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The Reduction of Nitroprusside by Hydrogen with *Proteus vulgaris*¹

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The hydrogenase activity of *Proteus vulgaris* is inhibited by several iron pentacyano compounds; it is not inhibited by the iron hexacyano compounds. The effects of some cyano complexes of cobalt, molybdenum and manganese have also been tested. The enzyme hydrogenase catalyses the reduction of nitroprusside by one mole of hydrogen. An intermediate blue compound forms which can be oxidized by oxygen, iodine and ferricyanide.

We have previously shown that the enzyme hydrogenase is inhibited by very low concentrations of nitric oxide.² In a search for other inhibitors we have tested the effect of nitroprusside, Na₂[Fe(CN)₅NO], and some other cyano complexes of iron, molybdenum, cobalt and manganese on the activity of hydrogenase.

Experimental

The effect of the various inhibitors was tested either on intact cells of *Proteus vulgaris* or on cell-free extracts.³ The enzyme activity was measured by the activation of molecular hydrogen as indicated either by the rate of the ortho-para hydrogen conversion or the rate of the exchange reaction between HDO and H₂.⁴ For the exchange reaction, the flask contained 0.5 ml. of D₂O (99.8%), the appropriate amount of bacterial suspension, and 0.15 M phosphate buffer pH 6.7, to make the volume 5 ml.; the gas phase was normal hydrogen. For the conversion reaction, D₂O was omitted and parahydrogen used as the gas phase.

The pentacyano-iron compounds were prepared according to Hofmann⁵; the cobalt, manganese and molybdenum compounds were prepared according to Brauer.⁶ All other compounds were commercially available preparations used without further purification.

Results

The Inhibition of Hydrogenase by Pentacyano and Hexacyano Complexes.—The inhibitory effect of nitroprusside on the exchange reaction is shown in Table I. The inhibition rises as the concentration of nitroprusside is increased from 10⁻⁵ to 10⁻⁴ M, then decreases until a concentration of about 10⁻² M is reached, then rises rapidly to become almost complete at 0.2 M. The same phenomena are observed in the effect of nitroprusside in inhibiting the ortho-para conversion by hydrogenase where there was 62% inhibition at 10⁻⁴ M nitroprusside and 48% inhibition at 10⁻² M. In neither case is the inhibition removed by the addition of sodium dithionite (Na₂S₂O₄), indicating that the inhibition is not due to oxidation or oxygenation of the enzyme³. This inhibition also was observed with cell-free extracts.

The inhibition is not due to the presence of nitric oxide, since no nitric oxide (mass 30) could be de-

(1) This work was aided by a contract between the Office of Naval Research, Department of the Navy, and Columbia University (ONR 28802). Reproduction of this article in whole or in part is permitted for any purpose of the United States Government.

(2) A. I. Krasna and D. Rittenberg, *Proc. Natl. Acad. Sci.*, **40**, 225 (1954).

(3) H. F. Fisher, A. I. Krasna and D. Rittenberg, *J. Biol. Chem.*, **209**, 569 (1954).

(4) A. I. Krasna and D. Rittenberg, *THIS JOURNAL*, **76**, 3015 (1954).

(5) K. A. Hofmann, *Ann.*, **312**, 1 (1900).

(6) G. Brauer, "Handbuch der Präparativen Anorganischen Chemie," Ferdinand Enke Verlag, Stuttgart, 1954.

TABLE I

INHIBITION OF THE EXCHANGE REACTION BY SODIUM NITROPRUSSIDE Na₂Fe(CN)₅NO

Each flask contained 1.0 ml. of a cell suspension of *Proteus vulgaris* (0.78 mg. N/ml.) made up to 5 cc. with 0.15 M phosphate buffer pH 6.7 and having a final D₂O concentration of 10%.

Nitroprusside concn., M	Rate constant (k ^a × 10 ³)	Inhibition, %
0	1.8	..
10 ⁻⁵	1.5	16
10 ⁻⁴	0.56	69
10 ⁻³	0.79	56
5 × 10 ⁻³	0.85	53
10 ⁻²	0.97	46
10 ⁻¹	0.25	87
2 × 10 ⁻¹	..	95

^a k is the first-order rate constant for the reaction.

tected by mass spectrometric analysis of the gas phase of an inhibited system.

