MEDWELL PUBLISHING
Journal of Animal and Veterinary Advances 8 (3): 603-607, 2009
ISSN: 1680-5593

Relative Contributions of Ruminal Bacteria, Protozoa and Fungi to Degradation of Forage Fiber Fractions

[1]A.D. Foroozandeh, [2]M. Rezaeian, [3]G.R. Balaly and [4]M. Alikhani
[1]Department of Animal Science, Faculty of Agriculture,
Science and Research Branch, Islamic Azad University, Tehran, Iran
[2]Department of Animal Health and Nutrition, Faculty of Veterinary Medicine, University of Tehran, Iran
[3]Department of Biology, Faculty of Science, University of Isfahan, Iran
[4]Department of Animal Science, Faculty of Agriculture, Isfahan University of Technology, Iran

Abstract: The relative capabilities of different rumen microorganisms to degrade forage fiber were compared *in vitro*. Forages were used, as the substrates in the culture of rumen microorganisms, were alfalfa hay and wheat straw. Microbial groups were separated by physical (sedimentation and centrifugation) and chemical (addition of various antibiotics) treatments of rumen fluid. Prepared microbial fractions were: Whole Ruminal Fluid (WRF), Bacterial (B), Protozoal (P) and Fungal (F) fractions; cocultures of B+P, B+F and P+F and a free-microorganisms rumen culture as Negative System (NS). Light microscopic observations of samples during the incubation period supported that fractionation method used for separating different microbial groups were satisfactory. The results indicated that WRF and cocultures were more effective than monocultures on dry matter loss of all forages. However, the effect of bacterial fraction on DM loss of alfalfa was equal to WRF and cocultures but more than the other two monocultures. The NDF and ADF losses of alfalfa and wheat straw were higher in the B+P than other cocultures (i.e., the P+F and B+F) and WRF. The ADL loss when averaged across both forages was highest (about 15%) due to WRF and lowest (about 8%) to protozoal fractions. In general, rumen bacterial fractions appeared to be the most active fiber degraders between rumen microorganisms. The protozoal and fungal fractions showed lower capability in this regards. However, cocultures of different rumen microbial fractions seemed to display synergetic interactions.

Key words: Rumen, bacteria, fungi, protozoa, fiber, degradation

INTRODUCTION

The rumen is a complex ecosystem that contains many different microbial species and has a great potential for *in vitro* microbial association. Bacteria, protozoa and fungi have all been shown to involved in plant cell wall degradation. The anaerobic fungi may account up to 20% of the total microbial biomass in the rumen of sheep (Rezaeian *et al*., 2004). Microscopy studies (Akin *et al*., 1983; Bauchop, 1979a) indicated that rumen fungi are better able than bacteria to colonize the lignocellulosic components of plants and that they effectively degrade and weaken lignified tissues (Akin and Rigsby, 1987). However, they were loss effective in converting lignin to gases and were less able to solubilize the lignin component compared with ruminal bacteria (Gordon and Phillips, 1989; Akin, 1986). Despite these findings and those showed the contribution of ruminal fungi (Lee *et al*., 2000; Akin *et al*., 1989) to fiber digestion, it appears that under many conditions bacteria still are prominent and active fiber degrading group in the rumen (Akin and Benner, 1988; Windham and Akin, 1984).

The rumen protozoa has been shown to attach to damaged regions of fresh plant materials undergoing digestion in the rumen of sheep (Bauchop and Clark, 1976) and to associate with and degrade tissues in alfalfa stems (Bauchop, 1979b) and mesophyl tissues of cool season grass leaves (Amos and Akin, 1978). However, the exact role and overall contribution of each microbial group and their interactions to degradation and fermentation of plant cell wall material requires further attention. Therefore, the objective of the present study, were: to determine the role of individual microbial groups in degrading fiber, using physical and chemical treatment to suppress growth of or to select for specific microbial population and to quantitate forages of various digestibilities for degradation by single group or mixed population of rumen microorganisms.

