Further Studies of the Van den Bergh Reaction

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Azobilirubin production from sera of patients with regurgitation jaundice* (acute hepatitis and obstructive jaundice) was found under the conditions of the modified Rappaport & Eichhorn method (1943) to be maximal within 30 min. in both the direct and the indirect reaction (Gray & Whidborne, 1946). Sera from the few cases of retention (haemolytic) jaundice studied gave two forms of azobilirubin production-time curve; in one, azobilirubin production in the direct reaction was low and delayed, while in the other it was high and more rapid, requiring only 0.5 hr. to reach a maximum. In both, azobilirubin production in the indirect reaction was maximal only after about 5 hr. The present paper presents further observations of azobilirubin production from sera from patients with regurgitation jaundice and describes the results given by a larger series of sera from patients with retention jaundice. These last results suggested that differences in the diazo reaction of bilirubin-containing sera might be due to the presence in some of a substance or substances able to catalyze the formation of azobilirubin. It therefore appeared to be of value to investigate the diazo reaction of bilirubin when added to sera likely to contain pathological constituents or physiological constituents in excess.

MATERIALS

The nature of the material investigated is summarized together with the corresponding results in Table 1. Preliminary investigations showed no difference in the behaviour of sera or plasma in the diazo reaction, and since greater yields of plasma

than of serum can be obtained from samples of blood, many of the experiments have been carried out on plasma. In the account which follows, the term serum will be used for the material investigated irrespective of whether it was in fact serum; plasma or pleural fluid. All materials contained at least 1.5 mg. bilirubin/100 ml. The nature of the sera to which bilirubin was added is indicated together with the results given by such mixtures in Table 3.

METHODS

The rates of azobilirubin production were determined by the modified Rappaport & Eichhorn procedure previously described (Gray & Whidborne, 1946) but using a Spekker photoelectric absorptiometer and 0.5, 1.0 or 2.0 cm. cells according to the amount of bilirubin present in the serum. With sera from patients with retention jaundice, azobilirubin production in the direct reaction (i.e. in the absence of caffeine and citric acid) usually attained its maximum in less than 5 hr., but a few sera failed to give a constant value within this time. They often became opalescent on further standing, and the reading for the direct reaction was therefore never followed for longer than 5 hr. More rarely, the azobilirubin solution would fade before the 5 hr. had elapsed and the extent of the direct reaction has then been calculated from the maximum reading of the optical density. In the indirect reaction (i.e. in the presence of caffeine and citric acid), azobilirubin production was usually maximal within 6 hr. but occasionally not until 18–24 hr. when readings were continued for this length of time.

From these readings we calculated the direct-indirect quotient (D.I.Q.) defined as:

$$\frac{\text{final amount of azobilirubin formed in the direct reaction}}{\text{final amount of azobilirubin formed in the indirect reaction}} \times 100.$$

For simplicity of presentation and discussion we have also introduced two additional terms which it is necessary to define:

$$(1) \text{ The direct-quotient (D.Q.)}_t = \frac{\text{amount of azobilirubin formed in the direct reaction at time } t}{\text{final amount of azobilirubin formed in the indirect reaction}} \times 100.$$

Graphs of (D.Q.)_t plotted against time therefore record the course of the direct reaction.

$$(2) \text{ The indirect-quotient (I.Q.)}_t = \frac{\text{amount of azobilirubin formed in the indirect reaction at time } t}{\text{final amount of azobilirubin formed in the indirect reaction}} \times 100.$$

Graphs of (I.Q.)_t plotted against time therefore record the course of the indirect reaction.

* In this article the nomenclature used will be that of Rich (1930) who classified jaundice into retention and regurgitation types. Retention jaundice corresponds to haemolytic jaundice but regurgitation jaundice is subdivided into mechanical and parenchymal types corresponding to obstructive and hepatogenous jaundice respectively.

In many experiments, azobilirubin production was also studied in the presence of the same buffers as ordinarily used but from which urea had been omitted. Urinary urobilin and urobilinogen were measured in a few cases by the method of Watson (1936). Bilirubin was added to serum by dissolving 3 mg. of pure bilirubin in 3 ml. of 0.2N-sodium carbonate at 80°. After cooling, 1 ml. of this solution was added to 9 ml. of serum. The bilirubin content of each serum was determined before and after the addition of the bilirubin, and the $(D.Q.)_t$ and $(I.Q.)_t$ followed at the usual intervals of time in the presence and absence of urea. Comparison solutions were prepared in which water replaced serum.

both the direct and indirect reactions occasionally requires between 0.5 and 1 hr. to reach its maximum instead of less than 0.5 hr. as previously reported but over 90 % of the maximum value has always been attained within 0.5 hr.

