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Enzyme Systems in Nitrogen Fixation

With Special Reference to the Role of Hydrogenase

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Philosophy in the University of London

by

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ABSTRACT

The primary object of any research into the biochemical fixation of nitrogen by soil bacteria, algae etc. is to establish the pathway whereby the nitrogen molecule is converted into amino acids and protein.

Nitrogen fixing bacteria, as well as many bacteria incapable of fixing nitrogen, contain a powerful hydrogenase which may be directly linked to the process, and one of the main topics has been to establish whether any of the possible intermediates of a reductive mechanism could be further reduced by this enzyme system. The mechanism considered is $\text{N}\equiv\text{N} \rightarrow \text{-N=N-} \rightarrow \text{N-N} \rightarrow \text{NH}_2$. Cell free fixation of nitrogen has not been achieved with certainty, although some claims to have obtained fixation in vitro have been made, and so the investigation had to begin with a study of the azo grouping. Particular attention has been focussed on the role of metal stimulation of the reduction of this grouping.

Various compounds have been prepared with the object of varying the electron density between the nitrogen atoms, and, as anticipated, a decrease in density causes a marked increase in the rate of reduction. The action of the enzyme has been compared with that of heterogeneous catalysts.

The study has been extended to include examination of the analogous ethylenic compounds and azomethines, and on the use of artificial carriers.

The Michaelis constant of nitrogenase has been redetermined by the use of isotopic techniques, and the higher value (as compared to the respirometric value) obtained by T.G.G. Wilson and E.R. Roberts in this laboratory has been confirmed. The constant had no significant temperature dependence, and the conclusions which can be drawn from this are discussed.

Attempts at purifying the hydrogenase enzyme from both *azotobacter vinelandii* and *desulphovibrio desulphuricans* have been carried out by using radioactive iron and cobalt, and these metals have been shown to be present at every level of purification which shows enzymic activity.

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Introduction

Hydrogenase may be defined as an enzyme capable of activating molecular hydrogen, either for participation in an isotopic hydrogen exchange reaction with water, or for the reduction of various acceptor molecules. All the evidence points to hydrogenase's being a necessary component of the systems responsible for hydrogen production.

Azotobacter vinelandii is the best example of an organism containing hydrogenase, but not oxidising or producing hydrogen during normal metabolism.

If the bacteria are cultivated in a medium containing ammonia or other forms of fixed nitrogen, a marked depression in hydrogenase content occurs, suggesting a close relationship between nitrogen fixation and hydrogenase activity (1, 2, 3). This relationship has already been suggested by other phenomena e.g. the competitive inhibition of nitrogen fixation (4, 5), and inhibition of light-stimulated hydrogen production by photosynthetic bacteria (6, 7).

Hydrogenase is also found in bacteria not capable of fixing nitrogen under the usual conditions (e.g. *E. coli*), but some strains of the anaerobic *Desulphovibrio desulphuricans*, which contains the most powerful hydrogenase yet discovered, have now been shown to be capable of fixing nitrogen (8), so that the correct conditions for its detection may not have been obtained in all cases.

In addition to the above examples, hydrogen^{ase} has been detected in organisms which neither fix nitrogen nor possess a normal hydrogen metabolism, for example in *Acetobacter peroxydans* (9) *Bacillus acidilactici* (10, 11, 12) and others. In addition Boichenko (13, 14, 15) has reported the presence of the enzyme in chloroplasts.

The properties of hydrogenase from *azotobacter* in cell-free extracts appear to be essentially similar to those of *E. coli* (2, 16). In *azotobacter*, practically all the activity is associated with insoluble cell wall material and mitochondria.

Acceptors

Reduction of acceptors in general may require the participation of several intermediate carriers, and, after activation of molecular hydrogen for the reduction of a carrier, electron (or hydrogen) transport could occur through widely different sequences, depending on the acceptor and the particular type of organism. Preparations from *azotobacter* oxidise hydrogen in the presence of dyes such as methylene blue and benzyl viologen, and also ferricyanide, cytochrome c and oxygen, but not in the presence of nitrate, fumarate, pyruvate, α -keto glutarate, DPN or riboflavin (2, 16).

Resting cells of photosynthetic bacteria can reduce e.g. oxygen, nitrate, sulphate, thiosulphate, ferricyanide, fumarate and dyes (17, 18, 19, 20).

The reduction of carriers is variously affected by inhibitors, the Knallgas reaction using *E. coli*, for example, is completely inhibited by cyanide, whereas the same reaction in *Bacillus delbrückii* is unaffected (21). Further work on this aspect of hydrogenase has confirmed that specificity for acceptors exists among the enzymes from different sources (22, 23), so that it cannot be regarded as a general reductase.

These considerations suggest that with a given terminal acceptor, the same overall reaction may involve different enzyme systems so that no single mechanism exists for the reduction of each particular acceptor.

Fumarate has often been quoted in the literature as being capable of reduction by hydrogenase, but the result of work carried out by the author has shown that reduction of this and other ethylenic systems by *Azotobacter vinelandii* and *Desulphovibrio desulphuricans* does not occur. This suggests the possibility of the existence of an enzyme system specific for the ethylenic linkage, and if so, it is presumably absent in the bacteria under consideration here.

Proteus vulgaris is capable of reducing fumarate, and the rate of reaction is increased 50-200% by the addition of this acceptor to the growth medium, although the rate of the exchange reaction is unaffected (24).

Partial inhibition of the reaction by crude cell-free extracts of *E. coli* is observed when e.g. α' -dipyridyl is added (25). The crude extracts contain suspended particles which are

the site of reduction of fumarate, nitrate and ferricyanide (26), fumarate reduction being inhibited by o phenanthroline but being partially reversed by the addition of Fe^{++} , Mn^{++} or Zn^{++} (27), although probably these ions were stimulants.

Reduction of methylene blue by cell-free preparations is unaffected by complexing agents, so that hydrogen transfer to this dye may be via a shorter sequence of processes than the fumarate reduction.

The identity of the enzyme system in fumarate reduction has not been elucidated, but could be succinic dehydrogenase acting in reverse, or fumaric reductase which is a flavin enzyme found as yet only in yeast, and capable of transferring hydrogen from reduced dyes to fumarate (28, 29).

Nitrate. This can be reduced by various strains of colon aerogenes bacteria either to nitrite or ammonia (30), and *Cl. welchii* and *Bact. coli* for example, are capable of reducing nitrate, nitrite and hydroxylamine to ammonia (31), although it has not been possible to demonstrate the intermediate formation of hydroxylamine in the reduction of nitrate or nitrite.

Many strains of colon aerogenes organisms can reduce nitrate to the nitrite stage, although in the presence of a suitable redox dye such as benzyl viologen, reduction of nitrite and hydroxylamine occurred. This suggests that certain bacteria do not possess the necessary intermediate carriers for the full reduction; however, work carried out by the author shows that transfer to nitrate via benzyl viologen does not occur, so that,

in the above example, the dye may not be acting as the terminal carrier.

Evans and Nason (32), working with a purified nitrate reductase from *Neurospora crassa*, showed that reduction of nitrate to nitrite in the presence of reduced TPN is mediated by FAD.

The exchange reaction

Hydrogenase activity may also be assayed by the so called exchange reaction in which the reaction $D_2 + H_2O \longrightarrow HD + HDO$ is catalysed, but owing to the relatively difficult experimental techniques, reduction of dyes is generally used. Since the exchange reaction can be carried out under strongly reducing conditions, highly purified preparations of the enzyme can be assayed which would be inactivated by other methods. Unfortunately, the method may be somewhat limited in its application since Burris (16) has shown that particulate preparations from *Azotobacter* catalysed the exchange reaction at a very slow rate while showing high activity with respect to hydrogen oxidation. No reasonable explanation was given by this worker, and the matter should certainly be given more attention.

The use of the radioactive isotope of hydrogen H_1^3 would probably be a useful improvement on the deuterium exchange method, although the possibility of isotopic discrimination might be an important factor. Franke and Münch (33), however, have shown that the uptake of hydrogen and deuterium in the reduction of fumarate by *E. coli* occurs at an identical rate. Similarly, Weller and

Mills (34) obtained no evidence of discrimination between deuterium and tritium in the catalytic hydrogen of quinone by copper acetate in isoquinoline solution. Assays based on tritium may therefore prove to be the standard method of the future for the detection of hydrogenase if the observations of Burris (16) can be resolved.

Hoberman and Rittenberg (35) observed that dilute suspensions of *P. vulgaris* could catalyse the exchange reaction, but were incapable of reducing methylene blue. Exchange did, however, occur in the presence of fumarate which was assumed to be able to substitute for the natural carriers required for hydrogenation, but not for exchange.

These workers also found that urethane inhibited methylene blue reduction but had no effect on the exchange reaction. These results suggest the possibility of two separate enzyme systems.

Enzyme preparations from the same source also show different properties e.g. soluble extracts from *E. coli* reduced methylene blue rapidly, but were inactive with respect to nitrate, fumarate and ferricyanide, unlike the particulate preparations from the same cells (36).

Joklik (36) also examined a series of redox indicators and found no dependence of the rate of reduction by extracts from *E. coli* on either chemical similarity or redox potential. Krasna and Rittenberg (37) made a similar, but more extensive study using various bacteria, all of which catalysed the exchange reaction, and reached the same conclusion, although the matter should be further clarified by an examination of the inhibitory effects of these

dyes before any definite statement can be made. Methylene blue has been shown to inhibit the hydrogenase from *Desulphovibrio desulphuricans* whereas benzyl viologen and methyl violet had little effect. Hydrogen uptake by the homogeneous system: CoCl_2/KCN was also inhibited by redox dyes in the order methylene blue > benzyl viologen > methyl violet (38).

Inhibitors

Carbon monoxide inhibits the enzymic reaction (39, 40), and Rittenberg (41) observed a reversal of this inhibition by light, although this could not be confirmed by other workers e.g. Stern (42), perhaps because the light used was not sufficiently intense.

Hydrogenase is inhibited by oxygen, the Knallgas reaction ($2\text{H}_2 + \text{O}_2 \longrightarrow 2\text{H}_2\text{O}$) only occurring if hydrogen is in large excess, although some bacteria are capable of carrying out the reaction in a mixture of hydrogen and oxygen in the ratio 2:1.

The inactivated enzyme can be reactivated by incubation under hydrogen or by the addition of donors such as glucose, pyruvate, formate, fumarate and succinate (41), although the action of the latter could not be confirmed by Lascelles and Still (43). Cyanide prevents this reactivation, so that 10^{-3} M KCN inhibits only when added in air and not when added under hydrogen.

Lascelles and Still (43, 44) found that the cell free enzyme from *E. coli* was not inhibited by sodium azide, which even stimulated the Knallgas reaction. They found that azide inhibition of the methylene blue reduction was small, and unaffected by the

state of oxidation of the enzyme, in contrast to the effect of hydroxylamine and hydrazine which evidently inhibit the conversion to the active reduced state. These inhibitors affect the Knallgas reaction much more than the methylene blue reaction.

Joklik (45, 46) has suggested that inactivation of the enzyme by oxygen can be explained by oxidation of sulphhydryl groups, on the evidence of inhibition of hydrogenase by e.g. o-mercuribenzoate, o-iodobenzoate, or phenyl mercuric acetate (PMA), the effect of the latter being reversed by thioglycollic acid. Under the experimental conditions, inhibition by PMA was only slight (28%). Thioglycollic acid caused 80% stimulation when added alone, subsequent addition of PMA having no effect.

The evidence for the dependence of oxygen sensitivity on the -SH groups is thus not conclusive, and Gest (20) contradicts the theory on the grounds that p-chloromercuribenzoate did not significantly affect the hydrogenase activity of cell-free extracts from *E. coli*, and that the partial oxygen inactivation was not corrected by addition of cysteine and other '-SH' reagents. The problem can be regarded as incompletely resolved as yet.

Joklik (46) suggests that the results of his experiments with carbon monoxide and cyanide on methylene blue reduction by cell-free extracts of *E. coli* are not consistent with the idea of an iron porphyrin prosthetic group. Carbon monoxide inhibition was not reversed by light, casting doubt on the proposal of Hobermann and Rittenberg (41), especially since objections have

been raised against an iron porphyrin group on the basis of oxidation-reduction potentials.

From these observations and inhibition studies, a reasonable assumption is that the enzyme is a ferrous heme compound which is inactive in the ferric state, and functions without a change of valency.

The role of iron in the action of the enzyme has been conclusively demonstrated by the work of Waring and Werkman (47), who showed that *Aerobacter indologenes* made deficient of iron by cultivation in a medium made free of metals by treatment with 8-hydroxy quinoline, showed only a very low hydrogenase activity. Replacement of e.g. copper to the medium failed to restore hydrogenase activity, but iron was effective in this respect.

However, its nature as a heme compound cannot be regarded as proved, since Gaffon (48) has shown that the hydrogenase of a green algae is inhibited by o phenanthroline, which is a powerful complexing agent with iron, and can remove it even from ferricyanide, but does not react with normal heme compounds in which the iron is inaccessible to the chelating influence of such reagents. The enzyme may in fact be a bile pigment type of compound, since the iron of these is relatively loosely bound.

Models for hydrogenase

Attempts have been made to correlate the action of hydrogenase with model systems consisting of heterogeneous or homogeneous catalysts. As early as 1934 Green and Stickland (49) showed that the enzyme from *B. coli* catalysed the reaction

$H_2 \rightleftharpoons 2H^+ + 2e$ in a reversible manner, and that at the same partial pressure of hydrogen and the same pH , the extent of reduction of methyl viologen with the enzyme and colloidal platinum was the same.

The mode of action of heterogeneous catalysts was originally considered to be relatively simple in view of the nature of the hydrogen molecule and inorganic catalysts, but the work of e.g. Farkas (50) has shown that the process is far from easily explained.

In the case of reduction of the ethylenic linkage, the weight of evidence lies in favour of an associative chemisorption at the double bond followed by reaction with chemisorbed hydrogen atoms or molecules. Couper and Eley (51) have shown that filling the partially empty d band of palladium by alloying it with gold destroys its catalytic activity, from which they argue that the exceptional behaviour of transition elements is due to their possessing vacant d orbitals of low energy and high numerical density.

As already mentioned, apart from acting as a reductase, the enzyme is also capable of catalysing the hydrogen - deuterium exchange and the ortho-para hydrogen conversion, and several systems have been discovered which carry out similar reactions.

The ortho-para conversion, for example, is strongly catalysed by many metals (51, 52) and by paramagnetic solids (53).

Copper acetate in pyridine or quinoline is capable of catalysing all three reactions (including the hydrogenation of quinone), and is probably the best model for hydrogenase at the present time (54).

Resonant systems similar to the porphyrins, as well as their metal derivatives can function as activators of molecular hydrogen above 200°. Calvin (55) and Eley (56) have shown that phthalocyanine and its copper derivative:

- a) enable deuterium to exchange with the hydrogen of water
- b) exchange part of their own hydrogen for deuterium
- c) catalyse the Knallgas reaction.

Unfortunately, the results were not reproducible, and not all their preparations were active (57). Phthalocyanines as well as porphyrins and haematins also catalyse the ortho-para exchange (58). Since the activation energy of the reaction $\text{H}_2 \rightarrow 2\text{H}$ in the presence of these catalysts is so small, the process might be observable at physiological temperatures if a sufficiently large surface area of these systems were to be used.

Nitrogen Fixation

Nitrogen fixation occurs in a wide variety of organisms e.g. Azotobacter, Clostridia, legume nodules, photosynthetic bacteria, chlorobium species, chromatium species, blue green algae, Nostoc muscorum and Anabaena cylindrica, and in soil yeasts such as Sacharromyces and Rhotorulla species (59).

Cell free nitrogen fixation has not yet been achieved with certainty, although some positive results have been claimed for Clostridia and Azotobacter.

Shug (60) using Clostridium extracts, showed a two-banded difference spectrum at 450 m μ and 390 m μ . on exposure to a gas mixture containing 0.2 atmospheres of hydrogen and 0.8 atmospheres of nitrogen. This was interpreted as reduction of endogenous flavin by hydrogen. Peaks in the Soret region at 450 and 425 m μ . were presumed to be reduced nitrogen products. A more recent report by Hamilton (61) pointed similarly to reduction of flavins and cytochromes a_1 , a_2 , b, c, by hydrogen in the presence of Azotobacter extracts, with subsequent specific oxidation by nitrogen.

Spectral examination of soybean nodule extracts indicated an electron carrier role for hemoglobin, since nitrogen gas oxidised endogenous hemoglobin to hemiglobin, whereas the reverse reaction was observed with hydrogen. These observations suggest a mechanism for nitrogen fixation for the first time, although the presence of trace amounts of oxygen in the nitrogen would invalidate any conclusions drawn from them. However, the usual precautions for removing oxygen were taken.

