37. THE MANOMETRIC ESTIMATION OF NITRITE IN SOLUTION AND IN TISSUE

BY J. BROOKS AND J. PACE

From the Low Temperature Research Station, University of Cambridge and Department of Scientific and Industrial Research, and Smithfield Laboratory (Department of Scientific and Industrial Research)

(Received 17 January 1940)

BAUMGARTEN & MARGGRAFF [1930] showed that nitrites can be estimated by measuring the volume of N_2 liberated by the action of excess amidosulphonic acid since the rate of reaction (I) is rapid compared with the rate of decomposition of HNO_2 (II).

It has been found that the range and convenience of the method are increased by the use of the manometric apparatus of Van Slyke or Warburg.

MATERIALS

Amidosulphonic acid. SO_2 was bubbled through a saturated solution of hydroxylamine hydrochloride [Raschig, 1887] for 48 hr. at room temperature and the mixture kept at -10° for a further 3–4 days. The crystals of NH_2SO_3H were recrystallized from H_2O ($50^\circ \rightarrow -10^\circ$).

Sodium nitrite. A.R. $NaNO_2$ was twice recrystallized and analysed volumetrically by the method of Adie & Wood [1900] as modified by Cool & Yoe [1933]. The solid contained $99.5 \pm 0.1\%$ $NaNO_2$; stock solutions were stable indefinitely. Other chemicals were of A.R. quality and contained no nitrite. Recalibrated pipettes were used for the delivery of $NaNO_2$ solutions.

Tissue. Pork muscle (loin), after rigor, was used; different samples showed no difference in behaviour. The mean water content was 76 g. H_2O /100 g. tissue.

Analysis of nitrite solutions

Warburg apparatus. The $NaNO_2$ solution was placed in the main compartment of the vessel [Warburg & Kubowitz, 1929] and the NH_2SO_3H solution¹ in the side bulb. When thermal equilibrium at 25° was reached the contents of the side bulb were tipped into the main compartment; the pressure increase reached a steady value in less than 10 min. Duplicate readings agreed to within 0.5 mm. Brodie solution, which, with the vessels used (volume about 19 ml. and v_F 1.4 ml.), corresponded to about 0.85 μ l. N_2 or 2.62×10^{-6} g. $NaNO_2$. Table 1 gives the results obtained with 1 ml. $NaNO_2$ solution containing 6.9×10^{-4} g. solid/ml. and 0.4 ml. 1.7% NH_2SO_3H solution. Only a slight excess of NH_2SO_3H (0.5–1.0 mol.) was required [Baumgarten & Marggraff, 1930] but the same results were obtained with more concentrated solutions.

¹ Hydrolysis of NH_2SO_3H in aqueous solution at room temperature is hardly detectable after several days [Baumgarten & Marggraff, 1930]. The dry solid kept over H_2SO_4 appears to be stable indefinitely.

Table 1

1 μ l. $N_2 = 3.08 \times 10^{-6}$ g. $NaNO_2$

Vessel	g. $NaNO_2$ found	% purity of $NaNO_2$
1	6.861×10^{-4}	99.45
4	6.874	99.63
6	6.866	99.51

The values for the percentage purity of the solid $NaNO_2$ are in good agreement with the results of the volumetric analysis and of similar accuracy. Results with more dilute solutions (taking the percentage purity as 99.5) are given in Table 2.

Table 2

1 ml. $NaNO_2$ solution: 0.4 ml. 1.7% NH_2SO_3H solution

g. $NaNO_2$ present	g. $NaNO_2$ found
3.42×10^{-4}	3.41×10^{-4}
1.37	1.37
0.68	0.69

With still more dilute solutions the percentage error becomes appreciable. The lower limit of concentration which can be analysed if an error of 3% is accepted is 10^{-5} g. $NaNO_2$ /ml., using 5 ml. $NaNO_2$ solution (the maximum permissible in the available vessels). The sensitivity of the method could be increased by the use of smaller vessels (thus halving v_G and keeping v_F constant would increase the sensitivity by about 100%) or of some other form of microrespirometer, e.g. that of Heatley *et al.* [1939].

