at 620 m μ . Under our conditions, 50 μ g. of glucose with 40 μ g. of DL-tryptophan gives a colour density of about 95 % of that of the sugar alone.

SUMMARY

1. The anthrone reaction has been investigated with a range of naturally occurring hexoses and pentoses.

2. The course of colour production varies widely with different sugars, and the test is much less sensitive for pentoses than for hexoses.

3. Sugar mixtures give results agreeing closely with those expected from the colour production of the individual sugars. Under the conditions used here there is no evidence of serious interaction of hexoses and pentoses.

4. For the estimation of soluble sugars in plant extracts the method yields results comparable with those obtained with the copper reagent, but includes the sugars of stable glycosides which may constitute a large proportion of the soluble carbohydrates in some plant tissues. Used in conjunction with chromatographic identification, the anthrone method is of particular value when limited quantities of tissue are available.

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Separation of the Bile Pigments of Serum, Bile and Urine

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Van den Bergh & Müller (1916) described two types of diazo reaction for bile pigment in serum. The sera of normal persons, and of patients with haemolytic jaundice, gave a red colour with diazotized sulphanilic acid only in the presence of ethanol. This was called an 'indirect' reaction, and was distinguished from the 'direct' reaction of bile, or sera from patients with obstructive jaundice, for which ethanol was unnecessary. The direct- and indirect-reacting components of serum were separated chromatographically by Cole & Lathe (1953). While the indirect-reacting pigment appeared to be bilirubin, it was suggested that the direct-reacting fraction was a mixture of two pigments. This has now been confirmed by separating these pigments on reverse-phase chromatograms. These pigments have also been found in human necropsy bile, and in the urine of patients with obstructive jaundice.

METHODS

Extraction of pigments. Pigments were prepared from the sera of jaundiced patients by adding, with stirring, to 1 vol. of serum, 0.18 vol. of saturated $(\text{NH}_4)_2\text{SO}_4$ and 2.5 vol. of ethanol. After standing for 30 min. the precipitate was removed by centrifuging. The supernatant was stored at -12° and was used as soon as possible, since the polar pigments were found to be altered after storing for several days at this temperature. Before use the supernatant was taken to dryness *in vacuo* at $<40^\circ$.

The pigments were separated from specimens of bile by the addition of 70 g. of $(\text{NH}_4)_2\text{SO}_4$ /100 ml. of bile. After vigorous mechanical stirring, the mixture was centrifuged and the resulting pellet of protein, pigment, etc., was lifted from the surface of the solution. It was stored in this form at -70° . Pigments and lipids were separated from the protein and salt of the pellet by grinding it with *n*-butanol:methanol (1:1, v/v) and re-extracting until an almost colourless solution was obtained. This procedure was then

repeated several times with 50% aqueous methanol until the extracting fluids became turbid. The residue, which was still pigmented, was discarded. The extracts were taken to dryness *in vacuo* at a temperature of $<40^{\circ}$.

The urine of patients with obstructive jaundice (24 hr. samples) was collected over CHCl_3 , at room temperature. The urine, while being stirred mechanically, was saturated with $(\text{NH}_4)_2\text{SO}_4$ (700 g./l.); during stirring, *n*-butanol was added to a final concentration of 1–2%. The turbid solution was then transferred to a separating funnel where the pigmented precipitate rose to the surface. After standing for about 1 hr. most of the lower liquid was drained off, the remainder was centrifuged, and the relatively compact pellet was removed. If a small amount of deeply coloured butanol separated it was taken off with the pellet. The whole was kept at -70° until required. The pigments were extracted from the urine deposit in the same way as from bile concentrates.

