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KINETICS OF THE FORMATION OF AZOBILIRUBIN

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The coupling reaction between bilirubin and p-diazobenzenesulphonic acid has been carried out in a mixture of ethanol, chloroform, and water, in which all the components of the reaction are soluble. One molecule of bilirubin reacts with two molecules of the diazonium salt to give two molecules of azobilirubin. The yield of the reaction, as determined colorimetrically, is close to 100 %. A kinetic analysis of the reaction has shown that it consists of two consecutive steps:

bilirubin + p-diazobenzenesulphonic acid → azobilirubin + + hydroxypyrromethene carbinol,

hydroxypyrromethene carbinol + p-diazobenzenesulphonic acid → → azobilirubin + CH_2O.

At 20° C, in a medium of 60 % by volume of ethanol, 30 % of chloroform, and 10 % of water, and containing 0.006 mole of HCl per litre, the first reaction constant $k_1 = 8._1 \times 10^3$ mole^{-1} l min^{-1} and the second constant $k_2 = 1.2 \times 10^3$ mole^{-1} l min^{-1}.

In the coupling of bilirubin with 2,4,6-tribromobenzene diazonium salt the main reaction is similar to that with p-diazobenzenesulphonic acid. The corresponding tribromoazobilirubin has been isolated with a yield of 78 %. It has been analysed, and its molecular weight has been determined by ebullioscopy in acetone. These data confirmed that the azodye formed contains only one-half of the bilirubin molecule. A side-reaction leads to a few per cent of a coupling product between one molecule of bilirubin and one molecule of the diazonium salt.

Introduction.

The coupling reaction between diazotized sulphanilic acid (p-diazobenzenesulphonic acid) and bilirubin has attracted a great deal of attention, because it is used in clinical chemistry for the determination of bilirubin [1] [2]). The kinetics of this reaction are used as a diagnostic aid, because they allow a differentiation between different sources of bilirubin in body fluids ("direct" and "indirect" bilirubin). A satisfactory kinetic description, however, has not yet been reached. Perhaps the main

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reason for the complicated course of this reaction is the fact that bilirubin is very insoluble in water [3], so that at the pH of most body fluids it has to be solubilized by proteins, bile acids or the like. In order to avoid these complications, we have investigated this reaction in a medium in which the reactants and the reaction products are all soluble. We found a mixture of ethanol, chloroform, and water very suitable.

Earlier investigations by *Fischer* and *Barrenscheen* [4], and by *Orndorff* and *Teeple* [5] showed that only a small percentage of the reaction leads to a monoazobilirubin in which one molecule of the diazonium salt is coupled to one molecule of bilirubin. The main product of the reaction is a compound containing two molecules of the diazonium salt per molecule of bilirubin. This compound was first thought to be a disazobilirubin, but *Fischer* and *Haberland* [6], working with mesobilirubin, have shown that it actually consists of half a molecule of

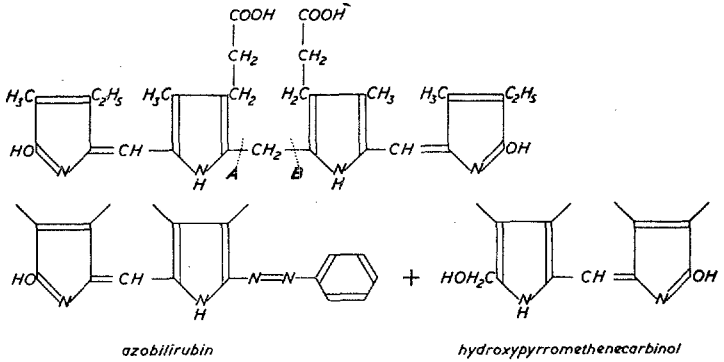

Fig. 1. Two ways of breaking of mesobilirubin. Reaction of one half to form "azobilirubin".

mesobilirubin coupled with one diazonium molecule. The main reaction can thus be represented as a hydrolysis of the bilirubin at the methylene bridge either at A or at B (see fig. 1), followed by reaction of the hydroxypyrromethene with the diazonium salt. The other half of the bilirubin molecule, a hydroxypyrromethene carbinol, according to *Fischer* and *Haberland*, probably condenses to a corresponding bilirubin.

