ton (*2*) published a modification of the fluorometric procedure which made possible the quantitative differentiation of epinephrine and norepinephrine in mixtures, further advancing the hope of developing a practicable chemical method for the assay of these hormones in biological fluids. Subsequent attempts to apply these procedures to the quantitative measurement of epinephrine and norepinephrine in plasma, however, were not uniformly successful, and a variety of modifications have been reported (*3*). In the course of a systematic evaluation of this method in our laboratory, some sources of error have been isolated which may possibly account for some of the difficulties experienced with this procedure.

The coefficients of the equations used to calculate the relative quantities of epinephrine and norepinephrine in a mixture were determined from separate measurements of standard solutions of epinephrine and norepinephrine, a Farrand photoelectric fluorometer, model A, being used to measure the fluorescence. It was found that the ratio of fluorescence of epinephrine to norepinephrine measured in this manner was not in agreement with the values calculated from the emission spectra of the epinephrine- and norepinephrine-ethylenediamine derivatives published by Persky and Roston. The fluorescent emission spectra of these derivatives were therefore investigated.

In these experiments, a Farrand spectrofluorometer (*4*) was used for determining the spectral distribution of fluorescence. Since it was found that the norepinephrine derivative was unstable and that the rate of decay of its fluorescence was a function of the intensity of

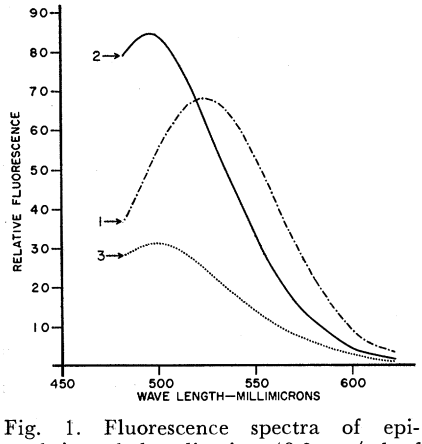

Fig. 1. Fluorescence spectra of epinephrine-ethylenediamine (0.2 μg/ml of isobutanol) and norepinephrine-ethylenediamine (0.2 μg/ml of isobutanol). Exciting wavelength is 436 mμ. Curve 1 is epinephrine; curve 2 is norepinephrine; curve 3 is the reagent blank. [Walter Reed Army Institute of Research]

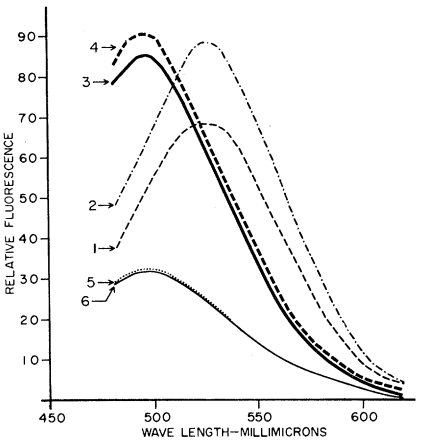

Fig. 2. Fluorescence spectra of epinephrine and norepinephrine condensed with ethylenediamine in acetic acid and in alumina-treated acetic acid. Exciting wavelength is 436 mμ. Curve 1 is epinephrine (acetic acid); curve 2 is epinephrine (alumina-treated acetic acid); curve 3 is norepinephrine (acetic acid); curve 4 is norepinephrine (alumina-treated acetic acid); curve 5 is reagent blank (acetic acid); curve 6 is reagent blank (alumina-treated acetic acid). [Walter Reed Army Institute of Research]

the exciting wave length, a Corning filter No. 3315, which passes the 436-mμ line, was used to decrease the intensity of the exciting light. The emission spectra of the ethylenediamine derivatives of epinephrine and norepinephrine (*5*) are shown in Fig. 1. The peak emission of the norepinephrine derivative is at 495 mμ, while the peak emission of the epinephrine derivative lies at 525 mμ. The ratio of fluorescence of epinephrine to norepinephrine, measured at 510 mμ, is 0.71, while the ratio measured at 600 is 4.00. These values are in agreement with the ratios determined with the Farrand model A fluorometer, Corning filters Nos. 5113 and 3389 being used in the primary, Corning filters Nos. 5433 and 3384 for the 510 mμ secondary, and Corning filter No. 2418 for the 600 mμ secondary.

