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Studies on the Microbiological Degradation of Wool. II. Nitrogen Metabolism

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INTRODUCTION

A previous paper from this laboratory (1) has discussed the sulfur metabolism of the dermatophytic fungus *Microsporium gypseum* in the degradation of wool. The present report is concerned with the changes in the nitrogen of the wool in the same process.

EXPERIMENTAL

A. Materials and Analytical Methods

Scoured sheep wool, treated as described in (1) was used. The fungus used was *Microsporium gypseum*, QM-196, which had been isolated from deteriorating woolen fabric. The basic medium for the shake-flask-technique studies was a suspension of wool in salt solution, made up as follows: $MgCl_2 \cdot 6H_2O$, 1.68 g.; K_2HPO_4 , 2.09 g.; KH_2PO_4 , 2.68 g., and 50.0 g. wool, made up to 1-l. with distilled water. The wool served as the only source of carbon, nitrogen, and sulfur.

Nitrogen was determined by the micro-Kjeldahl method; amino nitrogen by the Van Slyke manometric procedure (2); amino nitrogen on the solid wool residue was determined by the Dougherty and Ogg modification (3) of the Van Slyke manometric method. Amide nitrogen was determined after gentle hydrolysis with 1 N H_2SO_4 for 3 hr. in a boiling water bath. Reagents for ammonia nitrogen and amide nitrogen were those as described by Pucher, Vickery, and Leavenworth (4).

Fractionation of the protein in the digest was carried out by the method of Was-teney and Borsook (5) which involves precipitation of the protein by trichloroacetic acid, of the proteases by sodium sulfate at 33°C., and of the peptones by tannic acid, all under definitely fixed conditions.

B. Shake-Flask Technique for Study of Action of Fungus on Wool

This technique was described in detail in a previous publication (1). It consisted essentially of a system whereby sterile washed air was blown through a large 22-l. flask containing 5 l. of the basic salts medium and 5% wool. The ammonia evolved

from the main reactor vessel was absorbed in saturated boric acid. Each time a sample of the metabolic mixture was withdrawn for analyses, the absorbers were replaced by fresh ones.

To supplement and improve the accuracy of the results obtained from the above experiment, a series of eighteen 1-l. Florence flasks containing 150 ml. nutrient salt solution and 7.5 g. of chopped wool were prepared, sterilized, and inoculated with *M. gypseum*. At 0, 3, 6, 10, and 14 days, 4 flasks were removed and the wool plus mycelium residues of each of 2 flasks were thoroughly washed, dried, and analyzed separately. The remaining two flasks were used for duplicate determination of mycelium by digestion with 10% NaOH as previously described in (1).

RESULTS

A. Gross Nitrogen Changes During Degradation of Wool in Shake Flasks

Data in Fig. 1 and 2 show that the increase in alkalinity in the shake-culture is coincident with an accumulation of soluble-N compounds in the filtrate. The highest pH is noted at the 11th day, and after that

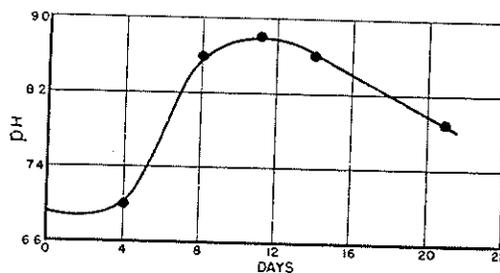


FIG. 1. pH changes in metabolic filtrate of *M. gypseum* growing on wool.

time the pH declines. As soon as the maximum pH is reached, active breakdown of the wool ceases as is indicated by no further loss of wool residue after the 14th day.

Figure 2 shows the trend of daily changes in the nitrogen of the wool after the growth of the fungus upon it. The curve relating to gases is plotted as nitrogen obtained by calculation from the ammonia evolved by the reaction, and is cumulative.

Subsequent studies on a series of filtrates have shown that about 50% of the total nitrogen of the filtrate was in the form of ammonia nitrogen. After the 14th day, the solubilization of wool was almost completely stopped.

