THE MECHANISM OF REDUCTION OF NITRATES AND NITRITES IN PROCESSES OF ASSIMILATION.

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The mechanism of reduction of nitrates of the alkali metals to ammonia and the formation of amino nitrogen in biochemical syntheses from inorganic nitrogen compounds have not been explained satisfactorily. Some investigators explain the transformation by assuming a direct reduction of the nitrates to ammonia by action of nascent hydrogen, while others assume an intermediate reduction of the nitrate to nitrite, from which, as the reduction proceeds, ammonia is produced. The exact chemical procedure by which bacteria or molds are able to produce ammonia or nitrates from alkali nitrates also has not been satisfactorily explained.

Schimper, the botanist, has been able to demonstrate experimentally that the reduction of nitrates in green leaves is connected intimately in some manner, not only with the influence of light, but also with the action of iron compounds in the leaves. This observation was so interesting to the writer that it led him to an investigation of the question whether iron actually takes part in the reduction of nitrates by means of bacteria. This work has proved very productive and, as a matter of fact, experiments have shown conclusively that the cholera bacillus, which possesses extraordinary reducing power for nitrates, has the ability to accumulate iron in its organism, and its reducing power may possibly be a function, not only of its ability to absorb oxygen by respiration, but also of its iron content.

The data revealed through this biological research, which was interrupted by the War and political disturbances in Europe,

1 Schimper, A. F. W., Bot. Z., 1890, xlvi, 73.
2 Unpublished data.
warranted a purely chemical investigation of the reduction of inorganic nitrates by means of iron salts. The opportunity to continue this work has now been offered to me, and, as a result, the study of this interesting problem has been taken up.³

An interesting paper by Menaul which recently appeared in this Journal,⁴ brings up for discussion a very important biochemical change. This investigator describes the action of formaldehyde on saltpeter in aqueous solution when exposed to sunlight, and the observation is made by him that in such solutions small quantities of hydrocyanic acid can be detected easily. This quite remarkable action of sunlight on nitrates was observed by the writer several years ago⁵ and this preliminary paper is now contributed to bring the results of this work before the American reader, and at the same time to present a summary of the principal results of his earlier investigations on the photochemical reduction of nitrates and the reduction of nitrates and nitrites with iron salts, which have appeared in various scientific publications during the last 10 years.⁶ This summary will be presented in three parts as follows: (1) Reduction of nitrates of alkali metals by means of light and also iron salts; (2) Reduction of nitrites of alkali metals by means of light and also iron salts; and (3) Synthesis of organic compounds containing nitrogen from inorganic compounds of nitrogen.

The Reduction of Nitrates.

In the course of researches on the photochemical decomposition and synthesis of nitrates and nitrites, the observation was made, for the first time, that one oxygen atom in nitrates must be bound in the molecule in a manner quite different from that of the oxygen atoms in nitrites. The writer has used the specific term "nitrate oxygen atom"⁷ to designate that oxygen atom which

³ Through cooperation with Prof. Treat B. Johnson, who has supplied rare research material, it has been possible to extend the field of investigation into the pyrimidine and purine series. The results of these researches, which are of immediate biochemical interest, will be published at a later date.

⁴ Menaul, P., J. Biol. Chem., 1921, xlvi, 297.
⁵ Baudisch, O., Ber. chem. Ges., 1916, xlix, 1151.
⁶ Baudisch, O., Ber. chem. Ges., 1911, xlv, 1009.
is easily split off from such salts either under the influence of light or of metallic iron. That we are dealing here with such a labile oxygen linking in nitrates is demonstrated to us by nature, in that the various nitrifying and denitrifying bacteria have the power to differentiate between nitrate and nitrite oxygen. The question naturally arises, under what influence or by means of what power an atom of oxygen can be split out of potassium nitrate, for example, with the formation of a nitrite. This problem was first attacked from a purely chemical standpoint, but it is now proposed to continue the study also from a biological point of view.

