On the Influence of Different Metal Salts on the Peroxidase Reaction.*

The 22nd Report of the Peroxidase Reaction

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The peroxidase reaction of Sato and Sekiya¹⁾ is called the copper method because of the use of copper sulphate solution. As they state,²⁾ the copper method is superior to other benzidine peroxidase methods, at least in one or more of the following points: 1) in simplicity, 2) in sensitivity, 3) in constancy of color and its contrast with the unstained red cells and 4) in the durability of the reagents used. They attribute all these features of the reaction to the use of copper solution. "The use of copper solution," they say, "not only makes unnecessary any fixation reagent such as formalin or alcohol etc., but also makes the fixation within a very short time, almost the instant a blood smear comes into contact with it. Thus we lose no time in fixation. Besides, copper solution is effective in keeping peroxidase granules, while mere water tends to loosen them. Thus the fixation as well as part of the peroxidase stain is done in almost no time. Thus, the time required for application of solution B is practically the whole time required for our peroxidase reaction." Before the publication of their copper method report, they had tested copper nitrate instead of the sulphate under the predication that the former might be better than the latter, because the second solution to be used together with the copper sulphate solution was benzidine. But the result was contrary to their expectation, the sulphate being far better than the nitrate. But they were unable to give the reason.

*) Part of paper read at the 7th General Meeting of the Tohoku Medical Society, Sendai, 1922, October.

1) A. Sato & S. Sekiya; Tohoku J. of Exp. Med., 1926, 7, 111.

2) A. Sato & S. Sekiya; Tohoku J. of Exp. Med., 1926, 7, 113.

The present paper will show which metal salts will give a similar influence to the copper sulphate on the peroxidase reaction.

Method of Investigation.

To an air-dried blood smear Solution I (see below) is applied, then most of it is poured off and immediately Solution II (see below) is applied and kept for two minutes. Then it is washed thoroughly with water. Solution II is prepared as follows: Rub 0.2 gm. of benzidine with a few drops of water in a mortar. Add 200 c.c. of water at room temperature and filter. To the filtrate add 4 drops of hydrogen peroxide (3 per cent). Solution I in this case is one of different metal solutions, which are made in a concent-

TABLE I.

Influence of different metal salts on the peroxidase reaction.

+ = of some influence.

— = of no or almost no influence.

Metal salts	Influence	Peroxidase granules stained
AgNO_3	—	
$\text{Al}_2(\text{SO}_4)_3 \cdot 18\text{H}_2\text{O}$	+	blue
$\text{Al}_2(\text{SO}_4)_3 \cdot \text{Na}_2\text{SO}_4 \cdot 24\text{H}_2\text{O}$	+	blue
$\text{Al}_2(\text{SO}_4)_3 (\text{NH}_4)_2\text{SO}_4 \cdot 24\text{H}_2\text{O}$	+	blue
$\text{Al}_2(\text{SO}_4)_3 \cdot \text{K}_2\text{SO}_4 \cdot 24\text{H}_2\text{O}$	+	blue
$\text{BaCl}_2 \cdot 2\text{H}_2\text{O}$	—	
$\text{Cd}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$	—	
$3\text{CdSO}_4 \cdot 8\text{H}_2\text{O}$	+	blue
$\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$	—	
$\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$	—	
$\text{Co}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$	+	
$\text{Cr}_2(\text{SO}_4)_3$	+	
$\text{Cr}_2(\text{SO}_4)_3 \cdot \text{K}_2\text{SO}_4 \cdot 24\text{H}_2\text{O}$	+	
$\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$	+	
$\text{Cu}(\text{C}_2\text{H}_3\text{O}_2)_2 \cdot \text{H}_2\text{O}$	+	
$\text{Cu}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$	+	
$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$	+	
$\text{Fe}_2(\text{SO}_4)_3 (\text{NH}_4)_2\text{SO}_4 \cdot 24\text{H}_2\text{O}$	+	blue (0.1% is good for use)
FeCl_3	—	
$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$	+	
HgCl_2	—	
$\text{Hg}(\text{NO}_3)_2$	+	
KCl	—	
KI	—	
K_2CO_3	—	
KNO_3	—	
K_2CrO_4	—	
$\text{K}_2\text{Cr}_2\text{O}_7$	+	blue (0.1% is better; solution affects also the red cells.)
K_4FeCy_6	—	
$\text{K}_3\text{FeCy}_6 \cdot 3\text{H}_2\text{O}$	—	
$\text{C}_4\text{H}_4\text{O}_6 \cdot \text{KNa} \cdot 4\text{H}_2\text{O}$	—	
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	—	
$\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$	—	
$\text{MnSO}_4 \cdot 5\text{H}_2\text{O}$	—	
NaCl	—	
$\text{NaI} \cdot 2\text{H}_2\text{O}$	—	
$\text{NaBr} \cdot 2\text{H}_2\text{O}$	—	
$\text{Na}_2\text{CO}_3 \cdot 10\text{H}_2\text{O}$	—	
NaNO_2	—	
NaNO_3	—	
$\text{Na}_2\text{SO}_4 \cdot 10\text{H}_2\text{O}$	+	blue
$\text{Na}_2\text{SO}_3 \cdot 7\text{H}_2\text{O}$	—	
$\text{Na}_2\text{S}_2\text{O}_5 \cdot 5\text{H}_2\text{O}$	—	
$2\text{Na}_3\text{C}_6\text{H}_5\text{O}_7 \cdot 11\text{H}_2\text{O}$	—	
NH_4Cl	—	
NH_4NO_3	—	
$(\text{NH}_4)_2\text{SO}_4$	+	blue
NH_4SCN	—	

