CLI. THE LYSINE CONTENT OF FEEDING STUFFS

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OSBORNE & MENDEL [1914] demonstrated conclusively that lysine was an essential amino-acid for normal growth, and drew attention to the importance of studying the qualitative as well as the quantitative aspects of proteins in nutrition. A year later Osborne *et al.* [1915] showed that the nutritive values of a number of proteins were closely related to their lysine contents. More recently attention has again been drawn to the importance of lysine by Morris & Wright [1933]. They state: "...when minimal quantities of protein are fed in the production rations of milking cows, a deficiency of lysine or tryptophan will lead to a marked reduction in milk yield."

The necessity of having some knowledge of the nature of proteins for nutritional studies has led to the publication of a large number of analyses of proteins isolated from feeding stuffs and protein concentrates. Thus Morris [1934] reports the results of analyses of a number of feeding stuffs used in Great Britain, including with his data values obtained by other workers. Almost all of the analyses, however, were made by the Van Slyke [1911] nitrogen distribution method, which has been widely criticized, particularly as a method for determining lysine [cf. Mitchell & Hamilton, 1929; and also Plimmer & Lowndes, 1938]. Recently Block [1934, 1] has shown that dilute alkali and dry heat produce some change in protein structure which is indicated by an abnormal and high value for lysine when determined by this method, so that most of the abovementioned results may be further criticized since the proteins were extracted from the feeding stuffs with dilute alkali. This procedure, furthermore, introduces large amounts of sodium chloride into the hydrolysate, and Thimann [1926] has shown that precipitation of the bases under these conditions is not reliable.

The isolation method of Kossel & Kutscher [1900], as modified by Vickery & Leavenworth [1928], offers definite advantages but requires large amounts of material. Block [1934, 2] adapted the method to small quantities of protein and this has been used, together with the Van Slyke and the original Vickery & Leavenworth methods, by Miller [1935] for the analysis of grass proteins. An examination of his data shows that the values obtained by the Van Slyke method must be considered extravagant. There is thus reason to doubt the accuracy of most of the existing values for the lysine content of feeding stuffs and for this reason, and because of the importance of lysine in the diet of animals, a reinvestigation of the subject has been made.

The isolation method of Block [1934, 2] was selected for preliminary studies. Experience showed, however, that even when all the precautions recommended by Miller [1935] are exercised, the method does not give reliable results with impure proteins. Its main faults are as follows.

(1) The precipitation of arginine and histidine as their silver salts, by introducing Ag^{+} as silver nitrate, leaves NO_3^{-} in the lysine filtrate, and at a

later stage, when the acidified solution is concentrated to 2 ml., there is danger of lysine being attacked by the nitric acid present. (2) The N recovered from the phosphotungstate precipitate is assumed to belong exclusively to lysine and the theoretical quantity of picric acid required to form lysine picrate is added. This is unsatisfactory, since as much as 40 % may be due to other substances, and the excess picric acid may form the soluble lysine dipicrate. The procedure of Calvery [1929] is preferable. (3) The lysine picrate is not recrystallized. Even with pure protein the product does not explode above 250° and Vickery & Leavenworth [1928] stress the fact that recrystallization is necessary. The loss on recrystallization is about 4 %.

It was found that if ammonia was removed immediately after hydrolysis and the silver salts of arginine and histidine removed together at *p*H 14 somewhat more satisfactory results could be obtained, some of which are given in Table I.

Table I. *Analyses of certain feeding stuffs by a modification of Block's procedure*

Feeding stuff	Wt. g.	N %	Total N g.	Lysine picrate obtained g.	Lysine N as % of total N
Earthnut cake	6·000	7·60	0·4560	0·2000	3·3
Wheat gluten	3·025	12·59	0·3815	0·0750	1·5
Blood meal	6·098	13·68	0·8342	0·8084	7·4

All weights and N values are expressed in % of the air-dry feeding stuff.