Van Slyke apparatus. In the analysis of a 2 ml. sample, one drop of octyl alcohol, 2 ml. $NaNO_2$ solution and 4.75 ml. H_2O were drawn into the gas chamber. Gas was extracted by shaking *in vacuo* for 5 min. with the Hg meniscus at the 50 ml. mark and the gas ejected. The chamber was evacuated till the solution was in the lower fourth and 0.25 ml. saturated NH_2SO_3H solution was added through a Hg seal. The contents of the chamber were shaken with the Hg meniscus at the 50 ml. mark for 5 min. and any traces of CO_2 absorbed in the usual way by the addition of 1 ml. air-free N NaOH. The gas was brought to a volume of 0.5 ml. (or 2.0 ml.) and the pressure p_1 read. The gas was ejected and the fluid meniscus lowered to the 0.5 ml. (or 2.0 ml.) mark and the pressure p_2 read. Then

$\text{vol. } N_2/100 \text{ ml.} = f(p_1 - p_2 - c)$ and $22.4 \text{ vol. } N_2/100 \text{ ml.} = 6.9 \times 10^{-4} \text{ g. } NaNO_2/\text{ml.}$ f is a factor tabulated by Peters & Van Slyke [1932, p. 325]; c is a small correction obtained from a blank analysis using 2 ml. H_2O in place of 2 ml. $NaNO_2$ solution, where $c = p_1' - p_2'$.

The means of duplicate analyses are given in Table 3; the meaning of the symbols S and a is defined by Peters & Van Slyke [1932, p. 282].

Table 3

2 ml. $NaNO_2$ solution analysed; $S = 7.0$ ml.

a ml.	Vol. $N_2/100$ ml. found	g. $NaNO_2$ /ml. found	g. $NaNO_2$ /ml. present
2.0	22.30	6.87×10^{-4}	6.86×10^{-4}
2.0	11.10	3.42	3.43
0.5	4.45	1.37	1.37
0.5	2.24	6.90×10^{-5}	6.86×10^{-5}

Duplicate analyses of 2 ml. samples agreed to within 0.03 vol./100 ml. corresponding to about 2×10^{-6} g. NaNO_2 ; the sensitivity therefore is similar to that of the Warburg apparatus. With a sample of 6 ml., $S=7$ ml. and $a=0.5$ ml. a concentration of 2×10^{-5} g. NaNO_2 /ml. can be analysed with an error of about 1%. It was found possible, however, to analyse a much larger sample, e.g. 25 ml. The procedure was similar to that described above except that one drop of octyl alcohol, 25 ml. NaNO_2 solution and 2 ml. H_2O were drawn into the gas chamber and gas was extracted and ejected three times (the period of shaking in each case was 5 min. with the Hg meniscus at the 50 ml. mark). Table 4 gives calculated values of f for $S=27.25$ ml. and $a=0.5$ ml. [cf. Peters & Van Slyke, p. 282] and the results of three analyses. Duplicate analyses agreed to within 0.002 vol./100 ml. corresponding to 1.5×10^{-6} g. NaNO_2 . With a 25 ml. sample therefore a concentration of 10^{-6} g. NaNO_2 /ml. can be estimated to within 3%.

Table 4

Sample = 25 ml. $S=27.25$ ml. $a=0.5$ ml.						
$^{\circ}\text{C.}$	15	16	17	18	19	20
f	0.00254	0.00253	0.00252	0.00251	0.00250	0.00249
	Vol. N_2 /100 ml.		g. NaNO_2 /ml. found		g. NaNO_2 /ml. present	
	0.178		5.48×10^{-6}		5.49×10^{-6}	
	0.0886		2.73		2.75	
	0.0365		1.12		1.10	

The specificity of the reaction

In agreement with Lutzenko [1937] it was found that the quantitative evolution of N_2 was not affected by the presence of NaCl , NaNO_3 , glucose, sucrose and tissue extractives. Analyses of NaNO_2 solutions containing these substances, using mainly the Warburg apparatus, are given in Table 5.

