

EVALUATION OF THE CONTRIBUTION OF FORAGE MATERIAL TO THE *IN VITRO* CULTURE OF ISOTRICHA

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ABSTRACT.—In order to define more clearly the contribution of forage materials to the *in vitro* culture of *Isotricha* several concentrations of dehydrated alfalfa meal were compared with similar concentrations of corn seedlings of unique chlorophyll status: normal, albino mutant, and etiolated. The ciliates were obtained from strained rumen fluid by gravitational procedures after incubation with fructose (3% w/v). A modified culture medium with no rumen fluid maintained *Isotricha* populations for as long as 30 days. A comparison of 0, 10, 30, and 50 mg of the alfalfa meal per day showed that population numbers and days of culture life were proportional to the amount of alfalfa provided. Mutant (one gene A/a), albino and etiolated corn seedling significantly depressed ciliate cultures as compared with alfalfa meal, which was significantly superior to normal corn seedling in maintaining these populations. The growth factors appear to be associated with chlorophyll or photosynthetic products.

The role of ciliate populations in the rumen has been difficult to evaluate because of the failure to obtain an axenic culture or to obtain growth in chemically defined culture media, as for example, has been possible in the case of *Tetrahymena*, Kidder (1951) with one exception, Samoya (1953). Those extended *in vitro* cultures of rumen ciliates reported to date contained chlorophyll material

or rumen fluid in the medium, as well as rumen bacteria. Insofar as the diet of the rumen fluid donor animal includes green plant material it can be assumed that the rumen fluid is also a medium rich in these constituents. It is evident that there is further need for clarification of the growth factors which are contributed by these constituents. It was, therefore, the objective of this investigation to further define the nature of the chlorophyllous plant material required for the *in vitro* culture of the holotrich genus, *Isotricha*. These ciliates of the order Trichostomatida have been cultured previously for limited periods of time; Sugden and Oxford (1952), Gutierrez (1958), Quinn *et al.* (1962), Purser and Weiser (1965), and Clark and Hungate (1966).

MATERIALS AND METHODS

Nearly pure populations of *Isotricha* were obtained by employing a procedure similar to that of Gutierrez (1958). Strained rumen fluid was diluted (1:1) with salt buffer solution and incubated at 40° C. with D-fructose (3% w/v) for 30 to 60 minutes in a separatory funnel. The resulting amylopectin-laden *Isotricha* possessed a higher specific gravity than the Entodiniomorphida. Conse-

TABLE 1.—*Isotricha* Culture Medium

Ingredients	Concentration/liter
K ₂ HPO ₄	1.0 grams
NaCl	3.0 grams
MgSO ₄ ·7H ₂ O	0.1 grams
CaCl ₂	0.1 grams
NaHCO ₃	5.0 grams
Streptomycin sulfate ...	20.0 mg
Na ₂ S·9H ₂ O	1.0 gram
Resazurin	0.01 grams
Starch (corn & wheat) ..	0.6 grams
Gas	100% CO ₂

quently, they could be separated gravimetrically in the separatory funnel and then by layering on a column about 1 M long containing warmed (40° C.), gassed (CO₂) salt solution. *Dasytricha* could be lysed by layering 8 cm. of distilled water over the salt solution.

The culture medium (Table 1) was derived mainly from Mah (1964) and Rahman *et al.* (1965). The NaHCO₃ solution was autoclaved separately from the other ingredients and mixed after cooling. The medium was warmed (40° C.), gassed (CO₂) and 25 ml were carefully pipetted into 40 ml round-bottom centrifuge tubes. The ciliates had been appropriately diluted with the warmed and gassed salt solution. Cultures were mixed well to assure uniform distribution and were added to the medium in 2 ml aliquots. Bacteria were introduced in 2 ml aliquots of fresh rumen fluid, centrifuged on the initial culture day to eliminate protozoa. Starch and streptomycin were added, the culture tubes were gassed with CO₂, 0.05% NaS·9H₂O was added, and the tubes were stoppered.

Forage Material

Alfalfa meal was pulverized in a Wiley mill and stored in a sealed container in the dark. The mutant corn (GC-100 Carolina Biological Supply Company) was a single gene A/a chlorophyll mutant (3:1). The plants were cultivated in washed sand in a growth chamber, and harvested two weeks after planting. The etiolated corn was obtained by growing normal dent corn in a darkened room for a similar period of time. The seedlings were harvested, air dried, pulverized in a Wiley mill, and stored in a sealed flask in the dark.

These plants differed in that the mutant had no chlorophyll and no protochloroplast whereas the etiolated had slightly modified chlorophyll (—2H) and contained a protochloroplast. Both had the carotenoid pigments.

Enumeration:

Counts of *Isotricha* were made by placing 1 ml of an appropriate dilution of the culture in a petri dish, in an area ringed with vaseline to contain the medium. The population was counted by placing the dish over a darkly lined grid. The ciliates were observed for motility and stained with Lugol's Iodine, facilitating their identification with a dissecting microscope (X 20).

Culture Maintenance

Cultures were maintained for longer periods by centrifugation at low speeds to concentrate the ciliates and then 12.5 ml of the supernatant was removed. Cultures could also be maintained by removing the top one-half of the undisturbed culture daily or on alternate days. The majority of ciliates remained near the bottom of the culture vessel. Twelve and one-half ml of fresh, warmed gassed medium was added to each tube, which was, in turn, gassed with CO₂ that had additional NaS·9H₂O. The tubes were then stoppered.

In the short-term comparisons of forage a similar procedure was followed except that the culture was thoroughly mixed by shaking and then one-half of it was removed and replaced by fresh medium.