(b) *Retention jaundice.* Only two sera and two pleural fluids were found in which the D.I.Q. as measured by the Rappaport & Eichhorn method was less than 50. One serum was from a patient who had been transfused with haemolyzed blood, the other from a patient with non-familial haemolytic jaundice. The $(D.Q.)_t$ time and $(I.Q.)_t$ time curves given by these materials are presented in Figs. 1a, b, c and d.

Fig. 1. Four observed $D.Q._t$ time and $I.Q._t$ time curves. D.I.Q. less than 50 with each serum. (a) Serum from patient transfused with haemolyzed blood. (b) Serum from patient with non-familial haemolytic jaundice. (c) Pleural fluid. (d) Pleural fluid.

Fig. 2. Four observed $D.Q._t$ time and $I.Q._t$ time curves. D.I.Q. greater than 50, $T_{75\% D.Q.}$ less than 0.5 hr.

Fig. 3. Four observed $D.Q._t$ time and $I.Q._t$ time curves. D.I.Q. greater than 50, $T_{75\% D.Q.}$ greater than 0.5 hr. (a) and (b) Sera from patient with non-familial haemolytic jaundice. (c) Pleural fluid. (d) Serum from patient with constitutional non-haemolytic jaundice.

Figs. 1-3. Ordinates = $(D.Q.)_t$ or $(I.Q.)_t$. Abscissae = time in hours. $D.Q._t$ time curve - - - - - $I.Q._t$ time curve —.

RESULTS

Form of the $(D.Q.)_t$ and $(I.Q.)_t$ time curves

(a) *Regurgitation jaundice.* Further study of cases of regurgitation jaundice has shown that when the bilirubinaemia is very high (i.e. above 15 mg./100 ml.) or when the jaundice has persisted for longer than 3 weeks the azobilirubin production in

All remaining materials gave D.I.Q. values between 50 and 100 but no correlation could be found between the value of the D.I.Q. and the type of fluid examined. In all but four (see below), the $(D.Q.)_t$ time curve was steep initially, $(D.Q.)_t$ reaching 75 % of its final value within 0.5 hr., the curves then flattening gradually as the $(D.Q.)_t$ approached its maximum and final value. Such curves resemble the $(D.Q.)_t$ time curves

observed with sera from patients with regurgitation jaundice. The (i.q.)_t curves have varied considerably ranging from a uniformly gradual slope (Fig. 2a) to the steep beginning and gradual termination of Fig. 2d, with the intermediate forms represented in Fig. 2b and c. The curves have been arbitrarily classified as types 2a, b, c and d according to whether the time ($T_{75\% \text{ i.q.}}$) taken for the (i.q.)_t to reach 75 % of its final value has been 3, 1.5-3, 0.5-1.5 or less than 0.5 hr.

The four exceptional materials referred to above in which the (D.Q.)_t failed to reach 75 % of its final value within 0.5 hr., although the D.I.Q. value was greater than 50, included sera from two cases of non-familial haemolytic jaundice, one of constitutional non-haemolytic jaundice and one pleural fluid. The four pairs of curves obtained are shown in Fig. 3a, b, c and d. In each there was delay in the rate of formation of azobilirubin in the direct reaction as well as in the indirect reaction.

Sera have been examined from seven cases of haemolytic jaundice following the transfusion of incompatible blood. At the height of the jaundice, i.e. 1 or 2 days after the transfusion, curves of the type 2a or b were obtained, but in the course of a few days the form of curve changed to type 2d resembling that obtained in regurgitation jaundice. Similar changes in the form of curve have been observed in two cases of severe haemolytic jaundice of very acute onset. The detailed distribution of the types of curve yielded by the various types of case is summarized in Table 1.

rubin production is also retarded. With sera from patients with regurgitation jaundice, the decrease in D.I.Q. usually amounted to only 5 % or less and the general form of curve was unaltered. One serum, however, showed a difference of 12 %. On the other hand, with sera from patients with retention jaundice giving forms of curve other than of type 2d, omission of urea greatly lowers the D.I.Q. and considerably retards the rate of azobilirubin production so that the general form of the (D.Q.)_t time curve is profoundly altered. The final concentration of urea in the direct reaction is 18.7 g./100 ml. which of course far exceeds the concentrations likely to be encountered in pathological sera. It has not been possible to compare accurately the effect of dilution by the phosphate buffer on the course of the direct reaction since when the diazo reaction was performed on undiluted sera an opalescence rapidly appeared preventing accurate photoelectric measurement of the azobilirubin produced under these conditions. As far as can be judged by eye the course of the direct reaction is not significantly altered by such dilution.

