LXXIII. THE CHEMISTRY OF MOULD TISSUE¹.

V. FRACTIONATION OF THE NITROGEN IN THE MYCELIUM OF *ASPERGILLUS FISCHERI*.

By HENRY JAN GORCICA, WILLIAM HAROLD PETERSON
AND HARRY STEENBOCK.

From the Departments of Agricultural Chemistry and Agricultural Bacteriology, University of Wisconsin, Madison.

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IN view of the enormous number of mould species and the high nitrogen content of the mycelium (4–10 %), it is surprising to find how meagre are the data on the nitrogenous constituents of such tissues. A review of the literature shows that only four or five species have been investigated and most of these only casually.

Small quantities of alkali-soluble proteins have been isolated by several investigators [Thomas and Moran, 1914; Takata, 1929, 2; Thomas, 1928; 1930; Clutterbuck *et al.*, 1932]. Such proteins have been uniformly low in nitrogen (12–13 %); some have been high in phosphorus (0.7 %); and some have given strong tests for carbohydrate. On the basis of such data Takata [1929, 1] suggested that a protein which he isolated might be regarded as a phosphoglycoprotein. Certain it is that the proteins of the mould pad are difficult to isolate and appear to be different in character from most of the proteins of plant and animal materials.

A number of amino-acids have been identified in the acid hydrolysate of the mycelium or proteins separated therefrom. Glycine, alanine, leucine, tyrosine, lysine, arginine, histidine, tryptophan, aspartic and glutamic acids have been reported by one or more investigators [Takata, 1929, 2; Clutterbuck *et al.*, 1932; Abderhalden and Rona, 1905; Vorbrodt, 1919; 1921]. As shown by Abderhalden and Rona [1905], the mould, *A. niger*, was able to synthesise the same amino-acids from either inorganic nitrogen (*e.g.* potassium nitrate) or organic nitrogen (*e.g.* glycine or glutamic acid).

Indirect evidence from feeding experiments with animals [Takata, 1929, 1; Skinner *et al.*, 1933] indicates either the absence of certain essential amino-acids or their presence in too low a proportion to bring about growth at an intake level of 15–18 % protein. Such feeding trials also indicate considerable variation in amino-acid content among different species of moulds and it may be even in the same species under different conditions of growth.

Besides amino-acids, xanthine, hypoxanthine, guanine, adenine, cytosine, glucosamine, choline, betaine and stachydrine have been reported [Takata, 1929, 2; Vorbrodt, 1921] among the hydrolysis products of *A. niger* and *A. oryzae*. Whether or not such compounds are found generally in mould mycelium cannot be decided on the basis of the data now available.

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In view of the incompleteness of the data, it seemed desirable (*a*) to separate the total nitrogen of mould pads into various fractions according to their solubilities and (*b*) to determine the partition of nitrogen in each of these fractions.

EXPERIMENTAL.

The mould tissue used in the following experiments was a pure culture of *A. fischeri* grown in batches of 25 kg. on a glucose-inorganic salts medium. The details of the procedure used in growing the mould have been reported in a previous paper [Peterson *et al.*, 1933].

Preliminary experiments.

Several preliminary nitrogen extraction experiments were conducted. While the data from these experiments cannot be presented here because of lack of space, the following statements give the more important results obtained.

Mould tissue which had been killed by steaming, then dried and ground and subjected to long extraction with hot alcohol to remove fat and sterols [Pruess *et al.*, 1932] proved to be unsatisfactory for nitrogen studies. Tissue that had been thus treated absorbed water and formed a slimy, jelly-like mass, which was not filterable. The extract remained turbid even after passage through a Sharples super-centrifuge. The nitrogen of the mycelium distributed itself among successive solvents as follows: water, 11.7 %; 2 % NaOH, 28.2 %; 5 % NaOH, 30.8 %; alkali-insoluble, 18.9 %.

Mould tissue which was killed by steaming at 99° for one hour, then dried for 48 hours at 65° and ground was more satisfactory in that it could be filtered by draining overnight from fluted filters. The solubility of the nitrogen in this material was as follows: water, 18.9 %; 2 % NaOH, 26.7 %; 5 % NaOH, 28.7 %; alkali-insoluble, 17.1 %.

