oxidation (Gallagher & Long, 1943), or to the oxidizing action of *A. faecalis* (Hoehn, Schmidt & Hughes, 1944); in both of these cases the C₃—OH is the last of three hydroxyl groups to undergo oxidation.

The bacterial cells of the *Proactinomyces* spp. evidently contain no enzymic mechanism capable of hydrolyzing ester-linkages, since cholesterol acetate under the above conditions was recovered unchanged, and androstenediol-17-benzoate yielded testosterone benzoate.

SUMMARY

Various of the more common hydroxy-steroids have been found to be oxidized to keto-derivatives by *Proactinomyces* spp., and the available evidence suggests the possible general nature of the method in the steroid series.

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Studies of the Van den Bergh Reaction

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Van den Bergh & Muller (1916) showed that the red pigment azobilirubin* was formed when a solution of diazotized sulphanilic acid was added to sera from patients with obstructive jaundice. On the other hand, with normal sera and sera from patients with other forms of jaundice the addition of ethanol was essential for the reaction to take place. The reaction occurring in the absence of ethanol was termed the direct reaction, while that occurring only in the presence of ethanol was termed the indirect reaction. The cause of the difference in behaviour of the two types of sera remains obscure, but possibilities which have been considered are (a) the existence of two forms of bilirubin in the two types of case (Van den Bergh, 1918; Grunenberg, 1923 *a, b*; Soejima, 1927; Newman, 1928; Fowweather, 1932), (b) combination of bilirubin with protein or other interfering substances in sera giving only the indirect reaction (Bollman, Sheard & Mann, 1927; Barron, 1931) and (c) the presence of catalytic substances in sera giving

the direct reaction (Acil & Goldgruber, 1932). Van den Bergh & Muller showed that sera giving the indirect reaction either failed completely to react with the diazo reagent in the absence of ethanol or reacted very slowly indeed (a delayed direct reaction), but they made no attempt to assign differences in clinical significance to these last two types. Feigl & Querner (1919), Lepehne (1920) and McNee (1922) subdivided the direct reaction into prompt and biphasic types. In contrast to the rapid completion of formation of azobilirubin in the prompt reaction, in the biphasic reaction formation of azobilirubin proceeded rapidly during the first minute after addition of the reagent but then continued at an obviously slower rate during the following 15 min. Such biphasic reactions were believed to be due to the presence of prompt reacting and delayed reacting bilirubins in varying proportions. After much controversy the general consensus of opinion was well expressed by Harrison (1937) who observed that 'the direct test (qualitative or quantitative) is of doubtful value and in routine work might be omitted'.

Malloy & Evelyn (1937) who determined total bilirubin by the formation of azobilirubin in the presence of methanol, replaced the latter by water, and claimed that the proportion of direct bilirubin could be determined by measuring azobilirubin 30 min. after addition of the diazo reagent. More-

* Established custom and the need for a short name are our reasons for using the term 'azobilirubin' for the red pigment produced by the reaction between bilirubin and an acid solution of diazotized sulphanilic acid. It is an unsatisfactory name, however, since Fischer and his colleagues have shown that the reaction consists of a splitting process as well as a coupling and that the red pigment is probably a mixture of isomeric diazotized hydroxy dipyrromethenes (Fischer & Orth, 1937).

over, by plotting colour development against time they attempted to distinguish between prompt, biphasic, and delayed direct types. Similar investigations were carried out by Lepehne (1942). Ducci & Watson (1945) recognized the existence of prompt direct reacting bilirubin (measured 1 min. after addition of reagents, in the absence of methanol) delayed direct reacting bilirubin (measured 14 min. later, believed by them to bear the same significance as indirect bilirubin) and true indirect bilirubin (15 min., methanol present) and regard the biphasic reaction as explained by the relative amounts present of prompt and delayed direct reacting bilirubin. However, Cabello-Ruiz (1943) has shown that the percentage of directly reacting pigment depends on the acidity at which the reaction is carried out and therefore regards such determinations as little more than semi-quantitative. The method of Rappaport & Eichhorn (1943) for the quantitative estimation of directly and indirectly reacting bilirubin in the presence of a buffer of an acidity of about pH 3.4 appeared worthy of more careful study especially for the investigation of the rate of formation of azobilirubin in the direct reaction.

