XCVII. A METHOD FOR THE ISOLATION OF GUANIDINE¹.

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A METHOD for the isolation of small amounts of guanidine in blood would be of interest in connection with several conditions, chiefly parathyroid tetany and diseases involving damage to the liver.

In the isolation of guanidine two main criticisms have been directed at methods previously put forward; (1) lack of separation of guanidine from creatine and creatinine and oxidation of the latter substances; (2) inadequate identification of guanidine salt. Later work has been confined to the estimation of a "guanidine substance" in blood by means of a colorimetric method. In all methods creatine and/or creatinine have been present when the final precipitation (or colour formation) was made.

In an endeavour to effect a complete separation of guanidine from creatine and creatinine, solutions of these substances were continuously extracted under reduced pressure with *isobutyl* alcohol, *amyl* alcohol, *n*-butyl acetate and toluene, but no separation occurred.

Whitehorn [1923] observed that treatment with sodium aluminium silicate (permutit) removed guanidine but left creatine and creatinine in solution. He makes the suggestion that all substances with a basic dissociation constant of 5×10^{-9} or greater are removed by permutit, while those substances with a smaller constant are left untouched.

Cannan and Shore [1928] state that the apparent dissociation constants for creatine and creatinine are 2.9×10^{-12} and 0.4×10^{-9} respectively, whilst guanidine has K_b 1.1×10^{-8} according to Whitehorn [1923] and 1.4×10^{-4} according to Morrell and Bellars [1907], the latter value appearing to be the more likely. If Whitehorn's suggestion is justified, then a separation of guanidine from creatine and creatinine seems possible, although arginine (K_b 1.3×10^{-5}) and histidine (K_b 1.0×10^{-5}) would be absorbed. Piffner and Myers [1926] state, however, that the absorption of guanidine and the methylated guanidines by permutit is not quantitative. I was unable to obtain a supply of permutit and used a similar preparation (doucil) procured

¹ The work contained in this paper formed part of a thesis which was accepted by the University of Durham for the degree of Ph.D.

from the British Drug Houses, Ltd. An analysis of doucil [Hilditch and Wheaton, 1925] is very similar to that of permutit in 1923 [Higgins and O'Callaghan, 1925].

	Na ₂ O	Al ₂ O ₃	SiO ₂
Doucil	13.2	21.8	65
Permutit	13.0	21.0	65

These approximate to Na₂O, Al₂O₃, 5SiO₂.

Guanidine was found to be completely removed from neutral aqueous solutions by doucil, while no retention of creatine or creatinine could be observed.

For the removal of bases from the silicate, a saturated solution of sodium chloride was found to be most convenient. The amounts recovered were not quantitative, especially with dilute solutions of guanidine.

As would be expected from the balanced action suggested by Whitehorn, the removal of guanidine from saline solutions was very much less efficient than from watery solutions. The percentage recovery of guanidine dropped from about 60 % (and higher) in water, to about 20 % in 1 % saline. Thus it seemed clear that, although a separation of guanidine from creatine and creatinine could be achieved by means of "doucil," some removal of sodium and/or concentration of solution was desirable before this observation could be applied effectively.

The most hopeful method seemed to be by the use of an adsorbing agent, and charcoal, already used by other workers, was investigated.

Preliminary experiments showed that

- (1) the greater the amount of guanidine to be adsorbed, the greater the amount of charcoal necessary:
- (2) the efficiency of a given weight of charcoal is increased by increasing the strength of sodium hydroxide up to about 0.02 *N*:
- (3) the efficiency of a given weight of charcoal is decreased by dispersion in a large bulk of fluid.

Thus the optimum amounts of charcoal and alkali depend on the volume of solution and the amount of guanidine. The amounts used in the final experiments were 4 g. of charcoal and 2 cc. of *N* NaOH for 100–250 cc. of fluid, when 20 mg. of guanidine base were adsorbed quantitatively.

