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Was Citrulline First a Laxative Substance?: The Truth about Modern Citrulline and Its Isolation

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Abstract: Citrulline is a non-protein amino acid and is produced by the enterocytes of the small bowel. The isolation though of citrulline is generally ascribed to the 1930s. In the present article, we demonstrated that before 1930, there was use of the term citrulline, signifying a resin produced by *Citrullus Colocynthis*. This citrulline is different from modern citrulline. However, neither was modern citrulline isolated in 1930 but somewhat earlier. Reviewing the original manuscripts, Koga and Ohtake (1914) did indeed isolate citrulline for the first time and at least half a dozen other researchers cite their work. Even though their work didn't lead to the determination of the structure and nature of citrulline, theirs was the first to isolate it. Our results have a certain historical and scientific significance and are discussed in extent.

Key words: citrulline, isolation, history, laxative

Introduction: Intestinal Failure and Citrulline

Intestinal failure (IF) occurs when there is reduced intestinal absorption so that macronutrient and water and electrolyte supplements are needed to maintain health or growth. Nutrient/fluid requirements determine whether IF is termed severe, moderate, or mild. Severe is when parenteral, moderate when enteral, and mild when oral nutritional fluid supplements are needed.[1,2)] The most common cause of IF is short bowel syndrome. Normal human small intestinal length, measured from the duodenojejunal flexure at autopsy or surgery, varies from about 275 cm to 850 cm, and tends to be shorter in women. After intestinal resection it is important to refer to the remaining length of small intestine measured at surgery or with an opisometer.[2)] In general, nutritional/fluid supplements are likely to be needed if less than 200 cm of small bowel remains. The most common reasons for a short bowel in adults are Crohn's disease, superior mesenteric artery thrombosis, and irradiation damage.[1)]

Citrulline's unique metabolism which involves production at the intestinal enterocyte has prompted suggestions that plasma citrulline level could be a reliable marker of gut function.[3)] This led to a hypothesis that citrulline may be a 'conditionally' essential amino acid in short bowel syndrome, even if it is not incorporated into proteins. Healthy patients with normal intestinal mucosa function and normal renal function have a citrulline level between 30 and 50 μmol/l with a median of 40 μmol/l.[4,5)] Although this range for plasma citrulline levels mainly comes from studies in Western Europe and North America, a Chinese study on 33 healthy Chinese subjects found a mean plasma citrulline level of 16.87±5.97 μmol/L (range 19–54) measured with HPLC.[6)] This finding is very interesting, becomes it creates a paradox in the multiple findings of the high diagnostic accuracy of citrulline level at 20 μmol/L.

Many researchers have examined whether citrulline levels are a marker of enterocyte mass or absorptive

function. Their study groups included patients with short bowel syndrome, celiac disease, Crohn's disease, patients who are on anti-neoplastic treatment or who have undergone intestinal transplantation. A recent meta-analysis of ours showed that the pooled correlation coefficient of citrulline levels with small bowel length in SBS patients was 0.697 while the correlation coefficient of citrulline levels with absorptive test was lower (0.326).[7)]

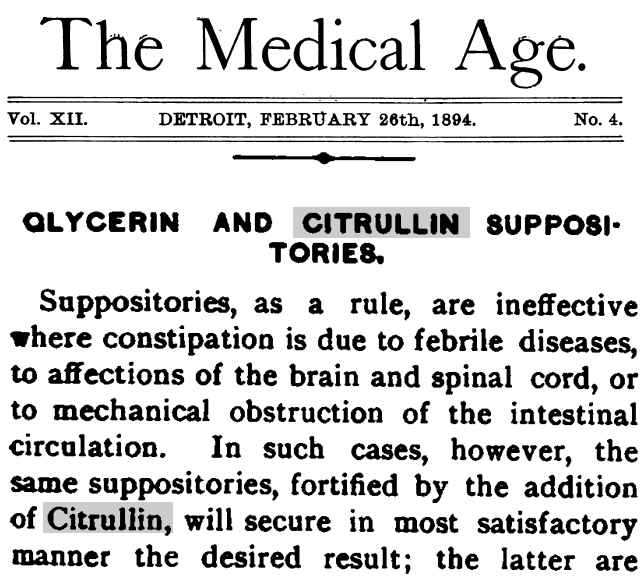
Historical Fallacies

"Citrulline was first isolated in 1930".[8)] This is a common phrase in the nutritional and gastroenterology literature regarding citrulline. Nevertheless, some interesting historical sources detail that the amino acid citrulline was discovered much earlier than that, while there was use of another citrulline at the late 19th century.

