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Gerald Klatskin, Victor A. Drill

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Research Article



# THE SIGNIFICANCE OF THE "ONE-MINUTE" (PROMPT DIRECT REACTING) BILIRUBIN IN SERUM [1]

By GERALD KLATSKIN and VICTOR A. DRILL [2]

*(From the Departments of Medicine and Pharmacology, Yale University School of Medicine, New Haven, Conn.)*

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The recent reports of Watson and Ducci (1, 2) have revived interest in the van den Bergh reaction (3), and in the physiological and chemical nature of serum bilirubin. These investigators have presented evidence to show that there are two types of bilirubin in serum, each having a distinctive chemical structure and physiological significance. Their work is based on the premise that the two types can be distinguished by their respective rates of diazotization in the direct van den Bergh reaction, and their observations have led to the conclusion that the prompt direct, or one-minute fraction, represents bilirubin which has been regurgitated from the biliary tree, and that the indirect fraction represents retained bilirubin. The difference in their behavior toward the diazo reagent is ascribed to the nature of their linkage with protein, the prompt direct fraction being a sodium salt loosely bound by adsorption, the indirect being firmly attached by a valence-bond. Another important physiological difference between the two fractions, according to Watson, is evident in their behavior in the kidney, direct bilirubin being excreted, indirect being retained.

These conclusions reaffirm the views of many early workers (4–8), but are not in accord with more recent opinion that there is only one type of bilirubin in serum (9–11), that there is no direct relationship between the renal excretion of bilirubin and the direct van den Bergh reaction (12), and that the van den Bergh reaction is of limited diagnostic significance (13). Watson (1) has emphasized two points which he believes may account for this divergence of opinion. First, almost all regurgitated bilirubin diazotizes within one minute in the direct reaction, and the increase in color observed beyond this point is due to diazotization of indirect bilirubin which reacts slowly in the absence of alcohol. In the past, direct bilirubin has been measured five to 30 minutes after the addition of diazo reagent, so that the results have been modified by the presence of indeterminate amounts of indirect bilirubin. Secondly, most of the experiments cited as proof of the non-existence of two forms of bilirubin simply demonstrate that all the serum bilirubin is bound to protein, but do not exclude the possibility that there are two types of protein-linkage, a suggestion first made by Coolidge (14).

In Ehrlich's original experiments on the diazo reaction in chloroform solutions of bilirubin, alcohol was found to be essential as a mutual solvent for the otherwise immiscible chloroform solution and aqueous reagent (15). Some years later van den Bergh and Müller (3a) discovered, quite by accident, that alcohol could be omitted in carrying out the reaction in bile, but not in aqueous solutions of sodium bilirubin. When they examined blood they found a similar difference in the behavior of the reaction in obstructive and hemolytic jaundice and applied the terms "direct" and "indirect" reaction respectively. The reaction appeared to be of value in differentiating hemolytic from other types of jaundice, but not in distinguishing extrahepatic biliary obstruction from hepatocellular damage, both of which were regarded as examples of obstructive jaundice in accord with the views of Eppinger (16). Later experience proved van den Bergh's observations to be sound, but because of its limitations the reaction was abandoned as a diagnostic test until it was revived by Watson (1). Since then his modification of the reaction has gained wide acceptance as a clinical laboratory test. While no claim has been made that it will differentiate extrahepatic biliary obstruction from hepatocellular jaundice, it is thought to be of value in estimating the degree of bile regurgitation in these two con-

[1] Aided by a grant from the Fluid Research Fund of the Yale University School of Medicine.

[2] Present Address: Department of Pharmacology, Wayne University College of Medicine, Detroit, Mich.

ditions. The evidence presented to prove that the one-minute fraction represents regurgitated bilirubin (17), and that its quantitation has greater diagnostic significance than the original qualitative van den Bergh reaction (1) is by no means conclusive.

The chemical basis for the two types of van den Bergh reaction remains obscure. Attempts to demonstrate two chemically distinct forms of bilirubin (18–20) have failed (6, 21, 22), but the original theory that the variable behavior of bilirubin depends on its linkage with protein survives, despite convincing evidence to the contrary. Van den Bergh and Müller (3a) considered the possibility that bilirubin gave an indirect reaction when combined with protein or lipid, but could not confirm this experimentally. They expressed the opinion that it was more likely that the behavior of serum bilirubin in the van den Bergh reaction was related to the presence or absence in serum of some normal constituent of bile. Later, van den Bergh revised this opinion and stated that the two types of diazo reaction were due either to minor chemical differences between direct and indirect bilirubin, or more probably to the linkage of indirect bilirubin to protein (3b). In 1923 Levi-Crailsheim (23) further elaborated this theory, and suggested that the bilirubin-protein complex was split in the liver cell before bilirubin was excreted into the biliary tree. This hypothesis has gained wide acceptance and has had a profound influence on the formulation of the modern concept of regurgitation and retention jaundice (1, 8, 24).

The failure to demonstrate unbound bilirubin in serum by dialysis (25), cataphoresis (14, 26–28) or ultrafiltration (10, 11, 14) casts doubt on the theory that the type of van den Bergh reaction is related to protein-linkage. Recent studies (9, 10), on the other hand, indicate that the type of diazo reaction may depend on the physico-chemical properties of serum rather than on the structure of bilirubin, a possibility first proposed by Adler and Strauss (29). Gray and Whidborne (9) suggest that the direct reaction may be due to the presence of a catalyst in serum specifically elaborated in regurgitation jaundice. Attempts to identify this catalyst with bile salts (14, 29, 30) and cholesterol (29, 30, 31) have generally failed.

The specificity of the diazo reaction is generally taken for granted, but Kerppola (32) has shown that the direct reaction may be due, in part at least, to the presence of non-bilirubin derivatives from bile. How important this factor may be is not known, as Kerppola's observations have not as yet been confirmed.

The factors governing the renal excretion of bilirubin are still poorly understood. There have been many attempts to explain the absence of bilirubinuria in some types of jaundice on the basis of differences in the physical or chemical properties of serum bilirubin. Thus, Hoover and Blankenhorn (33) tried to correlate the renal excretion of bilirubin with its filtrability through collodion; later, when appropriate collodion membranes were employed, it was shown that all serum bilirubin is protein-bound and non-filtrable (11). The advent of the van den Bergh reaction gave rise to a commonly held view that there is a renal threshold for direct bilirubin, but that the kidney is impermeable to the indirect type (1, 4, 8, 24). There is contradictory evidence, however, indicating that there is no causal relationship between biliuria and the type of van den Bergh reaction (12). Moreover, there is evidence to suggest that renal factors may be important in the excretion of bilirubin (34, 35).

It is evident from the foregoing that the conclusions regarding the chemical nature and physiological significance of the one-minute serum bilirubin fraction (1) are not in accord with other recent opinions. Since these conclusions have such an important bearing on both clinical and experimental studies of jaundice, there appeared to be a need for further study of this problem. The following investigation was undertaken to determine: (*1*) whether the reaction curves of azobilirubin development in serum warrant the conclusion that there are two types of bilirubin, (*2*) whether there is any relationship between the one-minute serum bilirubin level and the renal excretion of bilirubin, and (*3*) whether the one-minute serum bilirubin has diagnostic significance.

## METHODS AND MATERIAL

The rate of diazotization of serum bilirubin was determined in 90 freshly collected blood specimens drawn from patients suffering from a variety of diseases. An attempt was made to obtain sera with total bilirubin values of as wide a range and representing as many types of jaundice as possible.

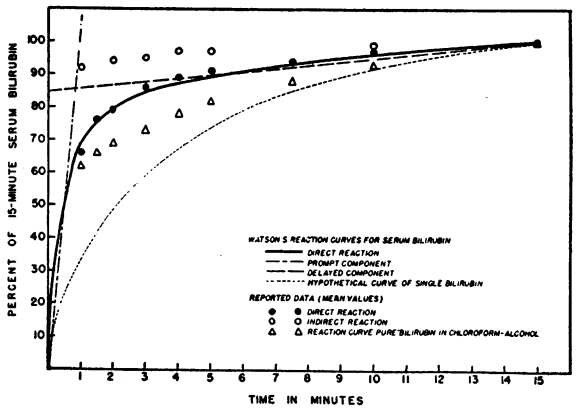


FIG. 1. RATE OF AZOBILIRUBIN DEVELOPMENT IN THE DIRECT AND INDIRECT REACTIONS OF SERUM AND IN THE DIRECT REACTION OF ALCOHOL-CHLOROFORM SOLUTIONS OF PURE BILIRUBIN MEASURED FOR 15 MINUTES

A modification of the Malloy and Evelyn method (36) was employed in studying the first group of 40 sera, the direct reaction being carried out on a mixture of 4 ml. of a 1:10 dilution of serum and 1 ml. of freshly prepared diazo reagent. The rate of diazotization was observed at frequent intervals for a period of 120 minutes in a Klett-Summerson photoelectric colorimeter with a number 54 filter. At the end of this period 5 ml. of absolute methyl alcohol were added to the mixture and the indirect reaction observed at intervals for 30 minutes. Galvanometer readings were converted to concentrations of bilirubin by means of a factor determined from the calibration curve obtained with serial ethyl alcohol dilutions of a chloroform solution of pure bilirubin (Eastman-Kodak).

The technique employed in Watson's laboratory (37) is essentially as outlined above with the following differences: (*a*) serum dilutions are varied from 0.1:10 to 0.5:10 depending on the concentration of bilirubin present, (*b*) the direct reaction is carried out on a mixture of 5 ml. of diluted serum and 1 ml. of diazo reagent, and the rate of diazotization observed for 15 minutes, (*c*) total bilirubin is determined 15 minutes after the addition of 6 ml. of absolute methyl alcohol to the reaction mixture, and (*d*) an Evelyn colorimeter is employed.

Because the azobilirubin development curves obtained on the first 40 sera studied were somewhat irregular, and since they differed so markedly from those obtained by Watson (1), a second group of 50 sera were studied in a similar manner, but with the following modifications in technique: (*a*) A Coleman Junior, model 6A, spectrophotometer with 19 mm. cuvettes and a wavelength setting of 540 mμ was employed instead of the Klett-Summerson colorimeter; the former is a single-cell instrument similar to the Evelyn colorimeter employed by Watson (37), and has proved to be a reliable and accurate instrument in this laboratory. (*b*) The calibration curve for azobilirubin was obtained with serial methyl alcohol dilutions of a chloroform solution of pure bilirubin (Hoffmann-LaRoche). (*c*) All volumetric pipettes and flasks employed were recalibrated to a tolerance of ± 0.1 per cent. (*d*) One ml. of serum was diluted sufficiently to keep all galvanometer readings during diazotization within the middle two-thirds of the scale; any diazotization curve with a transmittance value above 90 per cent or below 10 per cent in it was discarded. It was not possible, therefore, to include sera with normal bilirubin values, as had been done in the initial experiments. (*e*) Five ml. of diluted sera were transferred to each of two cuvettes; to one was added 1.0 ml. of freshly prepared diazo reagent, to the other 1.0 ml. of diazo blank. The rate of diazotization was then observed for a period of 120 minutes in the first tube, the second tube serving as a blank. Following this 6.0 ml. of absolute methyl alcohol were added to each tube and the period of observation continued for 30 minutes. This is essentially the procedure employed by Watson (37), except that the periods of observation in both the direct and indirect reaction were prolonged. For purposes of comparison with Watson's published curves (1), all the data have been presented for both the 15-minute and the longer observation periods employed in this experiment. Moreover, the concentrations of azobilirubin developed during the course of diazotization have been expressed as per cent of the 15-minute and 120-minute values in the direct reaction, and as per cent of the 15-minute and 30-minute values in the indirect reaction, to facilitate comparisons of diazotization rates in sera of different bilirubin concentrations.

