Peroxide Formation of Pharmacopoeial Tinctures.

32nd Report of the Peroxidase Reaction.

By

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1. Colour Reaction of Cow's Milk with Pharmacopoeial Tinctures.

In the course of an investigation into another matter I accidentally found a very distinct colour reaction of cow's milk with the use of a fresh guaiacum tincture and tincture of digitalis.¹⁾ The substitution of a number of other pharmacopoeial tinctures gave similar results, though there were differences of intensity and shade of colour. Though the survey of the works of De Clermont,²⁾ of Bach and Chodat,³⁾ of Moorl and Whitley,⁴⁾ of M. W. Onslow,⁵⁾ and of Gallagher,⁶⁾ concerning plant extract gave me the hint that the reaction in question might possibly be a peroxidase reaction, yet I was not sure of the true nature of it at that time, because tinctura cantharidis gave also a very distinct and beautiful reaction. This led me to study the reaction further with pharmacopoeial tinctures. Prof. Sato called the test Arakawa's "tincture-reaction" for the sake of simplicity. In our laboratory the reaction has now come to designate such a reaction, in which a fresh tincture of guaiacum and some one of the pharmacopoeial tinctures are used.

1) T. Arakawa and A. Sato, Tohoku J. Exp. Med., 1927, **9**, 646.

2) DeClermont, Compt. Rend. Acad. Sci., 1875, **80**, 1591.

3) A. Bach and R. Chodat, Berichte deutsch. chem. Ges., 1902, **35**, 2466.

4) B. Moorl and E. Whitley, Biochem. Journ., 1909, **4**, 136.

5) M. W. Onslow, Biochem. Journ., 1919, **13**, 1.

6) P. H. Gallagher, Bioch. J., 1923, **17**, 515.

2. Result of Tincture Reaction with Different Tinctures.

In the following Table I all the tinctures tested will be given according to the intensity of the reaction.

TABLE I.
Order of intensity of Arakawa's tincture reaction on cow's milk with different tinctures.

Pharmacopoeial tinctures	Intensity of reaction	Color of reaction
T. digitalis	##	bluish green
T. cantharidis	##	greenish blue
T. valerianae	##	blue
T. colchici	##	blue
T. colombo	##	bluish green
T. aconiti	##	blue
T. strophanti	##	blue
T. lobeliae	++	blue
T. zingiberis	++	blue
T. ipecacuanhae	++	dull green
T. chinae compositae	++	greenish blue
T. aromatica	+	green
T. asae foetidae	+	blue
T. cinnamomi	+	blue
T. strychni	+	greenish blue
T. aurantii	+	green
T. capsici	+	greenish blue
T. colocynthis	+	greenish blue
T. chinae	+	blue
T. cascarillae	+	greenish blue
T. quassiae	+	blue
T. valerianae etherea	+	greenish blue
T. aloes composita	+	greenish blue
T. aromatica acida	+	blue
T. cannabis indicae	+	green
T. opii benzoica	+	greenish blue
T. scopoliae	+	greenish blue
T. amara	+	greenish blue
T. catechu	— (= reaction negative)	
T. croci	—	
T. gallarum	—	
T. gentianae composita	—	
T. opii	—	

3. The True Nature of the Tincture Reaction.

All the tinctures above tabulated (Table I) had been obtained from the market. Then I prepared some tinctures according to the Pharmacopoeia Japonica. The first tincture used was the tinctura of digitalis prepared as follows: To 10 grms. of powdered digitalis leaves 100 c.c. of

spiritus dilutus (ca. 70%) were added and kept in a well stoppered bottle for seven days in a cool dark place with occasional shakings, and then filtered. Then reaction was performed with this fresh filtrate, but contrary to my expectation this gave not the slightest evidence of the reaction. Then the fresh tincture was left to stand at the room temperature and tested daily from then on with the results shown in Table II.

TABLE II.

