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[CONTRIBUTION FROM THE NEVADA AGRICULTURAL EXPERIMENT STATION.]

ENZYMES PRESENT IN ALFALFA SEEDS, ALFALFA INVESTIGATION, IV.[1]

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Any investigation aiming at a study of the life processes and metabolic changes of a plant will necessarily lead back to those changes which take place in the sprouting of the seed. The chemical changes involved in sprouting are conditioned not only by temperature and moisture, but by certain chemical complexes known under the name of enzymes.

These substances are frequently found in seeds, and it was the purpose of this investigation to characterize certain ones that might be present in the seeds of the alfalfa.

A good grade of *Medicago sativa* seeds was obtained from the *Skane Experiment Station*. To about 100 grams of the finely ground seed meal, 500 cc. of water were added, and the material allowed to soak for about 2 hours. The infusion was then filtered through muslin and the emulsion obtained used for the following tests, and will be spoken of as the seed extract.

Green,[2] Weis[3] and others have described the existence of proteolytic enzymes in germinating seeds, and Vines,[4] in his extensive researches, records their presence in many varieties of plants.

The alfalfa seed extract, mentioned above, was subjected to qualitative tests for protease, not only with the tryptophane reaction,[5] but by the digestion of casein and Witte-peptone, titrating the resulting amino acids according to the method of Sörensen.[6] All the tests showed the presence of a proteolytic enzyme, which will be discussed in detail further on.

[1] The present investigation was carried out in the laboratory of Prof. S. G. Hedin at Uppsala, Sweden; and I gratefully acknowledge Prof. Hedin's valuable counsel, as well as the use of his laboratory equipment. Professors Hammarsten and Mörner have also contributed new thoughts and suggestions.

[2] *Phil. Trans.*, **178B**, 58 (1887).

[3] *Comptes-rendus des travaux du Laboratoire de Carlsberg*, **5**, 133 (1903).

[4] *Annals of Botany*, years 1902–1906.

[5] *Ibid.*, **16**, 13 (1902).

[6] *Biochem. Z.*, **7**, 45.

Lipase.—The following experiments were carried out to determin if the seed extract contained a lipase:

Experiment 1.—(*a*) 0.26 cc. of Kahlbaum's ethyl butyrate + 4 cc. H_2O + 0.1 cc. toluene were heated together in a test tube for 5 minutes at 40°, after which 1 cc. of the seed extract was added and the resulting mixture titrated at once with 0.1 *N* sodium hydroxide, using phenolphthalein as indicator. 0.20 cc. was required for neutralization.

(*b*) In another tube, like amounts of the same substances were inserted and heated as above, but after adding the seed extract the tube was heated in a water bath at 40° for 15 minutes, after which the contents were titrated with 0.1 *N* sodium hydroxide, giving 0.25 cc. for neutralization.

Experiment 2.—(*a*) 26 cc. H_2O + 1 cc. seed extract + 10 drops toluene were measured into a bottle and heated in a thermostat at 37° for 24 hours, after which the contents were titrated with 0.1 *N* NaOH, giving 0.35 cc. for neutralization.

(*b*) 26 cc. H_2O + 1 cc. ethyl butyrate + 10 drops toluen, were heated together as in (*a*). The titration with 0.1 *N* NaOH gave 0.07 cc.

(*c*) 25 cc. H_2O + 1 cc. ethyl butyrate + 1 cc. seed extract + 10 drops toluene, heated together as in (*a*). The titration with 0.1 *N* alkali gave 0.36 cc.

Experiment 3.—(*a*) 26 cc. H_2O + 1 gram finely ground seeds + 10 drops toluene, heated in a bottle at 37° for 24 hours, after which the mixture was titrated with 0.1 *N* NaOH, giving 3.60 cc. for neutralization.

(*b*) 26 cc. H_2O + 1 cc. ethyl butyrate + 10 drops toluene, heated as in (*a*) and titrated with the same alkali, which gave 0.08 cc. for neutralization.

(*c*) 25 cc. H_2O + 1 cc. ethyl butyrate + 1 gram seed meal + 10 drops toluene, heated together as in (*a*) and then titrated with 0.1 *N* NaOH. 4.22 cc. were required for neutralization.

The first two experiments prove that no butyric ester lipase is present in the water extract of the alfalfa seeds, but the third shows that ethyl butyrate is hydrolyzed to a slight extent in the presence of the solid material. The positive results, however, are so small that one would hardly be justified in concluding that a lipase is present in the seeds of the alfalfa.

