ACTA CHEMICA SCANDINAVICA 15 (1961) 975–984

Crystalline Leghemoglobin

IV. Spectroscopic Studies of the Two Main Metleghemoglobin Components and Some of their Fatty Acid Complexes

NILS ELLFOLK*

Medicinska Nobelinstitutet, Biokemiska avdelningen, Stockholm, Sweden

The transition from acid to alkaline metleghemoglobin follows a monovalent dissociation curve. The pK values (at an ionic strength of 0.1) were determined spectrophotometrically and were found to be 8.16 for the electrophoretically faster moving component, and 8.34 for the slower one. No other hem linked groups were spectrophotometrically demonstrable between the pH values of 5.35 and 10.45. Acetate, propionate, butyrate and valerate were found to form reversible complexes with the two main metleghemoglobin components. The isosbestic points for the complexes were at 583 and 500–490 mμ. The pK values of the fatty acid complexes at pH 4.8 were $pK_{acetate} = 2.9$, $pK_{propionate} = 2.8$, $pK_{butyrate} = 2.4$ and $pK_{valerate} = 2.6$. The pK values were essentially independent of the sizes of the alkyl groups. This has led to the conclusion that the hem is not buried within the folds of the apoprotein, but is attached to the surface of it.

In 1939 Kubo [1] for the first time examined spectrophotometrically the pigment from soy bean root nodules and determined the positions of its absorption bands under different conditions. The oxygenated pigment showed absorption bands at 575 and 540 mμ, which on reduction were replaced by one band at 555 mμ. In the carbon monoxide compound, the bands were shifted to 570 and 535 mμ. The oxidized ferri-compound had bands at 625, 563, and 530 mμ. This compound was found to react with cyanide or fluoride to give compounds with bands at 540 or 610 mμ, respectively.

These original observations have been essentially confirmed in several laboratories. Keilin and Wang [2] found the absorption maxima of the oxygenated compound at 574 and 540 mμ. Little and Burris [3] found exactly the same maxima as Kubo, whereas Sternberg and Virtanen [4] reported maxima at 573 and 540 mμ. The maxima of the reduced leghemoglobin was found to be at 557 mμ by Keilin and Wang [2], whereas Little and Burris as well as Sternberg

* Permanent address: Research laboratories of the State Alcohol Monopoly, Helsinki, Finland.

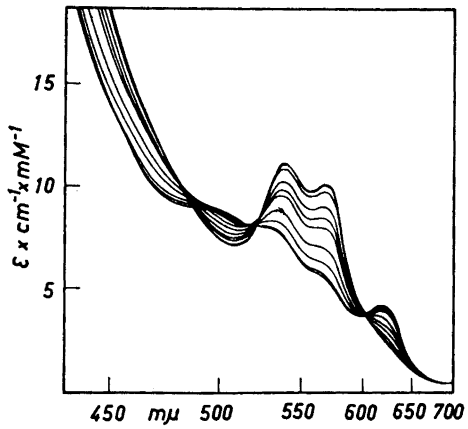

Fig. 1. The effect of pH upon the absorption spectra in the visible (700—450 mμ) of the electrophoretically faster component of metleghemoglobin in solutions of 0.1 μ ionic strength at + 20°C. The absorption curves corresponding to different pH values may be distinguished in the spectral region between 570—540 mμ, where the absorption increases with increasing basicity. At the pH values in parenthesis the spectral curves are superimposed: (5.35, 5.80, 6.40, 6.80), 7.47, 7.89, 8.31, 8.44, 8.75, 9.06, 9.50 (10.00, 10.45).

and Virtanen confirmed the value of Kubo. The maximum of the cyanide compound at 540 mμ, as determined by Kubo, was confirmed by Sternberg and Virtanen as well as by Little and Burris, with only a slight deviation (541 mμ). Greater differences were found between Kubo's values for the maxima of the carbon monoxide compound and those values determined by Keilin and Wang [2] (564 and 538 mμ), by Little and Burris [3] (565 and 539 mμ) and by Sternberg and Virtanen (565 and 538 mμ). The maximum of the fluoride compound was found to be at 605 mμ by Little and Burris as well as by Sternberg and Virtanen, and thus differed slightly from Kubo's value of 610 mμ.

