THE BIURET REACTION AND THE COLD NITRIC ACID TEST IN THE RECOGNITION OF PROTEIN

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In all books on physiological chemistry and clinical diagnosis one is recommended to place great reliance on the biuret reaction. In the course of some investigations on the comparative reliability of various tests for serum albumen I found that I obtained very contradictory results with this test, and therefore I think it would be of interest to give the results which I obtained in an exhaustive series of tests.

The usual method given in books for carrying out this test is: that one adds to the albumen solution some soda solution and then, drop by drop, a very dilute solution of copper sulphate, a blue precipitate appearing which, on shaking, dissolves with a pink tinge, finally changing to a reddish violet.¹ But on doing this I found that one could not be certain of obtaining the reaction in a urine which by other tests, such as cold nitric acid, acetic acid and heat, and potassium ferrocyanide and acetic acid, gave clear indications of the presence of albumen.

It was therefore necessary to make up a solution containing a definite quantity of serum albumen and to repeat the tests. On doing this I found that it was impossible to make certain of obtaining constant results unless one used solutions of soda and copper sulphate of definite strength, for the addition of the soda solution in varying amounts made a great difference in the result of the test.

It is generally recognised that it is necessary to add a dilute solution of copper sulphate, and I found that, although with a high percentage of albumen a 2½ per cent. solution of copper sulphate may be used, yet for all practical purposes the solution of copper sulphate should not be stronger than 1 per cent.

Another point on which the various text-books, which I have had an opportunity of consulting, lay little stress is on that of heating, and

1. Some text books advise adding the copper sulphate solution before the soda solution.

although one can obtain a more or less definite result in the cold, I found that the reaction is much more marked on heating. In fact, in a very weak solution of albumen the violet is not perceptible until the solution is boiled.

In consequence of these difficulties I decided to work with solutions of albumen, soda and copper sulphate of definite strength, and the original solutions which I made up were as follows:—

- I. Albumen solution. Distilled water containing 0·2 per cent. of albumen.
- II. A solution containing sodium hydrate 10 grammes and distilled water to 100 c.c.
- III. A solution containing re-crystallised copper sulphate 5 grammes and distilled water to 100 c.c.

Solution I.—Beginning with the original solution of 0·2 per cent. of albumen, I made an exhaustive series of dilutions with distilled water, these diluted solutions varying in strength from 0·05 per cent. to 0·00033 per cent. of albumen. A number of these dilutions are referred to in Tables I and II.

Solution II.—As a result of many tests in which I used varying percentages of sodium hydrate, in albumen solutions of different strengths, I found that a 10 per cent. solution of sodium hydrate gave the most constant results. The quantity of this soda solution used in each test—final result—is given in Tables I and II.

Solution III.—The 5 per cent. solution of copper sulphate was too strong, for even in the original solution of albumen (Solution I) a brown coloration with a white flocculent precipitate was obtained on the addition of one drop of this copper solution with soda. I therefore diluted to a $2\frac{1}{2}$ per cent. solution, but, after three or four estimations, I found this in turn too strong, and it was necessary to dilute to a 1 per cent. solution.

Table I shows results on using a $2\frac{1}{2}$ per cent. solution.

Table II „ „ • 1 „ „

For adding the copper sulphate and soda solutions drop by drop, I used an ordinary glass pipette, that is, ordinary glass tubing drawn to a small calibre at one end.

It was necessary to do a great many tests before being able to determine the best method of performing the test. I found as follows:—

(a) 10 c.c. of the albumen solution is the best amount to use.

(b) In all the albumen solutions of from 0·004 per cent. to the limit

of delicacy one drop of copper sulphate (1 per cent.) and five drops of sodium hydrate (10 per cent.) gave the best results.

In the albumen solutions stronger than 0·004 per cent. two to ten drops of copper sulphate (1 per cent.) can be added, according to the strength of the solution of albumen.

In the solutions from 0·05 per cent. to 0·012 per cent. of albumen I used $2\frac{1}{2}$ per cent. copper sulphate solution. (See Tables I and II.)

(c) The copper sulphate should be added first, then the soda solution. The contents of the test tube should then be mixed by inverting twice.

(d) After adding the copper and soda the solution should be heated to boiling, when the violet is intensified.

