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Metabolic Inhibitor(s) in Fractions of Orchardgrass *(Dactylis glomerata* L.) Detected by *in vitro* Rumen Fermentation Technique.* (31917)

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The possible presence of a metabolic inhibitor in orchardgrass (*Dactylis-glomerata* L.) hay grown on some Missouri farms was indicated by Hargus(1). Lambs fed either chopped orchardgrass or orchardgrass pellets supplemented with proteins, vitamins and minerals to meet the requirements set forth by the National Research Council failed to grow and showed symptoms of stiffness.

We conducted a series of investigations to find a suitable screening technique for detecting the active factor(s). One of the techniques selected for study was the *in vitro* rumen fermentation technique.

This technique has been used previously to study nonprotein nitrogen utilization(2,3), protein digestion(4), fatty acid production and utilization(5-7), cellulose digestion and factors affecting it (8-14), starch fermentation (15), and roughage evaluation(1,16,17).

This paper describes the technique developed as a screening tool to detect the presence of the metabolic inhibitor(s). Results of the preliminary fractionations are included. The *in vitro* rumen fermentation technique was modified from Garner(18). The inhibition of cellulose digestion by fractions of orchardgrass was considered as an indication of the presence of metabolic inhibitor(s) since the amount of cellulose digested reflects the metabolic activity of rumen microorganisms.

Preparation of nutrient solution and buffers: Nutrient solution was prepared by dissolving 1 g sodium carbonate, 920 mg vitamin free casein, 160 mg ammonium carbonate, 160 mg dextrose, and 160 μg cobalt chloride in 200 ml of distilled water. Equal parts of solutions A and B form a modified Hungate's artificial saliva(19); A was prepared by dissolving 3 g K_2HPO_4 in one L of distilled water; B by dissolving 6 g NaCl, 3 g $(NH_4)_2SO_4$, 3 g KH_2PO_4, 0.6 g $MgSO_4$ and 0.6 g $CaCl_2$ in one L of distilled water. Buffer solution C was prepared by dissolving 10 g $NaHCO_3$ in 100 ml of distilled water.

Preparation of substrate: Alfalfa and orchardgrass were ground through a 1 mm screen in a Wiley mill. Seven hundred mg of original substrate or a residue containing the same amount of cellulose as 700 mg hay was transferred into 50 ml glass tubes referred to as reaction tubes. To each reaction tube 5 ml nutrient solution, 5 ml Hungate's artificial saliva solution, 1 ml buffer solution C and 6 ml distilled water were added. Different levels of extracts of orchardgrass and extractants were added at the expense of distilled water. When organic solvent extractants or extracts were added, they were removed by evaporation over a water bath before other solutions were added. Alkali extract and extractant were neutralized to pH 7.0 before use. Reaction tubes were placed in a water bath at 39°C. Carbon dioxide, pretreated by passing it through nutrient solution, buffers

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and distilled water, was bubbled through the reaction tubes at an approximate rate of 40 bubbles per minute. The reaction tubes were inoculated with 3 ml strained, freshly prepared rumen fluid from a sheep fitted with a permanent rumen fistula and maintained on a daily ration of 1.70 kg good quality alfalfa. After an incubation period of 24 hours, the reaction was stopped by adding 1 ml of 5% H_2SO_4 to each reaction tube. The reaction tubes were centrifuged (2500-2700 rpm) for 15 minutes and the supernatent liquid was removed.

Cellulose was determined by a modification of the method of Crampton and Maynard (20). The modification consisted of placing the reaction tubes in a boiling water bath for 22 minutes instead of refluxing and adding 30 ml of previously prepared acid mixture (1635 ml acetic acid, 205 ml nitric acid and 410 ml distilled water). A standard alfalfa (cellulose digestibility 45%) was included in each assay set. Digestibility values of any day's run were corrected to the standard value.

Statistical comparisons of treatments were made with the control by the least significant difference method(21).

