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Studies on the Absorption Spectrum of Leghemoglobin, especially of Leghemiglobin

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While examining in this laboratory the chemical nature of leghemoglobin, the chromoprotein present in root nodules, we tried to isolate it in the pure state and to investigate in detail its composition and properties. The pigment is easily removed even from whole excised nodules, if they are steeped in water for some time, but the removal is accomplished most rapidly if the nodules are first crushed. In this latter case other substances present in the nodules are brought to solution in quantities greater than if the whole nodules are soaked. Leghemoglobin can be purified to a great extent by ammonium sulphate precipitation. By this method we usually obtained a preparation of leghemiglobin whose iron content was $\sim$ 0.27 % of dry matter [1], and only once [2] did we obtain a preparation with 0.34 % Fe, *i.e.* the same iron content as in blood hemoglobin and myoglobin. Ellfolk and Virtanen [3,4] have electrophoretically divided the leghemiglobin preparation with 0.27 % Fe into two components, the faster one containing about 0.35 % Fe and the slower one about 0.17 % Fe. The molecular weight of the former is about 17 000 and it contains one hemin group. It is probably pure leghemiglobin. The molecular weight of the slower component is much greater and the said authors presume it to be an absorption compound of an iron-free protein and leghemiglobin.

In this paper we shall present results of determinations of absorption spectra of leghemoglobin. Most of the determinations were made with preparations which contained 0.27 % iron. Their purity, judged by their iron and hemin content, was about 80 % leghemoglobin, the remainder being hemin-free protein. Our determinations were made in 1948—1949 but we were unable to publish the results earlier, as the composition of the preparations was not defined. The results of Ellfolk and Virtanen now justify their presentation. These authors also state that the spectrum of the low-hemin (slower) compo-

nent with trivalent iron closely corresponds to that of the pure leghemiglobin if the absorption is calculated on the basis of the hemin content of the preparation. For pure leghemiglobin, the faster component, separated by electrophoresis, we determined the spectrum only at pH 7.10.

Kubo [5] determined the absorption maxima of unpurified hemoglobin isolated from the root nodules of soybean, using the oxy-, carboxy-, cyanide-, and fluoride compounds. Later, Keilin and Wang [6] determined the absorption between 630 and 490 mμ of a hemoglobin preparation also isolated from soybean. Calculated on the basis of its hemin content the preparation contained about 40 % hemoglobin. The determinations included both oxy- and carboxyhemoglobin. Using raw preparations of leghemoglobin from soybean Little and Burris [7] also determined the absorption of its different derivatives.

We have been particularly interested in the absorption spectrum of leghemiglobin since the chemical composition of globin and the manner of linkage of the hemin group to globin seem to affect more strongly the spectrum of the hemi-form.

EXPERIMENTAL

The chromoprotein brought into solution by the action of water on the nodules contained its iron chiefly in trivalent form. Thus in the first place it was possible to isolate leghemiglobin from the nodules. By passing carbon monoxide into the water suspension of nodules both before and during crushing, solutions were obtained which contained much carboxy-leghemoglobin together with leghemiglobin. Leghemiglobin has been present in large or small quantities in all preparations, which were made by adding sodium azide to the solution before crushing the nodules. Therefore we isolated chromoprotein for our spectral determinations without adding any oxidation-preventing substances, *i.e.* in the form of leghemiglobin. The preparation thus obtained was purified by repeated precipitations with ammonium sulphate. The leghemiglobin content of the preparations was determined by comparing their absorption values with the corresponding values for blood hemiglobin at the point of intersection of the extinction curves, at 523 and 519 mμ. Similarly a comparison was made between the absorption maxima of the pyridine hemochromogen of leghemoglobin preparations and of blood hemoglobin. Reduced leghemoglobin, CO- and NO-leghemoglobin were prepared from the purified leghemiglobin preparation. It was difficult to prepare the O_2-leghemoglobin compound, since oxidation of iron to the trivalent form took place to some extent while the reduced leghemoglobin took up O_2. Only once did we obtain an O_2-leghemoglobin compound of such high purity that no leghemiglobin was present when judged by the spectrum. This was when a leghemiglobin solution containing much hemin-free protein was allowed to stand in a refrigerator after addition of dithionite. This preparation contained only about 40 % leghemoglobin and 60 % hemin-free protein.

