1948

Methemoglobin.

Herbert Friedman
University of Louisville

Follow this and additional works at: https://ir.library.louisville.edu/etd

Part of the Biochemistry Commons

Recommended Citation
https://doi.org/10.18297/etd/2043

This Master’s Thesis is brought to you for free and open access by ThinkIR: The University of Louisville's Institutional Repository. It has been accepted for inclusion in Electronic Theses and Dissertations by an authorized administrator of ThinkIR: The University of Louisville's Institutional Repository. This title appears here courtesy of the author, who has retained all other copyrights. For more information, please contact thinkir@louisville.edu.
UNIVERSITY OF LOUISVILLE

METHEMOGLOBIN

A Dissertation
Submitted To The Faculty
Of The Graduate School of The University of Louisville
In Partial Fulfillment of The
Requirements For The Degree
of Master of Science

Department of Bio-Chemistry

By

Herbert Friedman

Year

1948
NAME OF STUDENT: Herbert Friedman

TITLE OF THESIS: Methemoglobin

APPROVED BY READING COMMITTEE COMPOSED OF THE FOLLOWING MEMBERS:

Warren S. Rehm Jr.
Edward J. Van Loon
A. W. Homberger

NAME OF DIRECTOR: A. W. Homberger

DATE: 6-3-48
I wish to express my sincere appreciation to Doctor E. J. van Loon, Professor of Bio-chemistry, University of Louisville School of Medicine for his helpful criticism, valuable suggestions and direction, and to Doctor A. W. Homberger, Head of the Department of Bio-chemistry for the fellowship grant and the generous provision of the apparatus which made this paper possible.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Part</th>
<th>Title</th>
<th>Page Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Enzymiological Study</td>
<td></td>
</tr>
<tr>
<td></td>
<td>References</td>
<td>2</td>
</tr>
<tr>
<td>II</td>
<td>Methemoglobin</td>
<td></td>
</tr>
<tr>
<td></td>
<td>A. Preparation, Properties of and Methods of Determining MHb</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>B. Methemoglobinemia</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>C. Methemoglobin Formation</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>D. Oxidation Products of MHb - Producing Drugs</td>
<td>27</td>
</tr>
<tr>
<td></td>
<td>E. Regeneration (MHb to Hb) and Anti-Cyanide Ion Effects of MHb</td>
<td>31</td>
</tr>
<tr>
<td></td>
<td>F. Data and Discussion</td>
<td>37</td>
</tr>
<tr>
<td></td>
<td>G. Conclusions</td>
<td>48</td>
</tr>
<tr>
<td></td>
<td>H. References</td>
<td></td>
</tr>
</tbody>
</table>
PART I.

ENZYMIOLOGICAL STUDY
PART I.

Enzymological Study

The following chemical substances were prepared:

1. Hexose diphosphate (2)
2. Hexose monophosphate (2)
3. Phosphoglyceric acid (2)
4. Coenzyme I (9)
5. Hexokinase (6)
6. Cytochrome reductase (4)

Under the direction of Dr. O. Boyd Houchins these preparations were to have been used for the following:

1. To study the isolated cytochrome reductase enzyme system and the effect of α-tocopherol phosphate on that system according to the method of Haas, Horecker and Hogness (5).

2. To study the cytochrome reductase enzyme system in α-tocopherol deficiency according to the method of Potter and Dubois (2).

Due to a sudden illness of Dr. O. B. Houchins the above was terminated before any original research could be completed on the prepared substances.
2. Dubois and Potter J. Biol. Chem. 147, 41 (1943).
3. Dubois and Potter J. Biol. Chem. 150, 185 (1943).
PART II.

METHEMOGLOBIN
PART II.

Methemoglobin

For the sake of clarity, it is to be understood that the following abbreviations will be used throughout this thesis:

- Hb  reduced hemoglobin
- THb  total blood pigment
- HbO₂  oxyhemoglobin
- MHb  methemoglobin
- SHb  sulfhemoglobin
- NOHb  nitric hemoglobin
- PAP  p-aminophenol

A. Preparation, Properties of and Methods of Determining MHb.

The prosthetic group of the Hb compounds is an iron protoporphyrin termed heme. The reduced, or ferroheme, compound may be reversibly oxidized at the iron atom to ferriheme (hematin). The latter combines with acids to form ferriheme salts or with alkali to form ferriheme hydroxide. (199). Ferroheme may combine with certain nitrogenous substances (proteins, pyridine, cyanides) to form ferrohemochromogens while ferriheme forms a corresponding series of ferrihemochromogens. The combination of ferriheme and native globin has been performed in vitro and results in ferrihemoglobin, MHb. (170). Heubner recommends the term "Hemiglobin" as better than MHb. (100). Ferroheme and native globin forms ferrohemoglobin, Hb. The oxidation of Hb which results in MHb is to be contrasted with the
oxygenation of Hb which results in the formation of the addition complex, oxyferrohemoglobin, \( \text{HbO}_2 \).

Hb combines reversibly with oxygen, carbon monoxide and nitrous oxide. Through the action of strong acids or bases upon Hb the corresponding ferrihemes are formed. Oxidation with hydrogen peroxide in the presence of large amounts of cyanide or coupled oxidation with ascorbic acid results in the formation of pseudo-Hb. (15). Strong oxidation with a variety of agents changes the valence of the iron in Hb to form MHb which forms a reversible redox equilibrium with Hb. (73).

Hb and \( \text{HbO}_2 \) do not react with cyanides, flourides or azides while MHb combines reversibly with cyanides (17, 208), sulfides (200), peroxides (201), flourides (312, 382), azoimoid (337), and azides (337). Like any other ferriheme compound it may exist as the base or as the salt. (146). It does not combine with oxygen or carbon monoxide. (68, 94). Nitric oxide combines with Hb to form a very stable compound from which free Hb cannot easily be recovered. With MHb it forms an easily reversible compound which upon standing undergoes reduction to the stable Hb compound.

Colorimetric studies show that MHb in aqueous solution occurs as two forms in equilibrium. The equilibrium is dependent mainly on the hydrogen ion concentration, so that MHb may be regarded as an indicator with an interval of change between pH 6-10. (146).

While all investigators agree that MHb has less oxygen than \( \text{HbO}_2 \) and more than Hb, there is still a great difference of opinion as to whether the formula \( \text{HbO} \) or \( \text{HbOH} \) (equivalent to \( \text{Hb}_2\text{O} \)) is correct. Many observers (94, 146, 264, 268, 293, 310) have argued that MHb contains one-half the
oxygen of HbO₂. This is expressed by the formula HbO₂. On the other hand Kuester's (244) formulation of MHb as a ferri compound has been supported by the work of others. (70, 72, 245, 246, 383). In terms of oxygen content this means that MHb contains only one-fourth the oxygen of HbO₂ and was originally expressed by Kuester by the formula HbOH (Hb₂O). Conant and Fieser (70) state that the HbOH formula is in accord with their facts showing that 1 hydrogen equivalent of oxidizing agent and 1 hydrogen equivalent of reducing agent are used up in converting Hb + MHb and reversing the procedure, respectively.

The osmotic measurements of Adair (2) and the centrifuge methods of Svedberg (359) have shown that the Hb molecule contains 4 atoms of iron and has a molecular weight of 66,800. In MHb the iron is in the ferric state.

Neutral MHb has 4 absorption bands at wave length 631, 576, 540, 500. Alkaline MHb has 3 bands at wave lengths 600, 576, 540. (291). The deep seated difference in the spectrum relation of MHb in neutral and alkaline solutions is attributed to the very slight reversal to HbO₂. Denes (84) reported neutral MHb solution has a maximum absorption in the red portion of the spectrum. Ducou (94) reported that bands at wave length 633 in acid medium, 601 in alkaline solution and 607 in sodium flouride solution.

Crystalline MHb has been prepared by two methods. One depends on the oxidation of Hb by potassium ferricyanide and has been used by Hufner and Otto (177) and Jaderholm (185). The other depends on the autoxidation of HbO₂ in the presence of alcohol and has been used by Zeynek (420). Hufner and Otto (177) and Haurowitz (146) have prepared it by both methods. The starting material in either case was crystalline HbO₂ prepared by the
method of Terry and Green (365). MHb has been prepared by Hill and Holden (153) using ferric tartrate instead of potassium ferricyanide and also by Keilin and Hartree (201).

Crystals prepared by the above methods are microscopic, coffee brown, rhombic platelets appearing hexagonal because of incomplete growth at the acute angles. They are similar to HbO₂ crystals, are completely soluble in alkali and precipitated by neutralization. The solubility of the MHb on a number of phosphate buffers has been determined and shown to depend on the ionic strength of the buffer in the same manner as HbO₂. The MHb solutions remain stable in a cold room for several weeks. (226).

Various methods have been reported for the determination of blood pigments in the literature and for descriptive purposes may be divided into four categories:

1. Gasometric
2. Colorimetric
3. Gasometric and colorimetric
4. Spectrophotometric

1. Gasometric

The carbon monoxide capacity method was first suggested by Necloux and Fontes (265) and elaborated by Van Slyke and co-workers (374, 375). The method is given in detail by Peters and Van Slyke (286). The oxygen capacity method was improved by Conant and Fieser (71). The oxygen capacity is determined by the Van Slyke and Neill (377) method before and after reduction with sodium anthrahydroquinone beta sulfonate (71) or
tetanous tartrate (74).

