Reports

Dissimilation of Alfalfa Saponins by Rumen Bacteria

Experimental symptoms of bloat have been produced in sheep by the oral and intravenous administration of alfalfa saponins, demonstrating that saponins have more than one mode of action (*1*). However, the fate of the saponins, the action of microorganisms on them, and the mechanism in the production of bloat are still not known. Recently, Hungate *et al.* (*2*) and Jacobson and Lindahl (*3*) postulated that polysaccharide slime production by ruminal microorganisms might contribute to a stable froth formation which interferes with the normal eructation of fermentation gases in ruminants. The present report presents evidence that alfalfa saponins are utilized by certain rumen bacteria, with resultant production of acids, gas, and large amounts of slime.

Saponin-digesting bacteria were isolated from the rumens of six steers that were being fed a fresh alfalfa diet, by means of modifications of techniques described previously (*4*). Rumen contents were inoculated into two parallel dilution series of 20-percent-rumen-fluid agar shake-tubes, and one series was enriched with 0.5 percent alfalfa saponin. Organisms utilizing saponin were detected by the larger size of the colony as compared with that of the nonsaponin control. Organisms presumed to be saponin bacteria were observed on dilution series from all six animals, with a peak number of 680 million per milliliter of ruminal fluid. Microscopic examination of different colonies showed cells of variable morphology; the predominant type was a small, Gram-negative, curved, motile rod.

Pure cultures of several strains of the bacteria were grown in order to study their characteristics and to obtain quantitative evidence of saponin dissimilation from the chemical analyses of metabolic end products. Fermentation products from the breakdown of 200 mg of alfalfa saponins (*5*) by a representative strain in a yeast-extract peptone medium yielded (in millimoles): carbon dioxide, 1.9; acetic acid, 0.23; butyric acid, 0.15; formic acid, 0.52; lactic acid, 0.14; and traces of ethanol and propionic acid. The organism had a fermentation pattern similar to the atypical strains of *Butyrivibrio* described by Bryant (*6*). Large amounts of slime from the fermentation of saponins were harvested by centrifugation at the end of the incubation period, while scant growth occurred in control medium without saponin.

In the various experiments the dry-weight yield of viscous slime material was approximately 50 percent of the saponin provided as substrate. When the organism was grown on glucose, 10 percent of the substrate appeared as cellular material. Acid hydrolysis of the slime matter gave copious amounts of copper-reducing compounds, indicating its polysaccharide or other glycosidic nature. Heavy froth formation from the microbial degradation of saponins was readily demonstrated in rumen contents containing viable microorganisms, but froth was not produced from the saponins when the bacteria and protozoa were inactivated by mild heat. Rapid gas evolution formed a frothy foam layer 40 mm above the liquid phase 3 hours after 2-percent saponin was administered to rumen ingesta.

Our results support the theory that "frothy bloat" of ruminants maintained on rich forages, such as clover and alfalfa, may be caused by an increase in the production of bacterial polysaccharide slime. The latter forms stable foam in which the rumen fermentation gases are entrapped in the ingesta as numerous small gas bubbles and are prevented from escaping (*2*). In manometric experiments, Hungate (*2*) has observed a correlation between the foam production of rumen ingesta and the intensity of the bloat symptoms of cattle.

The evidence of a rapid microbial decomposition of legume saponins, with the concomitant production of gas and slime, indicates the interaction which exists between the saponins and certain rumen bacteria and demonstrates how these plant compounds may contribute to bloat. In addition, bacterial attack on soluble sugars in the plant materials probably enhances the quantity of slime in the rumen. It is noteworthy that Barrentine (*7*) has had some success in the control of bloat in cattle on rich pasturage through the administration of antibiotics.

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31 October 1957

Spectral Changes Accompanying Binding of Acridine Orange by Polyadenylic Acid

A recent article by Szent-Györgi and co-workers has cited evidence for formation of a complex between the fluorescent dye acridine orange and the adenine portion of adenosine triphosphate, as well as adenine itself (*1*). In view of this result, it was of interest to examine the interaction of this dye with synthetic polyribonucleotides. This has been done for polyadenylic acid with somewhat unexpected results.

Polyadenylic acid was prepared by the action upon adenosine diphosphate of the nucleotide polymerizing enzyme isolated from *Micrococcus lysodeikticus* by a method described elsewhere (*2*). Spectral and fluorescence studies were confined to concentrations of acridine orange sufficiently low ($< 5 \times 10^{-5} M$) so that the monomer-dimer equilibrium, as studied by Zanker (*3*), is displaced greatly in favor of the monomer. No spectral effect was observed upon addition of adenosine monophosphate. However, a quite marked effect was observed

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with polyadenylic acid. The addition of increasing amounts of polyadenylic acid to an acridine orange solution in 0.5*M* KCL, 0.01*M* NaOAc, *p*H 6.7, results in a very marked decrease in extinction at $\lambda = 495$ mμ, which also decreases relative to the 465 mμ peak, as Figs. 1 and 2 show. Under the above conditions, the relative decrease in extinction at 495 mμ is almost linear with respect to the mole ratio of adenosine monophosphate to acridine orange, and reaches its limiting value at a mole ratio close to 1:1, as Fig. 1 indicates. If the addition of polyadenylic acid is continued until quite large mole ratios of adenosine monophosphate units to acridine orange (70:1) are attained, the visible spectrum gradually undergoes a second alteration, d_{495} increasing to a relative value of 0.75. In view of the altered shape of the absorption spectrum it is unlikely that the decrease in extinction can be attributed to optical shielding effects alone. It is more likely that it at least partially reflects an altered resonance state of the bound dye molecule.

