

defect structure. There are also differences in the size distributions of the nitrogen platelets and the other precipitates.

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METAL-LIGAND BONDING IN IRON-PORPHYRINS AND OXYHAEMOGLOBIN

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THERE has been much discussion in recent years of the bonding in metalloporphyrins^{1,10} generally and of the bonding of oxygen in oxyhaemoglobin and oxymyoglobin in particular²⁻⁸. In this article we wish to outline some conclusions reached from recent physico-chemical studies, supported by molecular orbital calculations.

Before any general understanding of the affinity of haems for additional ligands can be gained, it is essential to distinguish between effects which are felt in the metal-ligand σ -bonds and those connected with the π -electron system of the molecule. It is also necessary to decide whether these effects originate in the porphyrin macrocycle or in the extra ligands themselves. When this kind of analysis is applied to oxyhaemoglobins it becomes obvious that the π -component of the iron-oxygen linkage is of great importance. As will be shown, this can be clearly seen by considering the affinities of oxygen for reconstituted haemoglobins⁹. It should be noted that effects which arise from the tertiary structure of the protein are beyond the scope of this analysis.

Bonding. Porphyrins can be regarded as tetraco-ordinating ligands which impose a near square-planar configuration on their metal chelates. In the iron chelates, two further ligands are readily co-ordinated to the metal in the z -direction (perpendicular to the xy porphyrin plane), giving essentially octahedral complexes such as the pyridine haemochromes. Overlap of metal hybrid orbitals with ligand orbitals may thus give rise to six co-ordinate σ -bonds, four in the plane of the porphyrin and two in a direction perpendicular to it.

The five orbitals of the $3d$ sub-shell are used in this way: the $3d_{z^2}$ and $3d_{x^2-y^2}$ orbitals (symmetry type e_g) are incorporated in metal hybrids which are involved in the metal-ligand σ -bonds, the $3d_{xy}$ orbital is occupied, but, by virtue of its symmetry, does not take part in bonding, while the $d\pi$ orbitals ($3d_{xz}$ and $3d_{yz}$ in this nomenclature) are of the correct symmetry to combine with $p\pi$ -orbitals of porphyrin nitrogen atoms and with the π -orbitals of perpendicular ligands as well. The latter are available, for example, in ligands like pyridine. In such a case the π -systems of the ligands and the porphyrin become linked via the $3d_{xz}$ and $3d_{yz}$ orbitals of the metal. Metal-ligand π -bonding of this type is expected to be most important when the d_{xz} and d_{yz} orbitals of the free metal ion

are filled; it leads to a flow of charge away from the metal.

Stabilities of haemochrome complexes. Table 1 sets out the stability constants¹⁰ of the haemochromes of meso-, proto-, deuterio-, and diacetyldeuterio-haems with pyridine, 4-aminopyridine and 4-cyanopyridine. Of these three ligands, 4-aminopyridine is by far the strongest base (pK 9.35) and 4-cyanopyridine the weakest (pK 1.8). It is commonly assumed that the factors governing the binding of a proton to the ligand are the same as those governing formation of the metal-ligand σ -bond. Accordingly, for a given haem, we expect the order of affinities of the ligands to be the same as the order of their basicities, provided that the σ -component of the bond between iron and pyridine dominates the bond strength, as usually seems to be the case. On the other hand, the π -type orbitals of these three ligands are able to mix with the $d\pi$ -orbitals of the iron atom; hence we expect the metal-ligand π -bond strength to be determined by the ability of the ligands to accept π -electrons. For these ligands electron accepting power is in the order 4-aminopyridine < pyridine < 4-cyanopyridine, so that the

Table 1. AFFINITY OF SUBSTITUTED PYRIDINES FOR Fe(II)-PORPHYRINS
A. Stability constants of bis-pyridine haemochromes* ($\log K_s$)

Ligand	Ligand pK_a	Meso-haem	Deuterio-haem	Proto-haem	Diacetyl-deuteriohaem
4-Aminopyridine	(9.35)	4.0	5.2	5.4	6.4
Pyridine	(5.17)	4.6	5.1	5.4	6.0
4-Cyanopyridine	(1.80)	5.8	6.7	6.5	5.8
pK_a of porphyrin		5.8	5.5	4.8	3.3

B. Electronic effects of substitution and stabilization of bis-pyridine complexes of mesohaem*

Ligand	Log K'_s	$S_f^\dagger$	$\sigma^\ddagger$
Pyridine	4.6	0	0
3-Aminopyridine	4.2	-2.1	-0.14
4-Aminopyridine	4.0	-8.4	-0.65
3-Cyanopyridine	5.1	+8.2	+0.64
4-Cyanopyridine	5.8	+7.9	+0.55
3-Methylpyridine	5.25	-0.3	-0.08
4-Methylpyridine	5.5	-0.8	-0.14

* Data from Lowe and Phillips, quoted in Falk¹⁰.

