# Conformational Changes in Sperm-Whale Metmyoglobin due to Combination with Antibodies to Apomyoglobin

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1. No ferrihaem was detected in the precipitate formed by metmyoglobin with an antiserum to apomyoglobin and the extinction at 410m$\mu$ of metmyoglobin, due to ferrihaem, was decreased by the univalent fragments of apomyoglobin antibodies. It was concluded that the combination of apomyoglobin antibodies with metmyoglobin caused the release of ferrihaem. As the removal of ferrihaem from metmyoglobin is accompanied by a conformational change, it was concluded that the conformation of metmyoglobin was altered by the apomyoglobin antibodies. 2. Antisera to metmyoglobin were divided into two groups; antisera of the first group revealed differences between the immunological reactivities of metmyoglobin and apomyoglobin, whereas no differences were detected with antisera of the second group. 3. Metmyoglobin was only partially re-formed by adding haematin to the precipitate produced by apomyoglobin with an antiserum of the first group, whereas complete re-formation of metmyoglobin was achieved in the presence of antisera of the second group. No metmyoglobin was formed on the addition of haematin to the precipitates produced by either metmyoglobin or apomyoglobin with the anti-apomyoglobin serum. 4. Immune precipitates formed by antisera to metmyoglobin dissociated at pH 1·8, whereas those formed by the anti-apomyoglobin serum did not dissociate. 5. These results suggest that apomyoglobin possessed different conformations when combined with metmyoglobin antibodies and apomyoglobin antibodies.

According to the classical theory of antigen–antibody interactions, an antigen combines with its homologous antibodies because the conformations of the antibody-combining sites are complementary to the patterns of the antigenic determinants. If this view is correct, changes in the conformation of an antigenic site should give rise to observable changes in the antigen–antibody reaction and, conversely, modifications of an antigen that cause no detectable alteration in immunological reactivity should have caused no structural changes in its antigenic determinants. Quite subtle changes in the conformations of certain proteins have been detected by using immunological techniques (Levine, 1962). For example, oxygenation of horse haemoglobin is associated with a conformational change (Perutz, Bolton, Diamond, Muirhead & Watson, 1964) and a change in immunological reactivity (Reichlin, Bucci, Antonini, Wyman & Rossi-Fanelli, 1964). Also, removal of the ferrihaem from metmyoglobin is accompanied by a conformational change (Breslow, Beychok, Hardman & Gurd, 1965; Crumpton & Polson, 1965; Harrison & Blout, 1965) and an alteration in the capacity of the protein to react with antibodies to metmyoglobin (Reichlin, Hay & Levine, 1963; Crumpton & Wilkinson, 1965).

On the other hand, it has been suggested (Najjar & Fisher, 1956; Najjar, 1963) that antibody may cause conformational changes in the antigen such that new or modified antigenic sites are formed. If this idea were shown, at least in some instances, to be correct, then the results of the application of immunological techniques to the detection of conformational changes in proteins might not be unequivocal. Some experimental evidence in support of this idea has recently been obtained. First, Pollock (1964) explained the effects of specific antisera on the enzymic activities of certain penicillinases by supposing that combination of antibody with the enzyme caused conformational changes that profoundly affected the properties of the enzyme active centre. It would appear that at least some of these changes occurred in parts of the enzyme molecule that contained no antigenic sites. Secondly, the results of the inhibition of muscle phosphorylase by univalent antibody fragments (Michaelides, Sherman & Helmreich, 1964) suggested that the conformation of the active centre was altered when the enzyme combined with

antibody. Thirdly, during a comparison of the reactivities of antisera to sperm-whale metmyoglobin with those of antisera to the haem-free protein (i.e. apomyoglobin) it became apparent that the precipitates formed by metmyoglobin with antisera to apomyoglobin did not contain any ferrihaem. The most plausible explanation of this phenomenon is that the apomyoglobin antibodies caused a conformational change in the protein moiety of metmyoglobin such that the ferrihaem was released. If these interpretations are correct, then antibodies may, at least in certain cases, possess the capacity to induce conformational changes in protein antigens. As a result, the use of immunological techniques to establish conformational identity and to detect changes in conformation should be critically evaluated.

The results of the comparison of the reactivities of anti-metmyoglobin sera with those of anti-apomyoglobin sera are described in the present paper, preliminary reports having already appeared (Crumpton, 1965, 1966).

## MATERIALS AND METHODS

Purified sperm-whale and horse metmyoglobins and sperm-whale apomyoglobin were prepared as previously described (Crumpton & Polson, 1965; Crumpton & Wilkinson, 1965). Twice-crystallized haemin (bovine, C grade) was purchased from Calbiochem (Los Angeles, Calif., U.S.A.) and was stored at 2°. Solutions of haematin were prepared immediately before use by dissolving a weighed sample (about 10mg.), which had been dried to constant weight *in vacuo*, in 1ml. of 0·1N-NaOH and diluting with water to 25ml.; the concentration was calculated from the weight of haemin used. A suspension of twice-crystallized papain (batch no. PAP5575; concn. 40mg./ml.) was purchased from Worthington Biochemical Corp. (Freehold, N.J., U.S.A.); solutions of the active enzyme were prepared by incubating, at 37°, 1vol. of suspension with 15vol. of 2·5mM-EDTA acid–0·01M-cysteine hydrochloride–0·1M-phosphate buffer, pH7·15. Sephadex G-75 (lot no. To–4372; bead form) was obtained from Pharmacia (Uppsala, Sweden) and rabbit serum albumin was kindly supplied by Miss E. M. Press.

*Antisera.* Antisera were prepared as described by Crumpton & Wilkinson (1965). Antisera to sperm-whale metmyoglobin and apomyoglobin are referred to as Ms and As respectively and antisera to horse metmyoglobin as Mh; this nomenclature differs from that previously used.

