

# NADH-Methemoglobin Reductase and Methemoglobinemia Among Leprosy Patients<sup>1</sup>

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The low level of methemoglobin usually detected in the blood is mostly due to the action of NADH-cytochrome b<sub>5</sub> reductase (EC 1.6.2.2), commonly named NADH-methemoglobin reductase. This NADH-dependent enzyme reduces cytochrome b<sub>5</sub> which, in turn, converts non-enzymically methemoglobin into hemoglobin (<sup>12, 17, 21</sup>). According to Tomoda, *et al.* (<sup>22</sup>), the methemoglobin reduction in the erythrocytes is also performed through two pathways: methemoglobin molecules are first transformed into half-oxidized hemoglobins (intermediate hemoglobins), and the latter are then changed into oxyhemoglobin after acceptance of four electrons per tetramer.

An almost complete deficiency of NADH-methemoglobin reductase is a rare event in most populations and is considered to be inherited as a recessive autosomal defect which determines congenital methemoglobinemia. Partial deficiency of this NADH-dependent enzyme is a condition exhibited by asymptomatic individuals who are most frequently found among relatives of cyanotic index cases with congenital methemoglobinemia due to severe deficiency of NADH-methemoglobin reductase (<sup>3, 7, 15, 18, 23</sup>).

In spite of being asymptomatic, persons with partial deficiency of NADH-methemoglobin reductase, who are believed to be heterozygous, are considered to have increased susceptibility to methemoglobin-producing chemicals and oxidant drugs. Thus, Cohen, *et al.* (<sup>4</sup>), investigating six North American soldiers who became cyanotic while taking antimalarial drugs, verified that all of them revealed a markedly decreased activity of NADH-methemoglo-

bin reductase in their erythrocytes. Among them, cyanosis due to methemoglobinemia, at levels between 20% and more than 30% of total hemoglobin, was associated with symptoms of toxic methemoglobinemia, that is to say, headache, fatigue, dizziness, nausea, vomiting and/or dyspnea.

Chloroquine (300 mg per day), primaquine (15 mg per day), and dapsone (25 mg per day) were each shown to provoke methemoglobinemia in doses that have no effect on normal persons. However, dapsone showed the greatest potential to cause an increased methemoglobin level.

Surveys on the frequency of individuals with partial NADH-methemoglobin reductase deficiency are scarce (<sup>7, 18</sup>) but suggest that such persons may be more common than has been realized on the basis of the very small incidence of the almost complete deficiency of this enzyme (congenital methemoglobinemia). Taking into account this suggestion and the fact that almost all leprosy patients ingest regularly a daily dose of 100 mg of dapsone, we decided to investigate the activity of NADH-methemoglobin reductase, as well as the level of methemoglobin, among Brazilian leprosy patients and healthy subjects.

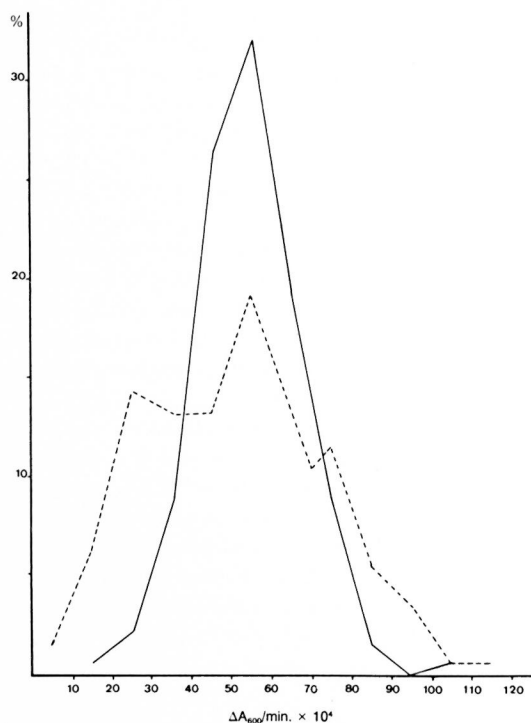
## MATERIALS AND METHODS

The levels of hemoglobin, methemoglobin, NADH-methemoglobin reductase, and sulfones were measured in the blood samples of 182 Brazilian adult leprosy patients, mostly of Caucasoid origin (17 Negroids), who were ingesting a daily dapsone dose of 100 mg. The same tests, except for the blood sulfone determinations, were applied to blood samples from 137 healthy Brazilian soldiers (128 Caucasoids and 9 Negroids). These healthy subjects had not ingested drugs by history at the time of this investigation.

