Acta Chemica Scandinavica B 33 (1979) 281—293

Bilirubin Acidity. Titrimetric and ^{13}C NMR Studies

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Acidimetric titration of bilirubin IX-α, dissolved in excess aqueous sodium hydroxide, showed that two protons are dissociated with pK values well below 7 and that one or several additional acidic groups titrate with pK around 12.9. Precipitation of the nearly insoluble acid precluded determination of the two lower pK values by titration in aqueous solution. In dimethyl sulfoxide solution, four acidic protons were demonstrated, titrating two by two without precipitation.

^{13}C NMR spectra of bilirubin IX-α were recorded and complete assignments were made by comparison with the spectra of bilirubin XIII-α and mesobilirubin *etc.* Such spectra, recorded after addition of 2 and 4 mol of base per mol of bilirubin IX-α, showed that both carboxyl groups are titrated by the first 2 mol of base, and both lactams by the following 2 mol of base. Cotitrations of bilirubin IX-α with other acids, *o*- and *m*-hydroxybenzoic acid and 2-pyridone, were used to determine relative pK values in dimethyl sulfoxide solution, and pK values for the four acidic protons of bilirubin IX-α in aqueous solution were calculated from the Born equation. Both carboxyl groups exhibited p$K = 4.4$, and both lactams p$K = 13.0$, in good agreement with values expected from the chemical structure of the bilirubin molecule.

The implications of these findings for understanding the mechanism of bilirubin neurotoxicity are discussed.

Bilirubin, a product of hemoglobin catabolism, is highly toxic to tissues, especially to parts of the central nervous system in the human neonate. In fatal cases, basal ganglia of the brain are stained yellow with bilirubin (kernicterus) while lasting brain damage may be seen in survivors. Toxicity is counteracted by conjugation and excretion of bilirubin, processes which are deficient in the newborn, and by a tight, reversible binding of the unconjugated bilirubin to plasma albumin. Toxic manifestations depend upon transfer of bilirubin from blood to nerve cells and are mostly seen in cases complicated by anoxia and acidosis, and on treatment with drugs competing for the bilirubin binding site on albumin. (For a review, see Ref. 1). A thorough knowledge of the thermodynamics and kinetics of this transfer is therefore essential, especially with respect to the effect of varying pH in acidosis, and this must be based on knowledge of the acidic properties, solubility, *etc.*, of bilirubin.

From the structure of bilirubin [2] (Fig. 1) it would seem that the two carboxyl protons should be released at pH values below neutral, and that two additional, weakly acidic protons are found in the lactam groups of the end rings. Lee *et al.*[3] have reported pK values for the carboxyl groups in agreement with this, while the acidity of the lactam groups has received little attention. Several authors [4–7] have published differing views and pK values around 7 or 8 for the carboxyl groups have been widely accepted (see Discussion). This issue is of biological importance and the present paper reports our efforts toward a definitive description of the acidic properties of bilirubin, utilizing acidimetric titration in water and non-aqueous media, and ^{13}C NMR spectroscopy. A study of the acidimetric stoichiometry of bilirubin binding with albumin is included and the implications of the findings for understanding the molecular mechanism of bilirubin neurotoxicity as a function of pH are discussed.

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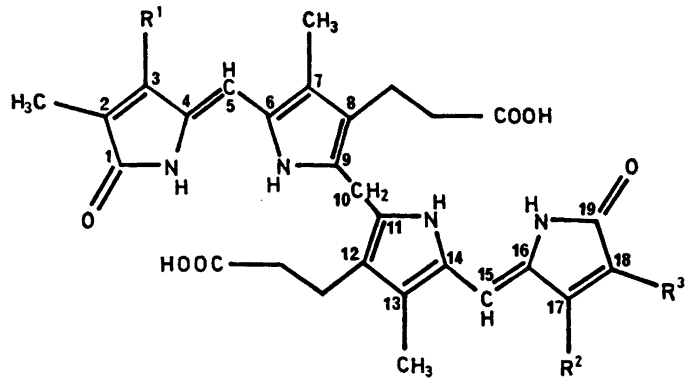

Fig. 1. The bilirubin skeleton.

	R^1	R^2	R^3
Bilirubin IX-α	$CH=CH_2$	CH_3	$CH=CH_2$
Bilirubin XIII-α	$CH=CH_2$	$CH=CH_2$	CH_3
Mesobilirubin	C_2H_5	CH_3	C_2H_5

RESULTS

Acidimetric titration of bilirubin

Titration in aqueous solution. Bilirubin, dissolved in an excess of aqueous sodium hydroxide was titrated with hydrochloric acid. The result is seen in Fig. 2a which also shows a blind titration without bilirubin. Light scattering was measured at intervals. From the difference of the two titrations the number of protons released per molecule of bilirubin was calculated as a function of pH and was plotted in Fig. 2b. In the pH interval from 11.5 to 8.0, it is seen that bilirubin is present as a divalent anion. Further addition of hydrochloric acid results in very little change of pH but causes a steep increase of light scattering, until, at pH 7.9, an average of 1.6 protons have been consumed by each bilirubin molecule. At this point the solution remains visibly clear in transmittent light but shows a strong Tyndall phenomenon, as quantified by the light scattering, indicating that the pigment has aggregated to large particles with an average negative charge of 0.4 electrons per bilirubin molecule. This colloid suspension is not stabile and, especially with a little more acid, soon forms a visible flocculation also revealed by irregular readings of light scattering. At lower pH values the average electric charge decreases further and finally, around pH 3.5, approaches zero.

The sharp transition occurs at pH 8.0 when the bilirubin concentration is 13 mM, as in Fig. 2, and is observed at lower pH values, approaching 7, when more dilute solutions are titrated. At a very low bilirubin concentration, 0.3 μM, this transition could not be observed and the increase of light scattering was slight and usually took place with a delay of more than 30 min.

