TECHNICAL NOTE

Alan L. Hatch,[1] *Ph.D.*

A Modified Reagent for the Confirmation of Blood

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ABSTRACT: Oxygen and pyridine compete for the same binding site on the heme molecule. Lowering the oxygen concentration in Takayama's reagent by addition of an oxygen scavenger such as dithiothreitol (Cleland's reagent) shifts this equilibrium in favor of pyridine, and increases the rate at which hemochromogen crystals are formed. This modification makes the confirmatory test for blood faster, and therefore easier to use. The absolute sensitivity and the specificity of the reagent appear unchanged.

KEYWORDS: criminalistics, blood, confirmation of blood, Takayama's reagent

The reaction of pyridine with hemoglobin to form hemochromogen crystals was first noted by Donogany [*1*], who also suggested its application to the problem of the medicolegal identification of blood. A number of reagents and/or methods have been suggested, as reviewed by Dilling [*2*] in 1910, and later by Mahler [*3*]. The method currently used by most workers was suggested by Takayama in 1912, but was not introduced into the European literature until 1922 by Strassmann [*4*]. The procedure has remained essentially unchanged to the present. Most of this early work is published in German. This paper is an analysis of conditions affecting the formation of the hemochromogen crystals.

Materials and Methods

Takayama's Reagent

The traditional Takayama reagent is made by combining 120 μL deionized water, 50 μL NaOH (10% W/V), 50 μL of a saturated dextrose (glucose) solution, and 50 μL of pyridine. This reagent is stable for several weeks if kept refrigerated. Note however, that low temperature increases the solubility of gases (including oxygen) in liquid.

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[1]Senior Criminalist, Tucson Police Department, Tucson, AZ.

"Oxygen Free" Takayama's Reagent

The aqueous components of Takayama's reagent were rendered oxygen free by boiling, then cooling in the absence of oxygen. Reagent grade pyridine was used without prior treatment. The oxygen free components were then combined in the same ratios as the traditional reagent. This reagent remains "oxygen free" only as long as it does not come in contact with atmospheric oxygen. No attempt was made to quantify its actual oxygen concentration.

Modified Takayama's Reagent

Oxygen scavengers such as dithiothreitol, sodium dithionite, mercaptoethanol, and dithioerythritol, react with and remove dissolved oxygen from solution. The chemically modified reagent contains 120 μL Cleland's reagent (0.05 M dithiothreitol), 50 μL NaOH (10% W/V), 50 μL of saturated dextrose solution, and 50 μL of pyridine. This chemically modified reagent remains "oxygen free" in the presence of atmospheric oxygen until the dithiothreitol is consumed. It was made fresh daily. The saturated dextrose solution and the NaOH solution can be kept at room temperature for extended periods of time, but Cleland's reagent is best stored frozen in small aliquots.

Results and Discussion

The heme prosthetic group has a central iron atom attached to four pyrrole groups [5]. The iron and the nitrogen atoms from the pyrrole groups all lie in the same plane. In this structure the iron (in the +2 oxidation state) can accommodate the binding of two more ligands, one above and one below the plane of the heme molecule. Several ligands are known to bind reversibly at these positions. During physiological transport by hemoglobin, an oxygen or carbon dioxide molecule occupies each of these binding sites. The binding of pyridine in these same sites is a prerequisite for the formation of hemochromogen crystals. This suggests that under certain conditions there can exist a competition between oxygen, carbon dioxide and pyridine for binding at the heme iron. Other non-physiological ligands which bind at this site include carbon monoxide and hydrogen cyanide (Fig. 1). In fact, the toxicity of these gases is thought to be due, at least in part, to the fact that they displace oxygen from this binding site. Both the strength with which a ligand binds (the affinity of the ligand for the site), and the number of ligands available for binding (the ligand concentration) affect which ligand binding will predominate. No attempt has been made to compare ligand binding strengths but we have found that conditions favoring binding of one ligand will reduce the amount of a second ligand bound. Four examples of this have been noted using, in each case, preformed hemochromogen crystals suspended in traditional Takayama's reagent (under a cover slip on a slide). First, directing a steam of compressed oxygen over these crystals causes them to dissolve. The same result is noted using oxygen formed from added hydrogen peroxide (by the peroxidase activity of the heme). Second, increasing the carbon dioxide concentration causes the preformed crystals to dissolve. This increase is achieved by addition of solid sodium bicarbonate which, by disassociation, acts as a source of carbon dioxide, and also changes the pH of the solution. Third, incubating the slide in an atmosphere containing carbon monoxide results in crystal dissolution. Carbon monoxide was produced by heating calcium carbonate with zinc granules. Fourth, addition of sodium cyanide (in 0.1 M NaOH) results in crystal dissolution. A model for the reversible binding of these ligands with heme is illustrated in Fig. 1. A prediction based on this model is that reducing the concentration of competing ligands capable of

