On the Assimilation of Nitrate Nitrogen by *Hansenula anomala*

By

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Hansenula anomala is a kind of film forming yeast which grows tolerably well in synthetic culure solution without addition of any growth substance. It has been reported by TANIDA (1935) and TERUI (1936) that nitrite is produced by this yeast in consequence of the nitrate reduction. In the previous paper the writers[1] communicated that molybdenum even in such a minute concentration as 2×10^{-3} M caused an accumulation of nitrite in the culture solution of this yeast, nitrate being applied as nitrogen source, while it was not the case without addition of molybdenum. This means that molybdenum has to do with the nitrate nitrogen metabolism. Recently it has been observed by some authors that molybdenum plays an important role for the assimilation of nitrate nitrogen by some kinds of moulds and higher plants, and the same thing is applicable to the fixation of free nitrogen by the nitrogen fixing bacteria.

In the present study it was aimed to investigate the early stage of the nitrogen assimilation by *Hansenula anomala*, with special reference to the action of molybdenum thereon. Having been manifested to have the ability of assimilation of free nitrogen[2], this yeast seems all the more to be a fitted material for our research purpose, because the study might so far develop in future as to see if any process exists common between the nitrogen assimilation from nitrate and that from free nitrogen.

The problems treated in the present study have intimate connection with the respiratory process, of which results will be communicated on other occasion.

Experiment 1

The present experiment was carried out in order to see what action

1) SAKAMURA and MAEDA (1947).
2) SCHANDERL (1940).

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will be done by molybdenum in a minute concentration on the culture of this yeast. For the purpose of culture of a long duration a synthetic solution of the following composition was used : KNO_3 6,31 g. KH_2PO_4 2,5 g. $MgSO_4 7H_2O$ 1,25 g. Glucose 45(M/4). H_2O 1000 cc.

In order to remove heavy metal impurity in the solution, the adsorption procedure was carried out, treating the solution with 0,5% Ca-phosphate, according to the method described in detail in the paper of SAKAMURA (1936). This method is not thorough enough to remove molybdenum. However, it was confirmed that the chemicals used in the present study did not contain molybdenum in trace as impurity. After the adsorption procedure, Fe, Zn, Cu, and Mn in the form of sulphates each in concentration 2×10^{-5} M, were added to the solution. When the action of molybdenum was examined, it was used in the form of ammonium molybdate in concentration 2×10^{-5} M. The cultures with and without molybdenum were indicated as + Mo and − Mo respectively. As culture vessels 100 cc ERLENMEYER flasks of Terex glass were used. 25 cc of the culture solution were put in each flask and sterilized for one hour in a steam sterilizer. The pH-value of the solution after the sterilization was 5,4.

The yeast used for the inoculation was taken from the preceding culture which had been conducted in one of the above mentioned solutions. The medium of two days' culture was thoroughly shaken and came to the inoculation; always to the new culture of the same sign. The culture vessels were kept in an incubator of 24°C.

After a certain duration the culture solution was centrifuged and the yeast cells were separated from the liquid. When the cells were used for further experiments, they were washed three times with redistilled water, and then cell suspension was made in 10 cc redistilled water. The relative growth rate was determined satisfactorily even by the comparison of the turbidity of the cell suspension. More exact results, however, could be obtained by the measurement of dry weight of cells.

In an incidental experiment in which the manometer measurement was conducted with the WARBURG manometer filled with hydrogen gas, it was confirmed that *Hansenula anomala* does not liberate free nitrogen from nitrate, i.e., this yeast has no power of denitrification.

In order to obtain a cell suspension in the required concentration, dry weight of yeast cells taken from a certain portion of the first prepared suspension was determined, and it was diluted according to the volume. By this method the suspension of the + Mo and − Mo material in the same density could be prepared. When growth and

chemical changes occuring in the culture solution were followed during the experiment, a series of cultures of the some kind was made in parallel, and these changes were regarded as similar in each vessel. Nitrite was determined with the GRIESS-ROMIJIN reagent and its estimated value was expressed roughly in numbers of the mark +. − 0. + 10^{-6} M. ++ 5×10^{-6} M. +++ 10^{-5} M. ++++ 5×10^{-5} M. +++++ 10^{-4} M. (cf. Table 1).

TABLE 1

Result	Mo	Days						
		2	3	4	5	6	8	10
pH	+	4,0	4,0	4,0	4,0	4,3	6,3	8,8
	−	4,0	3,8	3,9	4,0	4,2	6,0	8,2
NO'_2	+	++	++	++	++++	++++	++++	+++++
	−	Trace	−	Trace	+	++	++	++++
Glucose	+	++	++	+	Trace	−	−	−
	−	++	+	·	Trace	−	−	−
Growth	+				+++	+++	+++	+++
	−				++	++	++	++

After three days pH-value was always less in the −Mo culture than in the + Mo. In both the cultures pH-value decreased in accordance with the daily growth, but it increased again rapidly in the period of disappearance of glucose. Nitrite accumulated remarkably in the + Mo culture, while it was hardly detected in the − Mo. This difference has been found in our previous study too. The accumulation of nitrite tended to increase together with ascending pH-value. The consumption of glucose was much more in the −Mo culture than in the + Mo. No difference of growth was visible in the first three days, but after that period a better growth took place in the + Mo culture.