Antiserum Ms1 (antiserum WC; Crumpton, 1965, 1966) to sperm-whale metmyoglobin represented sera collected from nine rabbits that had been immunized with metmyoglobin in adjuvant, reimmunized 2 months later by three intravenous injections on successive days of 0·5ml. of a 1% solution of metmyoglobin and bled 1 week later.

Antiserum Mh2 (antiserum H2; Crumpton & Polson, 1965) to horse metmyoglobin represented sera collected from one rabbit after the injection of metmyoglobin in adjuvant and one or more courses of alum-precipitated metmyoglobin.

Antisera to sperm-whale apomyoglobin were prepared by immunizing six rabbits with apomyoglobin in adjuvant and with one and two courses (three intravenous injections on successive days) of alum-precipitated apomyoglobin (0·5ml. of 5mg./ml.). Three rabbits produced precipitating antibodies the properties of which were not significantly different. Antiserum As3 (antiserum Wg$\alpha$; Crumpton, 1965, 1966) represented sera collected from two of the three rabbits.

Normal rabbit serum was obtained by pooling samples of serum that had been collected from about 30 animals before immunization.

All sera were preserved with thiomersalate (0·01%), separated from any lipid material by centrifuging and stored at −20° and, when necessary, were diluted with normal rabbit serum.

*$\gamma$-Globulin and fragments of $\gamma$-globulin.* Rabbit $\gamma$-globulin was prepared from serum by precipitation with $Na_2SO_4$ according to the method of Kekwick (1940). Solutions of $\gamma$-globulin (about 25mg./ml.) or of purified antibody (about 3·5mg./ml.) in 2·5mM-EDTA acid–0·01M-cysteine hydrochloride–0·1M-phosphate buffer, pH7·15, were digested with papain (1% of the weight of $\gamma$-globulin) as described by Porter (1959). The digest was dialysed at 2° against 0·05M-phosphate buffer–0·15M-NaCl, pH7·0, and the Fc fragment, which was precipitated during dialysis, was separated by centrifuging. The clear supernatant, which contained mainly Fab fragment, was stored frozen at −20° and was used without any further treatment.

The nomenclature used for the fragments of $\gamma$-globulin is that recommended by the World Health Organisation (1964).

*Dissociation of myoglobin–antibody precipitates and isolation of antibodies.* This was carried out as described by Givol, Fuchs & Sela (1962). Myoglobin–antibody precipitates, containing about 16mg. of antibody, were washed with 0·9% NaCl and dissolved at 0° in 2·0ml. of 0·02N-HCl–0·15M-NaCl, pH1·8. This solution was added to a column (132cm. × 2·3cm.$^2$) of Sephadex G-75 which was then eluted with 0·02N-HCl–0·15M-NaCl at 2°; 3ml. fractions were collected. The extinctions at 280 and 215m$\mu$ of the eluate fractions were measured. The dissociated antibody was eluted from the column before myoglobin; fractions corresponding to the antibody peak were pooled. This pool was neutralized by the addition of 0·2N-NaOH dropwise with stirring, dialysed overnight against 0·05M-phosphate buffer–0·15M-NaCl, pH7·0, and then concentrated to about 3ml. by pressure dialysis against the above buffer at 2°. The small precipitate that formed during dialysis probably represented some incompletely dissociated antigen–antibody complex; this was removed by centrifuging and the supernatant was stored at −20°.

*Determination of protein concentration.* Concentrations of protein solutions were determined by measurement of their extinctions at 280m$\mu$ in a Unicam SP.500 spectrophotometer and a cell of 1cm. light-path. Specific extinction coefficients ($E^{1\%}_{1\,cm.}$) at 280m$\mu$ for sperm-whale metmyoglobin and apomyoglobin at neutral pH were 18·0 and 9·3 respectively (Crumpton & Wilkinson, 1965); the values for solutions in 0·1N-NaOH were the same as for those in water. Specific extinction coefficients at 410m$\mu$ for sperm-whale metmyoglobin in water and 0·1N-NaOH were 93·4 and 37·2 respectively; the latter value was determined within 30min. of the addition of alkali. The

Soret band of alkaline solutions of metmyoglobin was broader and flatter than that of neutral solutions and its wavelength of maximum absorption was at 400mμ compared with 410mμ at pH7 (cf. Theorell & Ehrenberg, 1951). Specific extinction coefficients at 280mμ for rabbit γ-globulin at neutral pH and in 0·1N-NaOH were taken to be 13·5 (Crumpton & Wilkinson, 1963) and 14·0 (Porter, 1957) respectively.

*Quantitative precipitin test.* This was carried out as described by Crumpton & Wilkinson (1965); antigen–antibody precipitates were dissolved in 0·1N-NaOH.

*Absorption spectra.* The absorption spectra of solutions of antigen–antibody precipitates were measured in a Unicam SP.700 recording spectrophotometer with microcells of 0·8ml. capacity and 2cm. light-path; the reference cuvette contained the control solution of the precipitin test that was derived from antiserum that had been incubated with 0·9% NaCl in place of the antigen solution. Measurements were made within 30min. of dissolving the precipitates since, when alkaline solutions of metmyoglobin–antibody precipitates were allowed to stand at room temperature, the absorption maximum at 410mμ decreased markedly; at 24hr. and 48hr. the extinctions were 60 and 40% respectively of that measured after 30min. (cf. Theorell & Ehrenberg, 1951).

Difference spectra were measured in a Unicam SP.500 spectrophotometer; when the reference cuvette contained serum the sensitivity of the instrument was adjusted so that the slit width at 410mμ was not greater than 0·6mm.

*Immunodiffusion.* Double diffusion in agar gel was carried out according to the method of Crumpton & Davies (1956). Antisera and antigen solutions were diluted so that the lines of antigen–antibody precipitate formed approximately midway between the antigen and the antiserum reservoirs.