Chromatography. Reverse-phase chromatograms were set up in 16 mm. (internal diameter) glass tubes using silicone-treated kieselguhr (prepared with Silicone Fluid M441 of Imperial Chemical Industries Ltd.) as described by Howard & Martin (1950). The following solvent systems were used: *A*— CCl_4 , 25 vol.; CHCl_3 , 25 vol.; CH_3OH , 38 vol.; 0.025M phosphate buffer pH 6, 12 vol. *B*—*n*-butanol, 50 vol.; 0.005M phosphate buffer pH 6, 50 vol. *C*—acetone, 25.9 ml.; $(\text{NH}_4)_2\text{SO}_4$, 17.0 g.; 0.05M phosphate buffer, pH 6, 62.5 ml. (sufficient 2.5N-NaOH, about 1.7 ml., was added to restore the pH to 6). Unless stated otherwise, all buffers were prepared as described by Cole (1933).

Columns with solvents *A* and *B* were made with 6 g. of kieselguhr and 3 ml. of stationary phase, while for system *C* half these amounts were used. All columns were run at room temperature (18 – 25°). The solvent was eluted from columns at a rate of 1 ml./3 min. (except for solvent *C*, 1 ml./10 min.). The depth of colour in the eluates was read in the microcell of a Spekker absorptiometer using Ilford filters no. 601 (maximum transmission about 430 $\text{m}\mu$).

Diazotization. The reagent was prepared by mixing 10 ml. of 0.1% (w/v) sulphilic acid in 0.25N-HCl with 0.3 ml. of 0.5% (w/v) NaNO_2 . Half a vol. of this solution was added to 1 vol. of the solution to be diazotized, followed by 1.5 or 4.5 vol. of ethanol, depending on the anticipated depth of colour. Extinctions were read in a 10 mm. cell using an Ilford green filter (no. 404 with maximal transmission at about 530 $\text{m}\mu$). Determination of the 'directness' of the diazotization was made on the aqueous layer after removal of *n*-butanol from the eluates by shaking them with 8 to 10 vol. of light petroleum.

For absorption curves of the azo compounds of pigments obtained from chromatographic columns, 0.2 ml. of diazo reagent was added to 3 ml. of the eluate, together with 0.3 ml. 3.6% $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ and ethanol (1.0 and 0.5 ml.) for columns using solvent systems *A* and *B* respectively. The rate of colour development under these conditions was observed and the absorption curves were plotted when the colour was maximal, using a Beckman quartz spectrophotometer.

Ultraviolet and visible absorption. A Unicam quartz spectrophotometer was used, with 10 mm. quartz cells. Turbidity was prevented in eluates from butanol columns by the addition of 0.2 ml. ethanol to 4 ml. of eluate. Readings were made generally at intervals of 10 $\text{m}\mu$. (2 $\text{m}\mu$. near peaks).

Solvents. *n*-Butanol, 'Laboratory Grade' and AnalaR grades of all other solvents were used without further purification.

Standards. A number of commercial samples of bilirubin were examined chromatographically with solvent system *A*. In each case one band of pigment predominated, though the amount of other pigments varied from specimen to specimen. Bilirubin from British Drug Houses Ltd., which contained only small amounts of these other pigments, was used as a standard in this work, and only the main pigment band on the column has been used for absorption studies.

RESULTS

Chromatographic separation of direct-reacting pigments. Cole & Lathe (1953) used solvent system *A*, on water-repellent kieselguhr, for the chromatographic separation of the bile pigments of serum into two components. In this system the polar materials sometimes appeared as two bands but these were always eluted together. For convenience these two polar pigments are called pigment I (the less polar) and pigment II (the more polar).

The polar pigments I and II can be separated on silicone-treated kieselguhr, using solvent mixture *B*. In Fig. 1, a chromatogram of serum from a case of hepatitis shows that in this system the more polar pigment II moved well ahead of the less polar, pigment I, which had an *R* value of about 0.3. Bilirubin and biliverdin had *R* values of 0 and 0.15, respectively, in this system.

The rate of movement of pigment II on this column was rather variable, possibly due to temperature changes, and usually it moved close to the solvent front (*R* value 0.7–0.95). Several more polar systems were tried in an endeavour to retard the pigment. Of these systems *C* was the most satisfactory, although the *R* value of pigment II was still rather variable, and tailing was apparent.