In the *North Carolina Medical Journal*, January 1883,[9)] we read the following passage:

> "The colocynthum purum prepared by Merck [...] produces watery stools with moderate tormina[10)] [...] There is also ***a resinoid substance called citrullin***, extracted from the colocynth fruit, insoluble in water, which, when taken internally in the dose of 5 milligrammes to 1 centigramme, or if administered hypodermically in the same dose [...] will produce the desired effect". (ref. 9, p. 44)

Similar excerpts appeared then in other journals, such as the *Medical Age*[11)] (Figure 1) and the *Journal of the American Medical Association*[12)], while even a significant gastroenterologist of that time, Ismar Boas (1858–1938),[13)] comments on citrulline's usefulness with difficult cases of constipation: "Laxatives may often be administered by the rectum in the form of small enemata [...] The most serviceable [...] for especially obstinate cases, are colocynthin and **citrullin**".[14)] To the reader of nutrition and gastroenterological articles, this early mentioning of the word citrullin[e] causes some surprise. That is because citrulline is



The Medical Age.

Vol. XII. DETROIT, FEBRUARY 26th, 1894. No. 4.

GLYCERIN AND CITRULLIN SUPPOSITORIES.

Suppositories, as a rule, are ineffective where constipation is due to febrile diseases, to affections of the brain and spinal cord, or to mechanical obstruction of the intestinal circulation. In such cases, however, the same suppositories, fortified by the addition of Citrullin, will secure in most satisfactory manner the desired result; the latter are

Figure 1. Passage from *The Medical Age* 1894; 12: 115, mentioning citrullin[e] suppositories

generally regarded to have been first discovered in the 1930s[15] and is part of the urea cycle taking place mainly in the liver; citrulline's pharmacological action has not yet been studied in humans, so this laxative effect also amazes us.

The purpose of this article is to answer whether citrulline had been discovered before the 1930s and much more whether it had been utilized therapeutically in someway we lack knowledge of - even today.

Early sources mentioning the term "citrulline"

We performed a systematic review of all texts before 1930, which mentioned the term "citrulline". A preliminary analysis had shown us that the synonym terms "citrullin" and "citrullinum" were also in use. Thus, we searched databases with extensive digitized volumes of rare books: Google Books (http://books.google.com), Internet Archive (http://www.archive.org), and Gallica (http://gallica.bnf.fr). These three databases are the largest online sources of mass digitization projects of books from a large range of American and English universities, while Gallica is a large-scale digitization program of the National Library of France. We used the search term "citrulline or citrullin or citrullinum" and time limit from 1800–1930. A source of bias might have been introduced, due to the fact that languages which do not use the Latin alphabet were not searched.

Our results revealed that 170 books and journal volumes have the word citrulline among their pages. This figure might increase to 254 volumes, if other editions are considered as well. Specifically, these were all published between 1882 and 1930 and include in their majority English, French, and German texts, while very few were in Dutch, Swedish, and Italian. A full account of all texts is given in Appendix A. All texts mention the results of two main articles, written by Hiller[16] and Kohlstock,[17] discussing subcutaneous and rectal injection of purgatives.

Is this citrulline, the modern citrulline?

This citrulline is a resin produced by the pulp of *Citrullus colocynthis*. *C. colocynthis* Schrad (family Cucurbitaceae), also known as colocynth or bitter apple, is a common weed found in countries of the Middle East and the Mediterranean (Figure 2). *C. colocynthis* has been used medicinally since ancient times. The fruits and seeds are used as purgative and have been suggested to possess antitumour activity.[18] From the pulp of *C. colocynthis*, α-elaterin, α-elaterin-2-D-glucopyranoside, citrullol, and an alkaloid with strong purgative action have been isolated,[19] while the other parts of this weed contain several more substances.[20] When overconsumed, it can cause colitis.[21] This citrulline is different from the modern citrulline, an amino acid which participates in the urea cycle.[5]

So, the term citrulline had been in use for about 40 years from 1882 reaching the early 1920s, referring to a strong purgative used occasionally in humans. This discussion was stimulated by two articles dealing with subcutaneous and rectal injection of purgatives.[16,17] In summary, both authors found that citrulline could be administered subcutaneously or rectally (dose: 5 mg to 20 mg) in cases of chronic constipation, but it was followed by adverse effects of severe pain, oedema and redness of the skin. Rectal injections were tolerated more easily than subcutaneous ones. Its use was most possibly hindered because of a high price or low availability,[22] while it was used more frequently in veterinary medicine.[23] It must have passed into oblivion due to the infrequent use and the ineffectiveness it presented as a laxative. A possibility is

Figure 2. A drawing of *C. colocynthis* from D. M. R. Culbreth. *A Manual of Materia Medica and Pharmacology*. 6th ed. Philadelphia: Lea & Febiger; 1917. p. 585

that it could have changed name over time, making it difficult to track in scientific literature.