The experiments strengthen the postulated hydrogenase-nitrogenase relationship; the hydrogenase content of cells has already been shown to be greater when fixing nitrogen (62), although in certain anaerobic nitrogen fixers e.g. C.pasteurianum no such obvious relationship has been demonstrated (63).

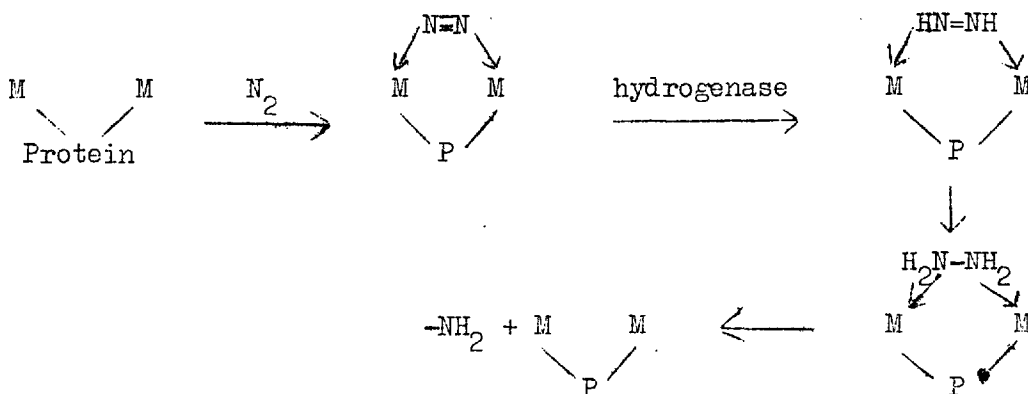
Rhodospirillum rubrum (an anaerobe) showed more hydrogenase activity when grown on nitrogen than on glutamate or ammonia (63).

Hydrogen is a competitive inhibitor of nitrogen fixation in *azotobacter*, other aerobic bacteria and in red clover nodules, and at high concentrations (0.8 atm.) inhibited the presumed nitrogenase extract from *C. pasteurianum* (60).

Recently, Hiai et al (64) showed competition by hydrogen of anaerobic nitrogen fixation by *Clostridia* with 60% decrease in fixation at 0.8 atm. The apparent dissociation constant of the hydrogen-enzyme complex was approximately 0.1 atm., a value similar to that for aerobic fixation when evaluated from respirometric data. These results are contrary to the older data of Rosenblum and Wilson (65) who indicated that the rate of anaerobic fixation was not affected by hydrogen. The present findings of Hiai serve to unify the general action of hydrogen as a competitive inhibitor of both aerobic and anaerobic nitrogen fixation.

It is possible that hydrogen is a donor in the fixation process, and at high concentrations competes with nitrogen for the active nitrogenase sites. Possible mechanisms for the interaction of hydrogenase and nitrogenase have been made by Winfield (66) and by Shug (60) involving chemisorption of nitrogen to a metallo-enzyme containing two metal atoms, possibly iron or molybdenum which can form covalent bonds to nitrogen. The hydrogenase would transfer hydrogen atoms to the chemisorbed nitrogen with ultimate release of ammonia.

The steps in the reaction would take the form:



Allison and Burris (67), in a kinetic analysis of N^{15} accumulated in the cells and medium of *Azotobacter*, concluded that the sequence of nitrogen fixation was via ammonia to glutamic acid. However, between the two and five-minute steady state exposures to N^{15} , glutamic acid attained a higher isotope concentration than free ammonia. This was attributed to interchange during harvesting and inactivation. No oxidised forms of nitrogen were isolated, and it was postulated that the fixation process is entirely reductive, occurring at sites on or near the cell surface.

If we assume a reductive mechanism, then the formation of a $-N=N-$ double bond is feasible, with subsequent reduction to $>N-N<$ etc. A series of azo compounds have ^s been prepared, and the ability of the enzyme to reduce them has been studied with particular regard to metal stimulation. The results of this investigation have led to a study of the corresponding $>C=C<$ and $>C=N-$ bonds.

Bacteriological methods

The *Azotobacter vinelandii* were grown from cultures used by previous workers in this laboratory, and the mannitol medium, sterilisation procedures etc., were essentially those described by them (68, 69).

The Michaelis Constant of Nitrogenase K_{N_2}

This constant was first determined by Lineweaver and Burk in 1934 (70, 71), a value of 0.02 atm. being obtained for *Azotobacter vinelandii* using the Warburg respirometric technique. A similar value was also obtained by Wilson (72) using the same system, and higher values for the nitrogen fixing system in root nodules (73) and in the symbiotic red clover system (74) have been observed. These experiments gave K_{N_2} as approximately 0.05 atm. whereas Burris (75) gave the value as 0.025 atm., similar to that of *Azotobacter* and the blue-green algae *Nostoc muscorum*.

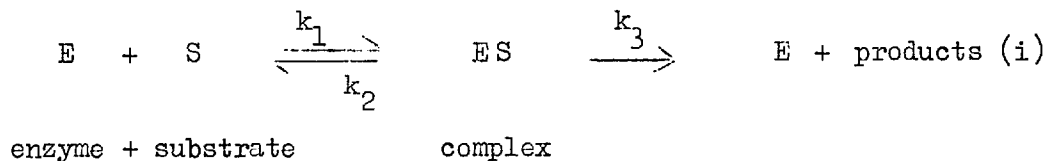
Wilson (76) in this laboratory was the first worker to make an accurate direct determination of the constant from nitrogen uptake using N^{15} . In view of lack of evidence to the contrary, it was formerly assumed that the nitrogen uptake was directly proportional to oxygen uptake, so that evaluation of rate constants of fixation indirectly in terms of respiration was considered justified.

Wilson's work has shown that the two methods give different values for K_{N_2} , the isotopic data giving a value approximately three times that obtained by the respirometric method.

It was decided, therefore, to repeat the determination by the isotopic method, and at the same time to measure the temperature variation in the value.

Theory

The enzymic reaction may be represented as



Let e , a , p , be the concentrations of enzyme, substrate and complex respectively. Then the rate of complex formation is given by:

$$\frac{dp}{dt} = k_1 (e-p)a - k_3p - k_2p \quad (\text{ii})$$

The rate of disappearance of substrate is

$$\frac{da}{dt} = -k_1 (e-p)a + k_2p \quad (\text{iii})$$

The overall reaction rate is given by the numeration of equations (2) and (3):

$$\frac{da}{dt} + \frac{dp}{dt} = -k_3p \quad (\text{iv})$$

At the steady state $\frac{dp}{dt} = 0$

$$\therefore \frac{da}{dt} = -k_3p \quad (\text{v})$$

∴ The overall reaction rate is directly proportional to the concentration of the enzyme substrate complex; also since

$$\frac{dp}{dt} = 0$$

$$k_1 (e-p)a = (k_3 + k_2)p \quad (\text{vi})$$

$$\begin{aligned} \frac{k_2 + k_3}{k_1} &= \frac{(e-p)a}{p} \\ &= \frac{[E][S]}{[ES]} = K_m \end{aligned} \quad (\text{vii})$$

∴ From equations (v) and (vii) the overall reaction rate is given by

$$\begin{aligned} v &= -\frac{da}{dt} \\ &= \frac{k_3}{K_m} \frac{ae}{K_m + a} \end{aligned} \quad (\text{viii}) \quad \left[\because P = \frac{ae}{K_m + a} \right]$$

The concentration of the enzyme substrate complex increases as a is increased, and the velocity of reaction increases hyperbolically with increasing substrate concentration until all the enzyme is bound as ES. i.e. $e \approx p$. Then equation (viii) becomes

$$v = \frac{V_{\max.} \cdot a}{K_m + a} \quad (\text{ix}) \quad \left[V_{\max.} = k_3 e \right]$$

$V_{\max.}$ being the maximum value of v attained when all the enzyme is bound as the complex.

In order to eliminate $V_{\max}$ from equation (ix) two values of v are required from separate experiments using different values of substrate concentration.

If k_1 and k_2 are the growth rate constants in two parallel experiments, these can be used in place of v in equation (ix) so that

$$k_1 = \frac{P_{N_2} (1) V_{\max}}{P_{N_2} (1) + K_{N_2}} \quad \text{where } P_{N_2} \text{ is the substrate concentration.}$$

$$k_2 = \frac{P_{N_2} (2) V_{\max}}{P_{N_2} (2) + K_{N_2}}$$

$$\therefore \frac{k_1}{k_2} = \frac{P_{N_2} (1)}{P_{N_2} (2)} \cdot \frac{P_{N_2} (2) + K_{N_2}}{P_{N_2} (1) + K_{N_2}} \quad (x)$$

from which K_{N_2} can be calculated.

As already indicated, the determination of growth rate constants based on respirometric data is of doubtful reliability, and the isotope method is much to be preferred.

During logarithmic growth, the rate of increase of cell nitrogen is given by

$$\frac{dN}{dt} = k [N]$$

$$\text{or } N = N_0 e^{kt}$$

where N_0 is the initial nitrogen content, and N is the content after time t . k is the growth rate constant.

The amount of labelled substrate found in the cells after time t will depend only on the relative increase in cell material and on the atom % excess of the isotope N^{15} in the substrate. If I is the N^{15} abundance in the substrate and E is the N^{15} abundance in the cells after time t , then

$$E = \frac{N - N_0}{N_0} I$$

$$= \frac{N_0 e^{kt} - N_0}{N_0 e^{kt}} I$$

from which

$$k = \frac{1}{t} \ln \frac{I}{I-E} \quad (\text{xi})$$

from this equation, the values of the rate constants can be obtained by measuring the N^{15} excess in the circulating atmosphere (substrate), the N^{15} concentration in the cells after the experiment, and the duration of the experiment. All these factors can be measured accurately, and the method is consequently much more reliable than determination of growth rates by e.g. measurement of the nitrogen content by analytical methods.

Experimental

The experimental determination of the Michaelis constant thus requires

- a) a gas circulating apparatus
- b) a method for converting the nitrogen in the cells to nitrogen gas
- c) a means of measuring the isotopic content of the gas.

The circulating apparatus was essentially that described by Lemin (77), only the essential details being mentioned here. The system consisted basically of a set of storage bulbs to contain oxygen, argon and isotopic nitrogen separately, a high vacuum line, and an electrically driven rubber diaphragm pump. The culture flasks were fitted with sintered glass inlet tube which ensured vigorous aeration of the bacterial culture.

Argon was obtained directly from the cylinder, pure oxygen was prepared by heating potassium permanganate at 250°, and isotopic nitrogen by the action of sodium hypobromite on ammonium chloride ($^{15}\text{NH}_4\text{Cl}$, derived from $^{15}\text{NH}_4\text{NO}_3$).

The circulating pump consisted of a hollowed-out polythene block with a sealed-on rubber diaphragm which could be compressed and depressed by means of a plunger attached to reciprocating electric motor. The apparatus was tested for leaks in the usual way by evacuating and examining with a tesla coil. This method did not permit the checking of the diaphragm pump, and as a final precaution the entire system was filled with argon, which was then circulated for about four hours. Examination of a small gas sample in the mass spectrometer then established whether any entry of atmospheric nitrogen had occurred.

For the isotopic run, a 25 ml., 48 hour shake culture was centrifuged to sediment the bacteria, which were then resuspended in 400 ml. of fresh sterile medium in a litre conical flask. A stream of moist, filtered air was drawn through the culture by means of a water pump, and the liquid was stirred magnetically to ensure

adequate aeration. This was continued for about 20 hours to ensure that the bacteria were in the log phase of growth.

The circulating apparatus had been designed so that two separate atmospheres could be circulated at the same time, and for the purpose in hand, these consisted of

- a) N^{15} ca. 0.04 atm., O_2 ca. 0.3 atm., A $\rightarrow$ 1.0 atm.
- b) N^{15} ca. 0.12 atm., O_2 ca. 0.3 atm., A $\rightarrow$ 1.0 atm.

The apparatus, which had been held under vacuum for several hours previously, was filled with the gases by means of the toepler pump, and 60 ml. of the 20 hours culture was introduced into each of the six aeration tubes (three for each atmosphere) which had been immersed in a thermostat. Each gas mixture was circulated through the respective cultures in parallel for approximately 4 hours, at the end of which time the bacteria were killed by the addition of dilute sulphuric acid. The bacteria were sedimented by centrifuging at about 3000 g. for 20 minutes, and washed once with distilled water by the same process. They were then transferred to Kjeldahl flasks and treated with 1.3 g. potassium sulphate and 2 ml. of catalyst (a suspension of mercuric sulphate in concentrated sulphuric acid). The mixture was heated for about 24 hours after becoming colourless, which ensured complete conversion of amines to ammonia. This is essential, since if any methylamine were present this would be converted to the CH_3N^+ ion in the mass spectrometer, and would be indistinguishable from the $N^{14}N^{15}$ peak.

0.02 atmosphere obtained respirometrically, and are in fairly close agreement with the value of 0.066 obtained by Wilson in this laboratory.

The results show that the isotopic method gives more consistent and reproducible values than those obtained respirometrically, and the higher value can safely be taken to be the correct one.

Significance of K_m (K_{N_2})

According to the original theory of Michaelis and Menten (78) and interpretations by other workers (70), K_m defines the equilibrium for the reversible formation of ES from E and S, regardless of the disturbance caused by the decomposition of the enzyme-substrate complex (ES), so that on this basis K_m defines an equilibrium which is characteristic of the affinity of the substrate for the enzyme.

However, kinetic measurements based solely on the rate of disappearance of the substrate never yield k_2/k_1 , since k_3 must have some value.

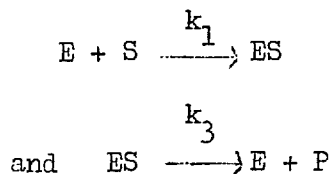
$$\text{The equation } K_m = \frac{[E][S]}{[ES]}$$

bears formal resemblance to an equilibrium expression, but it actually represents the condition for the steady state in two consecutive reactions, the first of which ($E + S \xrightleftharpoons[k_2]{k_1} ES$) is reversible.

Now if $k_2 \gg k_3$,

$$K_m \approx k_3/k_1$$

giving thermodynamic data representing the difference between the values for the two reactions



But if both k_2 and k_3 are significant then

$$K_m = \frac{k_2 + k_3}{k_1}$$

The activation energy of each separate reaction is given by the usual expression

$$v = P e^{E_{act}/RT}$$

where P is the collision frequency, but it does not follow that K_m can be used in the same way to obtain the thermodynamic data for the overall reaction.

The K_m value has been used to compare the relative affinities of the enzyme for the substrate, and it has been shown K_{N_2O} is approximately 0.15 atm. (obtained respirometrically) showing that the nitrogenase system has an affinity for nitrogen about seven times that for nitrous oxide (79).

Wilson (80) considers the poisoning of nitrogenase by physical adsorption is unlikely since the inhibiting effect of various gases such as helium, argon and ethane show no relationship to their Van der Waals' constants. However, in view of the stereochemical specificity of enzymes, the ability of the molecule to fit the active site may be of more importance. Nitrogenase may function by

means of chemisorption with a low activation energy permitting rapid adsorption and desorption. This would be in accord with the rapid response of rate of fixation to changes in nitrogen pressure.

Temperature Dependence of K_m

The interpretation of $\frac{dK_m}{dt}$ is rendered difficult in the absence of an exact physical concept of this constant (81), and several instances occur in the literature of incorrect deductions derived from this constant (82, 83, 84, 85).

The assumption has been made that K_m simply expresses the reversible formation of ES regardless of any shift in the equilibrium caused by its conversion to E + P, so that $\frac{dK_m}{dt}$ could be used in the Van't Hoff equation

$$\Delta G = -RT \ln K$$

to obtain the usual thermodynamic data. As already pointed out, these assumptions are not justified, and any attempts to interpret variations of K_m with temperature must be regarded with suspicion unless the limiting conditions can be shown to hold.

The experimentally determined value of K_{N_2} has been shown to have no temperature variation over a range of 10°, but this is not a sufficiently wide range to establish that $\frac{dK_{N_2}}{dt} = 0$. If the experimental technique can be sufficiently refined to allow determinations of K_{N_2} over the range 10°-40°, and if $\frac{dK_{N_2}}{dt}$ can then be shown to be zero, then assuming k_3 is relatively small, there would be some justification for concluding that the fixation process occurs by a physical adsorption mechanism, but at present this cannot be proven unequivocally.

Investigations using cell-free extracts

The preparation of cell-free extracts required the cultivation of a large quantity of bacteria in view of the relatively low activity of the extracts as compared with whole cells, and generally a 20 litre culture of *Azotobacter vinelandii* or *Desulphovibrio desulphuricans* gave sufficient material for several weeks' work.

