THE ESTIMATION OF AMINO ACID NITROGEN IN ANIMAL TISSUES.

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Of the existing methods for the determination of amino acid nitrogen in animal tissues, those of Folin and Denis (1) and of Van Slyke (2) are best known and most extensively employed. Both of them are long and tedious and call for rather large tissue samples. Also methyl alcohol containing zinc chloride is employed as an extractive by Folin and Denis and ethyl alcohol as a protein precipitant by Van Slyke. In both cases there are grounds for believing (1, 3–6) that some of the amino acids are lost in the treatment with alcohol. An attempt has therefore been made to develop a method which would be free from these disadvantages.

The one to be described is a modification of the Greenwald-Bock-Blau (5–7) technique for the determination of the amino acid content of blood. It has been used by the author primarily for the analysis of liver and muscle.

Procedure.

A portion of tissue, approximately 4 to 5 gm. in weight, is excised and dropped into a mortar containing liquid air. The sample is finely powdered, meanwhile being kept thoroughly frozen. A 3 gm. portion of the powder is then weighed out to the nearest mg. The use of a small weighing bottle and a chainomatic balance permits this to be done rapidly and reduces to a negligible amount the error from water condensation and air currents. The material is then transferred quantitatively with boiling 0.01 N acetic acid to a tube 8 × 1 inches, graduated to 50 cc. 30 to 35 cc. of the acetic acid are used in several portions of 5 to 10 cc. each. The tube is promptly immersed in a water bath and left for 7 minutes after
the temperature of the tissue suspension has reached the boiling point. The tube should be shaken from time to time. After 7 minutes boiling the tube is removed from the bath and allowed to cool. 3 cc. of 50 per cent trichloroacetic acid are now added and the volume accurately made up to 50 cc. with water. The suspension is mixed thoroughly and permitted to stand for 30 minutes. At the end of this time 2 gm. of infusorial earth are added. The mixture is shaken vigorously for 10 to 15 seconds and filtered. Of the filtrate (37 to 38 cc. are quickly obtained) 35 cc. are measured into a 100 cc. beaker and evaporated over a free flame or on a hot plate to about 10 cc. 10 per cent sodium hydroxide is now added until the solution is alkaline to phenolphthalein. Usually 10 to 20 drops are required. Boiling of the alkaline solution is continued for 2 minutes. It is then acidified by adding an excess of glacial acetic acid and the concentration continued with care to a final volume of about 1 cc. The residue is washed into the Van Slyke apparatus for determination of the amino acid nitrogen.

**DISCUSSION.**

**Preparation of the Sample.**

The use of liquid air presents several obvious advantages. In the first place the temperature of the sample may be lowered rapidly to a point where postmortem changes proceed immeasurably slowly. That this is an important consideration may be inferred from recent work on the inorganic phosphorus of muscle (8) and from the many investigations of lactic acid metabolism. Of equal importance is the destruction of the cells by freezing and thawing and the obtaining of a brittle mass which may be reduced readily to a very fine powder.

In most work on tissue analysis unfrozen material has been employed—being minced, finely ground with sand or powdered glass, or desiccated at 70–80° (9).

**Treatment with 0.01 N Acetic Acid.**

The purpose of this part of the procedure is to extract the amino acids, to remove most of the proteins by heat coagulation, and to destroy as rapidly as possible the tissue enzymes.
We have found by trial that the concentration of the acetic acid may be varied rather widely. Theoretically it might appear desirable for most complete heat coagulation to employ a buffer solution of pH 4 to 5. Probably with this end in view dilute mixtures of acetic acid and sodium acetate have been used by Autenrieth and Funk (10) and Mukai (11), while Hohlweg (12) and Richter-Quittner (13) employed mixtures of acetic acid and acid potassium phosphate for the same purpose. Since it was found by trial that 0.01 N acetic acid gave excellent coagulation under the conditions adopted, the use of a buffer was not attempted.

