[CONTRIBUTION FROM THE NEVADA AGRICULTURAL EXPERIMENT STATION.]

ALFALFA SAPONIN. ALFALFA INVESTIGATION VII.

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When alfalfa hay is extracted with hot 95% alcohol various substances are removed. Two of these, myristone[1] and alfalfone,[2] have already been described. When the filtered alcohol from the hay extraction was allowed to cool, a voluminous green precipitate resulted which was extracted with ether for several hours. The two above-mentioned ketones were obtained from the ether-soluble residue in the flask. An insoluble brown gummy substance remained in the extraction cartridge of the Soxhlet apparatus, of which it was deemed important to undertake a detailed investigation.

Some qualitative tests made upon the substance suggested that it might belong to the saponin group. It was very soluble in water, and when the solution was agitated a strong persistent foam was produced. It was also found to be strongly hygroscopic, and became semi-liquid and sticky when exposed to the air.

Some of the characteristic saponin tests were then applied, several of which resulted positively, so that scarcely any doubt remained that the substance was saponin, with the one exception that it contained nitrogen. The product was insoluble in most organic solvents, even in alcohol, which was used in its original extraction. Water and glycerin seemed, indeed, to be the only two solvents that dissolved the substance readily.

A fairly concentrated water solution of the saponin was poured slowly with constant stirring into a large volume of cold 95% alcohol, when a yellowish flocculent precipitate resulted which was filtered and washed with alcohol and then dried in a vacuum desiccator over sulfuric acid. The product was a yellowish very hygroscopic powder whose ash content amounted to between 11 and 12% in different lots prepared in this way. Repeated precipitations from alcohol did not appreciably lower the percentage of ash. One lot, however, prepared by several successive precipitations, gave an ash content of 9.45%.

In the long and tedious search for some method by which the substance could be purified, it was discovered that upon dialyzing a water solution of the saponin through parchment tubes it could be split up into two different substances, the diffusate having a light yellow color when dry, and an ash content of 21–22%. The dialysate was of a brownish color, when dry, with an ash content of 3–6% depending on the duration of the dialysis. When the dialysis was continued beyond 8 days the ash content of the dialysate remained nearly constant at 3.5–4%, although one lot which was dialyzed 12 days had an ash content of only 2.99%.

[1] THIS JOURNAL, 33, 2 (1911).

[2] *Ibid.*, **34**, 300 (1912).

Attention should here be called to the fact that the saponins described in the literature contain more or less ash.

A fractional precipitation of the alfalfa saponin was then undertaken to determine whether the nitrogen appeared in all of the fractions. Ten g. of saponin was dissolved in 100 cc. water, after which successive portions of 15–50 cc. of 95% alcohol were added, and the precipitate from each fraction filtered off and dried. Twelve fractions were made in this way and every one was found to contain nitrogen.

A determination of the amount of saponin present in air-dried alfalfa gave results indicating 2.0–2.3% of a product containing "Saponin X" (the yellow colored substance in the diffusate), but when this was removed by dialysis the remaining pure saponin did not amount to more than 1% of the original alfalfa.

Properties.—Alfalfa saponin dissolves readily in water and warm glycerine. It is slightly soluble in hot 95% alcohol and in glacial acetic acid, very slightly in ethyl acetate, carbon tetrachloride, phenol, nitrobenzene, and methyl alcohol. It is insoluble or almost insoluble in cold 95% alcohol, ether, chloroform, benzene and amyl alcohol. Dry saponin is colored brown and imparts a reddish color to its water and glycerin solutions. This color persists throughout the various purifications and chemical transformations. It is amorphous and a strong sternutatory in the dry and powdered state. When heated upon platinum foil it gives off white fumes of a decidedly characteristic odor. It has no definite melting point, but begins to darken and decompose at 280 to 300°. When pure it is almost tasteless, but before dialysis it has a slightly bitter taste, probably due to the yellow substance which accompanies it.