Light absorption spectra of aqueous bilirubin solutions proved to remain unchanged with varying pH in the range 8 to 11. At low bilirubin concentration, 0.3 μM, it was possible to obtain fairly reproducible spectra at pH as low as 7.0, when these were recorded promptly after dilution of the alkaline bilirubin solution with buffer. Such spectra were identical with those observed in more alkaline media. At pH values below 7, or, with higher bilirubin concentrations between pH 7 and 8, spectral changes occurred in the course of minutes or hours, with lowering of the maximum at 435 nm and formation of a new band around 510 and a simultaneous increase of light scattering, indicating aggregation of the pigment.[8]

In strongly alkaline solutions, pH increasing from 11.5 to 14, the maximum at 435 nm decreases and a new, with slightly lower extinction is formed at 410 nm. These changes are fast and reproducible and are fully reversed when pH is lowered to 11 within a minute. Fading of the colour is seen on standing at

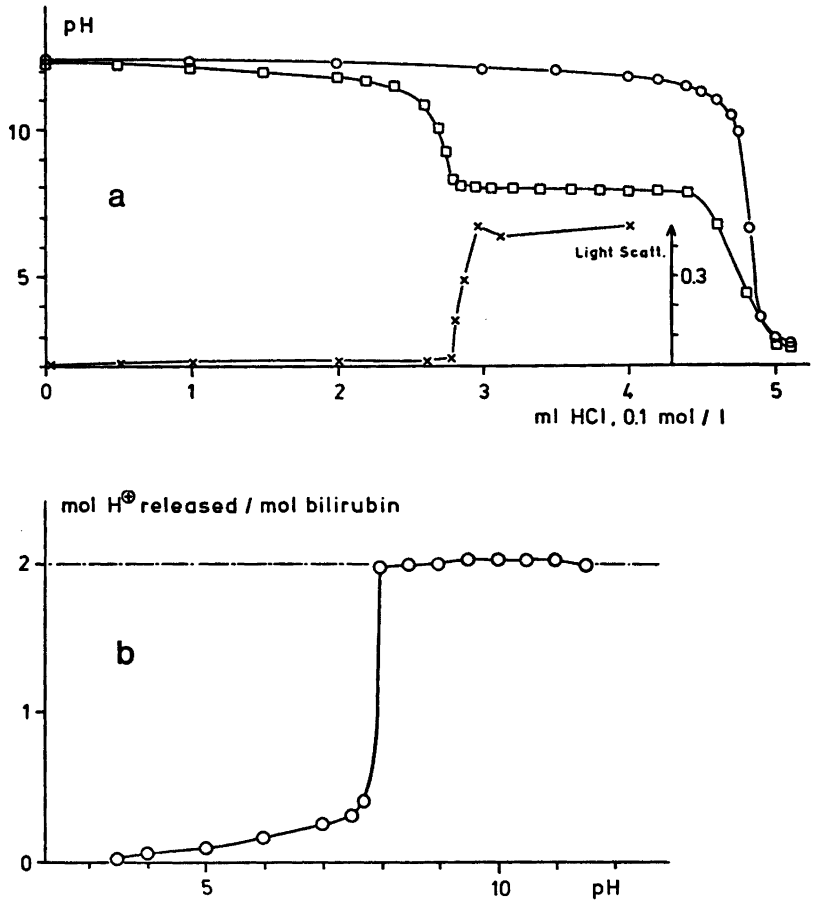

Fig. 2. a. Acidimetric titration of bilirubin IX-α dissolved in an excess of sodium hydroxide in water and titrated with hydrochloric acid, volume as shown along the abscissa. Left ordinate indicates pH with bilirubin present, □; and without, ○. Light scattering during titration of bilirubin is indicated on the right ordinate, ×, in arbitrary units. *b.* The average number of protons titrated per bilirubin molecule is plotted as a function of pH.

very high pH. The ratio of prompt absorbances at 435 and 410 nm was plotted as a function of pH (Fig. 3) and was found compatible with a simple acid dissociation with $pK = 12.9$ (at 24 °C, ionic strength 0.5 M).

In summary, bilirubin is soluble in water under alkaline conditions and forms a divalent anion which is stable in the range of pH 8 to 11. At pH values below 8, or 7 in dilute solutions, a slow process takes place, involving partial protonization and aggregation, finally resulting in precipitation of the acid. The divalent ion at higher pH shows weakly acidic properties, pK about 12.9.

Titration in non-aqueous media. Bilirubin was dissolved in chloroform, and ethanol and water were added to the ratio 3:6:1 (v/v). The light absorption spectrum is seen in Fig. 4 and remained unchanged by addition of hydrochloric acid. The spectrum in acid medium has maximum at 447 nm. This is reversibly transformed into a spectrum with maximum at 454 nm by addition of a small amount of sodium hydroxide, while a large base excess resulted in a spectrum with maximum at 414 nm. Even the latter change was reversible within a few minutes, but the strongly alkaline solutions bleached on standing.

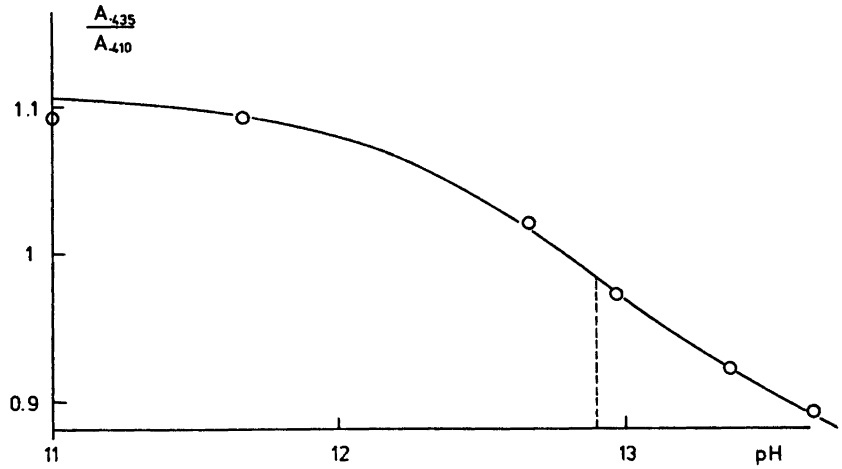

Fig. 3. Spectrophotometric titration of bilirubin IX-α in aqueous solution. The ratio of absorbances at 435 and 410 nm is plotted as a function of pH, O. The curve is calculated for titration of one or several acidic groups with pK 12.9.