It did appear likely, however, that neutral salts would facilitate extraction of the amino acids (when in high concentration by exosmosis) and assist coagulation of the proteins. Neutral salts, usually sodium chloride or sodium sulfate, as aids in heat coagulation and extraction were used by Liborius (4), Freund and Laubender (14), Fischer (15), Richter-Quittner (13), and many others. We investigated the effect of sodium chloride and calcium chloride additions but with results which showed their use to be unnecessary if not even inadvisable in this method. The amino acid values obtained on the samples treated with neutral salts were no greater than the control. Trouble was also experienced in the cases of high salt concentration through crystallization of the salt during evaporation of the filtrates.

The time of heating was fixed rather arbitrarily at 7 minutes. By gross observation of the tubes, coagulation appeared to be complete within 2 or 3 minutes. Long heating is undesirable because of the expenditure of time and the danger, small though it may be, of protein hydrolysis.

Treatment with Trichloroacetic Acid.

A portion of the tissue protein usually fails to be coagulated and must be removed by other means. For this purpose a great variety of precipitants has been applied to protein-containing fluids, frequently without any preliminary coagulation of the albumins and globulins. Uranium acetate was introduced by Kowalewsky (16) but has not been very extensively used. Sublimate (17) in acid solution enjoyed great popularity for 15 or 20 years. Other metallic salts, such as copper sulfate (18), lead acetate (19), and mercuric chloride (20), have been used considerably. Of the various alkaloidal reagents picric acid, meta-
phosphoric acid, phosphomolybdic acid, tannic acid—the latter being of particular service as a peptone precipitant—and phosphotungstic acid for more extensive precipitation have been widely used. Tungstic acid (21) has gained universal usage within recent years.

Finally there are three volatile precipitants which might appear to be most satisfactory, since in view of concentrating the filtrate to one-twentieth or less of its original volume it is desirable that an excess of the precipitant be capable of ready removal. These are ethyl alcohol, methyl alcohol, and trichloroacetic acid. The first has been used more extensively than any other protein precipitant. It has been of special value as a solvent for urea in the analysis of tissues for this substance. The use of methyl alcohol was instituted by Folin and Denis (1). Owing, however, to the precipitation of amino acids by these alcohols (1, 3–6), their use in the determination of amino acids in tissues does not appear advisable.

Trichloroacetic acid, on the other hand, is a specific protein precipitant. Because of its sensitivity it was first recommended for the detection of protein in urine (22) and rapidly gained favor as a reagent for blood and tissue analysis. It is a quantitative precipitant of the albumins and globulins and apparently does not precipitate proteoses, peptones, or polypeptides (23).

Relatively low concentrations of trichloroacetic acid (2 to 4 per cent) are effective. We investigated the effect of higher concentrations but with the development of serious difficulties in the later stages of the procedure, which rendered their use undesirable. The 35 cc. portions of filtrate containing 10 to 15 per cent of trichloroacetic acid spattered badly during concentration. Much of the excess trichloroacetic acid failed to be destroyed, during evaporation, and gave a final concentrate of rather high specific gravity. On being transferred to the Van Slyke pipette it settled in the narrow portion above the drainage tap where it failed to mix with the nitrous acid. The results obtained were low, not because of an increased precipitation by higher concentrations of trichloroacetic acid but because of failure of the concentrate to react in the Van Slyke apparatus. This explanation probably applies to a difficulty of a similar nature encountered by Bock (6).

The time of precipitation (30 minutes) is that used by Greenwald (5).
Treatment with Infusorial Earth.

Infusorial earth is used to facilitate filtration and to remove some nitrogenous substances which react with nitrous acid but are not simple amino acids. They are probably proteoses and peptones principally. In the absence of infusorial earth higher amino nitrogen values are obtained. That this is not due to the adsorption of amino acids by infusorial earth is to be inferred from the quantitative recovery of added amino acids and the observations of Michaelis, Pincussohn, and Rona (24), with mastic and kaolin. It is known, nevertheless, that these and other acid silicates are excellent adsorbents of proteins, proteoses, and peptones (25). Alumina cream and colloidal iron are other adsorbents commonly employed to the same end.