## RESULTS

A number of antisera to sperm-whale metmyoglobin were examined. Although each antiserum possessed some distinctive properties, their properties resembled, in general, those of either antiserum Ms1 or antiserum Mh2. Antisera to metmyoglobin were therefore classified into two groups and antisera Ms1 and Mh2 were selected as examples of these groups. The properties of antisera Ms1 and Mh2, and of the antiserum to apomyoglobin (As3), are summarized in Table 1.

### *Ferrihaem in metmyoglobin–antibody precipitates*

Antisera to metmyoglobin formed reddish-brown precipitates with metmyoglobin and white precipitates with apomyoglobin. In contrast, an antiserum to apomyoglobin formed white precipitates with both metmyoglobin and apomyoglobin.

*Antisera to metmyoglobin.* The absorption spectra of solutions of the precipitates produced by an anti-metmyoglobin serum are shown in Fig. 1.

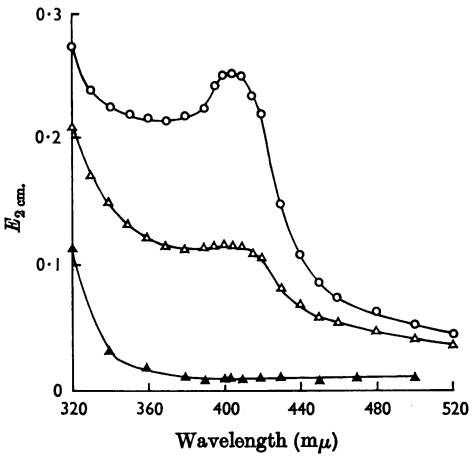


Fig. 1. Absorption spectra of solutions of the precipitates formed by 45μg. of sperm-whale metmyoglobin (○) and 45μg. of apomyoglobin (△) with 0·5ml. of antiserum Ms1 to sperm-whale metmyoglobin, and by 40μg. of apomyoglobin (▲) with 0·8ml. of a solution (950μg. of γ-globulin) of the purified antibody fraction of antiserum Ms1 that contained about 850μg. of precipitable antibody. The precipitates were dissolved in 1·2ml. of 0·1N-NaOH.

Table 1. *Properties of antisera*

The release of ferrihaem refers to that obtained from metmyoglobin by univalent antibody fragments. The re-formation of metmyoglobin refers to that obtained on the addition of haematin to the apomyoglobin–antibody precipitates. N.D., Not determined.

| Antiserum | No. of rabbits immunized | Antigen | Ferrihaem in metmyoglobin–antibody precipitate | Release of ferrihaem | Reactivities of metmyoglobin and apomyoglobin | Re-formation of metmyoglobin | Dissociation of immune precipitate |
|---|---|---|---|---|---|---|---|
| Ms1 | 9 | Sperm-whale metmyoglobin | Present | No | Same | Complete | Complete |
| Mh2 | 1 | Horse metmyoglobin | Present | N.D. | Different | Partial | N.D. |
| As3 | 2 | Sperm-whale apomyoglobin | Absent | Yes | Different | None | None |

The spectrum of the metmyoglobin–antibody precipitate possessed a peak with a maximum absorption at about 404mμ due to the presence of ferrihaem. The precipitate formed by apomyoglobin with antiserum Ms1 gave a small but definite absorption at 404mμ, which suggested that it also contained some ferrihaem. However, the precipitate produced by apomyoglobin with the purified antibody fraction of antiserum Ms1 failed to absorb at 404mμ; it was concluded that this precipitate did not contain any ferrihaem.

*Antiserum to apomyoglobin.* The absorption spectra of solutions of the precipitates formed by metmyoglobin and apomyoglobin with the antiserum to apomyoglobin are shown in Fig. 2; the concentrations of the solutions were similar to those whose spectra are shown in Fig. 1. The absorption spectra of the metmyoglobin– and apomyoglobin–antibody precipitates were identical and neither precipitate absorbed at 404mμ. A comparison of these spectra with those shown in Fig. 1 indicated that they resembled most closely the spectrum of the precipitate formed by apomyoglobin with the purified antibody fraction of antiserum Ms1. Absorption spectra of solutions of the precipitates produced by increasing amounts (6 to 70μg.) of metmyoglobin with 0·5ml. of antiserum As3 or the γ-globulin fraction of this antiserum were identical with those shown in Fig. 2. It was concluded that the precipitates formed by metmyoglobin with antibodies to apomyoglobin did not contain any ferrihaem.

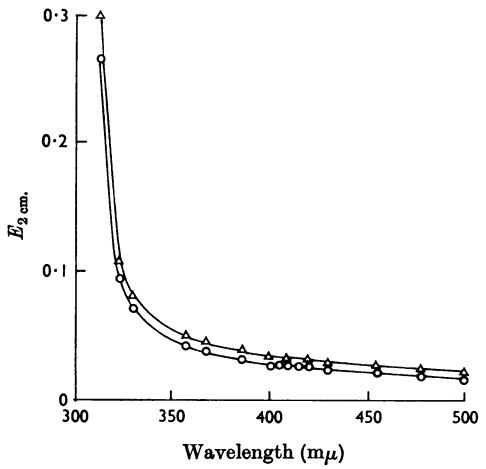


Fig. 2. Absorption spectra of solutions of the precipitates formed by 60μg. of sperm-whale metmyoglobin (○) and 60μg. of apomyoglobin (△) with 1·0ml. of antiserum As3 to sperm-whale apomyoglobin; the precipitates were dissolved in 0·9ml. of 0·1N-NaOH.