The history of modern citrulline's isolation

Modern citrulline is a non standard amino acid and in humans its plasma content is derived largely from the amount produced in enterocytes of the small bowel (Figure 3).[24] Citrulline's first isolation from the juice of the watermelon (*Citrullus vulgaris Schrad*) has generally been attributed to Mitsunori Wada,[5,8,25,26] who isolated citrulline and determined its chemical formula in 1930, naming the substance he isolated *citrulline.*[15] We would like to add further value to current literature on citrulline, by mentioning that it was isolated earlier than 1930, and specifically in 1914. We present the supporting evidence.

The first isolation of citrulline occurred in 1914 by Yotaro Koga and Ryo Ohtake,[27] who isolated citrulline from the juice of the watermelon and said that a substance was present in it with the chemical formula $C_6H_{13}N_3O_3$ (Figure 4). They didn't elucidate though the structure of this new substance nor did they name it. A possible inhibiting effect of this understanding may be the fact that it was written in Japanese, making it difficult for European and American scientists to grasp the significance of this paper. Although Japanese people had begun studying European languages from the early 19th Century, this was not the case with Europeans, whose knowledge of Japanese was rather scarce.[28] We have attached a translation of their article to Appendix B.

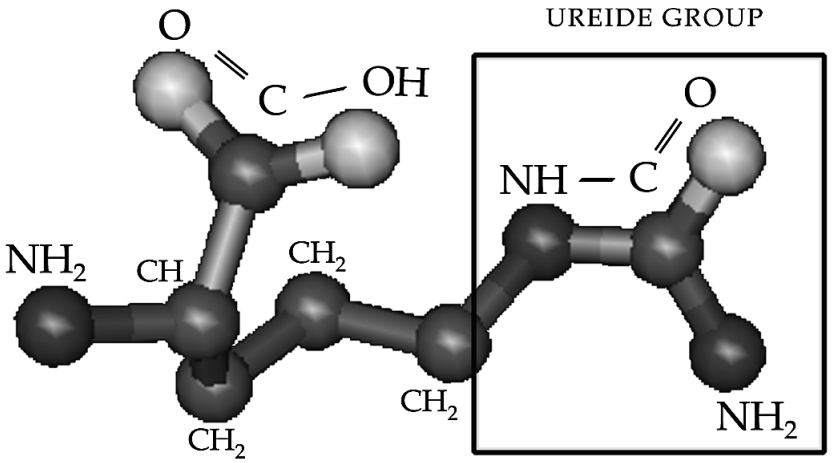
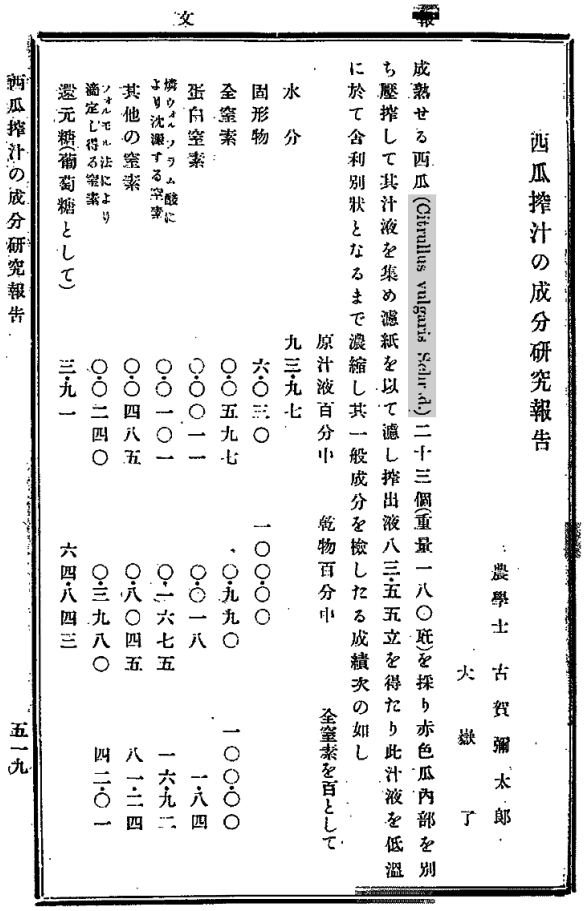


Figure 3. Citrulline structure. In a box is the characteristic group of citrulline, the ureide group (NH_2-CO-NH)


