# ON THE DIFFERENCES IN PHYSICO-CHEMICAL PROPERTIES OF VARIOUS PROTEINS IN PLANT SEEDS, (First Report).

## On the Differences in the Physico-chemical Properties of the Four Kinds of Rice-Protein.

By

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## Introduction.

It is impossible to classify the vegetable proteins by their chemical properties, because in these days our knowledge of the chemical structure of proteins is very incomplete. Many authors have placed the vegetable proteins in the four following classes according to their solubilities in different solvents, i. e. water, NaCl (10 %), alcohol (70 %) and alkali (0.2 %) soluble proteins and reported the chemical properties of each protein. But the relations between these four kinds of proteins have not been discussed. If we could make a clear difference amongst them in physico-chemical properties our experimental data would give not only important information for their classification but also the fundamental explanation of protein formation in plant seeds.

From the results of previous studies, the many physico-chemical relations between these four kinds of protein in the same plant seeds will be briefly reviewed in order to connect the authors' study with them. In the following elementary composition[1], a definite relation between the different kinds of protein in the same plant seed is observable.

| | Wheat | | | Soya-bean | | Indian-corn | |
|---|---|---|---|---|---|---|---|
| Kind of Protein | Leucosin | Glutenin | Gliadin | Legumelin | Glycinin | Glutelin | Zein |
| Element C | 53.0 | 52.34 | 52.72 | 53.06 | 52.12 | 51.26 | 55.20 |
| H | 6.8 | 6.82 | 6.86 | 6.94 | 6.93 | 6.72 | 7.26 |
| N | 16.8 | 17.69 | 17.66 | 16.14 | 17.53 | 15.82 | 16.13 |
| S | 1.3 | 1.08 | 1.03 | 1.17 | 0.79 | 0.90 | 0.60 |
| Solvent | Water | Alkali | Alcohol | Water | NaCl | Alkali | Alcohol |

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When the proteins in any of these plant seeds are arranged as water-, NaCl-, alkali- and alcohol-soluble, the percentage of nitrogen is seen to increase according to that order, while the contents of carbon and sulphur gradually decrease (excepting Indian-corn).

Osborne[3] reported the distribution of nitrogen in protein decomposition products. We could observe from his results that there is a definite relation between the four proteins of wheat, i. e. nitrogen of amide is increased and nitrogen of diamino decreased according to the above order of solubility of proteins.

| Solubility | Distribution of N | Amide N | Monoamino N | Diamino N |
|---|---|---|---|---|
| | Kind of Proteins | | | |
| Water | Leucosin | 1.16 | 11.83 | 3.50 |
| NaCl | Globulin | 1.42 | 9.83 | 6.82 |
| Alkali | Glutenin | 3.30 | 11.95 | 2.05 |
| Alcohol | Gliadin | 4.30 | 12.41 | 0.05 |

Osborne[3] determined also histidine-, arginine- and lysine-contents of different proteins in wheat, soya-bean and Indian-corn. We could observe from his results that the contents of these three components in the same plant seed always decreased gradually in the following order of water-, NaCl-, NaOH- and alcohol-soluble protein.

| Solubility | Kind of Protein | Histidine | Arginine | Lysine |
|---|---|---|---|---|
| Wheat | | | | |
| water | Leucosin | 2.83 | 5.94 | 2.75 |
| alkali | Glutenin | 1.76 | 4.72 | 1.92 |
| alcohol | Gliadin | 0.58 | 3.16 | 0.00 |
| Soya-bean | | | | |
| water | Legumelin | 2.04 | 5.37 | 4.91 |
| NaCl | Glycinin | 2.10 | 7.69 (?) | 3.39 |
| Indian-corn | | | | |
| alkali | Glutelin | 3.00 | 7.06 | 2.93 |
| alcohol | Zein | 0.83 | 1.35 | 0.00 |

Further, Rona and Michaelis[4] and Loeb[5] determined the iso-electric points of NaCl soluble protein (edestin) as pH 6.0–6.8 and of alcohol soluble protein (gliadin) as pH 9.23. We know that the former is slightly acid and the latter alkali. Osborne[6] determined the specific rotatory power of edestin as −42.3 and of gliadin −92.3 and we know that there

is an increasing tendency of this optical property according to the basic nature of proteins. Recently Csonka, Murphy and Jones[7] stated that the pH value of the iso-electric point of three kinds of protein is increased in the following order, i. e. albumin (pH 4–5), globulin (pH 5–5.5) and prolamin (pH 6–6.5), and the pH value of iso-electric point of the same kind of protein from different sources is nearly equal but if their solubilities are not equal then their iso-electric points are also different, i. e. among globulin thei so-electric point of $\beta$-form, which needs a greater quantity of ammonium sulphate for precipitation, shows a lower pH value than that of $\alpha$-form.

Rosenheim and Kajiura[8] detected in polished rice three kinds of protein, water-, NaCl- and alkali-soluble. The alkali soluble protein, named oryzenin by them, includes the greater part of the protein found in rice. Suzuki, Yoshimura and Fuji[9] reported that in rice seed, alcohol soluble protein was also detected and these authors investigated the amino acid content of these proteins. But there is no report which has discussed the relation of chemical properties amongst these four kinds of rice protein. On this point the following investigation was carried out.

## I. Isolation and Purification of the Four Proteins and Their Ash-Content.

### (1) Materials.

The samples of the authors' research were collected from different parts of Japan in 1925–26. The following hulled and unhulled rices were washed with water till the washings became clear, dried in the open air and then powdered with a stone mill. The powdered rice was extracted with ether in a Soxhlet's apparatus and again was dried in the open air.

| No. | Hulled or Unhulled | Locality producing the Rice |
|---|---|---|
| 1 | Hulled | Shigaken (Wataribune) |
| 2 | Unhulled | Aomoriken (Kamenoo). 1. |
| 3 | ,, | ,, 2. |
| 4 | ,, | ,, 3. |
| 5 | Hulled | Kiushu |
| 6 | Unhulled | ,, |
| 7 | Hulled | Yamagataken |

### (2) Preparation of Water Soluble Protein.

To 500 g. of the rice powder was added 2 liters of water and the mixture was shaken many times in a day by a shaking machine. After that it was placed over night in an ice chamber The upper liquid was decanted and the same treatment was repeated 3 times until the liquid gave no protein reaction. It was filtered several times until no iodine reaction to starch was observed by a Buchner's funnel which contained filter pulp of 1–2 cm. thickness.

