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Research Article

THE *IN VITRO* FORMATION OF AN OXIDIZING AGENT BY SURVIVING TISSUES AND SULFANILAMIDE

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Methemoglobin formation generally occurs during sulfanilamide therapy in humans (1, 2, 3, 4, 5). From the clinical statistics on methemoglobinemia it has been deduced that sulfanilamide is partially converted by the body into some active agent which can function as an oxidant (5). It is the purpose of this paper to determine whether the oxidation of hemoglobin is performed by sulfanilamide itself or whether the active agent is produced through the interaction of tissues and sulfanilamide.

METHODS

Normal animals were killed by a blow on the head followed by decapitation. The tissues were removed and slices of 0.2 to 0.4 mm. thickness were immediately prepared. These were suspended in 5 ml. Ringer-phosphate solution of pH 7.4 (6) containing 0.1 gram per cent glucose, varying concentrations of sulfanilamide, and the saline-washed erythrocytes from approximately 0.5 ml. of normal human blood. The mixtures were placed in 50 ml. Erlenmeyer flasks and were shaken constantly in a water bath at 37.5° C. for approximately 2 hours. The tissue slices were removed, dried, and weighed. The dry weight was generally between 30 and 50 mgm. The red cells were centrifuged, washed with physiological saline, hemolyzed with saponin, and diluted to 10 ml. with 1/20 M pH 7.4 phosphate buffer. Methemoglobin and total hemoglobin were determined spectrophotometrically by the change in extinction at 634 mμ upon conversion to cyanmethemoglobin (7). The presence of methemoglobin was confirmed by the disappearance of the alpha band upon the addition of cyanide, hydrosulfite, and ammonium hydroxide. Furthermore, the percentage of methemoglobin in the mixture as determined from the ratio of the extinctions at 575 mμ to 560 mμ (8) checked with the quantity as previously determined. Only methemoglobin could give these results. The bands of pigments other than methemoglobin and oxyhemoglobin were not detected with the hand spectroscope.

EXPERIMENTAL DATA

The incubation of erythrocytes with Ringer-phosphate solution containing from 0 to 250 mgm. per cent of sulfanilamide never caused the conversion of more than 1 per cent of the total hemoglobin to methemoglobin. The incubation of erythrocytes with tissue slices of the liver, kidney, muscle, spleen of the mouse, rabbit, rat, cat, and guinea pig, was likewise without appreciable effect on the hemoglobin. However, when liver slices were incubated with sulfanilamide and red cells, the formation of methemoglobin invariably occurred. A sample protocol is shown in Table I. Similar results were obtained with the livers of the cat, guinea pig, and rabbit. Other animals were not tried. These results are shown in Figure

TABLE I

*The formation of methemoglobin upon incubation of liver slices with sulfanilamide and red cells**

Preparation: Liver slice. Hemoglobin added: Human red cells (66 mgm. of oxyhemoglobin in mouse experiment. 87 mgm. of oxyhemoglobin in rat experiment). Duration of incubation: 2 hours.

Animal	Final sulfanilamide concentration	Weight of dry tissue	Methemoglobin		Conversion of total hemoglobin
	mgm. per cent	*mgm.*	*mgm.*	*mgm. per mgm. of dry tissue*	*per cent*
No tissue.....	100	0	0.5		0.8
Mouse.....	0	42	0.5		0.8
Mouse.....	25	31	12.0	0.4	18.2
Mouse.....	50	32	15.4	0.5	23.3
Rat.....	0	47	0.5		0.6
Rat.....	10	54	14.3	0.3	16.4
Rat.....	25	42	22.0	0.5	25.3
Rat.....	50	51	33.0	0.6	38.0
Rat.....	100	51	38.0	0.8	43.6

* Supernatant fluid after removal of tissue and red cells was colorless.