All the spectral determinations presented in this paper were made using the Beckman Quartz Spectrophotometer. The sample compartment of the apparatus was equipped with a thermospacer, hence the temperature of the solution under test could be maintained approximately constant. The slit of the spectrophotometer was very narrow for all determinations. The width of the spectral band was about 1 mμ within the wavelengths

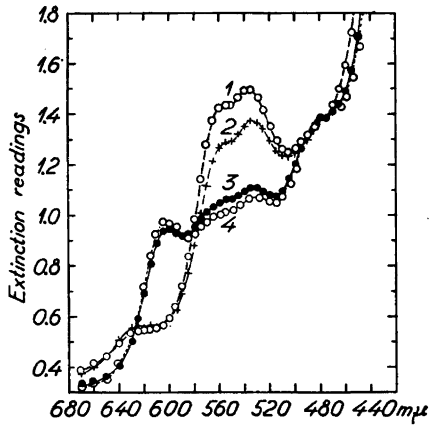

Fig. 1. Absorption spectra of crushed soybean nodules. Plants young, before flowering, nodules red. Determinations made about 1 hour after crushing the nodules. pH 6.5. 30—31 Aug., 1948.

1) 2.5 g nodules + 10 ml H_2O;
2) 2.5 » » + 10 » » + 0.4 mg $K_3Fe(CN)_6$;
3) 2.5 » » + 10 » » + 0.2 g NaF;
4) 2.5 » » + 10 » » + 0.2 » » + 0.4 mg $K_3Fe(CN)_6$.

of 600—450 mμ. The lengths of the absorption cells were each 1.00 cm. The extinction (density) values were determined usually at every 5th mμ, but near the absorption maxima as frequently as at every 2nd mμ. The curves were drawn to pass through every determination point. — All pH determinations were made immediately after the spectral determinations, using the glass electrode.

RESULTS AND DISCUSSION

Spectrum of leghemiglobin. Fig. 1 shows the spectrum of the water extract of the root nodules of soybean, both with and without potassium ferricyanide. The crushing and centrifugation were carried out rapidly. It appears from the spectrum that the chromoprotein brought into solution is almost entirely leghem*i*globin, since the potassium ferricyanide does not essentially affect the shape of the absorption curve. The spectrum also shows that the distinct maximum at 630 mμ, which is a characteristic of blood hem*i*globin, is faint for the leghem*i*globin solution isolated from the nodules and appears at about 620 mμ.

Addition of sodium fluoride to the water extract of crushed root nodules produces an absorption maximum at about 605 mμ thus showing the presence of leghem*i*globin. This maximum was formed regardless of whether or not ferricyanide was used as an oxidant (Fig. 1).

