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Immunochemistry of Sperm-Whale Myoglobins Prepared with Various Modified Porphyrins and Metalloporphyrins

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1. The preparation and characterization of manganese, iron, cobalt, nickel, copper and zinc metalloporphyrins is described. Ferrihaem was also esterified with pyrid-4-ylpropanol and the derivative characterized as the diester. 2. Complexes of these various porphyrins, as well as protoporphyrin IX, with apomyoglobin were formed and the resulting artificial myoglobins characterized. 3. Very little complex-formation was obtained with nickel, cobalt and manganese metalloporphyrins and apomyoglobin. 4. Myoglobin prepared with copper metalloporphyrin was immunochemically identical with native ferrimyoglobin. All the other artificial myoglobins were less reactive to varying degrees. 5. The changes in antigenic reactivities were attributed to conformational reorganization caused by the different co-ordination tendencies of the various metals or by the modification of the side chains of the haem.

It has been shown that, in haemoglobin and myoglobin, the haem group plays an important role in their immunochemistry. Although the haem group is not part of an antigenic site (Reichlin, Hay & Levine, 1963) it nevertheless plays an active part in providing the conformational requirements necessary for the antigenic specificity of myoglobin (Reichlin *et al.* 1963) and haemoglobin (Atassi, Brown & McEwan, 1965). In studies on modified antigens, the question arises whether any changes in the antigenic reactivity after modification are due to a direct involvement of the modified residue(s) or are the result of some drastic conformational changes. Physicochemical measurements on the protein solution will aid significantly in this connexion. It is, however, conceivable that our present techniques do not detect all possible conformational changes of a protein in solution. Since haem is not part of an antigenic site in haemoglobin and myoglobin, it appeared that these proteins would provide excellent systems for studying the effect of conformational factors on the antigen–antibody reaction. Myoglobins in which the iron of the haem was replaced by various metals have been prepared and characterized. Also, a ferrihaem derivative in which the carboxyl side chains were esterified with pyrid-4-ylpropanol has been synthesized and its corresponding myoglobin derivative has been prepared and studied. In addition, protoporphyrin IX–apomyoglobin complex was prepared. The immunochemistry of all these derivatives was investigated to determine the effect of conformational changes on the antigen–antibody reaction in myoglobin.

MATERIALS AND METHODS

Preparation of metmyoglobin and apomyoglobin. Mb* used for these studies was the major component (MbX) obtained on CM-cellulose chromatography (Atassi, 1964) of the crystalline protein. The apoprotein was prepared by a method similar to that described by Theorell & Åkeson (1955).

Preparation of metalloporphyrins. Iron was first removed from haem by a procedure similar to that of Fischer & Putzer (1926). A 3g. sample of haemin chloride (Eastman Organic Chemicals, Rochester, N.Y., U.S.A.) was heated under reflux with 98–100% formic acid (240ml.). Iron powder (3g.) was added in small portions over a period of 20min. and the mixture refluxed for a further 20min. when the addition of iron was complete. After cooling and centrifuging, the supernatant was poured into an equal volume of saturated ammonium oxalate solution. The protoporphyrin IX that separated out was collected by centrifuging, washed with water (six times) on the centrifuge and dried in a desiccator over P_2O_5.

* Abbreviations: Mb, sperm-whale metmyoglobin (this is preceded by a metal symbol to indicate a metalloporphyrin–apomyoglobin complex); proto-Mb, protoporphyrin IX–apomyoglobin complex; PPH, ferrihaem dipyrid-4-ylpropyl ester; PP-Mb, PPH–apomyoglobin complex; MbX, the major chromatographic component of sperm-whale myoglobin obtained on CM-cellulose chromatography (Atassi, 1964).

For insertion of the metal into protoporphyrin IX, a portion of the latter (400mg.) was dissolved in acetic acid (20ml.). Any trace of insoluble residue that did not dissolve on heating for 10min. was removed by centrifuging and discarded. The solution was mixed with 20ml. of acetic acid that had been saturated with the metal acetate. Usually the salt in which the metal has its higher oxidation state was used. At any rate the mixture of metal acetate and protoporphyrin IX in acetic acid was heated while shaking in air to ensure autoxidation of the metal to its higher oxidation state. After cooling, the mixture was evaporated down on a rotary evaporator to 1–2ml. Water (15–20ml.) was added to the solution; the precipitate was recovered by centrifuging, washed on the centrifuge several times with distilled water and dried in a desiccator.

