STUDIES ON THE NITROGEN METABOLISM OF PLANTS

VII. TOXICITY OF SOME OXIMES AND OXIMINO-ACIDS TO *AZOTOBACTER* AND THEIR UTILIZATION

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Summary

Twenty-two different oximino-derivatives were synthesized; they included ketoximes, dioximes, oximino-monocarboxylic acids, and oximino-dicarboxylic acids and esters of the last two groups of compounds. Toxicity of these compounds to *Azotobacter* was measured; esters, dioximes, and α-oximino-dicarboxylic acids were non-toxic at 5 x 10^{-3}M concentration, whilst the other oximino-derivatives were toxic at about 5 x 10^{-4}M. Toxicity was related to chemical structure and probable orientation in relation to membrane surfaces.

It is shown that *Azotobacter* readily uses both *cis-* and *trans-* modifications of both oximinosuccinic and α-oximinoglutaric acids as source of nitrogen at pH 6.8 in absence of atmospheric nitrogen or other source of nitrogen.

The possibility that these two oximino-acids might be intermediates in nitrate metabolism is discussed.

I. Introduction

Nitrogen added to plants either as ammonium or nitrate ion results in increases in amounts of both proteins and amino-acids and usually also in the amides asparagine and glutamine. However, evidence is accumulating which suggests that differences in metabolism occur according as the plants are provided with either ammonium salts or nitrates.

One of the most striking of these differences is in the relative amounts of organic acids, especially malic and citric acids, contained in the tissues. These acids decrease in amount as the concentration of ammonium salt in the external solution is increased (Clark 1936; Vickery *et al.* 1940).

In leaves of plants supplied with ammonium salts, Wood and Petrie (1938) showed that at a steady state the relationship between amino-acid and ammonium content within the leaves could be expressed by a logarithmic function of ammonia-N. The asymptotic part of the curve was ascribed to the fact that substrates providing the carbon-skeleton were limiting. It has long been held that oxy- and keto-acids are the immediate non-nitrogenous precursors of amino-acids. Wood, Mattner, and Symons in 1947 (unpublished data) supplied varying treatments of both ammonium salt and nitrate to oat plants under controlled environmental conditions; at the steady state they found that the same relationship between proteins and amino-acids held for both "ammonium" and "nitrate" plants. The ammonium plants showed the same relations between amino-acids

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and ammonia contents as described by Wood and Petrie (loc. cit.) ; but in nitrate plants the amino-acid content was independent of the ammonium content (which remained small) but increased as the nitrate supply was increased. In both sets of plants amide content showed similar relationship to ammonia-N content.

Pearsall and Billimoria (1939) asserted that light caused increased nitrate reduction as measured by increased production of organic-N in detached leaves floating on nutrient solutions.

Blackman and Templeman (1940) recorded gain of protein in leaves in full sunlight when plants were supplied with either ammonium salts or nitrate, but at lower light intensities they found that elaboration of protein was reduced and nitrate accumulated. They concluded that shaded leaves of grasses lose their ability to elaborate nitrate into organic-N, but their ability so to elaborate ammonium is unimpaired. Their ability to reduce nitrate was not dependent on carbohydrate content.

Burstrom (1943), using wheat seedlings at the two-leaf stage, found that nitrate in leaves was not reduced in darkness, but in light in amounts proportional to the light intensity. In this respect, he points out that leaves differ from roots, for in the latter, nitrate reduction occurs in darkness. Burstrom measured the rate of photosynthesis and also the amounts of soluble sugars in the leaves; he found, over a given time interval, that the amount of photosynthesis was greater than the increase in sugar content and that the difference between them was proportional to the amount of nitrate reduced. The amount of nitrate reduced was independent of rate of respiration; in the absence of nitrate he claims that all the carbon dioxide is converted into sugars. When ammonium salts were present these were assimilated by the leaf in both light and darkness at the expense of sugars. He concluded that nitrate was not reduced to ammonia since stored sugars were not drawn on in nitrate nutrition, and suggested that a direct carbon-nitrogen assimilate was formed in leaves in the presence of light, and also suggested, though without any experimental evidence, that a reduction product—nitrous acid, hyponitrous acid, or hydroxylamine—might combine with a photosynthetic product to form an oxime.

