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BLOOD ASSAYS IN THE FRESHWATER TURTLE,
***TRACHEMYS SCRIPTA*,**
WITH EMPHASIS ON METHEMOGLOBIN LEVELS

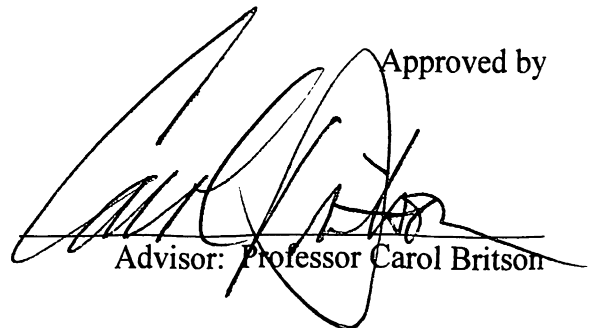
by

Erin Melissa Floyd

A thesis submitted to the faculty of The University of Mississippi in partial fulfillment of the requirements of the Sally McDonnell Barksdale Honors College

Oxford
May 2007

Approved by



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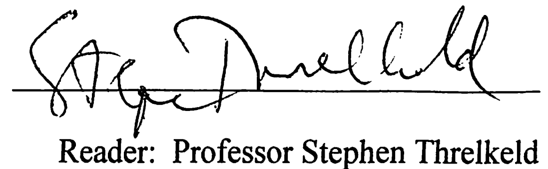

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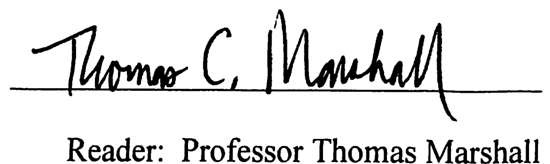

Reader: Professor Thomas Marshall

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ABSTRACT

ERIN MELISSA FLOYD: Blood Assays in the Freshwater Turtle, *Trachemys scripta*, with an Emphasis on Methemoglobin Levels
(Under the direction of Carol Britson)

Confined Animal Feeding Operations pollute water supplies with excess animal wastes containing different sources of nitrogen. How can we measure the damage being done? Methemoglobin percentages give the degree of toxicity of nitrates and nitrites in the environment. Methemoglobin is a variant of hemoglobin in which the ferrous ion has been oxidized to the ferric state. In this state, oxygen can no longer be transported by hemoglobin which is detrimental to the organism affected. Blood assays were conducted on the freshwater turtle, *Trachemys scripta.* In addition to methemoglobin analysis other assays including oxyhemoglobin saturation, red and white blood cell counts, hematocrit measurements and protein serum levels were completed in order to compare the blood of *Trachemys scripta* with other turtle standards. While methemoglobin results were inconsistent, assayed blood parameters were comparable to published values for other species of turtle.

TABLE OF CONTENTS

LIST OF TABLES AND FIGURES....................vi

INTRODUCTION....................1

MATERIALS AND METHODS....................6

RESULTS....................11

DISCUSSION....................12

LITERATURE CITED....................14

LIST OF TABLES AND FIGURES

Table 1 Permissible Limits for Nitrate-Nitrogen in Drinking Water....18

Table 2 Reported Cases of Nitrate Water-Induced Infant Methemoglobinemia Classified According to Nitrate-Nitrogen Concentration of Water Used in Feeding Formula....19

Table 3 Effect of Direct-Acting Agents on Methemoglobin Formation in Mink (*Mustela vison*) Erythrocytes....20

Table 4 Percent Methemoglobin Measurements in *Trachemys scripta*....21

Table 4a Variables for the equation, % of total Hb = 100 $[(A_{2a} - A_{2b} / A_{3a} - A_{3b})]$, in Turtle 1....22

Table 4b Variables for the equation, % of total Hb = 100 $[(A_{2a} - A_{2b} / A_{3a} - A_{3b})]$, in Turtle 2....23

Table 4c Variables for the equation, % of total Hb = 100 $[(A_{2a} - A_{2b} / A_{3a} - A_{3b})]$, in Turtle 3....24

Table 4d Variables for the equation, % of total Hb = 100 $[(A_{2a} - A_{2b} / A_{3a} - A_{3b})]$, in Turtle 4....25

Table 5 Percent Methemoglobin Measurements in *Equus caballus*, *Gallus domesticus*, and *Ovis aries*....26

Table 6 Percent Methemoglobin Measurements in Male Albino Mice (*Mus musculus*), Cr 1: CD-1 (ICR) BR Swiss COBS....27

Table 7 Determination of Hemoglobin Concentration (Hematocrit) in *Trachemys scripta*, *Equus caballus*, *Gallus domesticus*, and *Ovis aries*....28

Table 8 Blood Parameters for Other Species of Turtle and Red-eared Sliders (*Trachemys scripta*)....29

Figure 1 Chemical Structure of Hemoglobin....31

Figure 2 Molecular Structure of Hemoglobin....32

Figure 3 Chemical Structure of Methemoglobin....33

Figure 4a Absorbance Spectrum of Oxygenated and Deoxygenated Blood in Turtle 1 34

Figure 4b Absorbance Spectrum of Oxygenated and Deoxygenated Blood in Turtle 2 35

Figure 4c Absorbance Spectrum of Oxygenated and Deoxygenated Blood in Turtle 3 36

Figure 4d Absorbance Spectrum of Oxygenated and Deoxygenated Blood in Turtle 4 37

Figure 5a Absorbance Spectrum of Oxygenated and Deoxygenated Blood in *Ovis aries*....38

Figure 5b Absorbance Spectrum of Oxygenated and Deoxygenated Blood in *Equus caballus*....39

Figure 5c Absorbance Spectrum of Oxygenated and Deoxygenated Blood in *Gallus domesticus*....40

INTRODUCTION

Excess nitrogen in our water supply mainly comes from fertilizers, septic systems, and manure storage. Nitrogen not used by plants in fertilizer and manure is leached into the groundwater. Septic systems only remove half of the nitrogen in wastewater leaving the rest to mix in with ground water (McCasland et al. 2006). In the 1990's, elevated levels of nitrates were found in 1% of public water supplies, 9% of domestic water wells, and 21% of wells beneath farmlands (Johnson and Kross 1990).

Confined Animal Feeding Operations (CAFOs) are largely responsible for the production of America's meat products. They are the leading producers of turkey, pork, chicken, and most aquatic species (e.g., catfish). CAFOs, especially those dealing with hogs, also contribute greatly to pollution via excess animal wastes (Pagilla et al. 2000). In one case, the negative effects of the CAFO are unbelievable, according to Jeff Tietz, journalist for "Rolling Stone" (Tietz 2006). Smithfield Foods is the leading pork processor of the world. Raising 500,000 pigs at one branch alone, it is easy to understand how the company produces 26 million tons of waste products each year. Tietz (2006) asserts that many of Smithfield's contractors either inadvertently or in order to save money, allow the waste to pollute nearby water sources. When the lagoons fill, some of the waste is sprayed on surrounding fields. When heavy rain comes, the contents of the lagoons can overflow into the surrounding area (Tietz 2006).

This is an environmental problem because hog waste contains cyanide, ammonia, and nitrates. Specifically, in North Carolina, hog farms emit 300 tons of nitrogen into the air via ammonia gas, daily (Schlesinger and Hartley 1992). Nearly 70% of all global ammonia emissions are due to animal waste and biomass burning (Schlesinger and

Hartley 1992). Excess nitrogen in water supplies from manure kills fish and other aquatic creatures by depriving them of oxygen. Algal blooms form from nutrients in the waste and decomposers use up all of the oxygen in the water. Also, breathing polluted air with excess nitrogen could cause a number of human health related problems such as bronchitis and asthma (Tietz 2006). In 1995, eleven other waste lagoons from CAFOs in North Carolina were tested for nitrate levels. Six of the sites had exceeding levels of nitrate (EPA 1989). The EPA advisory level for nitrate is 10mg/L. Table 1 gives the permissible nitrate limits in drinking water for different age groups and species (Lee 1970).

Nitrates are not the only substance of concern. The substances fed to the hogs such as xenoestrogens and antibiotics are present in waste (Thu 1996). Life threatening pathogens are abundant. Elements such as phosphorus, sodium, potassium, copper, and zinc build up in soil that has been fertilized with manure. Other volatile compounds for which the effects are unknown are also present (Thu 1996).

According to Tietz in "Rolling Stone" (2006), in four years, Smithfield spilled 2 million gallons of waste into the Cape Fear River, 1.5 million gallons into its Persimmon Branch, 1 million gallons into the Trent River and 200,000 gallons into Turkey Creek. All of these rivers and streams supply human drinking water in North Carolina. In 1997, Smithfield was fined $12.6 million for 6,900 violations of the Clean Water Act in Virginia (Tietz 2006). Smithfield is also responsible for the biggest spill in the history of corporate hog farming (Tietz 2006). In 1995, the dike of a 120,000-square-foot lagoon ruptured, releasing 25.8 million gallons of effluvium into the headwaters of the New River in North Carolina (Tietz 2006). The waste had the ability to burn human skin

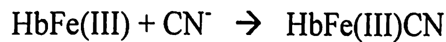
because it was so toxic (Tietz 2006). It took nearly two months to make its way sixteen miles downstream to the ocean (Tietz 2006). Every creature living in the river was killed (Tietz 2006).

Sometimes to prevent flooding of lagoons, farmers will spray excess wastes onto surrounding fields as fertilizer. Too much of this fertilizer can disrupt microbial nitrogen cycling, reduce species diversity, increase global warming, and increase acidification (Schindler et al. 1985). Disrupting the nitrogen cycle also increases eutrophication, increasing the amount of plant life using large amounts of oxygen causing fish kills and algal blooms dangerous to humans (Rabalais 2002).