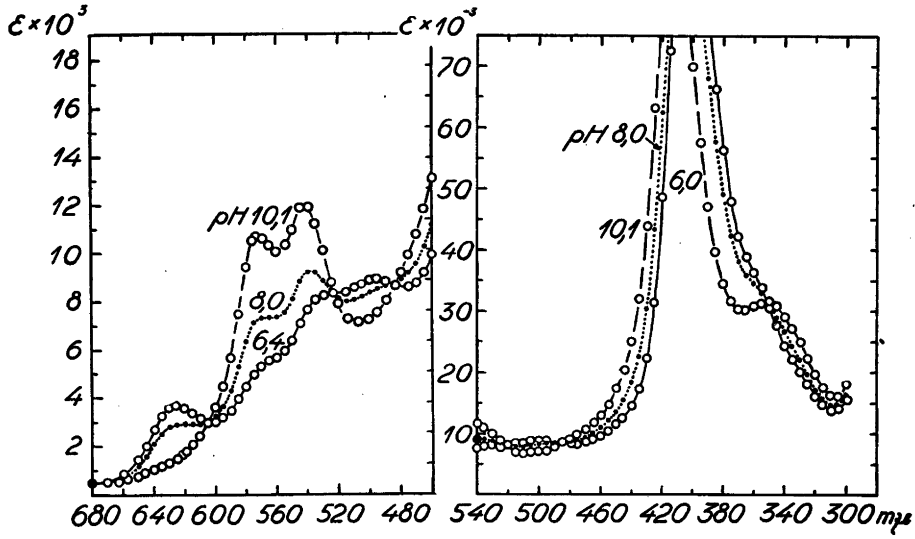

Fig. 2. Absorption spectra of purified leghemiglobin at pH 6.02, 6.40, 8.01, and 10.06, ionic strength in each solution 0.100. (Absorption curves practically the same at pH 6.02 and 6.4). Leghemiglobin concentration 0.66 mg/ml. Buffer: Na_2HPO_4-KH_2PO_4 (+NaCl) at pH 6.02—8.01; Na_2CO_3—$NaHCO_3$ (+NaCl) at pH 10.06, t = 22° C. The extinction curves intersect at 670, 605, 523, 484, about 410 (uncertain), and 352 mμ. 25 July, 1949.

The spectrum of the purified leghemiglobin preparation at different pH is shown in Fig. 2. For comparison, the spectrum of blood hemiglobin is presented in Fig. 3.

Purified leghemiglobin has a distinct maximum at 626 mμ. Hence, the diffuse band of the water extract of crushed nodules at about 620 mμ has

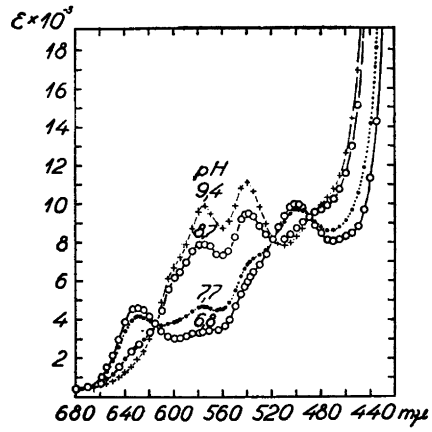

Fig. 3. Absorption spectra of blood hemiglobin (cow) at pH 6.41, 7.72, 8.70 and 9.43. O_2-hemoglobin oxidized with $K_3Fe(CN)_6$ with an amount not affecting the spectrum. Hemiglobin concentration 0.66 mg/ml. Buffer: Na_2HPO_4-KH_2PO_4 at pH 6.41—7.72, NaOH-glycine from pH 8.70 to 9.43. The extinction curves intersect at 670, 616, 519, 490, 414, and about 355 (uncertain) mμ. 22 Nov., 1948.

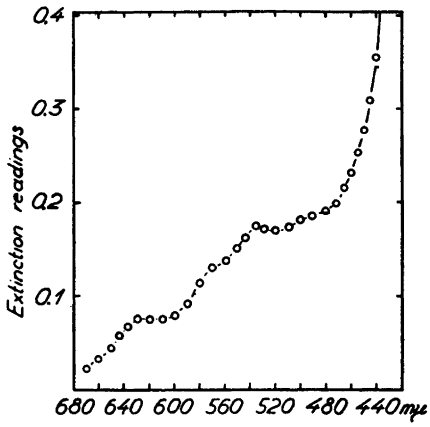

Fig. 4. Spectrum of electrophoretically purified leghemiglobin, faster component with I.P. 4.4, at pH 7.10 (Ellfolk and Virtanen). 5 May, 1950.

become denser and is shifted somewhat toward the red after the removal of foreign substances. In any case, the maximum, even with the purified leghemiglobin, is weaker than with blood hemiglobin.

Pure leghemiglobin isolated electrophoretically (the faster component with I.P. 4.4 and iron content 0.35 %) gave the spectrum illustrated by Fig. 4 according to the determinations of Ellfolk and Virtanen at pH 7.10. Due to an oversight no determinations were made with the preparation at other pH values.

The curve shows a great similarity between the spectrum of pure leghemiglobin and that of leghemiglobin of 80 % purity. First of all the curve confirms

Table 1. Intersection points of extinction curves of blood hemiglobin, leghemiglobin, and myohemiglobin determined at different pH values.