The curves obtained in the second set of experiments were very much smoother than those obtained in the first, but some irregularity was still evident. On inspecting the data it was found that these irregularities were usually due to changes in the galvanometer reading representing transmittance values of less than 0.5 per cent.

The diazotization curves in both sets of experiments were similar, especially when the very irregular curves obtained with sera of low bilirubin concentration were omitted. For the sake of brevity, therefore, the 40 curves

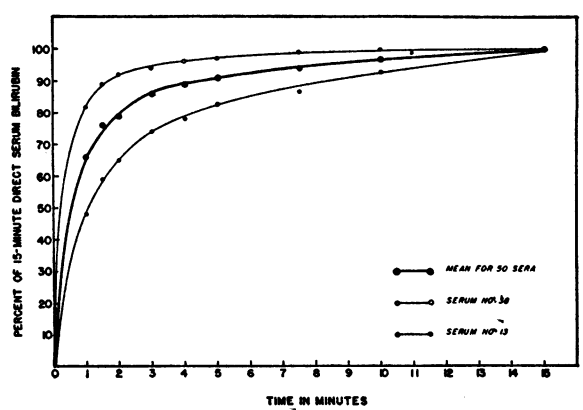


FIG. 2. RANGE OF DIAZOTIZATION RATES IN THE DIRECT REACTION OF 50 SERA OBSERVED FOR 15 MINUTES

obtained in the first group of experiments have been omitted from the discussion.

The rate of diazotization of pure bilirubin (Hoffmann-LaRoche) was studied in methyl alcohol dilutions of a freshly prepared chloroform solution containing 40 mg. per 100 ml. Dilutions were made with appropriate mixtures of alcohol and chloroform in volumetric flasks to attain a constant volume of chloroform (10 per cent), alcohol (80 per cent), and water in the form of diazo reagent (10 per cent) in the final reaction mixture for each concentration of bilirubin. Studies in this laboratory have indicated that the rate and completeness of diazotization are influenced by variations in the concentrations of these constituents of the reaction mixture (38).

TABLE I

*Rate of azobilirubin development in the direct reaction of serum followed for 15 minutes*

| Case | Serum bilirubin | | Serum dilution | Per cent of 15-min. direct bilirubin developed | | | | | | | | | Diagnosis |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 15-min. Direct | Total | | 1 *min.* | 1½ *min.* | 2 *min.* | 3 *min.* | 4 *min.* | 5 *min.* | 7½ *min.* | 10 *min.* | 15 *min.* | |
| | *mg. per cent* | *mg. per cent* | | | | | | | | | | | |
| 1 | 0.65 | 1.23 | 1:10 | 63 | 82 | 82 | 81 | 90 | 90 | 94 | 94 | 100 | Cirrhosis |
| 2 | 0.70 | 1.39 | 1:10 | 62 | 71 | 78 | 84 | 89 | 90 | 95 | 96 | 100 | Cirrhosis |
| 3 | 0.68 | 1.51 | 1:10 | 65 | 73 | 78 | 80 | 82 | 86 | 91 | 93 | 100 | Hepatitis, post-op. |
| 4 | 0.98 | 1.55 | 1:10 | 72 | 83 | — | 83 | 86 | 89 | 90 | 95 | 100 | Obstructive j.-cholecystitis |
| 5 | 0.69 | 1.61 | 1:10 | 66 | 71 | 77 | 77 | 83 | 82 | 91 | 95 | 100 | Cirrhosis |
| 6 | 1.05 | 1.83 | 1:10 | 57 | 70 | 78 | 84 | 88 | 89 | 93 | 99 | 100 | Obstructive j.-cholelithiasis |
| 7 | 0.96 | 1.92 | 1:10 | 62 | 69 | 74 | 79 | 82 | 84 | 89 | 94 | 100 | Cirrhosis |
| 8 | 1.04 | 2.07 | 1:10 | 69 | 78 | 83 | 86 | 89 | 90 | 93 | — | 100 | Cirrhosis |
| 9 | 0.97 | 2.11 | 1:10 | 59 | 65 | 75 | 77 | 79 | 80 | 89 | 93 | 100 | Cirrhosis |
| 10 | 1.71 | 2.46 | 1:10 | 73 | 83 | 86 | 90 | 93 | 93 | 96 | 99 | 100 | Metastatic carcinoma |
| 11 | 1.88 | 2.67 | 1:20 | 69 | 77 | 81 | 87 | 90 | 92 | 95 | 97 | 100 | Obstructive j.-carcinoma |
| 12 | 1.25 | 2.69 | 1:10 | 51 | 63 | 66 | 71 | 78 | 79 | 87 | 89 | 100 | Cirrhosis |
| 13 | 1.23 | 2.85 | 1:10 | 48 | 59 | 65 | 74 | 78 | 83 | 87 | 93 | 100 | Cirrhosis |
| 14 | 1.48 | 2.97 | 1:10 | 58 | 72 | 77 | 79 | 84 | 86 | 92 | 92 | 100 | Cirrhosis |
| 15 | 1.27 | 3.09 | 1:10 | 59 | 68 | 72 | 77 | 80 | 82 | 87 | 94 | 100 | Cirrhosis |
| 16 | 2.00 | 3.50 | 1:10 | 62 | 72 | 79 | 84 | 87 | 90 | 93 | 96 | 100 | Obstructive j.-cholelithiasis |
| 17 | 2.36 | 3.53 | 1:10 | 74 | 81 | 83 | 86 | 88 | 91 | 94 | 97 | 100 | Obstructive j.-cholelithiasis |
| 18 | 2.07 | 3.85 | 1:10 | 58 | 66 | 72 | 77 | 82 | 84 | 90 | 91 | 100 | Myelogenous leukemia |
| 19 | 3.07 | 3.99 | 1:10 | 69 | 79 | 84 | 89 | 92 | 94 | 96 | 97 | 100 | Metastatic carcinoma |
| 20 | 3.06 | 4.06 | 1:10 | 76 | 82 | — | 89 | 90 | 91 | 92 | 95 | 100 | Obstructive j.-cholelithiasis |
| 21 | 3.07 | 4.32 | 1:20 | 72 | 79 | 82 | 88 | 89 | 93 | 95 | 97 | 100 | Obstructive j.-carcinoma |
| 22 | 3.07 | 4.57 | 1:20 | 70 | 79 | 84 | 90 | 93 | 95 | 95 | 97 | 100 | Cirrhosis |
| 23 | 4.05 | 5.06 | 1:10 | 78 | 86 | 89 | 93 | 96 | 97 | 97 | 99 | 100 | Obstructive j.-cholecystitis |
| 24 | 3.42 | 5.29 | 1:10 | 75 | 84 | 89 | 92 | 94 | 95 | 96 | 98 | 100 | Massive hepat. necrosis (poison) |
| 25 | 3.81 | 5.48 | 1:20 | 62 | 75 | 78 | 84 | 87 | 89 | 93 | 96 | 100 | Obstructive j.-biliary atresia |
| 26 | 3.44 | 6.15 | 1:20 | 61 | 69 | 75 | 83 | 86 | 89 | 93 | 96 | 100 | Cirrhosis |
| 27 | 5.50 | 7.03 | 1:20 | 78 | 84 | 88 | 93 | 94 | 95 | 97 | 98 | 100 | Obstructive j.-carcinoma |
| 28 | 5.44 | 7.48 | 1:20 | 64 | 72 | 77 | 83 | 86 | 89 | 93 | 98 | 100 | Obstructive j.-biliary atresia |
| 29 | 5.18 | 7.77 | 1:20 | 61 | 75 | 77 | 88 | 89 | 92 | 95 | 97 | 100 | Cirrhosis |
| 30 | 5.06 | 7.84 | 1:20 | 63 | 74 | 79 | 85 | 89 | 91 | 95 | 96 | 100 | Cirrhosis |
| 31 | 4.96 | 8.19 | 1:10 | 73 | 80 | 87 | 91 | 93 | 94 | 96 | 97 | 100 | Massive hepat. necrosis (poison) |
| 32 | 5.76 | 9.00 | 1:20 | 70 | 79 | 84 | 88 | 91 | 93 | 97 | 97 | 100 | Obstructive j.-carcinoma |
| 33 | 6.23 | 9.91 | 1:20 | 68 | 77 | 80 | 87 | 90 | 91 | 95 | 97 | 100 | Hepatitis, recurrent |
| 34 | 6.95 | 9.91 | 1:20 | 58 | 69 | 75 | 82 | 86 | 89 | 93 | 97 | 100 | Obstructive j.-biliary atresia |
| 35 | 7.13 | 9.98 | 1:20 | 62 | 71 | 77 | 84 | 87 | 90 | 94 | 97 | 100 | Obstructive j.-biliary atresia |
| 36 | 7.73 | 10.01 | 1:20 | 73 | 83 | 88 | 92 | 94 | 95 | 97 | 99 | 100 | Hepatitis, homologous serum |
| 37 | 9.08 | 13.39 | 1:20 | 57 | 75 | 83 | 89 | 93 | 94 | 97 | 99 | 100 | Cirrhosis |
| 38 | 10.93 | 14.44 | 1:20 | 82 | 89 | 92 | 94 | 96 | 97 | 99 | 100 | 100 | Obstructive j.-carcinoma |
| 39 | 10.92 | 14.86 | 1:20 | 75 | 85 | 88 | 92 | 94 | 95 | 97 | 99 | 100 | Obstructive j.-carcinoma |
| 40 | 9.90 | 14.86 | 1:20 | 71 | 82 | 85 | 91 | 93 | 95 | 97 | 99 | 100 | Cirrhosis |
| 41 | 10.78 | 16.93 | 1:20 | 72 | 81 | 86 | 90 | 92 | 93 | 96 | 98 | 100 | Obstructive j.-carcinoma |
| 42 | 11.27 | 17.46 | 1:20 | 70 | 81 | 86 | 92 | 95 | 96 | 97 | 99 | 100 | Obstructive j.-carcinoma |
| 43 | 10.73 | 17.50 | 1:20 | 54 | 67 | 72 | 79 | 85 | 89 | 94 | 97 | 100 | Obstructive j.-carcinoma |
| 44 | 12.75 | 18.73 | 1:20 | 70 | 80 | 85 | 89 | 93 | 94 | 97 | 98 | 100 | Obstructive j.-carcinoma |
| 45 | 13.92 | 19.15 | 1:50 | 61 | 72 | 78 | 84 | 87 | 89 | 94 | 97 | 100 | Obstructive j.-carcinoma |
| 46 | 11.81 | 19.33 | 1:50 | 69 | 77 | 82 | 88 | 90 | 90 | 95 | 98 | 100 | Obstructive j.-carcinoma |
| 47 | 16.20 | 22.84 | 1:50 | 70 | 79 | 83 | 88 | 92 | 93 | 96 | 100 | 100 | Obstructive j.-carcinoma |
| 48 | 17.25 | 24.86 | 1:50 | 70 | 79 | 83 | 88 | 90 | 94 | 95 | 97 | 100 | Obstructive j.-carcinoma |
| 49 | 15.28 | 25.04 | 1:50 | 65 | 74 | 79 | 84 | 88 | 90 | 94 | 96 | 100 | Obstructive j.-carcinoma |
| 50 | 20.30 | 26.56 | 1:40 | 75 | 83 | 87 | 91 | 94 | 95 | 98 | 98 | 100 | Obstructive j.-carcinoma |
| | | | Mean: | 66 | 76 | 79 | 86 | 89 | 91 | 94 | 97 | 100 | |
| | | | Standard Deviation: | 7.3 | 6.5 | 11.8 | 5.3 | 7.1 | 4.3 | 2.9 | 2.4 | 0 | |