Among the leprosy individuals, 87 (46 males and 41 females) were outpatients living in Campinas, São Paulo (SP), Brazil,

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THE FIGURE. Distribution of 137 healthy subjects (—) and 182 leprosy patients (·····) according to their levels of NADH-methemoglobin reductase.

while the remaining 95 were males interned in a sanatorium (Hospital "Dr. Francisco Ribeiro Arantes," Itu, SP, Brazil). The period under dapsone therapy ranged from 1 month to 40 years among the outpatients (mean = 8.61; S.D. = 9.63), and from 4 months to 45 years among the patients interned in the sanatorium (mean = 16.89; S.D. = 12.23). The mean age was 59.96 (S.D. = 13.78) for the sanatorium leprosy cases, 43.3 years (S.D. = 14.55) for the outpatients, and 19 years (S.D. = 0.94) for the healthy subjects.

Venous blood samples of 3 ml were collected from all individuals and withdrawn into tubes containing 0.1 ml of 3% ethylenediaminetetra-acetic acid (EDTA) solution. Subsamples of 0.5 ml of blood were transferred to tubes containing 0.2 ml of acid citrate dextrose (ACD) which were maintained in a refrigerator for a maximum of 10 days, the tubes being shaken daily. These subsamples were to be used to assess the amount of NADH-methemoglobin reductase in the blood, since ACD is consid-

ered to maintain unchanged the activity of this reductase over a period of at least two weeks (<sup>18</sup>).

Hemoglobin and methemoglobin levels were measured 2–3 hr after blood collection. For this purpose, 0.1 ml from each sample containing 1 mg/ml of EDTA was hemolyzed in 4.9 ml of distilled water, after which 0.2 ml of the hemolysate was added to 1.8 ml of a phosphate buffered saline solution (0.01 M K<sub>2</sub>HPO<sub>4</sub> in 0.9% w/v NaCl solution, pH 7.3). The absorbance of this solution was determined in three different wavelengths—560, 576, and 630 nm—against a blank containing only the buffered saline solution. The oxyhemoglobin, deoxyhemoglobin, and methemoglobin levels in the hemolysate were obtained according to Benesch, *et al.*'s (<sup>2</sup>) method, using the corrected extinction coefficients published by Van Assendelft and Zijlstra (<sup>24</sup>). Thus, it was solved by the following matrix equation, where the coefficients correspond to the extinction coefficients:

$$\begin{bmatrix} 0.867 & 1.280 & 0.430 \\ 1.580 & 0.970 & 0.445 \\ 0.014 & 0.110 & 0.363 \end{bmatrix} \cdot \begin{bmatrix} \text{Oxyhemoglobin} \\ \text{Deoxyhemoglobin} \\ \text{Methemoglobin} \end{bmatrix} = \begin{bmatrix} A_{560} \\ A_{576} \\ A_{630} \end{bmatrix}$$

Methemoglobin values were further transformed to provide figures corresponding to Evelyn and Malloy's (<sup>6</sup>) method, since it is more frequently employed for the determination of the amount of methemoglobin. This transformation was obtained by applying the regression equation  $y = -0.008 + 1.11x$ , where  $x$  is the original value and  $y$  is the amount of methemoglobin in Evelyn and Malloy's (<sup>6</sup>) method. Taking into account that the initial 0.1 ml of blood was diluted 500 times in two successive steps (water and buffered saline solution), the levels of the hemolysate were multiplied by 500 to obtain the actual values for oxyhemoglobin, deoxyhemoglobin, and methemoglobin in the blood. Hemoglobin was expressed as g% and methemoglobin in percent of total hemoglobin.

The activity of NADH-methemoglobin reductase was measured as diaphorase according to a slightly modified Scott's (<sup>18</sup>) technique. At first, the erythrocytes of the blood conserved in ACD were washed 3 times at room temperature with 7 ml of phosphate buffered saline solution prepared

as described above. After each addition of buffered saline solution, the erythrocyte suspensions were centrifuged for 10 min at 2000 rpm. To the packed red cells the same volume of 1% sodium nitrite in phosphate buffered saline solution was added to cause the total conversion of hemoglobin into methemoglobin. This suspension was shaken and allowed to stand for 20 min at room temperature. The red cells were then washed 5 times with 7 ml of the phosphate buffered saline solution to remove excess nitrite. Then, to 0.05 ml of the packed cells 10 ml of distilled water was added and the absorbance determined at 600 nm, against distilled water, without the need of centrifuging the hemolysate.