METHODS

The diazo reagent was prepared according to the method of Harrison (1937) and was always used within 15 min. of preparation. All measurements of light absorption were carried out, except where otherwise indicated, with a Hilger Spekker photoelectric absorptiometer, with 1 cm. cells, and Ilford Spectral yellow green no. 605 filters (max. transmission 550 m μ), combined with Chance's Calorex glass. Interference by such factors as turbidity of the serum and presence of non-bilirubinoid pigment was avoided by measuring absorption densities relative to blanks containing serum, but with the diazo reagent replaced by approximately 0.15N-HCl. The blanks thus contained unchanged bilirubin, and therefore the calibration curves were made by measuring the absorption densities of the azobilirubin solutions relative to blanks containing corresponding amounts of bilirubin. pH was determined with a spiral glass electrode and a Unicam potentiometer. The electrode designed by Dr C. G. Pope of the Wellcome Physiological Laboratories, was similar to that described by MacInnes & Belcher (1933).

The crystalline bilirubin used for calibration was supplied by Hoffman La Roche Ltd., whose material Malloy & Evelyn found to yield more colour than that supplied by other manufacturers. We have found that bilirubin of equal chromogenic potency may be prepared along the lines suggested by Watson (1942). Crude bilirubin, dissolved in chloroform, was adsorbed on a column of alumina, which was then washed with chloroform and finally eluted with 1% acetic acid in chloroform. On evaporation of the eluate in an atmosphere of nitrogen and final drying of the residue *in vacuo* over potash, bilirubin was obtained which gave the same calibration curves as the Hoffmann La Roche preparation. This material so far has been obtained only in semi-crystalline condition.

Malloy & Evelyn method (1937). This has been employed unmodified except that the absorption of the final solution was measured in the Spekker instrument (a calibration curve for this instrument being made exactly as originally described). In a few cases the Malloy & Evelyn procedure has been used to determine the relative proportions of direct reacting and indirect reacting pigment.

Rappaport & Eichhorn method (1943). The buffers for the estimation of direct and indirect bilirubin were those described. The procedure was slightly modified. Serum (1 ml.) was measured into a test-tube, followed by 7 ml. of buffer and 1 ml. of the diazo reagent, and a blank was simultaneously prepared from 1 ml. of serum, 7 ml. of buffer and 1 ml. of 0.15N-HCl. The absorption of the first solution relative to this blank was then determined. Thus the only modification has been that we have doubled the amounts of buffer and diazo reagent relative to the amount of serum, to provide a larger volume of final solution without requiring an unduly large amount of blood. In most of the experiments designed to investigate the time-course of the direct reaction using sera from patients with obstructive jaundice or acute hepatitis, we measured absorption densities 15, 30 and 45 sec. and 1, 2, 3, 4, 5, 10, 15, 20, 25 and 30 min. after addition of the diazo reagent, using a photoelectric device of the Evelyn (1936) type and a Chance no. 5 green filter (max. transmission 520–540 m μ). In sera from patients with haemolytic jaundice the time-course of the reaction has been followed for 6 hr. or more, and the light absorption therefore measured less frequently in the Spekker, which was capable of greater accuracy but less convenient for repeated measurements at shorter intervals of time than the one photocell instrument. When total bilirubin was determined in the presence of indirect buffer, light absorption was measured at 30 min. in sera of patients with obstructive jaundice and acute hepatitis, but not until 6 hr. with sera of patients with haemolytic jaundice since maximum azobilirubin production is only attained at these times.

McNee & Keefer method (1925). The absorption of the final solution was measured relative to a blank, prepared in exactly the same way except that 0.15N-HCl was used instead of the diazo reagent. A special calibration curve was made with azobilirubin solutions prepared from pure bilirubin and adjusted to pH 4.8, the reaction of the final solution in the determination.

Haslewood & King method (1937). The absorption of the final solution was compared with the methyl red standard in the Spekker instrument, with water in the blank cell.