The modified procedure finally adopted in the present work, however, embodies certain interesting new features. The protein or feeding stuff is hydrolysed in the normal way with HCl, the hydrolysate diluted to such a volume that the HCl concentration is about 5 % and phosphotungstic acid added until no further precipitate is obtained. Humin and ammonia are thus precipitated together with the bases. After standing, the precipitate is filtered off, washed and decomposed in the presence of mineral acid by amyl alcohol-ether according to the usual Van Slyke procedure. Ammonium phosphotungstate, however, is not affected by this treatment and remains suspended together with the humin in the aqueous phase, which, of course, contains the free bases. After complete removal of the phosphotungstic acid in the usual way by the mixed organic solvents the aqueous phase is centrifuged and we thus obtain a clear, nearly colourless, solution of the bases without having had to perform any of the following operations incidental to the normal Block procedure—(1) removal of the mineral acid used for hydrolysis, (2) concentration of the hydrolysate at any stage and (3) removal of ammonia as gas from an alkaline solution. The resulting gain in time and saving of labour needs no emphasis.

Arginine and histidine are next removed together as silver salts at *p*H 14 by adding silver oxide instead of silver nitrate, the filtrate freed from reagents and the lysine precipitated as picrate by the procedure of Calvery [1929]. On recrystallization the first (and main) crop of picrate explodes at 266–267° and the second at 250°, thus satisfying the more rigorous conditions of Vickery & Leavenworth [1928].

Experimental

4–5 g. of protein are hydrolysed with five times the weight of 20 % HCl for 36–40 hr., and the hydrolysate quantitatively transferred to a 250 ml. pyrex centrifuge bottle, using as little hot water as possible for washing. The bases are precipitated by the addition of a saturated solution of phospho-24-tungstic acid

(A.R.) in water until an excess is present. Water is then added, with stirring, until the final concentration of HCl is 5 % by weight and the mixture kept at room temperature overnight.

The precipitate is centrifuged (to obtain a good separation it is sometimes necessary to stir before centrifuging), and washed twice by whipping up with 25 ml. quantities of a 2·5 % phospho-24-tungstic acid solution in 5 % (by weight) H_2SO_4 and centrifuging. The phosphotungstates are decomposed by disintegrating with 50 ml. of water (to which has been added 1 ml. of conc. H_2SO_4) and a liberal quantity of amyl alcohol-ether [Van Slyke, 1915] and the mixture centrifuged. The clear liquids (both aqueous and organic) are poured into a separating funnel. The residue, which consists of the acid-insoluble humin, the humin precipitated by the phosphotungstic acid and ammonium phosphotungstate, is washed twice, as before, using smaller quantities of acidified water and amyl alcohol-ether, the washings being added to the mixed liquids in the separating funnel. After a few minutes the aqueous solution, which should be water clear, is drawn off and shaken out three times successively with amyl alcohol-ether. The combined amyl alcohol-ether solutions are shaken once with a small quantity of water acidified with H_2SO_4, and the latter solution washed once with a fresh quantity of the amyl alcohol-ether. The aqueous solutions are then combined and given a final washing with a small quantity of the amyl alcohol-ether.

With the blood meal samples it was found more satisfactory to decompose the phosphotungstates with 50 ml. of acidified water and 75 ml. of the amyl alcohol-ether. The organic liquid forms the bottom layer on centrifuging and the clear aqueous layer may be syphoned off. The amyl alcohol-ether and insoluble precipitate are washed by stirring up three successive times with 25 ml. quantities of acidified water and centrifuging; the aqueous washings being siphoned off as before.

The final aqueous solution is run into a 500 ml. flask, reduced *in vacuo* to a small volume and quantitatively transferred to a 250 ml. pyrex centrifuge bottle. It is then heated in a boiling water bath and solid silver oxide added, in small quantities at a time, with rapid stirring, until an excess of silver is present (brown ppt. with cold saturated baryta). An excess of silver is readily obtained by this method, which is much quicker than shaking the cold aqueous solution with a suspension of silver oxide in water [Vickery & Leavenworth, 1928], and the final volume, for 5 g. of protein, is less than 200 ml. The solution is cooled and made distinctly alkaline to Nile blue, or alizarin yellow S, by the addition of warm saturated aqueous baryta. The precipitate is centrifuged and washed twice with small amounts of cold saturated aqueous baryta. The centrifugate and washings are transferred to a suitable Büchner flask, made acid to Congo red and the silver precipitated with H_2S under pressure. The mixture of $BaSO_4$ and Ag_2S is centrifuged and thoroughly washed with water. The clear aqueous solution is then concentrated *in vacuo* and made up to 100 ml., 1 ml. aliquots being withdrawn for estimation of total N.

