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Self-heating of Hay and Grain in Dewar Flasks and the Development of Farmer's Lung Antigens

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With an Appendix on Taxonomy by J. Lacey

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SUMMARY

In moist hay allowed to self-heat aerobically in Dewar flasks, the pattern of temperature change with time was affected considerably by the type of hay and duration of storage, but there was a relationship between water content and maximum temperature reached. Below 29 % water content there was little heating or antigen production; in the critical range of 29–34 % water content, different lots of hay self-heated to different temperatures between 33 and 55° and varied widely in their content of farmer's lung hay antigen complex (FLH), the wetter hays usually producing the more antigen; all samples with 40 % water heated to *c.* 65° and produced FLH antigen, associated with the presence of *Thermopolyspora polyspora*. Progressively less antigen, especially in the lower regions of the flasks, was produced as water content increased from 47 to 68 %. Moist barley and oat grain also self-heated and produced FLH antigen, usually only in the middle of the grain mass, where *T. polyspora* was most abundant; the drier upper layers and the lower regions where excess water accumulated were free from the antigen.

INTRODUCTION

The self-heating of baled hay has been studied in relation to the microbial and biochemical changes during moulding (Gregory, Lacey, Festenstein & Skinner, 1963) and to the development of antigens of immunological significance in farmer's lung disease (Gregory *et al.* 1964). Pepys *et al.* (1963) attributed the presence of these antigens (FLH complex) to the growth of the thermophilic actinomycetes *Thermopolyspora polyspora* and *Micromonospora vulgaris*.

The first laboratory experiments on self-heating of hay were made by Miehe (1907) who used a central wire container filled with hay surrounded by similar containers insulated with cotton-wool. Dewar flasks were used first by Hildebrandt (1927) and James, Rettger & Thom (1928) and later by Miehe (1930).

Our experiments in Dewar flasks began as studies on the effects of water and aeration on the self-heating process, in attempts to reach high temperatures. Hay very rich in FLH antigen was produced so the effect of water on antigen production

was examined in more detail. The chopped hay used in the wide-mouthed Dewar flasks was easily mixed with water, so avoiding sampling errors caused by uneven water distribution, which is unavoidable in baled hay. A few experiments were made with moistened barley and oat grain.

METHODS

Two batches of good quality air-dry hay were used in most experiments; 1961 hay from a timothy grass and fescue ley ('SB') hay, Gregory *et al.* (1963), used from March 1962 to November 1963, came from a stack stored in a barn; samples were periodically chopped into 1–3 cm. lengths and stored in polythene bags or in sacks at outdoor temperatures. The water content was 16 %, except for the last batch in November 1963, when it was 19 %.

The second batch of hay was baled in 1962 from a perennial ryegrass + meadow grass ley; a bale was chopped in November 1963 and used until March 1964. The water content, also 16 % at the start, fell to 9 % after storage in sacks in a heated greenhouse. The other hays were from bales made in 1962 (Yorkshire Fog + Agrostis ley) and 1963 (timothy + meadow fescue + white clover ley), referred to as 1962′ and 1963 hay, respectively.

A weighed quantity of chopped hay (usually 500 g.) was moistened in a polythene bucket to the required water content, thoroughly mixed by hand and then transferred to a 4 l. Dewar flask. A 1 l. and a 10 l. Dewar flask were also occasionally used, and in one experiment a barrel lagged with glass fibre sheet was filled with 15 kg. hay that had been moistened and mixed on a concrete floor. Water content is given as percentage wet weight, and weights of hay given all refer to the weight before adding water.

In experiments with barley (14 % water content), 2·5 kg. grain was used and water added to give a content of near 40 %, but the water was not all absorbed and some accumulated at the bottom of the 4 l. Dewar flask. In one experiment, as also in the experiment with oats (12 % water content), the grain was left to stand with the added water with occasional mixing, for 5 hr, before all the water was taken up.

A plug of cotton-wool about 2 cm. thick was placed on the surface of the hay or grain and one or two extra similar layers placed on top and overlapping the sides of the flask to lessen heat loss. A thermometer was inserted into the centre of the mass and the 4 l. flasks contained a second thermometer 2–3 cm. from the bottom of the mass 25 cm. deep. The thermometer in the barrel was in a metal tube filled with paraffin oil, to ensure good contact with the bulb, and also to diminish the temperature change, when it was raised by thread to be read.

Grain removed at the end of the experiments was subdivided into layers according to the visual appearance of microbial growth. The hay was occasionally subdivided in this way, but usually into three portions of approximately equal weight, designated upper, middle and lower layers; only the middle layer was usually examined microbiologically.

The samples were analysed as described by Gregory *et al.* (1963) and Gregory *et al.* (1964), except that the short chopped hay and grain to be examined in the wind tunnel was placed in muslin bags to prevent too much of the material being blown away. For immunological work the hay and grain samples were extracted with

phenol saline after defatting, and the extracts Seitz filtered, dialysed and freeze dried. All extracts were tested for precipitin reactions in agar gel against a set of twenty sera, using the double-diffusion method of Ouchterlony, and by immuno-electrophoresis, as described by Pepys *et al.* (1963) and Gregory *et al.* (1964).

RESULTS

Factors affecting self-heating

Quantity of hay. Figure 1 shows that 50 g. hay at 40 % water content in a 1 l. Dewar flask self-heated to only 28°, but 250 g. attained 49°. Larger quantities of hay did not become much hotter, 350 and 500 g. self-heating to 54° and 58°, respectively; 15 kg. in the lagged barrel also reached 58°. The self-heating patterns were very similar, with broad maxima and no subsidiary peaks.

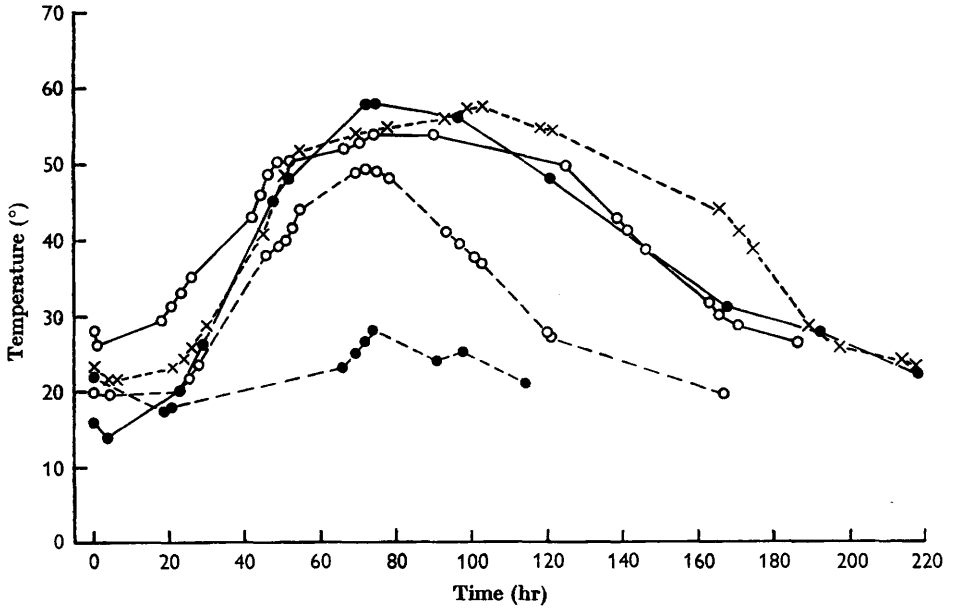

Fig. 1. Progress of self-heating of different quantities of 1961 hay, moistened to 40 % water content, (March 1962–January 1963). --●--, 50 g.; --○--, 250 g.; —○—, 350 g.; --×--, 500 g.; —●—, 15 kg.