Potentiometric titration of bilirubin with potassium hydroxide in dimethyl sulfoxide (Fig. 5) showed the presence of four titrable, acidic groups, dissociating two by two, well separated by a considerable difference of potential.

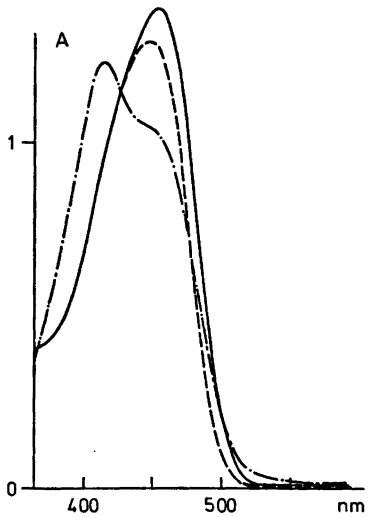

Fig. 4. Light absorption spectra of bilirubin IX-α, 20.4 μM, in chloroform–ethanol–water, 3:6:1 by vol. ---, with HCl, 40 μM; ——, with NaOH, 60 μM; —·—, with NaOH 88 mM. ---, the bilirubin acid (uncharged); ——, the bilirubin dianion (COO^-); and —·—, a mixture of the dianion and a tetra-anion in which the two lactam protons are partially dissociated.

The light absorption spectrum of bilirubin acid in dimethyl sulfoxide has a maximum at 435 nm and a shoulder at 412–422. This shoulder disappears on titration of two protons and the maximum is shifted to 457 nm on further titration of two protons.

The divalent bilirubin anion in aqueous solution undergoes reversible dimerisation[9] and the acid shows a pronounced tendency to aggregate. It was therefore considered necessary to examine whether di- or polymerisation takes place in dimethyl sulfoxide, before interpretations of titration curves could be made. Bilirubin was dissolved in dimethyl sulfoxide in concentrations ranging from 2 μM to 3 mM and light absorption spectra were recorded. The absorption maximum remained unchanged at 453 nm throughout this concentration range and the absorbance was proportional to the concentration. A maximum with lower extinction, located at 287 nm, likewise remained unchanged at bilirubin concentrations ranging from 1.2 to 18 mM. Dimerisation of the acid thus could not be demonstrated, or at least does not involve the chromophors as it does in water. Dimerisation of the ionic species is considered to be even less likely, due to ionic repulsion.

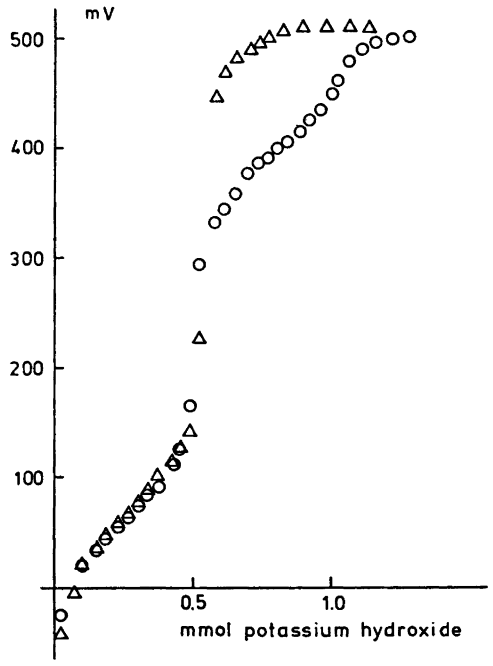

Fig. 5. Acidimetric titrations of bilirubin IX-α and of *m*-hydroxybenzoic acid in dimethyl sulfoxide. 0.25 mmol bilirubin IX-α, △; and 0.50 mmol *m*-hydroxybenzoic acid, ○; dissolved in 2.5 ml dimethyl sulfoxide, were titrated with 1.5 M potassium hydroxide in methanol, using glass and calomel electrodes.

The titration curves obtained in dimethyl sulfoxide thus demonstrates the existence of three species, *i.e.* the uncharged bilirubin acid, a divalent anion and a tetravalent anion. It appears likely that the three spectra obtained in chloroform – ethanol – water belong to the same species, while the spectra of aqueous solutions show the divalent anion at pH (7)8 – 11 and formation of a tri- or tetravalent anion in strongly alkaline solutions. The spectral differences from one medium to the other may be explained by different patterns of intramolecular hydrogen bonding.

A reasonable first guess would be that the divalent anion, irrespective of the solvent, has two carboxylate groups and that the lactam protons dissociate in strongly alkaline media. This was further investigated by ^{13}C NMR spectra.

^{13}C NMR studies

Assignment of bilirubin IX-α spectrum. The assignment (Table 1) is complex due to the slight asymmetry of the molecule. Comparisons were made with compounds containing structural elements similar to bilirubin IX-α. Chemical shifts in rings A and D were compared with those of 2-pyridone [10] (Table 2), chemical shifts in rings B and C with those in pyrrole [11]

Table 1. ^{13}C chemical shifts of bilirubin in dimethyl sulfoxide, relative to tetramethylsilane.