All text-books I have consulted mention that the colour obtained in the biuret reaction is pink or red, changing to violet. I was unable to obtain this play of colours, as all my positive tests gave the violet colour at once. I found that the stronger the solution of albumen the more marked was the violet colour—gradually diminishing with dilution—and, also, that in strong solutions of albumen the violet is intensified by the addition of copper sulphate solution drop by drop, boiling between drops.

In the very dilute solutions of albumen there is some difficulty in recognising the violet colour. I found that the colour was best seen as follows:—

1. In the meniscus.
2. On looking down the test tube—against white.
3. On comparing the albumen solution with a test tube containing an equal quantity of distilled water. The two tubes should be held side by side and compared:—

(a) On looking down both—against white.

(b) In the meniscus—

The best light is that which strikes from above. Both tubes should be slanted towards the operator at about 35°. One should hold the tubes above the head and look up through the meniscus from below, against a dark background.

TABLE I

Albumen per cent.	CuSO ₄ Solution $2\frac{1}{2}$ per cent.	NaOH Solution 10 per cent.	Result	Remarks
0·050	3 drops	5 drops	Violet	Reaction well marked
0·025	3 ..	5 ..	..	
0·016	2 ..	5 ..	..	Reaction marked "
0·012	1 drop	5 ..	..	

In the next dilution—solution of 0.008 per cent. of albumen—one drop of copper sulphate solution ($2\frac{1}{2}$ per cent.) and five drops of sodium hydrate solution (10 per cent.) were added, and the solution at once turned brown. On boiling, the brown colour deepened with the formation of a white flocculent precipitate. The result might be shown thus:—

Albumen per cent.	CuSO ₄ Solution 2½ per cent.	NaOH Solution 10 per cent.	Result	Remarks
0.008	1 drop	5 drops	No violet	Solution at once turns brown

In all the succeeding tests I used a 1 per cent. solution of copper sulphate.

TABLE II

Albumen per cent.	CuSO ₄ Solution 1 per cent.	NaOH Solution 10 per cent.	Result	Remarks
0.0080	2 drops	5 drops	Violet	Colour deepened on addition of one more drop CuSO ₄ solution.
0.0040	1 drop	"	"	
0.0026	"	"	"	
0.0020	"	"	"	
0.0016	"	"	"	
0.0013	"	"	"	
0.0011	"	"	"	
0.0010	"	"	"	
0.00088	"	"	"	Violet faint before boiling.
0.00080	"	"	"	
0.00072	"	"	"	
0.00066	"	"	"	Violet very faint before boiling.
0.00061	"	"	"	
0.00056	"	"	"	Using distilled water as comparison, violet is just perceptible before boiling.
0.00053	"	"	"	
0.00050	"	"	"	
0.00047	"	"	"	Violet imperceptible before boiling except on comparison with distilled water.
0.00044	"	"	"	
0.00042	"	"	"	
0.00040	"	"	"	Violet imperceptible before boiling even when compared with distilled water, and just perceptible after boiling when compared with distilled water.
0.00038	"	"	"	On standing for two minutes solution turns brown.
0.00036	"	"	"	On standing for one minute solution turns brown.
0.00034	"	"	"	On standing for twenty seconds solution turns brown.
0.00033	"	"	No violet	Solution at once turns brown, with the formation of a white flocculent precipitate.

From the results which I obtained in a watery solution of albumen, I decided to make further tests, using as my original solution urine and albumen. I therefore took a urine free from albumen and added albumen to make a 2 per cent. solution.

As in the previous tests, I made a great many dilutions with distilled water, and carried out investigations as before. The more or less abridged results are seen in Table III.

In these reactions I used sodium hydrate (10 per cent.) and copper sulphate (1 per cent.).

In the albumen solutions stronger than 0.04 per cent. more than one drop of copper sulphate solution was used, but in 0.04 per cent. and all the succeeding solutions to the limit of delicacy I found that one drop of copper sulphate solution gave the best results. In all cases five drops of sodium hydrate gave most constant results.

On account of the added colour of the urine, I found it more difficult to recognise the violet colour in this than in the first series of tests.