The difference between these amounts and those used by Weber [1927] is accounted for by the fact that Weber was dealing with small amounts of fluid and low concentrations of guanidine, for which the amounts he gives are adequate.

For the release of guanidine from charcoal, the conditions used in conjunction with those given above for adsorption were 100 cc. of 0.1 *N* sulphuric acid in 95 % ethyl alcohol for every 4 g. of charcoal used.

In confirmation of the work of Pffner and Myers [1930] it was found that the recovery was incomplete, being only 75–80 %.

These two processes were then combined, and some experiments performed

in order to find if reasonable recoveries could be made of guanidine added to aqueous solutions. The percentage recoveries were as shown in Table I.

Table I.

mg. of base per 100 cc. water	% recovery
46.5 (guanidine)	42.5
22.5 "	45
1.4 "	20
0.7 "	20
0.2 "	Nil
57.5 (<i>as</i> -dimethylguanidine)	25
22.5 "	25
3.6 "	36
1.8 "	6
0.9 "	Nil

These figures, while not high, are about what would have been expected from the previous experiments, as the recovery from charcoal is about 75 %, and that from doucil of about the same order. With *as*-dimethylguanidine, which is (presumably) a much weaker base, the recovery is not so high.

In order to ensure that creatine and creatinine were not broken down during these manipulations, solutions containing 10 mg. of creatine, 10 mg. of creatinine, and 5 mg. of each substance per 100 cc. water were subjected to exactly the same procedure as the solutions of guanidine, but no trace of any breakdown could be shown.

Thus from aqueous solutions it is possible to isolate reasonably small amounts of guanidine bases without any breakdown of creatine or creatinine.

Precipitation and identification of guanidine.

The precipitating agents which have been used previously are salts of mercury, silver, gold and platinum, phosphotungstic acid, picric acid and picrolonic acid. Zimmermann [1930] found that guanidine and methylguanidine are precipitated in neutral or acid solution by quinizarinsulphonic acid (1:4-dihydroxyanthraquinone-2-sulphonic acid). This acid also precipitates a number of other substances amongst which are ammonia, creatinine, arginine, histidine and lysine. Smorodinzew [1930] attempted, without success, to separate methylguanidine and creatinine by the differing solubilities of their reineckates. Neither of these substances seems suitable in view of their complexity and probable difficulties of analysis.

Of the substances mentioned previously, the use of silver and mercury salts has been justly criticised by Ewins [1916] and by Greenwald [1919]. Gold chloride is apparently easily reduced. Burns [1916], who used it, found small amounts of gold in his precipitates, and Beilstein [1922] says that methylguanidine chloroaurate is easily decomposed. Although platinum chloride has been used as a precipitant for the guanidine bases, the salts formed are apparently not very insoluble. Beilstein [1922] gives the solubility of methylguanidine chloroplatinate as 14.3 parts per 100 parts of water at 18–19°. He also states [1922] that *as*-dimethylguanidine chloroplatinate is easily soluble

in water. Phosphotungstic acid gives a precipitate with guanidine which is fairly insoluble (200 mg. per 100 g. water [Drummond, 1918]), but is soluble in excess [Kuen, 1927]. Apparently the precipitates with phosphotungstic acid are quite amorphous.

Picric and picrolonic acids have been used by various workers in this connection, the former by Sharpe [1920] and Greenwald [1924], the latter by Kuen [1927], Komarow [1929] and Ellis [1928]. Either of these acids seems to be suitable, and both were investigated in order to determine which would be more efficient.

In all precipitations an equal volume of saturated sodium picrate, or sodium picrolonate, was added to the solution containing the base to be precipitated.

Table II gives the limits of precipitation of guanidine and *as*-dimethylguanidine under these conditions. Guanidine picrate is bright yellow, and the picrolonate forms very characteristic small red balls. Both the dimethylguanidine salts are pale yellow and show delay in precipitation.