The diazo reaction of bilirubin added to serum

Some difficulty was encountered in the investigation of the solution of bilirubin in water since on the addition of the diazo reagent a brown precipitate appeared which in the case of the direct reaction settled to the bottom of the absorption cell and could not be induced to pass back into solution. A similar precipitate was obtained in the indirect reaction but

Table 1. *Materials investigated and their diazo reactions*

Type of jaundice	No. of cases	No. of sera	Type of curve					
			1	2a	2b	2c	2d	3
Infective jaundice	19	23	0	0	0	0	23	0
Obstructive jaundice	8	9	0	0	0	0	9	0
Chronic hepatitis	4	7	0	0	0	0	7	0
Homologous serum jaundice	2	3	0	0	0	0	3	0
Transfusion reactions	7	22	1	3	4	4	10	0
Familial haemolytic jaundice	11	11	0	1	1	8	1	0
Non-familial jaundice	9	18	1	2	5	6	2	2
Miscellaneous cases with definite haemolytic element but of doubtful origin	5	13	0	2	2	1	8	0
Constitutional non-haemolytic jaundice	1	1	0	0	0	0	0	1
Pleural fluids	11	12	2	2	3	1	3	1

Table 2. *Effect of urea (24 g./100 ml.) in the buffer used for the direct Van den Bergh reaction*

Type of jaundice	No. of cases	D.I.Q.	
		Urea present	Urea absent
Regurgitation	12	77 ± 1.9 (68-91)	75 ± 1.6 (68-84)
Retention	11	78 ± 4.8 (51-100)	35 ± 5.6 (8-78)

Effect of urea on the direct reaction

Table 2 shows that the omission of urea from the direct buffer decreased the D.I.Q. The rate of azobili-

this gradually redissolved during the experiment. It is therefore not surprising that the D.I.Q. amounted to only 27 and that the graphs (D.Q.)_t and (i.q.)_t plotted against time were similar to curve 1d.

Table 3. *Results given by bilirubin added to sera*

Nature of serum	Total bilirubin of serum (mg./100 ml.)		D.I.Q. with urea	$T_{75\% \text{ D.Q.}}$ with urea (hr.)	D.I.Q. without urea	$T_{75\% \text{ D.Q.}}$ without urea (hr.)	$T_{75\% \text{ I.Q.}}$ (hr.)
	Before addition of bilirubin	After addition of bilirubin					
Normal	1.0	11.2	74	1.0	21	2.8	4.1
Normal	0.8	10.8	72	1.5	19	2.0	5.0
Normal	0.4	11.0	67	1.25	20	2.0	4.7
Normal	0.4	11.7	84	1.0	17	2.0	5.5
Normal	0.4	11.7	80	0.8	20	2.0	5.5
Hypercholesterolaemia (nephritis)	0.4	10.6	66	1.9	15	2.0	2.8
Hypercholesterolaemia (nephritis)	0.4	9.8	84	0.5	18	2.0	5.0
Hypercholesterolaemia (myxoedema)	0.4	9.9	85	0.2	24	4.5	5.2
Haemolytic jaundice	3.3	14.8	80	0.1	12	2.0	4.75
Recovery stage of acute hepatitis	2.2	11.8	80	1.25	19	0.5	6
Acute hepatitis	9.8	20.2	57	0.75	41	0.3	2.5
	(D.I.Q. 70)						
Obstructive jaundice	10.2	20.1	66	0.5	40	0.25	2.25
	(D.I.Q. 84)						
Chronic liver damage (cirrhosis)	0.4	10.4	81	0.1	15	1.0	3.3
Chronic liver damage (cirrhosis)	3.7	14.2	80	0.1	22	0.25	2.6
Chronic liver damage (cirrhosis)	0.5	12.0	80	0.1	21	0.25	4.5

With bilirubin added to serum, no precipitation occurred and the results given by such mixtures are summarized in Table 3. The D.I.Q. was always greater in the presence of urea than in its absence, and except for the two experiments in which bilirubin was added to serum already containing an appreciable excess of bilirubin, the differences in D.I.Q. in the presence and absence of urea were of the order of those observed in retention jaundice. In all experiments, the $(\text{I.Q.})_t$ time curves were of the same form, maximum azobilirubin formation requiring from 3 to 8 hr.

