L. NECESSARY CONDITIONS FOR TESTING WITH THE REAGENT FOR VITAMIN C. (ANSWER TO H. D. KAY AND S. S. ZILVA.)

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(Received February 20th, 1924.)

KAY and ZILVA [1923], having studied the application of my reagent to the identification of vitamin C, found that the majority of antiscorbutic liquids gave the coloured reaction, but that a preparation obtained from lemon juice did not give it, though this preparation tested on animals still contained from 1/3 to 1/4 of the vitamin C present in the initial lemon juice. Secondly, these authors obtained a positive blue reaction with a commercial preparation obtained from yeast, "marmite," and also a positive reaction with the aqueous extracts of brewer's top fermentation yeast, first washed and dried at 37°. Kay and Zilva note that gentle oxidation, proved to be sufficient to destroy the vitamin C contained in lemon juice, also brings about the disappearance of the blue reaction, but they explain these facts by supposing the presence of a substance differing from vitamin C, which is destroyed by oxidation, just like this vitamin. We must add [Bezssonoff, 1922] that the action of oxidising enzymes and that of prolonged heating in the autoclave at 120° lead to the simultaneous disappearance of the blue coloration and of the antiscorbutic action. Recently, while replying to Wedgwood and Ford [1924] I confirmed [Bezssonoff, 1924, 2] my first supposition: the blue reaction is not given by the vitamin C itself, but by an unstable radical, which is split off from it.

In the article published in 1922 [Bezssonoff, 1922], I gave an example of a liquid not containing vitamin C and giving the blue reaction (urine of an animal fed with substances rich in vitamin C), and also that of a liquid containing vitamin C and not giving the reaction (cow's milk). As I have since shown [Bezssonoff, 1924, 2] by introducing a very simple complementary condition for testing with my reagent, the absence of vitamin from urine as well as its presence in a milk can actually be certified. The liquid to be tested is heated in a test-tube (boiled for about 15 sec.) or maintained in boiling water for 5 to 10 minutes. After cooling, the coloured reaction obtained is compared with that given by the same liquid unheated. It is best to use a weakly acidified liquid. If vitamin C was present in the liquid, the coloration in the heated tube is much more intense than in the unheated tube. It can be intensified

as much as four times. If the unheated liquid gives no coloration, it appears in the heated tube (example cow's milk [Bezssonoff, 1924, 2]). On the other hand, the blue reaction given by a solution not containing vitamin C diminishes or completely disappears in the heated tube. Kay and Zilva can easily convince themselves that their preparation which did not give the blue reaction though containing vitamin C, would give this reaction if first submitted to a short heating of 10 minutes in a water-bath. The intensification will be greater if they use an acidified solution (5 % HCl).

Quite different is the case concerning the observations of the authors on yeasts. The extracts and preparations obtained from yeast give, with my reagent, a yellow-brown coloration [Bezssonoff, 1924, 1] or none at all. Plum juice [Bezssonoff, 1922] gives the same reaction and it is known [Osborne and Mendel, 1920; Hess and Unger, 1918] that this fruit, like yeast, is rich in vitamin B and does not contain vitamin C. But this brown-yellow reaction can only be obtained with these substances if the reagent used has been prepared from sufficiently purified crystals of the acid $(\text{WO}_3)_{17} \cdot \text{MoO}_3 \cdot \text{P}_2\text{O}_5, 24 \text{ H}_2\text{O}$. By using a mixture or badly purified crystals, the yellow-brown coloration is not obtained but a blue one. I convinced myself that the aqueous extract of brewer's top fermentation yeast gave an absolutely yellow-brown coloration with my pure reagent. This same yeast washed, dried and digested in water according to the procedure followed by Kay and Zilva gave a weak yellow-brown coloration and an abundant precipitate. After separating the precipitate, the colour of the liquid could no longer be distinguished. Unfortunately, I could not get the commercial preparation obtained from yeast named "marmite." But using an aqueous extract of a French commercial preparation of dried yeast, I obtained a brown-yellow reaction. In all the cases just mentioned, by using a reagent prepared from badly washed crystals or simply the mixture which is used to prepare the crystals for the reagent, instead of the yellow-brown coloration, a blue reaction is obtained. Simonet [Bezssonoff, 1924, 1], testing with my reagent a preparation from yeast rich in vitamin B, obtained a yellow-brown coloration. I must add that a specially purified preparation of vitamin B obtained from yeast by Seidell's method, tested in the latter's presence, gave a pale yellow coloration hardly visible. Recently, a similar preparation, which had been tested on animals (kindly given me by Nakamoura, G. Bertrand's laboratory, Pasteur Institute) used in 1/2 % aqueous solution to which 5 % HCl had been added, was heated on the autoclave at 120° during 45 minutes. The cooled liquid tested with a diluted reagent (0.7 % of crystals = 6 drops to 2 cc.) gave a slight flocculation and a *distinctly* yellow-brown coloration which was slowly absorbed by the precipitate.

In a note published in this *Journal*, but overlooked by the authors [Bezssonoff, 1923], I insisted upon the necessity of preparing the reagent from crystals and I gave a simple method for doing it and for verifying the sufficient degree of purity of the crystals obtained. I must add that in testing the degree of purity of the crystals obtained in this way, it is important to be convinced that

the yellow-brown reaction given by weak dilutions of pyrogallol is sufficiently stable, *i.e.* can be preserved at least for two hours without turning blue. This change of colour is produced very rapidly in the case of badly washed crystals.

In 1922 [Bezssonoff, 1922] when I said that it was better to use a reagent prepared from crystals, I did not insist enough on this point and I described my first method of preparing the reagent, which consisted in making a mixture (modification of Folin's mixture). In my reagent are present the three acids which were first introduced in a reagent by Folin, but it is necessary to lay stress on the chief differences which exist between Folin's typical reagent and mine. Folin [Folin and Denis, 1912] expressed the opinion that in this reagent the reacting substance must be very complex. According to Wu [1920], this substance consists of a mixture of two tungsto-phospho-molybdic acids: $(\text{WO}_3)_{13} \cdot (\text{MoO}_3)_5 \cdot \text{P}_2\text{O}_5$ and $(\text{WO}_3)_{14} \cdot (\text{MoO}_3)_4 \cdot \text{P}_2\text{O}_5$. My reagent contains only one phospho-molybdo-tungstic acid of this series. In this acid, the molybdenum is present in the smallest proportions that can exist and, as indicated by Wu, the reduction (blue coloration) is obtained with more facility if the proportion of molybdenum in the phospho-molybdo-tungstic acids is greater. Thus we can easily understand that when the acid present in my reagent is not used in a sufficient degree of purity, the presence of other acids containing a greater proportion of molybdenum gives a blue coloration when it ought not to be produced.

CONCLUSIONS.

1. A complementary condition for testing antiscorbutic liquids with Bezssonoff's reagent is given; it consists in shortly heating the liquid before testing it for 5 to 10 minutes in a water-bath in neutral or slightly acid medium.

(a) When the unheated liquid, though containing the vitamin C, does not give the reaction, it appears in the heated (and then cooled) liquid.

(b) The colour reaction already given in the unheated tube is distinctly intensified in the heated tube, when the liquid contains vitamin C. It diminishes or completely disappears when the liquid does not contain vitamin C.

2. The reagent must be prepared from crystals sufficiently purified and tested for their degree of purity [Bezssonoff, 1923].

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