STUDIES IN THE BIOLOGICAL FIXATION OF NITROGEN

I. INHIBITION IN *AZOTOBACTER VINELANDII* BY HYDROXYLAMINE

by

B. A. PETHICA, E. R. ROBERTS AND E. R. S. WINTER

Department of Inorganic and Physical Chemistry, Imperial College of Science and Technology, London (England)

The metabolic path whereby amino acids are built up into protein, and which may be common to both fixing and non-fixing organisms, is less obscure than that by which nitrogen-fixing organisms convert molecular nitrogen to amino acids. In particular, the very first stages in the fixation process have produced considerable differences of opinion. The claim of ammonia to be the initial recognisable product has the support of much experimental evidence; so also has that of hydroxylamine. The nature of this evidence is so varied that we do not propose to review it in detail. It is drawn from studies on excretion, utilisation, and inhibition, augmented by investigations into cognate processes such as the haemoglobin–methaemoglobin conversion, the behaviour of enzyme systems believed to exist in nitrogen-fixing organisms, and the reactions of simple nitrogenous compounds with commonly occurring organic acids. In recent years, still further information has been provided by the application of isotope tracing methods.

Careful assessment of the large amount of data thus available leaves the authors with the conviction that strong as is the evidence in favour of ammonia, it is not of sufficient weight to exclude hydroxylamine, nor even such substances as hydrazine[1,2] and some other simple compounds[3]. The more important reasons for this conviction are, first, the existence of much contrary evidence[4], for which inadequate explanations have been offered; and secondly, the belief that the results of some experiments, designed to be crucial, are capable of alternative explanations. There is no doubt that ammonia plays a very important part, but the evidence does not prove it to be the first product of fixation. Moreover, although it is generally conceded that the fixation process, considered as a whole, is endothermic, and that it draws energy from the exothermic process of respiration, the possibility remains that some individual steps in fixation may be exothermic. All reasonable reactions that involve molecular nitrogen, and which are exothermic, are oxidative rather than reductive[4]. There seems to be no conclusive evidence that such an oxidative initial step does not occur; the recently reported behaviour of nitrous oxide in inhibiting fixation[5,6] may indicate that oxidised forms of nitrogen play an important part.

The need for further information about the early stages of the process led to the initiation here of studies in which the effects on fixation and respiration of possible intermediate substances have been measured. BURK AND HORNER'S[7] tenet that an intermediate may be considered specific to fixation if it occurs only in that process and

References p. 98/99.

not in the assimilation of fixed nitrogen does not imply that a substance may not be an intermediate in both processes; this point has been borne in mind in designing the present experiments. Additional studies have been made to determine whether the intermediates in question were utilised.

Inhibition of respiration has been measured using the Warburg technique[8]; inhibition of fixation has been determined by a direct method that makes use in a novel way of the isotopic tracing technique. The substances already studied include hydroxylamine, hyponitrous acid, nitrous oxide, nitrous acid, and hydrazine. This paper describes the methods employed and records the results obtained using hydroxylamine, the implications of which are discussed. Subsequent communications will record the results obtained with various other substances.

Use of ^{15}N in the determination of the rate of increase in cell nitrogen

If, to a culture in the logarithmic phase of growth taking up a given substrate, an isotopically-labelled substrate be supplied, it can be shown that, for a time interval, t,

$$k = \frac{1}{t} \ln \frac{I}{I-E} \tag{1}$$

(where k is a rate constant, I is the abundance of the isotope in the substrate, and E that in the cells after the time interval) or, for successive time intervals t_1 and t_2

$$k(t_2-t_1) = \frac{1}{t_2-t_1} \ln \frac{I-E_1}{I-E_2} \tag{1a}$$

(where the symbols have the corresponding meanings).

These relationships apply only to stable isotopes, but it is not difficult to modify them for application to radioactive species.

In the present research, the substrate was molecular nitrogen and k therefore measures the rate of increase in cell nitrogen resulting from fixation. Providing certain invalidating conditions are recognised, however, the method is applicable to the study of other substrates and other elements. The expressions would not hold if there were direct isotopic exchange between the substrate and the contents of the cells; nor if the labelled substrate were diluted by the excretion of intracellular substances; nor if it were adsorbed onto the cell without being actually incorporated into the contents. The results might also be untrustworthy in cases where the diffusion through the cell wall of ions or substances of low molecular weight were very rapid. In addition to these general factors, special considerations may arise in individual cases.

If, however, these conditions can be met, or allowed for, the method offers certain important advantages. First, the calculation of the rate constant, k, is made from measurements of time and isotope abundance ratios only. The latter, an intensive property, is independent of total cell nitrogen and is therefore free from sampling errors. Secondly, the rate of increase of a particular constituent may be measured independently of growth, *e.g.* during bacteriostasis. Thirdly, even in cases where the growth is not strictly logarithmic, the method will provide a mean logarithmic growth rate constant, so that an approximate kinetic study of, say, the late lag phase of growth is possible. Fourthly, k may be determined with considerable accuracy, often in experiments of short duration; unless the time is very short, its measurement involves negligible error, and the accuracy of k depends primarily on that of the abundance ratio measurements. Since suitable accuracy in measuring I may be assumed, the minimum permissible

References p. 98/99.

time interval is that necessary for E to attain a value sufficiently great for the desired accuracy of measurement. For nitrogen, in which the abundance ratios are readily obtained to within 1% for atom % excesses of ^{15}N between 1% and 30%, the accuracy of k is about 2%. In experiments here, using *A. vinelandii*, accuracies of this order were assured in experiments lasting only two hours.

The measurement of inhibition. The method above renders possible the determination of the rate of increase of cell nitrogen under conditions suitable either for fixation or for the assimilation of fixed nitrogen. In particular, if a source of fixed nitrogen is supplied as well as molecular nitrogen, the inhibition of the fixation process may be measured if only the molecular nitrogen be labelled. If the organism assimilates a fixation intermediate in preference to elementary nitrogen, a decrease of fixation rate in presence of a particular nitrogenous compound suggests that the latter is indeed a fixation intermediate; but this evidence is not conclusive, because the compound may inhibit fixation indirectly, *e.g.* by acting as a general poison. It is therefore necessary to measure also some other index of growth under the same conditions, so that such indirect effects may be detected. Respiration rate is a convenient metabolic index; it has, indeed, frequently been used as a measure of nitrogen fixation, the two processes having been shown to be closely parallel over short periods. Moreover, suitable treatment of respiration data affords information as to the nature of the inhibition.

