ENZYME STUDIES ON AZOTOBACTER

~~with special reference to the mechanism of nitrogen fixation.~~

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ABSTRACT

Further investigations have been carried out on the hydrogenase activity and nitrogen fixing ability of cell-free extracts, prepared by the ultrasonic disruption of 'continuous culture' grown cells of Azotobacter vinelandii.

Thick suspensions of stored bacteria have been disrupted both in phosphate buffer and young culture (medium) and the nitrogen fixing ability of the disruptates, and corresponding centrifugates (supernatants), have been determined by the $[^{15}N]$ technique.

The results of the experiments have been expressed on a quantitative basis, as increases of nitrogen in the disruptate or supernatant. The increase of nitrogen has been calculated from the atom% ^{15}N of the disruptate (or supernatant), the atom% ^{15}N of the 'exposing' atmosphere and the total (Kjeldahl) nitrogen of the disruptate (or supernatant). The basis of this calculation has been discussed.

Under the experimental conditions employed in this work it has been found that the supernatants do give rise to significant fixation of nitrogen but heat treated supernatants also gave comparable 'fixation'.

The appearance of such blanks has been examined

with respect to possible contributions from whole cells, from soluble and 'adsorbed' nitrogen and from other possible sources. A critical review of the [^{15}N] technique has been made with respect to these considerations.

The addition of acetyl phosphate, ADP, sodium borohydride and other possible requirements to disruptates and supernatants has been studied.

Mechanisms for hydrogenase activity and nitrogen fixation have been proposed.

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(I) INTRODUCTION

The beneficial effects of both nitrogenous fertilisers and legume crops on soil fertility have been appreciated for many centuries.

The realisation that nitrogen fixation, resulting from bacterial symbiosis, was responsible for the latter (1) and the subsequent discovery of the free living bacteria, Clostridium (2) and Azotobacter (3) capable of fixing nitrogen, held great promise for the future. However, this early promise was not fulfilled, partly as a result of the slow development of systematic bacteriological technique but principally the result of the rapid growth of industrial chemistry with the successful manufacture of artificial fertilisers from ammonia, synthesised from nitrogen and hydrogen (Haber process). Although the Haber process was costly, (it has been stated that an energy expenditure equivalent to 5 tons of coal is required for each ton of nitrogen fixed (4)), little effort was directed towards an understanding of biological nitrogen fixation. Indeed, until the late 1920's when Burk established the Wisconsin group, nitrogen fixation by bacteria was merely coupled with fixation by lightning (electric discharge) as an obscure part of the natural nitrogen cycle.

At the present time investigations on bacterial nitrogen fixation are being conducted in several countries although the principal effort remains in the U.S.A.,

by Wilson and Burris at Wisconsin and by the Dupont group at Wilmington. The range of bacteria, that have been shown to be "fixers", has been extended by the application of $[^{15}N]$ isotopic tracer technique. It has been reported that many heterotrophic and photoautrophic bacteria and a number of leguminosae are capable of utilising atmospheric nitrogen (5).

Over the past two decades, investigations were confined principally to whole cell studies on the anaerobe, Clostridium pasteurianum and the aerobe, Azotobacter vinelandii. Despite the application of the $[^{15}N]$ isotopic tracer technique, the pathway of fixation remained uncertain.

Recently (6) these investigations received new impetus, with the successful preparation of a cell-free nitrogen fixing system from Clostridium pasteurianum. This has resulted in an increased knowledge about the requirements of the fixing system of Clostridium, but unfortunately comparable success with Azotobacter has not been achieved. Cell-free fixation by extracts of Azotobacter have been reported (7) but their activity is slight in comparison with that of the clostridial extracts.

At Imperial College, the fixation of nitrogen by Azotobacter has been studied from a physico-chemical

approach for a number of years. In recent years more effort has been directed towards cell-free work, with particular emphasis on the role of hydrogenase in nitrogen fixation. The work reported in this thesis represents a continuation of this approach; in particular a continuation of work initiated by Stewart (8) and Smith (9) on hydrogenase activity and nitrogen fixation in cellfree extracts of Azotobacter.

(II) PATHWAYS PROPOSED FOR THE FIXATION OF NITROGEN BY AZOTOBACTER.

The fixation of atmospheric nitrogen by Azotobacter, first reported over sixty years ago, presents an intriguing problem to both the chemist and biochemist.

Although the stable isotope of nitrogen $[^{15}N]$ has been used in tracing experiments for some twenty years, progress in the elucidation of the mechanism of fixation has been slow and has not matched the success of Calvin et alia (10) in unravelling the pathway of the fixation of carbon dioxide with $[^{13}C]$ as tracer. In comparison the amount of knowledge on the conversion of the stable nitrogen molecule into organic nitrogen is disappointingly small and consequently the mechanism of fixation remains open to speculation.

The stability of the nitrogen molecule is well known. This is illustrated in TABLE I, (11) in which its bond dissociation energy is compared with that of hydrazine and the hypothetical di-imide.

TABLE I

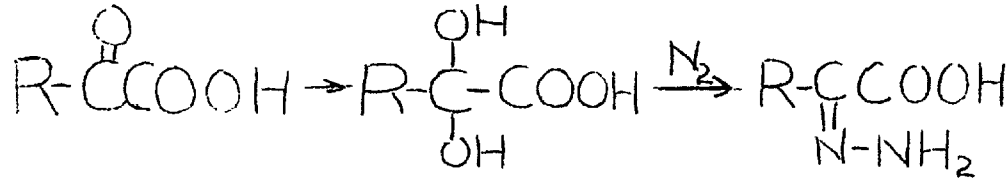
COMPOUND	FORMULA	BOND ORDER	BOND ENERGY kcal/mole	BOND LENGTH (N-N)s Å
NITROGEN	N_2	3	225	1.10
	$N_2^{\oplus}$	2.5	202	1.12
DI-IMIDE	N_2H_2	2	99	
HYDRAZINE	N_2H_4	1	38	1.47

It is apparent that the bond energy of nitrogen is greater than would be expected from a calculation based on the value for the N-N bond in hydrazine, since 3(N-N) = 114 Kcal/mole.

The extra stability has led to considerable speculation on the pathway of nitrogen fixation, particularly on the nature of the initial adsorption of nitrogen, whether this is physical or chemisorption.

On the following pages appears a brief historical review of the pathways that have been suggested for nitrogen fixation.

One of the earlier proposals for the incorporation of atmospheric nitrogen into organic compounds was made by Federov (12). Federov published a series of papers in the late 1940's on nitrogen fixation and proposed a direct incorporation into a keto-acid as shown below.



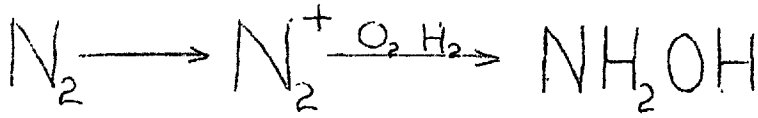
However a clear analysis of the mass of experimental data and particularly their relevance to the work in other laboratories has never been propounded by Federov.

Much of the earlier work was centred on the respective claims of ammonia and hydroxylamine as the first product of the fixing reaction. It was eventually concluded that ammonia was the key intermediate

in nitrogen fixation ..., (where "key intermediate" referred to the compound at which inorganic nitrogen metabolism ended and the nitrogen entered its organic pool)(13)(14).

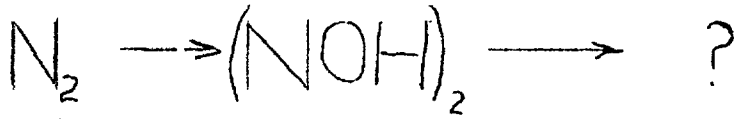
This, however, did not eliminate the possibility that the initial step in nitrogen fixation was oxidative or hydrolytic and several pathways involving such steps were postulated.

(A) VIRTANEN (15)



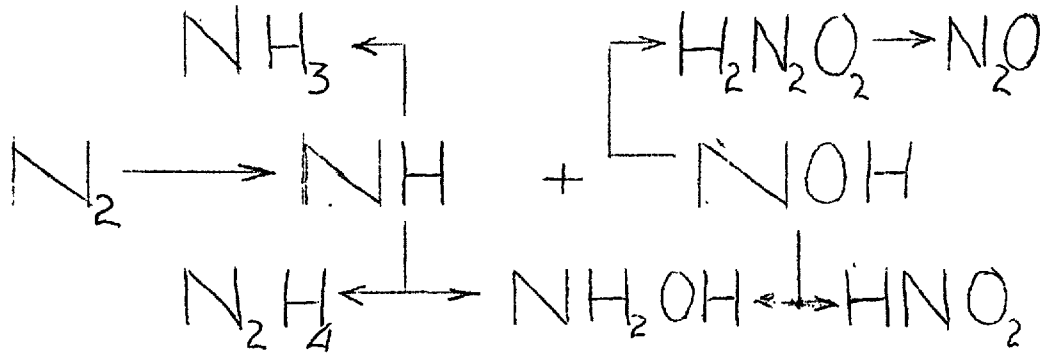
(B) WILSON (16)

(C) ROBERTS (17)



Roberts has also suggested a hydrolytic scheme which involves a dissociative initial step. This could give rise to a number of possible products of nitrogen fixation as is shown below (17) (18).

(D)



It is probable that (A) implies that an ionic mechanism may operate whereas (B) (C) (D) suggest free radical mechanisms.

The most widely accepted pathway at the present time is a purely reductive one and is thought to involve di-imide and hydrazine derivatives. A summary of possible pathways is shown in Figure 1.

The most favoured route is that involving bound diimide (V) and bound hydrazine (VI), with subsequent reduction to ammonia. It was proposed that an indirect reduction of hydrazine via P.C.A. (3,4 dihydro-pyridazinone 5-carboxylic acid) was more feasible. It was suggested that the P.C.A. was formed by the reaction between hydrazine and α-keto-glutarate(a reaction which can occur non-enzymatically), but the present experimental evidence for this route is not impressive.

It is worth mentioning that Yocum (19) attempted to

Possible pathways for nitrogen fixation

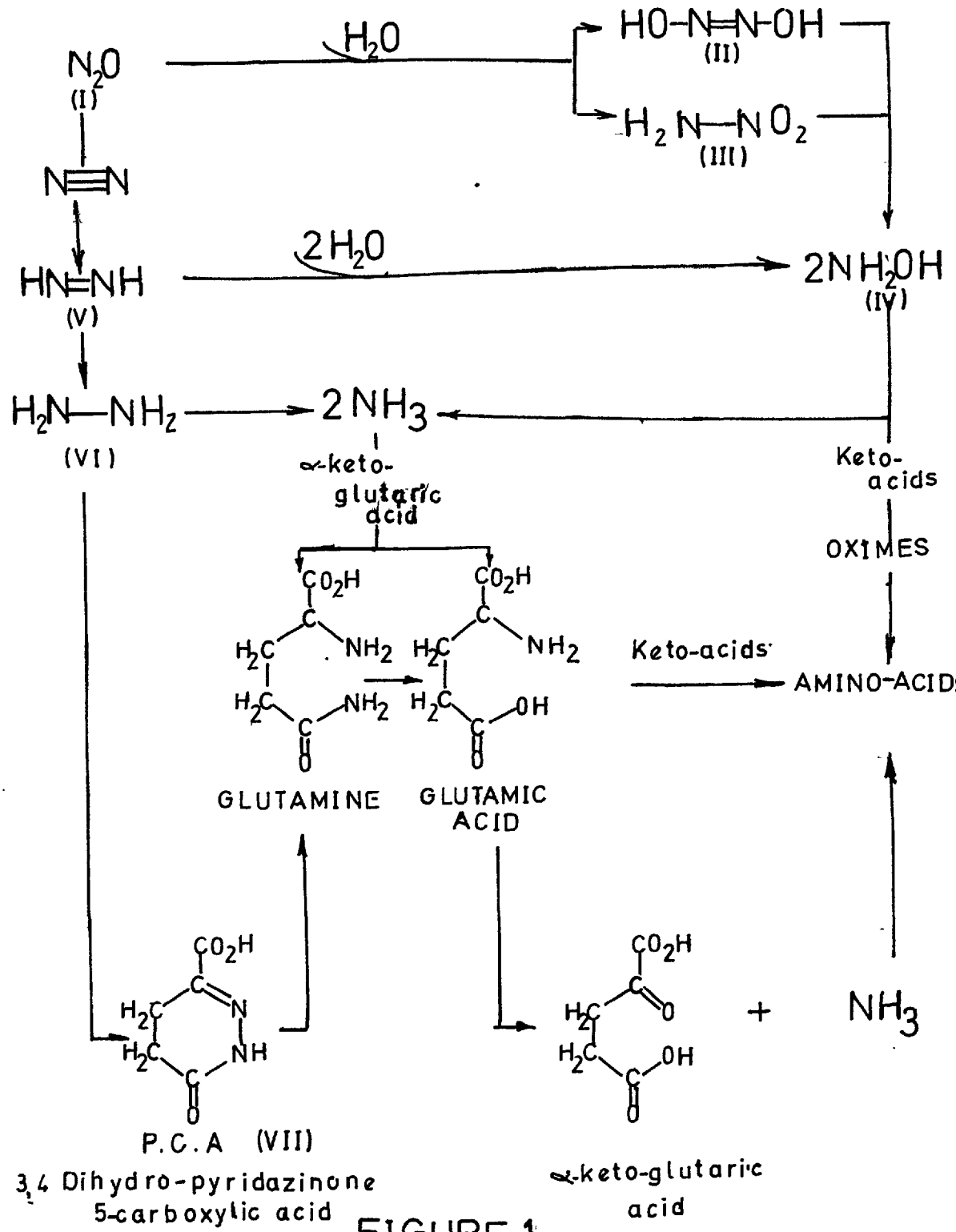


FIGURE 1

establish, on thermodynamic grounds, this pathway for nitrogen fixation by Clostridium pasteurianum. He suggested that nitrogen could be considered as a terminal acceptor analogous to oxygen in oxidative phosphorylation. He coupled nitrogen fixation to phosphorylation and found that this was thermodynamically favourable.

$$N_2 + 6[H] + ADP + P_i \longrightarrow 2NH_3 + ATP$$

The claims of nitrous oxide (I), hyponitrous acid (II), nitramide (III) and hydroxylamine (IV) as intermediates in nitrogen fixation have little experimental backing although they will receive further attention together with the claims of ammonia and hydrazine.

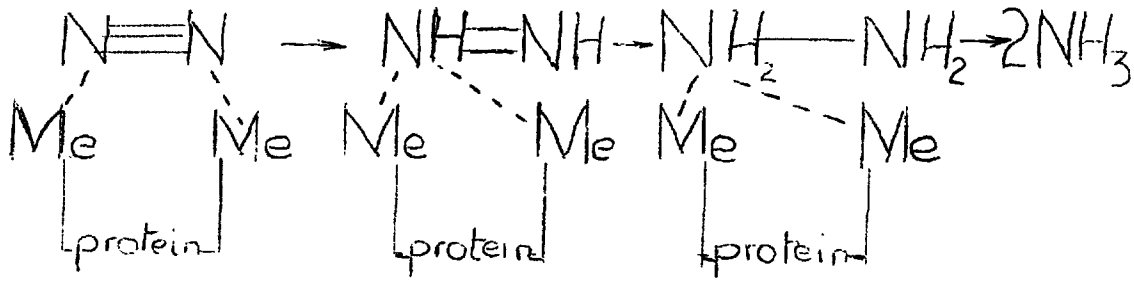
A recent communication (20), again on thermodynamic considerations, has proposed that nitrogen is directly incorporated into organic acids (cf. Federov). Pratt, with the energy requirements of the initial steps in mind, suggested that diazonium compounds may be intermediates. He represented aerobic fixation by

$$N_2 + \tfrac{1}{2}O_2 + BH_2 \longrightarrow BN_2 + H_2O$$

where BH_2 was an unspecified carboxylic acid.

This can be criticised on a number of grounds,

principally that it is difficult to visualise a mechanism for this and that no attempt has been made to enlarge on the initial step or to relate the subsequent steps to the critical experimental evidence. It does however suggest that the reduction of nitrogen may not be symmetrical, i.e. heterolytic reduction of the nitrogen molecule may occur. This point will receive further attention at a later stage when the possible mechanisms of nitrogen fixations are considered, particularly with respect to the evidence favouring a symmetrical reduction of the nitrogen molecule. Nason (21), several years ago, had implied that the symmetry of the nitrogen molecule may not be maintained in a reductive pathway. He proposed;



It can be seen from this brief review that only a tentative pathway of nitrogen fixation has emerged. It will become apparent that although a number of possible intermediates appear to have been eliminated, conclusive evidence may only arise from studies on 'nitrogenase', (the enzyme (5) responsible for nitrogen fixation,) in a cell-free state. Once cell-free fixation has been established on a suitable

scale it will be possible to employ model compounds to simulate the stages of reduction. At present there remain two outstanding questions, (a) how is the nitrogen adsorbed initially? and (b) what provides the reducing power for reduction to ammonia or derivatives of ammonia? It may well be that previous work has had only limited success because there has been a tendency to treat nitrogen fixation independently of cell metabolism, whereas in fact a cyclic pathway, similar to that in carbon dioxide fixation, exists.

(III) PREVIOUS STUDIES ON AZOTOBACTER

Azotobacter vinelandii, a gram negative aerobe, is characterised by its ability to fix molecular nitrogen and by its high respiratory activity. A vigorous aerobe, which produces large ovoid cells (c. 2μ diameter) it is able to utilise a variety of 'carbon' substrates (22) (23) and in stagnant cultures may be characterised by a yellow fluorescent pigment (24) (25).

Earlier studies have demonstrated that, under controlled cultural conditions, fixation of 15-20 μg. nitrogen/mg. sucrose can be consistently achieved (26). In large scale fermentation, with an increased carbon source (sucrose 6%), the need for an adequate oxygen supply has been emphasised. Under these conditions a velocity growth constant of 0.26 (mean generation time 3.1 hr) was achieved (27).

In this laboratory Wusteman (28) has devised a method for growing Azotobacter vinelandii under conditions of optimal aeration and obtained mean generation time of 1.7 hr. This equalled the previous best value for Azotobacter (29). With glucose as substrate, the oxygen consumption of resting cells of Azotobacter, has been shown to amount to 86% of the theoretical value for complete combusion (30). Williams and Wilson (31) have reported $Qo_2(N)$ values of over 10,000 with several substrates of the tricarboxylic acid cycle under high

partial pressure of oxygen, 0.5 atm.

In this laboratory Azotobacter vinelandii has been grown by continuous cultures (8) employing a 5% mannitol medium of the usual growth requirements. The latter include iron, molybdenum, magnesium, sodium, cobalt, sulphate, phosphate and chloride (26) and it has been reported that iron, molybdenum (32)and cobalt (33) are specifically required for nitrogen fixation.

The main pathway for the degradation of hexose appears to be the Entner-Doudoroff pathway. Cell-free extract work has established the breakdown of 6-phosphogluconic acid to pyruvate and glyceraldehyde 3-phosphate (34) (35) (36), the latter in part being converted to pyruvic acid and in part being recycled (35). Recent radio-respirometric data has supported this (37),although it was deduced that a small part of the glucose was catabolised by means of the pentose phosphate pathway (37)(38).

Cell-free work has also established the operation of a tricarboxylic acid cycle (39) (40)(41) (42) and recent work (43) has demonstrated the participation of the glyoxylic acid cycle when acetate was substrate.

A INTERMEDIATES IN NITROGEN FIXATION

In the early attempts to establish the pathway of nitrogen fixation, a variety of 'fixed' nitrogen compounds were supplied to Azotobacter. It was assumed that the

supply of an intermediate to a culture fixing nitrogen would result in its immediate and preferential use. Although little consideration was given to permeability and other transport factors, some progress was made. It was observed that ammonium ion was utilised without lag where as both nitrate and nitrite required adaptation (44). It was reported that urea, nitrate and nitrite were utilised via ammonia and it was claimed that ammonia was completely and immediately accepted as the source of nitrogen, to the exclusion of the 'nitrogen fixing' reaction (45) (46). Although this was strong evidence for ammonia as a key intermediate in the fixation of nitrogen, it did not eliminate the possibility that earlier intermediates (cf Figure I) existed and further utilisation experiments with suggested precursors of ammonia were conducted. A brief summary of this work follows.

