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The Molar Light Absorption of Pyridine Ferroprotoporphyrin (Pyridine Haemochromogen)

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Iron-protoporphyrin proteids are frequently and conveniently assayed spectrophotometrically after conversion to pyridine haemochromogen.

At this institute the determinations have since many years been made in a solution, 2.1 *M* in pyridine and 0.075 *M* in NaOH and with dithionite as reducing agent. A number of determinations of the molar absorption on different specimens of recrystallized haemin [1], made by different operators here during recent years, have given the value $\beta = 7.28—7.32 \times 10^7$ cm^2 × mole^{-1}, based on dry weight of recrystallized haemin, for the α-band at 556.5—557 mμ. de Duve [2] reported a similar value for pyridine haemochromogen obtained directly from haemo- and myoglobin ($\beta = 7.36 \times 10^7$ cm^2 × mole^{-1}).

Drabkin [3], when using 6.15 *M* pyridine and 0.0835 *M* KOH found the value $\beta_{558} = 7.14—7.22 \times 10^7$ cm^2 × mole^{-1} (recalc. from $\varepsilon = 31.05—31.40$ × mM^{-1} × cm^{-1}). Lemberg and Legge [4] found that liver catalase gave a haemochromogen spectrum in 1.25 % NaOH even without the addition of pyridine. Their value was $\beta_{558} = 7.14 \times 10^7$ cm^2 × mole^{-1} (recalc. from $d_{558} = 47.5$ for 1 mg catalase haemin per ml.).

We have occasionally observed, however, that the iron content of haemoproteins, calculated from pyridine haemochromogen determination ($\beta_{557} = 7.3 \times 10^7$) gave higher values than those found by direct determinations by the sulphosalicylic acid method [5,6]. This was especially observed in our recent attempts to prepare myoglobin of high purity. We have therefore redetermined the molar absorption of the α-band of the pyridine ferroprotoporphyrin.

EXPERIMENTS

Haemin with 8.26 % Fe was prepared from cow blood according to Fischer [1] but not recrystallized, since we have found that recrystallized material gives haemochromogen spectra with slightly flattened out minima and maxima.

A sample of recrystallized haemin was kindly put at our disposal by Professor Otto Warburg. Its iron content was 8.42 % according to our determination.

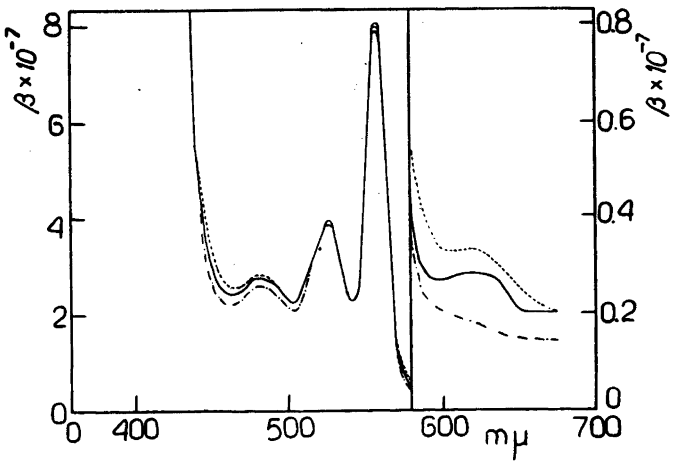

Fig. 1. Absorption spectra of reduced pyridine haemochromogens.
——— *non-recrystallized haemin*
····· *recrystallized haemin*
—.—.—.— *recrystallized myoglobin*

The absorption measurements were carried out and checked in different Beckman spectrophotometers which all were found to give the same values.

Variations in the alkali-concentration between 0.02 and 0.5 *M* NaOH at a constant pyridine concentration of 25 % (v/v) and in the pyridine concentration between 1.2 and 6.2 *M* at a constant alkali concentration of 0.1 *M* did not influence the light absorption of the haemochromogen.