$(\text{D.Q.})_t$ curves in the presence of urea. The $(\text{D.Q.})_t$ time curves given by bilirubin added to normal sera, to serum from a patient recovering from acute hepatitis and to the serum from one of the patients with hypercholesterolaemia, were of gentle slope and reached their maxima within 1.1 and 2.7 hr. Bilirubin added to the two other hypercholesterolaemic sera gave a slightly accelerated direct reaction. Bilirubin added to sera from three patients with chronic liver damage showed in each case a marked acceleration of the direct reaction comparable with that represented in Fig. 2*d*.

$(\text{D.Q.})_t$ curves in the absence of urea. Bilirubin added to the normal sera, to all three hypercholesterolaemic sera and to the serum from the patient with haemolytic jaundice gave the delayed reactions of type 1*d*. Addition of bilirubin to sera from two of the three patients with chronic liver damage caused markedly accelerated reactions, but on addition to the serum of the third patient and to that of the recovering hepatitis patient, the acceleration of the direct reaction, though still present was less striking.

The reactions of bilirubin added to the two sera already containing appreciable amounts of bilirubin are of particular interest (see below) and the $(\text{D.Q.})_t$ and $(\text{I.Q.})_t$ time curves have been presented in Fig. 4.

Fig. 4. Observed D.Q._t time curves of bilirubin added to two jaundiced sera already containing approximately 10 mg./100 ml. of bilirubin.

Ordinates = $(\text{D.Q.})_t$ or $(\text{I.Q.})_t$. Abscissae = time in hours.
 D.Q._t time curve ———. I.Q._t time curve - - - -.

DISCUSSION

The reasons for the differences in character of the Van den Bergh reaction as encountered in clinical medicine have long been obscure. Any theory based on one type of experiment has soon been succeeded by another supported equally well by experimental evidence of a different kind. Van den Bergh (1918) originally believed that in serum containing bilirubin giving a negative direct reaction the bilirubin was bound to the serum protein and that the addition of ethanol was therefore necessary to liberate bilirubin from the protein complex. Bennhold (1932) and Pedersen & Waldenström (1937),

however, showed by electrophoretic and ultracentrifuge studies that the bilirubin is bound to the plasma albumin whether the direct reaction is positive or negative. Coolidge (1940) confirmed this and postulated that the indirectly reacting bilirubin was chemically bound by valency bonds, whereas the direct reacting type is simply absorbed loosely. Others have postulated two different forms of bilirubin responsible for the two types of reaction (Fowweather, 1932; Davis & Sheard, 1937; Heilbrun & Hubbard, 1940), while the remaining possibility that the direct reaction might be due to the presence of catalysts in the serum itself has not been without its supporters (Acil & Golgruber, 1932). The present work lends some support to this last theory.

The diazo reaction in retention jaundice

In the early stages of these investigations the only types of direct reaction given by sera from patients with retention jaundice were those represented by Figs. 1*a* and 2*b* and the possibility was considered that the second type of reaction was caused by the presence in the serum of two forms of bilirubin one of which gave (D.Q.)_t and (I.Q.)_t time curves similar to Fig. 1*a* and the other the curves typical of regurgitation jaundice (Fig. 2*d*). Subsequent experience has shown that in retention jaundice many types of reaction occur varying imperceptibly from one extreme to the other.

Fig. 5. Theoretical D.Q._t time and I.Q._t time curves expected from materials giving curves of types 1*a* and 2*d*. (a) 100% 1*a*. (b) 66% 1*a*; 33% 2*d*. (c) 33% 1*a*; 66% 2*d*. (d) 100% 2*d*.

N.B. Ordinates = (D.Q.)_t or (I.Q.)_t. Abscissae = time in hours. D.Q._t time curve - - - - - . I.Q._t time curve — .