The following derivatives were prepared: manganese (manganic) metalloporphyrin (Found: Mn, 9·2. Calc.: 8·9%); iron (ferric) metalloporphyrin (Found: Fe, 9·0. Calc.: 9·1%); cobalt (cobaltic) metalloporphyrin (Found: Co, 9·8. Calc.: 9·5%); nickel (nickelous) metalloporphyrin (Found: Ni, 9·4. Calc.: 9·5%); copper (cupric) metalloporphyrin (Found: Cu, 10·1. Calc.: 10·2%); zinc metalloporphyrin (Found: Zn, 10·5. Calc.: 10·4%).

Esterification of ferrihaem with pyrid-4-ylpropanol. Haemin chloride (104mg.) was suspended in chloroform (6ml.), pyrid-4-ylpropanol (2ml.) was added followed by 2ml. of conc. HCl and the mixture was refluxed on a boiling-water bath for 2hr. It was then cooled, mixed with 0·5vol. of water and kept at 0–2° overnight. A crystalline product was obtained that was recovered by centrifuging, washed on the centrifuge with distilled water until the washings were colourless (eight washings) and dried in a desiccator. The derivative was insoluble in water but dissolved readily in 0·1N-NaOH or 10mM-Na_2HPO_4 containing KCN (0·01%). Analysis (Found: C, 67·2; H, 5·0; N, 9·4%) showed that it corresponded quite well with that of ferrihaem dipyrid-4-ylpropyl ester (Calc.: C, 67·4; H, 4·8; N, 9·4%).

Reconstitution of myoglobin Apomyoglobin (16mg.) was dissolved in 4ml. of water previously adjusted to pH7·0. The metalloporphyrin (or protoporphyrin IX) in 0·1N-NaOH (7mg. in 1ml.) was added drop by drop to the apomyoglobin solution at 0° and the pH maintained at 7·0–7·5 by the addition of 0·1N-HCl. The operation was carried out away from direct light. When the addition of the porphyrin was complete, the mixture was stirred in the ice bath for a further 2hr. and then dialysed against several changes of 10mM-phosphate buffer, pH7·2, containing KCN (0·01%). If cloudiness occurred during dialysis, the solution was cleared by centrifuging at 4900*g* at 0° for 30min.

For combination with apomyoglobin, PPH (8mg.) was dissolved in 4ml. of 20mM-Na_2HPO_4, containing KCN (0·02%), and added slowly to the magnetically stirred apomyoglobin solution (16mg.) in 4ml. of 20mM-phosphate buffer, pH7·2, containing KCN (0·02%). The operation was carried out at 0° and away from direct light. The complex was then treated as above.

Immunization. The preparation of rabbit and goat antisera to MbX has been described in detail by Atassi (1967). Rabbit antisera nos. 11, 17 and 100 and goat antiserum G1 were used for the present work.

Analytical methods. The metal content was determined on a Perkin–Elmer model 303 atomic absorption spectrophotometer. The concentrations of protein solutions were based on their nitrogen contents, which were determined in a micro-Kjeldahl apparatus similar to that described by Markham (1942). Spectral analyses were done in a Cary model 14 spectrophotometer. Double diffusion in 1% agar was by the method of Ouchterlony (1949); the lines were considerably sharper when gels contained KCN (0·05%). Precipitin experiments were carried out by the method of Heidelberger & Kendall (1935). Electrophoresis was on starch gel, at room temperature, in the discontinuous buffer system of Poulik (1957); the buffers contained KCN (0·05%) and the gels were stained with Amido Black (Smithies, 1959).