The medium used for the cultivation of the nitrogen fixing bacteria was that employed by previous workers in this laboratory

i.e.	Mannitol	15 g.
	K_2HPO_4	1.6 g.
	KH_2PO_4	0.4 g.
	$MgSO_4$	0.2 g.
	NaCl	0.2 g.
	$CaSO_4$	0.1 g.
	$FeSO_4 \cdot 7H_2O$	0.015 g.
	$NaMoO_4 \cdot 2H_2O$	0.001 g.
	H_2O	1 litre

The medium was made up in a 20 litre pyrex bottle, and was sterilised in an autoclave at 15 lbs./sq.in. for half an hour. The medium, after allowing it to cool, was inoculated with a 2-day 100 ml. shake culture, and a vigorous stream of moist, filtered air was drawn through the culture via a large sintered glass sparger. A rapid stream of air was essential to maintain adequate aeration, as stirring magnetically or otherwise presented some technical difficulties. The culture was maintained at approximately 30° in an

incubator for 2-3 days and the cells were harvested by centrifugation. This was originally carried out batchwise in 400 ml. polythene bottles in a large swing-out type centrifuge, but later on in the course of the work a Sharples centrifuge became available, enabling the operation to be carried out in 1-2 hours (as opposed to approximately 6 hours by the previous method).

The cells + precipitated phosphates were washed twice by centrifugation with phosphate buffer pH 7.0, giving a wet weight of about 40 g. of material, of which approximately 10 g. was the desired bacteria.

The product was transferred to two 50 ml. glass stoppered bottles with 15 ml. buffer, and about 40 g. of ballotini (small glass spheres) was added to each. The bottles were counterbalanced, flushed out with hydrogen, cooled in iced water for 10 minutes and shaken at the highest speed attainable with a microid flask shaker for 15 minutes, during which time the suspension became very viscous and frothy. The suspension was diluted with phosphate buffer and the bacterial suspension from each bottle was decanted from the heavy glass spheres into a 50 ml. plastic centrifuge tube. The two tubes were counterbalanced and the cell debris was sedimented at 3,000 g. for 1 hour.

The supernatant liquid was opalescent and pale red-brown in colour. This was carefully decanted into two more plastic centrifuge tubes and centrifuged at 20,000 g. for 1 hour at 0° in an M.S.E. high speed refrigerated centrifuge. This sedimented the mitochondria as a bright red precipitate, the supernatant fluid then being only

slightly opalescent. This solution will in future be referred to as the crude cell free extract, and was used for most of the hydrogenase reactions. The mitochondria have a very high hydrogenase activity, and a suspension of these in phosphate buffer was used in cases where a high concentration of enzyme was required. Both extracts were stored under hydrogen at 0-5° in small conical flasks.

Desulphovibrio desulphuricans

This anaerobe contains a very powerful hydrogenase system, and since it does not normally fix nitrogen it was considered desirable to investigate whether it differed from Azotobacter in its action on unsaturated linkages.

The bacteria were cultivated by the method described by Butlin et al (86) on the following medium:

K_2HPO_4	0.5 g. /litre
NH_4Cl	1.0 g.
Na_2SO_4	1.0 g.
$CaCl_2$	0.1 g.
$MgSO_4 \cdot 7H_2O$	2.0 g.
Sodium lactate	3.8 ml. of 70% solution
Yeast extract	0.1%

The iron solution consisted of a 1% solution of ferrous ammonium sulphate which was sterilized separately, and 50 ml. of which was added to the main culture solution when required.

For the preparation of a large quantity of bacteria, 50 ml. of culture medium in a glass stoppered bottle was inoculated with a few drops of the stock culture and the bottle was filled to overflowing with a further quantity of sterile medium. The bottle was then stoppered, so ensuring complete removal of air, and allowed to stand for a few days in the incubator until the appearance of a dense black colouration due to precipitated ferrous sulphide indicated that growth was proceeding satisfactorily. The whole of this culture was then transferred to 20 litres of sterile medium and the process was repeated. After 2-3 days the cells were harvested in the Sharples centrifuge and cell-free extracts were prepared in the manner described for *Azotobacter*. The crude cell-free extract was, however, very darkly coloured, probably due to the presence of colloidal ferrous sulphide.

The assay apparatus

The standard Warburg apparatus was used for this purpose. The system was arranged so that the set of flasks (usually five) could be alternately evacuated to approximately 1 centimetre of mercury pressure by means of a water pump, and filled with hydrogen from an aspirator. The hydrogen in the aspirator was obtained from a cylinder of the gas, but was freed from traces of oxygen by prior passage through a solution of chromous chloride.

The flask constants (i.e. the volume of hydrogen absorbed per millimetre drop in manometer fluid) were calculated by the mercury method described by Umbreit (87).

The two reactants were placed in their respective compartments of the Warburg flask, usually 10 ml. was placed in the main compartment and 0.6 - 0.7 ml. in the side arm.

In order to remove the last traces of oxygen from the system, a few drops of alkaline sodium dithionite solution were placed in the centre well of the flask, and a short strip of filter paper then inserted to increase the surface area of the solution.

The flasks were attached to their respective manometers and the system was evacuated rapidly in order to minimise inactivation of the dithionite solution. The flasks were then filled with hydrogen, and the process of evacuation and refilling was repeated three times to remove all traces of oxygen from the gaseous and aqueous phases. After filling the flasks, the manometers were attached to the side of a thermostated bath, the flasks being totally immersed in the bath, which was usually maintained at 30°.

The set of flasks were equilibrated by shaking for about half an hour, the level of the manometer fluid in the right hand arm was adjusted to the zero mark (by releasing gas if necessary), and the contents of the side arm were tipped into the main compartment. Shaking was resumed, and after certain time intervals the level in the right hand arm of the manometer was readjusted to the zero mark, and the level in the left hand arm noted.

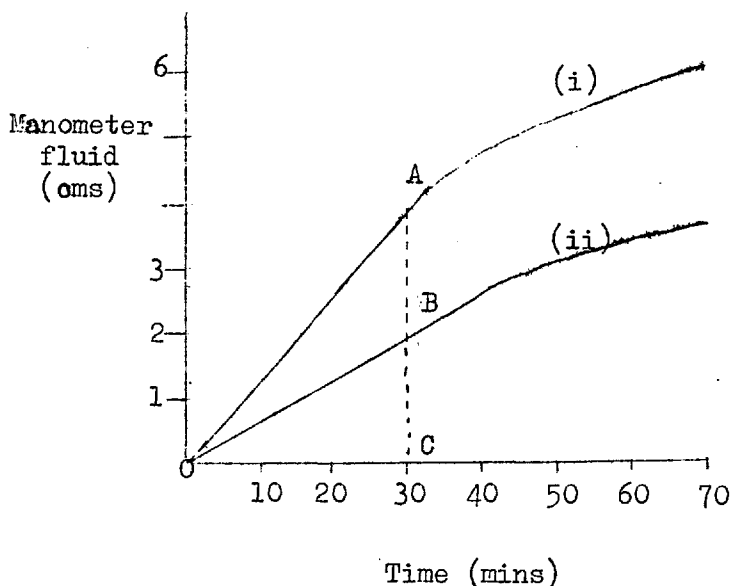
This gave the uptake in millimetres which was all that was necessary for the purpose of establishing reduction, comparison of reduction rates or stimulation of reduction. Sometimes it was necessary to know the exact amount of hydrogen absorbed and in these cases knowledge of the flask constant enabled the conversion to be made.

Benzyl viologen and methylene blue solutions

For assay purposes, the redox dyes were made up as 1% solutions in phosphate buffer.

A typical rate of reduction curve is shown in figure I:

Figure I.



Reduction of the acceptor is generally linear initially; but, towards the end, the curves tail off as shown [(i)] and [(ii).]

In some cases an induction period was observed (i.e. a short period at the beginning of the experiment when no reduction occurred, but this was usually less than 10 minutes. The linear portion of the graph was used for comparison of relative rates of hydrogen uptake, and in the example shown in figure I, the acceptor used in (i) is being reduced twice as rapidly as in (ii).

Investigations on enzymic reduction of the azo group

As discussed previously, the azo group could be formed in a reductive attack on the nitrogen molecule. The nitrogen molecule is remarkably stable, as is seen from the bond energies given by Pauling (88).

$\text{N} \equiv \text{N}$	225	Kcals/mole
$\text{N} = \text{N}$	98	"
$\text{N} - \text{N}$	38	"

Pitzer (89) has attempted to explain the stability of the nitrogen molecule on electronic considerations, but was unable to reach an adequate solution to the problem.

If, as is generally assumed, the nitrogen is adsorbed onto a metallic site (e.g. Mo or Fe) then this will result in decreased electron density between the two nitrogen atoms due to localisation of the lone pair, which might result in an increase in the rate of reduction of the bond. With this consideration in mind, the attempted reduction of azo compounds with varying degrees of electron density between the nitrogen atoms has been carried out, and particular regard has been made to metal stimulation. The simplest azo compound, diimide $\text{HN}=\text{NH}$ is not known to exist, which is unfortunate since this would be the most interesting compound for the purpose of the present study. Other azo compounds were therefore examined.

Azobenzene

This is the most readily available azo compound, but suffers from the disadvantage of being effectively insoluble in water. Acetone has little effect on the hydrogenase system and is frequently

used for precipitating the enzyme from cell-free extracts, so that an aqueous-acetone solution of the substrate was considered to be satisfactory.

The effect of acetone on the enzymic activity had first to be shown to be slight, and experiments showed that the rate of hydrogen uptake by methylene blue was effectively the same in phosphate buffer and in buffer containing 40% acetone.

This solvent, however, suffers from the disadvantage that it tends to froth during evacuation, so dioxan and ethanol were also tried, but both these solvents caused considerable inhibition at the concentrations required to ensure dissolution of the azobenzene. It was decided to use acetone as the solvent, and, in order to minimise frothing and loss of solvent during the evacuation process, the Warburg flasks were cooled in an ice bath during this operation. For work with azobenzene it was found satisfactory to use a solution of ca. 2 mg. of substrate in 1.0 ml. of a solution containing 40% acetone and 60% phosphate buffer pH 7.0. The azobenzene was made up to a standard solution in acetone, and the requisite volume of phosphate buffer was added to this solution when required.

If the enzyme is used in its usual form i.e. as a solution in buffer solution, then the heat of mixing causes expansion of the gas in the Warburg flask, and a further period of equilibration is required, during which time hydrogen uptake might have been occurring. To obviate this difficulty the enzyme solution was treated with acetone to give the same solvent concentration as the substrate solution, and no difficulties were experienced on mixing the two solutions.

Initial experiments were carried out using whole cells, concentrated cell debris, mitochondria and cell-free extracts, and the results of these experiments showed that direct reduction of azobenzene does not occur. Work on artificial carriers has demonstrated that it is possible to reduce the substrate indirectly (see later).

Water soluble derivatives of azobenzene

In order to avoid using acetone as a solvent, which involved some slight practical difficulties as described, it was decided to extend the investigation to include azobenzene-4-sulphonic acid and azobenzene-4-carboxylic acid. The acids were dissolved in water, neutralised with aqueous sodium hydroxide and adjusted to pH 7.0 before use. As with azobenzene, no evidence of direct reduction of hydrogenase was apparent.

Since the azo compounds might be inhibitors of hydrogenase, the effect of the three compounds on the reduction of methylene blue by azotobacter was examined. The assay was carried out in 40% acetone solution in which methylene blue is sufficiently soluble for the purpose, and the inhibiting effect of 10^{-2} M of azo compound was examined.

Results:

<u>Compound</u> at 10^{-2} M	<u>Inhibition</u>
Azobenzene	0
Azobenzene-4-sulphonic acid	~ 75%
Azobenzene-4-carboxylic acid	~ 67%

The experiment was repeated in 100% buffer solution using the sodium salts of the acid derivatives (azobenzene was excluded), and similar results were obtained.

Inhibition of a similar order was also observed during the reduction of benzyl viologen by cell free extracts of *Desulphovibrio desulphuricans*, so in view of the powerful inhibiting effect of the carboxylic and sulphonic acid groups, these compounds were not examined further.

Aliphatic azo compounds

The simplest azo compound, apart from diimide, is azomethane $\text{CH}_3\text{-N=N-CH}_3$ which is a stable gas, soluble in water. Sym-dimethyl hydrazine was prepared from hydrazine sulphate (90) and converted to azomethane by a similar method to that described by Ramsperger (91): Dimethyl hydrazine (2.5 g.) was dissolved in water (6 ml.) and allowed to drop slowly into a magnetically stirred solution of potassium chromate in water (15 ml.) in a 50 ml. conical flask cooled in an ice bath. The evolved gas was passed through a calcium chloride tube to remove water vapour, and condensed in a trap cooled with a cardice-ethanol mixture. After the reaction had ceased the pressure was gradually reduced to try and remove the gas dissolved in the reaction mixture.

The weight of the trap was accurately known, and reweighing after addition of exactly 1.0 ml. of water, in which azomethane is easily soluble, gave the weight of the product. The yield was only 0.16 g. so presumably a considerable amount of the product had remained trapped in the aqueous phase. However, the weight of

azomethane was quite sufficient for subsequent experiments, and for this purpose the 1.0 ml. of solution was further diluted so that 0.1 ml. contained approximately 1.0 mg. This standard solution was stored in a well-stoppered bottle at 0°, and for the hydrogenation experiments 0.1 ml. was diluted with phosphate buffer pH 7.0 to 0.6 ml.

During the evacuation of the Warburg flasks this solution (in the side arm) was frozen by means of an ethanol-cardice bath in order to prevent loss of the volatile material. After finally filling with hydrogen, the solid solution was allowed to melt, and the assay procedure was carried out in the normal manner.

Reduction was attempted using all the hydrogenase forms previously mentioned, but the results were uniformly negative even in experiments involving the use of artificial carriers and metal stimulators. The latter are discussed further in relation to other azo compounds.

The methyl group is electron repelling, so that the combined effect of the two groups in azomethane would result in a high electron density between the two nitrogen atoms. If the proposed reductive mechanism for nitrogen fixation is correct, then the failure to reduce this compound is explained.

To extend the examination of the aliphatic azo compounds, azopropane was prepared and investigated. The preparation followed that described by Lochte (92) except for the last stage, in which the use of copper oxide to convert sym-isopropyl hydrazine to the desired product is replaced by potassium chromate. This reduces the time of

oxidation from about 3 days to a matter of minutes.

Isopropyl hydrazine (10 g.) was dissolved in water (25 ml.) and added dropwise to a stirred solution of potassium chromate (25 g.) in water (50 ml.) over 10 minutes. The reaction mixture was steam distilled which completes the reaction, and the product was isolated from the distillate by ether extraction. The ether was removed by evaporation under reduced pressure and the residue was fractionally distilled, the product boiling at $89^{\circ}/760$ mm. being collected for experimentation. This amounted to 2 g. The entire product distilled in the range $87.5-89.0^{\circ}$ (literature $88.8^{\circ}/740$ mm.) the yield being 6.0 g.

The compound is only slightly soluble in water, so a standard solution was made up by dissolving 20 mg. in 0.5 ml. of acetone and making up to 10 ml. with phosphate buffer.

The substrate was examined fully in the manner described, but no evidence of reduction was obtained, and it found that no appreciable inhibition of methylene blue reduction by *Azotobacter*, or benzyl viologen reduction by *Desulphovibrio* occurred with the concentration of azopropane used in the attempted reductions.

Hence, the compound is not self inhibitory, and it may be concluded that the high electron density caused by the isopropyl group (the +I effect of $(\text{CH}_3)_2\text{CH}-$ is greater than that of the CH_3- group) prevents any reduction.

In view of the implications of metals as essential factors in the action of hydrogenase and nitrogenase, it was decided to examine the effect of the presence of metallic ions on the

reduction of various azo compounds. Since pyridine is capable of co-ordinating with many such ions, the obvious compound to examine was azopyridine.

The most convenient method of preparation is that described by Kirpal (93, 94) : 2-amino pyridine (2 g.) was dissolved in water (40 ml.) and sodium hypochlorite solution (100 ml., 12%) was added slowly with stirring to the ice-cooled solution (Kirpal carried out the reaction at 10°) over half an hour. A considerable amount of insoluble material and some tar was found, which was filtered off and the filtrate was extracted several times with ether. The ethereal extract was dried over sodium sulphate and evaporated under reduced pressure to give 0.25 g. of product M.P. 75°. The material was recrystallised from 80°/100° petrol to constant melting point. Yield = 0.2 g. M.P. 85° (Kirpal gives no yield for his reaction and reports the melting point as 81°).

2,2'-azopyridine is very soluble in water, and is much more readily studied than the previous azo compounds.

Initial experiments showed that the compound is easily reduced by cell-free extracts, absorbing 1 mole of hydrogen to form the corresponding hydrazopyridine. The reaction proceeds no further, so that reductive fission to amino pyridine does not occur.