Table II.

mg. base per cc.	Guanidine		<i>as</i> -Dimethylguanidine	
	Picrate	Picrolonate	Picrate	Picrolonate
1	Ppt. immediately	Ppt. immediately	Ppt. (2 hrs.)	Ppt. immediately
0.5	„	Trace in 2 hrs. greater overnight	Ppt. overnight	„
0.25	„	Nil	Nil	Trace in 2 hrs. greater overnight
0.125	„	Nil	Nil	Nil
0.0625	Ppt. on standing overnight	Nil	—	—

Under similar conditions a picrate is formed with 11.5 mg./cc. Na, 1.25 mg./cc. K, and 2.5 mg./cc. NH_4 (chlorides); the picrates of Mg and Ca are extremely soluble. Picrolonates are formed with 6 mg./cc. Na, 2.5 mg./cc. K, 2.5 mg./cc. NH_4 and 24 mg./cc. Mg. Calcium picrolonate is precipitated with 0.02 mg. Ca per cc., the solubility being less than that of calcium oxalate under similar conditions. The solubility of this salt has been investigated in detail, at the author's suggestion, by Robinson and Scott [1932].

Creatine (3 mg./cc.) and creatinine (0.75 mg./cc.) are precipitated by picric acid solutions, but neither substance appears to be precipitated by saturated sodium picrate solution.

Photomicrographs of the guanidine derivatives are shown in Plates II and III. Photomicrographs of some of these compounds have been published previously by Burns [1916], Sharpe [1916, 1925].

Attempts were made to crystallise guanidine picrate from various solvents other than water but without success. Methyl, ethyl, *isobutyl* and *amyl* alcohol gave crystals which were not so definite in shape as those obtained with water. Butyl acetate, in which the picrate is very slightly soluble, gave poor crystalline form. In ether, chloroform, light petroleum and benzene,

PICRATES.

Guanine picrate, $\times 340$.

Methylguanidine picrate, $\times 60$.

α -Dimethylguanidine picrate, $\times 60$.

PICROLONATES.

Guanidine picrolonate, $\times 210$.

Guanidine picrolonate, $\times 210$.

as-Methylguanidine picrolonate $\times 100$

as-Dimethylguanidine picrolonate, $\times 210$.

guanidine picrate is practically insoluble. The picrate is appreciably soluble in acetone, but the crystals obtained were very minute. Under these conditions, sodium picrate is in every way a better precipitating agent than sodium picrolonate. It is not only more efficient in precipitating guanidine, but is less easily precipitated by other bases.

Potassium and ammonia are the two most likely substances to be precipitated. The amounts of these substances in normal blood are [Harrison, 1930] K 150–250 mg. per 100 cc. and NH_3 0.1 to 0.25 mg. per 100 cc. Thus with normal bloods there is no likelihood of the precipitation of ammonia, but a distinct probability of the formation of potassium picrate. No way suggested itself of evading this difficulty excepting by analysis of the picrate.

In identifying picrates, methods used by previous workers have included melting-point, picric acid content, total nitrogen, and basic nitrogen [Greenwald, 1924; White, 1926–27]. Kuen [1927], using picrolonates, extracted the picronic acid with ether and weighed it. Of these the melting-point, basic nitrogen and picric acid content seem to be adequate. The estimation of total nitrogen is not suitable. It involves the use of a separate sample of picrate, and does not show such marked differences for different picrates as does the basic nitrogen. This latter can be estimated concurrently with the picric acid content.

Figures for the various picrates are given in Table III. As only small amounts of picrate would be available for analysis it is necessary to determine the smallest amount of picrate which would give a dependable result, and to ascertain the accuracy of the results.

Table III.