Amylase.—Tests were then made for amylase as a representative of the carbohydrases. The starch-iodine test could not be used, for the reason that the seed extract itself decolorizes iodine solutions. Resort was therefore had to the test for a reducing sugar in the digestion products.

Experiment 1.—(*a*) 5 cc. seed extract + 1 cc. "soluble" starch solution, heated together at 60° for 1 hour. The liquid was then heated to boiling and the coagulated proteins filtered off. To 3 cc. of the resulting filtrate were added 5 cc. H_2O and 60 drops of Fehling's solution, and the mixture boiled. A voluminous precipitate as well as a marked reduction of the copper resulted.

(*b*) 5 cc. seed extract + 1 cc. H_2O and the mixture treated in the same manner as in (*a*). A voluminous precipitate resulted, but no yellow color to indicate a reduction of copper.

(*c*) 5 cc. H_2O + 1 cc. starch solution, also treated as in (*a*). No reduction of copper took place.

Experiment 2.—All three parts of this experiment were carried out as in the previous one, except that the tubes were kept at room temperature for 3 hours instead of being heated at 60°. A marked reduction of copper was found in the (*a*) part, although not as pronounced as in the corresponding part of the previous experiment.

The starch solution used was quickly hydrolyzed by ptyalin as shown by a strong reduction of copper in the digestion products.

The above tests go to show that an amylase is present in the water extract of alfalfa seeds.

Coagulase.—Tests for a coagulase, on the order of rennin, were made with fresh milk, that had previously been treated with $^1/_{10}$ its volume of a 20% solution of calcium chloride.

Experiment 1.—(*a*) To 10 cc. of the prepared milk, 1 cc. of the clear neutral seed extract was added and the tube heated in a bath at 37°.

(*b*) 10 cc. milk + 1 cc. H_2O heated in the same bath simultaneously with (*a*).

At the end of $2^1/_2$ hours the milk in (*a*) was firmly coagulated but that in (*b*) exhibited no change.

Experiment 2.—(*a*) 10 cc. milk + 0.5 gram dry seed meal introduced into a test tube and heated in a bath at 37°.

(*b*) 10 cc. milk + 0.2 gram dry seed meal in another test tube and inserted in the bath at the same time.

(*c*) 10 cc. H_2O + 0.5 gram dry seed meal heated in a test tube like the above.

At the end of $1^1/_4$ hours the milk was firmly coagulated in (*a*). In (*b*) it was coagulated so that it could be poured with difficulty. In (*c*) the liquid had turned acid so that it required 0.37 cc. 0.1 *N* NaOH to neutralize it.

(*d*) 10 cc. milk + dilute acetic acid equivalent to 0.37 cc. 0.1 *N* NaOH measured into a test tube and heated at 37° for $1^1/_4$ hours.

(*e*) 10 cc. milk + the same equivalent of *n*-butyric acid, and the mixture heated as in (*d*).

At the end of the given time, the milk showed no signs of coagulation in either (*d*) or (*e*).

The above experiments go to show that the alfalfa seeds contain a coagulase which is soluble in water. Whether the coagulation of the milk is caused by an individual ferment of the nature of rennin, or is simply due to the protease present, cannot be ascertained at this time. The last two parts of the experiment show that the organic acids developed by the seeds during the heating could scarcely have caused the coagulation.

Emulsin.—This enzyme was tested for by the hydrolysis of amygdalin, as given in the following experiments:

Experiment 1.—(*a*) 25 cc. H_2O + 1 gram dry seed meal + 1 gram amygdalin were put into a bottle and heated in the thermostat at 37° for 24 hours.

(*b*) 25 cc. H_2O + 1 gram seed meal heated together in a bottle as in (*a*).

(*c*) 25 cc. H_2O + 1 gram amygdalin also heated together as in (*a*).

The digestion liquids in (*a*) and (*b*) were filtered and then tested for glucose and HCN. (*a*) had a strong benzaldehyde odor and gave the Prussian blue test for cyanide. It also gave a strong reduction of Fehling's solution. Neither (*b*) nor (*c*) gave any of the above tests.

Experiment 2.—The seed extract obtained by filtering through muslin was afterward filtered through filter paper and the clear liquid used for this experiment.

(*a*) 25 cc. H_2O + 2 cc. seed extract + 1 gram amygdalin were introduced into a bottle and heated 24 hours in a thermostat at 38°.