NITROUS OXIDE (I)

Nitrous oxide, isoteric with carbon dioxide, was found to be a competitive reversible inhibitor of nitrogen fixation (47) (48). Its utilisation by Azobacter is in dispute, since contrary to findings in this laboratory (49) Mozen and Burris (50) observed slight incorporation of $[^{15}N]$ from $[^{15}N]$ labelled nitrous oxide.

HYPONITROUS ACID (II)

This was also investigated in this laboratory. Chaudhary examined the decomposition of hyponitrous acid

over a range of buffers. Although in aqueous solution it decomposed slowly to nitrous oxide, it was found to act as a general poison to cultures of Azotobacter vinelandii (51).

NITRAMIDE (III)

Nitramide was found to decompose rapidly in aqueous solution. When supplied to Azotobacter culture, no uptake of $[^{15}N]$ from $[^{15}N]$ labelled nitramide was detected (52).

HYDROXYLAMINE (IV)

For several years hydroxylamine was the favoured intermediate in nitrogen fixation particularly as it offered an attractive pathway for fixation, via oximes, into cell material (15).

The detection of bound hydroxylamine compounds (53) and the identification of a hydroxylamine reductase(54) in Azotobacter supported this pathway. However work in this laboratory and Wisconsin did not support hydroxylamine as intermediate. Wilson et alia(55) reported that oximes were poorly utilised by Azotobacter cultures as was hydroxylamine. The extreme toxicity of the latter suggested that its failure to be utilised did not result from a failure to penetrate the cell. Pethica (56) found no evidence of hydroxylamine utilisation and reported that hydroxylamine inhibited both nitrogen fixation and respiration in Azotobacter.

Hydroxylamine was finally discarded as an intermediate in nitrogen fixation when Virtanen, its principal

proponent, detected bound hydroxylamine both in Azotobacter cultures fixing nitrogen and those utilising ammonia. This suggested that bound hydroxylamine was produced by the oxidation of ammonia or ammonium derivatives (57).

HYDRAZINE (VI)

Hydrazine, or a bound form of hydrazine has been increasingly favoured as an intermediate between nitrogen and ammonia. Azim has found that hydrazine inhibited both nitrogen fixation and respiration, although in low concentration (2×10^{-5}M), some stimulation of fixation was observed (58).

The principal work on the utilisation of hydrazine by Azotobacter cells appears to have been carried out under two distinct cultural conditions.

Bach (and subsequently Suzuki) investigated the disappearance of hydrazine from resting suspensions of Azotobacter. Using $[^{15}N]$ labelled hydrazine Bach (59) found that the organic azines were the principal products and by chromatographic comparison he deduced that the principal product was P.C.A. (3, 4 dihydropyridazinone 5-carboxylic acid, VII). He also claimed that these azines became more heavily labelled by $[^{15}N]$ when supplied with $[^{15}N]$ labelled nitrogen than with $[^{15}N]$ labelled ammonia. However the results were expressed only as atom % ^{15}N

excesses and not on a quantitative basis.

At that time Diamantis (60) had studied the disappearance of hydrazine from proliferating cultures and found that the hydrazine remained sequestrated in the medium. The hydrazine could be quantitatively recovered by acid hydrolysis of the medium. An accumulation of pyruvic acid was observed in the medium during the sequestration but this could not be related directly to hydrazine disappearance. In subsequent work Harris (61) isolated the picryl derivative of monoacetyl hydrazine and found that the disappearance of hydrazine was competitively inhibited by ammonia (and vice versa).

This was contrary to the findings of Suzuki (62) who found that, in heavy suspensions, hydrazine uptake was inhibited by ammonia in a non-competitive manner. In this work it was observed that the addition of respiratory substrates enhanced the disappearance of hydrazine. It was claimed that in cell-free extracts, when α-ketoglutarate was present, an increase of glutamic acid occurred. The latter claim coupled with the report by Bulen (63), that P.C.A. could be utilised by cell extracts but not by whole cells, would appear strong support for the P.C.A. pathway, but in neither case was detailed experimental evidence published.

Thus the experimental evidence suggests that hydrazine is merely sequestrated to form P.C.A. or an acetyl hydrazine derivative, according to the cultural conditions. In view of the reactivity of hydrazine with keto-compounds this is not surprising, but the salient point with respect to nitrogen fixation is that hydrazine, by direct or indirect means, has not been conclusively shown to be split to ammonia.

AMMONIA

Ammonia has been considered the key intermediate in nitrogen fixation for many years. From isotopic tracer experiments with $\left[{}^{15}N\right]$ a general pathway for the incorporation of nitrogen into cell materials has been proposed for the range of bacterial nitrogen 'fixers' (5).

$$^{15}N_2 \longrightarrow {}^{15}NH_3 \xrightarrow{\alpha\text{-ketoglutarate}} \text{Glutamic acid} \longrightarrow$$

This equation implies that a similar mechanism is operative over the different classes of 'fixers' and certainly presents the experimental evidence in an extremely simplified form. It must be remembered that this pathway has not yet been established on a completely quantitative basis since this would mean that both atoms of nitrogen are simultaneously converted to ammonia. It is possible that the nitrogen is split in a symmetrical manner to ammonia but certainly with Azotobacter the evidence at present does not substantiate this. There is strong support

for ammonia as the sole initial product of the fixing reaction from work on cell-free extracts of Clostridium, but the evidence does not rule out the possibility that the actual products of fixation of one molecule of nitrogen is ammonia and an alkaline labile compound. This work will be discussed at a later stage but it is important to note this possibility since a false conclusion is likely to mask the mechanism by which nitrogen is fixed, i.e. whether nitrogen is adsorbed or reduced in a symmetrical or unsymmetrical manner.

The evidence in support of ammonia as the important intermediate in nitrogen fixation is considered below.

(i) Whole cells of Azotobacter have been reported to utilise ammonia without lag (16) (44) (46) although Azim did detect a lag period in his studies an ammonia utilisation (64). Cell-free extracts have been shown to incorporate ammonia into amino acids (65). The latter, of course, merely demonstrates that a system capable of immediate ammonia utilisation is present.

It has been claimed that ammonium ion, (and compounds that are readily converted to ammonia,) are utilised to the virtual exclusion of molecular nitrogen (45) (46). These claims, which relate to a particular cultural condition and specific ammonium concentrations, would have been more acceptable if the results had been

had been presented on a quantitative basis rather than in terms of atom % ^{15}N excess.

The point will be enlarged upon in Section VII, Discussion.

In heavy suspensions, Newton et alia (66) have reported that nitrogen fixation and ammonia utilisation both occur. By exposing the culture to $[^{15}N_2]$ inhibition of fixation by ammonium ion was observed and calculations do show that under these particular conditions, the ammonium ion concentration was equivalent to about 20% of the total nitrogen of the culture. This is in contrast to the reported complete inhibition of fixation by ammonia in proliferating cultures in which the added ammonia concentration was about 6% of the total nitrogen of the culture (46). It is of course questionable whether 20% represents a high or low concentration of ammonia and whether Kjeldahl nitrogen is the most satisfactory reference for ammonia concentration.

The other principal finding of Newton et alia was that $[^{15}N]$ 'rapidly' appeared in the 'ammonium pool' when cell fixed $[^{15}N_2]$ in the presence of added ammoniumion and this was taken as strong support for ammonia as intermediate in nitrogen fixation.

Wusteman has been particularly critical of the

earlier work of Burris and Wilson, relating to the claim for the complete inhibition of fixation by ammonia(46). In his studies on the uptake of ammonia and other nitrogen compounds under conditions of optimal aeration, conditions which earlier work had tended to neglect, he found that fixation of atmospheric nitrogen and ammonia uptake occurred simultaneously (28). He reported the need for adequate provision to eliminate losses from aspiration when well aerated cultures were grown on ammonium acetate and deduced that the rate of uptake of nitrogen, from fixation and ammoniumion utilisation, greatly exceeded that of a culture growly solely on gaseous nitrogen.

Wusteman has pointed out (67) that Wilson and Burris had deduced complete inhibition from calculation based solely on growth rates of nitrogen assimilating cultures. They found that the calculated atom % ^{15}N excess, assuming that the increase in nitrogen (extrapolated from the data on nitrogen assimilation) was entirely labelled ammonia, corresponded to the experimental value. If in fact a higher rate of nitrogen uptake occured(as was found by Wusteman) then the apparent theoretical result could be explained on the basis that the enhanced $\left[^{15}N\right]$ uptake is countered (diluted) by fixation of atmospheric nitrogen. It should also be pointed out that this type of experiment is not suitable for assessing nitrogen fixation. In view of the large amount of total nitrogen(principally

$[^{14}N]$)present and the long exposure times it is not feasible to assess with any accuracy the amount of $[^{14}N]$fixed from calculation based on atom % ^{15}N excess. The calculation of results employing the $[^{15}N]$technique will receive attention at a later stage and in this respect further comment will be made on the work of Newton et alia.

(ii) Allison and Burris (68) in their short term studies on the incorporation of $^{15}N_2$ into Azotobacter cultures did attempt some quantitative assessment. It was found that the 'amide' fraction, a fraction obtained by the acid hydrolysis of the cells, contained 100% of the ^{15}N fixed (quantitative basis) after one minute exposure. These results suggested that ammonia was the product of fixation since, in the words of Allison and Burris, "when the percentage of the total $[^{15}N]$found in each fraction is plotted against time, the 'amide N' of the cell and ammonia N of the medium exhibit negative slopes characteristic of primary products of fixation". It must be pointed out that the amount of $[^{15}N]$in ammonia was only 20% of the total $[^{15}N]$present and it is clear that the particular method of liberating the ammonia, with respect to other labile amino compounds, may be an important factor. It was noted that after 5 minutes the medium contained a high quantity of $[^{15}N]$(40% on a quantitative basis) and that the glutamic acid contained a relatively high label (2.66 atom% ^{15}N excess) compared with ammonia (0.437 atom%

^{15}N excess) which increased with time. It has been suggested that glutamic acid is thus a precursor of ammonia in nitrogen fixation but the reverse remained more probable since the lower atom % $[^{15}N]$ excess in ammonia could result from dilution by intracellular ammonia during the isolation procedure.

This $[^{15}N]$ work does raise the question of the significance of atom % ^{15}N excesses. Wilson and Burris consider that a high concentration of ^{15}N (atom % ^{15}N excess) indicates the proximity in the reaction chain to the $[^{15}N]$ enriched compound supplied (16). A consideration of atom % ^{15}N excess alone is in most cases unsatisfactory, particularly if the atom % ^{15}N excess of the derivative is low in comparison to the source. In such cases it is impossible, solely from consideration of atom% ^{15}N excesses, to distinguish between primary products, secondary products and metobolic 'dead ends' since their rate of formation and decomposition and dilution by the isolation procedure are not usually known. It is advisable to refer to a quantitative basis.

(iii) Burma and Burris (69) carried out similar experiments to those of Allison and Burris with $[^{15}N]$ labelled ammonia. They reported a similar pattern of labelling, although the atom % ^{15}N excesses in purines were higher, but in the absence of a quantitative approach further conclusion is difficult.

(iv) A similar criticism can be applied to their work on transamination in extracts of Azotobacter with labelled ammonia in the presence of α-ketoglutarate (65). Although atom%. ^{15}N excesses in a number of acids have been given, further conclusion is not possible without reference to the number of nitrogen atoms in the particular amino acid and its total nitrogen value.

(v) Nicholas (70), in a review article, which summarised a lecture given to the Society of General Microbiologists, put forward strong evidence in support of ammonia being the initial product of fixation in Azotobacter. He reported that the ammonia, obtained (by a Conway Microdiffusion procedure) from a cell-free extract that had been exposed to $[^{13}N]$, contained a major part of the activity of the extract. However these values had not been given in his earlier paper on $[^{13}N]$ experiments (71) and have not been elaborated on since.

It is evident that the present experimental evidence does suggest that ammonia occupies an important place in nitrogen fixation, but its precise role has yet to be determined. In much of the previous $[^{15}N]$ work there has been an unfortunate tendency to place too much emphasis on atom% ^{15}N excesses alone, whereas they should be considered in their proper context and in most cases be converted to quantitative results. It is evident that successful cell-free work is required to establish the

role of ammonia and although much comparison has been made to the work on Clostridium it is advisable not to extend such comparisons further than the experimental evidence warrants.

B ROLE OF HYDROGENASE

Inhibition of nitrogen fixation in Azotobacter has been reported on a number of occasions, by orthophosphite (72), by fluoroacetate (73), by chloramphenicol (74), by trimethylamine oxide (75), by azide and by cyanate (76). The inhibition by the latter, by azide and cyanate, is of particular interest from steric consideration, since it has been suggested that inhibition by nitrous oxide and related molecules resulted from the blocking of the site of fixation, i.e. 'steric fit' (49). Azide and cyanate, like carbon dioxide, are isoteric with nitrous oxide and in Table II a comparison of their bond lengths appear.

TABLE II

	Formula	(N-N)s Å	(N-X)s Å	
NITROGEN	N_2	1.10		Linear
NITROUS OXIDE	N_2O	1.12	1.19	"
AZIDE	$N_3^{\ominus}$	1.15	1.15	"
CYANATE	$CN^{\ominus}$	(1.16)	1.16	"
CARBON DIOXIDE	CO_2	(1.16)		

However azide resembled nitrous oxide, since it was a more powerful competitive inhibitor whereas cyanate was found to inhibit nitrogen fixation weakly in a non-competitive manner. This suggested that other factors beside steric oneswere important.

The possibility that the enzyme hydrogenase, present in many nitrogen fixing bacteria, was the site of fixation has received serious consideration. Winfield (77) from chemical studies attempted to explain the mode of action of both nitrogenase and hydrogenase on the same site.

This view, that hydrogenase and nitrogenase are identical, has been criticised on the following grounds (78).
(a) Other organisms (e.g. Escherichia Coli) unable to fix nitrogen, contained hydrogenase.
(b) the hydrogenase activity of Azotobacter and Clostridium was equally active with molybdenum or with vanadium, whereas nitrogen fixation was inhibited by vanadium.
(c) a deficiency of iron caused inhibition of both nitrogen fixation and hydrogenase activity, whereas a molybdenum deficiency reduced nitrogen fixation but not hydrogenase activity.

The metal requirements of Azotobacter will be discussed later but it should be noted that the evidence on the possible replacement of molybdenum by vanadium is somewhat

ambiguous. In any discussion it is advisable to be specific about hydrogenase activity since this has been assayed by three principal methods, whose results are not easily correlated.

Method of assay

(a) reduction of 'two electron' dyes i.e. methylene blue (79).

(b) evolution of hydrogen from reduced viologen (Method of Gest) (80).

(c) isotopic exchange of hydrogen gas with deuterium labelled water (81).

The hydrogenase of Clostridium can be assayed by all three methods whereas that of Azotobacter can only be assayed conveniently by the 'methylene blue' method or with potassium ferricyanide as acceptor (82). Although it is difficult to correlate results from these methods for the same organism, it has been reported that some correlation of results from (b) or (c) is often observed (83). It might be concluded from these differences in the methods of assay that the hydrogenase enzyme is not identical in different organisms. However one might attribute these differences to variations of the 'potential' at the site in different organisms (aerobic v anaerobic), difference in potential of acceptors, difference of pH optima and different

metal requirements. For example, it has been reported that the purified hydrogenase from Desulfovibrio desulfuricans has a pH optimum of 5.5 for the exchange method and pH optimum of 8.4 for methylene blue reduction (84). There has already been some controversy as to whether hydrogen is split homolytically or heterolytically(83) (85) and it is clear that more investigation is needed on possible sites of hydrogenase activity. It is feasible that the site of hydrogenase activity is basically the same but that the above mentioned factors determine the actual mechanism. The evidence in support of a close association between hydrogenase activity and nitrogen fixation, is largely circumstantial.

(i) When Azotobacter cells are grown on ammonia or nitrate they exhibit a lower hydrogenase activity than when assimilating gaseous nitrogen (86).

(ii) Mutants of Azotobacter, which are not able to fix atmospheric nitrogen, have a less active hydrogenase (87).

Evidence from other organisms includes:

(iii)In the present of trichloromethyl sulphenyl benzoate, the growth rate of Clostridium was decreased more when growing on nitrogen than when growing on ammonia (88). It was observed that both nitrogenase and hydrogenase activity was reduced and that this inhibition could be reversed, in both cases, by the addition of sodium molybdate.

(iv) The existence of a hydrogenase system in symbiotic nitrogen fixation has been reported. When soy bean nodules were exposed to hydrogen a spectral shift was observed and exchange studies demonstrated the evolution of hydrogen (89). Further work with D_2 has confirmed this and it was found that hydrogen evolution was inhibited by nitrogen and nitrous oxide (90).

(v) The photoproduction of hydrogen by organisms of the Athiorhodaceae and Thiorhodaceae has been found to be inhibited by nitrogen or ammonia (91).

(vi) Cell-free extracts from Clostridium pasteurianum that are able to fix nitrogen, contain hydrogenase. However it has been reported that the role of hydrogenase in these extracts is restricted to hydrogen donation (6).

C. ROLE OF MOLYBDENUM AND OTHER METAL REQUIREMENTS

It has been shown that when fixing nitrogen, Azotobacter (and Clostridium) require extra molybdenum and iron (32) (92) (93). The role of molybdenum in nitrogen fixation remains obscure. Molybdenum has been shown to be a constituent of the assimilatory nitrate reductase of Neurospora Crassa (94) and it is possible that it is also the metal constituent of the inducible assimilatory nitrate reductase of Azotobacter (95). It has been reported that the hydrogenase of Clostridium pasteurianum is a molydoflavoprotein (96) (97), although it must be noted that molybdenum has not been

found in some purified hydrogenase preparations (98). When $[Mo^{99}]$ was supplied to a proliferating culture of Azotobacter vinelandii the majority of $[Mo^{99}]$ was found in a particulate fraction (99) (100). The ability of vanadium to replace molybdenum with respect to nitrogen fixation appears to vary with strain (101). The most recently published work, contrary to earlier work (95), reports that vanadium could effectively replace molybdenum in 19 out of the 20 strains of Azotobacter vinelandii examined (102).

Molybdenum cannot be replaced by tungstate although the latter was found to inhibit, competively, the uptake of $[Mo^{99}]$ (103) (104).

From studies employing $[Mo^{99}]$ and $[Si^{31}]$ it was deduced that a silico-molybdate complex was not involved in nitrogen fixation (105). Electron paramagnetic resonance spectroscopy (EPR) has been used in studies on extracts of Azotobacter (106). These studies have indicated that Mo^{5+}, Fe^{2+} (principally reduced non-haem iron) and some Fe^{3+}, and flavin semiquinones, were present. The signal associated with Mo^{5+} ($g = 1.97$), was observed in particles obtained by disrupting cells assimilating gaseous nitrogen in their culture medium. This well-defined signal was not found with cells grown on ammonia. The reduced iron (non haem) was associated with the signal of $g = 1.94$ and in recent studies this has been

confirmed (107). A fraction from butanol extraction of Azotobacter has been found to contain an iron protein. Although it was reported that the supernatant contained an unusual flavoprotein, the protein did not contain significant amounts of either haem or flavin.