Experiment 2

Except the following points, the experimental procedure was almost equal to the preceding experiment. For the inoculation of each culture one drop of suspension of the bottom yeast was used, which favored to remark the characteristic difference between the + Mo and −Mo culture.

Glucose 1125 mg. KNO_3 $6{,}25 \times 10^{-2}$ M. Nitrate was estimated by the phenol disulphonic acid method. (cf. Table 2).

TABLE 2

Days	Mo	pH	Dry weight of cells(mg)	Glucose (mg)		Econ. coeff.	$[NO'_3]$ $(10^{-3}$ M)			$[NO'_2]$ $(10^{-5}$M)
				Rest	Consumed		Rest	Consumed	Consumed per mg of dry cells	
3	+	4,3	60	362,5	762,5	0,079	5,04	1,21	0,0201	1
	−	4,4	52	310,0	815,0	0,064	5,04	1,21	0,0232	0,1
5	+	6,8	118	10,0	1115,0	0,106	0,86	5,39	0,0457	5,2
	−	4,1	62	0,75	1124,3	0,055	0,67	5,58	0,0900	0,1
7	+	7,8	124	−	1125,0	0,110	0,16	6,09	0,0491	5,2
	−	5,4	89	−	1125,0	0,079	0,30	5,95	0,0669	0,1

It was a noticeable fact that the growth and the consumption of glucose in the + Mo culture distinctly differed from those in the −Mo. The economical coefficient, which shows the relation between the growth and the sugar consumption, was remarkably less in the - Mo culture than in the + Mo. In connection with the absolute value of the consumed nitrate there was no marked difference between the culture with molybdenum and that without it. As the better growth, however, was recognized in the former case, the amount of nitrate consumed per mg of the dried cells was much higher in the latter culture. For the growth of the yeast in the −Mo culture it is naturally an unfavorable condition that glucose unavoidably wasted for the sake of unlimited reduction of nitrate, as visible in the following experiments.

Experiment 3

In the case of *Aspergillus*, molybdenum acts effectively on the utilization of nitrate nitrogen by the fungi, but the same thing cannot be applicable to the assimilation of ammonium nitrogen[1]. For the purpose to see if there will be such a difference between nitrate and ammonium salt in *Hansenula anomala* too, the present experiment was carried out. Some points concerning composition of the culture solution and experimental procedure, different from those in Experiment 1 were as follows: KNO_3 M/20. $(NH_4)_2CO_3$ M/40. One cc of cell suspension of a three days'

1) STEINBERG (1937), SAKAMURA (1947).

culture was diluted with 10 cc sterilized redistilled water and with one cc of the latter suspension the culture solution was inoculated. Duration of culture was three days. (cf. Table 3).

TABLE 3

N-source	Mo	pH	NO'_2	Dry weight of cells (mg)	Glucose
KNO_3	+	4,4	+	18	+
	−	4,2	−	15	+
$(NH_4)_2CO_3$	+	2,2	−	33	+
	−	2,2	−	40	+

Even if an ammonium salt of such a weak acid as carbonic acid was used, the acidity increased rapidly, and the growth was retarded. So far as such increase of acidity, which caused an accessory unfavourable influence of liberated acid, did not happen, ammonium nitrogen was more beneficial than nitrate nitrogen for the growth of the yeast. The role of molybdenum was noticeable in the case of the nitrate culture, but not in that of the ammonium culture. This point agrees with the result obtained from the experiment with *Aspergillus*.

Experiment 4

It has been several times observed that bacteria of different kinds produce nitrite and ammonia as a result of the reduction of nitrate in anaerobic culture[1]. According to STICKLAND (1931), free oxygen is ten times as active as nitrate for the oxidation of the reduced form of an intermediate acceptor caused by the toluene-treated cells of *Bac. coli*. That is, free oxygen is reduced ten times as rapidly as nitrate. Therefore, the reduction of nitrate should be inhibited by the presence of free oxygen in that case. The same thing has been ascertained by other authors[2]. It is out of question, and theoretically rather reasonable that the absence of free oxygen is favorable to the nitrate reduction. In the case of extremely aerobic bacteria such as *Bac. subtilis*, *Bac. mesentericus* and *Bac. megatherium* no nitrate reduction is visible[3]. However, anaerobiosis would not always be a necessary condition for this reaction[4], and moreover the opposite relation would be possible.

1) LAURENT, (1890), AUBEL et SALABARTAN (1925), QUASTEL, STEPHENSON and WHETHAM (1925), STICKLAND (1931), QUASTEL (1932).

2) STEPHENSON and STICKLAND (1931), YAMAGATA, (1937−1938), WOODS (1938), LEWIS and HINSHELWOOD (1948), KÉPES et LEMOIGNE (1948).

3) LAURENT (1890).

4) BLOM (1931), LEMOIGNE *et al.* (1947).

