A MICROMETHOD FOR THE DETERMINATION OF GLYCOCYAMINE IN BIOLOGICAL FLUIDS AND TISSUE EXTRACTS

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In the two following communications (1, 2) evidence is presented that glycocyamine is a normal precursor of creatine in the animal body. These studies required a satisfactory micromethod for the determination of glycocyamine. The most reliable method described in the literature consists in adsorption on Lloyd's reagent in acid solution, elution with baryta, removal of arginine from the eluate by repeated adsorption on permutit, and colorimetric determination of the remaining glycocyamine by means of the Sakaguchi reaction. There are only two substances which are common in biological fluids and which give an intense color in the Sakaguchi reaction. These are arginine and glycocyamine. This method was first introduced by Weber (3) and was modified by Bodansky (4) and by Davenport and Fisher (5).

In our hands even the latest version of the method, that described by Davenport and Fisher, had the following shortcomings: it was laborious and time-consuming, the adsorption of the glycocyamine on the Lloyd's reagent was incomplete, further losses of glycocyamine occurred in the repeated treatment with permutit (Davenport and Fisher report losing only 10 per cent in three adsorptions; with the permutit available to us we lost 80 per cent), and the color developed was unstable. Furthermore, the amount of glycocyamine lost on the permutit varied according to the amount of arginine present, the less arginine the greater the loss of glycocyamine.

All these disadvantages have been removed in the method described below. It is the first method in which glycocyamine

added to blood or urine can be determined quantitatively, even in concentrations as low as 0.1 mg. per cent. 2 to 5 ml. are sufficient for an analysis. An indication of the speed and convenience of the method is that twenty to forty analyses can be carried through simultaneously in about 2 hours.

Reagents—

0.02 M sodium-potassium phosphate solution at pH 7.0 (Sørensen buffer); or 0.3 per cent sodium chloride solution.

Permutit.

A stock solution of 0.2 per cent α-naphthol in absolute alcohol, diluted with water to 0.04 per cent before use.

40 per cent solution of urea in water.

Hypobromite solution made as follows: 0.66 ml. of liquid bromine (specific gravity 3.0) in 100 ml. of 5 per cent sodium hydroxide.

Procedure

Urine is diluted 5 to 10 times, according to its concentration. Blood may be deproteinized either by adjusting the pH to 6.0, 5-fold dilution with water, boiling and filtration, or by precipitation of the proteins with sodium tungstate and sulfuric acid according to the procedure of Folin and Wu, with the modification that for each ml. of blood, 2 ml. of water and 1 ml. each of the 10 per cent sodium tungstate and 0.66 N sulfuric acid are used. Tissue extracts are diluted with water until the final solution contains 1 gm. of tissue (fresh weight) in 40 ml. of suspension. The pH is adjusted to 6.0, and the suspension immersed in a boiling water bath for 10 minutes, cooled, and filtered. The analysis is carried out on the deproteinized filtrate.

Any arginine which may be present is removed by sending the solution through a column of permutit contained in the stem of a funnel made of two pieces of glass tubing. The dimensions are as follows: upper piece 100 mm. long, 15 mm. external diameter; lower piece 100 mm. long, 7 mm. external diameter (5 mm. internal diameter). The bottom end of the funnel is constricted slightly.

The dimensions of the lower narrow tubing should be as close as possible to those given above. If the tubing is narrower, the filtration will be slow; if it is much wider the reduced height of the

column of permutit may allow some arginine to escape adsorption. The lower end of the funnel is stoppered loosely with a small piece of absorbent cotton just sufficient to hold back the permutit. 0.9 gm. of permutit is poured in and tapped gently to remove entrapped air. It should form a column about 85 mm. tall. A test-tube graduated at 10 ml. is placed under the end of the funnel.

5 ml. of the solution to be analyzed, obtained as described above, are pipetted into the funnel. After all of this solution has passed below the top of the permutit column, 5 ml. of either the 0.02 M phosphate buffer or of the 0.3 per cent sodium chloride solution are pipetted in above the permutit. Each 5 ml. portion of solution takes about 15 minutes to pass through the column. When the second 5 ml. portion has passed through, the filtrate in the graduated test-tube below is made up to the 10 ml. mark with water and then shaken.

