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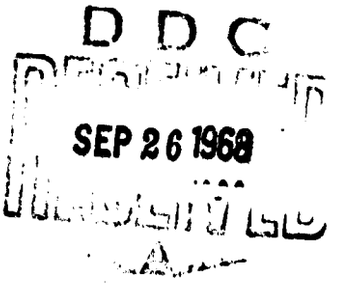

DEPARTMENT OF THE ARMY
Fort Detrick
Frederick, Maryland

Wachsmuth, H. and L. VanKoeckhoven. 1959. Contribution au dosage de la choline dans le serum sanguin (Contribution to the determination of choline in serum). Clinica Chimica Acta 4: 206-212, 1959

The determination of choline has been the objective of numerous investigations. The preliminary precipitation of choline, carried out in the absence of a specific color reaction, has the advantage of separating the product to be assayed from most of the interferring materials. For the precipitation, one can employ agents used for the precipitation of nitrogenous bases: chloroaurate, chloroplatinate, iodine, reinecke salt, phosphoric acid, and silicotungstic acid. Nevertheless, the precipitation of choline is not always quantitative. Because of the solubility coefficient and the lack of sensitivity of the reactions or the determinations carried out on the precipitates, determinations are difficult, sometimes impossible, especially if one is attempting to determine small quantities.

I. THE PRECIPATION OF CHOLINE WITH A POLYHETERO ACID

Methods of determination employing these acids are not very numerous (1,2). The precipitates obtained are relatively soluble and the methods require such large samples that they are not applicable to biological systems.

Nevertheless, one of us has described previously that in ethereal solutions, small quantities of nitrogenous bases are precipitated by certain acids or inorganic salts and that aqueous solutions of the latter are devoid of all reactivity - the choice of solvent is decisive and the nature of the precipitating agent of secondary importance. Silicotungstic acid has been considered in this connection. The sensitivity of silicotungstic acid for choline has been established and in aqueous solutions it is 1/3,200. It is still greater in an acid solution (N sulfuric acid). In an alcohol solution, the sensitivity in 1/8,000. In contrast, in ethereal

solutions, oplascent solutions are obtained at a concentration of 1/5,000,000.

It should be remembered that for A^+, the base or its salt is dissolved in alcohol and precipitated by an ethereal solution of silicotungstic acid, the final alcohol concentration of the ethereal solution being maintained at about 15 %. An aqueous solution of silicotungstic acid (under favorable conditions) forms a precipitate with A^+ having the structural formula and usually represented schematically by $SiO_4 \cdot 12\ WO_3 \cdot 4\ A^+$* .

From ethereal solutions, a precipitate has been obtained with the composition: 1 silicotungstic acid: 3 alcohols.

We have, moreover, established that to determine small quantities of choline, it is necessary to use an excess of reactants containing 50 times the theoretical quantities if one hopes to obtain precipitates of constant composition (1/3). The following technique has been adopted: an alcoholic solution of choline is evaporated in a centrifuge tube to about 0.2 ml. One slowly adds 10 ml of an ethereal solution of silicotungstic acid (5 mg/ml)**. After centrifugation, the supernatant is decanted off and the sediment is washed twice with 10 ml of ether containing 15 % alcohol. The precipitate is dissolved in 4 ml of water (warming slowly); the solution is cooled; and 2 ml of sodium hydrosulfite solution

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*It is noted that it is preferable to represent the structural formula by $12\ WO_3 \cdot SiO_4H_4 \cdot 4A^+ \cdot 2H_2O$ or better, by $H_8Si(W_2O_7)_6 \cdot 4A^+$ corresponding to a salt $K_8Si(W_2O_7)_4$ that can be transformed to the tetrapotassium salt : $SiW_{12}O_{42}K_4H_4$. If an excess of sulfuric acid is added to a solution of the neutral salt, there is formed among other things a tripotassium salt which gives rise to $SiW_{12}O_{42}K_3H_5$.

** Silicotungstic acid 50 mg; alcohol 1-1.5 ml; ether to 10 ml; filter.

(1 gm in 15 ml water).are added. The color that develops is stable for at least twenty minutes*.

Quantity of Choline precipitated mg	Choline estimated colorimetrically mg
0.20	0.21
0.20	0.19
0.41	0.40
0.61	0.62
0.81	0.80
1.02	1.02
1.22	1.24

We have controlled the composition of the precipitates effectively in the precipitation of 2.9 mg, 2.7 mg, and 1.45 mg of choline chloride, collecting the precipitates and comparing the results obtained by weighing with those obtained from colorimetric measurements on the precipitates. The gravimetric and colorimetric determinations assign the formula: 1 silicotungstic acid - 3 cholines.