Hansenula anomala aerobically grows vigorously in ordinary case, and it was pointed out by TANIDA (1935) that the aerobic condition is rather suitable for the nitrate reduction performed by this yeast. According to him, nitrite was found in the aerobic culture, but not in the anaerobic. From this fact he concluded that the nitrate reduction in the case of *Hansenula anomala* is a biochemical reaction which occurs accompanied by the aerobic respiration. TERUI (1936) who also has dealt with the research on the nitrate reduction by the same yeast, did not consider the aerobic respiration as a bearing factor, but stressed the action of a certain dehydrogenase for the explanation of mechanism of the nitrate reduction.

In the present experiment it was aimed to see first in what direction the process of the nitrate reduction will be influenced by free oxygen and to what extent it occurs. For this purpose it would not have been reasonable to adopt only the measurement of nitrite produced from nitrate as the criterion of the nitrate reduction, although this method has been used by TANIDA and TERUI. Nitrite produced from nitrate cannot remain unchanged, but will disappear by the further reduction. Therefore, we measured the amount of disappearing nitrate as the criterion of the reduction.

The following solution was prepared for the experiment: 0,5 cc yeast (three days' material) suspension. 0,5 cc M/6 glucose (M/30 after addition). 0,5 cc M/4 phosphate buffer. 0,5 cc M/20 $NaNO_3$ (M/100 after addition). 0,5 redistilled water. 2.5 cc in total. pH 5,4. Dry weight of yeast: 1,3 mg (+Mo and −Mo).

Cell suspension plus phosphate buffer solution was put in the main part of a THUNBERG-KEILIN tube, and glucose solution plus $NaNO_3$ solution in its retort. Some of the tubes thus prepared were evacuated with a vacuum pump to obtain anaerobic condition, and others remained aerobic without such a treatment. In the beginning, the solutions in both parts in each tube were thoroughly mixed together at the same time, and the tubes were kept in an incubator of 24°C. The duration of the experiment was 24 hours. (cf. Table 4).

TABLE 4

Mo	Condition	$[NO'_3](10^{-3}$ M)	
		Rest	Consumed
+	Aerobic	9,40	0,60
	Anaerobic	9,90	0,10
−	Aerobic	2,00	8,00
	Anaerobic	7,00	3,00

Without reference to the presence of molybdenum the disappearance of nitrate, namely its reduction, proceeded more rapidly in the aerobic culture than in the anaerobic. The favorable condition

of the presence of free oxygen for the nitrate reduction could not be doubted any more in this case, although the reduction could occur even anaerobically. With regard to the action of molybdenum it was found that the reduction happened more slowly in case of its addition than without it. The difference of velocity of the reduction between the aerobic and anaerobic condition was remarkable, especially in the -Mo culture. From this result it will be seen that as to the participation of oxygen the nitrate reduction in *Hansenula anomala* is distinguished from that of many bacteria. In what part of a range of reactions free oxygen participates will be analysed in the following experiments.

Experiment 5

The relation between the nitrate reduction and pH-value was examined. The method and condition were almost equal to Experiment 4, except that ordinary test tubes were used instead of THUNBERG-KEILIN tubes. Yeast was three days' material. Dry weight of yeast cells: 5 mg. (cf. Table 5).

TABLE 5

pH	$[NO'_3](10^{-3}$ M)		$[NO'_2]$ $(10^{-5}$M)
	Rest	Consumed	
7,2	5,2	4,8	3,0
5,4	5,1	4,9	0,6
3,8	4,7	5,4	—

TANIDA who determined the amount of nitrite as criterion of the reduction rate, has considered that it occurred most remarkably in the pH region of 7,0. From the result of Experiment 5, however, we can say the less the pH-value was, the more rapidly the reduction proceeded. Accumulation of nitrite in a large amount in the region of less pH-value seems to indicate that the reduction both of nitrate and nitrite was favored by high acidity.

Experiment 6

It was shown in the preceding experiments that the accumulation of nitrite occurred more noticeably in the + Mo culture than in the -Mo. This fact, together with the rapid disappearance of nitrate in the -Mo culture (Experiment 4), indicates that the reduction of nitrate as well as of nitrite is more or less inhibited by the presence of molybdenum. For the purpose further to confirm this fact, nitrite was used as nitrogen source and the influence of molybdenum on the velocity of its reduction was tested.

The test solution, method snd condition were almost the same as

in Experiment 5. THUNBERG-KEILIN tubes were used to keep yeast in anaerobic condition. $NaNO_2$ 3×10^{-5} M (++++). Carbon source: glucose and ethyl alcohol (each M/30). Temperature: 24°C. (cf. Table 6 A–D).

A. pH relation. Dry weight of yeast cells: 2,3 mg (+ Mo and -Mo). Carbon source: glucose. Duration 45 minutes. (cf. Table 6-A).

TABLE 6-A

Nitrite reaction

Mo	pH			
	6,2	5,4	4,6	3,8
+	+++	++	+	−
−	++	+	Trace	−

TABLE 6-B

Nitrite reaction

Mo	Aerobic	Anaerobic
+	++	++++
−	−	+

B. Influence of free oxygen. Dry weight of yeast cells: 2,0 mg (+ Mo and −Mo). Carbon source: glucose. pH 5,4. Duration: 30 minutes. (cf. Table 6-B).