Tincture reaction on cow's milk with use of fresh tincture of digitalis.

Days of lapse after filtration	1	2	3	5	7	10	15	30	50
T. digitalis	—	—	—	—	—	+	+	+	++

Other tinctures tested gave similar results, tinctura cantharidis among the rest. Thus it became probable that the reaction in question was a peroxidase reaction and that the peroxide formation of those tinctures were the cause of the positive tincture reaction. This is still further confirmed by the works of Tokué and Arakawa.⁷⁾ It is needless to add that cow's milk once boiled never gives the reaction.

4. Reaction with Tinctures Prepared with Alcohol of High Percentage.

10 grms. each of powdered radix ipecacuanhae and radix zingiberis were dried in an oven at 38° for two days and then kept in a desiccator for seven days. 5 grms. each of the dried powder were transferred into a well stoppered bottle containing 50 c.c. of 98% alcohol and kept at the room temperature for seven days with occasional shakings.

TABLE III.

Tincture reaction on cow's milk with specially prepared tinctures.

Days of lapse after filtration	1	5	7	30	37	44	74
Kinds of tinctures							
Tincture of valerian (specially prepared)	—	—	—	—	Water added in equal amount	—	—
Tincture of ginger (specially prepared)	—	—	—	—	Water added in equal amount	++	+++

7) K. Tokué and T. Arakawa, Tohoku J. Exp. Med., 1927, 9, 650.

The filtrate of each preparation was tested for given tincture reaction, but the result was negative after 30 days, even after two months, while an addition of an equal amount of distilled water to each filtrate made the reaction positive after the lapse of seven days as shown in Table III.

As shown above (Table III), tinctures prepared with alcohol of a high percentage are not suitable for the reaction, in spite of the fact that they contain in themselves such a substance as will make the reaction positive, because a mere addition of distilled water makes these inert tinctures active, if sufficient time is allowed to pass, and it will thus be seen that alcohol of a high percentage will act to retard, at least, the probable peroxide formation of tinctures.

Mere watery extracts do not give the reaction; I tried digitalis, cantharis, valerian, and ginger. After the lapse of three months they did not give the slightest evidence of the reaction.

5. Peroxide Formation Made More Probable.

If the probable peroxide formation of pharmacopoeial tinctures is true, then it will follow that if these are kept out of contact with air, they will remain inert. For the investigation of this matter I prepared tinctures of cantharis, ginger and valerian according to the Ph. Japonica, with the only difference that ca. 50% alcohol was used instead of ca. 70%. Each tincture was divided into two portions, one portion in direct contact with the air, and the other portion covered with liquid paraffin. The result is given in Table IV.

TABLE IV.

Tincture reaction on cow's milk with tinctures with and without paraffin.

Days of lapse after preparation Kinds of tinctures	1	5	7	10	20	30	60	6 months	1 year
T. cantharidis	—	—	—	+	+	++	++	++	+++
T. „ (with paraffin)	—	—	—	—	—	—	—	—	—
T. ginger	—	—	—	—	+	+	++	++	+++
T. „ (with paraffin)	—	—	—	—	—	—	—	—	—
T. valerianae	—	—	—	—	—	—	+	++	+++
T. „ (with paraffin)	—	—	—	—	—	—	—	—	—

The peroxide formation of those tinctures will be made more probable from the above experiment (Table IV). I also tried to make bubbles blown

by means of a suction pump in 200 c.c. of the tincture of colchicum which had remained inert, and tested the tincture reaction with a small portion of the tincture every hour during the whole 8 hours of bubbling. The portion taken after 7 hours showed a positive reaction (+), and that taken after 8 hours a distinct reaction (++) , while the original tincture which had not been treated remained inert of course.

6. *Some Factors Affecting the Probable Peroxide Formation of Pharmacopoeial Tinctures.*

a) Air (cf. 5).

b) Temperature.