Humans have a negative effect on the cycling of nitrogen every day. Nitrogen fertilizers and the combustion of fossil fuels aid in the disruption of the global cycle of nitrogen (Galloway et al. 1995). When this cycle is disrupted biological diversity is lost (Likens et al. 1996). Plants that use nitrogen efficiently die when the cycle is disrupted (Likens et al. 1996), and animals that feed off of these plants may also die. Greenhouse gases increase as do the amounts of smog (Likens et at. 1996). Important soil nutrients such as calcium and potassium are lost (Likens et al. 1996). Aquatic ecosystems see declines in number of plant and animal species (Vitousek et al. 1997). Wetlands serve as a nitrogen sink and when these are lost nitrogen is allowed to travel through streams and rivers (Leonardson 1994). All of this has occurred because humans have nearly doubled the rate of nitrogen put into the nitrogen cycle (Vitousek et al. 1997). Nitrate levels are easily tested from water sources. From 1965 to 1995 nitrate levels more than doubled in the Mississippi River (Turner and Rabalais 1991, Justic et al. 1995).

Methemoglobin percentages have been suggested as a biomarker for the presence of toxic levels of nitrate and nitrite in natural environments (Rodriguez-Moreno and Tarazona 1994). Methemoglobin is a variant of hemoglobin in which the ferrous ion has been oxidized to the ferric state. Figure 1 depicts the heme group in hemoglobin, Figure 2 depicts hemoglobin, and Figure 3 depicts methemoglobin. In the ferric state, oxygen can no longer be transported by hemoglobin (Dacie and Lewis 1968). The reaction is as follows:

$$HbFe(II) + Fe(III)(CN) \rightarrow HbFe(III) + Fe(II)(CN)$$



$$HbFe(III) + CN^{-} \rightarrow HbFe(III)CN$$

The Fe(II) of hemoglobin is oxidized to Fe(III) by ferricyanide and then converted into stable cyanmethemoglobin by addition of cyanide. Enzymes in the blood convert methemoglobin back to hemoglobin (e.g. Figure 3) and for this reason levels do not usually exceed 1%. Since infants have lower levels of these enzymes (methemoglobin reductase or its coenzyme reduced DPNH-dependent diphosphopyridine nucleotide, which is normally generated by glycolysis in the red blood cell), they usually have 1-2% methemoglobin levels (Lee 1970). Increased amounts result in methemoglobinemia. Some symptoms are tachycardia (rapid heart beat), dyspnea (shortness of breath), restlessness, vomiting, and diarrhea. When methemoglobin levels exceed 10%, cyanosis, digestive and respiratory problems may occur (Lee 1970). At levels exceeding 30% anoxia occurs, and at levels exceeding 70% brain damage or even death may be the final result (McCasland 2006). Table 2 gives reported cases of methemoglobin due to high concentration of nitrate-nitrogen in water (Lee 1970).

Methemoglobinemia is caused by aniline, nitrates, nitrites, and some sulfonamides that aid in the conversion of hemoglobin to methemoglobin (Dacie and Lewis 1968). Specifically, the effects of nitrates and nitrites will be discussed. Even more specifically, the disorder is commonly caused when excess nitrates are found in drinking water (McCasland 2006). When nitrate levels in water supplies are high, microorganisms in the digestive system may convert nitrate to nitrite (Vitousek et al. 1997). Nitrite is the intermediate compound of the nitrification process and occurs from the oxidation of ammonia to nitrate (Russo and Thurston 1991). Nitrite (NO_2) is formed from nitrate (NO_3) via reduction. Nitrates generally do not affect health until they are reduced to nitrites (McCasland 2006). Nitrite is absorbed into the bloodstream where it oxidizes hemoglobin to its ferric state (methemoglobin), which is incapable of transporting oxygen in the blood (Rodriguez-Moreno and Tarazona 1994; Vitousek et al. 1997).

Few nonhuman organisms have been tested for methemoglobin levels including rainbow trout (*Oncorhynchus mykiss*; Rodriguez-Moreno and Tarazona 1994) and mink (Calabrese et al. 1995). Rodriguez-Moreno and Tarazona (1994) found that in rainbow trout elevated nitrite levels in the blood were directly correlated to the fish having methemoglobinemia. After the fish were transferred to water containing no nitrite the disorder disappeared, presumably because of the working methemoglobin reductase systems. Thus, testing the effects of nitrite in the blood of rainbow trout proved to be an effective way to measure methemoglobin levels (Rodriguez-Moreno and Tarazona 1994). Calabrese et al. (1999) found from blood tests that mink were receptive to the following methemoglobin-forming agents: α-naphthol, nitrite, copper, p-dinitrobenzene, chlorite,

o-dinitrobenzene, and chlorate; nitrite being the most potent (Calabrese et al. 1995). Table 3 shows the effect of direct-acting agents on methemoglobin formation in mink erythrocytes.

Through experimentation, results similar to those found in rainbow trout and mink were expected to be found regarding the species *Trachemys scripta.* Potassium ferricyanide and potassium cyanide are the sources of nitrogen that will convert hemoglobin to methemoglobin (Tietz 1987). Ideally, blood levels would show methemoglobin concentration which would then be linked to methemoglobinemia (Rodriguez-Moreno and Tarazona 1994; Calabrese et al. 1995).

MATERIALS AND METHODS

The red-eared slider (*Trachemys scripta*) was chosen as the animal model in this study because it is easily found in or around wetlands in the southeastern United States and because it maintains site fidelity meaning it will stay at the site as it has access to food and water. Therefore, recent and chronic contamination in turtles can potentially be tested, whereas it might be more difficult in another animal model. Turtles are also easy to handle. The minimum number of organisms (4) chosen were necessary for conducting a Power-Efficiency Test (Martin and Bateson 1993, Siegel and Castellan 1988). This statistical test is used during preliminary research in order to determine the minimum number of samples (or organisms) needed to detect statistically significant results in later experiments.

Basic husbandry requirements for hatchling and juvenile turtles are as follows (Britson 1996): turtles were maintained under a 14L:10D photoperiod from the months

of April to July and 12L:12D during other months. Laboratory temperature was recorded daily. The turtles were exposed to both white florescent and incandescent light during the light part of the photoperiod. The turtles were housed in opaque, plastic chambers with dimensions of 32 in (L) x 14 in (W) x 12 in (H) with a maximum of one turtle per chamber. The housing chambers were filled with tap water to a depth of 1/3 to 1/2 chamber height. Basking structures were placed in the chambers to provide a dry area. The chambers were cleaned once per week and then refilled with water. The turtles were fed a rotating diet of Reptomin ® floating food sticks, commercially available goldfish, plant material (e.g., *Elodea* sp. and/or *Lemna* sp.) and freshwater shrimp. The turtles were fed five times per week. A calcium supplement (1g, tablet form) was added to the housing chambers once per week. Turtle length and mass was recorded weekly.

The turtles were given an extended amount of time to acclimate themselves to the new housing environment. Blood samples were drawn weekly during June, July, October, and November. The months of August and September were periods of rest. The blood sampling procedure required a team of two investigators. One investigator restrained the turtle and the second investigator collected the sample. The investigator collecting the sample located the crural vein which runs in the inner thigh muscles. A drop of blood was collected with a sterile syringe. EDTA was used as the anticoagulant. EDTA is effective in the hemolysis of reptile red blood cells (Harms et al 2000). In only a few instances did the collection site continue to bleed after the sample was taken. To stop bleeding, the first investigator applied direct pressure to the collection site and Liquid Bandaid™ was used to cover it. After samples were taken, the turtles were returned to their housing containers. The turtles were monitored daily for ill effects

physically and behaviorally. Ill effects were not anticipated from sampling (American Society of Ichthyologists and Herpetologists 1984) and were not observed. The last two weeks of the project involved observing the turtles for any latent effects.

The drop of blood was placed into a test tube containing 2mL of distilled water. Immediately, 2mL of potassium phosphate buffer (pH = 6.6) was added and mixed thoroughly. There were three designated cuvettes to be used in measuring the absorbance in the spectrophotometer (Britson 2003). The first cuvette served as the blank standard. It contained 1mL of phosphate buffer and 1mL of distilled water. 2mL of the hemolysate (blood, water, and phosphate buffer) were placed into the second and third cuvettes. To the third cuvette, one drop of potassium ferricyanide was added. Adding potassium ferricyanide oxidizes Fe(II) to Fe(III), which is the cause of the formation of methemoglobin. The cuvette was inverted with the use of Parafilm. The mixture was allowed to sit for ten minutes and the absorbance of the three cuvettes was measured at 630nm. These measurements were recorded as A_{2a} and A_{3a} for the second and third cuvettes, respectively. Next, one drop of potassium cyanide was added to all three cuvettes. Adding cyanide converts methemoglobin to cyanmethemoglobin. The cuvettes were inverted and allowed to sit for ten minutes. The absorbance of the three cuvettes was again measured at 630nm. These measurements were recorded as A_{3a} and A_{3b} for the second and third cuvettes, respectively. Methemoglobin percentages were also analyzed from stock chicken, horse, and sheep blood obtained from Carolina Biological Supply. The absorbance spectrum of methemoglobin gives a small peak anywhere from 630-635 nm (Tietz 1987). The following equation was used in determining the amount of methemoglobin measured (Tietz 1987):

$$\% \text{ of total Hb} = 100\ [(A_{2a} - A_{2b} / A_{3a} - A_{3b})]$$

which can better be explained as

$$\% \text{ of total Hb} = 100\ [(\text{normal Hb} - \text{metHb}/ \text{cyanmetHb} - \text{metHb})].$$

A_{2a}, A_{2b}, and A_{3a} were expected to be high numbers where A_{3b} was expected to be low in order to get 1% or lower methemglobin levels.

To be sure that the turtle blood was typical, several other tests were performed. First, an assay involving oxyhemoglobin saturation was performed on turtle, sheep, horse, and chicken blood. One drop of each sample was mixed with 8mL of distilled water in separate test tubes. 4mL of each test tube was poured into a second test tube for each sample. Two drops of sodium dithionite solution was added to the second test tube and mixed. The two solutions (one with sodium dithionite and one without) were transferred to two cuvettes. A third cuvette made up of distilled water was used as a blank. Absorbances for each cuvette were measured in increasing increments of 10nm from 500nm- 600nm. Absorption spectra for oxyhemoglobin and reduced hemoglobin were generated. Hemoglobin carrying no oxygen is deoxyhemoglobin and hemoglobin bound to oxygen is oxyhemoglobin (Britson 2003).

