An Exceedingly Sensitive Peroxidase Reagent for Human Milk.

28th Report of the Peroxidase Reaction.

By

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In the present paper a very sensitive peroxidase reagent will be reported. With respect to sensitivity it is almost essentially different from the reagents hitherto reported.

PEROXIDASE REAGENTS HITHERTO REPORTED.

According to Seligmann,¹⁾ it seems to have been Arnold (1881) who used for the first time guaiac tincture and hydrogen peroxide for the peroxidase of raw milk. Now there are a large number of reagents which have been used for peroxidase. As Bansi and Ucko²⁾ cite: "Zum Nachweis der Peroxydasen dienen zahlreiche Körper von Phenolcharakter, die farblos sind und bei der Oxydation in gefärbte Stoffe übergehen. Die gebräuchlichsten sind das Pyrogallol, das zu dem gelbbraunen Purpurogallin oxydiert wird (Struve, Bach, Chodat, Willstätter u. a.), das Guajacol (Kobert, Bertrand u. a.), das sich in braunes Tetraguajacol kondensiert, die verschiedenen Kresole (Bourquelot, Chodat), α -Naphthol (Loele), Adrenalin, Brenzcatechin, Kaffeesäure (Onslow), das Guajakharz (Scheunert c. s., Lubimenko, Palladin und Manskaja), Salicylaldehyd (Schmiedeberg 1875, Dony-Hénault), Guajaconsäure (Schaer), Hydrochinon, Phenolphthalin (Kastle, Shedd, Thomas), Dihydrodivanillin (Engler und Herzog).

1) E. Seligmann, Handbuch d. Milchkunde, von P. Sommerfeld, Wiesbaden 1909, p. 306.

2) H. W. Bansi and H. Ucko, Hoppe-Seyler's Zeitschrift, 1926, **157**, 192.

“Echte Peroxydasereaktion sind weiter die Benzidin-Methode, die Oxydation des Jodwasserstoffs zu Jod, die Entfärbung von Methylenblau, die Bildung von Malachitgrün aus Leucomalachit (Czyhlarz und Fürth).”

“Endlich gehört auch die Oxydation der Ameisensäure zu CO_2 zu den zum Nachweis der Peroxydase benutzten Methoden (Battelli und Stern).”

Besides these there have been used : Tetramethylenparaphenyldiamin, diphenylamin, mono- and paraphenyldiamin, barbados-aloin, anilacetate, mono- and dimethylanilin, phenol, o- and p-amidophenol, tyrosin or tannin, gallic acid, p-toluidin, xyloidin, thymol, resorcin, orcin, phloroglucin, benzoic acid, salicylic acid, creosote etc.

Many of the above mentioned are not sensitive enough for milk peroxidase, and even guaiac, benzidine, guaiacol, guaiaconic acid, p-phenylen-diamin, and α -naphthol etc. are not sensitive enough for milk samples which are not rich in peroxidase. It is thus quite natural that Seligmann³⁾ writes : “The indirect oxidases were found in cow’s, goat’s and donkey’s milk, but not in human and rabbit’s milk. They were however identified in the colostrum of the latter two.”

In 1913 Scheunert, Grimmer and Andryewsky³⁾ gave the following prescription which was to be used also for milk peroxidase :

5% guaiac tincture (freshly prepared)	100 c.c.
3% hydrogen peroxide	0.1–0.2 c.c.

In 1920 Marfan⁴⁾ used the following method : To 1 c.c. of human milk 1% aqueous solution of guaiacol, 3 or 4 drops of hydrogen peroxide are added, then a brick-red reaction ensues in the presence of peroxidase.

In 1926 Borinski⁵⁾ made a new reagent for milk peroxidase. His prescription is as follows : 0.85 guaiac resin is ground to power and dissolved in 85 parts of 70% alcohol by shaking, which will require from half to one hour. To the solution 10 c.c. of acid. carbol. liquefact. and 5 c.c. of 3% hydrogen peroxide are added. The reagent will keep mostly 3–4 weeks, sometimes longer. In 1924 Verway⁶⁾ used 1% dimethyl-p-phenyldiamin plus hydrogen peroxide which gave a blue color in the presence of milk peroxidase.