This direct reduction by cell-free extracts was very encouraging, since this enabled the possibility of metal stimulation to be examined fully.

In aqueous solution azopyridine forms a deep blue complex with ferrous ions, but this is relatively labile, as addition of

potassium phosphate buffer solution causes decolourisation and precipitation of iron. The precipitation of iron phosphates can be prevented by the addition of the equivalent amount of sodium citrate solution, and the blue complex can be thus stabilised.

Stoichiometry of the complex

The ferrous ion has the sp^3d^2 or the d^2sp^3 configuration i.e. has a co-ordination number of 6, so that on the assumption that both pyridine nitrogen atoms can co-ordinate with the ferrous ion, the complex would consist of three azopyridine groups surrounding the metallic ion.

The colour density of mixtures of aqueous solutions of ferrous sulphate and azopyridine was examined in a spectrophotometer and it was found that the greatest intensity was obtained with a ratio of 1:6 instead of the expected 1:3 ratio. However, in the stimulation experiments the maximum effect was attained with a 1:3 ratio, and for these experiments this ratio was adhered to.

For the investigations separate solutions containing 2.0 mg. azopyridine/0.2 ml. phosphate buffer and 1.42 mg. ferrous ammonium sulphate/0.1 ml. water were used. Ferrous ammonium sulphate was identical in its complex formation with azopyridine as ferrous sulphate, and was used because of its resistance to oxidation in aqueous solution. The iron solution was made up immediately before use, and was treated with the equivalent amount of sodium citrate.

0.2 ml. of azopyridine and 0.1 ml. of the ferrous solution were placed in the main compartment of the Warburg, and diluted to 1.0 ml. by the addition of phosphate buffer. The enzyme solution

(0.6 ml.) was placed in the side arm.

In comparison with a blank assay with no added iron, the rate of reduction was stimulated approximately 7 times, addition of more iron having no effect on the rate of reduction. If less than the theoretical amount of iron were added to the azopyridine solution, then the initial rate of hydrogen was very rapid, but fell off after the complex had been reduced, and the remaining free azopyridine then continued to absorb at the usual rate.

The blue complex is very soluble in water, and could not be extracted from the aqueous reaction mixture with any of the usual organic solvents. Chromatography on alumina in aqueous solution gave a very firmly adsorbed band at the top of the column which could not be eluted. However, since the structure of the complex was not of immediate importance, it was decided to postpone its isolation and characterisation.

In view of the success with iron, stimulation by other metals was investigated. The results are given in the following table.

<u>Metal</u>	<u>Ratio : metal : azopyridine</u>	<u>Stimulation</u>
Fe ⁺⁺	1 : 3	7 times
Co ⁺⁺	1 : 3	5-7 "
Cr ⁺⁺⁺	1 : 3	2
Mn ⁺⁺	1 : 3	0
Ni ⁺⁺	1 : 2	0
Hg ⁺⁺	1 : 2	3
Zn ⁺⁺	1 : 2	0
Cd ⁺⁺	1 : 2	0
Mg ⁺⁺	1 : 2	0
Mo (as NaMoO ₄)	1 : 4	0
Cu ⁺⁺	1 : 2	~ 100

The table shows that cobalt has a similar effect to iron, but that manganese and other transition elements give little or no stimulation.

Copper is extremely active, and with the usual concentrations of enzyme and substrate, the complex is reduced within the time of mixing, so that only an approximate value for the degree of stimulation can be obtained. If the enzyme is diluted sufficiently to give a measurable rate of hydrogen uptake in the presence of copper, then the unstimulated reduction is too slow to measure conveniently, and, in addition, the enzyme tends to become inactivated under such conditions.

The stimulated rate is also rendered uncertain, since with such high rates of hydrogen absorption, diffusion effects may be very great. However, the results are interesting, since the metal, which is not expected to have any connection with hydrogenase activity, is far more active than the transition elements which might conceivably be implicated.

The results with azopyridine suggested that replacement of one of the pyridine rings with an electron releasing group would lessen its rate of reduction, and to confirm this, phenyl azopyridine was prepared and investigated.

2-phenylazopyridine

This was prepared by the condensation of 2-aminopyridine and nitrosobenzene using the method of Campbell (95). The compound was purified by chromatography on alumina, and recrystallised from 40/60° petrol to give red needles M.P. 34° (Lit M.P. 34°).

As anticipated, this azo compound is less water soluble than azopyridine, but aqueous solutions of the required concentration were easily made.

Phenyl azopyridine was found to form a deep blue complex with ferrous salts in the same way as azopyridine, but no attempt was made to isolate it. The compound can be reduced directly with hydrogenase, but the rate of reduction is approximately half that of azopyridine, and the reaction likewise stops when one mole of hydrogen has been absorbed.

It was found that stimulation of reduction by iron, cobalt and copper occurred, the increase in rate being similar to that observed with azopyridine. No other metals were tried as stimulants.

The ability of the cell free extracts to reduce these two substrates was dependent on their age; after a few weeks' storage the crude enzyme was only about 20% as active as the freshly prepared extract, whereas the activity with respect to methylene blue and benzyl viologen had not decreased significantly.

In order to ascertain that the effect of metal stimulation was not merely due to an activation of the enzyme, the rate of reduction of methylene blue and benzyl viologen was determined in the presence of iron, cobalt, and copper in the concentrations used previously. Iron and cobalt had little or no effect on the rate of hydrogen uptake, but copper inhibited considerably.

Isomers of azopyridine

In order to determine whether the position of the nitrogen atom in the pyridine ring has any effect on the rate of reduction or on metal stimulation, two isomers were prepared: 3,3' azopyridine was prepared in a similar manner to that described for 2,2' azopyridine, starting from 3-amino pyridine. The product was recrystallised from 40/60° petrol to give orange yellow crystals M.P. 140° (lit M.P. 140°) but the yield was poor (5%); 4,4' azopyridine was synthesised from 4-amino pyridine to give yellowish crystals M.P. 108° (lit M.P. 108-9°).

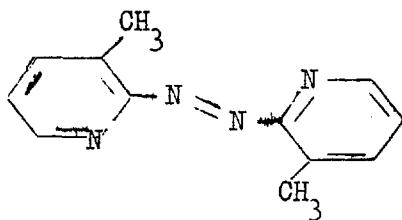
Both compounds are much less soluble in water than the 2,2' isomer, but sufficiently so to obtain solutions of the concentration required for the experiments in hand.

It was found that the rate of hydrogen uptake by the three isomers was identical, so that the position of the nitrogen atom in the ring is not a critical factor.

The 3,3' and 4,4' forms do not give any coloured products with ferrous ions, suggesting that no co-ordination takes place, presumably due to the increased separation of the ring nitrogen atoms. Neither isomer showed any evidence of increased rate of reduction in the presence of iron, cobalt or copper salt, which provides additional confirmation that the enzyme is not activated under these conditions.

Steric hindrance of the azo group

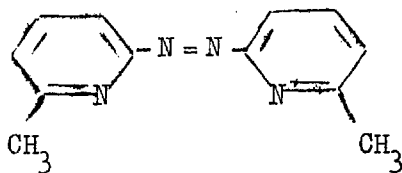
Steric hindrance might result in a decreased rate of reduction of the azo group, and to test this point 3,3' dimethyl 2,2' azopyridine



was prepared by the sodium hypochlorite method: 2-amino-3-methyl pyridine (13 g.) was dissolved in water (250 ml.) and treated with 12% sodium hypochlorite solution (500 ml.) in the manner previously described. Ether extraction yielded sticky orange material which was purified by chromatography on an alumina column using benzene as the developing solvent. This gave a lower diffuse yellow band, a narrower, better defined orange band, and a dark brown band firmly adsorbed at the top of the column. The bands were eluted with acetone, the lower band yielding only a few milligrams of sticky yellow material which was discarded. The middle band gave 0.58 g. of an orange crystalline solid M.P. 134°. Recrystallisation from 60°/80° petrol gave 0.5 g. M.P. 135°. (Found: C, 67.3; H, 5.8; N, 26.6. $C_{12}H_{12}N_4$ requires C, 67.9; H, 5.7; N, 26.4.)

The compound is not very soluble in water, so for reduction experiments it was necessary to use 20% aqueous acetone as solvent.

For comparison purposes the 6-methyl derivative:



was prepared from 6-methyl-2-aminopyridine (6 g.) giving 0.16 g. of the azo compound M.P. 130° after recrystallisation from 60/80 petrol. (Found C, 68.2; H, 5.7; N, 26.1).

The rate of reduction of both of these compounds is very slow being only 5% at most of that of 2,2'-azopyridine, but if a heterogeneous catalyst (e.g. palladium on carbon) is used, then the rates of reduction are identical. This latter observation is discussed later.

Stimulation of reduction by hydrogenase was not reproducible, but the results indicated that iron and copper were without effect, whereas cobalt increased the rate of reduction about four times.

The reduction of the 3-methyl- and 6-methyl derivatives is slow and it could only be demonstrated that the rates of reduction are approximately the same. The screening effect of the methyl groups in the 3 position may therefore be of secondary importance compared with the effect caused by the electron release of these groups.

Cis and trans isomers of azopyridine

To investigate the screening effect further, it was decided to prepare the cis form of azopyridine, as this might be more susceptible to attack by the hydrogenase system.

2,2'-Azopyridine is usually obtained as the trans form, and this was converted to the cis form by the method of Campbell (95): trans-azopyridine was dissolved in sodium-dried benzene/petrol and irradiated for 2 days with U.V. light from a Hanovia mercury vapour lamp. The solution was extracted with water to remove unchanged

trans-azopyridine, and the non-aqueous solution was chromatogrammed on a silica-gel column. The column was developed with benzene, when the trans form remained adsorbed at the top of the column and the cis form was gradually eluted. Evaporation at room temperature yielded cis-azopyridine M.P. 86° (lit 87°) which was used without further purification.

The compound is sparingly soluble in water, and experiments were carried out in 20% aqueous acetone. The rate of reduction was very slow, being only approximately 10% of that of the trans form under the same conditions.

No colour reaction was obtained with ferrous ions, and the rate of reduction was unaffected by the presence of iron, cobalt or copper.

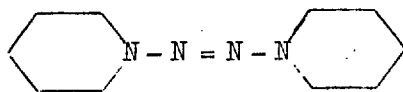
Indirect transfer can be achieved by the use of artificial carriers such as benzyl and methyl viologen.

Cis-azobenzene was obtained in a similar manner to that described for azopyridine, and, as expected, no transfer of hydrogen could be achieved except by the use of artificial carriers.

Quinones

The simple quinones i.e. p-benzoquinone, 1,2 and 1,4 naphthoquinone and anthraquinone (β -sulphonate), were all reduced by hydrogenase to the hydroquinones but with decreasing rapidity. Attempts to reduce 1,3 and 1,4 cyclohexanones did not succeed, with or without artificial carriers. Since the saturated ring appears to prevent reduction of the carbonyl system, it was decided to examine the effect of such a ring system on the azo grouping.

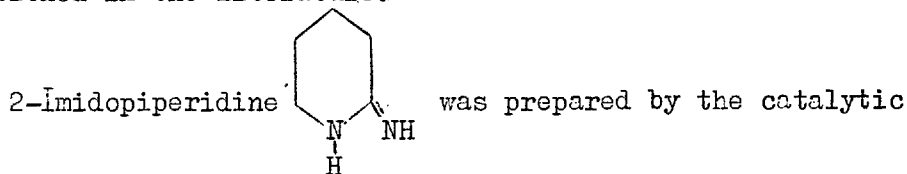
Little work has been reported on the azo-piperidines, the only well characterised example being N-azo-piperidine



which was prepared by the method of Knorr (96).

The compound is a white crystalline solid M.P. 45°, and was recrystallised twice from aqueous ethanol before use. It is only slightly soluble in water so experiments were carried out in aqueous acetone, but no reduction could be achieved directly, in the presence of iron, cobalt or copper, or indirectly via artificial carriers. The stability of the azo group is probably due to electron release from the ring.

In order to get a better comparison with the azopyridines the synthesis of corresponding azopiperidines was attempted: none are mentioned in the literature.



reduction of 2-amino pyridine as described by Graves (97); further reduction to the required 2-amino piperidine has not been reported.

The sodium hypochlorite method of oxidation was attempted, but none of the desired product was obtained, probably because of conversion of the imide to the 2-pyridone under the alkaline conditions used.

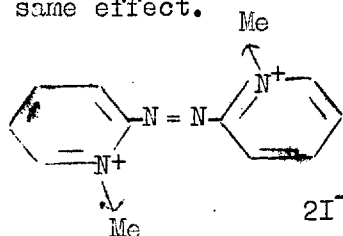
Oxidation with mercuric oxide (the method used for the final oxidation in the preparation of N-azo-piperidine) was also unsuccessful.

The 3- and 4-amino piperidines are known, and were prepared by the catalytic reduction of the corresponding amino pyridines. Treatment of these compounds with sodium hypochlorite resulted in an emulsion being formed from which a liquid was isolated, but in insufficient quantity to enable a characterisation to be carried out. Oxidation with mercuric oxide likewise was unsuccessful, and attempts to prepare the azopiperidines was postponed.

Effect of N-methyl co-ordination

Metal co-ordination has been shown to have a considerable stimulation effect on the rate of reduction of 2,2' azopyridine by hydrogenase, and it was decided to determine whether co-ordination to a methyl group would have the same effect.

The required compound



is not recorded in the literature, and attempts to synthesize it were carried out by reaction between azopyridine and methyl iodide. Only a small amount of azopyridine was available, so the reaction could only be investigated on a small scale.

The azo compound (0.15 g.) was refluxed with excess methyl iodide (1.2 ml.) for 25 minutes and the excess reactant was removed under reduced pressure. The product was black, and attempts to isolate the methiodide were unsuccessful, so for assay experiments the crude material was used: 20 mg. of the azo compound was refluxed with methyl iodide (1.0 ml.) for 10 minutes. The resulting black

solution was evaporated to dryness, and the residue was extracted with phosphate buffer pH 7.0, filtered, and made up to 5.0 ml. This gave a very dark solution with a concentration of azo pyridine of approximately 4 mg./ml.

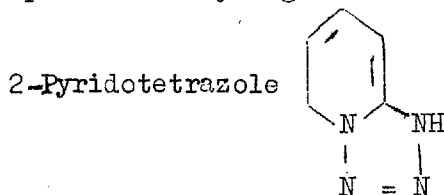
The rate of hydrogen uptake by this solution in the presence of hydrogenase was compared with the stimulation produced by copper ions.

The rate of reduction of the 'N-methyl azopyridine' solution was very rapid, being about one fifth of the rate of reduction of the copper complex.

In view of the fact that N-methyl azopyridine was not isolated and characterised, it cannot be stated definitely that the increased rate of reduction was due to the presence of the N-methyl group, but this would seem to be a reasonable conclusion which would be in accord with the effects of iron, cobalt and copper.

Cyclic azo compounds

The benzene ring is more resistant to reduction by heterogeneous catalysts than aliphatic unsaturated carbon-carbon bonds, and in view of this it was anticipated that hydrogen transfer to these compounds via hydrogenase would be difficult.



was prepared by the method of

Perkin and Robinson (98), and is sufficiently water soluble to enable all the transfer experiments to be carried out in 100% aqueous solution. However, despite the presence of the basic ring nitrogen

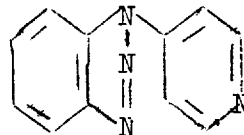
no reduction directly, in the presence of metallic ions or via artificial carriers was obtained, although the copper complex can be reduced by benzyl viologen.

The copper complex was prepared as follows: 2-pyridotetrazole (0.2 g.) was dissolved in ethanol (3 ml.) and treated with a solution of cupric chloride (0.7 g.) in ethanol (5 ml.) until precipitation of the apple-green complex was complete. This required approximately 2.0 ml. The precipitate was washed on a filter with ethanol until free from cupric chloride. Attempts at recrystallisation were unsuccessful, and the product was purified by reprecipitation from aqueous solution. Yield = 0.30 g. M.P. 215° (dec.). The material was not characterised.

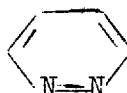
Direct hydrogen transfer by hydrogenase was not achieved, but reduced benzyl viologen is immediately decolourised, and reduction can be obtained by this means.

The cobalt complex was prepared in a similar manner; this is a pink solid with M.P. 310° (dec.). Transfer from reduced benzyl viologen does not occur.

