ON THE FORMATION OF AMINO ACIDS AND PROTEINS IN *TORULA UTILIS* ON NITRATE NUTRITION

by

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In this laboratory it was shown by ROINE[1] that low-nitrogen *Torula utilis* suspended in aerated ammonium sulphate solutions without sugar forms aminodicarboxylic acids, in particular glutamic acid, their amides and alanine from ammonium ions taken up by it. These nitrogen compounds constitute almost the entire soluble N-fraction. In low-nitrogen yeast the fraction of soluble nitrogen vigorously increased during the first 15 minutes when the protein synthesis was very weak. After this the protein synthesis considerably accelerated but still after two hours the soluble fraction contained about twice as much of the nitrogen taken up as the protein. These results speak in favour of the concept that the aminodicarboxylic acids arise as primary amino acids.

VIRTANEN AND CSÁKY[2] noted that low-nitrogen *Torula* suspended in nitrate solution is enriched with the same amino acids as in ammonium salt solution. Qualitatively the only noted difference between the nitrogen compounds formed is the fact that nitrate produces in yeast cells oxime nitrogen which is not produced by ammonium salts.

In quantitative respect the difference between various nitrogen fractions in low-nitrogen *Torula* is great depending on the nature of nitrogen feeding. For instance, the soluble N-fraction increases in nitrate yeast proportionally much less than in ammonium yeast, while the protein synthesis in ratio to the uptake of nitrogen is much more intensive in nitrate yeast than in ammonium yeast. This observation will be dealt with in this paper.

Torula utilis yeast was used in the experiments. The strain was the same as used in the previous experiments (ROINE[1], VIRTANEN AND CSÁKY[2]). The yeast was cultivated in the laboratory in wort agar tube and stored in an ice box to avoid frequent inoculation.

CULTIVATION OF INOCULATION YEAST

The inoculation yeast required for the cultivation of the actual yeast mass was grown under sterile conditions in four to eight 500 ml boiling flasks, each containing 50 ml nutrient solution of the following composition (solution A):

50 g cane sugar, 3 g $(NH_4)_2HPO_4$, 4 g $(NH_4)_2SO_4$, 1.5 g K_2SO_4, 1 g $MgSO_4.7H_2O$, 0.5 g $CaCl_2$, 1 l tap water.

The p_H of the solution was about 6–6.5. There was a heavy precipitate which, however, disappeared with the advance of the growth partly due to the lowering of the p_H, partly because the yeast consumed nutrient salts.

The flasks were inoculated with a loopful of yeast from the agar tube. The cultivation occurred in a shaking apparatus in 30° C water thermostat. Period of growth 3–4 days.

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CULTIVATION OF THE MOTHER YEAST

This phase as well as the subsequent ones was carried out in unsterile conditions. Into a 1.5 l Kluyver flask were placed 800–1000 ml of the above solution (solution A) and the total volume of the inoculation yeast (see above). A powerful stream of air was passed through the flask by means of a compressor. The air was purified by a light filter of cotton wool. The flask was kept throughout the course of cultivation in 30° C water thermostat. The p_H of the culture solution was kept at 4.5–5.0 by adding 1 *N* NaOH when needed. The p_H was controlled by Lyphan paper. The growth of the yeast mass was followed by taking at certain intervals a 10 ml sample to a centrifuge tube. After 10 min centrifugation (2500 rpm) the yeast was weighed. The cultivation took 10–12 hours. After this the whole yeast mass was separated by centrifugation, washed with tap water, weighed in a tared centrifuge tube and kept over night in an ice box.

CULTIVATION OF LOW-NITROGEN YEAST

This was also carried out in a Kluyver flask into which were measured 1000 ml of the following nutrient solution (solution B):

50 g cane sugar, 3 g KH_2PO_4, 1 g $MgSO_4.7H_2O$, 0.5 g $CaCl_2$, 1 l tap water.

The p_H was about 5, the solution only slightly turbid.

The nitrogen content of the mother yeast obtained in the above manner was reduced by suspending it in the solution B and by aerating it in 30° C water thermostat for 6–7 hours. The p_H was regulated as above. The yeast mass increased during this procedure with 1/3–1/2 of the initial fresh weight whereby its N-content simultaneously decreased. Low-nitrogen yeast was separated by centrifugation, washed with tap water and weighed. The mass was stored over night in a centrifuge tube in an ice box. With a longer storage the yeast was suspended in mineral salt solution, free from nitrogen nutrition and carbohydrates of the following composition (solution C):

3 g KH_2PO_4, 1 g $MgSO_4.7H_2O$, 0.5 g $CaCl_2$, 1 l tap water.