Corresponding Author: A.D. Foroozandeh, Department of Animal Science, Faculty of Agriculture, Science and Research Branch, Islamic Azad University, Tehran, Iran

MATERIALS AND METHODS

Preparation of forage substrates: Forages were collected from well managed fields near Isfahan and included wheat straw (Harvested once) and alfalfa (early bloom). Forage samples stored in plastic bags at -20°C and immediately before being used , these samples were dried at 50°C for 48 h. For *in vitro* dry matter digestibility and chemical analysis, the subsamples of all dried forages were ground to pass 1 mm screen.

Animals and collection of rumen contents: The ruminal contents removed though the cannula from 3 fistulated mature shull rams. The animals had been housed individually in metal pens. The ration consisted of 700 g alfalfa hay, 300 g wheat straw and 200 g barley grain per animal and fed twice a day in two equal meals. Water was available *ad libitum*. All samples isolated from the rumen just before morning rations were consumed. The collected digesta were transported by preheated (39°C) bottles to the laboratory and immediately strained through three layers of cheese clothes.

Separation of microbial fractions: As given in Fig. 1, the Strained Rumen Fluid (SRF) fractionated to 3 equal portion: About 500 mL of the SRF was intactly used as inocula to evaluate the activity of all microbial groups (WRF: Whole Ruminal Fluid), Protozoa + Fungal (P+F) and protozoa + bacterial (P+F) cocultures. The 2nd portion (about 700 mL) transferred to a stoppered separating funnel and allowed to stand for approximately 60 min at 39°C. The glass utensils gassed with CO_2 and pre-warmed to 39°C before use. Scum layer of small flocculant debris removed by suction and discarded. Most of the lower sedimental was collect and diluted by autoclaved rumen fluid (2X) and used to prepare Protozoal (P) fraction. The liquor fraction remaining after sedimentation does contain some of the smaller protozoa that have not settled. So, this part was recovered by centrifugation (1000 g for 10 min) and retentate was combined with the protozoal fraction. The remaining part was centrifuged at 1500 g for 25 min in order to remove protozoal cells. The supernatant was carefully collected and used to asses the effect of Fungal (F), Bacterial (B) fractions, coculture of Bacteria and Fungi (B+F) and a negative system. A summary of these separation procedure is given in Fig. 1.

The following antibiotics were prepared and added to bottles before inoculation with microbial groups: Antibacterial agents (1.25 mg penicillin G and 200 μg streptomycin mL^{-1} of broth) and antifungal agent (0.05 mg cycloheximide mL^{-1} of broth). Antibiotics were prepared such that 1 mL of solution was added to each bottle to

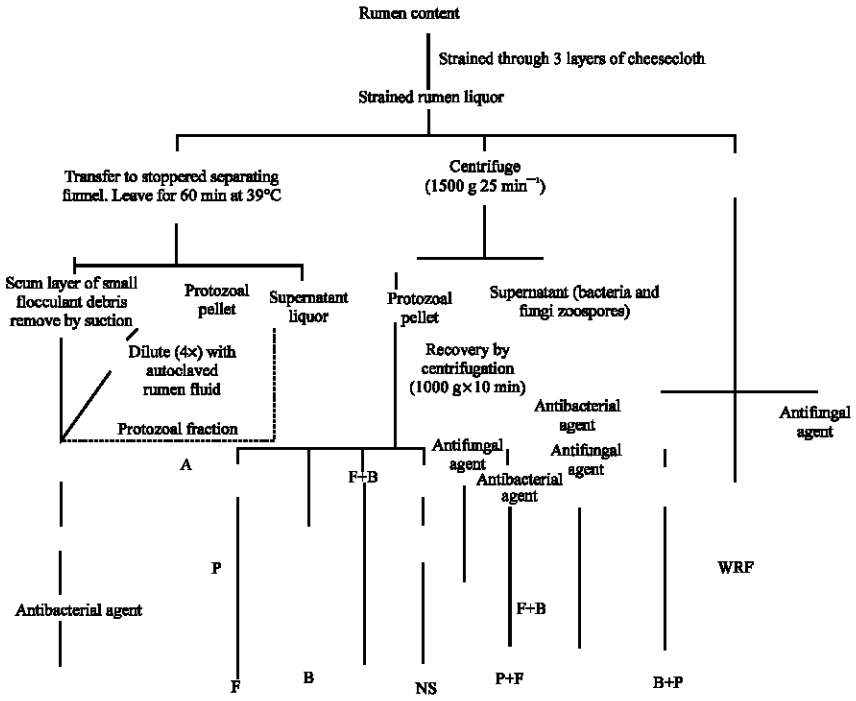

Fig. 1: Separation procedure for microbial fractions, F: Fungi; B: Bacteria; P: Protozoa, WRF: Whole Ruminal Fluid, NS: Negative System

give the desired concentration. In WRF or treatments with only one antibiotic, water was added to keep amount of additional liquids equivalent.