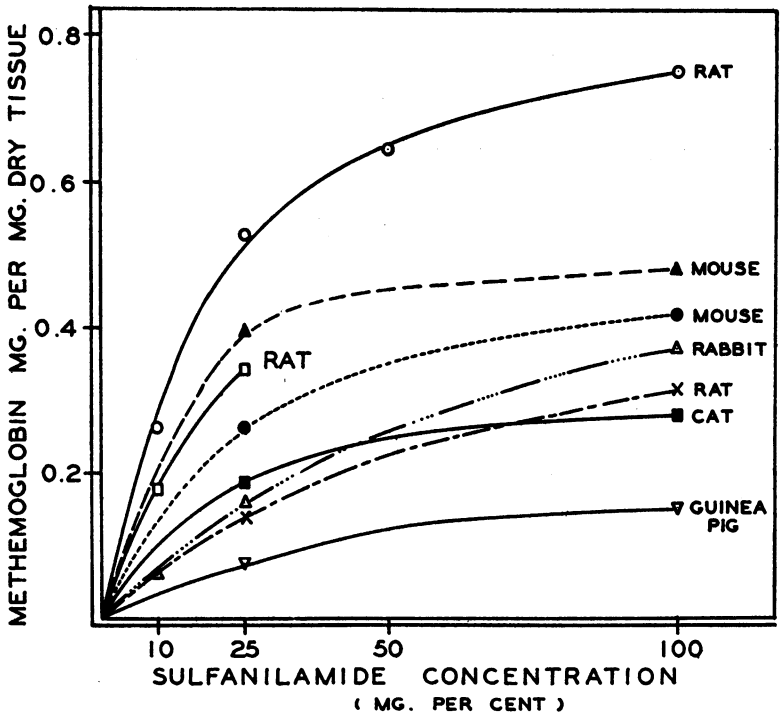

FIG. 1. FORMATION OF METHEMOGLOBIN UPON THE INCUBATION OF LIVER SLICES WITH SULFANILAMIDE AND ERYTHROCYTES

1 in which the quantity of methemoglobin formed per milligram of dry tissue is plotted against the sulfanilamide concentration used.[1] It is to be noted that the extent of methemoglobin formation increases with the sulfanilamide concentration, but tends to reach a maximum at approximately 100 mgm. per cent of sulfanilamide. The rate of methemoglobin formation is different for different animals of the same species.

Results obtained with the kidneys of the rat and mouse are shown in Figure 2. The mouse kidney is very effective, while the rat kidney is a much less efficient preparation. Unequivocal results were not obtained with the muscle or spleen of the cat, rat, mouse, rabbit, or guinea pig, nor with the kidney of the guinea pig or cat, nor with the brain of the rabbit or cat. Since the active agent is formed through the action of kidney, it is evident that this process is not related to the acetylation of sulfanilamide. The latter is not performed by the surviving kidney (9).

The intact liver cell is not absolutely necessary in the formation of the active agent since broken cell preparations (prepared by grinding liver with sand and passing the mixture through muslin)

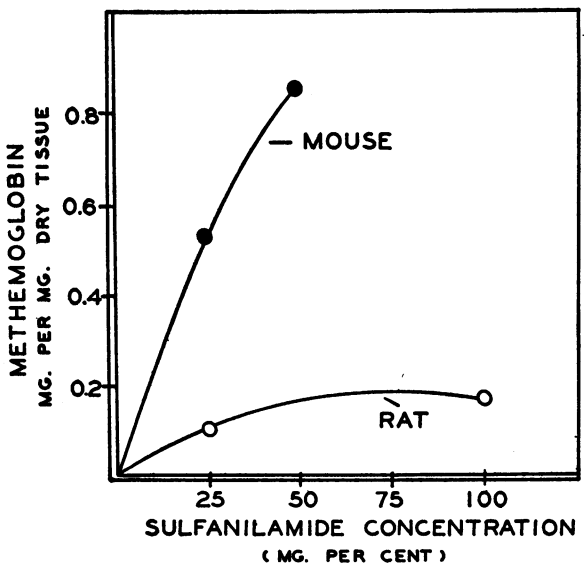

FIG. 2. FORMATION OF METHEMOGLOBIN BY KIDNEY SLICES *in Vitro*

[1] The sodium salt of sulfapyridine had a slightly smaller effect than sulfanilamide.

can effect the oxidation of hemoglobin when incubated with sulfanilamide. The effect, however, is very small. When a concentrated liver preparation of the rat was incubated with 100 mgm. per cent of sulfanilamide and 70 mgm. of hemoglobin, 5.5 mgm. of methemoglobin were formed as against 2.8 mgm. in the control without sulfanilamide. Dilute suspensions are ineffective.