All the experiments quoted above provide at least some grounds for concluding that alternative mechanisms may exist for the formation of organic-N, and especially of amino-acids, according as the plant is supplied with ammonium salts or nitrates. Accordingly we decided to investigate the utilization of some oximes by plants, and especially of oximino-acids which might be considered to be intermediaries in amino-acid synthesis. For preliminary investigations on toxicity and utilization we used *Azotobacter*, since use of a bacterium gave results in a short time and also because it has been claimed that oximes are produced as intermediaries in the metabolism of this organism.

Investigators concerned with the mechanism of N-fixation or nitrate reduction in N-fixing bacteria fall into two groups: those who consider ammonia to be the primary product of reduction, and those who assign this role to hydroxylamine. Arguments advocating ammonia as the primary product are necessarily

inconclusive since ammonia is always produced within the organism by deamination of amino-acids. The chief proponent of the hydroxylamine theory is Virtanen (1939). A critical review of the position has been made by Burris and Wilson (1945) who suggest theoretical pathways for N-fixation which may not be mutually exclusive.

Virtanen's arguments, derived from consideration of the relative amounts of oxalacetic and α-ketoglutaric acids formed by *Rhizobium* and their relative powers of condensing with hydroxylamine to form oximes, are unconvincing. By far the strongest claim for his theory is his reported isolation of *trans*-oximinosuccinic acid from nodules of *Rh. leguminosarum.*

Endres (1935) isolated a carboxime (amounting to 10 per cent. of the total-N fixed) from medium in which *Azotobacter* was growing. He found that this compound in concentrations from 1 x 10^{-5}M to 1.2 x 10^{-4}M (the highest concentration investigated) had no effect upon the oxygen uptake by the bacteria and concluded that it originated from N-fixation rather than from cell katabolism. Burk and Horner (1936) disagreed with this explanation on the ground that *Azotobacter* will not assimilate hydroxylamine even in non-toxic concentration and that the oxime was formed not only when molecular N was used as a source of N, but also when nitrate, nitrite, or ammonia was used. They considered, therefore, that the oxime was an unspecific by-product of metabolism.

Burris (1942) has shown that *Azotobacter* in air enriched with N^{15} produces the highest level of N^{15} in glutamic acid and the next highest in aspartic acid; findings which suggest the important roles of dicarboxylic acids and which he suggests are evidence for the "ammonia" theory, although of course the evidence is not exclusive. So far as *Azotobacter* is concerned it is possible that both mechanisms are operative, viz through an oxime or through ammonia. Virtanen (1947) has recently expressed the view that it is not impossible that hydroxylamine is chiefly reduced to ammonia and that oximes are formed to a small extent through by-reactions with hydroxylamine.

II. Experimental Methods

(*a*) *Synthesis of Oximino-Compounds*

With one or two exceptions it was necessary to synthesize and carefully purify all the oximes used by us. In some cases these syntheses, using methods adopted by other workers, proved difficult and gave poor yields, and we were compelled to try alternative procedures. Since many of these compounds have seldom been prepared we give brief accounts of our experience.

Acetoxime, 2-butanone oxime and 2-pentanone oxime were obtained from the corresponding ketones by the prolonged action of ice-cold aqueous alkaline hydroxylamine solution according to standard procedure. Methyl ethyl ketone was obtained readily by distillation of mixed calcium salts of acetic and propionic acids, but a mixture of calcium acetate and calcium butyrate gave negligible quantities of methyl propyl ketone which was obtained in quantity by alkaline

hydrolysis of ethyl α-ethylacetoacetate. Methylglyoxime was obtained readily from chloracetone and hydroxylamine according to the method of Hantzsch and Wild (1896). Merck's dimethylglyoxime was recrystallized and used as source of this compound. Diacetylmonoxime was readily obtained from methyl ethyl ketone by the method of Diels and Jost (1902).