*Release of ferrihaem from metmyoglobin*

The above result suggested that the combination of apomyoglobin antibodies with metmyoglobin caused a release of ferrihaem. If this view is correct, the extinction at 410mμ of metmyoglobin should be reduced by the apomyoglobin antibodies, since the extinction of free ferrihaem is less than that of an equimolar amount of metmyoglobin (see Fig. 2; Breslow *et al.* 1965). However, as antigen–antibody aggregates caused an increase in extinction due to light-scattering, measurements of the extinction at 410mμ could only be made before aggregation occurred.

The rate of aggregation of metmyoglobin (0·2ml. of 125μg./ml.) with 0·8ml. of antiserum As3 was followed at 22° by measuring the rate of change in extinction at 320mμ; no increase in extinction, due to light-scattering by aggregates, was detected until after 15min. The effect of antiserum As3 on the extinction at 410mμ of metmyoglobin was therefore determined within 15min. of mixing the reactants. The results are shown in Table 2. Antiserum As3 caused an 85% decrease in the extinction within 1min., whereas no significant decrease was detected with normal rabbit serum within 10min. Comparable results could not be obtained with antisera Ms1 and Mh2 since aggregation occurred too rapidly for measurements to be made. Under the conditions shown in Table 2, the γ-globulin fraction of antiserum As3, which possessed the same antibody activity as the whole serum, caused a very much smaller decrease in the extinction. The results suggested that the ferrihaem of metmyoglobin was released by the apomyoglobin antibodies before the formation of antigen–antibody aggregates. In this case, univalent Fab fragments of the apomyoglobin antibodies, which did not form aggregates, should also release ferrihaem.

Table 2. *Extinctions at 410mμ measured at 1 and 10min. after the addition of 0·2ml. of a solution of metmyoglobin (117μg./ml.) to 0·8ml. of 0·9% sodium chloride, normal rabbit serum and antiserum As3*

The reference cuvette contained either 0·9% NaCl or the respective serum with 0·9% NaCl in place of the metmyoglobin solution, the sensitivity of the spectrophotometer was adjusted so that the slit width was not greater than 0·6mm. and the temperature was 22°.

| Time after mixing (min.) | $E^{2\,cm.}_{410\,mμ}$ of metmyoglobin with 0·9% NaCl | Normal rabbit serum | Antiserum As3 |
|---|---|---|---|
| 1 | 0·438 | 0·430 | 0·066 |
| 10 | 0·434 | 0·426 | 0·060 |

Table 3. *Extinctions at 410mμ of metmyoglobin (0·2ml. of 135μg./ml.) that was incubated at 22° for various times with 0·8ml. of the Fab fragments of normal rabbit γ-globulin (18mg./ml.) of the purified antibody fraction of an antiserum to metmyoglobin (antiserum Ms1; 0·43mg./ml.) and of the γ-globulin fraction of the antiserum to apomyoglobin (antiserum As3; 20mg./ml.)*

The antibody activities of the amounts of Fab fragments of antisera Ms1 and As3 used were theoretically equivalent to 0·3ml. and 0·8ml. respectively of the original antisera. The reference cuvette contained, in each case, the respective Fab fragments with 0·2ml. of 0·05M-phosphate buffer–0·15M-NaCl, pH7·0, in place of the metmyoglobin solution.

| Time after mixing (min.) | $E^{2\,cm.}_{410\,m\mu}$ of metmyoglobin with Fab fragments of: Normal rabbit serum | Antiserum Ms1 | Antiserum As3 |
|---|---|---|---|
| 1 | 0·494 | 0·499 | 0·498 |
| 5 | 0·493 | 0·497 | 0·486 |
| 10 | — | — | 0·478 |
| 20 | 0·493 | 0·497 | 0·470 |
| 40 | — | — | 0·457 |
| 75 | — | — | 0·447 |
| 120 | 0·490 | 0·496 | 0·434 |
| 190 | 0·490 | 0·494 | 0·418 |
| 23hr. | 0·480 | 0·490 | 0·320 |

The effects of the Fab fragments of purified antibodies to metmyoglobin, normal rabbit γ-globulin and the γ-globulin fraction of antiserum As3 on the extinction at 410mμ of metmyoglobin were measured. The results, which are summarized in Table 3, indicated that the Fab fragments of apomyoglobin antibodies caused a decrease of 36% in the extinction in 23hr.; a decrease of 50% was obtained with 13·5μg. of metmyoglobin under the same conditions. In contrast, no significant decrease was detected with the Fab fragments of either normal rabbit γ-globulin or the antibodies to metmyoglobin. It was concluded that combination of the univalent fragments of the apomyoglobin antibodies with metmyoglobin gave rise to free ferrihaem.

A comparison of the above results indicated that the extinction of metmyoglobin decreased at a very much faster rate with antiserum As3 than with either the γ-globulin fraction or the Fab fragments of this serum. This may have been due to combination of the ferrihaem released by the whole antiserum with albumin and certain α- and β-globulins (cf. Allison & ap Rees, 1957; Shinowara & Walters, 1963). The rate of decrease in the extinction of metmyoglobin with the Fab fragments

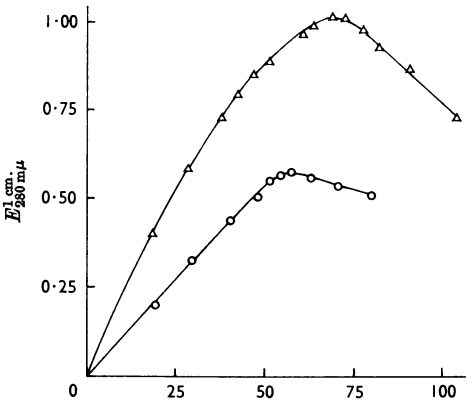


Fig. 3. Precipitin curves of sperm-whale metmyoglobin (○) and of apomyoglobin (△) with antiserum Mh2 to horse metmyoglobin. Increasing amounts of antigen in 0·2ml. of 0·9% NaCl were incubated for 1hr. at 37° and at 2° overnight with 0·5ml. of threefold-diluted antiserum and the washed precipitates were dissolved in 1·2ml. of 0·1N-NaOH.

was, however, not increased by the addition of 0·05ml. of a 10% (w/v) solution of rabbit serum albumin.