(*b*) 25 cc. H_2O + 2 cc. seed extract heated together as in (*a*).

(*c*) 25 cc. H_2O + 1 gram amygdalin also heated as in (*a*).

At the end of the given time a marked odor of oil of bitter almonds was noticed in (*a*) and the liquid showed a trace of hydrocyanic acid, but no copper reduction was observed. (*b*) and (*c*) gave negative results in all the tests.

Experiment 3.—(*a*) 25 cc. H_2O + 1 gram amygdalin + dilute acetic acid, equivalent to the acid developed in (*b*) of *Experiment 1*, and heated together at 37° for 24 hours.

(*b*) 25 cc. H_2O + 1 gram amygdalin + dilute *n*-butyric acid equivalent to the above amount of acetic acid, and heated together at 37° for 24 hours.

Neither (*a*) nor (*b*) gave the tests for a reducing sugar, hydrocyanic acid or benzaldehyde.

From these experiments I conclude that alfalfa seeds contain an emulsin that is very slightly soluble in water. Experiment 3 shows that there is not enough acid developed in the digestion of the seeds to affect hydrolysis of amygdalin. This enzyme is then a representative of the glucosidases.

Invertase.—Tests were made to find if the seeds and seed extract contained invertase. The Fehling tests indicated that no reducing sugar was present in the digestion products of the seed extract with cane sugar. An opalescence of the resulting filtrate rendered the polarimetric method useless. Invertase is probably not present in alfalfa seeds.

Peroxidase.—Oxidases and peroxidases are frequently encountered in seeds and plant juices, and it would not be surprising to find one or the other in the alfalfa seeds. Euler[1] describes an oxidase obtained from the stems and leaves of the alfalfa, which was isolated by the method of Bertrand.[2]

Experiment 1.—(*a*) 1 gram pyrogallol dissolved in water + 10 cc. of a 1% solution of H_2O_2 + 1 cc. of the seed extract and the whole made up to 50 cc. with water and allowed to stand in a tall glass cylinder. A reddish brown coloration of the solution developed immediately. The next morning a precipitate of the same color was found at the bottom.

(*b*) Equal amounts of the same substances except the H_2O_2 were introduced into another cylinder and also allowed to stand over night, but in this case no precipitate was found at the bottom, nor had the solution changed color.

Experiment 2.—Upon adding a small amount of the seed extract to a solution of *para*-cresol in dilute alcohol, the solution developed a milky opalescent character which, upon standing for several hours, resulted in a flocculent precipitate.

Experiment 3.—(*a*) To a guaiacum emulsion, made by dissolving the resin in alcohol and adding water till opalescence begins, was added 1 cc. of the seed extract, when a fine milky precipitate resulted. About 3 cc. of a 3% solution of H_2O_2 were then added. This caused a blue color to develop immediately.

(*b*) The same substances except the peroxide were introduced into another tube and allowed to stand. The next day no blue color had developed.

[1] *Z. physiol. Chem.*, **61**, 1.

[2] *Bull. soc. chim.*, [3] **17** (1897).

All the experiments cited point to the presence of a peroxidase, but not to a directly oxidizing enzyme in the seeds of the alfalfa.

Protease.—Qualitative tests indicated the presence of a proteolytic enzyme in the seed extract and it was considered important to ascertain to what class of the proteases it belonged, and something of its properties.

Fibrin Digestion. Experiment 1.—(*a*) 20 cc. seed extract + moist fibrin (about 0.5 gram) allowed to stand together in a bottle for 3 days, at room temperature.

(*b*) H_2O + fibrin as control.

(*c*) 25 cc. seed extract + 0.5 gram fibrin heated together 48 hours at 37°.

(*d*) H_2O + fibrin as control.

In all the bottles the fibrin remained unchanged. (*c*) did not give the tryptophane reaction. The others were not tested.

Albumin Digestion. Experiment 1.—(*a*) 20 cc. seed extract + 0.5 gram coagulated serum albumin, heated together 48 hours at 28°.

(*b*) The same substances and quantities heated together 48 hours at 37°.

At the end of the given time there was no apparent digestion.

Experiment 2.—(*a*) 25 cc. seed extract + 50 cc. neutral egg albumin solution, heated together in a bottle for 24 hours at 28°.

(*b*) 25 cc. H_2O + 50 cc. egg albumin solution heated as in (*a*).

(*c*) 25 cc. seed extract + 50 cc. H_2O heated as in (*a*).