The azo group in γ -pyridylbenzotriazole,



prepared by the method of Robinson (99), was resistant to reduction, steric hindrance presumably being a major factor. Similar results were obtained with pyridazine



Acetylenic and ethylenic linkages

It was decided to extend the transfer experiments to include the corresponding acetylene and ethylenes:

Undec-10-ynoic acid $\text{CH}_3 (\text{CH}_2)_7 \text{C}\equiv\text{C}-\text{COOH}$

This was used as the sodium salt. Reduction of this compound would show whether cis or trans hydrogen transfer occurred: a normal heterogeneous catalyst would give mainly the cis ethylene, and homogeneous transfer would give the trans product. Unfortunately, no enzymic reduction occurred with this substrate.

Fumaric acid has often been quoted in the literature as an acceptor of hydrogen in the presence of hydrogenase, but no evidence for its reduction by the two bacterial systems under examination was obtained. Similar negative results were obtained with crotonic acid.

It was hoped to adapt the enzymic system to reduce these unsaturated acids by incorporating them in the growth medium of the bacteria, but washed bacteria grown in this way still failed to act on the linkages.

If the electron density between the carbon atoms is a critical factor as already postulated for the azo compounds, then these results can be explained: in carbon an electron in a 2 s orbital approaches the nucleus more closely than one in a 2 p orbital. Walsh (100, 101) has suggested that the more s character a C-H bond has, the more negative will the carbon atom be.

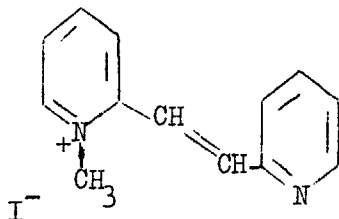
This trend is illustrated by consideration of methane, ethylene and acetylene:

CH_4	sp^3	(tetrahedral)	$\overset{+}{\text{C}} - \overset{-}{\text{H}}$
C_2H_4	sp^2	(trigonal)	$\text{C} - \text{H}$
C_2H_2	sp	(digonal)	$\overset{-}{\text{C}} - \overset{+}{\text{H}}$

i.e. a polarity reversal occurs in passing from methane to acetylene so that the carbon atom in acetylene has an effective negative charge, thus increasing the electron density around the carbon-carbon linkage.

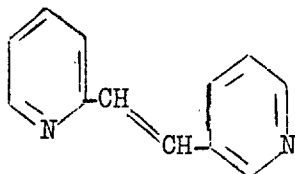
The pyridine ring has been shown to activate the azo group with respect to reduction by hydrogenase, and it was decided to determine whether this group attached to carbon-carbon double bond would enable enzymic reduction to take place.

Di- α -pyridylethylene was prepared as the monomethiodide



by the condensation of pyridine-2-aldehyde and α -picoline methiodide by the method of Phillips (102). The N-methyl group is advantageous in rendering the compound water soluble, and enables a comparison to be made with the effect of the N-methyl group in azopyridine.

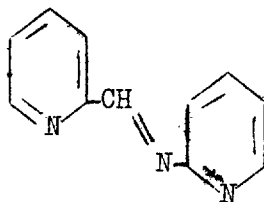
No transfer to the ethylenic linkage was obtained, and similar negative results were obtained with α - β dipyridyl ethylene



The -CH=N- grouping

This is present in the so-called Schiff's bases, and the dipyridyl derivative was prepared for examination.

The required compound



was prepared by the following method: pyridine-2-aldehyde (3.8 g.) was mixed with 2-amino pyridine (3.5 g.) and allowed to stand. The solution became warm and viscous, and after it had cooled to room temperature, ethanol (3 ml.) was added and the mixture was allowed to stand overnight at 0°. The crystalline mass was stirred with a little ethanol, filtered and recrystallised from aqueous ethanol. Yield = 2.5 g. M.P. 128°. (Found C, 72.0; H, 5.1; N, 22.7.

$C_{11}H_9N_3$ requires C, 72.2; H, 4.9; N, 22.9)

The compound forms a purple complex with ferrous salts, but it is very unstable, and the solution soon becomes decolourised.

Direct hydrogen transfer does not occur even in the presence of iron, copper or cobalt ions, but indirect transfer via benzyl or methyl viologen occurs slowly (see later).

Comparison between hydrogenase and heterogeneous catalysts

In order to ascertain if the hydrogenase system worked merely by physical adsorption of the substrate onto the enzyme site in the same manner as a heterogeneous catalyst, the rates of hydrogen transfer to the azo and other groups has been studied.

Catalysts. Initially, palladium adsorbed on strontium carbonate (103) was used but this had a tendency to fall out of suspension and to flocculate, so that reproducible rates of reduction could not be obtained. This catalyst was discarded in favour of 10% palladium on charcoal (104), and reproducible rates of reduction were easily obtained. For the reduction experiments 3 mg. of catalyst was suspended in 10 ml. of the solvent to be used, decanted from any heavy particles, and 1.0 ml. pipetted out for use.

The relative rates of reduction of the azo compounds was first studied with the following results:

<u>Compound</u>	<u>azobenzene</u>	<u>phenylazopyridine</u>	<u>azopyridine</u>
<u>Relative reduction rate</u>	2	1	1

i.e. azobenzene is reduced twice as fast as the other azo compounds.

The effect of two nitrogen atoms in the ring could not be ascertained because of the practical difficulty of making azo-pyrimidine.

The rates of reduction of azopyridine, 3-methyl-azopyridine and 6-methyl azopyridine are identical with Pd/C, but hydrogenase reduces the latter two compounds very slowly by comparison with the parent compound (see p.44).

Reduction of 2,2' azopyridine by Pd/C in phosphate buffer is stimulated approximately twice by ferrous ions in comparison with seven-fold stimulation of the enzymic rate.

Reduction of azobenzene in ethanolic medium by Pd/C is rapid, but is completely inhibited by cupric ions.

Ethylenic derivatives

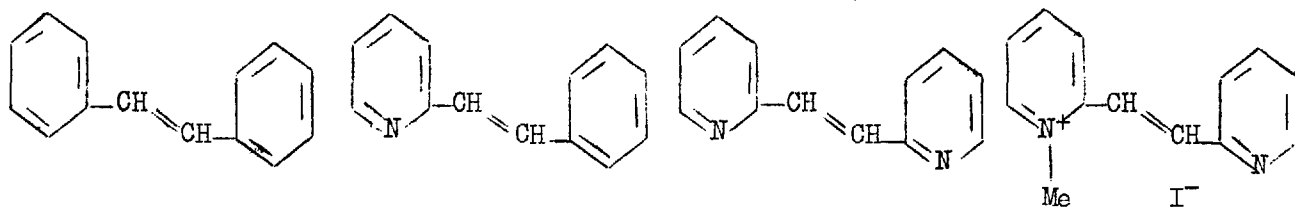
Di- α -pyridylethylene was prepared by the thermal dissociation of the methiodide at 250° in vacuo.

α -Pyridylstyrene was similarly obtained from the methiodide prepared by the method of Phillips (102).

Trans-stilbene was prepared as described in Organic Syntheses (105).

The compounds are not water soluble, so reduction was carried out in ethanol in which the methiodides are also sufficiently soluble for the purpose. As already stated, enzymic reduction does not occur, and only the relative rates of reduction by the heterogeneous catalyst could be studied. The results obtained were as shown:

Compound



Relative rates of reduction

20

2

1

0

It can be seen that the rates follow the same pattern as that observed for the azo compounds, and that stilbene is even more rapidly reduced relatively than azobenzene.

The N-methyl group reduces the rate of hydrogen uptake effectively to zero, which is the reverse effect to that observed with hydrogenase (acting on azopyridine).

Metal stimulation

A very brief study was made of the effects of metallic ions on the rate of heterogeneous transfer, and, in general, stimulation is of a low order compared with that observed for enzymic reduction of the azo group, or negligible.

The results can be summarised:

<u>Compound</u>	<u>Metal</u>	<u>Solvent</u>	<u>Stimulation</u>
22' azopyridine	Fe (II)	Buffer solution	2
azobenzene	Cu (II)	Ethanol	Inhibition
αα' dipyridyl ethylene	Fe (II)	Aq. acetone	2
"	Co (II)	Pyridine	2
"	Cu (II)	Pyridine	4
"	Cu (I)	Pyridine	0

The results of this investigation indicate that heterogeneous catalysts do not act as a good model for the action of hydrogenase.

Artificial carriers

Any substance which can accept hydrogen from a donor and then be reoxidised by a substrate may be regarded as a carrier. Natural hydrogen carriers (or electron carriers as they are usually referred to, although this term is misleading) include such substances as DPNH ($\rightleftharpoons$ DPN + H) and TPNH ($\rightleftharpoons$ TPN + H), and it was part of the purpose to examine hydrogen carriers with special regard to rate of transfer and specificity. This has special reference to enzymic reduction of such substances as for example nitrite, nitrate and hydroxylamine, since some enzymes (all of which exhibit classical hydrogenase activity) show specificity towards these substrates. Stickland (106), for instance showed that reduction of nitrate by *E. coli* does not proceed beyond the nitrite stage, but Lascelles (107) demonstrated that *E. coli* and *Cl. welchii* became capable of transferring hydrogen to all three substrates if a catalytic amount of an artificial carrier were present.

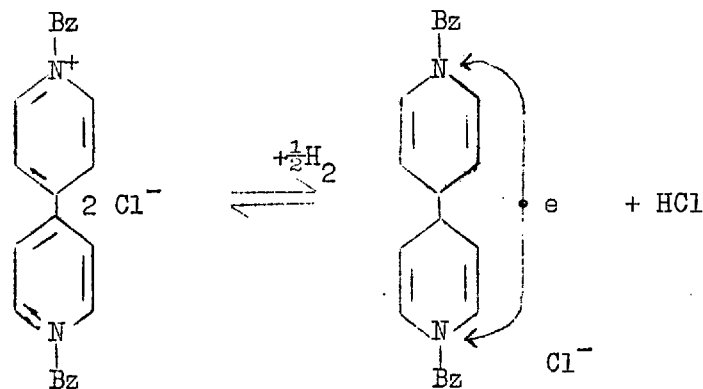
Senez (108, 109) showed that cell-free extracts of *Desulphovibrio desulphuricans* reduced hydroxylamine in the presence of molecular hydrogen by a process in which hydrogenase is the only enzymic factor. The mechanism consists of reduction of a natural hydrogen carrier, cytochrome C_3 , which is chemically reoxidised by hydroxylamine without the intervention of a specific reductase.

Benzyl viologen can be substituted for the natural carrier but the efficiency is lower, as is purified ferrocyclochrome from beef heart, and Senez suggests that any system containing hydrogenase will be capable of reducing hydroxylamine.

If a reductive mechanism for nitrogen fixation is correct, then it is possible that hydrogen transfer to the nitrogen molecule could occur if a suitable carrier could be devised. With this in mind, all the carriers examined have been tested for their ability to transfer to the azo group and the nitrogen molecule.

Benzyl viologen (110)

This is a dipyridyl derivative with a redox potential of -0.359 v. which is low enough to reduce a wide variety of compounds.



When reduced by half a molecule of hydrogen, the originally colourless salt forms a red-violet semiquinonoid free radical in which an electron oscillates between the two nitrogen atoms. In this state it is capable of reducing suitable acceptors. Methyl viologen behaves similarly giving a deep violet free radical, the redox potential being even lower than the benzyl derivative (-0.50 v.). The semiquinonoid forms can be reduced further by sodium dithionite to

nearly colourless compounds in which the octet of the nitrogen is complete. These compounds are easily oxidised to the free radical and thence to the colourless viologen.

When employed as artificial carriers, 0.10 ml. of a 1% solution in phosphate buffer was added to the enzyme solution in the main compartment of the Warburg flask, the substrate being in the side-arm. When the carrier had been completely reduced, the substrate was tipped into the main compartment, when generally the viologen was rapidly decolourised if any transfer was to take place.

The effect on azobenzene was first examined in aqueous acetone solution: as already demonstrated, direct enzymic reduction does not occur, but in the presence of benzyl or methyl viologen, transfer takes place. Transfer from the reduced carrier is very rapid, so that the reduction of the reoxidised carrier is the rate determining step.

The three isomers of azopyridine and phenylazopyridine are all reducible directly by the hydrogenase system, but transfer can also be achieved indirectly, as evidenced by the immediate decolourisation of the free radical on addition of these substrates.

The effect on azomethane, azopropane, N-azopyridine, undecynoic acid, crotonic acid, fumaric acid, dipyridyl ethylenes and azomethines was examined, but only in the latter instance was any transfer obtained.

Transfer to azoxybenzene followed the same pattern as azobenzene.

In order to test the ability of carriers to reduce molecular nitrogen, the aspirator of the Warburg apparatus was filled with a gas mixture containing 25-30% nitrogen, the remainder being hydrogen, and the usual procedure was followed.

With benzyl viologen no transfer to nitrogen was achieved; the presence of iron, cobalt or copper had no effect.

Methyl viologen has a lower redox potential than the benzyl derivative, and identical results were obtained using this carrier with the aforementioned potential acceptors, there being no evidence of specificity.

In order to ascertain the dependence of transfer on the group attached to the nitrogen atom, the parent compound 4,4'-dipyridyl dihydrochloride was examined, but this resisted enzymic reduction, and consequently its ability to act as an artificial carrier could not be established.

The functioning of the dipyridyl derivatives thus appears to be dependent on the presence of the N-alkyl grouping, and it was hoped to extend the study of this type of carrier to include the metal derivatives (if any), to determine whether they would function in the same manner as the alkyl derivatives.

To establish this point, attempts were made to reduce 4,4'-dipyridyl hydrochloride enzymically in the presence of ferrous, cobaltous and cupric ions, but no transfer was obtained.

2,2'-Dipyridyl forms metal complexes readily (this property being used e.g. for the colourimetric determination of iron), and it was hoped to form the free radical of a metal complex by partial

reduction. Enzymic hydrogen transfer did not occur at a measurable rate in the presence of ferrous or cobaltous ions, but with cupric ions hydrogen was absorbed slowly until half a mole had been taken up, when reduction ceased. The red copper complex was rapidly decolourised on exposure to air, so it is possible that a free radical was formed.

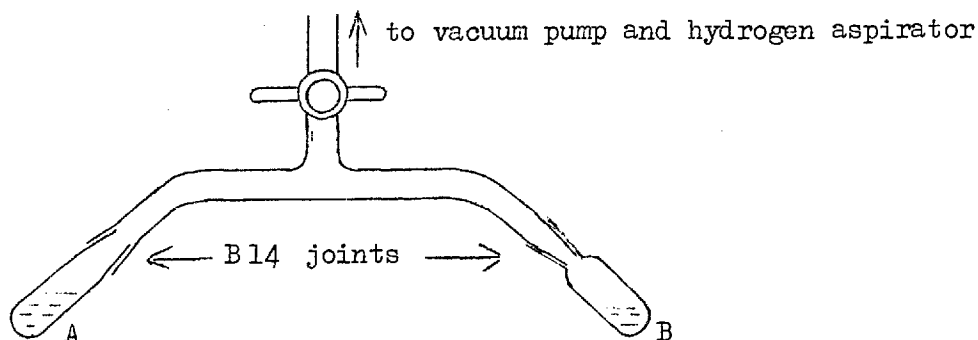
Attempts to use this copper complex as an artificial carrier were discouraging: azobenzene, fumarate, nitrite, nitrate, azide and molecular nitrogen all gave negative results.

During the course of this work it was found that other redox dyes could act as artificial carriers to the azo group e.g. neutral red ($E' = -0.31$ v.), phenosafranine ($E' = -0.25$ v.) and janus green ($E' = -0.23$ v.), although, due to the dependence of the overall reaction rate on the rate of reduction of the carrier, differing rates of transfer were obtained. A detailed comparison was made on the relative rates of enzymic reduction of benzyl viologen and neutral red. With neutral red an induction period of $1\frac{1}{2}$ hours (i.e. the period before which an observable rate of reduction could be detected) was observed as compared with 10 minutes for benzyl viologen under identical conditions. When reduction was proceeding steadily, the rate of transfer to benzyl viologen was approximately twice as rapid as that to neutral red.

The other redox dyes showed similar characteristics i.e. relatively long induction period followed by a relatively slower rate of reduction compared with benzyl viologen.

DPN was found to be capable of acting as a carrier to azobenzene, which was an unexpected discovery in view of the fact that this co-enzyme is present in crude cell-free extracts. As a result of this, cytochrome c, which is reported by Senez to act as a carrier in the enzymic reduction of hydroxylamine, was investigated, but this failed to function successfully with azobenzene.

To complete the work on artificial carriers it was necessary to show that the process was non-enzymic i.e. that the rate of transfer to the acceptor was not affected by the presence of the active enzyme. This was carried out by means of the simple apparatus shown in the diagram:



The enzyme solution plus the carrier was placed in the A side-arm and the solution of the acceptor placed in the B side-arm. The first system examined was benzyl viologen/2,2' azopyridine since this presents the least practical difficulty: the intensely coloured free radical which benzyl viologen forms on reduction becomes decolourised on reoxidation so that this can be easily observed, and azopyridine is easily soluble in phosphate buffer, which is also advantageous.