Treatment of the Filtrate.

The muscle filtrates have always been observed to be clear and colorless. The liver filtrates are always slightly yellow. If glycogen is present, they are faintly opalescent.

The original purpose of boiling for 2 minutes or so in alkaline solution was to remove ammonia arising from the hydrolysis of urea. Bock (6) added urease in the form of soy bean meal to the blood samples to bring about this hydrolysis. It is doubtful whether the precaution is desirable. Soy bean meal and most of the partly purified urease preparations contain appreciable quantities of amino nitrogen. We are inclined to agree with Blau (7) that the use of soy bean meal in this connection is not advisable. Although we have omitted, therefore, the destruction of urea, we have found it necessary to retain Bock's practice of boiling for a brief period in an alkaline solution. Ammonia, 13 to 17 mg. per 100 gm. of tissue, is given off. It will also be observed that the amino nitrogen values are unduly high if this precaution be omitted.

EXPERIMENTAL.

In much of this work the thigh muscles of rabbits were employed. Elsewhere livers and thigh muscles of albino rats were used. After 24 hours fasting they were killed by stunning and bled by decapitation (rabbits) or a deep incision through the thorax (rats).
Experiment I. Extraction with Neutral Salts.—To 3 gm. portions of the frozen powdered muscle of a young rabbit were added 5 cc. of a solution of sodium chloride or calcium chloride of the strength indicated. After 5 minutes at room temperature, the suspension was transferred to the dilute acetic acid and boiled for 7 minutes. The estimations were continued according to the method described. The results are presented in Table I.

Experiment II. Prompt Excision of Tissue Is Advisable.—Samples weighing 4 to 5 gm. were excised from the thigh muscles of an adult rabbit at intervals after killing of 2, 10, 18, and 33 minutes, respectively. The corresponding amino nitrogen values expressed in mg. per 100 gm. of tissue were 42.3, 45.1, 48.5, 47.8.

Experiment III. Treatment with Trichloroacetic Acid.—(a) Liver and muscle of an adult rabbit were used, and the routine procedure was altered by adding to the heat-coagulated 3 gm. portions, trichloroacetic acid in final concentrations of 3, 6, 9, and 12 per cent, respectively, and omitting the treatment with infusorial earth. The corresponding amino nitrogen values in mg. per 100 gm. of tissue were: muscle, 45.5, 45.8, 35.8, 19.0; liver, 55.6, 48.9, 43.7, 33.4.

(b) From the livers and thigh muscles of four male rats pooled samples of frozen liver and muscle, 15.16 and 15.15 gm. respectively, were weighed out. 150 to 175 cc. of the dilute acetic acid were employed in heat coagulation, trichloroacetic acid was added in a final concentration of 3 per cent, and each was diluted to 250 cc. After 30 minutes standing, infusorial earth being omitted, the samples were filtered. To 40 cc. portions of the respective filtrates trichloroacetic acid was now added in increasing quantities. Each portion was then diluted to 52 cc. and after a further

<table>
<thead>
<tr>
<th>Salt solution</th>
<th>Amino N, mg. per 100 gm. muscle</th>
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<tbody>
<tr>
<td>Routine procedure</td>
<td>23.5</td>
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<tr>
<td>1 per cent sodium chloride</td>
<td>23.3</td>
</tr>
<tr>
<td>10 &quot; &quot; &quot; &quot;</td>
<td>23.4</td>
</tr>
<tr>
<td>Saturated &quot; &quot; &quot; &quot;</td>
<td>20.4</td>
</tr>
<tr>
<td>1 per cent calcium &quot; &quot; &quot; &quot;</td>
<td>22.5</td>
</tr>
<tr>
<td>Saturated &quot; &quot; &quot; &quot;</td>
<td>23.4</td>
</tr>
</tbody>
</table>
30 minutes the newly formed precipitate which was very slight in quantity was removed by centrifuging. In this way the effect of trichloroacetic acid in concentrations of 3, 6, 9, 12, and 15 per cent was determined. The corresponding amino nitrogen values in mg. per 100 gm. of tissue were: muscle, 61.1, 58.2, 50.3, 41.4, 23.9; liver, 42.3, 34.4, 28.9, 22.7, 20.0.

