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ALFALFA HEMICELLULOSE

Constitution of the Hemicellulose of Alfalfa (*Medicago sativa*). Further Studies on the Acidic Components Produced by Hydrolysis

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The constitution of the hemicellulose of alfalfa (*Medicago sativa* var. Ranger) is being investigated in an attempt to ascertain the possible relationship between chemical structure and nutritional value for ruminants. Three aldobiouronic acids, 2-O-α-D-galactopyranosyluronic acid-L-rhamnose, 6-O-β-D-glucopyranosyluronic acid-D-galactose, and 2-O-α-D-glucopyranosyluronic acid-L-xylose, have been obtained by acid hydrolysis of alfalfa hemicellulose, in addition to the five acidic components and five neutral sugars reported previously. The results may be of value in ascertaining the role of hemicelluloses in animal nutrition.

THE HYDROLYSIS of the hemicellulose of alfalfa (*Medicago sativa* var. Ranger) leading to a mixture of neutral and acidic components was described in a previous communication (*7*). The neutral components were shown to be L-arabinose, D-xylose, D-galactose, D-glucose, and L-rhamnose. Of the seven acidic components encountered, five were identified as oxalic acid, D-galacturonic acid, 4-*O*-methyl-D-glucuronic acid, 2-*O*-(4-*O*-methyl-α-D-glucosyluronic acid)-D-xylose, and *O*-4-*O*-methyl-α-D-glucosyluronic acid-(1→2)-*O*-β-D-xylopyranosyl-(1→4)-β-D-xylose. The two other unidentified acids, which were thought to be galacturonic acid containing aldobiouronic acids, are shown herein to be 2-*O*-α-D-galactopyranosyluronic acid-L-rhamnose (I, R = H) and 6-*O*-β-D-glucopyranosyluronic acid-D-galactose (IV, R=H). Another acid, 2-*O*-α-D-glucopyranosyluronic acid-D-xylose (VIII, R=R_1=H), which brings the total to eight, was derived from an aldotriouronic acid which appears to be *O*-α-D-glucopyranosyluronic acid (1→2)-*O*-β-D-xylopyranosyl-(1→4)-D-xylose (VII), the analog of the previously identified aldotriouronic acid cited above which contained 4-*O*-methyl-D-glucuronic acid.

Experimental

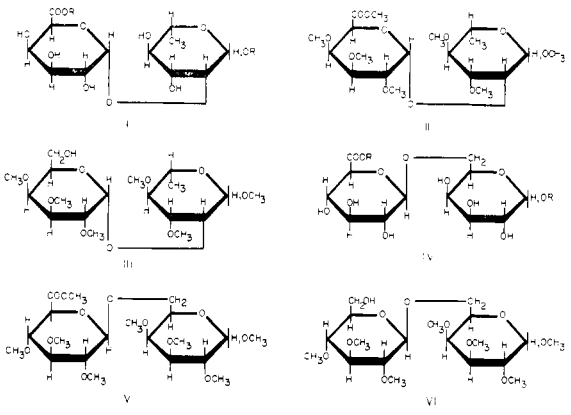
All evaporations were carried out in vacuo at 35° to 45° C. (bath temperature) unless specified otherwise.

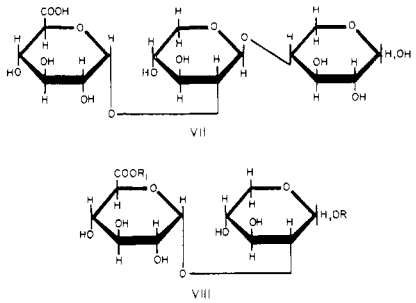
The ion exchange resins, Amberlite IR-120 (H^+ form) and Duolite A-4 (OH^- form), were used throughout.

The irrigating solvents used in chromatography were: *A*, *n*-butyl alcohol–acetic acid–water (2:1:1); *B*, ethyl acetate–acetic acid–formic acid–water (18:3:1:4); *C*, pyridine–ethyl acetate–water (1:2.5:3.5); *D*, *n*-butyl alcohol–ethyl alcohol–water (4:1:5); and *E*, butanone-water azeotrope.