The p_H was about 5, the solution only slightly turbid.

PERFORMANCE OF THE ACTUAL EXPERIMENT WITH LOW-NITROGEN YEAST

Into a Kluyver flask were placed 400–450 ml of the above solution C and 200–250 ml of a heavy suspension of low-nitrogen yeast (30–70 g fresh yeast). The flask was aerated as above at 30° C.

After 10 min a 100 ml sample was taken for analysis (0 sample). After 15 min to the suspension was added 100 ml of a solution which contained either 10 g $(NH_4)_2SO_4$ or 15.3 g KNO_3, *i.e.*, in either case 2.120 g N. This moment was taken for the start of the experiment. At certain intervals 100 ml samples were taken from the suspension for analysis. All samples were taken into tared centrifuge tubes, the centrifugate was washed with 100 ml of tap water, recentrifuged, and weighed.

When ammonium sulphate served as N-source the p_H of the solution was inclined to fall, and therefore 1 *N* NaOH was added to the solution to maintain its p_H at 4.5–5.0. When potassium nitrate formed the N-source the p_H had a tendency to rise. This was prevented by adding H_2SO_4.

ANALYSES

The nitrogen compounds of the yeast were separated into *soluble* and *insoluble fractions* by extracting the soluble substances at a low temperature with 8% trichloracetic acid (ROINE[1]). The centrifuged, washed and weighed sample of yeast was rinsed with 8% trichloracetic acid into a measuring cylinder and made up to a definite volume so that the total volume corresponded to 3–4 times the fresh weight of yeast. After shaking the suspension was let to stand over night in an ice box.

Total nitrogen. After standing over night an aliquot (2 ml) was taken from the trichloracetic acid suspension for determination of total nitrogen according to KJELDAHL using 5–6 hours combustion and oxidation with hydrogen peroxide.

Soluble nitrogen. The remaining trichloracetic acid suspension was centrifuged and filtered cell-free through a 3 G 4 Jena glass filter. Nitrogen was determined from an aliquot (2 ml) according to KJELDAHL using 1.5 hours combustion and oxidation with hydrogen peroxide.

Protein nitrogen (nitrogen of the insoluble fraction) was calculated as a difference between total nitrogen and soluble nitrogen.

Results which represent the amounts of total, soluble, and protein nitrogen in low-nitrogen yeast fed with ammonia and nitrate nitrogen are given in Tables I and II.

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TABLE I

DIFFERENT NITROGEN FRACTIONS OF *Torula* YEASTS FED WITH NH_4-N AND NO_3-N (CALCULATED PER 100 g FRESH YEAST). STRONG AERATION.

Time min	Total nitrogen		Soluble nitrogen		Protein nitrogen	
	mg	increase mg	mg	increase mg	mg	increase mg
			NH_4-experiment			
0	850		113		737	
45	1099	249	301	188	798	61
75	1194	344	366	253	828	91
180	1285	435	361	248	924	187
			NO_3-experiment			
0	816		98		718	
15	850	34	115	17	735	17
75	977	161	159	61	818	100
120	1010	194	154	56	856	136

TABLE II

DIFFERENT NITROGEN FRACTIONS OF *Torula* YEASTS FED WITH NH_4-N AND NO_3-N (CALCULATED PER 100 g FRESH YEAST). WEAKER AERATION THAN IN THE EXPERIMENTS IN TABLE I.

Time min	Total nitrogen		Soluble nitrogen		Protein nitrogen	
	mg	increase mg	mg	increase mg	mg	increase mg
			NH_4-experiment			
0	873		94		779	
45	1121	248	300	206	821	42
75	1167	294	306	212	861	82
195	1279	406	320	226	959	180
			NO_3-experiment			
0	879		95		784	
75	949	70	137	42	812	28
195	1053	174	149	54	904	120

The results recorded in Table I are illustrated by graphs in Fig. 1. The figure shows clearly the essential difference between the increase of soluble nitrogen and protein nitrogen in low-nitrogen yeast with ammonia or nitrate feeding. During 75 minutes ammonia nitrogen produced nearly three times as much soluble nitrogen as protein nitrogen (91 mg or 28.8% protein N of the total uptake 344 mg N) while nitrate nitrogen again produced nearly twice as much protein nitrogen as soluble nitrogen (100 mg or 62.1% protein N of the total uptake 161 mg N). The protein amount formed was thus practically equal in ammonia and in nitrate experiments. The results are quantitatively variable in different experiments, often even considerably, but in all of them the same difference of principle is noticeable.

