XLIII. THE AMINO-ACIDS OF TISSUES.
VI. DETERMINATION OF THE BASIC AMINO-ACIDS
IN SMALL QUANTITIES OF PROTEIN.

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IN order to analyse the protein of material which can be obtained only in very small quantities, it became necessary to develop suitable micro-chemical technique. Micro-chemical methods cannot always be a small scale copy of ordinary procedure, as errors in a method which are relatively insignificant when large amounts of material are employed, may be of sufficient magnitude to vitiate micro-chemical work altogether. This is particularly true of the Van Slyke method of estimating the basic amino-acids. The ordinary technique of this method has undergone various minor modifications since its description by Van Slyke [1911], but is recognised as not yet being absolutely perfect. Such errors as have not yet been overcome, or which perhaps are not yet realised, might well become accentuated in a micro-adaptation, so that before it is safe to apply the general outline of this method to a small scale, some of these errors demand careful consideration from the point of view of the general principles of micro-analysis, some of which have been clearly set forth by Pregl [1930].

Narayana and Sreenivasaya [1928] proposed a micro-modification of this method, in which they appear to have recognised that one difficulty would be the estimation of arginine. Plimmer [1916] showed that when arginine is estimated by boiling with caustic alkali, histidine may be decomposed to the extent of some 3 %. Such decomposition, although insignificant when examining most proteins in the usual way would give rise to too great an error for micro-chemical work. The difficulty with the suggestion of Narayana and Sreenivasaya, who use arginase extracts, lies in the fact that these extracts may be very far from pure, and that a number of blanks must be carried out to correct for ammonia liberated by these extracts. Work proceeding in this laboratory makes it clear that complete separation of histidine and arginine is not secured by means of flavianic acid to a sufficient degree of accuracy, when using very small quantities of protein material. We decided, therefore, that a direct determination of histidine would be more dependable. Plimmer and

Phillips [1924] have described a method for the direct determination of histidine by bromination during the ordinary Van Slyke process. They assumed that tryptophan would be destroyed sufficiently to prevent its causing appreciable error by absorbing bromine. Our own results, reported below show that whilst this is the case in the ordinary way, it is not so when small quantities are analysed. We have found that prolonged hydrolysis with acid is required in order that complete destruction of tryptophan may be achieved. When this destruction is incomplete, the diamino-fraction absorbs a greater quantity of bromine than would be required by the amount of histidine present.

The matter of prolonged hydrolysis was of special interest to us in view of the previous observations of one of us [Rosedale, 1929; Rosedale and Morris, 1930] that different tissues required different lengths of time for hydrolysis if concordant duplicates were to be obtained. Our present experiments have led us to the conclusion that bromination of histidine cannot be employed in the micro-procedure. Da Silva [1931] has shown, however, that histidine may be estimated in small quantities of protein by precipitation with mercuric sulphate after alkaline hydrolysis. We have therefore adopted the procedure of hydrolysing separate samples with acid and with alkali and estimating histidine by the latter process. An additional step in routine is usually unwelcome if a greater expenditure of material and time is involved, but we have found that the whole procedure is greatly simplified by the adoption of micro-methods and that it is possible to arrange the work so that no more time is expended than that required for the usual Van Slyke procedure.

EXPERIMENTAL.

Estimation of histidine by bromination. Fifty grams of gelatin were hydrolysed for 40 hours with 25 % HCl. After evaporating to dryness several times *in vacuo*, the hydrolysate was made up to 2 litres. Portions of 100 cc. were used for the Van Slyke determinations which were carried out according to the modification of Plimmer and Rosedale [1925]. The total N of 100 cc. was found to be 0.3416 g. Histidine in the diamino-fraction was also determined by bromination, following exactly the directions of Plimmer and Phillips [1924]. Our blank experiments required 12.2 cc. sodium thiosulphate, of which 1 cc. corresponded to 0.0128 g. of bromine; 10 cc. of the diamino-fraction required 12.0 cc. thiosulphate which shows an absorption of 0.00256 g. bromine and this corresponds to 0.006731 g. of histidine-N. Table I gives the average of 18 experiments.