Another blood analysis was the complete red and white blood cell counts. A dilution technique using hemacytometer pipettes was completed. The pipette with the red plastic float was used for counting red blood cells and the pipette with the white plastic float was used for counting white blood cells. Two blood samples were drawn to the 0.5 mark in the pipettes. Hayem's solution was drawn to the 100 mark for the red blood cells and Turk's solution was drawn to the 100 mark for the white blood cells. The tips of the pipette were covered with the investigator's fingers and the contents of the pipette were

shaken by smooth, alternating hand movements, like that of a see-saw. A coverslip was placed on the hemacytometer and 1 drop from the pipette was placed between the counting chamber and coverslip. The counting chamber filled by capillary action. After 3 minutes the blood cells were ready to be counted. The number of red blood cells and white blood cells were accounted for using different equations. To find the number of red blood cells per microliter of blood the following equation was used: (Number of cells counted x dilution (675) x 4000) / Number of small squares counted). To find the number of white blood cells per microliter of blood the following equation was used: (Number of cells counted x dilution (675) x 10) / Number of small squares counted) (Britson 2003).

A simple blood smear on a microscope slide was made for differential white blood cell counts. The sample was stained with Methylene Blue and left to dry overnight. The next the day the slide was analyzed using a microscope.

The microhematocrit value for all turtles, chicken, sheep, and horse samples were assayed. This was done by collecting a drop of blood in a capillary tube. The blood filled end was sealed with wax and the tubes were placed in a microhematocrit centrifuge for 5 minutes. The hematocrit ratio was found with the following equation: (Packed cell height in mm/ sample height in mm) x 100. (Britson 2003).

The last blood assay conducted was the measurement of plasma protein with a refractometer. A drop of blood was placed on the refractometer slide and the measure of plasma protein in g/dL was given.

RESULTS

Table 4 shows the results obtained in measuring methemoglobin percentages in *Trachemys scripta*. There are more measurements for a few of the turtles because a larger blood sample was collected. Tables 4a-d give the values of the variables found for use in the equation to find the percent methemoglobin measurements for each turtle. Table 5 shows the results obtained in measuring methemoglobin percentages in *Equus caballus*, *Gallus domesticus*, and *Ovis aries*. Table 6 shows methemoglobin levels measured *in Mus musculu* for comparison (Olson et al. 1997).

Table 7 gives the hematocrit values found for *Trachemys scripta, Equus caballus, Gallus domesticus*, and *Ovis aries*. Table 8 is a compilation of different blood parameters found in different species of turtle and other reptiles and amphibians. For each of our parameters, a blood assay was completed on the four experimental turtles. The table can be used for comparison of the turtles in our study with other species.

One of the blood assays completed involved finding the absorbance spectrum of oxygenated and deoxygenated blood in all four turtles and in livestock blood. The results of this assay and the curves given are shown in Figures 4a-d and 5a-c.

DISCUSSION

In Table 8 blood parameters found in different literature sources were compiled and compared to those found in *Trachemys scripta*. Measurements found for hematocrit, protein serum, red and white blood cells counts, and differentiated white blood cell counts in *Trachemys scripta* were well within the expected ranges based on other

published data. This shows that the quality of blood did not affect the results given for methemoglobin analysis.

The ultimate purpose of this experiment was to create a solid technique for measuring methemoglobin levels in potentially contaminated animals. As seen from Table 4, the results were inconsistent and out of range. The technique throughout the course of experimentation was manipulated. In trials 1, 2, and 3 standard procedure (see Materials and Methods) was followed. In trial 4, the pH of the phosphate buffer was changed to 8.8. In trial 5, the pH of the phosphate buffer was changed to 7.1. In trial 6, the pH of the phosphate buffer was 6.7. In trials 7-12 EDTA was added to the protocol. The binding and lysing times were also lengthened from 2 minutes to 10 minutes. In trial 13, 2 drops of potassium ferricyanide and potassium cyanide were used. In trial 14, 4 drops of potassium ferricyanide and potassium cyanide were used. In trial 15, 6 drops of potassium ferricyanide and potassium cyanide were used. The binding and lysing times of trials 13-15 were also 10 minutes. In trial 16, EDTA was used, only 2 drops of potassium ferricyanide and potassium cyanide were used, and the binding and lysing times were 10 minutes. In the end, none of these things gave the results expected. There was also the issue that the spectrophotometer being used may not have given completely accurate results. There were instances where the absorbances given were inconsistent within the same sample. This statement is not valid until the spectrophotometer is tested for accuracy.

Though the goal of the experiment was not reached, steps have been made for this goal to be obtained in the future. Possible things that could be done differently are developing a phosphate buffer with a different pH, collecting a cleaner sample,

centrifuging the sample after lysis, and possibly finding another spectrophotomer for use after the one used is tested for accuracy. If the experiment was repeated a spectra including the absorbances of hemoglobin, methemoglobin, and cyanmethemoglobin at different wavelengths could be formulated and possible problems would be seen.

When a technique for measuring methemoglobin percentages is developed it will be used in the future for examining the demographic parameters in organisms which exhibit varying degrees of association with the aquatic environment and which are likely to be affected differently by CAFO (confined animal feeding operation) -mediated impacts.

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Table 1. Permissible Limits for Nitrate-Nitrogen in Drinking Water (Lee 1970).

Consumer	Assumed weight, kg	Region	Maximum permissible dose of nitrate-nitrogen, mg	Approximate daily water intake, liters	Permissible limit of nitrate-nitrogen in water, ppm
Infant	3	England	12	0.5	24
Infant	3	Tropics	12	2	6
Adult	60	England	240	1	240
Adult	60	Tropics	240	5.3	45
Cow	500	Tropics	2000	45	45

Table 2. Reported Cases of Nitrate Water-Induced Infant Methemoglobinemia Classified According to Nitrate-Nitrogen Concentration of Water Used in Feeding Formula (Lee 1970).

<table>
<tr><th rowspan="2">State</th><th colspan="2">Methemoglobinemia</th><th colspan="6">Number of cases associated with indicated ranges Nitrate-Nitrogen Concentration (ppm)</th><th rowspan="2">Number of cases for which data are available</th></tr>
<tr><th>Reported Cases</th><th>Reported Deaths</th><th>0-10</th><th>11-20</th><th>21-30</th><th>31-50</th><th>51-100</th><th>100+</th></tr>
<tr><td>California</td><td>1</td><td>0</td><td>0</td><td>0</td><td>0</td><td>0</td><td>1</td><td>0</td><td>1</td></tr>
<tr><td>Georgia</td><td>6</td><td>3</td><td colspan="6">Information not available</td><td>0</td></tr>
<tr><td>Illinois</td><td>75</td><td>6</td><td>0</td><td>1</td><td>2</td><td>2</td><td>12</td><td>11</td><td>28</td></tr>
<tr><td>Indiana</td><td>1</td><td>0</td><td>0</td><td>0</td><td>0</td><td>0</td><td>1</td><td>0</td><td>1</td></tr>
<tr><td>Iowa</td><td>Several</td><td>11</td><td>0</td><td>0</td><td>0</td><td>0</td><td>1</td><td>1</td><td>2</td></tr>
<tr><td>Kansas</td><td>13</td><td>3</td><td>0</td><td>0</td><td>1</td><td>1</td><td>2</td><td>8</td><td>12</td></tr>
<tr><td>Michigan</td><td>7</td><td>0</td><td>0</td><td>0</td><td>0</td><td>0</td><td>0</td><td>7</td><td>7</td></tr>
<tr><td>Minnesota</td><td>139</td><td>14</td><td>0</td><td>2</td><td colspan="2">25</td><td>53</td><td>49</td><td>129</td></tr>
<tr><td>Montana</td><td>2</td><td>0</td><td>0</td><td>0</td><td>0</td><td>0</td><td>0</td><td>2</td><td>2</td></tr>
<tr><td>Nebraska</td><td>22</td><td>1</td><td>0</td><td>1</td><td>0</td><td>4</td><td>9</td><td>8</td><td>22</td></tr>
<tr><td>New York</td><td>2</td><td>0</td><td>0</td><td>0</td><td>0</td><td>0</td><td>1</td><td>0</td><td>1</td></tr>
<tr><td>N. Dakota</td><td>9</td><td>1</td><td>0</td><td>1</td><td>1</td><td>0</td><td>0</td><td>6</td><td>8</td></tr>
<tr><td>Ohio</td><td>0</td><td>0</td><td>0</td><td>0</td><td>0</td><td>0</td><td>0</td><td>0</td><td>0</td></tr>
<tr><td>Oklahoma</td><td>0</td><td>0</td><td>0</td><td>0</td><td>0</td><td>0</td><td>0</td><td>0</td><td>0</td></tr>
<tr><td>S. Dakota</td><td>Several</td><td>0</td><td colspan="6">Information not available</td><td>0</td></tr>
<tr><td>Texas</td><td>0</td><td>0</td><td>0</td><td>0</td><td>0</td><td>0</td><td>0</td><td>0</td><td>0</td></tr>
<tr><td>Virginia</td><td>1</td><td>0</td><td>0</td><td>0</td><td>0</td><td>0</td><td>1</td><td>0</td><td>1</td></tr>
<tr><td>Total</td><td>278+</td><td>39</td><td>0</td><td>5</td><td colspan="2">36</td><td>81</td><td>92</td><td>214</td></tr>
</table>

Table 3. Effect of Direct-Acting Agents on Methemoglobin Formation in Mink (*Mustela vison*) Erythrocytes (Calabrese et al. 1995).