If we suppose that there are two types of bilirubin in serum one of which gives the type of reaction 1*a* and the other the type 2*d*, we should expect mixtures of the two to give the series of summation curves represented in Fig. 5. Although one of these (5*b*) somewhat resembles the observed curves of type 3*b*, simple summation cannot account for the other observed curves unless the existence of the two forms of bilirubin in the same serum grossly modifies their individual behaviour under the conditions of the test, which appears unlikely. It is more probable that the gradation in form of the curve is due to the presence of some factor exerting its effect in varying degrees either by direct catalysis of the reaction or by interference with some inhibiting factor present

in sera giving reactions of type 1*a*. This factor appears to accelerate the reaction more readily in the absence of caffeine and citric acid than it does in their presence (i.e. in direct than in indirect reaction), but presumably as its effect becomes more marked the reaction in the presence of these reagents also becomes accelerated to varying degrees, thus accounting for the reactions seen in Fig. 2. With its maximum effect both (D.Q.)_t and (I.Q.)_t time curves are of the forms seen in regurgitation jaundice. Not only rate but also extent of reaction appears to be accelerated by this hypothetical factor (compare D.I.Q. in Figs. 1 and 2). It has long been considered by many workers (Watson, 1937) that haemolytic jaundice is rarely, if ever, purely haemolytic in origin, but that there is usually an associated impairment of hepatic function. As far as is known this impairment of function is usually confined to the function of pigment excretion and it appeared of value, therefore, to determine whether or not change in form of the diazo reaction was accompanied by an increased excretion of urinary urobilin and urobilinogen. The few determinations of these pigments we have been able to make have been consistent with this possibility. It therefore appears possible that in pure retention jaundice with intact hepatic function the direct reaction may follow the course depicted in Fig. 1*a*. If there is associated liver damage then the unknown factor may accumulate

in the blood stream and cause some degree of change in the reaction. This factor would appear to act most effectively in regurgitation jaundice in which direct and indirect reactions of the type 2*d* always occur. A similar series of reactions may be given by haemorrhagic pleural effusions into which the factor may also pass.

The effect of urea on the direct reaction

The difference between the measurements of direct bilirubin by the Malloy & Evelyn (1937) method and by that of Rappaport & Eichhorn (1943) was ascribed in our earlier paper to the difference in pH of the two reaction mixtures but it is now seen that the presence of urea in the latter method also is an

important factor. The effect of the presence of urea on the direct reaction given by sera from patients with regurgitation jaundice is minimal, and the application of our earlier results (Gray & Whidborne, 1946) to the interpretation of the direct reaction in this condition therefore requires no revision. The profound effect of urea on the direct reaction given by sera from patients with retention jaundice indicates that in this condition the results obtained with the Rappaport & Eichhorn technique will not necessarily correspond with the course of the ordinary direct Van den Bergh reaction as usually performed. For this reason the direct reaction of many of the materials used has been investigated both in the presence and the absence of urea, since it is believed that the direct reaction under the latter condition is more representative of the ordinary reaction. The differences observed in the direct reaction given by various haemolytic sera in the presence of urea, however, were sufficiently striking to be worthy of the consideration accorded them in the previous section.

The diazo reaction of bilirubin added to serum

Although in these experiments with bilirubin added to sera, the direct reaction in the absence of urea more closely resembles the customary Van den Bergh reaction, the results obtained in the presence of urea have also been presented in order that they may be considered relative to the earlier work on naturally occurring sera without added bilirubin in the presence of a buffer containing urea.

The addition of bilirubin to normal serum results in a mixture which gives a diazo reaction similar to that given by sera from some patients with retention jaundice, i.e. those giving reactions of type 3. Similar treatment of certain pathological sera as detailed below resulted in accelerated direct diazo reactions both in the presence and in the absence of urea. It is important to note that in the absence of urea neither was the D.I.Q. increased nor was the indirect reaction accelerated in the way characteristic of regurgitation jaundice.

Hypercholesterolaemia. Both ester and free cholesterol were increased in the sera used. On two occasions, there was some acceleration of the direct reaction in the presence of urea, but no effect in its absence, nor on the course of the indirect reaction. Increased cholesterol content cannot therefore be the cause *per se* of the accelerated direct reaction in regurgitation jaundice.

Haemolytic jaundice. The marked acceleration of the direct reaction in the presence of urea of bilirubin added to serum from the patient with haemolytic jaundice may possibly be related to the fact that this serum originally gave a similar direct reaction.

Acute hepatitis. Bilirubin added to serum from the patient recovering from acute hepatitis showed

acceleration of the direct reaction in the absence of urea but paradoxically not in its presence. No acceleration of the indirect reaction resulted, so that if the characteristic nature of the diazo reaction in acute hepatitis is due to the accumulation in the blood of an accelerating factor, this factor must disappear rapidly with the return of the blood bilirubin to normal levels.