TABLE II

*Rate of azobilirubin development in the direct reaction of serum followed for 120 minutes*

| Case | Serum bilirubin | | Serum dilution | Per cent of 120 min. direct azobilirubin developed | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 120 min. Direct | Total | | 1 min. | 1½ min. | 2 min. | 3 min. | 4 min. | 5 min. | 7½ min. | 10 min. | 15 min. | 20 min. | 30 min. | 45 min. | 60 min. | 75 min. | 90 min. | 105 min. | 120 min. |
| | mg. per cent | mg. per cent | | | | | | | | | | | | | | | | | |
| 1 | 0.84 | 1.23 | 1:10 | 49 | 64 | 64 | 63 | 70 | 70 | 73 | 73 | 77 | 79 | 83 | 90 | 91 | 93 | 97 | 99 | 100 |
| 2 | 0.92 | 1.39 | 1:10 | 48 | 54 | 59 | 64 | 68 | 69 | 72 | 73 | 76 | 78 | 83 | 89 | 92 | 97 | 99 | 98 | 100 |
| 3 | 0.84 | 1.51 | 1:10 | 52 | 58 | 63 | 65 | 66 | 69 | 73 | 75 | 80 | 80 | 85 | 91 | 94 | 96 | 96 | 97 | 100 |
| 4 | 1.08 | 1.55 | 1:10 | 66 | 76 | — | 76 | 78 | 81 | 82 | 86 | 91 | 92 | 94 | 96 | 98 | 97 | 98 | 100 | 98 |
| 5 | 1.01 | 1.61 | 1:10 | 45 | 48 | 53 | 53 | 57 | 57 | 63 | 65 | 69 | 71 | 78 | 88 | 90 | 95 | 95 | 99 | 100 |
| 6 | 1.33 | 1.83 | 1:10 | 46 | 56 | 62 | 67 | 70 | 71 | 74 | 79 | 79 | 82 | 85 | 88 | 92 | 96 | 96 | 97 | 100 |
| 7 | 1.35 | 1.92 | 1:10 | 44 | 49 | 53 | 56 | 58 | 60 | 63 | 67 | 71 | 73 | 79 | 88 | 92 | 96 | 98 | 99 | 100 |
| 8 | 1.40 | 2.07 | 1:10 | 52 | 58 | 62 | 64 | 67 | 67 | 70 | — | 75 | 78 | 80 | 87 | 91 | 92 | 96 | 97 | 100 |
| 9 | 1.44 | 2.11 | 1:10 | 40 | 44 | 50 | 52 | 53 | 54 | 60 | 62 | 67 | 69 | 78 | 85 | 88 | 94 | 96 | 97 | 100 |
| 10 | 1.87 | 2.46 | 1:10 | 67 | 76 | 79 | 82 | 85 | 85 | 88 | 91 | 91 | 95 | 94 | 98 | 98 | 98 | 98 | 100 | 100 |
| 11 | 2.04 | 2.67 | 1:20 | 64 | 71 | 75 | 80 | 83 | 84 | 88 | 90 | 92 | 94 | 96 | 99 | 99 | 99 | 100 | 100 | 100 |
| 12 | 1.62 | 2.69 | 1:10 | 39 | 49 | 51 | 55 | 60 | 61 | 66 | 69 | 77 | 80 | 83 | 89 | 92 | 94 | 94 | 98 | 100 |
| 13 | 1.93 | 2.58 | 1:10 | 30 | 38 | 41 | 47 | 50 | 53 | 55 | 59 | 64 | 67 | 75 | 82 | 88 | 86 | 95 | 98 | 100 |
| 14 | 1.99 | 2.97 | 1:10 | 43 | 53 | 57 | 59 | 63 | 64 | 68 | 68 | 74 | 77 | 83 | 88 | 92 | 93 | 97 | 100 | 99 |
| 15 | 1.71 | 3.09 | 1:10 | 44 | 50 | 53 | 58 | 60 | 61 | 65 | 70 | 74 | 80 | 85 | 90 | 93 | 95 | 97 | 98 | 100 |
| 16 | 2.55 | 3.50 | 1:10 | 49 | 56 | 62 | 66 | 69 | 71 | 74 | 76 | 79 | 82 | 86 | 91 | 94 | 96 | 97 | 100 | 100 |
| 17 | 2.74 | 3.53 | 1:10 | 64 | 70 | 71 | 74 | 76 | 78 | 81 | 83 | 86 | 90 | 93 | 97 | 99 | 100 | 100 | 100 | 99 |
| 18 | 3.13 | 3.85 | 1:10 | 38 | 44 | 48 | 51 | 54 | 56 | 60 | 60 | 66 | 69 | 75 | 84 | 87 | 92 | 95 | 96 | 100 |
| 19 | 3.31 | 3.99 | 1:10 | 64 | 74 | 78 | 83 | 85 | 87 | 89 | 90 | 93 | 94 | 96 | 97 | 99 | 99 | 99 | 99 | 100 |
| 20 | 3.36 | 4.06 | 1:10 | 70 | 74 | — | 80 | 82 | 82 | 84 | 87 | 91 | 93 | 95 | 99 | 100 | 100 | 99 | 99 | 99 |
| 21 | 3.44 | 4.32 | 1:20 | 64 | 70 | 73 | 79 | 80 | 83 | 85 | 86 | 89 | 91 | 94 | 97 | 98 | 98 | 96 | 99 | 100 |
| 22 | 3.65 | 4.57 | 1:20 | 59 | 67 | 71 | 76 | 78 | 80 | 80 | 81 | 84 | 89 | 91 | 94 | 96 | 97 | 99 | 100 | 100 |
| 23 | 4.27 | 5.06 | 1:10 | 74 | 81 | 85 | 88 | 91 | 92 | 92 | 93 | 95 | 96 | 97 | 98 | 99 | 99 | 100 | 100 | 100 |
| 24 | 3.82 | 5.29 | 1:10 | 67 | 75 | 80 | 82 | 84 | 85 | 86 | 87 | 89 | 91 | 92 | 95 | 97 | 97 | 99 | 100 | 100 |
| 25 | 4.18 | 5.48 | 1:20 | 57 | 68 | 71 | 76 | 79 | 81 | 85 | 88 | 91 | 93 | 97 | 97 | 98 | 99 | 100 | 99 | 100 |
| 26 | 3.90 | 6.15 | 1:20 | 54 | 61 | 67 | 73 | 76 | 79 | 82 | 85 | 88 | 90 | 94 | 97 | 99 | 100 | 100 | 100 | 100 |
| 27 | 5.99 | 7.03 | 1:20 | 72 | 77 | 81 | 85 | 87 | 88 | 89 | 90 | 92 | 93 | 95 | 97 | 98 | 100 | 100 | 100 | 100 |
| 28 | 5.97 | 7.48 | 1:20 | 59 | 66 | 71 | 75 | 79 | 82 | 85 | 89 | 91 | 93 | 96 | 99 | 99 | 100 | 100 | 99 | 99 |
| 29 | 5.72 | 7.77 | 1:20 | 55 | 68 | 70 | 80 | 80 | 84 | 86 | 88 | 90 | 93 | 96 | 98 | 100 | 99 | 100 | 99 | 99 |
| 30 | 5.94 | 7.84 | 1:20 | 54 | 63 | 67 | 73 | 75 | 77 | 81 | 82 | 85 | 89 | 91 | 93 | 95 | 97 | 97 | 99 | 100 |
| 31 | 5.64 | 8.19 | 1:10 | 64 | 71 | 77 | 80 | 82 | 83 | 84 | 85 | 88 | 89 | 92 | 94 | 97 | 97 | 99 | 99 | 100 |
| 32 | 6.50 | 9.00 | 1:20 | 67 | 70 | 75 | 78 | 80 | 82 | 86 | 86 | 89 | 92 | 94 | 97 | 98 | 99 | 99 | 100 | 99 |
| 33 | 7.02 | 9.91 | 1:20 | 61 | 68 | 72 | 77 | 80 | 80 | 84 | 86 | 89 | 90 | 94 | 97 | 97 | 98 | 100 | 100 | 100 |
| 34 | 7.74 | 9.91 | 1:20 | 52 | 62 | 68 | 74 | 78 | 80 | 84 | 87 | 90 | 92 | 95 | 97 | 97 | 98 | 99 | 99 | 100 |
| 35 | 7.83 | 9.98 | 1:20 | 56 | 65 | 70 | 76 | 79 | 82 | 86 | 89 | 91 | 93 | 95 | 97 | 98 | 99 | 100 | 100 | 100 |
| 36 | 8.25 | 10.01 | 1:20 | 68 | 78 | 82 | 86 | 88 | 89 | 91 | 92 | 94 | 94 | 96 | 97 | 98 | 99 | 99 | 99 | 100 |
| 37 | 9.61 | 13.39 | 1:20 | 54 | 70 | 79 | 84 | 87 | 89 | 91 | 93 | 95 | 96 | 98 | 99 | 99 | 100 | 100 | 100 | 100 |
| 38 | 11.51 | 14.44 | 1:20 | 78 | 84 | 87 | 89 | 91 | 92 | 94 | 95 | 95 | 97 | 98 | 99 | 100 | 99 | 100 | 100 | 99 |
| 39 | 11.73 | 14.86 | 1:20 | 70 | 79 | 82 | 86 | 88 | 89 | 90 | 92 | 93 | 95 | 96 | 98 | 98 | 98 | 99 | 99 | 100 |
| 40 | 10.48 | 14.86 | 1:20 | 67 | 77 | 81 | 86 | 88 | 90 | 92 | 93 | 94 | 96 | 98 | 98 | 98 | 99 | 99 | 100 | 100 |
| 41 | 11.62 | 16.93 | 1:20 | 67 | 75 | 79 | 83 | 85 | 87 | 89 | 91 | 93 | 94 | 96 | 98 | 100 | 99 | 100 | 99 | 97 |
| 42 | 12.24 | 17.46 | 1:20 | 63 | 74 | 81 | 84 | 87 | 88 | 89 | 91 | 92 | 92 | 94 | 95 | 98 | 100 | 98 | 99 | 98 |
| 43 | 11.91 | 17.50 | 1:20 | 49 | 61 | 65 | 71 | 77 | 80 | 85 | 87 | 90 | 92 | 96 | 97 | 99 | 99 | 100 | 99 | 99 |
| 44 | 14.22 | 18.73 | 1:20 | 64 | 71 | 76 | 80 | 83 | 84 | 87 | 88 | 90 | 91 | 94 | 96 | 100 | 97 | 98 | 97 | 97 |
| 45 | 15.32 | 19.15 | 1:50 | 56 | 65 | 71 | 76 | 79 | 81 | 85 | 88 | 91 | 93 | 96 | 97 | 99 | 99 | 99 | 100 | 99 |
| 46 | 12.95 | 19.33 | 1:50 | 63 | 71 | 75 | 80 | 82 | 82 | 87 | 89 | 91 | 93 | 96 | 97 | 100 | 100 | 100 | 100 | 100 |
| 47 | 17.69 | 22.84 | 1:50 | 64 | 71 | 76 | 80 | 84 | 85 | 88 | 91 | 92 | 95 | 97 | 98 | 99 | 100 | 100 | 100 | 100 |
| 48 | 18.48 | 24.86 | 1:50 | 65 | 74 | 78 | 82 | 84 | 86 | 89 | 91 | 93 | 95 | 97 | 98 | 99 | 99 | 100 | 100 | 99 |
| 49 | 16.59 | 25.04 | 1:50 | 60 | 68 | 73 | 77 | 81 | 83 | 86 | 89 | 92 | 95 | 96 | 98 | 99 | 99 | 100 | 99 | 99 |
| 50 | 20.86 | 26.56 | 1:40 | 73 | 80 | 85 | 88 | 91 | 92 | 95 | 95 | 97 | 98 | 99 | 99 | — | 100 | 100 | 100 | 99 |
| | | | Mean: | 58 | 66 | 70 | 74 | 77 | 78 | 81 | 83 | 86 | 88 | 91 | 95 | 96 | 97 | 98 | 99 | 100 |
| | | | Standard Deviation: | 3.1 | 4.5 | 4.2 | 10.9 | 10.6 | 10.6 | 12.8 | 9.7 | 8.6 | 9.7 | 6.7 | 4.6 | 3.5 | 2.8 | 1.7 | 1.0 | 0.8 |

