Estimation of the Nitric Oxide formed from Hydroxylamine by *Nitrosomonas*

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(Received 7 May 1964)

1. Nitric oxide that was produced by reducing nitrite with an excess of acidified potassium iodide under nitrogen in Warburg respirometer flasks was rapidly absorbed by a solution of permanganate in sodium hydroxide held in the side arm. A small amount of nitrous oxide (or nitrogen) that was also produced was not absorbed. 2. By using a quantitative method for the recovery of nitrite from samples of the alkaline permanganate, it was found that the sum of the nitrite N formed and the residual nitrous oxide N was equivalent to the nitrite N used to generate the gases. These results showed that alkaline permanganate completely oxidized nitric oxide to nitrite. The method was suitable for determining 0.4–20 μ moles of nitric oxide. 3. The technique was used to determine the nitric oxide content of the nitrogenous gas that was produced anaerobically from hydroxylamine by an extract of the autotrophic nitrifying micro-organism *Nitrosomonas* in the presence of methylene blue as electron acceptor.

Differential manometric techniques were used by Chung & Najjar (1956) and by Anderson (1964) for the determination of nitric oxide, together with nitrous oxide or nitrogen, formed by the anaerobic metabolism of inorganic nitrogen compounds. Potassium hydroxide in the centre well of one Warburg flask did not absorb the nitrogenous gases, but alkaline sulphite in the centre well of a second flask, which contained an identical enzymic reaction mixture, rapidly converted nitric oxide into the stable dinitrosulphite ($\text{Na}_2\text{SO}_3 \cdot 2\text{NO}$). Since the calculation of nitric oxide formation from the difference in volume assumes that the same amount of nitric oxide is produced in each flask, a direct determination is desirable.

Najjar & Allen (1954) found that a known amount of dinitrosulphite in acid quantitatively decomposed to nitrous oxide according to the equation:

This direct method seems to have been replaced by the differential technique (Chung & Najjar, 1956). The present paper describes a direct method in which an alkaline permanganate solution quantitatively oxidizes nitric oxide to nitrite; nitrite is completely recovered in samples after adding arsenite to precipitate the excess of permanganate.

The nitric oxide for these experiments was generated by reducing nitrite with an excess of acidified iodide according to the reaction:

Johnston & Giauque (1929) found the reaction produced almost pure nitric oxide. Since small amounts of nitrous oxide were also formed, it was necessary to make nitrogen-balance experiments by using Warburg respirometers.

Extracts of the autotrophic nitrifying micro-organism *Nitrosomonas* catalyse the anaerobic formation of nitric oxide and nitrous oxide or nitrogen from hydroxylamine with methylene blue as electron acceptor, but no nitrite is formed. The nitric oxide was estimated differentially by Anderson (1964). Falcone, Shug & Nicholas (1963) used extracts of the same strain of *Nitrosomonas* but did not find nitric oxide when the nitrogenous gas mixture was analysed in the mass spectrometer. Aleem & Lees (1963), who used an extract of a different strain of the micro-organism, found that a nitrogenous gas was produced in the Warburg, but no nitrite was formed; these workers assumed that the gas was nitrous oxide. In the present work the amount of nitric oxide found by using the direct method (alkaline permanganate) is compared with that obtained by using the differential method (Anderson, 1964).

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EXPERIMENTAL

Reagents. Nitrogen containing only 0.02% of oxygen was obtained from Air Products Ltd., London, W. 1. Reagents were analytical grade wherever possible. Water was de-ionized. Special reagents were prepared as follows: (a) alkaline permanganate was a freshly prepared solution of 125 mM-potassium permanganate in N-sodium hydroxide; (b) alkaline arsenite was a solution of 1.3% (w/v) sodium arsenite in 0.67 N-sodium hydroxide; (c) alkaline sulphite was a solution of 10% (w/v) sodium sulphite in 0.5 N-sodium hydroxide.

Recovery of nitrite from alkaline permanganate. Manganese compounds interfered with the colorimetric estimation of nitrite as azo dye. The following method for recovering nitrite from alkaline permanganate avoided this interference. Samples (0.2 ml.) of alkaline permanganate containing 0.2–20 μ moles of nitrite were mixed with 3 ml. of alkaline arsenite. The resulting brown precipitate of manganese salts (mainly the dioxide) was diluted to 5 ml. with 1.3 ml. of water and 0.5 ml. of a solution of 0.5 M-potassium dihydrogen phosphate adjusted to pH 11 with sodium hydroxide. The phosphate increased the rate of aggregation of the precipitate. After 10 min. the mixture was centrifuged at 3000 g for 5 min. Nitrite was estimated in samples (up to 1 ml.) of the clear colourless supernatant liquid according to the method of Anderson (1963). After subtraction of the value in a reagent blank, the recovery of nitrite was 96–105% of the theoretical.

