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On the Nitrogen Content of Russian Comfrey

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(Text-fig. 1; Tables 1–7)

In the previous work[1)] the author reported the crude protein content of Russian comfrey, especially that its content in leaves was much more than in stems. The crude protein content was calculated from total nitrogen content multiplied 6.25 as usual. In this paper nature and properties of many kinds of nitrogenous compounds are reported.

EXPERIMENTAL METHOD

Samples of fresh or dried leaves of Russian comfrey, which was cultivated in the field of Kawaguchi Farm, Department of Animal Husbandry, 1961, were used.

Text-fig. 1. Treatment of Sample.

Sample (100 *g* of fresh leaves or 10 *g* of dried leaves)

- Add H_2O 150 *ml*. Shake 1 hr. let stand 1 hr. separate by centrifuge, filter by suction, again repeat the same manipulation twice.
  - ↓ Filtrate (H_2O soluble fraction)
  - ↓ Residue
    - Add 10% NaCl 150 *ml*, repeat the same manipulation thrice above mentioned.
      - ↓ Filtrate (10% NaCl soluble fraction)
      - ↓ Residue
        - Add 70% EtOH 150 *ml*, repeat the same manipulation thrice above mentioned.
          - ↓ Filtrate (70% EtOH soluble fraction)
          - ↓ Residue
            - Add 0.3% NaOH 150 *ml*, repeat the same manipulation thrice above mentioned.
              - ↓ Filtrate (0.3% NaOH soluble fraction)
              - ↓ Residue
                - Add 0.3% NaOH alkaline 60% EtOH 150 *ml*, boil in water bath with condenser for 15 minutes. After cooling separate by centrifuge. Again repeat the same manipulation twice.
                  - ↓ Filtrate (0.3% NaOH 60% EtOH soluble fraction)
                  - ↓ Residue (Insoluble fraction)

After harvesting, the fresh leaves of Russian comfrey were cut into pieces of 2 or 3 *cm*, at once and further torn to pieces by a homogenizer. Dried leaves were prepared as follows: after cutting into 2 or 3 *cm*, the leaves were dried in the electric thermostat below 50°C and after drying ground up by pulverizer. The harvest time was the latter part of May and the early part of September.

The analytical method of nitrogen which is soluble in many kinds of solvents is applied correspondingly to the method that has been reported by KANDATSU[2]. 100 *g* of fresh leaves or 10 *g* of dried leaves were treated as shown in the figure and on the various solute, nitrogen was determined by GUNNING's modified method[3] and protein nitrogen by BARNSTEIN's method[3]. And the difference between the former and the latter was considered to be non-protein nitrogen.

RESULT AND DISCUSSION

The results obtained by the above mentioned method are as follows:

As shown in Table 1, fresh leaves contain 86% of moisture and 0.5% of total N. Dried leaves a and b contain about 7% of moisture and 4% of nitrogen, and a little difference of these elements can be recognized in different growth periods.

Table 1. Soluble Nitrogen (%)

Solvent	Fresh leaf	Dried leaf	
		a	b
H_2O	0.068	0.448	0.474
10% NaCl	0.065	0.255	0.207
70% EtOH	0.027	0.177	0.118
0.3% NaOH	0.051	0.415	0.502
0.3% NaOH 60% EtOH	0.100	0.564	0.597
Sum	0.311	1.859	1.898
Residue N	0.173	1.875	1.352
Total N	0.520	4.065	3.984
Moisture (%)	86.650	7.247	7.787

Table 2. Ratio of Soluble Nitrogen (%)

Solvent	Fresh leaf	Dried leaf	
		a	b
H_2O	21.9	24.1	25.0
10% NaCl	20.9	13.7	10.9
70% EtOH	8.7	9.5	6.2
0.3% NaOH	16.4	22.3	26.4
0.3% NaOH 60% EtOH	32.1	30.3	31.5
Sum	100.0	99.9	100.0
Total N	59.8	45.7	47.2

Note. a: Leaf cut in the latter part of May.
b: Leaf cut in the early part of September.