In explanation of the photochemical reduction of nitrates in aqueous solution, we may assume, according to Werner's theory of reaction, an activation of the residual valence of an oxygen atom of the nitrate and of the oxygen atom of the water, resulting in the attraction of molecules of water into the inner sphere of the nitrate molecule. There follows a dissociation of the nitrate molecule with the formation of oxygen as is expressed by the following equation:

\[
\begin{align*}
\text{K} & \left[ \begin{array}{c} 
\text{K} & \text{N} & \text{O} \\
\text{O} & \text{O} \\
\text{O} & \text{O} \\
\end{array} \right] \\
\text{light} & \rightarrow \text{K} \left[ \begin{array}{c} 
\text{N} & \text{O} \\
\text{O} & \text{O} \\
\text{O} & \text{O} \\
\end{array} \right] + \text{H}_2\text{O} \\
& \rightarrow \text{KNO}_2 + \text{O} + \text{H}_2\text{O}.
\end{align*}
\]

In other words, this reduction process takes place under the influence of light even in an atmosphere of oxygen, and stops at the nitrite stage. The reverse process can also take place and nitrite is readily formed from ammonia by photochemical oxidation without, however, any production of nitrate.

It is apparent from these facts that light can readily split off an atom of oxygen from nitrates of the alkali metals, without the presence or influence of either nascent or molecular hydrogen. This dissociation of nitrate into oxygen and nitrite can also be brought about by means of metallic iron as well as under the influence of the energy of light. If a neutral oxygen-free solution of potassium nitrate be shaken in a vacuum with active iron prepared by reduction with hydrogen, the supernatant liquor obtained...
after the iron powder has been allowed to settle will give every reaction applicable for the detection of nitrous acid. In other words, metallic iron will easily reduce potassium nitrate to potassium nitrite in the cold in the absence of every trace of oxygen, and under conditions such that neither the action on the iron by water nor the effect of nascent hydrogen can possibly come into consideration. These results lead to the assumption, therefore, that iron readily splits off an oxygen atom of the nitrate after having first entered into a loose combination with it. This change may be expressed by the following equation:

\[ \begin{array}{c}
K\left[ \begin{array}{c}
O \\
N \cdots \cdots \cdots O
\end{array} \right] + Fe \rightarrow K\left[ \begin{array}{c}
O \\
N \cdots \cdots \cdots Fe
\end{array} \right] \rightarrow KNO_2 + FeO.
\end{array} \]

From these examples it is seen that the photochemical reduction of nitrates to nitrites and their reduction by means of metallic iron are similar in nature, and in both cases there occurs either an activation or mobilization of the valence energy leading to the formation of an unstable addition product, which finally breaks down into the final products of reaction. Although it has been possible to show a relation between the photochemical reduction of nitrates to nitrites and the corresponding biological reduction in green leaves, it has not yet been possible to connect known chemical reduction processes with the biological reductions occurring naturally in bacteria or in molds.

It formerly appeared scarcely possible to attach any biological importance to ferrous hydroxide in these reduction processes because, as was commonly believed, the reaction proceeded stoichiometrically and ferric hydroxide was formed at the expense of the oxygen split off from the nitrate. In biological processes, however, one cannot speak of stoichiometrical reactions in connection with metals, because, as is well known, they are present only in traces. Their action must be explained, on the contrary, by a particular energy inherent in their molecules, and consequently the writer has been accustomed to consider the metals as functioning in biological processes either as "mobilizers" or "catalysts,"

having the power to bring into play the latent energy of certain organic molecules.

From the point of view of Werner's coordination theory, it is possible to conceive of a relation between the mobilizing power of such a catalytic agent, and the peculiar power expressed by residual valency, which, as is well known, has the ability to draw new atoms, molecules, and radicals into the sphere of action of the internal nucleus of the metal, resulting in the most varied types of reactions. In fact, it is well known that extremely finely divided metals, such as platinum, palladium, or iron, possess these valence powers to a large degree, and their specific action has often been placed in parallel with purely biological processes.