Fig. 1. Elution of polar pigments of icteric serum from chromatogram (solvent system *B*), showing depth of colour (●—●) and concentration of diazo-reacting material (×—×). Details in text, this page.

Absorption curves of serum pigments. The spectral absorption curves of serum pigments, and of a bilirubin standard, were determined in the solvent as they were eluted from the chromatography columns. In Fig. 2 (solvent system *A*) bilirubin is compared with the non-polar pigment obtained from the serum of a patient with haemolytic jaundice, and with a band of polar pigments from the serum of a patient with obstructive jaundice. Bilirubin and the non-polar pigment of serum share the same peak at 454 $m\mu$., while the curve of the polar pigments has a peak at 419 $m\mu$. A part of the ultraviolet absorption of this latter curve was due to contaminants, since it could be demonstrated in eluates prepared in an identical way from normal unpigmented serum. Contaminants may also have made a minor contribution to the ultraviolet absorption of the non-polar serum pigment.

The absorption curves of pigments I and II were compared after elution from a column using solvent system *B* (Fig. 3). The absorption curve of bilirubin, which is included here, was not obtained as an eluate, since bilirubin does not move on this column. The bilirubin (from a specimen known to be pure by its chromatographic behaviour) was dissolved in the dilute alkaline component of the pH 6 buffer of system *B*, together with the amount of *n*-butanol necessary to saturate it, and then the acid component of the buffer was added. The absorption maxima of bilirubin, pigment I, and pigment II were at 450, 452 and 448 $m\mu$. respectively. Absorption curves were also obtained from chromato-

graphed extracts of normal unpigmented serum. Those eluates which corresponded with pigment I had negligible ultraviolet absorption, while the more polar eluates, comparable with pigment II, showed considerable ultraviolet absorption.

Diazotization of pigments. The non-polar pigment of serum was known previously to give no diazo reaction in the absence of ethanol. In the present study, both pigments I and II gave an immediate, 'direct', reaction when diazotized in the absence of ethanol. The rate of colour development in the presence of ethanol was the same for all three pigments, as was the relationship between the amount of yellow colour and the amount of azo compound formed. The products of diazotization of all these pigments had maximal absorption at 526 $m\mu$., at pH 4. The shape of the absorption curves was similar above 450 $m\mu$., and changes in pH resulted only in a shift of the peak. Below 450 $m\mu$. absorption depended partly on the amount of original reactants present.

Pigments in bile and urine. The extracts of pigment from the urines of a number of patients with obstructive jaundice have been examined chromatographically. Some pigments I and II and small quantities of bilirubin were found. Pigment I was the more abundant of the polar pigments. Frequently there were larger amounts of a red-brown pigment, running slightly faster than pigment I on the column using solvent *B*.

Bile extracts had substantially larger proportions of the polar bile pigments than had urine extracts, and pigment II was more abundant than pigment I.

Fig. 2. Comparative absorption spectra of bilirubin (○- - -○), the non-polar pigment of icteric (haemolytic) serum (×- - ×), and the polar pigments (□- - -□) from icteric (obstructive) serum. The extinctions shown for the non-polar and polar pigments have no strict quantitative significance and are only of a comparative character.

Fig. 3. Comparative absorption spectra of pigment I (●- - ●) and pigment II (×- - ×), as eluted from a chromatogram of solvent system *B*, and of bilirubin (○- - -○) dissolved in the polar phase of solvent system *B*. For significance of the extinctions of pigments I and II see Fig. 2.

Bilirubin accounted for only a small proportion of the material giving a positive diazo reaction. Moreover, there were other pigments than bilirubin, pigments I and II, which gave this reaction.

