tube growth on nutrient agar was 40% greater than growth on plain agar at 8:30 A.M., and 236% greater

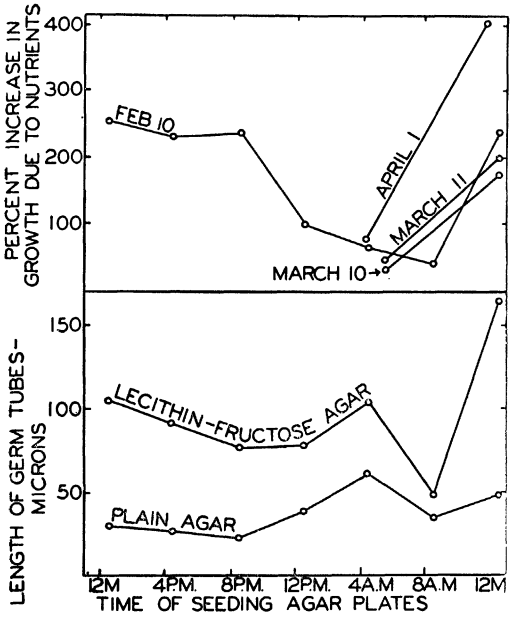

FIG. 1. Diurnal cycle of responsiveness of conidia of *Erysiphe graminis* to lecithin-fructose agar. Each value is the average of 20 germ tubes. Data of the 2 lower graphs are for February 10–11, 1949. The weather was cloudy before and during these tests and also during those of March 10 and 11. The weather was clear before and during the test of April 1.

than on plain agar at 12:30 P.M. In three subsequent trials, an attempt was made to compare only the approximate peak and trough of this diurnal cycle of responsiveness to nutrients. In these trials, the increase in germ tube length due to nutrients averaged 56% for seedings made from 4:15 to 5:30 A.M., and 244% for seedings made from 11:50 A.M. to 12:20 P.M. (see Fig. 1). In all trials, germination ranged between 25% and 92% and showed no apparent diurnal cycle and no apparent correlation with germ tube length.

Concurrent attempts to study in a similar way germ tube growth *in vitro* of *Erysiphe polygoni* from bean, and *Uncinula necator* from grape (both fungi show a clear diurnal cycle of conidiophore maturation, and the bean fungus also shows diurnal variation in sensitivity to copper sulfate [*3*]) failed to evince any marked response to the lecithin-fructose medium at any time tested. This indicates that there are specific differences between closely related species of powdery mildews with respect to nutrient response.

As the principal index of the nutrient value of a test chemical is the difference in growth of germ tubes on media with and without the test chemical, this diurnal responsiveness to nutrients, as in the germinability of the conidia of *E. polygoni* (*1*), is apparently the result of diurnal changes in the fungus while grown on the host plant before use in these tests. Diurnal responsiveness may be of considerable importance in studies of the growth *in vitro* of some powdery mildews.

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A New Method for the Study *in Vitro* of Rumen Digestion

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The utilization of cellulose has long been recognized as a primary factor in the nutrition of ruminants and particularly so among those subsisting largely on roughages. It is believed, therefore, that the rate of cellulose digestion *in vitro* by the microorganisms of the rumen may be used as a measure of how nearly it is possible to approach cellulose digestion *in vivo*.

A technique for the study of digestion in the rumen *in vitro* has recently been published by Marston (*3*). His procedure appears to simulate more closely the natural environment in the rumen than any earlier investigations. There is, however, one significant omission. No provision is made for the removal of nongaseous fermentation products which, as they accumulate, might be expected to slow the rate and eventually inhibit digestion.

To test the influence of such an accumulation of breakdown products, cellulose digestion was compared after incubation with fresh rumen fluid in a closed vessel (the technique of Marston), and in a semipermeable bag suspended in a large volume of aqueous growth medium.

Samples of rumen liquor were collected by scooping semisolid material from the lower regions of the rumen of a fistulated cow and straining this through three layers of cheesecloth, pressing the residue by hand directly into a glass container in which the air had previously been replaced by nitrogen or carbon dioxide. To minimize heat losses, the receptacle was wrapped in paper. After thorough mixing, 700-ml aliquots of this liquor were incubated with 12.0 (and later 20.0) g of Whatman #12 filter paper (ground in a Wiley mill to pass the 0.5 mm sieve), 15.0 ml of 1 M KH_2PO_4, and 7.5 ml each of 2 M $(NH_4)_2SO_4$, 1 M $MgSO_4$ and 0.5 M $CaCl_2$ in a glass bottle and in a semipermeable bag, using the type of apparatus presented in Fig. 1. Two fermentations were conducted at one time in duplicate apparatus, differing only in vessel *B*, which in one was a glass bottle, and in the other a "Visking" cellulose sausage casing of 4½ in. diam. In both, vessel *B* is closed by *a*, which is a wood stopper surrounded by a rubber gasket and coated with wax, and suspended in a 12 × 12-in. water bath *H*. Tubes *c* and *d* serve as inlets and outlets for gas, respectively; tube *e* is connected to a burette containing 0.5 M Na_2CO_3; *g* is a mercury-sealed shaft of an adjustable speed stirrer;