Separation of Uronic Acid Components. The three uronic acids, 2-*O*-α-D-galactopyranosyluronic acid-L-rhamnose (I, R = H), 6-*O*-β-D-glucopyranosyluronic acid-D-galactose (IV, R = H), and an unknown aldotriouronic acid (?VII), which were separated from the other uronic acid components of alfalfa hemicellulose by cellulose column chromatography as described previously (*7*), had approximately the same R_f value (0.16) using solvent *A*. Examination showed that the aldotriouronic acid could be separated from the mixture of the two aldobiouronic acids by electrophoresis (*2*) using Whatman No. 1 paper, 0.1*M* borate buffer, and *p*-anisidine–phosphoric acid spray reagent. Separation on a preparative scale was carried out as follows: The sirup (0.40 gram) was applied to eight pieces of Whatman No. 3MM paper (5 × 22 inches), and each paper was placed on the electrophoresis apparatus (*2*) for 4 hours using 0.1*M* borate buffer, 600 volts and 30 to 40 ma.

After each of the papers had been dried, a strip was cut from the center and sprayed with the *p*-anisidine–phosphoric acid reagent to locate the position of each component. The components were isolated by extracting the appropriate section of the paper with water, and each solution was passed through the cation exchange resin. The effluent was evaporated to dryness. The residue was then treated for 30 minutes with 0.5% methanolic hydrogen chloride at room temperature after which the solvent was evaporated at room temperature to remove borate (*2*). In this manner an aldotriouronic acid (130 mg.), possibly α-D-glucopyranosyluronic acid-(1→2)-D-xylose-(1→4)-D-xylose (VII), and a mixture, *F*, (180 mg.) consisting of the two aldobiouronic acids (I and IV, R=H) were obtained.





The mixture *F* obtained above was refluxed with 1% methanolic-hydrogen chloride (25 ml.) for 5 hours after which the reaction mixture was neutralized with silver carbonate, filtered, and evaporated to a sirup. The sirupy mixture was resolved by paper chromatography using five pieces of Whatman No. 1 paper (9 × 22 inches) and the irrigating solvent *C* to give methyl [methyl 2-*O*-(α-D-galactopyranosyl)uronate]-L-rhamnopyranoside (I, R=CH_3) (59 mg.) and methyl [methyl 6-*O*-(β-D-glucopyranosyl)uronate]-D-galactopyranoside (IV, R=CH_3) (41 mg.).

Identification of 2-*O*-α-D-galactopyranosyluronic Acid-L-rhamnose- (I, R = H). A portion of I (R=CH_3) (2 mg.) was heated at 100° C. with 1*N* sulfuric acid for 10 hours. The solution was neutralized with barium carbonate, filtered, passed through a cation exchange resin, and evaporated to dryness. Chromatographic analysis of the residual sirup with solvents *A*, *B*, and *C* indicated the presence of D-galactouronic acid and L-rhamnose.

The methyl ester, methyl glycoside (I, R=CH_3) (57 mg.), was methylated by the Kuhn technique (*4*) using *N*,*N*-dimethylformamide (8 ml.), methyl iodide (2 ml.), and silver oxide (1 gram). The mixture was stirred for 24 hours after which the residual salts were separated by filtration and washed with *N*,*N*-dimethylformamide (2 ml.), and then with chloroform (20 ml.). The chloroform filtrate was washed with 5% aqueous potassium cyanide (20 ml.). The aqueous layer was separated and washed with chloroform (5 × 15 ml.). The chloroform extracts were dried over anhydrous sodium sulfate, filtered, and evaporated to a sirup (50 mg.). This sirup was twice methylated by refluxing with methyl iodide (5 ml.) and silver oxide (0.5 gram) (*8*) for 24 hours. The reaction mixture was filtered and evaporated to give methyl [methyl 2-*O*-(2,3,4-tri-*O*-methyl-α-D-galactosyl) uronate]-3,4-di-*O*-methyl-L-rhamnoside (II) (50 mg.) as a sirup which had $[\alpha]_D^{22}$ +72° (c—concentration in grams per 100 ml. of solution—1.7 in chloroform).