The next step consisted of the addition of 0.2 ml of 0.012 M 2, 6-dichlorobenzene indophenol (DCIP) in 1 M Tris-HCl buffer, pH 7.6, containing 0.011 M disodium EDTA to 3 ml of the hemolysate. According to Scott<sup>(18)</sup>, the EDTA added to the solution is necessary in order to obtain reproducible rates of non-enzymic reduction of the dye, although EDTA has no effect on the enzymatic reaction. The enzyme reaction was started by adding 0.05 ml of 0.008 M NADH, and the absorbance at 600 nm was continuously recorded for 5 min at room temperature. The reagent blank was prepared using 3 ml of distilled water instead of the hemolysate. Enzyme activity was expressed as change in absorbance per minute, at 600 nm, multiplied by  $10^4$  ( $\Delta A_{600}/\text{min} \times 10^4$ ), assuming 0.2 as the initial optical density<sup>(18)</sup>.

The dapsone level in the patients' blood was determined by adapting Simpson's method described in an appendix to a paper written by Molesworth and Narayanaswami<sup>(16)</sup>. Thus, after hemolyzing 0.5 ml of blood containing EDTA in 2.5 ml of distilled water, 2.5 ml of 2 N HCl was added to the hemolysate to provide a higher solubility of dapsone and, therefore, a better recovery of this sulfone. This mixture was vigorously shaken before adding 2.0 ml of 12% trichloroacetic acid for deproteinizing the blood. After complete homogenization, the liquid was filtered through common filter paper.

From the clean, colorless filtrate 2.0 ml was transferred to a vial into which 0.05 ml of 0.3% sodium nitrite was added to diazo-

tize the dapsone. After mixing well, the solution was allowed to stand for 3 min; 0.05 ml of 1.5% ammonium sulphamate being added to remove excess nitrite in the solution. Once again the solution was mixed well and allowed to stand for 2 min more. Then 0.05 ml of 0.1% N-(1-naphthyl)-ethylene diamine hydrochloride was added to couple with the diazotized dapsone to develop a purplish-red color. The solution was mixed well and kept in the dark for 20 min at room temperature for full development of the color prior to being transferred to a spectrophotometer cuvette.

The absorbance was measured at 550 nm against a reagent blank prepared with 0.5 ml of distilled water instead of 0.5 ml of blood. The levels of dapsone were expressed as mg/l of the whole blood, after comparing the absorbances with those obtained by a standard curve of aqueous solutions of crystalline dapsone.

## RESULTS

The means and the standard deviations of the hemoglobin, methemoglobin, sulfones, and NADH-methemoglobin reductase levels found among the 182 leprosy patients and the 137 healthy subjects are shown in Table 1. Table 2 shows the results of the multiple regression analysis applied to the data of the leprosy patients when the activity of NADH-methemoglobin reductase was considered as the dependent variable. Table 3 presents the results of the same analysis applied to the same data, but in this table the amount of methemoglobin was taken as the dependent variable.

The Figure illustrates the distribution of the examined individuals according to their NADH-methemoglobin reductase levels.

## DISCUSSION

The data in Table 1 confirm previous findings that leprosy patients usually present significantly smaller amounts of hemoglobin than do healthy individuals, this difference being due, at least in part, to the hemolytic effect of dapsone<sup>(1, 5)</sup>.

In spite of the similar values of NADH-methemoglobin reductase activity exhibited by the leprosy patients and the healthy subjects (Table 1), these two groups should be considered as different concerning this trait. In fact, the individual measurements

TABLE 1. Means ( $\pm$ Standard Deviations) of the variables studied on 182 leprosy patients and 137 healthy subjects.