The gasometric methods are tedious and require experience and elaborate apparatus. The oxygen capacity method is specific for MHB, but there is always the danger of the reoxidation of the laked Hb to MHB during the aeration with oxygen. This tends to make the final result too low but the error is minimized by the use of tartanous tartrate as the reducing agent. As it has been pointed out (74), the carbon monoxide methods are disturbed by the presence of other pigments. After reduction both hematin and SHb may combine with carbon monoxide and thus appear in the results as MHB. In both methods MHB is calculated from the difference between two determinations thus magnifying the errors.

2. Combined Gasometric and Colorimetric Determinations

Active Hb is determined by the carbon monoxide or oxygen combining power. Total pigment is determined by a variety of methods: colorimetric cyano-MHB (399, 414), acid hematin (146), MHB (14) or total iron (77, 104, 108, 410). The difference between the active Hb and total pigment represents MHB.

These method are not specific. If the oxygen capacity is used for the determination of the active Hb and the total iron for the Tlb, the result will represent all the iron containing pigments, MHB, SHb, hematin and some non-Hb iron. In the case of the carbon monoxide methods a variable amount of SHb may combine with the gas and be calculated as active Hb. The difficulties of the colorimetric methods have been discussed by Schwentker (332). They require fresh standards which are difficult to prepare and must be constantly rechecked. Turbidity (148), hematin or
other colored substances interfere with the cyano-MHb and MHb methods. The iron analysis are more accurate but are more difficult to conduct and generally require preliminary ashing.

3. Spectrophotometric Methods

These methods are based upon the fact that the addition of cyanide ion quantitatively converts MHb to cyano-MHb without affecting the absorption spectra of either SHb or HbO₂. Because the spectrum of MHb varies with pH and ionic strength, it is necessary to buffer the solution to a known pH and to a reasonably constant salt concentration. The buffer is also necessary to prevent the cyanide ion from changing the clarity of the solution. With these restrictions the method is simple; they allow the use of uncleared solutions and may be used in the presence of other pigments which are unaffected by cyanide ion.

Variation of these methods for the determination of blood pigments can be found in the following references: (13, 14, 41, 42, 46, 51, 56, 71, 74, 77, 91, 101, 102, 103, 104, 108, 110, 114, 118, 152, 173, 175, 176, 188, 196, 203, 235, 244, 265, 275, 286, 301, 304, 332, 349, 361, 374, 375, 376, 377, 398, 408, 410, 414).
E. Methemoglobinemia

Methemoglobinemia (MHB) is a fairly well known substance now that is has been distinguished from SHb (370), and its presence in the blood stream can be affected by a multitude of different substances. Its presence in normal circulating blood is controversial however. Ammundsen (5) found inactive or non-oxygen combining Hb (MHB) to an average of 3.5% with a maximum of 14.5% in 82 persons who had not taken any MHB producing drugs. Jung and Isserkutz (196) reported a maximum of 8% of the Thb may be present as MHB in the normal blood. Altmeiers (4) spectroscopic examinations showed that normal blood contains 1.7% MHB. Paul and Kemp (281) tested 100 patients and MHB was found in the plasma of all but one. The values ranged from 0.01-0.5 gm.% and the mean MHB was 0.09 gm.%. Records of 20 blood donors showed values of MHB ranging between 0.03 and 0.13 gm.%. Clarke et al. (61) stated that MHB was not present in dogs in detectable amounts and Dognon (91) placed the amount at less than 1% in dogs and humans. Horecker (172) prepared calf Hb with no other impurity other than 0.6% MHB.

Investigations have shown that MHB is formed during the course of putrefaction (216), in the body after death (216, 327) and in sterile blood solutions (272) which are stored under the usual conditions. The formation in the body, according to Schmidt (327), is due to increased permeability of the membranes to MHB forming substances. In the sterile blood solutions if the Hb is reduced and stored in the absence of air, the formation of MHB is inhibited or wholly prevented (272).

Talma (362) and Stadie (350) found the abnormal pigment in the corpuscles. The former claimed that MHB is not to be found in the serum and the
latter, supported by Ottenberg and Fox (277), found the plasma to be free of the pigment. Stadie (350), however, stated that MHb may exist in the blood in two distinct conditions; in the plasma alone, a true methemoglobinemia but rare, or in the red blood cells a condition of polycythemia. The latter is the usual occurrence.

Smith and Wikoff (336) suggested that formation of MHb might destroy the erythrocytes. Adams et. al. (3) showed, however, that there was no increased fragility of the erythrocytes in dogs even after administration of MHb producing drugs for three months.

The formation of MHb after poisoning was shown by Kronig (213) to be accompanied by certain morphological conditions of the erythrocytes. Hence by staining it was hoped to identify the MHb. The methylene blue-eosin and acid hemotoxylin-eosin stain showed masses of Hb and masses differing from Hb which were taken up by the leucocytes after which the conversion to MHb took place.

MHb also has a noted clinical importance with cases of methemoglobinemia being reported as early as 1886 and 1890 (219). The presence of many MHb producing substances in commonly used preparations leads to frequent cases of methemoglobinemia. Many cases have been recorded in the literature. Acetanilid usually ingested in fairly large quantities for analgesia is the most frequent cause of methemoglobinemia (28, 44, 76, 86, 107, 157, 189, 236, 248, 257a, 260, 282, 305, 314, 321, 336, 339, 352, 354, 384, 411). The physiological action of acetanilid is said to reduce temperature by converting HbO2 → MHb and interfering with oxidation (305).

Eight cases of methemoglobinemia have been shown to be due to the wearing of newly dyed shoes, the dye containing nitrobenzene (355). Similar
cases were reported with aniline dyes (405). Malden (237) and others (6, 368) showed that both aniline and nitrobenzene workers showed symptoms of methemoglobinemia from time to time. Loeb et. al. (231) indicate a long line of nitrobenzol poisonings producing methemoglobinemia. Harrington (141) showed that poisoning by aniline and its derivatives may occur through the unbroken skin, by direct contact or from saturated clothing, by the inhalation of vapors or the swallowing of particles with food or saliva.

Clark and Paul (57) reported the case of a student who spilled a hot solution of metanitroaniline and metadinitrobenzene on arms and legs at noon. By 11:00 P.M. methemoglobinemia developed to a point of a 60% conversion of the THb.

Many other cases of methemoglobinemia are recorded in literature due to such varied causes as the sulfonamides (45, 258), antikamnia and phencatine (354), orangiene powders (254) and lobar pneumonia(284). While recovery in 24-48 hours is the general rule (135), some deaths have been recorded. (76, 254, 314, 321).

The symptoms of methemoglobinemia are numerous; the most common, however, is cyanosis. This cyanosis has been attributed to MHb (4, 390, 402), SHb, an unusual degree of unsaturation of venous blood (258), presence of aniline black (54, 238, 417) and the colored derivatives of the dyes ingested (11, 206). Clinically Garvin (113) reported cyanosis in 90% of the cases treated with sulfanilamide at the Cleveland City Hospital. Maximum MHb produced was 6.3 gm.% against a THb of 17.0 gm.%. Snodgrass and Anderson (345) reported cyanosis in 29.6% and Colebrook and Purdie (65) 50% in cases treated similarly. Snapper (344) reported severe cyanosis in patients with diseased livers who ingested phenacetin.
An early work on acetanilid indicates that MHb plays a role in the formation of cyanosis. Evidence has accumulated to show that MHb could be demonstrated in the blood several hours after ingestion of acetanilid and has been demonstrated spectroscopically (44, 85, 92) and by the oxygen carrying capacity method (236). At this stage phenols which can be detected in the blood stream are at their highest levels and cyanosis is deepest, while at a later stage it may be impossible to demonstrate the spectrum of MHb though cyanosis is still observed (282). The persistent cyanosis observed when these MHb producing drugs are ingested for long periods or in large doses have been described variously as livid blue, tending to chocolate or reddish brown (143, 283), grey or slaty in color, and gives the body an appearance similar to that seen under ultra-violet light. Following ingestion the livid cyanosis develops and at this stage MHb is undoubtedly a factor, while as the drug is eliminated the livid blue gives way to a muddy grey which persists long after the MHb has disappeared. Small amounts of PAP in the tissues have been repeatedly noticed and some observers account to it for this condition. (248, 417). Kobert (209) reported that if acetanilid is not ingested but introduced parentally, it may not be possible to detect the presence of MHb even though cyanosis is seen.

At this point it should be noted that the spectroscopic method of MHb detection is sometimes ineffective at lower levels of MHb concentration. Altmeyer (4) showed that non-cyanosed patients receiving suffanilamide had had 3% MHb; cyanosed at least 6.3%. Since the spectroscopic method is ineffective at these levels, it would appear that although evidence points to the cyanosis being due partly to metabolized products of the ingested drugs,
MHb probably plays a greater role than the foregoing observations would ordinarily indicate.

Stadie's (348) observations on cyanosis in cases of lobar pneumonia indicated that MHb played a very small role in its production. A definite relation between the degree of cyanosis and the per cent of arterial unsaturation was shown. When the former increases the latter increases, and the venous unsaturation varies similarly. Since the total oxygen capacity was high in all the cases, the author felt that the MHb played a minor role in the causation of cyanosis.

The following symptomatology has been described as characteristic of methemoglobinemia (61): Cyanosis and appearance of MHb in the blood, headache, dizziness, mental confusion, muscular weakness, and in more severe poisonings ataxia, impairment of consciousness and coma. The pulse is usually thready and weak, blood pressure usually decreased. Other symptoms have been described. (25).

Meulengracht and Lundsteen (248) and others (10, 92, 342, 417) claimed the development of an anemia which disappeared slowly when the drugs were discontinued. Malden (237) showed that aniline and nitrobenzene workers had normal or high red blood cell count with a decrease in Hb of 5-50% based on specific gravity tests. Production of anemia, however, is contested by Morgan and Anderson (257a).