Table 5

1 ml. solution contained 6.86×10^{-4} g. NaNO_2									
	NaCl					NaCl and NaNO_3			
g./100 ml.	3	6	10.8	25	NaNO_3 4.5	20	4.5	Glucose 5	Sucrose 5
g. NaNO_2 /ml. $\times 10^4$	6.90	6.86	6.86	6.90	6.85	6.83		6.86	6.79
found									Tissue extrac- tives*
									6.80

* 50 g. minced tissue were stirred with 50 ml. 10.8% NaCl solution for 2 hr. and the filtrate added to an equal volume of NaNO_2 solution.

When there is a marked difference between the concentrations of the solutions in the two compartments of the Warburg vessel a vapour pressure correction (obtained from a blank experiment with the solutions in the absence of NaNO_2) must be added to or subtracted from the observed pressure increase.¹ With the

¹ The pressure of H_2O vapour in the vessel is mainly determined by the vapour pressure of the solution in the main compartment. When the latter pressure is altered by the addition of $\text{NH}_4\text{SO}_3\text{H}$ solution the pressure increase is the sum of the change in vapour pressure in the vessel and the change due to the evolution of N_2 . With concentrated salt solutions in the main compartment the magnitude of the correction can be decreased by increasing the concentration of the solution in the side bulb, e.g. by using 0.4 ml. saturated $\text{NH}_4\text{SO}_3\text{H}$ solution.

dilute solutions of Tables 1 and 2 the correction was negligible (<0.2 mm. Brodie solution) but became appreciable with the solutions of Table 5. For the analysis of solutions of the latter type it is better to use the Van Slyke apparatus as the correction c is less dependent on the composition of the sample.

When a solution contained NO_2^- and HCO_3^- or $\text{CO}_3^{=}$, N_2 and CO_2 were liberated by $\text{NH}_2\text{SO}_3\text{H}$. In the Van Slyke apparatus CO_2 was absorbed by the routine addition of 1 ml. air-free NaOH and in the Warburg apparatus by the usual method of placing 0.3 ml. 5% KOH solution and a small roll of filter paper in the central tower of the vessel.

It should be noted that the estimation of nitrite contained in strongly buffered solutions may require the addition of increased amounts of $\text{NH}_2\text{SO}_3\text{H}$ since a low pH is required for the reaction.

The estimation of nitrite in tissue

A known amount of NaNO_2 was mixed with tissue and the mixture analysed; this usual test of an analytical method was complicated by reaction between nitrite and tissue constituents.

(A) *Van Slyke apparatus*. Nitrite was estimated by the analysis of an aqueous extract of the tissue (without preliminary removal of dissolved protein). Samples of 10 g. minced tissue were thoroughly mixed with 1 ml. of NaNO_2 solution (containing 6.86×10^{-3} g. NaNO_2) and the mixtures, after storage at 25° for various periods, were transferred to 500 ml. flasks. In each case the volume of tissue and washings was made up to 500 ml. with H_2O and the mixture gently shaken for 1 hr. After filtration, 25 ml. filtrate were analysed in the Van Slyke apparatus. In some cases 1 ml. NaNO_2 solution was added to 10 g. tissue previously mixed thoroughly with 1 g. solid NaCl . The mean of duplicate analyses is given in Fig. 1, the times give the interval between addition of NaNO_2 to the tissue and dilution with H_2O .