Determination of Choline in Serum

The serum is clarified with an alcohol-ether solution is the alcohol solution is evaporated. The residue is taken up in saturated $Ba(OH)_2$ solution and saponification is carried out for 2 hours. The excess $Ba(OH)_2$ is precipitated with dilute sulfuric acid. In the presence of phenolphthalein, N NaOH and N HCl are added drop-by-drop until the color disappears. The solution is centrifuged. One can evaporate the aqueous solution spontaneously or at temperature not exceeding 40°. The residue

* If one adds a moderate quantity of pyridine or N NaOH, the color becomes 2.6 times as intense. In aqueous solutions at 650 mμ, an absorbance of 0.1 as measured on a Lumetron spectrophotometer (tube diameter= 16 mm) corresponds to 0.80 mg of silicotungstic acid or 0.73 mg of silicotungstic acid anhydride

Foot note from previous page, contd.

With the addition of pyridine or NaOH (precipitate dissolved in 3.5 ml of water + 2 ml hydrosulfite + 0.5 ml 1 N NaOH = final volume of 6 ml), an absorbancy of 0.1 corresponds to 0.305 mg of silicotungstic acid.

Text, Contd..

is dissolved in alcohol and filtered or centrifuge. The alcohol solution is then reduced to a small volume (0.2 ml). The choline is precipitated with an alcohol-ether solution of silicotungstic acid.

Quantity of Choline in Serum

Reinecke Salt Method mg/ml serum	Silicotungstic acid Method mg/ml serum
0.10	0.11
0.61	0.64
0.21	0.22
0.11	0.10
0.20	0.21
0.34	0.34
0.56	0.54
0.24	0.23
0.86	0.90
0.19	0.21
0.40	0.42

Furthermore, the determinations carried out on bile and tissues give elevate (too high) results.

Finally, we have examined the action of various agents for saponification and have compared the results obtained by precipitating the cholie with reineckate with those obtained by the precipitation with silicotungstic acid.

Egg Phosphotides

Hydrolyzing Agent	Preciptation with reineckate mg	with silicotungstate mg
20 % HNO_3	3.74	2.40
N NaOH	3.25	2.40
Sat. $Ba(OH)_2$	3.88	3.15
	Phosphotides of "Animal Origin"	
20 % HNO_3	2.86	2.79
N NaOH	1.54	2.45
Sat. $Ba(OH)_2$	1.89	2.97

With the latter results, it is evident that not only does the precipitating agent affect the results, but that they are also affected by the hydrolyzing agent selected.

In addition, we have precipitated the choline with reineckate and redissolved the precipitate in acetone. The acetone solution is reduced to a small volume and the choline is precipitated with silicotungstic acid. The results of the colorimetric reaction obtained with silicotungstic acid indicates that the transformation of the reineckate to a silicotungstate is quantitative.

Silicotungstic acid appears, moreover, able to precipitate the phosphotides in ethereal solutions without having to attribute this precipitation to the presence of free choline. On the other hand, if one adds to an ethereal solution of phosphotides acetone containing silicotungstic acid, the phosphotides no longer are precipitated and, inversely, the precipitates obtained by the addition of acetone to an ethereal solution of phosphotides are redissolved by the addition of silicotungstic acid.

2. A COLORIMETRIC REACTION WITH REINECKE SALT

The precipitation of choline with reinecke salt constitutes a classical method of determination. The numerous modifications of this method sometimes involve the conditions of precipitation, and sometimes (usually) the determination of the reineckate recovered. The determination of nitrogen in the precipitate takes advantage of a favorable multiplication factor (7 times the quantity of choline). Nevertheless, when the Kjeldahl method is employed, there is the inconvenience of deficient results as wells as the lengthened time of assay as a result of the destruction and distillation required. Other investigators (3-5, 11) have measured the color of the reineckate. The dissolution of the precipitate in acetone and measurement of the optical density of the reineckate comprises a simple and rapid method (6-8). However, the determination becomes difficult or impossible with small quantities of reineckate. In addition, the color is unstable, especially in alkaline solutions. Attempts have been made to avoid these inconveniences by carrying out the determination in the ultraviolet (U.V.)(10) but this method is not applicable.

In the method that we propose here, we have attempted, on one hand, to reduce the solubility coefficient and, on the other hand, to carry out a colorimetric determination on extremely small quantities of reineckate.

Precipitation

This is carried out in N HCl. One adds a saturated solution of reinecke salt in N HCL to the solution until a faint rose color just develops. The mixture is then placed in the refrigerator for ½ hour and then filtered through fritted glass.