The pK value for the transitation from acid to the alkaline form of metleghemoglobin of the unresolved material was determined for the first time by Sternberg and Virtanen, and was found to be equal to 8.254, $\mu = 0.1$. Later, Thorogood [5,6] made determinations of the pK values of the two main components isolated by paper electrophoresis, and found them to be equal to 8.02 for the electrophoretically faster component, and 8.22 for the slower one at an ionic strength of 0.1.

In the present investigation, the pK's for the transition from the acid to alkaline forms of the two metleghemoglobins were determined. The reversible formation of acetate, propionate, butyrate and valerate complexes with the two metleghemoglobins was also investigated.

EXPERIMENTAL

Methods. Spectrophotometric measurements were made with a Beckman model DK 2 in the most experiments. The pH's of the solutions were measured with the glass electrode which was standardized against phthalate and borate solutions.

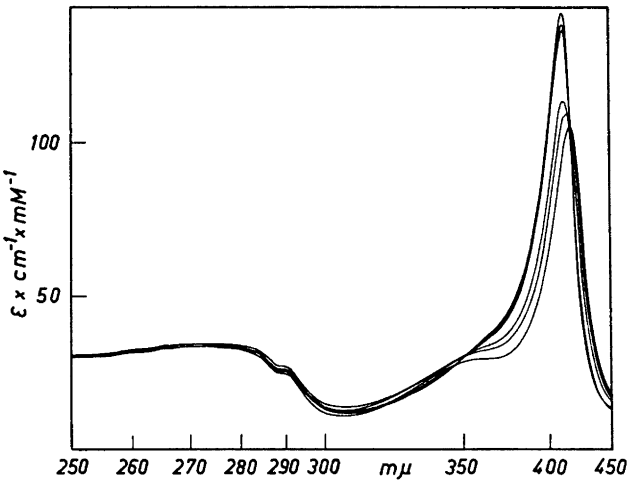

Fig. 2. The effect of pH upon the absorption spectra in the ultraviolet (450—250 mμ) of the electrophoretically faster component of metleghemoglobin in solutions of 0.1 ionic strength at + 20°C. The pH order may be followed in the spectral region between 403.5—412 mμ. The curves were obtained at pH: 5.35, 6.80, 7.47, 8.31, 8.75 and 9.50. The Soret band increases in intensity with increasing acidity.

Material. The two components of metleghemoglobin (metLhb or Lhb^+) were prepared as described previously [7,8]. Their electrophoretic homogeneity was always checked. Horse myoglobin I was prepared chromatographically [9].

RESULTS

Studies of the absorption spectra of the two main metLhb components in the visible and ultraviolet regions of the spectrum were carried out on solutions of the proteins in phosphate, veronal, and glycine-NaOH buffers of 0.1 ionic strength. Figs. 1 and 2 show the molecular extinction coefficients (250 to 700 mμ) of the faster component as a function of pH. In the visible region, all the curves intersect at four isosbestic points, which were found for both of the main metLbh components at 604—606, 523—525, 483—495 and 410 mμ. In the ultraviolet region, an isosbestic point was found for the two components at about 355—353 mμ. The Soret band was situated at 403.5 mμ at acid pH, and the height of the band decreased at alkaline pH with a concomitant shift to 412 mμ. From the figures, it can be concluded that only one hem linked acid group is demonstrable by spectrophotometric analysis between pH 5.35 and 10.45. Thorogood [5,6] has, however, reported the presence of a second porphyrin linked acid group which was titrable spectrophotometrically below pH 7. Thorogood found the isosbestic points of the dissociation in the acid range at 582, 503, and 484 mμ.