DISCUSSION

Cole & Lathe (1953) suggested that the non-polar pigment of serum was bilirubin, basing this on the identical behaviour of these two substances on the chromatogram and in the direct and indirect diazo reactions. Additional evidence is now provided by a comparison of their adsorption curves (Fig. 2). The main difference is in the ultraviolet range. However, the increased ultraviolet absorption shown by eluates containing the non-polar serum pigment is attributable to other materials which are present in normal serum, and which are eluted from the column in the same position as bilirubin. This feature of the ultraviolet absorption has also been noted by Claireaux, Cole & Lathe (1953) in similar fractions of jaundiced brain. Throughout the visible range, on the other hand, the absorption curves of the non-polar serum pigment and of bilirubin are almost identical. Thus, although not entirely conclusive, all the evidence points to bilirubin as the non-polar pigment of serum.

No specimen of mesobilirubin has been available for comparison with the serum pigments. However, its maximal absorption (in chloroform), 25 $m\mu$, closer to the ultraviolet, distinguishes it from bilirubin (Heilmeyer, 1943), and therefore from the non-polar serum pigment. On the other hand, mesobilirubin is unlikely to be one of the polar pigments, since Feruglio & Salvini (1947) have reported that mesobilirubin is indistinguishable from bilirubin in the diazo reaction. In its chromatographic behaviour mesobilirubin would be expected to be less, rather than more, polar than bilirubin, because it lacks two double bonds in the β substituents, and is known to be more soluble in chloroform than is bilirubin. In addition, mesobilirubin is more stable than the polar pigments. It seems probable then that mesobilirubin is not one of the serum bile pigments.

The component of serum bile pigment which reacts directly in the diazo test, and is prominent in the sera of patients with obstructive jaundice, can be separated into two pigments (I and II), both of which react directly. Considerable variations in the relative proportions of these two polar pigments have been noticed.

The two polar pigments are labile and have not been isolated in pure form. However, following chromatography they appear to be relatively free from other materials which absorb in the visible range. The spectral curves of pigments I and II, and bilirubin are all similar, though distinctive features are noticed. Differences between the spectra of

bilirubin and the polar pigments are noted in the polar phase of solvent A (Fig. 2), the peak of the polar pigments being 25 $m\mu$, towards the ultraviolet. The ultraviolet peak, in the region of 275 $m\mu$, seems to be most prominent in pigment II, in solvent B. This absorption peak is found in the corresponding eluates of normal serum, and may be due to contaminants. Although these materials are impure it seems probable from the similarity of their absorption curves, and of those of their diazo compounds, that they have the same tetrapyrrole structure as bilirubin. Whatever changes occur when the liver transforms bilirubin into pigments I and II, it does not greatly affect the chromogenic structure.

It has been known for a long time (van den Bergh, 1918; Andrewes, 1924) that direct-reacting sera or urines become indirect-reacting, like bilirubin, if they are allowed to stand at room temperature. It has been our experience that even relatively mild treatment of the polar pigments is likely to lead to the appearance of a band of bilirubin in a subsequent chromatogram. Moreover, a transformation of pigment I into pigment II and bilirubin can be produced by warming and evaporation. The reverse reactions have not been observed, and it has not been possible to change bilirubin into the polar pigments in experiments *in vitro*. Until more is known about their interrelation it is not possible to state how much of pigment I or pigment II may be produced from the other during manipulation. It is certain that bilirubin will be observed as an artifact after rigorous treatment of bile, and of some urines and sera.

It has been stated frequently that the main pigment of human bile is bilirubin. This now requires qualification. In earlier studies the preliminary treatment of the bile was probably sufficient to produce bilirubin from the polar pigments. The procedures outlined here preserve substantial amounts of these two pigments, and bilirubin occurs in only minor amounts. Other bile pigments have also been observed, and some of them gave a diazo reaction. Only three naturally occurring pigments have previously been reported to give the diazo reaction, namely, bilirubin, mesobilirubin and mesobilene- α or dihydromesobilirubin (Siedel, 1951). It seems probable that this series could be extended, or, alternatively, that in bile there occur a number of unstable pigments which are transformed to those giving a positive reaction.