A solution of the aforementioned sirup (II) in diethylether (10 ml.) was added dropwise with stirring to a solution of lithium aluminum hydride (0.15 gram) (*1*, *5*) in diethylether (10 ml.). The solution was allowed to stand at room temperature for 1 hour. Ethyl acetate was then added to destroy the excess lithium aluminum hydride. The solution was evaporated to dryness and the residue was heated at 90° to 100°C. with acetic anhydride (5 ml.) and sodium acetate (0.05 gram) for 3 hours. The excess of the acetic anhydride was evaporated in vacuo at 60° to 70°C. and the residue was shaken with 1*N* hydrochloric acid (20 ml.) and chloroform (20 ml.). The chloroform layer was separated from the aqueous layer which was re-extracted with chloroform (4 × 20 ml.). The chloroform extracts were combined, dried over anhydrous potassium carbonate, filtered, and evaporated to a sirup. The sirup was warmed at 40° C. with 0.5*N* sodium hydroxide for 1 hour. The solution was passed through the cation resin and the effluent evaporated to give methyl 2-*O*-(2,3,4-tri-*O*-methyl-α-D-galactosyl)-3,4-di-*O*-methyl-L-rhamnoside (III) (42 mg.) as a sirup which had $[\alpha]_D^{22}$ +76° (c, 1.4 in chloroform).

The methylated disaccharide (III) was heated at 100° C. with 1*N* sulfuric acid for 7 hours after which the hydrolyzate was passed through the anion resin. The effluent was evaporated to a sirup (35 mg.) which had $[\alpha]_D^{22}$ + 30° (c, 1.6 in chloroform). Paper chromatography (solvent *E*) indicated the presence of 2,3,4-tri-*O*-methyl-D-galactose and 3,4-di-*O*-methyl-L-rhamnose. The mixture was separated by paper chromatography (solvent *E*) using two pieces of Whatman No. 1 paper (8 × 22 inches) giving 2,3,4-tri-*O*-methyl-D-galactose (13 mg.) and 3,4-di-*O*-methyl-L-rhamnose (13 mg.). The latter crystallized when nucleated with an authentic sample. After recrystallization from diethylether–petroleum ether (boiling point 35° to 60° C.), it had a melting point and mixed melting point of 90° to 92° C. and $[\alpha]_D^{20}$ +20° (equilibrium value, c, 1.0 in water). Lit. (*3*): m.p. 92° C., $[\alpha]_D$ + 18.6° (in water).

The 2,3,4-tri-*O*-methyl-D-galactose was refluxed for 4 hours with ethyl alcohol (10 ml.) in the presence of aniline (30 mg.). The ethyl alcohol and excess aniline were evaporated. The residue was dissolved in ethyl alcohol (2 ml.) and treated with a small amount of charcoal; the solution was filtered and the filtrate evaporated to a sirup which crystallized when nucleated. After recrystallization from methanol, the *N*-phenyl-2,3,4-tri-*O*-methyl-β-D-galactosylamine had a melting point and mixed melting point of 163° to 166° C. and $[\alpha]_D^{25}$ −55° changing to +42° (c, 1.8 in methanol). Lit. (*9*): melting point 167° C. $[\alpha]_D^{18}$ −65° changing to +43° (c, 1.1 in methanol).

Identification of 6-*O*-β-D-Glucopyranosyluronic Acid-D-galactose (IV, R=H). The methyl ester, methyl glycoside (IV, R=CH_3) (2 mg.), was hydrolyzed as described above to give a sirup which was warmed with acetic acid and evaporated. Chromatographic analysis of the sirup with irrigating solvents *A* and *C* indicated the presence of D-glucuronic acid, D-glucurono-(6 → 3)-lactone, and D-galactose.

Methylation of IV (R=CH_3) (39 mg.) by the Kuhn (*4*) and by the Purdie (*8*) methods as described above for I, R=CH_3, gave methyl [methyl-6-*O*-(2,3,4-tri-*O*-methyl-β-D-glucosyl) uronate]-2,3,4-tri-*O*-methyl-D-galactoside (V) as a sirup which had $[\alpha]_D^{22}$ +48° (c, 1 in chloroform).

A solution of the sirup (V) in diethylether (10 ml.) was added dropwise with stirring to a solution of lithium aluminum hydride (0.15 gram) (*1*, *5*) in diethylether (10 ml.). After the solution had stood at room temperature for 1 hour, ethyl acetate was added to destroy the excess lithium aluminum hydride, and the solution was evaporated to dryness. Cold 1*N* hydrochloric acid (25 ml.) was added to the residue and the aqueous solution was extracted with chloroform (5 × 20 ml.). The combined chloroform extracts were dried over anhydrous potassium carbonate, filtered, and evaporated to give methyl 6-*O*-(2,3,-

4 - tri - *O* - methyl - β - D - glucosyl) - 2,3,4 - tri - *O* - methyl - D - galactoside (VI) which had $[\alpha]_D^{23}$ +47° (c, 1 in chloroform).