The reduction of nitrates with ferrous hydroxide assumed a new interest when it was discovered that this reagent alone does not split off nitrate oxygen as had been assumed, but reduces nitrates only under the influence of oxygen. As it is well known that white ferrous hydroxide is converted instantaneously into green ferrous hydroxide peroxide by the oxygen of the air, it is reasonable to say, therefore, that this polymolecular combination or peroxide is the active reagent which brings about this transformation of nitrates into nitrites.

\[
\begin{array}{c}
\text{O}_2 \\
\text{Fe} \\
\text{(OH)}_3 \\
\end{array}
\]

(Coordination formula for ferrous hydroxide peroxide.)

The mechanism of this reduction has not yet been explained, but the attempt will be made here to show that the free energy of ferrous hydroxide is increased enormously by a loose combination with an oxygen molecule, and that this increase in energy makes itself apparent both physically and chemically. The striking effect on the color of white ferrous hydroxide, which is caused by the smallest trace of oxygen, shows that the oxygen enters into the inner sphere of the iron nucleus. Schäfer has proved spectroscopically that such extraordinary alterations in color, either in the visible or in the invisible part of the spectrum, can only take place simultaneously with changes in the inner sphere.

of the molecule. It has, however, not yet been shown how many oxygen molecules are present in such a molecular combination. It may be possible that the observation of Meyer,\textsuperscript{12} who discovered that strongly magnetic substances were rich in absorption bands, whereas diamagnetic substances were poor in absorption bands, has some connection with these facts. This coincides completely with the action of the above mentioned iron compound, because, while the white ferrous hydroxide has practically no magnetic properties, the green to black ferrous hydroxide peroxide possesses magnetic properties which are almost equal to that of metallic iron. According to Hilpert,\textsuperscript{13} when a stream of air or oxygen is led through a precipitate of ferrous hydroxide, the magnetic properties of the precipitate increase rapidly. Quartaroli\textsuperscript{14} has also shown that oxidation with air converts the ordinary non-magnetic ferrous hydroxide into mixed ferro-ferri oxides, which possess a susceptibility almost a hundred times greater than ferric salts. In fact the magnetic susceptibility of Fe$_2$O$_4$ approaches that of the metal iron itself. It may be concluded, therefore, from our present knowledge, that there is a very close relationship between the physical properties of metallic iron and those of ferrous hydroxide peroxide, and it will be of the greatest interest and importance to determine whether there is also any direct relationship between the peculiar chemical properties of this peroxide and those of finely divided metals. The simplest explanation, therefore, for the reduction of nitrates is the assumption that freshly precipitated, colloidal ferrous hydroxide peroxide acts catalytically as a finely divided metal.

It seems very probable that the peculiar properties resulting from the colloidal nature of ferrous hydroxide peroxide and the properties of the metal resulting from its position as the central atom of a complex system actually coalesce. Furthermore, these characteristic properties apply only to the peroxide and not to ferrous hydroxide, because the latter compound is not only unable to bring about a reduction of nitrates, but also will not react to form polynuclear compounds. Not until brought under the influence of oxygen does the central iron nucleus of ferrous hy-

\textsuperscript{13} Hilpert, S., \textit{Ber. chem. Ges.}, 1909, xlii, 2248.
\textsuperscript{14} Quartaroli, A., \textit{Chem. Zentr.}, 1917, i, 729.
droxide or other ferrous salts acquire the property of attracting new molecules of ferrous hydroxide into its inner sphere. This coordination combination exists, according to Werner, not between iron and iron, but between the active hydroxyl oxygen atoms of the ferrous hydroxide, which are attracted to the central iron nucleus of the peroxide by its residual valency. It is also possible for the iron atoms of ferrous hydroxide to be held in combination through the peroxide oxygen atoms, as has been demonstrated in the case of cobalt compounds by the classical researches of Werner. The mechanism of the autooxidation of ferrous hydroxide and the formation of strongly magnetic Fe$_3$O$_4$·$x$H$_2$O may be expressed by the following formulas:

\[
\begin{align*}
\left[ \text{Fe(OH)}_6 \right] (\text{OH})_2 + O_2 & \rightarrow \left[ \text{FeO} \right] \text{O}_2 \left( \text{OH})_2 \right] (\text{OH})_2 + 2\text{Fe(OH)}_2 \\
\left[ \text{Fe(OH)}_6 \right] (\text{OH})_2 + O_2 & \rightarrow \left[ \text{FeO} \right] \text{O}_2 \left( \text{OH})_2 \right] (\text{OH})_2 + 2\text{Fe(OH)}_2 \\
\left[ \text{FeO} \right] \text{O}_2 \left( \text{OH})_2 \right] & = \text{Fe}_3\text{O}_4 + x\text{H}_2\text{O} + O.
\end{align*}
\]