Agent	N	Dose (mM)	Methemoglobin % ± 1 SD
Nitrite	10	0.0	0.9 ± 0.5
	8	0.25	4.2 ± 1.3
	10	0.50	9.2 ± 3.8
	10	1.0	21.9 ± 6.5
	10	1.5	33.5 ± 7.9
	10	2.0	42.9 ± 11.5
Chlorite	7	0.0	0.9 ± 0.6
	6	0.5	3.8 ± 1.2
	7	1.0	10.5 ± 1.8
	7	2.0	22.9 ± 2.8
	7	3.0	34.3 ± 5.5
	7	4.0	49.9 ± 10.2
Chlorate	10	0.0	0.9 ± 0.5
	10	7.5	1.5 ± 0.5
	10	15.0	2.3 ± 1.1
	10	22.5	7.1 ± 4.2
	10	30.0	24.9 ± 9.8
Copper	7	0.0	0.9 ± 0.6
	6	0.125	2.4 ± 1.5
	7	0.250	10.3 ± 4.8
	7	0.500	17.9 ± 8.7
	7	1.00	24.8 ± 10.8
	7	2.00	31.9 ± 11.0
p-dinitrobenzene	8	0.0	0.8 ± 0.2
	6	0.02	6.3 ± 1.9
	6	0.04	8.7 ± 3.6
	6	0.1	9.7 ± 2.3
	6	0.2	11.7 ± 4.3
	6	0.5	17.5 ± 3.8
	6	1.0	19.4 ± 4.6
o-dinitrobenzene	8	0.0	0.8 ± 0.2
	6	0.2	6.1 ± 2.4
	6	0.4	6.7 ± 1.0
	6	1.0	8.9 ± 1.6
	6	2.0	18.0 ± 9.1
	6	5.0	22.0 ± 7.7
	6	10.0	13.3 ± 8.4
α-napthol	9	0.0	0.8 ± 0.6
	8	0.06	10.0 ± 4.2
	9	0.13	18.2 ± 6.8
	9	0.25	35.5 ± 12.5
	9	0.5	51.9 ± 16.6
	8	1.0	79.8 ± 16.2

Table 4. Percent Methemoglobin Measurements in *Trachemys scripta.*

Trial	Turtle 1	Turtle 2	Turtle 3	Turtle 4
1	-42.9	-112.5	110.0	250.0
2	-866.7	13.7	112.5	121.4
3	55.3	16.7	-23.2	100.0
4	53.6	43.1	112.6	120.6
5	50.0	113.4	23.1	220.0
6	-21.1	72.9	138.1	36.8
7	393.1	600.0	540.7	400.0
8	131.5		370.4	
9	667.2		660.0	
10	604.8			
11	501.3			
12	443.2			
13	260.7	552.4	349.5	217.9
14	389.5		353.5	440.0
15	414.9		1075.0	623.1
16	235.0	109.2	144.7	152.0

Table 4a. Variables for the equation, % of total Hb = 100 [($A_{2a} - A_{2b} / A_{3a} - A_{3b}$)], in Turtle 1.

Trial	A_{2a}	A_{2b}	A_{3a}	A_{3b}
1	0.465	0.468	0.483	0.476
2	0.802	0.776	0.806	0.809
3	1.265	1.244	1.382	1.344
4	0.602	0.587	0.613	0.585
5	0.497	0.493	0.505	0.497
6	0.447	0.451	0.478	0.459
7	0.787	0.504	0.590	0.518
8	0.608	0.512	0.604	0.531
9	0.875	0.508	0.584	0.529
10	0.887	0.506	0.584	0.521
11	0.887	0.511	0.600	0.525
12	0.838	0.510	0.601	0.527
13	1.127	0.504	0.760	0.521
14	1.782	0.851	1.146	0.907
15	1.679	0.845	1.101	0.900
16	0.769	0.487	0.618	0.498

Table 4b. Variables for the equation, % of total Hb = 100 [($A_{2a} - A_{2b} / A_{3a} - A_{3b}$)], in Turtle 2.

Trial	A_{2a}	A_{2b}	A_{3a}	A_{3b}
1	0.426	0.435	0.451	0.443
2	0.969	0.962	1.070	1.019
3	0.510	0.530	0.508	0.518
4	1.115	1.056	1.220	1.083
5	0.611	0.501	0.616	0.519
6	0.611	0.466	0.697	0.498
7	0.621	0.495	0.527	0.503
8				
9				
10				
11				
12				
13	0.609	0.493	0.529	0.508
14				
15				
16	0.922	0.907	0.839	0.831

Table 4c. Variables for the equation, % of total Hb = 100 [(A_{2a} – A_{2b} / A_{3a} – A_{3b})], in Turtle 3.

Trial	A_{2a}	A_{2b}	A_{3a}	A_{3b}
1	0.563	0.541	0.597	0.577
2	1.230	1.167	1.288	1.232
3	1.554	1.590	1.815	1.660
4	0.789	0.795	0.728	0.741
5	0.512	0.506	0.534	0.508
6	0.525	0.467	0.527	0.485
7	0.797	0.505	0.569	0.515
8	0.804	0.504	0.601	0.520
9	0.698	0.500	0.537	0.507
10				
11				
12				
13	0.874	0.493	0.615	0.506
14	0.916	0.513	0.981	0.867
15	1.356	0.840	0.576	0.528
16	0.837	0.122	0.646	0.152

Table 4d. Variables for the equation, % of total Hb = 100 [($A_{2a} - A_{2b} / A_{3a} - A_{3b}$)], in Turtle 4.

Trial	A_{2a}	A_{2b}	A_{3a}	A_{3b}
1	0.520	0.530	0.551	0.555
2	1.098	1.064	1.148	1.120
3	0.620	0.637	0.614	0.631
4	0.677	0.636	0.709	0.675
5	0.506	0.495	0.503	0.498
6	0.462	0.455	0.487	0.468
7	0.559	0.499	0.520	0.505
8				
9				
10				
11				
12				
13	0.675	0.492	0.586	0.502
14	1.168	0.838	0.934	0.859
15	1.254	0.849	0.902	0.837
16	0.708	0.515	0.650	0.523

Table 5. Percent Methemoglobin Measurements from *Equus caballus*, *Gallus domesticus*, and *Ovis aries*

Trial	*Equus caballus*	*Gallus domesticus*	*Ovis aries*
1	19.7	115.2	10.3
2	14.6	622.5	11.4
3	12.3	508.3	8.7

Table 6. Percent Methemoglobin Measurements in Male Albino Mice (*Mus musculus*), Crl:CD-1 (ICR) BR Swiss COBS (Charles River Laboratories, Portage, MI, USA) (Olson et al. 1997) .

Validation Run	Sample	Automatic 1	Automatic 2	Manual
1	1	1.72	1.77	1.79
	2	4.33	4.09	4.70
	3	8.12	6.72	8.57
	4	14.08	13.05	15.69
	5	28.06	27.17	28.98
2	1	1.88	2.41	1.73
	2	6.37	7.25	6.15
	3	10.98	11.63	11.37
	4	21.73	21.68	21.93
	5	40.52	40.68	43.04
3	1	2.62	2.25	2.68
	2	6.40	6.44	7.63
	3	11.95	10.22	12.24
	4	20.84	20.85	22.15
	5	40.73	40.21	43.59
4	1	2.64	2.59	2.57
	2	7.06	6.04	7.45
	3	12.30	11.68	13.54
	4	22.23	21.12	23.65
	5	48.60	42.93	46.26
5	1	1.21	1.26	1.76
	2	4.97	4.06	5.62
	3	8.70	8.44	10.21
	4	17.60	16.03	18.80
	5	33.36	31.93	34.72

Table 7. Determination of Hemoglobin Concentration in Sample (Hematocrit) in *Trachemys scripta* and livestock blood.

	Drabkin's Solution	Blood	Absorbance at 540 nm	Hemoglobin Concentration (g/dL)
Blank	2 mL	None	-0.115	
Trachemys scripta 1	2 mL	20 µL	-0.234	37.6%
Trachemys scripta 2	2 mL	20 µL	-0.294	41.7%
Trachemys scripta 3	2 mL	20 µL	-0.324	30.8%
Trachemys scripta 4	2 mL	20 µL	-0.421	30.0%
Equus caballus	2 mL	20 µL	0.027	40.0%
Ovis aries	2 mL	20 µL	0.037	16.3%
Gallus domesticus	2 mL	20 µL	0.050	41.5%

Table 8. Blood parameters for other species of turtle and red-eared sliders (*Trachemys scripta*).

	Hemato-crit	Protein Serum	RBC estimate	WBC estimate	Neutrophils	Eosinophils	Basophils	Lympho-cytes	Mono-cytes	reference
Wild Male Bog Turtle	20%	2.8 g/dL	N/A	7000/uL	N/A	1%	15%	48%	5%	Brenner et al 2002
Wild Female Bog Turtle	20%	2.9 g/dL	N/A	7000/uL	N/A	3%	13%	55%	4%	Brenner et al 2002
Captive Bog Turtle	19%	2.5 g/dL	N/A	12,500/uL	N/A	0%	18%	66%	2%	Brenner et al 2002
Eastern Box Turtle 1	22%	3.8 g/dL	N/A	16,800/uL	N/A	N/A	6%	53%	N/A	De Voe et al 2004
Eastern Box Turtle 2	21%	2.6 g/dL	N/A	9800/uL	N/A	N/A	7%	12%	N/A	De Voe et al 2004
Green Iguana	30%	6.2 g/dL	N/A	N/A	N/A	N/A	N/A	N/A	N/A	Hernande: Divers et : 2005
Hermann Tortoises	27%	3.4 g/dL	$0.74 \times 10^{12}/L$	7950/uL	N/A	.13%	4.9%	60%	.13%	Neiffer et al. 200:
Captive Juvenile Mugger Crocodile	25%	3.1 g/dL	690000	8710/uL	N/A	6%	.11%	28%	1%	Stacy and Whitaker 2000
Captive Subadult Mugger Crocodile	23%	3.1 g/dL	720000	8610/uL	N/A	4%	.35%	35%	1%	Stacy and Whitaker 2000
Captive Adult Mugger Crocodile	26%	3.2 g/dL	800000	6970/uL	N/A	8%	0%	26%	2%	Stacy and Whitaker 2000
Viperid Snakes	15-37%	3.4-6.4 g/dL	N/A	1000-8000/uL	N/A	0-14%	0-6%	2-65%	22-34%	Sutton and Taylor 2003
Red-eared slider 1	38%	4.0 g/dL	1,980,000	1219/uL	57%	4%	3%	31%	5%	Present study