C. Carbon source. Dry weight of yeast cells: 2,0 mg (+ Mo and -Mo). (cf. Table 6-C).

TABLE 6-C

Nitrite reaction

pH	Minute	Mo	Glucose	Alcohol
7,2	60	+	++	++++
		−	+	+++
5,4	4,5	+	++	+++
		−	+	++

D. Production of ammonia. Dry weight of yeast cell: 2,5 mg (+ Mo and −Mo). Carbon source: glucose. $NaNO_3$ M/1000. pH 5,4. Cell suspension and test solution were mixed together in a test tube, and it was closed with a rubber stopper. The tubes were kept at 24°C. After two hours the stopper was removed, a small quantity of NaOH added, warmed, and the evaporating ammonia was tested with the NESSLER reagent. For this purpose, the opening of the test tube was covered with a cover glass on which a drop of the reagent was hanging. (cf. Table 6-D).

TABLE 6-D

Mo	NESSLER reaction
+	Slow
−	Rapid

From Experiment 6 (A–D) it will be seen that the reduction of nitrite occurs more rapidly in the −Mo material than the + Mo. The pH relation and influence of free oxygen were equal to the case of nitrate reduction. Ethyl alcohol was available as carbon source, but it was inferior to glucose as substrate for the nitrate reduction.

Experiment 7

In the previous experiments we have explained the disappearance of nitrite in the culture solution of *Hansenula anomala* as a result of its reduction. It might be, however, not impossible to assume that nitrite and ammonia would be oxidized to nitrate and nitrite respectively, through a route of the reverse reaction, because we have learned that in the present organism the reduction progresses rapidly rather in the aerobic condition. Moreover, as MATSUZAKI, one of our collaborators, has found that the oxygen-uptake by this yeast was increased when nitrate, nitrite or ammonium salt was supplied, the possibility of the re-oxidation of the reduction products mentioned above cannot be immediately denied. Although STICKLAND (1931) had already confirmed that such a re-oxidation of nitrite produced by the nitrate reduction does not happen in *Bac. coli*, it has been of significance to determine the question whether the oxidation would occur or not in the case of our yeast.

The method was almost similar to Experiment 5. If the re-oxidation would occur, there might be nitrite produced in the ammonium salt culture and nitrate in the nitrite culture respectively. $(NH_4)_2HPO_4$ M/50. $NaNO_2$ M/500. Dry weight of yeast cells: 2,0 mg (+ Mo and − Mo). pH 5,4. 24°C. Duration: 24 hours. (cf. Table 7).

TABLE 7

Mo	$+NaNO_2$	$+(NH_4)_2HPO_4$	
	NO'_3	NO'_3	NO'_2
+	−	−	−
−	−	−	−

From Table 7 it will be perceived that any partial reaction of the range of re-oxidations $NO'_3 \leftarrow NO'_2 \leftarrow NH_3$ did not take place. Therefore, we are able to say the reduction of nitrate and nitrite proceeds irreversibly. It is necessary now to look for an oxidative process in the later step of the continuous course of assimilation of nitrate nitrogen, namely any reaction which promotes the preceding nitrate reduction in equilibrium relation.

Experiment 8

According to AUBEL (1937), YAMAGATA (1937–1938, 1939) and EGAMI and SATO (1948) the nitrate (nitrite) reduction is performed by a combined action of nitrate (nitrite) reductase and dehydrogenase. Studies on the activity of dehydrogenases in *Hansenula anomala* were done by USAMI (1942), with intact cells and extracted enzyme preparation, using several kinds

of substrates. In the present experiment the activity of dehydrogenation was comparatively studied with the +Mo and −Mo living material.

The velocity of the decoloration of methylene blue (Mb) was measured, using THUNBERG-KEILIN tube. Test medium was prepared as follows: 0,5 cc yeast suspension. 0,5 cc M/6 glucose (final concentration M/30). 0,5 cc M/4 phosphate buffer (final concentration M/20). 0,5 cc M/5000 methylene blue (final concentration M/25000). 0,5 cc redistilled water. pH 7,2. Yeast: three days' material *a* and *b*[1]. Dry weight of cells: 1,9 mg. Glucose and methylene blue were placed in the retort and other liquid in the main tube. After evacuation the tubes were kept in a water bath of 24°C for three minutes, the content in the retort was removed into the main chamber, and the tubes came in the water bath. Time necessary for the decoloration was measured. For the purpose to compare activities of dehydrogenation between the yeast materials, especially when different amounts of cells were used, the reciprocal value of the product "decoloration time (minute) × dry weight (mg)" was calculated, and it was called "dehydrogenation value". (cf. Table 8).

