XLVII. GUANIDINE DETERMINATIONS ON SOME INVERTEBRATES BY A COLORIMETRIC PHOSPHOTUNGSTIC ACID METHOD.

By MAX MAPES ELLIS.

From the Physiological Institute, University of Glasgow.

(Received February 7th, 1928.)

INTRODUCTION.

It has been suggested [Jaffé, 1906; Paton, 1925] that guanidines may be detoxified in the vertebrate body by an acetylation through which they are transformed into creatine. In view of this possibility and the fact that the muscles of certain invertebrates have been reported as creatine-free [Suzuki, Joshimura and Irie, 1909; Okuda, 1912; Wilson, 1914], guanidine and creatine determinations have been made on molluscan muscle from the limpet, and guanidine determinations on various other invertebrate material.

METHOD.

1. *Extraction of tissue.* Except as noted under the individual analyses only freshly collected animals were used. The desired tissue was dissected out and minced fine immediately, weighed and put up in absolute alcohol in a ratio of 20 g. of mince to 80 cc. of alcohol. After 48 hours the fluid was drained off and the residue extracted three times with boiling absolute alcohol for three 30-minute periods. After the fluid from the final extraction had been removed, the residue was pressed into a Büchner funnel, sucked dry and then washed with a small quantity of absolute alcohol.

2. *Removal of the undesired extractives.* To the combined alcoholic extracts 15 cc. of distilled water were added and the whole reduced on a water-bath to approximately 10 cc. Sufficient magnesium hydroxide was then added to make the solution barely alkaline to litmus and the evaporation on the water-bath continued to dryness. By this treatment trimethylamine and ammonia are driven off, and methylguanidine is not destroyed [Kuen, 1927].

The residue was then taken up in approximately 30 cc. of absolute alcohol and the insoluble portion centrifuged down. After draining off the supernatant alcohol the precipitate was stirred up with 15 cc. of absolute alcohol and again centrifuged, and the supernatant alcohol added to that from the first centrifuging. The insoluble residue was discarded. The fluid could not be filtered at this stage, as guanidine compounds are adsorbed by filter-paper from both neutral and alkaline solutions. By means of this alcoholic extraction of the dry

residue, arginine (found in crayfish, crabs and lobsters, Ackermann and Kutscher [1907]), lysine (found in crabs, Ackermann and Kutscher; in lobsters, Suzuki, Joshimura and Fuji [1909]), most of the histidine (found in adductor muscle of *Mytilus edulis*, Jansen [1913]), most of the inorganic salts and some of the proteins are left behind in the residue (Guggenheim [1924]; Fränkel [1907]; Thierfelder [1909]).

To this alcoholic extract an equal volume of distilled water was added and the fluid reduced to approximately 10 cc. on the water-bath. The hot liquid was made up to approximately 20 cc. with warm distilled water, and 5 cc. of 2*N*/3 sulphuric acid were added. The fluid was then chilled to about 10° and allowed to warm up slowly to room temperature, when sufficient distilled water was added to make an even reading, and the exact volume noted. The liquid was next filtered through a damp, but well-drained filter-paper. This chilling of the acid solution separates the fatty substances and most of the lipochromes, both of which are caught by the wet filter-paper.

An aliquot part (as large as possible) of the clear filtrate was then carefully measured and 4 cc. of 10 % sodium tungstate solution were added for each 20 to 25 cc. of filtrate used. This gives the Folin-Wu [1919] precipitation reagent. If the protein precipitate did not settle quickly 1 cc. of 2*N*/3 sulphuric acid and 1 cc. of 10 % sodium tungstate were added. This was repeated until the protein precipitate separated quickly after shaking the fluid, after which 2 cc. of 2*N*/3 sulphuric acid in addition to that required for the protein precipitation were added to ensure an acid reaction, and the exact total volume was noted. This Folin-Wu precipitation completes the removal of the protein fraction and the lipochromes.

