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# On the Existence of a Mono-Vinyl *d*-Urobilin

**(*d*-urobilin 588/nuclear magnetic resonance/chromic acid oxidation)**

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**ABSTRACT** **Chromic acid degradation of a *d*-urobilin, obtained after incubation of bilirubin in fecal bacterial cultures, gave methylvinylmaleimide and methylethylmaleimide. The *d*-urobilin, molecular weight 588, $C_{33}H_{40}N_4O_6$, clearly showed the presence of both vinyl and ethyl resonances in the nuclear magnetic resonance spectrum. These results point unambiguously to a urobilin structure with one vinyl and one ethyl *beta*-substituent.**

The earlier history of the urobilinoids, including the *d*-urobilins, has recently been reviewed (1). A *d*-urobilin was first obtained in crystalline form by Schwartz and Watson (2) in 1942 from infected human bile. A *d*-urobilin was also later found by Sborov and Schwartz in dog feces (3) and by Sborov and coworkers (4) in the feces of patients receiving tetracycline. Combustion analyses (5, 6) could not distinguish between $C_{33}H_{40}N_4O_6$ and $C_{33}H_{42}N_4O_6$ for the urobilin, or $C_{33}H_{42}N_4O_6$ and $C_{33}H_{44}N_4O_6$ for the chromogen. An $H_{40}$ urobilin would be consonant with a monovinyl (*beta*-substituent) compound, whereas $H_{42}$ would agree with a diethyl urobilin. Later, Gray and Nicholson (7) isolated a considerable amount of a *d*-urobilin from the feces of a patient with hemolytic anemia due to thalassemia major and who had not received antibiotics. A detailed examination of this sample, including the observation that it consumed an extra mole of hydrogen on catalytic hydrogenation, led them to favor a *d*-urobilin ($H_{40}$) with one vinyl *beta*-substituent. However, as they noted, the data did not unambiguously exclude a structure having an extra unsaturation in an end ring, a formulation suggested earlier by Siedel (8).

Since these earlier observations, *d*-urobilins have often been isolated either from human feces or fecal bacterial cultures (9, 10). Their molecular weights, occasionally 588 ($H_{40}$) or more often 590 ($H_{42}$), were determined by mass spectrometry (9). A *d*-urobilin ($H_{40}$) has been produced on three occasions in separate cultures of human fecal flora with bilirubin, but not with mesobilirubin, as substrate (9, 10), and has also been isolated repeatedly from samples of dog feces (9, 10). It is of interest to note that a human strain of *Clostridium perfringens* isolated from ileostomy feces reduced bilirubin to a *d*-urobilin ($H_{42}$) (11) with either bilirubin or mesobilirubin as substrate, whereas a rat Clostridium species (12) has invariably produced *l*-stercobilin or *l*-half stercobilin, *in vitro* (10).

Abbreviations: mol. wt., molecular weight; *m/e*, mass to charge ratio; DMSO-d6, completely deuterated dimethyl sulfoxide; NMR, nuclear magnetic resonance.

Very recently Killilea and O'Carra (13) challenged the existence of an $H_{40}$ *d*-urobilin by disputing, *inter alia*, the interpretation of the mass spectral data in the molecular ion region. They invoked the already described disproportionation of *l*-stercobilin before volatilization in the ionization chamber of the mass spectrometer (9). When heated near its melting point, *l*-stercobilin (molecular weight, mol. wt. 594) apparently undergoes an oxidation and reduction reaction to yield volatile substances with molecular ions at *m/e* (mass to charge ratio) 592 and 596 with a standard electron impact source. Killilea and O'Carra explain the appearance of *m/e* 588 in the mass spectrum of *d*-urobilins in terms of disproportionation of an $H_{42}$ *d*-urobilin (mol. wt. 590). This apparently reasonable explanation is possibly flawed by the observation (9) that *d*-urobilin samples which show ions in the mass spectrometer at *m/e* 588 and 590 seldom show much intensity at *m/e* 592. When stercobilin (mol. wt. 594) undergoes disproportionation, it exhibits an early and relatively intense peak at *m/e* 596. The parallel behavior for an $H_{42}$ *d*-urobilin (mol. wt. 590) would be the appearance of an *early* intense peak at *m/e* 592. However, such a peak is not observed.

In the present work, we present conclusive evidence for the existence of an $H_{40}$ *d*-urobilin (mol. wt. 588) with one vinyl *beta*-substituent. The data are provided by chromic acid oxidation analysis, and nuclear magnetic resonance (NMR) spectroscopy.

