Kinetics of pH Change Accompanying CO Binding—The control experiments just described gave reasonable confidence in the possibility of following the kinetics of the pH change accompanying CO binding in an unbuffered solution of hemoglobin by making spectrophotometric measurements at 563 m$\mu$ in the presence of phenol red. As mentioned above, by following the reaction at 482 m$\mu$ and 563 m$\mu$, it is possible to isolate the optical density changes due, respectively, to the formation of CO hemoglobin and to the change in the degree of ionization of the dye. A typical experiment at pH$_{40}$ 7.15, reported in Fig. 1, shows that the rate of the optical density changes at the two wave lengths is the same, within experimental error, for the whole range of CO concentrations employed ($5 \times 10^{-4} M$ to $3.12 \times 10^{-4} M$). In Fig. 2, the optical density changes at 482 m$\mu$ are plotted against the optical density changes at 563 m$\mu$ over the same time intervals; this figure, from which time is eliminated as a parameter, brings out more specifically the relation between the processes measured at the two wave lengths. It is evident from this figure that the degree of ionization of the dye changes linearly with the amount of CO-hemoglobin formed. It may be noted that, for pH changes as small as those measured in these experiments, the change in pH may be assumed to be proportional to the degree of ionization of the indicator within the accuracy of the measurements. The total pH change calculated from the optical density change at 563 m$\mu$ and the final pH of the solution agrees well with the pH change measured statically in differential potentiometric titration going from deoxy- to CO-hemoglobin (2); under the conditions used the dye does not contribute significantly to the buffer power of the system.

A series of similar experiments was made over the pH range from less than 7 to 7.8, where the alkaline Bohr effect is operative. In some experiments the initial pH of the solutions was different from that of the experiment reported above, the other conditions remaining the same. In another experiment the temperature was changed from 11$^\circ$ to 29$^\circ$, with the effect of changing the rate of the CO combination and also the total observed pH change. This latter effect appears to agree with the change in the magnitude of the Bohr effect with temperature (8, 9). In one experiment the hemoglobin was dissolved in water and the reaction was allowed to take place at very low ionic strength ($10^{-4}$ to $10^{-6}$).

The results of all these experiments are essentially similar, within the accuracy of the measurements, to those described in more detail in Figs. 1 and 2. In most of them the total change in pH calculated from the optical density change at 563 m$\mu$ agrees within 10% with values determined statically under similar conditions.

The results just reported show that the procedure adopted is adequate to measure rapid pH changes associated with hemoglobin reactions. The sensitivity and accuracy of these kinetic pH determinations compare well with those of the static potentiometric measurements (2); thus, a wide range of applications of the method to the study of reactions of hemoglobin and other proteins may be foreseen.

It is worthwhile to discuss briefly the significance of the results obtained here on the kinetics of the Bohr effect. The time course of the pH change is the same as that of the uptake of CO over the whole range of CO concentrations examined; i.e. it is independent of the absolute velocity of this reaction. This result substantiates and gives an additional meaning to the linearity between the two processes observed in equilibrium measurements. It is to be noted that although kinetic proportionality implies the static proportionality as observed in equilibrium measurements (2), the converse is not true. If the Bohr effect reflects the conformational change associated with the reaction of the ligand with hemoglobin (3, 4), it would be expected that when the rate of CO uptake approaches that of the conformational transition, this would show up as a rate-limiting step in the kinetics of the proton liberation. Thus, as the rates of the two processes become the same, the pH change should follow the CO combination with a measurable delay. The results reported here clearly show that the speed of the conformational change cannot be rate-limiting within the range covered. Since the highest pseudo-first order rate constant for the reaction between CO and hemoglobin was about 80 sec$^{-1}$, it may be concluded that in hemoglobin the rate of the conformational transition leading to the liberation of the Bohr protons must be several times greater than this value.