Since one mole of benzyl viologen absorbs $\frac{1}{2}$ molecule of hydrogen to form the free radical which will then reduce $\frac{1}{2}$ molecule of azopyridine, the quantities required could be worked out: 0.2 ml. 0.2% azopyridine solution and 0.5 ml. phosphate buffer were placed in B, and 0.1 ml. 1% benzyl viologen solution, 0.5 ml. phosphate buffer and 5 drops of enzyme solution were placed in A.

The apparatus was connected to the aspirator and filled with hydrogen in the usual way, tube A then being allowed to stand at about 35° in a beaker of water for one hour to ensure complete reduction of the carrier. A duplicate apparatus was set up at the same time, and the enzyme was inactivated in one by heating tube A in a beaker of boiling water for ten minutes.

Both systems were evacuated, the taps were closed and the contents of A were tipped into B at the same time. The time for decolourisation of the dye (i.e. reoxidation) was noted; any difference between the two times would indicate that the process was partially, at least, enzyme dependent. The experiments were repeated using methyl viologen as the carrier; in this case 0.1 ml. of 0.6% solution was used. The amount of substrate used in all cases was approximately 80% in excess of that required by theory to ensure complete decolourisation of the carrier. The results obtained are shown in the table below:

<u>Substrate</u>	<u>Reoxidation time : Benzyl viologen Methyl viologen</u>	
<u>Trans</u> -azobenzene	Immediate	Immediate
<u>Cis</u> "	"	"
<u>Trans</u> -azopyridine	"	"
<u>Cis</u> "	"	"
NH ₂ OH.HCl	10 mins.	0.5 - 1.0 min.
KNO ₃	No transfer	No transfer
KNO ₂	10 mins.	1 min.
-CH=N-	25 mins.	1 min.
>C=C<	No transfer	No transfer

In the case of the azobenzenes sufficient acetone was added to ensure homogenous solution.

In all cases no difference in rate of transfer was observed when the enzyme was not inactivated, and so it can be concluded that transfer by this means is non-enzymic. Senez (108, 9) has demonstrated artificial transfer to hydroxylamine and nitrite.

It is seen that transfer from methyl viologen (when it occurs) is approximately 10 - 20 times as fast as that from benzyl viologen, presumably due to its lower redox potential, and that there is no evidence of specificity.

Thus, it can be seen that substrates not normally reducible by the enzyme directly can be reduced indirectly via an artificial carrier, so that the action of any reductase may in fact simply be due to the combination of the basic enzyme (hydrogenase) plus the necessary specific endogenous carriers. It would, therefore, not be necessary for the substrate to be adsorbed onto the so-called active site, and nitrogenase may act by this (carrier) mechanism.

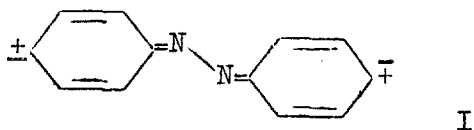
Nitrate and the ethylenic linkage could not be reduced directly or indirectly, which on this hypothesis suggests that the necessary carriers were not present in the enzyme extracts. Reduction of nitrate and fumarate has often been reported, so that the necessary carriers must exist, and it should be possible in the future to develop artificial carriers for this purpose.

Structure of the azo compounds

It is relevant here to discuss the structure of the azo grouping in the compounds.

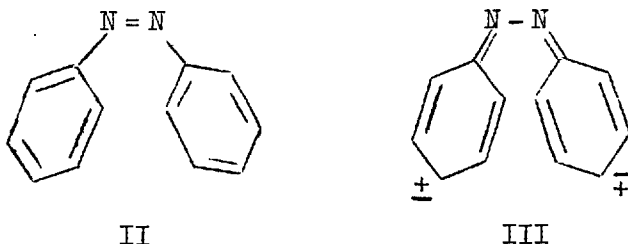
Azobenzene

The structure of the cis and trans forms has been determined by Robertson et al. using X-ray techniques (111, 112, 113) showing that the trans form is almost coplanar. The N=N distance is $1.23 \text{ \AA}$, C-N is $1.41 \text{ \AA}$, and C=N is $1.28 \text{ \AA}$ so that structures such as I must make some considerable contribution to the normal state:



i.e. the N=N bond must have some single bond character and the C-N bonds some double bond character. Also, the lone pairs on the nitrogen atoms must be shared to some extent with the ring system so that the electron density around the N=N linkage must be considerably less than the value expected in the absence of such conjugation.

Cis azobenzene II is not coplanar, since the effect of steric hindrance causes the benzene rings to be rotated about 50° from the planar position.



The N=N distance is $1.23 \text{ \AA}$ and the C-N distance is $1.46 \text{ \AA}$ i.e. almost the usual values, showing that the departure from coplanarity has led to a suppression of conjugation, and that structures such as III cannot contribute sufficiently to the resonant state.

This shows that the electron density around the N=N linkage must be less in the trans form than in the cis form. This is further substantiated by oxidation studies: the cis-form is oxidised to azoxybenzene ($\overset{\text{O}}{\text{N}}=\text{N}-$) much more rapidly than the trans form, so that the two electrons must be localised at one nitrogen atom. Assuming that the energy of localisation will be a large part of the activation energy, then the differing rates of oxidation are explained.

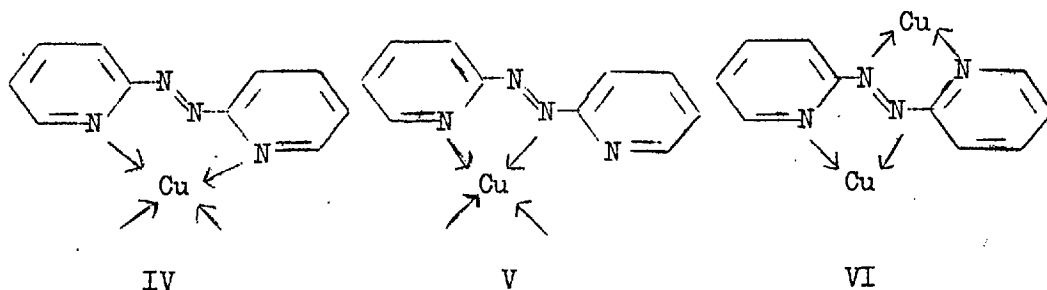
Experiments with hydrogenase and azoxybenzene gave no evidence of enzymic reduction, so that the localisation of the lone pair probably does not decrease the electron density sufficiently for this to occur. Transfer via artificial carriers, is, however, possible.

It is reasonable to assume from these considerations that azopyridine is also planar in the trans form, and this, coupled with the increased electronegativity of the nitrogen atom as compared with carbon, results in a large electron drift from the azo grouping. On the present hypothesis this would account for the rapid reduction rate observed with the azopyridines, and the relatively slow rate of reduction of cis-azopyridine by hydrogenase. In the case of N-azopiperidine, conjugation is not possible, so that reduction of the electron density around the azo group does not occur, and the linkage is thus entirely double bond in character.

Since the saturated cyclic compounds resemble aliphatic compounds in many respects, the similar negative results obtained with N-azopiperidine and azomethane and azopropane are probably related.

Piperidine forms complexes with many metals, and it was hoped that reduction of N-azopiperidine would occur in the presence of Fe^{++} , Cu^{++} or Co^{++} , but results were negative. If the nitrogen does co-ordinate with any of these ions under the experimental conditions, then the predominant factor in the enzymic reduction of the azo group would appear to be the ability of the molecule to undergo conjugation, co-ordination with metals having only a secondary effect on the electron density.

However, the stimulation of reduction of 2,2' azopyridine in the presence of Cu^{++} , Co^{++} , Fe^{++} is very high, the complexes formed possibly having one of the structures IV, V or VI:



In IV the ring nitrogens are probably too widely spaced to effectively co-ordinate with e.g. copper, so that V or VI seem more likely.

Experiments were carried out in which the rate of reduction of azopyridine by hydrogenase was measured in the presence of various concentrations of Cu^{++} . The maximum rate of reduction was obtained with a 1:2 ratio of copper to azopyridine; a lower concentration of copper caused an initial rate of reduction which was the same as the maximum rate, but this rapidly fell off before much reduction had occurred. With a 3:2 Cu: substrate ratio, the rate of reduction was the same as for the 1:2 ratio, which is unexpected since copper normally acts as a powerful inhibitor of hydrogenase. The results however, suggest formula V as the most likely structure, but as already mentioned in the case of the iron/azopyridine complex, these derivatives are difficult to purify, and it was not considered to be of sufficient importance to isolate and characterise them at the time.

If this structure is correct, then the enzyme or the reduced carrier could attack the azo grouping in the plane of the paper, or in a vertical plane. In the case of structure VI, the azo group is sterically hindered to such an extent as to permit attack only in a

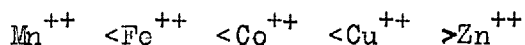
vertical plane.

The effect of N-metal co-ordination is to increase even further the electron drift from the azo group and this is reflected in the increased rate of enzymic reduction. The tendency of a metallic ion to draw electrons away from the attached group increases with the ionic charge and with decreasing ionic radius (due to the shielding effect of the outer electrons with increasing atomic number).

Electronegativity data for some metals have been given by Pauling and Haissinsky (88, 114, 115), who calculated the values from thermochemical data. The values obtained were:

Cu^{II}	2.3	
Mn^{VII}	2.4	
Co^{III}	>2.2	
Ni^{III}	>2.2	The electronegativity of hydrogen is
Fe^{VI}	>2.1	2.1 on this scale

These values are difficult to apply to the present problem; rather more useful is the electron affinity series given by Orgel (116).



Comparison with the table on p.42 shows a definite correlation between electron affinity and stimulation of enzymic reduction of 2,2' azopyridine: Cu^{++} has the greatest electron affinity and has a considerable effect on the rate of reduction of the azo group. Fe^{++} and Co^{++} cause less stimulation of reduction in accord with their lower electron affinity.

This effect is not confined to metallic ions since the present work has shown that there is some evidence for similar stimulation by N-methyl co-ordination.

Azomethane

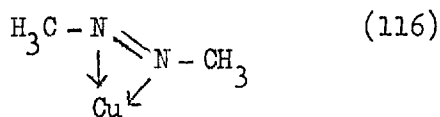
The structure of azomethane has been determined by Boersch (117),

$$\text{H}_3\text{C}-\text{N}=\text{N}-\text{CH}_3$$

using electron diffraction methods, the N=N distance having the usual value of $1.24 \pm 0.05 \text{ \AA}$.

The steric requirements for the adsorption of azobenzene and azopyridine on the active enzyme site (if this occurs) will presumably be very similar, and in view of the fact that in azomethane the azo group is relatively unhindered, the postulation that a steric fit of the azo group on the enzymic site is the basic requirement for reduction is unlikely.

Azomethane is reported to form complexes with e.g. Cu of the form



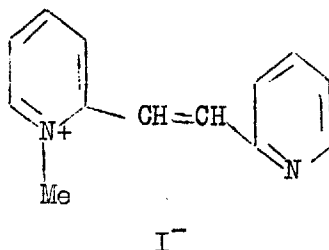
but no reduction by hydrogenase in the presence of Cu, Fe or Co occurred.

Ethylenic compounds

The structure of stilbene has been determined by Woodward using X-ray techniques (118), and shown to be planar, which is explained by conjugation of the double bond and the contribution which an alternative excited state makes to the normal state:

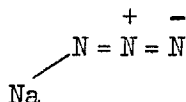
shown, the rate of reduction with cell free extracts are in the reverse order to that with palladium/charcoal.

A further interesting observation is that di- α -pyridyl-ethylene-N-methiodide is reduced at a very slow rate as compared with the parent compound, di- α -pyridyl ethylene, so that the N-methyl group may cause steric hindrance.



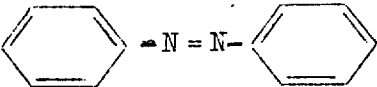
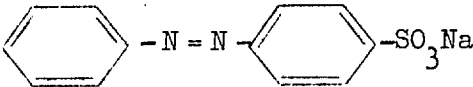
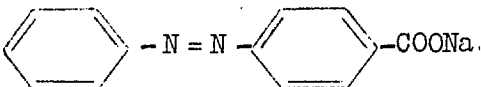
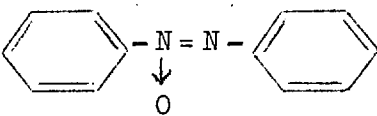
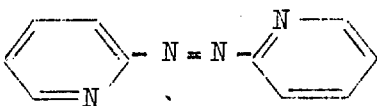
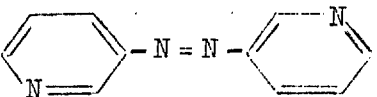
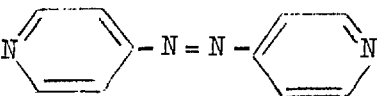
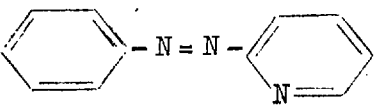
However, in view of the lack of data on the stereochemistry of this type of compound, and on the exact nature of adsorption onto catalytic surfaces, there is no advantage in making a hypothesis.

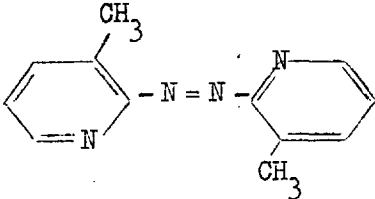
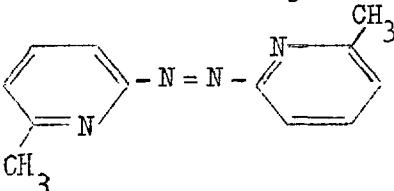
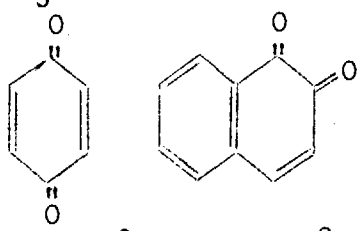
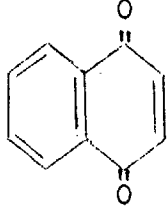
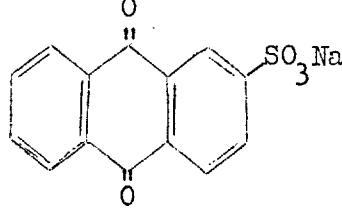
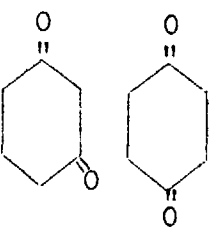
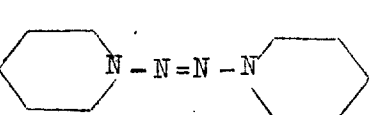
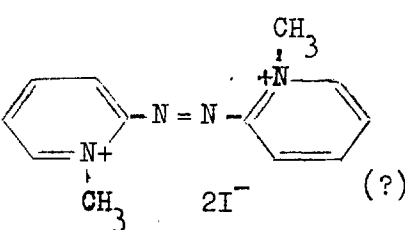
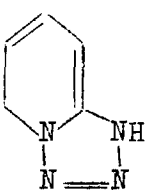
No reduction of the linkages in the azide grouping could be achieved. Sodium azide has the structure

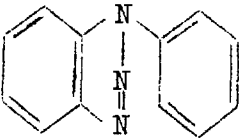
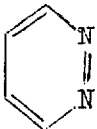
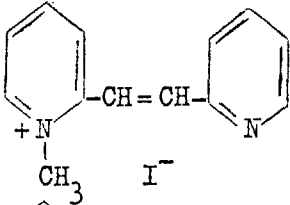
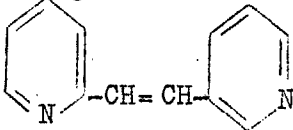
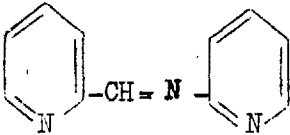


both N=N linkages having the same length (120), i.e. $1.17 \text{ \AA}$ which is less than the values for azo compounds.

Reduction of Substrates by Hydrogenase

<u>Substrate</u>	<u>Reduction : Direct Indirect</u>		<u>Remarks</u>
$\text{CH}_3\text{-N=N-CH}_3$	-ve	-ve	
$(\text{CH}_3)_2\text{CH-N=N-CH} \cdot (\text{CH}_3)_2$	-ve	-ve	
 (trans)	-ve	+ve	
" (cis)	-ve	+ve	
 -SO ₃ Na	-ve	+ve	} Considerable inhibition of enzyme
 -COONa	-ve	+ve	
	-ve	+ve	
 (trans)	+ve	+ve	Stimulation by Fe ⁺⁺ , Co ⁺⁺ , Hg ⁺⁺ , Cr ⁺⁺ , Cu ⁺⁺
" (cis)	+ve	+ve	Slow reduction (ca 10% of trans) Unaffected by metal ions.
	+ve	+ve	} Direct reduction rate identical with the 2,2' isomer, but no metal stimulation
	+ve	+ve	
	+ve	+ve	Reduction rate ca 50% of that of 2,2'-azopyridine. Stimulation by Fe, Co, Cu.