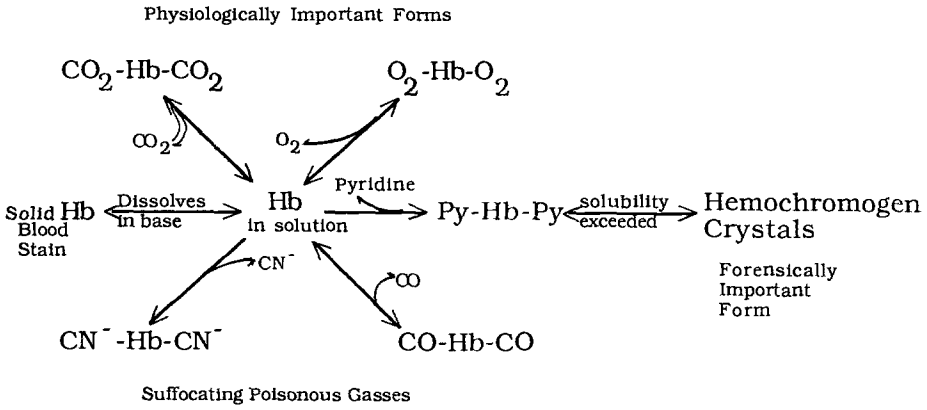

FIG. 1—*Binding of heme to various ligands. All pathways shown in this figure are reversible.*

binding to the heme iron would favor formation of hemochromogen crystals upon addition of pyridine.

Two ligands (capable of binding to heme) present in normal air are carbon dioxide and oxygen. The solubility of carbon dioxide in water (at 20°C, pH of 7, and 1 atm pressure) produces a total carbonate concentration of around 12 micromolar, of which, the majority exists as carbon dioxide. However, addition of sodium hydroxide to the level found in normal Takayama's reagent (~0.5 molar) increases the pH to >13, and shifts the equilibrium strongly in favor of the ionized carbonate species, thus reducing the carbon dioxide concentration by a factor of approximately twenty two million. Sodium hydroxide solution stored in contact with air strongly absorbs carbon dioxide, but the equilibrium is so far toward the ionized carbonate species, that until the pH of the solution is shifted, there is little effect on the concentration of dissolved carbon dioxide. This lowering of the carbon dioxide concentration, along with the fact that most proteins, including hemoglobin and other constituents of blood stains, are readily soluble in strongly basic solution, accounts for the presence of sodium hydroxide in Takayama's reagent.

Oxygen solubility, on the other hand, is not affected by the pH of the solution. We have found that conditions favoring low oxygen concentrations enhance the speed of formation of hemochromogen crystals. Liquids can be freed from dissolved oxygen by several methods. As shown in Table 1, "oxygen free" Takayama's reagent forms crystals more rapidly than the same reagent which is in equilibrium with atmospheric oxygen. Further, Takayama's reagent which has been briefly heated at 60°C under reduced pressure (which reduces the concentration of dissolved gasses) forms hemochromogen crystals almost as easily as the reagent rendered "oxygen free" by other means (data not shown). Since oxygen solubility in water decreases with increasing temperature, the basis for inducing the formation of hemochromogen crystals by gentle heating may be partially explained as reducing the concentration of dissolved oxygen.