EXPERIMENTAL

The isotope experiments

Materials and preliminary data

(i) *Preparation of cultures of A. vinelandii.* A culture was obtained from the National Collection of Type Cultures (Cat. no. 5073); later supplies of this organism were obtained from Rothamsted Experimental Station through the kindness of Dr. MEIKLEJOHN. The bacterium was grown in the following medium[9,10,11,12,13] (pH = 7.3):

Mannitol	15 g	$MgSO_4 \cdot 7H_2O$	0.2 g	$FeSO_4 \cdot 7H_2O$	0.015 g
K_2HPO_4	1.6 g	NaCl	0.2 g	$NaMoO_4$	0.001 g
KH_2PO_4	0.4 g	$CaSO_4$	0.1 g	Dist. water	1 litre

The iron is rapidly oxidised, and in experiments using hydroxylamine this was ensured by bubbling air through any freshly prepared medium. Stationary populations of 10^9 cells per ml were obtained. To obtain the maximum information from a number of virtually identical cultures, a large culture was subdivided, adding different concentrations of inhibitor to each portion, and aerating all of them simultaneously with a common atmosphere under identical conditions. The "master culture" was obtained by placing, without washing, suitable inocula from 5-day old cultures into up to 1 litre of fresh medium. Aeration for 20 hours ensured that the organisms were well into the early log phase. The success of this method is illustrated by the experiments on respiration; mathematical relationships of the same form hold for each set of data, although the constants differ from master culture to master culture.

(ii) *Preparation of isotopically-labelled atmosphere.* The isotopic nitrogen was prepared from $^{15}NH_4NO_3$* by conversion to $^{15}NH_4Cl$ and reaction with NaOBr, and the oxygen by heating pure potassium permanganate to 250° C. The oxygen was purified by passage through solid KOH and a trap cooled with liquid nitrogen.

(iii) *Hydroxylamine.* The low concentrations of hydroxylamine used eliminated the need for preparation of exceptionally pure material. The hydrochloride used contained 99.1% of $NH_2OH \cdot HCl$, as determined[14,15] by RASCHIG's method. Molar solutions were sterilised at 120° C for 5 minutes, no appreciable decomposition occurring. Solutions of the appropriate concentrations were prepared by diluting with sterile medium, having first adjusted the pH of the hydrochloride solution to 7 by the addition of 0.88 equivalents of NaOH per equivalent of hydroxylamine hydrochloride.

The presence in the culture medium of hydroxylamine introduces several complications. First, its rate of decomposition must be determined, so that its actual concentration during an experiment

* Supplied by Eastman Kodak Company, Rochester, N.Y., U.S.A.

References p. 98/99.

is known. Secondly, ammonia[16,17,18] and nitrite (vide infra) are among the products of decomposition and might themselves affect fixation. Thirdly, direct isotopic interchange[19,20,21] between hydroxylamine or nitrite or ammonia and nitrogen could invalidate the method, and must be tested for. Fourthly, hydroxylamine has a lytic action on Azotobacter, ammonia being one of the products; the extent of this action must be measured to ensure that it is unimportant. Fifthly, the analytical determination of hydroxylamine in very low concentrations is difficult. Finally, the possibility of utilisation of hydroxylamine had to be investigated. The findings on each of these points follow.

(a) Decomposition of hydroxylamine in the medium. Although[22,23,24] Lemoigne's modification of Blom's colorimetric method gave satisfactory results when used to determine hydroxylamine in pure aqueous solution, it indicated an apparently immediate decomposition, up to 50%, when applied to hydroxylamine dissolved in the medium. This arose from errors in the method itself, but normal decomposition curves were obtained when the following modified procedure was used. An aliquot of the test solution, containing about 5 γ (μg) of hydroxylamine-nitrogen was diluted with acidified medium to 10 ml, the final pH being 3. The solution was cooled under the tap, and 1 ml of the sulphanilic acid reagent and 1 ml of the iodine reagent added. After 15 minutes the iodine was *carefully* decolorised by thiosulphate, 1 ml of the naphthylamine reagent added and the colour allowed to develop for 15 minutes. An identical aliquot was treated in the same way (omitting the oxidation with iodine) to determine nitrite, and the difference taken as hydroxylamine.

Representing the decomposition reaction by $C = C_0 \cdot e^{-\lambda t}$, λ was found to be 0.0469 h^{-1} for $C_0 = 10^{-5}$ M and 0.0424 h^{-1} for $C_0 = 3 \cdot 10^{-4}$ M, thus showing first-order character over a thirty-fold range of concentration. The mean value of λ, from many experiments, was 0.048 h^{-1}, so that the decomposition of hydroxylamine is 13%, 25%, and 35% in 3, 6, and 9 hours respectively.

(b) Production of nitrite in decomposition of hydroxylamine. Determination of the residual nitrite in the above experiments revealed that nitrite is among the products of decomposition of hydroxylamine; other substances (such as hyponitrous acid) which might react as nitrite are not present in sufficiently large proportion to invalidate this conclusion. The higher proportion of hydroxylamine converted to nitrite at low concentrations (see Table I) may reflect the rapid decrease in the rate of reaction between nitrite and hydroxylamine as concentration decreases.

TABLE I

NH_2OH (*Mol/l*) *initially*	NO_2' (γ *of N/5 ml*)		*Increase in* NO_2' (*Mol/l/24 h*)	% NH_2OH *conv. to* NO_2*/24 h*
	initially	*finally*		
$3 \cdot 10^{-4}$	0.06	0.72	$4 \cdot 10^{-5}$	13
$3 \cdot 10^{-4}$	0.18	0.72		
10^{-4}	0.22	0.53	$2 \cdot 10^{-5}$	20
10^{-4}	0.30	0.53		
$3 \cdot 10^{-5}$	0.05	0.17	$9 \cdot 10^{-6}$	30
10^{-5}	0.14	0.30	$7.5 \cdot 10^{-6}$	75
10^{-5}	0.22	0.27		

For experiments lasting only a few hours the fraction of hydroxylamine decomposed is small, and even if the nitrite formed were immediately assimilated it would not be sufficient markedly to affect the rate of uptake of molecular nitrogen.

(c) Isotopic exchange between nitrogen and nitrogenous compounds. It has already been reported[25] that no interchange occurred between nitrogen and hydroxylamine in solutions at pH 3 and 7. Using the same technique, it was found that nitrite does not exchange nitrogen with gaseous N_2.