[1]) *A. A. Hijmans van den Bergh* and *I. Snapper*, Deut. Arch. klin. Med. **110**, 540 (1913); *A. A. Hijmans van den Bergh* and *P. Muller*, Biochem. Z. **77**, 90 (1916).

[2]) *C. L. J. Vink*, thesis Utrecht 1954.

[3]) *J. Th. G. Overbeek, C. L. J. Vink*, and *H. Deenstra*, Rec. trav. chim. **74**, 81 (1955).

[4]) *H. Fischer* and *H. Barrenscheen*, Z. physiol. Chem. **115**, 94 (1921).

[5]) *W. R. Orndorff* and *J. E. Teeple*, Am. Chem. J. **33**, 215 (1905).

[6]) *H. Fischer* and *H W. Haberland*, Z. physiol. Chem. **232**, 243 (1935).

In this paper we shall show that the hydroxypyrromethene carbinol also couples with the diazonium salt. This is only apparently contradictory to the results of *Fischer c.s.* They used only slightly more than one molecule of diazonium salt per molecule of bilirubin, and so the second reaction, which is slower than the first, could only have occurred to a very small extent in their experiments.

The two different ways of breaking up the bilirubin molecule give rise to two slightly different isomers of the azobilirubin and of the hydroxypyrromethene carbinol. In our work we could disregard the differences between these isomers, since our analysis is mainly based upon a spectrophotometric technique and apparently the absorption spectra of the two azobilirubins and the two carbinols are similar enough to appear identical.

We shall first give the analytical data on the stoichiometry of the reaction, and after that a kinetic analysis.

Reaction between bilirubin and 2,4,6-tribromobenzene-diazonium acid sulphate.

Following the example of *Orndorff* and *Teeple* [5]), we investigated the coupling between bilirubin and diazotized 2,4,6-tribromoanilin.

The reaction was carried out in a mixture of 9 : 1 chloroform-ethanol. The main products of the reaction were about 78 % of tribromobenzeneazohydroxypyrromethene (corresponding to the azobilirubin of fig. 1) and about 3 % of a monoazobilirubin. The identity of the reaction products was proved by analysis, especially for bromine, and, in the case of the main reaction product, by ebullioscopy in acetone, leading to a molecular weight (calculated 627) of 600 (standard deviation of average 40). For details see the experimental part at the end of this paper.

Reaction between bilirubin and p-diazobenzenesulphonic acid.

In coupling bilirubin and diazotized sulphanilic acid in a medium of 60 % by volume of ethanol, 30 % of chloroform, and 10 % of water, only one azo compound (actually two isomers) was formed. We obtained it in a pure state in 76 % yield and determined its absorption spectrum (see fig. 2). Using the extinction *) at 530 mμ as a measure of the concentration of azobilirubin, we could definitely show that one molecule of bilirubin reacts with two molecules of the diazonium salt to give two molecules of the azodye. The data of table I show that as long as the molar concentration of diazonium salt is less than twice the molar concentration of bilirubin, the diazonium salt is quantitatively converted into the dye. If an excess of diazonium salt is present, all the bilirubin is used up.

Table I.

Concentration of azobilirubin formed from bilirubin and diazotized sulphanilic acid in ethanol-chloroform-water (6 : 3 : 1), showing that one molecule of bilirubin reacts with two molecules of p-diazobenzene sulphonic acid.

Initial conc. of bilirubin in 10^{-5} mole/l	Initial *) conc. of diazonium salt in in 10^{-5} mole/l	Conc. azobilirubin formed in 90 minutes in 10^{-5} mole/l	conc. diaz. salt / conc. azobil.	conc. azobil. / conc. bilir.
5.13	15.3	10.0	—	1.95
„	12.2	10.2	—	1.99
„	10.2	9.91	1.03	
„	8.16	8.07	1.01	
„	6.53	6.74	0.97	
„	5.22	5.06	1.03	
„	4.08	4.16	0.98	
„	3.26	3.19	1.02	
„	2.61	2.62	1.00	
„	2.04	2.03	1.00	

*) A slow side-reaction with alcohol, which decomposes about 5 % of the diazonium salt in 30 minutes, has been roughly accounted for by diminishing the initial concentrations of the diazonium salt by 1 %.