	M.P. ° C.	Basic N %	Picric acid %
Guanidine picrate	318–323 (G.) 317–320 (W.)	14.8	79.5
Methylguanidine picrate	197 (G. and W.)	13.9	75.8
Dimethylguanidine picrate	225 (G. and W.) 230 (B.)	13.3	72.5
Ammonium	276–280 (G.) Volatilises (S. and P.)	5.7	93.1
Potassium	311–316 explodes (S. and P.)	Nil	85.8
Sodium	310–315 explodes (S. and P.)	Nil	91.2
G. = Greenwald [1924].		W. = White [1926–27].	
S. and P. = Silberrad and Phillips [1908].		B. = Beilstein [1929].	

The method of analysis adopted by Greenwald [1924] consisted in precipitating the picric acid, in a sample of guanidine picrate, with nitron, filtering through a Gooch crucible, and weighing the nitron picrate. The guanidine base in the filtrate he estimated by distilling with caustic soda, in a similar manner to the second stage of a Kjeldahl estimation. The smallest quantity on which a reasonably accurate analysis seemed feasible was 25 mg. From this amount of guanidine picrate, the weight of nitron picrate formed would be 47 mg., and the amount of ammonia distilled over would be

equivalent to 5.20 cc. of $N/20$ alkali. With a balance graduated to 0.0002 g., and a possible error of 0.0001 g. in each of four weighings (two in weighing the sample, and two in weighing the crucible) and each in the same direction, the difference would be 1.0 in the percentage of picric acid. In considering the estimation of the basic nitrogen, the calculation is not so simple, but by taking an actual example when 24.2 mg. of guanidine picrate were analysed, and calculating similarly the difference in the percentage nitrogen was 1.2. These calculations take a fairly extreme view of the probabilities of error, but such a view is necessary in endeavouring to determine the value of analytical figures. The differences in composition which would result from the admixture of 10 % of various picrates with pure guanidine picrate are given in Table IV.

Table IV.

Guanidine picrate mixed with	Difference in % basic N	Difference in % picric acid	Difference in m.p. of separate picrates
10 % potassium picrate	-1.5	+0.6	- 2°
10 % ammonium „	-0.9	+1.4	- 137°
10 % sodium „	-1.5	+0.6	- 3°
10 % inert impurity	-1.5	-8.0	—

From these figures it seems that impurities would be easily attributed to errors in analysis, but that analysis would establish the identity of the substance forming the major portion of the picrate analysed.

This method was then applied to quantities of blood to which guanidine and *as*-dimethylguanidine salts had been added, protein being removed by the method of Folin and Wu [1919]. The amounts of guanidine recovered were lower than those from aqueous solutions, as will be seen from Table V.

Table V.

		mg. base added to 100 cc.	% recovery	
			Water	Blood
Guanidine	(1)	22.5	45	11
	(2)	46.5	42.5	23
<i>as</i> -Dimethylguanidine	(1)	22.5	25	Nil
	(2)	57	25	15.5

As control experiments two samples, each of 100 cc. of defibrinated blood, were treated in the same way as those to which guanidine had been added. No insoluble picrate was formed in either case.

The analytical figures for these picrates, together with those of guanidine picrate and *as*-dimethylguanidine picrate, are given in Table VI.

These picrates were not recrystallised before analysis, but in each case the small amounts left after analysis were recrystallised and showed the form characteristic of the picrate anticipated.

These figures show that a failure to find guanidine bases in blood by this method indicates that the concentration of these bases is less than 20 mg./100 cc.

of blood in the case of guanidine and 50 mg./100 cc. of blood in the case of *as*-dimethylguanidine, although it seems probable that smaller amounts would be detected.

Table VI.

			M.P. ° C.	Basic nitrogen %	Picric acid %
Guanidine picrate			317-323	14.8	79.5
"	from water	(1)	318 (decomposes)	—	—
"	" blood	(1)	308 "	—	—
"	" water	(2)	316 "	14.5	78.3
"	" blood	(2)	306 "	15.8	77.0
<i>as</i> -Dimethylguanidine picrate			225	13.3	72.5
"	from water	(1)	227 (decomposes at 265)	—	—
"	" water	(2)	219 (decomposes at 275)	13.6	70.6
"	" blood	(2)	219 (decomposes at 260)	13.6	73.5

EXPERIMENTAL.

