creased the initial rate of formazan production. In the cases of isocitric and malic dehydrogenases, triphosphopyridine nucleotide[10] was required in addition to a purine. It was not possible to demonstrate tetrazolium reduction with the following substrates, in the presence or absence of cofactor: glycine, serine, threonine, methionine, aspartic acid, spermine, histamine and 1,4-butanediamine.

The activating effects of inosine with various substrates

Substrate	Activity O.D. 520/10 min	
	No Inosine	Plus Inosine
Tyramine	0·000	0·920
Isoamylamine	0·000	0·860
Isocitrate	0·000	1·030
L-Malate	0·000	0·200
Benzylamine	0·175	0·830
Tryptamine	0·210	0·900
Choline	0·060	0·400
Succinate	0·150	0·790

In order to determine the specifity of the cofactor effect AMP, purine, hypoxanthine, adenine and uric acid were tested with several of the substrates. It was found that with succinate the first three compounds act in the same way as inosine (Table) and that adenine and uric acid are inactive, as they are with amines. It was also desirable to learn whether the cofactor which functions in tetrazolium reduction is also involved in the reduction of other dyes. Thus far it has not been possible to demonstrate this for methylene blue, 2,6-dichlorophenolindophenol or ferricyanide, although hypoxanthine is required in the specific case of sulphite/methylene blue[1].

Discussion.—Tetrazolium dyes are being used increasingly in enzymological and histochemical investigations, but in spite of this the kinetics and mechanism of their reduction are incompletely understood. BRODIE and GOTS[12] have adduced evidence that tetrazolium salts accept protons from flavoproteins, but the present data indicate that the transfer system is probably more complex than this. The existence of a cofactor for tetrazolium reduction has been suspected previously. SPRINZ and WALDSCHMIDT-LEITZ[13] detected requirement of a cofactor for succinic dehydrogenase when measured with tetrazolium. Diphosphopyridine nucleotide satisfied the requirement but, according to the authors, is probably not the natural factor. Others[14] have made similar findings. The present results demonstrate, first of all, that certain purines, nucleosides and nucleotides, respectively, participate in the enzymatic reduction of tetrazolium salts; and, secondly, that this effect is specific for tetrazolium salts among the electron acceptors tested, i.e. it does not apply to the same enzyme preparations catalyzing the reduction of ferricyanide, methylene blue, 2,6-dichlorophenol indophenol or oxygen. The nature and function of the cofactor is still somewhat speculative, but two possibilities can be envisaged: (1) The cofactor serves in proton transfer, serving at the pyocyanine or tetrazolium level. Because the molar ratio, monoformazan/purine, ranges between 0·5 and 1·5 it is evident that the cofactor does not behave like the usual catalytic agents. It is conceivable that immediately after transferring protons to the pyocyanine or tetrazolium the cofactor differs somewhat from the original compound, and it is unable to serve in the enzyme system, but that it is slowly reconverted to the original structure[1]. This would account for molar ratios greater than unity, in that a portion of the cofactor would move through the entire cycle quickly enough to be utilized in the dehydrogenation more than once. (2) An alternative hypothesis is that the cofactor accepts the dehydrogenated substrate residue, resulting in a 'bound', inactive form of the cofactor. However, it is difficult to reconcile such a function with the variety of substrates and of purified compounds which are effective as cofactors.

From a comparison of the chemical structure of active substances it would appear that the 6 and 8 positions on the purine are of special importance. Thus, adenine and certain other 6-substituted analogues are inactive, as is azaguanine. The activity of adenosine and AMP may be explicable by their prior deamination, enzymes for which are found in the rat liver, although adenase is absent.

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T. L. SOURKES and J. R. LAGNADO

Allan Memorial Institute of Psychiatry, McGill University, Montreal (Canada), September 2, 1957.

Zusammenfassung

Nach der vorliegenden Untersuchung können verschiedene Purine und einige ihnen nahestehende Verbindungen an der enzymatischen Reduktion der Tetrazole als Zwischenprodukte teilnehmen.

