

over CaCl_2 and then distilled, was refluxed with stirring. When the mixture was homogeneous, MnO_2 (1.6 g) was added in one lot. Stirring under reflux was continued for 1 h. The warm mixture was filtered with suction through celite. Evaporation of CHCl_3 from the filtrate afforded a crystalline residue. The combined residues (3.5 g) from the oxidation of a total of 3.47 g of *3a* were recrystallized from benzene to give unchanged *3a* (2.19 g) and a crystalline residue (1.20 g), which was shown by TLC to be a mixture of the spirodiketone (*1*) and *2a*. This fraction was chromatographed on silica gel (24×2 cm) using benzene- CHCl_3 as eluent. Evaporation of the first fractions of eluate (85 ml) gave the spirodiketone *1* (0.14 g). A middle fraction (100 ml) contained a mixture of *1* and *2a*. Evaporation of the following fractions (140 ml) afforded mainly *2a* (0.75 g). Recrystallization of this fraction from ethyl acetate-light petroleum (1:3) gave pure *2a* (0.55 g); m.p. 121–122.5 °C. (Found: C 81.48; H 5.59. Calc. for $\text{C}_{17}\text{H}_{14}\text{O}_2$: C 81.58; H 5.64). IR (CCl_4): 1724 cm^{-1} (C=O).

Rearrangement of *2a*. A mixture of *2a* (0.032 g, 0.125 mmol), ethanol (1.25 ml, 96 %), H_2O (0.75 ml) and aqueous KOH (0.5 ml, 0.5 N) was refluxed for 1 min. The reaction mixture which contained a precipitate was diluted with H_2O (5 ml) and then cooled. The yield of *2b* was 0.0265 g (84 %); m.p. 196.5–199.5 °C.

The product was identical with the mono-reduction product (*2b*) of *1*¹ (TLC and mixed m.p.).

1,1',3,3'-Tetrahydro-2,2'-spirobi[2H-indene]-1-ol (*6*). A mixture of NaBH_4 (0.12 g, 3 mmol), ethanol (7 ml, 96 %) and the spiroketone (*5*)² (0.47 g, 2 mmol) was stirred at room temperature for 2 h. After cooling in ice, cold HCl (3 ml, 2 N) was gradually added followed by CHCl_3 . The organic phase was washed with water and saturated NaCl solution. After drying (CaSO_4), the CHCl_3 solution was evaporated, and the residue recrystallized from light petroleum to give *6* (0.40 g, 84 %), m.p. 70.2–73.4 °C. (Found: C 86.05; H 6.78. Calc. for $\text{C}_{17}\text{H}_{16}\text{O}$: C 86.40; H 6.83). IR (CCl_4): 3500 cm^{-1} (hydrogen bonded OH), 3660 cm^{-1} (free OH).

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Received December 21, 1975.

The Molar Absorptivity of Horseradish Peroxidase

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The absorption coefficient for the Soret band of HRP* has been given by various authors as $\epsilon_{\text{mM}} = 91 - 107$, and this uncertainty has hampered kinetic and stoichiometric analyses.^{1,2} HRP has also been stated not to obey Beer's law.³ The deviations were attributed to aggregations but no experimental support was given. We have redetermined the light absorption coefficients of HRP and present them together with some comments on storage and handling of HRP.

Materials and methods. HRP A2 (pI 3.9) and C2 (pI 8.8) were prepared as described⁴ and found to be homogeneous in disc electrophoresis and isofocussing. Samples of HRP were also purchased from Boehringer, Mannheim (grade I), Worthington Biochemical Corp., New Jersey (code HPOFF), Sigma Chemical Company, St. Louis (type IV), and Nutritional Biochemicals, Cleveland (catalogue No. 100963). The commercial preparations were dissolved in phosphate buffer pH 7.0, dialyzed overnight against distilled water, and centrifuged for 30 min at 48 000 g.