Table I.

	Arginine-N	Histidine-N	Lysine-N
Ordinary Van Slyke method	0.03453 g.	0.00601 g.	0.2526 g.
By bromination	0.03389 g.	0.006731 g.	0.2518 g.
Calculated as % of total N.			
Ordinary method	10.1 %	1.7 %	7.3 %
Bromination method ...	9.9 %	1.9 %	7.3 %

There is thus complete agreement between the two methods.

Two and a half grams gelatin + 50 mg. tryptophan were hydrolysed as above. After bromination 11.7 cc. thiosulphate were required, corresponding to an absorption of 0.0064 g. bromine instead of 0.00265 g. which would be expected from the previous experiments on gelatin alone. The extra amount of bromine, while only corresponding to about 1 mg. tryptophan, would cause error if calculated as histidine, as 1 mol. tryptophan absorbs 6 or 7 atoms of bromine and 1 mol. histidine absorbs 2 atoms. [Plimmer and Phillips, 1924; da Silva, 1931.]

Similar experiments were carried out with tryptophan added to gelatin, varying only the time of hydrolysis. It was found necessary to hydrolyse for 160 hours before we could be certain that the tryptophan would cease to exert any influence upon the bromination.

We considered therefore that the behaviour of tryptophan under the conditions of our experiments required further investigation.

Gortner and Holm [1917; Holm and Gortner, 1920] have discussed the formation of humin from tryptophan in the presence of aldehyde or carbohydrate. They divided the humin into three classes, insoluble in acid, soluble in acid, and phosphotungstic humin. They also drew the conclusion from their experiments that the indole portion of the tryptophan is responsible for humin formation, and carried out experiments showing that in the presence of aldehyde, pyrrole may contribute to the formation of humin. While Gortner and Holm do not provide a great deal of information about the phosphotungstic humin, the use of a sintered glass filter for separating the diamino-phosphotungstates has brought to our notice the frequency with which an insoluble brown material is left on the filter. As Gortner and Holm point out, some of this material is dissolved by the NaOH during the decomposition of the phosphotungstates. As it seems that the decomposition of tryptophan by reduction may take place in protein hydrolysis, it was essential for us to find out whether such decomposition products as indole and pyrrole could affect the bromination, and thus interfere with the estimation of histidine. 1 % solutions of indole in chloroform and of pyrrole in carbon tetrachloride when brominated according to our usual procedure absorbed 6 atoms of bromine per mol. of indole and pyrrole respectively. The only pyrrolidine compound available was proline and this absorbed no bromine. Since indole and pyrrole may be formed during acid hydrolysis, and since they brominate similarly to tryptophan, it is clear that estimation of histidine in this way would not be possible in a micro-method. Experiments were then undertaken by one of us with the view of determining tryptophan directly, so that its value might be deducted from the histidine. These experiments have already been reported [da Silva, 1931], and although they showed that an accurate estimation of tryptophan could not be assured, a direct estimation of histidine appeared to be possible.

The Van Slyke micro-procedure. As already stated, we determine histidine after hydrolysing with alkali. Acid hydrolysis is carried out upon a separate

sample of material, and from the two sets of data, information which is ordinarily given by Van Slyke's method is secured.