The molar absorption coefficients in Table 1 and in the corresponding absorption curves in Fig. 1 are based on iron determinations [6] on haemin solutions and not on the weights of the dissolved haemin samples. It was thus assumed that the light absorption at 557 mμ of the small amount of impurities which reduced the iron content of the preparations from the theoretical value of 8.57 % to 8.26 resp. 8.42 % was negligible under the experimental condi-

Table 1. Molar absorption coefficients ($\beta = \frac{1}{c} \times \frac{1}{d} \times \ln \frac{I_0}{I}$) *for reduced pyridine haemochromogens.*

	$\beta \times 10^{-7}$ cm² mole⁻¹					
Wave-length in mμ	Max. 557	Min. 540	Max. 526	Min. 503	Max. 480	Min. 460
Non-recrystallized haemin	8.04	2.30	4.03	2.26	2.78	2.44
Recrystallized haemin	7.88	2.28	4.03	2.29	2.83	2.60
Recrystallized myoglobin	7.95	2.29	3.97	2.10	2.55	2.21

tions. The absorption values for the pyridine haemochromogen prepared directly from recrystallized myoglobin were determined and the β calculated on the basis of the iron content. The results are given in Table 1 and Fig. 1.

As seen from the table the data for β_{557} agree with each other, but are considerably higher than those reported earlier in the literature. The true value is $\beta_{557} = 8.0$ rather than 7.3.

As seen from Fig. 1 there is a third band in the visible in addition to the well known α- and β-bands. Its maximum is at 480 mμ. In the regions of low light absorption above 580 mμ and around 460 mμ the recrystallized haemin (8.42 % Fe) gave somewhat higher absorption than the preparation crystallized only once. The opposite was true for the maximum at 557 mμ. Myoglobin gives a definitely better curve than both haemin preparations. This confirms our view that crystallization of Cl-haemin from glacial acetic acid + NaCl leads to some deterioration. The light absorption in the pyridine haemochromogen test is independent of the presence of protein, provided that alkali is present in excess. We determined β_{557} of pyridine haemochromogen for haemin before and after coupling to myoglobin apoprotein and found exactly the same value. It was also found that the addition of crystallized serum albumin to a concentration of 6 mg per ml did not influence the β_{557}.

Keilin and Hartree [7] gave the value 1.61 % haemin for their best horse radish peroxidase preparation, and concluded that they had reached a higher purity than for our crystallized preparations [8]. Their value was based on spectrophotometry of the pyridine haemochromogen. A sample of their preparation, kindly put at our disposal by Dr. E. F. Hartree, was examined for activity in the mesidine test [9] and found to have the same activity per haemin as our preparation. The haemin content of their preparation determined in our pyridine test and calculated on the dry weight value, submitted by Dr. Hartree, was found by us to be 1.36 % ($\beta_{557} = 8.0 \times 10^7$). The haemin content of horse radish peroxidase previously reported from our institute (1.47, 1.39 [8], 1.36 [10], 1.45, 1.38 [11]) had been calculated from the value $\beta_{557} = 7.3 \times 10^7$ cm^2 $\times$ mole^{-1}, and should thus be corrected by the factor 0.915 (=7.3/8). There is thus no reason to assume that the Keilin-Hartree preparation method for peroxidase leads to preparations essentially different from our original one.

SUMMARY

1. The molar absorption of the α-band of the pyridine ferroprotoporphyrin has been re-determined and found to be = 8.0×10^7 cm^2 $\times$ mole^{-1} and thus considerably higher than the values reported earlier in the literature.

2. Variations in alkali and pyridine concentrations within moderate limits have no influence on the spectrum.

3. The presence of myoglobin apoprotein and crystallized serum albumin in the solution does not change the light absorption.

4. The haemin content of Keilins' and Hartrees' horse radish peroxidase preparation was reexamined and found to be lower than reported, and in essential agreement with the values for our preparations.

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