Of the added NaNO_2 97% was recovered after 30 min. contact with tissue. There was a slow disappearance of nitrite; the rate was increased by about 70% by the presence of 1 g. NaCl /10 g. tissue. Extrapolation of the linear plots in Fig. 1 to zero time gave values of 98 and 99% recovery. The method therefore is reasonably accurate; the small initial deficit appears to be due mainly (*v. infra*) to a rapid reaction of nitrite with tissue constituents. Since a concentration of 10^{-6} g. NaNO_2 /ml. filtrate can be estimated to within 3% an initial concentration of about 50×10^{-6} g. NaNO_2 /g. tissue can be estimated with the same accuracy. The amount of tissue extracted could however be increased, thus using 50 g. tissue the limiting concentration giving an error of 3% would be 10^{-5} g. NaNO_2 /g. tissue.

(B) *Warburg apparatus*. With certain limitations nitrite in tissue can be estimated by the action of $\text{NH}_2\text{SO}_3\text{H}$ on the tissue. Pork muscle, after rigor, has a small residual respiration but the R.Q. is sufficiently near unity to give no pressure change in the Warburg vessel during a determination.¹

1 g. minced or thinly-sliced tissue was spread on the bottom of the main compartment and covered with 1 ml. NaNO_2 solution.² After 30 min. contact

¹ The O_2 uptake (in this case using KOH in the central tower) was found to be about $0.5 \mu\text{l.}/\text{g.}/\text{min.}$ at 25° . After the addition of $\text{NH}_2\text{SO}_3\text{H}$ the uptake was about $0.2 \mu\text{l.}/\text{g.}/\text{min.}$

² This procedure was equivalent to but more convenient than using 1 g. tissue containing added NaNO_2 and 1 ml. H_2O . Experiments showed that in 30 min. the uniform distribution of NaNO_2 between the solution and the H_2O of the muscle was at least 95% complete.

Fig. 1. Percentage recovery of NaNO₂ added to tissue (A) and to tissue containing 10% NaCl (B). The percentage recovery is given by $100(500 - 2.4)(\text{g. NaNO}_2/\text{ml. filtrate})/6.86 \times 10^{-3}$, where 2.4 ml. are taken as the volume of the non-aqueous portion of 10 g. tissue.

Fig. 2. Percentage recovery of NaNO₂ from 1 g. tissue (A) and from 1 g. tissue in the presence of 3×10^{-2} g. NaCl (B), 6×10^{-2} g. NaCl (C) and 10.8×10^{-2} g. NaCl (D).

0.4 ml. saturated $\text{NH}_2\text{SO}_3\text{H}$ solution¹ was added from the side bulb. At the pH of pork muscle the amount of bicarbonate present should be negligible; blank experiments in the absence of NaNO_2 gave a pressure decrease of 1 mm. Brodie solution (taken as the vapour pressure correction). The density of the tissue was taken as 1.05 in the calculation of v_F and v_G .

Ten experiments using 1 ml. solution containing 6.86×10^{-4} g. NaNO_2 gave a mean value of 97.5 % recovery. Experiments with longer periods of contact before the addition of $\text{NH}_2\text{SO}_3\text{H}$ (Fig. 2, curve A) showed that there was a linear rate of disappearance of nitrite of a similar magnitude (about 2 % per hour) to that previously observed. Extrapolation of the curve to zero time gave a value of 98.5 % recovery.

Table 6 gives the results obtained using different amounts of NaNO_2 ; the time of contact of tissue and NaNO_2 was 30 min. The values for the percentage recovery are not constant; this appears to be mainly due, not to experimental error, but to a rapid disappearance of nitrite of the order of 10^{-5} g. NaNO_2 /g. tissue (Table 6, column 4).