The clear filtrate thus obtained was very slightly acidified with dilute HCl whereupon the protein precipitated as a white amorphous mass. The upper solution was decanted off and the precipitate was washed by decantation until it gave no chlorine reaction. The precipitate of protein was placed in a dialyser and treated in running water for a few days and again the precipitate was centrifuged for the sake of removing the liquid. The protein was washed 3 times with absolute alcohol and 3 times with ether in a centrifugal machine and then dried in a $H_2SO_4$ desiccator of diminished pressure. The dried protein was powdered with an agate mortar and was again kept in a $H_2SO_4$ desiccator for 4 months.

### (3) Preparation of 10 % NaCl Soluble Protein.

The residue from which the water soluble protein was separated was mixed with 2 liters of water and NaCl and made up to 10 % solution of NaCl. The mixture was shaken for 30 minutes by a shaking machine. After that it was placed over night in an ice chamber. The upper liquid was decanted and the same treatment was repeated 3–4 times until the extract had no protein reaction. The upper liquid was collected and filtered several times until no iodine reaction to starch was observed by a Buchner's funnel and then NaCl soluble protein was precipitated by the same treatment as above mentioned. The NaCl soluble protein was also purified and dried in the same manner as was the water soluble protein.

### (4) Preparation of 70 % Alcohol and 0.2 % NaOH Soluble Proteins.

The residue from which were separated the water and the NaCl soluble protein, was washed several times until no chlorine reaction was observed and 2 liters of 0.2 % NaOH solution were added to it. The mixture was shaken for 30 minutes by a shaking machine and placed over night in an ice chamber. The upper liquid was decanted and the same treatment repeated 3 times. The upper liquid was filtered several times

until no iodine reaction to starch was observed by a Buchner's funnel and the alkali soluble protein was precipitated by the same treatment as above described. The precipitate was dialysed for one week to separate mineral substances and separated from the liquid by a centrifugal machine. The precipitate was mixed stirring with 70 % alcohol and placed over night in an ice chamber and the upper alcohol solution separated with a centrifugal machine. The same treatment was repeated 3 times until the upper alcohol solution had no protein reaction. The alcohol solution was filtered several times by a Buchner's funnel which contained a filter pulp until it became a transparent liquid. When the clear alcohol solution was evaporated at 35°C. under negative pressure, the evaporated residue became turbid. After addition of absolute alcohol again the evaporation was repeated until the alcohol soluble protein was attached to the bottom of the flask. The protein precipitate was washed by means of a centrifugal machine with absolute alcohol and ether and dried in a $H_2SO_4$ desiccator. The residual protein which was separated from 70 % alcohol soluble protein, was washed with absolute alcohol and ether and treated as above described. All these protein preparations were dried completely in a $H_2SO_4$ desiccator for 4 months. The ash content was determined by the ordinary method.

TABLE I.

*Water and ash content of samples.*

| Kind of Protein | Water (%) | Ash (%) | Ash (% in dry matter) | Proportion |
|---|---|---|---|---|
| No. 1. | | | | |
| Water soluble | 3.600 | 1.783 | 1.849 | 100.00 |
| NaCl Soluble | 2.920 | 0.800 | 0.824 | 44.56 |
| Alcohol Soluble | 3.243 | 0.536 | 0.554 | 29.96 |
| Alkali Soluble | 2.700 | 0.500 | 0.514 | 28.79 |
| No. 2. | | | | |
| NaCl Soluble | 2,760 | — | 0.786 | 100.00 |
| Alkali Soluble | 6.566 | — | 0.360 | 45.80 |
| No. 3. | | | | |
| NaCl Soluble | 7.592 | — | 0.808 | 100.00 |
| Alkali Soluble | 7.068 | — | 0.331 | 41.04 |
| No. 4. | | | | |
| NaCl Soluble | 6.715 | — | 0.762 | 100.09 |
| Alkali Soluble | 4.954 | — | 0.348 | 45.71 |
| No. 5. | | | | |
| NaCl Soluble | 5.948 | — | — | — |

| Kind of Protein | Water (%) | Ash (%) | Ash (% in dry matter) | Proportion |
|---|---|---|---|---|
| Alkali Soluble | 8.753 | — | — | — |
| No. 6. | | | | |
| NaCl Soluble | 8.188 | — | 0.688 | 100.00 |
| Alkali Soluble | 7.395 | — | 0.301 | 43.67 |
| No. 7. | | | | |
| NaCl Soluble | 4.814 | — | — | — |
| Alkali Soluble | 3.505 | — | — | — |

From the table it is observed that the ash content of four kinds of protein was decreased according to the following order i. e. water-, NaCl-, alkali-, and alcohol soluble protein and the quantity of ash of alkali soluble protein corresponds to 28.8 % that of water soluble protein, The ash content of alkali soluble protein also corresponds to 41–46 % that of NaCl soluble protein.

## II. Preliminary Experiment on the Iso-electric Point Estimation of the Four Protein Solutions.

### (a). Change of the Surface Tension of the Alkali Protein Solution Titration with HCl Solution.

One twentieth g. of each protein was dissolved in 10 c.c. of 1/50 n-NaOH solution. After standing for 24 hours (15°C.), 1 c.c. of the protein solution was diluted with 4 c.c. of pure water (ca. pH 7.0). The mixture was titrated with 1/500 n-HCl solution. The surface tension of the mixture was measured with a Nouy's apparatus, constructed on the principle of a torsion balance the torsion angle of which is proportional to the surface tension of the liquid. The following numbers are dyne per sq. cm. ($H_2O=75$) and are the mean of two experiments.

TABLE 2.

