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The Preparation and some Properties of Myoglobin Containing Meso- and Deutero-Haem

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Although experiments in which the protohaem of haemoglobin was replaced by other haems or porphyrins were first carried out by Hill & Holden (1926), the number of papers dealing with similar experiments is comparatively small, and even fewer have been concerned with the quantitative study of reactions of these 'synthetic' haemoproteins. Myoglobin, unlike haemoglobin, is free from effects due to haem–haem interaction and therefore seems particularly suited for work on the relation between the constitution of the haem group and the properties of the haemoprotein, yet little work has been reported. Drabkin (1945) has described the reconstitution of myoglobin with protohaem and has found that the reconstituted material has the same spectral absorption and crystal habit as the native protein. The preparation and properties of reconstituted myoglobin have recently been more fully described by Theorell & Åkeson (1955).

Since this work was completed a preliminary report has appeared from Rossi-Fanelli & Antonini (1957), who have prepared native human myoglobin apo-protein and have coupled it with protohaem and deuterohaem. They report the spectra of various derivatives, and found that there was a threefold increase in oxygen affinity on replacing protohaem with deuterohaem.

The object of the present experiments was to prepare myoglobins containing meso- and deuterohaem, to examine their spectra and to investigate the kinetics of their combination with carbon monoxide. A preliminary account has been given by Gibson & Smith (1957).

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EXPERIMENTAL

Haemins. The haemins used were prepared as described in the preceding paper (Smith, 1959).

Sodium dithionite. A sample of Manox dithionite was obtained from Holdman and Harden, Miles Platting, Manchester, 1.

Buffers. Phosphate buffers, pH 6·5–7·5 (Sörensen, 1909); for pH 5·0–6·3, 0·2N-citric acid–0·2N-NaOH (Britton & Welford, 1937); for pH 7·8–9·1, 0·2M-H_3BO_3 in 0·2N-KCl with 0·2N-NaOH (Clark, 1928).

Whale myoglobin. Whale meat from a local fishmonger was prepared both by the methods of Theorell & de Duve (1947) and of Bowen (1948). No crystals were obtained from the samples of whale meat available to us by either of these methods, although considerable yields of myoglobin were obtained in amorphous form.

Horse-heart myoglobin. Horse hearts were obtained fresh from the local abattoir and prepared by the method of Bowen (1948). Four hearts were used at various times, of which three yielded the typical wheatsheaf clusters of crystals at the final stages of the preparation. The fourth gave amorphous material only.

Preparation of native globin. Haematin was removed from the myoglobin molecule by a method devised by Dr F. W. J. Teale (personal communication). A solution of metmyoglobin was dialysed for 24 hr. against running distilled water in a rocking dialyser in 0·5 in. cellophan tubing. To 1 vol. of an approximately mM-solution of the salt-free myoglobin at 0°, 0·5 vol. of 2-butanone containing 10% (v/v) of 0·1N-HCl was added. The mixture was shaken gently and allowed to stand for 15 min. If a layer of 2-butanone had separated, it was removed. A second portion of acid butan-2-one equal in volume to the first was then added and the mixture again shaken and allowed to stand for 15 min. The process of removal of 2-butanone and its replacement with a fresh portion was continued until no further haematin passed into the upper layer. The lower solution of globin was dialysed against water or 0·02M-phosphate buffer, pH 7·1. The solution was stable for several days when stored at 0° and pH 7·1.

Reconstitution of myoglobin. A solution of the required strength of haematin (approximately 0·5 mM) in 0·02 N-NaOH was added gradually to a roughly equimolar solution of globin at 0° with stirring. The pH was measured with a glass electrode and a few drops 0·1 N-HCl were added, if necessary, to keep the mixture below pH 6·0 until all the haem had been run in. The mixture was then brought to between pH 7·0 and 8·0 by the addition of 0·1 N-NaOH. A precipitate which appeared on the first addition of the NaOH disappeared again as the addition of alkali continued. To precipitate denatured myoglobin and uncombined globin or haem the solution was next dialysed against half-saturated $(NH_4)_2SO_4$ solution, prepared by mixing equal volumes of saturated $(NH_4)_2SO_4$ and water and neutralizing with a few drops of aq. 7% (w/w) NH_3 solution. The precipitate was removed by centrifuging and the supernatant dialysed against distilled water or 0·02 M-phosphate buffer, pH 7·1, to remove $(NH_4)_2SO_4$. The resulting solutions were used either directly or after dilution for spectroscopic observation and for kinetic experiments.