HRP concentrations were based on pyridine hemochromogen determinations. ϵ for the α -band of this heme derivative has been given as 34.7⁵ and 33.9⁶ $\text{mM}^{-1} \text{cm}^{-1}$, $\Delta\epsilon$ (α -band-minimum at ~540 nm) as 24.6⁵ and 24.5⁶ $\text{mM}^{-1} \text{cm}^{-1}$, and the ratio $A_{\alpha\text{-band}}/A_{\text{min}}$ as 3.46–3.50⁵ and 3.60⁶. The values 33.9 and 24.5 give identical results when used to assay unknown solutions whereas 34.7 and 24.6 do not. For this reason, and because of the higher ratio and the complete absence of a plateau at 610–640 nm in the hemochromogen spectrum in the recent study⁶ we have used the value $\Delta\epsilon = 24.5 \text{ mM}^{-1} \text{cm}^{-1}$ in the present investigation.

Cytochrome *c* (Sigma, type VI) was reduced with dithionite in 50 mM sodium phosphate, pH 7.0, and filtered through Sephadex G-25 medium. $\Delta\epsilon_{550 \text{ red-ox}} = 19.6$ ⁷ $\text{mM}^{-1} \text{cm}^{-1}$.

Citric acid "nach Sørensen" (Merck, Darmstadt) was used. Pyridine (Mallinckrodt) was distilled over KOH. Water was distilled twice in all-glass vessels. Buffers, of ionic strength 0.1 unless otherwise stated, were filtered through Sartorius 0.2 μ membrane filters immediately before use. Ammonium sulfate *p.a.* (Merck, Darmstadt) was used for storage of HRP solutions.

* HRP, horseradish peroxidase (E. C. 1. 11. 1. 7, donor: H_2O_2 oxidoreductase). RZ, $A_{402.5}/A_{280}$.

Table 1. ϵ_{mM} for horseradish peroxidase A2 and C2 in 100 mM sodium phosphate, pH 7.0. Mean \pm S.D.

HRP A2 nm	ϵ_{mM}	HRP C2 nm	ϵ_{mM}
280	26.0 ^a	280	30.1 ^b
	± 0.6		± 0.6
310 ^c	12.9	313 ^c	13.1
402.5	109.1 ^a	402.5	102.1 ^b
	± 1.2		± 1.4
459 ^c	9.9	454 ^c	9.4
500	11.1	500	11.2
604 ^c	2.2	607 ^c	2.0
636	3.2	642	3.2

^a $n=6$, one preparation of HRP A2. ^b $n=10$, four preparations of HRP C2. ^c Minima.

Spectra were registered with a Beckman Acta III spectrophotometer, calibrated against a holmium oxide filter. Linearity in absorbance *vs.* concentration was checked by means of dilutions of a sodium dichromate solution in 5 mM H₂SO₄ for the range A_{267} 0.0085–1.620 (four values $A < 0.1$).⁸ HRP samples to pyridine hemochromogen and absorbance measurements were taken with the same pipette. All spectra were registered at 25 °C.

Results. Freshly prepared ⁴ HRP A2 and C2 give RZ 4.15–4.25 and 3.45–3.55; the values decrease by $\sim 2\%$ within a few days, and the initial RZ cannot be restored by rechromatography. The ratio $A_{\alpha\text{-band}}/A_{\text{min}}$ of their pyridine hemochromogen spectra remains, however, at 3.65–3.70. The values in Table 1 were obtained on material stored for weeks in saturated ammonium sulfate at +2 °C and then dissolved in, and dialyzed against 100 mM phosphate buffer, pH 7.0. The use of TRIS-HCl at pH 7.0 or citrate at pH 4.5 did not significantly change the absorption coefficients. ϵ and RZ were independent of the concentration of HRP at $\leq 20 \mu\text{M}$; higher concentrations were not tried. These results oppose those reported in Ref. 3, and HRP follows Beer's law (Table 2).

The value $\epsilon_{402.5} = 102.1 \text{ mM}^{-1} \text{ cm}^{-1}$ implies a further change in the direction wanted by Dunford.⁹ A solution of H₂O₂ was found to be 3.92 mM when standardized against Fe(II) cytochrome *c*, using $\Delta\epsilon_{550}$ (reduced-oxidized) = 19.6 mM⁻¹ cm⁻¹ and a trace of HRP as catalyst, and 3.93 mM when used to titrate Fe(III) HRP C2 to compound I. Thus $\epsilon_{402.5} = 102.1 \text{ mM}^{-1} \text{ cm}^{-1}$ is in reasonable agreement with two other numerical values.