Various affects on the urine have also been described. Nadler (260) claimed the presence of acetanilid though this is extremely doubtful. Probably acetanilid was detected in the body by the indophenol test on the urine. This is further borne out by the experiments of Smith (339) in which no acetanilid was found in the feces or urine indicating complete absorption.
The urine has been described to be normal in color, black (231), dark reddish brown (352), dark reddish amber (157) in various cases of methemoglobinemia. Payne (282) described conjugated PAP as forming a wine colored urine which turned dark brown or black upon standing. Young (417) noted changes in the urine after aniline ingestion from dark brown to dark red and then almost black.

The blood is usually brown or chocolate and turbid both in vivo and in vitro (38, 97, 107, 130, 141, 282, 417), does not become red upon shaking or aeration (277, 283, 350) and may be easily distinguished from normal oxygenated blood.
C. Methemoglobin Formation

MHb may be produced by a considerable number of drugs and toxic agents. (286, 400). The various MHb forming agents differ in the amounts of MHb they produce, as well as the speed with which they form the abnormal pigment. Formation also varies with the species.

Presented here is a Table based on a review of the literature of those compounds which have been tested for their ability for form MHb.

A. Benzamine Group


2. p-amino-propiophenone - (315, 363)

3. p-aminobenzophenone - (372)

4. p-aminoacetophenone - (372)

5. Aniline - (58, 61, 80, 82, 123, 143, 150, 152, 192, 223, 237, 262, 309, 311, 324, 331, 340, 350, 372, 378, 418, 289*, 418*, 419*)

6. 2 - aniloothanol - (19)

7. Dimethyl aniline - (80, 174, 309, -159*)

8. Alpha or beta - napthylamine - (80)

9. p-nitroaniline - (80)

10. Monoethyl aniline - (309)

11. Trichloroaniline - (309*)

12. Phenacetin - (39, 98, 123, 159, 184, 350, -159*)

13. Acetophenetidine - (159, 220, 221, 309, 373)

15. Hydroxylamines - (14, 45, 80, 130, 166, 183, 193, 223, 229, 287, 309, 313, 325, 350.)

16. Hydroxylamine hydrochloride - (309)

17. Phenylhydrazine hydrochloride - (262)


ortho - (79, 80, 131, 132, 159, 287.)

meta - (159)

19. Diazidophenyl sulfone, o-aminophenol - (131, 132)


21. Hydrazobenzene - (164)

22. Hydroxyacetamide - (1)

23. Toluidines - (309, 350)

24. 4:4 - Diaminophenyl sulfone - (309)

25. 4:4 - Diacetaminodiphenyl sulfone - (309)

26. Phenazine - (229)

27. p - phenylamine - (309*)

28. iso - amylamine - (309*)

B. Aromatic Nitro Compounds


   a. Nitrobenzol - (130, 80*)

   b. Meta-nitro-aniline - (57)

   c. Meta-dinitro-benzene - (57, 169)

- 16 -
2. Nitroglycerine - (130, 195, 276)
   a. Glycol dinitrate - (195)
   b. Glycol mononitrate - (195)
3. Nitrotoluenes - (195, -80*)
4. Nitroxylenes - (195)
5. Dinitro-phenol - (80*)

C. Sulfonamides
1. Sulfanilamide - (21, 45, 65, 109, 142, 181, 191, 212, 279, 309, 341, 381, 398, -49*.)
   a. Sulfapyridine - (212, 341, -50*.)
   b. Sulfathiazole - (212, -50*)
   c. Sodium sulfanilamide - (80*)
   d. Disulphanimide hydrochloride - (309*)
2. Prontosil - (64, 309, -48*.)
3. 2p-aminobenzenesulfonamide o-pyridine - (45)
4. Sulfanilic acid - (309*)
5. p-aminobenzoic acid - (309*)

D. Other Organic Compounds
2. Ether or chloroform - (97, 136, 146, -194*.)
3. Pyridine - (307)
4. Pyrogallol - (159, 180, 350, 358)
5. Azo-compounds (350)
   a. 3:3 - dinitroazoxybenzene - (156)
6. Oxidation product of oleic and linolic acids - (271)

   turpentine  
   cod liver oil  ) - (271)
   linseed oil  )

7. Adrenochrome - (379)

8. Formaldehyde - (350)

9. Plasmochin - (80)

10. Promin - (80)

11. Ferric tartrate - (153)

12. Sodium sulfanilate - (80*, 309*)

13. Aspirin - (39*, 382*, 416*)

14. Resorcinol - (159*)

15. Phloroglucinol - (159*)

16. Uliron - (309*)

17. Sinzedine - (309*)

E. Inorganic Compounds

1. Ferricyanides - (9, 67, 146, 153, 161, 167, 180, 245, 246, 250, 303, 350, 378, 379, 80*)


   a. Nitrates - (80, 195, 350)

3. Arsine - (114, 146, 229, 409*)

4. Ozone - (253, 350, 80*)

5. Peroxides - (201, 335, 350, 146*)

6. Iodine - (350)

- 18 -
7. Ammonium hydroxide - (225)
   a. Ammonium sulfate - (350)
8. Chromates and Dichromates - (242, 262, 294, 296.)
10. Sodium chloride - (350)
11. Permanganate - (146, 350)

F. Bacteria
   1. Pneumococcus - (143, 121, 126, 269, 283, 350.)
   2. Gaertner bacillus - (27, 350)
   3. Nitrobacilli - (384)
   4. Streptococcus viridans - (350)
   5. Cholera vibrio - (350)
   6. Peroxide forming bacteria - (270)

*(All references marked by an * have shown an inability to produce MHB under the experimental conditions of the particular observer).

Any attempt to discuss fully the entire experimental data represented in the preceding Table would be too extensive for this paper. Therefore, only the more significant works will be discussed.

I. Acetanilid

In the experimentation on MHB production by acetanilid three points stand out summarily describing its action in the body:

1. MHB is formed only after certain minimal doses are exceeded. The minimal or threshold dose varies with animals. (220).
2. The amount of MHb does not increase in direct proportion to increase in dose. (168, 330, 399).

3. There is an upper limit to the formation of MHb regardless of the dosage. (58, 85, 98, 330). Administration of amounts beyond that produce no greater concentration of MHb, but only increases the length of time the MHb persists.

Lester (220) arbitrarily placed the threshold dose for man at that amount of the drug which would produce an average maximum of 0.4 gm. of MHb corresponding to 2.7% MHb at a THb of 15.0 gm.

Since it has been conclusively proven that acetanilid produces MHb in vivo not not in vitro, it might be well to cite here some of the experimental data obtained in controlled poisonings of laboratory animals.

A group of rats were given 19 mg./kgm. of acetanilid for 13 weeks with no ill effects. With an increase in dosage to 400 mg./kgm., growth was retarded, MHb produced and death occurred in 9 out of 26 cases. (342).

The work of Van Loon, et.al. (373) showed no accumulation of MHb in dogs with administration of doses of acetanilid up to 72 mg./kgm. for 18 days. Larger doses, 250 mg./kgm., produced temporary methemoglobinemia and a decrease in THb. The lethal dose with intravenous injection, however, was previously shown to be between 350 and 500 mg./kgm. (60).

Helms (154) states that doses up to 1000 mg./kgm. are not fatal, and that the lethal dose for most laboratory animals is around 1500 mg./kgm. Twelve grains daily for 16 weeks had no effect upon human subjects.

Lester's (221) experiments on human subjects showed that acetanilid given in 3 doses of 0.5 gm. each day caused only a slight additive rise
in MHb within the day but a marked rise when given in 1 gm. doses. There was no accumulative formation of MHb when 1, 2 and 3 mg. were given daily for 5 weeks.

II. Aniline

The work of Clark, Van Loon and Morissey (61) would indicate a striking similarity of action between acetanilid and aniline. It was shown that progressively greater amounts of Hb were converted by increasing dosage to 100 mg./kgm. producing a maximum methemoglobinemia of about 70%. These responses are linear to the log of the dose but does not hold for larger doses. 200 mg. produced a maximum methemoglobinemia of 75% which was not increased by still larger doses of 400-1000 mgm. whether given orally or intravenously.

In general the larger the dose of aniline, the more rapid the increase and the longer the duration of the methemoglobinemia; thus doses of 200 mg. or larger maintained the concentration of MHb of 70-75% until death of the animal 10-20 hours later. Since the formation of MHb is reversible, it is indicated that the larger doses prolong the time during which MHb is formed and the reversal does not occur within the survival time of the animal.

It had been previously noted that the formation of MHb by aniline was a slow process, a reaction with a definite time log. Van Slyke and Vollmund (378) showed that no MHb was formed by 4 molecules at 38°C until after 2 hours and none by 2 molecules until after 5 hours in vitro. This would indicate that aniline, like acetanilid, does not produce MHb directly but rather from products formed from it by the liver. Aniline mixed
with whole blood without a tissue suspension does not form MHb. (252).

Maximum MHb production in dogs was found to be 70-75% of the THb at a minimum dosage of 200 mg./kgm. and is not increased by greater dosage. Somewhat higher concentrations have been produced in vitro. (61). It is suggested that the limitation on MHb formation is due to an equilibrium between the oxidation reduction potentials of the products of aniline (p-aminophenol-quinamine system) with the MHb-Hb system. (168).

III. Aminophenols

Experiments with the derivatives of o-, m-, and p-aminophenol show that the meta derivative is barely poisonous and the the ortho derivative is more poisonous than the para. (159, 287). The aminophenols can penetrate the red blood cell membrane to produce methemoglobinemia. (23).

