LXXIX. THE METABOLISM OF CREATINE. IV

A. IMPROVED DETERMINATION OF GLYCOCYAMINE

B. IMPROVED DETERMINATION OF ARGININE

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A. IMPROVED DETERMINATION OF GLYCOCYAMINE

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THE technique for the determination of glycocyamine in tissue extracts by Weber's method [1935], as described by Bodansky [1936], appears to possess two major disadvantages. In the first place, the tissue or extract taken for analysis has to contain approximately 1 mg. of glycocyamine, although the amount of glycocyamine needed for the final colorimetric measurement is only 0.025 mg. In the second place, Bodansky [1936] was able to recover only $75 \pm 5\%$ of added glycocyamine.

We have been able (method A below) to reduce the amount of glycocyamine needed for a determination to as little as 0.1 mg. and to recover $90 \pm 0.6\%$ of added glycocyamine. A simpler method (method B) is also described, which can be used in certain restricted circumstances, which recovers 100% of added glycocyamine.

Method A

Reagents required:

10% metaphosphoric acid.

Cold saturated baryta, and powdered barium hydroxide.

Fuller's earth. (B.D.H. "fuller's earth, levigated powder" is kept overnight in *N* HCl—200 ml. per 100 g., and then washed with water by decantation until the washings are Cl-free. The fuller's earth is filtered off by suction, dried on a water bath and ground until it passes a 90-mesh sieve.)

5*N* H₂SO₄.

Basic lead carbonate ("white lead powder").

Permutite. (We have found a white variety supplied by Harrington Bros. to be very satisfactory after washing with water to remove the finer particles.) 0.04% α -naphthol (stock solution of 0.2% in absolute alcohol, diluted with water).

10% NaOH.

40% urea solution.

Sodium hypobromite solution (2 g. Br in 100 ml. 5% NaOH).

The tissue to be analysed is minced finely under metaphosphoric acid, the mixture centrifuged and the supernatant liquid poured off. The residue in the tube is ground with a stout glass rod and extracted with more metaphosphoric

acid. After centrifuging and pouring off the extract, six further extractions are made, and the combined extracts are made up to an appropriate volume. In the instance of perfusates and other samples containing little protein, the protein is precipitated by adding enough metaphosphoric acid to give a final concentration of approximately 2%, the precipitate is removed by centrifuging and an aliquot of the supernatant fluid is taken for analysis.

Bodansky's [1936] procedure of autoclaving the tissue with HCl results in the liberation of large amounts of arginine from the tissue proteins, and arginine in large amounts interferes with the analysis. Metaphosphoric acid is preferred to trichloroacetic acid as a protein precipitant because trichloroacetic acid interferes with the colorimetric procedure and cannot readily be removed. Metaphosphoric acid can be removed as the Ba salt, as described below.

A measured volume of the combined extracts is placed in a centrifuge tube and 2 vol. of cold saturated baryta are added (where the concentration of glycocyamine is low, powdered $\text{Ba}(\text{OH})_2$ is stirred in at 40° until the supernatant liquid gives no precipitate on the addition of one drop of cold saturated baryta). The barium metaphosphate is centrifuged off and an aliquot of the supernatant liquid is transferred to a volumetric flask (e.g. 10 ml. extract taken, 20 ml. baryta added, 20 ml. supernatant transferred to 25 ml. flask). $5N \text{H}_2\text{SO}_4$ is added until the contents of the flask are acid to Congo red, and thereafter to make the final concentration $1.2N$. The contents of the flask are made up to volume and the BaSO_4 separated by centrifuging. A sample of the solution containing 0.1–0.5 mg. glycocyamine is placed in a 25 ml. centrifuge tube together with sufficient water to make the final volume 5 ml., 1 g. of the prepared fuller's earth is added and the tube is shaken for 10 min. in the apparatus of Fisher & Wilhelmi [1938]. The fuller's earth is centrifuged down and the supernatant liquid discarded. Six glass beads (5 mm. diam.) are placed in the tube, 5 ml. water are added and the fuller's earth is stirred up with a pointed glass rod. The tube is then shaken in the apparatus until the fuller's earth is uniformly suspended.

The fuller's earth forms a very compact sticky mass after centrifuging, and the above technique is essential for its resuspension at this and later stages of the determination.

The fuller's earth is centrifuged down again and the washings discarded. It is resuspended in 5 ml. water and saturated baryta is added, 0.5 ml. at a time, until the solution is just alkaline to Congo red, the tube being shaken in the apparatus between additions of baryta.

This is a crucial stage in the procedure. BaSO_4 is precipitated when baryta is added to the fuller's earth suspension, and we have been able to show that BaSO_4 adsorbs glycocyamine in the presence of any appreciable concentration of free $\text{Ba}(\text{OH})_2$. That is, an apolar adsorption appears to occur, of the type described by Phelps & Peters [1929] and Phelps [1929; 1931]. Since it also appears that glycocyamine adsorbed on BaSO_4 undergoes fairly rapid destruction, it is very important that the elution of glycocyamine from the fuller's earth should take place in the presence of a minimal excess of baryta. Bodansky [1936] elutes by grinding the fuller's earth with solid baryta, and we think it very probable that this procedure is responsible for his low recovery of glycocyamine.