Formation and absorption of nitric oxide. Reactions were performed in Warburg constant-volume respirometers with double-side-arm flasks (capacity 16 ml.). Reaction mixtures contained 1 m-equiv. of sulphuric acid and 0.2 m-mole of potassium iodide in 2.3 ml. of water. The first side arm contained 0.2 ml. of sodium nitrite solution; the second side arm contained 0.4 ml. of alkaline permanganate. In certain experiments sodium hydroxide solution was placed in the centre well. The volume used was 0.2 ml., which was added to a cylinder of filter paper made from an 18 mm. $\times$ 18 mm square. Alkaline permanganate was not used in the well since it rapidly attacked cellulose.

Flasks were gassed with nitrogen for 20 min. After temperature equilibration at 30° the nitrite was added and the flasks were shaken at 80 cyc./min. The formation of nitric oxide was complete in about 5 min. When the absorption was complete a small amount of residual gas was calculated as nitrous oxide.

Sampling technique. The flasks were opened for analysis 10 min. after the absorptions were complete. When absorptions were not complete the nitric oxide was removed by gassing with nitrogen for 10 min. A Pasteur pipette with a bent tip, which had been made by drawing out a thin-walled glass tube, was attached to a rubber bulb. The contents of the side arm were mixed by 'sucking and blowing' with the pipette and were then transferred to a test tube; 0.2 ml. samples were immediately analysed for nitrite.

The recoveries of nitrous oxide and the values of nitrite found in the permanganate were corrected by subtracting the amounts found in control experiments in which water replaced the nitrite solution added from the side arm.

Preparation of enzymic reaction mixtures. The enzymic reaction mixtures for the nitrogen-balance experiments were prepared in double-side-arm Warburg flasks in the manner described by Anderson (1964). For simplicity, all the gas

formations that are given in Fig. 1 are calculated as nitrous oxide.

RESULTS

Estimation of nitric oxide

Formation of nitrous oxide. The nitric oxide that was formed from 10 μ moles of nitrite and an excess of acidified iodide was slowly taken up at the rate of 0.05–0.1 μ mole/hr. in the absence of an absorbent. Experiments were therefore performed to find whether this was caused by reactions of nitrogen dioxide that might be formed from nitric oxide and the traces of oxygen (about 0.15 μ mole) in the atmosphere of nitrogen. Since nitrogen dioxide forms nitrite in alkali according to the equation:

tests for the gas were carried out by adding N-sodium hydroxide to the centre well. The rate of gas uptake was not increased and no nitrite was found in the well after 2 hr., showing that no nitrogen dioxide was formed. This gas was not produced during the absorption of nitric oxide by alkaline permanganate. The small amount of gas that remained was therefore nitrous oxide (or nitrogen).

The uptake of nitric oxide that was found in the absence of an absorbent was possibly caused by its reduction to nitrous oxide by the acidified iodide. Probably nitrous oxide was also formed during the rapid production of nitric oxide. In whichever way the nitrous oxide was produced, its recovery was taken into account in the following nitrogen-balance experiments.

Quantitative recovery of nitric oxide with alkaline permanganate reagent. When the uptake of nitric oxide by alkaline permanganate was complete, the sum of the recovery of nitrite N from the absorbent (9.3 μ g.atoms) and the nitrous oxide N (0.6 μ g.atom) was approximately equal to the nitrite N (10 μ g.atoms) used to generate the gases. These results show that alkaline permanganate quantitatively oxidizes nitric oxide to nitrite.

The recoveries (in μ g.atoms of N) of nitric oxide or nitrous oxide in the following experiments are expressed as a percentage of the nitrite N added. The absorptions were complete in 50 min. When nine experiments with 10 μ moles of nitrite were performed simultaneously, the recoveries of nitrous oxide N varied from 3 to 13%, but the recoveries of nitric oxide N plus nitrous oxide N were quantitative (97–105%). The recoveries of nitric oxide were 92–96%. When the nitrite was varied from 3 to 20 μ moles, the recoveries of nitric oxide N plus nitrous oxide N were also quantitative (95–106%). The recoveries of nitric oxide N and of nitrous oxide N were 91–101% and 1–12% respectively.

Fig. 1. Course of the formation of nitrous oxide and nitric oxide produced by the dehydrogenation of hydroxylamine by extracts of *Nitrosomonas*. Incubation was for 80 min. in double-side-arm Warburg flasks containing (in 2.5 ml.): 200 μ moles of tris buffer, pH 8.0; extract (approx. 3 mg. of protein); 20 μ moles of methylene blue. The reactions were started by adding 10 μ moles of hydroxylamine hydrochloride from the side arm. The atmosphere was nitrogen. The formation of nitrogenous gas (μ moles) was measured. Δ , Flask A, containing 0.2 ml. of 2 N-potassium hydroxide plus filter paper in the centre well; $\circ$, flask B, containing 0.4 ml. of alkaline permanganate in the second side arm; $\bullet$, flask C, containing 0.2 ml. of alkaline sulphite plus filter paper in the centre well.