The presence of the intact erythrocyte is not necessary to demonstrate this effect. Purified human hemoglobin was prepared by shaking washed erythrocytes with 1/7 volume of toluene, centrifuging off the hemoglobin solution, and dialyzing for several days against distilled water. After incubation with liver slices, or after incubation with Ringer-phosphate solution containing from 0 to 250 mgm. per cent of sulfanilamide for two hours, this solution showed 7 per cent of the total hemoglobin in the form of methemoglobin. When incubated with liver slices and 50 mgm. per cent of sulfanilamide, the methemoglobin rose to 13 per cent and with 100 mgm. per cent of sulfanilamide, it rose to 17 per cent. In these experiments 0.2 ml. of a solution containing 20.3 grams per cent of total hemoglobin were placed in a total volume of 5 ml. of sulfanilamide solution. Similar results were obtained with slices from the kidney of the rat and from the liver of the rabbit.

The fact that methemoglobin was formed whether or not the hemoglobin was contained within red cells suggested that a dialyzable agent was formed through the action of liver on sulfanilamide and that this agent then diffused through the erythrocyte membranes to oxidize the hemoglobin. The following experiments show that this conclusion is correct and that the simultaneous presence of tissue and hemoglobin is not necessary.

Liver slices were incubated with varying concentrations of sulfanilamide for two hours. At the end of that time, the tissue was removed and the fluid centrifuged at 3,000 r.p.m. for 15 minutes. The clear and colorless supernatant fluid was removed and incubated at 37.5° C. with human erythrocytes for 45 minutes. The results are shown in Table II. Methemoglobin formation occurs and the extent of the formation depends upon the concentration of sulfanilamide which was used during the incubation of the tissues.

This process may be repeated several times.

TABLE II

The formation of methemoglobin by the supernatant fluid of the reaction between liver slices and sulfanilamide

Sulfanilamide concentration	Volume of supernatant fluid	Hemoglobin of added red blood cells	Methemoglobin formed	Per cent conversion of total hemoglobin
mgm. per cent	*ml.*	*mgm.*	*mgm.*	*per cent*
0	5	87	0.5	0.6
0	5	87	0.5	0.6
10	5	87	4.4	5.1
25	5	87	10.0	11.5
100	5	87	21.0	24.2
200	5	87	35.0	40.3

Rat liver slices were suspended in Ringer-phosphate solution containing human red cells and sulfanilamide. After interaction for two hours, the colorless supernatant fluid obtained on centrifuging was again placed in contact with fresh red cells. A further formation of methemoglobin occurred (Table III–A). In other experiments, liver was incubated with sulfanilamide but without red cells for two hours. The super-

TABLE III

Methemoglobin formation upon repeated additions of red cells to a solution of the active agent

Tissue	Sulfanilamide concentration	Hemoglobin of added red cells	Methemoglobin formed	Conversion of total hemoglobin	Weight of dry tissue
	mgm. per cent	*mgm.*	*mgm.*	*per cent*	*mgm.*
		A			
1. Rat liver	0	52	0.5	1.0	42
2. ———	———	52	0.5	1.0	
3. Rat liver	10	52	9.9	19.0	57
4. ———	———	52	5.0	9.6	
5. Rat liver	25	52	14.8	28.5	43
6. ———	———	52	7.1	13.7	
		B			
7. Rat liver	25	0			53
8. ———	———	52	5.6	10.8	
9. ———	———	52	3.8	7.3	
10. Rat liver	100	0			53
11. ———	———	52	12.7	24.4	
12. ———	———	52	8.8	16.9	

Tissue incubated in 1, 3, 5, 7, and 10 for 2 hours. Supernatant fluid from these was incubated with fresh red cells for 45 minutes in 2, 4, 6, 8, and 11. Supernatant fluid of 8 and 11 was again incubated with fresh red cells for 45 minutes in 9 and 12.

natant fluid was incubated for 45 minutes with red cells and the formation of methemoglobin resulted. The supernatant fluid from this reaction was again incubated with fresh red cells and methemoglobin was again formed. The results are seen in Table III–B.