The spectra of HRP A2 and C2 differ significantly in ϵ_{280} and $\epsilon_{402.5}$ and also in the positions of the minima at 310/313 nm and the maxima at 636/642 nm. The positional difference in red is interesting since this part of the spectrum

Table 2. Absence of concentration effect on the molar absorption coefficients of horseradish peroxidase C2. Sodium phosphate, μ 0.1, pH 6.96, 25 °C.

HRP C2 μM	$A_{402.5}$	ϵ_{mM}
0.20	0.020	100.0
0.50	0.052	104.0
0.99	0.106	107.1
1.64	0.167	101.8
3.26	0.335	102.8
6.39	0.654	102.3
12.30	1.270	103.3
17.79	1.859	104.5

reflects the binding of the iron rather than the status in the porphyrin ring.¹⁰ $E_{m,pH,7}$ is -212 mV and -265 mV for these preparations of HRP A2 and C2.¹¹

RZ of the four commercial preparations ranged from 2.75 to 3.15 at $\leq 20 \mu\text{M}$ in sodium phosphate, pH 7.0, increasing slightly with concentration. $\epsilon_{402.5}$ was found to be 94, 97, 99, and 102 mM⁻¹ cm⁻¹ at pH 7, the three lower values being given by the lyophilized preparations. Variations in ϵ_{280} and RZ between preparation from the same manufacturer were sometimes noticed. Preparations with low RZ gave for some reason less reproducibility in both $\epsilon_{402.5}$ and ϵ_{280} at $< 3 \mu\text{M}$ concentrations in the citrate buffer. Otherwise these preparations also followed Beer's law.

HRP A2 and C2 in saturated ammonium sulfate solution can be kept for months at +2 °C and for a few days at room temperature without losses of activity or changes in spectrum. On the other hand we have occasionally found lyophilization or freezing ($-18 \text{ }^\circ\text{C}$) of buffered solutions to cause losses of activity and changes in spectrum, in agreement with published results on freezing.¹²

Acknowledgement. This study was supported by Statens medicinska forskningsråd (13X-4267).

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Received December 22, 1975.

Morpholine and Thiophene in Glow Discharge Reactions

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Organic molecules undergo a number of transformations in high frequency glow discharge reactions.¹ Ring-opening reactions of *N*-heterocyclic compounds often yield products in which the original *N*-heteroatom has become part of a nitrile group.² We have reexamined² the behaviour of morpholine under glow discharge conditions (27 MHz) using the apparatus shown in Fig. 1, and have confirmed morpholinoacetonitrile (*3a*) and dimorpholinomethane (*6*) as

major products. A third product was identified as α -morpholinopropionitrile (*3b*) whereas formation of β -morpholinopropionitrile has previously been reported.² The products obtained in the glow discharge reactions are explained by intermediate formation of formaldehyde, acetaldehyde and HCN which react further with other molecules of morpholine. The respective product paths were readily demonstrated in solution reactions. Formation of the acetonitrile *3a* has previously been explained by dehydrogenation in the plasma zone of β -morpholinoethylamine; the latter was assumed to be an intermediate reaction product.² Such an explanation fails to account for the formation of the propionitrile *3b*.

In the further study morpholine and its reaction products were admixed with the reactive intermediates from plasma reactions of thiophene by feeding morpholine and thiophene together [ratio 5:1 (v/v)] into the glow discharge zone. A solid was identified as the morpholine salt of morpholinocarbodithioic acid (*11*); the other major products were *N*-thioformylmorpholine (*10a*)² and *N*-thioacetylmorpholine (*10b*)⁴ besides the morpholine derivatives *3* and *6*.

The major solid products from thiophene alone were polymeric, whereas the volatile products were identified as S, H₂S and CS. Carbon monosulfide and thioketene may be the initial products in the reactions of thiophene and will react further with morpholine to yield the thioacyl derivatives *10*. Thioketene is highly reactive. It can be formed by pyrolysis of *tert*-butyl ethynyl sulfide but is polymerised above -80 °C.⁵ Photolysis of 1,2,3-thiadiazole has also been shown to yield thioketene and the isomeric ethynylthiol; photodecomposition of thioketene yields carbon mono-