Iron is now considered to be the main functional metal of hydrogenase (78) (98) (108). It has been reported that a purified preparation of hydrogenase, from Desulfovibrio desulfuricans contained iron but no molybdenum when examined by Xray Fluorescence spectroscopy (98). The most recent work on the hydrogenase from this organism suggested that ferrous iron is the important oxidation state (109). A requirement for calcium in nitrogen fixation was originally proposed but subsequent work suggested that this was more directly related to the synthesis of polymetaphosphate (32) (92). In this respect there has been one report that strontium and barium can replace calcium (110) but these studies have not appeared in detail. Nicholas has reported a cobalt or vitamin B_{12} requirement for nitrogen fixation. These were also found to be requirements in nitrate utilisation in Azotobacter and other microorganisms (33).

Gaseous inhibition.

The reports that hydrogen was an inhibitor of nitrogen fixation in a number of micro-organisms have been

cited as evidence for hydrogenase and nitrogenase as being identical (5). However recent work on Azotobacter has cast doubts on the role of hydrogen as an inhibitor to fixation. Parker (111) has found that under carefully controlled conditions hydrogen is not a competitive inhibitor of fixation. The results underlined the need to ensure adequate aeration and indicated that oxygen uptake is not a constant function of cell nitrogen under all conditions. In his earlier work (29) he had demonstrated that oxygen was a competitive inhibitor of nitrogen fixation. Inhibition of fixation by oxygen had already been indicated by the high Michaelis constant, reported by Wilson and Roberts (112), when measured at a higher oxygen pressure p^{O}_{2} = 0.3 atm. Parker observed that respiration reached a maximum of between 0.15 to 0.3 atm of oxygen whereas fixation was appreciably lower at 0.5 atm (113). He favoured the idea that respiration and nitrogen fixation could be considered as alternative pathways of oxidation. Dilworth subsequently reported that under high oxygen pressure an accumulation of pyruvic acid and α-ketoglutarate occurred (114). This is similar to the observation of Diamantis (60) that an accumulation of pyruvic acid in proliferating cultures occurred in the presence of hydrazine and may reflect the sensitivity of a pyruvic oxidase system in Azotobacter. In a recent communication Dilworth and Kennedy have

investigated the oxygen sensitivity in extracts containing enzymes concerned in acetate metabolism (43). This approach may prove to be extremely useful as it attempts to throw light on the oxygen requirement and inhibitors of nitrogen fixation in cell-free extracts.

D ENZYME LOCALISATION: LOCALISATION OF HYDROGENASE

Early studies (82) (86) on the hydrogenase of Azotobacter vinelandii had shown that it was essentially particulate and subsequent work has not improved, to any great extent, its 'solubilisation' (SECTION VII DISCUSSION). In their studies on the location of enzymes in Azotobacter Marr et alia(115) investigated the disruption of Azotobacter by ultrasonic disruption and osmotic shock methods. They observed that, during ultrasonic disruption, both cell count and turbidity decreased exponentially, although the latter only decreased 40% as rapidly as the former. The turbidity in excess of that due to remaining whole cells was attributed to large fragments of cells and in subsequent brief ultrasonic disruption the presence of hulls, cells emptied of cytoplasm, was reported. Since it was observed that the rate of release of glucose 6-phosphate dehydrogenase and RNA were identical with the rate of disruption, whereas the rate of release of hydrogenase, cytochrome and phospholipid was consistent with the rate of decreased turbidity, it was proposed that the former (glucose 6-phosphate

dehydrogenase, RNA) were originally present in the cytoplasm and that the latter (hydrogenase, cytochrome and phospholipid) were orignally present in the cell envelope. Electron microscropic studies (116) of the particulate fraction did indicate that it was not homogeneous but consisted of two particle sizes -

(i) RNA protein particles (from cytoplasm)

(ii) Irregular particles (from fragmentation of hull)

When the bacteria were disrupted by osmotic shock it was found that the respiratory enzymes and other characteristic components of the cell envelope (hydrogenase etc) were not found in small particles to any great extent but were located almost completely in the larger particles.

The most recent communications from these workers suggested that the intra-cytoplasmic membranes are the most probable locus of the respiratory enzymes of Azotobacter cells (117),(118).

However further localisation of hydrogenase is unlikely until further refinements of technique are developed in the disruption and examination of cell envelopes. This is particularly necessary with gram negative bacteria since it has been found that the cell wall structure of these is not so well defined as those in gram positive micro-organisms (119). Wusteman (28) summarised the distribution of enzymes in Azotobacter and this is reproduced

with slight modifications. (TABLE III).

TABLE III

ENVELOPE	CYTOPLASM
PART OF CITRIC ACID CYCLE Malic and Succinic dehydrogenases (120)	BULK OF CITRIC ACID CYCLE ENZYMES Aconitase, α-ketoglutaric oxidase (121) isocitric dehydrogenase (120)
PHOSPHOLIPIDS (116)	NUCLEOTIDE ENZYMES Adenosine deaminase (124)
AMINO ACID INCORPORATING SYSTEM (122)	CATALASE (124)
ELECTRON TRANSPORT AND OXIDATIVE PHOSPHORYLATING SYSTEMS DPNH oxidase, TPNH oxidase (119)	PHOSPHATASE (123) (124)
CYTOCHROMES	RNA protein particles (116)
HYDROGENASE (115)(116)	POLYMETAPHOSPHATE GRANULES(124)
(NITROGEN FIXING SYSTEM?)	
Fe, Mo ?	

The hydrogenase of Azotobacter has not been purified to any great extent and, unlike that of Desulfovibrio desulfuricans (98) has not been separated from the cytochrome components.

A number of publications have appeared on the cytochrome system of Azotobacter. The isolation and characterisation of two cytochromes, denoted c_4 and c_5 ,

was reported (125) (126) (127). It was reported that these only reacted with the cytochrome oxidase from Azotobacter and, unlike that reported by Wilson (128), resembled mammalian cytochrome c. In this respect Layne and Nason (129) have reported the preparation of a soluble cytochrome oxidase capable to oxidising reduced mammalian cytochrome c although at a somewhat slower rate than the reduced cytochrome c_4 and c_5.

The present of flavin, haem iron, non-haem iron and copper in ratio of 1:5:16:2 has been reported in a particulate system from Azotobacter (130). This was designated the 'electron transport particle' and catalysed the oxidation of succinate and DPNH.

Oxidative phosphorylation by washed particulate preparations from Azotobacter vinelandii has been studied by a number of workers. There appeared little correlation between the P/O ratios with respect to different substrates (DPNH, malate and succinate) but the presence of the culture medium (131) (132) or suitable electrolyte (133) (134) particularly Mg^{2+}, (and ATP . (133) (135)) appeared necessary.

In passing it is of interest to note that a requirement for a "medium factor" has been reported in other studies. Nicholas (136) has reported that a medium factor was necessary for cell-free fixation and a similar require-

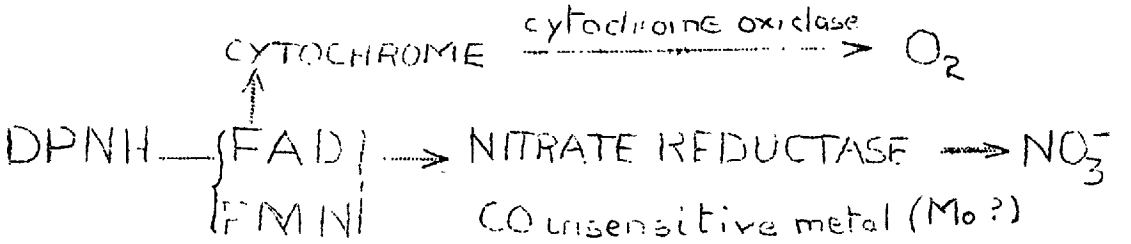
ment has been reported for the incorporation of ^{32}P into RNA in extracts of Azotobacter (137).

In oxidative phosphorylation it has been suggested that a necessary factor may be bound to a polynucleotide complex and that deactivation is a result of the dissociation of this complex on lowering the cation concentration (138). It was reported (131) that washed small particles (sedimented between 22,000g,30 min and 145,000g,60 min) have higher respiratory and $^{P}/_{O}$ ratios, with both succinate and DPNH as substrates, than large particles in extracts prepared by grinding with glass powder and ultrasonic disruption. However $^{P}/_{O}$ ratios remained small and variable and have not been visibly improved by altering the method of disruption; Ball mill, ultrasonic, French cell and grinding with alumina were among the methods tried (139) (140). Recently (141), there has been a suggestion that coenzyme Q, previously found in Azotobacter (142), was involved in the electron transport system and that flavin,haem iron, non-haem iron (in the ratio of 1:6:10) were present in these extracts.

An electron transfer sequence from DPNH for the particulate nitrate reductase of Azotobacter has been proposed (143). It has been mentioned earlier that the inducible assimilatory nitrate reductase of Azotobacter (143) (144) strongly resembled the nitrate reductase of

Neurospora crassa (95).

The latter has been shown to be a sulphydryl molybdoflavoprotein, the role of the sulphydryl being confined to holding DPNH in position (95). Although the metal constituent of the nitrate reductase from Azotobacter has not been characterised, the chief difference between the two is that the nitrate reductase from Neurospora is soluble whereas the nitrate reductase from Azotobacter, unlike its nitrate and hydroxylamine reductase (54), is particulate. Taniguchi (143) has reported that the nitrate reductase in Azotobacter resided in the large particles but only small amounts of DPNH hydroxylamine reductase and no DPNH nitrate reductase was detected in small particles. He proposed an electron transport sequence;



The particulate nature of the inducible nitrate reductase, the hydrogenase, cytochromes and oxidative phosphorylating systems gives an indication of the difficulties inherent in the preparation of cell-free extracts of particular enzymes. It is probable that nitrogen fixation is intimately connected with at least one of these systems and

that it would be more advantageous to extend studies in this direction rather than seek a separate enzyme system for nitrogen fixation.

One recalls the claim of Cheniae (145) that a relationship existed between nitrogen fixation and nitrate reductase in soybeans. Although there is no evidence that nitrate or similar oxidised nitrogen compounds are intermediates in nitrogen fixation it does not eliminate the possibility that nitrogen fixation and nitrate assimilation may occur at an essentially similar site. One recalls that nitrate requires adaptation and that the hydrogenase activity of Azotobacter cells growing on nitrate is lower than those assimilating gaseous nitrogen (87).

It would be helpful to understand 'adaptation' more fully since it may result from modification of enzyme system, the production of new enzyme systems or necessary cofactors. In this respect the adaptation of Azotobacter agile grown on sucrose to succinate and malate has been shown to be inhibited if the cells were washed in distilled water (146). A factor in the washings, subsequently isolated and identified as glucose or readily metabolised glucose polymer, when added back restored the original lag period.

Some thought must also be given to the reverse of nitrogen fixation, denitrification. Delwiche has

reported (147) that nitrogen and nitrous oxide produced by the reduction of labelled nitrate by Pseudomonas denitrificans contained only one labelled nitrogen. Pratt (20) has taken this as evidence in support of the heterolytic reduction of nitrogen.

It is evident that it is not possible to make any realistic assessment of the role of particular enzymes systems in nitrogen fixation at the present time. It is evident that further investigation on cell-free extracts closely linked to studies on nitrogen fixation should be made. Some advances in this direction have been made in extracts of Clostridium pasteurianum and in the following section the relevant work on cell-free nitrogen fixation in extracts of Clostridium pasterianum and Azotobacter vinelandii has been summarised.

(IV) NITROGEN FIXATION IN CELL-FREE EXTRACTS

A CLOSTRIDIUM PASTEURIANUM

Over the past few years much effort has been directed to the study of cell-free extracts from Clostridium. It is useful to summarise the salient features of this work for subsequent comparison to the work on Azotobacter. Several review articles have appeared on this topic and may be referred to for more detailed discussion (5) (6) (148)(149).

1. Method of disruption

The methods of disruption employed in the preparation of "cell-free fixing" extracts, included autolysis of dried cells, ultrasonic disruption and the Hughes press method. The autolysis method was favoured since it enabled large amounts (2-3 litres) of extract to be prepared. In this method wet cells were centrifuged down, washed and dried rapidly under vacuum without freezing. The subsequent extraction of enzyme was performed under strictly anaerbic conditions (under hydrogen) (150)(151).

2. It was reported that the nitrogen fixing system of dried cells was stable for periods over two months, provided that they were stored at $-15^{\circ}C$ in the absence of moisture(151)

3. Disrupted preparations contained from 2 to 5% of the original population of whole cells. After centrifugation at 144,000g for 90 minutes, no whole cells were detected(151).

4. Added pyruvate was essential for fixing activity. A

high ratio (100:1) of pyruvate utilised to nitrogen fixed was observed (152) and it was assumed that pyruvate provided the electronsnecessary for the reduction of nitrogen. The phosphoclastic reaction, the conversion of pyruvate to acetyl phosphate was found to be operative (153) and the following cofactors were essential (5) Fe^{2+}, Mg^{2+} (Mn^{2+}), thiamine, pyrophosphate, inorganic phosphate and CoenA. [A method for demonstrating cofactor requirements in nitrogen fixation has been published recently (286).]

5. It was reported that the optimum conditions for cell-free fixation were obtained at 0.015M phosphate and pH 6.3-6.5. An optimum ratio of 5:3 of pyruvate:protein was claimed (151).

6. Nitrogen fixing extracts were inactivated by exposure to air, although some protection from deactivation by oxygen was obtained on addition of sulphydryl compounds. On standing at $0^{o}C$ for 24 hrs 50% of the nitrogen fixing activity was lost (154).

7. Cell-free fixing extracts were tested for hydrazine, hydroxylamine and nitrite but no evidence in support of these compounds as intermediates in nitrogen fixation was found. No evidence for P.C.A. as intermediate was found (151).

8. Fractionation of extract. The nitrogen fixing system was separated into two components, a hydrogen donating system 'HDS', and a nitrogen activating system, 'NAS'.(6)

cf. Figure 2.

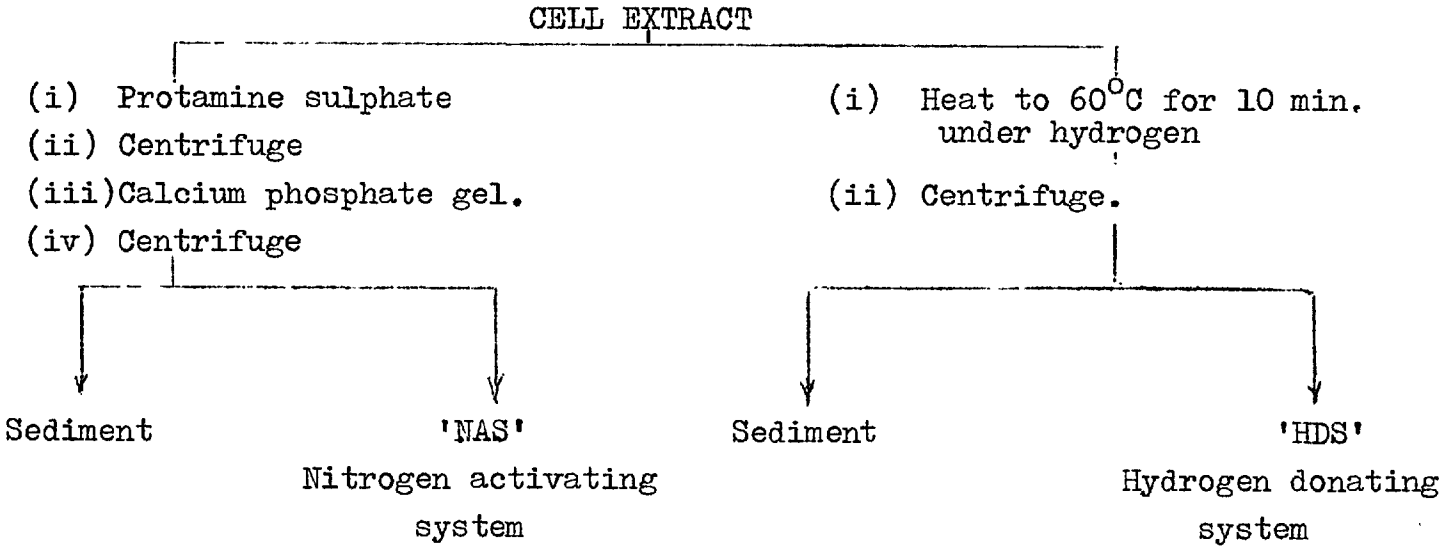

CELL EXTRACT
(i) Protamine sulphate
(ii) Centrifuge
(iii) Calcium phosphate gel.
(iv) Centrifuge
(i) Heat to 60°C for 10 min. under hydrogen
(ii) Centrifuge.
Sediment
'NAS'
Nitrogen activating system
Sediment
'HDS'
Hydrogen donating system

Figure 2

These were not separate fractions, but fractions obtained by different experimental procedures and which together gave a 'fixing' extract. In fact they contained several enzymes in common (148). The 'HDS' contained about 95% of the hydrogenase activity (assayed by methylene blue method) and most of the pyruvic dehydrogenase. The 'NAS' contained virtually no hydrogenase activity and the evidence in support of nitrogen activating is given below.

(i) In the original paper it was reported that the 'NAS' showed an increased absorption around 300 mμ under nitrogen. In freshly prepared extracts conditioned with sodium pyruvate or sodium α-ketobutyrate, when exposed to nitrogen an increase in absorption at 365 mμ was observed. In the absence of added pyruvate and α-ketoglutarate the nitrogen-induced spectral change was located around 300 mμ (of 'NAS') but was erratic in occurrence (151).

It must be noted that similar spectral changes had been previously reported (155). Shug observed changes in optical density on exposure of extracts of Clostridium to hydrogen and subsequently to nitrogen, which he attributed to the oxidation of a flavin by nitrogen. This shift could not be attributed to oxygen, but the extracts did not fix nitrogen.

Recent reviews have not placed much emphasis on the similar spectral responses in the extract and 'NAS' but

have supported the designation of 'NAS' on the following grounds.

(ii) Extracts, prepared from cells grown on ammonia, did not yield nitrogen fixing extracts by the above procedures (6) (156). Cross combination experiments showed that the 'NAS' fraction from ammonium grown cells was inactive, whereas combination of the 'HDS'. from ammonium grown cells with the 'NAS'. from nitrogen grown cells resulted in an active extract (although of reduced activity).

(iii)CO, an inhibitor of fixation,was found to inhibit the 'HDS'. reversibly. Although it was reported that pyruvic dehydrogenase was not affected by CO it was suggested that as well as the rapid reversible inhibition of hydrogenase, there was also inhibition of a component transferring electrons to nitrogenase. The 'NAS' was inhibited in an irreversible manner, 50% deactivation occurred after 15 minutes exposure and complete deactivation after 6 hrs exposure (6) (157).

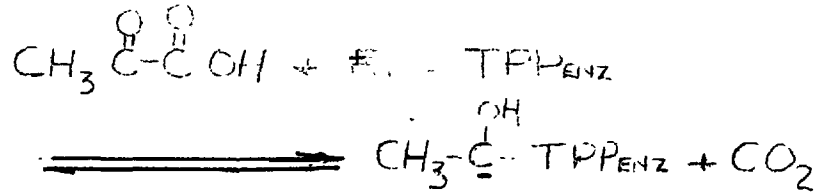
9. Ferredoxin, an electron carrier containing non-haem iron and able to couple pyruvate oxidation to hydrogenase has been isolated (153)(158). It was found to be essential to nitrogen fixation (159)(160) and to show some resemblence to a photosynthetic pyridine nucleotide reductase (161) isolated from spinach. This PPNR was found to transfer electrons from light-activated chloroplasts to NADP and could replace ferredoxin in the pyruvic dehydrogenase

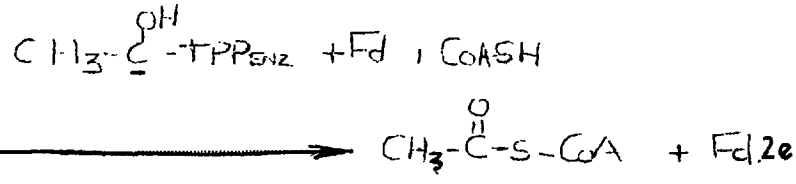
system Clostridium pasteurianum (153).