In this as well as all succeeding experiments where Sörensen's titration method[1] was employed to determin the extent of the protein digestion, the following procedure was adopted: 25 cc. of the liquid in the digestion bottles were drawn off and 15 cc. of water and 10 cc. of the formol phenolphthalein solution added. This mixture was then titrated at once with 0.5 *N* sodium hydroxide solution. The solutions were not neutralized before the formaldehyde was added because the controls in every experiment were considered more reliable for giving the influence of the separate liquids in the digestion. The titrations for the above resulted as follows: (*a*) 3.23 cc., (*b*) 1.05 cc. and (*c*) 2.33 cc. 0.5 *N* sodium hydroxide. The difference between (*a*) and the sum of (*b*) and (*c*) in cubic centimeters is therefore a relative index of the amount of the digestion. In this case the difference is within the limits of error of the experiment, and no digestion of egg albumin has taken place. (*a*) did not give the tryptophane reaction.

Legumin and Conglutin Digestion. Experiment 1.—Solutions of these proteins, obtained from Kahlbaum, were made, using a 10% sodium chloride solution as the solvent. The protein solutions were approximately 1%.

(*a*) 25 cc. seed extract + 50 cc. legumin solution, heated together 24 hours at 37°.

(*b*) 25 cc. seed extract + 50 cc. H_2O heated as in (*a*).

(*c*) 25 cc. H_2O + 50 cc. legumin solution also heated as in (*a*).

(*d*) 25 cc. seed extract heated in steam $^1/_2$ hour. After cooling, 50 cc. legumin solution added, and the mixture heated in a thermostat for 24 hours at 37°.

The formol titration with 0.5 *N* NaOH resulted as follows: (*a*) 2.29 cc., (*b*) 2.30 cc., (*c*) 0.58 cc., and (*d*) 1.73 cc. Therefore, no digestion of the legumin had taken

[1] *Loc. cit.*

place. A faint tryptophane reaction was given by (*a*), a little stronger by (*b*), but none by (*c*) or (*d*).

Experiment 2.—(*a*) 25 cc. seed extract + 50 cc. conglutin solution heated together 24 hours at 37°.

(*b*) 25 cc. seed extract + 50 cc. H_2O heated as in (*a*).

(*c*) 25 cc. H_2O + 50 cc. conglutin solution heated as in (*a*).

(*d*) 25 cc. seed extract heated in steam $^1/_2$ hour. After cooling, 50 cc. conglutin added and the mixture heated as in (*a*).

The formol titrations resulted thus: (*a*) 2.55 cc., (*b*) 1.89 cc., (*c*) 0.51 cc., and (*d*) 2.07 cc., showing that no digestion of conglutin had taken place. The tryptophane reactions were like those in experiment 1.

The conclusion to be drawn from the above experiments is that the protease in question is not a peptonizing one, since it is unable to digest a true protein.

Peptone Digestion. Experiment 1.—(*a*) 25 cc. seed extract + 50 cc. of a 2% H_2O solution of Witte peptone, neutral to litmus, and the mixture heated for 24 hours at 28°.[1]

(*b*) 25 cc. seed extract + 50 cc. H_2O heated as in (*a*).

(*c*) 25 cc. H_2O + 50 cc. Witte peptone solution also heated as in (*a*).

(*d*) 25 cc. seed extract heated in steam $^1/_2$ hour, then 50 cc. Witte peptone solution added and the mixture heated as in (*c*).

(*e*) 25 cc. seed extract + 50 cc. Witte peptone solution heated together 24 hours at 37°.

The formol titration resulted thus: (*a*) 8.35 cc., (*b*) 1.78 cc., (*c*) 4.22 cc., (*d*) 5.15 cc., and (*e*) 9.73 cc. The difference between (*a*) and (*d*) is 3.20 cc. 0.5 *N* NaOH, which is a relative measure of the digestion of Witte peptone by the seed protease at 28°. The difference between (*d*) and (*e*) is the approximate digestion at 37°. The tryptophane reaction resulted thus: (*a*) strong, (*b*) distinct, (*c*) none, (*d*) none, and (*e*) strong.

Experiment 2.—The seed extract in this experiment was prepared from seeds that had been allowed to germinate for a period of four days.

(*a*) 25 cc. seed extract + 50 cc. Witte peptone solution heated together for 24 hours at 28°.

(*b*) 25 cc. seed extract + 50 cc. H_2O heated as in (*a*).