Aeration. Aeration was usually by diffusion through the cotton-wool plug at the top of the flask, but in some experiments it was increased by passing air down a tube to the bottom of the flask. The flow rate was measured with a rotameter.

Additional aeration at 25 c.c./min. accelerated the cooling of 350 g. hay after the maximum temperature was reached, but had no effect on the self-heating pattern of 500 g. hay. Figure 2 shows the effect of varying the rate of airflow in a flask with 500 g. hay where entry of air by diffusion was prevented by closing with a wooden lid: the temperature rose to only 44° on aerating at 25 c.c./min., fell to 32° when aeration was stopped, and rose to 57° on starting it again at 40 c.c./min.

Aeration of 1250 g. hay in a 10 l. Dewar flask by diffusion through a cotton-wool plug only, was inadequate for maximum self-heating of the hay towards the bottom

of the flask. Maximum temperatures of 63°, 52° and 50° were attained in the upper, middle and lower parts, respectively; with additional aeration at 50 c.c./min., a maximum temperature of 68° was obtained at all levels. Temperatures found at the bottom of 4 l. flasks closed with cotton-wool plugs were usually a few degrees higher than those in the middle.

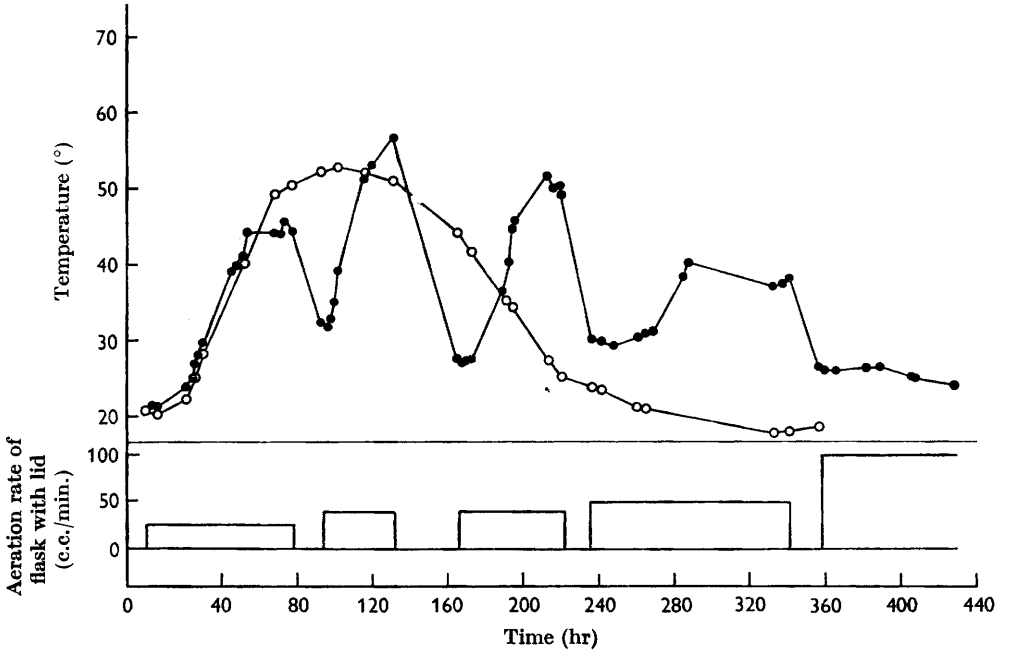

Fig. 2. Effect of aeration on self-heating of 1961 hay (500 g. moistened to 40 % water content; February 1963) ●, Dewar flask with fitting wooden lid aerated as shown; ○; flask with cotton wool plug, aerated by diffusion.

Water content, storage and type of hay. Figure 1 shows the self-heating pattern of 1961 hay in March 1962–January 1963, when there was one broad maximum only. Figure 3*a* shows that in September–October 1963 secondary peaks developed and these were particularly prominent in November 1963, especially at the larger water contents (Fig. 3*b*). Figure 3*c* shows heating patterns for 1962 hay, studied at different water contents, with mostly broad, single peaks as for the earlier 1961 hay. Two other hays examined at 40 % water content showed considerable differences, particularly in the times taken, 45 and 130 hr, respectively, to reach the maximum temperature (Fig. 3*d*). Increasing water content accentuated the heating pattern of any particular hay (Figs. 3*b*, *c*), hays with 30 % or less water content did not heat above 30°, hays with 57 and 68 % water content cooled slowly. Despite the differences in heating pattern, there is a general relationship between the maximum temperatures reached by the different hays and their water contents (Fig. 8).

Microflora in hays of different water content

Fungi. The total numbers of fungus spores released from the hay samples varied considerably (Fig. 4*a*) and peak concentrations reflected the abundance of different

species. Spores were abundant in hays of 28 to 29, 31, 34 and 47 % water content. The relative abundance of Aspergillus species gave a good guide to the initial water content of the hay. *Aspergillus glaucus* was most abundant in hay at 26 % water