It was determined that the quinimine form of p-aminophenol acted immediately to a maximal extent while the PAP took some time to do so. The former is believed to be the active MHb producer, and this oxidized form reacts with the Hb of the red blood cells while the PAP remains in the reduced stage. (23).

Since each molecule of aminophenols reacts several times with Hb molecules, the reaction between the oxidized form of PAP and Hb should result in the formation of more PAP and MHb. (159).

Toxicity studies in the laboratory were conducted by Brownlee and Gaddum (40) and show the following results on rats:

<table>
<thead>
<tr>
<th>PAP - mgm./kgm.</th>
<th>Mortality</th>
</tr>
</thead>
<tbody>
<tr>
<td>700</td>
<td>12/12</td>
</tr>
<tr>
<td>650</td>
<td>10/12</td>
</tr>
<tr>
<td>600</td>
<td>8/18</td>
</tr>
<tr>
<td>550</td>
<td>1/6</td>
</tr>
<tr>
<td>500</td>
<td>0/12</td>
</tr>
</tbody>
</table>
IV. Sulfanilamide

A total of 960 blood samples were taken from 476 patients receiving sulfanilamide and were examined for sulfanilamide and MHB. The bloods showed that the higher the content of the former, the greater the concentration of the MHB. The average MHB content was proportional to the sulfanilamide concentration. After a single dose of sulfanilamide the maximum MHB occurred several hours after maximum blood sulfanilamide peak. It seems that an active substance is normally produced in the course of sulfanilamide metabolism which causes the production of MHB. (142).

V. Ferricyanide

Ferricyanide produces MHB quite readily but is unable to pierce the membranes of the cells, therefore the blood must be laked. (378). Spectrophotometric and gasometric determinations have shown that 1 molecule of ferricyanide is required to expel 1 molecule of oxygen from HbO2. (9, 67, 246, 250, 303, 350, 378). The reaction may, therefore, be expressed:

\[
\text{Hb} + \text{K}_3\text{Fe(CN)}_6 + \text{H}_2\text{O} \rightarrow \text{HbOH} + \text{K}_3\text{HFe(CN)}_6 + \text{O}_2.
\] (303).

VI. Nitrites

Action of nitrite on Hb is extremely complicated. It is reported to vary with the molecular ratio of nitrite to Hb, pH, presence or absence of oxygen and reducing agents and possibly others. Among the products of reaction found under varying conditions in vitro are MHB, NOHb and NO-MHB. Widely different values have been reported for the amount of MHB formed to nitrite.
### Table

<table>
<thead>
<tr>
<th>Nitrite</th>
<th>MHb</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>0.5-0.7</td>
<td>0.5-0.7</td>
</tr>
<tr>
<td>0.5</td>
<td>0.5</td>
</tr>
</tbody>
</table>

A source of error in the above reports was lack of analyses for residual nitrite. The in vitro work of Greenberg, Lester and Haggard (125) indicated this. Their work can be summarized as follows:

1. Ratio of nitrite and MHb is 1:2. That is, 1 molecule of nitrite reacts with 2 molecules of Hb to produce 2 molecules of MHb.

2. Temperature, concentration of nitrite and pH are without influence upon the ratio of nitrite utilized to MHb formed.

3. In an acid medium the reaction of nitrite with Hb is complete in a short time.

4. In a neutral or slightly alkaline medium the reaction may take many hours for completion, depending on concentration of nitrite.

5. The amount of MHb formed per molecule of nitrite utilized cannot be determined directly in vivo, but it is probably the same as in vitro.

Hanzlik (137) reported that nitrite action on Hb is largely a function of pH with increasing efficiency in an acid solution up to pH 5 (with increasing acidity). The reaction was reported in a ratio of 1:1. At pH 7.0 he reported an increase in the amount of molecules of nitrite necessary to convert Hb to MHb and true alkalinity allows minute changes.

Brooks (31) reported that in the presence of a reducing agent, nitrite formed with Hb the same compound as results from the action of nitric oxide on reduced Hb: 1 molecule sodium nitrite completely converted 1 equivalent
of reduced Hb to NOHb. In the absence of both oxygen and a reducing agent, 1 molecule sodium nitrite combined with 2 equivalents of reduced Hb to produce 1 equivalent of MHB and 1 equivalent of NOHb. Meier (247) previously had also stated that nitrite action on HbO₂ was influenced by pH. He reported little change in an alkaline solution even if the nitrite was present in great excess. In a neutral or weakly acid reaction only MHB is formed if there is a definite molecular relationship between nitrite and blood pigment. Below this concentration relationship, HbO₂ is present along with the MHB, and above it both MHB and NOHb are present. Presence of NOHb as well as MHB in the reaction of Hb and nitrite had already been noted, (130, 145). In a molecular relationship where only MHB is produced about one-fourth of the oxygen bound to the HbO₂ is liberated, with a corresponding change in nitrite. The reaction may be expressed -

\[ 4 \text{HbO}_2 + 4 \text{HNO}_2 + 2 \text{H}_2\text{O} \rightarrow 4 \text{HbOH} + 4 \text{HNO}_3 + \text{O}_2 \]

The NOHb appearing in the presence of an excess of nitrite is due to a further breakdown of nitric acid into nitrite and nitric oxide with a relative deficiency of oxygen in which the MHB is reduced. In the absence of oxygen MHB and NOHb are formed from reduced Hb independently of the reaction. During the acute intoxication of cats with nitrite pronounced MHB formation occurs. Upon standing the blood contains only NOHb which is due to the fact that the reducing substances of the blood or bacterial oxygen becomes operative, reacting in the same way as a chemical reducing agent.

Marshall and Marshall (239), in experiments performed with crystallized ox HbO₂ in buffered solutions of sodium nitrite, reported the action
of the nitrite on blood shows three periods: induction, reactionary and stationary. The action is due to hydrolyzed nitrous acid, the primary effect being the formation of MHB which results from the hydrolysis of nitrogen peroxide. The formation of NOHB is a consecutive action resulting from the action of nitric oxide on reduced Hb. The action is facilitated by the presence of reducing agents which increase the nitric oxide pressure and cause reduction of the HbO₂ or MHB.

On the amount of MHB formed by nitrite in vivo, Wendel (399) reported that 30 mg./kgm. produced a 60-70% decrease in oxygen capacity in 60-100 minutes. Similar results were reported by Darling and Roughton (82). Hug (181) reported maximal appearance of MHB after injection of sodium nitrite in 15 minutes.

Nagazumi (262) and Jung and Bredow (195) reported sodium nitrite to be a more intense MHB former than many other substances. The former reported 87% MHB produced by sodium nitrite in white rats. The minimal lethal dose in rabbits was reported at .0876 gm./kgm. (297,300) with death occurring when 80% MHB was formed. Rappaport (300) reported 99% of Hb → MHB conversion with higher doses.
D. Oxidation Products of MHb - Producing Drugs

The amount of MHb found in the blood at any time is resultant of the breakdown of such substances as aniline and acetanilid and the production of their oxidation products in the body by the liver (24). Below is a diagram which indicates the direction of the normal breakdown of some of the benzamine group of drugs.

\[
\begin{align*}
\text{(Benzene)} & \rightarrow \text{(Phenol)} & \rightarrow \text{(Catechol)} & \rightarrow \text{(ortho-Quinone)} \\
\text{(Acetanilid)} & \rightarrow \text{(Aniline)} & \rightarrow \text{(PAP)} & \rightarrow \text{(Hydroquinone)} & \rightarrow \text{(para-Quinone)} \\
\text{(Phenacetin)} & \rightarrow \text{(para-Iminoquinone)}
\end{align*}
\]
It is well known that the presence of an amine group on the benzene ring greatly increases the ease with which the structure can be oxidized. The reactions are logical and sound chemically, but are theoretical physiologically in that they have not been proven to take place in the animal organism. The oxidation of acetanilid, aniline and phenacetin to PAP both in vivo and in vitro is well established as the compound has been isolated from the urine (124, 252, 417, 419) and blood serum (417, 419) of patients receiving these drugs. The oxidation of PAP to quinone is easily carried out in vitro but has not yet been demonstrated in vivo.

Many observers have stated p-aminophenol to be the active MHB producer in aniline or acetanilid poisoning (22, 83, 123, 138, 223, 251, 289, 302, 324, 326, 353, 404, 413). The work of Heubner and associates (159, 166) working with the simple aniline molecule most clearly substantiate this opinion. Although phenylhydroxylamine occurs as an intermediary in the oxidation of aniline, it is not considered to be the actual MHB former because of the ease with which it is irreversibly converted in vivo into azoxybenzene (45). This is contrary to the observers who attributed the MHB forming action to hydroxylamines (98, 122, 135).

When aniline was given to cats by subcutaneous injection of an aqueous solution, it was demonstrated that eight times the theoretical molar equivalent of MHB was formed at which stage an equilibrium was established when 37% of the total blood pigment was oxidized. This justifies Heubner's convictions that phenylhydroxylamine was not responsible, but that the oxidation reduction system set up by p-aminophenol and p-iminoquinone was
the effective agent. Iminoquinone would be capable of oxidizing 1 molecule of Hb to MHb being itself reduced thereby; it could again be oxidized in the blood when it would be available to start again.