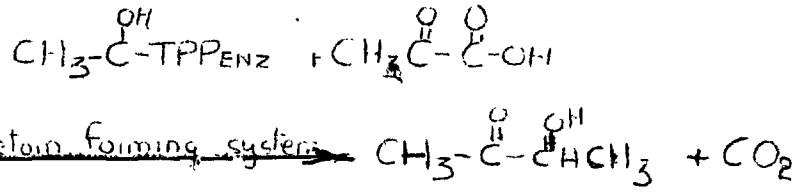
It has been reported that crystalline ferredoxin has 7 moles of iron and inorganic sulphide per mole of protein and that the seven iron atoms exist in two structurally non-equivalent environments, with at least two ferric atoms being present (162). A model for the active site of Ferredoxin has been proposed (163).

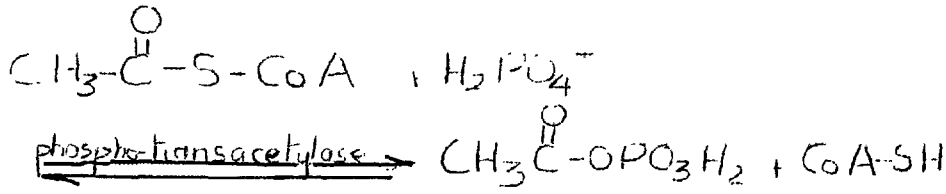
Although a cobalt requirement for nitrogen fixation, nitrite and hydroxylamine reductases in Clostridium pasteurianum has been reported (164), it apparently was not associated with Ferredoxin.

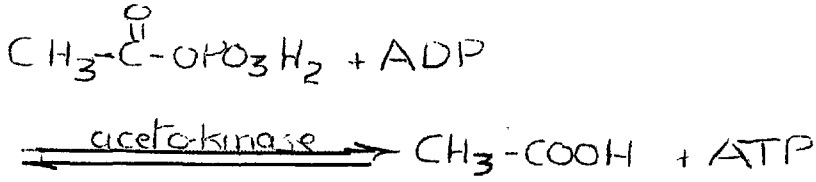
The following sequence of reactions has been proposed for the metabolism of pyruvate and the role of Ferredoxin in extracts of Clostridium (148)

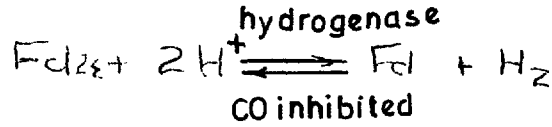












TPP = Thiomine pyrophosphate

Fd = Ferredoxin.

Methyl viologen was able to replace ferredoxin in some of its electron transfer reactions and ferredoxin has been found to effectively replace viologen in the assay of hydrogenase by the method of Gest (evolution of hydrogen from reduced viologen) (165). Ferredoxin has also been found to be required in the following reactions,

Fd = Ferredoxin

$NO_2^{\ominus}$ + 'Hase' + $3H_2$ + (Fe^{3+}) $\xrightarrow{Fd}$ $NH_4^{\oplus}$ (158)

NH_2OH + 'Hase' + H_2 $\xrightarrow{Fd}$ $NH_4^{\oplus}$ (166)

NADP + 'Hase' + H_2 $\xrightarrow{Fd}$ NADPH + $H^{\oplus}$ (167)

RIBOFLAVIN + 'Hase' + H_2 $\xrightarrow{Fd}$ reduced riboflavin (147)

FMN + 'Hase' + H_2 $\xrightarrow{Fd}$ $FMNH_2$ (148)

FAD + 'Hase' + H_2 $\xrightarrow{Fd}$ $FADH_2$ (148)

VANADATE + 'Hase' + H_2 $\xrightarrow{Fd}$ Vanadyl (148).

The most recent communication on ferredoxin reports its role as a reductant in pyruvate synthesis in a bacterial extract (168).

10. It was reported that nitrogen fixation in extracts was inhibited by high concentrations of ATP and acetyl phosphate (151). There was, however, some evidence that high energy phosphate was a requirement in nitrogen fixation.

McNary and Burris (169) reported that arsenate inhibited nitrogen fixation and suggested that this resulted from the prevention of acetyl phosphate formation and thus disrupting the generation of ATP. This was supported by the observation that glucose, in the presence of hexokinase, also caused inhibition in nitrogen fixation, presumably by depleting the ATP pool.

The possibility that the role of pyruvate in fixation was also to provide a continuous source of ATP has been considered (148) (159) (170) (171). It has been reported

that pyruvate may be replaced by an ATP generator and a reducing agent (159) (171) (172). Several workers have reported that hydrogen is capable of acting as the reducing agent (159)(172). Mortenson (159) has reported that 15 - 20 μmoles of ATP were required for each μ mole of ammonia synthesised. He suggested that ATP was required to activate nitrogen and overleaf is reproduced a recent scheme on the role of pyruvate in nitrogen fixation, Figure.3.

A further paper on the ATP requirement in nitrogen fixing extracts has appeared (287).

DUAL ROLE OF PYRUVATE IN BIOLOGICAL NITROGEN FIXATION (173)

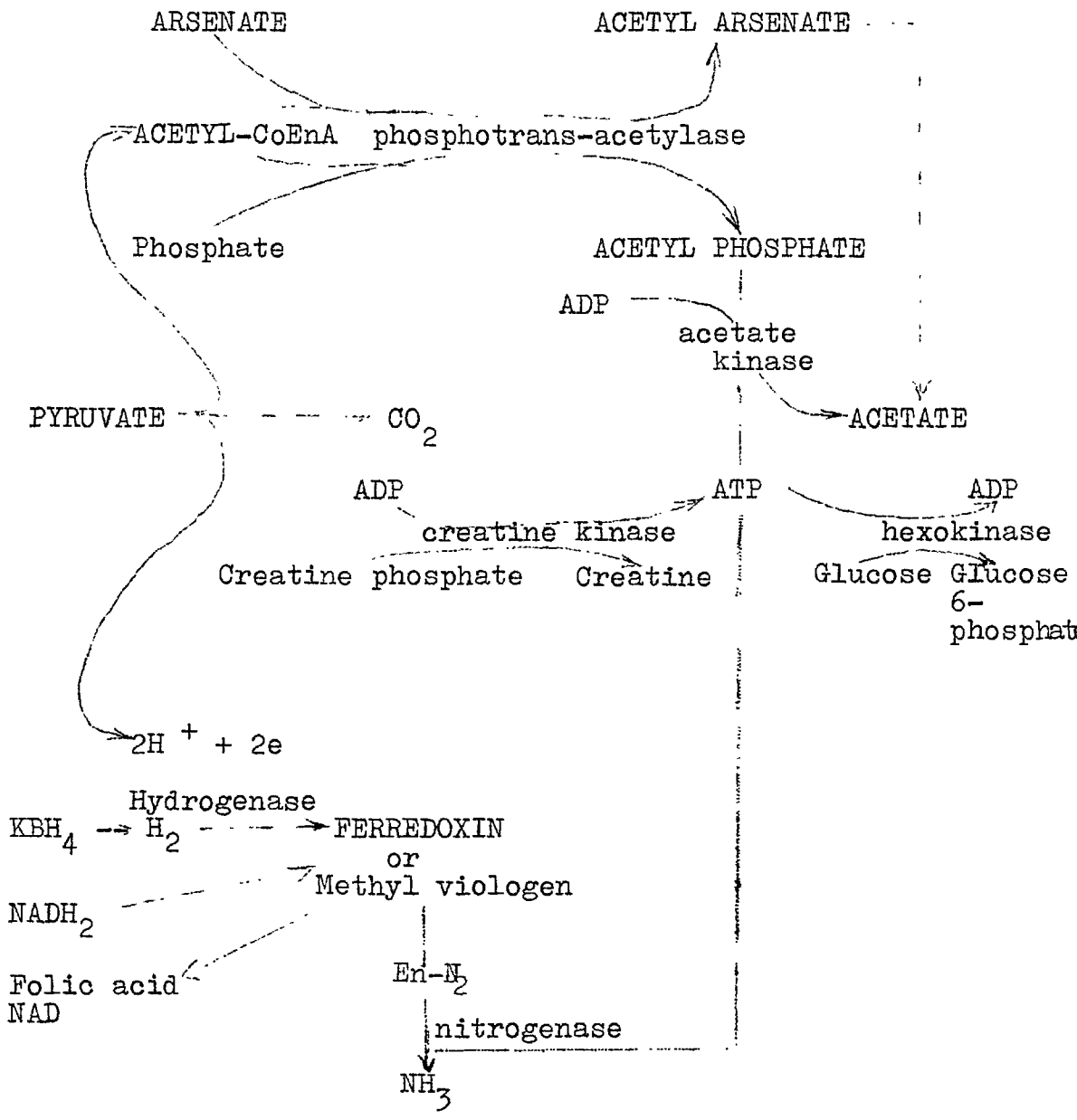


Figure 3

11. From the work on Clostridium it was concluded that ammonia was the first free product of the 'fixing' reaction. The principal support for this conclusion came from the high atom % ^{15}N excess (52 atom % ^{15}N) found in ammonia isolated from cell-free fixing extracts that had been exposed to $^{15}N_2$ (174) and from quantitative measurements. After the exposure it was reported that only 4% of the fixed nitrogen could be recovered as ammonia, whereas in the presence of an ammonium pool over 90% of fixed nitrogen in the form of ammonia (from alkaline distillation) was recovered (6)(150). The concentration of the ammonium pool (glutamine was equally effective) was high (120 μmoles) particularly when one remembers the earlier reports of inhibition of fixation in Azotobacter by ammonia (45)(46). In cell-free extracts, in which 15-20 mg protein N plus sodium pyruvate (100 mg) and buffer to 8 ml were exposed to 0.5 atm of 60 atom% ^{15}N nitrogen, the following inhibition was reported TABLE IV, (TABLE XII, (151)).

TABLE IV

AMMONIA INHIBITION OF NITROGEN FIXATION IN EXTRACTS

(by comparison of ^{15}N fixed with controls)

AMMONIA N added/flask	% inhibition
1.7 mg	10-20
4.0 mg	50-60
20 mg	94-98

It is not clear whether the above represented a comparison between atom% excesses ^{15}N of controls or a quantitative comparison

These results led to Mortenson (175) to develop a rapid method for measuring nitrogen fixation and this and the quantitative results will receive further attention in the discussion.

[B] AZOTOBACTER VINELANDII

Cell-free fixation in extracts of Azotobacter, have been reported on a number of occasions. Magee (176) in 1956 and Nason (177) in 1957, employing ultrasonic disruption and Jose (178) in 1959, employing lysis with lysozyme. However Nicholas (136) made the first claim for consistent cell-free fixation in Azotobacter. He reported that disruption had to be carried out in the original culture medium and attributed the previous inconsistent results to the absence of 'medium factors'

1. Method of Disruption

It has been reported that 'nitrogen fixing' extracts could be prepared by ultrasonic disruption (134) (179) (180) (71), by lysis with lysozyme (134) (179) and by phage lysis (181). In all cases young cultures (c 18 hr) were disrupted in their original culture medium. In one paper (71) it was reported that fixation could be conveniently detected by employing the radio active isotope of nitrogen [^{13}N.]

2. After ultrasonic disruption, the extracts were subjected to high speed centrifugation. It was reported that the extracts contained subcellular particles sedimented at 144,000g (1 hr.) but not at 25,000g (1 hr.). The particles were apparently not essential to fixation but did extend the storage life of fixing activity. No hydrogenase, assayed by methylene blue method, was detected in particle-free extracts.

3. As previously mentioned, EPR studies (106) indicated the presence of non-haem iron (not as ferredoxin), molybdenum, flavin and cytochromes b, c and a. Exposure to hydrogen and a medium factor reduced non-haem iron, molybdenum and the cytochrome. These components were reoxidised by nitrogen or oxygen but helium and argon were without effect.

Spectral shifts in extracts of Azotobacter exposed to nitrogen had been reported by Hamilton et alia (182). Nitrogen appeared to oxidise flavin and cytochrome b but the possibility that this might be due to oxygen, present as a contaminant, was not ruled out.

4. Oxygen, apparently was required for cell-free fixation.

5. Nicholas also reported (33) that cobalt and vitamin B_{12} were necessary for fixation in Azotobacter.

Further comments by Nicholas on nitrogen fixation appear in his two reviews of 1963 (7) (70).

In this laboratory Smith investigated the ultrasonic disruption of stored bacteria (9). He disrupted much thicker suspensions than Nicholas and obtained positive results in the presence of pyruvate or α-ketoglutarate. However their consistency left much to be desired.

The present work attempted to further this approach although also by combining the essential feature of the work of Nicholas, the presence of young culture medium.

(V) EXPERIMENTAL : APPARATUS AND TECHNIQUES

A. BACTERIOLOGICAL METHODS.

The organism used throughout this work was Azotobacter vinelandii (A. agilis var.vinelandii) strain "O", originally supplied by Professor Burris. This strain was characterised by the excretion of yellow green fluorescent pigment, readily visible in fully grown colonies on nitrogen-free agar-agar.

Stock culture.

Nitrogen free slopes were prepared from purified Azotobacter vinelandii "O" and stored at 4°C. It was customary to subculture every two months.

Purity.

Plating out procedures were carried out on nitrogen-free media (1.5% Agar-agar, 3% Mannitol in nitrogen-free medium) and on nutrient media ('Solmedia', Griffin and George). Liquid cultures were examined by nigrosin and gram-straining procedures.

Azotobacter vinelandii was grown in liquid culture on a modified Burk's medium containing 1.5% mannitol. The composition of the medium was that used by Wusteman (28).

Solution A	Concentration in medium
di-hydrogen potassium phosphate	(0.16 gm/litre)
di-potassium hydrogen phosphate	(0.64 " ")
Sodium Chloride.	(0.2 " ")
Sodium molybdate.	(0.002" ")

Solution B	Concentration
Calcium sulphate dihydrate	(0.05 gm/litre)
Disodium hydrogen citrate	(0.13 " ")
Ferrous sulphate	(0.015 " ")
Magnesium sulphate heptahydrate	(0.2 " ")

As is indicated above, the medium was made up from two (concentrated) stock solutions, which on combination and dilution gave the concentration shown at pH 7.2-7.3. Mannitol, to 1.5%, was added before sterilisation. The medium for continuous culture was essentially the same except that the mannitol concentration was 5%.

Sterilisation.

Media and apparatus, excluding the larger vessels of the continuous culture apparatus, were sterilised by autoclaving at 15 lb for 20 minutes. The large 10L. bottles and culture vessels required careful heating before autoclaving at 15lb for 40 minutes.

CONTINUOUS CULTURE APPARATUS

The continuous culture apparatus, a chemostat, had been built in the Chemistry Department at Imperial College for use in this laboratory. A detailed account of its construction appears in the thesis of Stewart (8). It can be seen from Figure 4 that it consists essentially of three parts, a medium reservoir, a culture vessel (cell) and a culture receiver. These are interconnected by Esco rubber tubing

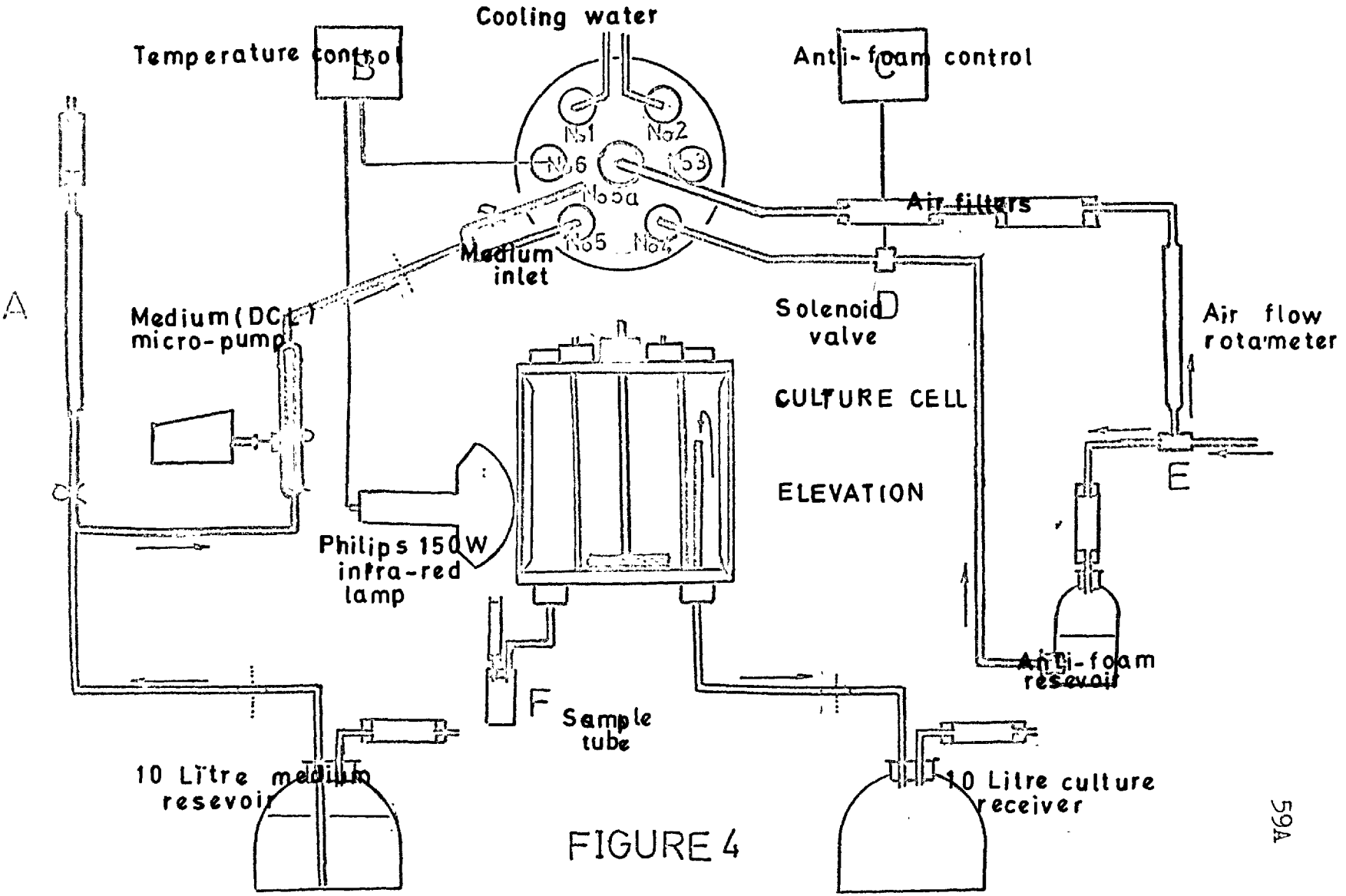

Cooling water
Temperature control
B
Anti-foam control
C
No1
No2
No6
No3
No4a
No5
No4
Air filters
Medium inlet
A
Medium (DC) micro-pump
Solenoid valve
D
Air flow rotameter
CULTURE CELL
ELEVATION
E
Philips 150W infra-red lamp
Anti-foam reservoir
F
Sample tube
10 Litre medium reservoir
10 Litre culture receiver