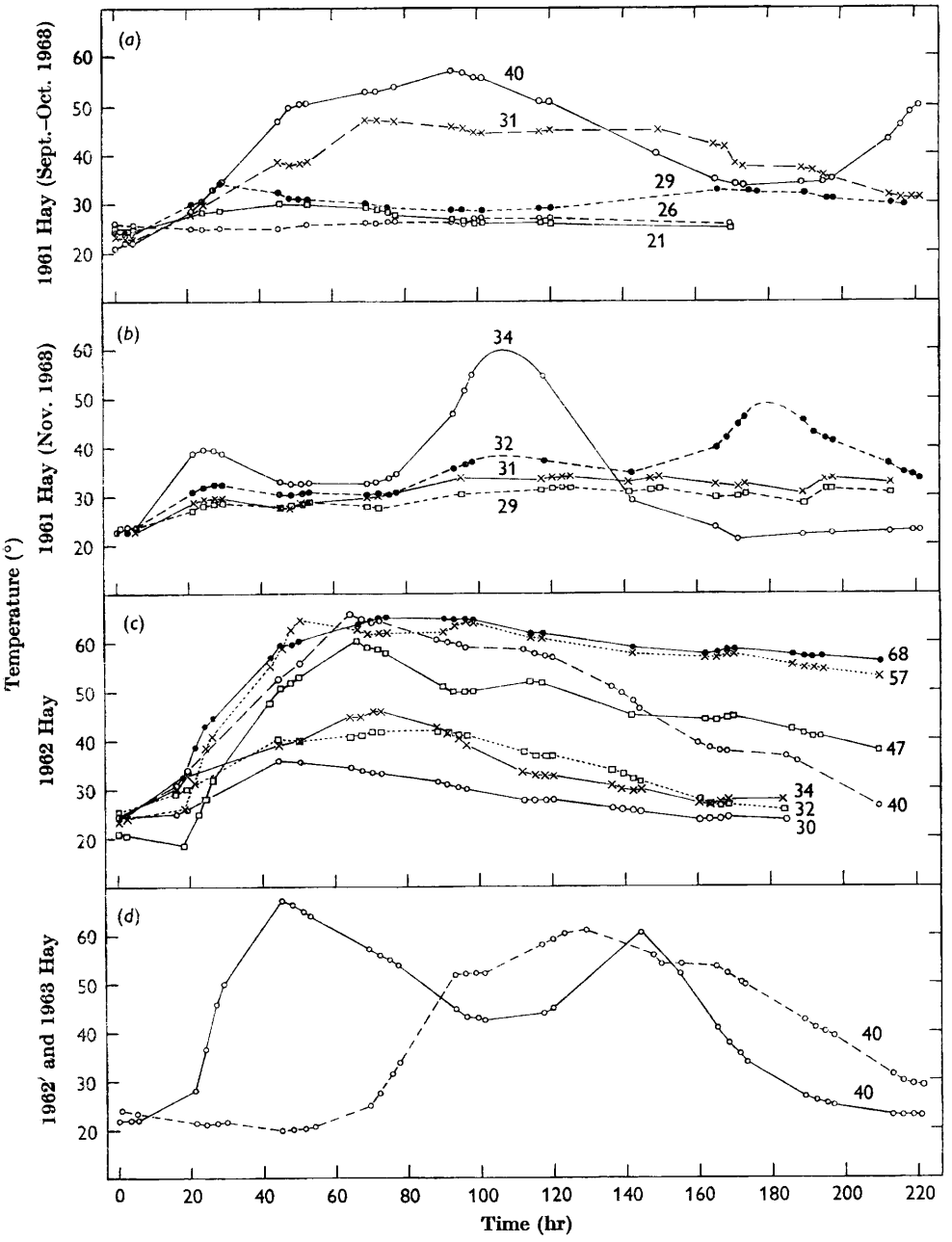

Fig. 3. Self-heating patterns of 500 g. quantities of different hays moistened to different water contents, also showing the effects of storage of 1961 hay (*a*, *b*); (*c*) 1962 hay; (*d*) ----1962′ hay; —— 1963 hay. Figures on curves show water contents.

content (Figs. 4*b*, 5). *A. versicolor* and *A. nidulans* accounted for the peaks of Aspergillus type spores at 28–29 and 31 % water content, respectively, and *A. fumigatus* was most abundant at 40 % water content. Mucor type spores, probably mostly *Absidia* sp. were most abundant in hay at 34 % water content (Fig. 4*b*) and *Humicola lanuginosa* and *Paecilomyces varioti* caused the peak at 47 % on Fig. 4.

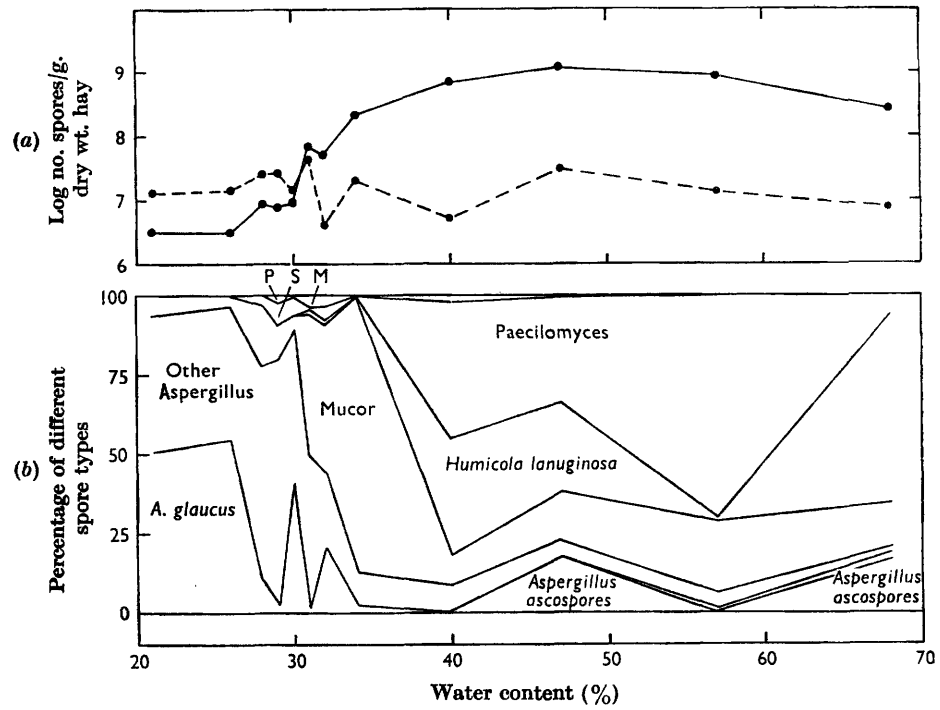

Fig. 4. Microflora in hays of different water content as determined by cascade impactor. (*a*), ---, fungi, ——, actinomycetes and bacteria; (*b*), proportions of fungal spore types. P = Paecilomyces, S = Scopulariopsis, M = miscellaneous.

Other species isolated included *Scopulariopsis brevicaulis* (most abundant at 28–29 % water content), *Mucor pusillus* (40–47 % water content) and occasionally *Malbranchea pulchella* var. *sulfurea*, *Aspergillus flavus*, *A. niger*, *A. terreus*, *Penicillium* spp., *Cladosporium* sp., and *Sporotrichum* spp. The values for the hay of 68 % water content are those in the upper layer, which had to be partly dried before wind tunnel examination and this may account for the apparent increases in spore numbers.

Actinomycetes and bacteria. Actinomycete spores and bacteria, as determined by the cascade impactor, were few in hay containing less than 30 % water (Fig. 4*a*); the hays of 40 % water content contained a mean of 430 million spores/g. dry wt. The most, 1220 million spores/g. dry wt. were at 47 % water content.

Bacteria growing at 25° and 60° were isolated in largest numbers from hay at 47 % water content, and those growing at 40° from hays of 34 % and 68 % water content. Up to 85 % of 40° bacteria from hays of 40 % water content were filamentous colonies of the *Bacillus licheniformis* type.

Andersen sampler plates incubated at 24° and 40° showed *Streptomyces fradiae* to be most abundant in hays at 31% water content (Fig. 6). Few colonies of *Micromonospora vulgaris* or *Thermopolyspora polyspora* were isolated from hays at less than 32% water content; most were isolated from hays at 68 and 47%, respectively. *Thermopolyspora glauca* occurred in a similar way to *T. polyspora*.