(1) *Extraction by partially miscible solvents under reduced pressure.* The apparatus introduced by Dakin [1920] was used. A solution containing 237 mg. creatine and 68 mg. guanidine in 250 cc. water was extracted with *isobutyl* alcohol for 36 hours. The extract contained 50 mg. creatine (20 %) and 17 mg. guanidine (25 %). Similar extractions were performed with *amyl* alcohol (which extracted a small percentage of guanidine), *n*-butyl acetate and toluene. The latter substances did not extract guanidine or creatine.

(2) *Experiments with doucil.* Doucil, a whitish granular solid, was kept in saturated sodium chloride solution (to remove calcium which was sometimes present). Before use it was washed thoroughly with distilled water and filled into a burette. A small reservoir was fitted to the top of the burette and the rate of flow of liquids regulated by an adjustable clip at the lower end. Prior to use the doucil was washed with dilute acetic acid (50-100 cc. of 1 %) and with water (200-300 cc.) until the filtrate was chloride-free and neutral to phenol red.

Solutions of guanidine were filtered through columns of doucil and followed by wash-water. In a few cases the filtrates were evaporated (with the addition of a little acid) to small bulk, and sodium picrate added. No precipitate resulted.

Attempts were made to remove guanidine from doucil by caustic soda and by alcohol, with no result. Washing with large volumes of water (400 cc.) gave no guanidine.

Guanidine was recovered from the doucil by washing with saturated sodium chloride solution, followed by an equal volume of water and, after evaporation to dryness, extracted from the sodium chloride with methyl alcohol, which is more efficient for this purpose than ethyl alcohol. The alcohol was evaporated and the residue extracted with methyl alcohol, which was again evaporated. The solid was taken up in water, but the solution contained a suspension (probably of silica) which could not be filtered or centrifuged. This was

removed by the addition of a little solid magnesium oxide, the solution being warmed to about 60°. A flocculent precipitate formed, which settled and was easily centrifuged. The clear liquid was decanted, neutralised, and an equal volume of saturated sodium picrate solution added.

Table VII.

Exp.	Fluid	Guanidine mg.	Doucil cc.	Saturated NaCl cc.	Recovery %
1	Water	20	34.5	50	57.5
				50	Tr.
2	"	20	10	50	100
3	"	10	13	50	40-50 (about)
4	"	4.5	13	50	Tr.
5	"	5	37	50	20-25
6	1 % saline	5	35	50	Nil
7	"	69	15	50	20 (a)
8	"	34.5	14	50	17.5 (a), (b)
9	"	33.5	33	50	33 } 41
				50	8 }
				50	Nil
10	"	28	14	50	11.5 p _H 8.2
				50	Nil
11	"	29	14	50	14.5 p _H 5.5
		<i>αs</i> -Dimethyl- guanidine mg.			
12	Water	22.5	12	55	68
13	"	10	12	50	59
14	"	6.5	11.5	50	Nil
15	"	3	10	50	Nil

Volume of fluid, 500 cc.

(a) Solid sodium chloride re-extracted with methyl alcohol—no guanidine.

(b) Magnesium oxide (and silicate) re-extracted with water—no guanidine.

Solutions containing creatine and creatinine were filtered through doucil, and the doucil was washed with water. By estimations on the original fluid and filtrate, it was found that no creatine (or creatinine) was removed. The doucil was in each case extracted with saturated sodium chloride solution, but no insoluble picrate was formed.

Table VIII.

Volume of solution	mg. base (creatine)	Volume of filtrate	mg. base in filtrate
80	98.5	186	93
95	188	250	191
		300	Nil
	(creatinine)		
190	249.5	299	242
		100	2
		200	0.5
		100	Nil
95	247	250	249
		200	Nil

Volume of doucil = 20 cc.