(d) Utilisation of hydroxylamine. The rates of disappearance of hydroxylamine from sterile and inoculated media were measured. The organism did not accelerate the rate of decomposition; indeed there seems to be some retardation. The observations of MITCHELL[26] suggested that this might be due to the reduction, by the bacteria, of Fe^{+3} to Fe^{+2}, the consequent reduced rate of disappearance of hydroxylamine masking any small amount of utilisation. Determination of both ferric and ferrous iron provided no evidence that this was so, although it revealed rapid removal of iron from the medium. In the presence of *A. vinelandii* that had undergone nine subcultures in a medium containing 10^{-4} M hydroxylamine, the retardation of decomposition was more pronounced. These observations indicate that the organism does not, and cannot be trained[27] to, use hydroxylamine; since the method of determination would, however, include acid-hydrolysed oximes, the evidence is not conclusive. The effect of hydroxylamine on the growth cycle of *A. vinelandii*, however, provides

References p. 98/99.

further support. Growth curves, obtained by direct cell counts of aliquots (corrected for evaporation in the incubator) show that no growth occurs until the concentration of hydroxylamine[28] has fallen to $5 \cdot 10^{-5}$ *M*. Repeated subculturing produced no reduction in the lag time; the appearance of a small morphological (coccoid) variant, which was shown not to be a contaminant and the proportion of which increased during 13 serial subcultures in 10^{-4} *M* hydroxylamine, was probably responsible for the abnormal growth curves obtained in later subcultures. (See Figs. 1 and 2).

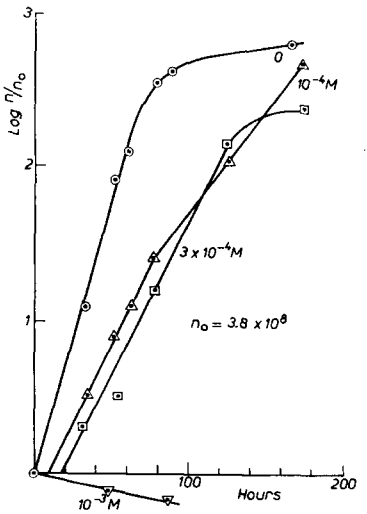


Fig. 1. Effect of hydroxylamine on growth cycle (first subculture).

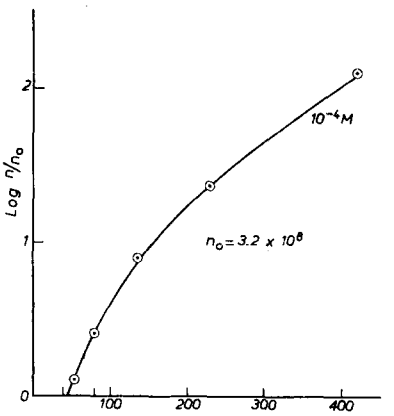


Fig. 2. Effect of hydroxylamine on growth cycle (thirteenth subculture).

(e) Lytic action of hydroxylamine. To test[29] VIRTANEN's suggestion that hydroxylamine is reduced to ammonia during fixation, cultures were made 10^{-3} *M* with respect to hydroxylamine, and the residual hydroxylamine and ammonia determined after five hours, the latter by means of Nessler's reagent after destruction of hydroxylamine with dichromate. Parallel experiments on hydroxylamine-free and on sterile media were made, and the residual ammonia found to be:

Test culture	220 γ NH_3-N
Sterile medium + NH_2OH	98 γ NH_3-N
NH_2OH-free culture	36 γ NH_3-N

Since the concentrations of hydroxylamine were sensibly identical in the first two cases, much of the increase in the test culture must have arisen from the liberation of nitrogenous substances from the cells. In accord with this observation, in some isotope experiments using high concentrations of hydroxylamine (and in which no fixation occurred), the total cell nitrogen showed progressive decrease with increasing concentration of hydroxylamine. It is clear that (i) the ammonia does not arise from the reduction of hydroxylamine; (ii) the extent to which lysis occurs, even at this relatively high concentration of hydroxylamine, is too small to invalidate the conclusions drawn from isotopic and respirometric data; the amount of nitrogen taken up during an experiment is very large compared with the amount of ammonia-N produced above.

Apparatus

Six flasks (Fig. 3) previously sterilised in an autoclave and the taps greased with sterile Apiezon M were so arranged that the synthetic atmosphere could be circulated uniformly through about 60 ml of culture in each; the use of sintered glass discs ensured good aeration. The flasks were attached to the circulating system by rubber connecters and each could be removed without affecting the circulation in remaining flasks. In later experiments alkathene was substituted for rubber. The apparatus was gas-tight, and pressures of 10^{-3} mm of mercury could be maintained over several days. Oscillating mercury piston pumps[30], with

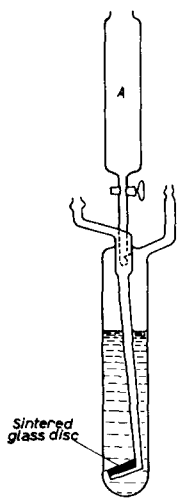


Fig. 3. Culture flask.

References p. 98/99.

the pistons covered with a layer of vacuum pump oil and operating through light, ground glass valves, were used; six such pumps in parallel produced circulation at the rate of about 100 litres per hour. The apparatus included a "ballast" of about 500 ml capacity to minimise changes in pressure during an experiment, soda lime tubes to maintain CO_2 concentration low, and suitable attachments for sampling. The cultures were admitted through A, oxygen passed through them for about two minutes to remove most of the atmospheric nitrogen, and the system evacuated, so becoming filled with water vapour. The prepared atmosphere was then admitted, and circulation allowed to proceed.

Early experiments were unsuccessful, partly because of lack of uniformity in aeration of the cultures, and partly because the organisms suffered oxygen starvation. The former difficulty disappeared when the sintered discs were introduced; the latter was avoided by (i) increasing p_{O_2} and (ii) raising the pumping rate to nearly 100 l per hour.

Results

The master culture was subdivided into six, each of about 10^{10} cells (equivalent to 2 mg of N); two were left free of hydroxylamine and the others made 10^{-5}, $3 \cdot 10^{-5}$, 10^{-4}, and $3 \cdot 10^{-4}$ *M* with respect to hydroxylamine. Circulation commenced, in all experiments, 1.5 hours after the initial standard inhibitor solution had been prepared. The atmosphere was $p_{N_2} = 0.24$ at. and $p_{O_2} = 0.60$ at. initially, the nitrogen containing 30.8 atom % excess of ^{15}N; further oxygen was admitted twice during the two-hour experiment to replace that lost in respiration, the rapid response to the additions indicating that equilibrium was reached. An approximate measure of the oxygen respired agreed well with the figure expected from the respiration data obtained with 80% oxygen atmospheres in the Warburg experiments (vide infra).