SUMMARY

1. A study of the absorption curve of the non-polar bile pigment, which predominates in the serum of patients with haemolytic jaundice, has provided additional evidence that it is bilirubin.

2. The serum of patients with obstructive jaundice has been shown to contain two pigments

reacting 'directly' in the van den Bergh test. Their absorption curves are similar, though not identical, and they differ from that of bilirubin.

3. The visible absorption curves of the azo compounds of these three pigments are indistinguishable.

4. The two direct-reacting pigments of serum occur in small amounts in the urine of patients with obstructive jaundice, and in large quantities in gallbladder bile obtained at necropsy, where they are more abundant than bilirubin.

5. The proportion between the two direct-reacting pigments of serum is variable.

6. There is evidence that other diazo-positive pigments occur in bile and urine.

We wish to express our thanks to the many clinical colleagues who have provided us with sera, and to Dr W. Hunt, St Mary's Hospital, Paddington, for obtaining specimens of autopsy bile. A grant from the Medical Research Council is also acknowledged by one of us (B.H.B.).

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Polyol Dehydrogenases

1. THE SPECIFICITY OF RAT-LIVER POLYOL DEHYDROGENASE

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The 'sorbitol' dehydrogenase of rat liver (Blakley, 1951) reacts with diphosphopyridine nucleotide (DPN) to catalyse the reversible oxidations:

Finding that glucose, gluconate, mannitol, dulcitol and D-iditol did not react with the enzyme, Blakley (1951) concluded that an oxidizable substrate must possess a primary alcohol group at C-1 adjacent to the site of oxidation (C-2) and a stereochemical pattern at C-2 and C-4 identical with that occurring in sorbitol and L-iditol.

Blakley's evidence eliminated the configuration at C-5 as a determinant of specificity but did not decide the importance of the configuration at C-3. In order to settle this point, and to gain more knowledge of the enzyme, we have investigated the oxidation of a wider range of polyhydric alcohols.

MATERIALS AND METHODS

Enzyme. The enzyme solution was the dialysed preparation B of Blakley (1951).

Coenzymes. Diphosphopyridine nucleotide (DPN) and triphosphopyridine nucleotide (TPN) were products of the

Sigma Chemical Co., St Louis, Missouri. The former was found to be 65% pure by the LePage (1947) assay and the latter 60% pure (assayed with glucose 6-phosphate dehydrogenase).

Acyclic polyols. Xylitol (m.p. 92–93.5°) and D-arabitol (m.p. 102°) were prepared by catalytic reduction of D-xylose and D-arabinose respectively, according to Carson, Waisbrot & Jones (1943). Allitol (m.p. 148–149°) was prepared by oxidation of divinyl glycol (Lespieau, 1946). Erythritol, ribitol and L-arabitol were obtained from Nutritional Biochemicals Inc., Cleveland, Ohio, U.S.A. Sorbitol monohydrate, D-mannitol and dulcitol were products (C.P. grade) of the Pfanstiehl Chemical Co., U.S.A. The following were gifts from Dr N. K. Richtmyer, National Institutes of Health, Bethesda, U.S.A.: D-gulitol, 6-deoxy-L-gulitol, D-talitol, volemitol, perseitol, β-sedoheptitol and L-gulo-D-gala-heptitol.

Cyclitols. Scyllitol (m.p. 347°) was prepared from scyllitol hexaacetate according to Posternak (1942). *meso*Inositol (C.P. grade) was obtained from the Pfanstiehl Chemical Co.; D- and L-inositol, L-viburnitol and scyllitol hexaacetate were gifts from Mrs E. Smith, Chemistry Department, University of Sydney.

Sugars. The sugars used were obtained from the following sources: D-xylose (C.P. grade), Pfanstiehl Chemical Co.; D-arabinose, Nutritional Biochemicals Inc.; D-mannoheptulose and D-glucoseheptulose, General Biochemicals Inc., Chagrin Falls, Ohio, U.S.A.; perseulose hemihydrate and