(3) *Experiments with charcoal.* To neutral solutions containing guanidine were added caustic soda and charcoal (Merck's, pure, dry, "for analysis"). The mixture was shaken at intervals, and allowed to stand for at least 2 hours. The charcoal was then filtered off and allowed to drain. The filtrate was neutralised (the difference between the amount of acid needed and the caustic soda originally added, being taken as the amount of soda adsorbed), acidified and evaporated, the guanidine being precipitated as picrate. The charcoal and filter-paper were returned to the original flask, sulphuric acid-alcohol was added, and the mixture allowed to stand overnight. The charcoal was again filtered, water added to the filtrate and the alcohol distilled off on a water-bath. The liquid was then neutralised with caustic soda (or barium carbonate) and the guanidine precipitated with sodium picrate.

Experiments on the conditions of adsorption are given in Table IX.

Table IX.

Guanidine mg.	Volume of solution cc.	Normality of NaOH in solution	Charcoal g.	Guanidine in filtrate mg.	Adsorption %
4.6	100	0.016, 0.01, 0.005	1	Nil	100
23	"	0.08	"	8.2	64
"	"	0.04	"	6.5	71
"	"	0.02	"	6.2	73
"	"	0.01	"	7.5	68
"	"	0.005	"	9.5	59
10	"	0.02	"	Tr.	99
"	"	"	2, 4	Nil	100
23	"	"	2	2	91
"	"	"	4	Nil	100
20	"	"	"	Nil	100
"	250	"	"	Nil	100
"	500	"	"	Tr.	99
"	1000	"	"	1	95
"	100	"	"	Nil	100
"	250	0.008	"	Nil	100
"	500	0.004	"	Tr.	99
"	1000	0.002	"	Tr.	99
23	100	0.02	"	Nil	100
14	"	"	"	Nil	(14 times) 100
20	"	"	"	Nil	(6 times) 100
40	"	"	"	1.5	96
80	"	"	"	16	80

(4) *Release of guanidine from charcoal.* The volume of acid alcohol for efficient extraction varies with the amount of charcoal. With a constant amount of charcoal, the percentage extracted increases with the strength of acid and with the volume of the extracting fluid. The substitution of methyl for ethyl alcohol decreases the efficiency. The precipitation of sulphuric acid by barium does not decrease the yield compared with neutralisation by caustic soda (Table X).

(5) *Charcoal and doucil.* Solutions of guanidine (and dimethylguanidine) in water were treated with charcoal and then filtered through doucil. The yields are given in Table I. One illustrative experiment is given.

Table X.

Guanidine in charcoal mg.	Charcoal g.	Volume of acid alcohol cc.	Normality of acid	Guanidine released %
16.5	1	50	0.02	52
21	2	"	"	46
23	4	"	"	20
23.5	4	"	0.33	69
"	"	"	0.17	67
"	"	"	0.08	59
"	"	"	0.04	60
"	"	"	0.04	51
(methyl alcohol)				
14	"	50	0.1	58 neutralised with NaOH
"	"	"	"	62 " BaCO ₃ (cold)
"	"	"	"	58 " BaCO ₃ (heat)
"	"	100	"	84 " NaOH
"	"	150	"	81 " NaOH

95 % ethyl alcohol. 4 g. charcoal contain 1.7 cc. *N* NaOH.