Culture conditions and *in vitro* incubations: The medium used in experimental cultures was based on medium 10 of Caldwell and Bryant (1966), except that agar was omitted and soluble carbon sources were replaced with forage substrates. Triplicate 500 mg samples of each substrate, for each of 8 inoculum sources, were placed in 50 mL serum bottles and 35 mL of sterile medium was added. Bottles were flashed with CO_2, stoppered with butyl rubber stoppers and aluminum crimp seals, autoclaved (121°C for 30 min) and stored at +4°C until used.

Each bottle was inoculated by 5 mL of microbial fractions through the septa. Samples were incubated for 72 h at 39°C with occasional shaking. Inoculated blanks for each microbial fraction were also run in duplicate.

Chemical analysis: Forage substrates were analyzed for DM by drying at 105°C for 24 h and CP was estimated as kjeldahl N × 6.25 (AOAC, 1995). All forage samples and *in vitro* fermentation residues were analyzed for Neutral-Detergent Fiber (NDF), Acid-Detergent Fiber (ADF) and 72% H_2SO_4 lignin (ADL), as described by Goering and Van Soest (1970). Loss of fiber components were calculated based on indigestible dry matter remaining.

RESULTS AND DISCUSSION

The chemical compositions of wheat straw and alfalfa are summarized in Table 1. The H_2SO_4 lignin values of wheat straw and alfalfa were comparable, the degree of lignification between these 2 differed due to amount of cell wall fiber. On the other hand, the amount of cellulose and hemicellulose in wheat straw were greater than alfalfa. These differences provide a comparative determination of the role of microbial groups play in degrading fiber components, especially lignin and lignocellulose (Windham and Akin, 1984).

Dry matter loss of all forage samples were significantly affected by microbial treatments (Table 2). Weight losses occurring in the absence of microbial activity (negative system) were about 8-36% of dry weight. Light microscopic monitoring of blanks, during first 24 h of incubations period, showed the absence of microbes in the negative controls. So, most of the weight loss in these groups can be attributed to the loss of soluble components (Akin and Benner, 1988; Akin *et al*., 1989).

With the monocultures (i.e., the bacterial, protozoal, or fungal fraction alone), overall degradation rate by the bacterial fraction was significantly highest ($p<0.05$). The rumen bacteria are believed to be responsible for most of feed digestion in the rumen because of their numerical predominance and metabolic diversity (Cheng *et al*., 1991). The results of studies using antifungal agents (e.g., Cycloheximide) showed that contribution of bacteria in degrading dry matter were equal to WRF and greater than fungi (Akin and Benner, 1988; Windham and Akin, 1984).

Table 1: Composition of forages used as substrate before incubation with microbial fractions

Component	Wheat straw	Alfalfa
Crude protein	5.5±0.50	19.1±0.3
Crude fat	1.1±0.50	1.0±0.04
NDF	69.2±0.30	43.5±0.4
ADF	48.4±0.30	34.7±0.2
ADL	9.9±0.06	8.4±0.08
Cellulose	38.5±1.00	26.3±0.5
Hemicellulose	20.8±0.30	8.7±0.5

Values represent averages of three measurments

In the present study, evaluation of dry matter loss and digestion of NDF, ADF and ADL did not indicate a major role for a fungal fraction as utilizers of lignin or degraders of lignified fiber. The only link between fungal activity and DM loss occurred with wheat straw (Table 2). The failure of fungi fraction to degrade cell wall components was enigmatic, since anaerobic fungus can produce a large amount of cellulase, hemicellulase and particularly proficient in producing Xylanasis (Akin and Borneman, 1990). Further, rumen fungi have been shown to attack and degrade tissues containing lignin, which are more resistant to rumen bacteria (Akin *et al*., 1983). Cheng *et al*. (1991) suggested that anaerobic rumen fungi show a marked lag in their *in vitro* ability to degrade cell wall materials. So, the lack of degrading might indicate that the fungi did not perform to their potential in current experiment. Further, Bauchop (1981) has suggested that fungal populations adopted to different diets could vary in invasiveness.