The relationship between absorption density and concentration over a very wide range was investigated in the following way: 10 mg. of bilirubin were dissolved in 50 ml. of chloroform. 20 ml. of this solution were treated with 100 ml. of ethanol and 10 ml. of diazo reagent. After 0.5 hr., 12 ml. of saturated ammonium sulphate were added with shaking. A lower layer and some ammonium sulphate separated out. The clear supernatant was evaporated *in vacuo* to a volume of about 10 ml. to remove as much ethanol and chloroform as possible. An equal volume of the Rappaport and Eichhorn indirect buffer was added and a series of solutions of known dilution prepared by the addition of further measured quantities of buffer. The absorption densities of each of these solutions were then measured with buffer in the control cell. In one experiment the weakest solution prepared showed an absorption density

of 0.378 and reference to the calibration curve for the Rappaport & Eichhorn method shows that this would correspond to a concentration of 0.58 mg. bilirubin/100 ml. of final solution. From this the strength of the more concentrated solutions could be calculated. The behaviour of azobilirubin solution prepared from sera was investigated by measuring the absorption densities of a series of known dilutions of an azobilirubin solution prepared from a serum of very high bilirubin content.

RESULTS

The quantitative aspects of bilirubin determination

The use of the Rappaport & Eichhorn procedure for our studies on the nature of the direct reaction necessitated its investigation from the quantitative point of view. Owing to the difficulty of preparing stable solutions of bilirubin in aqueous media at a physiological pH, few of the hitherto published methods have been investigated in so far as recoveries of bilirubin added to serum are concerned. Malloy & Evelyn, without giving details, report data indicating recoveries varying from 96 to 103 %, and we have confirmed that bilirubin in chloroform-methanol solution may be determined quantitatively when added to the Malloy & Evelyn reaction mixture. We have therefore regarded the method of Malloy & Evelyn as the method of reference.

Fig. 1. Calibration curves for serum bilirubin determination by Rappaport & Eichhorn, and Malloy & Evelyn methods.

Fig. 1 shows the calibration curve which we have obtained for the Malloy & Evelyn technique with the Spekker absorptiometer. Above 35 mg. of bilirubin/100 ml. of serum, the curve flattens slightly, but below this level absorption density is directly proportional to bilirubin concentration. Since the final dilution of serum was 1 in 25 and because we used cells of 1 cm. thickness, absorption densities of less than 0.05 were obtained for values of serum

bilirubin below 2.5 mg./100 ml. In our opinion it is not possible to measure accurately such low absorption densities with the Spekker instrument! Malloy & Evelyn, using test-tubes of $\frac{7}{8}$ in. diameter corresponding to a mean thickness of about 2 cm., could probably obtain accurate results with somewhat lower levels of bilirubin. With the Spekker instrument it has not been practicable to use a cell of greater thickness without increasing the final volume of solution, an increase which would be inconvenient. The Malloy & Evelyn technique when used with the Spekker absorptiometer is therefore unsatisfactory for values of bilirubin below 2.5 mg./100 ml. Malloy & Evelyn found their results to be higher than those obtained by other procedures, but apparently used their own calibration curve without adjusting the standard azobilirubin solutions to the pH of the reaction mixture of the particular method used. We have found that even if the acidities of the standard azobilirubin solutions were properly adjusted, the Malloy & Evelyn method still gave higher values than did the photoelectric modification of the method of McNee & Keefer.

Fig. 1 also shows the calibration curve we have obtained with the Rappaport and Eichhorn method using azobilirubin solutions suitably buffered to pH 3.2 and prepared from known amounts of bilirubin in chloroform-ethanol. It will be noticed that Beer's law is not followed above 15 mg./100 ml. An absorption density of 0.05 now corresponds to a bilirubin content of only 0.75 mg./100 ml. of serum.* Table 1a shows that the results with this method agree reasonably well with those obtained by the Malloy & Evelyn technique.

We have determined the bilirubin content of thirteen sera simultaneously by the methods of Malloy & Evelyn, Rappaport & Eichhorn and Haslewood & King, and found that whereas all three methods agree reasonably well for low values of serum bilirubin, with higher values the method of Haslewood & King gives results considerably lower than those of the other two methods; the latter agree moderately well (see Table 1b).