Since the changes in light absorption were maximal at 575 mμ, a series of determinations were made at this wave length and at different pH values for both of the two components. By plotting

$$\log \left[\frac{\varepsilon_{acid} - \varepsilon_{obs}}{\varepsilon_{obs} - \varepsilon_{alk}} \right]$$

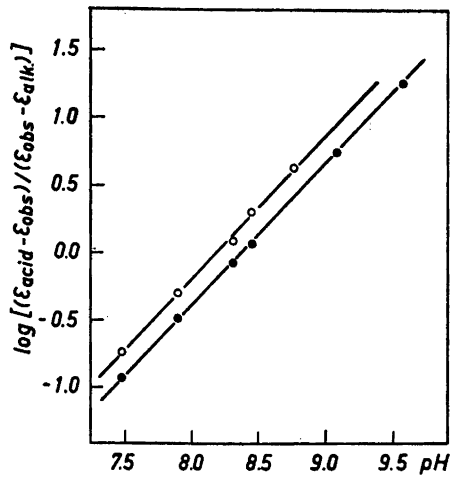

Fig. 3. Graphical determination of the pK-values of the transition from acid to alkaline metLhb's of the two metLhb components at an ionic strength of 0.1 and +20°C. ε_{acid} is the absorption of the total acid forms of the metLhb's when increased acidity caused no further change in absorption, and ε_{alk} the absorption of the total alkaline forms of the metLhb's, when increased alkality caused no further change in absorption, and ε_{obs} the absorption of the metLhb's at different pH values. All solutions contained buffers of ionic strength 0.1; (○) electrophoretically faster component of metLhb, pK = 8.16 (●) electrophoretically slower component of metLhb, pK = 8.34. The slopes of the lines equalled 1.

against pH a straight line was obtained with $n_{H^+} = 1$ (Fig. 3) for the two metLhb components. The equilibrium is illustrated by the equation

$$Lhb^+ \cdot H_2O \rightleftharpoons Lhb \cdot OH + H^+ \qquad (1)$$

The pH values when $[Lhb^+ \cdot H_2O] = [Lhb^+ \cdot OH^-]$, is equal to the p$K$, and was determined to be 8.16 for the electrophoretically faster component of

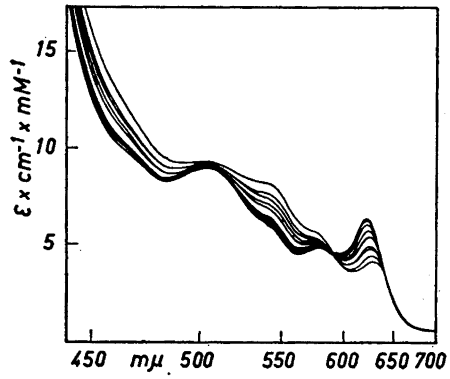

Fig. 4. Absorption spectra in the visible (700—450 mμ) of the electrophoretically faster component of metLhb at pH 5.50 in acetic acid buffers showing the effect of acetate concentration from 0 to 0.16 M at + 20°C.

metLhb and 8.34 for the electrophoretically slower one, both at an ionic strength of 0.1.

The pK values of the separated components agree well with the value of 8.254 determined by Sternberg and Virtanen [4] for the unresolved material. The pK values for the transition from acid to alkaline form of methemoglobins from different sources have values of 8.1—8.6 [10–15], and differ slightly from the values of 8.8—8.9 reported for the myoglobins [16,17].

When an acetic acid buffer is added to a solution of metleghemoglobin, a color change to the green is noticeable [8]. The spectral changes are illustrated in Fig. 4 with the electrophoretically faster component of metLhb in acetate buffers of different concentrations at pH 5.50, a pH where no hem linked groups are spectrophotometrically demonstrable in a phosphate buffer. The metleghemoglobin band at 625 mμ is increased in intensity and shifted to 618 mμ. The curves of the different concentrations of acetate intersect at two isosbestic points, one at 583 mμ and the other one at 500—490 mμ. From these findings it is evident that the acid dissociation reported by Thorogood [5,6] is caused by complex formation between metLhb and the acetate buffer used. Since changes in light absorption were maximal at 555 mμ, a series of determinations at this wavelength were carried out at different acetate concentrations but at constant pH. By plotting

$$\log \left[\frac{\varepsilon_0 - \varepsilon_{obs}}{\varepsilon_{obs} - \varepsilon_\infty} \right]$$ against $-\log$ [acetate], a straight line was obtained