Metal salts	In- fluence	Peroxidase granules stained
$(\text{NH}_4)_2\text{MnO}_4$	+	violet (0.1% is better than 1%; even 0.005% is good for use.)
$\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$	+	green
$\text{NiSO}_4 \cdot 7\text{H}_2\text{O}$	+	blue
$\text{Pb}(\text{NO}_3)_2$	+	green
$\text{Pb}(\text{C}_2\text{H}_3\text{O}_2) \cdot 3\text{H}_2\text{O}$	+	green
$\text{SnCl}_4 \cdot 3\text{H}_2\text{O}$	—	
$\text{SrCl}_2 \cdot 6\text{H}_2\text{O}$	—	
$\text{Sr}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$	—	
$\text{UO}_2(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$	+	blue (1% is better than 0.1%)
$\text{UO}_2(\text{C}_2\text{H}_3\text{O}_2)_2 \cdot 2\text{H}_2\text{O}$	—	
$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$	—	blue
P_4S_3	—	
$\text{Na}_2\text{WO}_4 \cdot 2\text{H}_2\text{O}$	—	
Na_2Mo_4	—	
NH_4HCO_3	—	
$\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$	—	
$\text{C}_2\text{O}_4(\text{NH}_4)_2 \cdot \text{H}_2\text{O}$	+	blue
$(\text{C}_6\text{H}_5\text{O}_7)_2\text{Na}_3 \cdot 4\text{H}_2\text{O}$	—	
$\text{C}_2\text{H}_3\text{O}_2\text{NH}_4$	—	
$\text{C}_2\text{O}_4\text{K}_2 \cdot \text{H}_2\text{O}$	—	
$\text{Na}_2\text{C}_2\text{O}_4 \cdot \text{H}_2\text{O}$	—	

ration of from 0,1 to 1 per cent according to their solubility.

Any worker with the copper method will consider not the sulphate, but the copper ion, as the effective ingredient in the reaction and will at the same time try, instead of the copper, the ferric ion (Fe^{+++}) which would, as he may think, be more effectual.

As shown in the above Table the fer-

ric ion has no influence on the color of the reaction. And it has become doubtful whether the cation would not contribute to change the color of the benzidine reaction. Therefore it will be necessary to observe the influence of the cation, especially those of the three anorganic cations, the chloride, the nitrate and the sulphate for simplicity sake.

TABLE II.

Influence of the cations on the peroxidase reaction.