The catalytic conversion, as shown below, would proceed until an equilibrium was reached.

\[
\begin{array}{c}
\text{NH}_2 \quad \text{NH} \quad \text{NH}_2 \\
\text{-H}_2 \quad + \text{Hb} \quad + \text{MHb} \\
\text{OH} \quad \text{O} \quad \text{OH}
\end{array}
\]

To fill the roll in this scheme a drug containing an aromatic amino group with a hydroxyl radical in the o- or p-position, or with a potential source, is required. (39).

Wendel (391) in his work on the production of MHb by methylene blue expounded a tentative theory on the mechanism of its production. Some reducing substance (inductor) present in the corpuscles is oxidized by methylene blue. The leuco-methylene blue forms a peroxide-like compound with HbO\(_2\). This complex peroxide effects the oxidation of lactic to pyruvic acid and is itself thereby converted to MHb, methylene blue and oxygen somewhat as follows:

\[
\text{Inductor} + 4 \text{MB} \rightarrow \text{Oxidized inductor} + 4 \text{MBH}_2
\]

\[
4 \text{MBH}_2 + \text{Hb (FeO}_2\text{)}_4 \rightarrow (\text{MBH}_2)_4 \text{Hb (FeO}_2\text{)}_4 \text{ (complex peroxide)}
\]

\[
(\text{MBH}_2)_4 \text{Hb (FeO}_2\text{)}_4 + 2 \text{lactate} \rightarrow 4 \text{MB} + \text{Hb (Fe}^{+++}\text{)}_4 + 2 \text{pyruvate} + 6 \text{H}_2\text{O} + 20^*
\]
The features of this scheme are:

1. An inductor, the oxidation of which by methylene blue forms carbon dioxide and the leuco-dye.

2. Reversible action of the dye.


4. The stoichiometric relation between lactic acid oxidized and Hb destroyed.

5. The sum of the oxygen required by these processes is one-half of the total oxygen consumption.
E. Regeneration ($\text{MHb} \rightarrow \text{Hb}$) and Anti-cyanide ion Effects of MHb

In almost all experimentation on the production of MHb in vivo there has been noted a marked regeneration of $\text{MHb} \rightarrow \text{Hb}$. This regeneration has caused the disappearance of MHb from the blood stream in times varying from 5 hours to 2 days. \cite{21, 57, 82, 118, 131, 169, 181, 228, 231, 295, 296, 311, 399}.

The lag in the accumulation of MHb in drawn blood was shown to be due to the intrinsic enzyme systems of the erythrocytes which reduce MHb to Hb. \cite{387}. It was discovered that the reducing agents working in vitro are formed primarily from glucose by the erythrocytes. Lactic acid, activated by an intraerythrocytic enzyme system, accounts for 25-50\% of the reduction. \cite{396}.

The mean rate of disappearance of MHb following injection of the sodium nitrite (0.5 cc of a 6\% solution/kgm.) is 11.2\% of the THb/hour. The standard deviation of the mean is $\pm$ 2.0\% of the THb/hour. The rate of MHb disappearance, expressed as per cent of the THb, is independent of the total pigment concentration and the MHb concentration. \cite{80}. Clark and associates \cite{61} showed the rate of regeneration in dogs poisoned with aniline to be 4-7\% of the THb/hour. Wendel \cite{399} placed the natural reducing system of red blood cells at about 10\% of THb/hour.

From many observations \cite{4, 35, 61, 79, 82, 90, 127, 144, 150, 151, 160, 288, 325, 351, 381, 397, 399, 407}, it has been found that intravenous injections of methylene blue accelerates reduction of $\text{MHb} \rightarrow \text{Hb}$. Methylene blue has also been shown to be effective by mouth although its action is slower and MHb can be prevented by oral administration of
0.5-1.0 gm./day. (399). Two possible sources of leuco-methylene blue in the body are: a) reduction of methylene blue in the erythrocytes by enzyme systems present there, and b) reduction of methylene blue in other tissues. The rate of the former is not rapid enough to account for all the MHb reduced, thus it appears the methylene blue is reduced in the tissues and returned as such to the erythrocytes. (399).

Decreased body temperature caused a decreased rate of reduction of MHb. Methylene blue was effective in accelerating reconversion of MHb → Hb at the lower body temperature. The slowness of the conversion was shown to be due to the temperature itself. (80). Reconversion in vitro also depended on temperature as well as the intactness of the red blood cells. Reconversion failed to occur when the blood was kept on ice or hemolyzed. (132).

Cox and Wendel (80) listed the following factors as not influencing the rate of reversion of MHb → Hb:

1. Blood sugar concentration - At comparable temperature in vivo and in vitro rates are the same and the elevation of blood sugar in vitro has no effect upon the rate during the period required for conversion of 50% of the MHb → Hb. Increase or decrease in vivo also has no effect between concentrations of 40-400 mg. per cent. However, successful use of dextrose solutions to revert MHb has been reported. Kosyakov (210) reported that insulin injection favors and glucose administration restricts MHb formation. Clark and Paul (57) and Reimann (302) suggested use of isotonic dextrose solutions to hasten MHb reversion. Brooks (36, 37) reported actual reduction of MHb by glucose injection (isotonic dextrose) and an inhibitory
effect on \textit{MHb} production if given before administration of sodium nitrite. Zierz (421), however, reported a negative effect of glucose upon \textit{MHb} reversion.

2. Prolonged methemoglobinemia - rate remains the same even after 4 complete reversions of \textit{MHb} to \textit{Hb} in 36 hours. Schlossman (325) after initial dose of sodium nitrite reported complete reversion of \textit{MHb} in 6-8 hours. With repetition for 8-13 weeks \textit{MHb} could be found 24 hours after the last injection.

3. Fasting - dogs fasting for 3 weeks show no change in ability to convert \textit{MHb} $\rightarrow$ \textit{Hb}.

4. \textit{MHb} concentration - when greater than 20%.

5. Total pigment.

6. Two or 3 degrees of fever.

Various other substances have shown an ability to reduce \textit{MHb} $\rightarrow$ \textit{Hb}. Sakurai (319) reported use of organ tissues in defibrinated blood having a reducing effect upon \textit{MHb}. Neill (269) reported that living pneumococci or their sterile extracts are capable of reducing \textit{MHb} after they have formed it from \textit{Hb}. Michel and Harris (253) reported reduction of \textit{MHb} by sodium hydrosulfite, ammonium sulfide (292), sodium anthrahydroquinone desulfurate, tetanus tartrate and Stokes' reagent.

Gutman et. al. (127) showed that either diphosphonucleotide or triphosphonucleotide, or both were necessary for the reduction of \textit{MHb} by lactate or adenosine diphosphate. Their work shows that:

a. When nicotinamide is present, a known suppressor of pyridine nucleotide hydrolysis, adenosine diphosphate or lactate can serve as a
substrate in the reduction of MHb in hemolysates as well as intact
erythrocytes, while glucose is no longer utilized after hemolysis.

b. MHb interacts with reduced diphosphonucleotide to yield Hb.
Both these reactions are greatly accelerated by methylene blue.

The reduction of MHb by glyceraldehyde, ascorbic acid (120, 380),
cysteine or by the enzymes in the blood, already mentioned here, has been
investigated. Keise's work (204) on the reduction of MHb was quite ex­
tensive.

The reduction of glyceraldehyde is proportional to the concentration
of both reactants and the pH. The reduction by ascorbic acid proceeds more
rapidly and reaches an asymptote with increasing ascorbic acid concen­
tration. The velocity increases very much more rapidly with a decrease in
hydrogen ion concentration beyond pH 8 than at pH 7. The reduction of MHb
by cysteine also proceeds asymptotically, reaching a maximum value with in­
creasing concentration of cysteine, and the velocity of the reaction is
maximal at pH 8. The reduction velocity in the intact red blood cells
attained a maximum at a 0.5% glucose concentration while fructose was less
effective below 0.5% concentration. A 0.001 M iodoacetate completely
inhibited the action of both glucose and fructose. If the cells were hemo­
yzed the MHb was no longer reduced by either glucose or fructose but only
by hexose mono-or diphosphate. The latter reaction is not inhibited by
iodoacetate.

The enzyme system involved in the reduction of MHb by hexose mono-
phosphate consists of at least two components. Besides the "Zwischen-
ferment", which in conjunction with coenzyme reduces hexose monophosphate,
a MHb reductase is also required. The reduction by lactate even of small
concentration is more effective than by glucose of similar concentration and a higher maximum velocity is obtained. The combined action of glucose and lactate exceeds the effect of 1 substrate. The reduction by lactate is not increased by reversibly oxidizable dyes, but by glucose and still more by hexose monophosphate. The catalytic action of dyes in the reduction of MHB by hexose monophosphate is due to the acceleration of the reaction between MHB and MHB reductase. The catalytic effect of the dyes was in the order:

naphthaquinone > Nile blue > methylene blue, and attains a maximum even in very low concentrations.

MHB appears in the literature fairly extensively as an antidote for cyanide ion poisoning. (52, 53, 96, 111, 117, 128, 178, 180, 257, 315, 316, 318, 335, 394, 396, 397, 398.).

Chen, Rose and Clowes (52) reported methylene blue as being able to oppose 2 minimal lethal doses of cyanide ion while amyl nitrite could oppose 4. Hug (178) showed that the formation of MHB by methylene blue, sodium nitrite or sodium thiosulfate increased survival time of dogs injected with cyanide ion threefold or more. Sahlin (318) showed that injection of methylene blue increased the survival time of white mice injected with cyanide ion from 30 minutes to 6 hours or more.