There are a number of weak reducing agents that react with dissolved oxygen and remove it from solution. These include dithiothreitol (Cleland's reagent), sodium dithionite, mercaptoethanol, and dithioerytritol. Addition of any of these to Takayama's reagent increases the speed with which it forms hemochromogen crystals to approximately that of the traditional reagent rendered "oxygen free" by physical methods. Table 1 compares the propensity for hemochromogen crystal formation of the traditional reagent to reagents

TABLE 1—*Time (s.) to form hemochromogen crystals.*

Dilution	Traditional Takayama	"Oxygen free" Takayama	Chemically modified Takayama
neat	>300	<5	<5
1:2	>300	<5	<5
1:4	>300	<5	<5
1:8	>300	160	12
1:16	>300	41	9
1:32	236	29	12
1:64	>300	28	20
1:256	>300	32	44
1:512	—	109	76
1:1024	—	130	150
1:2048	—	—	105

NOTE: Fresh whole venous blood (collected in an ACD vacutainer) was diluted as indicated with phosphobuffered saline. Five (5) microliters of the diluted sample were placed on a silanized glass slide, and dried in a 75°C oven under circulating air for 10 minutes. The resulting stain was cooled, covered with a piece of a coverslip, and reagent sufficient to flood approximately 90% of the stain was added. The stains (maintained at room temperature) were observed microscopically for five minutes, and then again at one hour. The number of seconds elapsing between the addition of reagent and the observation of crystals is given. It should be noted that at very high dilutions of blood, the amount of reagent added becomes critical. Excess reagent dissolves and dilutes the heme to the point where no hemochromogen crystals are observed.

made "oxygen free" by physical and chemical means. The advantage of using a reagent containing an oxygen scavenger is that it can remain in contact with air for several hours without losing its "oxygen free" reactivity. We chose Cleland's reagent as the oxygen scavenger because it is available in most laboratories engaged in forensic serology.

In fact, Cleland's reagent is used in forensic serology to reduce sulfide bonds in proteins. The suggestion has been made that part of the enhanced crystal formation may be due to Cleland's reagent reducing methemoglobin (iron in the +3 oxidation state) to hemoglobin (iron in the +2 oxidation state), thereby increasing the amount of hemoglobin available to form hemochromogen. This mechanism is probably not significant, as sodium dithionite, which converts oxyhemoglobin to deoxyhemoglobin without altering the oxidation state of the heme iron [*6*], produces an enhancement of hemochromogen crystal formation equivalent to that noted with Cleland's reagent. Further, this same enhancement can be obtained by physically removing the dissolved oxygen from traditional Takayama's reagent.

A second prediction based on the model in Fig. 1 is that increasing the pyridine concentration should increase the amount of hemochromogen crystals produced. This is not the case. Increasing the pyridine concentration does increase the amount of pyridine-heme complex formed, but it also increases the solubility of this complex (data not shown). The optimum pyridine concentration for formation of hemochromogen crystals from dried blood stains appears to be near the value found in traditional Takayama's reagent (2.3 molar).

The specificity of this chemically modified reagent was checked by reacting it with a variety of materials, including chemicals, plant extracts, and purified protein preparations. We have confirmed the finding of Blake and Dillon [*7*], that crystallized horseradish peroxidase forms crystals with this reagent (and with traditional Takayama's reagent) which strongly resemble those formed by the reaction with hemoglobin. With this exception, we have found no false positives.

Conclusions

Takayama's reagent forms hemochromogen crystals from dried blood stains faster in the absence of oxygen. Heat is not required to initiate this crystallization. Both these facts make the oxygen free reagent more convenient to use. It is also better for the analysis of small, or dilute blood stains, where diffusion losses incident to long incubation times, or heat induced fluid movements can reduce heme concentrations enough to prevent crystallization. Combining 120 μL Cleland's reagent (.05 M dithiothreitol), 50 μL NaOH (10% W/V), 50 μL saturated dextrose solution, and 50 μL pyridine produces a stable, oxygen free reagent for the confirmation of blood.

Acknowledgments

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Address requests for reprints or additional information to
Alan L. Hatch, Ph.D.
Tucson Police Dept.
P.O. Box 1071
Tucson, AZ 85702