(c) 15 gm. samples of rabbit liver and muscle were treated as in Experiment III, b except that infusorial earth was used after the first addition of trichloroacetic acid. When further quantities of trichloroacetic acid (up to 15 per cent) were added to portions of the resultant filtrates, no precipitates formed. The fluids remained perfectly clear. The material which is capable of precipitation by higher concentrations of trichloroacetic acid may also be adsorbed by infusorial earth.

(d) 15 gm. samples of rat liver and muscle were treated as in Experiment III, b. No precipitate formed when increasing quantities of trichloroacetic acid were added to portions of the respective filtrates. Nevertheless those portions now containing trichloroacetic acid in concentrations of 3, 6, 9, 12, and 15 per cent respectively were evaporated in the usual manner. The corresponding amino nitrogen values expressed in mg. per 100 gm. of tissue were: muscle, 50.0, 52.5, 49.3, 32.3, 36.4; liver, 44.7, 44.2, 34.5, 32.2, 20.9.

(e) A not unlikely explanation of the amino nitrogen decrease observed in Experiment III, d would be through anhydride formation, for as the concentrate became progressively more dense in the higher concentrations of trichloroacetic acid, the boiling point would be higher, and anhydride formation would proceed more rapidly. This possibility was now examined.

Three solutions of the following compositions were prepared: (A) 1 cc. of an amino acid solution (1.22 mg. of amino nitrogen) obtained by the total hydrolysis of egg albumin; 10 cc. of 50 per cent trichloroacetic acid; 22 cc. of water. (B) 1 cc. of the amino acid solution; 2 cc. of 50 per cent trichloroacetic acid; 30 cc. of water. (C) 5 cc. of the amino acid solution; 10 cc. of 50 per cent

1 The Van Slyke pipette was shaken occasionally while the concentrated sample was run in from the side burette. This produced a partial mixing of the materials and decreased the degree of settling of the concentrate in the bottom of the pipette.
trichloroacetic acid; 18 cc. of water. These solutions were concentrated in the usual way, Solutions A and B being reduced to about 2 cc. and transferred in toto to the Van Slyke apparatus, Solution C being similarly reduced but finally made up to exactly 10 cc. with water. Of the latter, 2 cc. samples were used for analysis in the Van Slyke apparatus. The analytical results were as follows: (A) 15 per cent trichloroacetic acid, 0.57 mg. of amino nitrogen, 47 per cent recovery; (B) 3 per cent trichloroacetic acid, 1.22 mg. of amino nitrogen, 100 per cent recovery; (C) 15 per cent trichloroacetic acid, 1.17 mg. of amino nitrogen, 96 per cent recovery. Solution A settled in the bottom of the Van Slyke pipette; Solutions B and C did not. Experiments III, a and III, e demonstrate quite clearly that trichloroacetic acid may not be used in concentrations greater than 6 per cent (Experiment III, d) in this analytical method.

Experiment IV. Treatment with Infusorial Earth.—15 gm. samples of frozen rat liver and muscle were suspended in 150 to 175 cc. of the boiling dilute acetic acid. After heat coagulation the suspensions were treated with trichloroacetic acid in a final concentration of 3 per cent and diluted with water to 250 cc. To 50 cc. portions of the respective filtrates, the following quantities of infusorial earth were added: 0.0, 0.5, 1.5, 3.0 gm., respectively. After 10 to 15 seconds of vigorous shaking, the mixtures were filtered. The amino nitrogen values corresponding respectively to the above quantities of infusorial earth were as follows: muscle, 68.1, sample lost, 51.8, 48.4; liver, 45.9, 38.2, 33.8, 33.9.