Chen, Rose and Clowes (53) tested various drugs for efficiency in antidoting cyanide ion poisoning. In order of their increasing effect was methylene blue, sodium thiosulfate, sodium tetrathionate, amyl nitrite, sodium nitrite, methylene blue and sodium tetrathionate, amyl
nitrite and sodium thiosulfate, sodium nitrite and sodium tetrathionate. The best combination, however, was sodium nitrite and sodium thiosulfate. In their combined action the following reactions probably take place:

1. $\text{NaNO}_2 + \text{Hb} \rightarrow \text{MHb}$
2. $\text{NaCN} + \text{MHB} \rightarrow \text{cyano-MHb}$
3. $\text{Na}_2\text{S}_2\text{O}_3 + \text{NaCN} + \text{O} \rightarrow \text{NaSCN} + \text{Na}_2\text{SO}_4$

The use of the two drugs together speeds the real detoxification to sulphocyanate.
F. Data and Discussion

A. Objective:

1. To note the effect of increasing amounts of PAP on blood.
2. To note the effect of increasing amounts of glucose (increments of 100 mgm. per cent) on the production of MHb by PAP in blood.

B. Procedure:

In the determination of the MHb and the THb the spectrophotometric method of Evelyn and Malloy (102) was used. All spectrophotometric measurements were made on the Coleman Universal Spectrophotometer, Model 11A.

The calculations for the determination of MHb and THb on the Coleman Spectrophotometer are based upon obtaining two calibration factors $F_M$ and $F_T$ which vary from one spectrophotometer to another. The procedure used in obtaining these factors is taken from the text "Practical Physiological Chemistry" by Hawk, Oser and Summerson, 12th Edition.

A normal sample of blood was obtained and its Hb content was measured by either the iron method of Wong (410) or the gasometric method of Van Slyke (374), using the procedure as outlined by Van Slyke and Neill (377).

The $F_M$ factor is obtained by dividing the THb, as obtained in the method of Wong or Van Slyke, by the difference in the $D_1$ and $D_2$ readings; the $F_T$ factor by dividing the THb by the $D_3$ reading as described in the method of Evelyn and Malloy (102). The $D_1$ reading is simply of the
diluted, buffered blood read at a wave length of 635. The D2 reading is the same except for the addition of neutralized sodium cyanide. The D3 reading is of the diluted blood in a potassium ferricyanide sodium cyanide buffered solution read at 540. The limit of precision of both the MHb and the THb analyses is about 0.1-0.2 mgm. per 100 cc.

The factors obtained for our spectrophotometer were $F_M = 42.3$ and $F_T = 49.6$. MHb and THb determinations are based on the formulae:

$$\text{MHb (gm %)} = (D_1 - D_2) \times F_M$$

$$\text{THb (gm %)} = D_3 \times F_T$$

The principle of this determination is that MHb has a characteristic light absorption at a wave length of 635. This is abolished in the presence of cyanide which converts the MHb to cyano-MHb. The difference in light absorption at a wave length of 635 before and after adding cyanide is a measure of the MHb present. THb is determined by converting all the Hb present to cyano-MHb and measuring the light absorption at a wave length of 540.

All cuvettes used were optically matched using a methyl red solution at a wave length of 480. All reference standards used contained the same amounts of PAP and buffer mixture as the diluted blood sample.

All blood samples were prepared by diluting 1 cc of blood to 100 cc with phosphate buffer of pH 6.6. Specific amounts of 0.01 M PAP were added to individual tests and their MHb producing ability was measured. On some samples, glucose in increments of 100 mgm. per cent was added to the blood, buffer and PAP solution. All solutions were incubated at 37°C.
The majority of samples lost their clarity after about 2 hours incubation, due probably to the oxidation products of PAP. In the advent of the lack of absolute clarity the solutions were centrifuged and the clear solution obtained was used. By comparison of D3 readings in identical centrifuged and clear non-centrifuged samples, it was determined that the centrifuging of the samples in no way interfered with the readings obtained.
TABLE I
0.25 cc PAP

<table>
<thead>
<tr>
<th>Time (hrs.)</th>
<th>THb</th>
<th>MHB</th>
<th>% MHB</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>11.98</td>
<td>1.32</td>
<td>11.0</td>
</tr>
<tr>
<td>3</td>
<td>2.21</td>
<td>17.6</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>4.28</td>
<td>35.8</td>
<td></td>
</tr>
<tr>
<td>23</td>
<td>5.40</td>
<td>45.2</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Time (hrs.)</th>
<th>THb</th>
<th>MHB</th>
<th>% MHB</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>13.24</td>
<td>2.18</td>
<td>16.5</td>
</tr>
<tr>
<td>3</td>
<td>2.84</td>
<td>21.4</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>5.42</td>
<td>40.8</td>
<td></td>
</tr>
<tr>
<td>23</td>
<td>9.45</td>
<td>71.3</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Time (hrs.)</th>
<th>THb</th>
<th>MHB</th>
<th>% MHB</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>13.62</td>
<td>1.39</td>
<td>10.2</td>
</tr>
<tr>
<td>3</td>
<td>2.81</td>
<td>20.6</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>5.16</td>
<td>37.9</td>
<td></td>
</tr>
<tr>
<td>23</td>
<td>6.15</td>
<td>45.2</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Time (hrs.)</th>
<th>THb</th>
<th>MHB</th>
<th>% MHB</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6.60</td>
<td>.91</td>
<td>13.8</td>
</tr>
<tr>
<td>3</td>
<td>1.57</td>
<td>23.8</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>3.27</td>
<td>49.5</td>
<td></td>
</tr>
<tr>
<td>23</td>
<td>4.87</td>
<td>73.7</td>
<td></td>
</tr>
<tr>
<td>Time (hrs)</td>
<td>THb</td>
<td>Mhb</td>
<td>% Mhb</td>
</tr>
<tr>
<td>-----------</td>
<td>-----</td>
<td>-----</td>
<td>-------</td>
</tr>
<tr>
<td>2</td>
<td>11.32</td>
<td>2.09</td>
<td>19.4</td>
</tr>
<tr>
<td>3</td>
<td>3.09</td>
<td>26.6</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>7.36</td>
<td>65.0</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>8.66</td>
<td>76.5</td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>5.88</td>
<td>52.0</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>13.69</td>
<td>2.58</td>
<td>18.8</td>
</tr>
<tr>
<td>3</td>
<td>3.73</td>
<td>27.2</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>8.13</td>
<td>59.3</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>11.40</td>
<td>83.1</td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>7.33</td>
<td>53.6</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>11.90</td>
<td>1.06</td>
<td>8.91</td>
</tr>
<tr>
<td>8</td>
<td>5.46</td>
<td>45.9</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>10.27</td>
<td>82.3</td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>10.80</td>
<td>90.6</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>11.41</td>
<td>.92</td>
<td>8.06</td>
</tr>
<tr>
<td>8</td>
<td>4.42</td>
<td>38.8</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>7.83</td>
<td>68.7</td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>6.06</td>
<td>53.0</td>
<td></td>
</tr>
</tbody>
</table>

[11]
### TABLE III.

1 cc of PAP

<table>
<thead>
<tr>
<th>Time (hrs.)</th>
<th>THb</th>
<th>MHB</th>
<th>% MHB</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>9.87</td>
<td>1.42</td>
<td>14.7</td>
</tr>
<tr>
<td>2</td>
<td>2.39</td>
<td>2.42</td>
<td>14.7</td>
</tr>
<tr>
<td>4</td>
<td>8.08</td>
<td>8.18</td>
<td>14.5</td>
</tr>
<tr>
<td>8</td>
<td>8.77</td>
<td>88.9</td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>5.13</td>
<td>55.1</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Time (hrs.)</th>
<th>THb</th>
<th>MHB</th>
<th>% MHB</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>11.67</td>
<td>2.34</td>
<td>20.2</td>
</tr>
<tr>
<td>2</td>
<td>4.10</td>
<td>35.1</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>5.80</td>
<td>49.7</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>7.22</td>
<td>61.8</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>10.87</td>
<td>93.2</td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>7.15</td>
<td>61.2</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Time (hrs.)</th>
<th>THb</th>
<th>MHB</th>
<th>% MHB</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>14.60</td>
<td>2.69</td>
<td>18.4</td>
</tr>
<tr>
<td>2</td>
<td>5.74</td>
<td>39.4</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>9.11</td>
<td>64.4</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>11.73</td>
<td>80.4</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>13.34</td>
<td>91.4</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>13.60</td>
<td>93.1</td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>9.32</td>
<td>63.8</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Time (hrs.)</th>
<th>THb</th>
<th>MHB</th>
<th>% MHB</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>13.55</td>
<td>2.79</td>
<td>20.6</td>
</tr>
<tr>
<td>2</td>
<td>4.12</td>
<td>30.3</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>6.62</td>
<td>48.7</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>8.89</td>
<td>65.5</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>11.68</td>
<td>86.2</td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>9.75</td>
<td>72.0</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Time (hrs.)</th>
<th>THb</th>
<th>MHB</th>
<th>% MHB</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.81</td>
<td>19.6</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>3.76</td>
<td>40.7</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>6.24</td>
<td>67.4</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>7.42</td>
<td>80.2</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>8.88</td>
<td>96.0</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>7.96</td>
<td>86.1</td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>6.43</td>
<td>69.6</td>
<td></td>
</tr>
</tbody>
</table>

- 42 -
TABLE IV.
5 cc PAP

<table>
<thead>
<tr>
<th>Time (hrs.)</th>
<th>THb</th>
<th>% MHb</th>
<th>MHb</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>17.6</td>
<td>2.31</td>
<td>13.1</td>
</tr>
<tr>
<td>2</td>
<td>7.20</td>
<td>40.8</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>12.95</td>
<td>73.5</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>13.72</td>
<td>78.0</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Time (hrs.)</th>
<th>THb</th>
<th>% MHb</th>
<th>MHb</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>17.7</td>
<td>2.77</td>
<td>15.7</td>
</tr>
<tr>
<td>2</td>
<td>8.26</td>
<td>46.6</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>14.35</td>
<td>81.0</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>15.90</td>
<td>89.8</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Time (hrs.)</th>
<th>THb</th>
<th>% MHb</th>
<th>MHb</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>16.5</td>
<td>3.64</td>
<td>22.1</td>
</tr>
<tr>
<td>2</td>
<td>8.13</td>
<td>49.3</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>14.58</td>
<td>88.8</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>15.30</td>
<td>92.6</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Time (hrs.)</th>
<th>THb</th>
<th>% MHb</th>
<th>MHb</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>17.0</td>
<td>3.92</td>
<td>23.2</td>
</tr>
<tr>
<td>2</td>
<td>8.44</td>
<td>49.6</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>15.78</td>
<td>93.5</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>16.10</td>
<td>94.7</td>
<td></td>
</tr>
</tbody>
</table>
### TABLE V.