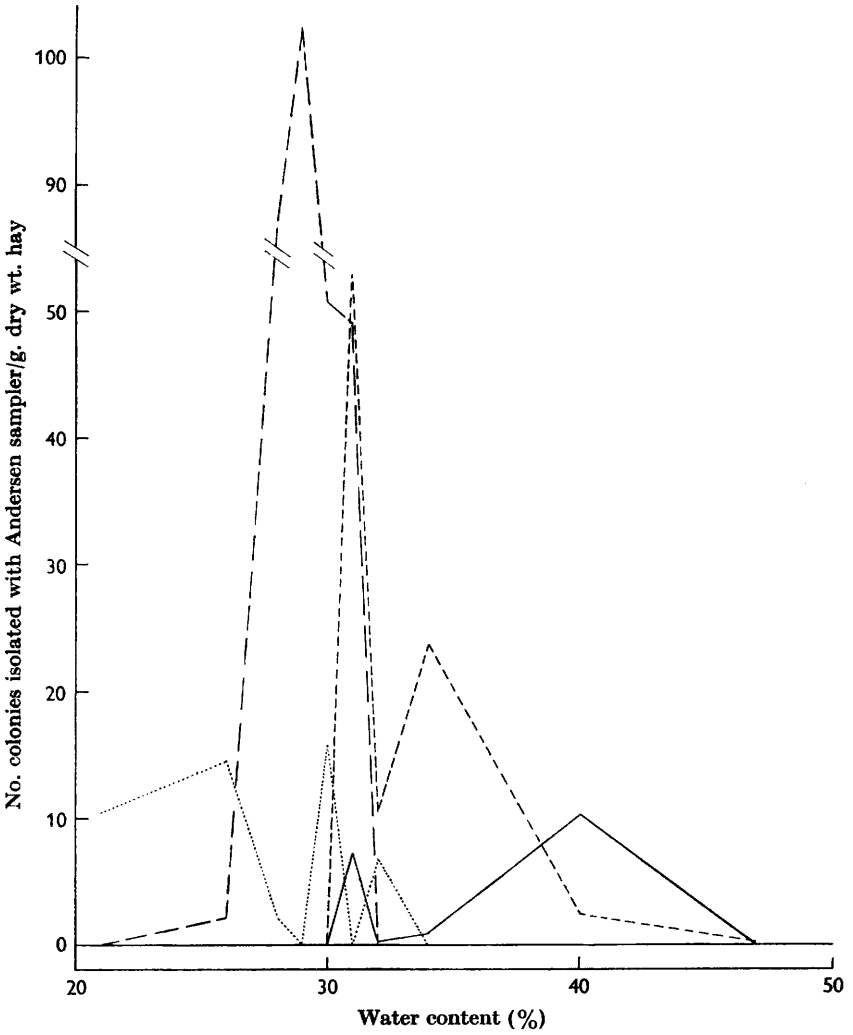

Fig. 5. Occurrence of Aspergillus species in hays at different water contents. —, *A. fumigatus*, — —; *A. versicolor*; ----, *A. nidulans*;....., *A. glaucus*.

FLH antigen

The early experiments with different quantities of hay at 40% water content showed that extracts of the samples of 250 g. and 15 kg. hay reacted in the double diffusion test with 17 and 19, respectively, of 20 sera tested. Extracts of samples of the upper and lower halves of the 350 g. hay reacted with 8 and 19 sera, respectively; the upper layer contained predominantly fungi, especially *Aspergillus* spp.

and *Humicola lanuginosa*, and the lower layer contained these plus many white actinomycetes, which were also observed in the other samples mentioned above.

Effect of water content. Table 1 shows results for 500 g. quantities of hay of water content of 40 % and less. The results are for the middle layers of the flasks only: extracts of the lower layer usually reacted with more sera than did those of the middle layer; extracts of the upper layer reacted with fewer sera, as with the 350 g. sample above.

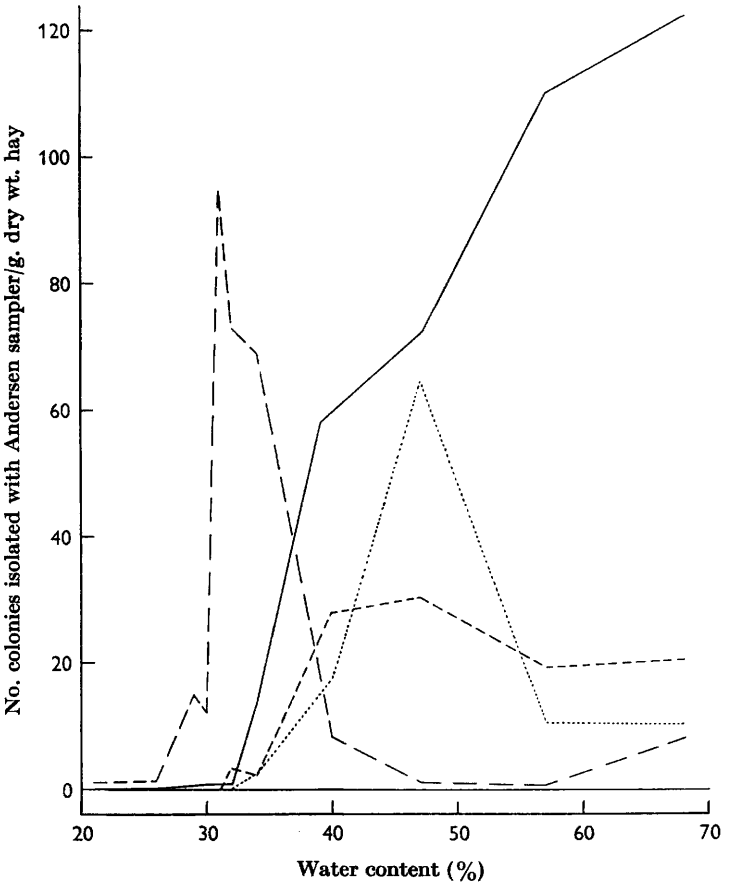

Fig. 6. Occurrence of actinomycete species in hays at different water contents. —, *Micromonospora vulgaris*; ——, *Streptomyces fradiae*; ----, *Thermopolyspora polyspora*; ·····, *Thermopolyspora glauca*.

The hay samples with 40 % water content reached maximum temperatures of 57–66°. Extracts of these samples reacted with 18–20 sera and gave typical immuno-electrophoretic patterns (Pepys *et al.* 1963). Extracts of some hays in the range of 29–34 % water content also gave typical patterns and these hays contained many *Thermopolyspora polyspora*, isolated by the Andersen sampler.

The hays of 29–34 % water content self-heated to 33–55° and differed considerably in their content of FLH antigen. Those hays with less than 29 % water self-heated little and their extracts reacted with few sera in the double-diffusion test.

Only occasional reactions were observed in the immuno-electrophoresis test and these consisted solely of the C line, which is typical of reactions given by fungi, particularly Mucor and by the actinomycete *Micromonospora vulgaris* (Jenkins, 1964). Very few *Thermopolyspora polyspora* were isolated from these samples.