RESULTS

Spectral properties. The spectral behaviour of various metalloporphyrins has been studied in detail by many workers (Haurowitz, 1935; Stern & Molvig, 1936; Falk & Nyholm, 1958; Falk & Perrin, 1961; Phillips, 1963) in several solvent systems. In the present work, the absorption spectra of protoporphyrin IX and manganese, iron, cobalt, nickel, copper and zinc metalloporphyrins were determined in 10mM-disodium hydrogen phosphate, containing potassium cyanide (0·01%). Since the spectra of these compounds have not been reported

Table 1. *Absorption maxima of various metalloporphyrins and other haem derivatives*

Solutions were in 10mM-Na_2HPO_4 containing KCN (0·01%).

Metalloporphyrin or haem derivative	$\lambda_{max.}$ (mμ)								
Mn porphyrin	255	—	—	339	390·5	—	540	570*	638*
Fe porphyrin	220	—	—	350	418·0	—	550	—	650*
Co porphyrin	—	—	305	345	433·0	—	546	—	647*
Ni porphyrin	237	267, 284	—	343	401·0	—	—	—	648
Cu porphyrin	244	—	—	315	397·5	—	532	570	649*
Zn porphyrin	232	—	—	—	396·5	502	545	575*	647
Protoporphyrin IX	237	—	—	350	399·5	505*	545	580*	650*
PPH	238	—	—	364	421·0	—	548	—	650*

* Absorption maximum very weak, mostly a small arrest in the curve.

in this solvent, it is sufficient to point out the positions of their absorption maxima, which are shown in Table 1. On the other hand, since this is the first report of the synthesis of PPH, both its absorption maxima (Table 1) and its spectrum are shown (Fig. 1).

The absorption spectra of the porphyrin–apomyoglobin complexes were also determined in 10mM-phosphate buffer, pH 7·0, containing potassium cyanide (0·01%). The positions of their absorption maxima and the ratios of the extinctions at some of these maxima are shown in Table 2. The values of these ratios suggested that very little complex-formation was obtained with nickel, cobalt and manganese metalloporphyrins. The apomyoglobin complexes of these metalloporphyrins had much higher extinctions in the ultraviolet than in the Soret region. These conclusions from spectral data were confirmed by the results of metal determinations (Found: Ni, 0·006; Co, 0·052; Mn, 0·086, respectively. Calc.: Ni, 0·330; Co, 0·331; Mn, 0·308%, respectively). All other metalloporphyrin–apomyoglobin complexes had metal contents similar to the expected values (±6%). Absorption spectra of PP-Mb showed an increase in the extinction at 544mμ relative to that at 422mμ. The slight variation in the relative intensities of these bands may be caused by the reversal of charge of the carboxyl side chains when the pyridyl group is introduced. The carboxyl groups of ferrihaem were esterified with pyrid-4-yl-propanol (instead of methanol or ethanol) in order to study the effect of charge reversal of the carboxyl side chains in haem on the properties of myoglobin and also to improve the solubility of the product. It should be pointed out that, to date, combination experiments have been carried out with methyl or ethyl esters of ferrihaem. These esters, unlike PPH, are insoluble in aqueous solvents and have to be dissolved in organic solvents before being mixed with apomyoglobin. Such a recombination procedure might yield a denatured haemoprotein.

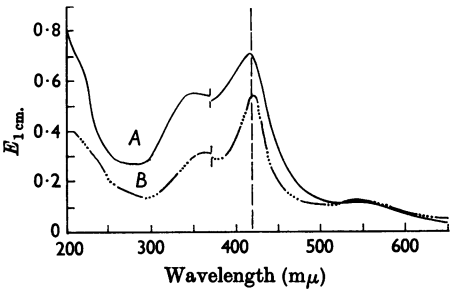

Fig. 1. Absorption spectra of ferrihaem (*A*) and PPH (*B*). Solutions were in 10mM-Na_2HPO_4 containing KCN (0·01%). Concentrations of solutions were not determined but were adjusted so that they had an identical extinction (0·122) at 549mμ.

Electrophoretic studies. Myoglobin derivatives containing protoporphyrin IX, iron, copper and zinc had identical electrophoretic mobilities on starch gel. PP-Mb, on the other hand, showed splitting into four bands with mobilities (relative to MbX = 1) of 1·92, 2·75, 3·52 and 4·03 (trace). This splitting might be related to the existence of myoglobin in various molecular species with identical chemical and immunochemical properties but differing only in charge (Atassi, 1964). Fe-Mb obtained from ferrihaem and apomyoglobin gave only one band in the position of MbX on starch-gel electrophoresis. This band was absent from the

Table 2. *Absorption maxima and ratios of extinctions at some of these maxima of apomyoglobin complexes with various haem derivatives*

All solutions were in 10mM-phosphate buffer, pH 7·0, containing KCN (0·01%).