An aliquot part (as large as possible) of the Folin-Wu filtrate was measured into a flask and approximately 0.5 g. of animal charcoal (purified by acid) added for each 20 cc. of filtrate used. The flask was heated quickly in a water-bath to about 90°, care being taken that the fluid did not boil, and the contents were filtered while hot through a small filter. The flask was rinsed with a small quantity of 2*N*/3 sulphuric acid, which was then poured on the filter. To the filtrate a second lot of animal charcoal was added (0.5 g. to 20 cc.) and the process repeated. If the creatine was to be determined the filter-papers and charcoal were preserved in 95 % alcohol, otherwise they were discarded. As each charcoal filtration removes 90 % or more of the creatine, as well as uric acid and phenols [Folin and Dennis, 1916], the double charcoal filtration reduced the creatine content of the filtrate to less than 1 % of the creatine content of the original extract.

After the charcoal filtrations the filtrate was first neutralised with 10 % sodium hydroxide, then made barely acid to litmus with as little hydrochloric acid as required, after which the fluid was evaporated to dryness on a water-bath. These adjustments of the reaction of the filtrate are necessary because either free sulphuric acid or alkali may decompose some of the guanidine on concentration. As the small quantity of excess hydrochloric acid required to

bring the solution to an acid reaction after neutralisation with sodium hydroxide is volatile, the solution may be evaporated on a water-bath with safety.

The residue from this evaporation was taken up in not more than 5 cc. of 5 % sulphuric acid, transferred to a centrifuge tube and the insoluble material allowed to settle. It is essential that the volume be kept as small as possible at this stage on account of the phosphotungstic acid precipitation. After standing for a few minutes, but without removing any of the material from the tube, 30 % aqueous phosphotungstic acid solution was added drop by drop until two drops gave no additional clouding after shaking the fluid at the top of the tube. The tube was then placed in an ice-chest, or otherwise kept cold (below 10°) for 18 hours, after which the preparation was centrifuged. The precipitate consisted essentially of guanidine, methylguanidine and betaine phosphotungstates, together with the small insoluble residue which formed on drying down the Folin-Wu filtrate.

3. *Determination of guanidine.* The final determination of the guanidines was made by means of the nitroprusside reaction, and Marston's reagent [1924] as modified by Weber [1927] was used. This reagent is prepared about 30 minutes before it is required by mixing equal volumes of 10 % solutions of sodium nitroprusside, potassium ferricyanide and sodium hydroxide. The stock solutions of nitroprusside and ferricyanide should be kept in the dark and made up fresh every few days. The nitroprusside reagent was chosen because of the presence of betaine phosphotungstate in the precipitate with the guanidines, and because of the small quantities of guanidines to be determined. As the solubility of betaine phosphotungstate in methyl alcohol is twice that of creatine phosphotungstate [Drummond, 1918], a gravimetric separation of guanidine and betaine phosphotungstate on the basis of their solubilities in methyl alcohol, like that used by Kühnau [1926] in his separation of guanidine and creatine phosphotungstates from vertebrate blood, is undesirable. It was found that betaine did not give a colour reaction with the nitroprusside reagent and that the small quantities of betaine carried over in the methyl alcohol extraction of the phosphotungstic precipitate did not influence the colour reactions of the guanidines.

After draining off all the fluid from the phosphotungstic precipitate 2 cc. of methyl alcohol (acetone-free) were added, the precipitate was mixed with the alcohol, and the insoluble portion centrifuged down. The methyl alcohol solution was drained off into an evaporating dish, the tube washed with 1 or 2 cc. of methyl alcohol (care being taken not to disturb the precipitate) and the washing added to the first methyl alcohol solution. This methyl alcohol contained the guanidine and methylguanidine phosphotungstates and a small quantity of betaine phosphotungstate. The recovery of the guanidines was quite complete, for 29.2 parts of guanidine phosphotungstate are soluble in 100 parts of methyl alcohol [Drummond, 1918] and 16 parts of methylguanidine phosphotungstate [Kühnau, 1926], as against 1.2 parts of betaine phosphotungstate and 0.6 part of creatine phosphotungstate [Drummond, 1918].

