THE NORMAL RATE OF REDUCTION OF METHEMOGLOBIN IN DOGS*

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Methemoglobin is not present in spectroscopically detectable amounts in freshly drawn blood of normal animals of the following and, presumably, other mammalian species: human, dog, cat, rabbit, rat, mouse, horse, monkey (Macacus rhesus), cattle, swine. Furthermore, methemoglobin is not demonstrable in incubated sterile blood from normal animals of these species for many hours after removal from the body. The lag in accumulation of methemoglobin in drawn blood was shown by Warburg, Kubowitz, and Christian (4) to be due to intrinsic enzyme systems of the erythrocytes which reduce methemoglobin to hemoglobin. These workers found that the reducing agents acting in vitro are formed primarily from glucose by the erythrocytes. Lactic acid, activated by an intraerythrocytic enzyme system, accounts for 25 to 50 per cent of the reduction (5). Other reductants have not been identified.

One objective of the experiments reported in this paper was to determine the extent to which the enzyme systems contained within the blood account for in vivo reduction of methemoglobin and, thus, for maintenance of the circulating hemoglobin in a functionally active form. Before this was undertaken, it was considered advisable to ascertain the normal physiological rate of methemoglobin reduction in vivo and the influence upon this rate of several physical and chemical factors. Incident to these studies we have determined the course of methemoglobin accumulation and disappearance following administration of a number of recognized methemo-

* Some of the data reported in this paper are taken from a thesis presented by one of the authors (W. W. C.) to the Graduate Committee of the University of Tennessee in partial fulfillment of the requirements for the degree of Master of Science, September, 1939. A preliminary report has appeared (1).

1 Recently several groups of workers (2, 3), using photoelectric, colorimetric methods or the carbon monoxide capacity method for methemoglobin estimation, have reported that samples of blood from normal animals of some of the above species may contain up to 15 per cent methemoglobin. Such quantities would be readily detectable by qualitative spectroscopic examination, which is sensitive to as little as 4 per cent of the total pigment. The common misstatement that methemoglobin is detectable spectroscopically only when its concentration is 15 to 20 per cent or more of the total pigment has been repeated in a recent paper by Ammundsen (3).
RATE OF REDUCTION OF METHEMOGLOBIN

globin-forming agents and several not hitherto tested. Also, the catalytic action of methylene blue in hastening reduction of methemoglobin to hemoglobin has been further explored.

Methods

Dogs which had been fasted for 18 hours or longer were used as experimental animals. If the administered substance was readily soluble in water, it was injected intravenously; if only sparingly soluble, it was suspended in olive oil, or mineral oil, and given by stomach tube.

In most instances methemoglobin was determined by a rapid spectroscopic method (6) which is referred to in this paper as the dilution method. Since this method is relatively new, we have compared values obtained by it with those given by the spectrophotometric method of Austin and Drabkin (7), and also with values given by the method of difference between total pigment and oxygen capacity (8). Total pigment was determined spectrophotometrically as cyanmethemoglobin (7). Comparative analyses of forty samples of blood containing methemoglobin due to thirteen different substances have shown that the method of difference between total pigment and oxygen capacity gives consistently the lowest methemoglobin concentrations. Values obtained by the Austin and Drabkin method average 4 per cent higher, and those by the dilution method 9 per cent higher. The divergences, however, are unrelated either to concentration of methemoglobin, which varied between 10 and 60 per cent of the total pigment, or to total pigment, which varied between 7 and 22 volumes per cent. In these comparison studies the analyses were so ordered that the results are not vitiated by in vitro changes in methemoglobin concentration in the samples.

Blood glucose was determined by the Shaffier-Hartmann method (9) on zinc filtrates (10).