Conclusion:

These data show that in the diazocoupling of bilirubin the main reaction (with diazotized sulphanilic acid the *only* reaction) is

1 bilirubin + 2 diazonium salt → 2 azobilirubin.

In order to obtain more information about the kinetics of the reaction, we determined the light absorption as a function of the time. The absorption spectra of the reactants and the reaction products were sufficiently well-defined to allow us to convert absorption data into concentrations.

Absorption spectra.

In the first place the molecular absorption spectra of bilirubin and azobilirubin were determined with a Beckmann model D.U. Quartz Spectrophotometer. The results are given in fig. 2. In the same figure the absorption of the hydroxypyrromethene carbinol (h.p.c.), the intermediate product in the formation of azobilirubin, is given. The preparation of h.p.c. is described in the experimental part. The p-diazobenzenesulphonic acid does not absorb measurably in the visible spectrum.

*) Where necessary, a small correction for the extinction of bilirubin was applied.

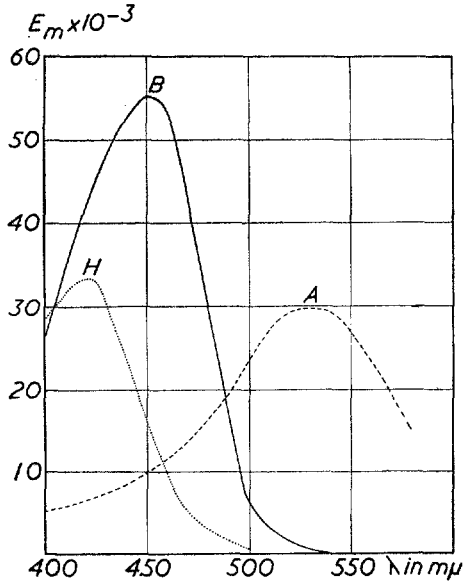

Fig. 2. Absorption spectra of bilirubin (B), azobilirubin (A), and hydroxypyrromethene carbinol (H) in a mixture of 60% (by volume) of ethanol, 30% of chloroform, and 10% of water, containing 0.006 mole of HCl per litre.

At the maximum of the absorption of azobilirubin ($\lambda = 530$ mμ) the absorption of bilirubin is very small and that of h.p.c. is negligible. Two other determinations are needed to enable the evaluation of the concentrations of all three products. For this purpose the wavelengths 425 mμ and 450 mμ have been selected, the maxima in the extinction of h.p.c. and of bilirubin respectively, although at these wavelengths the other component and the azobilirubin have a substantial absorption. The molar extinction coefficients are listed in table II.

Table II.

Molar extinction coefficients at three wavelengths.

	425 mμ	450 mμ	530 mμ
Azobilirubin (A)	6900	9800	29800
Bilirubin (B)	45800	55200	500
Hydroxypyrromethene carbinol (H)	32700	15800	—

Assuming that the extinctions are additive, we obtain the following equations, from which the concentrations of azobilirubin, bilirubin, and h.p.c. can be calculated.

$$C_A \times 10^5 = 0.02_5\ E_{425} - 0.05_1\ E_{450} + 3.36_7\ E_{530} \quad (1a)$$
$$C_B \times 10^5 = -1.46_6\ E_{425} + 3.03_4\ E_{450} - 0.65_8\ E_{530} \quad (1b)$$
$$C_H \times 10^5 = 5.10_6\ E_{425} - 4.23_8\ E_{450} + 0.21_2\ E_{530} \quad (1h)$$

where E_{425}, E_{450}, and E_{530} represent the extinction coefficients at the wavelengths 425 mμ, 540 mμ, and 530 mμ respectively.