<u>Substrate</u>	<u>Reduction : Direct Indirect</u>		<u>Remarks</u>
	+ve	+ve	Slow reduction (ca. 5% of 22' azopyridine). Identical rates if Pd/C is used. No stimulation with Fe^{++} , Cu^{++} , but +ve results with Co^{++} .
	+ve	+ve	
	+ve		Reduced with decroasin rapidly.
			
	+ve		
	-ve	=ve	
	-ve	-ve	Cu^{++} etc. had no effect.
	+ve		Very rapid reduction (ca. 20x that of 22' azopyridine)
	-ve	-ve	Indirect transfer can be obtained to the Cu complex, but not to the Co complex.

<u>Substrate</u>	<u>Reduction</u>	<u>Direct</u>	<u>Indirect</u>	<u>Remarks</u>
		-ve	-ve	
		-ve	-ve	
$\left. \begin{array}{l} \text{CH}_3(\text{CH}_2)_7\text{C}\equiv\text{COOH} \\ \text{CH}_3\text{CH}=\text{CH}\cdot\text{COOH} \\ \text{HOOC}\cdot\text{CH}=\text{CH}\cdot\text{COOH} \end{array} \right\}$	Na salts	-ve	-ve	
		-ve	-ve	
		-ve	-ve	
		-ve	+ve	
2,2' dipyridyl (Cu complex)		+ve		May form free radical (only $\frac{1}{2}$ mol. H_2 absorbed)
$\text{NH}_2\text{OH}\cdot\text{HCl}$		+ve	+ve	
KNO_2		-ve	+ve	
KNO_3		-ve	-ve	
NaN_3		-ve	-ve	

In all cases (except where otherwise stated) only 1 mole of hydrogen per mole of substrate was absorbed.

Purification of the Hydrogenase Enzyme.

The literature up to 1954 has been adequately reviewed by Gest (121), and the experiments of previous workers has shown that iron is necessary for activity of the enzyme from various sources. This was generally shown by the greatly reduced activity of hydrogenase when the bacteria were grown in media deficient in this element. This, of course, does not prove that hydrogenase (if it is a single enzyme) contains iron: this can only be accepted if iron can be shown to be present at all levels of purity, and it is true to say that this has not yet been achieved.

One of the problems in trying to establish this hypothesis has been the ready inactivation of highly purified preparations, either by the redox dye used for the assay procedure, so that the enzyme cannot be detected, or by atmospheric oxygen prior to assay. All purifications must be carried out as far as possible under an atmosphere of hydrogen at as low a temperature as possible, and in the presence of a low redox material such as cysteine or thioglycollic acid.

Desulphovibrio desulphuricans is the most active bacterium in respect of hydrogenase activity known at present, and as such is the most widely used for the preparation of cell-free extracts.

The most recent work on the purification of the enzyme from this bacterium is that of Sadana and Jagannathan (122). Their crude enzyme was extracted from acetone-dried cells, after which their purification procedure was as follows: The enzyme was precipitated by adjusting the buffer solution to pH 3.9 - 4.0 with acetic acid, redissolved in phosphate buffer solution, and heated to 60° for

10 minutes. The denatured protein was removed by centrifuging, and the supernatant solution was adjusted to pH 5.0 with acetic acid, and reheated at 50° for 1.5 minutes. The enzyme was precipitated from the supernatant solution by the addition of 3 volumes of acetone at -10°, and dried over phosphorus pentoxide in vacuo.

The enzyme was redissolved in phosphate buffer, and nucleic acids were removed with protamine sulphate solution. The enzyme was then adsorbed on calcium phosphate gel, eluted at pH 8.0 with phosphate buffer, evaporated to low volume, and impurities removed by passage through a column of I.R.C. 50 (NH_4^+ form). At this stage the enzyme was relatively unstable.

The enzyme was obtained in its final form by chromatography on cellulose, when the peak ratio 280 m μ : 260 m μ was 1.1.

The purified material was strongly coloured, and showed no absorption band characteristic of cytochromes, previously observed bands being shown to be due to this type of impurity. The spectrum showed absorption maxima at 555, 525 and 419 m μ in the reduced state.

The enzyme was completely inactive with respect to methylene blue unless iron was added, a concentration of 4.6×10^{-5} M. being required for $\frac{1}{2}$ maximum velocity of reduction. Addition of cytochrome C_3 , isolated from the bacteria, had no effect however, and similarly, activity could not be restored by the addition of Mn, Co, Ni, Zn, Pb, MoO_3^{--} , $\text{V}_2\text{O}_5^{--}$, WO_3^{--} , CrO_3^{--} and BO_3^{--} .

The work of Sadana and Jagannathan thus shows that the purified enzyme system required iron for hydrogenase action, but does not establish whether it contains iron. The only experimental evidence that this is so is that of Winfield (123) who claimed to have obtained a hydrogenase band by paper electrophoresis, which contained some of the radio iron added originally to the growth medium.

In view of the uncertainty surrounding this problem, it was decided to attempt the purification of the enzyme using radio iron as a tracer, and also to try and establish directly whether any other metal not normally associated with hydrogenase would follow the enzymic activity through the purification procedures. For this purpose radio cobalt was used.

Since the cultivation and preparation of cell-free extracts from *Azotobacter vinelandii* had been achieved successfully, it was decided to attempt the purification using this source of material initially.

The radio iron (Fe^{59}) was obtained as a 10 ml. solution containing ca. 30 $\mu\text{g. Fe}$ with an activity of ca. 100 $\mu\text{c.}$ which was the maximum activity permissible in this laboratory.

In order to avoid undue dilution of the isotope in the growth medium, it was first necessary to determine if the amount of iron normally added (0.015 mg. $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ /litre) could be reduced appreciably without affecting the growth of the bacteria.

Under normal conditions most of the iron is converted to insoluble phosphate in the medium, and cannot be separated easily from the bacteria by centrifuging. Therefore, for the purpose of determining the quantity of iron taken up by the bacteria during normal growth, the usual medium was made up, but containing sufficient citric acid (~ 40 mg./litre) to prevent precipitation of the iron. The medium was filtered before sterilisation, and a few μc of radio iron was added separately afterwards.

A five-litre culture was made up and aerated for 2-3 days in the usual manner, when a portion of the bacterial suspension was removed, centrifuged and the radioactivity (γ radiation) of the sedimented bacteria was determined using a scintillation counter. The quantity of iron in the bacteria was easily shown to be approximately 25% of the quantity added, so that it was considered satisfactory to add only 4 mg. of ferrous sulphate, as compared with the customary 15 mg. per litre. The bacteria grew perfectly well in this new medium, and no reduction in hydrogenase activity as compared with bacteria grown on the normal medium was observed. If the culture contains no added iron, some growth of bacteria occurs, with a reduction in total hydrogenase content, and a green fluorescent pigment is produced in the medium.

The bacteria were normally cultivated in 20 litres of medium, the radio iron solution being separately sterilised in a small flask so as to avoid loss should the 20-litre bottle break during autoclaving. The cooled medium was inoculated in the usual way, and aerated for 2-3 days, after which time the bacteria plus precipitated

phosphate was harvested in a Sharples centrifuge. The cell-free extract was obtained as previously described, and the radio iron plus magnesium, calcium etc., was recovered in about 90% yield from the cell debris by digestion with nitric acid.

After centrifuging at 20,000 g. for 1 hour, the cell-free extract contained about 5% of the total hydrogenase activity, since the enzyme is mainly concentrated in the cell wall of the bacteria.

Assay procedure

The most widely used redox dyes for the assay of hydrogenase are methylene blue, benzyl viologen and methyl viologen, of which the viologens are to be preferred for the assay of purified cell-free extracts, since, owing to their low redox potentials, there is less chance of inactivating the enzyme. There is the added advantage that the viologens being colourless in the oxidised state and highly coloured in the reduced state, show immediately any reduction that has occurred.

Methylene blue, however, has a high redox potential (+0.36), and as such can inactivate very highly purified preparations, and also suffers from the disadvantage that it is highly coloured in the oxidised state, so that reduction cannot be easily observed visually.

The reduction of the copper azopyridine complex by crude cell-free extracts has been shown to occur very rapidly, and it was hoped to use this complex to assay preparations of low hydrogenase activity, where the rate of reduction of the usual redox dyes would be too slow. Unfortunately, while reduction by crude extracts was extremely rapid, when purification had reached the stage of ammonium sulphate

fractionation, the preparations would no longer reduce the complex. This was not due to inactivation of the enzyme, as reduction of methylene blue in the presence of the complex was not affected; the inference is that loss of co-factors was responsible. It was therefore not possible to use this system for assay purposes, and the usual redox dyes had to be employed.

An examination of the relative rates of reduction of the three redox dyes by the crude cell-free extracts was made, and it was found that while transfer to methylene blue was satisfactory, that to the viologens was very slow indeed. Hydrogenase activity therefore had to be followed with methylene blue.

Purification procedure

a) Heat treatment: From a 20-litre culture 50-100 ml. of crude cell-free extract was obtained as a yellowish, faintly opalescent solution. This was transferred to a 250 ml. conical flask which was then flushed with hydrogen, stoppered and swirled in a water bath maintained at 60° for 10 minutes, during which time a greyish filamentous precipitate of denatured protein separated. The solution was rapidly cooled in an ice bath and transferred to 50 ml. plastic centrifuge tubes which were flushed with hydrogen, stoppered and spun at 3,000 g. in an angle head centrifuge for 45 minutes. It was found that even thick-walled soda-glass tubes frequently cracked at this speed, so that plastic tubes were always used except when volumes less than 10 ml. were being centrifuged, when glass tubes could be used with safety.

The clear straw coloured solution was decanted from the sediment, and stored in a 100 ml. conical flask under hydrogen at 2° until the next stage of purification was to be carried out.

Hydrogenase assays on this and the original cell-free extract showed its activity to have fallen to 50%, and radio counts on both solutions showed that the loss of iron was only about 13%.

Nucleic acid removal: nucleic acids are known to interfere with ammonium sulphate fractionations, and must be removed before this is attempted. There are two established methods for removal of nucleic acids: manganous chloride, used e.g. by Korkes (124), and protamine sulphate used e.g. by Sadana and Jagannathan (122). There is probably little to choose between these two methods, but since manganous chloride removes phosphate from the buffered solutions, and gives a less easily sedimentable precipitate, it was decided to use protamine sulphate.

This was dissolved in phosphate buffer pH 7.0, readjusted to this pH with dilute aqueous sodium hydroxide, and made up to a 2% solution with more buffer solution. Generally, a small amount of insoluble oily residue remained. The supernatant was added dropwise to the enzyme solution until no further precipitate was obtained, and the solution was then centrifuged in the usual way.

Hydrogenase assay and radio counts on the resultant clear enzyme solution showed that loss of enzymic activity was negligible, but that only 10-20% of the original iron content was present.

It was found that if the crude cell-free extract were treated with protamine sulphate before the heat treatment, then 50% of the hydrogenase activity was lost, and subsequent heat treatment caused no appreciable drop in enzymic activity. This suggests that the order of treatment is unimportant, but this was not investigated

further, and the original procedure was adhered to.

Ammonium sulphate fractionation

The enzyme solution (usually about 50 ml.) was treated with successive quantities of solid ammonium sulphate, and the precipitate formed at each stage was redissolved in the minimum volume of phosphate buffer. Each fraction was stored in stoppered test tubes with 0.05 ml. of 1% cysteine solution under hydrogen at 0-5° until required.

This fractionation procedure resulted in a total loss of activity of approximately 50% of the hydrogenase, and a similar loss in activity was observed in the enzyme obtained on complete precipitation by saturating the solution with ammonium sulphate.

A typical fractionation is reproduced below, having used about 10 μc Fe^{59} in the culture medium.

	<u>Hydrogenase activity</u>	<u>Fe^{59} (cpm)</u>
Original crude C.F.E.	100%	56,360
After heat and protamine treatment	50%	5,230
$(\text{NH}_4)_2\text{SO}_4$ precipitation:		
(i) 0-40% saturation	1.5%	1,980
(ii) 40-50% saturation	5%	1,440
(iii) 50-57% saturation	15%	600
(iv) 57-65% saturation	5%	870

The background on the scintillation counter was ca.250 cpm.

At this stage the most active fraction (iii) contains a little more than 1% of the original iron content, and 15% of the hydrogenase content, so that this represents only a 15-fold purification on an iron basis.

At this level of purity the enzyme was showing a tendency to become inactive due to exposure to air, and attempts to refractionate (iii) with ammonium sulphate resulted in complete inactivation.

Dialysis

The apparatus for dialysis consisted of glass tube approximately 8 cm. long and 1.5 cm. in diameter, closed at one end by a dialysis membrane. The solution to be dialysed was placed in the tube, which was then suspended inside a flask containing phosphate buffer pH 7.0, so that the membrane was just below the surface of the buffer solution. The flask was flushed out with hydrogen, and dialysis was allowed to proceed for 24 hours at 0-5°.

Dialysis of 0.5 ml. of fraction (iii) resulted in a loss of only 15% of the iron content, but 50% of the hydrogenase activity was lost.

In an attempt to recover any co-factors lost during the operation, dialysis was repeated, but against only 10 ml. of phosphate buffer, which was then freeze-dried. Addition of this material to the dialysed enzyme had no effect on its reduced activity, and there arose the possibility that the membrane was responsible for the partial inactivation. The enzyme was allowed to remain in contact with a strip of the membrane, of roughly the same area as that used in the dialysis apparatus, for several days, and its hydrogenase

activity was redetermined. The activity was unaffected by this treatment, showing that the membrane is not directly responsible for the deactivation.

Chromatography

The sample for column chromatography was prepared by dialysing fraction (iii) against phosphate buffer pH 6.2 for 24 hours, and then pouring it into 10 ml. of acetone at 0° to precipitate the enzyme. The enzyme was recovered by centrifugation, and redissolved in the minimum of buffer solution pH 6.2 (ca. 1.5 ml.).

The enzyme solution was run down columns of charcoal, 'cellite', cellulose and a polyalkylated cellulose, but none of these adsorbents was able to retain the enzyme. Zeokarb 225 (I.R.C. 50) prepared by the method of Hirs (125) was capable of adsorbing the enzyme, but only very loosely, and the hydrogenase activity was eluted rapidly by phosphate buffer pH 6.2.

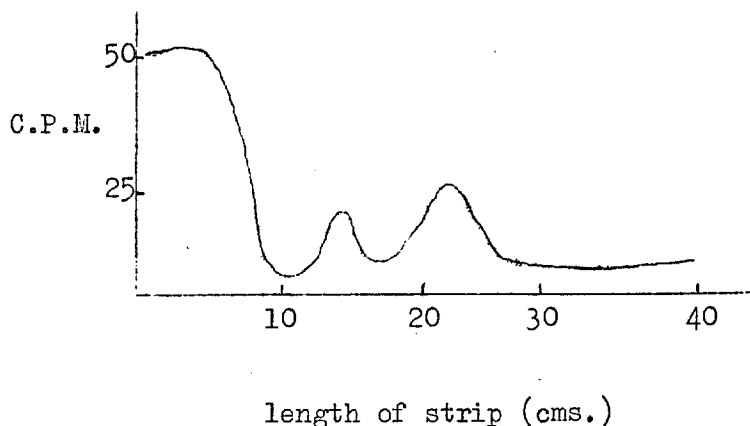
The resin removed a considerable amount of cytochromic material which remained adsorbed at the top of the column. The whole of the enzyme solution was thus partially purified by passage down a column of the resin approximately 25 cm. long and 1.5 cm. in diameter, and surrounded by a jacket containing crushed ice. The enzyme was eluted rapidly in a volume of ca. 15 ml., and was isolated by acetone precipitation as previously described. The enzyme was redissolved in 1.5 ml. of phosphate buffer pH 6.2. Both the hydrogenase activity and the iron content had dropped by 20% compared with the sample after dialysis. The iron activity corresponded to approximately 600 cpm, which was rather too low to follow a separation

by electrophoresis, so a further sample of hydrogenase was prepared by incorporating 50 μc Fe^{59} into the growth medium of a culture of Azotobacter. This gave a final fraction with a count of ca. 3,000 cpm.