TABLE III

Albumen per cent.	CuSO ₄ Solution 1 per cent.	NaOH Solution 10 per cent.	Result	Remarks
2.0	Five drops	Five drops	Violet	Colour deepened to maximum on addition of ten more drops of CuSO ₄ —drop by drop—boiling between drops.
0.20	"	"	"	Colour deepened to maximum on addition of five more drops of CuSO ₄ —drop by drop—boiling between drops.
0.040	One drop	Five drops	Violet	Reaction good.
0.020	"	"	"	
0.014	"	"	"	
0.010	"	"	"	
0.0066	"	"	"	
0.0050	"	"	"	
0.0040	"	"	"	
0.0033	"	"	"	Violet faint before boiling.
0.0028	"	"	"	
0.0025	"	"	"	
0.0022	"	"	"	Violet very faint before boiling.
0.0020	"	"	"	
0.0016	"	"	"	
0.0014	"	"	"	Violet perceptible before boiling only on comparison with distilled water.
0.0012	"	"	"	
0.0011	"	"	"	
0.0010	"	"	"	Violet imperceptible before boiling. After boiling violet just perceptible on comparison with distilled water.
0.0009	"	"	"	But changing almost at once to brown, with the formation of a white flocculent precipitate.

Having determined the delicacy of the test in a watery solution of albumen, and in a urine containing albumen diluted with water, I next tried to obtain the limit of delicacy in a urine containing albumen when urine free from albumen was used for dilution.

According to Hammarsten,¹ an excess of creatinin reduces copper sulphate, and Kellas and Wethered² have shown that an excess of uric acid and urates, as well as creatinin, causes a reduction of copper sulphate.

The first urine which I used for diluting contained an excess of uric acid, and in these solutions I was unable to obtain the violet colour except in the stronger solutions of albumen—0·2 per cent. and stronger.

I then did further tests with solutions made by diluting the albuminous urine with urines containing an excess of urates and creatinin, but was unable to obtain positive results except in the stronger solutions of albumen—0·2 per cent. and stronger.

While working on the biuret reaction it occurred to me that it would be of interest to determine the delicacy of the cold nitric acid test for albumen, as this test is so well known and so commonly used.

The limit of delicacy of this test given in text-books is 0·002 per cent., but I was able to obtain positive results in very much weaker solutions, as will be seen in Table IV.

To obtain satisfactory results in doing the test the following points should be noted:—

(a) 12 c.c. of albumen solution should be used.

(b) 2 c.c. of nitric acid should be used.

(c) A pipette should be used for adding the nitric acid to the albumen solution. This pipette is made as follows:—One end of an ordinary piece of glass tubing, 5 mm. diameter, is drawn to a calibre which allows the nitric acid to escape from the pipette by the drop and not in a continuous stream, when the finger is removed from the large end of the pipette.

The pipette should be not less than 28 cm. in length.

Technique of the Test.—Take 12 c.c. of albumen solution in a test tube. Draw up 2 c.c. of nitric acid in the pipette, place the finger over the end and carefully lower the pipette into the albumen solution until the point of the pipette is on the bottom of the test tube. Remove the finger from the pipette and allow the nitric acid to run in. When no more nitric acid runs in place the finger over the end of the pipette and carefully withdraw it. Care must be taken not to shake the test tube during the test.

By this method the line of demarcation between the albumen solution and the nitric acid is narrow and very distinct, thus facilitating the recognition of the albumen ring as it forms.

1. *Physiologischen Chemie.*

2. *The Lancet*, October, 1906.

My first series of tests was done with diluted solutions of my original albumen solution, that is, distilled water containing 0.2 per cent. of albumen. The results in a number of these solutions are given in Table IV.

One finds in text-books the statement that, for the cold nitric acid test for albumen to be positive, the albumen ring should occur within five minutes, but on referring to Table IV it will be seen that in very dilute solutions of albumen the ring did not occur until as long as fifteen minutes, and reached its maximum density in one hour after adding the nitric acid.