The importance of protozoa to host ruminant is not always apparent (Eadie and Gill, 1971). *In vitro* studies indicated that approximately a quarter to 1/3rd of fiber breakdown in the rumen was protozoal (Amos and Akin, 1978; Demeyer, 1981). The majority of protozoa appeared to resemble *E. ecaudatum* which have been show to actively attack and ingest cell walls of certain plants (Bauchop and Clark, 1976; Amos and Akin, 1978) and to associate with and degrade tissues in alfalfa stems of cool-season grass leaves (Bauchop, 1979a). However, the protozoa fraction alone did not progressively degrade the cell wall material in our experiment, which is in agreement with Lee *et al*. (2000) showing inability of protozoa fraction to ferment orchard grass cell wall extracts. Perhaps, initial degradation or physiological interactions with other microbes are required before protozoa can

Table 2: Dry weight loss and degredation rate of fiber components from alfalfa and wheat straw after incubation for 3 days with different microbial fractions

		Microbial treatment[a]								
Component	Substrate	F	B	P	WRF	B+F	P+F	B+P	NS	SEM
DM	ALF	39.97^{cd}	53.28^{ab}	42.71^{c}	58.47^{a}	54.76^{ab}	49.88^{b}	57.01^{a}	36.37^{d}	1.69
	WS	23.47^{d}	30.67^{c}	11.49^{e}	33.54^{b}	30.56^{c}	31.88^{bc}	40.08^{a}	7.76^{f}	2.20
NDF	ALF	9.94^{d}	24.37^{b}	10.85^{d}	32.43^{a}	25.39^{b}	18.55^{c}	30.09^{a}	1.63^{e}	2.19
	WS	3.51^{d}	7.72^{c}	1.59^{d}	13.7^{b}	14.27^{b}	12.63^{b}	14.81^{a}	0.48^{d}	1.40
ADF	ALF	4.51^{d}	19.47^{c}	4.80^{d}	29.39^{a}	24.66^{b}	19.81^{c}	30.17^{a}	0.917^{e}	2.30
	WS	4.13^{de}	5.58^{d}	1.40^{ef}	14.54^{bc}	12.36^{c}	15.9^{1b}	20.30^{a}	0.497^{f}	1.48
ADL	ALF	14.30^{d}	17.87^{cd}	14.97^{cd}	28.12^{a}	24.89^{ab}	21.35^{bc}	14.63^{d}	0.30^{e}	1.76
	WS	1.25^{d}	2.99^{d}	1.18^{d}	11.16^{b}	7.7^{c}	8.63^{bc}	15.57^{a}	0.19^{d}	1.11

[a]F: Fungi; B: Bacteria; P: Protozoa, WRF: Whole Ruminal Fluid and NS: Negative System. Means (n = 3) in the same row with different super scripts are significantly different ($p<0.05$)

degrade certain forages. However, higher DM and NDF loss of alfalfa with protozoal monoculture showed a substrate × treatment interaction ($p<0.05$). Windham and Akin (1984) suggested that degradation of more soluble carbohydrate components in DM and NDF fractions of alfalfa might be due to protozoa and especially holoterichs activity.

WRF and cocultures (B+P, B+F and P+F) were more effective compared to monocultures. The greatest overall dry matter and NDF losses (about 58 and 32%, respectively) from alfalfa occurred in WRF treatment. This would agree with the result of some experiments, using antibiotics to select for activity of certain group of rumen microbes (Akin and Benner, 1988; Akin *et al*., 1989; Lee *et al*., 2000; Windham and Akin, 1984).

The protozoal fraction alone failed to degrade cell wall material in our experiment, but in coculture with either Bacterial (B+P) or the fungal fraction (P+F) synergetically increased degradation of ADF from both forages compared to bacterial or fungal monoculture. *in vitro* the degradation on cellulose was improved when protozoa were added to a suspension of bacteria (Yoder *et al*., 1966), while Hidayate *et al*. (1993) reported the addition of protozoa to a bacterial suspension did not further increase fermentation and Lee *et al*. (2000) observed protozoa fraction inhibited the degradation rate of cell wall material by both the fungal and the bacterial fractions in cocultures. Moreover, Orpin (1984) reported that anaerobic fungi and rumen protozoa may be complementary rather than competitive in a natural system.