*Comparison of the immunological reactivities of metmyoglobin and apomyoglobin*

*Antisera to metmyoglobin.* The amounts of precipitate formed by the addition of increasing amounts of sperm-whale metmyoglobin and apomyoglobin to a constant volume of an antiserum to horse metmyoglobin (Mh2) are shown in Fig. 3. A comparison of the precipitin curves (Fig. 3) revealed that metmyoglobin and apomyoglobin possessed different immunological reactivities. In contrast, a comparison of the precipitin curves obtained with antiserum Ms1 (Fig. 4) showed that maximal precipitation required similar amounts of metmyoglobin and apomyoglobin and that the maximum amounts of precipitate contained the same quantity of antibody; amounts of antibody were calculated from the extinctions at 280mμ after subtracting the extinction due to the added antigen. Although metmyoglobin precipitated slightly more antibody than apomyoglobin in the region of antibody excess, it was concluded that their reactivities with respect to antiserum Ms1 were not significantly different.

Most of the antisera to metmyoglobin resembled antiserum Mh2 and revealed differences between the immunological reactivities of metmyoglobin

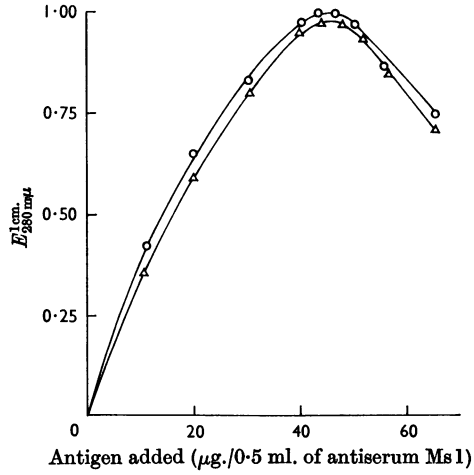


Fig. 4. Precipitin curves of sperm-whale metmyoglobin (○) and of apomyoglobin (△) with antiserum Ms 1 to sperm-whale metmyoglobin. Increasing amounts of antigen in 0·2 ml. of 0·9% NaCl were incubated for 1 hr. at 37° and at 2° overnight with 0·5 ml. of antiserum and the washed precipitates were dissolved in 1·2 ml. of 0·1 N-NaOH.

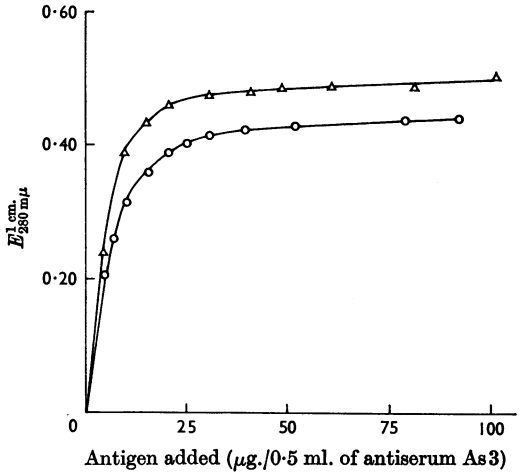


Fig. 5. Precipitin curves of sperm-whale metmyoglobin (○) and of apomyoglobin (△) with antiserum As 3 to sperm-whale apomyoglobin. Increasing amounts of antigen in 0·2 ml. of 0·9% NaCl were incubated for 1 hr. at 37° and at 2° overnight with 0·5 ml. of antiserum and the washed precipitates were dissolved in 1·2 ml. of 0·1 N-NaOH.

and apomyoglobin, whereas other antisera, which resembled antiserum Ms 1, revealed no differences.

*Antiserum to apomyoglobin.* The amounts of precipitate formed by the addition of increasing amounts of metmyoglobin and apomyoglobin to a constant volume of antiserum As 3 are shown in Fig. 5. The results indicated that antiserum As 3 detected differences between metmyoglobin and apomyoglobin.

The precipitin curves shown in Fig. 5 deviated from the classical pattern in that precipitation was not inhibited by excess of antigen. The supernatants recovered after the separation of the precipitates were tested for the presence of excess of antigen by diffusion in agar against fourfold-diluted antiserum Ms 1; lines of precipitate were formed by those supernatants that corresponded to the addition of 11 μg. or more of antigen. The amount of antigen present in each supernatant was determined by measuring the quantity of precipitate formed by a given volume of supernatant with 0·5 ml. of antiserum Ms 1. The results indicated that the supernatants contained an increasingly large fraction of the added antigen; for example, supernatants corresponding to the addition of 10·2 and 30·1 μg. of metmyoglobin gave the same amounts of precipitate as those produced by 3·9 and 19·0 μg. respectively of metmyoglobin with 0·5 ml. of antiserum Ms 1. The absorption spectra of the precipitates formed by the above supernatants revealed that they contained ferrihaem. As a result, in contrast with the precipitated metmyoglobin, which contained no ferrihaem (Fig. 2), it appeared that the non-precipitated metmyoglobin, which may be present as soluble antigen–antibody complexes, still contained ferrihaem.

### *Re-formation of metmyoglobin on the addition of haematin to apomyoglobin–antibody precipitates*

The precipitate formed by apomyoglobin with antiserum Ms 1 contained some ferrihaem (see Fig. 1). As the apomyoglobin added to the antiserum contained no ferrihaem, some metmyoglobin had probably been formed by transfer of ferrihaem from either or both methaemoglobin and other haematin–protein complexes present in the antiserum (Shinowara & Walters, 1963) to apomyoglobin (Banerjee, 1962*a*; Reichlin *et al.* 1963). It was therefore decided to determine whether complete re-formation of metmyoglobin could be achieved by the addition of haematin to the precipitates formed by apomyoglobin with both metmyoglobin antibodies and apomyoglobin antibodies.