[†] Stabilization factor, S_f , calculated from the data of Lowe and Phillips by da Silva and Calado¹¹. $S_f = (\log K'_s - 2 \log K'_a) - (\log K_s - 2 \log K_a)$. K_s and K'_s are the stability constants of the complexes of pyridine and substituted pyridine, respectively, and K_a and K'_a are the dissociation constants of the conjugate acids of pyridine and substituted pyridine, respectively.

[‡] Hammett's σ -factor for substituents.

tendency to form π -bonds is the reverse of that for σ -bond formation.

Reading down Table 1A from 4-aminopyridine to 4-cyanopyridine, one would expect haemochrome stability to increase or decrease according to whether variation in the π - or the σ -component of the metal-ligand bond is the more significant. In fact, both these situations are shown in the table. With mesohaem, the stability constants increase with decreasing ligand basicity, suggesting that the π -component of the bond is the major variable. This conclusion is supported by da Silva and Calado's analysis¹¹ of the experimental results shown in Table 1B. They obtained an excellent linear relationship between the Hammett σ -factor for the substituent in the substituted ligand and the expression $(\log K'_L - \log K_L)$, termed by them the "stabilization factor" (K'_L refers to the stability constant of the complex of the substituted ligand, K_L to that of pyridine itself).

In the diacetyldeuterohaem complexes in Table 1, on the other hand, σ -bonding seems the more important, since the stabilities of the complexes and the basicities of the ligands both increase in the same sense. Complexes of deuterohaem and protohaem appear to correspond to intermediate situations.

Electronic effects arising from the periphery of the porphyrin are also likely to influence the metal-ligand bond strength. The haems listed in Table 1 (meso-, deuterio-, proto- and diacetyldeutero-haems) differ in respect of the two groups at positions 2 and 4 which are ethyl, hydrogen, vinyl and acetyl respectively. The successive depletion of π -electrons from the inner atoms of the system as electron attraction in the side-chains increases is reflected by the steadily decreasing pK_3 values of the metal-free porphyrins themselves¹⁰. The two effects of this are (a) to increase metal-ligand bond strength by enhancing the ability of the central metal atom to accept σ -electrons from the perpendicular ligand, and (b) to decrease metal-ligand bond strength by reducing the donation of π -electrons to the perpendicular ligand. The latter occurs because the same $d\pi$ -orbitals of the iron atom are involved in bonding with the π -systems of the perpendicular ligands and of the haem, so that electron attraction by side-chains increases the strength of the iron to porphyrin nitrogen π -bonds at the expense of the iron to pyridine nitrogen π -bonds.

Reading across the table from mesohaem to diacetyldeuterohaem, one would expect haemochrome stability to increase or decrease according to whether the σ - or the π -component of the metal-ligand bond is the more significant. With 4-aminopyridine, variations in metal-ligand σ -bond strength appear more important than variations in π -bond strength. With 4-cyanopyridine the situation is more complex. The increase in stability from mesohaem to deuterohaem is presumably due to enhancement of the σ -bond while the decrease observed from deutero- to diacetyldeutero-haem seems to indicate that the expected increase in σ -bond strength has been offset by considerable weakening of the π -component as electron withdrawal by side-chains becomes more severe.

Representative results from a series of molecular orbital calculations for these compounds are given in Tables 2 and 3. The π -bond orders quoted are calculated by Coulson's formula from the results of Huckel molecular orbital calculations so that it is trends rather than absolute values which are of significance. The competition between iron-pyridine and iron-pyrrole π -bonds is clearly displayed in Table 2. The effect of the pyridine substituent on the π -electron density in the $d\pi$ -orbital of the iron is indicated in Table 3. The correlation of this π -electron density with the band I absorption maxima for these complexes may also be noted.

The molecular orbital calculations are consistent with the qualitative arguments used here to interpret the data of Table 1 in terms of the importance of π -bonding in haem type complexes. Further evidence of the significance

of the π -bond in these complexes is provided by the influence of pyridine substituents on the carbonyl stretching frequency in the pyridine-carbon monoxide haem complexes studied by Caughey *et al.*⁸.