The data relating to the clinical significance of the one-minute bilirubin and its relationship to the urinary excretion of bilirubin were obtained from routine, simultaneously collected, fresh blood and urine specimens submitted to the laboratory for examination. They were analyzed as soon as possible, but in many instances there was a delay of as long as three hours. One-minute and total serum bilirubin concentrations were determined by the method described for the first group of experiments in which the Klett-Summerson colorimeter was employed. Urine bile was determined by Sparkman's method (39). After these studies had been completed the accuracy of the urine bile method was checked against that of Watson and Hawkinson (40).

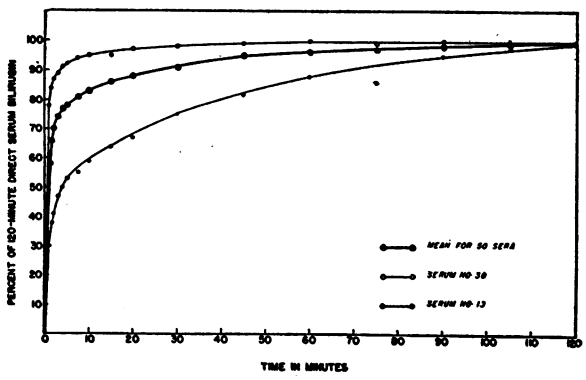


FIG. 3. RANGE OF DIAZOTIZATION RATES IN THE DIRECT REACTION OF 50 SERA OBSERVED FOR 120 MINUTES

The terminology suggested by Watson (1) has been adopted in this report, "one-minute" and "prompt direct" bilirubin being used interchangeably, and "total" referring to the value obtained after the addition of alcohol. The term "indirect," however, has been applied to the difference between the "total" and the "120-minute direct" bilirubin, and no account has been taken of the "delayed direct" fraction ("120-minute direct" minus the "one-minute"), since its significance is entirely dependent on the interpretation put on the diazotization curves under investigation. Watson regards the "delayed direct" and "indirect" fractions of bilirubin as identical, and, therefore, considers them as an entity ("total" minus "one-minute" fraction).

## RESULTS

*The rate of azobilirubin development.* The mean values for azobilirubin development observed for 15 minutes are plotted in Figure 1, and the reaction curves reported by Watson (1) are superimposed for purposes of comparison. The contour of the direct bilirubin curve observed by Watson has been described as a composite of two

TABLE III

*Rate of azobilirubin development in alcohol-chloroform solutions of pure bilirubin followed for 120 minutes*

| Bilirubin concentration | | Per cent of azobilirubin developed | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Reaction mixture* | Serum equivalent† | 1 min. | 1½ min. | 2 min. | 3 min. | 4 min. | 5 min. | 7½ min. | 10 min. | 15 min. | 20 min. | 30 min. | 45 min. | 60 min. | 75 min. | 90 min. | 105 min. | 120 min. |
| *mg. per cent* | *mg. per cent* | | | | | | | | | | | | | | | | | |
| 0.08 | 1.92 | — | 47 | 53 | 58 | 64 | 70 | 75 | 80 | 87 | 90 | 96 | 96 | 96 | 100 | 99 | 99 | 98 |
| 0.08 | 1.92 | 42 | 52 | 55 | 60 | 64 | 68 | 76 | 81 | 86 | 92 | 94 | 97 | 97 | 98 | 100 | 98 | 100 |
| 0.08 | 1.92 | 45 | 49 | 53 | 58 | 64 | 71 | 77 | 80 | 86 | 90 | 93 | 97 | 99 | 98 | 100 | 99 | 99 |
| 0.08 | 1.92 | 44 | 47 | 51 | 58 | 62 | 66 | 74 | 79 | 85 | 89 | 92 | 96 | 96 | 99 | 99 | — | 100 |
| 0.16 | 3.84 | — | 55 | 58 | 62 | 66 | 69 | 74 | 77 | 84 | 88 | 92 | 93 | 93 | 97 | 96 | 99 | 100 |
| 0.16 | 3.84 | 50 | 53 | 55 | 60 | 64 | 67 | 73 | 77 | 83 | 89 | 91 | 93 | 97 | 97 | 97 | 97 | 100 |
| 0.16 | 3.84 | 50 | 56 | 56 | 60 | 65 | 67 | 74 | 78 | 82 | 88 | 91 | 96 | 97 | 100 | 97 | 100 | 99 |
| 0.16 | 3.84 | 48 | 51 | 55 | 59 | 64 | 66 | 74 | 78 | 82 | 87 | 91 | 93 | 96 | 97 | 97 | 99 | 100 |
| 0.32 | 7.68 | — | 59 | 62 | 66 | 69 | 72 | 78 | 83 | 89 | 94 | 97 | 98 | 98 | 99 | 100 | — | 97 |
| 0.32 | 7.68 | — | 57 | 60 | 63 | 65 | 68 | 75 | 81 | 87 | 89 | 95 | 98 | 99 | 99 | 100 | 99 | 97 |
| 0.32 | 7.68 | 57 | 58 | 62 | 65 | 69 | 72 | 77 | 80 | 87 | 91 | 96 | 99 | 99 | 99 | — | 100 | 99 |
| 0.32 | 7.68 | 56 | 59 | 61 | 65 | 68 | 71 | 76 | 81 | 86 | 91 | 95 | 98 | 100 | 98 | 98 | 98 | 99 |
| 0.40 | 9.60 | — | — | 61 | 65 | 69 | 72 | 76 | 81 | 87 | 93 | 97 | 100 | 100 | 100 | 100 | 99 | 99 |
| 0.40 | 9.60 | 58 | 60 | 61 | 66 | 68 | 71 | 76 | 82 | 87 | 92 | 96 | 98 | 99 | 100 | 100 | 99 | 99 |
| 0.40 | 9.60 | — | 58 | 60 | 65 | 66 | 71 | 76 | 81 | 86 | 92 | 96 | 99 | 100 | 100 | 100 | 100 | 100 |
| 0.40 | 9.60 | 57 | 59 | 61 | 63 | 68 | 71 | 77 | 81 | 88 | 92 | 96 | 99 | 99 | 100 | 99 | 99 | 99 |
| 0.64 | 15.36 | 56 | 59 | 61 | 64 | 67 | 69 | 75 | 79 | — | 89 | 92 | 97 | 98 | 99 | 99 | 99 | 100 |
| 0.64 | 15.36 | 55 | 57 | 59 | 63 | 65 | 69 | 74 | 78 | 83 | 88 | 93 | 96 | 98 | 99 | 100 | 100 | 100 |
| 0.64 | 15.36 | 55 | 58 | 60 | 63 | 66 | 69 | 74 | 78 | 84 | 88 | 94 | 97 | 98 | 99 | 100 | 100 | 100 |
| 0.64 | 15.36 | 56 | 59 | 61 | 64 | 68 | 70 | 76 | 80 | 87 | 91 | 96 | 99 | 100 | 100 | 99 | 99 | 99 |
| 0.80 | 19.20 | — | 64 | 65 | 69 | 71 | 74 | 80 | 83 | 89 | 94 | 98 | 99 | 100 | 100 | 98 | 99 | 98 |
| 0.80 | 19.20 | 60 | 63 | 64 | 67 | 70 | 73 | 79 | 83 | 88 | 93 | 98 | 100 | 99 | 100 | 99 | 98 | 98 |
| 0.80 | 19.20 | 60 | 62 | 64 | 67 | 69 | 71 | 77 | 81 | 86 | 92 | 95 | 99 | 99 | 100 | 99 | 99 | 99 |
| | Mean: | 53 | 56 | 59 | 63 | 67 | 70 | 76 | 80 | 86 | 91 | 95 | 97 | 98 | 99 | 99 | 99 | 99 |
| | Standard Deviation: | 5.6 | 4.7 | 3.8 | 3.1 | 2.3 | 2.2 | 1.7 | 1.8 | 2.1 | 2.1 | 3.0 | 2.1 | 1.7 | 1.1 | 1.2 | 0.8 | 1.0 |

* Final concentration of reaction mixture: chloroform 10 per cent, methyl alcohol 80 per cent, water (diazo reagent) 10 per cent.

† Equivalent to concentration of total serum bilirubin determined by Watson modification (37) of Malloy-Evelyn method (36), employing an initial 1:10 dilution of serum, and a serum dilution of 1:24 in the final reaction mixture.

TABLE IV

*Rate of azobilirubin development in alcohol-chloroform solutions of pure bilirubin followed for 15 minutes*

| Bilirubin concentration | | Per cent of azobilirubin developed | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Reaction mixture* | Serum equivalent† | 1 *min.* | 1½ *min.* | 2 *min.* | 3 *min.* | 4 *min.* | 5 *min.* | 7½ *min.* | 10 *min.* | 15 *min.* |
| *mg. per cent* | *mg. per cent* | | | | | | | | | |
| 0.08 | 1.92 | — | 55 | 61 | 67 | 74 | 81 | 86 | 92 | 100 |
| 0.08 | 1.92 | 49 | 61 | 64 | 70 | 75 | 79 | 89 | 95 | 100 |
| 0.08 | 1.92 | 53 | 57 | 61 | 68 | 74 | 82 | 89 | 92 | 100 |
| 0.08 | 1.92 | 51 | 55 | 60 | 68 | 72 | 78 | 87 | 93 | 100 |
| 0.16 | 3.84 | — | 65 | 69 | 74 | 79 | 82 | 88 | 92 | 100 |
| 0.16 | 3.84 | 60 | 64 | 66 | 73 | 77 | 81 | 87 | 93 | 100 |
| 0.16 | 3.84 | 61 | 68 | 68 | 74 | 79 | 82 | 90 | 95 | 100 |
| 0.16 | 3.84 | 59 | 63 | 67 | 72 | 78 | 81 | 90 | 95 | 100 |
| 0.32 | 7.68 | — | 66 | 69 | 74 | 77 | 81 | 87 | 93 | 100 |
| 0.32 | 7.68 | — | 65 | 69 | 73 | 75 | 79 | 87 | 93 | 100 |
| 0.32 | 7.68 | 65 | 66 | 71 | 74 | 79 | 83 | 88 | 92 | 100 |
| 0.32 | 7.68 | 65 | 68 | 71 | 75 | 79 | 83 | 88 | 94 | 100 |
| 0.40 | 9.60 | — | — | 70 | 74 | 79 | 82 | 88 | 93 | 100 |
| 0.40 | 9.60 | 66 | 69 | 70 | 76 | 78 | 82 | 88 | 94 | 100 |
| 0.40 | 9.60 | — | 67 | 70 | 75 | 77 | 83 | 88 | 93 | 100 |
| 0.40 | 9.60 | 65 | 68 | 70 | 72 | 78 | 81 | 87 | 93 | 100 |
| 0.64 | 15.36 | 66 | 69 | 71 | 74 | 79 | 83 | 89 | 94 | 100 |
| 0.64 | 15.36 | 66 | 69 | 71 | 74 | 79 | 82 | 88 | 93 | 100 |
| 0.64 | 15.36 | 65 | 68 | 70 | 74 | 78 | 81 | 87 | 92 | 100 |
| 0.80 | 19.20 | — | 72 | 74 | 78 | 81 | 84 | 90 | 94 | 100 |
| 0.80 | 19.20 | 69 | 72 | 73 | 77 | 80 | 83 | 90 | 94 | 100 |
| 0.80 | 19.20 | 69 | 71 | 74 | 77 | 80 | 82 | 89 | 94 | 100 |
| Mean: | | 62 | 66 | 69 | 73 | 78 | 82 | 88 | 93 | 100 |
| Standard Deviation: | | 6.2 | 5.0 | 3.9 | 2.8 | 2.3 | 1.5 | 1.2 | 1.0 | 0 |

* Final concentration of reaction mixture: chloroform 10 per cent, methyl alcohol 80 per cent, water (diazo reagent) 10 per cent.