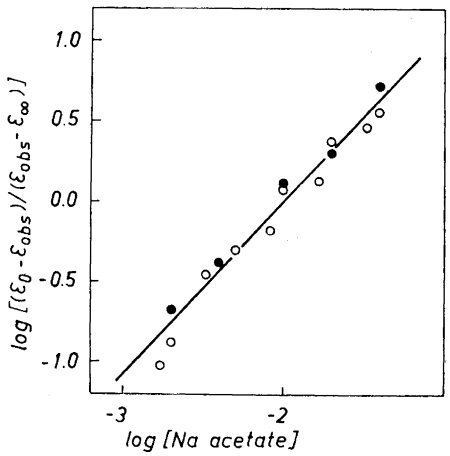

Fig. 5. Graphical determination of the average pK values for the acetate complexes from the absorption at 555 mμ of the two metLhb components in presence at varying concentrations of acetate. ε_0 is the absorption of metLhb in absence of acetate in phosphate buffer at pH 5.50. ε_{obs} is the absorption of metLhb at different concentrations of acetate, shown in the abscissa, and ε_∞ the absorption of metLhb when further addition of acetate did not cause further change in absorption. All solutions had the pH of 5.50 and temperature + 20°C. (●) electrophoretically faster component and (O) electrophoretically slower one; pK = 2.0. The slope of the line equalled 1.

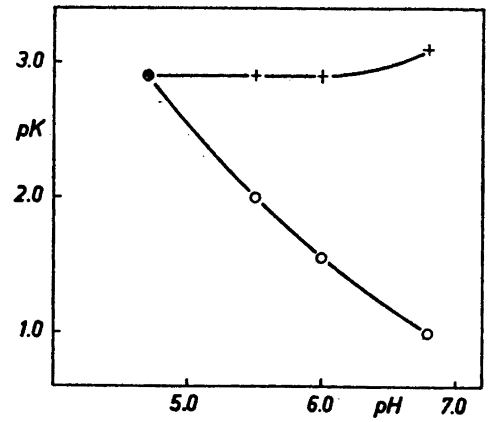

Fig. 6. The pH-dependence curve of the average pK values of the acetate or acetic acid complexes of the two metLhb components. Ordinate: pK value equal to -logarithm of the acetate (O) and acetic acid (+) concentrations, when [Lhb$^+$ acetate$^-$] or [Lhb$^+$ acetic acid] is equal to [Lhb$^+$H$_2$O]. Abscissa: pH.

for the two metLhb components with $n_{acetate} = 1$. This equilibrium is illustrated by the equation

$$Lhb^+ \cdot H_2O + CH_3COO^- \rightleftharpoons Lhb^+ \cdot CH_3COO^- + H_2O \qquad (2)$$

The value for $-\log$ [acetate] when [Lhb$^+$ · acetate$^-$] = [Lhb$^+$ · H$_2$O], is equal to the pK, and was determined to be 2.0 for the two metLhb components at pH 5.50 (Fig. 5).

In order to learn whether the complex involves acetate or undissociated acetic acid, the pH-dependence of the pK was investigated. The ligand concentration was calculated both as acetate and as undissociated acetic acid (Fig. 6). When the pK was calculated from acetate concentration it was found to vary with pH from 4.8 to 7, a region in which no hem-linked dissociation occurs. This behavior is not in accordance with eqn. (2) and is similar to that noted by Agner and Theorell [18] for catalase. On the other hand when the pK was calculated on the basis of undissociated acetic acid concentration it was found to be essentially pH independent. It therefore can be assumed that the formation of the complex below pH 7 involves the undissociated acetic acid molecule according to the following reaction:

$$Lhb^+ \cdot H_2O + CH_3COOH \rightleftharpoons Lhb^+ \cdot CH_3COOH + H_2O \qquad (3)$$

Several other fatty acids were examined for their ability to form complexes with both metLhb components. The experiments were conducted at pH 4.8, which is close to the pK_a values for the fatty acids studied. At this pH, the apparent pK calculated for the complex is the same whether one assumes that it is the fatty acid or the fatty acid anion, which is involved in the complex. The spectra of the acetate, propionate, butyrate and valerate complexes were