In one nitrate experiment also amino, amido, ammonia, aspartic acid, glutamic acid, asparagine, glutamine, and alanine nitrogens were determined in addition to soluble and total nitrogen.

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Amino N was determined by the Cu-method[3]. It was ascertained with several determinations, that amido nitrogen of glutamine and asparagine does not react in this method.

Amido N was determined according to PUCHER *et al.*[4] as well as *ammonia N*.

Amido N of glutamine was determined according to SCHWAB[5].

Amido N of asparagine was calculated by subtracting the amido N of glutamine from total amido N.

Alanine N was determined according to the principle of VIRTANEN *et al.*[6] by the ninhydrin oxidation through determining acetaldehyde in the bisulphite solution according to ROINE AND RAUTANEN[7].

Aminodicarboxylic acid N was determined according to FOREMAN. The precipitate was dissolved in 1 *N* acetic acid and the possible nucleotides precipitated with uranyl acetate (ROINE[1]). The nitrogen present in the solution after this was taken for aminodicarboxylic acid nitrogen.

Aspartic acid was determined according to ARHIMO[8] from the solution above. *Glutamic acid* was calculated by subtracting aspartic acid N from dicarboxylic acid N.

The experiment was performed as follows: 128 g of low-nitrogen yeast were suspended in 1400 ml mineral salt solution (solution C) free from carbohydrate and nitrogen sources, 25 g KNO_3 were added and at certain intervals 250 ml samples were taken from the solution for analysis. Table III gives the results.

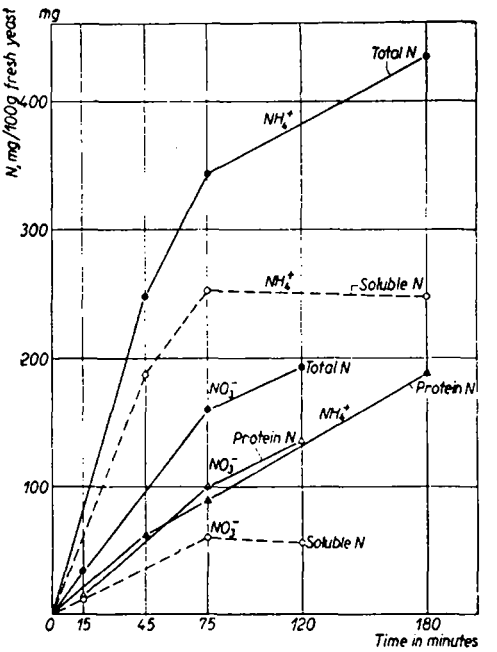

Fig. 1. Low-nitrogen *Torula* suspended in ammonia and nitrate solutions. Different N-fractions during 180 minutes (cf. Table I)

TABLE III

DIFFERENT NITROGEN FRACTIONS IN LOW-NITROGEN YEAST FED WITH NITRATE NITROGEN (CALCULATED PER 100 g FRESH YEAST)

Time min	Total N mg	Soluble N mg	Amino N					Amido N			Ammonia N mg
			total mg	dicarboxylic acid, mg	aspartic acid, mg	glutamic acid, mg	alanine mg	total mg	asparagine mg	glutamine mg	
0	714	62.4	39.7	18.92	5.82	13.10	2.53	2.35	1.25	1.10	0.23
15	925	77.3	49.7	25.2	6.23	18.97	5.63	8.6	3.52	5.08	0.58
30	947	92.8	58.0	26.1	6.04	20.06	8.79	12.9	4.58	8.32	1.01
60	1055	123.5	80.0	44.2	6.21	38.00	15.38	7.54	2.16	5.38	1.80
240	1112	118.2	82.8	58.3	6.05	52.25	8.94	5.08	3.22	1.86	0.41