Table 6

Side bulb—0.4 ml. saturated $\text{NH}_2\text{SO}_3\text{H}$ solution
Main compartment—1 g. tissue and 1 ml. NaNO_2 solution

g. NaNO_2 added	g. NaNO_2 found	% recovery	g. NaNO_2 lost
6.86×10^{-4}	6.69×10^{-4}	97.5	1.7×10^{-5}
3.43	3.33	97	1.0
1.37	1.30	95	0.7
0.69	0.62	90	0.7

The results obtained by methods A and B are in good agreement. Method B is more convenient but less sensitive than method A since the weight of tissue in the Warburg vessel could not be increased above 2 g.

The action of $\text{NH}_2\text{SO}_3\text{H}$ on tissue containing added NaCl

The action of $\text{NH}_2\text{SO}_3\text{H}$ on tissue containing NaNO_2 and high concentrations of NaCl gave unexpected results and nitrite could not be estimated by the addition of $\text{NH}_2\text{SO}_3\text{H}$ to such tissue. In these experiments 1 g. minced or sliced tissue was placed in the Warburg vessel together with 1 ml. solution containing 6.86×10^{-4} g. NaNO_2 and various amounts of NaCl. The side bulb contained 0.4 ml. saturated $\text{NH}_2\text{SO}_3\text{H}$ solution (the same results were obtained using 0.75 ml.). Vapour pressure corrections were obtained from blank experiments in the absence of NaNO_2 .

It was found (Fig. 2) that (a) the percentage recovery decreased with the time of contact of tissue and NaNO_2 , (b) the rate of disappearance of nitrite was increased by the presence of NaCl, (c) extrapolation of the linear plots to

¹ With 0.4 ml. $\text{NH}_2\text{SO}_3\text{H}$ solution of different concentrations in the side bulb the following values were obtained. The use of a larger volume of saturated $\text{NH}_2\text{SO}_3\text{H}$ solution did not increase further the percentage recovery. The lower values obtained with the more dilute solutions of $\text{NH}_2\text{SO}_3\text{H}$ may be due to the buffering power of the tissue:

% $\text{NH}_2\text{SO}_3\text{H}$	3.0	5.0	Saturated
% recovery	84.7	94.0	97.3

As would be expected the time required (30–45 min.) for the attainment of a steady pressure increase after the addition of $\text{NH}_2\text{SO}_3\text{H}$ was greater than that observed when $\text{NH}_2\text{SO}_3\text{H}$ was added to a nitrite solution.

zero time showed an initial deficit which was proportional to the amount of NaCl present. Table 7 gives the extrapolated values of percentage recovery at zero time.

Table 7

1 g. tissue plus 1 ml. solution			
g. NaCl/ml. (a)	% recovery at zero time	-Δ (% re- covery) (b)	b/a
0	98.5	—	—
3×10^{-2}	95.5	3.0	1.0
6	92.0	6.5	1.1
10.8	88.1	11.4	1.0

Further experiments showed that (a) the initial deficit was proportional to the concentration of NaCl and not to the amount, (b) the effect was reversible with respect to the NaCl concentration; thus the addition of H_2O to a tissue-NaCl- NaNO_2 - H_2O mixture after a given time increased the percentage recovery obtained on adding $\text{NH}_2\text{SO}_3\text{H}$ to a value corresponding to the lowered concentration of NaCl. The initial deficit therefore was not due to destruction of nitrite; this was confirmed by almost complete extraction of NaNO_2 with excess H_2O (99 % for zero time of contact) from tissue containing 10 % NaCl (Fig. 1). Further, since NaCl and tissue extractives had no effect on the quantitative evolution of N_2 in the absence of tissue (Table 5) it seems probable that part of the NaNO_2 present in a tissue structure containing relatively high concentrations of NaCl is not accessible to the action of $\text{NH}_2\text{SO}_3\text{H}$.

DISCUSSION

The manometric estimation of nitrite in solution is a convenient alternative to the usual volumetric methods [cf. Cool & Yoe, 1933] especially when the solution contains substances which interfere with volumetric analysis. Although small amounts of nitrite can be estimated manometrically the range of the method described is smaller than that of colorimetric analysis;¹ the procedure of the Association of Official Agricultural Chemists [1935] allows the estimation of as little as 0.3×10^{-6} g. NaNO_2 .