We concluded from these results that of the material removed by the infusorial earth, some contains amino nitrogen. 2 gm. of the adsorbent appear to be sufficient.

Experiment V. Concentration of the Filtrate.—(a) Of the filtrate obtained from rabbit muscle, equal portions were taken and concentrated to about 10 cc. They were then made alkaline in the usual manner and boiled for varying times; viz., 0.0, 0.1, 0.5, 1.0, 2.0, 4.0 minutes. At the ends of the specified intervals the fluids were acidified with acetic acid and the evaporation continued. The corresponding amino nitrogen values were 69.1, 60.0, 56.2, 54.9, 57.0, 57.1.

This indicates the presence in the filtrates of a readily volatile base (probably ammonia). The quantity, judging from the amino nitrogen values, is considerable.
(b) 2, 4, 6, and 8 gm. samples of rabbit muscle were each treated with 30 to 35 cc. of hot dilute acetic acid. The suspensions were boiled for 7 minutes, cooled, and treated with trichloroacetic acid. The volumes were made up to 50 cc. and filtered following the 30 minute interval for precipitation and the addition of infusorial earth. The filtrates were concentrated directly to 2 cc., except that of the 6 gm. sample which received the 2 minute boiling in alkaline solution. The amino nitrogen values expressed in mg. per 100 gm. of tissue were: 2 gm. sample, 47.1; 4 gm. sample, 47.0; 6 gm. sample, 25.2; 8 gm. sample, 44.7. This confirms the observation reported in Experiment V, a above.

(c) 40 cc. portions of the filtrates obtained by the routine procedure from 15 gm. samples of rat liver and muscle were concentrated to about 10 cc. Two liver and two muscle samples were made alkaline for 2 minutes, boiled, reacidified, and concentrated to about 2 cc. Two liver and two muscle samples were concentrated directly to 2 cc. without heating in alkaline solution.

Liver filtrate.

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<th>Amino N per 100 gm. tissue.</th>
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<tr>
<td></td>
<td>mg.</td>
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<tr>
<td>Boiled in alkaline solution</td>
<td>37.8</td>
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<tr>
<td>&quot; &quot; &quot; &quot; &quot; &quot; &quot; &quot;</td>
<td>37.4</td>
</tr>
<tr>
<td>Not boiled in alkaline solution</td>
<td>56.7</td>
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<tr>
<td>&quot; &quot; &quot; &quot; &quot; &quot; &quot; &quot;</td>
<td>56.5</td>
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Muscle filtrate.

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<th>Amino N per 100 gm. tissue.</th>
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<tr>
<td></td>
<td>mg.</td>
</tr>
<tr>
<td>Boiled in alkaline solution</td>
<td>48.1</td>
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<tr>
<td>&quot; &quot; &quot; &quot; &quot; &quot; &quot;</td>
<td>48.8</td>
</tr>
<tr>
<td>Not boiled in alkaline solution</td>
<td>59.3</td>
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<td>&quot; &quot; &quot; &quot; &quot; &quot; &quot;</td>
<td>59.3</td>
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(d) In the case of sixteen rats the ammonia given off during the 2 minutes boiling in alkaline solution was collected and estimated by Nesslerization.

<table>
<thead>
<tr>
<th>No. of samples.</th>
<th>Ammonia N, mg. per cent.</th>
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<tr>
<td></td>
<td>Average</td>
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<tr>
<td>16 muscle.</td>
<td>12.8</td>
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<td>15 liver.</td>
<td>17.2</td>
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(e) Owing to the fact that the ammonia values were of the same magnitude as accepted values for the urea nitrogen content of
tissues, we wondered whether the ammonia arose from total hydrolysis of tissue urea.