To the alcoholic solution of the phosphotungstate four volumes of distilled water were added (this produces a clouding due to the formation of a partial precipitate of the phosphotungstates), and the whole was reduced to about one-half the original volume on a water-bath. This fluid was then first neutralised with 10 % sodium hydroxide and next made just acid to litmus with as little hydrochloric acid as required, and the evaporation continued on the water-bath to dryness. This step is essential, as methyl alcohol gives a colour reaction with the nitroprusside reagent.

The residue was taken up in exactly 5 cc. of 2 % sodium hydroxide and without removing the solution from the evaporating dish exactly 1 cc. of the nitroprusside reagent was added. At the same time the nitroprusside reagent was added to the standard (see below) and the readings were made with a colorimeter after 10 to 15 minutes. As this method does not differentiate between guanidine and methylguanidine, and as the colorimetric values of the phosphotungstates of these two radicles are not the same, the final results have been reckoned both as guanidine and methylguanidine, the true values lying somewhere between these limits (see Table III).

4. *Standard for guanidine determinations.* As it was found that the shade of colour produced by guanidine and methylguanidine phosphotungstates with the nitroprusside reagent differed from the shade produced by the hydrochlorides of these radicles with that reagent, standards were first prepared from weighed quantities of repurified guanidine phosphotungstate. As this compound is difficult to prepare and does not keep well, after the preliminary standardisations were completed, the standards were prepared directly from dry, crystalline, guanidine hydrochloride (Merck) which has one molecule of water of crystallisation, as follows: to 1 cc. of 1 % aqueous solution of guanidine hydrochloride 1 cc. of 7 % phosphotungstic acid (Merck) was added and the resulting turbid fluid well shaken; after 5 minutes 3 cc. of 3.5 % sodium hydroxide solution were added and the mixture was again shaken until the precipitate was dissolved; to this solution 1 cc. of nitroprusside reagent was then added. The full colour develops in about 4 minutes and the standard is good for 30 minutes or longer, but readings were generally made after 10 to 15 minutes. This standard solution contains 5.202 mg. guanidine, and, as it contains the same amount of sodium hydroxide as the solution of the unknown, direct comparisons were made in the colorimeter. As guanidine hydrochloride is hygroscopic, samples used for standards were dried in a desiccator before being weighed. The aqueous solution of guanidine was not kept for more than 24 hours (*vide infra*).

Methylguanidine phosphotungstate when precipitated from methylguanidine hydrochloride (British Drug Houses, highest purity) gave approximately 3.2 times as much colour as guanidine phosphotungstate with the nitroprusside reagent. The quality of the colour, however, was the same as that given by guanidine phosphotungstate so that readings could be made against either a methylguanidine standard or a guanidine standard. Dimethylguanidine phos-

photungstate, when precipitated from dimethylguanidine hydrochloride (British Drug Houses, highest purity), gave a distinctly different shade of red with the nitroprusside reagent from that produced by either guanidine or methylguanidine. Accordingly this substance had to be compared with special standards.

5. *Limits of guanidine precipitation by phosphotungstic acid.* As this method depends critically on the completeness of the precipitation of the guanidines by phosphotungstic acid, and as two of the recent methods for guanidine determinations have also used phosphotungstic acid precipitations [Kühnau, 1926; Kuen, 1927], a series of recoveries of guanidines by phosphotungstic acid precipitation from known solutions of guanidines was studied.

The solutions of guanidine, methylguanidine and dimethylguanidine hydrochlorides were made up in 5 % sulphuric acid, as optimum precipitation with phosphotungstic acid is obtained from such a solution [Gulewitsch, 1899; Demjanowski, 1912; Drummond, 1918], and 30 % phosphotungstic acid was added in slight excess of the amount required for complete precipitation on the basis of molecular weights. These precipitation tests were made at two different temperatures, "room temperature"—around 18° dropping to possibly 10° at night, and "ice-chest temperature"—a constant temperature of 7°. The

Table I. *Averaged data for guanidine precipitations by phosphotungstic acid from 5 % sulphuric acid solutions.*