The best results were obtained when perfectly fresh untreated mycelium was used. Aqueous extracts of this material filtered readily and gave clear filtrates. However, complete extraction of water-soluble substances was obtained only after extraction of the residue with alcohol-ether following a first water extraction. When this was done, an additional quantity of water-soluble nitrogen, almost equal to that contained in the first extract, was obtained. 10 % sodium chloride solution or 1 % hydrochloric acid solution extracted about the same percentage of the total nitrogen as did water alone.

Extractions of the water-insoluble residue with alkaline (KOH, NaHCO₃, Na₂CO₃, Ba(OH)₂) solutions of various strengths and under different conditions of time and temperature were compared. 1 % KOH solution at room temperature followed by 4 % KOH solution at boiling temperature seemed to give the most regular and informative results.

On the basis of the preliminary experiments the final extraction procedure given below was adopted.

Method of extraction.

Mould tissue fresh from the pans of the steriliser-incubator was rinsed with distilled water and immediately ground to a fine paste in a "Nixtamal" mill. The ground pad was allowed to fall into a dish containing chipped ice and was stirred into the ice. When the pad had all been ground, it was emptied into a jar of iced water and stirred vigorously for 15 minutes with an electric stirrer. It was then filtered with suction on a cloth filter and the filtrate poured back upon the filter-cake and refiltered until it came through clear. The filter-cake was given two

more 15-minute extractions with iced water, and all three filtrates were combined and comprise the "First water-extract."

The filter-cake from the water-extract was thoroughly stirred into a large excess of alcohol-ether (1 : 1), and allowed to stand overnight. The alcohol-ether was filtered off, and the filter-cake was suspended in ether and allowed to stand several days before filtering. The filtrates were combined; they constitute the "Lipin fraction."

The ether-extracted filter-cake was spread out in a thin layer and air-dried before a fan for 2 hours. The dry powder was then stirred with water for 15 minutes and filtered. The filter-cake was re-suspended in water and allowed to soak overnight under protection of toluene. It was then filtered, and the two filtrates combined formed the "Second water-extract."

The residue from the water extraction was stirred into an excess of 1 % KOH solution and allowed to stand at room temperature for 2 days. The suspension was filtered with suction on a cloth filter, the filter-cake washed twice by stirring with water and filtering, and the three filtrates were combined. This is designated as "Cold 1 % KOH extract."

The filter-cake was boiled with 4 % KOH solution for one hour, filtered and washed twice with water, and the filtrates were combined to form the "Hot 4 % KOH extract."

The residue from the hot alkaline extraction was washed with 50 % alcohol, 95 % alcohol and ether and then dried. This fraction is called "Residue."

The extraction procedure as described above has been carried out a number of times, and two typical analyses (on batches 28 and 31) are presented in Table I.

Table I. *Nitrogen distribution of A. fischeri.*

Fractions	Percentage distribution				Percentage composition of fractions				Free amino-nitrogen. Percentage of total nitrogen in each fraction Batch 31
	Nitrogen Batch		Solids (ash-free basis) Batch		Nitrogen (ash-free basis) Batch		Ash Batch		
	28	31	28	31	28	31	28	31	
First water-extract	17.2	16.8	19.8	15.6	4.4	6.7	13.4	11.6	37.5
Lipins	1.2	1.2	9.5	8.5	0.9	1.1	0.0	0.0	†
Second water-extract	13.6	11.7	17.0	14.8	4.1	4.9	1.4	1.8	25.0
Cold 1 % KOH extract	39.7	41.2	21.1	22.9	9.6	11.1	35.0*	33.6*	9.4
Hot 4 % KOH extract	13.0	14.3	13.7	15.2	4.9	5.8	64.4*	54.1*	14.0
Residue	12.2	13.8	26.0	27.2	2.3	3.1	2.9	2.4	†
Total recovered	96.9	99.0	107.1	104.2	—	—	—	—	—
Whole mould	100.0	100.0	100.0	100.0	5.1	6.2	3.0	2.9	†

* Including ash from alkali.

† Not determined.

Nitrogen distribution.

As can be seen from Table I, 17.2 and 16.8 % of the total nitrogen of the mould pad of batches 28 and 31, respectively, were dissolved in the first water-extract. The nitrogen content of the extracted solids in batch 28 was slightly less (4.4 %) than that of the whole mould (5.1 %), whereas in batch 31 it was slightly more (6.7 % against 6.2 %).