The effect on the exchange reaction of some iron pentacyano compounds in which the NO radical is replaced by other groups is shown in Table II. The first six compounds listed contain ferrous iron and the last two ferric iron. The two ferric compounds showed lag periods longer than that of the control. The ferrous compounds, with the exception of the carbon monoxide compound, had approximately the same lag period as the control. The ferric compounds are reduced by hydrogenase and the extra lag period is the time required to bring about this reduction. The inhibition observed with the ferric compounds is undoubtedly due to the corresponding ferrous compounds which were formed by reduction. With the carbon monoxide compound, there was a lag period 65 minutes

TABLE II

INHIBITION OF EXCHANGE REACTION BY IRON PENTACYANO COMPOUNDS

Each flask contained 1.0 ml. of a cell suspension (1.06 mg. N/ml.).

Compd. tested (10 ⁻³ , M)	Inhibition, %	Lag period (min.)
Control	..	23
Na ₂ [Fe ^{II} (CN) ₅ NO]	60	41
Na ₃ [Fe ^{II} (CN) ₅ H ₂ O]	64	38
Na ₄ [Fe ^{II} (CN) ₅ NO ₂]	45	20
Na ₃ [Fe ^{II} (CN) ₅ NH ₃]	62	44
Na ₅ [Fe ^{II} (CN) ₅ SO ₃]	86	27
Na ₃ [Fe ^{II} (CN) ₅ CO]	0	87
Na ₂ [Fe ^{III} (CN) ₅ H ₂ O]	65	65
Na ₂ [Fe ^{III} (CN) ₅ NH ₃]	80	100

longer than the control. After this time there was no inhibition either in the light or the dark.

The effect of ferrous iron, ferric iron, ferrocyanide and ferricyanide on the activity of hydrogenase is shown in Table III. None of these compounds caused any inhibition. With all compounds, except ferricyanide, the lag period was about 30 minutes; with ferricyanide it was 80 minutes. This is similar to the effect observed with the ferro- and ferri-pentacyano complexes.

TABLE III

INHIBITION OF THE EXCHANGE REACTION BY IRON COMPOUNDS

Each flask contained 1.0 ml. of a cell suspension (0.3 mg. N/ml.).

Compd. tested (10^{-3} , M)	Rate constant ($k \times 10^3$)	Inhibition, %	Lag period (min.)
Control	3.2	..	30
$K_4Fe(CN)_6$	2.9	9	30
$K_3Fe(CN)_6$	2.9	9	80
Fe^{++}	2.9	9	24
Fe^{+++}	2.9	9	24
$Na_2Fe(CN)_5NO$	1.3	59	34

The enzyme is inhibited by iron pentacyano complexes, with the exception of the CO complex, but not by the hexacyano complexes. It is of interest that the sixth group of the pentacyano compounds is less firmly bound than the cyano groups of both the hexa- and pentacyano compounds. The inhibition could arise from the substitution of the sixth group of the pentacyano compounds by an active group of the enzyme.

The effects of some cobalt, molybdenum and manganese cyano compounds on the activity of hydrogenase are shown in Table IV.

TABLE IV

INHIBITION OF THE EXCHANGE REACTION BY OTHER CYANO COMPOUNDS

Each flask contained 0.07 ml. of a cell suspension (4.3 mg. N/ml.).

Compd. tested (10^{-3} , M)	Inhibition, %	Lag period (min.)
Control	..	16
$K_3[Co(CN)_6]$	10	18
$K_4[Mo(CN)_6NO]$	60	14
$K_3[Mn(CN)_6]$	45	16
$K_4[Mn(CN)_6]$	57	16
$K_3[Mn(CN)_5NO]$	12	18

Reduction of Nitroprusside.—While testing the inhibitory effect of nitroprusside on the exchange reaction it was observed that the solution which was originally a faint orange changed to blue, green, dark green, and finally to yellow brown in color. This suggested that the nitroprusside was being reduced by the hydrogenase system at the same time that it was partially inhibiting the enzyme. Quantitative determinations showed that nitroprusside is reduced with the consumption of one mole of hydrogen for every mole of nitroprusside (see Table V).