At the end of the run, the cells were killed by admission of acid, recovered by centrifugation, and samples of nitrogen prepared from them by Kjeldahl digestion, conversion to ammonia, and reaction of the latter with hypobromite. Recoveries both of ammonia and of nitrogen were measured as a check against contamination or leakage, and samples of the atmosphere at the beginning and end of each run were taken. The ^{15}N abundance was measured by means of the mass spectrometer. A set of typical results is given below.

TABLE II

Conc. of NH_2OH *(M)*	*% XS of* ^{15}N *over normal*	*Growth rate const., k* (h^{-1})
0	0.345	0.0059
10^{-5}	0.212	0.0037
$3 \cdot 10^{-5}$	0.145	0.0025
10^{-4}	0.065	0.0014
$3 \cdot 10^{-4}$	0.025	0.0004

Repetition of the experiments recently by Dr. M. A. AZIM, using a slightly lower oxygen pressure, a lower nitrogen pressure, and some argon has given almost identical values for the growth rates. The results are discussed later.

The respiration experiments

Since the inhibition of respiration does not vary markedly with density of culture, a density of 10^8 cells per ml was used, as in the isotope experiments. In the Warburg apparatus, inhibitions in air and in a mixture of 80% O_2 and 20% N_2 at atmospheric

References p. 98/99.

pressure were studied together, one in each set of seven manometers. One manometer in each set served as thermobarometer; two contained no inhibitor, and the remaining four contained different concentrations of hydroxylamine. In every case, 0.9 ml of culture was diluted with 0.1 ml of either sterile medium or hydroxylamine dissolved in sterile medium. As in the isotope experiments, the measurements began 1.5 hours after preparing the standard hydroxylamine solution, and continued for two hours.

Results from two typical sets of experiments are tabulated below; they are plotted as oxygen uptake against time (Figs. 4 and 5) and as the ratio of uptake (uninhibited) to uptake (inhibited) ($U_{O_2}/U^i_{O_2}$) against inhibitor concentration (Fig. 6).

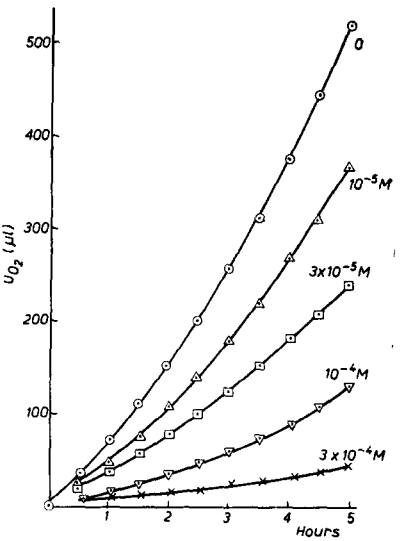


Fig. 4. Inhibition of respiration by hydroxylamine in air.

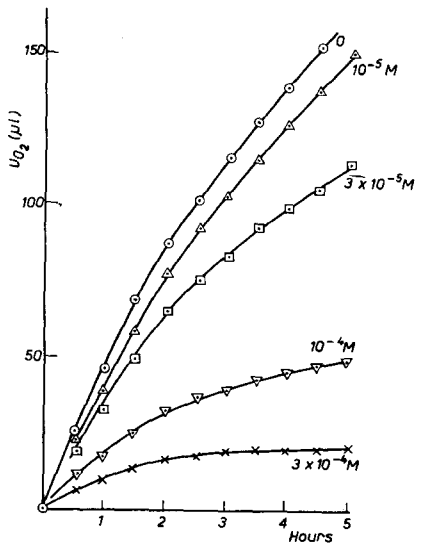


Fig. 5. Inhibition of respiration by hydroxylamine in 80% oxygen, 20% nitrogen.

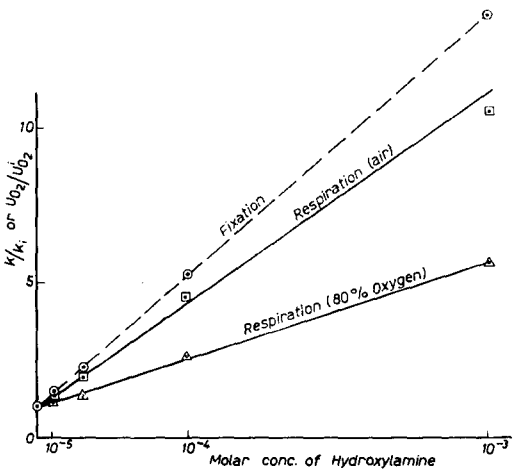


Fig. 6.

TABLE III

NH_2OH (M)	*I*				*II*			
	Residual resp. (%)		$U_{O_2}/U^i_{O_2}$		*Residual resp.* (%)		$U_{O_2}/U^i_{O_2}$	
	Air	*80% O_2*	*Air*	*80% O_2*	*Air*	*80% O_2*	*Air*	*80% O_2*
0	100	100	1	1	100	100	1	1
10^{-5}	68.2	79.6	1.47	1.26	69.2	90.0	1.44	1.11
$3 \cdot 10^{-5}$	51.1	—	1.96	—	50.0	74.6	2.00	1.34
10^{-4}	34.7	34.8	2.88	2.88	18.9	38.1	5.3	2.63
$3 \cdot 10^{-4}$	14.7	17.8	6.79	5.61	9.6	17.9	10.4	5.57

References p. 98/99.

Treatment of experimental results

Isotope data. A plot of the ratio of uninhibited to inhibited growth rate (k/k_i), calculated from the fixation data, against hydroxylamine concentration, c, gives a straight line, with unit intercept, in accordance with the relationship

$$k/k_i = 1 + \bar{K}_{N_2} \cdot c \qquad (2)$$

(where $\bar{K}_{N_2}$ is a constant). The value of the constant is $4.3 \cdot 10^4 M^{-1}$ (Fig. 6). It is more convenient to present the data as the plot of the empirical function

$$k_i = k^* - A \log c \qquad (3)$$

where k^* is the rate constant at the threshold concentration, c^*, below which hydroxylamine has no effect on fixation, and A is a constant. k^* is numerically equal to the uninhibited rate constant, and c^* is therefore determined by extrapolation of the plot to $k_i = k$. c^* is approximately $1 \cdot 10^{-6} M$. (Fig. 7).

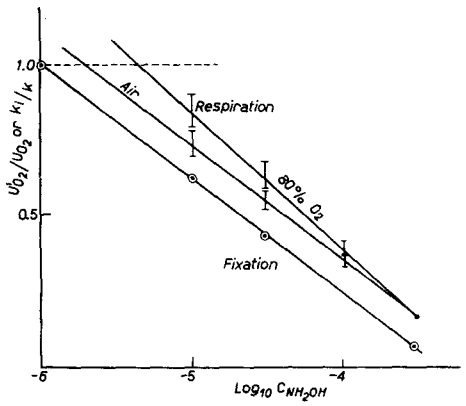


Fig. 7.