A. Influence of Final Volume

Volume ml	Quantity Precipitated mg	Quantity measured mg
2	1	1.07
4	1	1.04
6	1	1.04
8	1	0.98
10	1	0.98

B. Influence of Wash Liquids (on 1 mg choline)

Washed with 1.2 N HCl					Ether Wash				
3X with	1 ml	(3 ml)	detected	1.07 mg	3X with	2 ml	(6 ml)	detected	1.01 mg
3X "	3 "	6	"	0.95	3X "	4 "	8	"	1.01
3X "	6 "	18	"	0.86	3X "	8	16	"	1.01

C. Influence of Wash Liquid on Small Quantities of Choline

Quantity of choline (mg)	1.2 N HCl wash	Ether wash
0.90	1.14	1.01
0.66	0.76	0.76
0.45	0.44	0.41
0.23	0.09	0.25
0.12	0.00	0.09

The Colorimetric Reaction

In the classical determination, the reineckate is dissolved in acetone and from the color produced from the reinecke salt, one calculates the choline concentration. Moreover, for very small quantities of choline, this determination becomes impossible. We have asked ourselves if it would not be possible to transform the CN found in the molecule into BrCN first

and then to find a reaction of this with a pyridine derivatives. After a number of attempts, we adopted the following method of preparation: to 1 ml of acetone solution of the reineckate, one adds 2 ml of bromine water. It is reacted for several minutes after which 1.3 ml of sodium arsenite (2%) is added (there should be only a slight excess). To this solution is then added 1 ml of aasaturated aqueous solution of benzidine chlorohydrate; 2.7 ml of water (final volume 10 ml); and finally 2 ml of pyridine. The color develops immediately. A standard solution is employed which contains 40 µg of choline reineckate equivalent to 13.2 µg of choline chloride. However, the absorption maximum located at 490 mµ is shifted towards the longer wave lengths with time. In order to obtain linear values, it is necessary to choose for each measurement the wavelength representing the absorption maximum. In this way, when the measurements are carried out within 5 to 15 minutes, we have measured at wavelengths varying between 490 mµ at the beginning to 510 mµ at the end of the measurements.

Example: A serum is submitted to hydrolysis. After hydrolysis, an aliquot of the solution is filtered and the choline in this aliquot is precipitated with reinecke salt. After recovery, the precipitate is dissolved in acetone and varying amounts are subjected to a direct colorimetric reaction and to the BrCN reaction.

Direct Colorimetric Reaction : There was originally 2.08 mg of choline chloride in 3 ml of filtrate.

(see results on next Page)

Solution No.	Quantity in filtrate mg	Theoretical Quantity mg	Quantity Measured ~~Original Quantity~~ mg
1	3	2.08	2.08
2	1.6	1.11	1.13
3	0.8	0.55	0.53
4	0.4	0.27	0.13
5	0.2	0.14	0.00

BRCN Method: The acetone solutions can be diluted at the beginning in order to carry out the color reaction with BrCN.

Wavelength (mμ)	490	500	500	510	510
Time after appearance of color	3'	9'	12'	14'	16'
Standard 40μg/ml O.D.	0.700	0.685	0.665	0.650	0.650
Acetone solution					
No. 1 diluted 1/16		0.630			
No. 2 diluted 1/8		0.685			
No. 3 diluted 1/4			0.630		
No. 4 diluted 1/2			0.620		
No. 5 undiluted				0.525	

The measurements and calculations furnish the following results for the five solutions.

Solution No.	Quantity Calculated mg	Quantity measured mg
1	2.08	1.94
2	1.11	1.06
3	0.55	0.49
4	0.27	0.24
5	0.14	0.11

DETERMINATION OF CHOLINE WITH PURE CHOLINE

Sample Size mg	Quantity measured by direct colorimetry on reineckate precipitate mg	Quantity measured by the BrCN Method mg
1.30	1.32	1.03
0.57	0.54	0.50
0.28	0.25	0.25
0.14	0.00	0.11
0.07	0.00	0.05
0.28	0.29	0.26
0.14	0.06	0.12
0.07	0.00	0.06

DETERMINATION OF CHOLINE IN SERUM

	Quantity measured by direct colorimetry mg/ml	Quantity measured by the BrCN method mg/ml
	1.88	1.69
	0.52	0.42
	0.94	0.85
	1.08	0.96
	0.47	0.42
	0.21	0.21
	0.23	0.20
	0.42	0.41
in bile	0.35	0.34 -0.35 -0.38

From the results of our measurements, it is found that the color that we obtained is nearly 600 X as intense as the color of the reineckate of choline.

SUMMARY

In ethereal solution, choline is precipitated by silicotungstic acid. The sensitivity of this reaction is 1/5,000,000. Choline can be determined in the blood serum by reduction of the silicotungstate obtained; the amount of choline present is estimated from the intensity of the blue color obtained.

Determination by measuring the intensity of the color of choline reineckate is not possible in the case of very small amounts of precipitate. In the method proposed by the authors, use is made of a reaction which gives a color that is 600 times as intense as that of choline reineckate.

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