Variable	Leprosy patients	Healthy subjects	Difference
Hemoglobin (g%)	10.98 ( $\pm$ 1.78)	13.71 ( $\pm$ 1.45)	t = 14.654 <sup>c</sup>
Methemoglobin (%)	3.27 ( $\pm$ 2.65) <sup>a</sup>	2.44 ( $\pm$ 2.07) <sup>b</sup>	F = 1.64 <sup>d</sup> t' = 3.140 <sup>d</sup>
Sulfones in blood (mg/l)	4.19 ( $\pm$ 2.26)	—	
NADH-methemoglobin reductase ( $\Delta A_{600}/\text{min} \times 10^4$ )	50.62 ( $\pm$ 22.89)	53.58 ( $\pm$ 12.94)	F = 3.13 <sup>d</sup> t' = 1.461

<sup>a</sup> According to Benesch, *et al.*'s method (<sup>2,24</sup>) the values are: mean = 7.05%; S.D. = 3.83%.

<sup>b</sup> According to Benesch, *et al.*'s method (<sup>2,24</sup>) the values are: mean = 5.47%; S.D. = 3.67%.

<sup>c</sup> p < 0.001.

<sup>d</sup> p < 0.01.

of this enzyme among the leprosy patients were significantly more dispersed around the mean than those of the healthy subjects (F = 3.13; p < 0.01). As a consequence of this greater dispersion (The Figure), the proportion of individuals exhibiting a partial deficiency of NADH-methemoglobin reductase ( $\Delta A_{600}/\text{min} \times 10^4 < 30$ ) was significantly higher among the leprosy patients (22.5%) as compared to the healthy individuals (2.9%; corrected  $\chi^2 = 23.210$ ; p < 0.001). We cannot find a plausible explanation for these findings.

As can be seen in Table 2, the NADH-methemoglobin reductase activity among the leprosy patients was negatively correlated to the hemoglobin level and positively correlated to age. It is conceivable that the negative correlation to hemoglobin is a consequence of the hemolytic effect of dapsone, which increases the proportion of young erythrocytes in the blood (<sup>5</sup>). Since young

red cells exhibit a higher activity of NADH-methemoglobin reductase than older erythrocytes (<sup>9, 13</sup>), this could explain the negative correlation between the activity of this enzyme and the hemoglobin level. The weak correlation between age and NADH-methemoglobin reductase activity was confirmed by the small increase of the determination coefficient when age was included in the stepwise regression analysis.

Table 1 shows that the concentration of methemoglobin among the leprosy patients was slightly but significantly higher as compared to the healthy individuals. This increase of the methemoglobin level among the leprosy patients was influenced by the amount of sulfones in the blood (Table 3), which may generate methemoglobin as a consequence of the direct oxidation of hemoglobin. The increase of methemoglobin in the blood of the leprosy patients may also be due to the hemolytic effect of dap-

TABLE 2. Results of multiple regression analysis applied to data of 182 leprosy patients when activity of NADH-methemoglobin reductase is considered as the dependent variable.

Independent variable	Regression coefficient	Standard error	t <sub>(176)</sub>
Hemoglobin	-4.92	0.95	5.19 <sup>a</sup>
Methemoglobin	-0.68	0.61	1.11
Age	0.26	0.11	2.30 <sup>b</sup>
Years under sulfone therapy	0.001	0.16	0.01
Blood level of sulfones	0.006	0.71	0.01

<sup>a</sup> p < 0.001; determination coefficient = 0.20.

<sup>b</sup> p < 0.05; determination coefficient = 0.23.

TABLE 3. Results of multiple regression analysis applied to data of 182 leprosy patients when amount of methemoglobin is considered as the dependent variable.

Independent variable	Regression coefficient	Standard error	t <sub>(176)</sub>
Hemoglobin	0.20	0.19	1.05
Age	0.01	0.02	0.50
Years under sulfone therapy	0.01	0.03	0.33
Blood level of sulfones	0.35	0.13	2.69 <sup>a</sup>
NADH-methemoglobin reductase	-0.002	0.01	0.20

<sup>a</sup> p < 0.01; determination coefficient = 0.04.

sone, since hemolysis decreases the production of NADH. However, this possibility has not been investigated because the NADH-methemoglobin reductase activity was assessed in the hemolysates to which NADH was added.