FIGURE 4

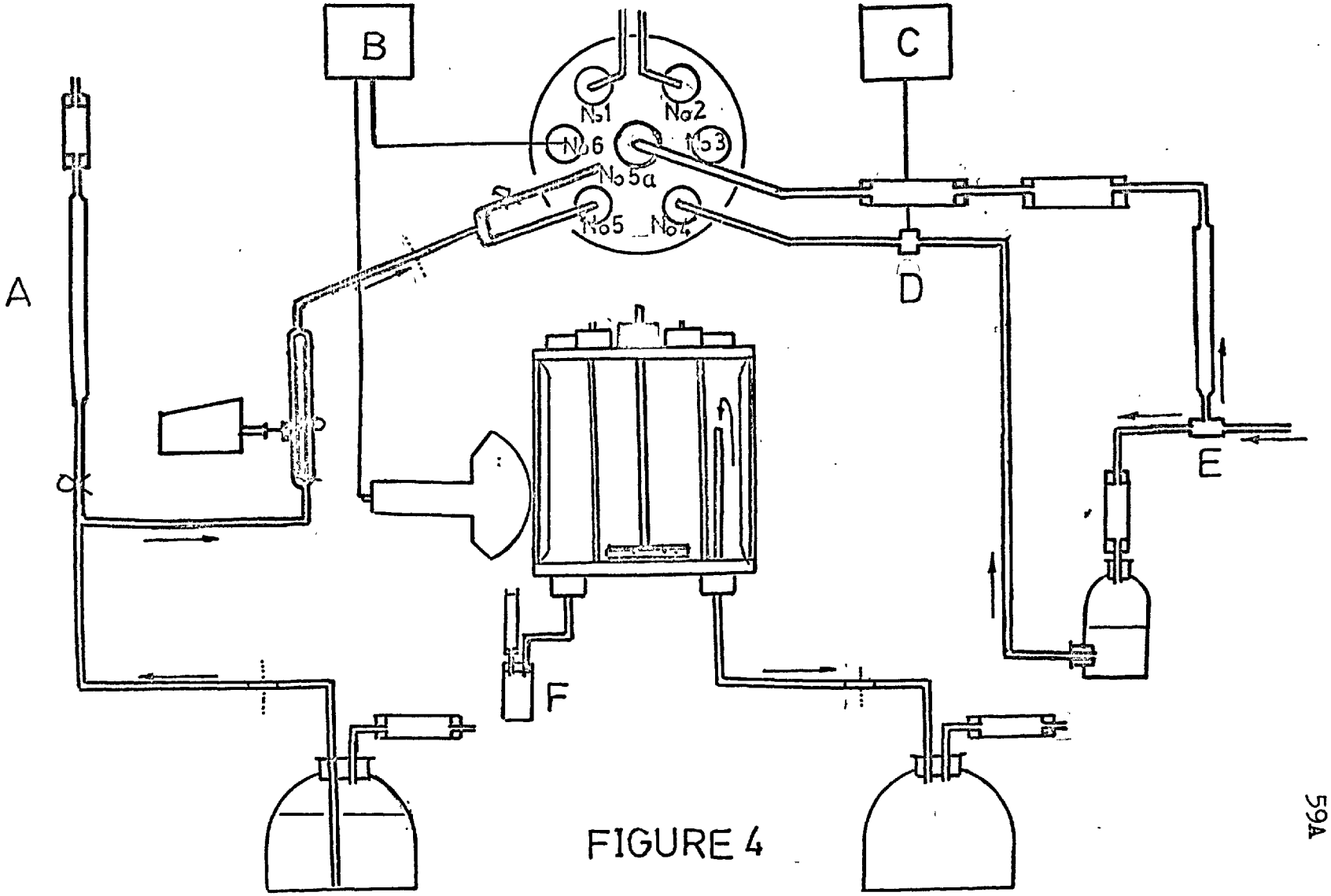


FIGURE 4

(a silicone rubber which can withstand sterilisation) and stainless steel snap connectors.

The culture cell was constructed of standard Q.V.F. glass pipeline (6" x 4") positioned between two stainless steel plates. It was fitted with a rotary stirrer and baffleplates to ensure efficient mixing of Azotobacter culture. Medium was pumped from the medium reservoir at a constant rate by the D.C.L. micropump and entered the cell via a capillary at No. 5. The cell was, however, initially filled through No. 5a to about 650 mls. The usual pumping rate was about 40 ml/hr with a volume of about 650 ml maintained by means of the overflow pipe, which led to the culture receiver. The pumping rate could be measured by means of the 10 ml pipette at A.

Air was supplied to the culture through the central stirrer gland to ensure adequate aeration. The filtered air was pumped in via the air rotameter E at 1 litre/min by a continuously rated Dymax Mark II air pump. This resulted in a backing pressure of 9 lb/sq.in. being available to aid antifoam addition. The air was filtered through two filters which were packed with superfine glasswool (Fibre Glass Co Ltd., St. Helens, Lancs).

To avoid extensive foaming regular additions of antifoam were made. The antifoam, M.S. Antifoam, Hopkins and Williams, was added through No. 4 in small doses

controlled by the solenoidvalve D and time circuit C.

The temperature of the culture was maintained at 30°C by means of the control circuit B. The efficient agitation raised the temperature above the required 30°C and thus a cooling coil (at No. 1 and No. 2) in combination with the infra red lamp was necessary. The temperature sensitive element inside the culture (at No. 6) was a thermistor (Stantel F.23) which was connected to B to enable the amount of heat supplied by the Philips 150 watt Infra Red lamp to be controlled to maintain the temperature at 30°C.

OPERATION

The apparatus was sterilisable in three parts;

(a) Medium reservoir and micropump connection.

(b) Culture cell and sampling tube (F).

(c) Culture receiver.

The medium in the culture was inoculated through the rubber serum cap (at No.3) with 5 ml of a 24 hr. 'shake' culture of Azotobacter vinelandii 'O.' The sterile syringe was filled with culture under aseptic conditions in a modified perspex box which was fitted with a low pressure mercury discharge tube. In setting up the continuous culture there were a number of points that required careful attention. These are discussed below.

(1) The culture cell and tubing required thorough cleaning before connecting up the apparatus for sterilisation.

In this respect the packing of the stirrer bearing was particularly important to ensure that the stirrer was smooth running but not a source of oil or contaminant. Initial washings of the cell with water invariably gave some diffuseness after running the stirrer. At this stage the temperature control should be checked.

(2) The packing of the air filters also required special consideration and will be discussed subsequently. Since these filters have to be sterilised in the autoclave it was advisable to reduce any interference, wetting etc., by allowing another filter (on overflow exit) to be the outlet for hot air. It was customary to cover the end of the filters with aluminium foil to eliminate steam condensation in the exit of the filters. It was necessary to examine filters after sterilization particularly the filter on the culture reservoir. If this was blocked it would result in a high back pressure in the cell with probable ejection of the rubber serum cap.

(3) It was important to ensure that a continuous thread of antifoam was maintained from the antifoam reservoir to the solenoid valve since serious delay would result if this was not the case. It was advisable to use fresh antifoam for each run.

(4) The simplest method of filling the culture cell was to raise the level of the medium reservoir above the level of the cell. By application of pressure to the liquid in

the medium reservoir the requisite quantity could be siphoned over and it was possible to eliminate air bubbles in the inlet tube. It must be noted that an air flow through the culture cell was necessary to eliminate the possibility of medium reaching the air filters. It was also evident that the filter on the medium reservoir must prevent contamination when air pressure is applied to the medium.

(5) A pure culture of Azotobacter vinelandii was of course essential for the inoculation.

During initial runs on continuous culture, both with nitrogen-free and ammonium media, persistant contamination of the Azotobacter by a gram negative rod-like organism occurred. In one run, Azotobacter growing on nitrogen - free medium, the contamination was observed after two days. A sample was taken several days later when the rods were in significant quantities and these were plated out. They were examined in the laboratory of the late Dr. Jacobs, and the rod-like organism was identified as a Bacillus. In subsequent runs although modifications were made to procedure and in particular to the air filters the appearance of small quantities of rods was not prevented.

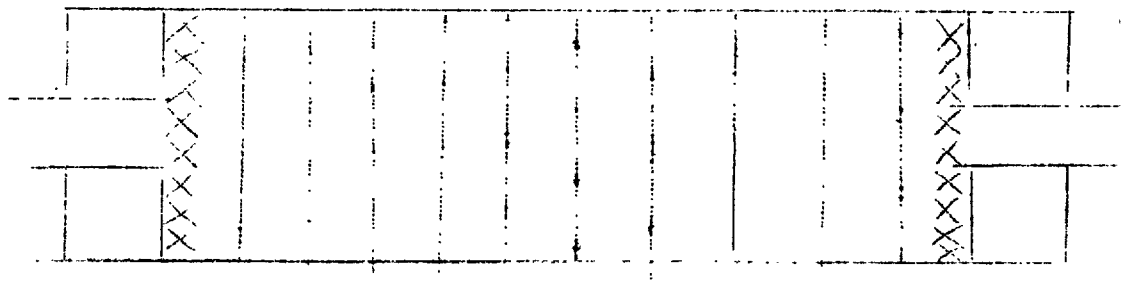
In Table V some measurements on bacteria taken from the continuous culture apparatus are shown. In this run, Azotobacter growing on nitrogen free medium, the appearance of rods was observed and the run curtailed.

TABLE V

AGE HR	Dilution rate	Nmg/ml	$Qo_{2(N)}$	$Q_{H_{2(N)}}$
0	0.0628	4.31	1,600	910
24	0.0671	1.62	1,400	1,650
50	0.0681	1.85	2,700	3,800
74	-	1.03	1,200	3,000
* 122	0.0651	2.38	3,400	900
142	0.0682	1.50	2,000	1,450

* Medium reservoir changed

The filters in this run were packed as shown below, on the advice of Dr. D. Herbert of Porton Down (183).



A glass tube c.1" diameter was packed tightly with discs of fibreglass wool (about 10-12 in number). Dr. Herbert recommended glass fibre wool (superfine,unfilmed) Type 2A,

density 0.5 lb cu.ft., for air filters and also pointed out that most piston type air compressors produced virtually sterile air. In the present work it might be expected that two such filters would have been adequate although it is evident that dry sterilization of filters would have been more desirable. Certainly further consideration of the air filters employed is desirable as is evident from reference to the recent review on the mechanism of air filtration (184).

It is possible that contamination could arise

(i) during the joining up of the medium reservoir, cell and culture receiver,

(ii) from an impure inoculum,

(iii) during the sampling procedure,

(iv) during the changeover of medium reservoir.

It is unlikely that (ii) and (iii) were the source of the contaminant and it is impossible to make an assessment of (i). It is probable that the changeover of medium reservoir, the removal of empty reservoir and its replacement by fresh medium, does present a contamination problem since it is not possible to maintain sterile conditions

during the changeover of the snap connectors. The results of Table V do show significant variations. The age of the culture was taken from 1hr after the stirring had commenced, since after inoculation the culture was allowed to grow with aeration but without stirring until a reasonable cell population was achieved. The dilution rate during the run was about 0.06 hr^{-1} somewhat higher than that of Stewart (8). The pumping rate varied between 42.5 and 45 ml/hr over 118 hrs with a bacterial population between 10^8 - 10^9 cells/ml.

It is difficult to account for the variation of $Qo_{2(N)}$ values although in the latter stages the contaminant was present. The variation in hydrogenase activity is more marked and the hydrogenase values $Q_{H_{2(N)}}$ are lower than values for 'shake' flasks. Stewart did observe some variation of hydrogenase which was particularly marked at the change-over point. During the 142 hrs, .79 portions of antifoam were added (c. 1ml) although not at regular intervals since some initial adjustment was made, one addition per 2-2½ hrs was a suitable addition rate.

It is evident that the present run did give a wider range of values than was hoped. It is possible that anti-foam concentration does affect respiration and hydrogenase measurement but it is likely that the continuous culture needs about 100 hrs to settle down. This was not achieved owing to the appearance of the contaminant.

[B] WARBURG TECHNIQUE

The hydrogenase and respiratory activities of whole cells and also cell extracts were measured by standard Warburg technique (185). Particular care was taken in cleaning Warburg flasks. It was customary to clean regularly with chrome nitric (overnight) with thorough washing with distilled water (x10) and with strong phosphate soln. After thorough washing with distilled water the flasks were dried in an oven at 100°C. This procedure was adhered with all the apparatus used in the subsequent extract work (Thunberg tubes etc). The flask constant of each Warburg flask was determined by the 'mercury' method and calculated for a liquid volume of 3.2 ml at 30°C (α hydrogen = 0.01619 α air = 0.00526).

The contents of the flask for hydrogenase and respiration determinations are summarised in Table VI.

TABLE VI

RESPIRATION		HYDROGENASE	
Main Compartment			
Azotobacter culture Mannitol medium	3ml.	Azotobacter culture/ disruptate/centrifugate	
		Pyrophosphate (0.1M) pH7.6	2.3ml
		Gelatin (8%)	0.5ml
Side arm.			
		Potassium ferricyanide	0.2ml
Centre well.			
Potassium hydroxide	0.2ml	Fieser's solution	0.2ml
	3.2ml		3.2ml
Gas phase	Air		Hydrogen

Fieser's solution (1835) has the composition, sodium hydroxide, sodium dithionite and sodium anthroquinone-β-sulphonate in ratio 75:90:9.

Hydrogenase activity was calculated from the uptake of hydrogen over a 40-45 minutes period after addition of ferricyanide The flasks were checked for leakages prior to this by a suitable period of equilibration at 30°C.

The rate of uptake of hydrogen should exceed 150 microlitres of hydrogen per hour since lower rates seriously affected the reproducibility of the method. It was not difficult to obtain a suitable rate of hydrogen uptake with extracts prepared from concentrated suspensions of whole cells but some trial and error was necessary with less concentrated suspensions of cells. In such cases it was desirable, where possible, to have duplicates for several concentrations of cells, since with lower rates of hydrogen uptake the reproducibility may not be better than 15%. In this respect Hyndman et alia (82) have recommended that the uptake of hydrogen, measured over a 30 minute period, should range between 20-50 microlitres/5 minute period.

[C] ULTRASONIC DISRUPTION

The bacterial suspensions were disrupted by the ultrasonic method of the previous workers in this laboratory.

A Titanium probe (End ratio 18:1) capable of generating 19-20 Kc/s was immersed about ¼" into the cell suspension (40ml)

The stored bacteria were disrupted either in 0.1M pyrophosphate (pH 7.6), 0.05M phosphate (pH 7.6) or young culture (medium), as recorded.

The time of disruption was 15 minutes and it was evident that during this time an efficient mixing occurred. This obviated the need for mechanical mixing.

The double walled vessel was cooled by circulating through it a methanol/cardice mixture, a means of cooling that had previously been shown to be adequate. The efficiency or reproducibility of this method of disruption is not readily apparent. In the experiments on cell-free nitrogen fixation it was standard procedure to disrupt 3 gm of stored bacteria in suspension. The soluble nitrogen varied from between 60-80% of the total (5 mgN/5ml) whereas the earlier experiments gave a wider range from 33% (6.43 mgN/ml) to 70% (0.6 mgN/ml) according to the cell density. The heterogeneity of the disruptate (enlarged upon in the discussion) and the difficulty in interpreting the % soluble nitrogen of stored cells in terms of efficiency of disruption makes a conclusion difficult. A further experiment to determine the effect of disruption time on % soluble nitrogen is shown in Figure 5. Relevant values from the experiments on cell-free nitrogen fixation are also plotted and this graph gives some idea of the reproducibility of the method.

ULTRASONIC DISRUPTION OF STORED BACTERIA (3 gm/40ml)

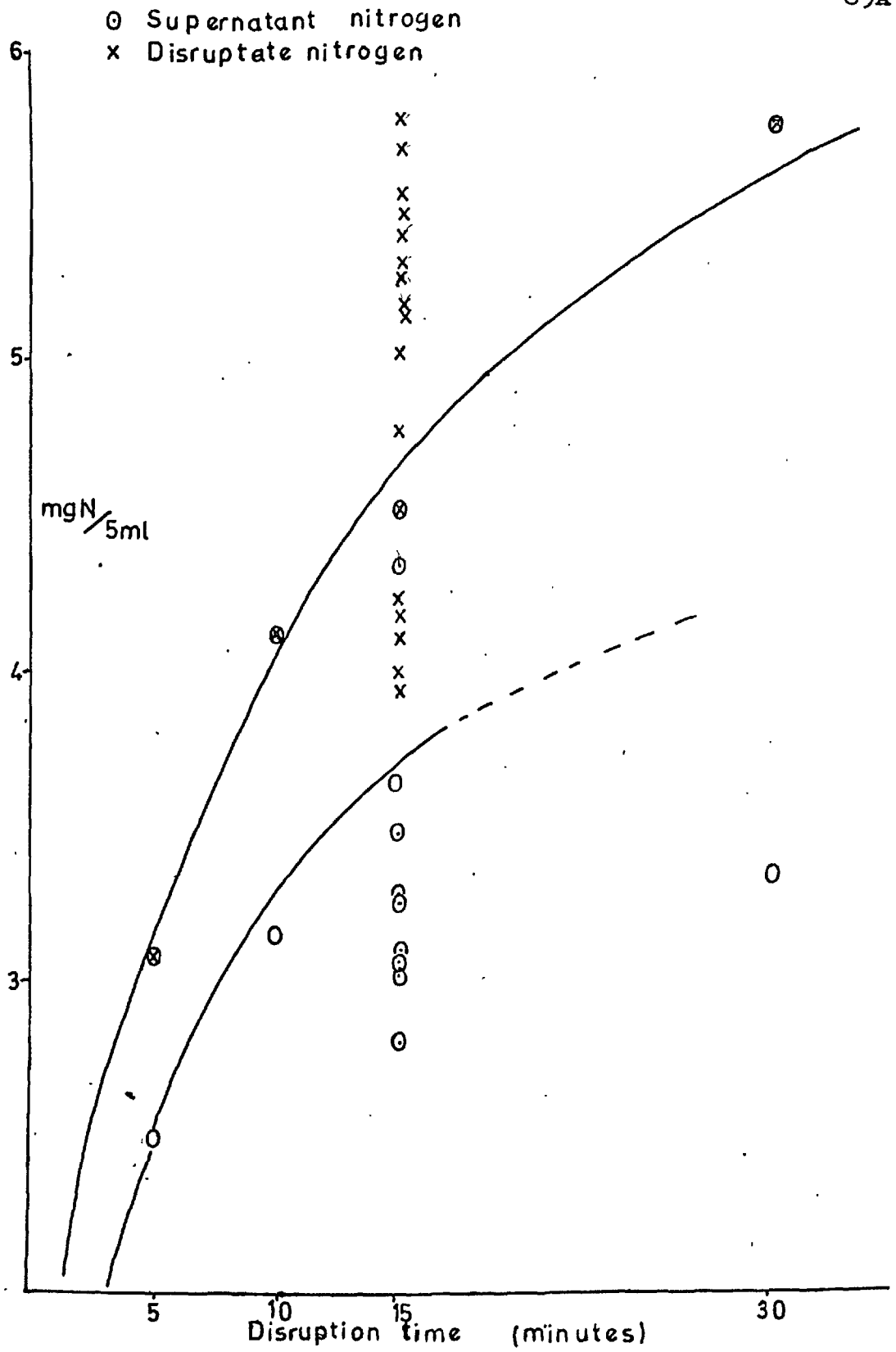


FIGURE 5

[D.] CENTRIFUGATION

The centrifugation (unless otherwise stated) was carried out in a Spinco Model L preparative ultracentrifuge (Beckman Instrument Inc., California, U.S.A) by courtesy of Professor W.O. James, Botany Department, Imperial College.

[E.] EXPERIMENTAL TECHNIQUE WITH ISOTOPIC NITROGEN [^{15}N]

The [^{15}N] labelled nitrogen used in this work was generated from [^{15}N] labelled ammonium sulphate (31.8 and 32 atom $\%^{15}N$) obtained from "Office National Industriel de l'azote", Toulouse, France. The nitrogen was generated by standard procedure by the oxidation of ammonia by sodium hypobromite. (187) (188) (288).

Preparation of sodium hypobromite.

Analar sodium hydroxide (10 gms) was dissolved in air-free water (40ml). After cooling in an ice-salt freezing mixture, liquid bromine (4ml) was added slowly. The pale yellow solution was transferred to a dark bottle, analar potassium iodide added to 0.1% W/V as recommended (189) (190), and stored in the refrigerators.