Kinetic measurements.

All the determinations of the kinetics of the coupling reaction have been carried out at 20° C in a medium of 60 % of ethanol, 30 % of chloroform, and 10 % of water, and containing 0.006 mole of HCl per litre. Table III shows the light absorption measured at the three wavelengths mentioned above during a reaction, and table IV gives the concentrations calculated from these data with the use of eq. (1). The last column of table IV gives a check on the stoichiometry of the reaction.

Table III.

Extinction coefficients at the wavelengths 425, 450, and 530 mμ during the reaction between 2.80×10^{-5} mole/l bilirubin and 10×10^{-5} mole/l p-diazobenzenesulphonic acid.

time in min	E_{425}	E_{450}	E_{530}
0	1.28	1.54	0.01
1	1.20	1.15	0.46
3	1.06	0.86	0.84
5	0.96	0.75	0.99_5
10	0.82	0.66	1.21
20	0.61	0.60	1.43_5
40	0.44	0.52 *)	1.59
∞	0.38_5	0.55	1.67

* Evidently too low.

It follows from the values of the coefficients in eq. (1) that small variations in the measured extinctions or in the molar extinction coefficients have a large influence on the calculated concentrations. The last decimal places in table IV therefore are not very accurate. The tendency for the sum of concentrations to be larger than

2.80×10^{-5} might be due to a somewhat high estimate for the molar extinction coefficient of h.p.c.

Table IV.

Concentrations of bilirubin, hydroxypyrromethene carbinol, and azobilirubin as calculated from the data of table III.

Time of reaction in min	bilirubin in 10^{-5} mole/l	hydroxy-pyrromethene carbinol in 10^{-5} mole/l	azobilirubin in 10^{-5} mole/l	bilirubin + ½ hydroxy-pyrromethene carbinol + ½ azobilirubin in 10^{-5} mole/l
0	2.80	(0.01)	(—0.01)	2.80
1	1.43	1.35	1.52	2.86_5
3	0.50	1.94_5	2.81	2.88
5	0.21_5	1.93_5	3.33_5	2.85
10	0.00	1.64_5	4.06	2.85
20	—0.02	0.87_5	4.81_5	2.82_5
40	$+0.00_5$ *)	0.21 *)	5.33_5	2.78*)
∞	$+0.00_5$	—0.01	5.60_5	2.80

*) Calculated with $E_{450} = 0.56$.

Evidently in the beginning of the reaction (less than 1 minute) one molecule of h.p.c. and one molecule of azobilirubin are formed for each molecule of bilirubin which disappears. In the end phase of the reaction (more than 10 minutes) there is practically no bilirubin left and the azobilirubin is formed exclusively from the h.p.c.

The reaction thus appears to be

1 bilirubin + 1 p-diazobenzenesulphonic acid → 1 azobilirubin + + 1 h.p.c.

1 h.p.c. + 1 p-diazobenzenesulphonic acid → 1 azobilirubin + CH_2O

with the following rate equations for the separate reactions

1st reaction $dx/dt = k_1 . b . d$ . . . . . (2)

2nd reaction $dy/dt = k_2 . h . d$ . . . . . (3)

where b, d, and h are the concentrations of bilirubin, p-diazobenzenesulphonic acid, and h.p.c. respectively, and x and y represent the concentrations of azobilirubin as formed by the first and the second reaction respectively.

As the general integration of these two equations is quite complicated, we have determined the constants k_1 and k_2 from special cases with extreme conditions of time and concentration.

Determination of the rate constant k_1 of the first reaction.

In the first stages of the reaction, when only a small amount of h.p.c. has been formed, the second reaction is practically absent. This

is still more pronounced when a large excess of bilirubin is used, because then the diazonium salt will be used up in the first reaction.

