from any biochemical system. It is likely that this type of reduction is more common than the present number of examples indicates and that additional instances will appear when the extreme lability of a Δ^4-3-hydroxysteroid to acid is appreciated. Rate studies of the dehydrations of II and pregn-4-3β-ol-20-one, under the mild conditions above, showed that the reactions were almost 75% complete within 5 min.

The reduction of the carbonyl group of a Δ^4-3-ketosteroid without prior reduction of the double bond reflects an additional degree of complexity in the metabolism of steroids. It will be worth while to test a Δ^4-3-hydroxysteroid as a substrate in various biochemical systems or *in vivo* to learn whether the double bond rearranges to form a Δ^5-3-hydroxysteroid. If this occurs, then the two steps—reduction of the 3-carbonyl group and rearrangement of the double bond—would constitute a reversal of the oxidation of a Δ^5-3-hydroxysteroid to a Δ^4-3-ketosteroid. This reversal has not been hitherto observed.

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Heme dissociation and autoxidation of myoglobin*

The autoxidation of HbO_2 and MbO_2 has been studied by BROOKS[1,2], GEORGE AND STRATMAN[3–5] and TSUSHIMA[6] among others. The evidence accumulated supports the concept that reduced, deoxygenated heme pigment reacts with oxygen to yield oxidized heme pigment. BROOKS[2] and GEORGE AND STRATMAN[4] showed that the rate constants for autoxidation of HbO_2 and MbO_2, respectively, increase with decreasing partial pressure of oxygen and go through a maximum at a partial pressure of oxygen corresponding to half saturation of the particular heme pigment. As WATTS[7] has pointed out, the differences in reduced heme pigment molecules which allow them

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to react with oxygen in one instance to become oxygenated and in another instance to become oxidized are completely unknown. KEILIN[8] has recently shown that there is no evidence for the presence of peroxides in solutions of HbO_2 undergoing autoxidation.

In oxygenated heme pigments, the bonding between heme iron and histidine of the protein is covalent. On the other hand, with reduced or oxidized heme pigments, iron is bound primarily by ionic bonds. This suggests the possibility that heme or hematin may be dissociated from protein when the pigment is reduced or oxidized. The concept of firm bonding, which is generally accepted as existing between heme prosthetic groups and apoprotein, apparently results from the inability to demonstrate free heme after dialysis, electrophoresis, or column chromatography of myoglobin or hemoglobin in aqueous solution. However, the environment of the heme group in myoglobin has been shown to be essentially non-polar due to side chains of the protein in the immediate vicinity of heme (KENDREW *et al.*[9]). This non-polar environment could be a factor in keeping heme from dissociating from apoprotein. Myoglobin may be thought of as an inclusion compound with the heme portion fitting into the apoprotein such that heme remains associated even though chemical linkages do not exist. Consequently, if one used an extracting solvent which is less polar than water, it may be possible to extract heme which is not chemically bonded to apoprotein. LEWIS[10] extracted various heme proteins with acetone and found that free hematin is extracted from metmyoglobin primarily in the pH range 4.5–4. This would indicate that there is very little free heme present in metmyoglobin at higher pH. However, similar experiments have not been done using reduced deoxygenated myoglobin.

The results reported here indicate that considerable heme can be extracted from Mb at pH 6.6, and the possibility that free heme is the reactant in autoxidation of MbO_2 is discussed.

Myoglobin was crystallized from beef skeletal muscle as previously described[11]. Metmyoglobin crystals were dissolved in distilled water and dialyzed against distilled water to obtain a salt-free metmyoglobin solution. The concentration of the metmyoglobin solution was determined by using a ε_{mM} of 11.3 for light absorption of the cyanide derivative of metmyoglobin at 540 mμ. A solution of metmyoglobin (0.070 mM) was prepared at pH 6.6 using NaCl as diluent to give an ionic strength of 0.05. The metmyoglobin solution was divided into two 5-ml portions. To one portion, approx. 3 mg of $Na_2S_2O_4$ was added to reduce the metmyoglobin. The Mb was immediately mixed with 10 ml of acetone at room temperature, and the mixture was centrifuged to remove precipitated protein. The acetone–water mixture containing extracted heme (which was immediately autoxidized to hematin) was acidified with 0.1 N HCl (0.1 ml acid added to 4.9 ml extract), and the absorbancy of the acid hematin was determined at 385 mμ. A second portion of metmyoglobin was extracted using the same procedure but with no added $Na_2S_2O_4$. The amount of acid hematin in the extract was determined using the extinction coefficient given by LEWIS[10] for acid hematin in aceton (absorbancy of 1 mg/ml haematin = 136).

Results showed that 40% of the heme present in Mb was extracted by acetone, but negligible amounts of hematin in metmyoglobin were extracted. The pH of both metmyoglobin and Mb solutions was 6.6.

The ease with which heme is extracted from Mb at relatively high pH tends to support the hypothesis that heme can dissociate chemically from Mb but still remains

physically associated with the apoprotein in aqueous solution. Presumably, such dissociated heme would be as labile to autoxidation as is free heme. Consequently, the autoxidation of MbO_2 may occur as follows:

$$MbO_2 \rightleftharpoons Mb + O_2$$

$$Mb \rightleftharpoons \text{heme} + \text{protein}$$

$$\text{heme} + O_2 \rightleftharpoons \text{hematin}$$

$$\text{hematin} + \text{protein} \rightleftharpoons \text{metmyoglobin}$$

Such a scheme would be compatible with the finding that rates of autoxidation increase with decreasing partial pressures of oxygen. The rate of autoxidation would depend upon concentrations of dissociated heme and O_2, and the dissociated heme concentration would be dependent on the concentration of Mb.

The relatively large amount of heme which was extracted by acetone (40%) should not be construed as indicating the equilibrium concentration existing at pH 6.6. Undoubtedly, addition of acetone displaces the equilibrium between Mb and dissociated heme towards more dissociation, since heme in acetone–water solution rapidly polymerizes, making it unavailable for recombination with apoprotein.

If autoxidation of MbO_2 is due to autoxidation of free heme, it may be that only those heme pigments in which the heme group can dissociate are autoxidizable. Unfortunately, relatively little is known of the heme linkage in most pigments. However, in cytochrome *c*, the heme is known to be firmly bound by iron–histidine bonds and thio–ether linkages to the vinyl side chains of heme. This firm binding of heme in cytochrome *c* may be the reason that it is not autoxidizable.

The hypothesis of heme groups chemically dissociating from reduced, deoxygenated heme proteins could have an important bearing on understanding of oxygenation reactions and the autoxidation of cytochrome oxidase. Consequently, the hypothesis warrants further investigation.

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