Preparation of orchardgrass fractions and extractants: Direct fractions of orchardgrass were prepared. The extractants used were cold distilled water, steam (bubbling steam for 3 hours through a mixture of 10 kg of orchardgrass and 100 L of distilled water), a boiling solution of 0.1% Tween 80§ (polyoxyethylene-(20)-sorbitan monooleate in distilled water), boiling 0.1 N $NaHCO_3$ containing 0.1% detergent Tween 80, and an azeotropic mixture of methanol and acetone.

Except for the steam extraction outlined above, all extractions were performed as follows. Five hundred ml of extractant were added to 50 g of ground orchardgrass. The mixture was continuously stirred with a glass rod for 15 minutes. It was strained through 4 layers of cheese cloth. The liquid obtained was again strained through 8 layers of cheese cloth and was vacuum filtered. The extract was concentrated in a vacuum evaporator at 60°C. Hot alkali-detergent extract was neutralized to pH 7.0 before concentration. Methanol-acetone (azeotropic) extract was prepared and concentrated in a soxhlet apparatus.

In the *in vitro* studies, the extractant was either concentrated in the same way as the extract or its concentration was proportionately raised to the same level as the extract in the control reaction tubes. The extracts are designated as E.

Residue: The residue obtained in the preparation of an extract was twice washed with the corresponding extractant and was dried in a vacuum oven at 65°C. The residue obtained in the preparation of methanol-acetone (azeotropic) extract was expressed to remove the solvent and was dried as mentioned above. All residues are designated as R.

Results. The effects of adding the several extracts of orchardgrass on cellulose digestibility of alfalfa, the cellulose digestibility of orchardgrass and residues are shown in Table I.

Extracts 1-E and 2-E showed no significant effect on cellulose digestibility of alfalfa. Both levels of extract 3-E markedly depressed ($P<.01$) cellulose digestibility of alfalfa. The 1X level of hot detergent extractant alone markedly enhanced ($P<.01$) cellulose digestibility of alfalfa. Cellulose digestibility of residue 3-R was markedly higher ($P<.01$) than that of orchardgrass itself. The 2X level of extract 4-E markedly depressed ($P<.01$) cellulose digestibility of alfalfa. Cellulose digestibility of residue 4-R was slightly but not significantly higher than that of orchardgrass. The 1X level of extract 5-E depressed ($P<.05$) cellulose digestibility of alfalfa. It was markedly depressed ($P<.01$) by 2X level of this extract. The 2X level of alkali detergent extractant alone markedly enhanced ($P<.01$) cellulose digestibility of alfalfa. Cellulose digestibility of residue 5-R was higher ($P<.05$) than that of orchardgrass. The 1X level of extract 6-E markedly depressed ($P<.01$) cellulose digestibility of alfalfa. The depression effect of 2X level of this extract was just statistically significant ($P<.05$). Both levels of the methanol-

§ Obtained from Nutritional Biochemicals Corp., Cleveland, Ohio.

TABLE I. Effect of Direct Extracts of Orchardgrass on Cellulose Digestibility* of Alfalfa, Cellulose Digestibility* of Orchardgrass and Its Residues.

Fraction No.	Extract Name	Extract Level	Cellulose digestibility %¶ — Alfalfa† + Extract	Alfalfa† + Extractant	Orchardgrass	Residue
1	Cold water	1X**	46.76 ± .88††	—	—	—
2	Steam	1X	46.75 ± 1.53	—	—	—
3	Hot detergent‡	1X 2X	40.38 ± 1.18§§ 36.36 ± 2.02§§	49.79 ± 1.57§§ 46.56 ± .58	22.86 ± 2.65	28.24 ± .40§§
4	Hot detergent§	1X 2X	44.61 ± 1.60 40.94 ± .77§§	49.79 ± 1.57§§ 46.56 ± .58	31.62 ± 2.02	33.16 ± .33
5	Hot alkali-detergent‖	1X 2X	42.51 ± 2.47‡‡ 39.77 ± 1.38§§	46.18 ± 3.02 47.73 ± .32‡‡	31.70 ± 1.74	34.67 ± 2.14‡‡
6	Methanol-acetone (azeotropic)	1X 2X	41.24 ± 1.79§§ 43.12 ± .69‡‡	44.59 ± 2.39 45.51 ± .42	31.70 ± 1.74	28.08 ± .20§§