RESULTS AND DISCUSSION

The initial experiments were designed to evaluate the rumen ciliate culture procedures attributed to Coleman (1960), Purser and Weiser (1965), Rahman *et al.* (1964), Hungate (1950) and Mah (1964). The number of ciliates and the longevity in days were used as criteria of success. Rumen fluid was essential only on the initial culture day probably as a bacterial inoculant. Subsequent additions produced more variability than if no rumen fluid was provided. Use of rumen fluid seemed undesir-

able also in view of its chemically ill defined nature.

A suction method was superior to centrifugation because less time was required, less aeration and cooling occurred, and the possibility of trapping ciliates in pellet debris was eliminated. *Isotricha* populations could be maintained *in vitro* for several weeks. The longest continuous culture was for 30 days and perhaps could have been extended further but was terminated because of other obligations. Population levels gradually declined suggesting that not all the requirements for the extended *in vitro* culture of this organism were met. It may also be a consequence, however, of an insufficiently rapid *in vitro* reproduction rate which has been tabbed at 48 hours by Gutierrez (1958), in which ciliates were lost by medium replacement. The 48 hour rate of reproduction does not appear to be realistic in view of rumen passage data rate. It is, however, conceivable that this situation can be modified by a chemotaxis stimuli which attracts the *Isotricha* to ingesta distal to the sulcus omissi.

A randomized complete block experimental design was employed to evaluate the effect of chlorophyll on the cultures (Table 2). Four forage treatments consisted of the chlorophyll mutant, etiolated and normal corn seedlings, and dehydrated alfalfa. Each treatment was provided at three levels (10, 30 and 50 mg per culture) and each level had four replications, in each of which the *Isotricha* were counted twice and the mean values recorded.

Insofar as numbers of *Isotricha* were fairly constant on the first three culture days, these counts were not

subjected to statistical analysis. Each successive day's treatment was performed separately in view of the substantial day-to-day population change. This was due, in part, to the culture dilution technique employed.

No significant differences could be demonstrated between any of the corn treatments on the fourth day, ($P > 0.05$). Alfalfa, however, supported larger numbers than the other treatments ($P < 0.05$) on the fourth day. On the fifth day no differences were evident between the mutant and etiolated corn. On each succeeding day, however, the etiolated corn was significantly superior, ($P < 0.05$). Similarly, normal corn and alfalfa were significantly superior to the non-chlorophyllous treatments on each following culture day, but not from each other on the fifth or sixth day. The alfalfa was clearly superior to the normal corn on the seventh and eighth day. A higher level of sugar in alfalfa than in corn may be responsible. The protein was higher (26% CP) in the corn than in the alfalfa (18% CP). It was also apparent that the 50 mg levels of the alfalfa and normal corn were more effective than other treatments in maintaining *Isotricha* populations, whereas 50 mg levels of mutant and etiolated corn were significantly superior to all lower levels of mutant corn and after day five were not different from 30 mg levels of normal corn. Similarly, 10 and 30-mg levels of etiolated corn did not differ from similar levels of normal corn except on day eight, ($P < 0.05$).

These data strongly suggested that chlorophyll, or perhaps some aspect of the porphyrin ring structure, is the first limiting nutrient in this *in*

TABLE 2.—Comparison of Four Forages at Three Levels 10, 30, & 50 mg per 40 ml Culture per day. Data Expressed as Isotrichs/ml

Block	Yellow			Etiolated			Green			Alfalfa			Day
	10mg	30mg	50mg	10mg	30mg	50mg	10	30	50	10	30	50	
A.....	400	188	264	208	249	255	212	198	210	172	390	476	4
B.....	360	310	360	250	284	254	338	516	555	326	611	657	
C.....	348	259	257	272	292	279	295	356	519	410	415	535	
Total.	1,108	757	881	730	825	788	845	1,070	1,284	908	1,416	1,668	
A.....	32	16	10	89	124	45	36	70	80	60	110	147	5
B.....	251	169	15	116	133	36	256	283	316	163	192	383	
C.....	160	87	19	97	117	33	123	188	208	131	138	159	
Total.	443	272	44	302	374	114	415	541	604	354	440	689	
A.....	0	1.0	0	36	24	3.0	4	16	20	4	35	96	6
B.....	69	42	0	43	58	1.0	72	84	100	74	74	110	
C.....	35	9	0	27	58	4.0	75	79	97	36	57	84	
Total.	104	52	0	106	140	8.0	151	179	217	114	166	290	
A.....	0	0	0	5	6	0	0	0	8	1	11	68	7
B.....	4	5	0	8	13	0	14	16	22	28	50	66	
C.....	4	0	0	9	11	0	7	13	14	15	29	40	
Total.	8	5	0	22	30	0	21	29	44	44	90	174	
A.....	0	0	0	0	1.0	0	0	0	0	0	4	11	8
B.....	0	0	0	0	0.0	0	2	3	4	8	16	23	
C.....	0	0	0	0	2.0	0	0	2	4	3	15	28	
Total.	0	0	0	0	3.0	0	2	5	8	11	35	62	

vitro Isotricha culture. The lack of stimulation effect of the etiolated corn at the 50-mg level indicated that some aspect of the complete chloroplast or a product of photosynthesis is also required. Further study regarding lipid or tocopherol requirements is, therefore, indicated. These data were somewhat confounded in that it is impossible to distinguish between bacterial and ciliate requirements. Bacteria, however, can be cultured without a chlorophyllous constituent, Caldwell and Bryant (1966) although *Bacteroides rumini-*

cola requires hemin Caldwell *et al.* (1966).

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