Assignment (see Fig. 1)	Bilirubin IX-α KOH added, molar ratio 0 ppm	2 ppm	4 ppm	Bilirubin XIII-α 0	Meso-bilirubin 0 ppm
COOH (8′)	173.89	178.88	178.85	173.90	173.91
COOH (12′)	173.89	178.88	178.85		173.91
C-1	171.30	172.21	180.80	171.27	171.93
C-19	170.36	171.20	178.85		171.53
C-17	141.84	141.63	140.57		140.84
C-3	140.41	140.74	139.11	140.35	147.14
C-11	131.39	133.00	130.42		130.28
C-9	130.63	132.37	128.53	130.71	129.40
C-16	128.31	127.98	144.11		129.15
C-4	127.54	127.33	142.76	127.43	127.86
C-α (3′)	127.34	128.33	129.43	127.29	–
C-α (18′)	127.03	127.58	129.43		–
C-12 [a]	124.01	123.91	127.63		122.90
C-8 [a]	123.31	123.17	127.39	123.33	122.43

Table 1. Continued.

C-2	123.31	123.35	120.20	123.21	122.43
C-18	122.47	122.70	119.69		130.21
C-14	122.31	123.35	127.09		121.85
C-6	122.12	123.17	125.85	122.02	121.99
C-β (18′)	121.95	121.44	116.17		–
C-13 [a]	119.79	120.81	119.04		119.23
C-7 [a]	119.59	120.62	117.84	119.49	119.23
C-β (3′)	117.12	116.75	114.26	117.01	–
C-15	99.96	100.84	99.55		97.96
C-5	99.12	100.39	98.71	99.11	97.67
$-CH_2COOH$ (8′) [b]	34.28	–	–		34.35
$-CH_2COOH$ (12′) [b]	34.28	–	–	34.17	34.35
$-CH_2-$ (10)	23.60	22.12	22.38	23.53	23.49
$-CH_2CH_2COOH$ (8′)	19.24	21.45	21.75		19.26
$-CH_2CH_2COOH$ (12)	19.24	21.45	21.75	19.20	19.26
CH_3 (2′)	9.36	9.57	10.57	9.35	9.09
CH_3 (18′)	9.18	9.57	9.69		9.09
CH_3	9.10	9.35	9.31	9.05	9.09
CH_3 (16′ and 12′)	9.10	9.35	9.31		7.99

[a] The assignments of C-12 and C-13 may possibly be interchanged, and likewise those of C-8 and C-7.
[b] The $-CH_2COOH$ signals are at most pH values hidden in the dimethyl sulfoxide signals.

Table 2. ^{13}C NMR chemical shifts for *o*- and *m*-hydroxybenzoic acid and 2-pyridone.

Compound	KOH added, molar ratio	C-1	C-2	C-3	C-4	C-5	C-6	COOH
		ppm	ppm	ppm	ppm	ppm	ppm	ppm
	o-Hydroxybenzoic acid							
	0	112.86	161.10	119.06	135.51	117.01	130.20	171.85
	0.5	116.82	161.63	117.89	133.75	116.55	130.47	172.40
	1	120.57	162.22	117.14	132.14	116.23	130.66	173.35
	1.5	123.03	163.66	117.44	131.23	115.07	130.48	174.06
	2	124.89	165.00	118.53	130.66	113.52	130.41	174.84
	m-Hydroxybenzoic acid							
	0	132.03	115.82	157.37	119.81	129.50	119.96	167.28
	0.5	136.66	116.16	157.34	118.13	128.79	119.90	169.00
	1	140.63	116.39	157.13	116.39	128.23	120.01	170.76
	2	140.64	119.39	166.03	119.03	127.70	114.75	173.63
	2-Pyridone							
	0	–	162.31	119.82	140.84	104.83	135.21	–
	0.5	–	167.14	117.31	139.28	105.69	140.83	–
	1	–	171.38	114.93	137.69	106.50	145.76	–

and substituted pyrroles.[12–14] The CH_2-CH_2-COOH part of the molecule was identified by comparison with chemical shifts observed in aliphatic carboxylic acids [15] and porphyrins.[16] Off-resonance $^{13}C-\{^1H\}$ decoupling experiments helped to identify the six olefinic carbons bearing hydrogens. The C-α and the C-β vinyl carbons could be distinguished by means of substituent effects on chemical shifts obtained from styrenes.[17] C-5 and C-15 were

identified as the olefinic carbons at highest field by comparison with porphyrins.[16] A comparison of chemical shifts in bilirubin IX-α and bilirubin XIII-α revealed that the chemical shifts in the latter are equal to half of the shifts in bilirubin IX-α. This feature was used to distinguish carbons in rings A and B from those in rings C and D. A comparison of chemical shifts in bilirubin IX-α and mesobilirubin decided the assignment of C-3, C-17, C-2 and C-18. The very large effects observed in going from a vinyl to an ethyl group are remarkable and are observed in benzene derivatives too. This comparison also supports the assignments of the vinylic carbons. C-4 and C-16, C-8 and C-12, C-6 and C-14 as well as C-9 and C-11 were assigned on observation of the splittings or broadenings caused by residual long range couplings in selective ^{1}H-decoupling experiments. The carboxylic acid carbons were characterized by their titration behaviour (*vide infra*). The carbonyl carbons, C-1 and C-19, were identified and distinguished by selective decoupling experiments as just described.

The assignments were furthermore substantiated by deuterium isotope effects on the chemical shifts as observed in spectra of solutions with methanol-d_4 added. This subject will be described in more detail in a forthcoming paper.[18]

The changes observed in the spectra during titration were compared with titration shifts of compounds containing elements similar to bilirubin IX-α. Comparisons were made with 2-pyridone (Table 2), pyrrole [17] and with aliphatic carboxylic acids.[15]

The assignments in mesobilirubin of carbon resonances C-6, C-8, C-12 and C-14 are tentative.