Authors	Source of hemiglobin	Concentration of the solution mg/ml	Intersection points of extinction curves * mμ
Austin and Drabkin [8]	blood	0.72	660 616 521
Hari [9]	»	?	616 518 487
Haurowitz [10]	»	0.19	619 519 490
Hicks and Holden [11]	»	?	418 358
Horecker [12]	»	2	672 617 521 488
Kubowitz [13]	»	?	680 621 521 490 414 355
Sternberg and Virtanen	»	0.96	670 616 519 490 ~ 414 ~ 355
Sternberg and Virtanen	root nodules	0.66	~670 605 523 484 ~ 410 ~ 352
Theorell and Ehrenberg [14]	muscle		626 523 495

* Calculated from published results or curves.

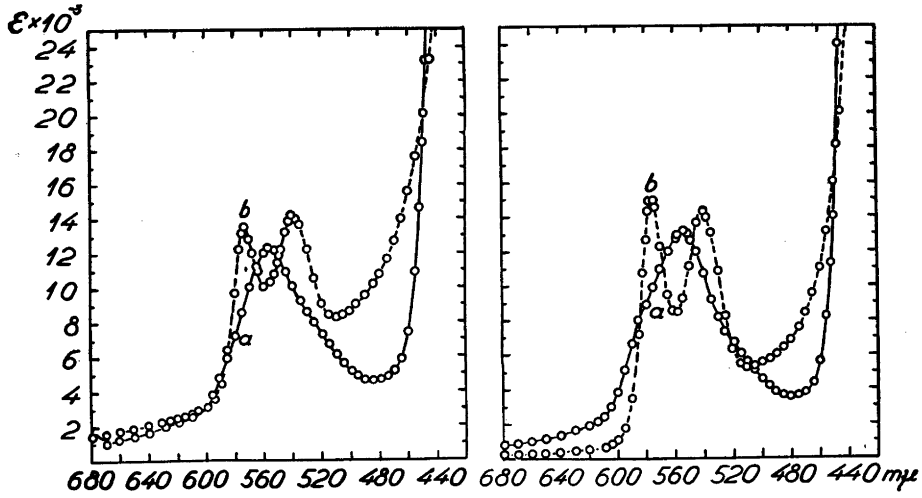

Fig. 5. Spectra of leghemoglobin (a) and legoxyhemoglobin (b), 0.65 mg/ml, 25 July. 1949 and 14 Oct., 1948.

Fig. 6. Spectra of bovine blood hemoglobin (a) and oxyhemoglobin (b), 0.65 mg/ml, 11 Nov., 1948.

the comparative weakness of the maximum at 626 mμ compared with the maximum of blood leghemiglobin at 630 mμ. The great dependence of the spectrums of hemiglobins on the pH of the solution makes it possible to compare the extinction curves of different hemiglobins only if they are determined at the same pH. Table 1 records the intersection points of the extinction curves of blood hemiglobin calculated from the values or curves of different investigators and the corresponding points for leghemiglobin on the basis of our own determinations.

Spectrum of leghemoglobin and legoxyhemoglobin. Leghemiglobin was reduced with sodium dithionite to leghemoglobin and the absorption curve determined (Fig. 5).

The purity of the legoxyhemoglobin preparation was as stated above and its spectrum is illustrated in Fig. 5. For comparison the spectra were also determined for blood oxyhemoglobin (cow) and for hemoglobin prepared from it (Fig. 6).

Spectrum of CO-leghemoglobin. Fig. 7 represents the spectrum of the carbon monoxide compound of leghemoglobin.

Spectrum of NO-leghemoglobin. Fig. 8 shows the spectra of the NO-compounds of leghemoglobin and blood hemoglobin.

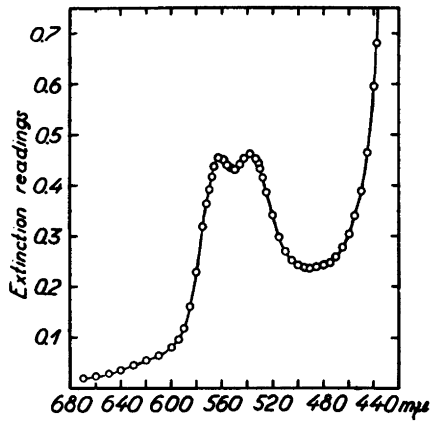

Fig. 7. Spectrum of CO-leghemoglobin, about 0.6 mg/ml, 2 Aug., 1949.