Red-eared slider 2	42%	3.0 g/dL	2,130,000	813/uL	56%	2%	4%	35%	3%	Present study
Red-eared slider 3	31%	2.5 g/dL	2,920,000	641/uL	61%	2%	2%	32%	3%	Present study
Red-eared slider 4	30%	3.0 g/dL	3,050,000	625/uL	63%	3%	2%	28%	4%	Present study

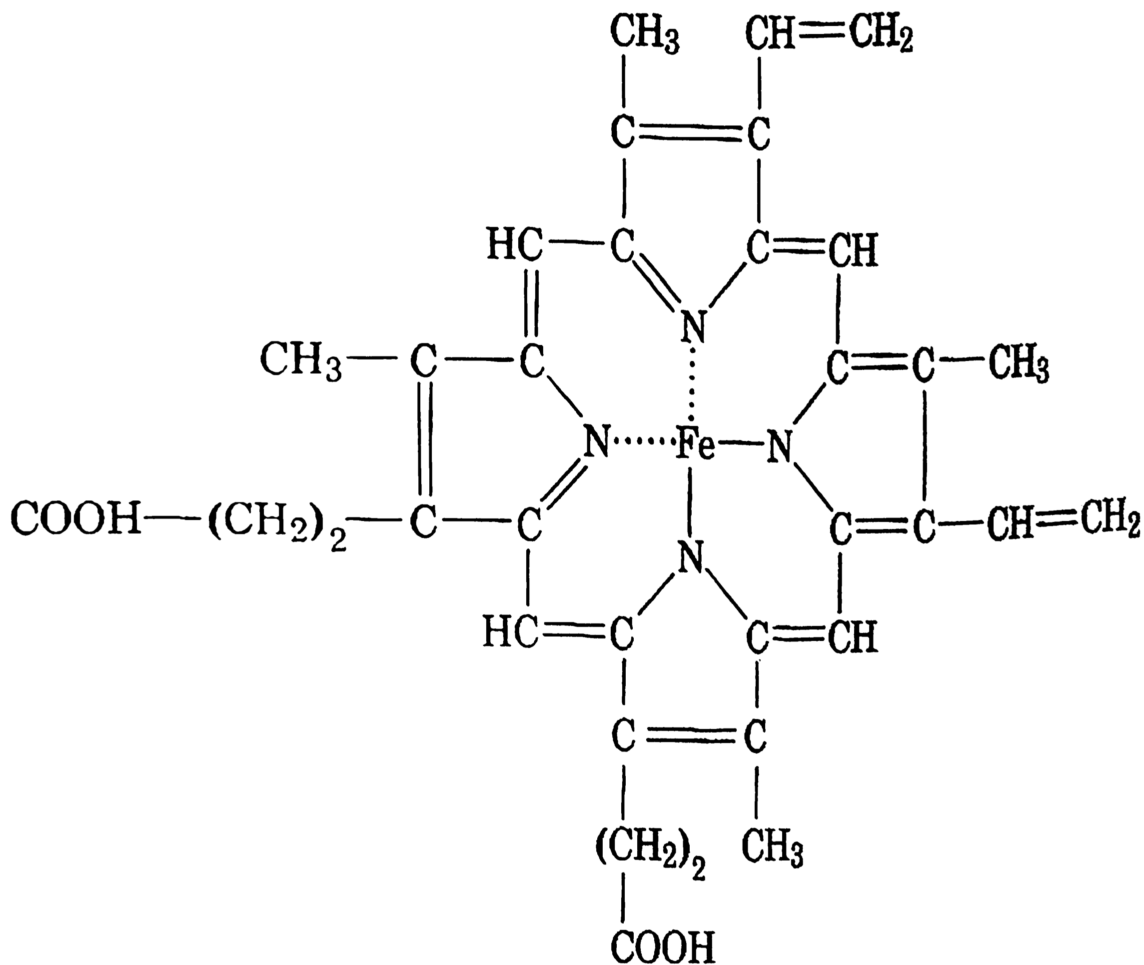

Figure 1. Chemical structure of hemoglobin as obtained from http://en.wikipedia.org/wiki/Hemoglobin.

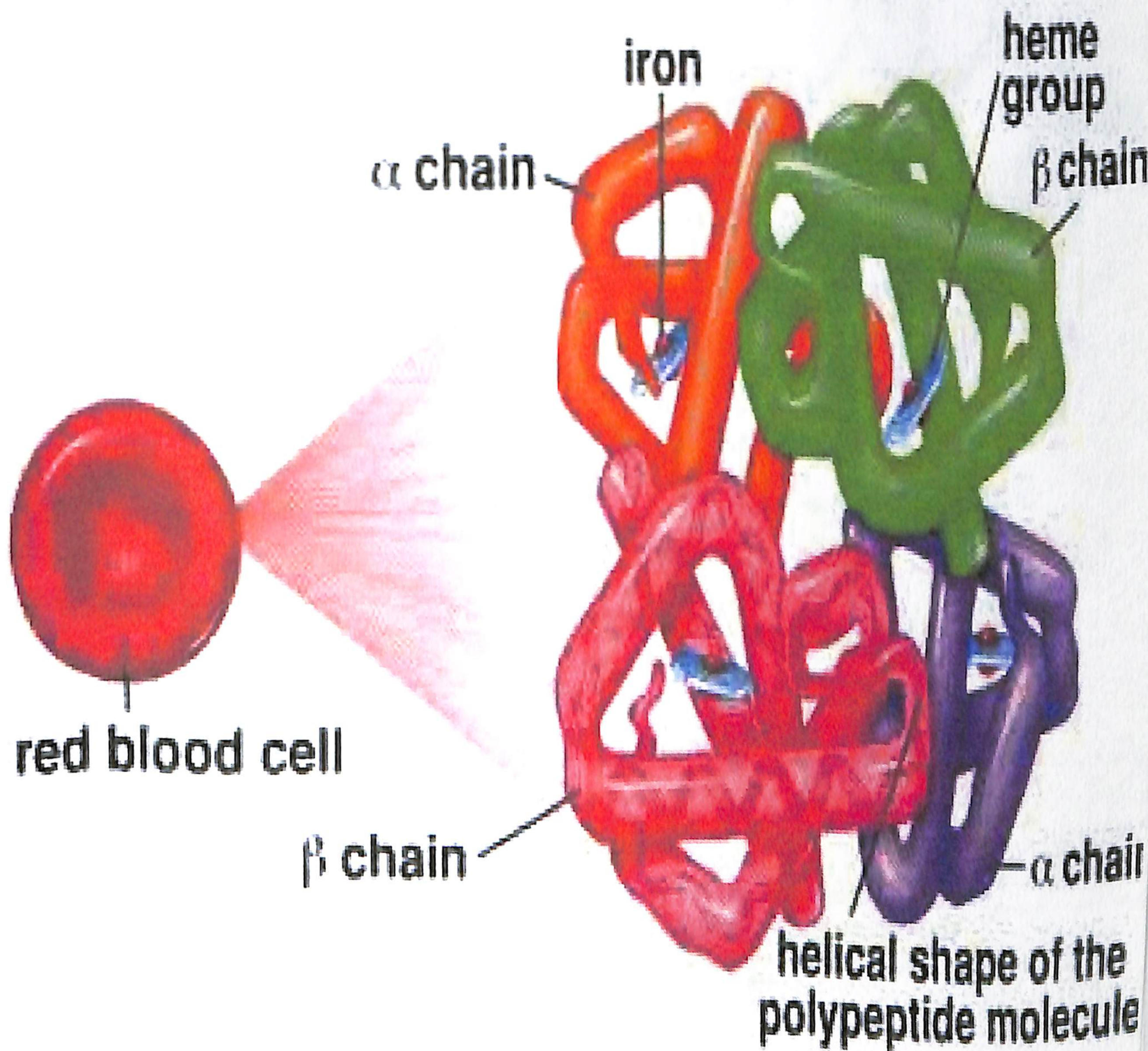

Figure 2. Molecular structure of hemoglobin as obtained from http://www.bloodless.it/

