ohne weiteres auf den Bohr-Effekt zurückzuführen sei. Erstens tritt eine auffällige Formänderung der Kurve auf, die beim einfachen Bohr-Effekt (auch in stark verdünnten Lösungen) fehlt. Zweitens ist der Histidin-Effekt, wie aus der Abbildung hervorgeht, am stärksten bei den niedrigen Konzentrationen, wo die pH-Verschiebung relativ gering sein muss. Da die Beziehung zwischen pH und t_{O_2} (50%) zwischen pH 7,50 und 6,50 bei menschlichem Hämoglobin linear ist (auch für verdünnte Hb-Lösungen!), würde man für den Einfluss des Histidins auf die Lage der Dissoziationskurve eine andere Beziehung erwarten, als in KLYNSTRAS Kurve in Erscheinung tritt. Selbstverständlich werden neue Experimente mit menschlichem Hämoglobin hier entscheiden müssen. KLYNSTRAS bisherige Versuche lassen weitgehende theoretische Schlussfolgerungen nicht zu.

H. P. WOLVEKAMP

Zoologisches Institut, Leiden, 3. April 1959.

Da der Hauptanlass zum vorausgehenden *Disputandum* die detaillierten theoretischen Überlegungen von F. KLYNSTRA waren und diese in ihren weitgehenden Schlussfolgerungen auch von Herrn Prof. WOLVEKAMP zurückgenommen wurden, können wir jetzt Schluss der Diskussion erklären. *M*

A Method for Purifying Rat Myoglobin for Kinetic Studies[1]

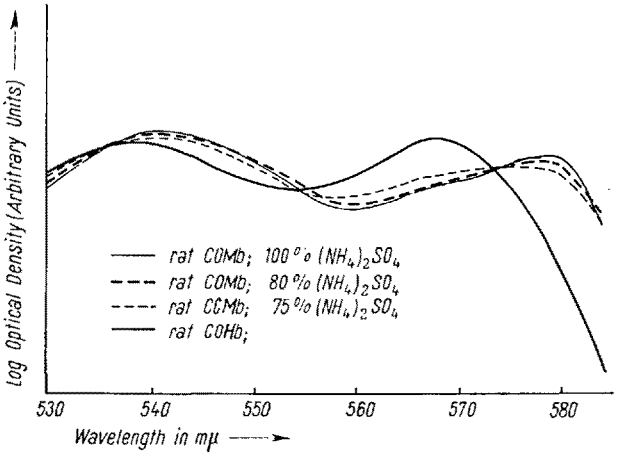
Recent investigations dealing with the functional role of myoglobin (Mb) during altitude acclimatization of rats necessitated the preparation of freshly purified Mb for use in kinetic studies[2]. While the chemistry of Mb obtained from other mammalian species has been adequately investigated[3,4], very little information is available on the purification of rat Mb[5].

The present report describes a relatively simple method for obtaining pure rat Mb which can be used for kinetic measurements. It is based on a procedure originally described by THEORELL[6] for purifying horse Mb.

The steps in the purification are as follows:

(1) Muscle obtained from rats is crushed while frozen in dry ice and the Mb is extracted with an equal volume of distilled water[7]. The extract is adjusted to pH 7·0 with 0·1 *M* NaOH.

(2) Extraneous proteins are then precipitated by the dropwise addition of saturated basic lead acetate (1/4 volume of solution to 1 volume of extract). After discarding the precipitate the excess lead ions are precipitated from the extract by the addition of a slight excess of Na_2HPO_4 and NaH_2PO_4. During precipitation, the pH is carefully maintained between 7 and 8. After centrifugation, the precipitate is discarded; the supernatant is readjusted to pH 7·0 and bubbled with carbon monoxide.



Comparison of the absorption spectra of rat COMb extracts (after dialysis against different saturations of ammonium sulfate solutions) with that of rat COHb. All solutions were in *M*/15 phosphate buffer, pH 7·8.