Compound in solution	Amount of salt in 1 cc. mg.	Amount of organic base in 1 cc. mg.	Base recovered by precipitation	
			Room temp. 18°-10° %	Ice-chest 7° %
Guanidine hydrochloride (Merck)	4	2.08	100	100
	2	1.04	100	100
	1	0.52	91	97
	0.6	0.31	85	95
	0.5	0.26	Trace	60
	0.4	0.21	None	22
Methylguanidine hydrochloride (B.D.H., highest purity)	0.2	0.10	None	None
	2	1.33	100	100
	1	0.67	100	100
	0.6	0.40	100	100
	0.5	0.33	100	100
	0.4	0.27	79	100
Methylguanidine hydrochloride (B.D.H., highest purity) kept as a 0.5 % aqueous solution for 1 week before testing	0.2	0.13	None	95
	2*	1.33*	—	23
	1*	0.67*	—	21
	0.6*	0.40*	—	11
	0.5*	0.33*	—	None
	0.4*	0.27*	—	None
Dimethylguanidine hydrochloride (B.D.H., highest purity)	2	1.40	100	—
	1	0.70	93	—
	0.6	0.42	82	—
	0.5	0.35	71	—
	0.4	0.28	66	—
	0.2	0.14	None	—

Probable error of the determinations does not exceed $\pm 3\%$.

* Amounts which should have been present had there been no decomposition of the salt in the aqueous solution.

precipitations were made from 5 cc. samples of various concentrations and in each case the tubes were allowed to stand for 18 hours before centrifuging. The determinations of the recoveries were made gravimetrically, colorimetrically and by titration (a method to be described elsewhere), and the averaged data are summarised in Table I.

From this table it is evident that the precipitation is more complete at a constant low temperature than at room temperature and that the differences are sufficient to make comparisons of determinations at room temperature unreliable for quantitative work. At ice-chest temperature the limits of "complete" precipitation, considering the probable error, seem to be near 0.75 mg. of guanidine and 0.20 mg. of methylguanidine per cc., and no determinations of guanidines from tissues have been considered as accurate in this report when less than 0.50 mg. of guanidine or 0.25 mg. of methylguanidine per cc. were found in the concentrate as taken up finally in 5 % sulphuric acid. One series of guanidine precipitations, not listed in the data, was made at -6° , and, as the recoveries did not differ essentially from those at $+7^{\circ}$, ice-chest temperature or 7° was adopted for this work.

In considering the reliability of standards one lot of 0.5 % aqueous methylguanidine solution was kept for a week at room temperature, sufficient 12.5 % sulphuric acid was then added a drop at a time to bring the whole up to 5 % sulphuric acid, after which the usual phosphotungstic acid precipitations were carried out at ice-chest temperature. This check series has been included in Table I, as the recoveries show that a 0.5 % aqueous solution of methylguanidine is unstable. Accordingly guanidine solutions more than 24 hours old were not used as standards.

An example of the result obtained in the determination of guanidine added to ox heart is given in Table II.

Table II. *Example of guanidine recovery by method described.*

Material	Guanidine hydrochloride added mg.	Guanidine added mg.	Guanidine found mg.	Guanidine recovered %
50 g. ox heart	None	None	0.648	—
50 g. ox heart	4.675	2.432	2.951	94.64

GUANIDINE CONTENT OF TISSUES AND ANIMALS EXAMINED.

In Table III the data from the various sets of determinations on invertebrate tissues have been summarised together with three determinations on vertebrate muscle. Owing to the small size of the invertebrates used it was not possible to get samples of muscle tissue alone large enough for guanidine determinations (samples of 100 g. or more were used), except in the case of the limpet. As the large muscular foot of this animal can be dissected away quickly and consists of almost pure muscle tissue, large samples were easily obtained. Two sets of limpet samples were extracted, the muscle tissue for one set ((a) in Table III) being taken from freshly collected animals within a few