Paper electrophoresis

Electrophoresis was carried out on Whatman no. 1 extra thick filter paper strips, approximately 3 x 60 cm. long, at pH 6.2. Below this pH there was a danger that the enzyme would precipitate from solution. The final fraction was added dropwise to the paper about 10 cm. from one end, each drop being dried by a jet of hydrogen, until 0.10 ml. had been transferred. The operation was done in duplicate, and the two papers were soaked in phosphate buffer and placed between the glass plates of the apparatus, so that ends of the papers dipped to a depth of 4-5 cms. in the electrode compartments. The 'spotted' ends were nearest the cathode. The paper was subjected to a potential difference of 1000 v. for 6 hours, when both papers were removed and allowed to dry at room temperature. One paper was sprayed with ninhydrin reagent prepared by the method of Stein and Moore (126), and developed with a jet of hot air.

The position of the developed spots was recorded, the paper was cut into 2 cm. strips, and the radioactivity of each was determined directly. The 'spectrum' of iron content obtained is shown in figure 3, from which it can be seen that most of the iron remains almost stationary at the origin.



Each peak of radioactivity corresponded to a positive ninhydrin reaction, and the respective strips on the second paper were eluted with phosphate buffer pH 7.0. None of these fractions showed any hydrogenase activity using methylene blue as acceptor, so it must be concluded that the enzyme is inactivated during electrophoresis, or that it is so sensitive afterwards, that it is inactivated by the redox dye.

The purification procedure was repeated with a cell-free extract from *Desulphovibrio desulphuricans*, since this shows high activity towards benzyl viologen, the advantages of which have already been mentioned.

The enzyme preparation showed high activity immediately before electrophoresis, but the fractions obtained subsequently from the paper were completely inactive. The reasons for this inactivation have not been fully investigated, but paper electrophoresis at a higher voltage (10 kv.) for a shorter period of time (1 hour) might yield an active product. Thus it cannot yet be proved beyond doubt that hydrogenase contains iron.

Since Fe^{59} is relatively expensive, with a half-life of 45 days, it was considered interesting to investigate the possibility of replacing iron by cobalt, and to follow the fractionation procedure using Co^{60} .

A 20 litre culture of *desulphovibrio desulphuricans* containing 300 μC Co^{60} (5 mg. $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ per litre) was prepared, and a cell-free extract obtained in the usual way.

The final product prior to electrophoresis showed high hydrogenase activity, and had a cobalt activity of 5,000 cpm. per ml.

Paper electrophoresis of this fraction gave a similar 'spectrum' to that shown in figure 3, with the ninhydrin spots corresponding to the peaks. None of the fractions eluted from the paper showed any hydrogenase activity with respect to benzyl viologen.

Since cobalt follows the fractionation procedure in a similar manner to that of iron, and a similar electrophoresis 'spectrum' is obtained, there is a definite possibility that cobalt could replace iron in the requirements for hydrogenase.

Summary

- a) The determination of the Michaelis constant of nitrogenase has been carried out using N^{15} , and shown to be approximately 3 times the value obtained from respirometric data. No variation in value could be detected over the temperature range employed.
- b) Direct reduction of unsaturated linkages by cell-free extracts has been shown to be limited to the azo group in three azopyridines and in phenylazopyridine.

Stimulation of hydrogen transfer is effected by metal ions, especially copper and iron, which is attributed to a decrease in the electron density of the azo linkage, and suggests a possible mechanism for nitrogenase action in which the electron density in the nitrogen-nitrogen triple bond is sufficiently reduced by chemisorption onto a metallic site (possibly molybdenum) to enable reduction by hydrogenase to take place.

The ease of reduction of the azo group has been shown to be dependent on the nature of the attached groupings: phenyl groups (azobenzene) which are highly polarisable prevent direct reduction, but indirect reduction via artificial carriers is possible. Electron releasing groups such as methyl and isopropyl prevent even indirect reduction. Cis-azopyridine is reduced very much more slowly than the trans isomer.

The ethylenic and acetylenic linkages could not be reduced under any conditions, but transfer to the azomethine group (when attached to pyridyl) could be achieved indirectly.

- c) The rate of reduction of azo compounds by a heterogeneous catalyst was shown to be the reverse of that by hydrogenase, indicating that hydrogenase does not act by a purely physical adsorption mechanism.
- d) Reduction could also be achieved by means of artificial carriers such as the viologens. Hydrogen transfer from the reduced carrier took place very rapidly to the aromatic azo compounds, but relatively slowly to hydroxylamine, nitrite, and the azomethines. The process was shown to be non-enzymic in nature. No transfer was obtained to the ethylenic linkage, nitrate, aliphatic azo compounds and the nitrogen molecule.
- e) Iron and cobalt have been shown to be present at all stages of purification of the enzyme, and it is possible that these two metals are interchangeable.

The preparation of a cell-free extract containing an active nitrogenase has now been reported (127, 128). If the same difficulties of purification are encountered as in the case of hydrogenase, then it is unlikely that the enzyme system will be characterised for some considerable time.

The results of the present work show that a reductive mechanism for nitrogenase is feasible, and that transfer of hydrogen to the nitrogen molecule might be achieved by a reduced carrier, possibly by prior adsorption of the gas onto a metal surface such as iron, cobalt, copper or molybdenum.

REFERENCES

1. Green, M., Alexander, M. and Wilson, P.W. Proc. Soc. Exp. Biol. Med., 1953, 82, 361-363.
2. Green, M. and Wilson, P.W. J. Bact., 1953, 65, 511-517.
3. Lee, S.B. and Wilson, P.W. J. Biol. Chem., 1943, 151, 377-385.
4. Wilson, P.W. The Biochemistry of Symbiotic Nitrogen Fixation, University of Wisconsin Press, Madison, Wisconsin (1940).
5. Wyss, O. and Wilson, P.W. Proc. Nat. Acad. Sci. U.S.A. 1941, 27, 162-168.
6. Gest, H. Bact. Rev., 1949, 15, 183-210.
7. Bregoff, H.M. and Kamen, M.D. Arch. Biochem. Biophys. 1952, 36, 202-220.
8. Le Gall, J., Senez, J.C. and Pichinoty, F. Ann. Inst. Pasteur, 1959, 96, 223-230.
9. Wieland, H. and Pistor, H.J. Ann. Chem., 1936, 522, 116-137.
10. Hobermann, H.D. and Rittenberg, D. J. Biol. Chem., 1943, 147, 211-227.
11. Bottomley, G.H., Cavanagh, B. and Polanyi, M. Nature, 1935, 136, 103-104.
12. Cavanagh, B., Horiuti, J. and Polanyi, M. Nature, 1935, 133, 797.
13. Boichenko, E.A. Compt. rend. Acad. Sci. U.S.S.R., 1946, 52, 521-524.
14. Boichenko, E.A. Biochimia, 1948, 13, 219-224.
15. Boichenko, E.A. Doklady Akad. Nauk. U.S.S.R., 1949, 64, 545-548.
16. Hyndman, L.A., Burris, R.H. and Wilson, P.W. J. Bact., 1953, 65, 522.
17. Bregoff, H.M. and Kamen, M.D. Arch. Biochem. Biophys., 1953, 36, 202-220.
18. Nakamura, H. Acta Phytochim (Tokyo), 1938, 10, 259-270.
19. Gest, H. Bact. Proc., 1950, 136-137.
20. Gest, H. J. Bact., 1952, 63, 111-121.

21. Yamagata, S., Nakamura, H. Acta Phytochim (Tokyo), 1938, 10, 297-311.
22. Gest, H. Proc. Nat. Acad. Sci. U.S.A., 1956, 42, 13-19.
23. Gest, H. Proc. Nat. Acad. Sci. U.S.A., 1956, 42, 180-185.
24. Farkas, L. and Fischer, E. J. Biol. Chem., 1947, 167, 787-805.
25. Gest, H. and Gibbs, M. J. Bact., 1952, 63, 661-664.
26. Gest, H. Phosphorus Metabolism, 1952, p.522.
27. Lascelles, J. and Still, J.L. Proc. Linnean Soc. N.S.W. 1947, 72, 49.
28. Fischer, F.G. and Eysenback, H. Ann. Chem., 1937, 530, 99-120.
29. " " " " Ann. Chem., 1942, 552, 203-242.
30. Lascelles, J. and Still, J.L. Austral. J. Exp. Biol. Med. Sci. 1946, 24, 159-167.
31. Woods, D.D. Biochem. J., 1938, 32, 2000-2012.
32. Evans, H.J. and Nason, A. Arch. Biochem. Biophys. 1952, 39 234.
33. Franke, W. and Mönch, J. Biochem. Z., 1948, 319, 174-183.
34. Weller, S. and Mills, G.A. J. Amer. Chem. Soc., 1953, 75, 769.
35. Hoberman, H.D. and Rittenberg, D. J. Biol. Chem., 1943, 147, 211.
36. Joklik, W.K. Austral. J. Exp. Biol. Med. Sci., 1950, 28, 321.
37. Krasna, A.I. and Rittenberg, D. Proc. Nat. Acad. Sci., U.S.A. 1956, 42, 181.
38. King, N.K. and Winfield, M.E. Biochem. Biophys. Acta, 1955, 17, 431-432.
39. Stephenson, M. and Stickland, L.H. Biochem. J., 1932, 26, 712.
40. " " " " Biochem. J., 1933, 27, 1517.
41. Hoberman, H.D. and Rittenberg, D. J. Biol. Chem., 1943, 147, 211.
42. Stern, K.J. Science, 1936, 83, 190.
43. Lascelles, J. and Still, J.L. Austral. J. Exp. Biol. Med. Sci. 1946, 24, 37-48.

44. Lascelles, J. and Still, J.L. Austral. J. Sci. Res., 1946, 2, 25.
45. Joklik, W.K. Austral. J. Exp. Biol. Med. Sci., 1950, 28, 321-329.
46. " " Austral. J. Exp. Biol. Med. Sci., 1950, 28, 331-338.
47. Waring, W.S. and Werkmann, C.H. Arch. Biochem., 1944, 4, 75.
48. Gaffon, H. Biol. Rev. Camb. Phil. Soc., 1944, 19, 1.
49. Green, D.E. and Stickland L.H. Biochem. J., 1934, 28, 898-900.
50. Farkas, L. J. Phys. Colloid Chem., 1951, 55, 1013.
51. Couper, A. and Eley, D.D. Quart. Rev., 1949, 3, 209.
52. Eley, D.D. Advances in Catalysis, 1948, Vol. I. p. 157.
53. Bonhoeffer, K.F. et al. Z. phys. Chem., 1933, 21B, 225.
54. Weller, S., Mills, G.A. J. Amer. Chem. Soc., 1953, 75, 769-777.
55. Calvin, M. et al. Trans. Faraday Soc., 1936, 32, 1436.
56. Eley, D.D. and Polanyi, M. Trans. Faraday Soc., 1936, 32, 1433.
57. Polanyi, M. Trans. Faraday Soc., 1938, 34, 1191.
58. Eley, D.D. Trans Faraday Soc., 1940, 36, 500.
59. Brown, M. and Metcalfe, G. Nature, 1957, 180, 282.
60. Shug, A.L. et al. Inorganic Nitrogen Metabolism, p.344.
61. Hamilton, P.B. et al. Proc. Nat. Acad. Sci., U.S.A., 1957, 43, 297.
62. Lee, S.B. and Wilson, P.W. J. Biol. Chem., 1943, 151, 377.
63. " " " " Inorganic Nitrogen Metabolism, p.298.
64. Hiai, S. et. al. J. Biochem (Tokyo), 1957, 44, 839.
65. Rosenblum, E.D. and Wilson, P.W. J. Bact., 1950, 59, 83.
66. Winfield, M.E. Rev. Pure Appl. Chem., 1955, 5, 217.
67. Allison, R.M. and Burris, R.H. J. Biol. Chem., 1957, 224, 351.
68. Diamantis, A. Ph.D. Thesis, University of London.

69. Rakestraw, J.A. Ph.D. Thesis, University of London.
70. Lineweaver, H., Burk, D. and Deming, W.E. J. Amer. Chem. Soc., 1934, 56, 225.
71. Lineweaver, H., et al. J. Amer. Chem. Soc., 1934, 56, 658.
72. Wilson, P.W., Burris, R.H. and Lind, C.J. Proc. Nat. Acad. Sci., U.S.A., 1942, 28, 243.
73. Burk, D. Ann. Rev. Biochem., 1941, 10, 587.
74. Wilson, P.W. J. Amer. Chem. Soc., 1936, 58, 1256.
75. Burris, R.H., Magee and Back. Ann. Acad. Sci., Fennicae, 1955, (A), No. 60, 190.
76. Wilson, T.G.G. and Roberts, E.R. Biochem. Biophys. Acta, 1954, 15, 390.
77. Lemin, P. Ph.D. Thesis, University of London.
78. Michaelis, M. and Menten, M.S. Biochem. Z., 1913, 49, 333.
79. Wilson and Roberts. Biochem. Biophys. Acta, 1954, 15, 568.
80. Molnar, D. et al. J. Amer. Chem. Soc., 1948, 70, 1713.
81. Stearn, A.E. Adv. Enzymol., 1949, 9, 25.
82. Kaufmann, S. et al. J. Biol. Chem., 1949, 177, 793.
83. Kiese, M. Biochem. Z., 1941, 307, 400.
84. Sizer, I.W. Adv. Enzymol., 1943, 3, 35.
85. Sizer, I.W. and Josephson. Food Res., 1943, 7, 201.
86. Butlin, K.R. et al. J. Gen. Microbiol., 1949, 3, 46.
87. Umbriet, W.W. et al. Manometric Techniques for the Study of Tissue Metabolism, Burgess, 1944.
88. Pauling, L. Nature of the Chemical Bond, Cornell University Press.
89. Pitzer, K.S. J. Amer. Chem. Soc., 1948, 70, 2140.
90. Org. Synth., II, 208.
91. Ramsperger, H.C. J. Amer. Chem. Soc., 1927, 49, 912.
92. Lochte, H. J. Amer. Chem. Soc., 1921, 43, 2597.

93. Kirpal, A. and Reuter, E. Ber., 1927, 60, 664.
94. Kirpal, A. Ber., 1934, 67, 70.
95. Campbell, N. J. Chem. Soc., 1953, 1281.
96. Knorr, L. Ann. 1883, 221.
97. Graves, T.B. J. Amer. Chem. Soc., 1924, 46, 1460-1470.
98. Perkin, W.H. and Robinson, R. J. Chem. Soc., 1913, 103, 1973.
99. Robinson, R. and Thornby, S. J. Chem. Soc., 1924, 125, 2170.
100. Walsh, A.D. Discuss. Faraday Soc., 1947, 2, 18.
101. " " J. Chem. Soc., 1948, 400.
102. Phillips, A.P. J. Org. Chem., 1947, 12, 333.
103. Martin, R.H. and Robinson, R. J. Chem. Soc., 1943, 491.
104. Vogel Text Book of Practical Organic Chemistry, 1948, 989.
105. Org. Synth. III, 786.
106. Stickland, L.H. Biochem. J., 1931, 25, 1543.
107. Lascelles, J. and Still, J.L. Austral. J. Exp. Biol. Med. Sci., 1946, 24, 159.
108. Senez, J.C. Biochem. Biophys. Acta, 1958, 27, 569.
109. " " Biochem. Biophys. Acta, 1958, 28, 355.
110. Michaelis, L. and Hill, E.S. J. Gen. Physiol., 1932-3, 16, 859.
111. De Lange, Robertson, J.M. and Woodward, I. Proc. Roy. Soc., 1939, A171, 398.
112. Robertson, J.M. ibid, p.232.
113. Hampson, G.C. and Robertson, J.M. ibid, 1941, 409.
114. Haïssinsky, M. J. Phys. Radium, 1946, 7, 7.
115. " " J. Chem. Phys., 1947, 15, 152.
116. Orgel, L.E. Biochem. Soc. Symp., 1958, 15, 16.
117. Boersch, H. Monatsh., 1935, 65, 327.

118. Robertson, J.M. and Woodward, I. Proc. Roy. Soc., 1937, A162, 568.
119. Robertson, J.M. ibid., 1935, 150, 348.
120. Hendricks, S.B. and Pauling, L. J. Amer. Chem. Soc., 1925, 47, 2904.
121. Gest, H. Bact. Rev., 1954, 18, 43.
122. Sadana, J.C. and Jagannathan, V. Biochem. Biophys. Acta, 1959, 32, No. 2, 592.
123. Winfield, M.E. Rev. Pure Appl. Chem., 1955, 5, 229.
124. Korkes, S. et al. J. Biol. Chem., 1951, 193, 721.
125. Hirs, C.H.W. Methods in Enzymology I, 113. Colowick and Kaplan.
126. Moore, S. and Stein, W.H. J. Biol. Chem., 1948, 176, 367.
127. Carnahan, J.E. et al. Biochem. Biophys. Acta, 1960, 38, 188.
128. Nicholas, D.J.D. and Fisher, D.J. Nature, 1960, 186, 735.