The method of Meyer and Jenny (1882) for α-oximinopropionic acid using aqueous solutions of sodium pyruvate and hydroxylamine gave very poor yields but the oximino-acid was obtained in 90 per cent. yield by treating an alcoholic solution of hydroxylamine hydrochloride with sodium ethylate and adding the filtered solution to an equivalent amount of pyruvic acid dissolved in absolute alcohol, both solutions being cooled to 0°C. On standing in a refrigerator overnight the acid was obtained as silky crystals.

α-Bromobutyric acid warmed with hydroxylamine hydrochloride in alkaline solution according to the method of Hantzsch and Wild (1896) gave negligible quantities of α-oximinobutyric acid. The acid was obtained in fair yield by hydrolysis of the ester obtained by passing nitrous gases through an alcoholic solution of ethyl α-ethylacetoacetate, a modification of Furth's (1883) method for α-oximinovaleric acid. The latter acid was prepared readily by Furth's (loc. cit.) method from ethyl nitrite and ethyl α-propylacetoacetate.

γ-Oximinovaleric acid was obtained in 60 per cent. yield according to the method of Muller (1883). However, the reaction is much slower than there suggested, and better yields were obtained by standing the reaction mixture overnight in a refrigerator.

γδ-dioximinovaleric acid was obtained in good yield from dibromolaevulinic acid according to the method of Wolff (1890).

Ethyl α-oximinopropionate was obtained by refluxing the silver salt of the acid with ethyl iodide. The ethyl esters of α-oximinobutyric and α-oximinovaleric were obtained in the course of preparation of the corresponding acids. The ethyl ester of α-oximinoacetoacetic acid was obtained in good yield from acetoacetic ester and $NaNO_2$ according to the method of Wolff (1902).

Trans-oximinosuccinic acid.* The direct action of alkaline hydroxylamine on oxalacetic (oxyfumaric) acid gave an uncrystallizable oil which rapidly became deep red. The acid was obtained in good yield by forming the Piutti ester by adding concentrated hydroxylamine solution to the sodium derivative of ethyl oxalacetate and finally completing the saponification according to the method of Cramer (1891). This oximino-acid is unstable in acid solution, rapidly decomposing into water and CO_2, and depositing deep red crystals of cyanoacetic acid; however, we found it quite stable in neutral or alkaline solutions.

Cis-oximinosuccinic acid† was obtained in about 50 per cent. yield from dinitrososuccinylsuccinic diethylester after saponification with sodium ethylate

*This acid is recorded by Beilstein as β-oximinosuccinic acid.

†This acid is recorded by Beilstein as α-oximinosuccinic acid.

and decomposition of the silver salt according to the method of Ebert (1885). The ethyl ester of this acid was obtained in the course of preparation of the acid.

α-Oximinoglutaric acids. Our experience generally had been that the direct addition of hydroxylamine to α-keto-acids or their esters gave rise to uncrystallizable oils so that the effect of direct addition to hydroxylamine to α-ketoglutaric acid was not immediately tried. Small quantities of an α-oximinoglutaric acid were synthesized by condensing ethyl β-iodopropionate with acetoacetic ester and then condensing the resulting diethyl α-acetoglutarate with ethyl nitrite to form the oxime ester (Wislicenus and Grutzner 1909). This method was abandoned owing to the small yields (about 15 per cent.) of α-acetylglutaric ester obtained. The oximino-acid was also prepared from dibromolaevulinic acid (the latter according to Heil and Kehrer (1884); the method of Wolff (1885) is too slow and unsatisfactory), through γδ-dioximinovaleric acid, furazanpropionic acid and δ-cyano-γ-oximinobutyric acid according to the methods of Wolff (1890). The lowest yield in this sequence was about 50 per cent., but owing to the long sequences of reactions involved, the amount of oximino-acid obtained was small. The acids obtained by these methods both melted at 156°C.

Finally, satisfactory yields (about 75 per cent.) of an α-oximinoglutaric acid were obtained by direct addition of a cold concentrated solution of hydroxylamine hydrochloride to a cold concentrated solution of α-ketoglutaric acid. We have found best conditions for synthesis of the latter to be synthesis of triethyl oxalosuccinate according to the method of Wislicenus (1911) (the method of Blaise and Gault (1911) gives very poor yields) followed by its decomposition by the method of Blaise and Gault (1911). The oximino-acid crystallizes readily in white rosettes. This acid melts at 140°C., whereas that formed by the first two methods melts at 156°C. We believe the latter to be the *trans-* form and the acid melting at 140°C. to be the *cis-* form.