All the reactions above mentioned will either not react with human milk or will give only a very faint reaction at most, or will give a positive reaction in the absence of peroxidase.

3) A. Scheunert, W. Grimmer, P. Andryewsky, *Biochem. Zeitschr.*, 1913, **53**, 300.

4) A.-B. Marfan, *Journ. de physiol. et de pathol. gén.*, 1920, **18**, 985.

5) P. Borinski, *Zeitschr. f. angew. Chem.*, 1926, **39**, 281.

6) A. Verway, *Med. Journ. of South Africa*, 1924, 20.

MY OWN REAGENT.

Here I desire to report my peroxidase reagent, which will show a very intense reaction even with human milk, while even the most sensitive reagent hitherto described will give only a very faint reaction with human milk even when very rich in peroxidase. Further my reagent consists of very simple substances, though it should be prepared with much precaution as is described below.

The Peroxidase Reagent.

My peroxidase reagent consists of the following four reagents.*

1. Reagent A.

Guaiacum resin	0.3-1.0
Acidum arsenicosum	0.02
Glacial acetic acid (Merck)	0.6
Sodium acetate (+3H ₂ O) (Merck)	1.36
Alcohol (99%)	ad 100 c.c.
Preparation is described below.	

2. Reagent B.

Benzidine (prepared as described below)	1.0
Sodium acetate (+3H ₂ O) (Merck)	1.36
Alcohol (99%)	ad 100 c.c.
Preparation is described below.	

3. Reagent C.

Pharmacopoeial tincture with 0.1% peroxide formed†	1.0
Guaiacol (Ph. Jap.)	2.0
Aceton (Merck)	ad 100 c.c.
Keep in a brown bottle.	

4. Reagent D.

This will be described in the 30th Report of the Peroxidase Reaction.
(Cf. This issue, p. 97.)

Method of Preparation.

Reagent A.

To the above mentioned amount of guaiacum resin, ground to powder in a mortar, add alcohol little by little, transfer the dissolved guaiacum to a 100 c.c. volumetric flask with the dissolved arsenic acid in it. All the resin should be dissolved in about 50 c.c. alcohol. All this is transferred

* These reagents have been improved over and over again.

† Cf. T. ARAKAWA, 32nd this issue 111 and 35th Reports of the Peroxidase Reaction.

to the 100 c.c. volumetric flask. Then add glacial acetic acid and sodium acetate, and fill up to the mark with alcohol.

Reagent A, thus prepared, is transparent and yellow with pH 5.0. It will keep for years in a cool dark place. Only a small portion should be placed on the table for daily use; if covered with black, it will keep for some months at the room temperature.

Reagent B.

1. Preliminary preparation of benzidine.

Add 20–30 c.c. of toluol to 10 grm. of benzidine (Kahlbaum or Merck) and stir with a glass rod. Decant the now colored toluol and add toluol anew. Repeat the procedure till the newly added toluol will not take any color, but remain colorless in spite of vigorous stirring.

Collect the benzidine thus treated on filter paper and cover with black paper and preserve in a desiccator.

Benzidine thus treated can be dissolved in alcohol with no or only faintly yellow color even in a high concentration, though, if kept in a colorless glass, it will become gradually colored on standing.

2. Proper preparation of Reagent B.

Dissolve 1.0 of the benzidine, prepared as above described, in a mortar, adding alcohol little by little, and transfer the dissolved benzidine to a 100 c.c. volumetric flask. All the benzidine should be dissolved in 50 c.c. of alcohol. All this is transferred to the volumetric flask after which add sodium acetate. Fill alcohol up to the mark.

Reagent B thus prepared is transparent and colorless or of a faint-yellow color with pH 8.0. It will keep for years, if kept in a cool place and entirely covered with black. Only a small portion should be placed on the table for daily use, though it will keep for some months.

Use of the Reagents.

The combination, Reagent A + Reagent C, forms the Peroxidase Reagent fit for qualitative estimation of milk peroxidase and for the approximative determination of its amount.

The combination, Reagent B + Reagent D, forms the Peroxidase Reagent for a precise quantitative estimation of milk peroxidase.