46.5 mg. guanidine base (carbonate neutralised with H₂SO₄) in 100 cc. water were treated with 4 cc. *N* NaOH and 8 g. charcoal, and, after standing 2 hours, filtered. Guanidine was extracted by 175 cc. acid alcohol (= 150 cc. 0.1 *N* acid) overnight. The alcohol was evaporated and, after heating, 1.45 g. BaCO₃ were added. The solution was neutralised with 5.2 cc. *N* NaOH made up to 100 cc. and filtered through 26 cc. doucil in 2 hours, the doucil being washed with 200 cc. water. Guanidine was removed from the doucil by 50 cc. saturated NaCl solution (2 hours 20 minutes) and washed with 50 cc. water. The filtrate was evaporated to dryness on the boiling water-bath and the salt extracted three times with hot methyl alcohol (abs.) (total volume 45–50 cc.). The alcohol was evaporated and the residue again extracted three times with hot methyl alcohol (total volume 15–20 cc.). After a third evaporation the residue was dissolved in 15 cc. water (3 portions of 5 cc.): solid magnesia was added to the warmed solution until a flocculent precipitate formed which was centrifuged. The clear liquid was neutralised (1 drop *N* HCl) and 15 cc. saturated sodium picrate solution were added. After standing overnight the picrate was centrifuged, washed with 2 cc. water, dried at 100° and weighed.

Yield. 99 mg., 42.5 %.

Analysis. Basic N, 14.5; picric acid, 78.3 %.

m.p. 316° with decomposition. Microscopically—guanidine picrate.

Solutions of creatine and creatinine (100 mg. per litre) were treated in an identical manner but no insoluble picrate was formed.

(6) *Analysis*. The picrates were analysed in a manner similar to that used by Greenwald [1924]. At least 25 mg. of picrate were dissolved in 30 cc. water, and 10 cc. of 1 % nitron in 0.5 % acetic acid added, drop by drop, to the heated solution. The solution was cooled in the ice-chest and filtered through a weighed Gooch crucible. The precipitate was then washed with a little ice-water and the crucible dried in an oven at 100° and weighed. Nitron was

removed from the filtrate by the addition of 1 cc. of nitric acid to the boiling fluid, and cooling in ice with stirring for at least 2 hours. After filtration through a Gooch crucible, and washing with a little ice-water, the liquid was transferred (300 cc.) to the distillation flask of a Kjeldahl apparatus. 100 cc. of 40 % caustic soda were added, and the whole was distilled into standard acid, until the volume (in the flask) was 50 cc. 300 cc. of water were added and distilled over, when the whole of the base was broken down. When the distillation was repeated a third time no base came over. Melting-points were determined, using paraffin wax. All the readings given are corrected for cooling of the mercury thread. The m.p. of pure guanidine picrate was 320–323° with decomposition. If the heating of the picrate were very slow, there was a tendency for it to char with no apparent melting.

(7) *Removal of protein from blood.* Protein was removed by the method of Folin and Wu [1919]. By use of this method the resulting dilution is about eightfold, but attempts to avoid this dilution by precipitating proteins with alcohol and sulphuric acid-alcohol gave no good results. The incomplete filtration with Folin's newer method [1930] makes its use inadvisable.

The completed method was then as follows.

(a) *Removal of protein.* 1 volume of blood diluted to 8 volumes with water. 1 volume of 10 % sodium tungstate solution added. 1 volume 0.66 *N* sulphuric acid added slowly with shaking. Stand 1 or 2 hours and filter (usually overnight).

(b) *Adsorption with charcoal.* Neutralise with caustic soda, add 2 cc. *N* NaOH and 4 g. of charcoal for every 250 cc. of liquid (and, if guanidine has been added, for every 20 mg. of guanidine). Shake at intervals and stand for 2 hours. Filter.

(c) *Release from charcoal.* Add 100 cc. of acid-alcohol (0.1 *N* sulphuric acid in 95 % alcohol) for every 4 g. charcoal and shake. Stand overnight. Filter. Remove alcohol by evaporation on boiling water-bath. Heat to boiling and add, while boiling, slightly less than the calculated weight of barium carbonate. Stand and centrifuge off the barium sulphate. Neutralise carefully (using phenol red).

(d) *Removal of guanidine by doucil.* Filter slowly through 25 cc. doucil (previously washed until neutral and chloride-free). Wash doucil with 200 cc. water.