A freshly prepared tincture of digitalis was divided into two portions. One was kept at the room temperature, and the other at about 8°C. The result is given in Table V.

TABLE V.

Tincture reaction on cow's milk with tinctures kept cold and at room temperature.

Tincture digitalis	Days of lapse after preparation	Day of filtration	5	10	17	50
Kept at room temperature	—	—	—	+	++	+++
„ at about 8°C	—	—	—	—	+	+

Other tinctures tested gave similar results, so that the room temperature accelerates the peroxide formation much more than a cold temperature.

c) Light.

To 200 c.c. of a freshly prepared tincture of digitalis 5 c.c. of 3.6% acetic acid was added and one portion of it was kept in a bottle with black paper all over it, while the other portion equal in amount was kept in a colourless transparent bottle. The result is shown in Table VI.

The portion in this colourless bottle was divided later (on the 50th day of filtration) into two portions again, and transferred each into a colourless bottle. One portion of these was kept at the room temperature within doors, while the other portion was exposed to the direct sun light on the roof for seven successive days (in the summer time).

The result was beyond my expectation ; the direct exposure for so long a time must indeed have destroyed the peroxide already formed, but when

the same bottle was kept later at the room temperature within doors for some time, the peroxide formation of that portion was much stronger than that in the other bottle which had been kept in the room since the beginning (Table VI).

TABLE VI.
*Tincture reaction on cow's milk with tinctures kept in
black and colorless bottles.*

<div>Days of lapse after filtration Kinds of bottles</div>	Day of filtra- tion	3	4	5	8	44	50	58	65
Black bottle	—	—	—	—	—	++	++	++	++
Colorless bottle	—	—	—	—	+	##	##	## (In room) + (on roof)	## (again in room)

Other tinctures tested gave similar results. Concerning the experiment of the influence of light, an influence of temperature must of course be taken into consideration. But as to the peculiar relation of the peroxide formation of the tincture exposed directly to the sunlight, further studies will be reported in the future.

d) Drying.

One portion of the tincture of valerian prepared with ca. 50% alcohol which had remained inert was evaporated to dryness over a water bath and then dissolved again in the original amount of ca. 50% alcohol.

This was compared with the other portion of the same tincture which had not been treated, as to the rapidity of peroxide formation (Table VII).

TABLE VII.
*Tincture reaction on cow's milk with tinctures with and
without special treatment.*

Days of lapse after treatment	1	5	7	20	1 month	2 months	6 months	1 year
Tinc. valerianae { Portion of the tincture treated specially	—	—	—	+	++	++	##	##
{ Portion of the tincture not treated	—	—	—	—	+	+	++	##

As shown in the above table (Table VII) drying has a most peculiar effect on the velocity of the peroxide formation. Later I performed a series

of investigations concerning the effect of drying ; the result will be reported in the near future together with the effect of the acidity on the peroxide formation.*

Remarks.

It was my first intention to utilize the reaction in question for the purpose of a peroxidase reaction of milk. During the course of this work I have been able to find a peroxidase reagent which is by far more sensitive than the reagents used in A r a k a w a's tincture reaction. By that reagent human milk even if diluted will give a very distinct reaction. (Investigations concerning the new peroxidase reagent have already been published in the 28th Report).

Summary.

1. A large number of pharmacopoeial tinctures give a more or less distinct colour reaction, when one of them is poured with the fresh guaiacum tincture on cow's milk (A r a k a w a's tincture reaction). Of the others *tinctura cantharidis* gives a very distinct colour reaction.

2. Those active pharmacopoeial tinctures are not active when freshly prepared. In the course of time they become active when they are left in contact with the air. Thus it is highly probable that those pharmacopoeial tinctures owe their activity to their peroxide formation.

3. The true nature of the tincture reaction is thus highly probably a peroxidase reaction, especially because boiled cow's milk does not give the slightest evidence of the reaction.

*) T. A r a k a w a, 36th Report of the Peroxidase Reaction.