Cl'		NO_3'		SO_4''	
NaCl	—	NaNO_3	—	Na_2SO_4	—
KCl	—	KNO_3	—	K_2SO_4	—
NH_4Cl	—	NH_4NO_3	—	$(\text{NH}_4)_2\text{SO}_4$	+
CaCl_2	—				
BaCl_2	—				
SrCl_2	—	$\text{Sr}(\text{NO}_3)_2$	—		
MgCl_2	—			MgSO_4	—
				CdSO_4	+
				ZnSO_4	+
				$\text{Al}_2(\text{SO}_4)_3$	+
				$\text{AlNa}(\text{SO}_4)_2$	+
				$\text{AlK}(\text{SO}_4)_2$	+
				$\text{AlNH}_4(\text{SO}_4)_2$	+
HgCl_2	—	$\text{Hg}(\text{NO}_3)_2$	+		
CuCl_2	+	$\text{Cu}(\text{NO}_3)_2$	+	CuSO_4	+
		AgNO_3	—		
		$\text{Pb}(\text{NO}_3)_2$	—		

Cl'		NO ₃ '		SO ₄ ''	
SnCl ₄	—			MnSO ₄	—
MnCl ₂	—			FeSO ₄	+
FeCl ₃	—			FeNH ₄ (SO ₄) ₂	+
				NiSO ₄	+
NiCl ₂	+				
CoCl ₂	—	Co(NO ₃) ₂	—	Cr ₂ (SO ₄) ₃	+
				CrK(SO ₄) ₂	+
		UO ₂ (NO ₃) ₂	+		

As in the above Table, the chlorides and the nitrates possess generally almost no influence, while the sulphates have mostly a decided influence. The following experiment will show this more clearly (Table III).

TABLE III.

Effect of sulphate on benzidine peroxidase reaction.

Salt solution used instead of copper sulphate.	Influence on brown peroxidase granules	
K ₂ SO ₄ (1%)	—	
MnCl ₂ ·6H ₂ O(1%)	—	
MnCl ₂ ·6H ₂ O(1%)+K ₂ SO ₄ (1%) in equal amount	+	blue
SnCl ₄ ·3H ₂ O(1%)	—	
SnCl ₄ ·3H ₂ O(1%)+K ₂ SO ₄ (1%) in equal amount	+	blue
Cd(NO ₃) ₂ ·4H ₂ O(1%)	—	
Cd(NO ₃) ₂ ·4H ₂ O(1%)+K ₂ SO ₄ (1%) in equal amount	+	blue

— no influence. + decided influence.

In Table III, if KCl or KNO₃ is used instead of K₂SO₄, then there is no influence exercised by either of them.

From Table I it will be seen that the bichromate salt is, as would be expected, of some influence, but it tends also to change the color of red cells.

Table IV shows the influence of anions.

TABLE IV.

Influence of anions on the benzidine peroxidase reaction.

NaCl	—	KCl	—	NH ₄ Cl	—
NaNO ₃	—	KNO ₃	—	NH ₄ NO ₃	—
Na ₂ SO ₄	—	K ₂ SO ₄	—	(NH ₄) ₂ SO ₄	+
Na ₂ SO ₃	—	K ₂ CO ₃	—	(NH ₄) ₂ MoO ₄	+
Na ₂ S ₂ O ₃	—	KI	—	NH ₄ SCN	—
NaNO ₂	—	K ₃ FeCy ₆	—		
Na ₂ CO ₃	—	K ₄ FeCy ₆	—		
NaI	—	K ₂ CrO ₄	—		
NaBr	—	K ₂ Cr ₂ O ₇	+		