*Change of the surface tension of alkali protein solution.*

| | Protein | | | |
|---|---|---|---|---|
| HCl (c.c.) (20°C.) | Water Soluble | NaCl Soluble | Alcohol Soluble | Alkali Soluble |
| 9.0 | 56.6 | 60.7 | 58.7 | 63.4 |
| 9.1 | 56.6 | 60.7 | 59.3 | 63.4 |
| 9.2 | 57.3 | 60.7 | 59.3 | 63.4 |

| | Protein. | | | |
|---|---|---|---|---|
| HCl (c.c.) (20°C.) | Water Soluble | NaCl Soluble | Alcohol Soluble | Alkali Soluble |
| 9.3 | 57.3 | 61.4 | 60.0 | 64.1 |
| 9.4 | 57.3 | 61.4 | 60.0 | 64.1 |
| 9.5 | 58.0 | 61.4 | 60.7 | 64.1 |
| 9.6 | 58.0 | 62.1 | 60.0 | 64.8 |
| 9.7 | 58.0 | 62.1 | 60.0 | 64.8 |
| 9.8 | 58.7 | 62.1 | 60.0 | 65.5 |
| 9.9 | 58.7 | 62.1 | 60.0 | 65.5 |
| 10.0 | 58.7 | 62 7 | 60.0 | 66.2 |
| 10.1 | 58.7 | 62.7 | 59.3 | 65.5 |
| 10.2 | 59.3 | 62.7 | 59.3 | 95.5 |
| 10.3 | 59.3 | 63.4 | 59.3 | 64.8 |
| 10.4 | 59.3 | 63.4 | 59.3 | 64.1 |
| 10.5 | 59.3 | 64.1 | 59.3 | 64.1 |
| 10.6 | 60.0 | 64.1 | 59.8 | 64.1 |
| 10.7 | 60.0 | 65.5 | 58.7 | 63.4 |
| 10.8 | 60.0 | 64.8 | 58.7 | 63.4 |
| 10.9 | 61.4 | 64.8 | 58.7 | 63.4 |
| 11.0 | 60.0 | 64.8 | 58.7 | 62.7 |
| 11.1 | 60.0 | 64.1 | — | — |
| 11.2 | 60.0 | 64.1 | — | — |

In the above table, the maximum point of surface tension shows a marked difference between the four kinds of protein solution i. e. water-, NaCl-, alkali- and alcohol-soluble protein solution. The maximum point of water soluble protein is more acidic than that of NaCl soluble protein and the latter is more acidic than alkali soluble protein which is still more acidic than alcohol soluble protein.

### (b). Change of the Turbidity of the Alkali Protein Solution in Titration with HCl Solution.

As in the above experiments, 0.04 g. of the protein was dissolved in 10 c.c. of 1/50 n-NaOH solution and 1 c.c. of the protein solution was diluted with 9 c.c. of pure distilled water. The mixture was titrated with a 1/500 n-HCl solution and the point of maximum turbidity was determined, using a Dubosq's nephelometer. These figures show the turbidity, i. e. the "−" sign indicates the emulsoid state, the "±" sign the mixture of emulsoid and suspensoid and the "+" sign indicates the suspensoid state.

TABLE 3.

*Change of the turbidity of the alkali protein solution.*

| | Protein. | | | |
|---|---|---|---|---|
| $HCl$,(c.c.) (20°C.) | Water Soluble | NaCl Soluble | Alcohol Soluble | Alkali Soluble |
| 9.0 | − | − | − | − |
| 9.1 | − | − | − | − |
| 9.2 | − | − | ± | − |
| 9.3 | − | − | ± | − |
| 9.4 | − | − | ± | − |
| 9.5 | − | − | + | − |
| 9.6 | − | − | + | − |
| 9.7 | − | − | + | ± |
| 9.8 | − | − | + | ± |
| 9.9 | − | − | + | ± |
| 10.0 | − | − | + | + |
| 10.1 | − | − | + | + |
| 10.2 | − | − | + | + |
| 10.3 | − | ± | + | + |
| 10.4 | − | ± | + | + |
| 10.5 | ± | ± | + | + |
| 10.6 | ± | ± | + | + |
| 10.7 | ± | + | + | + |
| 10.8 | ± | + | + | + |
| 10.9 | + | + | + | + |
| 11.0 | + | + | + | + |
| 11.1 | + | + | | |
| 11.2 | + | + | | |
| 11.3 | + | + | | |

According to the table, to obtain the maximum turbidity of the four kinds of protein solution a different quantity of acid is needed for each, i. e. the water soluble protein solution needs a greater quantity of acid than the NaCl soluble and the latter needs more acid than the alkali soluble which also needs more acid than the alcohol soluble protein solution.

From the results of the above two experiments, the iso-electric points of four kinds of rice protein could be distinguished from each other and their basity found to increase in the following order, i. e. water-, NaCl-, alkali- and alcohol-soluble protein.

## III. Sulphur Content of the Four Kinds of Protein.

One half g. of water free protein powder was taken and its sulphur content was determined by Denis-Benedict's[16] method. The following results were obtained.

TABLE 4.

*Sulphur content of the four kinds of rice-protein.*

| Kind of Protein. | | $BaSO_4$ (g.) | Sulphur (g.) | Sulphur (%) | Sulphur (Dry Substance %) | Average |
|---|---|---|---|---|---|---|
| Water Soluble | (1) | 0.0287 | 0.003802 | 0.7604 | 0.7889 | 0.8226 |
| | (2) | 0.0301 | 0.004127 | 0.8254 | 0.8592 | |
| NaCl Soluble | (1) | 0.0332 | 0.004552 | 0.9104 | 0.9376 | 0.9221 |
| | (2) | 0.0321 | 0.004402 | 0.8804 | 0.9067 | |
| Alcohol Soluble | (1) | 0.0072 | 0.001028 | 0.2056 | 0.2102 | 0.2199 |
| | (2) | 0.0081 | 0.001110 | 0.2222 | 0.2296 | |
| Alkali Soluble | (1) | 0.0197 | 0.002701 | 0.5402 | 0.5653 | 0.5532 |
| | (2) | 0.0192 | 0.002633 | 0.5266 | 0.5411 | |

In the above table, the sulphur content of the four proteins is quite different and diminishes gradually in the following order, i. e. from NaCl-, and water-soluble proteins to alkali and to alcohol soluble protein in the following proportion.

| NaCl Soluble Protein. | Water Soluble Protein. | Alkali Soluble Protein. | Alcohol Soluble Protein. |
|---|---|---|---|
| 100. | 89. | 60. | 24. |

## IV. On the Physico-chemical Differences between NaCl- and Alkali-Soluble Protein.

### (1). The Specific Rotatory Power of NaCl- and Alkali Soluble Protein.

The rotatory power of an organic compound is caused by the presence of asymmetric carbon atoms in its molecule and it is increased by the complexity of the molecule, by the saturation in molecular structure and by the special atomic groups. Therefore, it has great meaning for this

investigation to examine the rotatory power of the alkali solution of the four proteins. One tenth g. of each kind of protein was dissolved in 10 c.c. of 1/10 n-NaOH solution and the solution was examined by a Haensch-Schmidt half shadow polariscope.