(*c*) 25 cc. seed extract heated in steam for $^1/_2$ hour, after which 50 cc. Witte peptone solution were added and the bottle further heated for 24 hours at 28°.

(*d*) 25 cc. seed extract + 50 cc. peptone solution heated together for 24 hours at 37°.

(*e*) 25 cc. seed extract + 50 cc. H_2O heated as in (*d*).

(*f*) 25 cc. seed extract heated in steam for $^1/_2$ hour and then 50 cc. peptone solution added after which the mixture was heated as in (*d*).

The formol titrations resulted as follows: (*a*) 9.77 cc., (*b*) 2.33 cc., (*c*) 6.20 cc., (d) 11.60 cc., (*e*) 2.68 cc., and (*f*) 6.42 cc. 0.5 *N* NaOH.

The difference between (*a*) and (*c*) is 3.57 cc., and is slightly higher than the value obtained for the ungerminated seeds. (*d*) — (*f*) = 5.18 cc., which is also higher than the corresponding value for the ungerminated seeds. The tryptophane reactions were found to be: (*a*) strong, (*b*) ?, (*c*) none, (*d*) very strong, (*e*) ?, and (*f*) none.

From these experiments it is learned that the alfalfa seed protease

[1] All digestion mixtures containing Witte peptone and casein were treated with one to two cubic centimeters of toluene as disinfectant.

readily digests Witte peptone, and that 37° is a more favorable temperature than 28°.

Casein Digestion.—The casein solution used in the following experiments was prepared from Kahlbaum's casein made according to Hammarsten. Twenty grams were dissolved in water by first grinding small portions in a mortar with water and pouring the paste into a liter flask. The flask was then made nearly full with water and the contents shaken. Ten cc. of a normal sodium hydroxide solution together with a few cubic centimeters of toluene were added and the flask filled to the mark with water. It was then put in the thermostat at 37° and left over night. The next morning the solution, which was neutral to litmus, was filtered and ready for use.

Experiment 1.—(*a*) 25 cc. seed extract + 50 cc. casein solution were heated together for 24 hours at 37°.

(*b*) 25 cc. seed extract + 50 cc. H_2O heated as in (*a*).

(*c*) 25 cc. H_2O + 50 cc. casein solution heated as in (*a*).

(*d*) 25 cc. seed extract heated in steam for $^1/_2$ hour, after which 50 cc. casein solution were added and the mixture heated as in (*a*).

The formol titration resulted thus: (*a*) 5.56 cc., (*b*) 2.20 cc., (*c*) 1.27 cc., and (*d*) 2.12 cc. 0.5 *N* NaOH. The difference between (*a*) and (*d*) is 3.44 cc. and is considerably less than the value (4.58 cc.) obtained for the Witte peptone digestion under the same conditions. The tryptophane reactions resulted thus: (*a*) strong, (*b*) marked, (*c*) none, and (*d*) none.

Experiment 2.—(*a*) 25 cc. seed extract + 50 cc. casein solution heated together at 37° for 64 hours.

(*b*) 25 cc. seed extract heated in steam $^1/_2$ hour, then 50 cc. casein solution added and the mixture heated as in (*a*).

Formol titration: (*a*) 8.55 cc., (*b*) 2.83 cc. (*a*) — (*b*) = 5.72 cc. 0.5 *N* NaOH. Tryptophane reaction: (*a*) strong, (*b*) none.

Experiment 3.—(*a*) Same as *Experiment 2* (*a*) except heated at 28° for 64 hours.

(*b*) Same as *Experiment 2* (*b*) except heated at 28° for 64 hours.

Formol titration: (*a*) 7.40 cc., (*b*) 2.42 cc. (*a*) — (*b*) = 4.98 cc. 0.5 *N* NaOH. Tryptophane reaction: (*a*) marked, (*b*) none.

Experiment 4.—(*a*) Same as *Experiment 2* (*a*), except allowed to stand at room temperature for 64 hours.

(*b*) Same as *Experiment 2* (*b*), except allowed to stand at room temperature for 64 hours.

Formol titration: (*a*) 4.33 cc., (*b*) 2.32 cc. (*a*) — (*b*) = 2.01 cc. 0.5 *N* NaOH. Tryptophane reaction: (*a*) faint, (*b*) none.

The above experiments go to show that both time and temperature are important factors, influencing the casein digestion by alfalfa seed protease.

Autolysis.—It was noticed that in both the Witte peptone and casein digestion experiments, the results indicated a digestion of the protein contained in the seeds themselves. This autolytic action was further investigated in that some of the seed protein was obtained in solid form and subjected to the action of the protease.