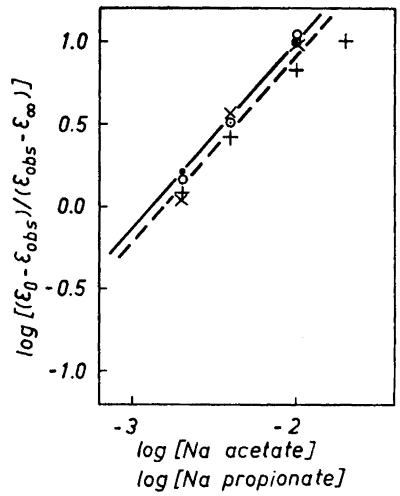

Fig. 7. Graphical determination of the average pK values of acetate and propionate complexes of the two metLhb components at pH 4.8 and + 20°C. The determinations were performed in a similar way as in Fig. 5 Acetate complex: electrophoretically faster component (●), electrophoretically slower component (○). The average pK value was 2.9 for the two components.
Propionate complex: electrophoretically faster component (×), electrophoretically slower component (+). The average pK value was 2.8 for the two components. In both cases the slopes of the lines equalled 1.

all similar (Fig. 5). The isosbestic points for these complexes were the same as those found for the acetate complex. By plotting

$$\log \left[\frac{\varepsilon_0 - \varepsilon_{obs}}{\varepsilon_{obs} - \varepsilon_\infty} \right] \textit{ versus } -\log [\text{ligand}]$$

at pH 4.8, a straight line was obtained (Figs 7 and 8). In all cases, the number of ligand groups involved in the complex was one. The average pK values for complexes with both of the Lhb components was 2.9 for the acetate complex, 2.8 for the propionate, 2.4 for the butyrate, and 2.6 for the valerate complexes.

As a comparison, horse myoglobin I was examined for its ability to form complexes with the fatty acids used above. No complex formation was observed under identical conditions of pH and concentrations.

DISCUSSION

In the trivalent state, the iron in hemoglobin as well as in myoglobin is able to combine with certain compounds, mainly anions. The compounds formed, are assumed to be complexes with the ferric iron. The iron in the center of the prophyrin nucleus has its six co-ordination valences occupied. Four of the valences lie in one plane, and link the iron atom to the nitrogen atoms of the pyrrol nuclei of the porphyrin. The remaining two valences, the fifth and sixth, are perpendicular to each side of the flat hem molecule and are pro-

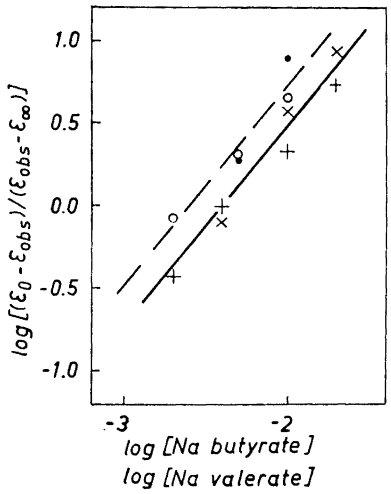

Fig. 8. Graphical determinations of the average pK-values of butyrate and valerate complexes of the two metLhb components at pH 4.8 and 20°C. The determinations were performed as described in Fig. 5. Butyrate complex: electrophoretically faster component (+), electrophoretically slower component (×). The average pK value was 2.4 for the two components. Valerate complex: electrophoretically faster component (O), electrophoretically slower component (•). The average pK value was 2.6 for the two components. In both cases the slopes of the lines equalled 1.

bably linked to an imidazole [19–23] moiety of a histidine residue of the protein molecule (position 5), and to a water molecule [22–25] (position 6).

At alkaline pH, a proton is dissociated from the water molecule (position 6) leaving the hydroxyl group bond to the iron. The dissociation of the water proton in the hemoglobins is found to occur within very narrow pH limits. The corresponding pK values for the fast and slow components of metLhb are very similar to these pK values, thereby indicating that the hem bound groups in hemoglobin and leghemoglobin probably are identical. The small differences between the pK's of the two main components is not necessarily an indication of differences in the hem bound group, but more probably reflect small differences in the protein structure close to the hemin ring, since amino acid analyses of the two main components do show small differences in their amino acid composition [26].

As to the position of the hemin ring in hemoglobin, St. George and Pauling [27] assumed it to lie within a fold or crevice of the globin. This assumption was based on the fact that hemoglobin combines more strongly with ethyl*iso*cyanide than with *tert*-butyl*iso*cyanide corresponding to a factor of 200 in equilibrium constants. Keilin [28] disagreed with this theory and proposed that the hem is situated on the surface of the globin. Recently, Lein and Pauling [29] arrived at the conclusion that the hem in myoglobin is also buried within the protein molecule. Strong additional support in favor of the view that the hem is buried within a fold of the peptide chains was obtained from X-ray analyses of crystals of hemoglobin [22] and myoglobin [23].