Characteristics of the Combination :

Reagent A + Reagent C.

The characteristics of Reagent A + Reagent C are as follows :—

1. *Its exceeding sensitivity and distinct color.*

Its sensitivity is very remarkable, and reacts very promptly with a beautiful, dark blue color with, for instance, saliva, human milk, cow's milk, rabbit's milk etc. and plant peroxidase.

It will give a distinct reaction with cow's milk (diluted 1 : 500–1000), saliva (diluted 1 : 250–500) and human milk (diluted 1 : 100–250). As to the pressed juices of vegetables it will give a still distinct reaction, with the giant radish, the common potato, the carrot, the onion etc., in the dilution 1 : 16,000 of the juice ; with spinach, the yam, the ginger etc., in the dilution of 1 : 33,000 ; with the sweet potato, in the dilution of 1 : 130,000.

2. *Its inertness on blood.*

The combination will not react at all with hemoglobin, so that it may be safely used for the peroxidase in the cases of pus with blood, of spinal fluid contaminated with blood, and of milk pressed from a breast with bleeding fissures. Of course my reagent does not react any more with milk, which has been treated for 15 minutes at 75°.

3. *Its durability.*

This has been already explained.

4. *The darkness of its color proportionate to the content of peroxidase of the material.*

This will be explained later.

Intensity of Arakawa's Reaction.

Prof. Sato has given the name of Arakawa's reaction for the sake of simplicity, to the reaction above described.

To give an idea of the intensity of the milk peroxidase reaction performed with my reagent, the comparison of the intensity of reaction in the 5 reactions will be given : a) Bourquelot's⁷⁾ reaction, which has often been used for human milk peroxidase in literature, b) Storch's⁸⁾ reaction,

7) M. Bourquelot, Journ. d. Physiol. et de Pathol. Gén. 1920, 18, 986. Cit. by A.-B. Marfan.

8) C. Storch, Handbuch der Milchkunde von H. Rievel, Hannover 1926, 3. Edit. p. 42.

TABLE.
Peroxidase reaction on human milk (same sample).
Reading done 5 minutes after the addition of respective reagents.

	Color of reaction	Human milk (not diluted)	Diluted 1:2	Diluted 1:4	Diluted 1:8	Diluted 1:16	Diluted 1:32	Diluted 1:64	Diluted 1:128	Diluted 1:256	Control (water without peroxidase)	Blood
a. Bourquelot's reaction	light brick red	+	+	+	+	+	+	+	+	+	+	+
b. Storch's reaction	purple	+	+	+	+	+	+	+	+	+	+	+
c. Rothenfusser's reaction	purple	+	+	+	+	+	+	+	+	+	+	+
d. Schroeter's reaction	yellow orange	+	+	+	+	+	+	+	+	+	+	+
e. Arakawa's reaction	dark blue	+	+	+	+	+	+	+	+	+	+	+

* Schroeter's reaction—orange color changed to blue, 30 minutes after addition of the reagent.

c) Rothenfusser's⁹⁾ reaction, d) Schroeter's¹⁰⁾ reaction and e) my own reaction (Table).

As shown in the above table (Table) Storch's and Rothenfusser's reaction cannot be used for the peroxidase, because it will become positive without the presence of peroxidase. And Bourquelot's reaction which has frequently been used for peroxidase of human milk reacts positive to blood also.

Schroeter's reaction does not react positive to blood. One who has seen the dark blue color promptly developed in cow's milk by Schroeter's reagent will presume that it will give a strong blue reaction to human milk too, but the fact is different.

9) S. Rothenfusser, Handbuch der Milchkunde (Ergänzungsheft) von P. Sommerfeld, München 1926, p. 24.

10) G. Schroeter, Berichte d. gesamt. Physiol., 1929, 50, 37.

It will give only a faint orange color in the case of human milk as shown in the Table. My reaction is ca. 60 ($=256/4$) times as strong as Schroeter's reaction (or Bourquelot's reaction which will react to blood too).

SUMMARY.

An exceedingly sensitive peroxidase reagent is described which is 64 times as sensitive as Bourquelot's and Schroeter's reagents. It will not react to blood in spite of its exceeding sensitivity.