At any rate, dapsona ingested in a daily dose of 100 mg was not able to provoke signs or symptoms of toxic methemoglobinemia either among the 141 leprosy patients (77.5%) with normal activity of NADH-methemoglobin reductase or among the 41 patients (22.5%) with partial deficiency of this enzyme. On the other hand, the mean level of methemoglobin among the patients with partial deficiency of NADH-methemoglobin reductase—3.20%; S.D. = 3.06%—has not differed significantly from that found among the patients exhibiting normal activity of this enzyme—3.18%; S.D. = 2.35%. (According to Benesch, *et al.*'s method<sup>2,24</sup>), the values are 6.63%; S.D. = 3.99% and 7.17%; S.D. = 3.79%, respectively.) The present data are, therefore, in disagreement with Cohen, *et al.*'s<sup>(4)</sup> findings that even low doses of dapsona (25 mg) are able to induce toxic methemoglobinemia in people with partial deficiency of NADH-methemoglobin reductase.

Among the healthy individuals no significant correlations could be found between methemoglobin and hemoglobin levels ( $r = 0.15$ ), between NADH-methemoglobin reductase activity and hemoglobin level ( $r = -0.16$ ), or between NADH-methemoglobin reductase activity and methemoglobin level ( $r = -0.14$ ). The latter result agrees with that of Kanazawa, *et al.*<sup>(14)</sup> who observed that the positive correlation between the methemoglobin level and NADH-methemoglobin reductase activity seen in newborn children does not prevail among normal adult individuals.

Another important point concerns Scott's method<sup>(18)</sup> for evaluating the NADH-methemoglobin reductase activity. By applying this method to Indian and Eskimo isolates from Alaska, among whom is found a high frequency of congenital methemoglobinemia, Scott<sup>(18)</sup> found a trimodal distribution which pointed out the occurrence of three groups of persons.

One of them exhibited an almost complete deficiency of NADH-methemoglobin reductase ( $\Delta A_{600}/\text{min} \times 10^4 \leq 5$ ) and were

considered to be recessive abnormal homozygotes. A second group showed a partial deficiency of this enzyme ( $\Delta A_{600}/\text{min} \times 10^4$  between 13 and 20) and were considered to be heterozygotes. Finally, a third group included persons with a high activity of NADH-methemoglobin reductase ( $\Delta A_{600}/\text{min} \times 10^4 \geq 30$ ) and were considered to be normal homozygotes.

We believe that the trimodal distribution observed by Scott<sup>(18)</sup> was a consequence of biased sampling, since he studied whole families of persons affected with congenital methemoglobinemia as well as their near consanguineous relatives. When Scott's<sup>(18)</sup> method is applied to samples randomly collected which do not include persons with congenital methemoglobinemia, a unimodal instead of the expected bimodal distribution appears. In agreement with our findings, Fialkow, *et al.*<sup>(7)</sup>, analyzing the NADH-methemoglobin reductase activity of 49 blood bank donors, found a unimodal distribution in spite of the fact that two persons (4.1% of their sample) had enzyme levels that would have led Scott<sup>(18)</sup> to classify them as heterozygotes. It is also important to stress that no significant difference is detected when the mean values of NADH-methemoglobin reductase activity observed by Fialkow, *et al.*<sup>(7)</sup> (mean = 55.6; S.D. = 13.8) and by us among healthy individuals (mean = 53.6; S.D. = 12.9) are compared ( $0.30 < p < 0.40$ ).

Besides depending upon a multi-enzyme system, as first suggested by Petraghani, *et al.*<sup>(17)</sup>, methemoglobin reduction is also a consequence of the action of nonenzyme substances such as ascorbic acid and glutathione<sup>(8)</sup>. This multi-enzyme system includes two NADH- and two NADPH-dependent methemoglobin reductases<sup>(20, 21)</sup>. Even considering the fact that one of the NADH-dependent methemoglobin reductases (NADH-cytochrome  $b_5$  reductase) is responsible for 61% of the erythrocytic capacity of methemoglobin reduction<sup>(20)</sup>, it seems likely that in Scott's<sup>(18)</sup> method the reduction of DCIP by the hemolysate might be influenced by the other NADH-methemoglobin reductase, the NADPH-methemoglobin reductases<sup>(11)</sup>, and by reduced glutathione<sup>(19)</sup>.

Taking into account these considerations, it seems plausible to accept that Scott's<sup>(18)</sup>

method is not appropriate for screening persons who are heterozygous for the NADH-cytochrome b<sub>5</sub> reductase deficient gene. The same is probably true with respect to Hegesh, *et al.*'s<sup>(10)</sup> method, in which methemoglobin itself is used as an acceptor for electrons. As a matter of fact, by applying his and Hegesh, *et al.*'s<sup>(10)</sup> method to 34 persons, Scott<sup>(19)</sup> obtained identical results. A specific method using cytochrome b<sub>5</sub> as the final acceptor of electrons is therefore needed for identifying heterozygous individuals for the gene which determines the NADH-cytochrome b<sub>5</sub> reductase deficiency.