Spectrum of pyridine hemochromogen from leghemoglobin and blood hemoglobin. The curve in Fig. 9 shows the identity of the spectra of the pyridine hemochromogens prepared from both hemoglobins.

Spectrum of the green pigment. Virtanen [15] and Virtanen *et al.*[16] have found that leghemoglobin is changed to a green pigment when nitrogen fixation ceases. This pigment, legcholeglobin, easily gives biliverdin in acetic acid solution and has no marked absorption maxima between 600 and 420 mμ.

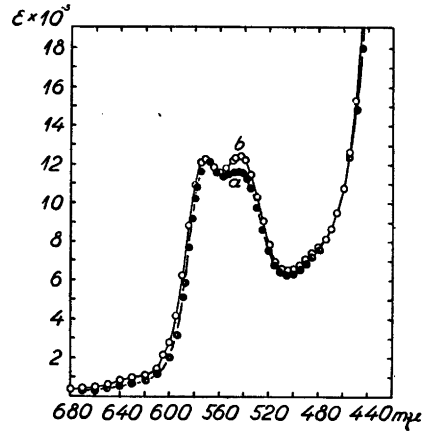

Fig. 8. Spectra of NO-leghemoglobin (a) and NO-hemoglobin of bovine blood (b), 0.65 mg/ml, 27 July, 1949 and 25 Nov., 1948.

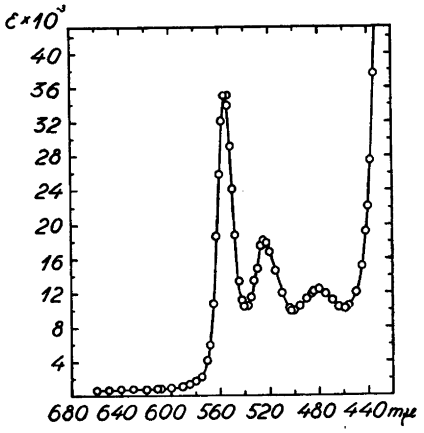

Fig. 9. Spectrum of pyridine hemochromogen from leghemoglobin (26 July, 1949) and blood hemoglobin (16 Nov., 1948). Concentration corresponds to 0.73 mg hemoglobin per ml.

Table 2.

Compound	Absorption maxima and minima of leghemoglobin and its compounds, mμ							Author
	Max.	Min.	Max.	Min.	Max.	Min.	Max.	
O_2-leghemoglobin	575		540					Kubo [5]
»	574		540					Keilin & Wang [6]
»	575		540					Little & Burris [7]
»	573	559	540	510				This paper
Leghemoglobin	555							Kubo
»	557							Keilin & Wang
»	555							Little & Burris
»	555	485						This paper
CO-leghemoglobin	570		535					Kubo
»	564		538					Keilin & Wang
»	565		539					Little & Burris
»	562	551	538	492				This paper
NO-leghemoglobin	570	557	545	504				This paper
Globin hemochrome	555		525					This paper
Pyridine leghemochrome	557		530					Kubo
» »	555	539	525	501	481	460		This paper
Leghem*i*globin, pH 7	625		563		530			Kubo
»	627							Little & Burris
» » 6.02	626	602			496	477		This paper
» » 7.10	625	610	570?		535	520		Ellfolk & Virtanen [3]
» » 8.01			570?		537	514		This paper
» » 10.06			572	560	541	508		This paper
CN-leghem*i*globin	540							Kubo
»	541							Little & Burris
» pH 6.48	540	504					417	This paper
F-leghem*i*globin	610							Kubo
»	605							Little & Burris
»	605							This paper

Table 2 summarizes the earlier determinations of the absorption maxima and minima of leghemoglobin compounds and also those determined in this work.