Alfalfa saponin is not toxic to animals or fish, and does not hemolyze blood. Its water solution has a high surface tension and minute quantities in water will produce decided foaming under agitation. To show the degree of surface tension produced, a fairly concentrated solution of alfalfa saponin was prepared (about 1 part to 25 parts of water). Bubbles were blown with this solution, using a side neck test-tube, as soap bubbles are blown. We succeeded in obtaining bubbles at least four inches in diameter, using this pure alfalfa saponin solution. When a Merck saponin solution of the same strength was tried scarcely half as large bubbles could be blown. Some further tests of the foam-producing property of alfalfa saponin were made at one of the soda fountains of the city, by mixing minute portions of the material with a few of the more common soft drinks, and then forcing charged water through them in the ordinary way. The foam produced was so strong and persistent that nothing had been seen like it.

Its optical properties could not be determined, since a 0.1% solution did not admit sufficient light through a 20 cm. tube. It is neutral to litmus

and phenolphthalein, but is slightly alkaline to sodium alizarine sulfonate. It is colored red by conc. sulfuric acid. A solution of potassium ferricyanide to which a drop of ferric chloride has been added is colored green by the saponin, changing to blue, and a solution of potassium permanganate is slowly reduced by it. The saponin is precipitated by neutral as well as basic lead acetate, by barium hydroxide and by picric acid. Its water solution does not reduce Fehling's solution, but if the former has first been heated with a dilute mineral acid the Fehling solution is strongly reduced. The heating with mineral acids produces glucose and a sapogenin. Its empirical formula is $C_{27}H_{37}NO_{16}$.

Composition.—A sample of saponin that had been dialyzed for 8 or 9 days, reprecipitated from 95% alcohol, filtered, washed, and dried, was subjected to elementary analysis by the Liebig combustion method. Nitrogen determinations were made on the same product by the Kjeldahl method.

Subst., 0.1714, 0.1740: CO_2, 0.3087, 0.3132; H_2O, 0.0872, 0.0878; ash, 0.0065, 0.0065.

Subst., 0.5101, 0.4988: N, 0.011208, 0.011348.

Calc. for $C_{27}H_{87}NO_{16}$: C, 51.32; H, 5.91; N, 2.22. Found: C, 51.06, 51.00; H, 5.92, 5.87; N, 2.19, 2.23; ash, 3.79, 3.73.

It has already been mentioned that a substance insoluble in water results when saponin in solution is heated with a mineral acid, and an attempt was made to determine the nature of this sapogenin. Saponin was hydrolyzed with boiling 12% hydrochloric acid in a flask supplied with a thimble reflux condenser for 4 hours, and the resulting precipitate carefully washed with water and dried.

Subst., 0.1408, 0.1527: CO_2, 0.2762, 0.2957; H_2O, 0.0565, 0.0599; ash, 0.0011, 0.0012.

Subst., 0.3841: N, 0.0133095.

Calc. for $C_{18}H_{18}NO_{10}$: C, 52.92; H, 4.45; N, 3.43. Found: C, 53.92, 53.23; H, 4.53, 4.36; N, 3.47; ash, 0.75.

It has already been mentioned that when alfalfa saponin is hydrolyzed with mineral acids a reducing sugar is found in the filtrate. After the hydrolysis of 2 g. saponin, the filtrate from the sapogenin was concentrated on the water bath, treated with sodium acetate and phenylhydrazine hydrochloride, and heated according to the conditions of the osazone test. Yellow needles separated in about 6 minutes, having a melting point of 204–205°. This established the identity of glucose.