Since the data in Fig. 2 do not account for all the nitrogen, a somewhat different experiment was set up to simplify and possibly reduce the errors that come from sampling one large vessel over a period of 21 days. A series of liter flasks containing 150 ml. salt solution plus 7.5 g. of chopped wool were set up and inoculated. The simple shake-flask technique replaced the constant air stream used in the former experiment. At intervals flasks were taken off and the contents of each ana-

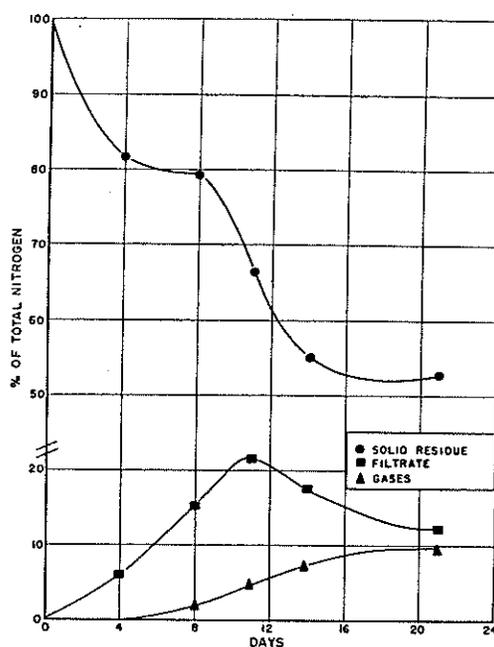


Fig. 2. Nitrogen distribution during wool breakdown by *M. gypseum*.

lyzed separately. The results appear in Table I. All of the values are calculated to an ash-free, moisture-free, basis.

In order to make the calculations in Table I on the basis of the *wool residue* rather than the *wool plus mycelium residue*, analyses of three batches of *M. gypseum* mycelium, grown on glucose-peptone-mineral salts medium for 10 days, were made. Average values, calculated to an ash-free, moisture-free, basis gave N, 6.28%; amino N, 0.1%; S, 0.327%; and cystine, 0.276%. It is assumed that the N and the S of

TABLE I

Analytical Values on Wool Residue at Various Stages in Digestion by M. gypseum

Days	Weight of wool remaining	Weight of mycelium	Amino N	N	S	Cystine	Ratio	
							N/S	S/Cystine
0	7.090	0.000	<i>mg./g.</i> 3.78	% 16.50	% 2.99	% 8.55	5.51	0.350
3	6.499	0.201	3.60	16.53	3.10	9.26	5.33	0.335
6	5.829	0.541	3.29	16.38	3.34	9.95	4.90	0.335
10	4.956	0.644	3.08	16.68	3.66	10.45	4.56	0.350
14	4.654	0.546	2.91	16.38	3.48	10.29	4.70	0.338

the mycelium do not vary significantly during the phases of the growth cycle on wool. It is further assumed in the following calculations that these values for mycelium grown on glucose-peptone medium are of essentially the same order of magnitude as when the organism is grown on wool. However, it is recognized that the composition of the mycelium may vary within fairly wide limits and even with a variation by a factor of 2, the S and N values given in Table I would not vary significantly.

The assumptions used in reporting the values in Table I may be best indicated by a sample calculation:

10-Day Digestion Sample

$$\begin{aligned} \text{Total residue (wool + mycelium)} &= 5.600 \text{ g.} \\ \text{Wt. of mycelium} &= 0.644 \text{ g.} \\ \text{Wool residue} &= 4.956 \text{ g.} \end{aligned}$$

Nitrogen calculations:

$$\begin{aligned} 5.600 \times 0.1549^1 &= 0.8674 \text{ g. N in total residue} \\ 0.644 \times 0.0628 &= 0.0404 \text{ g. N in mycelium} \\ &= 0.8270 \text{ g. N in wool residue} \\ \frac{0.8270}{4.956} \times 100 &= 16.68\% \text{ N in wool residue alone.} \end{aligned}$$

Sulfur and cystine calculations were made in a similar manner.

It also may be pointed out that a linear relationship exists between the percentage of S and the log of time, and the N percentage does

¹ Total N/g. wool residue plus mycelium.

not change. The correlation coefficient between the former equalled 0.85. Thus there is a significant increase in the S percentage of the wool residue with time, but this is not true for the N values.