The methylated disaccharide (VI) was heated at 100° C. with 0.5*N* sulfuric acid for 8 hours after which the solution was passed through anion exchange resin and evaporated to a sirup which had $[\alpha]_D^{21}$ +56° (c, 0.7 in chloroform). This was shown by paper chromatography (solvent *E*) to contain 2,3,4-tri-*O*-methyl-D-galactose and 2,-3,4-tri-*O*-methyl-D-glucose.

The mixture of tri-*O*-methylhexoses was separated by sheet-paper chromatography (solvent *E*) giving 2,3,4-tri-*O*-methyl-D-galactose (10 mg.) and 2,3,4-tri-*O*-methyl-D-glucose (7 mg.). The 2,3,4-tri-*O*-methyl-D-galactose (10 mg.) was treated with aniline in ethyl alcohol as described above to give crystalline *N*-phenyl - 2,3,4-tri-*O*-methyl-D-galactosylamine with a melting point and mixed melting point of 166° to 167° C. and $[\alpha]_D^{22}$ −63° changing to +43° (c, 1.5 in methanol).

The 2,3,4-tri-*O*-methyl-D-glucose (7 mg.) was heated at 75° to 80° C. with *p*-nitrobenzoyl chloride (30 mg.) and pyridine for 1.5 hours. Water was added to the cooled reaction mixture. The reaction mixture was kept at room temperature for 10 minutes before being poured with stirring into cold saturated sodium bicarbonate. The aqueous solution was extracted with chloroform (3 × 25 ml.) and the combined chloroform extracts were washed with water. The chloroform layer was dried over anhydrous potassium carbonate, filtered, and evaporated to a sirup. After recrystallization from methanol, the 2,3,4-tri-*O*-methyl - β - D - glucose - 1,6 - di - *p* - nitrobenzoate had a melting point and mixed melting point of 138° to 139° C.

Preparation of authentic 2,3,4-tri-*O*-methyl - D - glucose - 1,6 - di - *p* - nitrobenzoate. An authentic sample of 2,3,4-tri-*O*-methyl-D-glucose (30 mg.) was treated with *p*-nitrobenzoyl chloride as described above to give 2,3,4-tri-*O*-methyl - β - D - glucose - 1,6 - di - *p* - nitrobenzoate, melting point 138° to 139° C. (not increased by further recrystallization) and $[\alpha]_D^{22}$ −12° (c, 1.2 in chloroform). Anal. Calcd. for $C_{23}H_{24}O_{12}N_2$: C, 53.08; H, 4.65; N, 5.35. Found: C, 52.90; H, 4.84; N, 5.30.

Hydrolysis of the Aldotriouronic Acid and Identification of 2-*O*-α-D-Glucopyranosyluronic Acid-D-xylopyranose (VIII, R=R_1=H). A portion of the aldotriouronic acid (10 mg.) was hydrolyzed and lactonized as above; the resulting sirup was examined by paper chromatography with solvents *A*, *B*, and *C*. The presence of D-xylose, D-glucuronic acid, and D-glucurono-(6 → 3)-lactone was indicated as well as 2-*O*-α-D-glucopyranosyluronic acid-D-xylose (VIII, R=R_1=H) which had R_F 0.24 with solvent *A*.

The aldotriouronic acid (VII) (120 mg.) was refluxed with 4% methanolic-hydrogen chloride (25 ml.) for 20 hours after which the solution was neutralized with silver carbonate and filtered. The filtrate was evaporated to a sirup and warmed to 40° C. with 0.5*N* sodium hydroxide for 1 hour. The solution was passed successively through cation and anion resins and the effluent was evaporated and hydrolyzed (1*N* sulfuric acid, 10 hours at 100° C.). Neutralization of the solution with barium carbonate followed by filtration and evaporation gave a product identified as D-xylose by paper chromatography (solvents *C* and *D*). The methyl glycoside (VIII, R=CH_3, R_1=H), which was absorbed on the anion resin, was displaced from it with 1*N* sodium hydroxide; the alkaline solution was passed through cation resin and the effluent evaporated to give methyl 2-*O*-(α - D - glucopyranosyluronic acid)-D-xylopyranoside (VIII, R=CH_3, R_1=H) as a sirup. The methyl glycoside (VIII, R=CH_3, R_1=H) was refluxed with 2% methanolic-hydrogen chloride (10 ml.) for 4 hours. The solution was neutralized with silver carbonate, filtered, and evaporated to give methyl [methyl 2 - *O* - (α - D - glucopyranosyl)uronate]-D-xylopyranoside (VIII, R=R_1=CH_3) (68 mg.).