The experiments carried out in this study on the combination of different fatty acids with metleghemoglobin provide support for a picture of leghemoglobin which differs from that of myoglobin. Acetic, propionic, butyric and valeric acids combine almost equally tightly with metleghemoglobin, but at the same concentrations do not combine with myoglobin. It is evident, therefore, that the hem in leghemoglobin is much more accessible to ligands than

the hem in myoglobin, and forms equally strong complexes with fatty acids regardless of the size of the groups attached to the fatty acid carboxyl.

Although it has been well known that hemoglobin and myoglobin combine reversibly with molecular oxygen, it appears that no satisfactory explanation has yet been proposed to account for the remarkable property of combining reversibly with molecular oxygen, while at the same time protecting its divalent iron from oxidation. The mere formation of a co-ordination bond between a donor group of globin and the ferrous iron in hem is clearly inadequate to prevent the irreversible oxidation of the latter by molecular oxygen.

Wang *et al.*[30] have recently put forward a plausible theory which explains how the globin acts in order to let the hem combine reversibly with molecular oxygen. It is assumed that the hemin groups in hemoglobin and myoglobin are not completely exposed to water because of the low dielectric environment of the individual binding sites in the protein. Hydrophobic groups of the globin cover them at least partially. As a result, the average dielectric constant of the local environment of each binding site is much lower than that of freely exposed hem. This effectively retards the irreversible oxidation process without interferring with the reversible binding and release of molecular oxygen. Wang [31] has devised a synthetic model of hemoglobin to substantiate the picture of the binding sites. A film was prepared with a derivative of hem, 1-(2-phenylethyl)-imidazole carbonmonoxy hem diethylester, imbedded in a matrix of an amorphous mixture of polystyrene and 1-(2-phenylethyl)-imidazole. After a complete removal of the bound carbon monoxide by flushing with nitrogen, the film was exposed to air. A rapid combination with oxygen occurred, giving a product which displayed an oxyhemoglobin type of spectrum. The oxygenation was found to be reversible because oxygen was again removed by flushing the film with nitrogen. This cycle could be carried out repeatedly. Another film, in which the diethyl ester derivative of the hem was replaced with free hem, was immediately oxidized to the ferric state after exposure to air. This seemed to suggest that in contrast to the ester, free hems are not imbedded in the hydrophobic matrix but are quite exposed. The theory of Wang for the basis of oxygenation of hemoglobin and myoglobin is in good agreement with the concept of the imbedded position of the hem group as proposed by Pauling.

The strong autooxidizability of leghemoglobin may be explained satisfactorily by the uncovered position of the hem ring in a similar way as in Wang's model when the free hem on the surface of the polystyrene film was irreversibly oxidized but not oxygenated.

It has been established that the capacity of the leguminous root nodules to fix atmospheric nitrogen is parallel to the concentration of the hem proteins found in the nodules [32,33]. The action of leghemoglobin may be due to its ability to store oxygen and thereby promote the respiration of the nodule bacteria. However, the properties of Lhb make it less suited to function as an oxygen carrier only. Other functions for leghemoglobin have also been proposed. Hamilton *et al.*[34] reported recently an oxidation of leghemoglobin to metleghemoglobin when an extract of soybean root nodules had been exposed to nitrogen gas. The opposite reaction was observed with hydrogen gas.

Leghemoglobin was therefore suggested to act as an oxidoreduction catalyst in the symbiotic nitrogen fixation.

Bauer and Mortimer [35-37] recently proposed that the secondary attachment of N_2 to the hem group in Lhb is the first step in the symbiotic N_2-fixation. This hypothesis was based on the fact that a small amount of N_2 can be taken up by Lhb. This confirms the observation of van Slyke *et al.*[38] that oxygenated Hb takes up N_2 to a small extent.