TABLE 5.

*Showing the rotatory power of various proteins dissolved in alkali.*

| No. of Sample and Protein. | After 5 hrs. | After 24 hrs. | After 48 hrs. | After 72 hrs. | After 96 hrs. | After 1 week | After 5 weeks |
|---|---|---|---|---|---|---|---|
| No. 1. Alkali Soluble | — | 2.4 | — | — | — | 2.0 | 1.5 |
| NaCl Soluble | — | 2.3 | — | — | — | 1.9 | 1.3 |
| No. 2. Alkali Soluble | 2.5 | 2.7 | 3.0 | 2.5 | 2.4 | 2.2 | — |
| NaCl Soluble | 2.3 | 2.6 | 2.8 | 2.3 | 2.2 | 2.0 | — |
| No. 3. Alkali Soluble | 2.5 | 2.7 | 3.0 | 2.6 | 2.5 | 2.3 | — |
| NaCl Soluble | 2.3 | 2.6 | 2.8 | 2.5 | 2.4 | 2.1 | — |
| No. 4. Alkali Soluble | 2.5 | 2.8 | 3.1 | 2.5 | 2.4 | 2.2 | — |
| NaCl Soluble | 2.4 | 2.7 | 2.9 | 2.4 | 2.3 | 2.1 | — |
| No. 5. Alkali Soluble | 2.4 | 2.6 | 2.9 | 2.6 | 2.4 | 2.1 | — |
| NaCl Soluble | 2.2 | 2.5 | 2.7 | 2.5 | 2.3 | 2.0 | — |
| *Showing the specific rotatory power of various proteins dissolved in alkali.* | | | | | | | |
| No. 1. Alkali Soluble | — | −83.04 | — | — | — | −69.02 | −51.60 |
| NaCl Soluble | — | −79.58 | — | — | — | −65.74 | −44.98 |
| No. 2. Alkali Soluble | −64.88 | −70.07 | −77.85 | −64.88 | −62.28 | −57.09 | — |
| NaCl Soluble | −59.69 | −67.47 | −72.66 | −59.69 | −57.09 | −50.19 | — |
| No. 3. Alkali Soluble | −64.88 | −70.07 | −77.85 | −67.47 | −64.88 | −59.69 | — |
| NaCl Soluble | −59.69 | −67.47 | −72.66 | −64.88 | −62.28 | −54.50 | — |
| No. 4. Alkali Soluble | −64.88 | −72.66 | −80.45 | −64.88 | −62.28 | −57.09 | — |
| NaCl Soluble | −62.28 | −70.07 | −75.26 | −62.28 | −59.69 | −54.50 | — |
| No. 5. Alkali Soluble | −62.28 | −67.47 | −75.26 | −67.47 | −62.28 | −54.50 | — |
| NaCl Soluble | −57.09 | −64.88 | −70.07 | −64.88 | −59.69 | −50.19 | — |

The rotatory power of the alkali solution of the alkali soluble protein shows higher values than that of the NaCl soluble. The rotatory power of the alkali protein solution increased at first, reached the maximum and then decreased gradually after a long time. The rate of decrease of the rotatory power of the NaCl soluble protein solution is greater than that of the alkali soluble one, so the molecule of the former must be

destroyed more easily than the latter.

### (2). The Determination of the Quantities of HCL Combined with the NaCl- and Alkali-Soluble Protein.

As the simplest case of the combination of an inorganic acid with a protein occurs in the reaction $\left[R\begin{smallmatrix}NH_2\\COOH\end{smallmatrix}\right. + HCl = \left[R\begin{smallmatrix}NH_2HCl\\COOH\end{smallmatrix}\right.$, it may be said that in a given concentration of HCl, the HCl combining with the added protein is proportional to the free amino groups of the protein. However, according to Robertson[11], the -COHN- group of keto-form polypeptide linkage has a neutralizing effect, and enol-form changes the valence of N of the -C(OH)N- group from three to five and combines with acid according to the formula $-CO:N- + HCl = (COH)^{++} + :N\begin{smallmatrix}H\\Cl\end{smallmatrix}$. These considerations lead elsewhere than to the difference of the quantity of free amino nitrogen of the alkali soluble and the NaCl soluble proteins. Another difference of combining power with acid must exist in both kinds of proteins.

Cohnheim[12] proposed a method of calculating the combination degree of HCl with protein as follows. The inversion of sucrose by pure HCl is a monomolecular reaction and follows the formula $\log\frac{A}{A-X} = Kt$, where A is the concentration of sucrose before inversion, X the same after inversion for a given time t, and K is a constant which is a variable of the hydrogen ion concentration of the solution. If two inversions of sucrose by HCl with and without protein were carried out, the quantity of HCl combined with the protein may be calculated from the formula $\frac{C}{C'} = \frac{\log A - \log(A-X)}{\log A - \log(A-X')}$, where C represents the hydrogen ion concentration of pure HCl, C′ the same of a solution of protein in HCl, A the quantity of sucrose used, X and X′ the quantities of sucrose inverted by pure acid and protein acid solution respectively. C and C′ are figures proportional to the hydrogen ion concentration of the solution.