Respirometric data

The rate constant calculated from

$$k = \frac{1}{t} \cdot \ln \frac{\text{rate at time } t}{\text{rate at time O}}$$

for uninhibited cultures was 0.208 hr^{-1}, but in the presence of hydroxylamine the respiration curves do not increase regularly, and values of k_i from 0.11 to 0.6 may be obtained from different parts of the log plot. This is not entirely due to the continuous decomposition of inhibitor, since a plot of

$$k/k_i = 1 + \bar{K} \cdot f(t)$$

where $f(t)$ is the known-time-concentration relationship for hydroxylamine does not show the expected increases in k_i; the anomalous values may reflect the influence of another factor, possibly the conversion of the inhibitor to less toxic oximes.

The respiration data may conveniently be analysed in terms of total oxygen uptake. Thus when $U_{O_2}/U^i_{O_2}$ is plotted against c (Fig. 6), a straight line, with an intercept of unity is obtained, both for air and for 80% oxygen, in accordance with the equation

$$U_{O_2}/U^i_O = 1 + \bar{K}_{O_2} \cdot c \qquad (4)$$

where $\bar{K}_{O_2}$ is a constant. The values of $\bar{K}_{O_2}$ are lower for the high-oxygen atmosphere than for air, indicating competitive inhibition; thus although oxygen decreases the respiration rate it still competes in the usual way with another inhibitor and causes a decrease in the relative inhibition with increase in oxygen pressure. The only other case in which similar observations have been reported is the inhibition of *Azotobacter* respiration with cyanide.

As in the isotope data, it is advantageous to employ a function of the form

$$U^i_{O_2}/U^*_{O_2} = 1 - B \log c,$$

References p. 98/99.

$U^*_{O_2}$ being the oxygen uptake at the threshold concentration c^*, and B being a constant. From the plot (Fig. 7), c^* is found to be $2.3 \cdot 10^{-6}M$ in air, and $4.7 \cdot 10^{-6}M$ in 80% oxygen, both values considerably higher than the threshold concentration obtained from the nitrogen fixation data.

The plot of $\log \frac{U^i_{O_2}}{U_{O_2} - U^i_{O_2}}$ against $\log c$ is[31] in every case an unbroken straight line, clearly indicating that the inhibition is simple; there is no differential effect by hydroxylamine on "resting" and "active" respiration. The possibility that hydroxylamine may restrict cell growth processes and indirectly reduce respiration is also unlikely; it is known to inhibit the cytochrome system (and there is considerable[32] evidence that the respiratory system in Azotobacter is a modification of this), while direct anabolic inhibition, implying a double inhibitory effect, would be revealed by the above test. Further, the response to carbon monoxide shows that fixation may be completely inhibited without appreciable effect[33,34,35,36,37,38] on respiration, suggesting that the latter process can go on independently of fixation, and by implication, of growth.

These considerations lead to the conclusion that hydroxylamine inhibits respiration first and that any direct inhibition of fixation will be superimposed on this.

DISCUSSION

In the absence of quantitative thermodynamic data on fixation and of activation energies for the various possible steps in the process, it is impossible to infer whether the initial step is oxidative or reductive, except from a consideration of indirect evidence. The nitrogen-fixing and the nitrite- (and nitrate-) assimilating systems respond to carbon monoxide in a similar way, but the effect of this inhibitor on the assimilation of reduced nitrogen, *e.g.* ammonia, glutamate, etc.[33,35,36,39], is quite different. An oxidative initial step is therefore feasible, and if such were the case the oxidising agent may be a product of respiration or quite independent of this process. Were the latter true, the only reasonable agent would be oxygen itself, but this substance is not specific to fixation; it is concluded that if the first stage in fixation is an oxidation, then the oxidising agent must be a product of respiration, and must, of course, carry the necessary energy. A reductive first step would be endothermic, so that again an energy-carrying agent is essential. In both cases, a direct link between respiration and fixation would seem to require a high-energy product of the former.

Since only a small fraction of the oxidisable substrate is used to provide energy for fixation[39], the Fisher test applied above would scarcely detect the presence of a second respiratory system. No other oxygenating system has been proposed, apart from BURRIS AND WILSON's suggestion[33,39] that *Azotobacter* hydrogenase (assumed by them to be reversible) acts in a respiratory capacity through an unspecified oxygen carrier. The observed reversibility of hydrogenase[40] lends support to this idea, but much other evidence indicates that the oxygen-activating system in hydrogenase is the cytochrome-cytochrome oxidase system itself. We propose to disregard the possible existence of an additional respiratory system.

The adherence of both respiration and fixation data to equations of the form

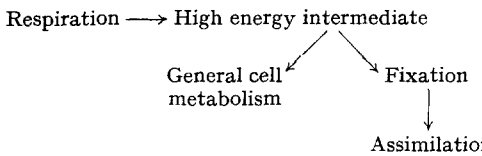
$$V/V_i = 1 + k \cdot c$$

(where V and V_i are uninhibited and inhibited rates respectively, k is a constant, and

References p. 98/99.

c is the concentration of hydroxylamine) demonstrates that the inhibition is in each case reversible, at least over short periods. The dependence of the value of the constant on oxygen pressure, in respiration, shows that inhibition of respiration is competitive. Hydroxylamine inhibits[24] the uptake of fixed nitrogen, but specific inhibition of the nitrogen-fixing system may be inferred from the relative total inhibitions of respiration and fixation considered in terms of a linked enzyme system.

The value of $\bar{K}_{O_2}$ in equation (4) is $1.55 \cdot 10^4 M^{-1}$ when p_{O_2} is 0.8 atmos. and $2.60 \cdot 10^4 M^{-1}$ when p_{O_2} is 0.2 atmos. By extrapolation, its approximate value at $p_{O_2} = 0.6$ atmos. (the oxygen pressure used in the fixation experiments) is $1.9 \cdot 10^4 M^{-1}$. This is significantly smaller than the value of $\bar{K}_{N_2}$ in equation (2) ($4.3 \cdot 10^4 M^{-1}$), indicating that the inhibition of fixation is much greater than that of respiration; this difference, moreover, is not the result of the use of the hydroxylamine as an alternative source of nitrogen, since it is not utilised. This result becomes clear in the light of a simple model, which may be regarded only as a convenient guide. A suitable scheme would be:



The nature of the high energy intermediate is unknown, e.g. it might be "energy-rich phosphate", hydrogen peroxide, free radicals, etc. Since respiration continues after fixation has been suppressed[21], the intermediate may be assumed to be diverted to general metabolic processes.

