Chemical Studies with Purified Metmyoglobin

Preparation of Purified Metmyoglobin In order to study the reactions of myoglobin derivatives with ascorbic acid and sodium nitrite, it was necessary to isolate and purify myoglobin or metmyoglobin. Beef muscle which had been frozen with dry ice and ground to a powder with a hammer mill was used as the source of myoglobin. The method of isolation was that of Theorell (*11*), using Morgan's (*9*) and certain other modifications. The early steps in the procedure are the same as those used in the determination of myoglobin.

After removal of the hemoglobin and other proteins precipitated by the 3M phosphate, the supernatant was dialyzed against water to remove the phosphate, which was found to interfere with further purification. The supernatant was dialyzed twice against cold, neutral saturated ammonium sulfate and once against cold, neutral 80% saturated ammonium sulfate. The use of the saturated ammonium sulfate at this point reduced the yield of purified metmyoglobin but was effective in precipitating large quantities of extraneous proteins. The metmyoglobin was selectively dissolved in the 80% ammonium sulfate and was then precipitated by dialysis against cold, neutral saturated ammonium sulfate. The harvested metmyoglobin crystals were then dissolved in the smallest amount of water possible and dialyzed against water to remove residual ammonium sulfate. Concentration and purity of the solution were calculated on the basis of 0.32% iron content and spectrophotometric

data assuming an extinction coefficient of 11.5 (*3*) at 540 mμ for cyanmetmyoglobin. Metmyoglobin preparations obtained with this procedure ranged from 90 to 100% purity and did not contain hemoglobin.

The chromatographic procedure of Helwig and Greenberg (*4*) for the removal of hemoglobin during the isolation of myoglobin was also investigated. The phosphate precipitation (3M at pH 6.6) described by Morgan (*9*) was equally effective and less time-consuming.

Conversion of Metmyoglobin To Myoglobin The purified metmyoglobin, obtained by the procedure outlined, was used in the investigation of the conversion of metmyoglobin to myoglobin at pH 7.0 and 5.8 using ascorbic acid as the reducing agent. These reactions were studied in evacuated Thunberg tubes.

Five milliliters of solution containing 0.15 millimole of metmyoglobin and 1 ml. of 2M phosphate buffer at pH 7.0 or 5.8 were placed in a Thunberg tube, while 0.1 millimole of ascorbic acid (pH adjusted to 5.3) was placed in the bulb of the stopper. The tubes were evacuated and the absorbances at 555 and 635 mμ (absorption maxima for myoglobin and metmyoglobin, respectively) were read using the Coleman Junior spectrophotometer.

An increase in absorption at 555 mμ and a decrease at 635 mμ were the criteria used for the formation of myoglobin and disappearance of metmyoglobin.

After the initial readings were made, the ascorbic acid solution was tipped into the tube and mixed with the metmyoglobin solution. The changes in absorbance at the two wave lengths were observed. A plot of typical results is presented in Figure 1.

It can be seen that the pH difference did not markedly affect the rate of conversion of metmyoglobin to myoglobin by ascorbic acid. When the absorbance at 555 mμ reached a maximum, the spectral curves obtained (450 to 670 mμ) showed that essentially complete conversion of metmyoglobin to myoglobin had occurred.

Conversion of Myoglobin To Nitric Oxide Myoglobin After the metmyoglobin had been converted to myoglobin and the spectral curves determined, the subsequent formation of nitric oxide myoglobin at pH 5.8 in the presence of sodium nitrite was studied. The Thunberg tubes were opened, 0.2 millimole of nitrite (pH 5.8) was introduced into the bulb of the stopper, and the tubes were quickly re-evacuated. After initial readings of absorbance at 545, 555, and 575 mμ, the nitrite was mixed with the myoglobin and the changes in absorbance at these three wave lengths were
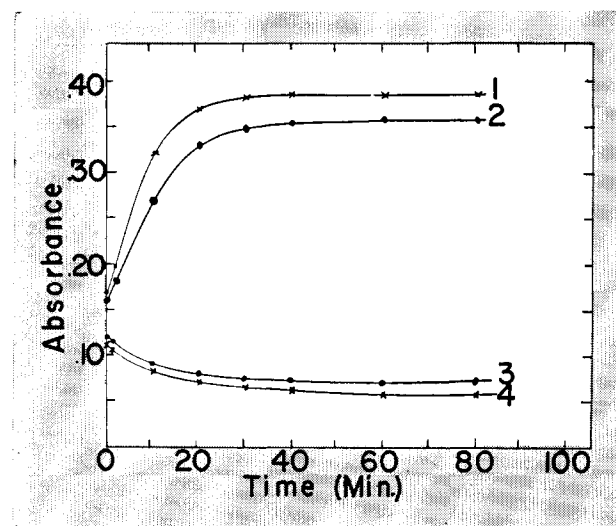
Figure 2. Spectral curves (Beckman) of myoglobin (1), metmyoglobin (2), and nitric oxide myoglobin (3) observed at pH 5.8

Concentration, 0.025 millimole per ml. Millimolar extinction coefficients. Myoglobin, 12.8 (555 mμ), metmyoglobin, 16.4 (500 mμ), and nitric oxide myoglobin, 14.2 (575 mμ) and 15.9 (544 mμ)

Figure 1. Rate of conversion of metmyoglobin to myoglobin

After addition of ascorbic acid at pH 5.8 and 7.0 based on absorbance changes at 555 and 635 mμ (see text)