We determined the percentage of directly reacting bilirubin in the sera of eight patients by the Rappaport & Eichhorn method and by that of Malloy & Evelyn. The percentage of direct bilirubin was always higher when measured by the Rappaport & Eichhorn method, and this was presumably due to the difference in pH at which the direct reaction was carried out in the two methods.

* If sufficient quantities of serum and reagents in the same proportions as in the original Rappaport & Eichhorn technique are used, e.g. 2 ml. of serum, 7 ml. of buffer and 1 ml. of diazo reagent, an absorption density of 0.05 will correspond to a value of serum bilirubin of 0.4 mg./100 ml. We have not employed these proportions in the work reported here.

Table 1a. Comparison of results of serum bilirubin determinations by Malloy & Evelyn and Rappaport & Eichhorn methods

Malloy & Evelyn method	Rappaport & Eichhorn method
Bilirubin concentration (mg./100 ml.)	
1.0	1.3
1.0	0.8
2.0	1.5
3.0	2.6
3.4	3.3
4.1	3.8
4.2	4.1
5.1	4.9
5.5	5.6
6.2	5.9
7.2	7.9
10.8	11.5
11.8	12.1
12.8	12.8
14.3	14.9
16.3	16.8
17.8	17.5
17.9	19.6
20.0	21.1
21.4	23.8
25.8	24.2
29.4	24.5
55.6	55.5

Table 1b. Comparison of results of serum bilirubin determination by three different methods

Malloy & Evelyn method	Rappaport & Eichhorn method	Haslewood & King method
Bilirubin concentration (mg./100 ml.)		
1.0	1.9	0.8
2.2	2.2	1.5
2.4	1.3	1.0
2.4	3.0	2.2
5.0	4.6	2.1
5.2	5.2	2.3
5.9	4.4	2.6
5.9	6.0	2.6
8.4	8.2	3.7
14.2	15.5	8.8
18.5	18.0	13.5
21.8	23.5	13.0
23.4	24.4	15.5

The rate of colour development in the direct Van den Bergh reaction

(a) *Sera from cases of obstructive jaundice and of acute hepatitis.* In these sera, the absorption density of the azobilirubin formed in the presence of direct buffer reached a maximum within 30 min. in both the original and modified Rappaport & Eichhorn procedures. By plotting absorption density against time, it was expected that the shape of the curve would reflect the type of direct reaction as shown in Fig. 2a. We have examined sera from sixty-seven patients with obstructive jaundice and with acute hepatitis but found no difference in behaviour of

Fig. 2. Time course of the direct Van den Bergh reaction. (a) Hypothetical curves of absorption density, plotted against time. (b) Observed curves of absorption density, plotted against time. (c) Observed curves of percentage of total bilirubin reacting to form azobilirubin, plotted against time.

sera from these two types of case. Fig. 2b, which gives the curves obtained with eight of these sera, shows that the top two curves are somewhat of the type we expected to be associated with a prompt reaction, while the next four curves are somewhat of the biphasic type. The sera which gave the top four curves were described as giving a prompt direct Van den Bergh reaction as judged visually. The reason for this discrepancy will become obvious

Fig. 3. Curves representing the time course of the direct and indirect Van den Bergh reactions of sera from two different cases of haemolytic jaundice.

later. The similarity in form of the curves is greater than expected, and all were biphasic in that an initial rapid increase of absorption density slowed down gradually over a period of minutes until the maximum was reached. As will be shown later, Beer's law is not followed with azobilirubin solutions corresponding to more than 1.6 mg./100 ml. of final solution, and a more significant comparison of the curves can be made if we plot against time the percentages of the total bilirubin which have been converted to azobilirubin (Fig. 2c). The similarity in form of these curves is striking.