Myoglobin derivative	$\lambda_{max.}$ (mμ)					Ratio of extinctions at absorption maxima	
	A	*B*	*C*	*D*	*E*	*A*/*D*	*D*/*E*
Mn-Mb	278	289*	340–350	417	540–550	5·11	3·94
Fe-Mb	278	290*	358–363	423	540	0·325	9·51
Co-Mb	266	—	355–360	419	550	7·66	5·00
Ni-Mb	267	283	—	425·5	—	17·46	1·37
Cu-Mb	278–279	—	355–360	421	543	0·448	9·59, 9·47
Zn-Mb	276	—	356	423	544	0·348	9·84
Proto-Mb	270–280	290*	350	410	540	0·381	8·03
PP-Mb	266	—	359	422	544	0·302	7·38
Mb	278	290*	358	422·7	540	0·328	9·62

* Absorption maximum only an arrest in the curve.

PP-Mb preparation. There is therefore a strong indication that the carboxyl side groups in haem are important in stabilizing the molecule in its major electrophoretic form.

Immunochemical studies. In agar double-diffusion experiments, all the myoglobin derivatives were immunochemically identical with native Mb since the precipitin lines fused completely, forming no spurs or intersections with the precipitin line of the native protein. However, the lines due to proto-porphyrin IX, Mn-Mb, Co-Mb, Ni-Mb and Zn-Mb were weaker than those given by Cu-Mb, Fe-Mb, PP-Mb and native Mb when solutions with similar protein concentrations were tested against a given antiserum.

It should be pointed out at the outset that Fe-Mb that had been prepared by re-forming the complex of apoprotein with resynthesized haem (from ferric iron and protoporphyrin IX) was immunochemically indistinguishable from native Mb by all immunochemical tests.

In quantitative precipitin experiments, proto-Mb was antigenically less efficient than native Mb, reaching maximum precipitation at a much larger antigen concentration. In addition, it was not an effective precipitation inhibitor in the region of antigen excess (Fig. 2). The experiments in Fig. 2 were done with rabbit antiserum no. 17, but similar results were obtained with rabbit antisera nos. 11 and 100. Fig. 2 also shows, in addition to the homologous reaction, the reactions of the antiserum with the apoprotein, Mn-Mb and PP-Mb. The reactivity of Mn-Mb was almost identical with that of the apoprotein. Similar results were obtained with antisera nos. 11 and 100. Fig. 2 suggests that the apoprotein reacts less efficiently than Mb with the antiserum to MbX. This finding is in agreement with that of Reichlin *et al.* (1963) for reaction of rabbit antibodies to horse myoglobin with its apoprotein. The results of Crumpton & Wilkinson (1965), on the other hand, showed very little difference in the reactivities of Mb and its apoprotein with rabbit antisera to native Mb. This conflict might be the result of differences in antisera specificities caused by differences in injection schedules; differences in specificity may also depend on the animal that was immunized. I have myself obtained antisera that behaved similarly to those of Crumpton & Wilkinson (1965) with regard to their reactions with Mb and apomyoglobin (e.g. antiserum G1; see Fig. 3*b*).

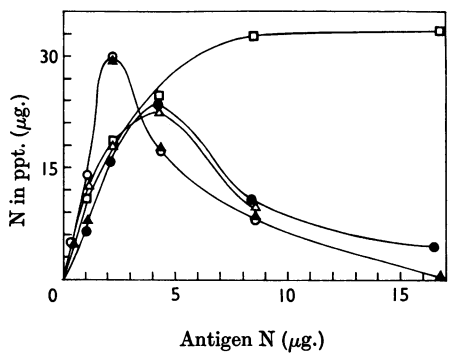

Fig. 2. Precipitin analyses with rabbit antiserum no. 17 that had been diluted with 0·15M-NaCl (1:1) and various artificial myoglobins. ○, Mb obtained by recombination of apomyoglobin with ferrihaem; ▲, PP-Mb; △, Mn-Mb; ●, apomyoglobin; □, proto-Mb.