Twenty mg. of protein were dissolved in 5 c.c. of 1/10 n-HCl solution solution and the whole was stood overnight. To the protein solution was added 25 c.c. of ca. 5 % sucrose solution. The mixture was inverted for exactly 5 minutes in a boiling water bath. At the end of the time, the solution was cooled to room temperature and the quantity of inverted sugar

was estimated by Bertrand's method. The quantity of sucrose was calculated by multiplying the obtained results by the factor 0.9499. The quantity of HCl in free state is calculated as follows, where 1/10 n-HCl solution contains 18.235 mg. Concentration of HCl in 5 c.c. and the concentration of sucrose used was 1230.165 mg. in 25 c.c.

TABLE 6.

*Combining power of proteins with HCl.*

| Kind of Protein | $KMnO_4$ (c.c.) | Cu (mg.) | Invert Sugar (mg.) | Sucrose (mg.) | Sucrose in 25 c.c. (mg.) |
|---|---|---|---|---|---|
| No. 2. Alkali Soluble | 35.45 | 372.39 | 204.26 | 194.0409 | 1164.245 |
| NaCl Soluble | 35.80 | 376.07 | 206.39 | 196.0643 | 1176.386 |
| No. 3. Alkali Soluble | 35.50 | 372 92 | 204.23 | 194.0124 | 1164.074 |
| NaCl Soluble | 35.85 | 376 60 | 206.70 | 196.3588 | 1178.153 |
| No. 4. Alkali Soluble | 35.85 | 373.44 | 204.84 | 194.5919 | 1167.551 |
| NaCl Soluble | 35.85 | 376.60 | 206.70 | 196.3588 | 1178.153 |
| No. 5. Alkali Soluble | 35.40 | 371.87 | 203.98 | 193.7749 | 1162.649 |
| NaCl Soluble | 35.78 | 275.86 | 206.68 | 196.3398 | 1178.039 |
| HCl Only | 36.20 | 380.27 | 209.08 | 198.6197 | 1191.718 |

$$\frac{C}{C'} = \frac{\log A - \log(A-X)}{\log A - \log(A-X')}$$

| | | |
|---|---|---|
| log A = 3.0900 | log(A−X) = 1.5849 | log A − log(A−X) = 1.5051 |
| No. 2. Alkali Soluble | „ = 1.8190 | „ = 1.2710 |
| NaCl Soluble | „ = 1.7306 | „ = 1.3594 |
| No. 3. Alkali Soluble | „ = 1.8201 | „ = 1.2699 |
| NaCl Soluble | „ = 1.7161 | „ = 1.3739 |
| No. 4. Alkali Soluble | „ = 1.7966 | „ = 1.2934 |
| NaCl Soluble | „ = 1.7161 | „ = 1.3739 |
| No. 5. Alkali Soluble | „ = 1.8294 | „ = 1.2606 |
| NaCl Soluble | „ = 1.7175 | „ = 1.3725 |

| Kind of Protein | HCl in Free State (mg.) | HCl in Combined State (mg.) |
|---|---|---|
| No. 2. Alkali Soluble | 15.399 | 2.836 |
| NaCl Soluble | 16.470 | 1.765 |
| No. 3. Alkali Soluble | 15.385 | 2.850 |
| NaCl Soluble | 16.645 | 1.590 |
| No. 4. Alkali Soluble | 15.670 | 2.565 |

| Kind of Protein | | HCl in Free State (mg.) | HCl in Combined State (mg.) |
|---|---|---|---|
| | NaCl Soluble | 16.645 | 1.590 |
| No. 5. | Alkali Soluble | 15.273 | 2.962 |
| | NaCl Soluble | 16.889 | 1,346 |

In the case of the alkali soluble protein, the quantity of HCl combined is always greater than in the case of the NaCl soluble protein.

### (3). The Silver Salt of Various Proteins.

Hitherto it has been repeatedly said that a protein or its decomposition product combines with heavy metals to make its salt. Hirschstein[13a & b] reported, especially concerning casein, that an insoluble precipitation obtained by adding silver nitrate to an ammonium solution of casein always contains a constant quantity of silver oxide that is chemically equivalent. The authors undertook the following examinations to compare the difference of the two kinds of protein in combination with silver.

Eight-tenths g. of protein was dissolved in 50 c.c. of 1/25 n-NaOH solution, the solution was neutralized with nitric acid using phenolphthalein as an indicator. Eight c.c. of 20 % silver nitrate solution were added to the mixture to form a precipitate. The precipitate was filtered, washed, dried and weighed in the usual way. The total nitrogen was estimated according to Kjeldahl's method using 0.2 g. of the dried dark brownish precipitate. 0.4 g. of the substance was fused with a fusing mixture, dissolved in nitric acid and the silver of the solution was determined as silver chloride by the gravimetric method. The results are tabulated in Table 7.

TABLE 7.

*Combination of silver with the two kinds of protein.*

| | Alkali Soluble N % in Dry Matter | NaCl Soluble N % in Dry Matter |
|---|---|---|
| Sample (1) | 15.3176 | 12.3288 |
| (2) | 14.5704 | 13.0760 |
| | Average 14.9440 | Average 12.7014 |
| | AgCl % in Dry Matter | AgCl % in Dry Matter |
| (1) | 7.8750 | 16.6520 |
| (2) | 7.2000 | 15.0000 |
| | Average 7.5370 | Average 15.8210 |

In respect to the quantity of nitrogen the silver salts of the alkali soluble protein predominate over those of the NaCl soluble protein, but in the quantity of silver the former is about half of the latter. This indicates that the carboxyl group which combines with silver is more abundant in the NaCl soluble protein than in the alkali soluble protein.

### (4). Elementary Composition of the NaCl and the Alkali Soluble Protein.

The authors estimated carbon and hydrogen by the ordinary method using an electric furnace. Nitrogen was estimated by Kjeldahl's method and sulphur by Denis-Benedict's method.

TABLE 8.