The structure of this polynuclear combination may be expressed graphically as follows:

From these graphic representations it is seen that ferrous hydroxide is converted by the absorption of oxygen into a peroxide of greater potential energy, whose iron nucleus, as experience has shown, possesses the property of intensifying the activity of and of entering into loose combination with the residual valence of oxygen atoms in other molecules of ferrous hydroxide present.
With the coordination theory of Werner as a basis, it becomes apparent from the foregoing that ferrous hydroxide may be transformed by the absorption of oxygen into a complex salt whose iron nucleus, just as finely divided metals, may enter into a wide range of reactions. A nitrate oxygen atom may be split off as well by means of light as by means of metallic iron or by ferrous hydroxide peroxide. All three of these processes of reduction may be considered to depend upon the same principle; namely, the mobilization or activation of the energy in the residual valence of the reacting materials. Ferrous hydroxide peroxide reacts most probably in a very similar manner to finely divided iron or platinum.

The Reduction of Nitrites.

Aqueous solutions of potassium nitrite containing easily oxidizable substances, such as alcohols, aldehydes, sugars, starches, etc., suffer a comparatively rapid reduction and decomposition under the influence of diffused daylight, and the change may be expressed very simply as follows:

\[ \text{KNO}_3 = \text{KNO} + \text{O}. \]

The presence of potassium nitrosyl in solution may be detected by means of its condensation reaction with aldehydes (Angeli's aldehyde reaction).\(^{15}\) Hydroxamic acids are formed as products of reaction, and as is well known, these acids give characteristic complex salts with iron which are colored a deep reddish violet. This reduction of potassium nitrite to potassium nitrosyl can also be accomplished by means of complex iron salts. The smooth reduction of potassium nitrite via potassium nitrosyl to ammonia by means of glucose, in the presence of very small quantities of iron, possesses particular biological interest. The system, glucose + iron + alkali, which is a fundamentally new reducing combination, does not attack in the least the alkali salts of nitric acid.\(^{16}\) It is therefore possible to make a quantitative separation


\(^{16}\) A method, based on this observation, has been developed for the quantitative determination of nitrites and nitrates in the presence of other nitrogen compounds in soil extracts. Pfeifer, T., and Simmerbacher, W., *Land. Versuchsstat.*, 1916, xeciii, 65; *J. Soc. Chem. Ind.*, 1919, xxxviii, 507.
between nitrate and nitrite by means of a grape sugar-iron-alkali solution. Grape sugar, which is absolutely free from iron, does not cause the slightest reduction, even on heating, of nitrites; and also chrysarobin (1,8-dioxy-3-methyl-anthranol), which occurs quite widely in the vegetable kingdom, has not the ability of reducing the salts of nitrous acid. On the other hand, the addition of traces of any iron salt to an alkaline solution of either of these substances enables them to reduce immediately the nitrites by way of nitrosyl to free ammonia. The particular part that iron plays in these reactions remained a mystery for a long time, but seems now to have been explained quite satisfactorily.