(e) *Removal of guanidine from doucil.* Filter 50 cc. of saturated sodium chloride solution, for every 20 mg. guanidine, slowly through the doucil, and wash with 50 cc. of water. Evaporate salt solution and wash-water to dryness on boiling water-bath. Extract solid sodium chloride three times with hot methyl alcohol (10–15 cc. each time). Evaporate methyl alcohol, and extract this salt three times with 5 cc. hot methyl alcohol. Evaporate off methyl alcohol. Dissolve residue in 15 cc. of hot water in a large centrifuge-tube. Put the tube in a boiling water-bath and add solid magnesia. When the liquid clears, cool and centrifuge.

(f) *Precipitation of guanidine.* Decant liquid into another centrifuge-tube, neutralise and add 15 cc. saturated sodium picrate solution, and stand overnight. If any precipitate is formed, centrifuge, wash with 2-3 cc. cold water and centrifuge again. Dry at 100° and weigh.

Details of the recoveries of guanidine from blood are given in Table XI. Analyses of the picrates are given in Table VI.

Table XI.

Base added to 100 cc. defibrinated blood	Vol. of filtrate cc.	N NaOH to neutralise cc.	Adsorption		Re-lease cc. acid-alcohol	Neutralisation		Vol. fluid cc.	Doucil		Vol. on adding picrate	Recovery
			N NaOH cc.	Char-coal g.		BaCO ₃ g.	N NaOH cc.		cc. (time)	Saturated NaCl (time)		
22.5 mg. guanidine	600	1.5	2	4	100	1	1.0	50	29 (1 hr.)	55 (1 hr.)	20	12 mg.-11 % *
46.5 mg. guanidine	750	0.6	14	20	400	3.36	5.7	100	25 (2 hrs. 15 mins.)	50 (2 hrs.)	15	54 mg.-23 %
										50	15	Nil
22 mg. dimethyl-guanidine	600	1.0	2	4	100	1	2.5	60	29 (1 hr. 20 mins.)	55 (45 mins.)	20	Nil
57 mg. dimethyl-guanidine	750	0.7	14	20	400	3.3	0.5	100	28 (2 hrs.)	50 (2 hrs.)	15	32 mg.-15.5 %
										50	15	Nil
Nil	600	2.5	2	4	100	1	2.5	50	27 (1 hr.)	55 (1 hr.)	20	Nil
Nil	750	0.7	14	20	400	3.3	0.7	100	29 (1 hr. 30 mins.)	55 (3 hrs.)	15	Nil
										50 (1 hr. 30 mins.)	15	Nil

* MgO re-extracted—nil.

SUMMARY.

A method is worked out for the estimation of guanidine bases in blood, particular attention being directed to the separation of these bases from creatine and creatinine.

An attempt to effect this separation was made by extraction with isobutyl alcohol, which extracts both substances partially, amyl alcohol, butyl acetate, and toluene, which do not extract either substance. The separation is effected by the use of doucil, a sodium aluminium silicate, which absorbs guanidine, but neither creatine nor creatinine. The removal of guanidine from doucil is incomplete, saturated sodium chloride solution being used, and the guanidine extracted from the salt by methyl alcohol. A preliminary concentration of the guanidine by adsorption on charcoal is found to be necessary, and the optimal conditions for adsorption and release of guanidine are worked out. Again adsorption is complete but release (by acid-alcohol) is only about 80 %.

The guanidine is precipitated by saturated sodium picrate and the solubilities of picrates of the commoner metals and guanidine are given, together with photomicrographs showing their crystalline appearance. The picrates are identified by melting-point, picric acid content and basic nitrogen.

With the completed method on aqueous solutions, 2 mg./100 cc. of guanidine or dimethylguanidine could be detected, although the recovery was only about 25 %.

For determinations in blood proteins were removed by the method of Folin and Wu. The percentage recovery from blood however dropped from 45 % (in water) to 25 % with 20 mg. guanidine per 100 cc.

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