*Elementary composition of various proteins.*

| Kind of Protein | | Sample (g.) | $CO_2$ (g.) | $H_2O$ (g.) | C(%) | H(%) | Sample (g.) | N(%) |
|---|---|---|---|---|---|---|---|---|
| No. 2. | Alkali soluble (1) | 0.0667 | 0.1190 | 0.0463 | 52.0940 | 7.4728 | | |
| | (2) | 0.0635 | 0.1135 | — | 52.0466 | 7.5766 | | |
| | Average | | | | 52.0703 | 7.5247 | | |
| | NaCl Soluble (1) | 0.0478 | 0.0903 | — | 53.5375 | 7.0801 | | |
| | (2) | 0.0526 | 0.0992 | 0.0343 | 53.4635 | 7.0927 | | |
| | Average | | | | 53.5005 | 7.0864 | | |
| No. 3. | Alkali Soluble (1) | 0.0694 | 0.1215 | 0.0481 | 51.3742 | 7.4419 | | |
| | (2) | 0.0788 | 0.1384 | 0.0546 | 51.5648 | 7.4378 | | |
| | Average | | | | 51.4695 | 7.4399 | | |
| | NaCl Soluble (1) | 0.1304 | 0.2356 | 0.0859 | 53.3987 | 7.0078 | 0.05 | 15.2842 |
| | (2) | 0.1688 | 0.3068 | 0.1108 | 53.3968 | 6.9985 | | |
| | Average | | | | 53.3978 | 7.0032 | | |
| No. 4. | Alkali Soluble | | | | | | 0.05 | 16.7652 |
| | NaCl Soluble | | | | | | ,, | 15.5024 |
| No. 5. | Alkali Soluble | | | | | | ,, | 16.2228 |
| | NaCl Soluble | | | | | | ,, | 15.8976 |
| No. 7. | Alkali Soluble | | | | | | ,, | 16.2228 |
| | NaCl Soluble | | | | | | ,, | 14.9717 |

TABLE 9.

*Sulphur content of various proteins.*

| Kind of Protein | Sample (g.) | $BaSO_4$ (g.) | S (g.) | S (air dry %) | S (dry matter %) |
|---|---|---|---|---|---|
| No. 2. Alkali Soluble | 0.2083 | 0.0111 | 0.001524 | 0.7473 | 0.7998 |
| NaCl Soluble | 0.2055 | 0.0156 | 0.002142 | 1.0424 | 1.1290 |
| No. 3. Alkali Soluble | 0.2031 | 0.0104 | 0.001428 | 0.7322 | 0.7879 |
| NaCl Soluble | 0.2144 | 0.0164 | 0.002252 | 1.0504 | 1.1378 |

The above data are summarized in the following table.

TABLE 10.

*Elementary composition of proteins by percentage.*

| Kind of Protein | Nitrogen | Hydrogen | Carbon | Sulphur |
|---|---|---|---|---|
| Alkali Soluble | 16.6389 | 7.4823 | 51.7698 | 0.7938 |
| NaCl Soluble | 15.4572 | 7.0448 | 53.4491 | 1.1334 |

On the elementary composition of two kinds of protein in the same plant seed, the alkali soluble protein is greater in nitrogen content than the NaCl soluble while the latter is greater in carbon and sulphur content than the former.

### (5). The Separation and Determination of Amino Acids.

The factor which controls the iso-electric point of a protein is the dissociation constant of amino acids and polypeptides which compose the protein molecule; thus, the protein's iso-electric point is effected according to the predominance of dissociation constants as acids or bases. In mono-amino acids the dissociation constants as acid are predominant, while in diamino acids and amides which decompose to ammonia easily, the dissociation constants as bases are predominant. Accordingly, it is very important for a comparison of proteins to separate and determine the quantity and kinds of amino acids of a protein.

A difference of the amino acids of the two proteins both in quantity and kind may be expected from the data of elementary analysis, which show

that the quantities of the nitrogen and carboxyl groups differ markedly.

The authors thus undertook the following experiments according to Van Slyke's method[14]. To a given quantity of sample was added 20 times its weight of 20 % HCl, the mixture was boiled and hydrolyzed for 8 hours on a sand bath under a reflux condenser. Next, almost all the HCl of the solution was driven off under diminished pressure at below 40°C. The residue was neutralized and made slightly alkaline by the addition of 10 % solution of calcium hydroxide. The ammonia nitrogen liberated was distilled into a standard sulphuric acid under diminished pressure at below 40°C. The remaining fluid was filtered and separated from melanine nitrogen. The melanine nitrogen was determined by Kjeldahl's method. The filtrate was acidified with HCl and evaporated under diminished pressure at below 40°C. To the concentrated filtrate were added 18 c.c. of conc. HCl and 15 g. of phosphotungstic acid and the diamino acids were precipitated. After 48 hour's undisturbed standing, the precipitate was filtered by suction and treated with a mixture of amylalcohol and ether according to Van Slyke's modified method[15]. The diamino acid nitrogen was determined separately. Using Van Slyke's micro-apparatus the free amino nitrogen was determined.

TABLE 11.

*Kind of nitrogen of dry matter % found in the NaCl and the alkali soluble proteins.*

| Kind of N / Kind of Protein | Total N. | Ammonia N. | Melanine N. | Monoamino N. | Diamino N. | Arginine N. | Lysine N. | Histidine N. | Cystine N. |
|---|---|---|---|---|---|---|---|---|---|
| NaCl Soluble (1) | 17.096 | 1.074 | 0.318 | 9.740 | 5.964 | 4.018 | 1.300 | 0.365 | 0.281 |
| (2) | 16.979 | 1.033 | 0.300 | 9.640 | 6.000 | 4.000 | 1.331 | 0.396 | 0.272 |
| Average | 17.038 | 1.054 | 0.309 | 9.693 | 5.982 | 4,009 | 1.316 | 0.381 | 0.277 |
| Alkali Soluble (1) | 17.311 | 1.780 | 0.336 | 9.042 | 6.133 | 3.315 | 0.885 | 1.716 | 0.216 |
| (2) | 16.997 | 1.819 | 0.396 | 8.814 | 5.968 | 3.183 | 0.962 | 1.617 | 0.206 |
| Average | 17.154 | 1.799 | 0.376 | 8.928 | 6,051 | 3.249 | 0.924 | 1.667 | 0.211 |

TABLE 12.