TABLE 8

Substrate	Mo	Yeast fed (*a*)		Yeast starved (*b*)	
		Decolor. time (Minute)	Dehydrog. value	Decolor. time (Minute)	Dehydrog. value
Glucose	+	18	0,029	111	0,005
	−	13	0,041	27	0,019
—	+	40	0,013	136	0,004
	−	35	0,015	44	0,012

The dehydrogenation proceeded more rapidly in the −Mo material than in the +Mo, regardless of the addition of substrate or nutrition state of the yeast. One of the writers (M.) has, in her other study, observed that the yeast of the ammonium culture fell below that of the nitrate culture in the power of glucose-dehydrogenation, without reference to molybdenum. From this fact it seems likely that a powerful adaptive enzyme was active in the material obtained from the nitrate culture, especially without molybdenum, where the vigorous reduction

1) Yeast cells were suspended in a phosphate buffer solution (+Mo and −Mo), and the cell suspension was airated for 12 hours. The thus treated material was indicated as *b*.

of nitrate happened.

Experiment 9

The dehydrogenation caused by living yeast cells and its bearing on pH-value were examined. Test solution and method were almost equal to Eperiment 8. Substrate: M/30 glucose. Dry weight of yeast cells (three days' material): 1,7 mg. Time necessary for the decoloration of methylene blue was shown in minutes in Table 9.

TABLE 9

Mo	pH		
	7,8	7,2	5,4
+	43	63	>190
−	38	50	90

The larger the pH-value was, the more rapidly the dehydrogenation proceeded. This relation resembles to the case of alcohol-dehydrogenase prepared from yeast cells of the same kind, though no remarkable difference was visible in the pH region between 7,2 and 7,8 in the experiment with this enzyme preparation[1]. It is a noticeable fact that the nitrate reduction was favored by a relatively high acidity, while the dehydrogenation, which is indispensable for the accomplishment of the nitrate reduction, showed an opposite relation to the acidity.

Experiment 10

Hydrogenase which activates hydrogen molecules, has been found by some authors[2] in bacteria, and free hydrogen acts as substrate for the reduction of nitrate, nitrite and hydroxylamine in the case of *Bac. coli* and *Clostridium Welchii*[3]. It will be of interest to see whether the same thing would be seen also in our material of *Hansenula anomala*.

TABLE 10

Mo	Substrate		
	Hydrogen	Glucose	−
+	105	60	105
−	105	50	87

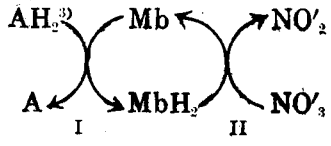
After evacuation of a THUNBERG-KEILIN tube containing the cell suspension, it was filled with hydrogen gas generated from a KIPP apparatus. As a control of substrate a M/30 glucose solution was used. Dry weight of yeast cells (three days' material): + Mo 2,5 mg. −Mo 2,6 mg. pH 7,2. Decoloration time was shown in minutes in Table 10.

1) USAMI (1942).
2) STEPHENSON and STICKLAND (1931, 1932).
3) WOODS (1938).

The result of Experiment 10 shows that hydrogen gas was unavailing as donator for the reduction of methylene blue, and in the case of the −Mo material, the decoloration was more or less retarded. There was no indication of presence of hydrogenase in *Hansenula anomala*.

Experiment 11

The coexistence of nitrate causes inhibition of the reduction of methylene blue performed by some kinds of bacteria[1]. As interference of the reduction would presumably occur between nitrate and methylene blue as in the case of that between free oxygen and nitrate. If the nitrate reduction is in predominance to that of methylene blue, the decoloration of the latter may be retarded, and leucomethylene blue may be oxidized by addition of nitrate in anaerobic condition[2]. These relations may be illustrated in the following schema:



The interference between methylene blue and nitrate means the competition between the coupled reactions I and II, and the oxidation of leucomethylene blue occurs according to the predominance of the latter reaction. An experiment along this line was conducted here.

Thunberg-Keilen tubes were used. Medium solution was prepared almost similarly to Experiment 8, except that 0,5 cc of M/3 $NaNO_3$ was applied instead of water in the present case. Concentration of $NaNO_3$ was M/15 after the dilution. In order to investigate the oxidation of leucomethylene blue, $NaNO_3$ solution was placed at first in the retort, and after complete decoloration of methylene blue it was removed into the main tube where the dye, yeast cells and donator were contained mixed. After 24 hours incubation at 24°C the density of the recovered color was determined. The initial color density: ++++. Dry weight of yeast cells (three days' material): +Mo 2,3 mg. −Mo 1,7 mg. (cf. Table 11 A, B).

1) Stickland (1981), Nakamura (1938), Yamagata u. Nakamura (1938), Yamagata (1937—1938).
2) Quastel and Whetham (1924), Aubel (1937).
3) Donator.