minutes after they were removed from the rocks on which they were living, while the muscle tissue for the other set ((b) in Table III) was dissected from limpets which had been removed from the rocks and kept alive and unattached in the laboratory for 24 hours before the dissection was begun. When the limpet is unattached the foot-muscle contracts violently and is drawn up into a central, saucer-shaped mass in a state resembling tetanic contraction. As the guanidine content of blood from man and various other mammals is reported as high in certain tetanies [Paton, 1925; Kühnau, 1926] it seemed desirable to compare these molluscan muscles following this natural "tetany" with normal muscles from freshly collected limpets, but neither creatine nor guanidines were detected in either series of limpet muscles. These results agree with the previous findings of Wilson [1914] in the scallop and periwinkle for creatine. Betaine was present in considerable quantity in the limpet muscle and was recovered as a phosphotungstate, although no quantitative determinations for betaine were made. Considering these results on muscle from the limpet in connection with the suggestions of Jaffé [1906] and Paton [1925] that guanidines may be detoxified into creatine, two interpretations are possible, either that there are no guanidines produced by the molluscan muscle to be acetylated to creatine, or that the guanidines if formed in molluscan muscle are rapidly and completely transformed into some other compound. Betaine may have some significance in this connection, as Hunter [1922] has suggested betaine as possibly the evolutionary precursor of creatine.

Table III. *Guanidine content of tissues and animal examined.*

Systematic name	Common name	Part used	Guanidine per 100 g. of fresh tissue	
			As guanidine mg.	As methyl- guanidine mg.
Coelenterata— <i>Metridium dianthus</i>	Sea anemone	Entire animal	None	None
Echinodermata— <i>Asterias rubens</i>	Common starfish	Entire animal	None	None
Mollusca <i>Patella vulgata</i> (a)	Limpet	Foot-muscle	None	None
" (b)	"	"	None	None
<i>Purpura lapillus</i>	Dog whelk	Foot-muscle plus some viscera	5.01	1.67
Arthropoda <i>Calanus helgolandicus</i>	Copepod	Entire animal	10.27	3.21
<i>Balanus balanoides</i>	Acorn barnacle	" "	None	None
<i>Ligia oceanica</i>	Marine isopod	" "	12.87	4.01
<i>Gammarus marinus</i>	Amphipod	" "	None	None
<i>Carcinus moenas</i>	Shore crab	Legs and claws	2.62	0.82
Vertebrata	Ox	Skeletal muscle	2.20	0.69
	"	Heart muscle	1.29	0.43
	Dog (pt.)	Skeletal muscle	2.12	0.66

(a) Muscle taken from freshly collected animals. (b) Muscle taken from animals in which the foot had been strongly contracted for 24 hours. (pt.) Parathyroidectomised dog.

All invertebrates were collected October 30 and 31, and November 1, 1927, except *Calanus*, which was taken May 25, 1925. Samples averaged 100 g. or more.

In preparing the tissue from the dog whelk for extraction the muscular foot was dissected out as in the limpet, but owing to the smaller size of the whelk and the differences in the arrangement of the viscera the samples of whelk tissue were not pure molluscan muscle, but muscle plus some viscera. The exact amount of the latter was difficult to estimate, but certainly did not exceed 20 % of the material. The analyses of these samples showed the presence of guanidines to the extent of 1.67 mg. of methylguanidine per 100 g. of tissue. Of course it must be recognised that these figures are open to two objections. The determinations may not represent guanidines actually produced in the body of the whelk, as the guanidines found may have been present in either plant or animal tissue included in the intestinal detritus with the viscera which were not completely dissected away, although certainly the total amount of such material was not large. It is also possible that the substances determined were not guanidines but some other unknown substances having the same separation and precipitation characteristics as the guanidines and giving the nitroprusside reaction.

Similarly the sample of tissue from the shore crab, although very largely muscle, contained some other tissues, as they were prepared by grinding the legs and claws into a fine mince for extraction. By weighing, drying and ashing various samples, the actual weight of the tissue extracted independent of the shell was computed and the values for the guanidines found have been reckoned on the basis of the weight of the fresh tissue alone. Suzuki, Joshimura and Irie [1909] in their extractions of the fresh shell of lobsters report 0.042 % of lysine, the other extractives being present in traces only, and, as lysine was excluded from the extractions of crab material discussed above, it seems unlikely that the presence of the shell materially affected the extraction of the other tissue.