^{13}C NMR acidimetric titration. Spectra were further recorded after addition of methanolic potassium hydroxide to bilirubin IX-α in dimethyl sulfoxide in molar ratios 2 and 4 (Table 1). Addition of 2 mol of base per mol of bilirubin caused a considerable change of the chemical shifts of the two coinciding lines at the low-field extreme, assigned to the carboxylic carbons. These observations confirm the assignment of the carboxylic carbons and demonstrate that the first and second acidic protons are those of the carboxyl groups. Further evidence is obtained by comparing the changes of the CH_3COOH carbons with results on ionization of aliphatic carboxylic acids.[15] Also the small changes seen at CH_2CH_2COOH support this conclusion. Moderate alterations of the chemical shifts, especially of C-11, C-9, C-18, C-6, C-14, C-12, C-8 and the two vinyl C-α, may be explained by conformational changes in the bilirubin molecule, caused by the deprotonations [3] or partly by solvent effects due to the addition of methanol. The small magnitude of the changes in the shifts of C-1 and C-19 indicate that the lactam form is still predominant in the dianion.

Further addition of base until 4 mol per mol of bilirubin resulted in major changes in C-1, C-19, C-16 and C-4. These demonstrate that the third and fourth acidic protons originate from the lactam groups.

Determination of relative pK values in dimethyl sulfoxide by cotitrations. Bilirubin was mixed with acids of known p$K(H_2O)$ and titrated in dimethyl sulfoxide (cotitration) in order to relate the pK's of bilirubin to known ones. For a conversion of pK's in H_2O to pK's in dimethyl sulfoxide, see the next paragraph.

Potentiometric cotitration of bilirubin and *m*-hydroxybenzoic acid revealed that the carboxylic acid pK's of bilirubin and *m*-hydroxybenzoic acid are identical in dimethyl sulfoxide within experimental error (Fig. 5).

Cotitrations using ^{13}C NMR as monitor have also been used. The main advantage of the NMR technique is that close-lying pK values can be monitored independently. NMR is thus well suited to follow the titration of two independent species simultaneously. In order to use ^{13}C NMR to determine relative acid strengths in aprotic solvents it must be established that mixing the two acids does not change the shifts of either. No such changes were observed by addition of *o*- or *m*-hydroxybenzoic acid or 2-pyridone to bilirubin.

The ^{13}C NMR spectrum of a mixture of bilirubin and *m*-hydroxybenzoic acid (1:2) to which was added potassium hydroxide in the molar ratio 0.8 gave the following shifts of bilirubin, COOH: 175.77 ppm, C-11: 132.07 ppm and C-9: 131.34 ppm, and of *m*-hydroxybenzoic acid, COOH: 168.11 ppm and C-1: 133.83 ppm. The bilirubin chemical shifts are very close to the values obtained by addition of base in the molar ratio 0.4 and the *m*-hydroxybenzoic acid chemical shifts are, as seen