Several attempts were made under a variety of conditions to obtain β-oximinoglutaric acid from acetonedicarboxylic acid and hydroxylamine according to the method of Emery (1890), but in all cases an uncrystallizable oil was obtained.

In all preparations, and especially where hydroxylamine was used in preparation, it was necessary to purify the compounds carefully before testing for toxicity to *Azotobacter*. Purification was ensured by a final recrystallization from ether.

(*b*) *Cultural Technique*

The organism used was *Azotobacter chroococcum*, obtained from the Waite Agricultural Research Institute and labelled Strain C. Active cultures were maintained by sub-culturing once a week and inoculations of experimental media were made from one-day-old cultures.

The medium was prepared as follows: 0.8 g. K_2HPO_4, 0.2 g. KH_2PO_4, 0.2 g. $MgSO_4.7H_2O$, and 0.1 g. $CaSO_4.H_2O$ were dissolved in a litre of water and the mixture allowed to settle. To 210 ml. of the supernatant solution 0.125 g. lactic acid neutralized with NaOH, 2.0 g. sucrose, and a trace of $FeCl_3$ and $Na_2MoO_4.2H_2O$ were added and the solution made up to 250 ml. Lots of this solution, each of 20 ml., were placed in Erlenmeyer flasks and autoclaved. Where oximino-acids were to be added to the medium these were first dissolved in water, neutralized with NaOH solution, sterilized in a Seitz filter, and the appropriate amount added to the medium in the flasks. The final reaction of the medium was pH 6.8. The medium was inoculated with 2 drops (approximately 0.08 ml.) of a one-day-old culture from a Pasteur pipette and cultured at 24°C. for 2-3 days.

For estimation of toxicity at different concentrations of oximes and oximino-acids, the sole criterion was whether or not growth occurred—visible growth being readily detected by turbidity in the medium. All treatments were replicated.

In determining utilization of oximino-acids by *Azotobacter*, culture flasks were prepared as above containing different concentrations of the substances under examination. After inoculation these were placed in a large desiccator which was evacuated, refilled and washed with cylinder oxygen (which contains too much nitrogen to use as the experimental atmosphere), the desiccator again evacuated and partially filled with pure oxygen obtained by heating pure MnO_2 and $KClO_3$. The pressure of oxygen in the desiccator was approximately 0.5 atm.

The amount of nitrogen utilized under these conditions was determined by precipitating the organisms with 10 per cent. $Al_2(SO_4)_3$ followed by successive centrifugations and washings according to the procedure of Horner and Allison (1944); the precipitate was then digested and the nitrogen estimated by micro-Kjeldahl.

III. Results and Discussion

(*a*) *Toxicity of Oximino-Derivatives to* Azotobacter

Results of the toxicity of these compounds, as measured by failure of *Azotobacter* to grow, are set out in Table 1.

It is clear that so far as toxicity is concerned, the compounds examined fall into four groups:

(1) Hydroxylamine, toxic at a concentration of 1×10^{-5}M.

(2) Ketoximes, toxic at concentrations of about 10^{-3}M.

(3) α-Oximino-monocarboxylic acids, toxic at about 10^{-4}M.

(4) Substances non-toxic at 5×10^{-3}M and in some cases non-toxic at 10^{-2}M. These compounds include esters of α-oximino-mono- and di-carboxylic acids, α-oximino-dicarboxylic acids, diacetyl monoxime, and the dioximes methylglyoxime, dimethylglyoxime and γδ-dioximinovaleric acid.