The ADL loss when averaged across both forages was highest (about 15%) due to WRF and lowest (about 8%) to protozoal fraction. In addition, total loss of ADL in cocultures were more than monocultures (except for B+P with in alfalfa). These data suggest a biological interaction of rumen microbes to degradation of lignin. Fungi monoculture in our study did not preferentially degrade the lignin component. In agreement Akin and Benner (1988), utilizing [^{14}C] lignocellulose from cord grass, found that ruminal fungi colonized the lignocellulosic tissues but did not preferentially degrade the lignin component. As they suggested, 1.25 and 14.3% loss of ADL (with in wheat straw and alfalfa, respectively) we observed in this study are be due to solubilization from plant cell wall.

Total weight loss and loss of individual components (NDF, ADF and ADL) where higher with in alfalfa compare to wheat straw. It is well known that histological and chemical differences between grass and legume cell walls (Jones and Wilson, 1987; Wilson, 1990) are responsible for the extent of degradation rate of forages used as substrate in this experiment.

CONCLUSION

Rumen bacterial fractions appeared to be the most active fiber degraders between rumen microorganisms. The protozoa and fungal fractions showed lower capability in this regards. However, cocultures of different rumen microbial fractions seemed to display synergetic interactions.

REFERENCES

Akin, D.E., 1986. Interaction of ruminal bacteria and fungi with southern forages. J. Anim. Sci., 63: 962-977. DOI: 10.1002/jsfa.2740490403. http://jas.fass.org/cgi/content/abstract/63/3/962.

Akin, D.E. and R. Benner, 1988. Degradation of polysaccharides and lignin by ruminal bacteria and fungi. Applied Environ. Microbiol., 54: 1117-1123. PMID: 3389808. http://aem.asm.org/cgi/reprint/54/5/1117.

Akin, D.E. and W.S. Borneman, 1990. Role of rumen fungi in fiber degradation. J. Dairy Sci., 73: 3023-3031. PMID: 2178175. www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd.

Akin, D.E. and L.L. Rigsby, 1987. Mixed fungal population and lignocellulosic tissue degredation in bovine rumen. Applied Environ. Microbiol., 53: 1987-1995. PMID: 2823705. www.ncbi.nlm.nih.gov/pubmed/2823705.

Akin, D.E., G.L.R. Gordon and J.P. Hogan, 1983. Rumen bacterial and fungal degredation of Digitaria pentzii grown with and without sulfur. Applied Environ. Microbiol., 46: 738-748. PMID: 239343. http://aem.asm.org/cgi/reprint/46/3/738.

Akin, D.E., C.E. Lyon, W.R. Windham and L. Rigsby, 1989. Physical degradation of lignified stem tissues ruminal fungi. Applied Environ. Microbiol., 55: 611-616. PMID: 184168. http://www. pubmedcentral.nih.gov/articlerender.fcgi?artid=184168.

Amos, H.E. and D.E. Akin, 1978. Rumen protozoal degradation of structurally intact forage tissues. Applied Environ. Microbiol., 36: 513-522. PMID: 243077. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=243077.

AOAC, 1995. Official Methods of Analysis of AOAC international. Arlington. 16th Edn. VA AOAC International. Arlington, VA, USA, Vol. 2.

Bauchop, T., 1979a. The rumen ciliate Epidinium in primary degredation plant tissue. Applied Environ. Microbiol., 37: 1217-1223. PMID: 243382. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=243382.

Bauchop, T., 1979b. Rumen anaerobic fungi of cattle and sheep. Applied Environ. Microbiol., 38: 148-158. PMID: 243449. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=243449.

Bauchop, T., 1981. The anaerobic fungi in the rumen fiber digestion. Agric. Environ., 6: 338-348. http://www.fao.org/agris/search/display.do?f=./1982/v804/XE8182943.xml;XE8182943.