Hydrolysis with alkali. An amount of substance corresponding to 0.3 g. protein is placed in a small Jena glass flask and hydrolysed with 20 % NaOH for 18–20 hours. In accordance with the directions of Folin and Ciocalteu [1927] the alkali is added in the proportion of 4 g. NaOH per g. protein. A few drops of butyl alcohol are added and the boiling is carried out in an air-bath. At the end of the period the condenser is removed and the butyl alcohol boiled off. The solution is then neutralised by adding, drop by drop, a solution of 14N H_2SO_4 , and allowing to cool. The hydrolysate is then made approximately normal with respect to H_2SO_4 . After filtration, the precipitate is well washed with N H_2SO_4 . If the washing is sufficiently thorough loss does not occur, and the final volume of the solution and washings amounts to 25–30 cc. After making exactly normal with H_2SO_4 , the solution is placed in 15 cc. centrifuge-tubes, about 8 cc. to each tube. To each tube 1 cc. of a 15 % HgSO_4 solution is added and after standing for 2–3 hours the tubes are centrifuged and the supernatant liquid is decanted. The precipitate is well washed with a 1.5 % solution of HgSO_4 , again centrifuged and the supernatant liquid discarded. The precipitate is stirred up with N HCl, 10 cc. being added to each tube, and after warming in the water-bath for 5 minutes the mercury precipitate is decomposed with H_2S . The tubes are again centrifuged and the liquid is filtered through a small filter, washing being carried out with 0.1 N H_2SO_4 . The filtrate and washings are concentrated and made up to 5 cc. in a graduated flask.

Two cc. of this solution are used for the determination of amino-N, using the micro-form of Van Slyke's apparatus, and 1 cc. is used for the determination of total N by Pregl's Kjeldahl method [1930]. In view of the likely presence of some tryptophan in this solution, oxidation must be prolonged, and mercuric sulphate must be added to the oxidising tube.

Histidine is then calculated from the data by the formula

$$\text{Histidine-N} = 3 (\text{Total N}) - 6 (\text{Amino-N}) \text{ [da Silva, 1931].}$$

Hydrolysis with acid. An amount of material equal to the quantity used for the alkaline hydrolysis is hydrolysed with 25 % HCl for 36 hours. After this time the hydrolysate is concentrated 5 or 6 times *in vacuo* in order to remove as much of the HCl as possible.

Determination of the amide-N and humin-N. The acid hydrolysate is made alkaline with the smallest quantity of finely ground CaO and distilled *in vacuo* in a 50 cc. Claisen flask to remove ammonia. In carrying out this procedure we have used a silver condensing-tube as used by Pregl for the micro-Kjeldahl distillation. Instead of using standard alkali for the titration, we have distilled the ammonia into 2 cc. of a saturated solution of boric acid, using bromocresol purple as indicator. The ammonia is then titrated directly with 0.01 N HCl. The residue from the distillation is filtered through a 4 cm. paper and well washed with water. The humin-N is determined by estimating the

total N in the precipitate and paper. In carrying out this, and all determinations of total N, the method of Pregl [1930] has been followed exactly, except that we use the boric acid solution instead of standard alkali as stated above. This does away with the necessity of using quartz receivers and boiling off the CO_2 which is necessary in the procedure as described by Pregl, and also does away with the frequent preparation of the standard alkali, a necessity imposed upon us by climate owing to the growth of moulds. In applying Pregl's method to this work it is necessary to take care that the oxidation is sufficiently thorough.

Precipitation with phosphotungstic acid. After many experiments we have found it desirable to add 1 cc. HCl to the filtrate from the humin and to concentrate to a volume just below 10 cc. 1.5 g. nitrogen-free phosphotungstic acid are then dissolved in the minimum of water and added to the solution which is made up exactly to 10 cc. and heated as described in the ordinary procedure in order to dissolve the phosphotungstates so as to obtain a precipitate which is easy to filter. This heating only takes a few minutes, after which the precipitate is allowed to stand for 48 hours.

Treatment of the phosphotungstates. The precipitate is collected on a Jena sintered-glass funnel (G 4 size) of 1 cm. diameter and washed 5 times with 1 cc. 10 % HCl. The filtrate is finally filtered again through a 4 cm. folded paper into a 25 cc. measuring flask and made to volume. The precipitate of the diamino-phosphotungstates upon the filter is dissolved in the smallest possible quantity of 2 N NaOH, the filter being washed with water and sucked dry. The solution is then filtered through the same folded paper as used in filtering the mono-amino-portion, into a 10 cc. graduated flask, and made to mark. Estimations of total N and amino-N are made upon both the diamino- and the monoamino-portions. Total N is estimated in 1 cc. portions and amino-N in 2 cc. portions.