To arrive at a quantitative relation between the saponin and the liberated dextrose, the following experiments were performed:

(*a*) 3.0234 g. saponin was heated in a pressure bottle with 100 cc. of a 10% sulfuric acid solution (by weight), for three hours at the boiling point of water. The resulting precipitate was collected on a filter paper and washed with water. The washings combined with the original sulfuric acid solution. After removing nearly all the acid with

barium, $^1/_4$ of the solution was taken for the dextrose determination, yielding 0.3674 g. copper by the Kendall method. This is equivalent to 256.9 mg. dextrose per g. of saponin

(*b*) 1.0042 g. saponin (another lot) was hydrolyzed by boiling for 5 hrs. with 100 cc. of a 5% hydrochloric acid solution, in a flask connected with a thimble reflux condenser. The resulting liquid yielded 0.4850 g. copper or 255.2 mg. dextrose per g. saponin.

(*c*) 1.0024 g. saponin was similarly hydrolyzed with 10% hydrochloric acid, yielding 0.4812 g. reduced copper, or 253.7 mg. dextrose per g. saponin.

The average of these three determinations is 255.3 mg. Cu per g. of saponin or 25.53% dextrose in the molecule. According to theory, considerably more dextrose should have been obtained provided all were split off, but the heating may not have continued long enough to complete the hydrolysis.

A few well known saponins contain galactose and pentose in addition to glucose; therefore experiments were carried out to determine whether one or both of these sugars were present. The galactose experiments all resulted negatively, but when the saponin was distilled with hydrochloric acid, furfural was obtained. Two quantitative experiments were carried out, and in both cases one gram of saponin was taken. The first yielded 0.2097 g. furfural phloroglucide and the second 0.2106 g., or an average of 0.21015 g. This is equivalent to 0.2117 g. pentose or 21.17% of the weight of the saponin taken.

As in the case of the dextrose determination, the yield of pentose was below that which was expected from theoretical considerations, but since the causes for these discrepancies may be difficult to determine it was deemed undesirable to investigate the same at this time.

In the process of the investigation it was discovered that alfalfa saponin could be acetylated, yielding a definite product. This acetyl derivative was made by heating one part saponin with one part sodium acetate and four parts acetic anhydride for one hour with a reflux condenser. The insoluble product was washed on the filter paper, then dissolved in glacial acetic acid and reprecipitated with water, and the substance again washed and dried. It was slightly darker in color than the original saponin and did not melt below 300°.

The acetyl determination upon this product was made by saponifying 0.5 g. with *N* sodium hydroxide for 30 minutes, acidifying with conc. sulfuric acid and distilling. The distillate was titrated with 0.1 *N* sodium hydroxide and 0.1674 g. acetic acid was found to be present, which is equivalent to 0.1199 g. CH_3CO, or 23.98% of the acetylated saponin.

The combustion values of the acetylated product would indicate that the acetylation had taken place in the sapogenin part of the molecule and that the remainder was removed. A regenerated saponin with the same properties could not be obtained from the acetylated product. It is not

beyond the range of possibility that the glucose radical had been eliminated in the process of preparation and purification of the acetylated product.

Stutz[1] also found that the empirical formula for quillajic acid, which was regenerated from an acetyl product, differed from the formula of the original saponin; and Kobert has proved definitely that assamin regenerated from the acetyl product has no similarity to the original assamin. Following are the combustion values obtained upon the acetylated product of alfalfa saponin:

Subst., 0.2017, 0.1558, 0.1734: CO_2, 0.4066, 0.3086, 0.3428; H_2O, 0.0778, 0.0614, 0.0737.

Found: C, 54.98, 54.02, 53.82; H, 4.31, 4.41, 4.74. Average (excluding last H value): C, 54.27; H, 4.36.

If we assume that alfalfa sapogenin, $C_{18}H_{18}NO_{10}$, was the substance that was acetylated instead of saponin, and that four acetyl radicals had entered the molecule, the nitrogen occupying the place of the fifth, we find that the theoretical composition of the compound would be C = 54.14% and H = 4.55%. If this assumption is correct the saponification of the product mentioned above could not have been carried to completion, but the combustion values obtained are in close agreement with the theoretical values for $C_{18}H_{14}NO_{10}.(CH_3CO)_4$.