The above model postulates three reactions:

$$\mathrm{E} \longrightarrow \mathrm{I} \qquad \text{(i)}$$

$$N + I \longrightarrow \text{Fixation} \qquad \text{(ii)}$$

$$M + I \longrightarrow \text{General cell metabolism} \qquad \text{(iii)}$$

where E represents the respiratory system, N the nitrogenase system, and M the enzyme system involved in other metabolic reactions; I is an unspecified, high energy product of respiration capable of reacting in each of the other systems.

For (i) we may write

$$V_E/iV_E = 1 + \bar{K}_E \cdot c$$

where the V's refer to rates of production of I and c is the concentration of hydroxylamine. Using the corresponding symbols for the other reactions, the general equations relating the rates are:

$$V_E = V_N + V_M \qquad \text{(a)}$$

$$iV_E = iV_N + iV_M \qquad \text{(b)}$$

or, in terms of the stationary concentration of the intermediate,

$$V_E = \frac{V_N^* [I]}{K_N + [I]} + \frac{V_M^* [I]}{K_M + [I]} \qquad \text{(c)}$$

$$iV_E = \frac{V_N^* [I]_i}{K_N + [I]_i} + \frac{V_M^* [I]_i}{K_M + [I]_i} \qquad \text{(d)}$$

where the asterisks denote maximum rates, and K_N and K_M are the MICHAELIS[41] constants

References p. 98/99.

of N and M with respect to I. Solution of (c) and (d) would permit comparison between the ratios V_N/iV_N and V_E/iV_E, but leads only to cumbersome quadratics in $[I]$ and $[I]_i$. It is convenient, therefore, to impose a limiting condition, *viz.* K_N and K_M are large compared with $[I]$, and effect a special solution. This condition is not unreasonable in view of the sensitivity of rates of fixation and general metabolism to changes in the rate of respiration.

Then

$$V_E = \frac{V_N^*}{K_N} \cdot [I] + \frac{V_N^*}{K_M} \cdot [I] \tag{e}$$

and

$$\frac{V_N}{V_E} = \frac{V_N^*/K_N}{V_N^*/K_N + V_M^*/K_M} \tag{f}$$

Let us now consider three cases:

I. E alone is inhibited; it may be shown that, since $V_E/iV_E = 1 + \bar{K}_E \cdot c$,

$$V_N/iV_N = V_E/iV_E \tag{g}$$

II. E and N are inhibited, E as in case I., and N according to the relation $V_N/iV_N = 1 + \bar{K}_N \cdot c$. This applies to both competitive and non-competitive inhibition if $\bar{K}_N$ is much greater than $[I]$. Then we have

$$iV_E = \frac{V_N^*/\bar{K}_N}{1 + \bar{K}_N \cdot c} \cdot [I]_i + \frac{V_N^*}{\bar{K}_M} \cdot [I]_i$$

and

$$\frac{V_N}{iV_N} = \frac{V_E}{iV_E} \cdot \frac{(V_M^*/\bar{K}_M)\,(1 + \bar{K} \cdot c) + V/\bar{K}_N}{V_M^*/\bar{K}_M + V_M^*/\bar{K}_N}$$

Since the fraction of oxidisable substrate used in fixation is very small, $V_N^*/\bar{K}_N$ is much smaller than $V_M^*/\bar{K}_M$, so that this relationship approximates to

$$\begin{aligned} V_N/iV_N &= (1 + \bar{K}_N \cdot c) \cdot V_E/iV_E \\ &= (1 + \bar{K}_N \cdot c)\,(1 + \bar{K}_E \cdot c) \\ &= 1 + (\bar{K}_N + \bar{K}_E) \cdot c \end{aligned} \tag{h}$$

if small, c^2, terms are neglected.

III. E, N, and M are inhibited; E and N as above, and M according to the relation $V_M/iV_M = 1 + \bar{K}_M \cdot c$. By similar reasoning we then find

$$\frac{V_N}{iV_N} = \frac{V_E}{iV_E} \cdot \frac{1 + \bar{K}_N \cdot c}{1 + \bar{K}_M \cdot c} \tag{j}$$

These relationships imply that inhibition of respiration alone leads to proportional inhibition of fixation; inhibition of all three systems may give rise to inhibition of fixation which is greater than or less than or equal to the inhibition of respiration, depending on the values of $\bar{K}_N$ and $\bar{K}_M$; inhibition of respiration and fixation only leads to greater inhibition of the latter than of the former—the form actually obtained in the present experiments. If the model is significant, therefore, the major effect of hydroxylamine is to inhibit respiration and fixation in series, with little concomitant effect on other cell reactions closely related to respiration. This being so, the constant

References p. 98/99.

$\bar{K}_{N_2}$ ($= 4.3 \cdot 10^4 M^{-1}$) becomes the sum of $\bar{K}_{O_2}$ and $\bar{K}_N$, where the latter defines the specific inhibition of the nitrogenase system by hydroxylamine, and has therefore the value $2.4 \cdot 10^4 M^{-1}$ when $p_{O_2} = 0.6$ atmos. Reference to equation (h), however, shows that when c is of the order $10^{-4} M$, the c^2 terms are no longer negligible, and the continuation of the straight line relationship beyond this point is surprising. In the unlikely event that no competition occurs between the postulated intermediate I and hydroxylamine, $\bar{K}_N$ would be reciprocal of the Michaelis constant of the nitrogenase-hydroxylamine complex; its value would indicate 50% inhibition of the nitrogenase system alone when the concentration of the hydroxylamine was about $4 \cdot 10^{-5} M$.

The possibility that the processes of respiration and fixation are linked through a high-energy intermediate of the former provokes speculation as to the nature and mode of action of this substance. The fact that both hydroxylamine and carbon monoxide specifically inhibit fixation suggests that a heavy metal catalyst (probably a haemoprotein) is operative in the nitrogen-fixing system, since ferrous haemoproteins are sensitive to carbon monoxide[42,43,44] and hydroxylamine is a common inhibitor of ferric haemoproteins[44,45,46,47]. There is also much evidence of the formation of hydrogen peroxide during respiration[44,48,49].