Spectrophotometry. The concentration of myoglobin solutions was determined with a Unicam SP. 600 spectrophotometer, Drabkin's (1947) millimolar extinction coefficient $\epsilon^{mM}_{540\,m\mu} = 11{\cdot}3$ being used for metmyoglobin cyanide. Drabkin (1942) found that the maximum extinction coefficients for several derivatives of meso- and copro-haem had the same numerical values as those for the corresponding derivatives of protohaem. It seemed reasonable to assume that the extinction coefficients for meso- and deutero-metmyoglobin cyanide had also the numerical value $\epsilon^{mM} = 11{\cdot}3$. The wavelengths used were 530 mμ for mesometmyoglobin cyanide and 534 mμ for deuterometmyoglobin cyanide. Similarly, the value of Hogness, Zscheile, Sidwell & Barron (1937) for the extinction coefficient of haematin $\epsilon^{mM}_{490\,m\mu} = 6{\cdot}55$ was used for all three haematins, readings being taken at 482 mμ for meso- and deutero-haematin. In determining the concentration of globin, $\epsilon^{mM}_{286\,m\mu} = 16{\cdot}2$ was used (Dr F. W. J. Teale, personal communication).

Kinetic experiments. These were carried out with the apparatus and methods described by Gibson (1956).

RESULTS

Spectral findings

The wavelengths of maximum absorption and the millimolar extinction coefficients for natural and reconstituted myoglobins are shown in Table 1. Additional columns have been included which give the results of Theorell (1934), Kiese & Kaeske (1942) and Bowen (1949), who have published figures for protomyoglobin. Except for the results of Kiese & Kaeske, as quoted by Bowen, the agreement between the various authors is satisfactory. Theorell's (1934) values are particularly close except for the γ-band of metmyoglobin. These values have been calculated from the specific extinction coefficients given by Theorell, taking the molecular weight of myoglobin as 17 300. Theorell & Åkeson (1955) have given new values for the molar extinction coefficients of protomyoglobin, all of which are in satisfactory agreement with those given here. They have at the same time recalculated the earlier figures, to express these as molar extinction coefficients. The recalculated figures are now about 10% lower than those given in Table 1, and are at the same time about 10–15% lower than the latest figures.

This divergence appears to be due to the interpolation of two different values for the molecular weight of myoglobin in Theorell's calculations. The 1934 figures appear to have been recalculated with an earlier value (Theorell, 1932) of 16 500 for the molecular weight (the present authors used 17 300), whereas the 1955 values are apparently based on the latest value of 18 800 for the molecular weight. This distinction is not made clear by Theorell & Åkeson, and largely explains the difference between individual values which they have attributed to iron impurities in the 1934 preparation.

The figures given by the present authors in Table 1 represent in every case the means of at least three determinations with different preparations of myglobin. In no case did the range between different preparations exceed 10% of the quantity measured, and was usually considerably less.

Kinetic results

Protomyoglobin. Experiments were first carried out to see if the native and reconstituted materials would yield similar values for the combination velocity constant for carbon monoxide. Both whale-muscle and horse-heart myoglobins have been used, but in view of the failure to obtain crystalline myoglobin from whale muscle, fewer experiments were carried out with it. The results of reconstitution experiments with whale myoglobin are given in Table 2. The reconstituted material combined slightly less rapidly than the native protein. With three preparations of horse-heart myoglobin the results were variable, the reconstituted myoglobin giving a markedly lower velocity constant than the native protein in two cases. In comparing the meso- and deutero-myoglobin with protomyoglobin, therefore, direct comparison of the three reconstituted proteins was preferred to comparison of native protomyoglobin with meso- and deutero-myoglobin. When the splitting and reconstitution procedure was repeated, with separate portions of a single preparation of native myoglobin, each portion of reconstituted myoglobin gave the same velocity constant for combination with carbon monoxide. The differences between preparations recorded in Table 2 are believed to be due to differences in the original myoglobin preparations rather than to variations of the experimental procedures for splitting and reconstitution.