These effects might occur because all of the oxidizing agent had not been utilized in an irreversible reaction by the first addition of red cells. However, a consideration of the rate of reaction shows that methemoglobin formation tends to reach a maximum at the end of 45 to 60 minutes even when high concentrations of sulfanilamide are used. This is shown in Figure 3 where the supernatant fluid from the mixture in Ringer-phosphate containing 100 mgm. per cent of sulfanilamide was incubated with red cells for varying lengths of time. Nevertheless, if fresh red cells are incubated with the supernatant fluid (see Table III), an additional methemoglobin formation to an extent of 2/3 of the original intensity occurs. Furthermore, the supernatant fluid from the incubation of liver slices in a solution containing 100 mgm. per cent of sulfanilamide caused the formation of 21 mgm. of methemoglobin in 60 minutes in the presence of red cells having a total of 65 mgm. of oxyhemoglobin. The same supernatant fluid, however, caused the production of 34 mgm. of methemoglobin in the same period of time when incubated with double the quantity of red cells. The ratio of oxidized hemoglobin to total hemoglobin thus remains relatively the same. These results seem best to be explained by the action of a redox system on the hemoglobin-methemoglobin system.

The most intense methemoglobin formation per mgm. of sulfanilamide occurred when liver slices were incubated with red cells in 5 ml. of a solution containing 10 mgm. per cent of sulfanilamide, 14 mgm. of methemoglobin being formed. This quantity of sulfanilamide, if converted into a monovalent oxidizing agent, would be equivalent to 48.5 mgm. of hemoglobin. Therefore 29 per cent of the theoretical yield was obtained. It is probable, however, that most of the sulfanilamide is not converted into an active agent. It has been found that the destruction of sulfanilamide by the surviving liver as tested by the method of Marshall (10) is negligible (9). Unless the amino group is held intact during the formation of the active agent, the substance must be autoxidizable and must act as a catalytic reversible redox system. Furthermore, when the supernatant fluid from the incubation of rat liver slices in sulfanilamide solutions is mixed with

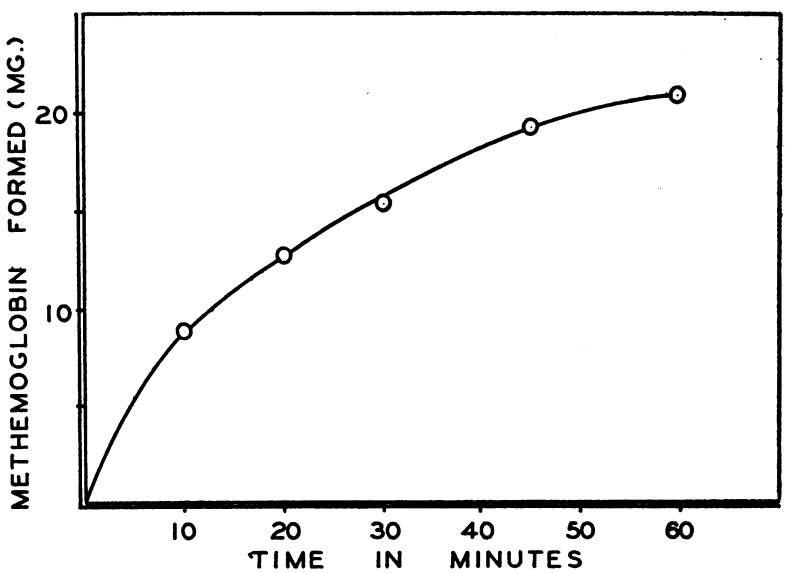

FIG. 3. RATE OF FORMATION OF METHEMOGLOBIN IN RED BLOOD CELLS INCUBATED WITH THE SUPERNATANT FLUID OF THE REACTION OF RAT LIVER SLICES ON SULFANILAMIDE

red cells under anaerobic conditions, very little methemoglobin is formed as compared to the result of the same experiment under aerobic conditions. This suggests but does not prove that the continual reoxidation of the agent by air ordinarily occurs. These problems are being investigated more thoroughly.