Several saponins react with cholesterine to yield insoluble cholesterides, which are easily purified, and the saponin regenerated with apparently the same properties. Consequently, several attempts were made to obtain a cholesteride with alfalfa saponin, but all of them failed. With alcohol the reaction requires at least 1% solution of the saponin, but it was impossible to make even a 0.1% solution.

Metallic derivatives were made with both lead and barium. The salts resulting were well defined amorphous bodies, but no solvents could be found to dissolve them. The salts were both dark colored, but the lead salt was of a more pronounced yellow. After washing the salts repeatedly by decantation and on filters quantitative determinations of the metals were made, but the results were not uniform. A slight difference in concentration of the saponin solution or the temperature at which the precipitation took place may have been responsible for the variation. Two determinations of lead in the lead salts gave 51.75% and 52.75% Pb. The barium determinations in the barium salts yielded 41.39% and 40.02% Ba. No significance is considered to attach to these results, although some definite connection might be established if it were thought to be worth while to put more work on the problem.

Pharmacological.—As a class saponins are poisonous substances and the toxicity seems to be specific to fish. Consequently, experiments along this line were tried with alfalfa saponin.

[1] *Ann.*, **218**, 255 (1863).

(*a*) A black bass 5 inches long died inside of 7 hours when put into 3.5 liters of a saponin solution of concentration 1 to 35,000.

(*b*) A black bass of about the same size died inside of 15 hours when put into 25 liters of a saponin solution of concentration 1 to 35,000.

(*c*) Another black bass of the same size as the two previous died in about 12 hours after being put into 25 liters of a saponin solution whose concentration was 1 to 25,000.

The above experiments would indicate that alfalfa saponin is toxic to fish, for the same volume of fresh water would keep a fish of this size alive for many days. The saponin solution at the above concentration would foam to some extent when agitated, and it had a slightly yellowish tint.

Before drawing definite conclusions regarding the toxicity of the saponin to fish, another experiment was tried using a carp (gold fish) obtained from the warm water ponds at Bower's Mansion, Nevada.

(*a*) 25 liters of an alfalfa saponin solution, of a concentration 1 to 35,000 was prepared, and a gold fish weighing 13 g. was put into it at 2 P.M. on Aug. 21. On the 22d the fish appeared normal and on the 23rd food was supplied in the form of flies, of which a number were eaten. On the 23rd and 24th, the fish came to the surface of the solution every minute or two, with the apparent purpose of getting oxygen from the air. A fresh saponin solution was prepared on the 24th, and in the interim the fish was transferred to two liters of fresh water. During the time that elapsed he did not come to the surface for air a single time, nor did he come to the surface after being put into the fresh saponin solution until the second day, as far as we could observe. On the 28th of August the fish was removed from the saponin solution in an apparently normal condition.

This experiment suggested that the special effect of saponin upon fish might be due to a physical condition of the solution preventing the normal supply of air, rather than to toxic properties. The black bass did not have the instinct of going to the surface for air and consequently succumbed, while the gold fish secured air from above the surface when the solution had become exhausted of oxygen.

The question then arose as to whether this explanation could be made of the effect of other saponins upon fish. Unfortunately a variety of saponins are not to be obtained upon the market. We succeeded, however, in obtaining a small bottle of saponin prepared by Merck & Co. It was a white amorphous powder giving all the characteristic saponin reactions, including the hemolysis of blood.

(*b*) 25 liters of a solution of this saponin was prepared with a concentration of 1 to 35,000; and the gold fish used in the previous experiment was put into it. This was at 4 P.M. Aug. 28. At 4 P.M. Aug 30, the fish was removed from the solution in a normal condition.

(*c*) A duplicate solution of Merck's saponin was prepared and another gold fish weighing 10 g. was put into it on August 30, and kept there until the evening of Sept. 8, when the fish was removed apparently healthy and well. During the 10 days the fish was in the solution he was fed flies and fish food from time to time.