When the reduction of nitroprusside was effected in weakly buffered solutions, the pH at the end was lower than at the beginning. Back titration with alkali showed that one equivalent of hydrogen ion

TABLE V

OXIDATION OF REDUCED COMPOUNDS WITH IODINE

Each flask contained 0.3 ml. of a cell suspension (10.6 mg. N/ml.) made up to a total volume of 3 ml. with 0.15 M phosphate buffer pH 6.7.

Nitroprusside added (μ mole)	Color of reduced product	H ₂ consumed during enzymatic reduction (μ mole)	I ₂ consumed by reduced product (μ equiv.)
3	Brown ^a	2.9	..
5	Brown ^a	4.9	..
10	Brown ^a	9.8	32.7
10	Brown ^a	9.7	29.9
15	Brown ^a	13.0	..
10	Blue ^b	4.5	11.6
10	Blue ^b	5.14	16.0
20	Blue ^b	8.9	24.9
20	Blue ^b	9.8	27.5

^a These reductions were allowed to proceed to completion and then excess iodine solution was immediately added and titrated with thiosulfate. ^b These reductions were stopped in the middle by opening the flasks to air. Excess iodine was then added immediately and titrated with thiosulfate.

was produced per mole of hydrogen consumed (Table VI).

TABLE VI

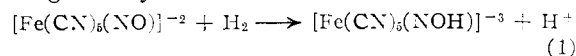
ACID PRODUCED DURING REDUCTION OF NITROPRUSSIDE

The buffer concentration in these experiments was 0.025 M and the total volume of the system was 3 ml.

Nitroprusside added (μ mole)	Color of reduced product	H ₂ consumed during enzymatic reduction (μ M.)	Acid produced during reduction (μ eq.)
10 ^a	Brown	7.8	7.2
10 ^b	Brown	8.4	7.6
20 ^c	Blue	5.0	4.8

^a The system contained 0.6 ml. of a suspension of *Proteus vulgaris* (6 mg. N/ml.) and the initial pH was 7.22. After reduction was complete, the pH was 6.84 and 1.73 ml. of 0.0042 N NaOH was required to bring the pH back to the original value. ^b This system contained 0.6 ml. of a cell suspension (6 mg. N/ml.) and the initial pH was 7.58. After reduction was complete, the pH was 7.00 and 1.8 ml. of 0.0042 N NaOH was required to bring the pH back to 7.58. ^c This system contained 0.2 ml. of a cell suspension (10.6 mg. N/ml.) and the initial pH was 7.96. When the reduction was stopped, the pH of the system was 7.58 after atmospheric oxidation and 0.9 ml. of 0.0053 N NaOH was required to bring the pH back to 7.96.

In the nitroprusside ion, $[Fe(CN)_5NO]^{-2}$, iron is in the ferrous state and the NO group is present as the positively charged nitrosyl group, NO^+ .⁷ On the basis of this formula it seems improbable that the reduction involves the iron since it is already in the ferrous state, but it is probable that the nitrosyl radical is reduced. The data suggest the reaction for the reduction of nitroprusside by the hydrogenase system to be



The reduced product is oxidized by neutral iodine (see Table V), requiring three equivalents of iodine per mole of compound. The oxidized product gives a test for nitrite by the method of Griess.⁸ Two equivalents of iodine are required to oxidize the nitrogen at the oxidation level of NOH (hyponitrous

(7) N. V. Sidgwick, "The Chemical Elements and their Compounds," Oxford University Press, 1952, p. 1343.

(8) P. Griess, *Ber.*, **12**, 427 (1879).

acid) to nitrite; the other equivalent must be used to oxidize the iron to the ferric state. All these data suggest that the oxidized compound has the composition $\text{Na}_3[\text{Fe}^{\text{III}}(\text{CN})_5\text{NO}_2]$.

The different colors observed during the reduction of nitroprusside by hydrogenase indicate that the reduction takes place in stages. The first compound produced during the reduction is blue. The final compound is brown. During the intermediate states the solution goes through various shades of green which probably are due to mixtures of the blue and brown compounds. The same sequence of colors is observed when nitroprusside is reduced by hydrosulfite.