REFERENCES

Some Properties of Purified Valyl and Lysyl Ribonucleic Acid Synthetases from Yeast
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The valyl and lysyl ribonucleic acid synthetases from yeast have been purified to apparent homogeneity as judged by sedimentation analyses and starch gel electrophoreses. The purification procedure involved ammonium sulfate fractionation and chromatography on DEAE-cellulose, Amberlite XE-64, and hydroxylapatite, and gave for the valine enzyme an increase of specific activity from 9 in the crude extract to 12,000 in the most purified fraction. The corresponding figures for the lysine enzyme were 140 and 63,000, respectively. One unit of enzyme activity was defined as the amount of enzyme that would catalyze the formation of 1 mmole of aminoacyl RNA in 30 min at 37$^\circ$ under standard conditions. Specific activity was defined as activity per mg of protein.

Molecular weights of 116,000 and 113,000 were calculated for the valyl- and lysyl-RNA synthetases from approach to equilibrium runs assuming a partial specific volume of 0.75. The
ratios of absorbance at 280 nm to that at 290 nm for the two enzymes were 1.70 to 1.75. When valyl-RNA synthetase was incubated with ATP, Mg++, and valine, a valyladenosine monophosphate-enzyme complex was formed that could be isolated by chromatography on Sephadex G-25 or Amberlite XE-84. In an experiment where valine-\(^{14}C\) and ATP-\(^3H\) were used, the ratio of valine to AMP in the isolated valyl-AMP-enzyme complex was 0.95 (Fig. 1).

The isolated valyl-AMP-enzyme complex could transfer its valine to tRNA\(^\text{f}\) from yeast or Escherichia coli. This transfer reaction is reversible as shown by the formation of valyl-AMP-enzyme when valyl-RNA (from yeast) was incubated with enzyme and AMP. There was no formation of valyl-AMP-enzyme in the absence of AMP. The transfer reaction proceeded in both directions without the addition of Mg++. The velocity of the reaction, measured in the forward direction, was much lower with E. coli RNA than with yeast RNA (1). The results of some typical experiments are summarized in Table 1.

The result of Experiment 1 in Table 1 shows that under the conditions used we did not obtain a quantitative yield of valyl-AMP-enzyme from the enzyme. The reason for this discrepancy is presently being investigated.

The valyl-AMP-enzyme isolated from Sephadex was contaminated by small amounts of inorganic pyrophosphate present in the phosphate buffer eluent. When valyl-AMP-enzyme labeled with \(^{14}C\) and \(^3H\) in the valine and AMP moieties, respectively, was incubated at 37° with Mg++, 0.5 mmole of valyl-AMP-enzyme per ml in 0.6 M potassium phosphate buffer, \(pH\) 6.5, and \(10^{-4}\) M Mg++, there was a rapid breakdown of valyl-AMP-enzyme with the formation of an equivalent amount of ATP and valine according to the reverse of Equation 1 below.

Under these conditions the breakdown was complete in less than 1 min. The ATP formed was identified by its chromatographic behavior on paper and on Dowex 2.

In the absence of Mg++, the complex is fairly stable. There was less than 3% breakdown after 3 hours in 0.6 M potassium phosphate buffer, \(pH\) 6.5, at 0°, and no detectable breakdown after incubation at 37° for 8 min.

The results of this investigation are summarized in Equations 1 and 2.

Valine + ATP + enzyme + Mg++ \(\rightarrow\) valyl-AMP-enzyme + PP\(_i\); (1)

Valyl-AMP-enzyme + RNA \(\rightarrow\) valyl-RNA + AMP + enzyme (2)

The formation and properties of a corresponding enzyme-substrate complex are presently being studied with the lysine enzyme.

Norris and Berg (2), with a highly purified isoleucyl-RNA synthetase from E. coli, and Allende et al. (3), who worked with a partially purified threonyl-RNA synthetase from the same source, have recently reported on the isolation of isoleucyl- and threonyl-AMP-enzyme complexes. Their general results are in good agreement with our own although direct evidence for the reversibility of Equation 2 has so far not been reported for the E. coli enzymes. It is interesting to note the close agreement between the molecular weight of 112,500 reported by Norris and Berg for the isoleucyl-RNA synthetase and the molecular weights of the yeast enzymes given above.

REFERENCES

\(^1\) The following abbreviation is used: tRNA, transfer ribonucleic acid.
Some Properties of Purified Valyl and Lysyl Ribonucleic Acid Synthetases from Yeast
Ulf Lagerkvist and Johan Waldenström