EXPERIMENTAL

In searching for substances which might be useful in producing methemoglobin, we have tested twenty-five compounds. Single doses of the following substances (the dose, in mg. per kilo, is given after each substance and the mode of administration is indicated by V, intravenous, or T, stomach tube) regularly produced methemoglobin: acetanilide, 200, T; o-aminophenol, 20, V; p-aminophenol, 20, V; aniline, 50, V and T; dimethylaniline, 50, T; hydroxylamine, 5, V; α- or β-naphthylamine, 200, T; p-nitroaniline, 15, V; nitrobenzene, 200, T; nitroglycerin, 10, V; sodium dichromate, 60, V; sodium nitrite, 30, V. The following substances produced methemoglobin only after several daily administrations of large doses: bismuth subnitrate, 1000, T; plasmochin, 1, T; and promin, 600, T.
Single doses of H acid (30, V), hydroquinone (30, V), o-nitrophenol (700, T), and p-nitrotoluene (50, T) caused no accumulation of methemoglobin. Dinitrophenol, sodium chlorate, sodium ferricyanide, sodium sulfanilate, and sulfanilamide gave negative results even after repeated administrations of large doses.

The course of accumulation of methemoglobin in the blood and its subsequent disappearance following administration of single doses of some of these substances are illustrated in Fig. 1. Each curve represents an average result of several experiments. The rates of accumulation as well as the rates of disappearance of methemoglobin vary greatly. Thus, intrave-

![Fig. 1. Representative variations in the accumulation and disappearance of methemoglobin. Acetanilide, dimethylaniline, α-naphthylamine, and nitrobenzene were administered orally. Other substances were injected intravenously.](http://www.jbc.org/)

In contrast to oral administration of most of these substances, intravenous administration of several of them yields quite reproducible results. Table I summarizes the results of thirty-three experiments in which methemoglobin-forming substances were injected intravenously. The rates of disappearance of methemoglobin formed by intravenous injection
of o-aminophenol, p-aminophenol, or sodium nitrite proved to be the same and are the most rapid yet observed. Methemoglobin produced by aniline or p-nitroaniline, perhaps, disappears more slowly.

As judged by twenty experiments on fourteen dogs, the mean rate of disappearance of methemoglobin following injection of sodium nitrite (0.5 cc. of 6 per cent solution per kilo) is 11.2 per cent of the total pigment per hour. The maximum and minimum rates were, respectively, 16.1 and 7.3 per cent of the total pigment per hour. The standard deviation of the mean is ±2.0 per cent of the total pigment per hour. In these experiments total pigment ranged in different dogs between 22.0 and 6.2 volumes per cent. However, the rate of methemoglobin disappearance, expressed as per cent of the total pigment, is independent of the total pigment concentration and the methemoglobin concentration. Thus, in one animal methemoglobin disappeared at the rate of 12.2 per cent per hour when the total pigment was 16.0 volumes per cent. After this animal was made anemic by repeated hemorrhage (total pigment = 6.2 volumes per cent), the rate of disappearance was unchanged.

Means of Altering Rate of Reversion of Methemoglobin to Hemoglobin

**Methylene Blue**—From observations on humans, dogs, and rabbits it has been found previously (11-13) that intravenous injection of methylene blue accelerates reduction of methemoglobin to hemoglobin when the methemoglobinemia is induced by aniline, nitrite, p-bromoaniline, nitrobenzene, p-aminophenol, or the sulfonamides. Methemoglobinemia in dogs due to acetanilide, o-aminophenol, dimethylaniline, α- or β-naphthylamine, o- or p-nitroaniline, also, we now find to respond to methylene blue injections.