The fuller's earth is kept suspended in the solution for 10 min. and is then centrifuged down. The eluate is transferred to a 25 ml. volumetric flask, and two further elutions are performed, the fuller's earth being suspended in 5 ml. of water for 10 min. in each instance. $5N \text{H}_2\text{SO}_4$ is then added to the combined eluates until the solution is acid to Congo red (one drop should suffice). The contents of the flask are then made up to volume and approximately 10 ml. are

poured into a 15 ml. centrifuge tube containing about 1 g. of basic lead carbonate. The tube is shaken until the contents are alkaline to Congo red and then 0.4 g. permutite is added and the tube shaken for 10 min., preferably by turning over and over slowly. The contents are centrifuged and the supernatant liquid is transferred to another 15 ml. centrifuge tube and again shaken with 0.4 g. permutite. After a third shaking with permutite the liquid is centrifuged to remove any fine permutite particles and 5 ml. taken for colorimetric determination.

We have carried out the colorimetric determination by means of the Sakaguchi reaction in a manner very similar to that described by Bodansky [1936]. We used neutral grey as a permanent standard, but, as we have found a linear relation between the amount of glycocyamine present and the neutral grey reading for amounts of glycocyamine between 0.02 and 0.10 mg., and as the colour obtained is stable up to 30 min. or more, simple colorimetry with a glycocyamine standard should be as accurate as our method. We find that:

$$\text{Glycocyamine (mg.)} = 0.00888 R_{10},$$

where R_{10} is the length in mm. of the column of neutral grey which balances a 10 mm. column of the unknown solution, comparison being made through filter No. 5 on the Leitz colorimeter ($509 \text{ m}\mu$). This relation holds, however, only when the following procedure is adhered to: 1 ml. 0.04% α -naphthol and 1 ml. 10% NaOH are added to 5 ml. test solution and the mixture placed in ice for 15 min.; then 0.5 ml. sodium hypobromite solution is added with stirring, followed exactly 15 sec. later by 1 ml. ice-cold 40% urea solution. 10 ml. ice-cold distilled water are then added as rapidly as possible, and the colorimetric comparison is made.

We have found it convenient to use an apparatus consisting of two pipettes of appropriate size connected by means of T-tubes and taps to a syringe for filling and to a pressure bottle for expulsion of the solutions. A separate lead from the pressure bottle to a glass tube drawn out to a fine tip provides a means of stirring the solutions. It is also advisable to have a jacket round the urea pipette which can be filled with ice-water. With this apparatus we obtained the relation between colour development (in terms of R_{10} ; see above) and the time interval between the addition of hypobromite and urea which is illustrated in Fig. 1. It is clear that the maximum reproducibility is to be expected when the flatter portions of the curves are used, and we have therefore chosen 15 sec. interval.

Despite the apparent complexity of the procedure, Table I shows that very considerable regularity in the recovery of glycocyamine submitted to the procedure can be obtained. The probable error of individual determinations is only $\pm 2.1\%$ of the glycocyamine added, or $\pm 2.3\%$ of the glycocyamine recovered. However, as we have adopted the practice of using quadruplicate determinations for all analyses, the figures in the last column of Table I are the most important. They show that the recovery calculated from the mean of quadruplicate analyses is $89.9 \pm 0.6\%$. Taking into account the size of the series from which these statistics are calculated, there is less than one chance in fifty of the observed recovery differing by more than $\pm 2.4\%$ from 89.9% .

Fig. 1.

Observations on the amount of glycocyamine left in a solution after treatment with fuller's earth and on the amount of glycocyamine found in the water used for washing show that approximately 10 % of the glycocyamine used can be found in these situations, so that it appears that 90 % constitutes the maximum practicable recovery.

Table II illustrates the specificity of the method. The figures in the column "glycocyamine found" are obtained by dividing the mean amount recovered by 0.899 (i.e. by the mean recovery). It will be seen that, so far as the two most probable interfering substances are concerned, the method is very highly specific. We have not had the opportunity to test possible interfering substances other than arginine and creatine, but it seems highly probable that those substances which Bodansky [1936] found to be without effect on the original Weber technique will be without effect on our method. These substances were: sarcosine, choline, betaine, creatinine, glycine, alanine, histidine, guanidine and methylguanidine.