西瓜搾汁の成分研究報告

農學士　古賀彌太郎
大嶽　了

成熟せる西瓜(Citrullus vulgaris Schr.ds)二十三個(重量一八〇瓩)を採り赤色瓜内部を別ち壓搾して其汁液を集め濾紙を以て濾し搾出液八三・五五立を得たり此汁液を低溫に於て含利別狀となるまで濃縮し其一般成分を檢したる成績次の如し

	原汁液百分中	乾物百分中	全窒素を百として
水分	九三・九七		
固形物	六・〇三〇	一〇〇・〇〇	
全窒素	〇・〇五九七	〇・九九〇	一〇〇・〇〇
蛋白窒素	〇・〇〇一一	〇・〇一八四	一・八四
燐ウォルフラム酸により沈澱する窒素	〇・〇〇一〇一	〇・一六七五	一六・九二
其他の窒素	〇・〇四八五	〇・八〇四五	八一・二四
ソルモル法により適定し得る窒素	〇・〇二四〇	〇・三九八〇	四二・〇一
還元糖(葡萄糖として)	三・九一	六四・八四三	

Figure 4. The first page from the article by Koga and Ohtake (1914) in the *Journal of the Tokyo Chemical Society*. To be read from right to left and from up to down

Thus a few years later, the Japanese scientist M. Wada, repeated their experiment in 1930 in the same laboratory (Agricultural Chemical Laboratory, Tokyo Imperial University). He isolated this new amino acid, defined its chemical formula and structure and proved that his observations concerning this new amino acid were correct by synthesizing citrulline. He named this amino acid "citrulline".[15,29] He further demonstrated its isolation from the tryptic digestion of casein and possibly arginine.[30] He published his findings in a significant German journal of that period *Biochemische Zeitschrift*[15] and in the *Proceedings of the Imperial Academy* in English.[29] Publishing in German and English was of great significance since it enhanced the swift acceptance of his results from fellow scientists.

The first isolation though by Koga and Ohtake[27] was acknowledged by other researchers over the following years. They attributed to Koga and Ohtake the first isolation from the juice of watermelon and denote that Wada performed the first sound study determining the chemical properties and structure of citrulline, as well as the name "citrulline".

First of all, Wada[15] himself acknowledges their contribution to the first isolation of citrulline by saying "Sixteen years ago in this laboratory, Y. Koga and S. Odake[31] isolated from the juice of watermelon a nitrogenous compound as colorless prisms, different from arginine and Glycocoll betaine ... The analysis yielded the empirical formula $C_6H_{13}N_3O_3$. Since then, the structure has not been elucidated".

Ackermann,[32] who has been referenced elsewhere as the co-discoverer of citrulline with Wada,[25] mentioned in reference to citrulline, that, "such a substance had already been isolated from the watermelon, Citrullus vulgaris, by Y. Koga and S. Odake, and its structure had been fully described by Wada".

A few years later, Vickery[33] mentions "In 1914, Koga and Odake described the isolation of a substance $C_6H_{l3}N_3O_3$ from the juice of watermelon. Aside from the fact that it formed a copper salt, little else was recorded. In 1930, Wada prepared the substance again and showed that its properties were best explained on the assumption that it is L-carbamido ornithine". Vickery[34] repeats his views once more several years later in an article on the discovery of amino acids.

Krebs and Henseleit[35] demonstrated that citrulline is an intermediate in the mechanism whereby urea is formed in the liver, signifying the importance of this amino acid in nitrogen metabolism,[36] citing first the work by Koga and Ohtake,[27] and then that of Wada.[15]

Fearon notes that "the carbamido-acid citrulline, isolated by Koga & Odake in 1914 from the watermelon, attracted no general attention until Wada (1930) established its constitution as α-amino-δ-carbamidovaleric acid".[36]

Finally, Impellizzeri et al.,[37] while claiming that citrulline has been detected in a variety of plant sources and presumably is universal in plants (because of its role in arginine biosynthesis), cite the work by Koga and Ohtake[27] as the first to have isolated citrulline from the juice of watermelon.

Conclusion

In the present article, we demonstrated that before 1930, when modern citrulline, was first believed to be isolated, there was use of the term citrulline, signifying though a resin produced by *C. Colocynthis*. This citrulline is different from modern citrulline. However, neither was modern citrulline isolated in 1930 but somewhat earlier. Reviewing the original manuscripts, Koga and Ohtake[27] did indeed isolate citrulline for the first time and at least half a dozen other researchers cite their work. Even though their work didn't lead

to the determination of the structure and nature of citrulline, theirs was the first to isolate it. Our results have a certain historical and clinical significance since they lead to a hypothesis: Could this old citrulline-resin be considered by physicians as an alternative clinical useful purgative, when given subcutaneously or rectally?