When 0.5–2.5 μ moles of nitrite were used the recoveries of nitrous oxide N and of nitric oxide N plus nitrous oxide N were 5–50% and 81–124% respectively. The wide range of recoveries of nitric oxide N plus nitrous oxide N was probably caused by errors in measuring the small amounts of nitrous oxide. Since the recoveries of nitric oxide were large (74–86%) it seems likely that all of the nitric oxide present was oxidized in 50 min. The recovery of nitric oxide that was formed from 0.5 μ mole of nitrite was 0.37 μ mole. The approximate range of the estimation of nitric oxide is thus 0.4–20 μ moles.

Formation of nitric oxide from hydroxylamine by *Nitrosomonas*

Recoveries with direct and differential methods. The anaerobic formation of the mixture of nitric oxide and nitrous oxide (or nitrogen) from 10 μ moles of hydroxylamine and 20 μ moles of methylene blue in the presence of an extract of *Nitrosomonas* at pH 8 was complete in 30 min. (flask A in Fig. 1). The nitric oxide that was formed in parallel experiments was rapidly absorbed by alkaline permanganate (flask B in Fig. 1) or alkaline sulphite (flask C in Fig. 1). The analysis of the gas mixture recorded in Table 1 shows that similar amounts of nitrous oxide remained in flasks B and C. The amount of nitric oxide recovered by using the direct method was 3.5 μ moles (flask B in Table 1).

The nitric oxide formation, which is represented by the difference between the maximum reading in flask A and the residual nitrous oxide in flask B or C (Fig. 1), was calculated by using the equation given with the differential method (Anderson, 1964). The amount of nitric oxide found by comparing the readings of flasks A and B was 3.3 μ moles, which was in agreement with the 3.2 μ moles found by comparing the readings of flasks A and C (Table 1). These values were slightly smaller than 3.5 μ moles. The three values of the recovery of nitric oxide N plus nitrous oxide N were approximately equal to the 10 μ moles of hydroxylamine added (Table 1).

Table 1. Recoveries of nitric oxide and nitrous oxide found by using the direct and differential methods

The results were obtained from the experiments given in Fig. 1.

Absorbent used	Method used for estimating nitric oxide	Recovery of nitric oxide (μ g.atoms of N)	Recovery of nitrous oxide (μ g.atoms of N)	Total recovery (% of theoretical, 10 μ g.atoms)
Alkaline permanganate (flask B in Fig. 1)	Differential (flasks A and B)	3.3	6.2	95
	Direct (flask B only)	3.5	6.2	97
Alkaline sulphite (flask C in Fig. 1)	Differential (flasks A and C)	3.2	6.4	96

When the extract was replaced by boiled extract, nitrous oxide was slowly produced (0.4 μ mole/80 min.) but no nitric oxide was formed.

Uptake of nitric oxide. A slow absorption (approx. 0.3 μ mole/hr.) followed the formation of nitric oxide in flask A (Fig. 1). Since no nitrogen dioxide was formed, it seems likely that the uptake was caused by the reduction of nitric oxide to nitrous oxide by reduced methylene blue. The reaction might be catalysed by an enzyme derived from the small concentrations of heterotrophic bacteria that contaminate the whole-cell suspensions of the autotroph.

DISCUSSION

Alkaline permanganate quantitatively oxidized nitric oxide to nitrite. When small amounts of nitrite (0.5–2.5 μ moles) were used for gas production the recoveries of nitric oxide were slightly less than the theoretical. The smallest amount of gas that can be estimated by this direct method is probably 0.4 μ mole. Differential methods cannot estimate such small amounts. If alkaline permanganate could be used in the centre well the absorption of the gas would be more rapid, and greater recoveries might be obtained. It would be necessary however, to use a support other than filter paper, which is rapidly attacked by the permanganate.

In using the differential method to estimate the nitric oxide that was formed by the anaerobic dehydrogenation of hydroxylamine, no account was

taken of the uptake of the gas during its absorption by the alkaline sulphite or alkaline permanganate reagent. If the uptake were caused by a reduction of nitric oxide to nitrous oxide, a low recovery of the former and a high recovery of the latter gas would be obtained. In the present work the uptake of nitric oxide (0.3 μ mole/hr.) was negligible compared with the rates of its absorption, and the recovery of total gaseous N was approximately the theoretical of 10 μ moles. The amount of nitric oxide N formed corresponded to slightly more than 30% of the hydroxylamine N.

The direct method was performed by using only one flask. An enzymic reduction of nitric oxide would therefore increase the recovery of nitrous oxide without affecting the recovery of nitric oxide N plus nitrous oxide N. This was the result obtained.

I thank Dr C. F. Phelps for helpful discussions.

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