* *In vitro.*
† Average cellulose digestibility in control reaction tubes was 45.42%.
‡ Prepared from orchardgrass of relatively low cellulose-digestibility.
§ Prepared from orchardgrass of relatively high cellulose-digestibility.
‖ Neutralized to pH 7.0.
¶ Mean of cellulose digestibility values in 3 reaction tubes.
** X equivalent to 700 mg.
†† Standard deviation. L.S.D. extracts and extractants vs. control (.05) = 2.28, L.S.D. extracts and extractants vs. control (.01) = 3.02, L.S.D. residues vs. orchardgrass (.05) = 2.49, L.S.D. residues vs. orchardgrass (.01) = 3.36.
‡‡ $P < .05$.
§§ $P < .01$.

acetone extractant alone showed no significant effect on cellulose digestibility of alfalfa. Cellulose digestibility of residue 6-R was markedly lower ($P < .01$) than that of orchardgrass.

Discussion. The depressing action of extracts 3-E to 6-E on *in vitro* cellulose digestibility shows that a cellulose digestion inhibitor is present in orchardgrass and that it can be extracted with hot detergent, hot alkali-detergent, and an azeotropic solution of methanol and acetone. This information will allow a more rapid search for the active principle(s). The results of extract 3-E *vs.* 4-E suggest that the metabolic inhibitor(s) is more concentrated in relatively low cellulose-digestibility orchardgrass than relatively high cellulose-digestibility orchardgrass.

The higher cellulose digestibility of residue 3-R and 5-R as compared to orchardgrass support the idea that the metabolic inhibitor(s) is extractable with hot detergent and hot alkali detergent. The results of 3-R and 4-R show that the marked improvement in the cellulose digestibility of residue 3-R cannot be attributed to the stimulating effect of traces of detergent left in the dried residue, but due to the removal of metabolic inhibitor(s). Residues 3-R and 4-R contained about the same amount of detergent. The markedly low cellulose digestibility of residue 6-R may be attributed to either extraction of some cellulose digestion aiding factor which might have been extracted along with the inhibitor(s) or an intensification of activity of inhibiting factor(s). Since it is possible that more than one inhibitor may be present in orchardgrass, the activity of some could be intensified by methanol-acetone (azeotropic) extraction procedure, while the others are extracted with this treatment.

The absence of any depressing effect on cellulose digestion by the experimental levels of any extractants alone confirms that the presence of activity in various extracts cannot be attributed to various extractants themselves. Extractants containing detergent had a stimulating effect on cellulose digestion of alfalfa. It may be due to either an effect of the detergent on microorganisms or an indirect effect because cellulose was made more available to microbial attack.

It is concluded that the *in vitro* rumen fermentation technique can be used as a screening tool for detecting at least part of the metabolic inhibitor(s) in various fractions of orchardgrass.

Summary. Cellulose digestion determined by the *in vitro* rumen fermentation technique was used as a criterion to detect the presence of metabolic inhibitor(s) in various fractions of orchardgrass. The metabolic inhibitor is extractable with hot detergent, hot alkali-detergent, and azeotropic solution of methanol and acetone. The inhibitor appeared to be more concentrated in relatively low cellulose-digestibility orchardgrass than relatively high cellulose-digestibility orchardgrass.

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Effects of Erythropoietin on ^{14}C-Formate Uptake by Spleen and Bone Marrow Nucleic Acids of Erythrocyte-Transfused Mice.* (31918)

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It has been shown that erythropoiesis is almost completely suppressed in spleen of erythrocyte-transfused mice(1). ^{14}C-formate incorporation into the RNA and DNA of spleen of these animals is markedly depressed, while in marrow only a slight decrease is observed(2). A single injection of erythropoietin induces a wave of erythropoiesis that proceeds in an orderly fashion from proerythroblast to reticulocyte in the spleen of mice made polycythemic by transfusion(3). With such a system it is thus possible to study the incorporation of precursors into nucleic acids at different stages of erythropoiesis, at various times after erythropoietin injection.

It has been suggested that erythropoietin (EP) acts on the erythropoietic mechanism by stimulating an early synthesis of RNA (4,5). The experimental basis for this has

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