1. 555 mμ, pH 7.0
2. 555 mμ, pH 5.8
3. 635 mμ, pH 5.8
4. 635 mμ, pH 7.0
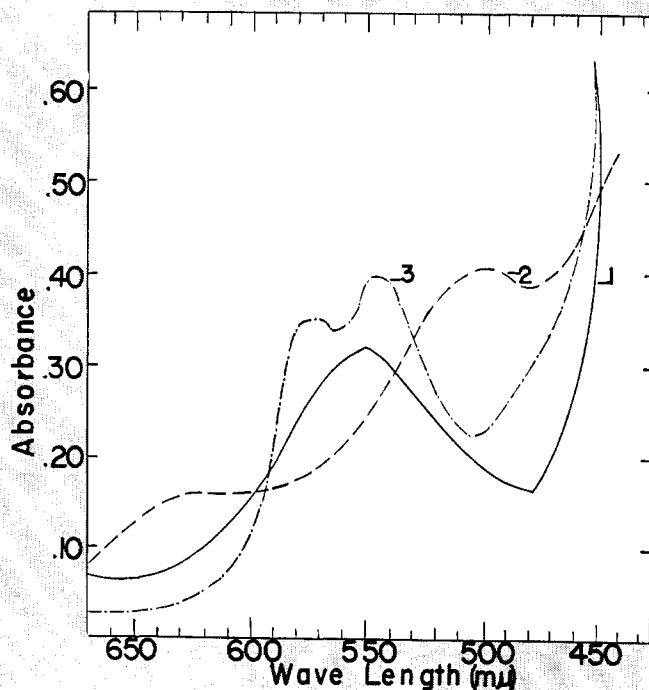
observed on the Coleman Junior spectrophotometer. These wave lengths were chosen because nitric oxide hemoglobin is reported (3) to have absorption peaks at 545 and 575 mμ. Hence, an increase in absorbance at 545 and 575 mμ and a decrease at 555 mμ would be indicative of the formation of nitric oxide myoglobin and the disappearance of myoglobin. The absorbance at 545 and 575 mμ increased as expected; however, instead of a decrease at 555 mμ, there was an increase. The maximum absorbance change was obtained within 5 minutes after addition of nitrite.

When the reaction was apparently complete, the spectral curve was determined using the Beckman DU spectrophotometer (Figure 2). The spectral curves for the isolated metmyoglobin and myoglobin (formed after treatment of metmyoglobin with ascorbic acid) are also presented. These data show that nitric oxide myoglobin has absorption peaks at 544 and 575 mμ and the increase in absorption at 555 mμ observed with the Coleman Junior spectrophotometer was due to the inability of this instrument to resolve the peaks of maximum absorption. While the absorption peak at 544

mμ agrees with that presented by Kiese and Kaeske (7) for nitric oxide myoglobin from horse heart, the peak at 575 mμ is at variance with the position they present for it at 584 mμ. Further studies on these and other chemical reactions of myoglobin and related derivatives are being carried out.

Summary A procedure slightly modified from that described by Theorell, Morgan, and Drabkin has been used for the preparation of purified metmyoglobin. The conversion of metmyoglobin to myoglobin was investigated by following spectral changes after the addition of ascorbic acid. No difference in the rate of conversion was observed when the reaction was carried out at pH 5.8 as compared to pH 7.0. Myoglobin was converted to nitric oxide myoglobin in less than 5 minutes at pH 5.8 when nitrite was added. Absorption curves for metmyoglobin, myoglobin, and nitric oxide myoglobin are presented.

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EVALUATION OF MALT

Relation of Alpha-Amylase and Limit Dextrinase of Barley Malt to Production of Ethyl Alcohol from Grains

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THE DISTILLING AND MALTING INDUSTRIES have long recognized the desirability of predetermining the performance of barley malts used in the production of ethyl alcohol from grain. Barley malt potentialities have usually been measured in terms of the activity of one of the enzymes involved in the hydrolytic degradation of starch. There is little or no agreement at present as to the total number of enzymes involved in this degradation, but two principal types have been accepted generally—β-amylase, a saccharifying enzyme, and α-amylase, a dextrinizing enzyme. Recent evidence has revealed a third enzyme with an action that appears to be additive to those of α- and β-amylase. Kneen (3) in 1945 termed this enzyme "limit dextrinase" and hypothesized that it hydrolyzes the complex dextrins which remain after α- and β-amylase have acted on grain starch.

The diastatic power test, which is principally a measurement of β-amylase, was at one time the most commonly used criterion for measuring the potential performance of barley malts used in the distilling industry. Four research laboratories (2) collaborated in a program to study the relationship between alcohol yield and such factors as diastatic power, α-amylase, wort nitrogen, Kjeldahl nitrogen, and proteolytic activity. The data showed that the diastatic power test is not a reliable index and that, of the factors investigated, α-amylase is the most significant criterion for predicting alcohol yield. Limit dextrinase activity was not measured in those studies, be-

Table I. Distribution of Malt Samples According to Enzyme Activities

α-Amylase (Sandstedt Method)		α-Amylase (ASBC Method)		Limit Dextrinase	
Units	No. of samples	Units	No. of samples	Units	No. of samples
75–80	3	50 and above	2	300 and above	4
70–74	2	45–49	16	280–299	6
65–69	8	40–44	16	260–279	9
60–64	9	35–39	28	240–259	10
55–59	12	30–34	18	220–239	14
50–54	20	25–29	8	200–219	19
45–49	20	20–24	6	180–199	12
40–44	10	15–19	4	160–179	9
35–39	6	10–14	1	140–159	4
30–34	4			120–139	6
25–29	4			100–119	7
20–24	3			Below 100	1