### SUMMARY

The NADH-methemoglobin reductase activity as well as hemoglobin and methemoglobin levels were investigated in blood samples of 182 adult leprosy patients and 137 Brazilian army enlisted men. The level of sulfones in the blood samples of the leprosy patients, all of them ingesting a daily dose of 100 mg dapsone, was also investigated.

The mean value of NADH-methemoglobin reductase activity exhibited by the leprosy patients did not differ from that observed among the healthy individuals. However, the variance of the former group was significantly higher than that observed among the healthy subjects. As a consequence, the proportion of individuals showing a partial deficiency of NADH-methemoglobin reductase was significantly higher among the leprosy patients (22.5%) than among the healthy individuals (2.9%). The activity of this enzyme among the leprosy patients was negatively correlated to the hemoglobin level and slightly positively correlated to age.

The concentration of methemoglobin among the leprosy patients was slightly but significantly higher as compared to the healthy individuals. The increase of the methemoglobin level among the leprosy patients was influenced by the amount of sulfones in the blood. However, no case in which dapsone was ingested in a daily dose of 100 mg presented the signs or symptoms of toxic methemoglobinemia.

### RESUMEN

Se midieron los niveles sanguíneos de la actividad de la NADH-metahemoglobina reductasa, de la he-

moglobina y de la metahemoglobina en 182 pacientes adultos con lepra y en 137 soldados brasileños. También se investigaron los niveles de sulfonas en la sangre de pacientes, todos ellos con una ingesta diaria de 100 mg de dapsona.

El valor medio de la actividad de NADH-metahemoglobina reductasa exhibida por los pacientes con lepra no difirió de la observada entre los individuos sanos. Sin embargo, la varianza del primer grupo fue significativamente mayor que la observada entre los sujetos sanos. Como consecuencia, la proporción de individuos que mostraron una deficiencia parcial de la NADH-metahemoglobina reductasa fue significativamente mayor entre los pacientes con lepra (22.5%) que entre los individuos sanos (2.9%). La actividad de esta enzima entre los pacientes con lepra correlacionó negativamente con el nivel de hemoglobina y en forma ligeramente positiva con la edad.

La concentración de metahemoglobina entre los pacientes con lepra fue ligera-pero significativamente mayor que en los individuos sanos. El aumento del nivel de metahemoglobina entre los pacientes con lepra estuvo influenciado por la cantidad de sulfonas en la sangre. Sin embargo, en ninguno de los casos que ingerían dapsona en una dosis diaria de 100 mg se encontraron síntomas o signos de metahemoglobinemia tóxica.

### RÉSUMÉ

Dans des échantillons de sang provenant de 182 malades de la lèpre adultes, et de 137 recrues de l'armée brésilienne, on a déterminé les niveaux de la NADH-méthémoglobine réductase, de même que les taux d'hémoglobine et de méthémoglobine. On a également étudié les taux de sulfone dans les échantillons sanguins des malades de la lèpre, qui tous absorbaient une dose quotidienne de 100 mg de sulfone.

La valeur moyenne de l'activité en NADH-méthémoglobine réductase chez les malades de la lèpre n'était pas différente de celle observée chez les individus sains. Néanmoins, chez ces malades, la variance était significativement plus élevée que celle relevée chez les témoins. En conséquence, la proportion d'individus présentant une déficience partielle en NADH-méthémoglobine réductase était significativement plus élevée chez les malades de la lèpre (22.5%) que parmi les témoins sains (2.9%). L'activité de cette enzyme chez les malades de la lèpre présentait une corrélation significative avec le taux d'hémoglobine, et une corrélation légèrement positive avec l'âge.

La concentration de méthémoglobine chez les malades de la lèpre était légèrement, mais significativement, plus élevée, que chez les individus sains. L'augmentation du taux de méthémoglobine chez les malades de la lèpre était influencée par la quantité de sulfones dans le sang. Néanmoins, aucun malade absorbant une dose de sulfones de 100 mg par jour n'a présenté de symptômes toxiques de méthémoglobinémie.

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