Equilibrium constant of acid and alkaline leghemiglobin. The pronounced change in the spectrum of hem*i*globin solutions which accompanies the pH-change is due to the amount of acid and alkaline hem*i*globin at different pH values. Spectrophotometric studies of Austin and Drabkin [8] with blood hem*i*globin have shown that at intermediate pH values (from 6.0 to 9.4) the conversion from the acid to the alkaline form closely conforms to the equation

$$\mathrm{pH} = \mathrm{p}K + \log \frac{Hi \text{ alkaline}}{Hi \text{ acid}} \qquad (1)$$

Using the equation

$$\frac{1 + 10^{pK-pH_1}}{1 + 10^{pK-pH_2}} = \frac{\Sigma |E_2 - E_0|}{\Sigma |E_1 - E_0|} \qquad (2)$$

where E_0 is the extinction value of the acid form, and E_1 and E_2 the extinction values at pH_1 and pH_2, all measured at the same wavelength, we obtain directly the weighted average of pK within the wavelength region examined (presuming that the limit of error is approximately the same at every E-value). $\Sigma|E_2-E_0|$ is the sum of all absolute E_2-E_0 values. Such E-values must be excluded, which measured at the same wavelength (near the intersection points of the curves), possibly give values of opposite signs for E_2-E_0 and E_1-E_0. Using the equation (2), the pK of leghemiglobin was calculated from the extinction values of the leghemiglobin solutions examined (99 values at 33 different wavelengths within 650—490 mμ) and the value 8.261 was obtained assuming that at pH 6.02 leghemiglobin is exclusively in acid form. This, however, is not exactly the case. According to equation (1) using the pK value 8.261 it can be calculated that alkaline leghemiglobin is present at different pH values in the following percentages: at pH 6.02 = 0.57 %, at pH 8.01 = 36.0 % and at pH 10.06 = 98.4 %.

Considering these values we obtain the corrected pK value 8.254 $\pm$ 0.04 which, in consequence, is the equilibrium constant for acid and alkaline leghemiglobin at 22° C and ionic strength of 0.100.

With a leghemiglobin preparation of lower purity (about 40 %) the pK value of 8.18 was found at an ionic strength of 0.050 and 13° C. By employing the equation introduced by Austin and Drabkin [8] and Coryell *et al.*[17] for the dependence of the pK value of blood hemiglobin on the ionic strength (μ): $\Delta\ pK = 0.59 \cdot \Delta \sqrt{\mu}$ we obtain 8.235 for the pK value of 40 % leghemiglobin at an ionic strength 0.100, *i.e.* practically the same as for 80 % leghemiglobin. The effect of temperature on the pK of hemiglobin is unknown.

For the hemiglobin of dog's blood Austin and Drabkin found pK = 8.12 $\pm$ 0.01 and for bovine hemiglobin Coryell *et al.* found pK = 8.07 $\pm$ 0.02, at 22—26° C both at the ionic strength 0.100. Theorell and Ehrenberg [14] have recently found for ferrimyoglobin (myohemiglobin) pK = 8.95 at ca 20° C and at ionic strength 0.10. The pK of leghemiglobin is much closer to the pK values of blood hemiglobin than to those of myohemiglobin. The dependence of the pK of leghemiglobin on the ionic strength is, according to our determinations, of the same order as that determined by Austin and Drabkin with blood hemiglobin and hence, much greater than that of myohemiglobin according to Theorell and Ehrenberg. It must be borne in

mind, however, that our determinations were made with leghemiglobin preparations of varying purity (80 % and 40 %) and at different temperatures.

The noticeable parallelism of the leghemoglobin content of root nodules with their nitrogen-fixing ability is apt to draw special attention to this pigment. Our knowledge of the structure of leghemoglobin has substantially increased of late. The protein component of leghemoglobin is very different from that of the hemoglobin of blood, as revealed by the great difference in the amino acid composition and isoelectric points. On the contrary, there is a great similarity between the absorption spectra. Pronounced differences have, however, been noted in the spectra of blood hemiglobin and leghemiglobin. Not only the absorption maxima but also the positions of the intersection points of the absorption curves where the absorption is independent of pH are partly different (Table 1).

The pK values of leghemiglobin and blood hemiglobin do not seem to differ much from each other. Thus leghemiglobin would noticeably differ from myohemiglobin. The linkages between iron and protein can be presumed to be different, though nothing definite can be said in this connection on the basis of our results. On the other hand, it is questionable whether the differences noted in the spectra of leghemiglobin and blood hemiglobin are entirely due to the different structure and molecular weight of the corresponding proteins or whether even then the different linkage of iron is in question. The identical spectra of pyridine hemochromogens of leghemoglobin and blood hemoglobin confirm the finding of Kubo that the hemins of leghemoglobin and blood hemoglobin are identical.