Washing the permutit column with the phosphate or sodium chloride solution removes the small amount of glycocyamine which remains in the permutit column after the 5 ml. of the solution under analysis have passed through. The dilute phosphate buffer and the sodium chloride solution are equally effective. We prefer the sodium chloride solution, because it is simpler to make up and also because a faint turbidity is sometimes obtained in the final filtrate when the phosphate solution is used.

Different batches of permutit may differ in their affinity for arginine and glycocyamine. We have mentioned above that Davenport and Fisher lost only 10 per cent of the glycocyamine present in three successive treatments with fresh permutit, whereas we lost 80 per cent. It may be advisable therefore before a fresh batch of permutit is used in the procedure described to ascertain the height of the column and amount of permutit necessary to remove all the arginine without loss of glycocyamine. The prescription we have given allows some latitude and it may cover all the variations in permutit now sold in this country.

The permutit can be regenerated after it has been used by the following procedure. It is washed several times with tap water, thrown on a Buchner funnel, an equal volume of 25 per cent sodium chloride solution passed through, and then washed with distilled water until the wash water is chloride-free.

The development of the color by means of the Sakaguchi reaction

and the colorimetry may be carried out with any convenient aliquot so long as the proportions of the reagents are kept the same. The quantities of reagents given below are for 2 ml. aliquots which we have been using. Twenty to forty analyses can be carried through simultaneously. Test-tubes containing the aliquots of the solutions to be analyzed, inserted in a rack, are

TABLE I

Determination of Glycocyamine in Aqueous Solutions Containing Different Amounts of Interfering Substances

All values are concentrations measured in mg. per cent.

The concentrations of the added substances were those in the final mixtures before they were sent through the permutit columns. The final filtrates contained only half the concentrations of glycocyamine shown. Accordingly the standards were diluted 1:1 with 0.02 M phosphate buffer solution.

Arginine added	Creatine added	Glycocyamine added	Glycocyamine found by analysis
35	10	3.0	3.02
35	5	3.0	3.01
35	1	3.0	3.01
35	0	3.0	3.00
35	10	1.0	1.01
35	5	1.0	1.02
35	1	1.0	1.00
35	0	1.0	1.00
35	10	0.1	0.10
35	5	0.1	0.10
35	1	0.1	0.10
35	0	0.1	0.10
70	25	0.1	0.10
35	25	0.1	0.10
18.5	25	0.1	0.09
0	25	0.1	0.09

placed in an ice water bath. In this bath also are the α-naphthol (0.04 per cent), urea, and hypobromite solutions. After 5 to 10 minutes 0.4 ml. of the α-naphthol solution is added to each tube, the contents shaken, 0.4 ml. of the chilled urea solution added, and the contents shaken again. After another 5 minute interval 0.2 ml. of the chilled hypobromite solution is added, the contents immediately mixed by vigorous shaking, and the tube replaced in the ice bath.

The color develops slowly, attaining its maximum intensity in about 20 minutes, after which (so long as the solution is in the ice bath) it remains constant for at least 1 hour. Before the color is measured, the solution is warmed to room temperature by shaking in water for ½ minute and the dissolved gas removed by vigorous tapping for 20 seconds. If the colored solution is left at room temperature for ½ hour or more, the color slowly fades.

TABLE II

Determination of Glycocyamine in Ringer's Solution and in Rat Heart and Kidney Extracts to Which Different Quantities of Arginine and Glycocyamine Were Added

All values are concentrations measured in mg. per cent.

The concentrations of added glycocyamine and the experimental values given were those in the final filtrates; *i.e.*, after they had passed through the permutit and were diluted with an equal volume of washing solution. The concentrations of added arginine were those before the solutions had passed through the permutit.