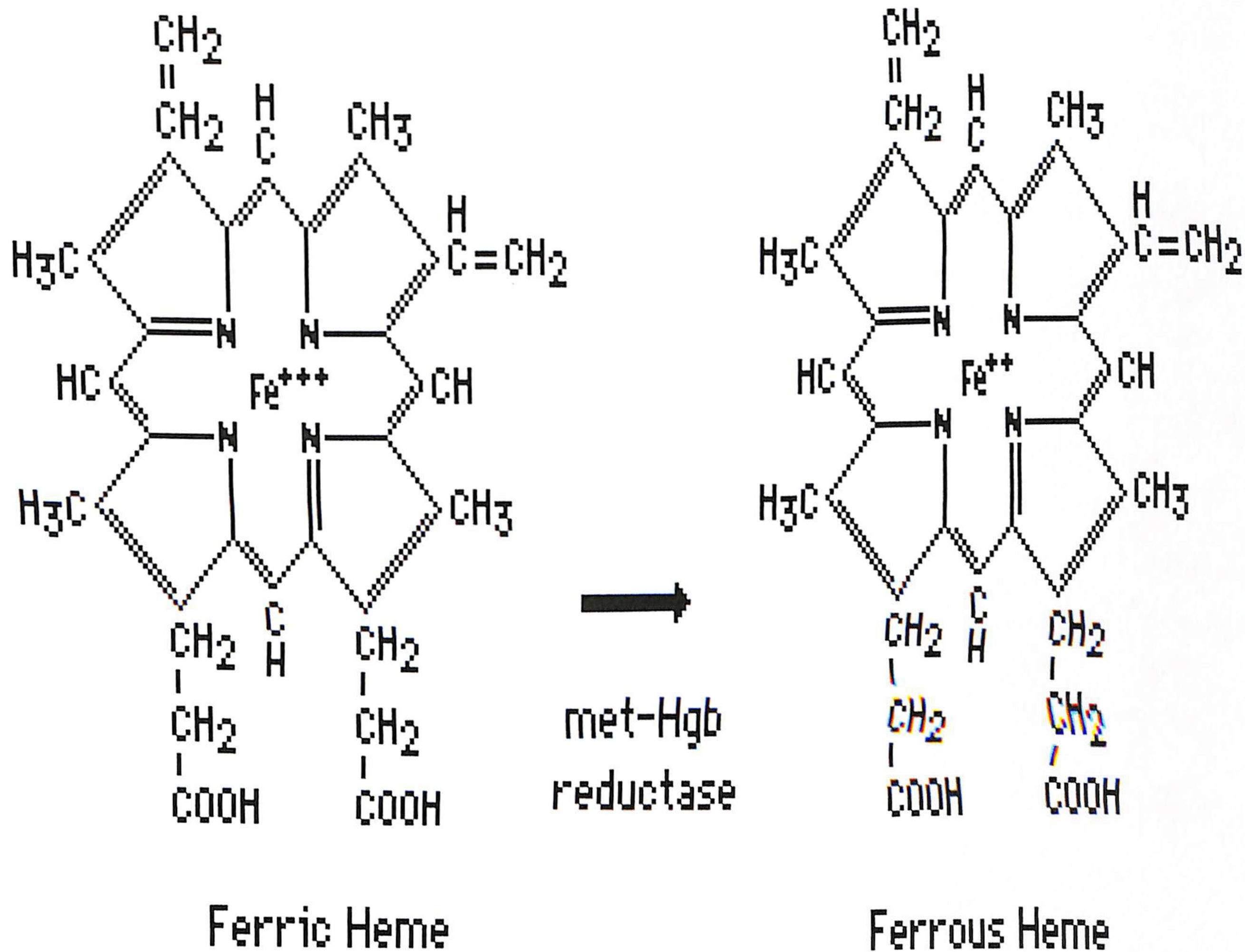

Figure 3. Chemical structure of methemoglobin (on left) as obtained from <http://www.med-ed.virginia.edu/courses/path/innes/rcd/enzyme.cfm>. Also shown is the methemoglobin reductase reaction to convert methemoglobin back to hemoglobin.

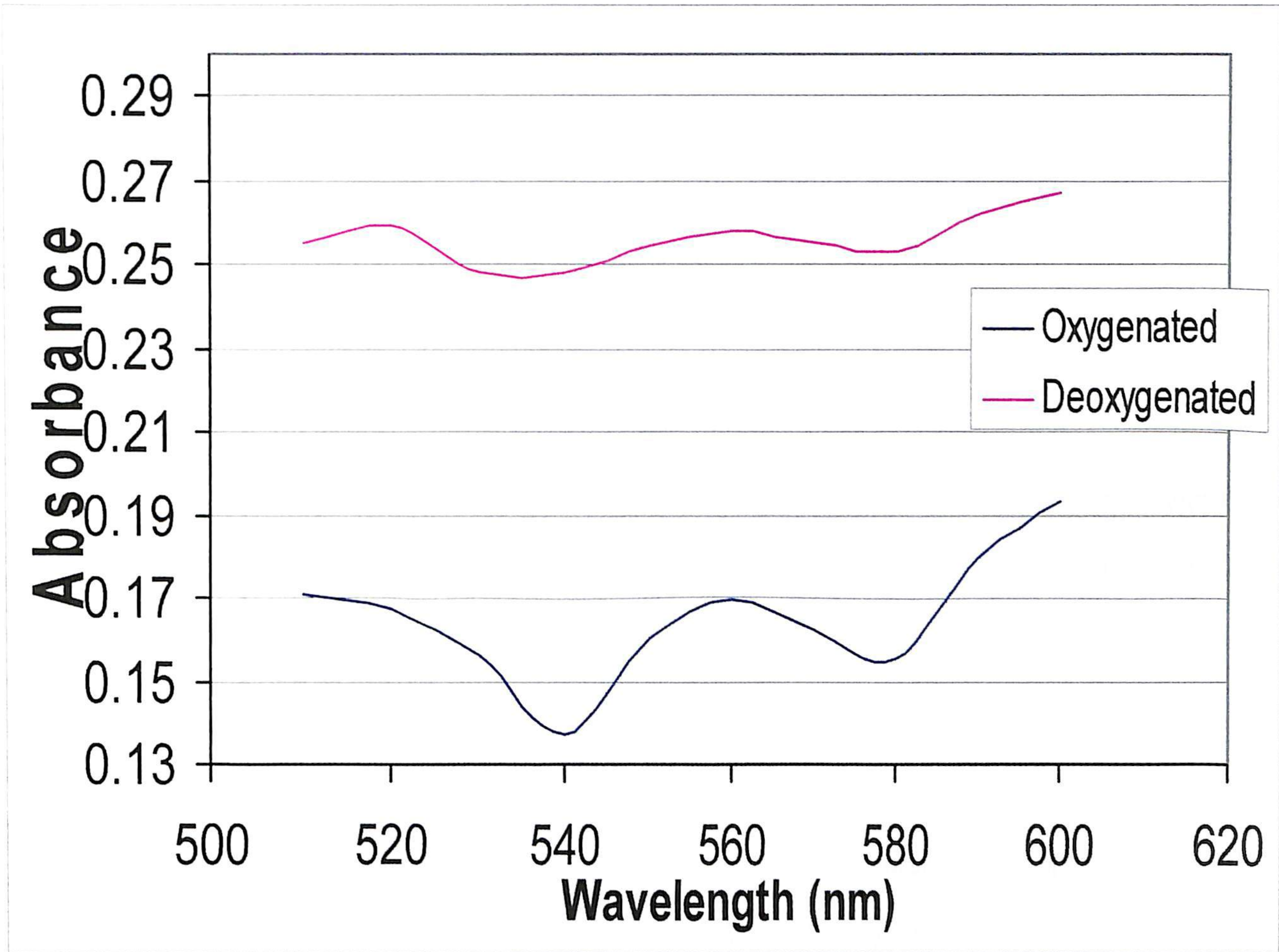

Figure 4a. Absorbance spectrum of oxygenated and deoxygenated blood in Turtle 1.

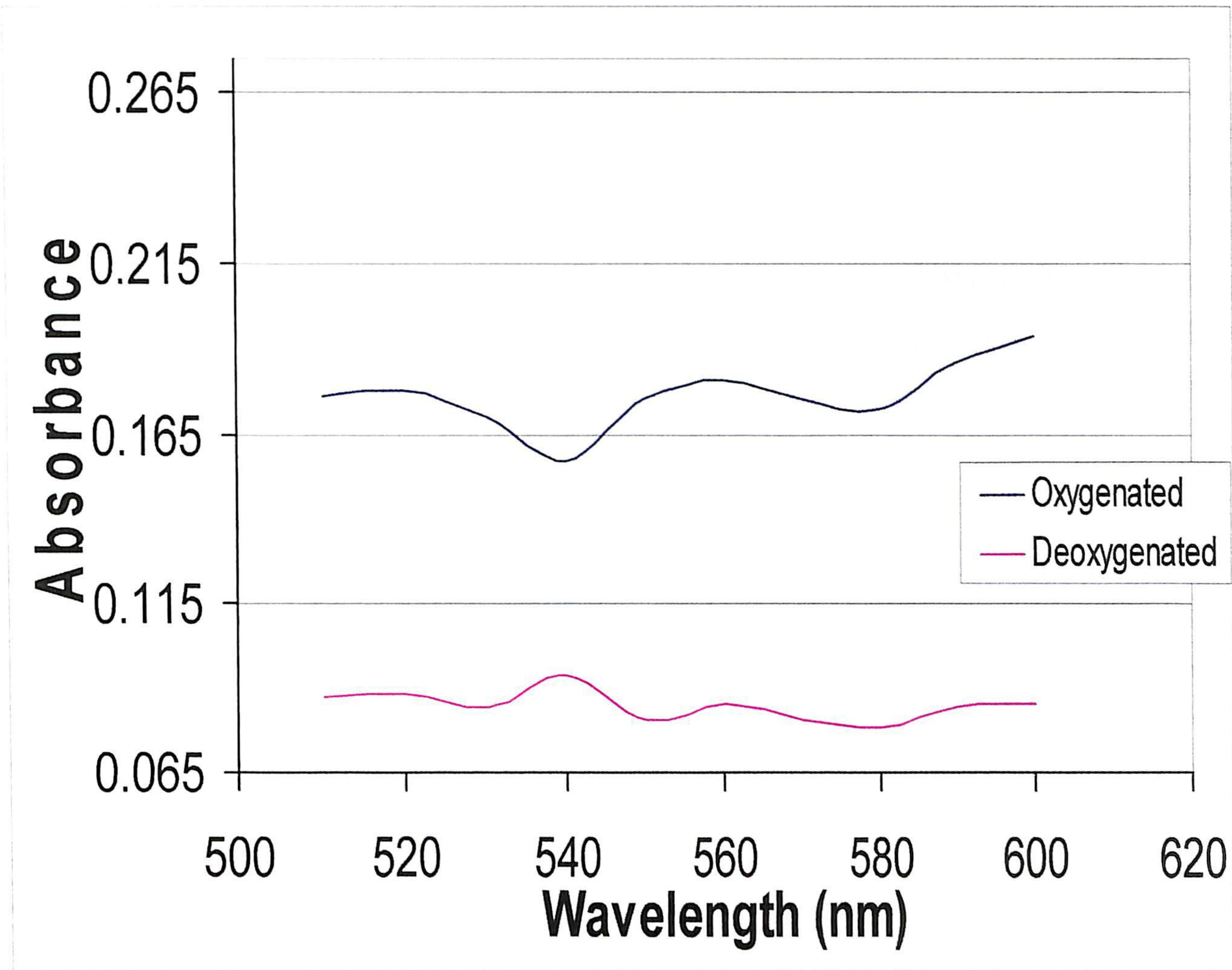

Figure 4b. Absorbance spectrum of oxygenated and deoxygenated blood in Turtle 2.