TABLE 1

TOXICITY OF OXIMINO-DERIVATIVES TO AZOTOBACTER

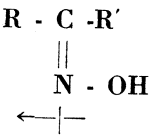
Oximino-Derivatives	Molarity									
	1×10^{-2}	5×10^{-3}	2×10^{-3}	1×10^{-3}	5×10^{-4}	2×10^{-4}	1×10^{-4}	5×10^{-5}	1×10^{-5}	5×10^{-6}
Hydroxylamine		—		—				—	—	+
Ketoximes										
Acetoxime		—		—		+	+	+	+	+
2-propanone oxime		—		—		+	+	+	+	+
2-butanone oxime	—		—	—	±	+	+	+	+	+
Diacetyl monoxime	+		+	+	+	+	+	+	+	+
Methylglyoxime	—		+	+	+	+	+	+	+	+
Dimethylglyoxime	(not soluble)		+	+	+	+	+	+	+	+
Oximino-monocarboxylic acids										
Oximinopropionic				—		—	—	+	+	+
α-Oximinobutyric				—		±	+	+	+	+
α-Oximinovaleric	—			—		—	±	+	+	+
γ-Oximinovaleric				—		+	+	+	+	+
γδ-Dioximinovaleric										
Esters										
Ethyl oximinopropionate	±	+		+			+	+	+	+
Ethyl α-oximinobutyrate		+		+			+	+	+	+
Ethyl α-oximinovalerate	+		+	+			+	+	+	+
Ethyl oximinoacetoacetate	±	+		+			+	+	+	+
Ethyl oximinosuccinate	—	+		+			+	+		
Oximino-dicarboxylic acids										
Cis-oximinosuccinic	—	±		+			+	+	+	+
Trans-oximinosuccinic	±	+		+			+	+	+	+
Cis-α-oximinoglutaric		±		+			+	+	+	+
Trans-α-oximinoglutaric	—	+	+	+			+	+	+	+

\+ growth occurred — no growth ± growth incipient

Before suggesting possible mechanisms of inhibition of growth it is necessary to show that penetration of the cells by the oximes occurs. Taking mortality as an indication of penetration it is clear that the ketoximes are approximately equally effective in spite of a marked increase in lipophilic properties in the series, 2-butanone oxime being only sparingly soluble in water. A similar argument, so far as approximately equal toxicity is concerned, holds for the α-oximino-monocarboxylic acids, but here supporting evidence of penetration is obtained from the fact, to be discussed later, that oximinopropionic acid and also the oximino-dicarboxylic acids in non-toxic concentration can be utilized as a source of nitrogen by plants.

It should be pointed out here that α-oximino acids are relatively strong acids, comparable to monochloracetic acid, for example. The following are the

dissociation constants (K x 10^5) of some of the acids used in this investigation: α-oximinopropionic, 51.4; α-oximinobutyric, 69; α-oximinovaleric, 68.5; *trans*-oximinosuccinic, 110; *cis*-oximinosuccinic, 372. Dissociation constants for the corresponding unsubstituted acids are propionic, 1.43; butyric, 1.49; n-valeric, 1.61; succinic (K_1), 6.8 γ-Oximinovaleric acid on the other hand is a weak acid (K x $10^5 = 2.3$). It is clear, therefore, that the oximino group as a substituent is definitely acylous, i.e. electron-attracting; it is analogous in this respect to the amino group which, though basic, is definitely acylous as a substituent, as might be expected in view of the fact that the nuclear charge on the nitrogen atom is greater than that on the carbon. It is also apparent that as usual the dissociation constant is determined by the distance of the substituent from the carboxyl group. In oximes both trivalent N and OH are dipoles and dipoles are therefore directed as follows:



The oximino group is not ionized though the oximino-acids are strongly ionized.

According to modern views, the living cell surface consists of a lipoid-protein film with the lipoid as layers of molecules with their hydrophilic groups directed towards the water and their lipophilic chains directed inwards and interlocking; the proteins are spread at the surface of the lipoids. It also seems probable that there is a definite spatial arrangement of enzymes in the cell and that some of these are formed by proteins on either side of the lipoid layer, e.g., succinic dehydrogenase.

If this view is correct, interpretation of the inhibition of growth brought about in *Azotobacter* by oximino compounds may be explained by orientation and combination or coordination of the polar molecules or ions of the oximes with specific centres of the enzymes or proteins, although these may be modified by the structure of the oximes themselves. It is possible, for example, that the oxime group may coordinate with semi-polar $> \overset{+}{C} - \overset{-}{O}$ groups in the specific protein.