Table 3. Protein N (%)

Solvent	Fresh leaf	Dried leaf	
		a	b
H_2O	0.043	0.415	0.355
10% NaCl	0.049	0.178	0.121
70% EtOH	0.024	0.177	0.188
0.3% NaOH	0.051	0.355	0.433
0.3% NaOH 60% EtOH	0.095	0.546	0.578
Sum	0.262	1.671	1.605

Table 4. Ratio of Protein N (%)

Solvent	Fresh leaf	Dried leaf	
		a	b
H_2O	16.4	24.8	22.1
10% NaCl	18.7	10.7	7.5
70% EtOH	9.2	10.6	7.4
0.3% NaOH	19.5	21.2	27.0
0.3% NaOH 60% EtOH	36.2	32.7	36.0
Sum	100.0	100.0	100.0

Table 5. Non-Protein N (%)

Solvent	Fresh leaf	Dried leaf	
		a	b
H_2O	0.025	0.033	0.119
10% NaCl	0.016	0.077	0.086
70% EtOH	0.003	0.0	0.0
0.3% NaOH	0.0	0.060	0.069
0.3% NaOH 60% EtOH	0.005	0.018	0.019
Sum	0.049	0.188	0.293

Table 6. Ratio of Non-Protein N (%)

Solvent	Fresh leaf	Dried leaf	
		a	b
H_2O	51.0	17.5	40.6
10% NaCl	32.6	41.0	29.4
70% EtOH	6.1	0.0	0.0
0.3% NaOH	0.0	31.9	23.5
0.3% NaOH 60% EtOH	10.2	9.6	6.5
Sum	99.9	100.0	100.0

Ratios of each soluble nitrogen content in many kinds of solvents to the sum of soluble nitrogen are shown in Table 2. In fresh leaves ratio of 0.3% NaOH alkaline 60% EtOH soluble nitrogen fraction is 32% the greatest, H_2O soluble nitrogen and 10% NaCl soluble nitrogen fractions are 21.9 and 20.9% the next, 0.3% NaOH soluble nitrogen is still less, and EtOH soluble nitrogen is 8.7% the least.

In dried leaves a and b, ratios of 0.3% NaOH alkaline 60% EtOH soluble nitrogen are 30.3 and 31.5%, and these ratios are almost similar. Ratios of H_2O soluble nitrogen of dried leaves are 24.1 and 25.0% and ratios of NaOH soluble nitrogen are 22.5 and 26.4% also, and both ratios in H_2O and NaOH soluble nitrogen are much more than those in fresh leaves. Against ratios of NaCl soluble nitrogen are 13.7 and 10.9% and considerably much less than that of fresh leaves. Ratios of EtOH soluble nitrogen of dried leaves are 6 and 9% and the variation is not distinct as compared to the ratio of fresh leaves. These uniform tendencies are visible in the results in case of protein nitrogen content also.

The difference of soluble nitrogen content in many kinds of solvents between fresh and dried leaves is derived from the difference of protein nitrogen content, and this variation of solubility (prosperity of activity) in soluble nitrogen is considered to be denaturation of protein by many kinds of factors.[4] Further, the difference of protein nitrogen content between a and b is not remarkable, and in consideration of the kinds of plant protein, the component of Russian comfrey is almost uniform although the harvest period is different.

Russian comfrey does not wither in a summer of high temperature and little precipitation, and its constituent is almost uniform in both seasons, such as spring and summer. So therefore Russian comfrey is recongnized to be a suitable feed crop in the region of the south western part of Japan.

STOIKOFF[5] investigated the concentration of protein and its identification in legumes and reported that the solubility of protein nitrogen and non-protein nitrogen in H_2O were 2.85 and 0.96%, respectively; in 7% NaCl, 0.83 and 0.00% and in 0.2% NaOH, 0.45 and 0.00%. Though protein content and concentration of solvents such as NaCl and NaOH, are not uniform, in Russian comfrey, both fresh and dried leaves, non-protein nitrogen in NaCl and NaOH soluble fractions is contained. It is obvious that difference of nitrogen content is recognized by the species of plant.

The content of other nitrogenous compounds determined by the usual method is as follows:

Table 7. Some other Nitrogen (%)

	Dried leaf	
	a	b
NH_3-N	0.006	0.005
NO_3-N	0.312	0.220
NH_2-N	0.236	0.329
Amide-N	0.037	0.026
Peptide-N	1.434	1.740

Though NH_3-N is useful to produce amino acid in the tissue of plant, its content is very little and uniform in both a and b leaves.