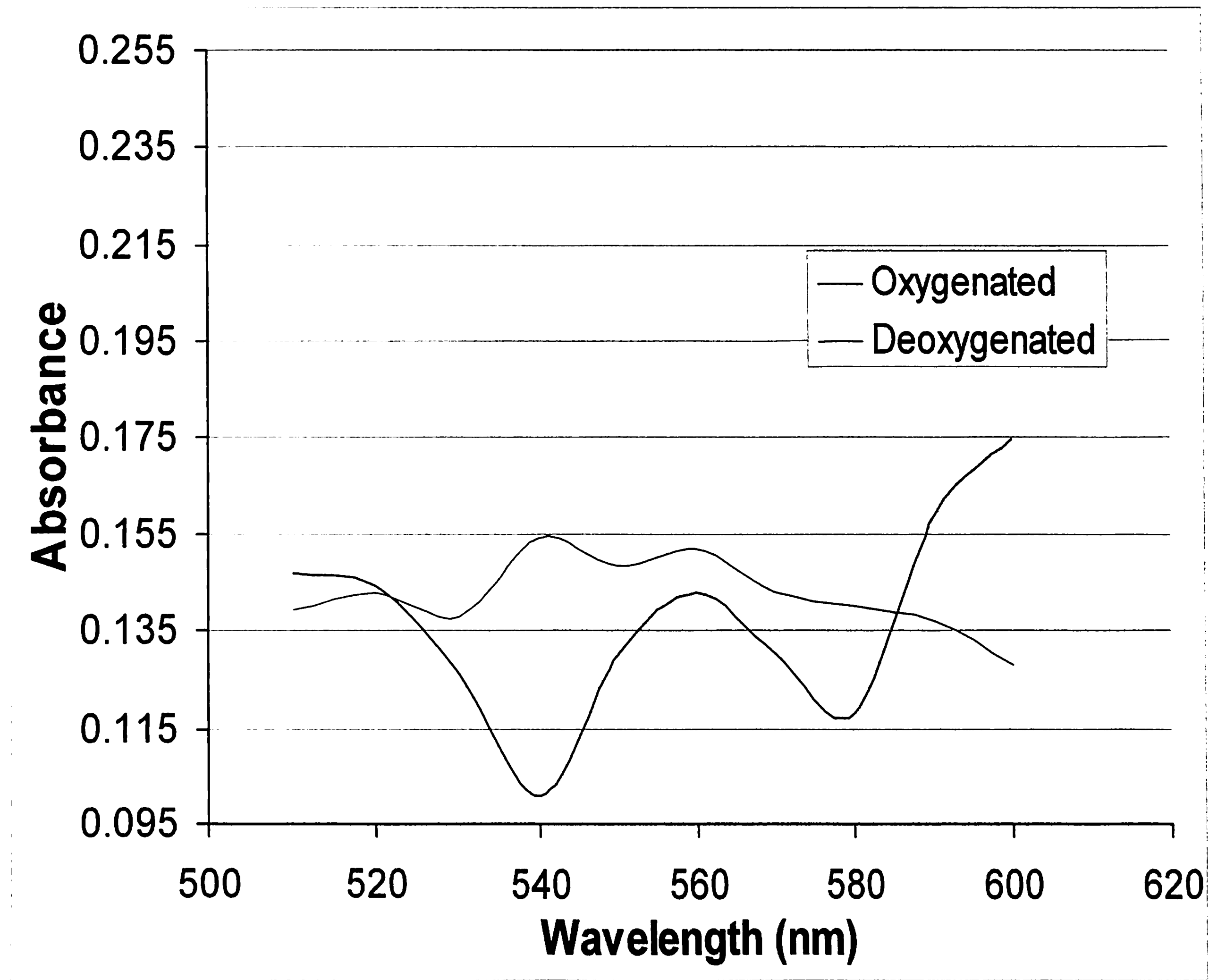

Figure 4c. Absorbance spectrum of oxygenated and deoxygenated blood in Turtle 3.

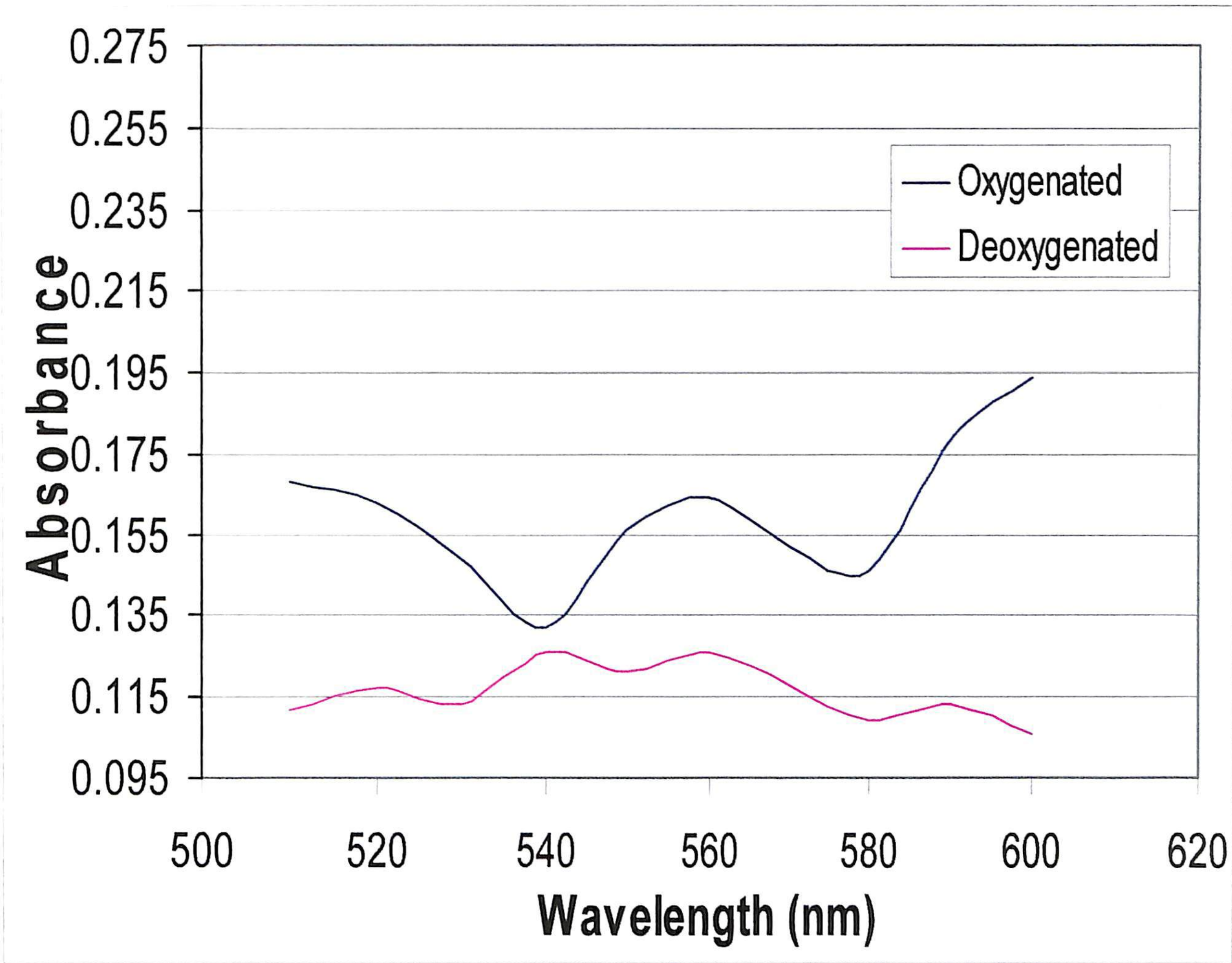

Figure 4d. Absorbance spectrum of oxygenated and deoxygenated blood in Turtle 4.