The first water-extract was a yellowish green solution of p_H 4.6. It gave negative biuret, Millon and Hopkins-Cole tests and a positive xanthoproteic test. It gave no precipitate with trichloroacetic, acetic or hydrochloric acid, but gave a slimy brown precipitate when made alkaline with KOH, and a further precipitate upon the addition of baryta to the filtrate from the KOH precipitation. Neither of these two precipitates contained as much as 1 % of nitrogen. The KOH precipitate contained 56 % ash and the baryta precipitate about 90 %. Since the extracted solids contained about 12 % ash, it is evident that the water extraction removes most (71 %) of the ash of the mould pad. The barium precipitate consisted of about 12 % barium sulphate and about 88 % barium phosphate.

Several peculiar results were noted in making the colour tests on this first water-extract: the development of a deep green colour with the biuret reagents, and a blue colour with phosphotungstic acid-sodium carbonate. On investigation of the reasons for the blue colour test, it was found that Hunter's [1928] colour test for ergothioneine was strongly positive. Attempts to isolate ergothioneine by two different procedures [Tanret, 1909; Williamson and Meldrum, 1932] failed.

The second water-extract (following the removal of the lipins) differed in a few particulars from the first. It gave a negative biuret test, but positive Millon, Hopkins-Cole and xanthoproteic tests. No proteins were present, however, as was shown by the absence of any precipitate with trichloroacetic acid or upon heating.

Extraction with cold 1 % KOH solution removed about 2/5 of the nitrogen originally present in the mould pad (39.7 %, batch 28; 41.2 %, batch 31). The extract gave positive biuret, xanthoproteic, Millon and Hopkins-Cole tests. A heavy white precipitate was formed upon acidification. A considerable amount of carbohydrate was present in the extract as was evidenced by the fact that the acid hydrolysate of the extract strongly reduced Fehling's solution.

Various procedures were used in an attempt to isolate the protein from the extract, including fractional precipitation by alcohol and acetone, saturation with zinc sulphate, precipitation by trichloroacetic and tungstic acids; but none of these methods gave a protein preparation free from carbohydrate. In fact, the highest nitrogen content found in any fraction was only 12.3 %. It may be that the carbohydrate and protein were combined as in the case of glycoproteins. Other investigators have reported similar low nitrogen figures for mould proteins, 12–12.9 % [Thomas and Moran, 1914; Clutterbuck *et al.*, 1932].

The extraction with boiling 4 % KOH removed a quantity of material which was rather low in nitrogen (4.9–5.8 %). The extract was coloured dark brown, and the precipitates obtained from it were also very dark. Part of the alkali used in the extraction was not accounted for on titration with acid, presumably because of the presence of acidic groups in the extracted material.

The nature of the resistant, alkali-insoluble residue has been discussed elsewhere [Norman and Peterson, 1932]. Hydrolysis for 24 hours with 20 % HCl brought 78 % of its nitrogen into solution. Glucosamine hydrochloride readily crystallised out of the acid hydrolysate and accounted for 62 % of the nitrogen in the resistant residue.

Partition of the water-soluble nitrogen.

The ammonia-nitrogen of the water-extract (batch 28) was very low, only 3.9 % of the total. It is quite obvious that the mould, although it may absorb nitrogen in the form of ammonium salts, does not store it in the hyphae for any length of time in that form.

A rough attempt was made to determine the amount of urea present by precipitation with xanthidrol and determination of the nitrogen content of the precipitate. This was found to be 3.9 % of the total nitrogen in the extract.

The amounts of amide-nitrogen found varied within wide limits according to the method used. The lowest figure, 3.5 %, was obtained by hydrolysis with 2*N* H₂SO₄ for 6 hours [Vickery, 1931]. The highest figure, 14.8 %, was found after hydrolysis with 20 % HCl for 24 hours. The difference between these two figures (11.3 %), is formed from unknown compounds during the severe hydrolysis with 20 % HCl. The maximum figure is slightly less, however, than that reported by Vorbrodts [1926] who found 20.9 % amide-nitrogen in the hydrolysate of the water-extract of *A. niger*.