To explain this reduction process and its application for the quantitative separation of nitrates and nitrites, we may assume that the unsaturated, trivalent nitrogen atom of the nitrite molecule enters, through its activated residual valency, into a loose combination with the central iron nucleus of whatever complex salt is present, and then dissociates through the intermediate formation of nitrosyl into NO and K. The residual valency of the nitrate oxygen atom is not sufficiently active or powerful to displace the molecules or radicals already present in the inner sphere of the iron nucleus, and therefore these compounds are not attacked. That these reductions may all be considered as complex salt reactions, or in other words "nuclear exchange or displacement reactions" may be shown by the following example: A solution of 1 gm. of K₄Fe(CN)₆, 1 gm. of NaNO₂, and 5 gm. of sodium carbonate in 200 cc. of water, is distilled in a stream of oxygen. After interrupting the stream of oxygen, it is possible to detect nitrous acid in the distillate. The oxygen, under the influence of heat, has displaced the cyanogen group from the inner sphere, and in its place a molecule of nitrite has entered. The nitrite, however, decomposes and its scission product, NO, which at first takes its place in the inner sphere of the iron nucleus, is in turn displaced by the oxygen, and finally passes over into the distillate where it is easily detected as nitrous acid.

The fact that this remarkable reaction may not only be influenced by daylight, but in some cases will not take place except under the influence of daylight, is of particular chemical and biological interest. For example, if a freshly prepared solution of potassium ferrocyanide be treated with an aqueous alcohol
solution of nitrosobenzene and placed under the influence of diffused daylight, the solution which in the beginning possesses a weak, greenish yellow color, changes in a few minutes to a deep reddish violet. The mechanism of the reaction may be expressed as follows:

\[
\left[ \text{Fe(CN)}_6 \right]^2- + \text{C}_6\text{H}_5\text{NO} \rightarrow \left[ \text{Fe(CN)}_6 \right]^3+ + \text{KCN}.
\]

On treating the aqueous solution of this reddish violet compound with an excess of potassium nitrite and again placing the solution under the influence of diffused daylight the following decompositions take place:

\[
\begin{align*}
\text{KNO}_3 & \rightarrow \text{KNO} + \text{O} \\
\text{KNO} & \rightarrow \text{K} + \text{NO} \\
\text{H}_2\text{O} + \text{K} & \rightarrow \text{KOH} + \text{H}
\end{align*}
\]

\[
\left[ \text{Fe(CN)}_6 \right]^3+ + \text{KNO}_2 \rightarrow \left[ \text{Fe(CN)}_6 \right]^{4+} \text{K}_3 + \text{C}_6\text{H}_5\text{NO}.
\]

The deep violet color of the solution disappears very rapidly and the reddish yellow or potassium nitroprusside takes its place. By means of this reaction, the reduction of sodium nitrite by means of a complex iron salt and light is demonstrated.

These processes of reduction of nitrates by way of nitrosyl to ammonia, may be drawn into intimate relation with biological reductions of nitrite, particularly as sugar, or its products of decomposition, and iron constantly accompany the nitrates in plants or in bacteria. Kostyschew and Tswekowa\(^{17}\) state that the reduction of nitrate to nitrous acid takes place without the presence of any sugar, but that the further conversion of the nitrous acid, at least in the case of \textit{Mucor racemosus}, is accomplished only in the presence of sugar. It seems likely, from investigations with cholera bacteria, that nitrates are reduced to nitrites by way of nitrosyl, because it was possible to detect the alkaline decomposition products of nitrosyl, for example, NO and NH\(_3\) (the latter as a reduction product of NO), in the volatile portions of alkaline cholera peptone cultures.\(^{18}\)


\(^{18}\) Baudisch, O., \textit{Ber. chem. Ges.}, 1916, xlix, 1148.
The assumption that nitrite is converted into nitrosyl finds a
further support in the qualitative and quantitative composition
of the gases which are produced during the photochemical reduc-
tion of nitrites in the presence of formaldehyde and during the
biological reduction. For example, those bacteria which have
the property of decomposing nitrates, produce a fermentation
gas which consists of about 65 to 72 per cent N₂O.⁶ In addition
to the nitrous oxide, there is also always formed a little nitrogen,
traces of NO, and also of prussic acid. A formaldehyde-potassium
nitrite solution produces a gas under the influence of diffused
daylight, which contains 64 per cent N₂O, as well as very small
quantities of NO and HCN. The latter two gases were detected
qualitatively by sensitive reactions. Clawson and Young¹⁹ have
detected prussic acid in cultures of Bacillus pyocyaneus and of
other bacteria.