Another leghemoglobin compound whose spectrum differs considerably from the spectrum of the corresponding blood hemoglobin compound is carboxyleghemoglobin. The maxima of CO-leghemoglobin are at 562 and 538 mμ, the corresponding maxima of CO-hemoglobin of blood being, according to our estimations, at 569 and 539 mμ. The maxima of O_2-leghemoglobin and blood (bovine) O_2-hemoglobin are at 574, 540 mμ, and at 576, 540 mμ respectively. The distance of the α-maxima of O_2- and CO-leghemoglobin in Å (Span) is 110, and for blood (bovine) hemoglobin it is 80. Keilin and Wang [6] have found the value of 100 for the Span of a leghemoglobin preparation of about 40 % purity.

SUMMARY

Determinations of absorption spectra were made with purified leghemoglobin preparations, which, with the exception of the O_2-compound, contained about 20 % foreign protein. Particular attention was given to the absorption spectra of leghemiglobin at different pH values. The absorption curve of

leghem*i*globin differs noticeably from the corresponding absorption curves for blood hem*i*globin and myohem*i*globin. The pK value of acid and alkaline leghem*i*globin lies near the corresponding pK value for blood hem*i*globin but differs considerably from that for myohem*i*globin. The ionic strength, too, has a distinctly different effect on the pK values of leghem*i*globin and myohem*i*globin.

Since the amino acid composition of leghemoglobin is essentially different from that of blood hemoglobin and myohemoglobin the structural differences of globin may cause some differences in the absorption spectra. A different linkage of globin to the hemin group is also possible as suggested by the considerable differences noted in the pK values of leghem*i*globin and myohem*i*globin. Whether both or only one of these factors is concerned cannot be decided on the basis of our experiments.

Addition concerning the nomenclature. In this paper as well as in the previous ones from this laboratory the nomenclature suggested by Lemberg for hemoglobin and its derivatives has been used because of its brevity. However, differentiation of *o* and *i* by italics is very unwieldy and unclear. The nomenclature introduced by Pauling and Coryell, from which the valency of iron distinctly appears, is perhaps preferable (*e.g.* methemoglobin (Keilin), hem*i*globin (Lemberg), ferrihemoglobin (Pauling)).

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REFERENCES

1. Virtanen, A. I., Jorma, J., and Laine, T. *Suomen Kemistilehti* **B, 18** (1945) 49.
2. Virtanen, A. I., Jorma, J., Linkola, H., and Linnasalmi, A. *Acta Chem. Scand.* **1** (1947) 90.
3. Ellfolk, N., and Virtanen, A. I. *Acta Chem. Scand.* **4** (1950) 1014.
4. Ellfolk, N., and Virtanen, A. I. *Acta Chem. Scand.* **6** (1952) 411.
5. Kubo, H. *Acta Phytochim.* **11** (1939) 195.
6. Keilin, D., and Wang, Y. L. *Nature* **155** (1945) 227.
7. Little, H. N., and Burris, R. H. *J. Am. Chem. Soc.* **69** (1947) 838.
8. Austin, J. H., and Drabkin, D. L. *J. Biol. Chem.* **112** (1935) 67.
9. Hari, P. *Biochem. J.* **103** (1920) 271.
10. Haurowitz, F. *Z. physiol. Chem.* **138** (1924) 68.
11. Hicks, C. S., and Holden, H. F. *Australian J. Exp. Biol. Med. Sci.* **6** (1929) 175.
12. Horecker, B. L. *J. Biol. Chem.* **148** (1943) 173.
13. Kubowitz, F. *Z. ges. inn. Med.* **3** (1948) 501.
14. Theorell, H., and Ehrenberg, A. *Acta Chem. Scand.* **5** (1951) 823.
15. Virtanen, A. I. *Nature* **155** (1945) 747.
16. Virtanen, A. I., Laine, T., and Linkola, H. *Suomen Kemistilehti* **B, 18** (1945) 36.
17. Coryell, C. D., Stitt, F., and Pauling, L. *J. Am. Chem. Soc.* **59** (1937) 633.

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