† Equivalent to concentration of total serum bilirubin determined by Watson modification (37) of Malloy-Evelyn method (36), employing an initial 1:10 dilution of serum, and a serum dilution of 1:24 in the final reaction mixture.

curves, the change from the almost vertical to the more horizontal component representing a change in the order of reaction and indicating the presence of two compounds (1). This interpretation is the basis for the partition of serum bilirubin at one minute, and depends on the assumption that the reaction of a single bilirubin would describe a parabolic curve.

It can be seen that the mean values for azobilirubin developed in the direct reaction of 50 sera, observed for an arbitrary period of 15 minutes, fall on a diphasic curve similar to that described by Watson. However, when the diazotization reaction curves for the individual sera were plotted (Figure 2 and Table I) they were found to exhibit marked variations in their contour. This was even more apparent when diazotization was allowed to go on to completion during a two-hour period. It is evident from Figure 3 and Table II that there is no characteristic curve for the diazotization of serum bilirubin in the direct reaction, that the curves are more or less diphasic, but variable in contour, and that the change in the order of reaction described by Watson (1) is neither constant at one minute nor clearly discernible in all sera. Moreover, a comparison of Figures 2 and 3 illustrates the necessity for allowing diazotization to go on to completion in studying the reaction curve of bilirubin. In the present experiments on serum, diazotization was usually complete in one to two hours, and, in many instances, was followed by a slight decrease in azobilirubin concentration, possibly due to fading of its color. This phenomenon was also observed during diazotization of alcohol-chloroform solutions of pure bilirubin (Table III). A number of

jaundiced sera and solutions of bilirubin were observed for periods longer than two hours following diazotization. In none was there a significant increase in azobilirubin concentration after two hours.

When the mean values for azobilirubin development in alcohol-chloroform solutions are plotted (Figure 1) they fall on a diphasic curve similar to that found in the direct reaction of jaundiced sera, rather than on the hypothetical parabolic curve of a single bilirubin suggested by Watson (1). The individual curves exhibited some degree of variation in their contour (Tables III and IV), but not as great as that seen in the direct reaction of serum.

Similarly the diazotization curve for indirect

TABLE V

*Rate of azobilirubin development in the indirect reaction of serum on the addition of alcohol following completion of the direct reaction*

| Case | Serum bilirubin | | Per cent of indirect bilirubin | | | | | | | Serum bilirubin | | Per cent of indirect bilirubin | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Indirect* | Total† | 1 min. | 2 min. | 3 min. | 4 min. | 5 min. | 10 min. | 15 min. | Indirect‡ | Total§ | 1 min. | 2 min. | 3 min. | 4 min. | 5 min. | 10 min. | 15 min. | 20 min. | 30 min. |
| | mg. per cent | mg. per cent | | | | | | | | mg. per cent | mg. per cent | | | | | | | | | |
| 1 | 0.39 | 1.23 | 86 | 87 | 96 | 96 | 94 | 96 | 100 | 0.39 | 1.23 | 86 | 87 | 96 | 96 | 94 | 96 | 100 | 96 | 93 |
| 2 | 0.47 | 1.39 | 85 | 89 | 91 | 95 | 97 | 100 | 96 | 0.47 | 1.39 | 85 | 89 | 91 | 95 | 97 | 100 | 96 | 95 | 95 |
| 3 | 0.59 | 1.43 | 80 | 80 | 85 | 88 | 93 | 99 | 100 | 0.67 | 1.51 | 77 | 77 | 81 | 84 | 88 | 94 | 95 | 100 | 94 |
| 5 | 0.60 | 1.61 | 89 | 95 | 97 | 97 | 98 | 98 | 100 | 0.60 | 1.61 | 89 | 95 | 97 | 97 | 98 | 98 | 100 | 95 | 95 |
| 6 | 0.50 | 1.83 | 92 | 92 | 94 | 94 | 94 | 100 | 100 | 0.50 | 1.83 | 92 | 92 | 94 | 94 | 94 | 100 | 100 | 100 | 99 |
| 8 | 0.67 | 2.07 | 86 | 91 | 91 | 95 | 95 | 100 | 98 | 0.67 | 2.07 | 86 | 91 | 91 | 95 | 95 | 100 | 98 | 95 | 94 |
| 10 | 0.59 | 2.46 | 89 | 89 | 93 | 94 | 100 | 98 | 98 | 0.59 | 2.46 | 89 | 89 | 93 | 94 | 100 | 98 | 98 | 98 | 94 |
| 11 | 0.63 | 2.67 | 85 | 92 | 95 | 97 | 99 | 100 | 99 | 0.63 | 2.67 | 85 | 92 | 95 | 97 | 99 | 100 | 97 | 97 | 95 |
| 12 | 1.07 | 2.69 | 86 | 89 | 90 | — | 93 | 100 | 99 | 1.07 | 2.69 | 86 | 89 | 90 | — | 93 | 100 | 99 | 100 | 99 |
| 13 | 0.89 | 2.82 | 92 | 93 | 96 | 96 | 97 | 100 | 99 | 0.92 | 2.85 | 91 | 92 | 95 | 95 | 96 | 99 | 99 | 98 | 100 |
| 15 | 1.38 | 3.09 | 84 | 89 | 91 | 94 | 94 | 99 | 100 | 1.38 | 3.09 | 84 | 89 | 91 | 94 | 94 | 99 | 100 | 100 | 99 |
| 16 | 0.88 | 3.43 | 95 | 97 | 97 | 100 | 99 | 100 | 100 | 0.95 | 3.50 | 94 | 96 | 96 | 98 | 97 | 98 | 98 | 100 | 98 |
| 17 | 0.79 | 3.53 | 94 | 94 | 97 | 97 | 98 | 100 | 100 | 0.79 | 3.53 | 94 | 94 | 97 | 97 | 98 | 100 | 100 | 97 | 97 |
| 18 | 0.72 | 3.85 | 91 | 93 | 93 | 94 | 96 | 100 | 100 | 0.72 | 3.85 | 91 | 93 | 93 | 94 | 96 | 100 | 100 | 99 | 98 |
| 19 | 0.68 | 3.99 | 97 | 97 | 98 | 99 | 99 | 100 | 100 | 0.68 | 3.99 | 97 | 97 | 98 | 99 | 99 | 100 | 100 | 99 | 99 |
| 20 | 0.70 | 4.06 | 97 | — | 98 | 98 | 100 | 98 | 99 | | | | | | | | | | | |
| 21 | 0.88 | 4.32 | 93 | 96 | 99 | 99 | 100 | 100 | 99 | 0.88 | 4.32 | 93 | 96 | 99 | 99 | 100 | 100 | 99 | 99 | 97 |
| 22 | 0.92 | 4.57 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 0.92 | 4.57 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 98 | 89 |
| 23 | 0.79 | 5.06 | 96 | 98 | 98 | 99 | 99 | 100 | 100 | 0.79 | 5.06 | 96 | 98 | 98 | 99 | 99 | 100 | 100 | 100 | 100 |
| 24 | 1.31 | 5.13 | 95 | 97 | 97 | 98 | 99 | 100 | 100 | 1.47 | 5.29 | 92 | 94 | 94 | 95 | 95 | 96 | 97 | 97 | 100 |
| 25 | 1.30 | 5.48 | 95 | 96 | 97 | 99 | 99 | 100 | 94 | 1.30 | 5.48 | 95 | 96 | 97 | 99 | 99 | 100 | 94 | 97 | 92 |
| 26 | 2.18 | 6.08 | 82 | 86 | 90 | 93 | 94 | 99 | 100 | 2.25 | 6.15 | 81 | 85 | 89 | 91 | 93 | 98 | 99 | 99 | 100 |
| 27 | 1.04 | 7.03 | 96 | 97 | 100 | 100 | 100 | 100 | 100 | 1.04 | 7.03 | 96 | 97 | 100 | 100 | 100 | 100 | 100 | 100 | 97 |
| 28 | 1.51 | 7.48 | 82 | 90 | 97 | 97 | 100 | 94 | 95 | 1.51 | 7.48 | 82 | 90 | 97 | 97 | 100 | 94 | 95 | 91 | 92 |
| 29 | 2.05 | 7.77 | 97 | 99 | 100 | 100 | 100 | 100 | 100 | 2.05 | 7.77 | 97 | 99 | 100 | 100 | 100 | 100 | 100 | 100 | 98 |
| 30 | 1.90 | 7.84 | 99 | 100 | 100 | 100 | 100 | 100 | 100 | 1.90 | 7.84 | 99 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 97 |
| 33 | 2.59 | 9.61 | 86 | 89 | 90 | 93 | 93 | 96 | 100 | 2.89 | 9.91 | 83 | 86 | 87 | 90 | 90 | 93 | 97 | 98 | 100 |
| 35 | 2.15 | 9.98 | 97 | 99 | 99 | 99 | 99 | 100 | 100 | 2.15 | 9.98 | 97 | 99 | 99 | 99 | 99 | 100 | 100 | 100 | 99 |
| 36 | 1.76 | 10.01 | 94 | 97 | 96 | 97 | 97 | 100 | 99 | 1.76 | 10.01 | 94 | 97 | 96 | 97 | 97 | 100 | 99 | 100 | 96 |
| 37 | 3.78 | 13.39 | 96 | 97 | 98 | 99 | 100 | 100 | 100 | 3.78 | 13.39 | 96 | 97 | 98 | 99 | 100 | 100 | 100 | 100 | 99 |
| 38 | 2.93 | 14.44 | 99 | 99 | 99 | 100 | 100 | 100 | 98 | 2.93 | 14.44 | 99 | 99 | 99 | 100 | 100 | 100 | 98 | 97 | 95 |
| 39 | 3.13 | 14.86 | 99 | 99 | 100 | 99 | 100 | 99 | 97 | 3.13 | 14.86 | 99 | 99 | 100 | 99 | 100 | 99 | 99 | 99 | 97 |
| 40 | 4.38 | 14.86 | 89 | 92 | 91 | 92 | 93 | 100 | 100 | 4.38 | 14.86 | 89 | 92 | 91 | 92 | 93 | 100 | 100 | 94 | 93 |
| 42 | 5.05 | 17.29 | 87 | 87 | 87 | 88 | 88 | 89 | 100 | 5.22 | 17.46 | 86 | 86 | 86 | 87 | 87 | 88 | 99 | 100 | 100 |
| 43 | 5.59 | 17.50 | 99 | 99 | 99 | 100 | 100 | 100 | 100 | 5.59 | 17.50 | 99 | 99 | 99 | 100 | 100 | 100 | 100 | 100 | 100 |
| 44 | 4.51 | 18.73 | 97 | 98 | 98 | 98 | 96 | 96 | 100 | 4.51 | 18.73 | 97 | 98 | 98 | 98 | 96 | 96 | 100 | 99 | 98 |
| 46 | 4.06 | 17.01 | 94 | 97 | 99 | 100 | 100 | 100 | 100 | 6.38 | 19.33 | 83 | 85 | 86 | 88 | 86 | 87 | 86 | 86 | 100 |
| 47 | 5.15 | 22.84 | 95 | 96 | 96 | 97 | 97 | 100 | 100 | 5.15 | 22.84 | 95 | 96 | 96 | 97 | 97 | 100 | 100 | 100 | 96 |
| 49 | 7.45 | 24.04 | 84 | 88 | 90 | 92 | 94 | 97 | 100 | 8.45 | 25.04 | 81 | 85 | 87 | 88 | 91 | 94 | 96 | 97 | 100 |
| 50 | 5.70 | 26.56 | 98 | 99 | 99 | 100 | 100 | 100 | 100 | 5.70 | 26.56 | 98 | 99 | 99 | 100 | 100 | 100 | 100 | 100 | 100 |
| | Mean: | | 92 | 94 | 95 | 97 | 97 | 99 | 99 | | | 91 | 93 | 95 | 96 | 96 | 98 | 98 | 98 | 97 |
| | Standard Deviation: | | 5.6 | 4.8 | 3.8 | 3.3 | 2.9 | 2.2 | 1.5 | | | 6.2 | 5.4 | 4.6 | 4.1 | 3.9 | 3.0 | 2.4 | 2.8 | 2.9 |