Bauchop, T. and R.T.J. Clarke, 1976. Attachment of the ciliate Epidinium crawley to plant fragment in the sheep rumen. Applied Environ. Microbiol., 32: 417-422. PMID: 170080. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=170080.

Caldwell, D.R. and M.R. Bryant, 1966. Medium without rumen fluid for nonselective enumeration and isolation of rumen bacteria. Applied Microbiol., 14: 799-801. PMID: 1058417. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1058417.

Cheng, K.J., H. Kudo and S.H. Duncan *et al*., 1991. Prevention of fungal colonization and digestion of cellulose by the addition of methyl cellulose. Can. J. Microbiol., 37: 484-487. PMID: 1913353. http://www.ncbi.nlm.nih.gov/pubmed/1913353?dopt=Abstract.

Demeyer, D.I., 1981. Rumen microbs and digestion of plant cell walls. Agric. Environ., 6: 295-337. http://md1.csa.com/partners/viewrecord.php.

Eadie, J.M. and J.C. Gill, 1971. The effect of the absence of rumen ciliate protozoa on growing lambs fed on a roughage-concentrate diet. Br. J. Nutr., 26: 155-167. PMID: 4999056. http://www.ncbi.nlm.nih.gov/pubmed/4999056.

Goering, H.K. and P.J. Van Soest, 1970. Forage fiber and analyses (apparatus, reagents, procedures and some applications). U.S. Department of Agriculture Handbook 379. U.S. Department of Agriculture. Washington, D.C. www.getcited.org/pub/101564920.

Gordon, G.L. and M.W. Phillips, 1989. Degradation and utilization of cellulose by 3 different anaerobic fungi from the ovine rumen. Applied Environ. Microbiol., 55: 1703-1710. PMID: 202938. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=202938.

Hidayat, H.K., C.J. Newbold and C.S. Stewart, 1993. The contributions of bacteria and protozoa to ruminal fermentation of forage *in vitro*, as determined by microbial gas production. Anim. Feed Sci. Technol., 42: 193-208.

Jones, D.I.H. and A. Wilson, 1987. Nutritive Quality of Forage. In: Hacker J.B. and J.H. Ternouth (Eds.). The Nutrition of Herbivores. P65. Academic Press, Orlando, FL. ISBN: 0127500529. http://siris-libraries.si.edu/ipac20/ipac.jsp?uri=full=3100001~!328965!0#focus.

Lee, S.S., J.K. Ha and K.J. Cheng, 2000. Relative contributions of bacteria, protozoa and fungi to *in vitro* degredation of orchard grass cell walls and their interactions. Applied Environ. Microbiol., 66: 3807-3813. PMID: 10966394. http://aem.asm.org/cgi/content/full/66/9/3807.

Orpin, C.G., 1984. The role of ciliate protozoa and fungi in the rumen digestion of plant cell walls. Anim. Feed Sci. Technol., 10: 121-143. http://scholar.google.com/scholar?

Rezaeian, M., G.W. Beakes and D.S. Parker, 2004. Distribution and estimation of anaerobic zoosporic fungi along the digestive tracts of sheep. Mycol. Res., 108 (10): 1227-1233. PMID: 15535073. http://linkinghub.elsevier.com/retrieve/pii/S0953756208604463.

Wilson, J.R., 1990. Influence of Plant Anatomy on Digestion and Fiber Breakdown. In: Akin, D.E., L.G. Ljungdahl, J.R. Wilson and P.J. Harris (Eds.). Microbial and Plant Opportunities to Improve Lignocellulose Utilization by Ruminant. Elsevier. New York, pp: 99. ISBN: 0-444-01581-7. http://www.amazon.co.uk/Microbial-Opportunities-Lignocellulose-Utilization-Ruminants/dp/0838563120.

Windham, W.R. and D.E. Akin, 1984. Rumen fungi and forage fiber degradation. Applied Environ. Microbiol., 48: 473-476. PMID: 241550. http://aem.asm.org/cgi/reprint/48/3/473.

Yoder, R.D., A. Trenkle and W. Burroghs, 1966. Influence of rumen protozoa upon cellulose digestion *in vitro*. J. Anim. Sci., 25: 609-612. PMID: 4961305. http://jas.fass.org/cgi/reprint/25/3/609.