An additional source of error was observed in the procedures used for determining the relative quantities of epinephrine and norepinephrine after these substances have been isolated by adsorption on alumina. A difference was observed in the fluorescence of epinephrine and norepinephrine condensed with ethylenediamine in standard acetic acid solutions as compared with the fluorescence of epinephrine and norepinephrine condensed with ethylenediamine in acetic acid which has been passed over an alumina column. It has been reported that the fluorescence of the epinephrine derivative is 100 to 150 percent greater in the acetic acid treated with alumina (*6*). In Fig. 2 the fluorescence of epinephrine and norepinephrine condensed with ethylenediamine in acetic acid is compared with the fluorescence of epinephrine and norepinephrine condensed with ethylenediamine in acetic acid which has been passed through an alumina column. It can be seen that the intensity of the fluorescence of both the epinephrine and norepinephrine derivatives is increased, the increase for the epinephrine derivative being greater than that for the norepinephrine derivative. It is also evident that there is no proportionate increase in the fluorescence of the reagent blank.

In a range of 0.02 to 0.20 μg, the average increase in the fluorescence of the epinephrine derivative measured at 510 mμ is 30 percent, while the average increase in the fluorescence of the norepinephrine derivative amounts to 9 percent. Figure 2 also indicates that the difference in the spectral distribution of the fluorescence of the derivatives prepared in acetic acid and in alumina-treated acetic acid is of a quantitative rather than a qualitative nature.

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14 June 1957

Bilirubin Glucuronide Formation in vitro; Demonstration of a Defect in Gilbert's Disease

Cole and Lathe (*1*) succeeded in isolating preparations of bilirubin which, free of protein, yielded either direct (immediate) or indirect (delayed) reactions with diazotized sulfanilic acid (van den Bergh reaction). More recently, Billing and Lathe (*2*), Schmid (*3*), and Talafant (*4*) have demonstrated that direct-reacting bilirubin is the glucuronide of bilirubin. A substance present in boiled liver extract, later shown to be uridine diphosphate glucuronic acid (UDPGA), has been found to enhance the formation of glucuronides of various receptors by rat liver homogenate (*5*). An enzyme (transferase) present in the microsomal fraction of rat liver has been shown to

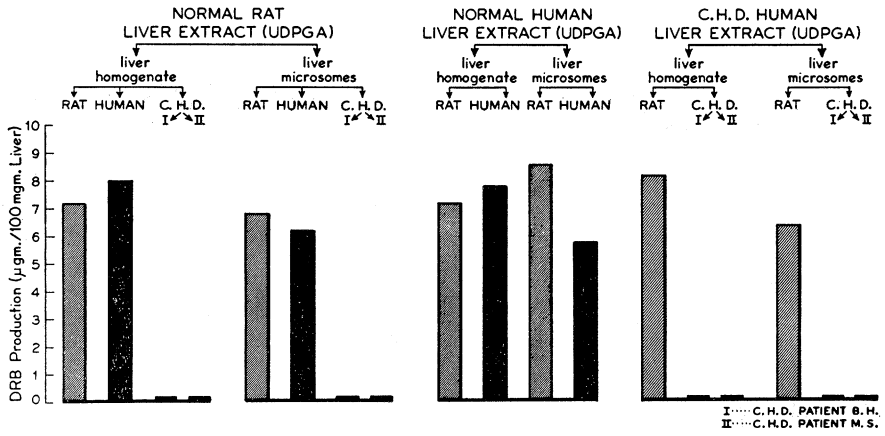

Fig. 1. Direct-reacting bilirubin glucuronide production by liver preparations from normal rats and human beings and from patients with constitutional hepatic dysfunction (Gilbert's disease).

catalyze the transfer of glucuronic acid from UDPGA to various receptors (*5, 6*). On the basis of these observations, an *in vitro* system has been developed which can convert indirect-reacting bilirubin to direct-reacting bilirubin glucuronide.

This system consists of (i) bilirubin, (ii) an extract of human or rat liver which presumably supplies UDPGA, and (iii) a homogenate of human or rat liver, or microsomes prepared from the homogenate. On fractionation of the whole homogenate, virtually all of its transferase activity is found in the microsomal fraction. With this system it has been possible to assay the effectiveness of liver tissue of normal human beings and of patients with constitutional hepatic dysfunction (CHD) in serving as a source of UDPGA and of transferase activity.