*Antisera to metmyoglobin.* A twofold molar excess of haematin (relative to the amount of apomyoglobin) was added to antiserum Ms 1 either before the addition of apomyoglobin or after the formation of the apomyoglobin–antibody precipitate; control tubes contained either metmyoglobin in place of apomyoglobin or water instead of the haematin solution. The extinctions at 280 and

Table 4. *Extinctions at 280 and 410 mμ of solutions of the precipitates formed by metmyoglobin and apomyoglobin (45 μg. in 0·20 ml.) with 0·5 ml. of antiserum Ms 1 in the presence and absence of ferrihaem*

The amount of haematin (0·024 ml. of 146 μg. of haemin chloride/ml. of 0·004 N-NaOH) added was equivalent to a twofold molar excess relative to apomyoglobin. The washed precipitates were dissolved in 1·2 ml. of 0·1 N-NaOH.

| | Without ferrihaem | | With ferrihaem | |
|---|---|---|---|---|
| Antigen | $E^{1\,cm.}_{280\,m\mu}$ | $E^{2\,cm.}_{410\,m\mu}$ | $E^{1\,cm.}_{280\,m\mu}$ | $E^{2\,cm.}_{410\,m\mu}$ |
| Metmyoglobin | 1·00 | 0·248 | 1·01* | 0·250* |
| Apomyoglobin | 0·975 | 0·115 | 1·01* | 0·254* |
| Apomyoglobin | — | — | 1·02† | 0·252† |

* Haematin added to the antiserum before the addition of antigen.
† Haematin added after the antiserum and apomyoglobin had been incubated for 1 hr. at 37°.

Table 5. *Extinctions at 280 and 410 mμ of solutions of the precipitates formed by metmyoglobin and apomyoglobin with antiserum Mh 2 in the presence and absence of ferrihaem*

The amounts of metmyoglobin and apomyoglobin (56 and 70 μg. respectively in 0·2 ml.) added to 0·5 ml. of threefold-diluted antiserum Mh 2 were those that produced the maximum quantities of precipitate (see Fig. 3). The amount of haematin (0·037 ml. of 146 μg. of haemin chloride/ml. of 0·004 N-NaOH) added was equivalent to a twofold molar excess relative to apomyoglobin. The washed precipitates were dissolved in 1·2 ml. of 0·1 N-NaOH.

| | Amount of antigen | Without ferrihaem | | With ferrihaem | |
|---|---|---|---|---|---|
| Antigen | (μg.) | $E^{1\,cm.}_{280\,m\mu}$ | $E^{2\,cm.}_{410\,m\mu}$ | $E^{1\,cm.}_{280\,m\mu}$ | $E^{2\,cm.}_{410\,m\mu}$ |
| Metmyoglobin | 56 | 0·580 | 0·206 | 0·583* | 0·208* |
| Apomyoglobin | 70 | 1·04 | 0·125 | 1·06* | 0·204* |
| Apomyoglobin | 70 | — | — | 1·08† | 0·211† |

* Haematin added to the antiserum before the addition of antigen.
† Haematin added after the antiserum and apomyoglobin had been incubated for 1 hr. at 37°.

410 mμ of the solutions of the immune precipitates are shown in Table 4. The extinctions of the precipitate formed by apomyoglobin in the presence of ferrihaem were identical with those of the precipitate produced by the same amount of metmyoglobin and were greater than those of the precipitate formed by apomyoglobin in the absence of ferrihaem; the addition of a fivefold molar excess of haematin caused no further increase in the extinctions of the apomyoglobin–antibody precipitate. No differences were detected between the extinctions of the precipitates formed by metmyoglobin in the presence and absence of ferrihaem; detectable amounts of ferrihaem were therefore not bound non-specifically by immune precipitates. It was concluded that metmyoglobin was completely re-formed on the addition of haematin to the precipitate produced by apomyoglobin with antiserum Ms 1.

The extinctions of the precipitates formed by metmyoglobin and apomyoglobin with antiserum Mh 2 in the presence and absence of ferrihaem are given in Table 5. The extinction at 410 mμ of the precipitate formed by 70 μg. of apomyoglobin with ferrihaem (0·211) was not significantly different from that of the precipitate produced by a smaller amount (56 μg.) of metmyoglobin (0·206). Consequently, the amount of metmyoglobin re-formed was probably the same as that which gave the maximum quantity of precipitate with antiserum Mh 2 (56 μg.; see Fig. 3). If all of the apomyoglobin in the precipitate had been transformed into metmyoglobin the extinction at 410 mμ should have been greater than 0·211. It was concluded that metmyoglobin was only partially re-formed on the addition of haematin to the precipitate produced by apomyoglobin with antiserum Mh 2.

*Antiserum to apomyoglobin.* The addition of a twofold molar excess of haematin to antiserum As 3 caused no detectable increase in the extinctions at 410 mμ of the precipitates that were formed by metmyoglobin or apomyoglobin. The same result was obtained on the addition of haematin to the immune precipitates. It was concluded that no metmyoglobin was re-formed.

Amounts of haematin greater than a twofold

molar excess also gave no increase in the extinction at 410mμ of the precipitates. Slightly less precipitate was, however, formed by metmyoglobin and apomyoglobin under these conditions; a tenfold molar excess of haematin caused a decrease of not more than 10% in the amounts of precipitate.