From the data secured in this way, knowing the amount of histidine-N from the alkaline hydrolysis, the arginine-N and lysine-N can be calculated.

Determination of cystine. When cystine is present it must be estimated. Plimmer and Lowndes [1927] find that about 40 % of the total cystine is precipitated by phosphotungstic acid. If it is proposed to weigh the sulphur as BaSO_4 , the phosphotungstic acid must be got rid of. This, together with the fact that we are dealing with solutions of the diamino-acids, and not with solid substances, has caused Pregl's method of sulphur estimation to be rather inconvenient and, in view of the fact that cystine is present in proteins like caseinogen to so small an extent as 0.2 %, renders the method impracticable in such work. We have accordingly attempted estimations of sulphur upon the acid hydrolysate direct, before precipitating with phosphotungstic acid, and have carried out our oxidation by boiling with a mixture of 3 parts HNO_3 and 1 part HClO_4 according to a method described by Chatron [1931]. Boiling is carried to dryness in a small flask, and the heating continued for a short time in order to complete the oxidation. After cooling we have dissolved the residue in a few cc. of water and transferred it to a 7 cm. glass dish, precipitated with BaCl_2 and proceeded with the technique as described by Pregl. ..

One cc. of a solution containing 1 mg. cystine per cc. was added to 2 cc. of a gelatin hydrolysate from our first experiments described above. This corresponds to 0.2 % cystine in the gelatin. After carrying out the above procedure we obtained on the first occasion, 1.874 mg. BaSO_4 , on the second, 1.863 mg. so that our errors are — 1.0 % and — 1.5 %, which would doubtless be increased had we attempted to estimate some 40 % of such a small quantity in a diamino-fraction, after removal of phosphotungstic acid. We have contented ourselves, therefore, with attempting micro-sulphur estimations upon the proteins used in our experiments below, but have failed to secure amounts of BaSO_4 that could be weighed upon a micro-balance. We have therefore neglected cystine in these experiments. We consider that it would be important to improve the method for cystine estimation before our general micro-method could be applied to keratins, although we believe that any error involved by its neglect in the estimations of ordinary tissue proteins is insignificant. We hope to return to the study of this point on a future occasion.

Experiments with proteins. Our first experiments using the micro-method were carried out with gelatin and caseinogen as their composition is well known, and could therefore be used as a check upon the method.

Acid hydrolysis: 13.17 g. gelatin were hydrolysed for 36 hours with 25 % HCl. After concentration *in vacuo* several times, the residue was dissolved in water and made up to 100 cc. Total N was 21.0833 mg./cc. The micro-procedure was carried out as already described on 1 cc. portions, and the results are given in Table II.