Preparation of [^{15}N] labelled 'atmosphere'.

The atmospheres in this work were made up with composition

40% nitrogen

40% Argon

20% Oxygen

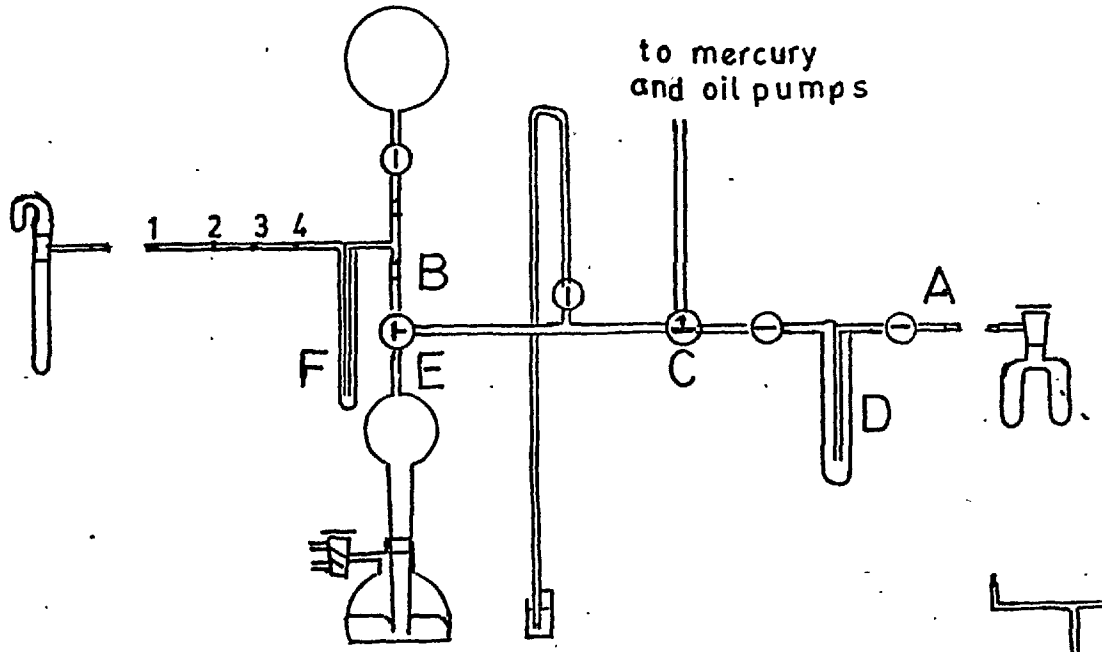
The atmospheres were made up on the general vacuum line

(designed by B.A. Allsop) and stored in 250 ml bulbs. The vacuum line (also fitted with exposure apparatus) is illustrated in Figure 6.

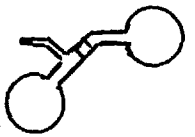
The calculated amount of labelled ammonium sulphate (c.1.2 gm) was weighed out and placed in suitable portions in one arm of the nitrogen generator. The nitrogen generator was either the Rittenberg tube or the large scale apparatus (I), illustrated in Figure 6.

The ammonium sulphate was dissolved in the minimum quantity of water, an excess of hypobromite added to the other arm of the generator, and the contents frozen in a methanol-cardice mixture. The nitrogen generator was fitted to the vacuum line at A and the whole system, 250 ml flask on B, Toepler pump and nitrogen generator, evacuated. When a high vacuum was obtained tap C was turned to cut out the pumping system and the content of the nitrogen generator were allowed to warm up. A cardice trap was fitted around trap D and the nitrogen generated by careful mixing of the hypobromite with the ammonium sulphate solution. The nitrogen was transferred to the storage bulb with the aid of the Toepler and its pressure (bulb to tap C) was recorded. This procedure was repeated until the desired pressure of nitrogen had been achieved.

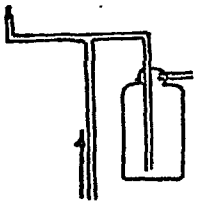
Argon was added to the bulb in portions from a cylinder by the arrangement shown in Figure 6,(II).



[I]



[II]



Gas (cylindre)

GENERAL VACUUM LINE FOR PREPARATORY AND EXPOSURE. WORK

FIGURE 6

The Toepler bulb was filled with a known pressure of argon before being added to the storage bulb. This was repeated until the required total pressure (argon and nitrogen) for 40% argon was obtained.

The oxygen from a cylinder was added by the same procedure until the gas in the storage bulb had the required pressure (c74cm).

EXPOSURE OF EXTRACT TO LABELLED ATMOSPHERE.

The extracts (5 mls) were exposed to the ^{15}N labelled atmosphere in Thunberg tubes (25 ml capacity) by the method employed by Smith (9).

The exposure apparatus is shown in Figure 6. It can be seen that Thunberg tube holder (for four Thunberg tubes) is fitted at B, with the gas storage bulb in position. The contents of the Thunberg tubes were frozen in methanol/cardice and the Thunberg tubes fitted in their respective sockets 1,2,3,4. The Thunberg tubes and Toepler bulb were evacuated and pumps cut off by taps C and E. A cardice trap was fitted at F and the gas from the storage bulb was allowed into the Thunberg tubes. By means of the Toepler the pressure of gas in Thunberg was adjusted to about atmospheric pressure (c74cm). The Thunberg tubes, after removal from the vacuum line, were shaken at 30°C, for the required period.

Removal of atmosphere.

The contents of the Thunberg tubes were frozen and the atmosphere was pumped into a residual storage flask by the reverse procedure. The contents of the flask were allowed to warm up under vacuum before removal for Kjeldahl nitrogen analysis.

[F] KJELDAHL PROCEDURE.

The Kjeldahl procedure used in this work was essentially that of Willit and Ogg (191) although a longer digestion time was used. The extracts were digested in analar concentrated sulphuric acid (1.5 ml) with a B.D.H. 'mercury' tablet (1 gm sodium sulphate, 0.1 gm mercury) as catalyst. After the digestion had proceeded eight hours a further porous chip was added and mixture refluxed for 1 hr. The clear solution was allowed to cool and the white residue was taken up in a small quantity of distilled water.

The ammonia was liberated by addition of 10 ml sodium hydroxide (40%) and 5 ml sodium hydroxide (40%)— sodium thiosulphate (5%) solution and steam distilled into 10ml of boric acid solution (2%). The boric acid solution contained two drops of bromocresol green (0.1%) and the steam distillation was continued 7 minutes after the ammonia appeared (indicator blue). The flask was released from the tip of condensor and removed after a further 2 min distillation. The ammonia was titrated against standard hydrochloric acid.

Pre-reduction techniques.

(i) Friedrich Method (192)

Glucose (100mg) and constant boiling hydriodic acid (1ml) were added to the sample in a 50ml Kjeldahl flask and the mixture gently refluxed for 45 minutes. The volume of the solution was reduced by careful distillation and on cooling potassium sulphate (500mg), water (1ml) and sulphuric acid (1.5ml) were added. The iodine was steam distilled off, several additions of water being necessary. On cooling the 'mercury' catalyst tablet was added and the contents of the flask digested in the usual manner.

(ii) Pre-reduction by Zn/Fe couple (193)

98% Formic acid (0.2ml), concentrated hydrochloric acid (0.1 ml) were added to the sample in a 50 ml Kjeldahl flask. After warming to 80°C, zinc dust (80mg) was added with shaking. Iron powder (40 mg) was added and the flask shaken for two minutes. Concentrated hydrochloric acid (0.1ml) and ethanol (0.1 ml)were added and flask warmed. Further concentrated hydrochloric acid (0.1ml) was added at five minute intervals until the iron dissolved. Concentrated sulphuric acid (1m) was added and the flask warmed. After addition of further concentrated sulphuric acid (0.5 ml) and a 'mercury' catalyst tablet the mixture was digested in the usual manner.

Accuracy

Although the accuracy of Kjeldahl method may differ with the particular catalyst and Kjeldahl apparatus used, its reproducibility depends on,

(a) the reproducibility of the conversion of organic nitrogen to ammonia and the quantitative isolation of ammonia from the acid digest.

(b) the determination of ammonia.

The latter (b) procedure should not be responsible for significant errors ($< 0.1\%$) particularly as the errors from this source have been thoroughly discussed by Conway (194). It is difficult to assess the errors resulting from (a) since they are specific to a particular procedure. In earlier studies with a series of amino acids, arginine hydrochloride, glutamic acid, L-histidine hydrochloride; D.L.-Leucine and tyrosine, the overall accuracy of the Kjeldahl procedure was about 1%. This referred to the procedure with 10 mgN and thus in the extract work, which related to nitrogen values of between 0.5-5 mgN, the accuracy should have remained in the range 1-2%. Further consideration of the Kjeldahl procedure is made in the discussion Section VIII).

[G] ANALYSIS FOR [^{15}N] IN THE MASS SPECTROMETER

The atom % ^{15}N values reported in this thesis were measured on a Nier-type mass spectrometer. The mass spectrometer, originally constructed in this laboratory by

Davidson (195) had a constant magnetic field, maintained by a 60°C sector magnet, and employed potential scanning.

The isotopic abundance of $[^{15}N]$ in a gas sample can be conveniently calculated from the ratio of the intensities of the ion currents resulting from the species $(^{14}N - ^{14}N)^+$, 28, and $(^{15}N - ^{14}N)^+$, 29. It has been shown that if the atom % $^{15}N = 100 \alpha$, then α = fraction ^{15}N (on an atom basis)

$$\alpha = \frac{1}{1 + 2R}$$

where R is the ratio of intensities of ion currents $\frac{28}{29}$.

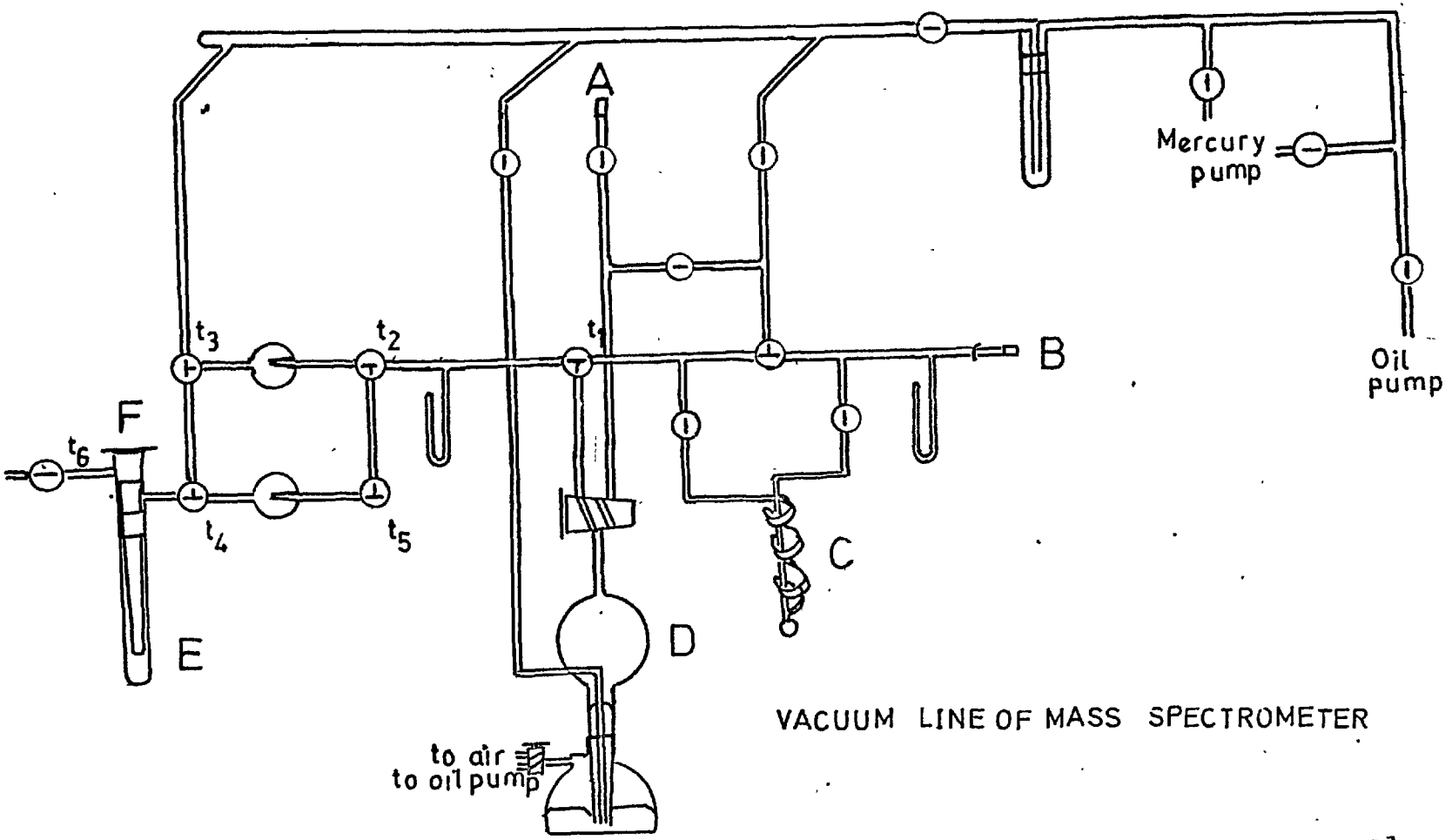
α can also be determined from the $\frac{28}{30}$ and $\frac{29}{30}$ ratios since in equilibrated nitrogen gas the species $(^{14}N - ^{14}N)$, $(^{15}N - ^{14}N)$, $(^{15}N - ^{15}N)$ are present in the ratio $(1 - \alpha)^2 : 2\alpha(1 - \alpha) : \alpha^2$

for $\frac{29}{30}$ ratio $\quad \alpha = \frac{2}{2 + R}$

for $\frac{28}{30}$ ratio $\quad \alpha = \frac{1}{1 + \sqrt{R}}$

The measurement of $\frac{29}{30}$ and $\frac{28}{30}$ provides a useful check on α calculated from the $\frac{28}{29}$ ratio particularly with respect to air leakage and when small amounts of nitrogen are measured. In the latter case small amounts of atmospheric nitrogen may appreciably effect the $\frac{28}{29}$ value and it is often necessary to apply a correction.

The vacuum system of the mass spectrometer is illustrated in Figure 7). Sample nitrogen was converted to gaseous nitrogen by the hypobromite procedure in a Rittenberg tube at B



VACUUM LINE OF MASS SPECTROMETER

FIGURE 7

and stored in the Toeppler bulb D. Gas samples (from storage bulbs) could be directly introduced into the Toeppler at A.

Operating procedure

(1) After checking the cold trap of the mercury pump in the mass spectrometer, the pressure was measured on a Pirani gauge. If this was 10^{-5}mm the ion gauge was switched on. If a suitable pressure was shown (c. 10^{-6}mm) the filament was switched on for at least 30 minutes before use (filament current 0.2 milli-amp)

(2) The sample vacuum line was pumped out with careful degassing of the trap E and Toeppler bulb D. The residual values of 28 and 29 peaks were measured and the main trap E was then incorporated in the mass spectrometer system by turning the main tap at F, at the same time cutting trap E out of the vacuum line by means of tap t_4. This resulted in an increase in the value of the residuals of 28 and 29 peaks. After several minutes, when the residuals had returned to their initial values a liquid nitrogen trap was placed at E and the mass spectrometer was ready to receive a sample.

(3) The sample was introduced to the Toeppler via A or B. The ammonia sample from the Kjeldahl procedure (c.3ml) was placed in one arm of a Rittenberg tube which contained an excess of alkaline sodium hypobromite in the other arm. The Rittenberg tube was placed on the vacuum line at B and

the contents frozen. After evacuation the contents were allowed to warm and 'degas'. After re-freezing and evacuation, the contents were allowed to warm up and the nitrogen generated on mixing.

(4) The gas was allowed to expand into the Toeppler. After cutting out the Rittenberg tube, by suitably turning tap t_1, the Toeppler was adjusted to give a suitable gas pressure behind the leak. (1-2cm for the top leak, 4-5 cms for the lower leak).

(5) After the residuals had been measured the gas was leaked into the mass spectrometer either by the top leak ($t_2 - t_3 - t_4$) or the lower leak ($t_2 - t_5 - t_4$). This was accomplished by first opening tap t_2 to check the gas flow through the selected leak. If this was unhindered tap t_4 was opened and the vacuum line cut off by turning tap t_3 or t_4. This procedure reduced the initial inflow of gas into the mass spectrometer and enabled the 28 and 29 values to build up steadily.

(6) When the maximum value of the 28 was reached two readings of the values of the 28 and 29 peak heights were recorded. A careful check on the baseline at the 29 peak was advisable.

(7) The sample was removed from the mass spectrometer by shutting off the tap at F and allowing the mass spectrometer tube to be pumped down. The cold trap was removed from E and this trap and the Toeppler were opened to the pumping

system and thoroughly degassed.

(8) This procedure was carried out with nitrogen generated from ammonium chloride at least once a day (with a comparable quantity of nitrogen to that generated from the samples). It was usual to check the mass spectrometer against nitrogen from 'normal' ammonium chloride every 3-4 samples when the mass spectrometer was working well.

Operational trouble.

During the initial period of operation the mass spectrometer had persistent vacuum trouble. Since the work of Smith the vacuum line had been renewed with the exception of the main tap at F. After the replacement of this tap the vacuum markedly improved and subsequently caused no great difficulty. A further tap t_6 was inserted between the mass spectrometer and the main tap at F to enable the main tap at F to be periodically greased without shutting down the mass spectrometer.

Some trouble was also experienced with the leaks, several being fitted before a suitable one was obtained. It was also essential to grease the taps carefully since excess grease or other debris ultimately would block the leaks. Owing to the humid conditions of the laboratory during the summer (temperature c.25°C) two types of grease were used. Apiezon grease "T", a thicker grease, proved useful for the two way taps instead of the usual Apiezon grease "N".

Accuracy of measurement:

The absolute determination of atom % ^{15}N depends on a number of factors, e.g. the vacuum, the age of the filament, amplifier stability and residual peak interference.

In Table VII is summarized the range of ions which could result in the interference of measurement on 28, 29, 30 peaks.

It is evident that abnormal values for the 29 peak will result if methylamine is present. This underlines the need for a complete Kjeldahl analysis, employing suitable catalyst and digestion time (196), particularly as it has been reported that methylamine 'sticks' in the mass spectrometer and requires 'baking' out (197).

A similar effect on the 28 peak will be observed if carbon dioxide, carbon monoxide or even acetone are present in significant amounts.