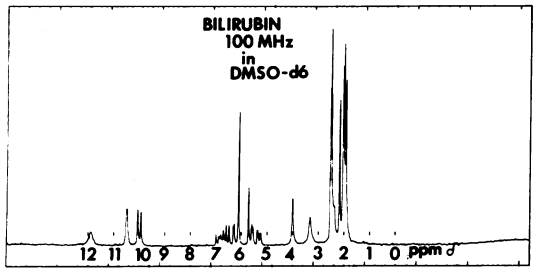
## MATERIAL AND METHODS

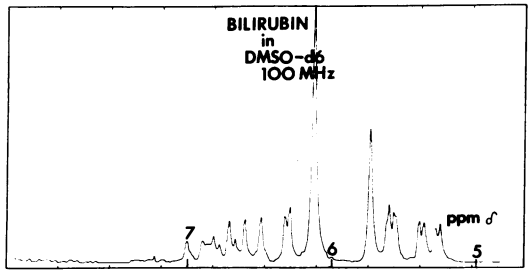
*d*-Urobilin (*m/e* 588 as mol. wt.) was obtained from the fecal bacterial reduction of bilirubin IX$\alpha$ *in vitro* (10). The bilirubin IX$\alpha$ used in these experiments was purchased from Nutritional Biochemicals Corporation. NMR spectra were determined on a Varian XL-100 spectrometer equipped with rapid pulse Fourier analysis capabilities, or on a Varian 300 MHz instrument in the continuous wave mode. The bilirubin used in these studies was purchased from Sigma. Mass spectra were measured on a Varian MAT 311 spectrometer at 70 eV. The chromic acid oxidations were carried out according to Rüdiger's method (14).

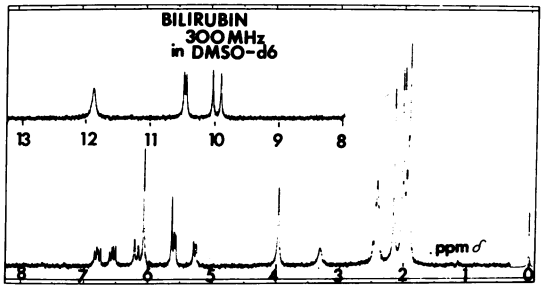
## RESULTS AND DISCUSSION

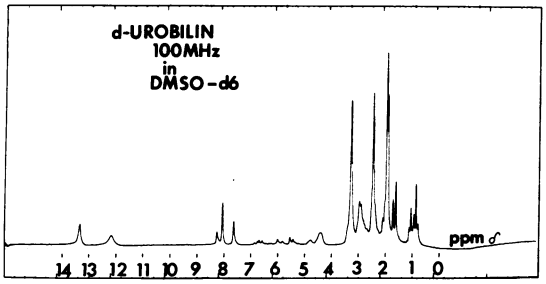
### The nuclear magnetic resonance data

A careful examination of the NMR spectrum should reveal whether a *d*-urobilin (588) possesses a vinyl *beta*-substituent



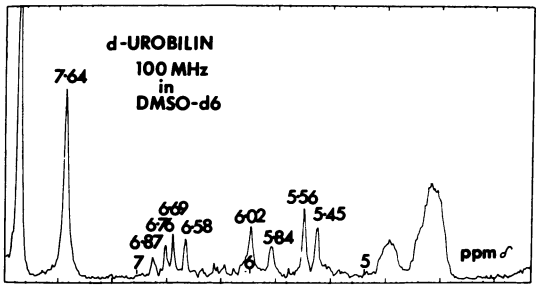




Fig. 1. NMR spectrum of bilirubin IX$\alpha$ in DMSO-d6 (completely deuterated dimethylsulfoxide) at 100 MHz.

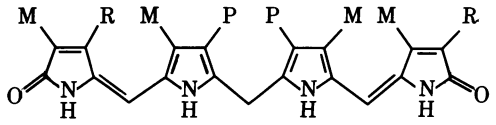
Fig. 2. Expansion of the vinyl proton region of the NMR spectrum of bilirubin IX$\alpha$ in DMSO-d6 at 100 MHz.

Fig. 3. NMR spectrum of bilirubin IX$\alpha$ in DMSO-d6 at 300 MHz. (The somewhat less legible trace in the region of 2$\delta$ is irrelevant in the present context.)

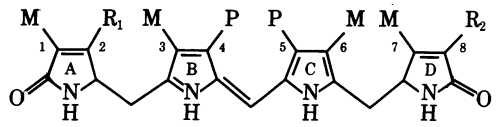
Fig. 4. NMR spectrum of *d*-urobilin (mol. wt. 588) in DMSO-d6 at 100 MHz.