The sirup (VIII, R=R_1=CH_3) was heated at 90° to 100° C. with acetic anhydride (8 ml.) and sodium acetate (0.13 gram) for 4 hours. After the acetic anhydride was evaporated in vacuo at 60° to 70° C., the residue was shaken with chloroform (20 ml.) and water (20 ml.). The chloroform layer was separated; the aqueous layer was then extracted with chloroform (3 × 20 ml.). The combined chloroform extracts were dried over potassium carbonate, filtered, and evaporated to give methyl [methyl 2-*O*-(2,3,4-tri-*O*-acetyl - α - D - glucosyl) - uronate] -3,4 - di-*O*-acetyl-D-xyloside which crystallized spontaneously. After recrystallization from ethyl alcohol, it had a melting point and mixed melting point of 255° to 257° C. and $[\alpha]_D^{24}$ +103° (c, 1.9 in chloroform). Lit. (*6*): melting point 255° to 257° C. $[\alpha]_D^{22}$ +103° (c, 1.2 in chloroform).

Discussion

The mixture of unidentified uronic acid–containing oligosaccharides referred to in the previous communication (*7*) was separated from the other acidic components of the alfalfa hemicellulose by cellulose column chromatography using *n*-butyl alcohol–acetic acid–water as the solvent. By paper electrophoresis using a borate buffer the unknown mixture was separated into two components—one, an aldotriouronic acid; the other, a mixture of two aldobiouronic acids. The mixture of the two aldobiouronic acids was treated with methanolic hydrogen chloride to give ester glycosides. These glycosides were separated by paper chromatography using a pyridine–ethyl acetate–water solvent to give methyl [methyl 2-*O*-(α-D-galactopyranosyl)uronate] - L - rhamno - pyranoside (I, R=CH_3) and methyl [methyl 6 - *O* - (β - D - glucopyranosyl)-uronate]-D-galactopyranoside (IV, R=CH_3).

The aldotriouronic acid (VII) gave on acid hydrolysis D-xylose, D-glucuronic acid (accompanied by the derived and readily identified D-glucurono-(6 → 3)-lactone), and 2-*O*-α-D-glucopyranosyluronic acid-D-xylose (VIII, R=R_1=H). When the aldotriouronic acid was subjected to methanolysis, methyl D-xyloside and methyl [methyl 2-*O*-(α-D-glucopyranosyl)uronate] - D - xyloside (VIII, R=R_1=CH_3) were formed. The latter was separated in the free acid form (VIII, R=CH_3, R_1=H) on an anion exchange resin. Reformation of the methyl ester (VIII, R=R_1=CH_3) followed by acetylation provided the characteristic crystalline methyl [methyl 2-*O*-α-D-glucopyranosyl)uronate] - D - xyloside pentaacetate (*7*).

The mobility of this oligouronic acid on paper chromatograms and electrophoretograms and the fact that on hydrolysis it gives xylose and the aldobiouronic acid, 2-*O*-α-D-glucopyranosyluronic acid-D-xylose (VIII, R=R_1=H), strongly suggest that the oligouronic acid is an aldotriouronic acid, α-D-G*p*A-(1→2)-D-xyl*p*-(1→?)-D-xyl (VII). The linkage between the two xylose units remains to be established; but by analogy with the aldotriouronic previously isolated and identified (*7*) from alfalfa hemicellulose, namely, 4-*O*-methyl-α-D-G*p*A-(1→2)-β-D-xyl*p*-(1→4)-D-xyl, it would appear that this linkage may be of the β-(1→4)type. (G*p*A = glucosyl uronic acid; xyl*p* = xylopyranosyl).

The 2-*O*-α-D-galactopyranosyluronic acid-L-rhamnose (I, R=H) was identified by complete methylation to give methyl [methyl 2-*O*-(2,3,4-tri-*O*-methyl - α - D - galactosyl)uronate] -3,4 - di-*O*-methyl-L-rhamnoside (II). Reduction of the latter with lithium aluminum hydride (*1*, *5*) afforded methyl 2 - *O* - (2,3,4 - tri - *O* - methyl - α - D - galactosyl) - 3,4 - di - *O* - methyl - L - rhamnoside (III), which upon hydrolysis gave 2,3,4-tri-*O*-methyl-D-galactose (identified as its characteristic aniline derivative) (*9*) and 3,4-di-*O*-methyl-L-rhamnose (*3*) which crystallized directly. The relatively high positive specific optical rotation of the aldobiouronic acid and its derivatives strongly suggests that the biose linkage is of the α-type.