When small quantities of nitrogen (c. 200 µg) are analysed in the mass spectrometer some correction for contamination by air is necessary. Two methods have been used to estimate the amount of nitrogen from the air (a) the measurement of the oxygen peak at 32 and hence $\frac{28}{32}$ ratio and (b) the measurement of the nitrogen:argon ratio $\frac{28}{40}$ (187)(198). The latter (b) has the disadvantage that argon is present in very small quantities and that errors in its

TABLE VII

Mass Number	Ionised molecule or fragment	Source
46	$^{16}O\,^{12}C\,^{18}O$ (+)	carbon dioxide
45	$^{16}O\,^{13}C\,^{16}O$ (+)	"
	$^{15}N\,^{14}N\,^{16}O$ (+)	nitrous oxide
44	$^{14}N\,^{14}N\,^{16}O$ (+)	nitrous oxide
	$^{16}O\,^{12}C\,^{16}O$ (+)	carbon dioxide
40	$(A)^{(+)}$	Argon
32	$^{16}O_2$ (+)	Oxygen
30	$^{12}C\,^{18}O$ (+)	carbon dioxide
	$^{15}N\,^{15}N$ (+)	nitrogen
	$^{14}N\,^{16}O$ (+)	nitric oxide
29	$(CH_3)_2N$ (+)	methylamine
	$^{15}N\,^{14}N$ (+)	nitrogen
	$^{13}C\,^{16}O$ (+)	carbon dioxide carbon monoxide
	Me_2CO (++)	acetone
28	$^{14}N\,^{14}N$ (+)	nitrogen
	$^{12}C\,^{16}O$ (+)	carbon dioxide carbon monoxide
22	$^{16}O\,^{12}C\,^{16}O$ (++)	Carbon dioxide
18	H_2O (+)	water
17	OH (+)	"
16	$^{16}O_2$ (++)	oxygen
	^{16}O (+)	"
15	$^{15}N\,^{14}N$ (++)	nitrogen
$14\frac{1}{2}$	$^{15}N\,^{14}N$ (++)	"
14	$^{14}N\,^{14}N$ (++)	"

measurement are magnified by the high ratio of nitrogen to argon in air (≈ 73). The former method (a) is rendered inaccurate if any oxygen results from the decomposition of hypobromite. The formation of oxygen by such decomposition is a possibility, particularly as this decomposition has been shown to be catalysed by cupric ion (199). In practice potassium iodide is added to hypobromite since no detectable release of oxygen was found when 0.1% potassium iodide is present (190). Sims and Cocking (190) also suggested that the other by-products which interfered with the measurement of nitrogen at mass numbers 28, 29 and 30, carbon dioxide and nitrous oxide, could be frozen out after the nitrogen has been generated by immersing the reaction vessel in liquid air. Capindale and Tomlin (198) have pointed out that the peak at mass number 44, which is sometimes used to correct for the contribution of carbon dioxide to the peak at mass number 28, more likely results from nitrous oxide, particularly as the reaction between alkaline hypobromite and ammonium salt, has been shown to produce nitrous oxide (200). It is, in any event, advisable to have a liquid air trap between the generated nitrogen gas and the mass spectrometer to eliminate such contaminants. It is evident that in measuring the $[^{15}N]$ content in small samples of nitrogen special consideration is required. In this respect a recent article, (201), has discussed these problems in relation to the measurement of $[^{15}N]$ in soil microbiological research.

Figure 8 shows the residual chart for the mass spectrometer, the peak heights for the range of mass numbers 16 - 32 for the 'empty' mass spectrometer. It can be seen that the highest residual occurs at mass number 18, resulting from water vapour, although the true ratio between the peaks is not apparent as the 18 peak has been measured on shunt 3 and not, as in the case of the others, on shunt 1. Thus the peak height for mass number 18 should be multiplied by the ratio of shunt 3: shunt 1 to bring it to the same order of magnitude.

The residual chart shows small peaks at mass numbers 16, 29, and 32 and a slight peak at mass number 27. This chart was run with a vacuum of 2.2×10^{-6} mm of mercury. When a sample was leaking into the mass spectrometer the pressure increased to $3\text{-}4 \times 10^{-6}$ mm of mercury.

Natural abundance of nitrogen and reproducibility of mass spectrometer.

For the purpose of the theoretical calculations in this thesis the natural abundance of nitrogen was taken as 0.38 atom % ^{15}N. The natural abundance of nitrogen (from measurement on $\frac{28}{29}$ ratios) has been quoted as 0.37 (196), 0.359 (source normal ammonium chloride) (198) and 0.368 (source ammonium sulphate) (202). Huser (201) using an Atlas mass spectrometer (Type $CH_3/15$ IV) gave the following values for purified nitrogen gas and nitrogen gas generated from ammonium chloride. Table VIII.

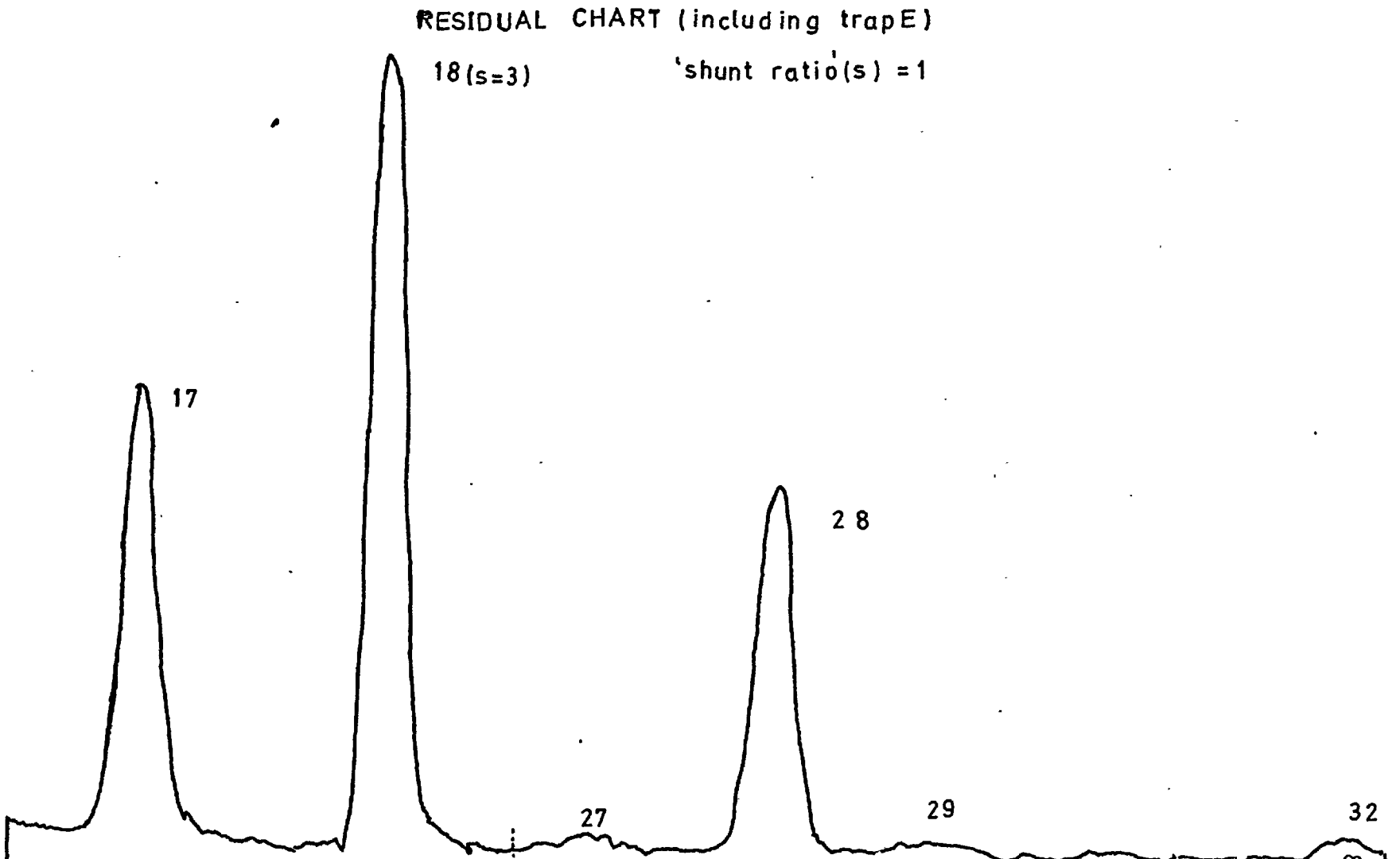

RESIDUAL CHART (including trapE)
18(s=3)
'shunt ratio'(s) = 1
17
28
27
29
32

FIGURE 8

TABLE VIII

	Nos. of analyses	Year	Mean	%
Gas standard	18	(1960)	0.3611 ± 0.0020	0.55
	26	1961	0.3629 ± 0.0020	0.55
	40	1962	0.3619 ± 0.0021	0.58
Ammonium chloride standard	13		0.3609 ± 0.0019	0.52

In the present work the initial values of the natural abundance of nitrogen were high owing to the vacuum trouble. After this had been corrected the mass spectrometer behaved particularly well except during periodic troubles with amplifier instability.

The mass spectrometer was not capable of the precision of that of Huser. An analysis of the atom % ^{15}N values of nitrogen from ammonium chloride was made over the final two months when the mass spectrometer had been fitted with a new filament. This resulted in a mean value for the natural abundance of 0.390 ± 0.016 (46 measurements) through the lower leak and by measuring the 28 peak on the 100 shunt and 29 on the 3 shunt (i.e. shunt ratio $\frac{100}{3}$). All values were included in this analysis. When only the maximum values were taken a mean value of 0.380 (mean deviation 0.015) was obtained (28 measurements).

In the calculation of increase in nitrogen (nitrogen fixed) the important quantity was not an absolute measurement

of atom % ^{15}N but the atom % ^{15}N excess (over the natural abundance) in the sample. It was standard practice to determine the atom % ^{15}N excess by subtraction of the value for nitrogen from ammonium chloride (on the same day), from the measured atom % ^{15}N. Thus relative error rather than absolute error was important and this lay between 2-3% over the principal range of atom % ^{15}N values measured. The calculation of increase in nitrogen from atom % ^{15}N is elaborated upon in the discussion.

Acetyl phosphate

Acetyl phosphate was prepared by the method of Lipmann and Tuttle (203)(204) on ½ scale. It was isolated as the di-silver salt and stored in a dark bottle in the refrigerators.

Preparation

(a) Tri-silver phosphate was prepared by adding a 20% solution of silver nitrate to a solution disodium hydrogen phosphate (containing 103 gms). The nitrate solution (250 ml.) was added to the hot solution of disodium hydrogen phosphate (600 ml), the suspension boiled, filtered and the yellow trisilver phosphate dried at 100°C overnight.

Yield 41 gm (%Ag 64.8)

(b) The trisilver phosphate was converted to monosilver phosphate by the procedure set out below. Trisilver phophate (32 gm) and 88% phosphoric acid (10 ml) were well mixed in a 100 ml bolt-head flask. The grey mixture was cooled

and suspended in ether.

(c) Acetylation was carried out by allowing acetyl chloride (15 ml), in an equal volume of ether, to drop onto the grey suspension. The suspension was brought to pH 3.5 by careful addition of cold sodium carbonate (1M), silver chloride filtered off and the filtrate neutralised by adding sodium hydroxide (33%), with phenol red as indicator. The bulk of the sodium phosphate was removed by filtration after cooling. After the pH had been adjusted to pH8 silver phosphate and silver chloride were precipitated out by addition of an excess of silver nitrate (25%). Disilver acetyl phosphate was precipitated by further addition of silver nitrate. The white precipitate was washed with a few mls of ice cold water and a small quantity of alcohol. After further washing with alcohol (twice) and ether (twice) it was dried in vacuo over phosphorus pentoxide.

The crude salt was taken up in a slight excess of sodium chloride solution (0.25M) After removing the silver chloride, silver nitrate was added carefully and any yellow precipitate was discarded. The disilver acetyl phosphate was reprecipitated, washed and dried as described above.

Yield 4.1 gm.

%	Found	Calculated
% Ag	59.8	61
% P	8.9	8.8
% Acetic Acid	19.8	16.9

Silver was determined by titration against standard thiocyanate (205), phosphate by a modified method of Fiske and Subbarow (206) (207) or by ammonium molybdate precipitation (205).

Determination of acetyl phosphate

Acetyl phosphate was determined colorimetrically as the Ferric-complex of the hydroxamate (204).

Reagents:

(1) Hydroxylamine solution.

A 28% aqueous solution of hydroxylamine hydrochloride half neutralised by adding an equal volume of 14% sodium hydroxide. This was prepared daily.

(2) Acetate solution.

This was a solution of acetic acid and sodium acetate (0.1M), (2:8), with pH 5.4.

(3) Hydrochloric acid. This was prepared by dilution of concentrated hydrochloric acid (1 volume) with water (3 volumes)

(4) Trichloracetic acid solution. A 12% aqueous solution of trichloracetic acid.

(5) Ferric chloride solution. This was made up as a 5% solution of ferric chloride (hydrated) in hydrochloric acid (0.1N).

Procedure

The test solution (0.5 —1ml) was mixed with 1 ml of hydroxylamine solution. Acetate buffer (1 ml) was added

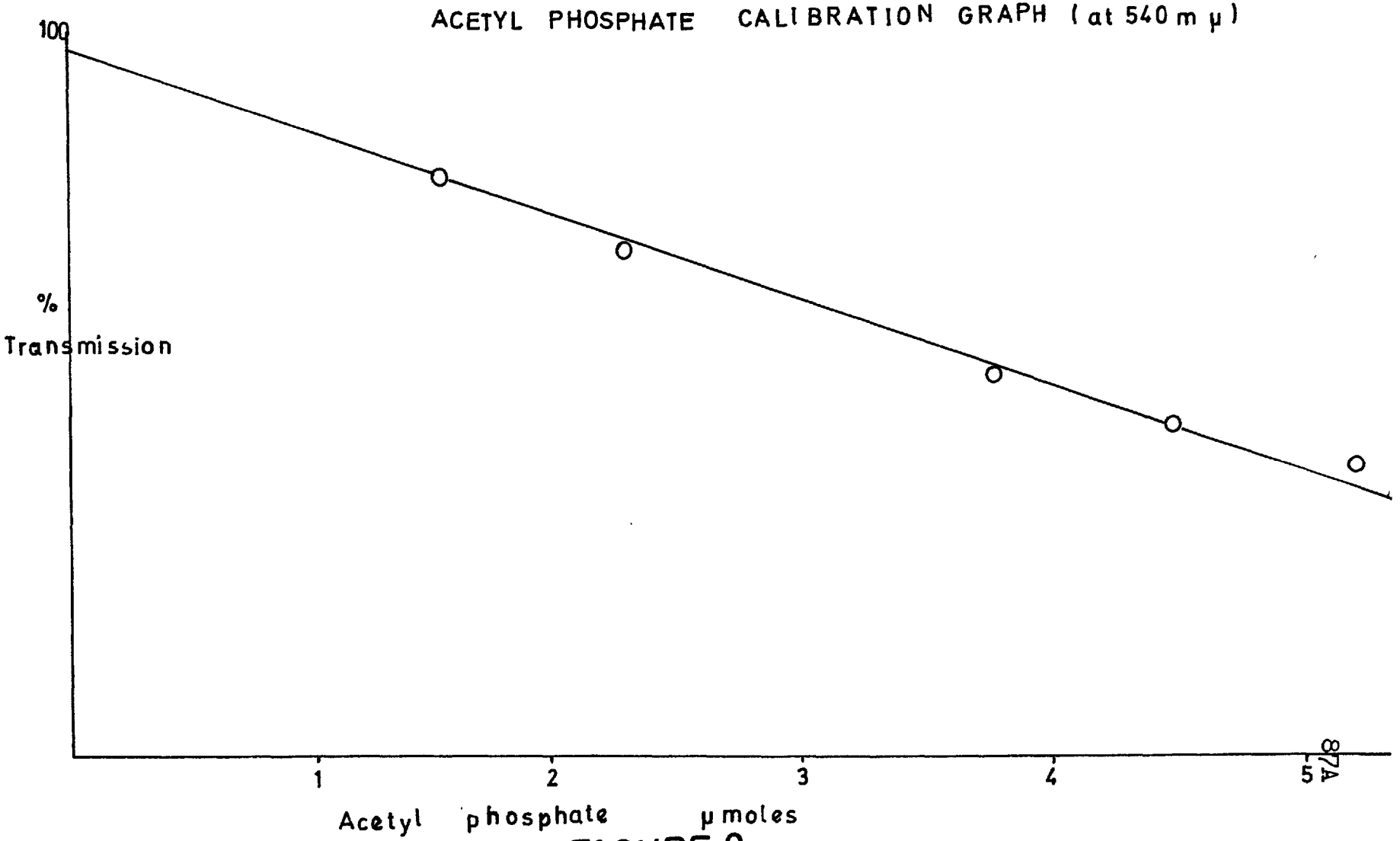

ACETYL PHOSPHATE CALIBRATION GRAPH (at 540 mμ)
100
% Transmission
1
2
3
4
5
Acetyl phosphate μ moles
87A

FIGURE 9

and volume adjusted to 3 ml.

The solution was allowed to stand 10 minutes. Hydrochloric acid (1 ml), trichloracetic acid solution (1 ml) and ferric chloride (1 ml) solutions were added. After 20 minutes, the % absorption at 540 mμ with respect to a blank was measured in a Unicam S.P. 500 spectrophotometer. The calibration graph is shown in Figure 9 .

The biochemicals used in the nitrogen fixing experiments are summarised below.

ADENOSINE 5' DIPHOSPHORIC ACID DILITHIUM SALT (ADP)
(B.D.H. Biochemicals).

COCARBOXYLASE
(B.D.H. Biochemicals).

DIHYDRO-NICOTINAMIDE ADENINE DINUCLEOTIDE DISODIUM SALT (NADH)
(B.D.H. Biochemicals).

α-KETOGLUTARIC ACID
(Mann Research Laboratories Incorp, New York).

NICOTINAMIDE ADENINE DINUCLEOTIDE (NAD)
(B.D.H. Biochemicals)

PYRIDOXAL 5' PHOSPHORIC ACID
(B.D.H. Biochemicals)

SODIUM PYRUVATE
(Mann Research Laboratories Incorp, New York).

DL-THIOCTIC ACID (DL- LIPOIC ACID).
(B.D.H. Biochemicals)

(VI) EXPERIMENTAL: RESULTS

[A] RESULTS : HYDROGENASE

TABLE IX Preliminary extraction of hydrogenase.

	μlH_2/ml/hr	mgN/ml	μlH_2/mgN/hr
(1) Disruptate	6,100		
Supernatant	170	0.65	260
"Red layer" extract	1,270		
(2) Disruptate	7,500	2.73	2,740
Supernatant	620	1.44	430
"Red layer" extract	1,800	0.24	7,500
(3) Disruptate	11,000	3.06	3,600
Supernatant	2,400	0.86	2,800
"Red layer" extract	1,800	0.14	12,800
(4) Disruptate	11,900	3.12	3,800
Supernatant	2,000	1.63	1,200
"Red layer" extract	6,000	0.59	10,200
(5) Disruptate	10,900	3.29	3,300
Supernatant	3,400	2,37	1,400
"Red layer" extract	4,200	0.50	8,400
(6) Disruptate	9,900	6.43	1,500
Supernatant	1,460	2.16	670
"Red layer" extract	2,700	0.40	6,750

μlH_2/mgN/hr = uptake of hydrogen (in microlitres) per milligram nitrogen (Kjeldahl) per hour.

SUPERNATANT refers to the supernatant after centrifuging the disruptate at 15,000 r.p.m. for 1 hr.

"Red layer" extract refers to the buffer extract of the upper red layer of the sediment (obtained by centrifugation of disruptate at 15,000 r.p.m. for 1 hr.).

TABLE X The Hydrogenase activities of the disruptates.

	$\mu l\ 1H_2$/ml/hr	Nmg/ml	$\mu l H_2$mgN/ml
(1)	1,170	0.58	2,000
(2)	3,000	0.59	5,100
(3)	3,100	0.90	3,500
(4)	3,100	1.40	2,200
* (5)	6,120		
(6)	7,500	2.73	2,700
* (7)	11,000	3.06	3,600
(8)	11,900	3.12	3,800
(9)	10,900	3.29	3,300
(10)	13,500	3.88	3,500
(11)	9,900	6.43	1,400

* Prepared from "freshly stored" bacteria c.3 months (some contaminant present).

TABLE XI Hydrogenase activity of particulate fraction 10,000 -20,000 r.p.m. (6,590-76,300 g. $r = r_{av}$)

	$\mu l H_2$/ml/hr.	mgN/ml.	$\mu l H_2$/mgN/hr.
(1) Disruptate	3,000	0.59	5,100
Supernatant (10,000 r.p.m.,1hr.)	1,700	0.42	4,000
Supernatant (20,000 r.p.m.,1 hr.)	780	0.38	2,060
Fraction (10,000-20,000 r.p.m.)	1,120	0.11	10,200
(2) Disruptate	13,500	3.88	3,500
Supernatant (10,000 r.p.m.,1hr.)	7,900	2.71	2,900
Supernatant (20,000 r.p.m.,1hr.)	4,500	1.75	2,600
Fraction (10,000-20,000 r.p.m.)	5,900	0.38	16,000

TABLE XII Differential centrifugation of disruptate.