In some batches of mould the first water-extract contained some substance which had the power of catalytically decomposing nitrous acid to nitric oxide with extreme rapidity. Consequently, attempts to determine the α -amino-nitrogen of these extracts by the Van Slyke method were attended with great difficulty. The apparent amino-nitrogen of these extracts varied from 80 to 110 % of the total nitrogen in the extract. However, in the water-extract of a batch that behaved normally the free amino-nitrogen before acid hydrolysis was 37.5 % of the total nitrogen in the extract. (Table I.)

The nitrogen partition of the water-extract from batches 31 and 34 is given in Table II. The basic nitrogen in the acid-hydrolysed extract varied from 14.9 %

Table II. *Nitrogen partition of water-extract.*

	Batch 31	Batch 34
	% of total N	% of total N
Ammonia-N	18.7	18.1
Humin-N	6.6	5.4
Phosphotungstic acid precipitate-N	14.9	20.0
Phosphotungstic acid filtrate, amino-N	58.9	58.4
Phosphotungstic acid filtrate, non-amino-N	1.5	0.3
Total recovered	100.6	102.2

of the total (batch 31) to 20.0 % (batch 34). Vorbrodts [1926] found 6.2 % basic nitrogen in the acid hydrolysate of the water-extract of *A. niger*.

Although the water-extract gave no clearly positive test for peptides, it contained a large proportion of peptide-nitrogen. Before hydrolysis, the α -amino-nitrogen was 30.2 % and after hydrolysis 67.7 %, giving a difference of 37.5 % as combined amino-nitrogen.

The lipin fraction contained a small amount of nitrogen which was not investigated further.

Fractionation and partition of the alkali-soluble nitrogen.

The cold alkaline extract was found to contain two types of protein—one soluble in acid solution, the other precipitable on acidification. The acid filtrate, however, also contained considerable amounts of carbohydrate, and the acid-soluble protein and carbohydrate had almost identical solubilities, so much so that it was exceedingly difficult to separate the two. For example, tungstic acid, which completely precipitated the protein, was found to precipitate the carbohydrate also.

The procedure finally adopted is shown in the following scheme: precipitation of first protein fraction with acid at p_H 4.8–5.2, precipitation of second protein

fraction with copper sulphate at p_H 6.2–6.5, with most of the carbohydrate remaining in the filtrate.

Fractionation of nitrogen in first alkaline extract (batch 34).

Explanation: Figures above the line and on the right indicate percentage of nitrogen in each fraction (ash-free basis). Figures below the line on the right show the percentage of the total nitrogen which was contained in each fraction. Figures below the line on the left indicate the percentage of the total solids (ash-free) found in each fraction.

The first protein precipitate was a tan-coloured powder; it contained 1.2 % ash, 11.8 % N and 0.4 % P. It was soluble in dilute alkalis, precipitated by acidification with mineral acids (partly dissolved by an excess), acetic, trichloroacetic, phosphotungstic and tungstic acids. It gave all the usual protein colour tests and in addition reduced Fehling's solution after acid hydrolysis.

The copper sulphate precipitate dissolved in excess acid with the formation of a greenish blue colour, and in excess alkali with the formation of a deep purple colour. It contained 12.3 % N and 2.4 % P (ash-free basis), and varying amounts of ash, 12 to 37 %. This preparation was precipitated from acid solution by phosphotungstic and tungstic acids. The acid hydrolysate of this preparation reduced Fehling's solution.

The filtrate from the copper sulphate precipitate no longer gave any protein colour tests but contained 3.2 % N (ash-free basis), of which 36 % was free amino-nitrogen. After acid hydrolysis, it strongly reduced Fehling's solution. The distribution of the nitrogen and of ash-free solids in the three fractions is shown in the scheme.