Table 1. *Wavelengths of absorption maxima and millimolar extinction coefficients for myoglobin derivatives*

	Horse-heart protomyoglobin						Meso-myoglobin		Deutero-myoglobin		Whale proto-myoglobin	
	Theorell (1934)*		Kiese & Kaeske† (1942)		Bowen (1949)		Gibson & Smith‡ (1957)					
	$m\mu$	ϵ^{mM}	$m\mu$	ϵ^{mM}	$m\mu$	ϵ^{mM}	$m\mu$	ϵ^{mM}	$m\mu$	ϵ^{mM}	$m\mu$	ϵ^{mM}	$m\mu$	ϵ^{mM}
Metmyoglobin	630	4·0	630	2·7, 7·2	630	3·7	630	3·9	—	—	626	—	630	4·7
	500	8·8	500	8·9, 17·0	500	9·8	500	9·5	492	—	500	10·7	500	9·6
	407	116·0§	—	—	—	—	408	153·0	394	102·0	392	130·0	410	148·0
	280	28·9	—	—	—	—	—	—	—	—	—	—	—	—
Metmyoglobin cyanide	—	—	—	—	540	11·3‖	540	11·3‖	530	11·3‖	534	11·3‖	540	11·3‖
							422	117·1	412	102·8	410	96·2	422	119·0
Reduced myoglobin	555	11·2	555	13·4, 29·8	555	12·9	552	13·0	546	13·0	542	12·1	556	14·9
	435	107·2	—	—	—	—	432	114·0	422	117·5	422	95·5	434	123·8
CO–myoglobin	579	12·6	580	11·6, 17·5	577	12·9	580	12·3	556	12·2	560	12·2	578	15·1
	540	12·3	540	12·2, 19·2	540	14·8	542	15·0	528	15·3	530	15·2	540	17·0
	424	162·6	—	—	—	—	422	167·0	409	111·0	408	176·0	426	187·0
	340	20·8	—	—	—	—	—	—	—	—	—	—	—	—
	275	36·3	—	—	—	—	—	—	—	—	—	—	—	—
Oxymyoglobin	582	12·6	586	17·0	582	15·1	582	13·4	—	—	—	—	582	15·2
	542	12·3	547	17·5	544	14·6	544	13·9	—	—	—	—	545	15·2
	418	95·1	—	—	—	—	420	112·0	—	—	—	—	418	129·5

* Theorell (1934) gives the following specific extinction coefficients $\left(\log \frac{I_0/I}{cd}\right.$, where c = g./l. and d = 1 cm.$\left.\right)$: reading down the column, 0·23, 0·51, 6·7, 1·67, 0·65, 6·2, 0·73, 0·71, 9·4, 1·2, 2·1, 0·73, 0·71 and 5·5. The millimolar extinction coefficients are calculated from these, taking the mol.wt. of myoglobin as 17 300, after Bowen (1948).

† The first column gives values calculated by us as described in the text; the second column is taken from Bowen (1949).

‡ Values of the extinction coefficient in these columns are obtained by first finding the concentration of the solution as the ferric cyanide compound, Drabkin's (1947) value of 11·3 being used for the millimolar extinction coefficient at 540 $m\mu$.

§ George & Irvine (1952) give a value of 163·5 for this coefficient.

‖ Drabkin's (1947) millimolar extinction coefficient of 11·3 at 540 $m\mu$ is commonly accepted.

Table 2. *Reactions of native and reconstituted myoglobins with carbon monoxide*

Figures are the velocity constants [M^{-1} sec.$^{-1}$ × 10^{-6}] for the reaction myoglobin + CO → myoglobin–CO, measured by flash photolysis. The columns refer to separate preparations of myoglobin, and the figures in parentheses give the numbers of reconstitution experiments or separate experimental determinations averaged in each case. Protein concentration 20 μM; CO concentration 200 μM. Temperatures were between 18° and 22° in each case. pH 7·1, 0·2 M-phosphate buffer.