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Conflict of interest statement: No competing interest

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10) Acute Abdominal Pain
11) 'Glycerin and Citrullin Suppositories', *Medical Age*, 12 (1894), 115.
12) 'Queries and Minor Notes', *Journal of the American Medical Association*, 53 (1909), 310.
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17) P. Kohlstock, 'Ueber subcutane und rectale Anwendung von Abführmitteln', *Charité-Annalen*, 17 (1892), 283–294.
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29) Mitsunori Wada, 'On the Occurrence of a New Amino Acid in Watermelon', *Proceedings of the Imperial Academy*, 6 (1930), 15–17.
30) Mitsunori Wada, 'Isolierung des Citrullins (δ-Carbamido-ornithin) aus tryptischen Verdauungsprodukten des Caseins', *Biochemische Zeitschrift*, 257 (1933), 1–7.
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Appendix A

A bibliographic list of the sources mentioning the term "citrulline" from 1882 to 1930, before the discovery of the modern amino acid citrulline

No.	Source
	Dutch Texts
1	Nederlands tijdschrift voor geneeskunde. Houten: Bohn Stafleu van Loghum; 1894. vol. 38, p. 1010
2	Ritsema IC, Sack J, Greshoff M. Index phytochemicus. Amsterdam: Uitgave van het Laboratorium; 1905. p. 12
	English Texts
3	Morse WH. New therapeutical agents. Detroit: Davis; 1882. p. 183
4	The Cincinnati Lancet-Clinic. Cincinnati: J. C. Culbertson; 1882. vol. 48, p. 136
5	The London Medical Record. London: Hart; 1882. vol. 10, p. 490
6	American Medical Digest. New York; 1882. p. 156
7	The Louisville Medical News. Louisville: Ky; 1882. pp. 117–8
8	Medical Record. New York: W. Wood; 1882. vol. 22, p. 604
9	Chicago Medical Review. Chicago: Chandler & Engelhard; 1882. vol. 5–6, p. 563
10	The Medical Press & Circular. London; 1882. p. 268
11	The North Carolina Medical Journal. Charlotte, N. C.; 1883. vol. 11, p. 45
12	The Medical Times and Register. Philadelphia: Medical Publishing Company; 1883. vol. 13, p. 71
13	Pharmaceutical Journal and Transactions. London: Churchill; 1883. p. 343
14	Therapeutic Gazette. G. S. Davis; 1883. vol. 7, p. 380
15	Lescher FH. Recent materia medica: notes on their origin and therapeutics. London: Churchill; 1884. p. 27
16	Bruce JM. Materia medica and therapeutics, 1st ed. London: Cassell; 1884. p. 297
17	Medical and Surgical Reporter. Philadelphia; 1889. vol. 60, p. 498
18	Wood's Medical and Surgical Monographs. New York: W. Wood; 1889. vol. 3, p. 504
19	Meyer Druggist. St. Louis; 1891. p. 358
20	Braithwaite JO. Yearbook of Pharmacy. London: J.&A. Churchill; 1893. p. 220
21	American Veterinary Review. USA; 1893. vol. 17, p. 138
22	The Medical Age. Detroit: E. G. Swift; 1893. vol. 11, p. 322
23	Therapeutic Gazette. G. S. Davis; 1893. vol. 17, p. 704
24	Bulletin of Pharmacy. E. G. Swift; 1893. vol. 7, pp. 181, 279, 286
25	American Journal of Pharmacy. Philadelphia: Philadelphia College of Pharmacy; 1893. vol. 65, p. 225
26	The Medical Bulletin. Philadelphia: F. A. Davis; 1893. vol. 15, p. 310
27	Proceedings of the American Pharmaceutical Association at the Annual Meeting. Scio, Ohio; 1893. vol. 41, p. 450
28	Western Druggist. Chicago; 1893. vol. 15, p. 150
29	Chemist & Druggist. London: Morgan Brothers; 1893. vol. 42, p. 380
30	Yearbook of Pharmacy. London: J.&A. Churchill; 1893. p. 220
31	Pacific Medical Journal. San Francisco; 1893. vol. 36, p. 562
32	The Cincinnati Lancet and Clinic. Cincinnati: Culbertson; 1893. vol. 70, p. 540
33	The Western Medical Reporter. Chicago; 1893. vol. 15, issue 1, p. 278
34	The Journal of the British Homoeopathic Society. London; 1893. vol. 1, p. 101
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38	Annual of the Universal Medical Sciences. Philadelphia: F. A. Davis; 1894. vol. 5, p. A76
39	The Year-book of Treatment. Lea Brothers; 1894. vol. 10, p. 124
40	The Veterinary Magazine. Philadelphia; 1894. vol. 1, p. 223
41	Shoemaker JV. A practical treatise on materia medica and therapeutics: with especial reference to the clinical application of drugs, 3rd ed. Philadelphia: F. A. Davis; 1895. p. 359
42	Boas I. Diseases of the intestines (Trans. S Basch). New York: Appleton; 1901. p. 253
43	Therapeutic Monthly. Philadelphia: Medical Journal Union; 1901. vols. 1–2, p. 88
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No.	Source
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Appendix B