Arginine added	Glycocyamine added	Glycocyamine					
		Ringer's solution		Kidney extract		Heart extract	
		Found	After subtracting blank value	Found	After subtracting blank value	Found	After subtracting blank value
0	0			0.04	0	0.08	0
35	0	0	0	0.04	0	0.08	0
35	1.50	1.53	1.53	1.59	1.55	1.56	1.48
0	0.75	0.765	0.765	0.79	0.75	0.85	0.77
35	0.75	0.80	0.80	0.82	0.78	0.84	0.76
35	0.375	0.41	0.41	0.44	0.40	0.45	0.37
35	0.075	0.08	0.08	0.11	0.07	0.15	0.07

With each group of analyses the color is developed simultaneously in four standard solutions containing 0, 0.25, 0.5, and 1.0 mg. per cent of glycocyamine.

To measure the color we have used a spectrophotometer or a colorimeter. With either instrument the best light is that at approximately 0.525 μ. There is a linear relationship between the concentration of glycocyamine up to 1 mg. per cent and the intensity of the color when it is measured with light near to 0.525 μ.

Tables I to IV indicate the sensitivity and reliability of the method described above. Table I gives some typical results

with prepared mixtures, in aqueous solution, containing varying amounts of glycocyamine and of two interfering substances, arginine and creatine.

Table II summarizes some tests of the method with Ringer's solution and two tissue extracts. These tests were necessary, even after the results in Table I were obtained, because the equilibrium relation between the glycocyamine adsorbed on permutit and that remaining in solution is affected by the concentration of

TABLE III

Determination of Added Glycocyamine and of Added Arginine in Human Urine (Diluted 5-Fold with Water)

All values are concentrations measured in mg. per cent.

The concentrations of added glycocyamine and the experimental values found were those in the filtrates; *i.e.*, after they had passed through the permutit and were diluted with an equal volume of washing solution. The concentrations of added arginine were those before the solutions had passed through the permutit.

Glycocyamine added	Arginine added	Glycocyamine	
		Found	After subtracting amount originally present
0	0	0.21	0
0	22	0.21	0
1.0	0	1.26	1.05
0.5	0	0.72	0.51
0.5	30	0.71	0.50
0.5	22	0.70	0.49
0.5	10	0.69	0.48
0.25	0	0.46	0.25

salt. The reliability of most of the experimental results described in the next communication (1) depends on the accuracy of this analytical method. The data in Table II show that the agreement between theoretical and experimental values is satisfactory.

The determination of glycocyamine in urine is one of the most severe tests to which an analytical method for glycocyamine can be subjected. Urine contains relatively high concentrations of interfering substances such as urea, ammonia, creatinine, and amino acids including arginine. The rigor of the test was in-

creased by adding to the urine varying amounts of arginine and glycocyamine. Table III shows that the same satisfactory agreement is obtained between theoretical and experimental values as in simple aqueous solutions. This is also the case with blood (Table IV).

TABLE IV

Determination of Glycocyamine Added to Human Blood

All values are given in mg. per cent.

Varying amounts of a 10 mg. per cent glycocyamine solution and of a 20 mg. per cent arginine solution were added to 4 ml. aliquots of whole human blood. To these mixtures water was added so that the final dilution of the blood was the same in each case; *i.e.*, 4 ml. of blood and 8 ml. of water or of the combined glycocyamine and arginine solutions. 4 ml. of 10 per cent sodium tungstate followed by 4 ml. of 0.66 N H_2SO_4 were then added to each mixture. The analyses were carried out on the filtrates. The concentrations of glycocyamine and arginine in these filtrates were further reduced by one-half in the analytical procedure.

Concentration ÷ 2 in Folin-Wu filtrate		Concentration of glycocyamine in Folin-Wu filtrate	
Glycocyamine added	Arginine added	Found	After subtracting amount originally present
1.0	0	0.99	0.95
0.5	0	0.55	0.50
0.2	0	0.24	0.19
0.1	0	0.15	0.10
0	0	0.05	0
0.5	2.0	0.55	0.50
0.5	1.5	0.55	0.50
0.5	1.0	0.54	0.49
0.5	0.5	0.54	0.49
0	2.0	0.05	0

SUMMARY

A micromethod is described for the determination of glycocyamine in biological fluids and tissue extracts. The advantages of this method over those previously described are that added glycocyamine is recovered quantitatively; it is faster and more convenient.

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