*Dissociation of immune precipitates*

Attempts were made to isolate the antibodies of antisera Ms1 and As3 by elution of solutions of the precipitates formed with metmyoglobin from a column of Sephadex G-75 with 0·02N-hydrochloric acid–0·15M-sodium chloride, pH1·8 (Givol *et al.* 1962); the elution patterns obtained are shown in Fig. 6. The solution of the precipitate formed by antiserum Ms1 was resolved into two peaks (Fig. 6*a*), the positions of which were identical with those of normal rabbit γ-globulin and metmyoglobin eluted separately from the same column under the above conditions; the γ-globulin was eluted first. In contrast, the solution of the precipitate produced by antiserum As3 gave one peak only, in the position of γ-globulin (Fig. 6*b*). When a pool of the fractions corresponding to this peak was adjusted to pH7 and dialysed, a precipitate formed, the amount of which was similar to that which was initially added to the column. On the other hand, similar treatment of the γ-globulin peak given by antiserum Ms1 produced negligible amounts of precipitate; in this case about 85% of the amount of antibody calculated to be present in the initial precipitate was recovered as soluble γ-globulin, of which about 90% was specifically precipitated by metmyoglobin. These results indicated that the metmyoglobin–antibody precipitate produced by antiserum Ms1 dissociated almost completely at pH1·8 whereas that formed by the anti-apomyoglobin serum did not dissociate under the same conditions.

A comparison of the elution pattern shown in Fig. 6(*a*) with that obtained by Givol *et al.* (1962) for a lysozyme–antibody precipitate indicated that the position of the metmyoglobin peak corresponded to that of an aggregate; the results of ultracentrifugal analyses also suggested that metmyoglobin is aggregated at pH1·8. A pool of the fractions corresponding to the metmyoglobin peak (Fig. 6*a*) was adjusted to pH7, dialysed against 0·05M-phosphate buffer–0·15M-sodium chloride, pH7·0, and concentrated to 3ml. As the solution of the recovered material did not absorb at 410mμ, it was concluded that it contained no ferrihaem. Metmyoglobin was, however, formed from this material by the addition of haematin. These results suggested that metmyoglobin is denatured at pH1·8 and that solutions of metmyoglobin in 0·02N-hydrochloric acid–0·15M-sodium chloride contain ferrihaem and an aggregate of the apoprotein (Theorell & Ehrenberg, 1951; Breslow & Gurd, 1962).

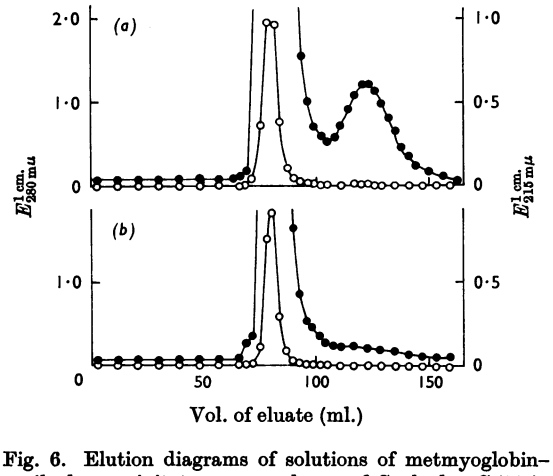


Fig. 6. Elution diagrams of solutions of metmyoglobin–antibody precipitates on a column of Sephadex G-75 in 0·02N-HCl–0·15M-NaCl, pH1·8. ○, $E^{1\,cm.}_{280\,m\mu}$; ●, $E^{1\,cm.}_{215\,m\mu}$. Precipitates were formed by 900μg. of metmyoglobin with (*a*) 10ml. of antiserum Ms1 and (*b*) 15ml. of antiserum As3. Experimental details are given in the text.

## DISCUSSION

The removal of ferrihaem from metmyoglobin is accompanied by a change in the conformation of the protein moiety (Breslow *et al.* 1965; Crumpton & Polson, 1965; Harrison & Blout, 1965); the original conformation is restored on the addition of an equimolar amount of haematin to apomyoglobin (Theorell & Åkeson, 1955; Breslow *et al.* 1965; Harrison & Blout, 1965). As the iron–histidine bond makes a small contribution only towards the binding of ferrihaem by the apoprotein, the difference in conformation between metmyoglobin and apomyoglobin is probably determined by the hydrophobic interactions between the protein moiety and the protoporphyrin portion of ferrihaem (Breslow & Koehler, 1965). Consequently, any conformational change in the apoprotein that disrupts the hydrophobic interactions will result in a release of ferrihaem.

*Comparison of antisera to metmyoglobin.* Antisera to metmyoglobin could be divided into two groups. Most of the antisera were included in the first group; these antisera detected differences between the reactivities of metmyoglobin and apomyoglobin (Reichlin *et al.* 1963; Crumpton & Wilkinson, 1965) that are, undoubtedly, related to the different conformations of the proteins. In contrast, antisera

of the second group revealed no differences between the reactivities of metmyoglobin and apomyoglobin.

The two groups of antisera appeared to differ also with respect to the extent to which metmyoglobin was re-formed on the addition of haematin to apomyoglobin–antibody precipitates. Complete re-formation of metmyoglobin was achieved on the addition of a twofold molar excess of haematin (relative to apomyoglobin) to the precipitate formed by an antiserum of the second group (antiserum Ms 1), whereas metmyoglobin was only partially re-formed by adding haematin to the precipitate produced by an antiserum of the first group (antiserum Mh 2). The combination of ferrihaem with the apomyoglobin in the precipitate formed by antiserum Mh 2 may have been inhibited by antibody molecules. However, as the re-formation of metmyoglobin is accompanied by a conformational change, it is also possible that the conformation of some of the precipitated apomyoglobin was such that ferrihaem was not bound. If this interpretation is correct, the conformation of the apomyoglobin that was transformed into metmyoglobin was the same as that of metmyoglobin. In this case, the conformation of apomyoglobin may have been altered by combination with antibody. Alternatively, if solutions of apomyoglobin contain molecules with different conformations in continuous interchange (cf. Lumry & Eyring, 1954), the antibodies may have selected and stabilized those molecules with complementary conformations.