Translation of the article of Koga and Ohtake (1914) from Japanese into English.

Koga Y, Ohtake R. Study report on the constituents of squeezed watermelon.
Journal of the Tokyo Chemical Society [*Tokyo Kagaku Kaishi*] 1914; **35**: 519–28.
古賀彌太郎，大嶽　了．西瓜搾汁の成分研究報告．東京化學會誌 1914; **35**: 519–528.

Study report on the constituents of squeezed watermelon

by Bachelors of Agriculture

Yotaro Koga
Ryo Ohtake

Red-coloured interiors of 23 ripe watermelon (Citrullus vulgaris Schrad) (weight 180 kg) were separated and pressed to get juice from them. After filtering through paper, 83.55 litres of liquid were left. The liquid was concentrated at a low temperature until it had a sherbet-like condition. The general results of its analysis are as follows:

	Original juice	Dried provision	All nitrogens
	100%	100%	100%
water	93.97000		
Solid (substance)	6.03000	100.0000	
all nitrogens	0.05970	0.9900	100.00
protein nitrogen	0.00110	0.0180	1.84
nitrogen which is precipitated by phosphorus wolfram acid	0.01010	0.1675	16.92
other nitrogens	0.04850	0.8045	81.24
nitrogen which can be measured by Formol method	0.02400	0.3980	42.01
reducing sugar (as grape sugar)	3.91000	64.8430	
inverted sugar	4.54000	75.2900	
cane sugar	0.59900	9.9340	
pentose	0.06416	1.0640	
ash content	0.18370	3.0460	
acidity original juice 100 millilitre equivalent to 0.0844g caustic baryta as malic acid	0.06600	1.0940	

Quantitative table of ash content

	original juice (100%)	dried provision (100%)	
ash content	0.1837	3.4660	100.000
K_2O	0.1222	2.0270	66.522
Na_2O	0.0030	0.0497	1.634
CaO	0.0081	0.1340	4.355
MgO	0.0071	0.1180	3.865
Fe_2O_8	0.0003	0.0049	0.164
P_2O_5	0.0108	0.1790	5.879
SO_3 (sulfuric acid)	0.0074	0.1230	4.028
SiO_2 (silicic acid)	0.0032	0.0530	1.742

Experimentation

1. Separation of acids

A spoonful of the mentioned sherbet-like substance was diluted to make a litre of liquid. In the liquid, basic acetic acid lead solution was added until precipitation was yielded. After being separated from the liquid and cleaned with water, the precipitation was distributed thoroughly in the proper amount of water and decomposed through hydrogen sulphide. Lead sulphide precipitation yielded from the decomposition was removed and then free hydrogen sulphide was eradicated at low temperature.

The same operation was repeated again to get colourless free acid liquid and then the liquid was highly concentrated and left in a cool place for some days. As no crystal was yielded from the concentrated liquid, a part of it was diluted with water, added with copper hydroxide, boiled and filtered. The filtered solution then formed big pillar shaped crystals (0.9 g) through concentration. This substance was not carbonized by heat. By removing copper by hydrogen sulphide and adding nitric acid and molybdenum acid liquid to free acid solution, it had the reaction which was peculiar to phosphoric acid. Therefore, it was proved that they were crystal forms of copper phosphate.

After confirming that new crystal form was no longer separated out from the mother liquid from which copper phosphate crystal forms had already been removed, copper was precipitated by hydrogen sulphide and removed. All the acid liquids were then brought together and concentrated. When thick ammonia solution was added to them, white pillar-shaped ammonium phosphate (0.5 g) was formed and filtered. Sometimes the filtered solution was then added with ammonia water. While being stirred, the solution was concentrated to sherbet-like condition. After being added with alcohol and left as it was, it formed pillar shaped crystals (0.8 g) and melted at 178 degrees Celsius (no correction). It was dried at 100 degrees Celsius in a vacuum and analyzed as follows:

0.1565 g substance 0.1839 g carbon dioxide 0.0836 g water
0.1178 g substance 9.5 ml nitrogen (23 degree Celsius, 760 mm)

	carbon	hydrogen	nitrogen
calculated figure ($C_4H_9NO_5$)	31.79	5.96	9.27
experimental figure	32.04	6.13	9.04

Namely the analysis showed the same figure that acid ammonium salt of malic acid had. As the crystals' mother liquid had quite a small amount, more specific experiment was not done.