$\left\{ \begin{array}{l} \text{CaCl}_2 \\ \text{MgSO}_4 \end{array} \right.$	$\begin{array}{c} - \\ - \end{array}$	$\left\{ \begin{array}{l} \text{SrCl}_2 \\ \text{Sr(NO}_3)_2 \\ \text{SnCl}_4 \end{array} \right.$	$\begin{array}{c} - \\ - \\ - \end{array}$	$\left\{ \begin{array}{l} \text{BaCl}_2 \\ \text{Cd(NO}_3)_2 \\ \text{CdSO}_4 \end{array} \right.$	$\begin{array}{c} - \\ - \\ + \end{array}$
$\left\{ \begin{array}{l} \text{CuCl}_2 \\ \text{CuNO}_3 \\ \text{CuSO}_4 \end{array} \right.$	$\begin{array}{c} + \\ + \\ + \end{array}$	$\left\{ \begin{array}{l} \text{AgNO}_3 \end{array} \right.$	$\begin{array}{c} - \end{array}$	$\left\{ \begin{array}{l} \text{HgCl}_2 \\ \text{HgNO}_3 \end{array} \right.$	$\begin{array}{c} - \\ - \end{array}$
$\left\{ \begin{array}{l} \text{Cu(C}_2\text{H}_3\text{O}_2)_2 \\ \text{Al}_2(\text{SO}_4)_3 \\ \text{AlK}(\text{SO}_4)_2 \end{array} \right.$	$\begin{array}{c} + \\ + \\ + \end{array}$				
$\left\{ \begin{array}{l} \text{AlNa}(\text{SO}_4)_2 \\ \text{AlNH}_4(\text{SO}_4)_2 \end{array} \right.$	$\begin{array}{c} + \\ + \end{array}$				
$\left\{ \begin{array}{l} \text{SnSO}_4 \end{array} \right.$	$\begin{array}{c} + \end{array}$	$\left\{ \begin{array}{l} \text{Pb(NO}_3)_2 \\ \text{Pb(C}_2\text{H}_3\text{O}_2)_2 \end{array} \right.$	$\begin{array}{c} - \\ - \end{array}$	$\left\{ \begin{array}{l} \text{CoCl}_2 \\ \text{Co(NO}_3)_2 \end{array} \right.$	$\begin{array}{c} - \\ + \end{array}$
$\left\{ \begin{array}{l} \text{FeCl}_2 \\ \text{FeSO}_4 \end{array} \right.$	$\begin{array}{c} - \\ + \end{array}$	$\left\{ \begin{array}{l} \text{NiCl}_2 \\ \text{NiSO}_4 \end{array} \right.$	$\begin{array}{c} + \\ + \end{array}$	$\left\{ \begin{array}{l} \text{MnCl}_2 \\ \text{MnSO}_4 \end{array} \right.$	$\begin{array}{c} - \\ - \end{array}$
$\left\{ \begin{array}{l} \text{FeNH}_4(\text{SO}_4)_2 \end{array} \right.$	$\begin{array}{c} + \end{array}$				

As shown in Table IV, the ferric ion is of no influence, while the iron alum exercises a decided influence, which may be explained from the fact that the latter is a sulphate compound. The most remarkable thing in Tables III and IV is that the copper sulphate has the combined advantage of both the copper and the sulphate ion; all the copper salts and most of the sulphate salts exercise decided influence on the benzidine peroxidase reaction. Sato and Sekiya accidentally and very happily selected the right salt as Solution I of their peroxidase reaction; after all my investigations I can say there is no better salt for the purpose. And it was for this reason that they wondered why they could not get a better or even an equal result with the copper nitrate.

Remarks.

Of many salts tabulated above which give positive influence on the benzidine peroxidase reaction, only the two salts chrome alum and ammonium molybdate are to be selected as Solution I, if the following conditions are to be fulfilled: 1. Solution I should fix fresh blood smears, (viz. red cells as well as leucocytes), 2. it should remain without any influence on the naive color of red cells, and 3. the peroxidase granules should be so colored that the color stands in a conspicuous contrast to the remaining unstained red cells. Chrome alum stains the granules a beautifully dark blue color, and ammonium molybdate, dark violet.

It would seem that the peroxidase granules are stained blue in the copper method of Sato and Sekiya because of the blue color which develops when Solution I and II are mixed.¹⁾ The fact is different.

1) Cf. Arnold and Mentzel, Ber. d. dtsh. chem. Ges. 1902, 35, II, 1328.

There are indeed a number of salts above tabulated which produce a blue color when added to Solution II of their peroxidase reagent, but not all of them will stain the peroxidase granules blue when used in combination with Solution II. Nay, neither of those two salts—ammonium molybdate, 0.1 percent and chrome alum, 1 percent—which may be used instead of copper sulphate in the peroxidase reaction, will produce any remarkable change of color when poured with Solution II on the blood smear, but nevertheless they stain the peroxidase granules, dark blue and dark violet respectively.

By way of appendix the effect of a double use of metal salts, which are of no influence in themselves when used independently as shown in Table I, will be shown in Table V.

TABLE V.