Nitrite in tissue is usually estimated colorimetrically after extraction with H_2O and removal of dissolved protein. The procedure of Lewis & Blake [1932, p. 433], applied to mixtures of tissue and nitrite and to samples of bacon gave results which agreed to within 7 % (the error of the colorimetric method is from 5 to 10 %) with those obtained by the manometric technique. In these analyses the NaNO_2 content was greater than 50×10^{-6} g./g. tissue; the colorimetric method can be used to estimate concentrations of the order of 10^{-6} g. NaNO_2 /g. tissue.

The results indicate that nitrite reacts rapidly with tissue constituents in amounts of the order of 10^{-5} g. NaNO_2 /g. tissue; reaction with haemoglobin and myoglobin in the muscle would probably account for a large part of this initial nitrite loss. There is also a slow disappearance of nitrite in contact with

¹ Numerous modifications of the Griess-Ilosvay colour reaction have been suggested. In some cases the reagents, sulphanilic acid and α -naphthylamine, are used in acetic acid solution. It was found when the nitrite solution contained NaCl that the depth of colour developed depended on the concentration of NaCl. When HCl was substituted for acetic acid, as in the methods of the A.O.A.C. [1935] and Lewis & Blake [1932, p. 420], the presence of up to 25 % NaCl in the nitrite solution had no effect on the estimation.

tissue but the cause is unknown. Using the Warburg apparatus, a small gas absorption was detectable over long periods of contact of nitrite with tissue (<1 mm. Brodie solution/hr.) but the amount was too small to account for the disappearance on the basis of atmospheric oxidation of NaNO_2 catalysed by a tissue enzyme.

SUMMARY

1. Manometric methods for the estimation of nitrite are described. The methods are based on the reaction of $\text{NH}_2\text{SO}_3\text{H}$ with nitrite in the Van Slyke or Warburg apparatus.

2. The quantitative evolution of N_2 from nitrite and $\text{NH}_2\text{SO}_3\text{H}$ is not affected by the presence of NaCl , NaNO_3 , sucrose, glucose and muscle extractives.

3. Nitrite in muscle can be estimated by the manometric analysis of a H_2O extract of the tissue. With certain limitations nitrite in muscle can be estimated directly by the action of $\text{NH}_2\text{SO}_3\text{H}$ on tissue contained in the Warburg apparatus.

4. On mixing muscle and NaNO_2 there is both a rapid disappearance of a small amount of nitrite (of the order of 10^{-5} g. NaNO_2 /g. tissue) and a slow disappearance of nitrite.

The work described above was carried out as part of the programme of the Food Investigation Board, and is published by permission of the Department of Scientific and Industrial Research.

REFERENCES

- Adie & Wood (1900). *J. chem. Soc.* **77**, 1076.
Association of Official Agricultural Chemists (1935). *Methods of Analysis*, p. 506. Washington D.C.: A.O.A.C.
Baumgarten & Marggraff (1930). *Ber. dtsh. chem. Ges.* **63**, 1019.
Cool & Yoe (1933). *Industr. Engng Chem. (Anal. Ed.)*, **5**, 112.
Heatley, Berenblum & Chain (1939). *Biochem. J.* **33**, 53.
Lewis & Blake (1932). *Allen's Commercial Organic Analysis*, 5th ed. **9**, 420, 433. London: Churchill.
Lutzenko (1937). *Zh. prikl. Khim., Leningr.*, **10**, 948.
Peters & Van Slyke (1932). *Quantitative Clinical Chemistry*, **2**, 282, 325. London: Baillière, Tindall and Cox.
Raschig (1887). *Ber. dtsh. chem. Ges.* **20**, 584, 1158.
Warburg & Kubowitz (1929). *Biochem. Z.* **214**, 5.