The blue solution formed after reduction with half a mole of hydrogen is bleached by air to yield a yellow brown solution. If nitroprusside is half reduced, air admitted to bleach the blue solution and the reduction now continued, the total amount of hydrogen consumed is not greater than when the reduction is completed in one step (see Table VII). The oxidation of the blue solution yields a compound other than nitroprusside. This solution, after oxidation by air, gives a test for nitrite ion; nitroprusside does not. The blue color is discharged when oxidized by ferricyanide or iodine and the test for nitrite becomes positive.

TABLE VII

REDUCTION OF NITROPRUSSIDE IN STAGES BY HYDROGENASE

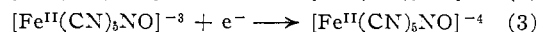
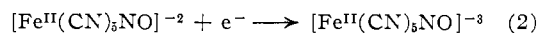
Cell concn. (mg. N)	Nitroprusside (μmoles)	Hydrogen consumed when reduction is interrupted (μmole)	Hydrogen consumed (μmoles)		Total
			Before opening to air	After opening to air	
0.62	3.0	2.45	1.16	1.00	2.16
0.62	5.0	3.80	2.20	1.40	3.60
1.24	5.0	4.20	1.98	2.18	4.16

It would appear that the blue compound is $[\text{Fe}^{\text{II}}(\text{CN})_5\text{NO}]^{-3}$ in which the nitrosyl radical, NO^+ , of the nitroprusside is reduced to the neutral NO. The product formed by air oxidation of the blue compound is not further oxidized by a neutral iodine solution. Oxidation of the blue compound by neutral iodine consumes approximately three equivalents of iodine per mole of hydrogen consumed (see Table V). Oxidation of the blue compound, $[\text{Fe}^{\text{II}}(\text{CN})_5\text{NO}]^{-3}$, to $[\text{Fe}^{\text{II}}(\text{CN})_5\text{NO}_2]^{-4}$ would require two equivalents of iodine per mole of hydrogen

consumed. The consumption of more than this quantity of iodine suggests that the iodine also oxidized the $[\text{Fe}^{\text{II}}(\text{CN})_5\text{NO}_2]^{-4}$ to $[\text{Fe}^{\text{III}}(\text{CN})_5\text{NO}_2]^{-3}$. This oxidation would require another two equivalents of iodine per mole of hydrogen consumed. The uptake of less than this quantity of iodine is probably due to partial oxidation of the blue compound by air. (The order of this oxidation may be the reverse, *i.e.*, the iron of $[\text{Fe}^{\text{II}}(\text{CN})_5\text{NO}]^{-3}$ may first be oxidized and then the NO group.) Since the compound formed by air oxidation of the blue compound is not further oxidized by iodine it seems likely that the compound obtained by air oxidation also is $[\text{Fe}^{\text{III}}(\text{CN})_5\text{NO}_2]^{-3}$.

When the reduction was interrupted after the nitroprusside was only partly reduced, it was found that one equivalent of hydrogen ion was formed per mole of hydrogen consumed (see Table VI). It appears that the hydrogen ion is formed during the uptake of the first equivalent of hydrogen by the nitroprusside. The formation of this quantity of hydrogen ion is also consistent with the structure assigned to the air oxidized compound.

Kolthoff and Toren⁹ have studied the reduction of nitroprusside at the dropping mercury electrode and from their data have postulated that the reduction proceeds according to the equations



Our findings on the enzymatic reduction are essentially in accord with their suggestion.

Both the blue compound as well as the final product of reduction are unstable at room temperature. After a few hours a blue precipitate forms which is probably prussian blue.

Nitroprusside is reduced by the hydrogenase system of *Desulfovibrio desulfuricans* but not by that of *Rhodospirillum rubrum*.

There was no reduction of nitroprusside (no color change) when platinum oxide was used in place of the bacteria.¹⁰ Nitroprusside appears to be reduced, as evidenced by the appearance of a dark blue color, by sodium hydrosulfite, sodium formaldehyde sulfoxylate and sodium borohydride.

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(9) I. M. Kolthoff and P. E. Toren, *THIS JOURNAL*, **75**, 1197 (1953).

(10) "Gmelins Handbuch der Anorganischen Chemie," 8th Edition, Vol. 59B, Berlin, 1932, p. 736.