Table 1. *Occurrence of FLH antigen in self-heated hay, classified according to immuno-electrophoretic analysis*

All experiments with 500 g. hay (initial weight). Results for middle layers of Dewar flasks.

Hay reference 1 = 1961 hay (for *a*, *b*, see Fig. 3) 2 = 1962 hay	Water content (%)	Max. temp. (°)	Number reacting sera (max. 20)	Number *T. polyspora* colonies/g. dry wt. hay	pH
		A, B and C lines			
		(i) Strong pattern			
1*a*	40	57	18	31·1	7·0
2	40	66	18	6·0	8·0
1962′ hay	40	61	20	—	7·2
1963 hay	40	67	19	47·2	7·7
1*b*	34	55	19	3·9	8·5
1*b*	32	47	17	6·3	7·6
		(ii) Weak pattern			
2	34	46	11	0·9	7·4
1*b*	31	34	14	0	6·9
1*b*	30	35	14	0	6·9
1*b*	29	32	10	0	6·9
		C lines only			
2	32	42	11	0·4	7·8
1*a*	31	47	6	0	7·3
1*a*	29	35	6	0	7·1
1*a*	28	36	6	0	7·3
		No lines			
1*b*	30	33	7	0·9	7·1
2	30	36	6	0·5	7·5
1*a*	26	31	5	0	7·3
1*a*	21	27	5	0	6·9

Table 2 shows the results for the wetter hays of 47–68 % water content, which self-heated to 61–65°. The more water the hay contained, the less FLH antigen was present, particularly in the lower part of the flask. *Thermopolyspora polyspora*, however, was abundant in all such hays, including the upper layer of the wettest hay, an extract of which reacted with only 8 sera and gave a weak immuno-electrophoretic pattern.

Microfloral succession and development of FLH antigen in hay of 40 % water content

Sequential events during self-heating were studied by filling each of eight flasks with 500 g. 1962 hay and emptying one flask every day. The flasks differed slightly in heating pattern, probably because of differences in packing, but the general

pattern was similar and the series can be regarded as sequential samples in the same experiment (Fig. 7).

Fungi. Fungal spores increased rapidly during the first 2 days when yeasts were predominant (Fig. 7). After 4 days numbers had declined to fewer than 1 million spores/g. dry wt. hay. They remained few until the 9th day, when numbers of *Humicola lanuginosa*, Paecilomyces, Mucor and Aspergillus type spores all increased.

Table 2. *Occurrence of FLH antigen in self-heated 1962 hay of water content between 47 and 68 %*

Water content (%)	Layer of Dewar flask	pH	No. reacting sera (max. 20)	No. *T. polyspora* colonies/g. dry wt. hay	Immuno-electro-phoretic pattern
47	Upper	7·5	18		Strong
	Middle	7·3	18	30	Strong
	Lower	6·7	19		Weak
57	Upper	6·8	18		Weak
	Middle	6·8	16	19	Very weak
	Lower	6·1	1		Negative
68	Upper	7·6	8	20	Weak
	Middle	7·8	4		C lines only
	Lower	7·1	1		Negative

Bacteria. Dilution-plate counts showed that bacteria able to grow at 25° increased from about 1×10^6 to $3{\cdot}7 \times 10^9$ organisms/g. dry wt. hay during the first day (Fig. 7). The colonies were almost all punctiform, buff or orange-yellow and consisted of Gram-positive cocci referrable to Micrococcus, Subgroup 6 (Baird-Parker, 1962). For the first 5 days this type of microflora remained essentially unchanged; although the numbers of bacteria increased still further; thereafter, the numbers declined but the proportion forming filamentous colonies, mostly *Bacillus licheniformis*, increased. After 6 days, filamentous colonies constituted over 68 % of the total, falling to 44 % on the 8th day, when the total numbers had declined to $1{\cdot}5 \times 10^8$/g. dry wt. hay.

On plates incubated at 40°, these filamentous colonies formed a significant proportion of colonies from most samples, though colonies of micrococci were also numerous. Numbers of filamentous colonies changed little throughout the experiment, but their proportion of the total increased up to the fifth day and then declined.

Thermophilic bacteria growing at 60° occurred only in the last three samples and formed 21 % of the total colonies in the last two; most colonies were thermophilic actinomycetes. The Andersen sampler detected a few thermophilic bacteria after 2 days and more from four days onwards.

Actinomycetes. Actinomycete spores and bacteria increased rapidly during the first 2 days, but, after a decline, numbers were still increasing at the end of the experiment (Fig. 7). Andersen-sampler plates indicated that most of the organisms during the early period were bacteria. Few *Micromonospora vulgaris* were isolated at 60° until the third day, but numbers rapidly increased to a maximum after 5 days. *Thermopolyspora polyspora* was first detected after 2 days at 60°, but colonies were