Metal salts	Influence on peroxidase granules	
$\left\{ \begin{array}{l} \text{SnCl}_2(1\%) + \text{NH}_4\text{C}_2\text{H}_3\text{O}_2(1\%) \text{ in equal amount} \\ \text{Cd}(\text{NO}_3)_2(1\%) + \text{NH}_4\text{C}_2\text{H}_3\text{O}_2(1\%) \text{ in equal amount} \\ \text{MnCl}_2(1\%) + \text{NH}_4\text{C}_2\text{H}_3\text{O}_2(1\%) \text{ in equal amount} \\ \text{Na}_2\text{S}_2\text{O}_3(1\%) + \text{NH}_4\text{C}_2\text{H}_3\text{O}_2(1\%) \text{ in equal amount} \\ \text{SnCl}_2(1\%) + (\text{C}_6\text{H}_5\text{O}_7)_2\text{Na}_3 \cdot 4\text{H}_2\text{O}(1\%) \text{ in equal amount} \\ \text{Cd}(\text{NO}_3)_2(1\%) + (\text{C}_6\text{H}_5\text{O}_7)_2\text{Na}_3 \cdot 4\text{H}_2\text{O}(1\%) \text{ in equal amount} \\ \text{MnCl}_2(1\%) + (\text{C}_6\text{H}_5\text{O}_7)_2\text{Na}_3 \cdot 4\text{H}_2\text{O}(1\%) \text{ in equal amount} \\ \text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}(1\%) + (\text{C}_6\text{H}_5\text{O}_7)_2\text{Na}_3 \cdot 4\text{H}_2\text{O}(1\%) \text{ in equal amount} \end{array} \right.$	+	blue
$\left\{ \begin{array}{l} \text{SnCl}_2(1\%) + \text{C}_2\text{O}_4\text{K}_2 \cdot \text{H}_2\text{O}(1\%) \text{ in equal amount} \\ \text{Cd}(\text{NO}_3)_2(1\%) + \text{C}_2\text{O}_4\text{K}_2 \cdot \text{H}_2\text{O}(1\%) \text{ in equal amount} \\ \text{MnCl}_2(1\%) + \text{C}_2\text{O}_4\text{K}_2 \cdot \text{H}_2\text{O}(1\%) \text{ in equal amount} \\ \text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}(1\%) + \text{C}_2\text{O}_4\text{K}_2 \cdot \text{H}_2\text{O}(1\%) \text{ in equal amount} \end{array} \right.$	+	blue
$\left\{ \begin{array}{l} \text{SnCl}_2(1\%) + \text{C}_2\text{O}_4\text{Na}_2(1\%) \text{ in equal amount} \\ \text{Cd}(\text{NO}_3)_2(1\%) + \text{C}_2\text{O}_4\text{Na}_2(1\%) \text{ in equal amount} \\ \text{MnCl}_2(1\%) + \text{C}_2\text{O}_4\text{Na}_2(1\%) \text{ in equal amount} \\ \text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}(1\%) + \text{C}_2\text{O}_4\text{Na}_2(1\%) \text{ in equal amount} \end{array} \right.$	+	blue
$\left\{ \begin{array}{l} \text{SnCl}_2(1\%) + \text{C}_2\text{O}_4\text{Na}_2(1\%) \text{ in equal amount} \\ \text{Cd}(\text{NO}_3)_2(1\%) + \text{C}_2\text{O}_4\text{Na}_2(1\%) \text{ in equal amount} \\ \text{MnCl}_2(1\%) + \text{C}_2\text{O}_4\text{Na}_2(1\%) \text{ in equal amount} \\ \text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}(1\%) + \text{C}_2\text{O}_4\text{Na}_2(1\%) \text{ in equal amount} \end{array} \right.$	—	—

÷ decided influence. — no influence.

Summary.

The significance of the copper sulphate as Solution I of the peroxidase reagent of Sato and Sekiya has been studied, together with the influence of other metal salts. The copper salt is always of decided influence, to whichever cation it may be combined, and the sulphate salt is generally of decided influence too, to whichever anion it may be combined. Most chlorides and most nitrates are of no influence. The copper sulphate has, thus, the double advantage in itself. This will explain why the copper peroxidase reaction is so sensitive and gives so distinct a picture.