As was pointed out previously, intravenously injected methylene blue may produce only a temporary reduction of methemoglobin in humans receiving the sulfonamide drugs (12, 13). The same is true in dogs which

<table>
<thead>
<tr>
<th>Substance Injected</th>
<th>Amount Injected</th>
<th>No. of Experiments</th>
<th>Average Time to Reach Maximal Methemoglobin Concentration</th>
<th>Rate of Methemoglobin Disappearance</th>
<th>Standard Deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>o-Aminophenol</td>
<td>20 mg. per kg.</td>
<td>3</td>
<td>45 min.</td>
<td>11.2 per cent total pigment per hr.</td>
<td>±3.0 per cent total pigment per hr.</td>
</tr>
<tr>
<td>p-Aminophenol</td>
<td>20 mg. per kg.</td>
<td>5</td>
<td>45 min.</td>
<td>11.8 per cent total pigment per hr.</td>
<td>±1.4 per cent total pigment per hr.</td>
</tr>
<tr>
<td>Aniline</td>
<td>50 mg. per kg.</td>
<td>5</td>
<td>200 min.</td>
<td>9.6 per cent total pigment per hr.</td>
<td>±2.9 per cent total pigment per hr.</td>
</tr>
<tr>
<td>Sodium nitrite</td>
<td>30 mg. per kg.</td>
<td>20</td>
<td>45 min.</td>
<td>11.2 per cent total pigment per hr.</td>
<td>±2.0 per cent total pigment per hr.</td>
</tr>
</tbody>
</table>
have received large amounts of acetanilide, dimethylaniline, or nitrobenzene orally. Fig. 2 illustrates this behavior and shows also, as compared with the first injection of methylene blue, a diminished effectiveness of two subsequent injections. The latter phenomenon is probably a result of an increase in concentration of the active pigment-oxidizing agent, owing to prolonged absorption of the drug.

Temperature—The rate of disappearance of methemoglobin in one dog with normal body temperature was found to be 10.3 per cent of the total pigment per hour (Fig. 3). When the animal’s body temperature was lowered about 10° by packing in ice, the rate decreased to 3.3 per cent per hour.
hour. Methylene blue was effective, however, in accelerating reconversion of methemoglobin to hemoglobin at the lowered body temperature. That the slowness of disappearance of methemoglobin at lowered body temperature is not due to prolonged methemoglobin formation is indicated by the fact that a sample of blood drawn 1 hour after injection of nitrite was free of nitrite, as judged by the starch-iodide test. Three other animals gave similar results. Nembutal was used to maintain light anesthesia in these experiments.

### Table II

**Comparison of Rates of Disappearance of Methemoglobin in Vivo and in Vitro and Effect of Blood Sugar Concentration in Vitro**

<table>
<thead>
<tr>
<th>Dog No.</th>
<th>Blood glucose concentration at 0 time</th>
<th>Methemoglobin concentration at intervals after maximum concentration is reached, per cent total pigment</th>
<th>Average rate of disappearance of methemoglobin per cent total pigment per hr.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>In vivo</strong></td>
<td><em>mg. per cent</em></td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>&quot; <em>vivo</em></td>
<td>93 54 49 44 41 37 33 29 25</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>&quot; <em>vivo</em> + glucose&quot;</td>
<td>293 54 49 45 41 38 33 29 25</td>
<td>8</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>In vivo</strong></td>
<td>293 76 67 61 54 48 43 39 35</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>&quot; <em>vivo</em> + glucose&quot;</td>
<td>293 76 67 60 51 46 41 36 31</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>&quot; <em>vito</em></td>
<td>493 76 67 60 52 45 42 38 35</td>
<td>10</td>
</tr>
</tbody>
</table>

* Time of maximum methemoglobin concentration.

**Factors Which Do Not Affect Rate of Reversion of Methemoglobin to Hemoglobin**

**Blood Sugar Concentration**—Dog 1 (Table II) was given sodium nitrite intravenously. At the end of an hour 50 cc. of blood were drawn, defibrinated, and divided equally between two flasks. 50 mg. of glucose were added to the blood in one of the flasks and the two samples were then incubated at 37.5° with gentle rocking. The concentration of methemoglobin was determined from time to time in the incubated samples as well as in further samples drawn from the dog. The same procedure was followed in Dog 2, except that this animal received 5 gm. of glucose per kilo orally before nitrite was injected. Results of these two experiments show that at comparable temperatures the *in vivo* and *in vitro* rates of disappearance of methemoglobin are the same, and that elevation of blood sugar *in vitro* has no effect upon the rate during the period required for conversion of 50 per cent of the methemoglobin to hemoglobin.
The experiments summarized in Table III show a lack of effect of increased blood sugar concentration upon the rate of disappearance of methemoglobin in vivo. In Experiments 1 and 2 the rate was determined while the animal's blood sugar was at normal levels. In Experiments 3 and 4