	Horse			Whale	
Native	0·53 (1)	0·46 (1)	0·43 (1)	0·72 (3)	0·74 (4)
Protomyoglobin	0·52 (3)	0·28 (2)	0·14 (5)	0·54 (1)	0·64 (7)
Mesomyoglobin	—	0·24 (2)	0·17 (6)	—	0·66 (1)
Deuteromyoglobin	1·29 (4)	1·16 (1)	0·69 (2)	—	1·25 (4)

The results for the native protein confirm the species difference suggested by Gibson (1956), the velocity constants for horse-heart myoglobin being appreciably smaller than those for whale myoglobin. Millikan's (1936) finding that the rate constant is independent of pH within the range 7·0–9·0 has been confirmed. It has been found, however, that in more acid solutions there is a moderate increase which reaches 50% at pH 5·0.

Mesomyoglobin. The kinetic results with mesomyoglobin given in Table 2 show that the change from vinyl to ethyl side chains on the porphyrin molecule has apparently had no effect on the rate constant. The reconstituted protomyoglobin combines at the same speed as mesomyoglobin in each of the samples examined.

Deuteromyoglobin. The results of experiments with deuteromyoglobin are also included in Table 2. In this case it has been found that the replacement of the vinyl groups of protohaem by hydrogens to give deuterohaem has led to a marked increase in the rate of reaction with carbon monoxide. The average increase in rate is between 2½- and 3-fold, as compared with the reconstituted protomyoglobin from the same preparation.

DISCUSSION

Up to the present only two series of hybrid haemoproteins, in which the native protein is combined with various haems, appear to have been described. These are the reconstituted haemoglobins (Hill & Holden, 1926; Warburg & Negelein, 1932) and the reconstituted peroxidases (Gjessing & Sumner, 1942; Theorell, Bergstrom & Åkeson, 1942). Although Drabkin (1945) has previously reported the reversible splitting of protomyoglobin, this is believed to be the first time that myoglobins other than those occurring naturally have been prepared. Because of its theoretical simplicity myoglobin is often used as a model on which to base theories of haemoglobin reactions, and it is thus of considerable interest to know that myoglobin, too, will form a series of hybrid compounds.

The spectral data presented for meso- and deutero-myoglobin show a general shift of the bands towards the blue, behaviour which is parallel to that shown by simpler haem compounds (Drabkin, 1942) and with the findings of Hill & Holden (1926) for mesohaemoglobin. The figures given for protomyoglobin agree well with Theorell's findings (1934; Theorell & Åkeson, 1955). Kiese & Kaeske (1942) have published tracings obtained with a recording spectrophotometer for (among other compounds) carbon monoxyhaemoglobin, nitric oxide haemoglobin, methaemoglobin and haemoglobin; carbon monoxymyoglobin, nitric oxide myoglobin, metmyoglobin and myoglobin. Their records were taken in pairs and compared directly the corresponding derivatives of myoglobin and haemoglobin. Bowen (1949) has calculated extinction coefficients from these curves, reproduced in Table 1, which differ widely from those of other authors. These discrepancies appear to be due to errors in the labelling of the ordinates of the spectrophotometric records. If, instead of calculating the extinction coefficients from the ordinates, the extinction coefficients for the haemoglobin derivatives are taken from Lemberg & Legge (1949), then the curves for myoglobin may be evaluated with the ratio of haemoglobin concentration to myoglobin concentration given by Kiese & Kaeske. The results so obtained have been added to Table 1 and are in reasonable agreement with Theorell's, Bowen's and our own figures, making it clear that the discrepancy is of arithmetic rather than of observational origin. The source of Bowen's entry for Kiese & Kaeske's observations on oxymyoglobin is obscure since this compound was not among the derivatives they examined.