*Nitrogen percentage.*

| Kind of N. / Kind of Protein | Total N. | Ammonia N. | Melanine N. | Monoamino N. | Diamino N. | Arginine N. | Lysine N. | Histidine N. | Cystine N. |
|---|---|---|---|---|---|---|---|---|---|
| NaCl Soluble | 100.000 | 6.186 | 1.814 | 56.890 | 35.109 | 23.529 | 7.724 | 2.236 | 1.626 |
| Alkali Soluble | 100.000 | 10.487 | 2.207 | 52.401 | 35.274 | 18.940 | 5.386 | 9.718 | 1.230 |

TABLE 13.

*Percentages of nitrogen contained in different amino acids as found in the NaCl and the alkali soluble proteins. The maximum quantity is taken as 100.*

| Kind of N. / Kind of Protein | Ammonia N. | Melanine N. | Monoamino N. | Diamino N. | Arginine N. | Lysine N. | Histidine N. | Cystine N. |
|---|---|---|---|---|---|---|---|---|
| NaCl Soluble | 58.99 | 82.19 | 100.00 | 99.53 | 100.00 | 100.00 | 23.01 | 100.00 |
| Alkali Soluble | 100.00 | 100.00 | 92.11 | 100.00 | 80.50 | 69.73 | 100.00 | 75.65 |

Thus it is seen that in the NaCl soluble protein there is a predominance of monoamino, arginine, lysine and cystine nitrogen, while in the alkali soluble protein there is an excess of ammonia, melanine, diamino and histidine nitrogen. The quantities of ammonia nitrogen in the NaCl soluble protein are about 59 % of those of the alkali soluble protein, histidine nitrogen 23 %, and melanine nitrogen about 82 %.

### (6). Determination of the Free Amino Nitrogen.

It is a widely accepted assumption that the substances giving a biuret reaction, i. e. those combining more than two amino groups of -CONH-, -CSNH-, -C(NH)NH-, or -CHNH-, contain small quantities of free amino nitrogen. At the same time, Van Slyke and Birchard[16] estimated the quantities of free amino nitrogen of all kinds of proteins and proposed that they are proportional to lysine nitrogen of protein. The authors know the lysine nitrogen is predominant in the NaCl soluble protein by a ratio to alkali soluble of 100 : ca. 70 (see Table 14). So it may be considered that the quantity of free amino nitrogen of the NaCl soluble protein is superior

to that of the alkali soluble protein.

As Wilson[17] has asserted that Sörensen's method[18] excelled Van Slyke's for the determination of free amino nitrogen, the authors adopted the former for the purpose.

One tenth g. of protein was dissolved in 25 c.c. of 1/5 n-NaOH solution. To 20 c.c. of the solution were added 10 c.c. of neutral formalin (50 c.c. of formalin of purchase was titrated with 1/5 n-NaOH solution using 1 c.c. of 1 % phenolphthalein as indicator until the solution was colored slight pink). The solution was titrated with 1/5 n-HCl solution until it became slightly pink in colour. With 20 c.c. of water, 5 c.c. of 1/5 n-NaOH solution and 10 c.c. of neutral formalin, a control experiment was carried out and necessary corrections were made. 1 c.c. of 1/5 n-NaOH solution corresponds to 0.0028 g. of free amino nitrogen.

TABLE 14.

*Content of free amino nitrogen in various proteins.*

| No. of Sample | Alkali Soluble Protein | | | NaCl Soluble Proetin | | |
|---|---|---|---|---|---|---|
| | n/5 NaOH (c.c.) | Nitrogen (g.) | % of dry matter | n/5 NaOH (c.c.) | Nitrogen (g.) | % of dry matter |
| No. 2. | 0.55 | 0.00154 | 2.0603 | 0.35 | 0.00098 | 1.2728 |
| No. 3. | 0.45 | 0.00126 | 1.6949 | 0.30 | 0.00084 | 1.1374 |
| No. 4. | 0.55 | 0.00154 | 2.0095 | 0.40 | 0.00112 | 1.5008 |
| No. 6. | 0.40 | 0.00112 | 1.4508 | 0.25 | 0.00070 | 0.9193 |
| No. 7. | 0.50 | 0.00142 | 1.8898 | 0.30 | 0.00084 | 1.1164 |
| Average | | | 1.8211 | | | 1.1697 |
| Ratio | | | 100.0000 | | | 64.2300 |

According to the table, the content of free amino nitrogen of the alkali soluble protein is always superior to that of the NaCl soluble protein. When this fact is compared with the case of lysine nitrogen, there is great evidence against Van Slyke and Birchard's suggestion. So we must consider further the free amino groups in the polypeptide linkage of the protein molecule.

### (7). Determination of the Tyrosine and the Histidine.

For the determination of tyrosine and histidine of a protein, Hanke[19] has proposed the colorimetric method of diazo reaction. One half g. was

mixed with 30 c.c. of water and 5 c.c. of $H_2SO_4$ in a 100 c.c. flask. The flask was attached to a reflux condenser and the mixture was boiled for 24 hours, cooled, transferred to a 1000 c.c. flask, diluted to 500 c.c. heated over a water bath, and treated with a hot solution of 30 g. of $Ba(OH)_2$ in 150 c.c. of water. The mixture was then adjusted with dilute solutions of $Ba(OH)_2$ and $H_2SO_4$ until it contained a slight excess of the sulphate ion. Digested for several hours and filtered, while hot, through a Buchner's funnel. The $BaSO_4$ was throughly washed with hot water. The filtrate transferred to a glass dish and condensed on a water bath.

The semisolid residue was transferred to a 300 c.c. flask with 20 c.c. of water. Silver sulphate (200 c.c. of an 0.8 % solution) was added and mixed thoroughly. It was then treated with a warm solution of 10 g. of $Ba(OH)_2$ in 30 c.c. of water. The brown mixture was transferred immediately to an ice bath where it was allowed to settle for 1/2 hour. Under this condition the mixture remained brown; there was no noticeable deposition of metallic silver. The cold mixture was centrifuged. This effects a sharp separation in a cold medium in a minimum space of time. The clear, colorless, supernatant liquid was decanted into a 1000 c.c. flask and immediately acidified with 20 % $H_2SO_4$. The residue was washed once, by centrifugalization, with 20 c.c. of a cold, saturated solution of $Ba(OH)_2$. The washings were added to the main bulk of the silver filtrate which contains all of the tyrosine. This silver filtrate must be acidified with $N_2SO_4$ before it gets warm to avoid the oxidation of tyrosine by silver which is invariably present in small amounts. A sufficient excess of $H_2SO_4$ was added to cause the $Ba(OH)_2$ to settle readily. Normal HCl was then added, drop by drop, until the precipitate of silver chloride was no longer obtained. The liquid should give a faint test for chlorine ion. It was heated on a steam bath and adjusted with $Ba(OH)_2$ and $H_2SO_4$ until a slight excess of sulphate ion was present. Digested for several hours, filtered, transferred filtrate and washings to a glass dish and concentrated them.