TABLE 11-A[1]

pH	Mo	− $NaNO_3$		+ $NaNO_3$	
		Decolor. time (minute)	Dehydrog. value	Decolor. time (minute)	Dehydrog. value
7,8	+	24	0,019	33	0,014
	−	24	0,024	33	0,018
7,2	+	24	0,019	34	0,013
	−	24	0,024	34	0,017
5,4	+	44	0,003	159	0,002
	−	33	0,018	59	0,004

TABLE 11-B

Oxidation of leucomethylene blue after 24 hours.
Recoloration was indicated with the mark +.

pH	Mo	$-NaNO_3$	$+NaNO_3$
7,8	+	−	+
	−	−	+
7,2	+	−	++
	−	−	++
5,4	+	−	+++
	−	−	+++

From the above tables it will be seen that the coexistence of nitrate retarded the reduction of methylene blue, especially in high acidity, but it was not remarkable in the region of alkaline reaction. This would be explicable also by the fact that the reduction of methylene blue was favored rather in high pH, as indicated in Experiment 9. The oxidation of leucomethylene blue in accordance with the simultaneous reduction of nitrate could be recognized, and the higher the acidity was, the more rapidly it took place. From this result it may be concluded that one of the coupled reactions I and II illustrated in the above schema dominates the other, according to pH. That is to say, the higher the acidity is, the more intensely the reductase exceeds the dehydrogenase in the action, and *vice versa*. In Table 11-B we are not able directly to compare the rate of oxidation of leucomethylene blue between the + Mo and − Mo material, because they were not taken in equal amounts, One of the writers (M.), however, has confirmed in another work that the nitrate reductase, prepared from the − Mo cells of *Hansenula anomala*, showed no change in its activity, when molybdenum was added. Therefore, it may be very probable to assume that

1) In the exderiment, of which result is shown in Table 11-A, the amount of yeast was intentionally adjusted so as to make the decoloration time equal between the + Mo and − Mo material. Therefore, it will be recommended rather to compare the dehydrogenation values, if the object is to see any action of molybedenum.

molybdenum takes no part as a constituent of reductase, or that it plays no role in the formation of the enzyme.

Experiment 12

According to YAMAGATA (1939) the action of nitrate reductase prepared from *Bac. pyocyaneus* is inhibited by urethane. AUBEL and EGAMI (1935, 1936) have demonstrated that phenylurethane acts inhibitorily upon the nitrate reduction performed by living bacteria, when *l*-alanine or *d.l*-lactic acid is given as donator. However, in the case of *Bac. coli* the urethane inhibition does not remarkably occur[1].

By the method of oxidation of leucomethylene blue and by means of the test of nitrite and ammonia produced from nitrate by the reduction, the influence of urethane upon the nitrate reduction was investigated.

$NaNO_3$ M/15. $NaNO_2$ M/100. Urethane 1 M. No influence of the incubation was visible. Dry weight of yeast cells (three days' material): 2,2 mg (+ Mo and − Mo).

Since the substrate and methylene blue needed to be placed in the main part of the THUNBERG-KEILIN tube mixed with cell suspension, from the beginning, the decoloration was unavoidably in progress already during the evacuation.

The retort contained nitrate or nitrite solution at the beginning, and with addition of urethane in case of need. After the decoloration

TABLE 12

	Mo	Oxidation of MbH_2						After 18,5 hours			
		5 min		2,5 hours		18,5 hours		NO'_2		NH_3	
		pH 7,2	pH 5,4	pH 7,2	pH 5,4	pH 7,2	pH 5,4	pH 7,2	pH 5,4	pH 7,2	pH 5,4
$NaNO_3$	+	−	−	−	−	−	−	++++	++++	++	++
	−	−	−	−	−	−	−	−	−	+++	+
$NaNO_2$	+	−	+	−	+++	−	++++	Much	Much	++	++
	−	−	+	−	+++	+	+++	Much	Much	+++	+
$NaNO_3$ + Urethane	+	−	+	−	+	−	−	−	−	+	+++
	−	−	−	−	−	−	−	−	−	++	+
$NaNO_2$ + Urethane	+	−	++	−	++++	+	++++	Much	Much	+	+
	−	−	+	−	+++	++	++++	Much	Much	++	+++

1) LASCELLS and STILL (1946).

of methylene blue the contents of the main tube and of the retort were thoroughly mixed together in the former part, and the tubes were kept in a water bath of 24°C. Color density recovered in a definite duration were compared with each other. Ammonia was caught in a HCl solution by the airal distillation and tested with the NESSLER reagent. (cf. Table 12).

Urethane inhibited the reduction process $NO'_3 \rightarrow NO'_2 \rightarrow NH_3$. This was shown also in aerobic condition in other experiments. The principal part of the reductive reaction, where urethane inhibitorily acts, seems to be the dehydrogenase system. The reaction participated by nitrate and nitrite reductase, which can be demonstrated by the oxidation of leucomethylene blue, was not inhibited by urethane, but tended rather to be accelerated. The last mentioned relation is explicable by the assumption that the oxidation of leucomethylene blue, might be promoted by the urethane inhibition of the reaction I in the schema illustrated in Experiment 11, which causes the prevailing occurrence of the reaction II. The nitrate reduction as a whole can be regarded as inhibited by urethane, but at least in the case of *Hansenula anomala* it cannot be justified to conclude that the inhibition occurs in the reductive reaction itself, for which the activity of reductase is responsible.

Some different points between the +Mo and −Mo material were seen, respecting the influence of urethane, but any conclusion, which might explain the action of molybdenum, could not be derived from these facts.

Experiment 13

According th TANIDA (1935) 0,02% monoiodo-acetic acid inhibits the oxygen respiration of *Hansenula anomala* about 12 per cent, and it does the alcoholic fermentation perfectly, but never the nitrate reduction. However, AUBEL and GLASER (1938) have reported that the reduction of nitrate by bacteria was not influenced by this inhibitor.