#### PAP + Increments of Glucose

<table>
<thead>
<tr>
<th>Time (hrs.)</th>
<th>THb</th>
<th>MHB</th>
<th>% MHB</th>
<th>Time (hrs.)</th>
<th>THb</th>
<th>MHB</th>
<th>% MHB</th>
<th>Time (hrs.)</th>
<th>THb</th>
<th>MHB</th>
<th>% MHB</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>9.29</td>
<td>1.98</td>
<td>21.3</td>
<td>1</td>
<td>7.82</td>
<td>1.28</td>
<td>16.4</td>
<td>2</td>
<td>11.90</td>
<td>1.59</td>
<td>13.4</td>
</tr>
<tr>
<td>2</td>
<td>2.16</td>
<td>23.2</td>
<td></td>
<td>2</td>
<td>1.97</td>
<td>25.2</td>
<td></td>
<td>4</td>
<td>2.13</td>
<td>17.7</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>5.35</td>
<td>53.5</td>
<td></td>
<td>4</td>
<td>4.17</td>
<td>53.3</td>
<td></td>
<td>8</td>
<td>5.67</td>
<td>17.6</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>8.10</td>
<td>87.0</td>
<td></td>
<td>8</td>
<td>5.73</td>
<td>73.3</td>
<td></td>
<td>16</td>
<td>11.30</td>
<td>94.9</td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>6.00</td>
<td>64.5</td>
<td></td>
<td>24</td>
<td>4.53</td>
<td>58.0</td>
<td></td>
<td>24</td>
<td>10.90</td>
<td>91.3</td>
<td></td>
</tr>
</tbody>
</table>

Based on 200 mgm. glucose
(See Table 12) (28)

<table>
<thead>
<tr>
<th>Time (hrs.)</th>
<th>THb</th>
<th>MHB</th>
<th>% MHB</th>
<th>Time (hrs.)</th>
<th>THb</th>
<th>MHB</th>
<th>% MHB</th>
<th>Time (hrs.)</th>
<th>THb</th>
<th>MHB</th>
<th>% MHB</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>11.85</td>
<td>.78</td>
<td>6.58</td>
<td>2</td>
<td>18.60</td>
<td>3.04</td>
<td>16.3</td>
<td>2</td>
<td>18.60</td>
<td>2.93</td>
<td>15.7</td>
</tr>
<tr>
<td>4</td>
<td>4.99</td>
<td>16.8</td>
<td></td>
<td>4</td>
<td>5.61</td>
<td>31.5</td>
<td></td>
<td>4</td>
<td>5.45</td>
<td>29.2</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>6.15</td>
<td>51.9</td>
<td></td>
<td>8</td>
<td>9.54</td>
<td>51.3</td>
<td></td>
<td>8</td>
<td>10.67</td>
<td>57.3</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>9.28</td>
<td>73.3</td>
<td></td>
<td>12</td>
<td>14.18</td>
<td>76.2</td>
<td></td>
<td>12</td>
<td>14.36</td>
<td>77.0</td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>8.56</td>
<td>72.5</td>
<td></td>
<td>24</td>
<td>10.79</td>
<td>58.0</td>
<td></td>
<td>24</td>
<td>10.71</td>
<td>57.6</td>
<td></td>
</tr>
</tbody>
</table>

Based on 150 mgm. glucose
(See Table 13) (29)

<table>
<thead>
<tr>
<th>Time (hrs.)</th>
<th>THb</th>
<th>MHB</th>
<th>% MHB</th>
<th>Time (hrs.)</th>
<th>THb</th>
<th>MHB</th>
<th>% MHB</th>
<th>Time (hrs.)</th>
<th>THb</th>
<th>MHB</th>
<th>% MHB</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>11.85</td>
<td>.78</td>
<td>6.58</td>
<td>2</td>
<td>18.60</td>
<td>3.04</td>
<td>16.3</td>
<td>2</td>
<td>18.60</td>
<td>2.93</td>
<td>15.7</td>
</tr>
<tr>
<td>4</td>
<td>4.99</td>
<td>16.8</td>
<td></td>
<td>4</td>
<td>5.61</td>
<td>31.5</td>
<td></td>
<td>4</td>
<td>5.45</td>
<td>29.2</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>6.15</td>
<td>51.9</td>
<td></td>
<td>8</td>
<td>9.54</td>
<td>51.3</td>
<td></td>
<td>8</td>
<td>10.67</td>
<td>57.3</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>11.90</td>
<td>37.3</td>
<td></td>
<td>12</td>
<td>14.18</td>
<td>76.2</td>
<td></td>
<td>12</td>
<td>14.36</td>
<td>77.0</td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>8.56</td>
<td>72.5</td>
<td></td>
<td>24</td>
<td>10.79</td>
<td>58.0</td>
<td></td>
<td>24</td>
<td>10.71</td>
<td>57.6</td>
<td></td>
</tr>
</tbody>
</table>

Based on 300 mgm. glucose
(See Table 14) (30)

<table>
<thead>
<tr>
<th>Time (hrs.)</th>
<th>THb</th>
<th>MHB</th>
<th>% MHB</th>
<th>Time (hrs.)</th>
<th>THb</th>
<th>MHB</th>
<th>% MHB</th>
<th>Time (hrs.)</th>
<th>THb</th>
<th>MHB</th>
<th>% MHB</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>11.85</td>
<td>.78</td>
<td>6.58</td>
<td>2</td>
<td>18.60</td>
<td>3.04</td>
<td>16.3</td>
<td>2</td>
<td>18.60</td>
<td>2.93</td>
<td>15.7</td>
</tr>
<tr>
<td>4</td>
<td>4.99</td>
<td>16.8</td>
<td></td>
<td>4</td>
<td>5.61</td>
<td>31.5</td>
<td></td>
<td>4</td>
<td>5.45</td>
<td>29.2</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>6.15</td>
<td>51.9</td>
<td></td>
<td>8</td>
<td>9.54</td>
<td>51.3</td>
<td></td>
<td>8</td>
<td>10.67</td>
<td>57.3</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>11.90</td>
<td>37.3</td>
<td></td>
<td>12</td>
<td>14.18</td>
<td>76.2</td>
<td></td>
<td>12</td>
<td>14.36</td>
<td>77.0</td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>8.56</td>
<td>72.5</td>
<td></td>
<td>24</td>
<td>10.79</td>
<td>58.0</td>
<td></td>
<td>24</td>
<td>10.71</td>
<td>57.6</td>
<td></td>
</tr>
</tbody>
</table>

Based on 450 mgm. glucose
(See Table 15) (31)

<table>
<thead>
<tr>
<th>Time (hrs.)</th>
<th>THb</th>
<th>MHB</th>
<th>% MHB</th>
<th>Time (hrs.)</th>
<th>THb</th>
<th>MHB</th>
<th>% MHB</th>
<th>Time (hrs.)</th>
<th>THb</th>
<th>MHB</th>
<th>% MHB</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>11.85</td>
<td>.78</td>
<td>6.58</td>
<td>2</td>
<td>18.60</td>
<td>3.04</td>
<td>16.3</td>
<td>2</td>
<td>15.55</td>
<td>2.24</td>
<td>14.4</td>
</tr>
<tr>
<td>4</td>
<td>4.99</td>
<td>16.8</td>
<td></td>
<td>4</td>
<td>5.61</td>
<td>31.5</td>
<td></td>
<td>4</td>
<td>3.24</td>
<td>20.8</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>6.15</td>
<td>51.9</td>
<td></td>
<td>8</td>
<td>9.54</td>
<td>51.3</td>
<td></td>
<td>8</td>
<td>3.68</td>
<td>23.6</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>11.90</td>
<td>37.3</td>
<td></td>
<td>12</td>
<td>14.18</td>
<td>76.2</td>
<td></td>
<td>12</td>
<td>4.06</td>
<td>26.1</td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>8.56</td>
<td>72.5</td>
<td></td>
<td>24</td>
<td>10.79</td>
<td>58.0</td>
<td></td>
<td>24</td>
<td>3.48</td>
<td>22.4</td>
<td></td>
</tr>
</tbody>
</table>

Based on 100 mgm. glucose
(See Table 9) (25)