Integration of the first rate equation with neglect of the second leads to

$$k_1 = \frac{2.30}{(b_1 - d_1)t} \log \frac{d_1 b_2}{d_2 b_1} \quad . \quad . \quad . \quad . \quad . \quad . \quad (4)$$

where b_1 and d_1 are the initial concentrations, and b_2 and d_2 are calculated by subtracting the azobilirubin formed at time t from the initial concentrations of bilirubin and the diazonium salt. The azobilirubin is simply determined by its light absorption at 530 $m\mu$, allowing where necessary for the small absorption by bilirubin.

As eq. (4) is expected to describe the actual reaction better the earlier the stage of the reaction, extrapolation of k_1 calculated with eq. (4) to zero time should give a reliable value for k_1. Table V gives a few examples.

Table V.

Extrapolation of k_1 to zero time.

Initial concentration in 10^{-5} mole/l							
p. diazobenzenesulphonic acid	bilirubin	k_1 calculated with eq. 4 in 10^3 mole^{-1} l min^{-1}					k_1 extrapolated to t=0 in 10^3 mole^{-1} l min^{-1}
		t=1/4 min	t=1/2m	t=3/4m	t=1m	t=1 1/4m	
20	1.70	8.30	8.71	—	11.4	—	8.0
20	3.45	8.45	8.93	—	10.9	—	8.1
20	5.13	8.24	8.75	—	10.2	—	7.9
8	3.40	8.10	8.30	—	8.72	—	7.9
4	3.40	7.83	7.95	—	8.10	—	7.8
1.91	38.7	8.22	8.27	8.6	—	8.3	8.2

Giving a somewhat larger weight to the last experiment with the high excess of bilirubin, we propose the following value for k_1

$$k_1 = 8._1 \times 10^3 \ mole^{-1} \ l \ min^{-1}.$$

Determination of the rate constant k_2 of the second reaction.

The second reaction becomes more and more preponderant towards the end of the reaction, especially when the diazonium salt is in large excess.

a. The data of tables III and IV for the longer reaction times can

be interpreted as a second-order reaction between h.p.c. and the diazonium salt. The reaction constant is calculated from

$$k_2 = \frac{2.30}{(t_2 - t_1)(d_1 - h_1)} \log \frac{d_2 h_1}{d_1 h_2} \quad . \quad . \quad . \quad . \quad . \quad (5)$$

where the subscripts 1 and 2 at d and h indicate the concentrations at the times t_1 and t_2 respectively. The concentration of the diazonium salt in this case is equal to (10×10^{-5} — conc. azobilirubin). The concentrations of h.p.c. have not been taken directly from table IV, because these values are admittedly inaccurate. For long reaction times ($t = 10, 20, 40$) a better estimate of the concentration of h.p.c. is obtained by putting it equal to 5.60×10^{-5} — conc. azobilirubin. The results are given in table VI.

Table VI.

Calculation of the reaction constant k_2 from the azobilirubin data of table IV.

t_1 in min	t_2 in min	k_2 from eq. (5) in 10^3 mole^{-1} 1 min^{-1}
10	20	1.22
20	40	1.11

Average value $k_2 = 1.2 \times 10^3$ mole^{-1} 1 min^{-1}.

b. With a very large excess of the diazonium salt the reaction simplifies to two consecutive reactions of the first order. If x and y are the concentrations of azobilirubin formed by the first and the second reaction respectively, the kinetic equations are

$$\frac{dx}{dt} = k_1 d_1 (b_0 - x) = k_1' (b_0 - x) \quad . \quad . \quad . \quad . \quad (6)$$

$$\frac{dy}{dt} = k_2 d_2 (x - y) = k_2' (x - y) \quad . \quad . \quad . \quad . \quad . \quad (7)$$

where b_0 represents the initial concentration of bilirubin, and d_1 and d_2 the concentrations of the diazonium salt during the first part and the second part of the reaction. Choosing d_1 and d_2 equal to the concentration of the diazonium salt when the reaction is ¼ and ¾ completed seems an easy way to account for the small, but not quite negligible variation of this concentration.