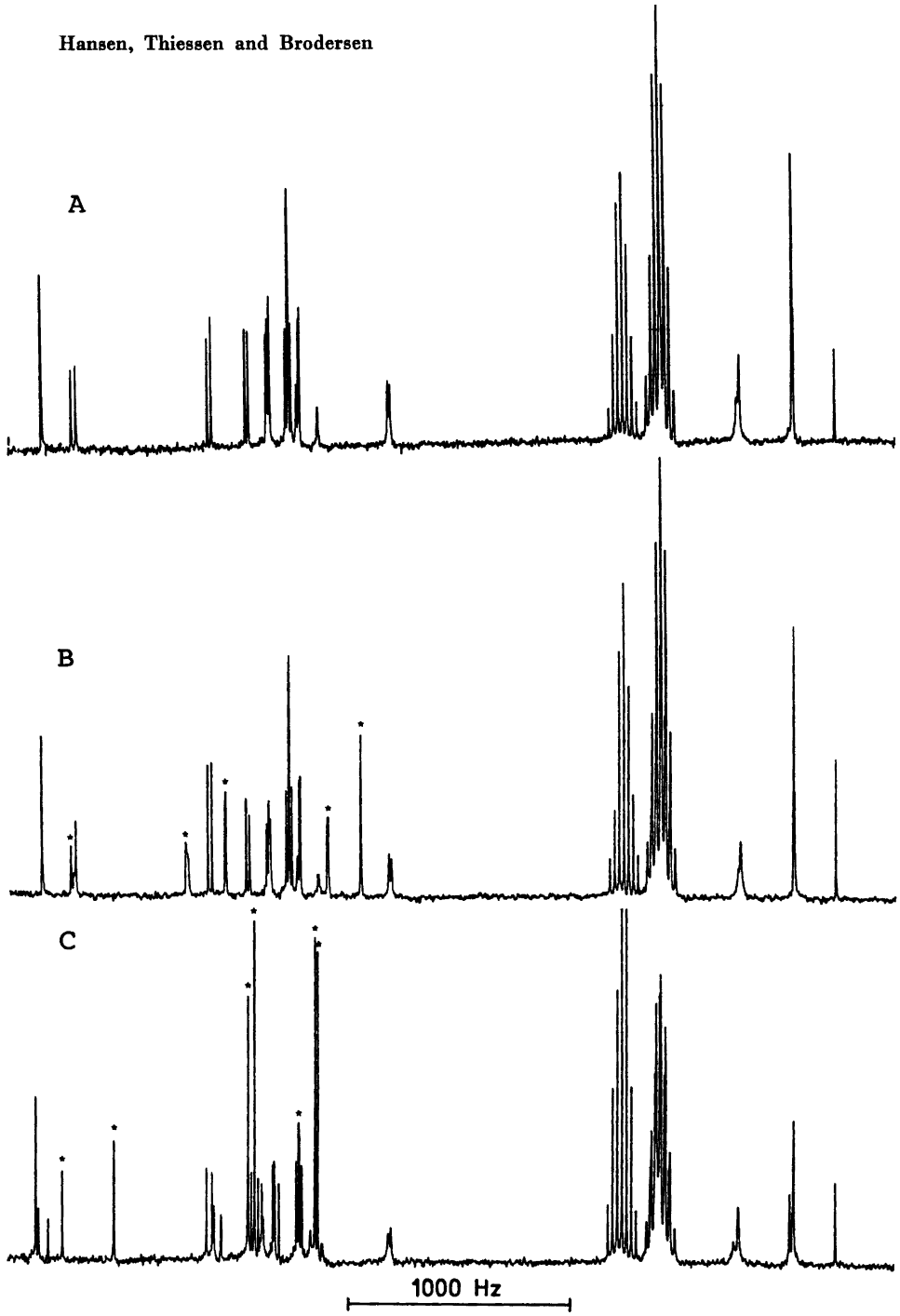

Fig. 6. ^{13}C NMR spectra of bilirubin and the standard acids, *o*-hydroxybenzoic acid and pyridine-2-one partially titrated with potassium hydroxide. ^{13}C NMR spectra recorded at 20.0 MHz. The solvent was in all cases d_6-dimethyl sulfoxide. A, bilirubin IX-α plus 2 mol KOH per mol bilirubin. B, bilirubin IX-α plus 2 mol 2-pyridone plus 4 mol KOH per mol bilirubin. C, bilirubin IX-α plus 2 mol *o*-hydroxybenzoic acid plus 6 mol KOH per mol bilirubin. The scale is given below. The line at highest field is in all spectra the carbon signals of tetramethylsilane. Signals marked with asterisks are assigned in B to 2-pyridone and in C to *o*-hydroxybenzoic acid.

from Table 2, also consistent with addition of approximately 0.4 equivalent of base. The ^{13}C NMR experiment supports the finding from the potentiometric titration, that the carboxylic acid protons in bilirubin and *m*-hydroxybenzoic acid titrate simultaneously and thus have equal pK values in dimethyl sulfoxide.

The pK's of the lactam groups were investigated by ^{13}C NMR cotitrations with 2-pyridone and with *o*-hydroxybenzoic acid. Addition of 4 equivalents of base to a mixture of 2-pyridone and bilirubin IX-α (2:1) resulted in exclusive titration of the amide proton of the former (see Figs. 6A and 6B). Similarly, addition of 6 equivalents of base to a mixture of *o*-hydroxybenzoic acid and bilirubin (2:1) titrated only the carboxylic acid group in the former, as well as both of the carboxylic acid and both of the lactam protons in bilirubin IX-α (see Figs. 6A and 6C). The pK's of both lactam protons in bilirubin must therefore be higher than that of the amide proton of 2-pyridine, and lower than the pK of the phenolic proton of *o*-hydroxybenzoic acid. The differences of pK must in both cases be about 1.3 or more.

Calculation of pK *values in aqueous solution.* In order to relate the relative pK determinations in aprotic solvents to pK values in water, the changes in going from dimethyl sulfoxide to water were calculated. These changes were estimated from the Born equation as described by Kolthoff and Bruckenstein.[19]

$$\ln [K(\text{solvent 1})/K(\text{solvent 2})] = [-Ne^2/2RT]\text{-}[1/r_{H^+} + z_B^2/r_B - z_{HB}^2/r_{HB}][1/D_2 - 1/D_1] \quad (1)$$

where r_{H^+} is the radius of the proton, r_{HB} is the radius of the acid, r_B is the radius of the corresponding base, z is the charge and D is the dielectric constant of the solvent. It is assumed that the radii of the acid and the conjugated base are identical, that the ions are spherical, and that the solvent is a continuum. The radii for bilirubin IX-α, *o*- and *m*- hydroxybenzoic acid, 2-pyridone and the solvated proton were set to 7, 2, 2, 2 and 2 Å, respectively. Furthermore, changes in the dielectric constant of the solvent due to addition of the base dissolved in methanol have not been taken into account. The pK values calculated from eqn. (1) are given in Table 3. In these calculations it was assumed that the acidic groups on the two parts of the bilirubin molecule do not interact. This is probably justified since the carboxylic acid groups are at least 7 Å apart. The same assumption was made for the lactam groups.

First, the pK values (DMSO) of *m* and *o*-hydroxybenzoic acid and 2-pyridone were calculated from known values in water. The pK(DMSO) of the carboxylic protons in bilirubin is identical with that of *m*-hydroxybenzoic acid carboxyl, 5.1. The p$K(H_2O)$ of the bilirubin carboxyls was finally calculated from eqn. (1) which gave 4.4. This is the average pK of the two carboxylic protons in bilirubin. However, no difference of pK between the two groups could be detected.

The pK values (DMSO) for the lactam protons in bilirubin, according to the above findings, must be below pK(DMSO) of the phenolic proton of *o*-hydroxybenzoic acid, 15.5, less 1.3, *i.e.* below 14.2. On the other hand, the lactam

Table 3. Calculation of bilirubin pK values from eqn. (1), at 32 °C.

	p$K(H_2O)$ [a]	ΔpK calc.	pK(DMSO) calc.	pK(DMSO) by cotritation	ΔpK calc.	p$K(H_2O)$
m-Hydroxybenzoic acid carboxyl	4.0	+1.1 [b]	5.1			
Bilirubin carboxyl				5.1	−0.7 [b]	4.4
o-Hydroxybenzoic acid hydroxyl	13.4	+2.1 [c]	15.5			
2-Pyridone lactam	11.5	+1.1 [b]	12.6			
Bilirubin lactam				14.0	−1.0 [d]	13.0

[a] pK values from Ref. 17. [b] Uncharged acid. [c] Monoanion. [d] Formally treated as a monoanion.

pK's(DMSO) should be higher than pK(DMSO) for 2-pyridone, 12.6 plus 1.3, *i.e.* above 13.9 The pK(DMSO) of both lactam protons in bilirubin is thus close to 14.0. The value of pK(H_2O) of the lactams, calculated from this result using equation (1), is 13.0.