Alkaline hydrolysis: 135 mg. gelatin were hydrolysed with NaOH and the

Table II. *Percentage of total N.*

	Amide-N	Humin-N	Diamino-fraction					Monoamino-fraction		Total
			Total N	Amino-N	Arginine-N	Histidine-N	Lysine-N	Total N	Amino-N	
Gelatin	1.48	0.16	17.46	11.44	7.41	0.69	9.35	73.29	60.59	92.31
	1.48	0.15	17.68	12.33	6.52	0.69	10.46	74.04	66.07	93.37
	1.50	0.18	22.18	12.33	12.50	0.69	8.97	73.03	63.07	96.89
	Mean...	1.48	0.16	19.1	12.03	8.81	0.69	9.59	73.45	63.22
Caseinogen	11.16	1.16	27.0	18.54	3.45	8.85	10.08	63.24	53.49	102.1
	10.04	1.00	23.1	12.79	5.99	8.85	8.35	63.79	53.44	98.54
Mean...	10.60	1.08	25.0	15.66	4.72	8.85	9.21	63.51	53.45	100.32
Oysters:										
2nd pepsin digest	3.11	11.44	23.36	11.52	10.33	3.79	5.47	57.82	57.80	95.75
	3.12	11.15	23.49	11.62	12.44	3.79	7.24	57.83	57.75	95.68
Mean...	3.11	11.29	23.42	11.57	11.38	3.79	6.35	57.82	57.77	95.71
3rd pepsin digest	5.70	14.49	22.59	11.38	14.01	2.06	4.72	57.32	27.47	100.1
	5.66	14.63	23.64	11.89	13.82	2.06	7.74	57.34	27.68	101.2
Mean...	5.68	14.56	23.61	11.63	13.91	2.06	6.23	57.33	27.57	100.6
Trypsin digest	6.26	3.66	18.50	7.32	6.96	8.0	3.53	71.55	34.18	99.97
	5.79	3.66	25.07	13.37	7.87	8.0	8.58	74.62	64.01	109.14
Mean...	6.02	3.66	21.78	10.34	7.41	8.0	6.05	73.08	49.09	104.05
<i>Torula utilis</i>	5.06	10.79	22.79	7.09	16.36	4.93	1.36	58.99	53.09	97.66
	5.05	10.71	22.79	7.14	16.49	4.93	1.37	58.99	53.13	97.56
Mean...	5.05	10.75	22.79	7.11	16.42	4.93	1.36	58.99	53.11	97.61

procedure described above followed. The results are given for histidine in Table II.

Caseinogen was treated similarly. 16.187 g. caseinogen were used for the acid hydrolysis. After making up to volume the total N was 25.900 mg./cc. The results of these experiments have been collected in Table II.

For the alkaline hydrolysis, 171 mg. were used. Total N of the histidine portion = 2.703 mg. Amino-N = 0.9478 mg. From the formula [da Silva, 1931] histidine-N = 2.423 mg., consequently the amount of histidine-N calculated for the acid hydrolysis is 2.293 mg.

It will be seen from Table II that the same degree of concordance between duplicates as is usually obtained with Van Slyke's method may be secured with our procedure.

We have used this method for a further examination of oysters. Rosedale and Morris [1930] recorded data for oysters, their data being obtained from the filtrate of a peptic digest. It was of interest to see how complete the digestion had been, and also to note the difference, if any, between the types of amino-acids that took longer to be released by pepsin. Rosedale and Morris had found in the first peptic digest a large amount of histidine. We therefore submitted the residue from their peptic digest to two further digestions with pepsin, each lasting 17 days, filtering off the solid material and using the filtrate for our experiments. The residue was finally digested with trypsin. These three digests have been submitted to our micro-procedure and our results are given in Table II. As Rosedale and Morris had already made one peptic digest with this material, we have called our first digest "pepsin 2."

Opportunity has been taken of examining the protein of *Torula utilis*. The organism was grown upon the following medium which is free from organic nitrogen.

Glucose	50.0 g.	MgSO ₄ , 7H ₂ O	0.50 g.
NaNO ₃	2.0 g.	FeSO ₄ , 7H ₂ O	0.01 g.
KH ₂ PO ₄	1.0 g.	Agar-agar	20-30 g.
KCl	0.5 g.	Water	1000 cc.

After incubation the cells were washed with saline, then water, and after centrifuging were dried over CaCl₂. An analysis gave the following results: nitrogen 7.05 %, ash 1.09 %, fat 0.01 %, moisture 80.03 %. The dried material was extracted with 1 % acetic acid and digested with pepsin in the usual way. The filtrate from the peptic digest was used for our experiments. Quantities of 300 mg. of the dried material were weighed out and digested for each experiment. The micro-procedure as detailed above was carried out and the results are given in Table II.

SUMMARY.

1. A micro-method for the estimation of the basic amino-acids is described.
2. Data secured by its use upon gelatin, caseinogen, oysters and *Torula utilis* are presented.
3. Some further evidence of the probable interference of tryptophan with the Van Slyke method has been brought forward.

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