(3) Having converted the Mb to carbonylmyoglobin (COMb)[8], the extract is dialyzed against 60% saturated $(NH_4)_2SO_4$, pH 7·0, for 18 h. Next the resulting precipitate is centrifuged down; the supernatant is reconverted to COMb and dialyzed against 80% saturated $(NH_4)_2SO_4$, pH 7·0, for 12 h[9]. The light red precipitate contains all spectroscopically detectable hemoglobin, some Mb, and other proteins. The precipitate is removed by centrifugation at 14000 *g*.

(4) The supernatant is converted to COMb and dialyzed against *M*/15 K_2HPO_4. After 12 h of dialysis the purified extract contains O_2Mb with a minimal amount of metmyoglobin.

On a number of occasions attempts were made to form O_2Mb by oxygenation of either reduced Mb or COMb. The oxygenation of reduced Mb was unsatisfactory since it consistently resulted in the formation of much metmyoglobin. In contrast, the oxygenation of COMb sometimes yielded pure O_2Mb, though generally some metmyoglobin was also present. The latter method was not used since it was more time consuming than the dialysis method and usually gave only slightly less metmyoglobin contamination.

The unstable nature of Mb necessitates that it be purified as quickly as possible and that the purified solutions be used for kinetic measurements within a few hours after preparation. After storing Mb at 5°C for a few days, metmyoglobin could not be converted to O_2Mb.

The purification of Mb for kinetic experiments necessitates the removal of any protein impurity which might influence the measurement of the reaction rates. Hemoglobin and cytochrome *c* are probably the only protein impurities that would interfere with measurements of O_2Mb kinetics. The absence of a band at 550 mμ (Figure) shows that cytochrome *c* was not present in the purified solutions.

[1] This research was aided by the U.S. Air Force School of Aviation Medicine, Randolf AFB, under contract AF 18(600)-940 and the National Institutes of Health under grant RG-5112(RI). This is paper No. 2342 in the Pennsylvania Agricultural Experiment Station.

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[4] A. ROSSI-FANELLI and E. ANTONINI, Exper. *13*, 477 (1957).

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[6] A. H. T. THEORELL, Biochem. Z. *252*, 1 (1932).

[7] *Mb* must be kept around 0–10°C throughout the purification. On several occasions when *Mb* solutions warmed to 25°C for several hours, changes were detected in the 530–585 mμ region of the COMb spectrum.

[8] During purification Mb should be kept as COMb, which seems to be less susceptible to denaturation than O_2Mb. In order to form COMb, a pinch of $Na_2S_2O_4$ is added to the solution after it has been saturated with CO.

[9] The 80% saturated $(NH_4)_2SO_4$ should have a specific gravity of at least 1·205 at 25°C to assure removal of hemoglobin.

The absence of spectrally detectable hemoglobin after the 80% saturated $(NH_4)_2SO_4$ dialysis was established in the following way. In the region 530 mμ to 585 mμ, carbonyl hemoglobin (COHb) and COMb have different absorption spectra so that a change in the percentage of COHb in the solution results in changes in the absorption spectrum of the mixture. If the log optical density (OD) is plotted against wavelength in the 530–585 mμ region, the shape of the curve will depend only on the percentages of each absorbing component present. From the Figure it can be seen that after the 80% saturated $(NH_4)_2SO_4$ dialysis the percentages of COHb and COMb remained unchanged upon dialysis against more saturated $(NH_4)_2SO_4$ solutions. Since hemoglobin starts to precipitate at a lower ionic strength than Mb, it is probable that the curves obtained from the supernatant after the 80% and 100% saturated dialysis were identical because all spectroscopically detectable hemoglobin was removed.

Although the above procedure yielded purified Mb extracts which proved suitable for kinetic experiments, several additional attempts were made to carry the purification one step further by crystallizing Mb. The purified Mb solution was fractionated against more concentrated $(NH_4)_2SO_4$. After several days the resulting precipitate contained particles which resembled the globuliform crystals described by ROSSI-FANELLI *et al.*[10]; however, no macroscopic crystals were obtained on continuing the dialysis for six weeks.