PP-Mb was identical with native Mb in its reactivity with rabbit antiserum no. 17 (Fig. 2). This suggested that esterification of the carboxyl side groups in haem with pyridyl groups, which leads to the reversal of their charge, has no effect on the antigenic specificity of the protein. In other words, it would appear that no drastic conformational changes take place here. This conclusion was rather surprising, especially in the light of proposals that the carboxyl side groups play an active part in the linkage of haem to globin in haemoglobin and myoglobin (Lemberg & Legge, 1949; O'Hagan, 1961). The reaction of PP-Mb was then tested further with rabbit antisera nos. 11 and 100 and goat antiserum G1. With all these three antisera, the reactivity of PP-Mb was even poorer than that of apomyoglobin. Fig. 3 shows the reaction of PP-Mb with antisera nos. 11 and G1. These results present an example of specificity differences between antisera from the same species. It also stresses the point that conclusions should always be based on experiments with more than one antiserum.

Less antibody nitrogen was precipitated by Co-Mb than by Fe-Mb. Since very little complex-formation was achieved between cobalt metalloporphyrin and the apoprotein, no conclusion should be made here because large amounts of apomyoglobin were present in the reaction mixture. In contrast, complex-formation of copper and zinc metalloporphyrins with apomyoglobin was complete and the results are significant. The Cu-Mb precipitated as much antibody nitrogen as the homologous antigen when allowed to react with antisera nos. 11, 17 and 100. The precipitin reaction of Cu-Mb with antiserum no. 17 is shown in Fig. 4. Fig. 4 also shows the precipitin reaction of Zn-Mb with antiserum no. 17. Zn-Mb reacted poorly with the antiserum to Mb; in fact, it precipitated less antibody nitrogen than the apoprotein. Results with antisera nos. 11 and 100 and Zn-Mb were similar to those obtained with antiserum no. 17.