The second aldobiouronic acid (IV,

R=H) was shown by acid hydrolysis to be composed of D-glucuronic acid and D-galactose. Complete methylation of the methyl ester methyl glycoside (IV, R=CH_3) gave methyl-[methyl-6-*O*-(2,3,4 - tri - *O* - methyl - β - D - glucosyl) uronate] - 2,3,4 - tri - *O* - methyl - D - galactoside (V) which upon reduction with lithium aluminum hydride (*1, 5*) yielded methyl 6-*O*-(2,3,4-tri-*O*-methyl-β - D - glucosyl) - 2,3,4 - tri - *O* - methyl - D-galactoside (VI). The structure of the latter was deduced from the observation that upon hydrolysis it afforded about equal amounts of 2,3,4-tri-*O*-methyl-D-glucose (identified as the 1,6-di-*p*-nitrobenzoate) and 2,3,4-tri-*O*-methyl-D-galactose (identified as its aniline derivative).

These findings emphasize the highly complex structure of the alfalfa hemicellulose. Thus 4-*O*-methyl-D-glucuronic acid was previously shown to be attached by α-(1→2) linkages to D-xylose units. In the present paper, D-glucuronic acid is shown to be attached to D-xylose by α-(1→2) linkages and to D-galactose by β-(1→6) linkages. The present work also revealed that D-galacturonic acid units are an integral part of the hemicellulose molecular complex and that they are joined by α-(1→2) linkages to L-rhamnose. D-Glucuronic acid units linked by β-(1→6) bonds to D-galactose and D-galacturonic acid units joined by α-(1→2) linkages to L-rhamnose are structural features common to the plant gums and mucilages (*10*).

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FEED SUPPLEMENTS

Enzyme Supplementation of Rations for Dairy Calves

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An evaluation was made of the effects of adding supplementary digestive enzymes to calf starter rations, containing either steam-rolled or dry-cracked grains. Measurements were made of blood glucose level, rumen pH, proportion of rumen volatile, fatty acids, feed consumption, and growth rate of calves during a 16-week period. Neither the addition of supplementary enzymes nor steaming of the grains had any appreciable effect on feed consumption or growth rate to either 8 or 16 weeks of age. The average blood glucose level of all groups of calves declined from approximately 80 mg. per 100 ml. at 11 days of age to a level typical of an adult ruminant by 32 days of age. Differences among groups with respect to proportions of rumen volatile, fatty acids were of insufficient magnitude to be reflected in growth rate.

DURING THE LAST few years it has been shown (*3, 5, 7, 9, 18*) that very young animals secrete a limited amount of certain digestive enzymes known to be necessary for the utilization of rations ordinarily consumed by mature animals of the same species. Moreover, Larson *et al.* (*13*) observed that very little digestion of starch occurred in calves which were about 9 months of age when different experimental diets were fed directly into the omaso-abomasal cavity, presumably because insufficient amylase was secreted to utilize the starch in these diets efficiently.

In the evaluation of supplemental enzymes for calves, most investigators have added the enzymes to milk replacers or gruel mixtures which upon ingestion would be expected to pass directly into the abomasum (*6, 14, 20, 27*). In these studies, the addition of pepsin, malt diastase, papain, animal diastase, or pancreatin powder to rations containing various ingredients has been without benefit for dairy calves.

More recently, enzymes have been added to the rations of cattle and sheep under conditions where the ingested feed would pass into the rumen. Burroughs *et al.* (*2*) and Nelson and Catron (*17*) have reported experiments in which the addition of various supplementary enzyme preparations to rations for beef steers increased the rate of gain and improved feed conversion efficiency. Other workers (*12, 19, 21, 24, 25*) have observed no substantial benefit from such additions to rations for fattening lambs or beef cattle. In the work by Burroughs *et al.* (*2*), the digestibility of dry matter, organic matter, protein, and cellulose was not influenced by the addition of a combination of proteolytic and amylolytic enzymes (Agrozyme); however, Grainger and Stroud (*8*) observed an increase in the digestibility of dry matter by wethers upon the addition of gumase, amylase, or a combination of these enzymes along with protease, to a semipurified ration. The addition of the three enzymes separately, or in combination, resulted in a significant increase in crude cellulose digestibility, while only the combina-

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