(b) *Sera from cases of haemolytic jaundice.* Figs. 3a and b show the types of curve we have found with sera from cases of haemolytic jaundice; of these, type (a) is the more common. In these cases the time curves of the indirect reactions are also shown. It will be noticed that maximum pigment production is reached only about 6 hr. after the addition of reagents and that in both cases the time curves for directly reacting bilirubin are very different from those obtained with sera from cases of obstructive jaundice and of acute hepatitis. In curve 3b it appears as if the direct reaction is almost completely negative, but in 3a the curve is typically biphasic, but not in the usually accepted sense. The initial more rapid direct reaction continues for $\frac{1}{2}$ hr. and is then succeeded by a much slower reaction. Some curves intermediate in character between the two forms represented in Fig. 3 have been encountered.

The light absorption of solutions of azobilirubin

Fig. 4 (in which abscissae refer to concentrations of bilirubin equivalent to the azo pigment in the final solution) shows the relationship between absorption density and concentration over a very wide

Fig. 4. Absorption densities of solutions of azobilirubin, *p*-hydroxy-azobenzene, phenol red and thymol blue plotted against concentration.

range. The divergence from Beer's law is just obvious at 1.6 mg./100 ml., probably becomes significant at 2.5 mg./100 ml. and is gross above 4 mg./100 ml., and above this level absorption density increases relatively little with concentration. This failure of Beer's law at such relatively low concentrations of azobilirubin is also encountered with solutions derived from sera. The divergence is so great that we wondered if we were dealing with a true Beer's law effect, and if such strong solutions of azobilirubin contained an appreciable quantity of uncoupled bilirubin, but increase in concentration of reagents

made no difference to the absorption density, and calculation showed that the reagents were present in great excess. It has not been possible to prepare crystalline azobilirubin and examine the absorption densities of solutions of varying concentration, but Fig. 3 also shows the absorption densities of other highly coloured substances plotted against concentrations from which it is seen that this gross failure of Beer's law at high absorption densities is common to them all. It is possible that this is a molecular interference phenomenon of a nature allied to 'quenching' in ultra-violet fluorescence. In our opinion, the divergence from Beer's law of the absorption properties of azobilirubin solutions is not necessarily due to incomplete coupling of bilirubin.

DISCUSSION

The Rappaport & Eichhorn method in both original and modified forms for the photoelectric determination of serum bilirubin is very satisfactory, provided that the final solution is suitably diluted with buffer when the concentration is such that Beer's law is no

of azobilirubin formed from bilirubin, in sera from patients with obstructive jaundice and acute hepatitis, whether these sera gave prompt, biphasic or delayed direct reactions, was unexpected. However, the failure of solutions of azobilirubin to follow Beer's law in concentrations encountered during the performance of the direct Van den Bergh reaction provides a complete explanation of these various types of visual direct reaction (see Table 2). If a serum with a bilirubin content of 18 mg./100 ml. is diluted with $\frac{1}{2}$ vol. of diazo reagent, the bilirubin concentration will be only 12 mg./100 ml. Our curves show that in the first 30 sec. about 35 % of the bilirubin reacts to form azobilirubin, i.e. $12 \times 35/100 = 4.2$ mg./100 ml. In the next 30 sec. a further 25 % reacts, i.e. 3.0 mg./100 ml. During the first 30 sec. there is, therefore, a very rapid development of colour slowing down towards the end of that time when concentrations of azobilirubin are obtained at which Beer's law is no longer held. During the next 30 sec. although a further 3.0 mg. bilirubin react, the increase in colour corresponding to this is very little because of the gross divergence

Table 2. *Analysis of the direct Van den Bergh reaction*

	Serum 1	Serum 2	Serum 3
Concentration of bilirubin in serum	18 mg./100 ml.	9 mg./100 ml.	3 mg./100 ml.
Ditto after dilution with $\frac{1}{2}$ vol. diazo reagent	12 mg./100 ml.	6 mg./100 ml.	2 mg./100 ml.
In 1st 30 sec., about 35 % of the total bilirubin reacts to form azobilirubin	4.2 mg./100 ml.	2.1 mg./100 ml.	0.7 mg./100 ml.
Appearance to naked eye during 1st 30 sec.	Rapid development of much colour slowing markedly at end of 30 sec.	Fairly rapid even development of colour	Slow development of colour
In 2nd 30 sec. a further 25 % of the total bilirubin reacts to form azobilirubin	3.0 mg./100 ml.	1.5 mg./100 ml.	0.5 mg./100 ml.
Appearance to naked eye during 2nd 30 sec.	No obvious increase of colour	Slower development of colour	Slower development still
Description	Prompt	Biphasic	Delayed