In the present experiment the action of this inhibitor was reexamined, being used the method of decoloration of methylene blue and oxidation of leucomethylene blue and test of produced nitrite, which enabled us to see what part of the course of the reduction would be inhibited by this inhibitor. Glucose and ethyl alcohol M/30. $NaNO_3$ M/15. $NaNO_2$ 3×10^{-5} M. Monoiodo-acetic acid M/1000. pH 7,2. Dry weight of yeast cells (three days' material) : 30 mg in the case of nitrate reduction, 2,05 mg in the case of nitrite reduction. All the test tubes

were kept at 24°C. When the decoloration of of methylene blue was measured, nitrate or nitrite were not added. Color density of the dye recovered by the re-oxidation and nitrite produced from nitrate were determined after 24 hours. pH 7,2. (cf. Table 13 A, B).

TABLE 13–A

+ $NaNO_3$

Substrate	M.I.A.[1]	Mo	Decolor. time (Minute)	Oxidation of MbH_2	NO'_2 produced
Glucose	+	+	12	+	Trace
		−	25	+	Trace
	−	+	12	+++	++
		−	25	++	+
Alcohol	+	+	51	−	Trace
		−	45	−	Trace
	−	+	37	−	+
		−	25	−	++

The yeast material in the case of alcohol was different from that in the case of glucose.

It was shown in this experiment that the decoloration was not inhibited by monoiodo-acetic acid, when glucose was used as subtrate, while a clear inhibition appeared in the case of alcohol as substrate. This fact coincides with the finding of USAMI (1942) that alcohol-dehydrogenase of this yeast is inhibited by monoiodo-acetic acid.

TABLE 13–B

+$NaNO_2$. Substrate: Glucose.

M.I.A.	Mo	Decolor. time (Minute)	Oxidation of MbH_2
+	+	12	++
	−	9	++
−	+	11	++
	−	9	++

Though, according to TANIDA (1935), the nitrate reduction in *Hansenula anomala* is said not to be inhibited by monoiodo-acetic acid, in our case the partial reactions, of which the whole process of the reduction consists, should be affected by it more or less variably. When glucose was used as hydrogen donator, with which no inhibition of the dehydrogenation was caused by monoiodo-acetic acid, this inhibitor retarded the oxidation of leucomethylene blue, $NaNO_3$ being given, while it was

1) Mnoiodo-acetic acid.

not the case with $NaNO_2$. The decrease of the production of nitrite from nitrate in the former case means that the partial reaction $NO'_3 \rightarrow NO'_2$ is affected by monoiodo-acetic acid, and the accumulation of nitrite in less amount in the −Mo yeast than in the +Mo material is due to a rapid progress of the reduction of nitrate, namely to its rapid disappearance. From the present experiment it may be assumed that the reaction catalysed by nitrate reductase is sensitive to monoiodo-acetic acid, while that which is catalysed by nitrite reductase is insensitive to it.

Experiment 14

It has been demonstrated by some authors[1] that cyanide inhibits the nitrate reduction by bacteria, and the interfering action of nitrate on the reduction of methylene blue can be eliminated by it. From the fact that the nitrate reduction is inhibited by cyanide, it might be possible to assume that there would exist an enzyme containing heavy metal which may be inhibited by cyanide. The preparation of nitrate- and nitrite-reductase of bacteria was realized by YAMAGATA (1937/1938, 1939), and he has proved the inhibition of these enzymes by cyanide. Also EGAMI and SATO (1948) have attained to obtain nitrate reductase from various kinds of plant materials. It has been already confirmed by TERUI (1936) that the reduction also in *Hansenula anomala* is inhibited by cyanide.

An almost similar procedure was conducted in this experiment as in the previous, excepting the use of KCN instead of urethane or monoiodo-acetic acid.

Glucose (substrate) M/30. $NaNO_3$ M/15. $NaNO_2$ 3×10^{-5} M. KCN M/1000. pH 5,4. Dry weight of yeast cells (three days' material): 2,3 mg (+Mo and −Mo). (cf. Table 14).

An addition of KCN caused the decrease of the production of nitrite from nitrate and also the decrease of the disappearing amount of the given nitrite, that is KCN inhibits the reductive reaction both of nitrate and nitrite.

TABLE 14

Mo	NO'_2 produced (after 5 hours)		NO'_2-rest (after 1,25 hours)	
	+KCN	−KCN	+KCN	−KCN
+	−	+++	++++	++
−	−	++	+++	−

Experiment 15

From the previous experiments it seems probable that the favorable

1) STICKLAND (1931), AUBEL (1937), YAMAGATA (1937/1938).

condition of aerobiosis for the nitrate reduction by this yeast is indirectly due to a certain oxidative reaction. Since the nitrate reduction is often used to be followed by the synthesis of organic nitrogenous compounds, it may be expected that such an oxidative reaction occurs after the amino acid formation, and molybdenum has influence on the region of this synthesis.