* Total minus 120-minute direct bilirubin.
† Total bilirubin read 15 minutes after addition of alcohol.
‡ Total minus 120-minute direct bilirubin.
§ Total bilirubin read 30 minutes after addition of alcohol.

bilirubin in serum, presumably a single compound, is diphasic rather than parabolic (Figure 1, Table V). The initial phase of the reaction, however, was very much more rapid than that of either direct serum bilirubin or pure bilirubin in alcohol-chloroform solution.

In an analysis of the factors responsible for the variability of the reaction curves of bilirubin in the direct reaction of serum, the speed of diazotization appeared to be related to the total bilirubin concentration and to the type of jaundice (Table VI). Alcohol-chloroform solutions of pure bilirubin showed a similar increase in the rate of diazotization as the concentration of bilirubin rose (Tables III and VI), but it was obvious from the wide range of rates at both high and low concentrations in the direct reaction (Table II), and from the unusually rapid rates in the indirect reaction at very low concentrations (Table V), that other factors must have been important in deter-

TABLE VI

*Relation of azobilirubin development rate to bilirubin concentration and type of jaundice*

| | Per cent of azobilirubin developed | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 *min.* | 1½ *min.* | 2 *min.* | 3 *min.* | 4 *min.* | 5 *min.* | 7½ *min.* | 10 *min.* | 15 *min.* | 20 *min.* | 30 *min.* | 45 *min.* | 60 *min.* | 75 *min.* | 90 *min.* | 105 *min.* | 120 *min.* |
| *Direct Reaction in Serum* | | | | | | | | | | | | | | | | | |
| *Total bilirubin Concentration* | | | | | | | | | | | | | | | | | |
| 1.23–4.57 *mg. per cent* | | | | | | | | | | | | | | | | | |
| Mean: | 52 | 59 | 61 | 66 | 69 | 70 | 73 | 76 | 79 | 82 | 86 | 91 | 94 | 96 | 97 | 99 | 100 |
| Standard Deviation: | 11.1 | 11.4 | 10.4 | 11.0 | 10.7 | 10.7 | 9.9 | 10.1 | 8.9 | 8.8 | 6.9 | 5.0 | 3.9 | 3.2 | 1.7 | 1.3 | 0.6 |
| 5.06–26.56 *mg. per cent* | | | | | | | | | | | | | | | | | |
| Mean: | 63 | 71 | 76 | 80 | 83 | 85 | 87 | 89 | 91 | 93 | 96 | 97 | 98 | 99 | 99 | 99 | 99 |
| Standard Deviation: | 7.2 | 6.2 | 5.9 | 5.0 | 4.6 | 4.2 | 3.4 | 3.1 | 2.6 | 2.3 | 1.9 | 1.5 | 1.2 | 1.1 | 0.8 | 0.7 | 1.0 |
| t* | 3.94 | 4.40 | 5.67 | 5.43 | 5.60 | 6.05 | 6.20 | 5.58 | 5.78 | 5.56 | 6.50 | 5.36 | 4.54 | 4.29 | 4.72 | — | 1.43 |
| *Type of jaundice* | | | | | | | | | | | | | | | | | |
| Parenchymatous† | | | | | | | | | | | | | | | | | |
| Mean: | 52 | 60 | 64 | 68 | 71 | 73 | 76 | 78 | 81 | 83 | 87 | 92 | 94 | 96 | 98 | 99 | 100 |
| Standard Deviation: | 10.4 | 11.3 | 11.7 | 12.3 | 11.9 | 12.1 | 11.1 | 10.8 | 9.5 | 9.1 | 7.3 | 4.8 | 3.7 | 3.2 | 1.8 | 1.0 | 0.3 |
| Obstructive‡ | | | | | | | | | | | | | | | | | |
| Mean: | 61 | 69 | 73 | 78 | 81 | 83 | 86 | 88 | 90 | 92 | 95 | 97 | 98 | 99 | 99 | 99 | 99 |
| Standard Deviation: | 7.1 | 5.7 | 5.4 | 4.7 | 4.3 | 4.0 | 3.6 | 3.5 | 3.2 | 2.8 | 2.6 | 2.3 | 1.7 | 0.3 | 1.3 | 0.8 | 0.9 |
| t* | 3.12 | 3.06 | 3.10 | 3.19 | 3.44 | 3.40 | 3.78 | 3.78 | 3.93 | 4.11 | 4.52 | 4.06 | 4.33 | 4.06 | 1.93 | — | 1.43 |
| *Alcohol-Chloroform Solutions* | | | | | | | | | | | | | | | | | |
| *Concentration (in reaction mixture)* | | | | | | | | | | | | | | | | | |
| 0.08–0.32 *mg. per cent* | | | | | | | | | | | | | | | | | |
| Mean: | 49 | 54 | 57 | 61 | 65 | 69 | 75 | 80 | 85 | 90 | 94 | 96 | 97 | 98 | 98 | 99 | 99 |
| Standard Deviation: | 5.1 | 4.2 | 3.6 | 2.8 | 2.1 | 2.2 | 1.5 | 1.8 | 2.1 | 1.9 | 2.1 | 2.1 | 1.8 | 1.2 | 1.5 | 0.9 | 1.1 |
| 0.40–0.80 *mg. per cent:* | | | | | | | | | | | | | | | | | |
| Mean: | 57 | 60 | 62 | 65 | 68 | 71 | 76 | 81 | 87 | 91 | 96 | 98 | 99 | 100 | 99 | 99 | 99 |
| Standard Deviation: | 1.9 | 2.2 | 1.8 | 1.9 | 1.7 | 1.6 | 1.8 | 1.7 | 1.7 | 2.0 | 1.9 | 1.6 | 0.9 | 0.5 | 0.7 | 0.7 | 0.8 |
| t§ | 3.91 | 4.08 | 4.03 | 3.81 | 3.53 | 2.44 | 1.40 | 1.33 | 2.40 | 1.18 | 2.30 | 2.51 | 3.25 | 5.09 | 1.92 | — | — |

* Values greater than 2.75 correspond to a P value of less than 0.01 and indicate a highly significant difference between means.

† Twenty cases in which diagnosis was established with reasonable certainty. Mean total serum bilirubin 5.49 ± 4.1 mg. per cent.

‡ Nineteen cases in which diagnosis was established with reasonable certainty. Mean total serum bilirubin 11.79 ± 7.6 mg. per cent.

§ Values greater than 2.85 correspond to a P value of less than 0.01 and indicate a highly significant difference between means.

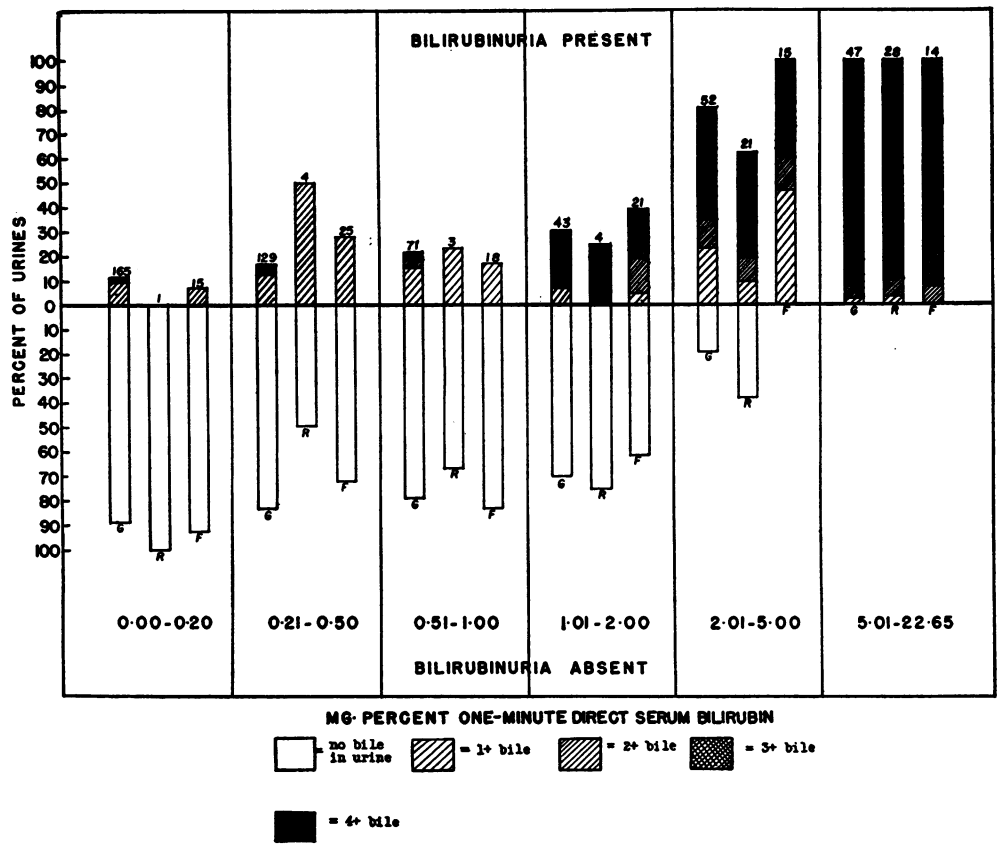


FIG. 4. RELATION OF ONE-MINUTE SERUM BILIRUBIN CONCENTRATION TO THE CONCENTRATION OF BILIRUBIN IN URINE

G = group as a whole. R = jaundice increasing. F = jaundice subsiding.
Number of urines tested recorded at head of each column.

mining the speed of diazotization. The more rapid diazotization of serum in obstructive than in parenchymatous jaundice was probably related to the significantly higher concentration of total bilirubin in the former (Table VI).