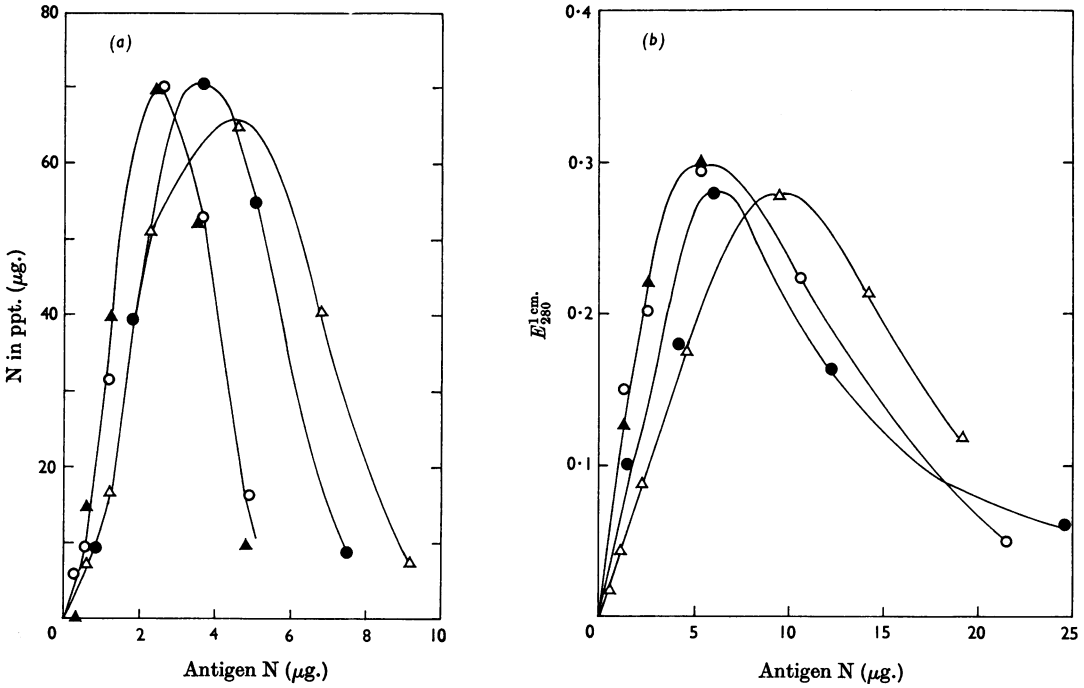

Fig. 3. Precipitin studies on PP-Mb. ○, Mb; ▲, Mb obtained by recombination of apomyoglobin with ferrihaem; ●, apomyoglobin; △, PP-Mb. (*a*) Reactions with rabbit antiserum no. 11 that had been diluted with 0·15M-NaCl (1:1); (*b*) reactions with goat antiserum G1. The differences in scales and units of (*a*) and (*b*) should be noted. The immune precipitates in (*b*) were dissolved in 0·5ml. of 0·5N-NaOH and the volume was made up to 3ml. with 0·15M-NaCl.

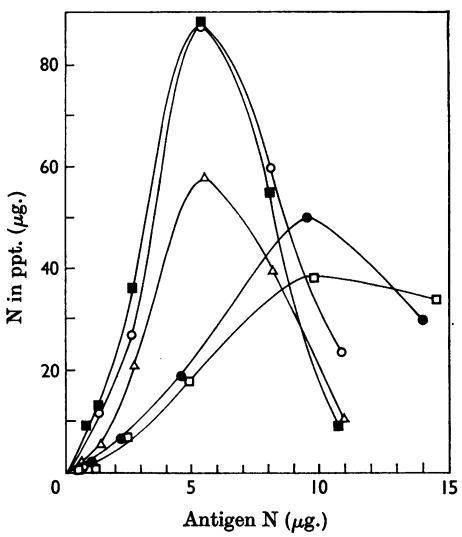

Fig. 4. Precipitin analyses with rabbit antiserum no. 17 and some artificial myoglobins. ○, Mb obtained by recombination of apomyoglobin with ferrihaem; ■, Cu-Mb; △, Co-Mb; □, Zn-Mb; ●, apomyoglobin.

DISCUSSION

The preparation of protoporphyrin IX–apohaemoglobin complexes has already been reported (Hill & Holden, 1926; Rossi-Fanelli, Antonini & Caputo, 1959). The combination is highly specific (Rossi-Fanelli, Antonini & Caputo, 1964), giving a product of stability comparable with that of the haem–apoprotein complex and which does not combine further with haem (Teale, 1959). This suggests that haem and protoporphyrin IX bind to the same protein sites. Of course other explanations are possible, which depict a conformational reorganization on combination of protoporphyrin IX with globin of such nature that further uptake of haem will not be possible. The similarities of the spectra of Fe-Mb and proto-Mb suggest identical binding regions. Also, the results of Breslow & Koehler (1965) suggested that the conformations of proto-Mb and Mb were essentially similar; however, proto-Mb, as expected, had one more titratable histidine residue, its tyrosine residues had a slightly higher pK_a and it was less stable to acid denaturation than Mb. My immunochemical

results suggest that these somewhat slight differences can be sufficient to bring about a change in antigenic reactivity. The absence of the iron–apomyoglobin linkages probably gives rise to a 'looser' structure than that of myoglobin.

In the manganese protoporphyrin, the complex will be of the d^4 type. Manganic complexes have a high-spin configuration, $(t_{2g})^3(eg)$, and an appreciable Jahn-Teller effect in a low ligand field. (Information on these types of complexes and the Jahn-Teller effect may be found in: Orgel, 1960; Griffith, 1961; Jones, 1964; Falk, 1964.) Both manganous and manganic complexes may add two extra ligand molecules, one above and one below the porphyrin plane, to form distorted octahedra. In bispyridine, bisnicotine and bispicoline complexes of manganous, manganic, cobaltous or cobaltic mesoporphyrin, in aqueous solution, no competition apparently exists between OH^- ions and extra ligand even at pH 13 (McConnel, Overell, Petrow & Sturgeon, 1953). What this might mean in terms of myoglobin structure is that the E7 histidine residue (histidine 64 in the E helix; Kendrew *et al.* 1961) might become directly linked with the metal in the metalloporphyrin and not through a water molecule as normally occurs with ferrihaem. Alternatively, no co-ordination with the distal histidine residue may occur and the sixth co-ordinate position of the octahedron will be occupied by CN^-. At any rate, the effect on the conformation of the protein will probably be appreciable and the conformational difference would be expected to be detectable by immunochemical studies. However, complete complex-formation with manganese prophyrin was not obtained and the results should be interpreted with caution.