<table>
<thead>
<tr>
<th>Time (hrs.)</th>
<th>THb</th>
<th>MHB</th>
<th>% MHB</th>
<th>Time (hrs.)</th>
<th>THb</th>
<th>MHB</th>
<th>% MHB</th>
<th>Time (hrs.)</th>
<th>THb</th>
<th>MHB</th>
<th>% MHB</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>11.85</td>
<td>.78</td>
<td>6.58</td>
<td>2</td>
<td>18.60</td>
<td>3.04</td>
<td>16.3</td>
<td>2</td>
<td>15.55</td>
<td>2.24</td>
<td>14.4</td>
</tr>
<tr>
<td>4</td>
<td>4.99</td>
<td>16.8</td>
<td></td>
<td>4</td>
<td>5.61</td>
<td>31.5</td>
<td></td>
<td>4</td>
<td>3.24</td>
<td>20.8</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>6.15</td>
<td>51.9</td>
<td></td>
<td>8</td>
<td>9.54</td>
<td>51.3</td>
<td></td>
<td>8</td>
<td>3.68</td>
<td>23.6</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>11.90</td>
<td>37.3</td>
<td></td>
<td>12</td>
<td>14.18</td>
<td>76.2</td>
<td></td>
<td>12</td>
<td>4.06</td>
<td>26.1</td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>8.56</td>
<td>72.5</td>
<td></td>
<td>24</td>
<td>10.79</td>
<td>58.0</td>
<td></td>
<td>24</td>
<td>3.48</td>
<td>22.4</td>
<td></td>
</tr>
</tbody>
</table>

Based on 200 mgm. glucose
(See Table 10) (26)

<table>
<thead>
<tr>
<th>Time (hrs.)</th>
<th>THb</th>
<th>MHB</th>
<th>% MHB</th>
<th>Time (hrs.)</th>
<th>THb</th>
<th>MHB</th>
<th>% MHB</th>
<th>Time (hrs.)</th>
<th>THb</th>
<th>MHB</th>
<th>% MHB</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>11.85</td>
<td>.78</td>
<td>6.58</td>
<td>2</td>
<td>18.60</td>
<td>3.04</td>
<td>16.3</td>
<td>2</td>
<td>15.55</td>
<td>2.24</td>
<td>14.4</td>
</tr>
<tr>
<td>4</td>
<td>4.99</td>
<td>16.8</td>
<td></td>
<td>4</td>
<td>5.61</td>
<td>31.5</td>
<td></td>
<td>4</td>
<td>3.24</td>
<td>20.8</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>6.15</td>
<td>51.9</td>
<td></td>
<td>8</td>
<td>9.54</td>
<td>51.3</td>
<td></td>
<td>8</td>
<td>3.68</td>
<td>23.6</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>11.90</td>
<td>37.3</td>
<td></td>
<td>12</td>
<td>14.18</td>
<td>76.2</td>
<td></td>
<td>12</td>
<td>4.06</td>
<td>26.1</td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>8.56</td>
<td>72.5</td>
<td></td>
<td>24</td>
<td>10.79</td>
<td>58.0</td>
<td></td>
<td>24</td>
<td>3.48</td>
<td>22.4</td>
<td></td>
</tr>
</tbody>
</table>

Based on 300 mgm. glucose
(See Table 11) (27)
C. Interpretation

Table I - Use of 0.25 cc of 0.01 M PAP results in a rapid initial production of MHB. After the large initial production, the rate of formation seems to taper with the maximum amount not yet attained at the end of 23 hours. The THb seemingly has no effect upon either the rate of production or the percentage of MHB produced within 24 hours. This is shown by comparing Tables 2, 3 and 4.

Table 2 has a THb of 13.24, an initial production of 16.5% and a maximum production of 71.3% at the end of 23 hours. Table 3 with a similar THb of 13.62 has an initial rate of 10.2% and a total production at the end of 23 hours of only 45.2%. Table 4 which has a THb of about one-half the previous has an initial rate of 13.8% and a total production within 23 hours of 73.7%.

Table II - Use of 0.5 cc of 0.01 M PAP brings the peak of MHB production within 24 hour range in most cases. Tables 6, 9 and 10 show the maximum reached from the 6th to the 24th hour; Tables 5, 7, 8, 13 and 14 narrow this limit to the 12th - 24th hour.

Table 12 shows the peak at about the 16th hour while in only 2 cases, Tables 11 and 15, the peak of production had not yet been reached at the end of 24 hours. The initial rate varies from one sample to another, and again the evidence is that the THb has no effect either upon the initial rate or the amount of MHB produced.
Table III - With the increase of the 0.01 M PAP to 1 cc the time of maximum production is again shortened from the 16 hours of Table II to about 9-11 hours. The initial speeds are about 20% for samples varying in THb from 9.25-14.6. (Tables 17, 18, 19, 20). The per cent of conversion at the maximum point is greater than with the use of 0.25 cc or 0.5 cc of 0.01 M PAP as used in Tables I and II.

Table I (14) showed a maximum at 23 hours of 73.7%; Table II (12) showed maximum production of 93.0% at 16 hours. In Table III (20) the 8 hour maximum was a 96% conversion.

Table IV - With the use of 0.5 cc of 0.01 M PAP the time interval is only slightly shortened. From the 4 and 8 hour readings obtained it would appear that the maximum production produced by this amount of PAP occurs in about 7-9 hours. Comparison here between initial rate, per cent of production and THb continues to give no correlation between the total pigment and the initial rate or per cent of production.

Table V - This Table presents tests using 0.5 cc PAP identical with some of those previously run but with glucose, in increments of 100 mgm.%, added. This does not account, however, for the normal glucose content of the blood which is unknown.

Table 25 - 100 mgm. of glucose added to test described in Table 9 results in an increased initial speed and a relative similarity thereafter.

Table 26 - 100 mgm. glucose shows a decrease in initial speed and a slight decrease in MHb production.
Table 27 - 200 mgm. glucose shows an increase in initial speed of production and an increase in total MHb production.

Table 28 - 200 mgm. glucose. Slight increase in initial production but a large decrease in total MHb production.

Table 29 - 150 mgm. glucose. No change in initial rate but an increase in the total production.

Table 30 - 300 mgm. glucose - same as Table 29.

Table 31 - 450 mgm. glucose. Increase in initial rate; no change in the total MHb production.

Table 32 - 400 mgm. glucose. Initial rate is doubled and the total production is halved.

Table 33 - 400 mgm. glucose. Initial speed is greatly increased but the total production is decreased to less than one-half.

Addition of small amounts of glucose (100-400 mgm.) to MHb producing solutions in vitro provide a variety of results with both initial speed and the total production. Only when the addition of glucose has reached proportions accounted for by the addition of 400 mgm. or more of glucose plus the original unknown content of the blood does any definite result occur. This addition of glucose greatly increases the initial rate of production but seemingly inhibits the total production. The effect of still greater amounts of glucose remains for further experimentation.

All Tables showing a decrease in the per cent of MHb after the peak has been reached must not be attributed to the enzymatic action of the blood. Since the methods of determination require the taking of the blood, it is probable that when the maximum production of MHb is reached
there is a breakdown of the oxidation products of PAP and the MHb-Hb equilibrium system. Therefore, those values only demonstrate that the peak of MHb production has been passed rather than any reversal of that production.

D. Conclusions

1. Increasing doses of PAP have no definite effect upon the initial rate of MHb production in vitro.

2. Increasing doses of PAP serve to decrease the time required for maximum production in individual samples and convert a larger percentage of Hb → MHb. As the dose increases, the shortening of the time interval becomes less and the increase in the percentage conversion becomes smaller. It appears that there is a maximum level at which there would be no further increase in total production and the conversion would occur in minimum of time.

3. There seems to be no correlation between initial rate and total production of MHb and the THb of the sample.

4. Addition of glucose up to 400 mgm. per cent seems to have no definite effect upon either the initial rate or the total production of MHb.

5. Glucose content well above 400 mgm. per cent causes a change in the system resulting in a higher initial speed of production and an inhibitory effect upon the total production of MHb.
68. Conant, J. B.: Harvey Lectures. 28, 159-83 (1933).


89. Dittrich, : Arch. Exptl. Path. u Therap. 29, 247 (1892).


110. Fraser, and Davis: Med. Times & Gazette. 239 (1862).


   C. A. 32, 8569 (1938).


   C. A. 38, 1561 (1944).


   C. A. 8, 718 (1914).


   C. A. 33, 3881 (1939).

   C. A. 18, 1306 (1924).


   C. A. 18, 857 (1924).


41, 321-7 (1937).

25, 445 (1940).

144, 1989 (1905).

1, 1056 (1923).

102, 164 (1929).


110, 489 (1938).

2, 27 (1887).

74, 1717 (1920).

188, 15-21 (1934).


1, 109-11 (1936).

92, 275 (1925).

6, 728 (1924).

93, 1373 (1925).

8, 71 (1926).

181, 823-6 (1925). C. A.

41, 299-313 (1925).

41, 535-l 9 (1925).

41, 551-60 (1925).

41, 561-70 (1925).

183, 319-62 (1936).

   C. A. 35, 7436 (1941).

   C. A. 32, 9291 (1938).

   479-81 (1938).


   (1944).


   16, 375-94 (1912).

   Williams & Wilkins (1932).

   C. A. 37, 4797 (1943).

   C. A. 39, 3593 (1945).

   (1909).

   C. A. 37, 6742 (1943).

   C. A. 17, 1250 (1923).


   C. A. 17, 1250 (1923).


333. Seekles, L. and Sjollema, B.: Acta Bevia Neerlandica - Physiol.,
C. A. 26, 3842 (1932).

C. A. 31, 7531 (1937).


C. A. 16, 3691 (1922).

C. A. 19, 2087 (1925).


371. Van der Bergh, and Gutterink, : Berliner Klinische Wochenschrift. 43, 7 (1906).