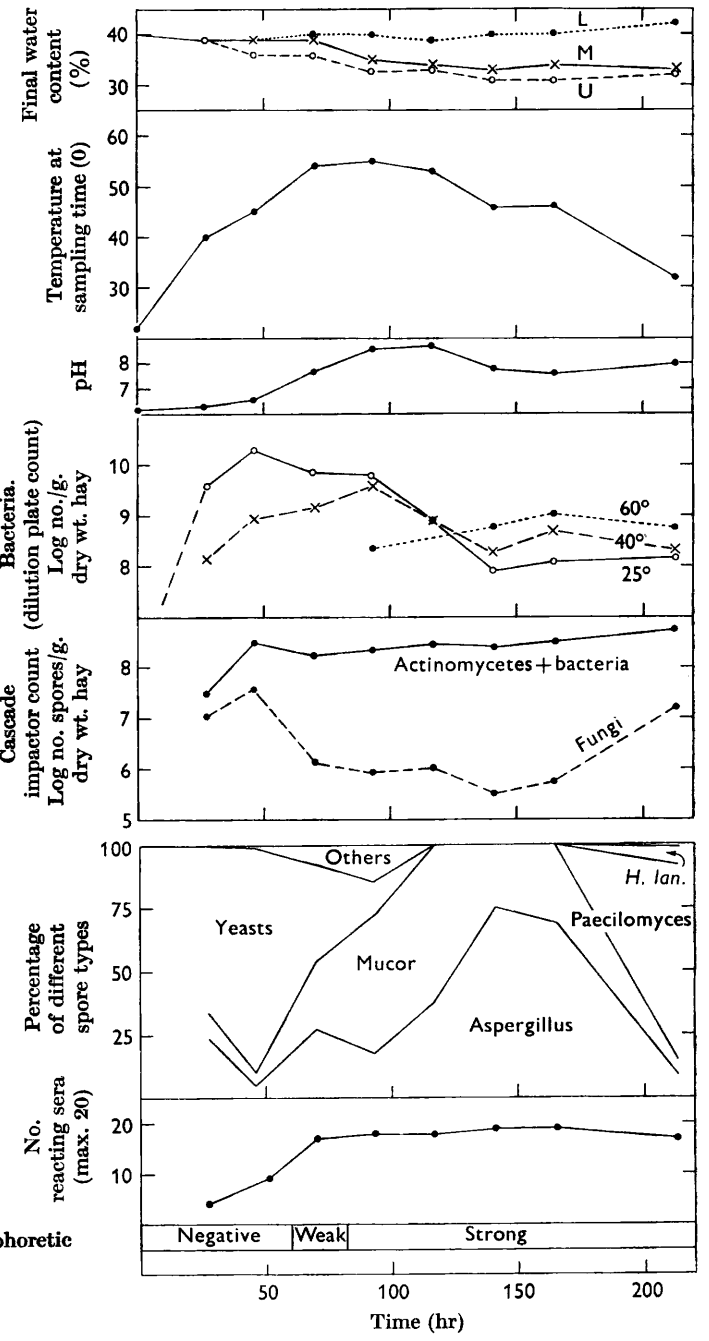

Fig. 7. Microfloral succession and development of FLH antigen in hay of 40% water content. Maximum temperatures of 62–63° were reached in 69–75 hr. L, M and U correspond to the lower, middle and upper layers of the flasks, respectively.

rare until the fourth day and reached maximum numbers on the seventh day. FLH antigen was first detectable on the third day, at the same time as the numbers of actinomycetes isolated became significant. The presence of strong immuno-electrophoretic patterns on the fourth and subsequent days relates well with the concomitant appearance of significant numbers of *T. polyspora*.

Effect of heating and growth of fungi on Thermopolyspora polyspora antigen

Gregory *et al.* (1964) found that the FLH antigen content of sequential samples of baled hay fluctuated. A possible cause of this could have been that extensive development of fungi destroyed the antigen and this possibility was studied in experiments with 50 g. quantities of 1961 hay in 2 lb. Kilner jars, as used by Pepys *et al.* (1963). Sterile ammoniated hay was inoculated with *Thermopolyspora polyspora*, incubated at 40° for 13 days and then inoculated with a mixture of *Absidia* sp., *Aspergillus fumigatus*, *Humicola lanuginosa* and *Mucor pusillus*, and incubated again for 20 days at 40°. Samples both with and without added fungi had a pH of 7·5 and extracts reacted with seventeen sera in the double-diffusion test. Typical immuno-electrophoretic patterns (A, B and C lines) were observed, though the extract of the hay inoculated with fungi was slightly less reactive than the extract of the hay inoculated with *T. polyspora* alone.

Thermopolyspora polyspora grows unevenly on hay in jars, so to ensure its equal distribution, several jars were incubated for 6 weeks at 40°, when areas of hay showing good growth were bulked, well mixed and re-distributed in four jars. Propylene oxide was added to these jars in the usual way to sterilize them and the vapour allowed to disperse (Pepys *et al.* 1963). Two of the jars were then inoculated with the mixed fungi as before, the third treated with sterile water and all three jars incubated at 40°. One jar containing the fungi was removed after 1 week and the other two after 3 weeks. The hays from all these jars as well as from the unincubated jar had similar FLH antigen contents, suggesting that prolonged incubation at 40° with or without concomitant fungal growth, did not alter FLH antigen in any way.

Self heating of barley and oat grain

In the first experiment with barley at a nominal 35% water content, the temperature slowly increased to 58·5° in 13 days, remained between 55 and 59° until 42 days, when it slowly fell to 46·5° at 55 days. There were up to 90 million Aspergillus type spores/g. dry wt. in the upper layers, mostly *A. flavus*. *Humicola lanuginosa* was most abundant in the middle layers (up to 29 million spores/g. dry wt.) but few colonies were isolated and there were few fungi in the lower layers of grain. Actinomycete spores and bacteria were most abundant in the middle and lower layers, with *Streptomyces fradiae* predominant near the surface and *Thermopolyspora polyspora* and an unidentified white actinomycete in the middle. Most *Micromonospora vulgaris* colonies were isolated towards the bottom, but this species was otherwise not abundant.

Immuno-electrophoresis showed that FLH antigen was present in extracts of barley taken from the middle layers of the Dewar flask, with pH 7·2–7·4 (Table 3). The layers above were more alkaline (pH 8·1 and 8·4) and extracts contained less

antigen; extracts of the lowest layers, with 53 and 58 % final water content, did not contain detectable amounts of antigen, but gave a non-specific reaction with all sera tested.

A second experiment with barley at 33 % water content which ended after 7 days, showed a similar slow rise of temperature as the first to 43·5°. *Aspergillus flavus* was similarly abundant in the upper layer, but the number of Penicillium colonies

Table 3. *Occurrence of FLH antigen in self-heated barley and oats grain*

Weight of sample (g.) (final wet weight)	Final water content (%)	pH	No. reacting sera (max. 20)	Immuno-electrophoretic pattern
(Expt. 1) 2·5 kg. barley (14 % water content) + 800 ml. water				
104 (top)	12	6·3	6	Negative
155	18	6·9	10	Negative
140	17	7·5	13	Negative
333	23	8·1	16	Very weak
176	27	8·4	16	Weak
212	24	7·4	17	Strong
168	26	7·2	17	Strong
291	26	7·3	18	Strong
170	38	7·3	14	Very weak
229	53	6·9	12	Negative
389 (bottom)	58	6·9	1	Negative
Control	14	6·6	—	—
(Expt. 2) 2·5 kg. barley + 700 ml. water				
242 (top)	—	—	—	—
471	30	6·4	13	C line only
1690	31	6·3	14	C line only
446	44	6·4	11	C line only
280 (bottom)	46	6·5	10	Negative
(Expt. 3) 2·5 kg. barley soaked with 1000 ml. water				
153 (top)	—	—	—	—
246	20	7·1	10	Negative
1326	33	7·1	19	Strong
1106 (bottom)	40	7·1	18	Strong
2·5 kg. oats (12 % water content) soaked with 1000 ml. water, 2·767 kg. moistened oats placed in Dewar flask				
32 (top)	—	—	—	
84	—	6·3	17	Very weak C line only
100	—	6·3	15	Very weak C line only
492	20	6·5	19	Weak C line only
293	23	6·5	19	Weak
756	28	6·6	15	Weak
239 (bottom)	38	6·6	11	Negative
Control	12	6·4	12	Negative

isolated increased with increasing depth and probably accounted for most of the 19 million/g. Aspergillus and Penicillium type spores in the lowest layer. *Absidia* sp. was also abundant in the upper layers. Actinomycetes increased with increasing depth nearly to the bottom of the flask, but decreased again in the lowest layer. *Streptomyces fradiae* and unidentified white and grey colonies were most abundant, with a few *Micromonospora vulgaris* towards the bottom. FLH antigen was again

not detectable in extracts of the wettest lower region, with 46% final water content. Extracts of the other layers yielded only C lines which, considered in conjunction with the few *M. vulgaris* isolated, and the low pH values of the grain, may have been fungal in origin.