2. Separation of nitrogen containing compounds

After excessive lead was removed by passing hydrogen sulphide through the filtrate which had been yielded by the mentioned basic lead acetate, the filtrate was concentrated in low pressure and temperature. Then its' hydrogen sulphide was driven out from the filtrate and amount of the filtrate was about 700 ml.

While sodium carbonate was added to the filtrate for neutralization, mercury acetate solution was added to it by slow degree until limit of precipitation formation in it. Then alcohol was added to it to sink all the precipitation in it and the filtrate was filtrated with suction and the precipitation was washed well with alcohol. Then the precipitation was distributed in water and resolved by hydrogen sulphide. For resolving the precipitation of mercury in it, hydrogen sulphide was used a few times until complete decomposition. After that all the filtrate was collected and vaporized. Hydrogen sulphide was driven away from the filtrate, and its' amount was made to be about 500 ml. Sulphuric acid was added to it to have 5% density solution and also phosphorus wolfram acid was added to make precipitation in it.

After being filtrated with suction and washed well, the precipitation was decomposed with baryta by an ordinary method. Excessive baryta was precisely removed with sulphuric from the filtrate. Then the filtrate was concentrated, added with picric acid and cooled down with ice to make a lot of crystals (3.6 g yielded amount). When they were recrystallized from warm water, they had beautiful needle-like shapes at 204 melting point (no correction). They were dried and analyzed as follows.

0.0994 g substance 0.1299 g carbon dioxide 0.0442 g water
0.1813 g substance 37.6 ml nitrogen (14 degree Celsius, 748 mm)
0.1567 g substance 0.0889 g picric acid

	carbon	hydrogen	nitrogen	picric acid
calculated figure ($C_6H_{14}N_4O_2C_6H_3N_3O_7$)	35.73	4.21	24.32	56.82
experimental figure	35.64	4.94	23.91	56.73

Namely, it matched with arginine picrate.

It was found that when the mother liquid of arginine picrate was concentrated in low temperature, a lot of crystals were precipitated. 24 hours later the crystals were collected and washed well with alcohol ether, but this substance did not combine with picric acid and was precipitated in a free state. Therefore, the substance was dissolved further in water and picric acid was removed by shaking the solution with ether.

With boneblack the solution was decoloured and alcohol (in the same container) was added and left as it was. Then, by low degrees, white pillar-shaped large crystals were yielded (yielded amount 0.8 g). In 168 degree Celsius (no correction), it was decomposed with bubbles. In a vacuum (100 degree Celsius) it was dried and analyzed as follows.

I	0.1804 g substance	30.8 ml nitrogen (19 degree Celsius, 760 mm)	
	0.0960 g substance	0.1274 g carbon dioxide	0.0635 g water
II	0.1100 g substance	21.16 ml nitrogen (28 degree Celsius, 752 mm)	
	0.1094 g substance	0.1440 g carbon dioxide	0.0677 g water

		carbon	hydrogen	nitrogen
calculated figure (as $C_4H_{10}N_2O_3$)		35.84	7.46	20.89
experimental figure	I	36.19	7.35	19.55
	II	35.88	7.04	20.89

This substance had the mentioned experimental figure. Its' water solution had neutrality and no property of an organic base. When it burned, no ash content was left. When it was boiled with ninhydrin solution, it had the blue-coloured reaction as an amino acid did. When it had red heat in a narrow tube and a piece of hydrochloric acid soaked-wood was closed near, the substance turned red like pirol. And when the substance was boiled with copper hydroxide, copper salt was yielded. At 232 degree Celsius, the substance turned to red with bubbles and decomposed itself. When it was heated with 10% hydrochloric acid, it separated ammonia. Other properties were not examined further due to material meagre.

To the mentioned substance's mother liquid, sulphuric acid was added to make it become acid. To remove picric acid from the liquid, it was shaken several times with ether. And sulphuric acid was precisely removed by baryta from it. When the liquid was concentrated and boiled with copper hydroxide, it yielded copper salt which hardly melted in water. When the copper was collected and recrystalized from boiling water, large panel-shaped crystals were yielded. They turned to red colour at 257 degree Celsius, producing bubbles. They were dried and analyzed in a vacuum (100 degree Celsius) as follows.