Chronic liver damage. Addition of bilirubin to sera of patients with chronic liver damage resulted in considerably accelerated diazo reactions in the presence of urea and in two cases in the absence of urea, but again the (I.Q.)_t time curves were unaltered.

These experiments thus suggest that the factor causing the characteristic Van den Bergh reaction in regurgitation jaundice must be fairly specifically retained in the blood in this condition.

The results obtained with bilirubin added to sera already containing appreciable amounts of bilirubin require consideration in some detail. Fig. 4 shows that the (D.Q.)_t and (I.Q.)_t time curves resemble closely the theoretical curves obtained by a mixture of equal amounts of bilirubin giving two different types of reaction (i.e. curves midway between those represented in Fig. 5*b* and *c*). These results alone in the present series of investigations support the view that there may be two different forms of bilirubin. However, the D.I.Q. of the mixture was less than the average of the values for the two materials.

It is not easy to correlate these results with those of Cantarow (1944) who observed that with the Malloy & Evelyn technique, when obstructive jaundice serum was mixed with haemolytic jaundice serum the observed proportion of direct bilirubin was greater than the theoretical. However, his haemolytic jaundice serum contained only 2.3 mg. bilirubin/100 ml. and of this only 13–22 % took part in the direct reaction. In our opinion, such measurements approach the limit of accuracy for the method and it is questionable whether the difference between theoretical and observed values for direct bilirubin are significant. Barron (1931) observed that the addition of bilirubin to normal sera caused the direct reaction to be negative, biphasic or prompt according to the amount of bilirubin added. Our earlier experiments have shown that in regurgitation jaundice, either delayed, biphasic or prompt reactions were obtained according to the concentration of bilirubin in the serum. Our experiments have also shown that even in haemolytic jaundice a weak direct reaction is always obtained and all the recent literature dealing with measurements of the alleged proportions of direct and indirect bilirubin show that at least 10 % of the total bilirubin takes part in the direct reaction in this condition. Thus, if the amount of bilirubin added is sufficiently large (over 15 mg./100 ml.) 1.5 mg./100 ml. or more, will take part in the direct reaction, and in the less diluted solution in

which the direct reaction is performed the reaction will appear by a qualitative test to be positive or even prompt. The observations of Barron (1931) are thus explicable.

SUMMARY

1. The rates of azobilirubin production from bilirubin-containing body fluids have been determined under various conditions.

2. In the presence of a phosphate buffer containing urea, all sera from patients with regurgitation jaundice, all but five of sixty-five sera from patients with retention jaundice and all but three of the twelve pleural fluids examined, formed azobilirubin rapidly on treatment with the diazo reagent.

3. In the presence of a phosphate buffer but not containing urea, the diazo reagent formed azobilirubin rapidly from all sera of patients with regurgitation jaundice, but much more slowly from most sera of patients with retention jaundice.

4. High concentrations of urea catalyze and increase azobilirubin production to a greater extent in retention jaundice than in regurgitation jaundice.

5. The difference between measurements of direct bilirubin by the Malloy & Evelyn method and by that of Rappaport & Eichhorn is due not only to the difference in pH at which the measurements are made, but also to the presence of urea in high concentration in the buffer used in the latter method.

6. In the presence of sodium citrate, citric acid, caffeine and urea, the diazo reagent formed azobilirubin rapidly from all sera from patients with regurgitation jaundice and about one-third of the sera from patients with retention jaundice, but more

slowly from the remaining two-thirds. Haemorrhagic pleural fluids behaved similarly to the sera from patients with retention jaundice.

7. In retention jaundice, when azobilirubin production is plotted against time, a series of curves is obtained, some of which are identical with those observed in regurgitation jaundice.

8. These experiments suggest that the rapid formation of azobilirubin on the addition of diazo reagent to sera from patients with regurgitation jaundice is due to the presence of catalysts in the sera and not the existence in regurgitation jaundice of a form of bilirubin different from that in serum of patients with retention jaundice.

9. The behaviour of the diazo reagent towards bilirubin added to normal sera and to sera containing pathological constituents, or physiological constituents in excess, suggests that the catalytic substance or substances possibly in combination with the bilirubin may be specifically elaborated in regurgitation jaundice.

10. The terms direct and indirect bilirubin should be abandoned because there is no proof that two different forms exist.

11. The use of the term direct-indirect quotient is recommended since it is merely an index of the extent of the direct reaction.

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