Since the amino-N values are of certain usefulness in our interpretation, they are given in Table II, and were values obtained on the same samples used to obtain the data in Table I. They were similarly corrected as above to give values for the wool residue, and not wool plus mycelium residue. Since only negligible amounts of amino N could be detected in mycelium, it was assumed that all was present in the wool and calculated as such.

Additional data showing an increase in the sulfur and cystine in the wool residue during digestion is shown in Table III, which compares

TABLE II
Changes in Amino-Nitrogen Content of Wool During Digestion by M. gypseum

Days	Amino N		
	Mgs./g. of wool residue	Per cent of total N in wool residue	Change
0	3.78	2.29	<i>per cent</i> —
3	3.60	2.26	— 1.3
6	3.29	2.24	— 2.2
10	3.08	2.15	— 6.1
14	2.91	2.03	— 11.4

the action of a cell-free metabolic filtrate having high activity on *ball-milled* wool, as well as a commercial trypsin and a crystalline trypsin sample. Other experiments have confirmed the fact that the difference in degree of digestion between the fungal enzyme extract and crystalline trypsin is due to the presence of additional proteinases in the former. It is suggested that this is the same type of digestion as goes on with the more intact chopped wool.

The initial amino-N value of 3.78 mg./g. (Table I) is high in comparison with that of "normal" wool. This wool differed from the "normal" wool since it had been autoclaved, as described in the *Methods* section. Since a consistent value of 2.96 mg./g. for nonautoclaved wool

TABLE III
 Analysis of Residues From Digestion of Ball-Milled Wool With Enzyme
 Extracts of *M. gypseum* or by Trypsin^a

Enzyme	Digestion of ball-milled wool	Cystine, %		Sulfur, %		S/Cystine ratio
		Residue	Lost on digestion	Residue	Lost on digestion	
None	—	8.17	%	2.78	%	0.34
Enzyme extract from <i>M. gypseum</i>	83.0	9.28	79.7	4.7	69.8	0.50
Enzyme extract from <i>M. gypseum</i>	88.1	12.67	81.6	3.8	83.8	0.30
Crude trypsin, 0.05%	76.1	11.96	65.0	—	—	—
Crystalline trypsin, 0.01%	61.5	12.83	40.0	4.45	38.5	0.35
Crystalline trypsin, 0.05%	60.5	—	—	—	—	—

^a After 4 days incubation.

was found, it was of interest to compare our normal value with other normal values for wool as found in the literature. They are as follows:

Wool	Mg./g.	Reference
Intact	3.05	(6)
Chopped	3.30	(3)
Chopped	3.16	(7)

B. Nitrogen Partition of the Metabolic Filtrate

Results of a more detailed gross-N partition of the metabolic filtrate are summarized in Table IV.

Since it was known that a certain portion of the chopped wool was soluble in the salt solution alone, a control nitrogen partition was determined. However, this sample contained only 2% of the nitrogen of the inoculated sample.

Table IV shows that an amino nitrogen value of 0.44 mg. N/ml. was found. The total protein, proteose, peptone, and residual nitrogen was 0.97 mg. N, of which 71% or 0.69 mg. was the residual nitrogen fraction, which would be the nitrogen of the amino acids and dialyzable peptides. The amino nitrogen would then represent 45.4% of this total nitrogen. This figure may be considered reasonable in view of the large portion of the amino acids such as arginine, histidine, lysine, and proline which make up 23% of the amino acid content of wool and contain other nitrogen than amino nitrogen. Furthermore, a portion of the nitrogen of this fraction is linked as peptide nitrogen.