I	0.0764 g substance	13.2 ml nitrogen (19 degree Celsius, 764 mm)	
	0.0889 g substance	0.1125 g carbon dioxide	0.0543 g water
	0.1357 g substance	0.0267 g copper	
II	0.0713 g substance	0.0895 g carbon dioxide	0.0438 g water
	0.1026 g substance	18 ml nitrogen (21 degree Celsius, 751 mm)	

		carbon	hydrogen	nitrogen	copper
calculated figure (as $C_{12}H_{28}N_6O_6Cu$)		34.64	6.34	20.21	15.30
experimental figure	I	34.51	6.78	19.88	15.70
	II	34.23	6.82	19.53	

Then this copper salt was decomposed and made to become an extricated substance. The substance was analyzed as follows.

0.1024 g substance	0.1510 g carbon dioxide	0.0680 g water
0.1209 g substance	24.75 ml nitrogen (25 degree Celsius, 756 mm)	

	carbon	hydrogen	nitrogen
calculated figure (as $C_6H_{15}N_6O_?$)	40.68	8.47	23.72
experimental figure	40.21	7.37	22.56

This substance had a sweet taste and its' properties were very much like the mentioned substance. A part of the nitrogen was able to be titrated by the Formol method. The copper salt was melted in water more difficultly than the mentioned one. Water solution of the extricated substance showed reddish purple colour by copper sulphate solution and caustic soda solution. It precipitated copper salt for a day or two. The precipitation of phosphorus wolfram acid had beautiful pillar-shaped crystals and melted easily by an excess of the phosphorus wolfram acid. It was also found that the precipitation had much relation with sulphuric acid's concentration then.

By the above reason, the authors added baryta to the filtrate of the precipitation of phosphorus wolfram acid to remove the phosphorus wolfram acid and sulphuric acid. After removing precisely excessive baryta by sulphuric acid, they concentrated it and continued to add sulphuric acid to it until it had 7% density. When phosphorus wolfram acid was added gradually to it, it was found that in addition to crystalline precipitation, oily substance was yielded. Therefore quickly these 2 kinds of precipitations were separately filtrated with suction and the crystalline precipitation was decomposed by baryta by an ordinary method. After the baryta in it was removed by sulphuric acid, the solution was concentrated. Totally the same crystals as the mentioned substance was yielded (total yielded amount 0.7 g).

As the oily precipitation became solidified when it was left for some time, it was crystallized after being separated from filter paper, decomposed by baryta, concentrated and added with alcohol. White needle-shaped crystals (amount 0.5 g) were yielded. They were decomposed at 180 degree Celsius while bubbles were produced. They were the copper salt which had salty taste and melted in water easier than the copper salt of the mentioned substance. It turned red with bubbles at 248 degree Celsius. The copper salt was dried and analyzed in a vacuum (100 degree Celsius) as follows.

0.1510 g substance	0.1896 g carbon dioxide	0.0755 g water
0.1083 g substance	18.3 ml nitrogen (20 degree Celsius, 769 mm)	

	carbon	hydrogen	nitrogen
calculated figure (as $C_{12}H_{28}N_6O_6Cu$)	34.64	6.34	20.21
experimental figure	34.24	5.55	19.44

And the analysis of the extricated substance showed as follows.

0.1420 g substance	27.3 ml nitrogen (17 degree Celsius, 766 mm)
0.1056 g substance	20.7 ml nitrogen (20 degree Celsius, 766 mm)

	nitrogen
calculated figure (as $C_6H_{15}N_3O_?$)	23.72
experimental figure	22.42, 22.50

Namely the analysis showed that it matched with the mentioned substance. But as the other properties did not match, it could be determined that both substances were isomers.

As the properties of the mentioned 3 substances were very much like a polypeptide, the authors planned to hydrolyze and form ester. But the materials for it were trifling and satisfied results could not be achieved. The authors hope that in the future, further study on it will be done. Phosphorus wolfram acid and sulphuric acid were removed from the filtrate of the phosphorus wolfram acid precipitation by baryta and excessive baryta was removed precisely by sulphuric acid from the filtrate. The filtrate was concentrated and a small amount of sherbet-like substance was produced from it. Then water was added to it for having alkalinity in it. Drops of dimethyl sulphate were added for methylation. Then when picric acid was added, glycol betaine picric acid salt (0.05 g) was gotten at 180 degree Celsius melting point. Its' yielded amount was trifling and analysis on it was not done.

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