The nickel and copper metalloporphyrins represent a rather interesting case. First, the nickelous complex possesses the d^8 configuration and shows in its ground state, $(t_{2g})^6(eg)^2$, a considerable Jahn-Teller distortion. The distortion is so great that Ni^{2+} forms square planar four-co-ordinate complexes. The Ni^{2+}–porphyrin chelate, like the Cu^{2+}–porphyrin chelate, has generally low affinity for extra ligands and therefore low-spin octahedral complexes should not exist (Orgel, 1961). Therefore Ni^{2+}–apomyoglobin co-ordination is not possible. Nevertheless, interaction through porphyrin carboxyl side groups can still take place and binding may occur through side chains (O'Hagan, 1961). In three attempts, however, I have failed to prepare the Ni-Mb complex in any appreciable concentration; only a very negligible amount of complex-formation occurred as concluded from the spectral and analytical data.

On the other hand, the cupric complex, which is of the d^9 type, also possesses a considerable Jahn-Teller distortion and therefore its complexes are mostly square planar. However, cupric complexes often have two ligands in the axial direction at a greater distance. The lone electron in Cu^{2+} is involved in bonding to porphyrin nitrogen atoms and is in the $3dx^2$–y^2 orbital and not elevated to the $4d$ level (Falk & Nyholm, 1958; Havemann, Haberditzl & Mader, 1961). Therefore the attainment of the octahedral co-ordination is probably by some weak bonding to the two extra donor atoms. This suggests that Cu-Mb complex-formation may take place especially with the contribution of globin–porphyrin side-chain interactions. The final complex most likely has the same configuration as the (ferric) Fe-Mb complex, as suggested from immunochemical studies.

The zinc complex possesses the d^{10} configuration. This is a completely filled system and therefore the behaviour related to an incompletely filled d orbital is not exhibited. The co-ordination number of Zn^{2+} is four and it prefers tetrahedral ligand distribution. Therefore some weakening of the bonding orbitals might be expected on co-ordination with the square-planar porphyrin. The Zn^{2+}–porphyrin chelates are diamagnetic (Falk & Nyholm, 1958). The Zn^{2+} ion has enough electronegativity to allow the addition of a fifth but not a sixth ligand (Falk, 1964). Falk & Phillips (1964) suggested that, on addition of one extra ligand, the bond to one of the porphyrin nitrogen atoms is released and the metal ion is able to approximate the tetrahedral sp^3 hybridization that it prefers. In terms of myoglobin structure this will imply that linkage is only to one of the histidine residues (say the F8 histidine residue; Kendrew *et al.* 1961). With nothing to attach the E7 histidine residue to the neighbourhood of the porphyrin, the E helix will probably drift further away from the F helix than is normally permissible in Fe-Mb. Such an appreciable configurational change should be detectable by immunochemical studies and might account for the decrease in antigenic reactivity of Zn-Mb.

Finally, if the porphyrin carboxyl side groups are important in the haem–apomyoglobin linkage, then the effect of their esterification on the antigenic reactivity should be apparent. The effect on the configuration of the protein might even be more pronounced when the charge of the side groups is reversed, as happens when these are esterified with groups containing a pyridine nucleus. In fact, with three out of four antisera tested, the antigenic reactivity of the derivative had decreased relative to the homologous antigen. It is, however, noteworthy that one serum seemed 'indifferent' to the change.

In conclusion, the discussion presented above has proposed that the differences observed between

the various artificial myoglobins are mainly due to conformational alterations. With this in mind, it is evident that immunochemical methods can be used to study and follow conformational changes in proteins. However, the possibility should not be excluded at the moment that the porphyrin analogues may conceivably mask some of the antigenic sites of myoglobin.

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