[10] This compound, unlike DPN, cannot serve as cofactor in the test system containing amine as substrate.

[11] E. SHELTON, in *Proceedings of the Histochemical Society*, J. Histochem. Cytochem. *4*, 427 (1956).

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Oxygen Equilibrium of Human Myoglobins (Mb I and Mb II)[1]

In previous research, we demonstrated that at least three components (Mb I, Mb II, Mb III) are present in the human muscle and in preparations of crystallized myoglobin (Mb). These components differ from one another in their electrophoretic behaviour[2].

In this note, we give the results of experiments aimed at studying the oxygen equilibrium of Mb I and Mb II. It has not been possible to make experiments with Mb III, because of the small quantities in which it is present in Mb solutions.

We have found these studies interesting to investigate the possible physiological meaning of these pigments,

[1] Aided by a grant from the Rockefeller Foundation.

[2] A. ROSSI-FANELLI and E. ANTONINI, Arch. Biochem. Biophys. *65*, 578 (1956).

and to establish, as seemed to be necessary in the course of a systematic research on the O_2 equilibrium of human Mb[3], whether the dissociation curves of non-homogeneous crystallized Mb were different, and in which way, from those obtained with homogeneous preparations.

Preparation of crystallized human Mb.—Mb has been crystallized according to ROSSI-FANELLI[4]. The crystals were dissolved in H_2O and the solution obtained was dialyzed until the ammonium sulphate disappeared, and if necessary, the solution concentrated by freeze-drying.

Separation of the Mb components.—Components I and II were separated through preparative zone electrophoresis, using starch-gel supporting medium. The method used for the preparation of gel and for the elution of substances was basically that described by SMITHIES[5].

Starch gels 23 × 12 × 1 cm in size, in borate buffer pH 8·6, I 0·05 were used. The separation was obtained in 8 to 10 h, with a voltage gradient of 6–8 V/cm. It is possible to separate in each electrophoresis from 1 to 2 ml of a solution of Mb 1 – 3%. As can be seen by the experiment shown on Figure 1 (made in accordance with a technique described in a previous work[2]), the separated components are electrophoretically homogeneous.

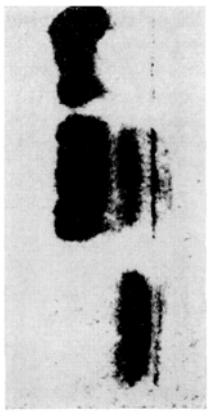

Fig. 1.—Paper electrophoresis of homogeneous Mb I (1), Mb II (3) and unhomogeneous crystallized myoglobin (2).

Determination of the O_2 dissociation curves.—The borate buffer solutions of the components I and II, obtained by elution from starch-gel, are brought from pH 8·6 to pH 8 with diluted acetic acid. Mb I is obtained in concentrations of 0·5–1%, and Mb II in 5 or 6 times weaker concentrations. The isolated components were reduced through the enzymatic system previously described[6]. The dissociation curves were determined by means of a spectrophotometric method which we have developed and which is particularly suited in the case of small quantities of pigment and of diluted solutions[3].

Since Mb II can only be obtained in diluted solutions ($0{\cdot}5 \times 10^{-4}$ *M*), we made a series of preliminary tests in order to ascertain the influence of the concentration of Mb on the O_2 dissociation curve. These experiments, which were made either on non-homogeneous Mb or on Mb I, showed that the Mb concentration has no appreciable influence on the O_2 dissociation curve from $0{\cdot}2$ to $4{\cdot}5 \times 10^{-4}$ *M*. The dissociation curves of Mb I have therefore been determined using the same concentration of Mb as used in the case of Mb II.

The spectrophotometric readings were taken, with a light path of 2 mm, in the visible region (λ = 510, 540, 560, 580) and mainly for the diluted solutions, in the Soret zone (λ = 415, 430).