The incubation system (*5*) consisted of 0.3 ml of 0.5*M* potassium phosphate buffer (*p*H 7.4); 0.1 ml of 0.3*M* magnesium chloride; either $3.5 \times 10^{-5}M$ commercial bilirubin or $1.4 \times 10^{-4}M$ orthoaminophenol and $10^{-3}M$ ascorbic acid, suspended in 1 ml of 0.4 percent albumin solution. The total volume of each sample was 3.4 ml. Liver extract was prepared from homogenized, freshly obtained human or rat liver, which was gently boiled in isotonic potassium chloride and was subsequently centrifuged. The homogenates of human or rat liver were prepared in nine volumes of isotonic alkaline potassium chloride. Microsomes of human or rat liver were prepared by the Novikoff (*7*) and Schneider-Hogeboom (*8*) procedures, or in a medium of alkaline isotonic potassium chloride. Human and rat liver extracts and homogenates or microsomes were incubated interchangeably with bilirubin (Fig. 1) or orthoaminophenol. Incubation was carried out for 40 minutes at 37.5°C, in room air, at *p*H 7.4. Orthoaminophenol glucuronide was determined by the method of Dutton and Storey (*5*). Direct-reacting bilirubin production was determined by measuring the concentration of the resulting azopigment after centrifugation (*9*) and by subsequent chromatography, as has been described by Schmid (*3*). Mild acid hydrolysis or treatment with beta glucuronidase converted the direct-reacting azopigment to the indirect-reacting azopigment with the liberation of 1 mole of glucuronic acid per mole of converted azopigment. The glucuronic acid was identified by the carbazole reaction (*10*) and by paper chromatography.

Liver tissue was obtained at surgery from normal adults and from two patients with constitutional hepatic dysfunction (Gilbert's disease). The first patient (B.H.) was 21 years of age; the second (M.S.) was 43 years of age (*11*). The serum bilirubin concentrations were 8.8 and 18.8 mg percent, respectively, virtually all of which reacted only indirectly with diazotized sulfanilic acid. Histologic examination of the liver specimens revealed no abnormalities. Bile was aspirated from the gall bladder of the second patient, and its bilirubin was virtually all direct-reacting bilirubin glucuronide.

Figure 1 indicates that normal human or rat liver extract (UDPGA) and normal human or rat liver homogenate or microsomes (transferase) are capable of effecting the conjugation of bilirubin and glucuronic acid. The liver extracts of patients with constitutional hepatic dysfunction can effect conjugation on incubation with normal rat liver homogenates or microsomes. The liver homogenates or microsomes of the patients with constitutional hepatic dysfunction, however, are markedly defective in promoting conjugation on incubation with normal rat liver extract as well as with CHD liver extract. In other studies, no inhibition of bilirubin conjugation by CHD liver homogenates was detected (*12*). The defect in the livers of patients with constitutional hepatic dysfunction resides, therefore, in a deficiency of transferase activity. This deficiency is not limited to bilirubin conjugation, for the formation of orthoaminophenol glucuronide is similarly impaired. The finding of direct-reacting bilirubin glucuronide in the bile of one of the patients with constitutional hepatic dysfunction is perhaps explicable on the basis of a slight degree of transferase activity. The possibility may be raised, however, of an alternate pathway of glucuronide formation in man.

These studies demonstrate that the formation of bilirubin glucuronide in human, as well as rat, liver involves the transfer by an enzyme in liver microsomes of glucuronic acid from uridine diphosphate glucuronic acid to bilirubin. Grodsky and Carbone (*13*) and Schmid (*14*) have shown independently that this mechanism is present in normal rat liver. The deficiency in transferase activity in the liver microsomes of patients with constitutional hepatic dysfunction accounts for the difficulty in the excretion of bilirubin in this disease. An analogous defect is observed in the congenital hyperbilirubinemia of rats (*12, 14, 15*). The defective formation of several other glucuronides in these rats and in patients with constitutional hepatic dysfunction (*14*) is probably ascribable to a deficiency in transferase activity (*16*).

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