The results recorded in Table III and illustrated graphically in Figs. 2 and 3 show that when low-nitrogen *Torula* takes up nitrate nitrogen the entire increase of soluble nitrogen fraction during the first 15 minutes is practically composed of aminodicarboxylic acids, chiefly of glutamic acid, their amides, alanine, and small amount of ammonia. Besides, some oxime-N is formed which, however, is insignificant in quantitative sense[2]. The small amount of ammonia in the soluble fraction may partly originate from a slight decomposition of glutamine. During the next 45 minutes also other nitrogen compounds are formed to some extent, for the nitrogen of the said amino acids, amides and ammonia then constitute only 77% of the soluble nitrogen. These results are in

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good agreement with the findings by ROINE on the uptake of ammonia nitrogen by low-nitrogen *Torula*. The very low amount of soluble nitrogen compared with protein nitrogen in nitrate yeast is illustrated in Fig. 2.

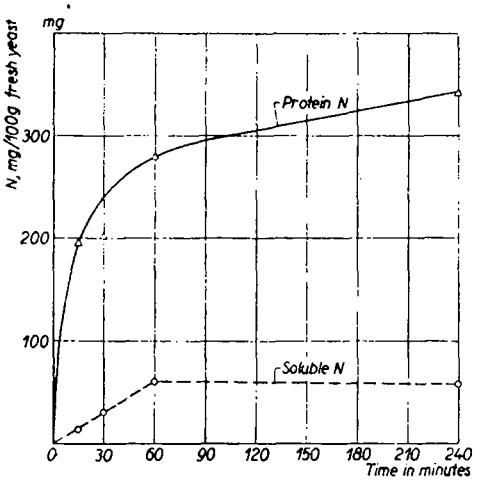

Fig. 2. Low-nitrogen *Torula* suspended in nitrate solution. Protein-N and soluble N during 240 min (cf. Table III)

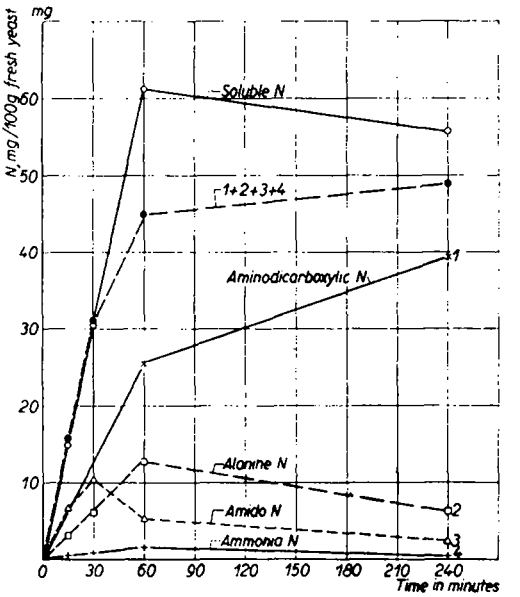

Fig. 3. Composition of soluble N-fraction in *Torula* suspended in nitrate solution (cf. Table III and Fig. 2)

DISCUSSION

The observations made on low-nitrogen *Torula* indicate that yeast fed either with nitrate or ammonia nitrogen produced the same amino acids and amides which in both cases constitute, during the short experimental time, practically the entire increase in the soluble nitrogen fraction. The increase of this fraction in yeast suspended in ammonium salt solution is much higher than in nitrate solution. Nevertheless, the protein synthesis may reach the same level in both cases. This indicates that 1) the synthesis of aminodicarboxylic acids and alanine takes place more rapidly from ammonia nitrogen than from nitrate nitrogen and that 2) the ample formation of aminodicarboxylic acids and alanine does not as such guarantee an intensive protein synthesis; this depends also on other factors, for instance, possibly on the velocity of the formation of the other amino acids. This with the proviso that when protein is formed all its structure components are present. Whether this holds good, is, however, still unproved. If it does hold good, the results reveal that the synthesis of aminodicarboxylic acids in low-nitrogen yeast takes place with superoptimal velocity in ammonium salt solution, exceeding noticeably the velocity of protein synthesis. In the nitrate solution, again, the synthesis of aminodicarboxylic acids would be approximately optimal corresponding in a high degree to the velocity of protein synthesis. If it is presumed that the synthesis of amino acids occurs according to scheme 1 the accumulation of aminodicarboxylic acids, their amides, and alanine during the short experimental time would be due to the slowness of the synthesis of other amino acids which in turn would retard the protein synthesis.