Table III. *Nitrogen partition of the combined alkaline extracts (batch 31).*

	Extract % of total N	H ₂ SO ₄ ppt. % of total N	CuSO ₄ ppt. % of total N
Ammonia-N	6.6	4.9	6.7
Humin-N	8.2	7.7	7.9
Phosphotungstic acid precipitate-N	24.4	20.5	31.6
Phosphotungstic acid filtrate, amino-N	51.5	61.5	55.0
Phosphotungstic acid filtrate, non-amino-N	6.1	6.9	0.0
Total recovered	96.8	101.5	101.2

Table III shows the nitrogen partition of the combined alkaline extracts of batch 31, in which 55.5 % of the total N of the mycelium was alkali-soluble. This material differed markedly from the water-extract in that only one-third as much

ammonia was liberated by acid hydrolysis. The alkali-soluble nitrogen also contained a considerably larger amount of basic nitrogen than did the water-extract. The copper sulphate precipitate contained 50 % more basic nitrogen than the acid precipitate, which fact may be due to the presence in the former fraction of most, if not all, of the mould nucleic acid.

This difference between the two fractions was still more marked in the nitrogen partition (Table IV) carried out on the two fractions from the cold 1 % KOH

Table IV. *Nitrogen partition of protein fractions from first alkaline extract (batch 34).*

	H ₂ SO ₄ ppt. %* of total N	CuSO ₄ ppt. % of total N
Ammonia-N	4.9	8.7
Humin-N	5.8	13.8
Phosphotungstic acid precipitate-N	22.8	38.0
Phosphotungstic acid filtrate, amino-N	60.2	36.5
Phosphotungstic acid filtrate, non-amino-N	3.9	1.8
Total recovered	97.6	98.8

* Average of two determinations.

extract (containing 43.2 % of the total mycelial nitrogen) of batch 34. In the copper sulphate precipitate of this extract 38 % of the nitrogen was basic and only 36.5 % monoamino-nitrogen. This fraction would very likely serve as a convenient source of mould nucleic acid.

SUMMARY.

The solubility and partition of the nitrogenous constituents in the mycelium of *A. fischeri* have been determined. The mould-nitrogen was most easily extracted when fresh, unaltered mould tissue was used. The mycelium contained about 30 % water-soluble non-protein-N, 55 % of alkali-soluble protein-N, and 12 % of alkali-insoluble residue-N.

The alkali-soluble protein consisted chiefly of two fractions, one precipitated by acids, the other acid-soluble but precipitated by copper sulphate.

The acid-insoluble protein contained 1.2 % ash, 11.8 % N and 0.4 % P. Of the N in this protein, 22.8 % was basic N and 60.2 % was monoamino-N.

The acid-soluble protein contained a variable, but generally high, ash (12-37 %), 12.3 % N (ash-free basis), and 2.4 % P (ash-free basis). This protein contained 38.0 % of basic N and only 36.5 % of monoamino-N.

The resistant residue contained 2.3 % N. Of the nitrogen in this residue, 62 % has been isolated as glucosamine-N.

REFERENCES.

- Abderhalden and Rona (1905). *Z. physiol. Chem.* **46**, 179.
- Clutterbuck, Lovell and Raistrick (1932). *Biochem. J.* **26**, 1907.
- Hunter (1928). *Biochem. J.* **22**, 4.
- Norman and Peterson (1932). *Biochem. J.* **26**, 1946.
- Peterson, Pruess, Gorcica and Greene (1933). *Ind. Eng. Chem.* **25**, 213.
- Pruess, Peterson and Fred (1932). *J. Biol. Chem.* **97**, 483.

- Skinner, Peterson and Steenbock (1933). *Biochem. Z.* **267**, 169.
Takata (1929, 1). *J. Soc. Chem. Ind. Japan*, **32**, 155 B.
—— (1929, 2). *J. Soc. Chem. Ind. Japan*, **32**, 243 B.
Tanret (1909). *J. Pharm. Chim.* **30**, 145.
Thomas (1928). *Amer. J. Bot.* **15**, 537.
—— (1930). *Amer. J. Bot.* **17**, 779.
—— and Moran (1914). *Compt. Rend. Acad. Sci.* **159**, 125.
Vickery (1931). *J. Biol. Chem.* **90**, 179.
Vorbodt (1919). *Bull. Intern. Acad. Polonaise, Classe Sci. Math. Nat. B.*, 71.
—— (1921). *Bull. Intern. Acad. Polonaise, Classe Sci. Math. Nat. B.*, 223.
—— (1926). *Bull. Intern. Acad. Polonaise, Classe Sci. Math. Nat. B.*, 517.
Williamson and Meldrum (1932). *Biochem. J.* **26**, 815.