet free from vitamin A. The same amount of this oil equalled 10 blue standard units when mixed with 1 cc. of the reagent, and measured in a layer of 10 mm. thickness. As a preliminary indication, therefore, of the value of an unknown oil in vitamin A, it may be taken that 10 blue standard units correspond to one "rat unit" of vitamin A when the colorimetric comparison is made under the indicated conditions.

The following procedure, the outcome of two years' experience, is recommended for carrying out the test.

(a) *Arsenic chloride test.* The oil is measured by means of a Wright's capillary micro-pipette [Wright and Colebrook, 1921], graduated with mercury to hold 25 mm.³ and delivering 20 mg. oil into a test-tube of clear white glass of 10 mm. internal diameter. 1 cc. of the reagent (pure AsCl_3) is delivered from a standard 10 cc. burette into the test-tube and a reading taken in the colorimeter immediately (time limit 30 seconds). The results are expressed in standard units of blue. Five consecutive tests are made in each case, differing on the average by ± 0.10 unit.

(b) *Antimony chloride test.* By means of a standard pipette 1 cc. of the oil is measured into a measuring flask of 5 cc. capacity. The pipette is rinsed out three times with chloroform, and the solution made up to volume with the same solvent. In order to make the results comparable with the AsCl_3 and the biological test, 0.2 cc. (= 40 mm.³ oil) of the solution is measured into a test-tube (10 mm. diameter) and mixed with 2 cc. of a 30 % antimony chloride solution in chloroform, delivered from a standard burette. Readings are taken immediately as above, but, owing to the slightly increased stability of the SbCl_3 reaction, a longer time limit is generally permissible, readings taken within 1 minute showing no greater difference than ± 0.10 unit as the average of five consecutive estimations. Results obtained on the same oils by different observers at different times agreed within ± 0.50 unit, showing the usual variations due to differences in colour perception of the observers.

Identical values are obtained by the use of either AsCl_3 or SbCl_3 as a reagent for the colour production. The latter reagent is conveniently prepared by dissolving the required amount of antimony trichloride (B.D.H.) in ordinary B.P. chloroform by rapidly warming to about 50° on a water-bath. A perfectly clear solution results, which keeps unchanged when stored in well-stoppered bottles. A stock solution, prepared 12 months ago and kept in a clear glass bottle under ordinary laboratory conditions, gave constant results when tested at intervals on the same oil. Since such a solution is more convenient in use than undiluted AsCl_3 , it is preferable for general work, except in the rare cases where an extremely low result requires confirmation by the AsCl_3 reagent [Rosenheim and Webster, 1927; see also Wilson, 1927]. This lack of sensitiveness in the antimony reagent is clearly due to the dilution with chloroform of both the oil and the reagent. The explanation suggested by Wokes and Willimott [1927, 1] that a separation of the chloroform solution into two layers may have been the cause of the discrepancy observed is unwarranted, since such has never occurred in our experience when the solution is prepared and kept as above described. It might be pointed out in view of the attempts to increase the accuracy of the reaction [Wokes and Willimott, 1927, 2] that this need is not apparent, since the error of the colorimetric test is out of all proportion smaller than that of the biological animal experiment, which may be as much as 40 % with the technique at present in use.

Further applications. In applying the instrument to any of the colorimetric methods in use, it is only necessary to construct a reference curve by plotting on the ordinate the number of colour units, determined by the estimation of the colour value of solutions containing known amounts of the substance to be analysed, the latter being plotted on the abscissa. As an illustration may be given the curve (Fig. 2) obtained for cystine by the use of the phosphotungstic reagent of Folin and Looney [1922]. I am indebted to Mr W. K. Anslow for the construction of this curve.

The accuracy of the method is proved by the analysis of a solution containing 0.19 % cystine, the amount being unknown to the observer. The result

was 0.19 % by the colorimetric method and by calculation from the sulphur content.

It may further be suggested that the apparatus might be easily adapted in routine work to colorimetric p_H determinations, the colour values of various indicators expressed in Lovibond's standard units, covering a p_H range of 1.2-9.0, being available [Taub, 1927].

Fig. 2.

ADDENDUM. BY EDGAR SCHUSTER.

The apparatus consists of a wooden box, on the front of which is mounted a metal plate, painted a dead black. This plate with some of the surrounding woodwork is shown in the photograph (Fig. 1). Near its upper edge is a circular recess in which are two windows. A blackened tube with an eye-piece (not shown) fits this recess and serves to exclude extraneous light. The upper window is for viewing the coloured solution to be tested, and the lower one for the combination of coloured glasses with which its colour is matched. The liquid to be tested is contained in a standard test-tube (or cell) which is supported in a mounting so that its lower part comes between the upper window and the source of light. The top of the test-tube may be seen in the photograph. Behind the lower window are seven horizontal frames supported in a rack as closely as possible one behind the other. In the first frame are nine glasses, which, in combination with a blank space in the frame, provide a series of ten degrees of blueness differing by whole units: *i.e.* 0, 1, 2, 3, 4, 5, 6, 7, 8, 9. In the second frame the glasses form a scale of blueness rising by increments of one-tenth of a unit from 0 to 0.9. The third and fourth frames give units

and tenths of units respectively of yellow, and the fifth and sixth frames, of red. The seventh frame holds a blank space and three glasses, each having a tint of 10 units, and being blue, yellow and red respectively. Lateral movement of the frames is controlled by the seven knobs, which can be slid to and fro in the seven slots seen in the photograph between the circular recess and the bottom of the plate. The two upper knobs are coloured blue, and control the first and second frames. Between them is a scale, by which the tint of the glasses appearing behind the window may be read. As shown in the photograph the upper knob is opposite the 1 on this scale, and the lower knob, the 2. This means that the glasses shown are the 1 unit blue and the 0.2 unit blue, giving in combination 1.2 units of blueness. The next two knobs are coloured yellow and control the frames containing the yellow glasses, and the fifth and sixth knobs are coloured red and operate the red glasses. Scales similar to that between the two blue knobs are fitted between the two yellow and the two red. The lowest knob is black and moves the seventh frame; whether one of the three glasses or the blank space is showing is indicated by the row of coloured discs—white, blue, yellow or red which are fixed above the slot.

The mechanical arrangement by which the knobs control the movement of the frames is as follows: each knob is screwed to the middle of a long strip of steel, lying in the inside of the apparatus and supported horizontally by two guides carried on vertical steel bars. A descending member of the correct length is fixed to each end of each frame and its lower end is connected with the corresponding horizontal strip by a suitable distance piece. It is obvious that the number of frames and of standard glasses carried in each may be modified to suit special requirements.

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