A third sample of barley was allowed to stand with water for 5 hr to increase its water content to 40%. It heated more rapidly than the previous samples; the temperature exceeded 60° on the eighth day and reached a maximum of 66° after 12 days. It was still over 60° when the experiment ended after 19 days. Aspergillus, Mucor and *Humicola lanuginosa* spores were all abundant in the upper layers and many *Aspergillus fumigatus* were isolated. Actinomycete spores and bacteria were most abundant in the middle layers, *Streptomyces fradiae* near the top, *Thermopolyspora polyspora* in the middle and *Micromonospora vulgaris* most abundant near the bottom of the flask. FLH antigen was not detectable in the upper layer, but strong reactions were obtained with the remainder of the grain. The lowest layer (pH 7·1) had 40% final water content and despite its large bulk (1106 g.), was uniform in appearance to the bottom of the flask. Extracts of this and the next layer above it reacted with 18 and 19 sera respectively in the double-diffusion test and both extracts gave typical patterns in the immuno-electrophoresis test.

A sample of oat grain was also allowed to absorb water to reach a water content of 37%. The temperature, which rose faster than in any of the barley samples, reached 60° after 3 days and a maximum of 64° after 10 days. It remained near 60° for a further 23 days, but then fell below 50° during the next 3 days. The pH of the grain at the end of the experiment was 6·3–6·5. *Aspergillus fumigatus* was abundant in the upper half of the flask, whereas *A. nidulans*, *Absidia* sp. and *Humicola lanuginosa* were most numerous (up to 14 million spores/g. dry wt.) in the middle layers. Actinomycetes, also most abundant in the upper half of the flask, were more abundant (up to 568 million/g. dry wt.) than in any of the barley samples. *Streptomyces fradiae* was again most abundant in the upper layers, whereas *Thermopolyspora polyspora* and *Micromonospora vulgaris* were most numerous towards the middle, with the maximum population of *M. vulgaris* slightly higher up the flask than that of the *T. polyspora*. The lowest layer with 38% final water content contained no detectable antigen and the upper layers gave reactions in the C region only in the immuno-electrophoresis tests. FLH antigen giving A, B and C lines was found in the middle of the flask. As with the second barley experiment, the scarcity of *M. vulgaris* and the low pH suggested that the C lines may have been fungal in origin.

DISCUSSION

The initial studies on the conditions favouring self-heating in Dewar flasks led to the choice of 500 g. hay in a 4 l. flask plugged with cotton-wool as the most suitable for studying the effects of varying the water content.

The importance of oxygen for the self-heating process was first shown by Miehe (1907) and also by Hildebrandt (1927); Carlyle & Norman (1941) found that a cotton plug gave satisfactory aeration of oat straw in a quart Dewar flask, with least likelihood of heat loss. The efficiency of the cotton-wool plug in allowing diffusion of air was shown by the experiment where the wooden lid was substituted

(Fig. 2). The effects of alternately starting and stopping the air flow were large, as noted by Glathe (1952) in similar experiments with chopped hay.

The maximum temperatures of 57–67° reached by 500 g. quantities of hay at 40 % water content (Fig. 3) were similar to those in field experiments with bales of 40 % water content and a stack of 30 % water content (Gregory *et al.* 1963). There was no special advantage in using 1250 g. hay in a 10 l. flask, which self-heated to similar temperatures, but required extra aeration for the hay in the lower part to heat adequately.

Table 4. *Predominant fungus and actinomycete species in hays allowed to mould at different water contents*

Water content (%)	Predominant species
26	*Aspergillus glaucus* group
28–29	*A. versicolor*, *Scopulariopsis brevicaulis*
31	*A. nidulans*, *Absidia* sp., *Streptomyces fradiae*
40	*A. fumigatus*, *Humicola lanuginosa*
47	*Humicola lanuginosa*, *Thermopolyspora glauca*, *T. polyspora*
57	*Micromonospora vulgaris*, *Paecilomyces varioti*

The final products of the Dewar flasks hays with 40 % water content were all visibly mouldy throughout the mass. The pH values of the hays were usually near 7, or above (Table 1), resembling hay from mouldy bales (Gregory *et al.* 1963) rather than brown hay from the centre of a self-heated stack, with pH values of 5 or below, which does not contain FLH antigen (Gregory *et al.* 1964). Miehe (1911) described the hay from his laboratory experiments as also quite unlike the brown hay of self-heated haystacks. The findings from our experiments, therefore, seem to apply to wet bales rather than to wet stacks, except for those particular regions in the stacks where mould had developed (Gregory *et al.* 1963).

Hildebrandt (1927) was the first to examine the microflora of self-heated hays from Dewar flasks and he isolated *Actinomyces thermophilus*, *Aspergillus fumigatus* and *Bacillus calfactor*, all previously identified by Miehe (1907) in hay. Many more species were isolated in our experiments, although *A. thermophilus* and *B. calfactor* were not recognized. Inocula of most species must be widely distributed on hay, because the same type of moulding occurred in different hays wetted to the same water content. Table 4 shows the predominant species arising at the different water contents. Thus from a knowledge of the *Aspergillus* spp. and actinomycetes present on a sample of mouldy hay, the initial water content and maximum temperature during self-heating can be assessed.

Water content is also critical for producing FLH antigen and Fig. 8 relates all the results in terms of water content. Hay of 40 % water content invariably produced FLH antigen (Table 1) and very many *Micromonospora vulgaris* and *Thermopolyspora polyspora*. Hay of 29–34 % water content gave FLH antigen in only some experiments; this range of water content is critical, because the maximum temperature reached, the numbers of thermophilic actinomycetes and the antigen content all increased greatly with small increases in water content. Hay of less than 29 % water content, which developed only C lines, showed no *T. polyspora* but there

were a few *M. vulgaris*. Hay with less than 25 % water seems unlikely to develop FLH antigen. These conclusions accord with the results of field experiments of Gregory *et al.* (1964), who found that hay baled at less than 30 % water content did not develop FLH antigen readily.