Kinetic studies on haematin compounds have been concentrated on materials such as haemoglobin, catalase and peroxidase, and myoglobin has received little attention. The classical paper on myoglobin kinetics is that of Millikan (1936), which until quite recently gave the only published value for the velocity of combination of carbon monoxide with native myoglobin. With a flow method he obtained a value of $(0{\cdot}3 \pm 0{\cdot}1) \times 10^6$ M^{-1} sec.$^{-1}$ at 20°. More recently Gibson (1956) obtained a value of $(0{\cdot}55 \pm 0{\cdot}03) \times 10^6$ M^{-1} sec.$^{-1}$ at 19° for whale myoglobin. In the present studies native whale myoglobin was found to give a velocity constant of $(0{\cdot}69 \pm 0{\cdot}04) \times 10^6$ M^{-1} sec.$^{-1}$ at 18–22°, whereas three preparations from horse heart gave a mean value of $(0{\cdot}47 \pm 0{\cdot}03) \times 10^{-6}$ M^{-1} sec.$^{-1}$ at 20·5°. Gibson pointed out the differences between his result and that of Millikan, and suggested that a species difference might exist. Although the present experiments have confirmed Gibson's previous observations on whale myoglobin it now appears that the species difference is less than had been supposed, the result with horse-heart myoglobin being somewhat higher than that suggested by Millikan. Since Millikan's experiments were carried out with a carefully checked and very reliable flow method, it is likely that his lower values reflect real differences in the preparations, some of which were stored for considerable periods before use.

One of the chief difficulties in the present work has been the occurrence of erratic and unpredictable changes in myoglobin preparations on storage. One sample of crystalline whale myoglobin prepared by Dr S. R. Elsden and Dr F. W. J. Teale in 1955, and examined at intervals by flash-photolysis

and by flow methods, has given the same velocity constant from the time of its preparation up to the time of writing. This sample has been stored as a crystalline paste at $-12°$. On the other hand, a sample of crystalline horse-heart myoglobin also stored at $-12°$ showed a decrease of 50% in the rate of combination with carbon monoxide after 6 months of storage. This material showed no sign whatever of changes in the absorption spectrum which might be associated with denaturation. In general our experience has been that most samples gave lower combination rates after storage, and some preparations have shown a significant decrease after storage for as little as 2 weeks. These effects cannot easily be explained, but may perhaps be due to small differences in the preparative procedure initiating molecular changes which proceed further in the isolated protein even in the cold. It appears that changes in the rate of combination with carbon monoxide provide a much more sensitive means of detecting incipient denaturation in the protein than do spectral changes or other properties of the material.

In many cases the splitting and reconstitution procedures have caused similar changes in the velocity constant for the combination of myoglobin. The extent of these changes seems to have depended more on the initial state of the myoglobin than upon the splitting and reconstitution procedure itself, since in several samples the reconstituted material has had the same or almost the same velocity constant as the original material. Further, where considerable changes have taken place on splitting and reconstitution, these changes have remained constant in size from one batch of the same preparation to another and so are probably due, not to variations in the procedures themselevs, but to degenerative changes which had taken place in the myoglobin preparations used.

Although it was the exception rather than the rule to obtain a reconstituted material with the same kinetic properties as the native protein, the fact that such a result could be obtained at all is evidence that the haem group can be returned to an environment little different from that from which it was taken. This result has some bearing on the 'crevice' theory of St George & Pauling (1951), suggesting that the 'crevice' is more in the nature of a dimple, since the haem molecule could scarcely be expected to penetrate deeply within the body of the myoglobin molecule unless the removal of the haem group is associated with unfolding of the peptide chain, which is reversed on resynthesis of the haemoprotein. The suggestion that the site of the haem group is rather near the surface appears to be in agreement with the latest results of the detailed X-ray studies of Kendrew, Bodo, Dintzis, Parrish & Wyckoff (1958).