longer followed. An additional reading of absorption density taken 1 hr. after mixture of the reagents will indicate whether the case is one of haemolytic jaundice and whether it is necessary to wait 6 or more hours before obtaining the final reading. In regard to the discrepancies between the results given by the McNee & Keefer method and by the Haslewood & King method, it has long been known that low results may be caused by adsorption of pigment on precipitated protein, particularly with sera from cases of obstructive jaundice. We find such adsorption also occurs in sera from cases of acute hepatitis. It is therefore possible that methods which do not avoid protein precipitation are accurate only for the determination of the bilirubin content of normal sera and of sera from cases of haemolytic jaundice.

The remarkable similarity in form of the curves obtained by plotting time against the percentages

from Beer's law for concentrations of azobilirubin corresponding to more than 4 mg. bilirubin/100 ml. The reaction, therefore, appears prompt because the naked eye is ordinarily unable to appreciate this further slight increase in colour. If the serum contains 9 mg./100 ml., dilution with diazo reagent will give a concentration of 6 mg./100 ml. In the first 30 sec. about 2.1 mg./100 ml. will react, in the second 30 sec. another 1.5 mg./100 ml. There will be an even and rapid production of azobilirubin during the first 30 sec., and because within the range up to 2.5 mg./100 ml. Beer's law is fairly satisfactorily held, there will be an even and rapid development of colour with this production. During the second 30 sec. azobilirubin increases from a concentration corresponding to 2.1 mg. of bilirubin/100 ml. to one corresponding to 3.6 mg./100 ml. Not only is less azobilirubin produced (during that

30 sec. period) but the increase in intensity of colour is not so great in proportion to the amount of azobilirubin formed since at this level Beer's law is no longer followed. However, the divergence from Beer's law at this range is not so great as when the concentration is above 4 mg./100 ml., and the eye is able to appreciate this slower increase in intensity of colour. The reaction therefore appears biphasic. On the other hand, if the serum contains only 3 mg./100 ml., diluted it will contain 2 mg./100 ml. with 0.7 mg./100 ml. and 0.5 mg./100 ml. reacting in the first and second 30 sec. periods respectively. To the eye the development of colour will appear fairly uniform and the reaction will be described as 'delayed'.

The type of direct Van den Bergh reaction in obstructive jaundice and in acute hepatitis therefore depends solely on the concentration of bilirubin in the serum, a conception which is in agreement with the work of Stein (1941), whose findings we have confirmed in that on dilution of serum of high bilirubin content, prompt, biphasic and delayed reactions occur according to the extent of the dilution. The results of Delgado (1943) and of Ducci & Watson (1945) demonstrating that the type of direct Van den Bergh reaction depends upon the relative amounts of prompt and delayed direct bilirubin present may be explained equally well by regarding the type of reaction as depending on the amount of total bilirubin. The findings in this paper demonstrate the accuracy of Van den Bergh's original observations and the confusion which has arisen from the attempts of others to elaborate upon the types of reaction which bears his name. There is little doubt that direct Van den Bergh reactions should be described only as positive and negative, with the possible provision for a third category, the delayed reaction in which colour development occurs only after about 15 min. This delayed reaction is of precisely the same significance as a negative direct reaction. Such an interpretation is already accepted in practice (Lichtman, 1942). Thus positive direct Van den Bergh reactions in which colour development occurs at any time during the first 15 min. may indicate either obstructive or hepatogenous jaundice, irrespective of the degree of promptness, of delay, or of its biphasic appearance, and it is not possible to distinguish between these types of jaundice by the nature of the Van den Bergh reaction.