These experiments tend to overthrow the generally accepted theory

that saponins are poisonous to fish. Time did not permit the preparation of other saponins for further work along this line.

Having established that alfalfa saponin was harmless to fish except in so far as it acts in an asphyxiating manner, it was decided to ascertain whether or not it hemolyzed blood, and was toxic to warm-blooded animals.

A 2% alfalfa saponin solution was prepared; ten test-tubes, each containing 5 cc. of a physiological salt solution together with one cc. of a 5% washed sheep-corpuscle suspension. Into these test-tubes were pipetted 1.0, 0.9, 0.8, 0.7, 0.6, 0.5, 0.4, 0.3, 0.2, and 0.1 cc. respectively of the above saponin solution. The tubes were then shaken and allowed to stand at room temperature. In 24 hours the corpuscles had settled out, leaving a colorless solution at the top in all the tubes. The tubes were allowed to stand for 6 days, but no change was observed.

A duplicate set of experiments was tried by using defibrinated sheep blood instead of the corpuscles, but with the same results. Another set of experiments with the saponin and defibrinated blood was carried out as above, but the tubes were kept at 37°, instead of at room temperature. The results, however, were the same.

Alfalfa saponin, then, does not hemolyze blood.

Two sets of experiments like the above were performed using sheep corpuscles and defibrinated blood, together with Merck's saponin of the same concentrations. Hemolysis took place in all the tubes, though at a somewhat slower rate in the more dilute solutions.

The following experiments were performed to determine whether saponin, and especially alfalfa saponin, is poisonous to warm-blooded animals.

0.5 g. of alfalfa saponin in water solution was given to one rabbit and 1.0 g. to another *per os*. The rabbits were kept under observation for 4 weeks without any abnormal symptoms developing. 0.5 g. of the same saponin in water solution was given to a guinea pig, and the animal kept under observation for the same length of time without showing any signs of poisoning.

The alfalfa saponin was next tried upon a sheep, by giving it 19 g. dissolved in water. During the three days that the sheep was kept under observation no apparent physiological disturbances were manifested, but a 0.75% diminution in the number of red blood corpuscles was recorded. This change is so slight, however, that it might easily have been due to other causes or to an error in the count.

To compare the effect of Merck's saponin upon the same kinds of animals, 0.2 g. in water solution was given to a guinea pig *per os*. In a few hours the animal developed symptoms of gagging, as if trying to expel something from the stomach. It showed signs of stupor and would not eat, and died on the sixth day. The autopsy showed a slightly congested liver. Gastro-enteritis was pronounced.

A rabbit was given 0.3 g. of the same Merck saponin in water solution *per os*. For 10 days the rabbit suffered no ill effects.

0.1 g. Merck's saponin was given to a guinea pig, and 0.15 g. to a rabbit intravenously. Death was almost instantaneous in both cases.

0.2 g. alfalfa saponin was given subcutaneously to a rabbit weighing 900 g. At the end of twenty days the rabbit was well and weighed 1140 g.

0.3 g. alfalfa saponin was given the same way to another rabbit, but in this case death resulted in about 24 hours. A duplicate of this experiment seemed desirable; therefore, the same amount of substance in aqueous solution was given subcutaneously to a third rabbit. In three days this rabbit was also dead. In the last two cases the

autopsy revealed an extensive local irritation and a gelatinous infiltration of a sanguinous nature. There was also congestion of the lungs in both cases.

Merck's saponin was tried in the same way. A rabbit was given 0.15 g. in water subcutaneously. In the afternoon of the same day the rabbit was dull and uncomfortable, but ate a little. At 8 A.M. on the second day respiration was very weak and shallow, and at 9 A.M. the animal died. Autopsy revealed the same sanguinous infiltration around the point of injection. There was considerable ascitic fluid in the abdominal cavity, also indications of acute peritonitis. The blood was not clotted.