No difference was found between the oxygenation percentage, either when the readings were made in the visible region or in the Soret zone; and no appreciable difference between Mb I and Mb II in the extinction and maxima of the peaks of the oxygenated and deoxygenated derivatives.

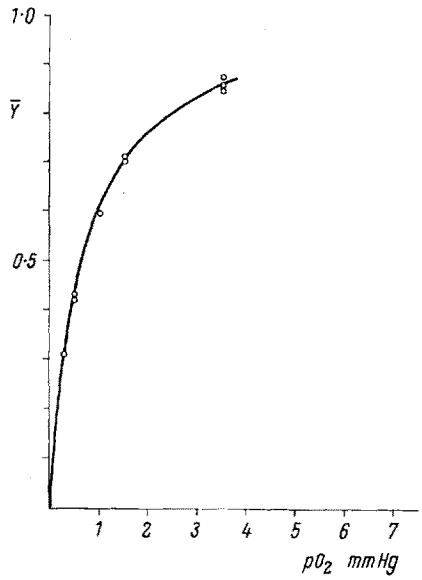

Fig. 2.—Oxygen dissociation curve of human Mb I (5×10^{-5} *M*) at 20° and pH 8; p ½ = 0·66 mm Hg; $Y = MbO_2/Mb + MbO_2$.

Shape of the O_2 dissociation curve.—As shown in Figure 2 and 3, Mb I as well as Mb II have hyperbolic dissociation curves which follow the Hill equation:

$$MbO_2/Mb = K\,(p\,O_2)n \quad \text{with } n = 1.*$$

This result is very interesting, particularly as far as the minor component is concerned, because this property shows that it is a true myoglobin.

Oxygen affinity.—The experiments reported in Figure 2 and 3 show that at 20° and at pH 8 the two Mb have a very similar O_2 affinity. Actually, many dissociation curves for Mb II have given p ½ values slightly lower than those of Mb I and non-homogeneous Mb. These differences, however, are not far from the limit of the experimental error of the method.

Influence of temperature on oxygen affinity.—The influence of temperature on the O_2 dissociation curves of Mb I and Mb II was studied between 20°C and 40°C.

The Table and Figure 3 give the variation of the log. K and p ½, with temperature, both for Mb I and Mb II.

It can be seen that the O_2 affinity at different temperatures is similar for both Mbs.

[3] A. ROSSI-FANELLI and E. ANTONINI (in press).

[4] A. ROSSI-FANELLI, *Haemoglobin* (Butterworth's Sci. Publ., London 1949), p. 115.

[5] O. SMITHIES, Biochem. J. *61*, 629 (1955).

[6] A. ROSSI-FANELLI, E. ANTONINI, and B. MONDOVÌ, Arch. Biochem. Biophys. *68*, 341 (1947).

* MbO_2 and Mb denote concentrations.

The overall heat of reaction calculated by the Van't-Hoff equation between 20° and 40° was found to be: for Mb I $\Delta H = -13{\cdot}500$ cal., and for Mb II $\Delta H = -13{\cdot}300$ cal.

The results we have obtained show that the dissociation curves for Mb I and Mb II are very similar. The figures given for the minor component (Mb II) are highly interesting. This pigment shows all the properties

t °C	log K		p ½ (mm Hg)	
	Mb I	Mb II	Mb I	Mb II
20	+ 0·18	+ 0·19	0·66	0·65
25	+ 0·03	+ 0·04	0·93	0·91
30	− 0·13	− 0·11	1·35	1·29
35	− 0·28	− 0·23	1·91	1·70
40	− 0·47	− 0·42	2·95	2·63

Values of log K and p ½ for human Mb I and Mb II at different temperatures. $K = MbO_2/Mb \cdot pO_2$; p ½ = oxygen pressure (at 20° C) for 50% saturation.

of a myoglobin: reversible combination with oxygen, shape of the dissociation curve, and affinity for O_2, so that we must definitely give up the thought that we are dealing with an impurity or an artefact.