Oxygenation of haemoglobin. For the present argument haemoglobin may be regarded as ferro-protoporphyrin with a nitrogen atom of a histidine residue of globin bonded to the iron on one side of the plane⁵; apparently the site opposite the histidine is vacant¹³. Addition of an oxygen molecule changes the high-spin ferro-haemoglobin to diamagnetic oxyhaemoglobin. Not only has spin-pairing been induced in the iron by the act of combination with oxygen, but in addition the two unpaired electrons of oxygen itself have become paired.

Oxygen affinities of a number of native oxygen carriers have been studied in various media and under various experimental conditions by Rossi-Fanelli, Antonini, *et al.*¹². The same workers have also obtained oxygen affinity data for reconstituted haemoglobins in which native globin and various haems other than protohaem are combined⁹. Among these reconstituted haemoglobins, effects caused by variation in the macromolecular environment are minimized so that the nature of the haems must be the main factor affecting the oxygen affinities. The results are shown in Table 4.

It is clear that there is a general trend towards lower oxygen affinities as π -electron density is withdrawn from the middle of the porphyrin towards electron-attracting substituents at the periphery. The obvious explanation for this is analogous to that given in the previous section for the similarly decreasing affinities of these same haems for the strongly π -bonding ligand 4-cyanopyridine.

The correlation between oxygen affinity of the haemoglobin and, say, the pK_3 of the corresponding porphyrin is not as exact as was apparent in the data used by Falk¹⁰, who compared reconstituted meso-, deuterio-, and proto-haemoglobins with native chlorocruorin and obtained an essentially straight line relationship. The more recent study of a reconstituted haemoglobin made by combining chlorocruorohaem with apohaemoglobin has produced a figure for the oxygen affinity quite different from that of the native compound⁹, indicating the importance of the tertiary structure of the protein. Effects of the macro-

Table 2. CALCULATED π -BOND ORDERS FOR FURTHER COMPLEXES OF HAEMS

Ligand	Mesohaem		Diacetyldeuterohaem	
	Iron-pyridine $d\pi-p\pi$ bond	Iron-pyrrole $d\pi-p\pi$ bond	Iron-pyridine $d\pi-p\pi$ bond	Iron-pyrrole $d\pi-p\pi$ bond
4-Aminopyridine	0.362	0.499	0.360	0.492
Pyridine	0.369	0.486	0.361	0.491
4-Cyanopyridine	0.404	0.473	0.389	0.481

Table 3. SPECTRAL DATA AND CALCULATED ELECTRON DISTRIBUTION DATA FOR FURTHER COMPLEXES OF HAEMS

Ligand	Mesohaem	Deutero-haem	Protohaem	Diacetyldeuterohaem
4-Aminopyridine	0.801 (547 $m\mu$)	0.798	0.786 (557 $m\mu$)	0.775 (575 $m\mu$)
Pyridine	0.848 (546 $m\mu$)	0.844	0.830 (555 $m\mu$)	0.815 (571 $m\mu$)
4-Cyanopyridine	0.862 (545 $m\mu$)	0.859	0.849 (554 $m\mu$)	0.834 (569 $m\mu$)

The figures in the table refer to the electron density in the $d\pi$ -orbital of iron which interacts with the two pyridine ligands both in the same plane. The wavelengths ($m\mu$) of the absorption maxima of band I are given in brackets.

Table 4. OXYGEN AFFINITIES OF NATIVE AND RECONSTITUTED HAEMOGLOBINS^{9,12}

A. Reconstituted haemoglobins and myoglobins				
Haem	pK_3 of porphyrin*	Haemoglobin $\log p_{1/2}$ †	n ‡	Myoglobin $\log p_{1/2}$ †
Meso	5.85	-0.35	1.6	—
Haemato	—	+0.03	1.0	—
Deutero	5.50	+0.14	1.7	-0.63
Proto	4.80	+0.36	2.7	-0.13
Chlorocruoro	3.75	+0.20	1.5	—
B. Native oxygen carriers				
	pK_3 of porphyrin*	$\log p_{1/2}$ †	n ‡	
Protohaemoglobin	4.80	+0.35	2.7	
Chlorocruorin	3.75	+1.5	4.5	
Protomyoglobin	4.80	-0.18	1	

* See ref. 10.

† $\log p_{1/2} = \log pO_2$ for 50 per cent oxygenation.

‡ n Refers to the exponent term in the Hill equation.

molecular environment may also be reflected by values of the parameter n of the Hill equation greater than unity. The two results for myoglobin where $n=1$ (see Table 4) are particularly valuable and it is to be hoped that additional data on this system will become available.