The two forms of curve encountered in the study of sera from cases of haemolytic jaundice will be discussed more fully in a subsequent publication. It may be mentioned, however, that the biphasic character of the direct reaction in cases whose curve of the direct reaction is represented in Fig. 3a may well be due to the presence of a mixture of two forms of bilirubin. Visual observation would have de-

scribed these reactions as greatly delayed, and they would never have been classified as biphasic in the usually accepted sense.

The work described in this paper has been carried out on sera containing more than 1 mg. of total bilirubin/100 ml. and we have not attempted to confirm the investigations (by the Malloy & Evelyn method) of Cantarow, Wirts & Hollander (1942) on the proportion of direct and indirect bilirubin in sera of normal bilirubin content, since their results were not confirmed by Ducci & San Martin (1943). In our opinion, not only is it difficult to obtain accurate measurements with such low optical densities but other factors such as imperfections in the test-tube cells used by them may play an important part in producing apparent differences in the percentage of direct bilirubin. Cantarow, Wirts & Hollander also found an increased proportion of direct reacting bilirubin in most cases of hyperbilirubinaemia associated with cirrhosis, hepatitis or biliary obstruction. Biphasic reactions have always been regarded as classical examples of direct reactions due to mixtures of directly and indirectly reacting bilirubin. Such sera when investigated in detail behave like sera giving prompt reactions. We have also found that all sera from cases of haemolytic jaundice require 5-6 hr. for complete coupling of the bilirubin, yet the so-called indirect fraction encountered in the other types of case complete the reaction well within the usual 30 min. For this reason we do not subscribe to the view that the so-called indirect bilirubin of non-haemolytic cases is the same as that from cases of haemolytic jaundice, and consider that measurements of directly and indirectly reacting bilirubin by methods employed by Malloy & Evelyn, by Cantarow, Wirts & Hollander and by Ducci & Watson (1945) can have little precise meaning. It should be realized that such measurements are quite arbitrary, and the terms direct and indirect bilirubin in such investigations should be abandoned.

SUMMARY

1. The Rappaport & Eichhorn (1943) method in slightly modified form gives the same results for the determination of serum bilirubin as the method of Malloy & Evelyn (1937), whereas other methods give lower results owing to adsorption of pigment on the precipitated proteins.

2. The modified Rappaport & Eichhorn procedure has been used to investigate the rate of formation of azobilirubin in the direct Van den Bergh reaction.

3. Beer's law is not obeyed by solutions of azobilirubin in concentrations corresponding to more than 1.6 mg. of bilirubin/100 ml. of final solution. In concentrations corresponding to more than 4 mg.

bilirubin/100 ml. of final solution, absorption density increases relatively little with concentration.

4. Prompt, biphasic and delayed direct reactions encountered in obstructive jaundice or acute hepatitis depend solely upon the quantity of bilirubin present and are explained by this failure of Beer's law.

5. In the Rappaport & Eichhorn method total bilirubin in sera from cases of haemolytic jaundice requires 5-6 hr. for complete coupling, instead of less than 30 min. required for bilirubin in sera from cases of obstructive jaundice and acute hepatitis. In haemolytic jaundice two types of direct reaction are encountered: (1) a very greatly delayed and exceedingly weak development of colour, and (2) a biphasic response in which the less slow phase occurs during the first hour of the reaction.

6. Reasons are given for disregarding most measurements of direct and indirect bilirubin hitherto reported in the literature.

7. The significance of these findings is discussed.

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ADDENDUM

Since this work was completed, cells of 2 cm. thickness and of only 15 ml. capacity have become available for use with the Spekker instrument and low concentrations of bilirubin may be measured more accurately with them than with the 1 cm. cells.

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Problems Associated with the Acid Hydrolysis of an Impure Protein Preparation

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Products of the hydrolysis of proteins, the amino-acids, were being listed in qualitative and quantitative studies of protein composition long before the introduction of the peptide theory (Fischer, 1902; Hofmeister, 1902) of protein structure pro-

vided a satisfactory theoretical basis for the practice. In general, acid hydrolysis has been more widely used than alkali hydrolysis and, since its introduction by Ritthausen (1868), hydrochloric acid has tended to replace sulphuric acid as hydrolyzing