Experiment 1.—(*a*) The protein used for this part of the experiment was obtained by heating the filtered and clear seed extract in steam, and separating, washing and drying the resulting precipitate. 25 cc. seed extract + 25 cc. H_2O + 0.5 gram of the air-dried protein, heated together in a bottle for 24 hours at 37°.

(*b*) The protein for this part was obtained by adding about 3 volumes of 92° alcohol to the filtered and clear seed extract and separating, washing and drying the resulting precipitate. 25 cc. seed extract + 25 cc. H_2O + 0.5 gram air-dried protein, heated together for 24 hours at 37°.

(*c*) 25 cc. seed extract + 25 cc. H_2O + large amount (about 3 gram) of seed protein obtained as in (*b*), and the mixture heated together for 24 hours at 37°.

(*d*) 25 cc. seed extract + 25 cc. H_2O heated together at 37° for 24 hours.

The formol titration resulted in the following values: (*a*) 3.13 cc., (*b*) 3.10 cc., (*c*) 5.05 cc., and (*d*) 2.85 cc. of 0.5 *N* NaOH. Marked tryptophane reactions were obtained in all four parts of the experiment.

These results indicate a small but certain digestion of the protein present in the alfalfa seeds, and, incidentally, that this protein is not on the order of albumin or conglutin but rather of the casein type.

Water vs. Sodium Chloride Solution as Solvents for the Protease.—An experiment was carried out to determin whether or not a 1% solution of sodium chloride is a better solvent for the protease than water.

Two portions of 31 grams each of the finely ground seeds were extracted, the one with 300 cc. water and the other with the same volume of a 1% sodium chloride solution. The extracts were first filtered through muslin and then through filter paper. The two series of experiments were started with equal volumes of the two extracts, to digest equal volumes of a 2% casein solution. Without giving the five parts of the experiment in detail, it may suffice to say that, without exception, the titration values were slightly higher in those parts containing the seed extract from the sodium chloride solution.

From these results it is evident that a 1% sodium chloride solution is a slightly better solvent for the seed protease than water.

Reaction Influence.—Heretofore the digestions have been carried out in a neutral or slightly acid solution, the latter condition being the natural acidity of the seed extract. It was therefore decided to find what effect a change of reaction would have upon the digestion of casein by the seed protease.

Experiment 1.—(*a*) 25 cc. of the seed extract were heated in steam $^1/_2$ hour, then 50 cc. of the casein solution added and the mixture heated 24 hours at 37°.

(*b*) 25 cc. seed extract + 50 cc. H_2O heated 24 hours at 37°.

(*c*) 25 cc. H_2O + 50 cc. casein solution heated as in (*b*).

(*d*) 25 cc. seed extract + 50 cc. casein solution heated as in (*b*).

(*e*) 25 cc. seed extract + 50 cc. casein solution + 0.5 gram $CaCO_3$, heated together as in (*b*).

(*f*) 25 cc. seed extract + 50 cc. casein solution + Na_2CO_3 in solution to 0.5% and the mixture heated as in (*b*).

(*g*) 25 cc. seed extract + 50 cc. casein solution + Na_2CO_3 to 1% and heated together as in (*b*).

(*h*) 25 cc. seed extract + 50 cc. casein solution + Na_2CO_3 to 2% and heated together as in (*b*).

At the end of the digestion period the exact equivalent of hydrochloric acid was added to neutralize the sodium carbonate used for the alkalinity. At the time of titration, correction was made for the dilution of the solutions resulting from the addition of the alkali and acid.

In this case the formol titration was carried out with 0.1 *N* BaO_2H_2, and the results obtained were the following: (*a*) 4.50 cc., (*b*) 3.80 cc., (*c*) 1.55 cc., (*d*) 8.95 cc., (*e*) 8.40 cc., (*f*) 10.05 cc., (*g*) 8.05 cc., (*h*) 7.60 cc. The figures indicate that the 0.5% Na_2CO_3 solution is the most favorable for the digestion, although it is not inhibited by a 2% Na_2CO_3 solution. The tryptophane reaction, however, did not appear in the 2% Na_2CO_3 solution, and only a trace in the 1% solution. This reaction was also weaker in the 0.5% Na_2CO_3 solution than in those of (*d*) and (*e*).

Experiment 2.—(*a*) 25 cc. seed extract + 50 cc. casein solution + HCl to 0.05% of the volume of the whole liquid, heated together for 24 hours at 37°.