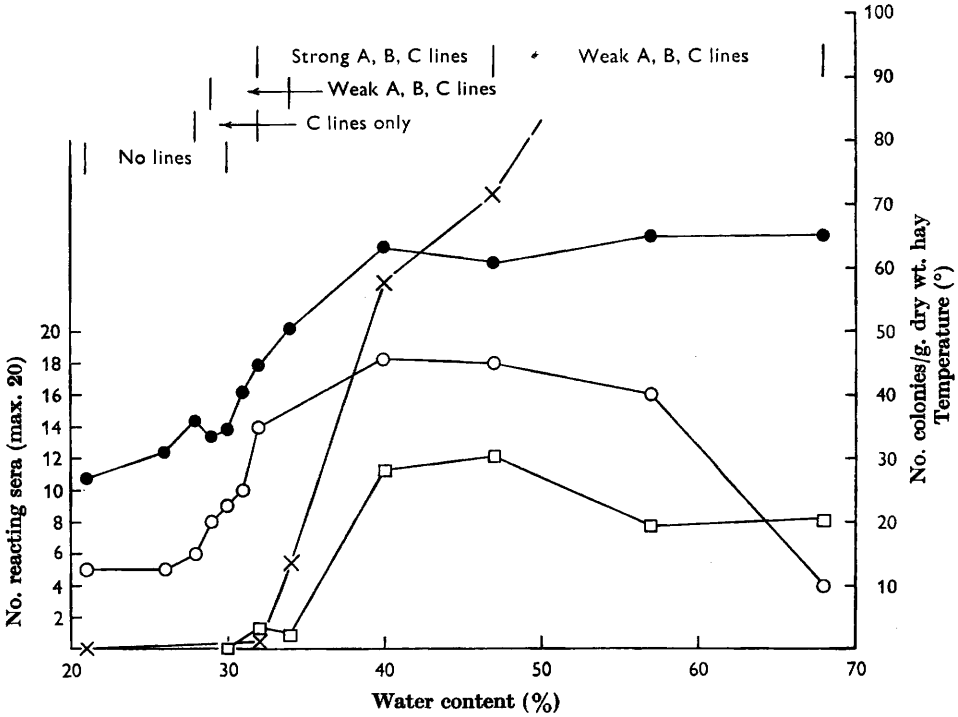

Fig. 8. Summary of results in relation to water content of hay and occurrence of FLH antigen. ●, maximum temperature; □, *T. polyspora*; ×, *M. vulgaris*; ○, No. reacting sera.

With the hays of water content of 40 % and less, extracts from the lower regions of the flask reacted with more sera than those from higher up. The upper half of the flasks dried faster than the lower half (Fig. 7), so the upper layers may have become too dry to support microbial growth. In contrast, with hays of 47–68 % water content, the wetter the hay, the less FLH antigen was produced (Fig. 8), particularly in the bottom of the flask (Table 2). Restricted aeration and more water in the lower layers may have adversely affected the growth of *Thermopolyspora polyspora*, which were fewer the wetter the hay (Fig. 8), but the decrease in FLH antigen seems large by comparison. *Micromonospora vulgaris* was most abundant in these wet hays.

Differences in heating pattern of hay apparently influence FLH antigen production. Table 1 shows that the old 1961 hay with multi-peaked heating pattern produced antigen more readily than 1962 hay with only one temperature maximum (Fig. 3). This may be a function of the type of hay, its age and manner of storage, or the amount of inoculum present. Fig. 3 shows that the wetted 1962′ hay took as long as 70 hr before it began to heat, but the sequential sampling study with 1962 hay (Fig. 7) showed that FLH antigen appeared after 70 hr, when the temperature

had reached 54° and was still rising. Temperature is obviously a better criterion than time after wetting of the hay to assess when antigen is likely to develop. In their field experiments done with fresh grass after cutting and partial drying, Gregory *et al.* (1964) showed that FLH antigen was detectable 4–6 days after baling, which fits in well with the results for the Dewar flask experiments done with rewetted hay.

FLH antigen can be produced in as little as 250 g. hay (Fig. 1), so that even small pockets of hay in a bale or stack, given the right conditions of water content, aeration and thermal insulation, may mould and produce antigen.

Grain can also self-heat when wet and develop a considerable microflora, including the thermophilic actinomycetes forming FLH antigen. Evidently, as with hay, FLH antigen occurs in a critical range of water content. The minimum water content for moulding of various materials including cereals, meat and textiles is generally accepted as that in equilibrium with a relative humidity of 75% (Milner & Geddes, 1946). The equilibrium water content depends on the nature of the material and Snow, Crichton & Wright (1944*a*, *b*) estimate the safe levels for short periods of aerobic storage for wheat and oat grain and hay, as 15·7, 14·5 and 12·6% respectively and as 14·6, 13·4 and 11·0% respectively for long periods (at 18–20°). Christensen & Linko (1963) suggest 14·3% as the highest water content for storing wheat safely. Waite (1949) emphasizes the importance of temperature, which affects mould growth. Hay kept at a relative humidity of 85% (22% water content) and a temperature of 6° (winter conditions) moulded only after 120 days, whereas at 15° (summer temperature) it moulded after 2 days; under summer conditions, 75% relative humidity (15% water content) and 15°, the hay did not mould. Barley and oat grain will start moulding at similar water contents to those for hay, and the safe minimum water-content for grain, to avoid producing FLH antigen, is probably the same as for hay, assessed as 25%, but this has yet to be determined.

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APPENDIX ON TAXONOMY

BY J. LACEY

A comment is necessary on the taxonomy of some of the species isolated. To aid comparison with our earlier publications on moulding of hay and farmer's lung disease, the same names have been used throughout, although we recognized that for some species other names are preferable. Apinis (1963) and Pugh, Blakeman & Morgan-Jones (1964) propose that *Humicola lanuginosa* (Griffon & Maublanc) Bunce 1961 should be referred to *Thermomyces lanuginosus* Tsiklinsky, 1899. However, Griffon & Maublanc (1911) and Bunce (personal communication) regarded Tsiklinsky's description as insufficient to delimit the species, and Cooney & Emerson (1964) point out that the photographs, if the stated magnification is correct, show spores smaller than 3·6 μ diam. instead of the 6–10 μ of *H. lanuginosa*. Also, the photographs show only smooth spores, which are either immature, or if mature are more like *H. grisea* var *thermoides* Cooney & Emerson than the sculptured aleuriospores of *H. lanuginosa*. Tsiklinsky (1899) proposed the name *Thermomyces lanuginosus* provisionally 'en attendant que j'en aie étudié la morphologie' but no subsequent description by her has been found. Identification of isolates with *T. lanuginosus* would seem to be largely conjectural, the few known species of thermophilic fungi, and the abundance of *H. lanuginosa*, making it most likely that Tsiklinsky isolated the same species. It is questionable, however, whether such conjecture is a good basis for a taxon, and we follow Cooney & Emerson (1964) in retaining the name *Humicola lanuginosa* proposed by Bunce (1961).

A new species *Mucor miehei* was described by Cooney & Emerson (1964). This has not been distinguished from *M. pusillus* Lindt in our work, although we have now isolated it from hay. The Absidia species is probably referable to *A. corymbifera* although some isolates resembling *A. ramosa* were found and a range of intermediate forms.

Micromonospora vulgaris Waksman, Umbreit & Cordon, 1939 and *Thermoactinomyces vulgaris* Tsiklinsky 1899 are synonymous (Küster & Locci, 1964) and the latter name is generally preferred, although some of the same objections can be raised to it as to *Thermomyces lanuginosus*.

Thermopolyspora glauca Corbaz, Gregory & Lacey, 1963 is a synonym of *Thermomonospora viridis* (Schuurmans *et al.*) Küster & Locci, 1963, but the colour of the aerial mycelium could perhaps be better described as grey-blue rather than grey-green, and some of our isolates also produce black or dark brown soluble pigment. Isolates described by Corbaz *et al.* (1963) as *Thermopolyspora polyspora* have been examined by Henssen who considers it to be distinct from *T. polyspora* Henssen, 1957. It should probably be referred to *Micropolyspora* Lechevalier, Solotorovsky & McDermont 1961.

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