Hydroxylamine is in a different category from the other oximes studied here; first, it is a kationic substance, and second, it may form oximes by condensation with keto groups. Endres (1935) found that oxygen uptake by *Azotobacter* was depressed by about 60 per cent. by 10^{-5}M hydroxylamine, figures which agree with our growth studies. It is also of interest to note here that Nakamura (1938) found 95 per cent. inhibition of photosynthesis in *Chlorella* by 10^{-4}M hydroxylamine, and Gaffron (1942) has indicated that it affects an oxygen-liberating enzyme and that its effect is not due to oxime formation but to specific interaction with the photochemical enzyme.

We suggest that the =NOH group of the ketoximes coordinates with groups on the specific protein, and in any case these molecules would be oriented with the hydrophilic =NOH toward the protein and their lipophilic hydrocarbon chains directed inward.

Similar considerations apply to the α-oximino-monocarboxylic acids, which, however, are strongly ionized and contain in addition to an =NOH group the hydrophilic —COO^- group with a tendency to coordinate with, for example, NH_2 groups on the protein. The hydrocarbon chains, however, will be directed towards the lipoids, as in the case of ketoximes.

In α-oximino-dicarboxylic acids, however, the situation is different. These compounds are strong acids, highly ionized, and with two —COO^- groups, one at each end of the molecule which is consequently less polar. The ion will therefore tend to coordinate with kationic groups on the specific protein and to orient itself parallel to the surface of the protein, a state of affairs unlike that of the oximino-monocarboxylic acids with their ions approximately at right angles to the protein surface. In the dicarboxylic acids, whether an =NOH group can bond or not with an appropriate surface group will depend upon the topography of the active surface. Such an explanation offers a reasonable basis for the relative toxicities of the oximino derivatives of the mono- and di-carboxylic acids.

However, this theory does not explain the non-toxicity of other substances examined, viz. esters of oximino-acids, dioximes, and diacetylmonoxime. It is true that in most of these compounds (except the esters) the presence of hydrophilic groups at each end of the molecule will cause it to lie on the protein surface, but does not explain why coordination of the oxime group with the protein may not occur.

We suggest that the relative non-toxicity of these compounds is due to their structure in which chelation occurs. Chelation—the inter- or intra-molecular coordination in systems containing donor and acceptor units—has been shown by infra-red spectroscopy to occur in dioximes (eg. in dimethylglyoxime and benzil dioxime) and in keto-oximes (e.g. benzil monoxime). Since chelation is similar to that leading to simpler open-chain coordination complexes, this process will compete with coordination of oximino groups with any active centres of a specific protein and accounts for the non-toxicity to *Azotobacter* of methyl- and dimethylglyoxime, γδ-dioximinovaleric acid and diacetyl monoxime. We suggest that the lack of toxicity of the esters of oximino-acids is also due to chelation since their structure is akin to that of a keto-oxime like diacetyl monoxime, for the introduction of the ethyl group into the carboxyl destroys the resonance of the latter. Pertinent structures are shown in Figure 1.

(*b*) *Utilization of Oximino-Acids as Source of Nitrogen*

The highest non-toxic concentration of hydroxylamine and oximino-propionic acid at which growth of *Azotobacter* occurred was respectively 5×10^{-6}M and 5×10^{-5}M; these correspond respectively to 0.0014 mg. N and

0.014 mg. N per 20 ml. culture solution. To determine whether utilization of these compounds occurred in complete absence of other sources of nitrogen is beyond the limits of accuracy of the methods employed. In a subsequent paper it will be shown that these two compounds are utilized by oat plants in non-toxic concentrations.