*Comparison of the antiserum to apomyoglobin with antisera to metmyoglobin.* The antiserum to apomyoglobin differed in five respects from antisera to metmyoglobin: (1) precipitates formed with metmyoglobin contained no ferrihaem; (2) the extinction at 410 m$\mu$ of metmyoglobin was decreased by the univalent fragments of apomyoglobin antibodies; (3) the precipitin curves deviated from the classical pattern in that precipitates were insoluble in the region of antigen excess; (4) no metmyoglobin was re-formed on the addition of haematin to immune precipitates; (5) immune precipitates did not dissociate at pH 1·8. It seems likely that these distinctive properties of the anti-apomyoglobin serum are related to each other.

The reason for the insolubility of antigen–antibody complexes in the presence of excess of antigen is not known, but this may depend on the conformation of the antigen in the complexes. With respect to the ease of dissociation of immune precipitates, it appeared that the apomyoglobin antibodies possessed a higher avidity than the metmyoglobin antibodies. Also, as a conformational change in the antigen is apparently necessary for dissociation to occur (Bennett & Haber, 1963), the apomyoglobin antibodies were probably more effective in maintaining the conformation of the antigen.

*Release of ferrihaem from metmyoglobin by apomyoglobin antibodies.* The precipitates formed by metmyoglobin with the antiserum to apomyoglobin contained no ferrihaem and the extinction at 410 m$\mu$ of metmyoglobin, due to ferrihaem, was decreased by the anti-apomyoglobin serum, and by the $\gamma$-globulin fraction and the Fab fragments of this serum. It was concluded that combination of the apomyoglobin antibodies with metmyoglobin gave rise to free ferrihaem and apomyoglobin–antibody complexes.

A comparison of the rates of decrease in extinction due to antiserum As 3 and its Fab fragments (Tables 2 and 3) indicated that the Fab fragments caused a very much less rapid decrease than the whole antiserum. This decrease in rate may have been due to a decrease in the rate of the metmyoglobin–antibody reaction. However, the immediate decrease in extinction that occurred with antiserum As 3 was probably due to the very rapid removal of the free ferrihaem by combination with albumin and $\alpha$- and $\beta$-globulins of the serum (Allison & ap Rees, 1957; Shinowara & Walters, 1963). On the other hand, in the absence of these serum components, the extinction at 410 m$\mu$ of the ferrihaem released by the Fab fragments would have decreased slowly as a result of aggregation (Inada & Shibata, 1962). Consequently, the rate of decrease in extinction due to the Fab fragments may be a measure of the rate of aggregation of the free ferrihaem rather than of its formation.

There would appear to be two possible explanations for the formation of free ferrihaem and apomyoglobin–antibody complexes from metmyoglobin and antibodies to apomyoglobin. First, although it is generally accepted that the ferrihaem of metmyoglobin is firmly bound to the apoprotein (Snyder, 1963), it would appear that metmyoglobin dissociates to a very small extent; the dissociation constant was calculated to be about $10^{-15}$ M at 25° and at pH 7 (Banerjee, 1962*a*,*b*). Consequently, the apomyoglobin antibodies may have reacted preferentially with the very small amount of apomyoglobin that is in equilibrium with metmyoglobin. Secondly, the antibodies may have combined with metmyoglobin and induced a conformational change in the protein moiety such that the ferrihaem was no longer bound. This explanation is regarded as being more likely than the first for the following reason. The rate constant for the association of ferrihaem and apomyoglobin will be less than the maximum value for a diffusion-controlled reaction ($1{\cdot}5 \times 10^{9}$ M$^{-1}$ sec.$^{-1}$; Froese & Sehon, 1965) and is likely to be similar to that for the association of a hapten with its specific antibody (e.g. $1{\cdot}8 \times 10^{8}$ M$^{-1}$ sec.$^{-1}$; Froese & Sehon, 1965). If

the latter value is assumed and the dissociation constant of metmyoglobin is $10^{-15}$M, then the rate constant for the dissociation will be $1{\cdot}8 \times 10^{-7}$sec.$^{-1}$ and, if the dissociation is a first-order reaction, the half-time for dissociation will be about $3{\cdot}9 \times 10^{6}$sec. (45 days). As a result, if the rate of reaction of the apomyoglobin with antibody is faster than the rate of dissociation of metmyoglobin, the release of ferrihaem would be half complete in about 45 days. The rate of release of ferrihaem from metmyoglobin during the reaction with the Fab fragments of the apomyoglobin antibodies (Table 3) was at least as fast as the rate of decrease in the extinction at 410m$\mu$. Further, as ferrihaem was probably not released from all of the metmyoglobin under these conditions and as the aggregates of ferrihaem contributed to the extinction at 410m$\mu$, the release of ferrihaem was probably at least half complete in 23hr. Thus ferrihaem was released very much faster than would have occurred if its release had been due to the reaction of the antibody fragments with the very small amount of apomyoglobin in equilibrium with metmyoglobin. Consequently, it was concluded that the antibodies to apomyoglobin had induced a conformational change in metmyoglobin.

The reason why the apomyoglobin antibodies induced a conformational change in metmyoglobin is not known, but it seems likely that this property was related to the specificities and avidities of the antibodies. The induction of conformational changes in globular proteins by specific antisera is not a universal phenomenon; for example, as the conformations of metmyoglobin and apomyoglobin can be distinguished immunologically, the difference between their conformations must be preserved when they are combined with some antibodies. It is, however, evident from the above results that the use of immunological techniques to detect changes in the conformations of globular proteins may not yield unequivocal results and that the interpretation of the results of immunological investigations may be questionable.

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