Acidimetric stoichiometry of bilirubin binding with albumin. Bilirubin was dissolved in a small excess of aqueous sodium hydroxide and added to a non-buffered solution of human serum albumin, previously adjusted to a selected pH value, 7.4 or 9.0. The molar ratio of bilirubin to albumin was 0.5 so that practically all bilirubin was bound to one site on the protein molecule.[21] The increase of pH, about 0.1, was measured and was compared with the increase obtained when the excess amount of sodium hydroxide alone was added. The number, m, of protons released per molecule of bilirubin was calculated. The over-all process, starting with solid bilirubin, is

Bilirubin (solid acid)	+ Albumin (dissolved at selected pH)	→ Complex (dissolved, same pH)	+ mH$^+$

Determination of m in 28 experiments at pH 7.4 gave a median value of 2.6 (range 2.3 to 3.0). At pH 9.0, 33 determinations gave a median m value of 2.3 (range 1.7 to 2.8). Blind experiments indicated that carbon dioxide caused a variable error, increasing m by an average of 0.2, even though a low-carbonate sodium hydroxide was used and the determinations of pH were done under nitrogen. Corrected median values of m were thus 2.4 and 2.1, respectively at pH 7.4 and 9.0. At these values of pH, bilirubin acid thus releases two protons on binding to albumin. The stoichiometry of the process is equivalent to binding of the bilirubin dianion.

DISCUSSION

Acidimetric titrations of bilirubin in water and non-aqueous solvents have often been reported. Considerable controversy exists, however, over the identification of the ionic species involved, as summarized below. The present paper reports a novel, partial titration of weakly acidic groups in aqueous solution besides complete titrations of four protons, dissociating two by two, in dimethyl sulfoxide, and documents spectra of the acid, dianion and tetra-anion in non-aqueous media. These species are identified as the acid, the dicarboxylate ion, and a tetra-anion resulting from further loss of two protons from the lactam groups, by means of ^{13}C NMR spectra. The pK values are determined by cotitrations with known substances, potentiometrically, and monitored by ^{13}C NMR. In water, both carboxyls have pK = 4.4, as expected for simple carboxylic acids. The pK of both lactam groups, 13.0, is found to be in good agreement with that obtained by partial titration in water, 12.9.

The NMR assignments made in the present paper are different from other reports,[22,23] but are in accordance with partial assignments recently published.[24]

Overbeek *et al.*[4] in 1955 assumed that the two carboxyl groups in bilirubin have pK values around 5 and explained the titration behaviour by the very low solubility of the acid. On this basis they calculated the titration curve and found excellent agreement with the observed one, which again was similar to the one reported here (Fig. 2a).

Gray *et al.*[5] performed spectrophotometric titration of bilirubin IX-α in aqueous solution and reported a pK about 7.1. These authors also noted that readings changed during one hour and that, because of instability of the pigment, an irregular behaviour below pH 5, and small increments of optical density with pH, the pK value was not accurate. This figure has, however, been widely accepted and is often cited in pediatric literature.

Lee *et al.*,[3] as a result of acidimetric and spectrophotometric titrations of bilirubin in various organic solvents, reported that four acidic groups could be demonstrated. They suggested that ionization of the carboxyl groups, although apparently isolated from the chromophores, can affect the absorption spectra through a conformational change. By comparison with a series of aliphatic dicarboxylic acids, which were titrated in *N*,*N*-dimethylformamide as was bilirubin, and ranging the results directly with known pK values of the aliphatic acids in water, they found pK_1(H_2O) = 4.3 and pK_2(H_2O) = 5.3 for bilirubin. Although no attempt was made to consider the effect of the differing ionic radii, these results are in reasonably good agreement with the present

findings. Lee *et al.*[3] assigned the third and fourth acidic functions to a more complicated process, involving either of the central pyrroles and leading to abstraction of the active hydrogen from the central methane bridge. The latter conclusion is at variance with our findings.

These authors also pointed to the importance of the solubility aspects and explained formerly reported pK values between 7 and 8 by the insolubility of the acid.

Knell *et al.*[6] in 1975 supposed that the two lactam groups dissociate about pH 7.5 with formation of a tetra-anion in alkaline solution.

Krasner and Yaffe [7] in 1973 reported a titration curve in aqueous medium, similar to that of Ref. 4 and of the present paper (Fig. 2a) and, disregarding the influence of low solubility of bilirubin in neutral and acid solution, concluded that both carboxyls have pK = 7.55 at 26 °C. This interpretation of the titration curve, however, is erroneous as was already evident from the work of Overbeek *et al.*[4] in 1955. The shape of the curve does not correspond to titration of an acid in a homogeneous solution, and colloid aggregation and later precipitation of the acid takes place during titration.

Titration in *N*,*N*-dimethylformamide of bilirubin with a strong base has also been reported [25] and it was found that the two carboxyl protons were removed first and, with more base and addition of complexing metal ions, two further acidic protons could be removed from the pyrrol nitrogens.

In consequence of the experimental results, bilirubin in aqueous solution must occur almost totally as a dinegative carboxylate ion throughout the range of pH from neutral to 11. This ion is in equilibrium with a minute concentration of the electroneutral acid. Since the pK values of both carboxyls are considerably below 7, the concentration of the acid, at pH above 7, must be proportional to the square of the hydrogen ion concentration, at a constant bilirubin level. The solubility of the acid, which is extremely low, is exceeded at pH values below 7 to 8, depending upon the bilirubin, concentration, and aggregation of the acid takes place. The solubility of bilirubin in aqueous buffers should accordingly be inversely proportional with the squared hydrogen ion concentration.[4] This relation has been experimentally verified within a range of pH 7.35 to 8.65,[26] a finding which can be taken as experimental evidence for the presence of the bilirubin dianion in aqueous equilibrium systems at these pH values. The UV spectra demonstrate the presence of the dianion at a pH as low as 7.0, albeit only for a short period of time. At pH 7.0 the solubility of bilirubin is about 1 nM (determined by extrapolation)[26] and is thus too low for spectroscopic identification of the dissolved ionic species.