TABLE IV
Fractional Analysis of Metabolic Filtrate

	Uninoculated control	Fungal digest		
			Per cent of total N	Per cent of (A)
Total N	mg. N/ml. 0.0563	mg. N/ml. 2.68	—	
Ammonia N	0.003	1.62	60.4	
Amide N	0.000	0.09	3.4	
Amino N	—	0.44	16.4	45.4
Protein, proteose, peptone, and amino acid N (A)	0.0560	0.97		100
Protein N	0.0040	0.04		4.1
Proteose N	0.0004	0.18		18.6
Peptone N	0.0072	0.06		6.2
Residual N (amino acid N and peptide N)	0.0444	0.69		71.1

The fact that wool is not so strongly acid despite its large amounts of aspartic and glutamic acid indicate the presence of acid amides. The usual amino acid analyses of wool seldom include analyses for either amide-N or glutamine or asparagine. Thus, the presence of a small (3.4% of the total), but definite amount of amide-N in the fungal filtrate indicates release of either asparagine or glutamine as such from the wool or a synthesis of this linkage by the action of the microorganism. This value should be considered a minimum figure since the wool was autoclaved for 5 min. at 120°C. to obtain a sterile substrate for the

action of the fungus and it has been shown by Hamilton (8) that glutamine at pH 6.5 is converted to pyrrolidonecarboxylic acid during autoclaving. This N would not be released by gentle acid hydrolysis, and as such would not be detected in an amide-N determination.

DISCUSSION

The following observations regarding wool residue at various stages of digestion by *M. gypseum* can be made from the data in Table I:

1. The nitrogen content does not show a significant variation with time.
2. The sulfur content shows a significant constant increase with time of digestion.
3. The S/cystine ratio stays fairly constant. This fact indicates that the sulfur is still linked in the cystine of the wool as before any microbial action. The above results may be interpreted as meaning that since the sulfur and cystine apparently increased, this increase was brought about by a differential hydrolysis of the wool protein. Thus a nitrogenous component with less cystine per original unit weight of wool is split off first. This is reflected in Table I as keeping the nitrogen constant and the sulfur increasing. A further example to illustrate this point may be given by taking the 14-day nitrogen value in wool residue from Fig. 1 and comparing it with the 14-day sulfur value for wool residue, given in a previous publication (1), and part of the same general experiment. The amount of the total nitrogen remaining in the wool residue was 55% as compared with 75% of the original sulfur.
4. It appears that deamination takes place primarily at the expense of the polypeptide after hydrolysis of the keratin molecule. Direct deamination of the intact keratin molecule accounts for little, if any, of the ammonia liberated. These statements are based on the analyses which show only 0.25% loss of amino nitrogen in the wool residue whereas there is 9.6% of the total nitrogen found as ammonia nitrogen in the filtrate after 21 days.

It was also found that a significant increase in the initial amino nitrogen of wool occurred as a result of autoclaving wool. This is probably due to a splitting of the polypeptide chains. This increase may have some significance in explaining the much greater susceptibility of autoclaved wool to microbiological degradation (9). More specifically,

it may suggest that the rate-governing step in the sequence of reactions involved in the degradation of wool is the initial cleavage of the peptide bond in the keratin molecule.

Mention might be made of the presence of a nondialyzable brown pigment always found present in the filtrate. In the Tiselius electrophoresis apparatus, the pigment and other small amounts of protein migrate faster than a main protein component. We have found this main protein fraction to have definite proteolytic activity against such substrates as ball-milled wool, gelatin, and casein.

SUMMARY

1. Degradation of wool by the action of the fungus *M. gypseum*, observed by means of shake-culture technique, was followed by means of nitrogen distribution studies.
2. At about 11 days, growth had stopped, and the quantities of soluble organic nitrogen and ammonia became fairly constant. The maximum digestion of the wool was reached in 14 days while the maximum liberation of soluble organic nitrogen into the filtrate occurred in 11 days.
3. The total nitrogen in the wool residue at various stages of digestion by *M. gypseum* does not significantly vary from its initial value. However, the sulfur values showed a constant increase with time of digestion over a period of 14 days, indicating a differential hydrolysis of wool protein.
4. A nitrogen partition of the metabolic filtrate showed that the 36% organic nitrogen was distributed as follows: 4.1% soluble-protein-N, 18.6% proteose-N, 6.2% peptone-N, and 71.1% amino acid- and peptide-N. The inorganic-N comprising 60.4% of the total N was ammonia.
5. Wool is not significantly deaminated until degradation of the molecule has proceeded to the stage of polypeptides and amino acids.
6. In the microbiological degradation of the wool molecule, there is a differential initial splitting away of non-sulfur portions.

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