(*b*) 25 cc. seed extract + 50 cc. casein solution + HCl to 0.1%, heated as in (*a*).

(*c*) 25 cc. seed extract + 50 cc. casein solution + HCl to 0.2%, heated as in (*a*).

(*d*) 25 cc. seed extract + 50 cc. casein solution + HCl to 0.3%, heated as in (*a*).

(*e*) 25 cc. seed extract + 50 cc. casein solution + HCl to 0.4%, heated as in (*a*).

The formol titration of the foregoing solutions was carried out with a 0.1 *N* NaOH solution, and the results obtained were the following: (*d*) of *Experiment 1*, 8.95 cc., (*a*) 8.14 cc., (*b*) 7.45 cc., (*c*) 6.35 cc., (*d*) 5.21 cc., and (*e*) 4.83 cc. As the HCl concentration increases from 0.05% to 0.4% the amount of digestion decreases. The tryptophane reaction was strongest in (*a*) and diminished in regular gradation to (*d*) which gave only a trace.

Digestion Inhibited by Egg Albumin.—Since egg albumin and blood serum are not digested by the protease, it was to be expected that they might inhibit the action of the enzyme on casein and peptone by combining with part of the enzyme, as Hedin[1] has found to be true, in his experiments with other proteases. It was therefore interesting to learn if the action of the alfalfa protease is also checked by the same substances.

Experiment 1.—(*a*) 25 cc. seed extract + 10 cc. H_2O + 50 cc. casein solution were mixed and the mixture titrated with formol and 0.5 *N* NaOH without time for digestion.

(*b*) Same as (*a*), but digested 24 hours at 37°, and then titrated.

(*c*) 25 cc. seed extract + 10 cc. neutral egg albumin solution + 50 cc. casein solution and the mixture titrated with formol and 0.5 *N* NaOH without time for digestion.

(*d*) Same as (*c*), except digested for 24 hours at 37°, and then titrated.

(*e*) 25 cc. seed extract + 10 cc. albumin solution, allowed to stand together $^1/_2$ hour, then 50 cc. casein solution added and the mixture titrated without time for digestion.

(*f*) Same as (*e*), except digested for 24 hours at 37°, and then titrated.

(*g*) 25 cc. seed extract + 10 cc. albumin solution, allowed to stand together 2 hours, then 50 cc. casein solution added and the mixture titrated without time for digestion.

(*h*) Same as (*g*), except digested for 24 hours at 37°, and then titrated.

(*i*) 25 cc. seed extract + 10 cc. albumin solution, allowed to stand together

[1] *Z. physiol. Chem.*, 50, 497; 52, 412.

15 hours at 28°, then 50 cc. casein solution were added and the mixture titrated without time for digestion.

(*j*) Same as (*i*), except digested for 24 hours at 37° and then titrated.

The formol titrations in the above experiments resulted as follows:

(*a*) 2.04 cc., (*b*) 4.44 cc. (*b*) — (*a*) = 2.40 cc. 0.5 *N* NaOH.
(*c*) 2.25 cc., (*d*) 4.29 cc. (*d*) — (*c*) = 2.04 cc. 0.5 *N* NaOH.
(*e*) 2.13 cc., (*f*) 4.19 cc. (*f*) — (*e*) = 2.06 cc. 0.5 *N* NaOH.
(*g*) 2.26 cc., (*h*) 4.05 cc. (*h*) — (*g*) = 1.79 cc. 0.5 *N* NaOH.
(*i*) 2.64 cc., (*j*) 4.10 cc. (*j*) — (*i*) = 1.46 cc. 0.5 *N* NaOH.

The effect of the egg albumin on the casein digestion comes out in a striking manner in these results. It will be noticed that the difference in the titration values of the digested and undigested portions, where albumin has not been added, is larger than the differences where the albumin has been added, and that the differences grow smaller as the time of action between the albumin and protease increases.

Experiment 2.—This experiment was carried out to ascertain whether the time or the temperature, at which the albumin acts on the protease, is the controlling factor in producing the inhibition.

(*a*) 25 cc. seed extract + 10 cc. egg albumin solution left to act together for 2 hours at room temperature, then 50 cc. casein solution were added and the mixture titrated with formol and 0.5 *N* NaOH without time for digestion.

(*b*) Same as (*a*), except digested for 24 hours at 37° after adding the casein.