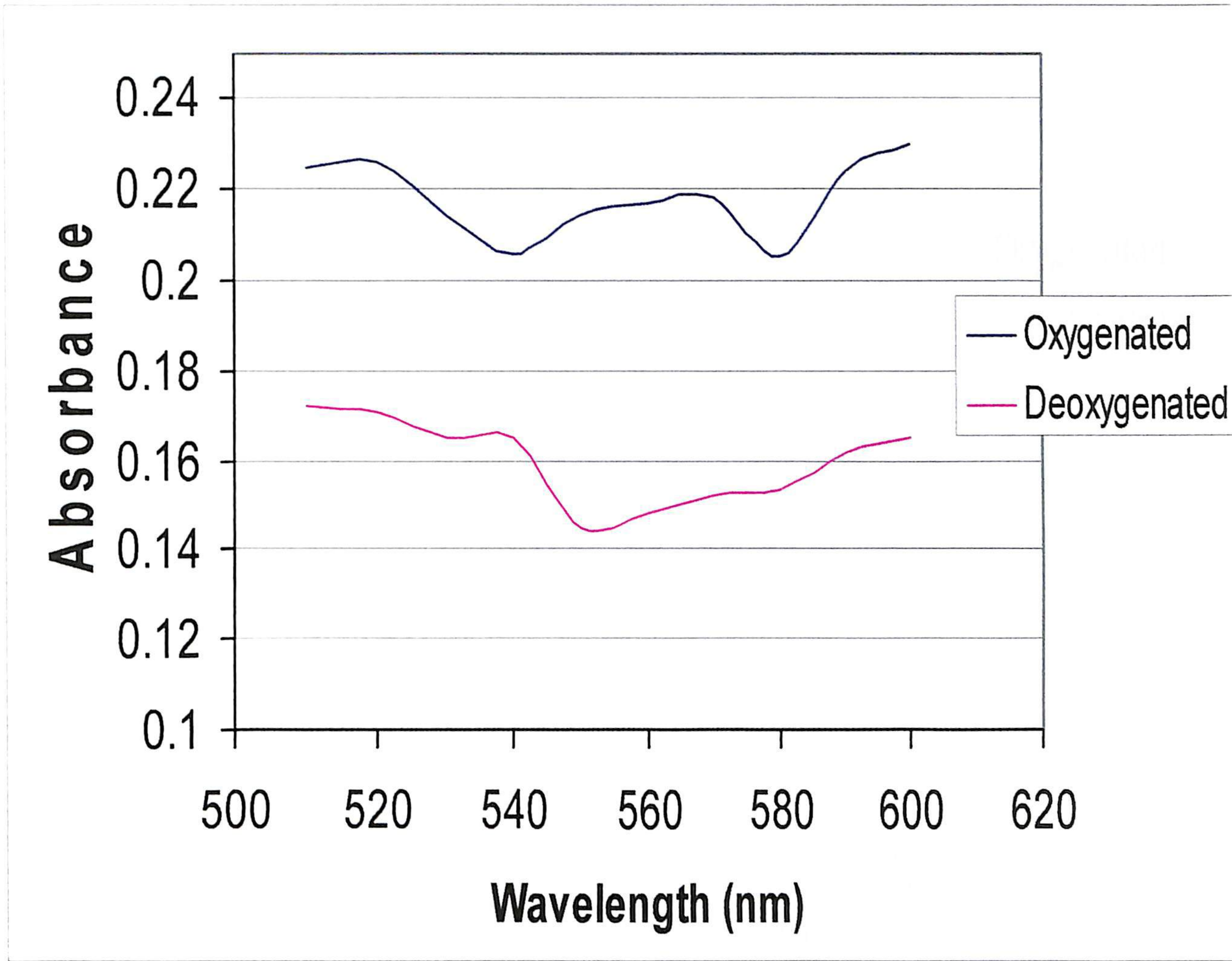

Figure 5a. Absorbance spectrum of oxygenated and deoxygenated blood in *Ovis aries*.

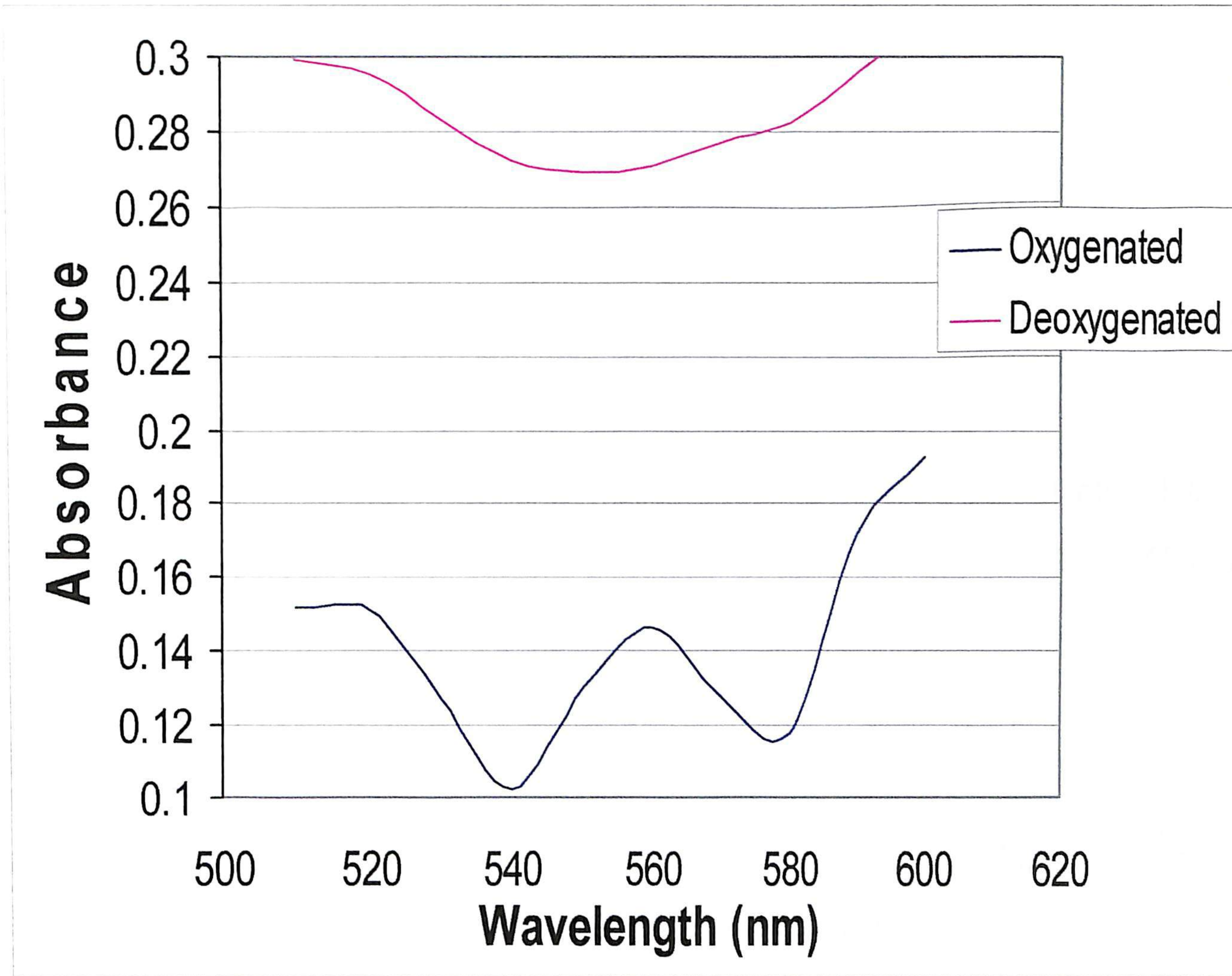

Figure 5b. Absorbance spectrum of oxygenated and deoxygenated blood in *Equus caballus*.

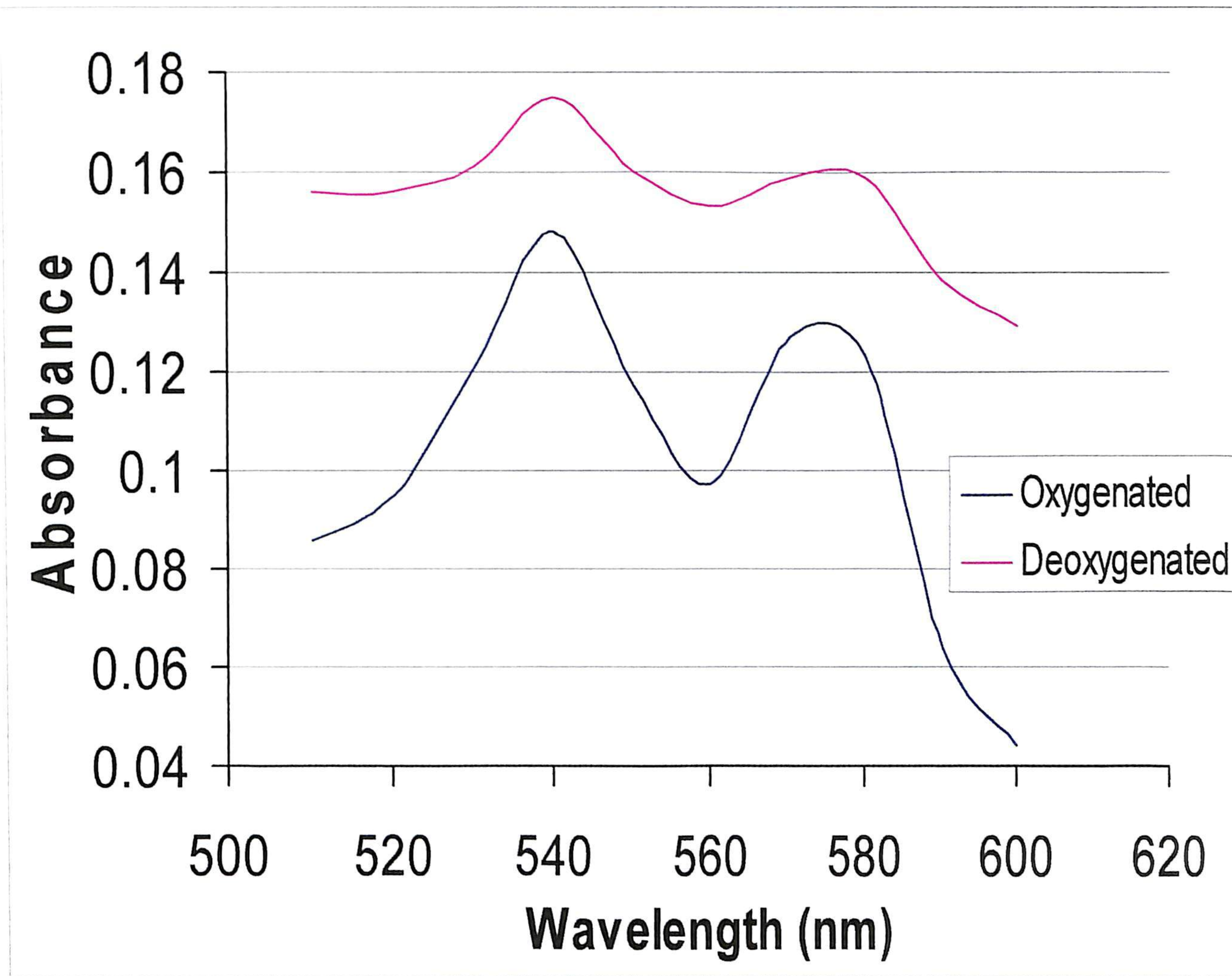

Figure 5c. Absorbance spectrum of oxygenated and deoxygenated blood in *Gallus domesticus*.