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Blood glucose concentration at 0 time</th>
<th>Methemoglobin concentration at intervals after maximum concentration is reached, per cent total pigment</th>
<th>Average rate of methemoglobin disappearance per cent total pigment per hr.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg. per cent</td>
<td>0 min.*              30 min.              60 min.              90 min.              120 min.              150 min.              180 min.</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Normal†</td>
<td>54                   47                   40                   33                   26                   20                   14</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>&quot;</td>
<td>55                   50                   42                   34                   27                   14</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>120</td>
<td>53                   45                   36                   31                   26</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>245</td>
<td>66                   60                   53                   47                   41                   34                   27</td>
<td></td>
</tr>
</tbody>
</table>

* Time of maximum methemoglobin concentration.
† Not determined but found to be normal on other occasions.

**Fig. 4.** Lack of influence of low blood sugar concentrations upon rate of disappearance of methemoglobin. The figures below the points are blood glucose concentrations in mg. per cent.

the rate was determined while the blood sugar was elevated by oral administration of glucose.

To test the possible effect of hypoglycemia upon the rate of disappearance of methemoglobin, a dog was given an injection of 30 units of insulin at the same time that nitrite was injected. Results of this experiment are illustrated in Fig. 4, where the connected points represent the methemoglobin concentration at various intervals after injection of nitrite and the
figures below the points are the blood glucose concentrations at the indicated times. The rate of methemoglobin disappearance is not significantly influenced by the lowered blood sugar.

**Prolonged Methemoglobinemia**—In order to determine the effect of protracted methemoglobinemia upon the ability of blood to reduce methemoglobin, a dog was given intravenous injections of sodium nitrite (30 mg. per kilo) at intervals of 5 to 6 hours for 36 hours. Following the first injection, subsequent injections were made when the methemoglobin concentration had fallen to about 20 per cent. The rate of disappearance was determined after each of the seven injections. The data in Table IV indicate the lack of effect of continuously high concentration of methemoglobin upon the rate of disappearance. It is to be noted that at the average rate of disappearance shown by this animal all of the blood pigment must have under-

**Table IV**

*Effect of Successive Injections of Sodium Nitrite on Rate of Methemoglobin Disappearance in Vivo*

<table>
<thead>
<tr>
<th>Nitrite injection</th>
<th>Time since first injection</th>
<th>Maximum methemoglobin concentration</th>
<th>Rate of disappearance of methemoglobin following injection</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>min.</td>
<td>per cent total pigment</td>
<td>per cent total pigment per hr.</td>
</tr>
<tr>
<td>1st</td>
<td>0</td>
<td>65</td>
<td>10</td>
</tr>
<tr>
<td>2nd</td>
<td>345</td>
<td>63</td>
<td>10</td>
</tr>
<tr>
<td>3rd</td>
<td>675</td>
<td>61</td>
<td>11</td>
</tr>
<tr>
<td>4th</td>
<td>975</td>
<td>64</td>
<td>10</td>
</tr>
<tr>
<td>5th</td>
<td>1305</td>
<td>61</td>
<td>11</td>
</tr>
<tr>
<td>6th</td>
<td>1560</td>
<td>52</td>
<td>9</td>
</tr>
<tr>
<td>7th</td>
<td>1835</td>
<td>62</td>
<td>11</td>
</tr>
</tbody>
</table>

gone oxidation and reduction at least four times in the period of 36 hours during which methemoglobin was constantly present in the blood.

**Fasting**—Two animals which were fasted for 3 weeks showed no change in ability to convert methemoglobin to hemoglobin.