The nitrate cultures of short duration were carried out, using relatively large amount of the yeast cells, and the products, namely nitrite, ammonia, amino acid and amide were estimated. The measurement of amino acid was conducted by the VAN SLYKE method and amide by the SACHS-method. The culture solution in each flask was of the following composition: M/6 glucose 5 cc (150 mg in total). M/4 phosphate buffer 5cc. $NaNO_3$ solution 5 cc ($2{,}09 \times 10^{-4}$ M after mixing). Redistilled water 5 cc. Dry weight of yeast cells (three days' material): 7,78 mg (+Mo and −Mo). Duration: 15 hours. 24°C (cf. Table 15).

TABLE 15

Mo	pH	Dry weight of cells (mg)	Increase of dry weight (mg)	$[NO'_3]$	$[NO'_2]$	Nitrogen consumed (mg)	$[NH_3]$	[Amide-N]	[Amide-N] per mg of yeast	[Amino-N]	Glucose
+	4,6	18,3	10,6	35	0,1	9,502	0,573	2,8	0,153	2,6	+
−	4,5	17,7	10,0	29	0,03	10,111	0,502	16,5	0,932	2,7	+

The concentration unit 10^{-4} M in all the cases.

Ammonium nitrogen was richer in the + Mo culture than in the −Mo in every case, though this difference was not very remarkable. With regard to the amount of amide there was a clear difference between both kinds of yeast materials, namely it was prominent in the −Mo culture, especially when the amide nitrogen per mg of the dried cells was calculated. No marked result was obtained to judge the action of molybdenum upon the accumulation of amino acid. From the result of this experiment we may say that less ammonia and more amide were found in the −Mo culture than in the + Mo. Since the culture was of short duration an error could be avoided which might be caused by the different growth rates of yeast cells.

Experiment 16

The purpose of the present experiment was almost equal to Experiment 15, but the culture duration was longer. The culture medium

was inoculated with one drop of solution containing bottom yeast cells of the previous culture of same sign. KNO_3 6.25×10^{-4} M. Yeast cells: three days' material. (cf. Table 16).

TABLE 16

Days	Mo	pH	Dry weight of yeast	$[NO'_3]$	$[NO'_2]$	$[NH_3]$	[Amide-N]	[Amide-N] per mg of yeast	[Amino-N]	Glucose
3	+	4,3	128	41,4	0,3	0,026	3,35	0,026	0,106	+
	−	4,2	82	41,4	—	—	3,80	0,046	0,212	+
5	+	4,4	203	25,6	1,01	0,277	2,04	0,010	0,207	+
	−	4,0	160	26,2	Trace	0,188	2,33	0,014	0,064	+

The concentration unit 10^{-4} M in all the cases.

The dissimilar growth rates made it difficult to see the difference of the amounts of the products between the + Mo and −Mo culture. Nevertheless, there could be found a tendency towards the production of less ammonia and of more amide nitrogen in the −Mo culture. As to the amount of amino acid any conclussive difference between both cultures was scarcely detected.

Also in another experiment where ammonium chloride was used as nitrogen source, an accumulation of more amide nitrogen was detected in the −Mo culture.

Conclusion and Summary

The reduction of nitrate by *Hansenula anomala* which previously had been cultured with addition of molybdenum in minute concentration (2×10^{-6} M), took place more slowly than without it. This relation seems to be contrary to the facts found in the case of *Aspergillus*, at least superficially. In the + Mo nitrate-culture nitrite accumulated as the reduction product, while it was not at all or hardly detected in the −Mo. Molybdenum limited the consumption of glucose which is needed by the nitrate reduction, and this restrictive action favored rather the growth of the yeast, and it resulted in the increase of the economical coefficient. In this relation molybdenum can be regarded as a regulator which limits the waste of free energy accompanied by the nitrogen metabolism. Such an action of molybdenum was not visible in the ammonium salt culture. The nitrate reduction occurred more rapidly in the aerobic condition than in the anaerobic. No re-oxidation of the

reduction products happened. Among the partial reaction of the nitrate reduction the dehydrogenation system of the −Mo yeast was more active than that of the +Mo, but no influence of molybdenum appeared on the reductase system. Between the +Mo and −Mo culture, supplied with nitrate or ammonium salt as nitrogen source, there was found a remarkable difference in the accumulation of amide and ammonia produced from these nitrogen sources. From the results obtained in the present investigation it may be concluded that there is a certain oxidative reaction after the synthesis of amino acid which has a far-reaching equilibrium influence on the velocity of the nitrate and nitrite reduction. As such a reaction we may presumably consider a reaction connected with the formation of amide which is regarded to need free oxygen in several organisms. The above mentioned action of molybdenum seems to happen on this assumed reaction, but not on the reductase reaction itself, though the dehydrogenase system is not indifferent to the action of this metal.

The higher the acidity was, the more intensely the reductase exceeded the dehydrogenase in the action, and *vice versa*. Urethane did not inhibit the nitrate reduction itself, but had influence on the dehydrogenation. When glucose was used as a hydrogen donator, the reductive reaction catalysed by nitrate reductase was retarded by monoiodo-acetic acid, while the reaction depending on the nitrite reductase was insensitive to it. Cyanide inhibited the reduction both of nitrate and nitrite, as demonstrated in bacteria by many authors.

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