Fig. 5. Expansion of the vinyl proton region of the NMR spectrum of *d*-urobilin (mol. wt. 588) in DMSO-d6 at 100 MHz.

(at C-2 or C-8). Such an examination is facilitated by locating the expected positions of the vinyl hydrogen resonances in a suitable model compound, such as bilirubin.




**1a** bilirubin IX$\alpha$, R = V
**1b** mesobilirubin IX$\alpha$, R = E




**2a** $R_1$ = V, $R_2$ = E
**2b** *d*-urobilin (588) $R_1$ = E, $R_2$ = V
M, —$CH_3$; E, —$CH_2CH_3$; P, —$CH_2CH_2COOH$; V, —$CH{=}CH_2$

Hence, the 100 and 300 MHz NMR spectra of bilirubin IX$\alpha$ (**1a**, vinyl groups at C-2 and C-8) were determined (Figs. 1–3). This was done as follows. The bilirubin spectrum (Fig. 1) was compared with the spectrum of mesobilirubin IX$\alpha$ (**1b**), which has ethyl instead of the vinyl groups at C-2 and C-8. The set of resonances between 5 and 7$\delta$ (ppm, tetramethylsilane) in the bilirubin spectrum were not present in the mesobilirubin spectrum. Therefore that region was assigned to the vinyl groups at C-2 and C-8 of bilirubin, and it agrees in location with the well-established assignments for the vinyl resonances of butadiene and styrene (Table 1). In the case of bilirubin, the vinyl resonances in the expanded vinyl region of Fig. 2 (100 MHz) were more completely resolved into two ABC patterns by going to 300 MHz (Fig. 3 and Table 1). It is not possible to ascertain which ABC pattern is to be associated with position 2 and which with position 8 on the basis of the coupling constants and chemical shift data alone.

We are now in a position to identify the presence of a vinyl group in the NMR spectra of a *d*-urobilin (588) (Figs. 4 and 5). Those NMR spectra clearly show resonances in the same vinyl region [5–7$\delta$ (ppm)] as found with bilirubin (Figs. 1–3). Furthermore, from an examination of the data of Table 1, it may be concluded that the resonances between 5 and 7$\delta$ (ppm) in Figs. 4 and 5 have the correct chemical shifts, coupling constants and pattern (AMX) consistent with either formulation **2a** or **2b** for *d*-urobilin (588).

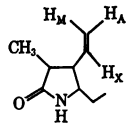
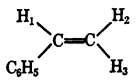
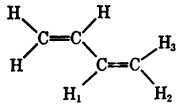
### The chromic acid oxidation

The existence of a vinyl group in the *d*-urobilin (588) under discussion, is completely substantiated by the detection of methylvinylmaleimide following chromic acid oxidation (13). As expected, methylethylmaleimide is isolated from this oxidation in roughly 50% of the yield obtained after chromic acid oxidation of mesobilirubin (**1b**).

## CONCLUSION

These data clearly indicate the presence of both vinyl and ethyl *beta*-substituents in at least one of our *d*-urobilins (588), **2a** or **2b**. They do not, however, indicate that *all d*-urobilin (588) compounds have a vinyl group, for the unsaturation in question might alternatively be located in one of the end rings of a "*d*-urobilin" (588) molecule with two ethyl groups (8).

TABLE 1. *NMR coupling constants and chemical shifts for vinyl hydrogens of bilirubin IXα, d-urobilin (mol. wt. 588), styrene, and 1,3-butadiene*

| System | Coupling constants in Hz | | Chemical shift, ppm from tetramethylsilane |
|---|---|---|---|
|  | $J_{AM}$: <1<br>$J_{AX}$: 11<br>$J_{MX}$: 18 | | $H_A$ 5.51<br>$H_M$ 5.93<br>$H_X$ 6.73 |
| Vinyl groups in bilirubin IXα | $J_{AB}$: 2.4<br>$J_{AC}$: 14<br>$J_{BC}$: 17.6 | $J_{A'B}$: <1<br>$J_{A'C}$: 14<br>$J_{B'C}$: 18 | $H_A$ 5.27; $H_{A'}$ 5.58<br>$H_B$ 6.17; $H_{B'}$ 5.63<br>$H_C$ 6.79; $H_{C'}$ 6.79<br>or 6.55 or 6.55 |
|  | [a] $J_{2,3}$: 1.3<br>$J_{1,2}$: 10.7<br>$J_{1,3}$: 17.5 | | |
|  | [b] $J_{2,3}$: 1.7<br>$J_{1,2}$: 10.2<br>$J_{1,3}$: 17.1 | | |

[a] Ref. 15.
[b] Ref. 16.

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