Following the failure to obtain crystalline Mb by $(NH_4)_2SO_4$ fractionation, the samples obtained after step 3 in the above procedure were chromatographed on Amberlite IRC 50. Some non-chromoprotein contaminants were separated by this ion exchange resin. The results of this exploratory investigation are supported by similar findings for Mb from other animal species[11] and suggest that ion exchange chromatography may be used for further purification. An additional method of Mb purification which merits consideration is continuous paper electrophoresis, which was recently used in purifying fish Mb[12]. However, it is our belief that the dialysis technique described in the present report is simpler and more likely to yield larger amounts of Mb which can be used in spectroscopic measurements of kinetic reactions.

E. H. STRICKLAND, A. ANTHONY, and E. ACKERMAN

Biophysics Laboratories, Pennsylvania State University, University Park (Pennsylvania), February 26, 1959.

Résumé

Une technique est décrite pour préparer de la myoglobine pure par fractionnement avec sulfate d'ammonium.

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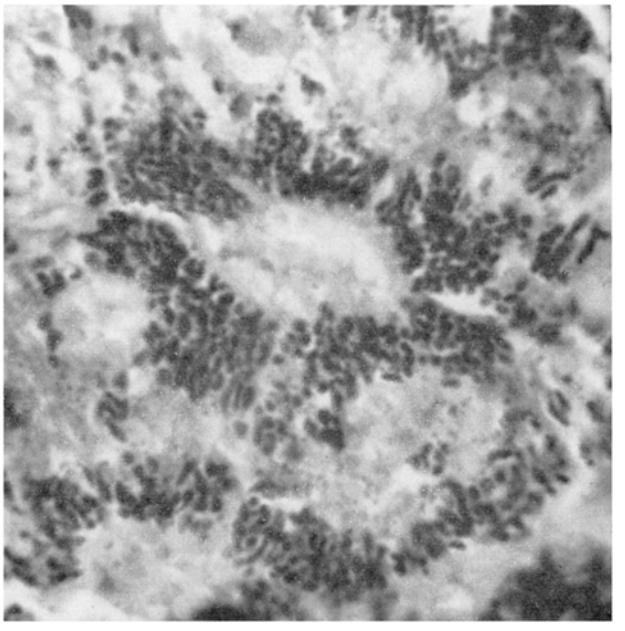
PRO EXPERIMENTIS

Rapid Staining of Mitochondria

Several methods have, up till now, been suggested for the demonstration of mitochondria in permanent mount tissues; but most of them, requiring special fixatives, mordants and lenght of time, are not suitable for routine laboratory work.



Mitochondria in hepatic cells of Guinea pig (× 1500).

I have obtained excellent results with the following rapid and simple method, and I advice it not only for routine but also for special histological study (pathological changes as cloude swelling, and so on).

Fixation: fix exclusively in 10% formalin. Embed in paraffin.

Method of staining

(1) After removal of the paraffin in the usual way, rinse in water and place the sections into a warm normal solution of hydrochloric acid (about 60°C) for 3 min.
(2) Rinse in water.
(3) Place the sections in fuchsin acid aqueous solution 1% for 30 sec.
(4) Rinse in water.
(5) Place the sections in light green aqueous solution 1% for 1 to 3 min.
(6) Rinse in water.
(7) Dehydrate in 95% and absolute alcohol.
(8) Clear in xylol and mount in balsam.

Mitochondria stand out sharply, stained purple-red with peripheral wall green, chromatin and collagen green, muscle tissue purplish, erythocites brilliant red.

A. NOVELLI

Department of General Pathology of the University of Genova (Italy), March 6, 1959.

Riassunto

Viene descritta una nuova semplice tecnica per la colorazione rapida dei mitocondri nelle sezioni di tessuti: i pezzi devono essere fissati in formalina, le fettine, dopo sparaffinamento, vengono immerse in una soluzione calda (60°C) di acido cloridrico normale per 3 min e poi colorate con fucsina acida (1%) per 30 sec e con verde luce (1%) per 1–3 min. I mitocondri risaltano intensamente colorati in rosso con una sottile parete colorata in verde.