In the light of these facts, and of the catalysis of polymerisation and oxidation reactions by the ferrous ferric-hydrogen peroxide system[50,51,52,53,54,55] (which occurs through the agency of hydroxyl radicals), and also of the observation that catalase[56,57,58,59] and methaemoglobin[60] act by a change in the valency of their iron and the production of hydroxyl radicals[50], it seems reasonable to postulate that the fixation process is brought about by such free radicals, *e.g.*

$$N_2 + 2OH \longrightarrow N_2(OH)_2$$

It is envisaged that the radicals form a complex, $E_N \cdot (OH)_2$, with the nitrogenase, and oxidise molecular nitrogen. The presence of OH radicals would, of course, lead to affinity for hydrogen, and a scheme may be formulated to include the competitive inhibition by this gas:

$$E_N + 2OH \longrightarrow E_N \cdot (OH)_2 \xrightarrow{N_2} E_N(OH)_2N_2 \longrightarrow E_N + \text{fixation products}$$

$$E_N \cdot (OH)_2 \xrightarrow{H_2} E_N \cdot (OH)_2H_2 \text{ (inactive)}$$

Although this scheme is in some respects similar to one of those put forward by WILSON AND BURRIS[39], which involved "hydroxylated" enzyme, these workers preferred to account for hydrogen competition on the basis of a "hydrogenated" enzyme, $A_h \cdot H$, the competition being written

$$2A_h \cdot H + H_2 \longrightarrow 2A_h \cdot H_2 \text{ (not catalysed)}$$
$$2A_h \cdot H + E_NN_2 \longrightarrow E_N + 2A_h + \text{fixed nitrogen (fixation)}$$

It can be shown, however, that competititon at normal p_{H_2} and p_{N_2} would not be observed if the Michaelis constant of nitrogenase is as low as published data suggest. Nor, if the first reaction were catalysed, would the case be different, since the only likely catalyst (the hydrogenase) again has a very low Michaelis constant.

In the scheme now put forward, the significance of the Fe^{+3}/Fe^{+2} couple is that it allows oxidation of nitrogen by hydroxyl radicals; it accounts for hydrogen competition

References p. 98/99.

in terms of direct competition for nitrogen-activating sites; and it is in accord with the non-competitive inhibition of fixation by carbon monoxide and the slight response of the nitrate- and nitrite-assimilating system to this gas.

One further feature of Azotobacter physiology requires explanation, namely the dependence of the production of the adaptive enzyme hydrogenase on the utilisation of gaseous nitrogen[61]. One of WILSON's[39] schemes assigns a role to hydrogenase, but this does not account for the above dependence; moreover, if, as he suggested, radicals arose from a reversed Knallgas reaction the link with respiration is obscured. The hypothesis given above requires that the radical producer must be closely linked with the nitrogenase, and it is reasonable that it may indeed by hydrogenase, which is known to undergo Fe^{+3}/Fe^{+2} changes[64]. Indeed it is not unlikely that hydrogenase and nitrogenase are the same enzyme with two sets of active sites.

The postulated formation of a compound of formula $N_2(OH)_2$ suggests that hyponitrous acid may be the earliest product of fixation, and this notion appears to receive support from the competitive inhibition of fixation by nitrous oxide. Further work on this aspect of the problem will be reported shortly.

ACKNOWLEDGEMENT

The authors wish to express their indebtedness to the Royal Society for a grant with which the isotopic nitrogen used in these experiments was purchased, and Messrs. B. LAPORTE, Ltd., for a gift of pure 80% hydrol. One of us (B.A.P.) is indebted to the Ministry of Education and the University of London for financial assistance during the course of this work.

SUMMARY

A method is described whereby the rate of increase in cell content of a particular material may be measured isotopically. The method is free from sampling errors, and gives accurate values in short experiments. It has been applied to the study of the effect of hydroxylamine on fixation of nitrogen and on respiration in *Azotobacter vinelandii*. Respiration and fixation are inhibited in series. The results have been interpreted in terms of a linked enzyme system, and the linkage is believed to occur through the medium of a high-energy product of respiration, possibly hydrogen peroxide acting as a source of hydroxyl radicals.

In the course of preliminary investigations, the rate of decomposition of hydroxylamine in aqueous solution and in medium (both sterile and inoculated with *A. vinelandii*) were measured. The reaction is first order over a wide range of concentration; nitrite and ammonia appear among the decomposition products. Nitrogen does not interchange with nitrite or hydroxylamine. Hydroxylamine is not utilised by the organism.

RÉSUMÉ

Les auteurs décrivent une méthode isotopique qui permet de mesurer la vitesse d'accroissement de la teneur des cellules en un produit donné. Cette méthode ne comporte pas d'erreurs d'échantillonnage et donne des valeurs précises dans des expériences courtes. Elle a été appliquée à l'étude de l'action de l'hydroxylamine sur la fixation de l'azote et sur la respiration chez *Azotobacter vinelandii*. La respiration et la fixation sont inhibées en série. Ces résultats s'expliqueraient par l'existence d'un système enzymatique couplé, la liaison se produisant par l'intermédiaire d'un produit de la respiration riche en énergie, qui pourrait être l'eau oxygénée servant de source de radicaux hydroxyles.

Au cours d'expériences préliminaires, la vitesse de décomposition de l'hydroxylamine en solution aqueuse et dans un milieu (stérile ou inoculé avec *A. vinelandii*) a été mesurée. La réaction est du premier ordre dans un domaine de concentration étendu; parmi les produits de décomposition figurent les nitrites et l'ammoniac. L'azote ne s'échange pas avec les nitrites ou l'hydroxylamine. L'hydroxylamine n'est pas utilisée par l'organisme étudié.

References p. 98/99.

ZUSAMMENFASSUNG

Es wird eine Methode beschrieben mit der die Geschwindigkeit der Zunahme von Zelleninhalt an einem besonderen Stoff isotopisch gemessen werden kann. Die Methode wird nicht beeinflusst durch Fehler bei der Probeentnahme und gibt in kurzen Versuchen genaue Werte. Sie wurde bei der Untersuchung der Wirkung von Hydroxylamin auf die Fixierung von Stickstoff und auf die Respiration in *Azotobacter vinelandii* angewendet. Die Respiration und die Fixierung werden in Serie gehemmt. Die Ergebnisse werden interpretiert als ein Ausdruck eines verbundenen Enzymsystems und es wird angenommen, dass die Bindung mittels eines energiereichen Atmungsproduktes erfolgt und wahrscheinlich Wasserstoffsuperoxyd als Quelle für das Hydroxylradikal verantwortlich ist.

Im Laufe von vorhergehenden Untersuchungen wurde die Zersetzungsgeschwindigkeit von Hydroxylamin in wässriger Lösung und im Medium, die beide steril und mit *A. vinelandii* geimpft waren, untersucht. Sie zeigt sich in einem grossen Konzentrationsbereich als eine Reaktion erster Ordnung; Nitrit und Ammoniak treten als Zersetzungsprodukte auf. Stickstoff ist nicht vertauschbar mit Nitrit oder Hydroxylamin. Hydroxylamin wird vom Organismus nicht verbraucht.