That π -bonding is important in the binding of oxygen to haemoglobin was first suggested by Pauling¹⁴ and interaction between the $d\pi$ -orbitals of iron and the $p\pi$ -orbitals of the closer of the two oxygen atoms is a feature of his model. Because of conjugation between the metal-oxygen π -system and that of the porphyrin, and presuming that the metal-oxygen $d\pi-p\pi$ overlap is not small, a structure of this type is in good agreement with the explanation of oxygen affinities given here. The same may be said of the more recent model due to Griffith² in which the oxygen molecule is considered to be parallel to the haem plane, permitting interaction of iron $d\pi$ -orbitals with $p\pi$ -orbitals of both oxygen atoms.

Numerous physical techniques^{1,6} have been applied to oxyhaemoglobin, but it is still not possible to say which of the two models is correct. Current X-ray structural evidence¹⁵ seems to favour Griffith's model. A study of the oxygen adduct of Vaska's iridium compound¹⁶, which binds oxygen reversibly, shows that the oxygen molecule is bound in the manner suggested by Griffith. The increase in the oxygen atom-oxygen atom bond length, as well as the changes in other physical

properties, are strongly suggestive of marked metal-oxygen π -interaction⁷ which, if present in oxyhaemoglobin would favour the explanation of oxygen affinities given in this paper.

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EFFECT OF BRADYKININ ON THE HUMAN ISOLATED SMALL AND LARGE INTESTINE

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UNTIL recently there has been very little investigation of the human alimentary tract by standard pharmacological techniques *in vitro*. This was probably because of the difficulty of obtaining healthy specimens and of oxygenating so thick a tissue. The muscle strip preparation which is easily obtained at operation has proved to be a useful method of investigation. The problem of achalasia of the cardia stimulated work on the human oesophagus^{1,2}, the lower third of which is composed of smooth muscle. This was followed by observations on the behaviour of the aganglionic muscle in Hirschsprung's disease (congenital megacolon) by Trounce and Nightingale³. Since then there has been some attempt to describe the normal pharmacology of the human gastro-intestinal tract. The circular⁴⁻⁶ and the longitudinal muscles^{7,8} of the human colon have been investigated, and both preparations regularly exhibit relaxation to various nicotinic compounds without evidence of a cholinergic component and both show the remarkable inhibitory effect of 5-hydroxytryptamine. The behaviour of similar strips from the human jejunum^{9,10} and ileum^{5,11} has also been described and shown to be different from the colon. What emerges from this work is that there are certain regional differences both in the response to substances which act directly on smooth muscle and in the response to agents which stimulate the ganglia. This article describes the effect of bradykinin on different parts of the human alimentary canal and again shows that the response of the bowel wall varies from region to region.

Longitudinal and circular muscle strips about 20 mm long and 1-2 mm wide were taken from the human jejunum, ileum and distal half of the colon. Muscle strips were taken only from regions that appeared healthy and had not been obstructed. None of the strips reported in this article came from patients with inflammatory disease

of the bowel (for example, ulcerative colitis or Crohn's disease). The strips consisted of full thickness bowel wall with the mucosa and submucosa removed. They were collected in the operating theatre and immediately placed in Krebs solution at room temperature and quickly taken to the laboratory. There they were set up in an isolated organ bath in Krebs solution gassed with 95 per cent oxygen and 5 per cent carbon dioxide at 37° C and placed under a tension of 1.5 g. The preparation was left for 15-30 min by which time spontaneous activity had usually developed. Recording was made on a smoked drum with a direct-writing isotonic lever. The details of method have been previously described⁶. Synthetic bradykinin (Sandoz) was used in all experiments and the bradykinin placebo provided by Sandoz was frequently tested for activity.

Jejunum. Nineteen longitudinal and seven circular strips were investigated. These were taken from sixteen patients, twelve of whom had duodenal or gastric ulceration and four had neoplasms of the pancreas. All the longitudinal strips contracted to acetylcholine (usually maximal responses were obtained with 10-100 ng/ml.) and this could be antagonized by atropine (20 μ g/ml. 5 min) but was not affected by procaine (30 μ g/ml. 30 min) or hexamethonium (50 μ g/ml. 5 min). All nineteen strips contracted in response to bradykinin and fifteen of them gave maximal responses with 0.2-1.0 ng/ml. Of the remainder, two were more sensitive giving maximal contractions at 0.005 ng/ml. and two less sensitive requiring 20 ng/ml. Most strips showed tachyphylaxis. The response to bradykinin could not be antagonized by atropine (Fig. 1), hexamethonium or procaine. The concentration of procaine used (30 μ g/ml. 30 min) was found to be sufficient to antagonize the action of nicotine on the human ileum and colon⁶. The placebo was inactive