**DISCUSSION**

The average rate at which accumulated methemoglobin within the circulating erythrocytes of dogs is replaced by functionally active hemoglobin is $11.3 \pm 2.0$ per cent of the total pigment per hour. This rate, observed following injection of such different chemical substances as sodium nitrite, o-aminophenol, and p-aminophenol, would appear to be the true physiological rate of this vital reduction reaction. The slower rate of disappearance and consequent apparent slower rate of reduction seen when certain other substances are administered are probably explained by pro-
longed formation of methemoglobin, which masks the reduction reaction. In the case of nitrite, methemoglobin formation is rapid and nitrite is equally rapidly destroyed. 60 minutes after intravenous injection of 30 mg. of sodium nitrite per kilo, methemoglobin formation ceases and nitrite is no longer detectable in the plasma by the very sensitive starch-iodide test.

The rate of reduction of methemoglobin is independent of the following factors: (1) methemoglobin concentration, at least when this is greater than 20 per cent of the total pigment; (2) total pigment, i.e. hemoglobin plus methemoglobin; and (3) glucose concentration, between 40 and 400 mg. per cent. The rate is not influenced by several weeks of fasting and is sustained in vivo for a period of at least 36 hours, during which time all of the pigment in the circulating blood passes through methemoglobin at least four times. Since the rate of reduction in vivo is the same as that in vitro, the influence of other body tissues appears to be negligible. The rate of reduction is retarded by low body temperature, but is uninfluenced by 2 or 3 degrees of fever produced by injection of dinitrophenol.

The recognized role of glucose as the principal source of reducing agents for methemoglobin in vitro led Brooks (14) to test the value of intravenous injection of glucose in hastening reduction of methemoglobin in vivo. On the basis of experiments with rabbits, injected with nitrite to produce methemoglobin, Brooks concluded that intravenous administration of 10 to 20 mg. of glucose per kilo (Brooks gave 1 to 2 cc. of 1 per cent solution per kilo) greatly accelerates the reconversion of methemoglobin to hemoglobin. In consequence Brooks (15) has recommended intravenous administration of glucose in treatment of human methemoglobinemia. Our experiments on dogs, to which we gave amounts of glucose which produced significant and measured increases in blood sugar concentration, failed to demonstrate any accelerating action of glucose in this species.

The rate of methemoglobin reduction shows no correlation with total pigment only when the rate is expressed as per cent of total pigment per unit time. When the rate is expressed in absolute units, e.g. volumes per cent per hour, there is a significant decrease in the rate as the total pigment decreases. Thus, one dog before hemorrhage, when the total pigment was 16.0 volumes per cent, showed a rate of reduction of 12.2 per cent of total pigment per hour or 2.0 volumes per cent per hour. After hemorrhage, when the total pigment was 6.2 volumes per cent, the rate expressed as per cent of total pigment was unchanged. Expressed in terms of volumes per cent per hour, the rate had decreased to 0.7.

Finally, the rate of reduction of methemoglobin is much more constant from animal to animal and in a given animal at different times when the percentage mode of expression is employed than when the rate is expressed in absolute units. This is evidenced by the fact that the standard devia-
tion of the mean is approximately twice as great in the latter case as in the former.

There appears to be ample evidence that the maintenance of hemoglobin in a functionally active state is accomplished mainly if not entirely by enzyme systems contained within the circulating erythrocytes.

SUMMARY

Methemoglobin contained within circulating erythrocytes of dogs is reduced to hemoglobin at a constant average rate of 11.3 per cent of the total pigment per hour. This rate, therefore, represents the maximum resistance of this species to accumulation of methemoglobin. Reduction of intracorpuscular methemoglobin is solely a function of enzyme systems contained within the erythrocytes. Ability to reduce methemoglobin is impaired by low body temperature. It is not affected by severe hypoglycemia or by blood sugar concentrations several times the normal. Capacity to convert methemoglobin to hemoglobin is not diminished even after all of the blood pigment has been converted to methemoglobin four times in a relatively short period.

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