NH_2-N contents are 0.236% in a and 0.329% in b, and its content in b is more than in a. Amide-N is 0.037% in a and 0.026% in b, more in a than in b. In both nitrogen contents some differences can be recognized in different growth periods.[6] BRADY[7] reported that amino nitrogen concentration increased rapidly during wilting and ensilage of short rotation of ryegrass and white clover, and that the concentration of other fractions depended on the particular conditions prevailing.

Though amino acid production in plant tissue is increased by fertilization,[8)9)10] amino acid content varies in a part of the plant.[10] WILDING[11] reported that red clover from which flowers had been removed had 40–50% more amino acids than the controls, and also free amino acids in alfalfa showed a 20% increase from August to December in the hardiest variety with a 31% increase in acids, and the nonhardy variety showed little change in these fractions. MACGREGOR[9] studied the Minnesota alfalfa proteins, comparing with results from other areas, which tended to be consistent in amino acid content unless soil nutritional deficiencies or climatic conditions were considerably different, and there was no apparent difference in the amino acid levels of the unfertilized and fertilized alfalfa, but the Minnesota levels were generally slightly higher than those reported from other areas.

It is well known that peptide is a condensate of amino acid. Peptide nitrogen fraction of Russian comfrey is 1.434% in a and 1.740% in b. ROHRLICH[12)13] studied air dried samples, finding that there was an increase of total nitrogenous material and a decrease of water soluble nitrogen compounds in the course of development of wheat and rye seeds. And chromatographs of the proteins occuring during development showed the presence of peptides comprising 2–20 amino acids. And he isolated from water extracts of flour and analyzed; a tripeptide containing serine, glutamic acid, and valine, and a tetrapeptide containing 3 mols glutamic acid and 1 of valine. Though authors detected many kinds of amino acids from Russian comfrey by paperchromatography, what sort of peptide would depend upon the future investigation.

Nitrate nitrogen in feed crops has an important effect upon the physiological condition of livestock and causes public discussion usually. In Russian comfrey NO_3-N content is 0.312% in a and 0.220% in b.

Usually feed crops contain nitrate,[14)15] and the nitrate content of the crops has a direct dependence on rates of nitrogenous fertilization. In rye grass[16] the maximum value is 14.96 *mg* nitrate N/*g* dry matter. The value for fodder rye is 10.67, for white mustard 25.6, and for Liho rape 16.11. The mean nitrate content of 133 samples[17] of meadow hay is 0.12% nitrate on dry matter (minimum 0.02, Maximum 0.43% nitrate). In 12 samples of silage (corn, clover, and mixture) the mean nitrate content is 0.05%. And in an other study the average nitrate nitrogen of 48 samples of grass[18] is 0.02%. KAGEYAMA[19] and others examined a method using NO_3-N content in leaves as an indicator to establish a suitable rapid method for diagnosing the N-nutritional status of vegetables.

The concentration of nitrate in forages is influenced greatly by species,[14][17][20][21] part of the plant,[15][20] stage of maturity,[20] level of nitrogenous fertilization,[16][17][20] and light intensity,[16][20] time of season,[20] daily low temperature[15] and variations between daily low and high temperatures,[15] and least affected by closely related species,[14][18][20] varieties,[20] time of nitrogenous fertilization,[21] kind and placement of nitrogenous fertilizer,[20] and lack of certain plant nutrients.[20]

On the kinds of nitrogenous fertilizer, GRIFFITH[22] reported that when nitrogen was applied as $(NH_4)_2SO_4$ at increasing rates up to 12 hundred wt./ acre, nitrate content of grass mixtures, also increased, reaching 2,000 p.p.m. 6 weeks after the heaviest treatment, and rape contained high levels of nitrate, there was no difference between Nitrochalk and $(NH_4)_2SO_4$ in respect of nitrate accumulation. ERIKSSON[23] observed that fertilizing with $Ca(NO_3)_2$ had a relatively strong influence on the nitrate content.