To 3 gm. samples of frozen rabbit muscle increasing quantities of urea were added. The routine procedure already described was then followed. The ammonia given off during concentration of the filtrates was collected and estimated.

<table>
<thead>
<tr>
<th>Amount of Urea Added</th>
<th>Ammonia, mg. per 13 gm. tissue</th>
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<tbody>
<tr>
<td>No urea added</td>
<td>0.435</td>
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<tr>
<td>1 cc. urea solution added</td>
<td>0.448, 0.660</td>
</tr>
<tr>
<td>2 &quot; &quot; &quot; &quot;</td>
<td>0.492, 0.885</td>
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</table>

* Assuming all of the added urea to be hydrolyzed.

It appears therefore that the ammonia is not formed by the destruction of urea in the course of the analysis.

(f) The ammonia given off may not represent preformed tissue ammonia. (1) Ammonia reacts slowly with nitrous acid. The 2 minute boiling in alkaline solution removes 13 to 17 mg. per cent of ammonia N (Experiment V, d) but also causes a decrease in the amino nitrogen of 11 to 19 mg. per cent (Experiment V, e). (2) Blood, which contains a negligible quantity of ammonia, suffers the same decrease in the amino nitrogen value as a result of 2 minutes boiling of the filtrate in alkaline solution.

Dog blood was treated according to the routine procedure described for muscle and liver. Freezing with liquid air was omitted. Of two portions of the filtrate, one was concentrated to 2 cc. directly; the other was submitted to the routine treatment with sodium hydroxide. The latter gave an amino nitrogen value of 8.0 mg. per cent (the accepted order of magnitude for blood amino nitrogen); the former gave a value of 16.4.

Experiment VI. Recovery of Added Amino Acids.—(a) To four 2 gm. portions of frozen rabbit muscle were added 0.0, 1.0, 2.0, and 3.0 cc. respectively, of an aqueous solution of dL-alanine (0.254 gm. per 100 cc.). The samples were then transferred to boiling tubes with the hot dilute acetic acid. The rest of the procedure was according to the method described. The following amino nitrogen values expressed in mg. per 2 gm. of muscle were obtained: 0.819,
1.125, 1.532, 1.848. The corresponding calculated values were: 0.819, 1.219, 1.619, 2.019. Per cent recovery, 93, 94, 92.

(b) The above experiment was repeated with a solution of monoamino monocarboxylic acids obtained by butyl alcohol extraction of totally hydrolyzed casein (26) (0.405 gm. of monoamino acids (0.038 gm. of amino N) per 100 cc.). Amino nitrogen values obtained: 0.673, 1.022, 1.358, 1.743; calculated, 0.673, 1.059, 1.442, 1.816; per cent recovery, 96, 94, 96.

Experiment VII. Amino Nitrogen Content of Rat Liver and Muscle.—(a) Five female rats (150 to 180 gm. in weight) were fasted for 24 hours. They were then killed by stunning and bleeding through a thoracic incision. The liver and the thigh muscles of one side were rapidly excised and analyzed according to the routine procedure. The following amino nitrogen values expressed in mg. per 100 gm. of tissue were obtained: liver, sample lost, 40.3, 45.2, 36.6, 43.3; muscle, 51.1, 50.5, 57.6, 50.3, 49.3.

(b) Five female rats (150 to 170 gm. in weight) were similarly treated. Infusorial earth was omitted. The higher values which follow were obtained: liver, 45.6, sample lost, 47.2, 51.2, 51.9; muscle, 59.3, 59.7, 57.5, 61.1, 59.3.

Part of this work was done in the Biochemical Laboratory of the University of Toronto.

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13. Richter-Quittner, M., Biochem. Z., 1919, xcvi, 179.
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