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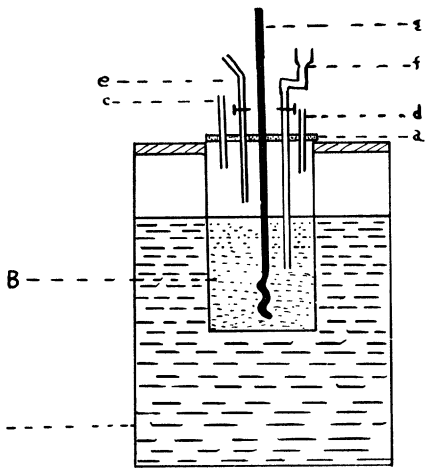

FIG. 1. Apparatus for study of rumen digestion *in vitro*.

and *f* is a tube fitted with a stopcock and a reservoir into which fluid can be drawn for pH determinations. The temperature of the water baths was thermostatically controlled to a temperature of 39° C. After Experiment 1, a current of nitrogen gas replaced the motor-driven stirrer in the bath carrying the semipermeable bag. This ensured adequate mixing as well as improved anaerobic conditions.

Prior to commencing an experiment, tube *c* was pushed well down into the fluids in *B* and a rapid current of nitrogen containing 5% carbon dioxide passed through for about 3 min, after which the tube was raised above the surface of the fluid and the gas flow maintained at a low rate for the duration of the experiment. The pH of the fluids in *B* was then adjusted to 6.8, by running in alkali through *e* while stirring. The inorganic salt concentrations and pH were adjusted in the water bath to the levels inside the semipermeable bag, and the fermentation allowed to proceed at 39° C in both the glass bottle and in the semipermeable bag for a period of 24 hr at or near a pH of 6.8.

The frequency and amount of stirring to which the fermenting fluids were subjected varied with the different experiments, as indicated in Table 1. The pH was adjusted to about 6.8 at 2-hr intervals. As the pH seldom dropped below 6.3 in the course of 2 hr, more frequent adjustments were deemed unnecessary.

TABLE 1

In Vitro DIGESTION OF CELLULOSE

Experiment	Semipermeable bag			Glass bottle		
	Wt in g of cellulose			Wt in g of cellulose		
	At start	Residue	Digested	At start	Residue	Digested
1*	11.89	5.00	*6.89*	11.89	6.91	*4.98*
2*	11.58	3.77	*7.81*	11.58	6.56	*5.02*
3†	19.07	3.42	*15.65*	19.07	10.26	*8.81*
4‡	19.36	6.10	*13.26*	19.36	8.12	*11.24*

* Stirred for 1 min at 1-hr intervals.
† Stirred for 1 min at ½-hr intervals.
‡ Stirring in glass bottle the same as in Experiment 3; stirring in the bag for 3 min at ½-hr and for 10 min at 2-hr intervals.

At the end of 24 hr, the "brews" were made up to definite volumes, well shaken, and aliquots, usually 50 or 100 ml, taken for the determination of residual cellulose using the method of Crampton and Maynard (*1*). The cellulose content of the rumen liquor before incubation was also estimated.

The production of volatile fatty acids was determined in the final experiment, 4. The volatile acids in the steam distillates were transferred to 25 ml of chloroform containing 10% butanol (CB_{10}), according to a method followed by Elsden (*2*). Total acids were then titrated with 0.1 *N* $Ba(OH)_2$ solution, using phenol red as indicator. Finally, 1-ml aliquots of the CB_{10} extracts were used to separate the total volatile acids chromatographically, according to the method of Peterson and Johnson (*4*), into a propionic acid fraction representing the portion eluted by benzene, and an acetic acid fraction representing the portion eluted by CB_{10}.

Some preliminary data on cellulose digestion during 24 hr are presented in Table 1. In comparison, calculations from Marston's (*loc. cit.*) data showed that he obtained cellulose digestion to the extent of 5–7 g.

The variable, so far as could be determined, was the type of container in which the digestions were conducted. Therefore, the differences in favor of the semipermeable bag recorded in Table 1 for all four trials appear to be due only to an inhibitory influence of fission products accumulating in the glass bottle. And if this is so, differences in rates of fermentation may be expected to be greater, the higher the activity of the particular sample of rumen liquor incubated. This is borne out by the results of Experiments 1–3. Precisely what led to these differences in activity in samples of rumen fluid collected, admittedly on different dates, but at the same time of day from the same animal on the same dietary regime, it is not possible to say at present. Mention must be made, however, of the probability that the ideal, according to which the transfer of the rumen liquor from the living animal to the artificial environment should be undertaken with the least delay, exposure to air, and loss of heat, was progressively approached from Experiments 1–3.