The semisolid residue, which usually includes well refined crystals of tyrosine, was transferred to a 100 c.c. flask with 25 c.c. of water. One half c.c. of glacial acetic acid and 1 g. of mercury acetate were added and the mixture was boiled for 10 minutes under a reflux condenser. A small amount of precipitate is always obtained. The mixture was cooled, treated with 2.5 g. of NaCl and placed in an ice chest for 2 hours; transferred to a centrifuge tube with 10 c.c. of 10 % NaCl and centrifuged for 5 minutes. Decanted and discarded the clear supernatant liquid. The solid was washed once with 10 c.c. of 10 % NaCl solution. The solid was transferred back

quantitatively, to the boiling flask with 15 c.c. of hot 20 % HCl. The mixture was heated on the steam bath for 30 minutes. The liquid was treated with 20 c.c. of water, saturated with $H_2S$, heated on the water bath for 30 minutes, and filtered through a small folded filter. Filtrate and washing were collected evaporated and dissolved in unit volume and the tyrosine measured by colorimetric method.

The silver precipitate was transferred to a 150 c.c. flask with 20 c.c. of normal $H_2SO_4$ and 50 c.c. of water. The mixture thus obtained was treated with 1 c.c. of 37 % HCl, agitated until it became homogeneous, and finally warmed on the steam bath, for 15 minutes, to complete the conversion of the silver compound into silver chloride. When conversion is complete, silver chloride becomes unmistakely homogeneous and it is usually light colored. Filtered into a 250 c.c. volmetric flask, washed the precipitate with water until the washings were free from chlorine, neutralized the filtrate with 20 % HCl and diluted to the unit volume and determined histidine by colorimetric method.

TABLE 15.

*Showing tyrosine and histidine content of various proteins.*
*Figures are the percentage of dry matter.*

| | Tyrosine | Histidine |
|---|---|---|
| Alkali Soluble Protein | 0.182 | 0.0042 |
| NaCl Soluble Protein | 0.195 | 0.0027 |

In the above table, there is not much difference between tyrosine content of alkali soluble and NaCl soluble protein, while histidine content of the former is twice that of the latter.

## SUMMARY.

From the above experimental results, we could summarize the following differences in the physico-chemical properties between the four kinds of rice protein.

(1) The ash contents of the four proteins decrease according to the following order, i. e. water-, NaCl-, alkali- and alcohol-soluble protein. The quantity of alkali soluble protein corresponds to about half of the NaCl

soluble.

(2) There is a difference between the quantities of HCl needed to obtain the maximum surface tension and turbidity of the four protein alkali solutions. To obtain their maximum point, the water soluble protein solution needs a greater quantity of acid than the NaCl soluble and the latter needs more acid than the alkali soluble which in turn needs more acid than the alcohol soluble protein solution. So the iso-electric point of the four kinds of rice protein could be distinguished and their basity shown to increase in the following order, i. e. water-, NaCl-, alkali-, and alcohol-soluble protein.

(3) In the elementary composition, the sulphur content of the four proteins is decreased according to the following order, i. e. from water-, and NaCl soluble proteins to alkali soluble and to alcohol soluble. The sulphur content of the alkali soluble protein corresponds to about half the quantity of NaCl soluble and the carbon quantity is less than the latter while nitrogen is the contrary.

(4) Rotatory power of organic compounds is increased by asymmetric carbon atoms, complexity of molecular structures, saturation compound and especially by the presence of -COR groups. In alkali protein solution, this group is decreased by the neutralization of carboxyl groups by the transition of keto-form to enol-form in molecular structure. The higher rotatory power of the alkali soluble protein and the lower rotatory power of NaCl soluble must be caused by the above complexity in their molecular structure.

(5) Using Cohnheim's method, the quantity of HCl combined with the alkali soluble protein is greater than that of the NaCl soluble and the quantity of the latter corresponds to half of the former. This is due to the abundance of amino groups of the former which combine with HCl more easily in the former than in the latter.

(6) The silver salts of various proteins were prepared. The quantity of silver of the alkali soluble protein is about two-thirds that of the NaCl soluble protein. This is due to the fact that the NaCl soluble protein contains more carboxyl groups than the alkali soluble.

(7) On the determination of amino acids by Van Slyke's method, it is observed that in NaCl soluble protein, there is a predominance of mono-amino-, lysine-, and cystine-nitrogen content while in the alkali soluble protein, there is an excess of amide-, melanine-, and histidine-nitrogen. The quantity of ammonia nitrogen in the NaCl soluble protein is about 59 % of that of the alkali soluble protein, histidine nitrogen 23 %, melanine nitrogen 82 %. This is due to the fact that the alkali soluble protein having

a predominance of amide and diamino acids has a more basic nature in its amino acids components and polypeptide linkage than does the NaCl soluble which predominates in monoamino acids and needs a large quantity of HCl for precipitation from its alkali solution.

(8) The free amino nitrogen was determined by Sörensen's method. The quantity in the alkali soluble protein predominates over that of the NaCl soluble protein. This fact coincides with the predominance of basity in the iso-electric point and amide of amino acids components of the former. But when we compare the quantity of free amino nitrogen to lysine content in protein molecule, there is inverse proportionality in them, so it is a great argument againt Van Slyke and Birchard's suggestion.

(9) The contents of tyrosine and histidine were estimated without any distinct difference between tyrosine content in two kinds of protein. Great superiority in histidine content of the alkali soluble protein over the NaCl soluble was found.

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