Integration of eq. (6) gives

$$x = b\{1 - \exp(-k_1't)\} \quad . \quad . \quad . \quad . \quad . \quad . \quad (8)$$

The second equation can then be integrated, giving

$$y = b\left\{1 + \frac{k_2'}{k_1' - k_2'} \exp(-k_1't) - \frac{k_1'}{k_1' - k_2'} \exp(-k_2't\right\} \quad . \quad (9)$$

or:

$$x + y = [\text{azobilirubin}] = b\left\{2 - \frac{k_1' - 2k_2'}{k_1' - k'_2} \exp(-k_1't) - \frac{k_1'}{k_1' - k'_2} \exp(-k_2't)\right\} \quad (10)$$

In table VII we compare the data on an experiment with a large excess of diazonium salt with eq. (10), using $k_1' = 8.1 \times 10^3 \times 39.1 \times 10^{-5} = 3.17$ and $k_2' = 1.2 \times 10^3 \times 37.3 \times 10^{-5} = 0.447$. The agreement is quite satisfactory.

Table VII.

Reaction between 40×10^{-5} molar p-diazobenzenesulphonic acid and 1.81×10^{-5} molar bilirubin.

Time in min	Azobilirubin in 10^{-5} mole/l	(x + y) calc. with eq. (10)
0	0	0
0.25	1.01	1.05
0.50	1.65	1.62
1	2.28	2.21
3	3.07	3.07
6	3.47	3.46

Conclusion.

The diazo reaction of bilirubin with p-diazobenzenesulphonic acid proceeds in two consecutive steps, both of the second order. At 20° C in a medium of 60 % of ethanol, 30 % of chloroform, 10 % of water, and 0.006 molar in HCl the reaction constants are:

$$k_1 = 8.1 \times 10^3 \text{ mole}^{-1} \text{ l min}^{-1}$$
$$k_2 = 1.2 \times 10^3 \text{ mole}^{-1} \text{ l min}^{-1}$$

It is quite probable that the diazo reaction as used in clinical chemistry follows the same pattern, but as bilirubin is very insoluble in water, and can only be present in a reactive form in that medium if solubilized by proteins, bile acids or the like, it is not astonishing

that the kinetics of the reaction in these circumstances are more complicated than in a homogeneous medium.

Experimental.

Bilirubin. The bilirubin used was obtained from Hoffmann-La Roche and was recrystallized from chloroform. It contained no biliverdin and was pure, as shown by its nitrogen content, its equivalent weight in titration with NaOH, and its absorption spectrum.

Chloroform. In order to avoid oxidizing contaminations, chloroform was purified by washing with thiosulphate solution and with water, drying over sodium sulphate, and distillation in the presence of a crystal of $Na_2S_2O_3$.

2,4,6-Tribromobenzenediazonium acid sulphate. 2,4,6-Tribromoanilin acid sulphate was nearly quantitatively diazotized with amyl nitrite in acetic acid [7]). The tribromobenzenediazonium acid sulphate crystallized after addition of ether to the reaction mixture.

Coupling of bilirubin and 2,4,6-tribromobenzenediazonium acid sulphate. 500 mg of pure bilirubin (0.855×10^{-3} mole) were refluxed with 450 ml of chloroform until solution was complete. After cooling to 5° C, 50 ml of absolute alcohol were added. Then 1.71×10^{-3} mole of freshly prepared tribromobenzenediazonium acid sulphate in a few ml of cold water was added to the bilirubin solution, which was thoroughly shaken. After 24 hours' standing, four products of the reaction could be distinguished: a small amount of biliverdin, 2 azodyes, and a yellow product, probably the hydroxypyrromethene carbinol. Biliverdin (an oxidation product of bilirubin) could be adsorbed on a chromatographic column of Na_2CO_3. The remaining mixture was washed with water. As a result some monoazobilirubin (I) precipitated. The filtrate was shaken with a solution of potassium hydroxide, which took up the two azodyes and the yellow product. This solution is washed with a small amount of chloroform and acidified. The precipitate is washed with water, dried, and extracted in a Soxhlet extractor with ethyl acetate as described by *Orndorff* and *Teeple* [5]). Monoazobilirubin stays behind (II). The "disazobilirubin" and the yellow product are dissolved. This solution is evaporated *in vacuo* to dryness, dissolved in chloroform ethanol (9 : 1), and passed through a column of Na_2CO_3, on which the yellow product stays slightly behind the red "disazodye". The solution of the "disazodye" is washed with water, evaporated *in vacuo* to dryness, and the pigment is recrystallized from ethyl acetate. The yield of tribromobenzeneazohydroxypyrromethene ("disazobilirubin") was 78 %, assuming two molecules of the dye for each molecule of bilirubin.