For reasons not connected with this work the ration of the cow was changed about a week prior to Experiment 4, from hay plus a concentrate to hay only. Judged by the frequency of certain characteristic churning movements set up periodically in the fermenting liquors by gas accumulating in the cellulose settling gradually to the bottom of the containers and then escaping to the surface, the rumen sample obtained after this change of diet was particularly active. This conclusion was supported by the comparatively high rate of cellulose breakdown registered for the glass bottle in Experiment 4. The rate of fermentation in the semipermeable bag again exceeded that in the glass bottle but not to the extent that might have been expected from results of Experiment 3. An explanation may be the amount of stirring used, as noted in Table 1. The higher rate of stirring applied to the semipermeable bag may have had an adverse effect, in

view of Marston's observation that rapid and frequent stirring caused decreased fermentation.

Results of the analysis of the fermentation products obtained in Experiment 4 are presented in Table 2. These data indicate that: (1) acetic and propionic acids are

TABLE 2

VOLATILE ACIDS IN "BREWS" AND DIALYZATE AFTER 24-HR FERMENTATION (G)

Acid	Introduced with 700 ml rumen liquor	Glass bottle	Semipermeable bag		
			Inside	Outside	Total
Propionic ..	1.56	5.11	2.58	2.64	5.22
Acetic	2.26	5.28	2.22	2.38	5.60
Total	3.82	10.39	4.80	6.02	10.82

formed in the same proportions, irrespective of whether fermentation is carried out in the glass or cellophane container; (2) apparently less total volatile acid/g of cellulose digested is formed in the semipermeable bag than in the glass bottle, and (3) acetic acid diffuses at a greater rate from the bag than does propionic acid.

In conclusion it may be mentioned that there was no microscopic evidence of attack by the ruminal organisms on the cellophane bag. This is indeed fortunate, and may be explained on the basis that attack on cellulosic materials usually begins at broken or torn ends and no such surfaces were exposed in these experiments.

This work is being continued to explore ways of improving the technique.

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A Simple Universal Dairy Products Phosphatase Test

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Dairy products vary quite widely in character and composition. For example, there is the extreme contrast that exists between whole milk and ripened cheese. This variability has affected the applicability of standard phosphatase methods to various dairy products. Until recently, the Kay-Graham phosphatase test could not be used for ripened cheese or for chocolate milk products because interfering substances in them turned blue with the Folin-Ciocalteu reagent, resulting in false positive readings.

In addition, in all present-day phosphatase tests, including Sanders and Sager's (*3*), the concentration of the buffer and precipitating agent has to be shifted for many of the dairy products having different buffer capacities. This has led to a great deal of added effort and much confusion.

TABLE 1

SENSITIVITY OF THE CORNELL PHOSPHATASE METHOD WITH FRESH WHOLE MILK AND 15-MONTH-OLD CHEDDAR CHEESE

Milk	Phenol γ/0.5 ml*	Cheddar cheese	Phenol γ/0.25 g*
Pasteurized†	1.3	Made from	
" + 0.1% raw	14.2	pasteurized	
" + 0.4% raw	47.0	milk	1.2
" + 1.0% raw	64.0	" + 0.1% raw	8.4
		" + 0.4% raw	22.0
		" + 1.0% raw	30.0

* Tentatively, any value over 5.0 γ phenol/0.5 ml milk or 0.25 g cheese is considered underpasteurized.

† Pasteurized milk for this test is milk heated to at least 143° F for 30 min.

During the past year, an ether extraction technique has been evolved for the elimination of all interfering substances in dairy products when using the Kay-Graham phosphatase method with the Folin-Ciocalteu reagent (*1*). This work also brought out the fact that the barbital buffer used in the Kay-Graham test was unsatisfactory. As a result, a highly concentrated sodium carbonate-sodium bicarbonate buffer was substituted, with excellent results (*2*).

Though the extraction process proved highly satisfac tory in improving the Kay-Graham test, information obtained during this investigation pointed toward the development of a very simple procedure for all dairy products without extraction when using 2,6-dibromoquinonechlorimide (BQC) as the color indicator.

The method used in this new test is to incubate dairy product samples with a sodium carbonate-sodium bicarbonate buffer substrate at pH 9.5–9.7 for a period of time, then to precipitate the protein with an acid precipitant. After filtration, the filtrate is brought back to an alkaline reaction with carbonate, and BQC is then added. Blue color is produced if sufficient phosphatase was present in the milk during incubation to split the added disodium phenyl phosphate in the buffer substrate.

This test differs from others in that (1) the buffer and precipitating agent are of different nature, (2) only one concentration of buffer and precipitating solutions is required for all dairy products, and (3) it is not necessary to heat-inactivate the phosphatase enzyme after incubation, since the precipitating agent reduces the milk-substrate solution to pH 1–2.

The details of the method are briefly outlined as follows: 1 ml of milk or fluid dairy product or 0.5 g of cheese or other solid dairy product is mixed with 10 ml of warm carbonate buffer substrate (sodium carbonate 11.5 g; sodium bicarbonate 10.5 g; disodium phenyl phosphate 1.09 g/l).

The test tubes are then incubated at 32–37° C for 18–24 hr. After incubation, 1 ml of an acid precipitant