The remaining solution is transferred to a 250 ml. centrifuge bottle, the major part of the H_2SO_4 removed as $BaSO_4$, the precipitate washed and the filtrate and washings concentrated to a small volume *in vacuo* in the presence of a small excess of $BaCO_3$. The $BaCO_3$ and $BaSO_4$ are then removed by filtration through a No. 42 Whatman filter and the filtrate concentrated *in vacuo* to 2 ml. The detachable head of the flask and the capillary are then washed down with 5–10 ml. of 93 % alcohol, one or two drops of water being added if the mixture becomes more than faintly turbid. For each mg. of N present in solution, half the quantity of picric acid required to form the lysine picrate is then added. Crystals

should form on stirring the solution, which is left in the ice chest overnight. The lysine picrate is filtered off under reduced pressure on a sintered glass crucible, washed with cold absolute alcohol and the filtrate tested for unprecipitated lysine by addition of a further quantity of picric acid dissolved in warm absolute alcohol [Calvery, 1929]. The crude lysine picrate is filtered off on the same sintered glass crucible as the first crop, and the combined crops dried and weighed. The material is then dissolved in hot water, the solution sucked through the crucible and concentrated until crystals appear on the surface. The solution is cooled in the ice chest, the lysine picrate filtered off, washed with absolute alcohol, dried in a steam oven and weighed. The filtrate and washings are concentrated to a small volume, cooled and the crystals collected as before. The final volume of the mother liquor from this crop is measured, and a correction of 3·4 mg. per ml. applied for the solubility of the lysine picrate. The first crop should explode at a temperature of 266–267° and the second crop above 250° [Vickery & Leavenworth, 1928].

The data given in Table II were obtained by this method. The casein used was a sample of Kahlbaum's Hammarsten casein, and contained 14·5 % N (dry weight). Blood meal A was a sample used in a study of the relationship between

Table II. *Lysine content of casein and blood meals*

	Wt. of sample (air-dried) g.	Total N g.	Wt. of lysine picrate: 1st crop g.	Wt. of lysine picrate: 2nd crop g.	Solubility correction g.	Lysine N as % of total N
Casein	5·8620	0·8180	0·792	0·0690	0·0170	8·03
,,	5·2790	0·7364	0·702	0·0410	0·0170	7·74
Blood meal, sample A	3·7520	0·4724	0·437	0·0560	0·0180	8·00
,, ,, B	4·0660	0·5120	0·453	0·0773	0·0153	7·97
,, ,, C	5·0770	0·7362	0·786	0·1040	0·0136	9·18
,, ,, D	4·9865	0·7050	0·778	0·0400	0·0136	8·83

milk yield and the lysine content of feeds [Bartlett, 1936]. This meal contained 12·6 % N and was probably dried at a high temperature during the course of manufacture, for it contained a very large quantity of extremely hard particles which were most difficult to hydrolyse. Samples C and D were prepared from blood clot. Sample C contained 14·5 % N, was water-soluble and was spray-dried at 134° F. Sample D contained 14·1 % N and consisted of the "fines" screened from meal dried in a steam-jacketed pan at 220° F. The values obtained for the lysine content of casein agree very well with that (7·72 %) obtained by Vickery & White [1933].

The new method is probably the shortest that can be devised for the determination of lysine, using phosphotungstic acid as a precipitant. The time required for a complete analysis, in duplicate, is about 3 days. The method when applied to a feed containing a low percentage of lysine (wheat gluten) was not successful. It is believed that this is due to the very high percentage of proline in the feed. The proline would be precipitated with the bases, and, since it would not be removed as the silver salt, would be present in the final lysine fraction and thus prevent the formation and isolation of pure lysine picrate.

The values found for the lysine content of the blood meals vary somewhat, but in no case approach the high value obtained by Morris [1934] for a sample of blood meal analysed by the Van Slyke procedure. There is little doubt that the treatment of blood meal in course of manufacture will determine its value as a source of protein and lysine. As pointed out above, some of the meals were

extremely hard and almost impossible to reduce to a fine powder in a heavy mortar, and required prolonged digestion with 20% HCl to effect complete hydrolysis. It is likely that such meals would be of little value as feeding stuffs, even though they were shown to be rich in lysine by chemical analyses.

Summary

1. A shorter method for the determination of lysine in proteins is described, and is applied to the analyses of blood meals.

2. Values found for lysine in blood meals by the isolation method are considerably below published values obtained by the Van Slyke procedure.

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