To summarize, the photochemical reduction of the alkaline
nitrites proceeds by way of the reactive intermediate product,
potassium nitrosyl, which may be detected by means of aldehydes,
as well in the case of the photochemical reduction of nitrites as
in the case of a reduction with the system, grape sugar + iron
+ alkali. Certain complex iron salts possess the property of
reducing nitrites, whereas under these conditions nitrates remain
unchanged. The reduction of the alkali nitrites by means of
complex iron salts depends most likely upon the residual valency
of the central iron nucleus and in all these changes light as well
as heat exercises a very fundamental influence.

Synthesis of Organic Compounds Containing Nitrogen from Inorganic
Compounds of Nitrogen.

Nitrosyl, which is formed in the reduction of alkali nitrates,
interacts readily with formaldehyde with formation of formhy-
droxamic acid (Angeli’s aldehyde reaction). This reaction
proceeds, as was shown by the writer and Coert,²⁰ through the
intermediate formation of nitroso methyl alcohol. Formhydrox-
amic acid is then formed from this by molecular rearrangement,
and may be detected easily by means of its characteristic iron
and copper salts, which are highly colored.

¹⁹ Clawson, B. J., and Young, C. C., J. Biol. Chem., 1913, xv, 419.
Formhydroxamic acid, under the influence of light, loses an atom of oxygen and is converted into formaldoxime, which is also characterized by its great reactivity. The stable form of this compound is altered under the influence of light and also alkali, and is transformed into an extremely labile modification. This transformation may be expressed as follows:\(^{21}\)

As is well known, formaldoxime exhibits a strong tendency to polymerize with the formation of three-carbon chain compounds. Its labile form is also capable of reacting further with aldehydes with the formation of three-carbon compounds, and furthermore under the influence of light combines with formaldehyde to form cyclic combinations containing both nitrogen and carbon. Under the influence of light formaldoxime undergoes, in part, a Beckmann rearrangement with formation of formamide, and, in part, a complete dissociation into prussic acid, water, and ammonia. These changes are expressed below:

The small quantities of prussic acid that are always found accompanying the treatment of formaldehyde in nitrate solutions with light, or by the reduction of nitrates with bacteria, may have been formed in accordance with the above reaction from the aldoximes. The photochemical formation of nitroso methyl alcohol (or hydroxamic acid) from formaldehyde, methyl alcohol, and nitrosyl may be the chemical counterpart of a possible biochemical formation of carbon-nitrogen-containing organic substances from inorganic nitrogen. The next step to amino nitrogen is simpler and may either take place by reduction or, as in the case of aldoxime, by simple rearrangement. It seems probable that the proof of the biological importance of nitroso methyl alcohol or formhydroxamic acid is found in its marked reactivity and in the pronounced tendency which it has to rearrangement, to polymerization, and to the formation of complex salts, particularly of iron. It seems extremely possible to introduce amino nitrogen into the higher alcohols, sugars, starches, etc., by means of nitrosyl which is formed photochemically from the nitrites and which is capable of entering into such widely different reactions.

Recent work\textsuperscript{10} has revealed the fact that light may be replaced in certain cases by means of ferrous hydroxide and oxygen. Fer-
rous hydroxide peroxide oxidizes alcohols to aldehydes simultaneously with the reduction of nitrites to nitrosyl, and therefore synthesis of formhydroxamic acid or formaldoxime takes place. In fact, in these solutions of ferrous sulfate, containing bicarbonate and nitrite, three dissimilar reactions proceed simultaneously—oxidation, reduction, and synthesis.

To summarize, nitrosyl is formed from alkali nitrites photochemically and by reduction with glucose in the presence of iron and by reduction with ferrous hydroxide in the presence of oxygen. The formation of carbon and nitrogen organic compounds in green plants and bacterial cultures from inorganic nitrogen, and the production of \( \text{N}_2\text{O}, \text{N}_2, \text{NO}, \) and HCN during fermentation and photochemical reduction may be explained by the intermediate formation of nitrosyl \( \text{H}[\text{NO}^\cdot] \) and its subsequent reaction with aldehydic combinations.

\(^{22}\) Bracket indicates labile character.
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