*Relation of the one-minute bilirubin level in serum to the renal excretion of bilirubin.* The results of 507 simultaneous blood and urine analyses are summarized in Figures 4 and 5. Considering the group as a whole (column G, Figure 4) it can be seen that bilirubinuria occurred with increasing frequency and intensity as the blood level of one-minute bilirubin rose, but there did not appear to be a renal threshold for one-minute bilirubin, since bilirubinuria occurred, in some instances, at blood levels within the normal range, and failed to appear in others at more than 10 times that level. Since it has been suggested that the threshold may be variable, and in hepatitis, at least, related to the stage of the disease (1), the relationship between serum one-minute bilirubin and bilirubinuria was compared in individuals with increasing and decreasing jaundice. It can be seen in Figure 4 (columns R and F) that there is no significant difference between the two groups. The same is also true when hepatitis, cirrhosis and extrahepatic biliary obstruction are compared (Figure 5). It is to be noted that when the serum level of one-minute bilirubin exceeded 5.0 mg. per cent bilirubin invariably appeared in the urine. The significance of this observation will be discussed later.

All the urine analyses in this laboratory are carried out by a well-trained technician employing the method of Sparkman (39). Only rarely have weakly positive tests for bile been observed in normal individuals. To test the reliability of the method, however, especially in those instances

where the test was positive for bile in the presence of a normal one-minute serum bilirubin level, and to compare these results with Watson's, a series of 350 successive urines were tested by both Sparkman's technique and by the Watson-Hawkinson barium-strip method (40). In 249 instances both tests were negative for bile, in 46 both tests were positive, and in 55 the test was positive by Sparkman's method and negative by the barium-strip method. That the discrepancy between the two methods was due to the greater sensitivity of Sparkman's technique, rather than to an inordinate number of false-positive results is suggested by the following: (*1*) all the urines tested were obtained from patients with proved or suspected liver disease, (*2*) when both tests were positive, Sparkman's invariably gave the stronger reaction, and (*3*) of the 40 individuals with a positive Sparkman and negative barium-strip test who had simultaneous serum bilirubin determinations, 20 had abnormally high bilirubin levels, six with one-minute values over 1.0 mg. per cent (maximum 8.4), and nine with total bilirubin levels over 2.0 mg. per cent (maximum 16.73).

It is well known that urine bilirubin decomposes on standing, especially in sunlight. Sivó and Forrai (41) have found that with mild bilirubinuria the Gmelin test may become negative within three hours in the shade, and within one hour in sunlight. Most of the urines in the present study were exposed to ordinary room light in the laboratory for one to three hours, so that mild degrees of bilirubinuria may have escaped detection. It is doubtful that elimination of this error would have influenced the results to any great extent, since adding one-plus positive at the expense of negative tests would not have changed

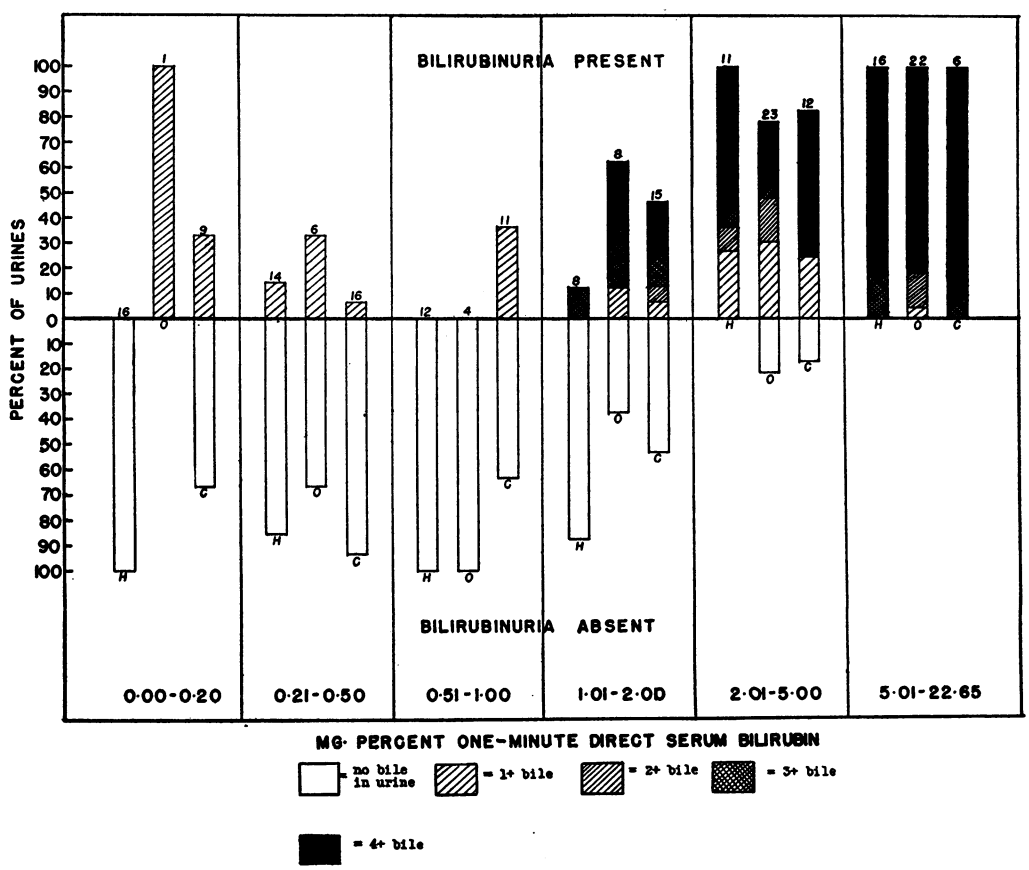


FIG. 5. RELATION OF ONE-MINUTE SERUM BILIRUBIN CONCENTRATION TO THE CONCENTRATION OF BILIRUBIN IN URINE IN VARIOUS TYPES OF JAUNDICE

H = hepatitis. O = obstructive jaundice. C = cirrhosis.

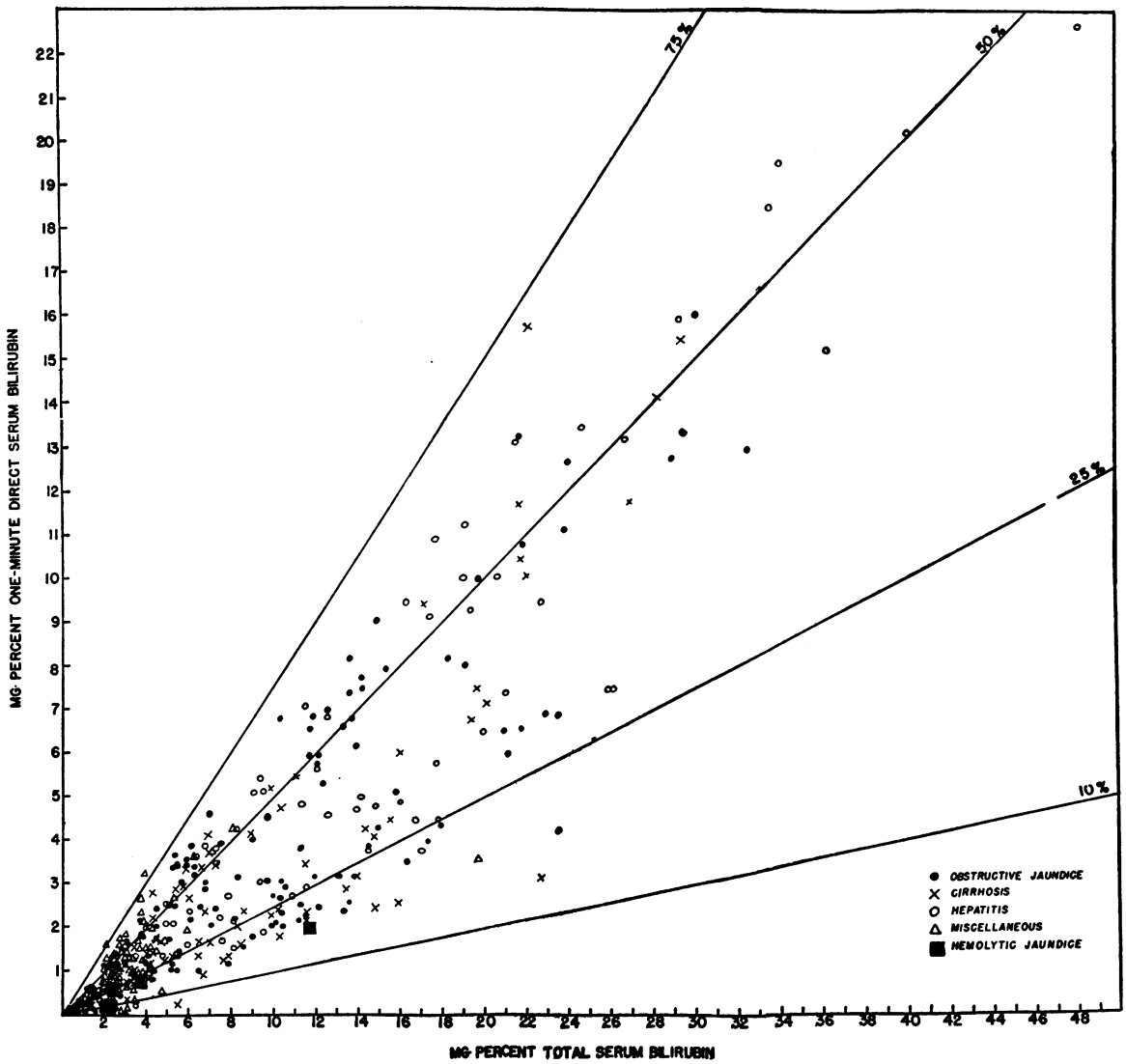


FIG. 6. RELATION OF ONE-MINUTE TO TOTAL SERUM BILIRUBIN CONCENTRATION IN VARIOUS TYPES OF JAUNDICE

the range of one-minute serum bilirubin values over which bilirubinuria occurred.

*The clinical significance of the one-minute serum bilirubin level.* In Figure 6 are plotted the values for one-minute and total serum bilirubin in 400 random blood samples submitted to the laboratory for analysis during a two-year period. Several hundred other observations made during the same period are not included because the values fell within the normal range, or slightly above, and could not be plotted without obscuring the details of the figure. It is evident that over a wide range of values there is a more or less direct relationship between the one-minute and total serum bilirubin levels, and that the range of ratios between the two is wide at low concentrations and tends to narrow at high concentrations. There is a suggestion, moreover, that the ratio tends to increase with the concentration of total bilirubin, although the number of observations at very high levels is too small to establish this point with certainty.

It is also evident from Figure 6 that there is no significant difference in either the concentrations of one-minute bilirubin or in the one-minute: total bilirubin ratios in extrahepatic obstruction, cirrhosis, hepatitis, and a variety of miscellaneous

conditions, including congestive failure, anemia, and infection. Unfortunately only four bloods from patients with hemolytic jaundice were available for study. All showed relatively low one-minute bilirubin concentrations and low one-minute: total bilirubin ratios. However, it can be seen from Figure 6 that these values fall within the lower limits of the range observed in other types of jaundice, and so are of little value in differentiating this type of jaundice from the others.