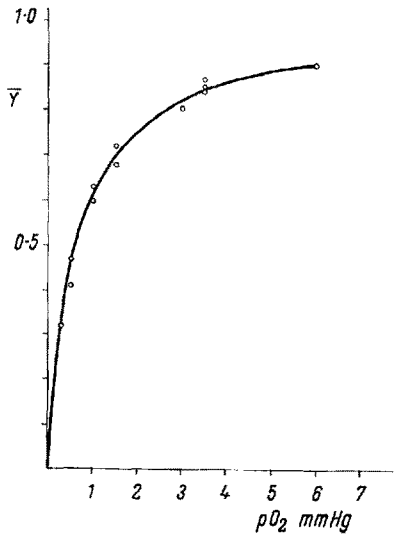

Fig. 3. – Oxygen dissociation curve of human Mb II (4×10^{-5} M) at 20° and pH 8; p ½ = 0·65 mm Hg; $\bar{Y} = MbO_2/Mb + MbO_2$.

As far as the two Mb are concerned, and we have demonstrated their presence also in the muscle extract, we can state that they are perfectly equivalent in their most typical physiological property: that is, the reversible combination with O_2. We must reject the hypothesis that Mb II is a residue of foetal Mb; experiments which we have carried out demonstrate that foetal Mb cannot be separated in electrophoresis from adult Mb, and that the preparations of foetal Mb show the presence of several components which have electrophoretic characteristics similar to those found in adult Mb.

The above results show that studies (also with spectrophotometric methods) on the equilibrium between human Mb and O_2 can be rightly based on non-homogeneous crystallized pigment particularly because Mb I and Mb II have identical absorption spectra.

Research on the structure of the pigment must, on the other hand, be based on homogeneous preparations. Results of the preliminary research which we have

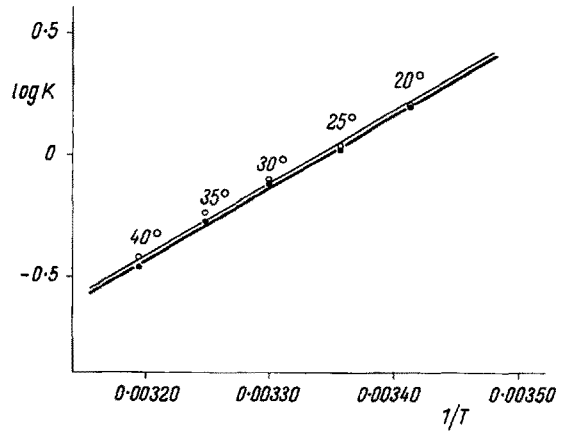

Fig. 4. – Influence of temperature on oxygen affinity of human Mb I = ● and Mb II = ○; pH 8, borate buffer I 0·05; $K = MbO_2/Mb \cdot pO_2$.

carried out lead us to think that the two Mb have different chemical natures, as seems to be the case with horse-Mb[7].

Research along this line is in progress in our Institute.

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Institute of Biological Chemistry, University of Rome (Italy), August 5, 1957.

Riassunto

Dalla mioglobina umana cristallizzata sono stati isolati, a mezzo dell'elettroforesi su gel di amido, due componenti puri ed omogenei: Mb I e Mb II. Dei due componenti sono state determinate, in varie condizioni sperimentali, le curve di dissociazione per l'ossigeno.

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Über die Alkaloide von *Rauwolfia ligustrina* R. & S. Raugustin, ein neues reserpinähnliches Alkaloid*

Von den amerikanischen Rauwolfia-Arten sind bis heute ausser *R. sellowii* Muell.-Arg.[2], *R. grandiflora* Mart.[3] und *R. schueli* Speg.[4] hauptsächlich die nach der neuen Klassifikation von Rao[5] unter der Bezeichnung

* 30. Mitteilung über Rauwolfia-Alkaloide[1].

[1] 29. Mitteilung siehe C. F. Huebner und E. Schlittler, J. Amer. chem. Soc. *79*, 250 (1957).

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