REFERENCES

1 M. V. FEDEROV, *Mikrobiologiya*, 15 (1946) 509.
2 M. V. FEDEROV, *C.r. acad. sci. U.R.S.S.*, 52 (1946) 81.
3 M. V. FEDEROV, *C.r. acad. Sci. U.R.S.S.*, 49 (1945) 675.
4 R. H. BURRIS AND P. W. WILSON, *Ann. Rev. Biochem.*, 1945.
5 D. MOLNAR *et al.*, *J. Am. Chem. Soc.*, 70 (1948) 1713.
6 T. G. G. WILSON AND E. R. ROBERTS, *Chem. and Ind.*, (1952) 87.
7 D. BURK AND C. K. HORNER, *Soil Science*, 41 (1936) 8; *Trans. 3rd Intern. Congr. Soil Sci.*, Oxford, 1 (1935) 148.
8 W. W. UMBREIT, R. H. BURRIS AND J. F. STAUFFER, *Manometric Techniques for the Study of Tissue Metabolism*, Burgess, 1944.
9 E. B. FRED AND S. A. WAKSMAN, *Laboratory Manual of General Microbiology*, 1928.
10 C. K. HORNER *et al.*, *J. Agric. Res.*, 65 (1942) 173.
11 S. J. BUREMA AND K. T. WIERINGA, *Chem. Zentr.*, (1943 II) 130.
12 H. L. JENSEN, *Proc. Linnean Soc. N.S. Wales*, 72 (1948) 299.
13 S. I. MATYASHVIA, *Mikrobiologiya*, 16 (1947) 19.
14 F. RASCHIG, *Ann.*, 241 (1887) 161.
15 A. D. MITCHELL, *Sutton's Volumetric Analysis*, 1935.
16 K. A. HOFFMANN AND F. KNOLL, *Ber.*, 57B (1924) 937.
17 A. KURTENACKER AND F. WERNER, *Z. Anorg. Allgem. Chem.*, 160 (1927) 387.
18 M. BODENSTEIN, *Z. Electrochem.*, 47 (1941) 501.
19 Y. NISHINA *et al.*, *J. Chem. Phys.*, 9 (1941) 571.
20 T. H. NORRIS *et al.*, *J. Chem. Phys.*, 9 (1941) 726.
21 G. G. JORIS, *J. Chem. Phys.*, 9 (1941) 775.
22 J. BLOM, *Ber.*, 59 (1926) 121.
23 M. LEMOIGNE *et al.*, *Bull. soc. chem. biol.*, 18 (1936) 841, 868.
24 W. SEGAL AND P. W. WILSON, *J. Bact.*, 57 (1949) 55.
25 B. A. PETHICA *et al.*, *J. Chem. Phys.*, 18 (1950) 996.
26 A. D. MITCHELL, *J. Chem. Soc.*, (1926) 336.
27 P. R. LEWIS AND C. N. HINSHELWOOD, *J. Chem. Soc.*, (1948) 824.
28 R. NOVAK AND P. W. WILSON, *J. Bact.*, 55 (1948) 517.
29 A. I. VIRTANEN, *Biol. Rev.*, 22 (1947) 258.
30 E. R. ROBERTS, H. J. EMELEUS AND H. V. A. BRISCOE, *J. Chem. Soc.*, (1939) 41.
31 K. C. FISHER AND J. R. STEARN, *J. Cell Comp. Physiol.*, 19 (1942) 102.
32 S. B. LEE *et al.*, *Proc. Soc. Exptl. Biol. Med.*, 50 (1947) 96.
33 R. H. BURRIS AND P. W. WILSON, *Bot. Gaz.*, 108 (1946) 256.
34 C. J. LIND AND P. W. WILSON, *J. Am. Chem. Soc.*, 63 (1941) 3511.
35 C. J. LIND AND P. W. WILSON, *Arch. Biochem.*, 1 (1943) 59.
36 P. W. WILSON AND C. J. LIND, *J. Bact.*, 45 (1943) 219.
37 P. W. WILSON, *Ergeb. Enzymeforsch.*, 8 (1939) 13.
38 E. R. EBERSOLE *et al.*, *Arch. Biochem.*, 3 (1944) 399.
39 P. W. WILSON AND R. H. BURRIS, *Bact. Rev.*, 11 (1947) 41.
40 B. A. PETHICA *et al.*, *Research*, 3 (1950) 382.
41 M. MICHAELIS AND M. L. MENTEN, *Biochem. Z.*, 49 (1913) 1333.
42 H. D. HOBERMAN AND D. RITTENBERG, *J. Biol. Chem.*, 147 (1943) 211.
43 S. B. LEE *et al.*, *ibid.*, 144 (1942) 273.

[44] C. OPPENHEIMER AND K. G. STERN, *Biological Oxidation*, The Hague, 1939.
[45] M. G. SEVAG *et al.*, *Biochem. Z.*, 228 (1936) 41; *J. Biol. Chem.*, 144 (1942) 711.
[46] C. N. HINSHELWOOD, *Biol. Rev.*, 19 (1944) 150.
[47] J. LASCELLES AND J. L. STILL, *Austr. J. Expl. Biol. Med. Sci.*, 24 (1946) 37.
[48] C. K. HORNER AND H. LINEWEAVER, *J. Cell Comp. Physiol.*, 1 (1932) 435.
[49] J. B. SUMNER AND G. F. SOMERS, *Enzymes*, New York, 1947.
[50] J. WEISS, *J. Phys. Chem.*, 41 (1937) 1107.
[51] F. HABER AND J. WEISS, *Proc. Roy. Soc.*, 147A (1934) 332.
[52] J. H. BAXENDALE *et al.*, *Trans. Faraday Soc.*, 42 (1946) 155, 668.
[53] J. WEISS AND C. W. HUMPHREY, *Nature*, 163 (1949) 691.
[54] M. G. EVANS, *J. Chem. Soc.*, (1947) 266.
[55] J. H. MERZ AND W. A. WATERS, *Discussions Faraday Soc.*, 2 (1947) 179.
[56] J. WEISS AND H. WEIL-MALHERBE, *Nature*, 144 (1939) 866.
[57] D. KEILIN AND E. F. HARTREE, *Proc. Roy. Soc.*, B124 (1937) 397.
[58] R. LEMBERG AND E. C. FOULKES, *Nature*, 161 (1948) 131.
[59] P. GEORGE, *ibid.*, 160 (1947) 41.
[60] D. KEILIN AND E. F. HARTREE, *ibid.*, 166 (1950) 514.
[61] J. B. WILSON *et al.*, *J. Biol. Chem.*, 144 (1942) 265.

Received December 11th, 1953