Phosphorous fertilizer[16] shows an influence only at medium nitrate content, and nitrate contents over 8–10 *mg* nitrate N/*g* dry matter are not effected by increasing phosphorous application. Double rates of potassium application[16] caused an increased nitrate concentration.

Hemoglobin in blood of animal is a sort of Haemprotein, and consists of both protheme and simple protglobin, and assumes a red color. Usually haem iron exists as a condition of two valence, and can combine reversionary with molecular oxygen owing to partial pressure of oxygen, and physiologicaly discharges an important part of the carrier of oxygen.

Hemoglobin which is added oxygen is called Oxyhemoglobin (HbO_2) and it also combines with carbon monoxide (CO) in addition to oxygen. When one treats hemoglobin by an oxidizing agent, heme iron is oxidized and becomes a condition of three valence. It is called methemoglobin (Hemiglobin) and has no efficiency to combine with oxygen or carbon monoxide already. Especially excessive concentration of nitrate in feed crops and heavy fertilization under drought conditions may cause fatal poisoning in cattle by methemoglobin. Generally Methemoglobinemia is well known as caused by methemoglobin.

MOORE[24] described the occurrence of poisoning in 3 cows caused by eating freshly dug mangold plants containing 2.5% nitrate. HYMAS[25] investigated the effects of nitrate to yearling cattle. A concentrate of nitrate salts mixture which contained 17.52% of NO_3-N when given via stomach tube caused death at 928 and

737 *mg* NO_3-N/*kg*, but doses of 640 and 455 *mg/kg* resulted in no ill effects. SIMON[26] showed that lesions in the fetuses and fetal membranes of three heifers given 100 *g* of KNO_3 daily, which aborted, were comparable to those observed in marshland abortions. KATHEIN[27] investigated nitrate poisoning with 8 fatalities and 3 recoveries, and established on the basis of autopsy findings and chemical tests. Since symptoms of methemoglobinemia were present in 11 cases, the incrimination of the plant was justified. KAMEOKA[28] fed goats and rabbits with feed which contained $NaNO_3$ in the amount of 2%, and examined the metabolism of nitrate and nitrite. According to his investigation nitrate was completely absorbed, but only 50% of it was excreted in the urine. He presumed that nitrate was partly reduced to nitrite by microorganisms and animal tissues.

LINDNER[29] also fed cattle with wilted sugar beet tops, containing 0.2–0.8% (wet. wt.) of NO_3 poisoning. Nitrate as $KN^{15}O_3$ was added to the rumen by WANG [30] and others to trace the formation, absorption and elemination of nitrate. Nitrate, nitrite and NH_4 ions were absorbed in considerable quantity directly from the rumen into the blood. The highest concentration of N^{15} in the NH_4 ions in the blood appeared 3–4 hrs. after $KN^{15}O_3$ was added to the rumen, whereas the highest N^{15} concentration in the nitrate plus nitrite fraction appeared in the blood after 5–6 hrs. The formation of methemoglobin in the blood followed the time course of nitrite formation in the rumen rather closely, suggesting that nitrite was passed rapidly and directly from the rumen into the blood.

JAMIESON[31] studied the effects of adding moderate amounts of nitrate (up to 25 *g*/day of KNO_3) to the rumen of the grazing sheep, and a slight stimulation of rumen function after continuous daily nitrate dosing was indicated. The sheep exhibited methemoglobinemia under 35% conversion of total hemoglobin to methemoglobin. MORRIS[32] observed the test animals fed on roughage were not affected by the nitrates in the forage, even though the roughage in some cases contained 3% of KNO_3.

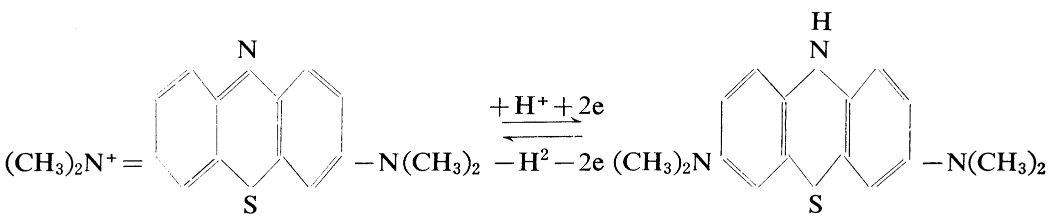
In many results of studying on some aspects of nitrate intoxication in livestock, it is clear that nitrate concentration influences methemoglobinemia.