(*c*) 25 cc. seed extract + 10 cc. egg albumin solution allowed to act together for 2 hours at 37°, then 50 cc. casein solution added and the mixture titrated without time for digestion.

(*d*) Same as (*c*), except after the casein was added the mixture was digested for 24 hours at 37°.

The formol titrations resulted as follows:

(*a*) 2.38 cc., (*b*) 4.63 cc. (*b*) — (*a*) = 2.25 cc. 0.5 *N* NaOH.
(*c*) 2.55 cc., (*d*) 4.80 cc. (*d*) — (*c*) = 2.25 cc. 0.5 *N* NaOH.

These results show that it is the time, and not the temperature, which is the controlling factor.

Experiment 3.—It was desirable to learn if an egg albumin solution also inhibits the action of seed protease on Witte peptone.

The different parts of the experiment were similar to those of *Experiment 1*, and the results indicate that the same rule is followed, although the differences in the titration values are somewhat smaller than those obtained in *Experiment 1*.

Experiment 4.—This experiment was carried out to find if blood serum also inhibits the action of the protease on casein.

(*a*) 25 cc. seed extract + 10 cc. H_2O + 50 cc. casein solution added together and titrated without time for digestion.

(*b*) Same as (*a*), except that the mixture was heated in the thermostat at 36° for 24 hours.

(*c*) 25 cc. seed extract + 10 cc. ox serum + 50 cc. casein solution, and the mixture titrated without time for digestion.

(*d*) Same as (*c*), except that the mixture was digested for 24 hours at 36°.

(*e*) 25 cc. seed extract + 10 cc. ox serum, and the mixture allowed to stand for 2 hours, after which 50 cc. casein solution were added, and the mixture titrated without time for digestion.

(*f*) Same as (*e*), except after the casein was added the mixture was allowed to digest for 24 hours at 36°.

(*g*) 25 cc. seed extract + 10 cc. ox serum, and the mixture allowed to stand for 15 hours, after which 50 cc. casein solution were added and this mixture titrated without time for digestion.

(*h*) Same as (*g*), except after the casein was added the mixture was digested for 24 hours at 36°.

Following are the results of the formol titrations:

(*a*) 2.08 cc., (*b*) 5.31 cc. (*b*) — (*a*) = 3.23 cc. 0.5 *N* NaOH.
(*c*) 2.93 cc., (*d*) 5.80 cc. (*d*) — (*c*) = 2.87 cc. 0.5 *N* NaOH.
(*e*) 3.09 cc., (*f*) 5.24 cc. (*f*) — (*e*) = 2.15 cc. 0.5 *N* NaOH.
(*g*) 3.74 cc., (*h*) 4.77 cc. (*h*) — (*g*) = 1.03 cc. 0.5 *N* NaOH.

From these values it is evident that ox serum, as well as egg albumin, inhibits the digestion of casein by the alfalfa seed protease.

Conclusions.

The above investigation establishes that the alfalfa seeds contain enzymes that have the power of hydrolyzing starch and amygdalin, like amylase and emulsin, respectively; an enzyme that coagulates milk, like rennin; an enzyme that precipitates purpurogallin from a pyrogallol solution with hydrogen peroxide, like the ordinary peroxidases; and an enzyme that has the power of digesting casein and Witte peptone, like a protease.

The investigation further established that this protease is a vegetable erepsin, for it will not begin the digestion of egg albumin, serum, legumin or conglutin; and that its digestion of casein and Witte peptone is inhibited to some extent by the presence of small quantities of egg albumin and serum.

The seeds, in all probability, do not contain invertase, and if a lipase is present, it is not soluble in water. It is not probable that the acidity developed in the seed extract, when allowed to stand, is due to a lipase, for the clear extract, having no solid present, also turns acid in the given time; and that this extract is unable to hydrolyze ethyl butyrate.

I am hoping to be able to carry out similar experiments with extracts from the roots, stems and leaves of the alfalfa plant.

NEW BOOKS.

Les Atmosphères des Planètes. By DR. SVANTE ARRHENIUS. Address given before the Société de Chimie Physique, 8 March, 1911. A. Hermann et fils 6, Rue de la Sorbonne, 6, Paris. Price, 1 franc.

In order that a planet may be said to have an atmosphere, it must show a sharp discontinuity of density. Within the solar system this is certainly true of Mercury, Venus, the Earth and Mars. Accordingly, the prevailing atmospheric conditions upon these planets (and the Moon) are discussed, together with an outline of the steps through which each