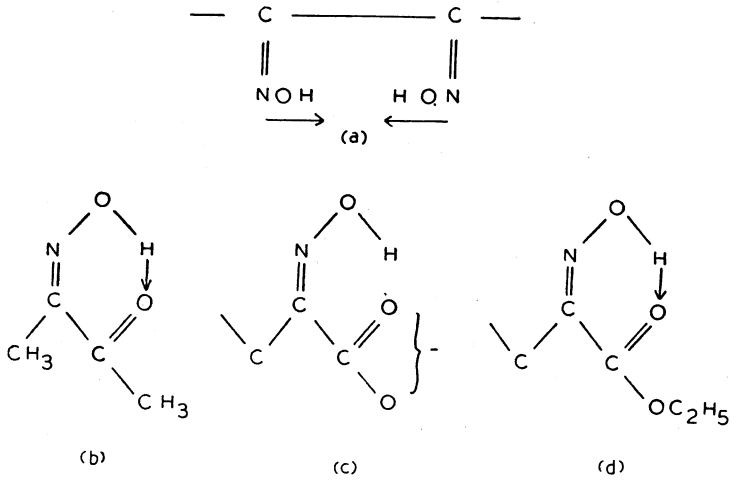


Fig. 1.—Illustrating the structural formulae of (a) dioximes; (b) diacetylmonoxime; (c) ion of an α-oximino-acid; (d) ester of an α-oximino-acid.

In *Azotobacter* cultures, the question whether the organism can utilize oximino-acids when deprived of all other forms of nitrogen was restricted to the oximinosuccinic and oximinoglutaric acids, where, owing to their relative non-toxicity, much larger concentrations can be employed. The organisms were grown, under the conditions described earlier, under a partial pressure of pure oxygen, in media containing the oximino-acids in 5 x 10^{-3}M, 1 x 10^{-3}M and 5 x 10^{-4}M concentration; these concentrations correspond to 1.4 mg., 0.28 mg., and 0.14 mg. of nitrogen per 20 ml. of culture solution. The N-contents of organisms grown at different concentrations at different incubation times are given in Table 2. The figures represent the amount of N derived from the oximino-acid and were obtained by deducting the N-content of the controls (which contained no added N in the medium) from that of the organisms grown on the oximes; the value of the controls in each experiment was approximately 0.05 mg. N.

Table 2 shows that the oximino-acids are utilized as a source of nitrogen and that the amount of organic nitrogen elaborated by the organism tends to increase both with concentration of the oximino-acid and with time. The experiments also indicate that *trans*-oximinoglutaric acid is more readily utilized than *trans*-oximinosuccinic acid and both of these more readily than *cis*-oximinoglutaric acid.

TABLE 2

AMOUNTS OF ORGANIC NITROGEN (MG. N) ELABORATED BY AZOTOBACTER GROWN ON 20 ML. CULTURE SOLUTION OXIMINO-ACIDS AT VARIOUS CONCENTRATIONS FOR DIFFERENT TIMES; PH 6.8.

Culture Solution	Age (hr.)	Molarity of Oximino-acid In Oxygen			In Air	
		5×10^{-3}	1×10^{-3}	5×10^{-4}	5×10^{-3}	Nil (Control)
Trans-oximinosuccinic acid	48	0.03	0.02	nil	0.18	0.17
	72	n.d.*	0.03	0.02	n.d.	n.d.
	72	0.03	0.05	n.d.	0.17	0.14
	84	0.13	0.09	n.d.	1.53	1.43
	120	0.13	0.09	0.11	0.38	0.47
	120	0.50	0.09	0.09	1.01	0.46
Trans-oximinoglutaric acid	48	0.09	0.02	nil	0.24	0.17
	72	0.37	0.06	0.02	0.78	0.26
	72	0.12	0.05	0.02	0.58	0.14
	84	0.41	0.16	0.01	1.80	1.43
Cis-oximinoglutaric acid	120	0.03	0.09	0.05	0.06	0.10
	120	nil	0.09	0.12	0.50	0.47

*n.d.—not determined.

The fifth column in Table 2 shows the amount of N fixed in air by cultures, run at the same time as those in the absence of atmospheric N, but with the addition of 5×10^{-3}M oximino-acid. These figures show that, at 5×10^{-3}M concentration N-synthesis from the oximino-acids is considerably less in oxygen than in air.

The sixth column in Table 2 gives N-content of organisms grown in air with no added N. In all cases differences between columns 5 and 6 are small compared with the total amount of N fixed, but generally are positive, which may mean that a small amount of N from the oximino-acids is utilized even when the organism is fixing N in air.

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