For the other extractions entire animals were minced, but the findings give the guanidine content of the whole animal on the basis of the weight of the fresh tissue alone, corrections being made for the weight of the shell or exoskeleton. Two of the species used, the shore isopod and the pelagic copepod, contained guanidines or guanidine-like substances, the other species examined did not. Here again the possibility that these findings from whole animals may have been based on guanidines present in the intestinal contents rather than on guanidines produced by the animals themselves must be borne in mind.

The three determinations on vertebrate muscle included in Table III are taken from another series of analyses bearing on other experiments, and are added to show that the method as described can be applied to vertebrate muscle as well. The ox tissues examined were ordinary butcher's beef and ox heart, and the dog muscle was obtained from a parathyroidectomised dog which had been given calcium so that its tetany was reduced. All these values for guanidine are much lower than those reported by Smorodinzew [1914] for sheep flesh, but as he used a baryta-silver method it is possible that some of the creatine of the muscle was changed into methyl-

guanidine by the reagents [Ewins, 1916], and included in the determinations as guanidine. The results for vertebrate muscle given in Table III, however, are higher than those given by Weber [1927] for normal human blood (0.07–0.15 mg. per 100 cc.) or even for hypertension cases (0.3–0.6 mg. per 100 cc.).

SUMMARY.

1. A colorimetric phosphotungstic acid method for the determination of guanidines in invertebrate and other tissues is described.

2. Pure samples of molluscan muscle from freshly collected limpets and from unattached limpets, in which the foot muscle had been in prolonged contraction, were found to be both creatine- and guanidine-free.

3. Guanidines or substances giving the separation, precipitation, and nitroprusside reactions of guanidines, were found in four invertebrates (the dog whelk, *Purpura lapillus*; the pelagic copepod, *Calanus helgolandicus*; the marine isopod, *Ligia oceanica*; and the shore crab, *Carcinus moenas*), in which animals tissues other than muscle alone were extracted.

The writer is very grateful to Prof. D. Noël Paton, at whose suggestion this work was undertaken, and Prof. E. P. Cathcart, for the privileges of the Physiological Institute of the University of Glasgow, and to Mr Richard Elmhirst, Director of the Millport Station, for his assistance in obtaining and identifying the marine material used.

REFERENCES.

- Ackermann and Kutscher (1907). *Z. Unters. Nahrungs. Genussm.* **13**, 180.
Demjanowski (1912). *Z. physiol. Chem.* **80**, 212.
Drummond (1918). *Biochem. J.* **12**, 5.
Ewins (1916). *Biochem. J.* **10**, 103.
Folin and Dennis (1916). *J. Biol. Chem.* **26**, 498.
Folin and Wu (1919). *J. Biol. Chem.* **38**, 81.
Fränkel (1907). *Descriptive Biochemie* (Wiesbaden).
Guggenheim (1924). *Die biogenen Amine* (Berlin).
Gulewitsch (1899). *Z. physiol. Chem.* **27**, 178.
Hunter (1922). *Physiol. Reviews*, **2**, 586.
Jaffé (1906). *Z. physiol. Chem.* **48**, 430.
Jansen (1913). *Z. physiol. Chem.* **85**, 231.
Kuen (1927). *Biochem. Z.* **187**, 283.
Kühnau (1926). *Arch. exp. Path. Pharm.* **115**, 75.
Marston (1924). *Austral. J. Exp. Biol. Med. Sci.* **1**, 99.
Okuda (1912). *J. Coll. Agric. Tokyo*, **5**, 25.
Paton (1925). *Glasgow Med. J.* Dec., 1.
Smorodinzew (1914). *Z. physiol. Chem.* **92**, 221.
Suzuki, Joshimura and Irie (1909). *Z. physiol. Chem.* **62**, 1.
Suzuki, Joshimura and Fuji (1909). *J. Coll. Agric. Tokyo*, **1**, 77.
Thierfelder (1909). *Hoppe-Seyler's Handb. chemischen Analyse* (Berlin).
Weber (1927). *Proc. Soc. Exp. Biol. Med.* **24**, 712.
Wilson (1914). *J. Biol. Chem.* **18**, 17.