Table I. *Recovery of glycocyamine by method A*

Glycocyamine added (mg.)	Glycocyamine recovered (mg.)	% recovery	
		Individual determinations	Mean of quadruplicates
0.101	0.090	89.2	89.7
	0.090	89.2	
	0.091	90.2	
	0.091	90.2	
0.157	0.137	87.3	90.6
	0.145	92.4	
	0.140	89.2	
	0.147	93.4	
0.202	0.182	90.1	90.3
	0.182	90.1	
	0.182	90.1	
	0.184	91.1	
0.270	0.245	90.7	—
	0.238	88.2	
0.321	0.284	88.5	89.2
	0.286	89.1	
	0.287	89.4	
	0.288	89.7	
0.404	0.362	89.6	89.5
	0.358	88.6	
	0.358	88.6	
	0.369	91.3	
Means		89.8 ± 2.1	89.9 ± 0.6

Table II. *Specificity of method A*

Glycocyamine added (mg.)	Arginine added (mg.)	Creatine added (mg.)	Glycocyamine found (mg.)
0.281	0.100	—	0.286
0.221	0.415	—	0.222
—	0.415	—	0.000
0.206	0.415	0.848	0.206
—	0.415	0.848	0.000

Method B

In tissue extracts, in which the final salt concentration is not more than 0.4 %, small amounts of arginine and creatine can be removed by treatment with permutite, and it is therefore possible to dispense with the adsorption on

fuller's earth. The shortened procedure is as follows: The metaphosphoric acid used as a protein precipitant is removed with baryta, the excess baryta is removed with H_2SO_4 , and an aliquot of the supernatant fluid is treated with basic lead carbonate and permutite, all the steps being carried out as in method A.

The quantitative character and specificity of this method are illustrated in Tables III and IV respectively.

Table III. *Recovery of glycocyamine by method B*

Glycocyamine (mg.)		
Added	Recovered	% recovered
0.101	0.100	
	0.101	
	0.101	
	0.101	99.75
0.126	0.124	
	0.127	
	0.127	
	0.128	100.05
0.252	0.253	
	0.252	
	0.251	
	0.253	100.10
0.375	0.367	
	0.376	
	0.378	
	0.378	99.95
Mean		99.97 ± 0.15

Table IV. *Specificity of method B*

Glycocyamine added (mg.)	Arginine added (mg.)	Creatine added (mg.)	Glycocyamine recovered (mg.)
0.250 mg. in Ringer-Locke (salt conc. about 1%)	0.015	—	0.251
	0.030	—	0.263
	0.050	—	0.272
	0.070	—	0.273
	0.087	—	0.270
	0.024	—	0.156
0.155 mg. in muscle extract (salt conc. about 0.4%)	—	0.510	0.155
	—	—	0.155

As we were able to show that, in the absence of added glycocyamine, perfusates and heart extracts prepared in the manner described by Davenport *et al.* [1938] gave no colour when submitted to method B, we have used this method in the investigation of the relation between glycocyamine disappearance and creatine appearance during perfusion of isolated hearts which is described in the paper referred to above.

B. IMPROVED DETERMINATION OF ARGININE

By R. B. FISHER and A. E. WILHELMI

The procedure of Jorpes & Thorén [1932] for the colorimetric estimation of arginine which we adopted earlier [Fisher & Wilhelmi, 1937] has been modified in the light of facts elicited in the development of the method for glycocyamine

[Part I above] to give (a) more accurately reproducible, and (b) more stable colours. The changes in procedure consist first, in adding the urea *exactly* 15 sec. after the addition of hypobromite, by the method described above, and second, in adding 10 ml. ice-cold distilled water immediately after the addition of the urea, as recommended by Weber [1935] for glycocycamine. Further, we have found that the samples need be kept in ice for only 15 min. after the addition of alkali and α -naphthol before the colour is developed. After this interval no further change with time (up to 2 hr.) seems to occur, so that long series of consecutive estimations may be run quite safely.

Colorimetry is done with neutral grey solution as a standard in the manner described by Fisher & Wilhelmi [1937]. The relationship between amount of arginine present and depth of neutral grey for a range extending from 25 to 110 γ is

$$Y = 5.60x^{1.06},$$

where Y is the amount of arginine in γ , and x is the matching depth of neutral grey in mm. when the unknown is set at 10 mm. The standard error of an individual estimation is $\pm 2.3\%$, showing a marked improvement over the former method.

Table V. *Change in apparent arginine with increasing time after development of colour*

Arginine present (γ)	Arginine found (γ)				Total change (γ)
	Min. 3	25	45	60	
27.1	27.6	30.8	30.3	33.8	+6.2
27.1	28.6	28.6	29.0	29.2	+0.6
81.4	84.4	81.9	78.4	77.5	-6.9
81.4	82.9	79.4	78.9	77.0	-5.9

The stability of the colour is illustrated in Table V. It is apparent that the colours are stable enough to allow time for the preparation of standards in instances where some method of absolute colorimetry is not at hand. Although the arginine-colour relationship is not strictly linear, over short ranges departure from linearity is not serious, and no very great error is introduced in using standards of nearly the same strength as the unknown. Since the colours are stable enough to allow time for the preparation and development of an appropriate standard, the estimations can, if necessary, be carried out with reasonable accuracy by simple colorimetry.

SUMMARY

Improvements in Weber's method for the determination of glycocycamine are described which result in increased sensitivity and reproducibility. The probable error of the mean of quadruplicate analyses is $\pm 0.6\%$.

A shorter and less specific method is described for use in certain restricted circumstances. The probable error of the mean of quadruplicate analyses is $\pm 0.15\%$.

The method of Jorpes & Thorén for determination of arginine has been modified to give increased stability of colour and an accuracy of $\pm 2.3\%$.

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