It is possible that the active agent may owe its effect to an anticatalase action. Accumulation of hydrogen peroxide during the metabolism of the red cells might result and might cause the oxidation of hemoglobin to methemoglobin. To eliminate this possibility, the following experiment was performed. Liver slices were incubated in Ringer-phosphate solution containing 100 mgm. per cent of sulfanilamide. The supernatant fluid after centrifugalization was incubated for 30 minutes with human red cells. The mixture was then hemolyzed with saponin. The same procedure was followed in another vessel save that the supernatant fluid of liver slices incubated in Ringer-phosphate solution without sulfanilamide was used. To each mixture of hemolyzed cells and supernatant fluid was added a small amount of hydrogen peroxide and the oxygen evolution was measured manometrically in the Warburg apparatus. The rate and extent of the catalase action were found to be identical in both mixtures. The extent of methemoglobin formation in the preparation containing sulfanilamide was then compared with another similar preparation which had not been mixed with hydrogen peroxide. No change in the amount could be detected although both had much more methemoglobin than did the control without sulfanilamide. The entire experiment was then repeated with the exception that the red cells were not hemolyzed before the addition of the peroxide, and the same results were obtained. If the active agent acted through anticatalase action, the oxygen evolution after the addition of hydrogen peroxide should have been slower in the solutions containing sulfanilamide and an increased formation of methemoglobin should have resulted in those solutions when peroxide was added.

Using the technique of testing the supernatant fluids (from the incubation of tissue and sulfanilamide) with red cells, it has been found that precipitation of the protein with trichloracetic acid does not remove the active substance. The agent is dialyzable through ordinary viscose membranes, is relatively stable at 0°, and is destroyed by boiling. The substance can exert its action on red cells when they are suspended in serum.

DISCUSSION

The formation of an oxidizing agent from sulfanilamide by tissues adequately explains the methemoglobinemia which has been found in patients treated with this drug. Clinical methemoglobemia has been noted to depend upon the concentration of sulfanilamide in the blood, and to reach a peak several hours after the sulfanilamide concentration of the blood had reached its peak (5). The methemoglobinemia of patients was also found to depend slightly upon the concentration of circulating hemoglobin. All these results have been repeated with the use of surviving tissues *in vitro*.

It is to be noted that all the supernatant fluids from the incubation of tissue with sulfanilamide were colorless. Nevertheless, these preparations were more active in forming methemoglobin than some of the blue solution prepared by the action of ultraviolet light on sulfanilamide solutions by the method of Ottenberg and Fox (11). It is therefore very improbable that the active agent formed by tissues is identical with the blue photooxidation product. If this were true, the color should have been easily detected in the supernatant solutions. In addition, a marked destruction of sulfanilamide (at least of the amino group) occurs during the action of ultraviolet light whereas practically no destruction can be detected by the same method when sulfanilamide is incubated with tissue.

Main, Shinn, and Mellon (12) have suggested that a substance having an anticatalase action is formed by bacteria from sulfanilamide and that the resultant accumulation of hydrogen peroxide may explain the therapeutic action of the drug. The formation of methemoglobin by tissues and sulfanilamide does not depend upon a similar action and the agent reported herein is not identical with the anticatalactic substance because (*a*) the red cells after incubation with the agent retain their catalase activity, (*b*) the oxygen uptake of red cells in the absence of an autoxidizable carrier is so small that, even though all the oxygen

were transformed to hydrogen peroxide, not more than 1 mgm. of methemoglobin would have been formed under the conditions of our experiments, (*c*) the activity of the agent persisted after preliminary interaction of red cells, which should have absorbed any anticatalase present.

Rimington (13) has suggested that the agent causing methemoglobinemia may also account for the excess porphyrin excretion seen during sulfanilamide therapy. The proof of this possibility and the relation of the agent to other toxic manifestations must await the isolation of larger quantities of the active agent.

The oxidizing agent may account for some of the therapeutic properties of the drug. Levaditi and Vaisman (14) have shown that sulfanilamide which has been absorbed *via* the gastro-intestinal tract seems to be more potent than locally applied sulfanilamide, and they believed that the gastro-intestinal tract or viscera caused some change in the drug. It is planned to study the bactericidal activity of the agent.[2]

SUMMARY

It has been demonstrated that sulfanilamide cannot function as an oxidizing agent on hemoglobin. Upon the interaction of certain tissues with sulfanilamide, an oxidizing agent is formed which can cause the production of methemoglobin. This process has been discussed in relation to the clinical findings in patients on sulfanilamide therapy.

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[2] Since this paper was accepted for publication, the theoretical importance of oxidation products of sulfanilamide with high oxidizing potentials has been admirably stressed by P. A. Shaffer. (The mode of action of sulphanilamid. Science, 1939, **89**, 547.)