## COMMENT

From the data presented it appears that the one-minute bilirubin level is an arbitrarily selected point on the ascending limb of a variable diphasic curve representing azobilirubin development in the direct reaction of serum. While the ratio of one-minute: total bilirubin may serve as an index of the speed of the reaction during its initial rapid phase, the evidence does not support the contention that the one-minute fraction is a measure of a distinct type of bilirubin. Gray and Whidborne (9) and more recently Deenstra (42) have come to essentially the same conclusion.

The conclusion that there are two types of bilirubin in serum was based on the observation that the reaction curve for direct azobilirubin development was diphasic, and on the assumption that the reaction curve for a single bilirubin would be parabolic (1). The clearly diphasic character of the reaction curves for known single types of bilirubin in the experiments being reported, either in the form of alcohol-chloroform solutions of pure bilirubin, or as indirect bilirubin in alcohol-serum mixtures, while not necessarily identical with that of a hypothetical single bilirubin in serum alone, casts doubt on the validity of this assumption. Unfortunately the solubility of bilirubin is such that the preparation of a simple solution in serum is not possible without the introduction of strong alkali, alcohol or chloroform, all of which may influence the rate of diazotization.

As for the diphasic character of the reaction curve of azobilirubin development in the direct reaction of serum, it has been shown to be inconstant, and, when present, to be of variable contour. This is difficult to explain on the basis of varying proportions of two types of bilirubin in serum. If such were the case, each type might be expected to exhibit a distinctive reaction curve. Yet, both in the present experiments and in those of Gray and Whidborne (9), indirect bilirubin, one of the two types, showed a variability in its speed of diazotization similar to that of the presumed mixture of two bilirubins in the direct reaction. A number of other experiments may be cited as further evidence against the theory that fractionation of serum bilirubin based on the van den Bergh reaction measures two types of bilirubin in serum. Thus, Cantarow (10) found that when two sera containing different proportions of direct and indirect bilirubin were mixed, the final concentrations observed were different from those predicted for solutions containing two types of bilirubin. Gray and Whidborne (9) demonstrated that the rate of diazotization of pure bilirubin added to serum depended on the clinical state of the patient from whom the serum was obtained. These and other experiments suggest that the rate of diazotization may depend on chemical or physical factors in the serum, rather than on differences in the chemical nature of bilirubin. The striking difference in the rate of diazotization of bilirubin in alcohol-chloroform solutions and in serum-alcohol mixtures (indirect reaction) observed in the present experiments illustrates the importance of the solvent in which the reaction occurs.

The factors governing the speed of diazotization of bilirubin in serum are not fully understood. It is well recognized that the concentration of total bilirubin is an important factor in determining the type of visual or *qualitative* van den Bergh reaction obtained (43). Thus, a prompt direct reaction can be converted to a biphasic, and then to a delayed direct by simple serial dilution of serum (44, 45), suggesting a decreased rate of diazotization. However, Deenstra (42) has pointed out the important fact that the color development apparent to the naked eye depends both on the speed of diazotization and on the concentration of bilirubin present, so that a prompt direct reaction may occur either with a high concentration of bilirubin reacting slowly, or with a low concentration reacting rapidly. Moreover, Gray and Whidborne (43) have shown that, in the dilution employed in the *qualitative* van den Bergh reaction, concentrations of azobilirubin may be attained which fall outside the range in which Beer's law holds, and have pointed out the impor-

tance of this factor in determining the type of visual reaction observed. In *quantitative* studies of azobilirubin development employing a photoelectric colorimeter, however, the serum is always diluted sufficiently to avoid this effect. It must be recognized, therefore, that the results of the visual and colorimetric van den Bergh determinations are not comparable. Nevertheless, the results of the present experiments suggest that the concentration of bilirubin is also a factor in determining the speed of diazotization when the reaction is studied colorimetrically, although there are reasons to believe that other factors play an important role.

The physiological basis for differentiating two types of bilirubin in serum appears to be as uncertain as the chemical. The evidence indicating that the prompt direct type represents regurgitated bilirubin is largely circumstantial. In experimental obstructive jaundice the *qualitative* van den Bergh reaction is indirect early and changes to biphasic and then to prompt direct as the jaundice deepens (46–48), a finding to be expected in the light of Gray and Whidborne's work (9), but difficult to explain if the prompt direct reaction measures regurgitated bilirubin. In Gonzales-Oddone's experiments (17) there was a prompt increase in the one-minute bilirubin fraction in thoracic duct lymph following biliary obstruction. While this has been cited as evidence that a special type of bilirubin was being regurgitated from the biliary tree into the lymph and thence into blood (37), the possibility cannot be excluded that bilirubin was passing from the sinusoids to the lymph directly, without passing through the liver cells or bile capillaries, and that its rapid diazotization rate, indicated by the marked increase in the one-minute fraction, was due to the appearance or accumulation in hepatic lymph of other substances affecting the speed of diazotization. Similarly, the high proportion of one-minute bilirubin in fistula bile, cited as evidence for the specificity of excreted bilirubin (37), might well be related to the high concentration of bilirubin in bile and to other chemical and physical properties of bile affecting its rate of diazotization. Convincing clinical evidence that the one-minute serum bilirubin fraction has been regurgitated from the biliary tree is likewise lacking. Even if hepatocellular damage were always accompanied by some degree of bile regurgitation, a significantly higher proportion of prompt direct bilirubin might be expected in the serum of obstructive than in hepatocellular jaundice. Yet, no such difference has been demonstrated in the present study, based on one-minute: total serum bilirubin ratios, or in other investigations based on the *qualitative* van den Bergh reaction (3, 4, 45).

The mechanisms underlying the renal excretion of bilirubin are still obscure. Since all of the bilirubin in serum is protein-bound (11, 14, 26–28) and that in urine protein-free (11), it is probable that excretion occurs predominantly by way of the tubules rather than by glomerular filtration (34). The most perplexing problem is the relationship of the urinary excretion of bilirubin to its concentration in blood. The most striking feature of this relationship, usually expressed as the "renal threshold," is its extreme variability which is evident not only in the several types of jaundice (1, 3, 4), but also during the development and recession of jaundice (1), in individual (1) and species (49) differences, and in relation to the functional state of the kidneys (50). Attempts have been made to correlate the renal excretion of bilirubin with its concentration in serum, with its chemical or physical state in serum, and with the functional capacity of the kidney. Since van den Bergh's time a relationship between serum concentration and urinary excretion of bilirubin has been recognized, and several "threshold" values have been reported, depending on the methods of analysis employed (3, 4, 12). The failure of bilirubin to appear in the urine in hemolytic jaundice, even at high serum levels, has made it clear that serum concentration cannot be the only factor involved, and it has been suggested that there is a "renal threshold" for direct bilirubin but that the kidney is impermeable to indirect bilirubin (3, 4, 7). This conclusion, based on the *qualitative* van den Bergh reaction, has not been confirmed by either With (12) or Watson (1), but these investigators differ in their interpretation of the findings, especially in hemolytic jaundice. With (12, 13, 51) has found the "renal threshold" for total serum bilirubin to be approximately 5.0 mg. per cent, and has come to the conclusion that the absence of bilirubinuria in hemolytic jaundice may be related to the rarity of such levels in that disease, and that there is in-

adequate evidence to prove that indirect bilirubin is not excreted by the kidney. Watson (1), on the other hand, has observed several cases of hemolytic jaundice with total bilirubin levels well above the "threshold" reported by With without bilirubinuria, and has concluded that the original suggestion that there is a "renal threshold" for direct serum bilirubin but not for indirect is essentially correct, but that the failure to find a good correlation in the past has been due to the method of measuring direct bilirubin. More recently Watson has asserted that there is no fixed renal threshold for bilirubin, but that the "prompt reacting bilirubin, or sodium bilirubinate, gains access to urine with relative ease, whereas the bilirubinglobin probably does not pass through the glomerulus at least from those concentrations at which it is found in the circulating blood (52)."

The failure of bilirubin to appear in the urine in occasional cases of obstructive jaundice, accompanied by severe impairment of renal function (50) suggests that, under some circumstances at least, the "renal threshold" for bilirubin may be related to the functional capacity of the kidney. This has led to attempts to correlate the variability of the "renal threshold" of bilirubin in various types of jaundice with renal function. Thus, Rissel (35) has reported a greater renal clearance of bilirubin in mechanical obstructive than in hepatocellular jaundice, which can be correlated with differences in creatinine clearance. This difference in bilirubin clearance has been confirmed by Enachesco, Comanesco and Zamfiresco (53), but not by With (12), who has also failed to find a significant correlation between bilirubin and urea clearances (34).

Another factor which may be of some importance in connection with the renal excretion of bilirubin is the concentration of bile salts present in blood. Intravenously injected bilirubin is ordinarily not excreted into the urine (54, 55) unless bile salts are injected simultaneously (56). The action of bile salts in facilitating the urinary excretion of bilirubin is not well understood, and has been attributed both to its effect on the conversion of indirect to direct bilirubin in serum (57), and to a direct effect on the kidney (58).

In the present study the range of one-minute serum bilirubin concentrations at which urinary excretion of bilirubin occurred was so wide that it is difficult to regard the former as a "threshold substance." Although bilirubin excretion occurred with increasing frequency and intensity as the one-minute serum bilirubin level rose, there was a concomitant increase in the concentration of total bilirubin and conceivably of other substances which might affect the kidney directly, so that the evidence for a functional relationship between the one-minute serum bilirubin level and its excretion by the kidney is by no means convincing.

As indicated above neither the absolute values of one-minute bilirubin nor its ratio to total serum bilirubin is of value in the differential diagnosis of jaundice, except that a high ratio almost certainly excludes uncomplicated hemolytic jaundice. Watson agrees that the one-minute bilirubin determination is of little value in the differential diagnosis of jaundice, but has recently expressed the opinion that its real value lies in the diagnosis of early biliary obstructive and parenchymal liver disease, conditions in which he frequently finds a two- to four-fold increase in the one-minute fraction, even before there is a significant rise in the total serum bilirubin level, and in chronic liver and biliary tract disease without jaundice, in which an elevated one-minute serum bilirubin concentration may be even more decisive in the diagnosis than that of the total (37). There have been too few instances of an elevated one-minute bilirubin level without an increase in total serum bilirubin in this laboratory to permit an evaluation of such observations. We would agree with Deenstra (42), however, in interpreting such an increase as indicative of a change in the serum accelerating diazotization, rather than of an accumulation of regurgitated bilirubin.

## CONCLUSIONS

1. The evidence presented does not support the view that the one-minute serum bilirubin fraction is a chemically distinct entity, representing regurgitated bilirubin, which can be differentiated from indirect bilirubin by its physico-chemical properties and by its physiological behavior in the kidney.

2. The one-minute bilirubin level is an arbitrarily selected point on the ascending limb of a variable diphasic curve representing the rate of azobilirubin development in the direct van den Bergh reaction of serum.

3. The one-minute: total serum bilirubin ratio is an index of the rate of diazotization in the direct reaction and tends to be low in hemolytic jaundice, but is of limited value in the differential diagnosis of jaundice because of the wide range of overlapping values in biliary obstruction and hepatocellular disease.

4. The evidence suggests that the rate of diazotization is determined by the concentration of bilirubin and by chemical or physical factors in serum, and not by the presence of variable mixtures of two types of bilirubin having different reaction rates.

5. The urinary excretion of bilirubin does not appear to be determined by the one-minute serum bilirubin level.

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