Analysis *):

Found:	N 8.95, 8.48; Br 37.8
Calculated for $C_{16}H_{17}N_2O_3(N_2C_6H_2Br_3)$ (M.W. = 627):	„ 8.93; „ 38.2

*) The analyses have been carried out by the Organic Chemical Institute T.N.O. at Utrecht and by the microanalytical section of the Laboratory for Organic Chemistry of the University of Amsterdam.

Elevation of the boiling point in acetone solutions of tribromobenzeneazohydroxypyrromethene.

Azodye in g	Acetone in g	Elevation of the boiling point in °C	Molecular weight
0.022	6.60	0.008	710
0.030	6.50	0.015	530
0.036	6.50	0.017	560
0.045	6.30	0.020	610
		average	600
		standard deviation of the average	40
		calculated	627

The monoazobilirubin (fraction I + II) was recrystallized from chloroform and was only obtained in a 3% yield, insufficient to determine the molecular weight.

Analysis:

Found: C 45.9, 45.6; H 4.38, 4.35; N 8.46, Br 25.4.
Calc. for $C_{33}H_{35}N_4O_6(N_2C_6H_2Br_3)$ (925): „ 50.61; „ 4.03; „ 9.08, „ 25.95

The analysis points to a rather impure product, but especially the Br content shows that it is indeed a monoazobilirubin.

p-Diazobenzenesulphonic acid. A concentrated aqueous solution of sulphanilic acid was diazotized with a slight excess of $NaNO_2$ at 0° C by slowly adding HCl to the reaction mixture. The diazonium chloride, which is only sparingly soluble, precipitates and is recrystallized from a mixture of water and ethanol (1 : 1). It is highly sensitive to friction (explosive). The purity was checked by quantitative coupling with β naphthol.

Reaction between bilirubin and p-diazobenzenesulphonic acid. On adding an aqueous solution of 180 mg of p-diazobenzenesulphonic acid to a solution of 200 mg of bilirubin in chloroform-ethanol, a red colour developed readily. After 24 hours, water is added until phase separation occurs. The red azodye goes into the aqueous solution, which is further purified by repeated shaking with chloroform. After evaporation in a vacuum, a small amount of a yellow product is separated on a column with Al_2O_3 in ethanol solution. After evaporation of the ethanol the yield of pure azobilirubin was 76%.

Analysis.

Calculated for $C_{22}H_{22}N_4O_6S$ (470,5): N 11.48
Found: „ 11.40, 11.34.

Preparation of hydroxypyrromethene carbinol. An aqueous solution of p-diazobenzenesulphonic acid is added to a solution of bilirubin in ethanol-chloroform in a molecular ratio of 1.30 : 1. At the end of the reaction all the bilirubin has disappeared, and the reaction mixture therefore only contains the azodye and h.p.c. They were separated from each other by the same method described above for the

[7]) *A. Hantzsch* and *E. Jochem*, Ber. **34**, 3339 (1901).

preparation of azobilirubin. On the chromatographic column with Al_2O_3 the h.p.c. is adsorbed as a yellow band. This is dissolved in 60 % of ethanol, 30 % of chloroform, and 10 % of water (0.006 molar in HCl), and the absorption spectrum is determined. The total amount of h.p.c. is calculated, assuming that all the bilirubin has reacted and that no losses have occurred, so that conc. h.p.c. = 2 × original concentration bilirubin — original concentration diazonium salt. A correction of a few % is applied for the decomposition of the diazonium salt by the ethanol (5 % in 30 min).

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