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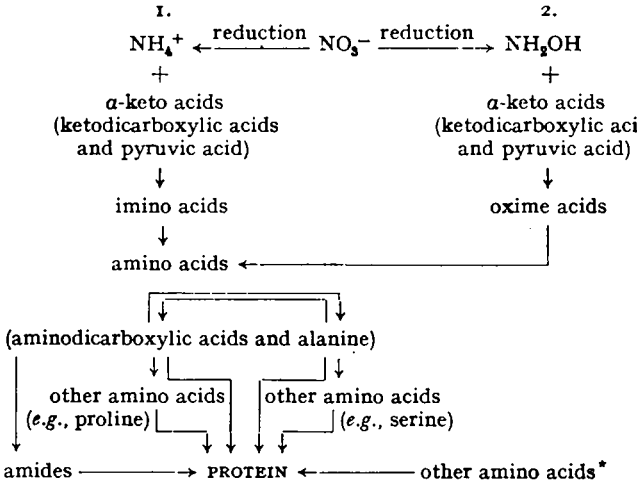
In the presented scheme besides aminodicarboxylic acids, alanine, too, has been placed to the primary position. It may entirely arise from aminodicarboxylic acids through transamination (VIRTANEN *et al.*[9], ROINE[1]) but the possibility for its primary formation from pyruvic acid and ammonia is very great since transamination may not be sufficiently rapid to explain alone its formation. Which of the aminodicarboxylic acids, aspartic or glutamic acid, is the primary one has not been dealt with in the scheme.



* Formed from a source other than aminodicarboxylic acids and alanine.

In the above the complete reduction of nitrate to ammonia has been taken for granted. The rapid formation of oxime from nitrate nitrogen in *Torula* indicates, however, that the hydroxylamine formed in the reduction of nitrate also reacts with the > CO group. The maximum amount of oxime N is found according to VIRTANEN AND CSÁKY[2] in *Torula* yeast already 10 min after feeding with nitrate nitrogen. The synthesis of amino acids may, accordingly, take place at least partly over oximes (scheme 2). As far as the synthesis of amino acids proceeds this way to a large extent its different velocity depending on the nitrogen source (ammonia or nitrate) would be easily explicable. The alike velocity of protein synthesis in both cases would even then remain obscure.

SUMMARY

Low-nitrogen *Torula utilis* takes rapidly up nitrogen in aerated nitrate solution. Both soluble and insoluble nitrogen ("protein nitrogen") are increased in the cells. Soluble nitrogen was extracted from the cells with 8% trichloracetic acid. The increase of the soluble N-fraction was much smaller than in the corresponding experiment in which *Torula* was fed with ammonia nitrogen instead of nitrate nitrogen. On the other hand, the accumulation of protein in the cells was in many experiments equal in either case. This difference between the influence of nitrate and ammonia nitrogen has been dealt with at a greater length in the Discussion.

In the nitrate experiment the increase of the soluble N-fraction during the first 15 min contained aminodicarboxylic acids, in the first place glutamic acid, their amides, alanine, and some ammonia as well as a very small quantity of oxime nitrogen. These nitrogen compounds correspond roughly to the total soluble N. With a prolonged experimental time the total soluble N was considerably higher than the sum of the nitrogen in the said amino acids and amides. ROINE arrived previously in similar results in regard to the amino acids and amides in soluble N-fraction when examining the uptake of ammonia nitrogen by *Torula* yeast. To alike result led the investigations by RAUTANEN[10] on green plants.

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RÉSUMÉ

Torula utilis pauvre en azote dans une solution de nitrate aérée accumule rapidement de l'azote. La quantité d'azote soluble ainsi que d'azote insoluble (azote protéique) augmente dans les cellules. L'azote soluble a été extrait des cellules avec l'acide trichloracétique à 8%. L'augmentation de la fraction en azote soluble a été beaucoup plus faible que dans une expérience correspondante où *Torula* a été cultivée dans un milieu contenant de l'azote ammoniacal au lieu d'azote nitré. Mais pourtant dans plusieurs expériences l'accumulation de la protéine dans les cellules a été pareille dans les deux cas. Cette différence entre l'influence de l'azote nitré et de l'azote ammoniacal a été traitée plus en détail dans la Discussion.