Acknowledgements. The author wishes to express his thanks to Professor Hugo Theorell for encouraging interest in the work and to Mr. Å. Åkeson for a sample of myoglobin I. The investigation has been supported by a grant from the *Finnish State Commission of Natural Sciences* (*Statens Naturvetenskapliga Kommission i Finland*) which is gratefully acknowledged.

REFERENCES

1. Kubo, H. *Acta Phytochim. (Japan)* **11** (1939) 195.
2. Keilin, D. and Wang, Y. L. *Nature* **155** (1945) 227.
3. Little, H. N. and Burris, R. H. *J. Am. Chem. Soc.* **69** (1947) 838.
4. Sternberg, H. and Virtanen, A. I. *Acta Chem. Scand.* **6** (1952) 1342.
5. Thorogood, E. *Chromoproteins from Soy bean Nodules.* Diss. University of Pennsylvania, Philadelphia, 1955.
6. Thorogood, E. *Science* **126** (1957) 1011.
7. Ellfolk, N. *Acta Chem. Scand.* **13** (1959) 596.
8. Ellfolk, N. *Acta Chem. Scand.* **14** (1960) 609.
9. Åkeson, Å. and Theorell, H. *Arch. Biochem. Biophys.* **91** (1960) 319.
10. Austin, J. H. and Dabkin, D. L. *J. Biol. Chem.* **112** (1935) 67.
11. Coryell, C. D., Stitt, F. and Pauling, L. *J. Am. Chem. Soc* **59** (1937) 633.
13. Theorell, H. *Arkiv Kemi, Mineral Geol.* **A16** (1943) No. 14.
14. George, P. and Hanania, G. *Biochem. J.* **55** (1953) 236.
15. Havemann, R. and Mahling, A. *Z. physik. Chem.* **204** (1955) 60.
16. Theorell, H. and Ehrenberg, A. *Acta Chem. Scand.* **5** (1951) 823.
17. George, P. and Hanania, G. *Biochem. J.* **52** (1952) 517.
18. Agner, K. and Theorell, H. *Arch. Biochem.* **10** (1946) 321.
19. Wyman, J. *Advances in Protein Chem.* **4** (1948) 407.
20. Coryell, C. D. and Pauling, L. *J. Biol. Chem.* **132** (1940) 769.
21. Keilin, J. *Nature* **187** (1960) 365.
22. Perutz, M. F., Rossmann, M. G., Cullis, A. F., Muirhead, H., Will, G. and North, A. C. T. *Nature* **185** (1960) 416.
23. Kendrew, J. C., Dickerson, R. E., Strandberg, B. E., Hart, R. G., Davies, D. R., Philips, D. C. and Shore, V. C. *Nature* **185** (1960) 422.
24. Haurowitz, F. *J. Biol. Chem.* **193** (1951) 443.
25. Keilin, D. and Hartree, E. F. *Nature* **170** (1952) 161.
26. Ellfolk, N. *Acta Chem. Scand.* **15** (1961) 545.
27. St. George, R. C. C. and Pauling, L. *Science* **114** (1951) 629.
28. Keilin, D. *Nature* **171** (1953) 922.
29. Lein, A. and Pauling, L. *Proc. Natl. Acad. Sci. U.S.* **42** (1956) 51.
30. Wang, J. H., Nakahara, A. and Fleischer, E. B. *J. Am. Chem. Soc.* **80** (1958) 1109.
31. Wang, J. H. *J. Am. Chem. Soc.* **80** (1958) 3168.
32. Virtanen, A. I., Jorma, J., Linkola, H. and Linnasalmi, A. *Acta Chem. Scand.* **1** (1947) 90.
33. Virtanen, A. I., Erkama, J. and Linkola, H. *Acta Chem. Scand.* **1** (1947) 861.
34. Hamilton, P. B., Shug, A. L. and Wilson, P. W. *Proc. Natl. Acad. Sci. U.S.* **43** (1957) 297.
35. Mortimer, R. G. and Bauer, N. *J. Phys. Chem.* **64** (1960) 387.
36. Bauer, N. and Mortimer, R. G. *Biochim. et Biophys. Acta* **40** (1960) 170.
37. Bauer, N. *Nature* **188** (1960) 471.
38. van Slyke, D. D., Dillon, R. T. and Margaria, R. *J. Biol. Chem.* **105** (1934) 571.

Received December 23, 1960.