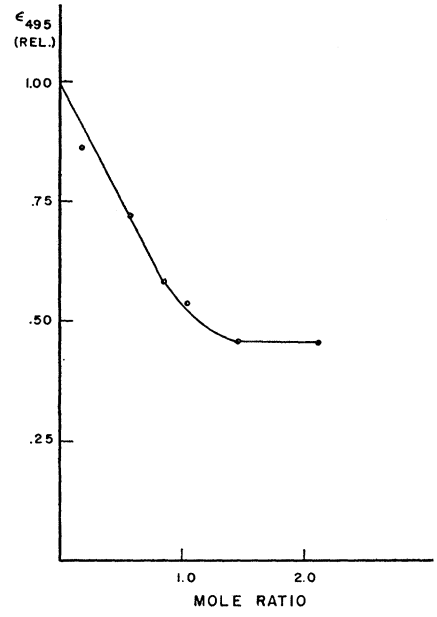

Fig. 1. Effect of addition of increasing amounts of polyadenylic acid to an acridine orange solution of molarity 4.7×10^{-6}. The *p*H is 6.7. The solvent is 0.01*M* NaOAc, 0.5 *M* KCl.

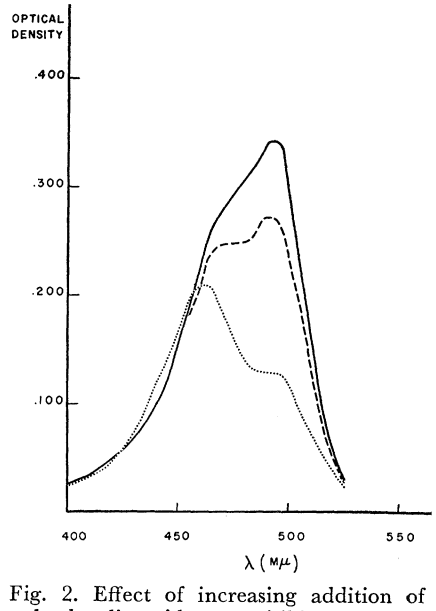

Fig. 2. Effect of increasing addition of polyadenylic acid upon visible spectra of acridine orange. The solvent is 0.01*M* NaOAc, 0.5*M* KCl. The *p*H is 6.7. The molarity of acridine orange is 6.7×10^{-6}. Solid line, no added polyadenylic acid; broken line, $2.6 \times 10^{-6}M$ (adenosine monophosphate units) polyadenylic acid; dotted line, $2.1 \times 10^{-5}M$ (adenosine monophosphate units) polyadenylic acid.

These phenomena were paralleled by a pronounced decrease in the intensity of the fluorescence of the acridine orange ($\lambda_{max.} = 550$ mμ). The intensity of fluorescence decreases to a value of 0.27 relative to that for free dye at about a 1:1 mole ratio of dye to monomer unit (for a total dye concentration of 6.2×10^{-6}) and slowly increases as the nucleotide-dye ratio becomes very large, reaching a relative value of 0.95 at a ratio of 70:1.

Pretreatment of the polyadenylic acid by formaldehyde according to the method of Fraenkel-Conrat results in abolition of both the spectrophotometric and fluorescence quenching effects (*4*).

The preceding results are consistent with the conclusion that acridine orange is bound in a one-to-one manner by the constituent adenosine monophosphate units of polyadenylic acid at low nucleotide-dye ratios. The subsequent alteration of the spectral and fluorescence behavior at high nucleotide-dye ratios reflects the competitive formation of a second complex, favored by the sharing of bound dye by an excess of polyadenylic macromolecules.

In view of the abolition of the spectral and fluorescence effects by blocking the 6-amino group of adenine with formaldehyde and by titration of the adenine groups, it appears that the adenine ring is probably involved in the interaction process. These results thus provide additional evidence for the availability of the adenine group of polyadenylic acid at alkaline *p*H's, as suggested earlier (*4*). In contrast, deoxyribonucleic acid isolated from *M. lysodeikticus* gave no spectral change.

These effects are of considerable interest in view of the known properties of acridine orange as a vital stain and bactericidal agent (*5*).

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5. The opinions expressed here are ours and are not to be construed as official or as reflecting the views of the Navy Department or the naval service at large.

23 October 1957

Use of Mononitrophenols Containing Halogens as Selective Sea Lamprey Larvicides

The invasion of the upper Great Lakes by the sea lamprey and its destructive attacks upon important fishes have engendered a considerable effort to control the numbers of this predator. Means for combating this pest may be applied effectively and economically during those parts of its life-cycle that are spent in streams (*1*). Destruction or diversion of the adults during their spawning runs (the terminal stage of the life-cycle) is a practicable procedure. This is being accomplished by electrical barriers which kill, or repel, the adults before they reach the spawning grounds (*2*). A serious shortcoming of this control technique is the time lag which occurs before the effect of such measures can become apparent. At least six generations of larval lampreys, already resident in stream habitats, continue to grow, transform into adults, and migrate into the lakes to begin their existence as parasites. Thus it may be as much as seven years before the lamprey population of a lake basin shows any marked decrease after the blocking of all streams suitable for spawning.

The life-cycle of the sea lamprey may also be broken effectively at the larval stage by the regulated application of selectively toxic chemicals. This treatment of streams inhabited by sea lampreys should produce more immediate control of the species than is being achieved by present techniques. In the course of screening various chemicals in a search for selective sea lamprey larvicides (*3*), we discovered six mononitrophenols containing halogens which are significantly more toxic to sea lamprey larvae than to other aquatic organisms (*4*). We also found that the conversion of four of these substances to their respective sodium salts increases their general utility without significantly affecting their useful biological activity (Table 1).

Laboratory experiments were conducted in 10-lit glass battery jars, each containing 5 lit of water. These vessels were aerated by means of standard stone air-breakers and were maintained at a constant temperature by immersion in