Dans l'expérience de nitrate la fraction augmentée en azote soluble pendant les 15 premières minutes contenait des acides aminodicarboxyliques, principalement de l'acide glutamique, des amides correspondantes, de l'alanine et un peu d'ammonium ainsi qu'une quantité minime d'azote d'oxime. Ces composés azotés correspondent en gros à l'azote total. Dans le cas où la durée de l'expérience a été prolongée, la quantité d'azote soluble totale a été sensiblement plus élevée que la quantité totale d'azote contenue dans tous ces acides aminés, amides et alanine. En examinant la rétention de l'azote ammoniacal par la levure *Torula* ROINE est arrivé aux mêmes résultats en ce qui concerne les acides aminés et les amides dans la fraction d'azote soluble. Les expériences effectuées avec des plantes vertes par RAUTANEN ont donné les mêmes résultats[10].

ZUSAMMENFASSUNG

Die stickstoffarme *Torula utilis* nimmt in durchgelüfteter Nitratlösung schnell Stickstoff auf. Sowohl löslicher als unlöslicher Stickstoff ("Proteinstickstoff") nimmt in den Zellen zu. Die Zunahme der löslichen N-Fraktion, die aus den Zellen mit 8%-iger Trichloressigsäure extrahiert wurde, war viel kleiner als in dem entsprechenden Versuch, in welchem *Torula* in Ammoniumsulfatlösung suspendiert war. Dagegen war die Akkumulation von Protein in den Zellen in vielen Versuchen gleich gross in beiden Fällen. Dieser Unterschied zwischen dem Einfluss des Nitrat- und Ammoniakstickstoffs ist in der Diskussion ausführlicher behandelt worden.

In dem Experiment mit Nitrat enthielt die Zunahme der löslichen N-Fraktion im Lauf der ersten 15 Minuten Aminodicarbonsäuren, an erster Stelle Glutaminsäure, deren Amide, Alanin und auch etwas Ammoniak sowohl wie eine sehr kleine Menge von Oximstickstoff. Diese Stickstoffverbindungen entsprechen im grossen und ganzen dem totalen löslichen Stickstoff. In einer verlängerten Versuchszeit war der lösliche Stickstoff bedeutend höher als die Summe des Stickstoffs in den genannten Aminosäuren, Amiden und Alanin. ROINE ist früher zu ähnlichen Resultaten mit Hinsicht auf die Aminosäuren und Amide in löslicher N-Fraktion gekommen, während er die Aufnahme von Ammoniumstickstoff bei der *Torula*-Hefe untersuchte. Zu diesem Schluss haben desgleichen die Untersuchungen von RAUTANEN[10] mit grünen Pflanzen geführt.

REFERENCES

1 P. ROINE, *Ann. Acad. Sci. Fennicae, Ser. A. II. Chem.*, No. 26 (1947).
2 A. I. VIRTANEN AND T. Z. CSÁKY, *Nature*, 144 (1948) 597.
3 C. G. POPE AND M. F. STEVENS, *Biochem. J.*, 33 (1939) 1070.
4 G. W. PUCHER, H. B. VICKERY, AND C. S. LEAVENWORTH, *Ind. Eng. Chem., Anal. Ed.*, 7 (1935) 152.
5 G. SCHWAB, *Planta*, 25 (1936) 579.
6 A. I. VIRTANEN AND T. LAINE, *Nature*, 142 (1938) 754; *Skand. Arch. Physiol.*, 80 (1938) 392; A. I. VIRTANEN, T. LAINE, AND T. TOIVONEN, *Z. physiol. Chem.*, 266 (1940) 193; A. I. VIRTANEN AND N. RAUTANEN, *Biochem. J.*, 41 (1947) 101.
7 P. ROINE AND N. RAUTANEN, *Acta. Chem. Scand.*, 1 (1947) 854.
8 A. A. ARHIMO, *Suomen Kemistilehti*, B, 12 (1939) 6.
9 A. I. VIRTANEN AND T. LAINE, *Nature*, 141 (1938) 748.
10 N. RAUTANEN, *Acta Chem. Scand.*, 2 (1948) 127.

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