Although the difficulties in comparing the native with the reconstituted myoglobins are very considerable, it seems possible to draw definite conclusions from the comparison of the three synthetic myoglobins, as, in spite of variations in the rate of combination of any one myoglobin with carbon monoxide, due presumably to the state of the globin used in its preparation, the comparative picture has remained the same. Proto- and mesomyoglobin have similar rates of combination, whereas deuteromyoglobin reacts considerably faster, the average value for the increase in rate on substituting deuterohaem for protohaem being about $3\frac{1}{2}$-fold. In the preceding paper (Smith, 1959) it is shown that in the carbon monoxide haemochromogens the rates of reaction of the proto-, meso- and deutero-haem compounds with carbon monoxide are in the ratio 1:2·5:3·1. The observed rate must be influenced by the side chains at positions 2 and 4 of the porphyrin ring, since this is the only point of difference between the compounds. The side chains concerned are vinyl, ethyl and hydrogen in proto-, meso- and deuteroporphyrin respectively, and the electron-attracting power of these groups decreases in the same order. Thus the side chains of deuterohaem have the least influence on the valency electrons of the iron atom, whereas the vinyl groups of protohaem may exert a stronger attraction on them through the structure of the haem. The former might therefore be expected to show a faster reaction rate than the latter, since the combining electrons of the iron would be more accessible to the ligand molecule in this case. Although this is observed experimentally in the haem compounds mentioned above, in the myoglobins mesomyoglobin forms an exception to the simple rule suggested. There is no reason why the behaviour of the haemoprotein must reflect that of the simpler compounds. The problem is exceedingly complex and is unlikely to be resolved until further information is available about the nature of the myoglobin molecule and of the haem–globin linkage. From the functional point of view, the results suggest that a fairly specific haem structure is required to maintain the normal combination rates, and it may be tentatively supposed that in the myoglobins it is the removal of one or more of the carbon atoms belonging to the side chains in positions 2 and 4 of the porphyrin nucleus, rather than the exchange of an unsaturated for a saturated grouping, which has the greatest effect on the reaction rate. This suggests that in this case a steric rather than an electromeric effect is the more important. The order of effect found with the different forms of myoglobin is similar to the changes obtained when meso- and deutero-peroxidase were compared with protoperoxidase by Theorell *et al.* (1942), who found

activities of 57 and 63% respectively as compared with that of the native protein.

SUMMARY

1. The preparation and spectra of native, reconstituted proto-, meso- and deutero-myoglobin are described.

2. The rates of combination of these proteins with carbon monoxide have been measured. In most cases the rate for the reconstituted proto-myoglobin was lower than that for the native protein.

3. The relative rates for reconstituted proto-, meso- and deutero-myoglobin were 1:1:3·5.

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The Lipids of Whole Blood

1. LIPID BIOSYNTHESIS IN HUMAN BLOOD *IN VITRO*

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In recent years the erythrocyte has been shown [Caffrey, Tremblay, Gabrio & Huennekens (1956), and Lowy, Ramot & London (1958)] to possess at least some of the normal metabolic pathways, contrary to the older view which considered the cell simply as an inert carrier of haemoglobin (for a recent review see Prankherd, 1955). Little detailed study, however, has been made of lipid turnover, though Lovelock (1955) suggested on the basis of physical measurements that the structural integrity of the erythrocyte is maintained by its own metabolic activity. Altman, Watman & Salomon (1951) described the incorporation *in vivo* of [*Me*-^{14}C]acetate into the stroma of the rabbit erythrocyte and suggested that metabolically active lipid constituents of the stroma (with a life span of 3–4 days) exchange with chemically identical plasma components. This type of exchange had previously been demonstrated by Muir, Perrone & Popják (1951) to occur with erythrocyte cholesterol. Altman (1953) reported the incorporation *in vitro* of [*carboxy*-^{14}C]acetate into the stroma of the rabbit erythrocyte during the incubation, either of whole blood or of erythrocytes in saline; approximately 1·9% of the total activity was found in the stroma after 6 hr. Activity of a comparable order was also found in the fatty acids of the plasma lipids. Similar incorporations *in vitro* of labelled acetate were demonstrated to occur in human blood by Altman & Swisher (1954).

We have extended this work and have demonstrated the ability of the cellular components of blood to synthesize a wide variety of fatty acids, to incorporate these acids into both triglycerides and phospholipids and to exchange both lipids with