Binding of bilirubin with albumin at physiological pH values may accordingly take place as binding of the dianion, in agreement with the general tendency for binding of anions to this protein. This, in fact, is the most probable mechanism since the stoichiometry of the process, as shown above, is binding of the dianion without involvement of protons and since the binding constant is independent of pH in the range from about 7 to 9.3.[27,28]

Animal experiments [29] and work with cell cultures,[27,30] as well as clinical experience [31] has demonstrated a marked increase of bilirubin toxicity on lowering of pH. It was originally pointed out by Silberberg *et al.*[30] that this could be explained, either by increased binding of bilirubin to the target nerve cell, or by decreased binding affinity of bilirubin to albumin, at a low pH. The latter possibility was tentatively proposed by Diamond and Schmid [29] and has been accepted by many pediatricians. According to several authors, bilirubin in acidosis is released from albumin as a result of decreased binding affinity at low pH, and consequently enters the central nervous system. The finding of unchanged binding affinity with varying pH [27,28] speaks against this mechanism. Also, the observations reported in the present paper are contradictory to this concept and favour an explanation by increased binding of bilirubin to the tissues at low pH, as seen in the following.

Exchange of bilirubin between its complex with albumin and the tissues probably depends upon the following reversible processes,

$$\text{Bilirubin acid} \rightleftharpoons \text{Bilirubin}^{2-} + 2\text{H}^+$$
$$\text{Bilirubin}^{2-} + \text{Albumin} \rightleftharpoons \text{Complex}$$

Hydrogen ions are not involved in the latter process and this equilibrium as such is therefore independent of pH. The concentration of

bilirubin acid, on the other hand increases fourfold when pH is decreased by 0.3, as seen from the acid dissociation equilibrium. Since the acid has a very low solubility in water, deposition of this substance in the tissues is likely in acidosis.

At present, it seems that all experimental findings and clinical experience can be explained by increased binding of bilirubin acid to the target on lowering of pH, in agreement with the alternative model of Silberberg *et al.*[30] and as later proposed by Nelson *et al.*[27] It is less likely that an acid dissociation of bilirubin with pK between 7 and 8, and decreased binding affinity to albumin on lowering of pH, should determine the neurotoxicity of bilirubin in case of acidosis, as often stated in the pediatric literature.

MATERIALS AND METHODS

Materials. Bilirubin was obtained from Sigma Chemical Company. E_{454nm} 1 % in chloroform was 1.02×10^3. This preparation contained mainly the IX-α isomer, with traces of III-α and XIII-α as revealed by thin-layer chromatography.[32] Acidimetric titrations in aqueous medium and recording of light absorption spectra of the different ionic species in water and non-aqueous solvents were done with this and a purified bilirubin preparation,[33] with identical results.

Bilirubin XIII-α was prepared from bilirubin IX-α [34] and purified by column chromatography on alumina. Mesobilirubin was prepared from bilirubin IX-α by catalytic hydrogenation with 10 % palladium on charcoal.[35]

Human serum albumin was obtained from AB Kabi. *o*-Hydroxybenzoic acid (Fluka), *m*-hydroxybenzoic acid (B.D.H.) and 2-pyridone (Fluka) were used without further purification. Methanol (Merck, ≤ 0.1 % water) were stored above molecular sieves, 4A. Deuterated solvents (99.9 %) were purchased from Stohler Isotope Chemicals, Annenberg, Switzerland.

Acidimetric titration of bilirubin in aqueous medium. Bilirubin, 0.1 mmol, was weighed out, dissolved in 0.48 mmol low-carbonate sodium hydroxide (from 50 % NaOH solution), diluted to 5 ml, and titrated with hydrochloric acid, 0.1 mol/l, in a nitrogen atmosphere at room temperature. The pH and the light scattering of the reaction mixture was measured at intervals, using an Aminco-Bowman spectrophotofluorometer, incident and emitted wavelength 546 nm.

Titration in non-aqueous solvent. A solution of 0.25 mmol bilirubin in 2.5 ml dimethyl sulfoxide was titrated with 1.5 M potassium hydroxide in methanol. Acidity was monitored by a Radiometer pH-meter 26 with a glass electrode No. G-2222-B and a reference electrode K-901. The latter is a calomel electrode with a salt bridge containing a saturated, aqueous solution of lithium chloride, especially suited for titration in non-aqueous medium. The titration was performed under N_2-atmosphere in the dark at 20 °C. The standard acid, *m*-hydroxybenzoic acid, was titrated similarly.

Light absorption spectra. Spectrophotometric spectra were obtained on a Beckman Spectrophotometer Acta M-VI. (Beckman Instruments, Fullerton, Ca, USA). Special precautions were necessary in order to obtain reproducible spectra of bilirubin in dilute aqueous solutions. An alkaline solution of bilirubin was added to tris buffers, pH 7, 8, and 9, 10 cm optical cells, preheated to 37 °C to a final bilirubin concentration 300 nM and spectra were recorded promptly. The work was carried out with exclusion of day-light and using incandescent light bulbs, covered with a double layer of orange cellophane, absorbing at least 95 % of light at the wavelengths absorbed by bilirubin.

NMR experiments. ^{13}C NMR spectra were run in the Fourier Transform mode on a Varian XL-100 instrument at 25.2 MHz or at a Varian CFT-20 instrument at 20.0 MHz. Usually 36 000 transients were accumulated during 15 h. Dimethyl sulfoxide-d_6 was used as internal lock. The temperature was 32 ± 2 °C. Concentrations of 50 – 100 mg bilirubin per ml with 150 mg KOH per ml of methanol-d_4 were used. The chemical shifts are given in ppm relative to tetramethylsilane (internal standard).

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Received November 15, 1978.