TABLE IV

Albumen per cent.	Nitric Acid	Result	Time	
0.20	2 c.c.	Positive	In 20 seconds	
0.0020	"	"	" 30 "	
0.0010	"	"	" 1 minute	
0.00066	"	"	" 1 "	
0.00050	"	"	" 1½ minutes	
0.00040	"	"	" 2 "	
0.00033	"	"	" 2½ "	
0.00027	"	"	" 3 "	
0.00025	"	"	" 3½ "	
0.00022	"	"	" 3½ "	
0.00020	"	"	" 4 "	
0.00018	"	"	" 4½ "	
0.00016	"	"	" 5 "	
0.00015	"	"	" 5½ "	
0.00014	"	"	" 6 "	
0.00013	"	"	" 6½ "	
0.00012	"	"	" 7 "	
0.000117	"	"	" 7½ "	
0.000111	"	"	" 8 "	
0.000105	"	"	" 9 "	
0.000100	"	"	" 10 "	Ring most marked in 15 minutes
0.000095	"	"	" 11 "	" " 20 "
0.000090	"	"	" 12 "	" " 25 "
0.000086	"	"	" 13 "	" " 30 "
0.000083	"	"	" 14 "	" " 35 "
0.000080	"	"	" 14 "	" " 40 "
0.000076	"	"	" 14 "	" " 45 "
0.000074	"	"	" 15 "	" " 50 "
0.000071	"	"	" 15 "	" " 1 hour
0.000068	"	"	" 15 "	" " 1 hour. Very faint
0.000063	"	"	" 15 "	" " 1 hour. Very faint
0.000060	"	"	" 15 "	" " 1 hour. Very faint

For my next series of tests I used urine containing 2 per cent. of albumen—made by taking a urine free from albumen and adding albumen to make 2 per cent. Taking this as my original solution I made a large number of dilutions, using for dilution a urine free from albumen.

The results in a number of these solutions are given in Table V.

In doing the cold nitric acid test in albuminous urine containing an excess of uric acid, after adding the nitric acid a cloudiness appears in a few minutes, gradually increasing in density all through the urine from the top of the solution down to the nitric acid ring. To differentiate between this cloudiness, due to uric acid, and the albumen ring, the urine should be heated gently just above the nitric acid ring. The cloudiness due to uric acid disappears and the albumen ring remains. There will then be seen in such cases, in order from below, the nitric acid ring, the white albumen ring, a clear space, and above this more or less cloudiness.

It must be remembered in this connection that albumose may be present also. If so, on gently heating just above the nitric acid ring, the depth of the white precipitate will diminish, since the albumose is dissolved and the albumen is left. By this means one can obtain a rough idea as to the quantity of albumose present.

In Table V it will be seen that in very weak solutions of albumen the albumen ring did not appear until ten minutes after the addition of the nitric acid.

TABLE V

Albumen per cent.	Nitric Acid	Result	Time	
0.04	2 c.c.	Positive	In 1 minute	
0.0040	"	"	" 1½ minutes	
0.0020	"	"	" 2 "	
0.0013	"	"	" 2½ "	
0.0010	"	"	" 3 "	
0.00080	"	"	" 3½ "	
0.00060	"	"	" 4 "	
0.00057	"	"	" 4½ "	
0.00050	"	"	" 5 "	
0.00044	"	"	" 5½ "	
0.00040	"	"	" 6 "	
0.00036	"	"	" 6½ "	
0.00033	"	"	" 7 "	
0.00030	"	"	" 7 "	
0.00028	"	"	" 8 "	
0.00026	"	"	" 8½ "	
0.00025	"	"	" 9 "	
0.00023	"	"	" 9½ "	
0.00022	"	"	" 9½ "	Faint
0.00021	"	"	" 10 "	
0.00020	"	"	" 10 "	Very faint

CONCLUSIONS

A. *Biuret Reaction.—Limit of Delicacy.*

I. In a watery solution of albumen is 0.0004 per cent. or four parts of albumen in 1,000,000 parts of distilled water.

II. In albuminous urine diluted with distilled water is 0·001 per cent. or one part of albumen in 100,000 parts of urine and distilled water.

III. In albuminous urine diluted with urine free from albumen.

The biuret reaction is very difficult to obtain in concentrated urines containing an excess of uric acid, urates or creatinin. In such cases it is only in the stronger solutions—0·2 per cent.—that the reaction is good. In the weaker solutions of albumen the violet colour may be obtained, but almost at once the colour changes to brown.

In most cases no violet colour is obtained, the solution at once turning brown with the formation of a white flocculent precipitate.

B. *Cold Nitric Acid Test.—Limit of Delicacy.*

I. In a watery solution of albumen is 0·00006 per cent. or six parts of albumen in 10,000,000 parts of distilled water.

II. In albuminous urine diluted with urine free from albumen is 0·0002 per cent. or two parts of albumen in 1,000,000 parts of urine.

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