By CASE[33] NO_3 content of over 0.5% in rations is unsafe, and NO_2 is 10–15 times as toxic as NO_3. Further by SCHINK[34] complete reduction by bacteria of 2.6 *g* nitrate/*kg* and resorption of the nitrite in a 30 *kg* hog causes fatal methemoglobinemia; 25% reduction of the nitrate in 1 *kg* of feed containing 6–7 *g/kg* of KNO_3 may be dangerous.

Methemoglobin can be reversible to hemoglobin by a reducing agent. The rate of nitrate and nitrite reduction in rumen ingesta is closely correlated with the basic diet. The addition of carbohydrate and vitamin A[33] help reduce the toxicity of the total NO_3 content of the total ration is not over 1.5%. The presence of glucose promotes the rapid reduction of nitrate to nitrite. Supplement of sugar increases the tolerance for nitrate.[35][36]

The oral administration of nitrate or nitrite to grazing sheep induces changes in the composition of the rumen volatile fatty acids.[37] This alteration in the volatile acid content of the rumen is considered to be responsible for one of the known variable nitrate content and the other the protein content of the pasture. Lower fatty acids do not act as H donators for the reduction of nitrate by rumen micro-organisms.[38] H_2 is a very active donator for this reaction leading to the formation of NH_3 from nitrate, nitrite or NH_2OH. Formate, succinate, lactate, citrate, glucose, malate, or mannitol, some of which may be present in the rumen, are also H donators.

Intravenous injection of 50 *ml* of 4% methylene blue solution is quite effective in counteracting the toxicant.[24] Methylene blue indicates extreme high redox-potential. This effect is considered to be the reason that the redox-potential in organs of living thing is lower than that of Methylene blue, and Methylene blue is harmless compared to redox-potential enzyme.



More nitrate effects the duration of the active state in skeletal muscle and reduces tetanic tension.[39][40]

As above mentioned, Russian comfrey contains 0.3–0.4% of nitrate, and its content is within the limit of concentration of nitrate in many other feed crops, though some differences in nitrate concentration are recognized in different growth periods. So it is not considered that Methemoglobinemia is caused in livestock by such as the nitrate concentration in Russian comfrey.

SUMMARY

The nitrogen content which is soluble in many kinds of solvents, in fresh and dried leaves of Russian comfrey was investigated. Harvest periods of Russian comfrey are in the latter part of May and in the early part of September. Ratios of H_2O and 0.3% NaOH soluble nitrogen are increased and 10% NaCl soluble nitrogen decreased in dried leaves compared to fresh leaves. These tendencies are observed in protein nitrogen fraction of dried leaves. The variation of solubility is considered to be caused by denaturation of protein. NH_3-N, NO_3-N, NH_2-N, Amide-N, and Peptide-N are determined in dried leaves, and some considerations of these nitrogenous compounds, especially nitrate poisoning effect on livestock, are issued.

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Russian comfrey の窒素化合物

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Russian comfrey の新鮮葉 100 *g* 風乾葉 10 *g* に水，10％食塩水，70％アルコール液，3.3％苛性ソーダ溶液，0.3％苛性ソーダ60％アルコール液を順次10倍量宛加え，同じ操作を3回繰返しその合一沪液について窒素を測定した．各種溶剤可溶の窒素総量は全窒素量に対し新鮮葉は60％，風乾葉は47％で稍少ない．また試料の風乾により各種溶剤可溶窒素は総量に対し，10％食塩水可溶窒素量はその割合を減少するが，0.3％苛性ソーダ可溶窒素量はその割合を増加する．純蛋白質においても同様の傾向がみられる．これは乾燥のため蛋白質に変性をきたしたもので試料採取の時期を異にしても同様な結果を示す．なおアンモニア態，硝酸態，アミノ態，アミド態，ペプチド態窒素をも測定し，特に硝酸と家畜の Methemoglobinemia との関係を検討した．