0.1 g. Merck's saponin was given subcutaneously to a guinea pig weighing 430 g. Symptoms similar to those of the rabbit developed and the animal was found dead the next morning. A sanguinous infiltration at the point of injection was also observed in this animal. The blood did not clot.

For the present it seemed unnecessary to carry these experiments further, as the results obtained give a fairly definite idea of the physiological action of saponin as well as of alfalfa saponin.

Summary and Conclusions.

Alfalfa saponin obtained from dry alfalfa hay by extraction with alcohol is very similar to other saponins as far as physical and chemical properties go, but differs somewhat in its toxicological properties. It does not hemolyze blood, whereas other saponins do. Saponins are non-nitrogenous with the exception of solanin. Alfalfa saponin is also nitrogenous, and these two substances, then, consitute the connecting links between the true saponins and the alkaloids.

Alfalfa saponin has the empirical formula $C_{27}H_{37}NO_{16}$, and hydrolyzes to a sapogenin having the formula $C_{18}H_{18}NO_{10}$, besides yielding a glucose derivative. One pentose radical is present in each molecule of saponin. The acetyl product was made and characterized, indicating that the acetylation takes place in the sapogenin part of the molecule.

In the generally accepted sense alfalfa saponin is toxic to fish, but this property is doubtless due to the physical property by which it prevents access of air to the water. This saponin does not seem to have any physiological action when administered to animals *per os*, but causes severe local irritation and death when injected subcutaneously. Alfalfa saponin is a colored substance having no sharp melting point, but contains a small amount of ash. It is very hygroscopic but a strong sternutatory in the dry state. Alfalfa saponin solutions have a very high surface tension. Bubbles four inches or more in diameter can be blown with it. It carries with it a yellowish substance, "Saponin X," which is very difficult to remove. The fact that this saponin is not poisonous might render it of some commercial value.

Besides "Saponin X," three other well defined substances have been isolated from alfalfa and set aside for future investigation. Two of these are proteins and one of them is a bitter principle.

Alfalfa is one of the best forage crops, mainly on account of its high

protein content. For this reason it would be of considerable interest to investigate these two proteins, and if possible to establish some definite relation which they bear to the plant's power of abstracting nitrogen from the air.

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RENO, NEV.

[CONTRIBUTION FROM THE CHEMICAL LABORATORY OF THE UNIVERSITY OF ILLINOIS.]

CYCLIC ETHERS FROM *o*-ALLYL PHENOLS; METHYLENE COUMARANES.

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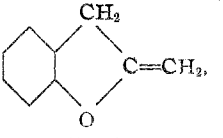
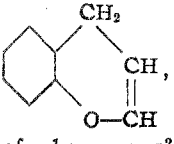
o-Allyl phenols, which are so easily prepared by the rearrangement of allyl-phenyl ethers, offer an admirable starting point for the production of various cyclic ethers. Claisen[1] has shown, for example, that pyridine hydrochloride converts them quantitatively into methyl coumaranes. The present investigation proves that methylene coumaranes,  , may also be readily produced from *o*-allyl phenols by the following succession of reactions: (1) acetylation of the *o*-allyl phenol, (2) addition of bromine to the double bond, (3) treatment with alcoholic potash. All three reactions run smoothly and good yields are obtained. Moreover, the method has been applied with success to 6 different *o*-allyl phenols so that the reaction is unquestionably a general one.

The research was originally undertaken in an attempt to synthesize the 6-membered unsaturated cyclic ethers of the following general formula,  , which may be called chromenes from analogy to the name of chromanes[2] given the corresponding saturated ethers. These substances present interest not only because they have not hitherto been synthesized but also because they may be looked upon as the basic substances of the large and important class of natural dyes, the flavones and their derivatives.

[1] *Chem. Zentr.*, [2] 1213 (1914); *D. R. P.* 279,864.

[2] *Ber.*, **38**, 855 (1905); **39**, 2856 (1906).