	μlH_2/ml/hr.	mg/N/ml	μlH_2/mg/hr.
(1) Disruptate	3,100	1.40	2,200
Supernatant (12,500 r.p.m.,1hr.)	1,650	1.08	1,530
Supernatant (17,500 r.p.m.,1hr.)	1,080	1.01	1,070
Fraction (12,500-17,500 r.p.m.)	1,240	0.21	5,900
(2) Disruptate	3,100	0.90	3,400
Supernatant (17,500 r.p.n.,1hr.)	400	0.62	650
Supernatant (30,000 r.p.m.,1hr.)	170	0.51	330
Fraction (17,500-30,000 r.p.m.)	150	0.05	2,900
(3) Disruptate	1,170	0.58	2,000
Supernatant (30,000 r.p.m.,1hr.)	195	0.45	430
Supernatant (40,000 r.p.m.,1hr.)	130	0.41	320
Fraction (30,000-40,000 r.p.m.)	80	(0.03)	(2,700)

TABLE XIII Hydrogenase of stored disruptates

	Additions	$\mu l H_2$/ml/hr.	%
Disruptate		10,900	
Disruptate (15 months storage)		2,500	23
Disruptate (15 months storage)	0.5 ml 10^{-2}M sodium dithionite	4,700	43

TABLE XIV Hydrogenase of stored particles.

	Additions	$\mu l H_2$/ml/hr	%
Fraction 10,000 r.p.m. - 20,000 r.p.m. (2 months storage)		2,400	
Fraction 10,000 r.p.m. - 20,000 r.p.m. (24 months storage)	0.5 ml 10^{-2}M sodium dithionite	800	33

TABLE XV Effect of heat treatment on Hydrogenase

	μlH_2/ml/hr.	μlH_2/mgN/hr.	%
(1) Supernatant (20,000r.p.m., 1hr).	1,500	3,000	
Supernatant as above Placed in water at 100°C for 10min.	60		4
(2)Supernatant (30,000r.p.m., 1hr).	460	1,150	
Supernatant as above Placed in water at 100°C for 10 min.	46		10

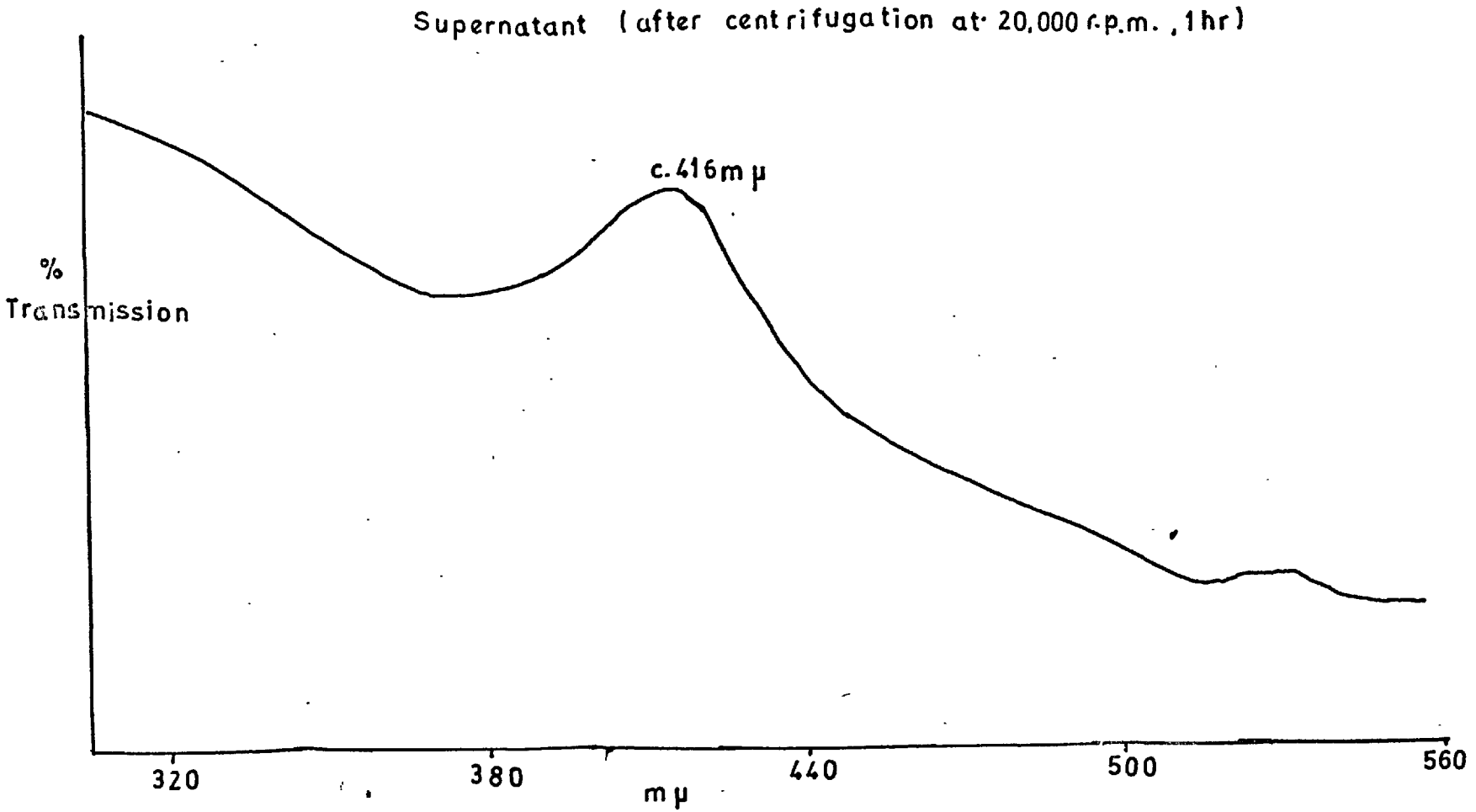

Supernatant (after centrifugation at 20,000 r.p.m., 1 hr)
c. 416 mμ
%
Transmission
320
380
440
500
560
mμ

FIGURE 10

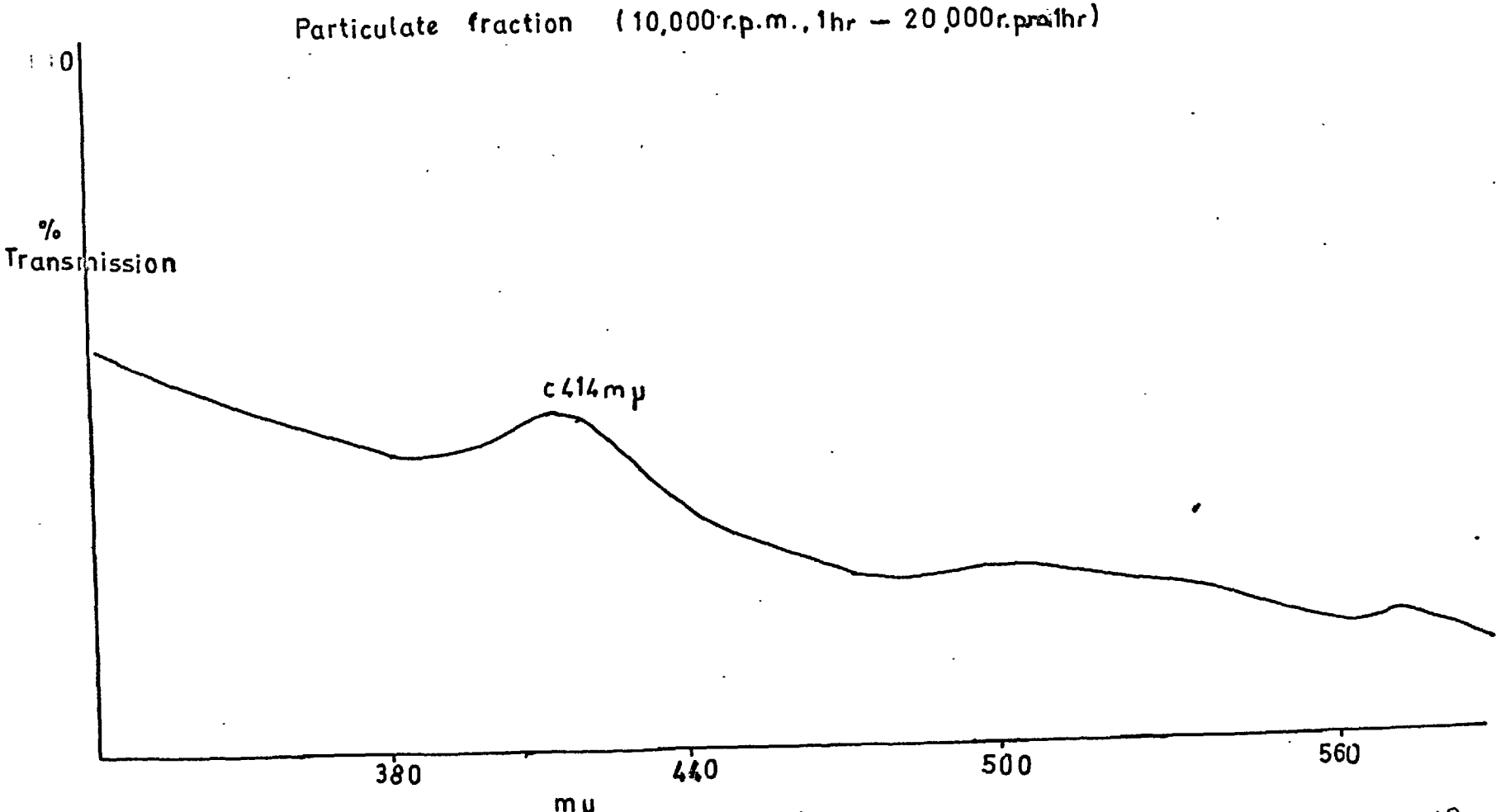

Particulate fraction (10,000 r.p.m., 1hr – 20,000 r.p.m. 1hr)
100
% Transmission
c 414 mμ
380
440
500
560
mμ

FIGURE 11

[B] RESULTS: CELL-FREE NITROGEN FIXATION

EXPERIMENT (1)	atom% ^{15}N Excess	Total Nitrogen mg	Increase in nitrogen µg
(1) Supernatant (5ml.)	0.77	3.00	75
(2) Supernatant (5ml.) **	1.81	3.26	192
(3) Supernatant (5ml.)	0.79	4.78	123
(4) Supernatant (5ml.) **	0.73	4.45	105

3gm. stored bacteria 40 ml. 0.05M Phosphate (pH 7.6.)

Disruption : 15 min.

Centrifugation M.S.E.Minor centrifuge 30 min.4,350 r.p.m. (2,730g)

** Also filtered through an oxoid membrane filter.

Exposure : 18 hr.

Atmosphere : 31.8% ^{15}N.

Plates : All positive.

EXPERIMENT (2)	atom% ^{15}N Excess	Total Nitrogen mg	Increase in Nitrogen µg.
(1) Supernatant (5ml.)	0.53	1.74	30
(2) Supernatant (5ml.)	0.54	3,51	61
(3) Supernatant (5ml.) **	0.41	2.32	31
(4) Supernatant (2.5 ml.) **	0.93	0.83	25

3gm. stored bacteria 40ml. culture medium. (20 hr. culture centrifuged 30 min, 4,350 r.p.m.)

Disruption : 15 min.

Centrifugation : 30 min. 4,350 r.p.m. (2,730)

Exposure : 21 hr. ** Filtered through an oxoid membrane filter.

Atmosphere : 30.6% ^{15}N

Plates A few whole cells on each plate.

EXPERIMENT (3)	atom% ^{15}N Excess	Total Nitrogen mg	Increase in Nitrogen µg
(1) Supernatant (5ml)	0.41	4.39	59
(2) Supernatant (2.5ml)	0.75	1.96	48
(3) Supernatant (5ml)**	0.56	1.81	33
(4) Disruptate (5ml)	0.29	5.54	52

** Disruptate placed in water at 100°C for 10 min before centrifugation.

3 gm stored bacteria in 40 ml. Potassium phosphate (0.05M) pH 7.6

Disruption : 15 min

Centrifugation : 1 hr. Spinco Model L ultracentrifuge 10,000 r.p.m. (6,590 $r=r_{av}$)

Exposure : 6 hr. pressure 71.7 cms.

Atmosphere : 31.8% ^{15}N (40% N)

Plates (1) + ve, (3) +ve.

EXPERIMENT (4)	atom % ^{15}N excess	Total nitrogen mg	Increase in nitrogen µg
(1) Supernatant (5ml)	0.91	3.08	91
(2) Supernatant (5ml)	0.40	2.77	36
(3) Supernatant (5ml)*	0.57	2.15	40
(4) Disruptate (5ml)	0.37	4.10	49
(5) Disruptate (5ml)**	0.01	4.20	00

* Disruptate heated to 100°C before centrifugation.
**Not exposed to ^{15}N

3gm. stored bacteria in 40 ml. pyrophosphate (0.1M, pH 7.6)

Disruption : 15 min

Centrifugation : 1 hr. 10,000 r.p.m. (6,590g $r=r_{av}$)

Exposure : 6 hr. pressure 75 cm.

Atmosphere : 31.8% ^{15}N (44%N)

Plates (1) +ve.

EXPERIMENT (5)	atom % ^{15}N Excess	Total Nitrogen mg.	Increase in Nitrogen µg.
(1) Supernatant (5ml)	0.53	3.47	60
(2) Supernatant (5ml)	0.36	3.22	38
(3) Supernatant (5ml)**	1.02	0.56	19
(4) Disruptate (5ml)	0.25	4.76	39

** Disruptate placed in water at 100°C for 10 min before centrifugation.

3 gm. stored bacteria	40 ml. culture medium 19 hr. culture membrane filtered.
Disruption:	15 min.
Centrifugation:	1 hr. 10,000 r.p.m. (6,590g, $r=r_{av}$)
Exposure:	6 hr. pressure 66.3 cm.
Atmosphere:	31.8% ^{15}N (40.5%N)
Plates:	(1) +ve(4) + ve

EXPERIMENT (6)	Atom% ^{15}N Excess	Total Nitrogen mg.	Increase in Nitrogen µg.
(1) Supernatant (5ml)	2.48	0.34	29
(2) Supernatant (5ml)	2.06	0.35	25
(3) Supernatant (5ml)*	2.22	0.31	23
(4) Supernatant (5ml)	0.08	0.37	1

* Placed in water at 100°C for 10 min.

Shake culture (78 hr) 40 ml.

Disruption: 15 min.

Centriguation: Spinco Model L Ultracentrifuge 12,500 r.p.m. (10,300g $r=r_{av}$)

Exposure: 6 hr. 52.8cm. pressure.

Atmosphere: 29.4% ^{15}N

Plates: (1) + ve (3) + ve

EXPERIMENT (7)	Atom% ^{15}N Excess	Total Nitrogen mg	Increase in Nitrogen µg.
(1) Supernatant (5ml)	0.13	2.18	9
(2) Supernatant (5ml) Pre-reduced HI/sucrose	0.37	2.48	30
(3) Supernatant (5ml) Pre-reduced Zn/Fe.	0.22	2.33	17
(4) Supernatant (5ml) Pre-reduced Zn/Fe.	0.52	1.37	23
(5) Supernatant (5ml)*	0.30	1.49	14.
(6) Supernatant (5ml) (Diluted)	1.00	0.74	24
(7) Supernatant (5ml*) (Diluted)	0.83	0.84	22
(8) Original culture(5ml) medium	10.8	0.06	21
(9) Original culture(5ml) medium + added ammonium chloride	0.37	1.55	18
(10) Disruptate (5ml)	0.14	5.28	24

* Placed in water at 100°C for 10 min.

3 gm. stored bacteria 40 ml. 18 hr. culture medium (Supernatant 15,000 r.p.m, 25 min)

Disruption: 15min.

Centrifugation: 1 hr. 15,000 r.p.m. (14,830g r-r_{av})

Atmosphere: 32% ^{15}N (39%N)

Exposure: 6hr. 73 cm. pressure.

Plates: (2) +ve (6) +ve (8) +ve.

EXPERIMENT (8)	Atom% ^{15}N Excess	Total Nitrogen mg	Increase in Nitrogen µg.
(1) Supernatant (5ml)	0.27	4.32	40
(2) Supernatant (5ml) Pre-reduced Zn/Fe	0.51	4.48	78
(3) Supernatant (5ml) Pre-reduced Zn/Fe	0.45	3.40	52
(4) Supernatant (5ml) Pre-reduced HI/sucrose	0.53	3.53	63
(5) Supernatant (5ml Pre-reduced HI/sucrose	0.41	3.83	54
(6)Supernatant (5ml)**	0.26	3.69	34
(7) Original culture medium (5ml)	0.50	1.54	37
(8) Disruptate (5ml)	0.24	5.20	72

** Placed in water at 100°C for 10 min.

3 gm stored bacteria 50 ml. 17 hr. culture medium (20,000 r.p.m. 30 min)

Disruption : 15 min.

Centrifugation : 1 hr. 20,000 r.p.m. (26,300g $r=r_{av}$)

Exposure : 6 hr. pressure 72 cm. (1)-(6)
51 cm. (7)-(8)

Atmosphere : 29.4% ^{15}N
18.4% ^{15}N

Plates : (2) +ve, (7) +ve.

EXPERIMENT (9)	Atom% ^{15}N Excess	Total Nitrogen mg	Increase in Nitrogen µg.
(1) Supernatant (5ml)	0.78	3.13	93
(2) Supernatant (5ml) Pre-reduced HI/sucrose	0.31	1.98	23
(3) Supernatant (5ml) Pre-reduced Zn/Fe.	0.64	3.49	85
(4) Supernatant (5ml)*	0.25	3.61	33
(5) Original culture (5ml)	1.06	1.39	56
(6) Disruptate (5ml)	0.40	5.10	77

* Placed in water at 100°C for 10 min.

3 gm. stored bacteria 40 ml. 18 hr. culture medium (10,000 r.p.m)

Disruption : 15 min.

Centrifugation : 1 hr 30,000 r.p.m. (59,310g $r=r_{av}$)

Exposure : 3 hr pressure 68.7 cm (1)-(4)
73.8 cm.(5)-(6)

Atmosphere : 25.4% ^{15}N

Plates: (5) +ve.

EXPERIMENT (10)	Atom% ^{15}N Excess	Total Nitrogen mg .	Increase in Nitrogen µg
(1) Supernatant (5ml)	0.36	1.85	27
(2) Supernatant (5ml)	0.27	1.76	19
(3) Supernatant (5ml) Pre-reduced Zn/Fe.	0.27	1.55	17
(4) Supernatant * (5ml)	0.14	1.56	8
(5) Supernatant** (5ml)	0.15	1.09	6
(6) Disruptate (5ml)	0.14	4.24.	24

(1) Not frozen during exposure to $[^{15}N]$

* Placed in water at 100°C for 10 min.

**Disruptate placed in water at 70°C for 10 min before centrifugation.

3 gm. stored bacteria 40 ml. 0.05M phosphate (pH7.6)

Disruption : 15 min.

Centrifugation : 1 hr. 30,000 r.p.m. (59,310g $r=r_{av}$)

Exposure : 3 hr. pressure 74.5 cm.

Atmosphere : 23.9% ^{15}N

Plates: (1) +ve.

EXPERIMENT (11)	Atom% ^{15}N Excess	Total Nitrogen mg .	Increase in Nitrogen . µg
(1) Supernatant (5ml) Pre-reduced HI/sucrose.	1.93	0.28	20
(2) Supernatant (5ml) Pre-reduced Zn/Fe	3.06	0.39	44
(3) Supernatant (5ml)	2.98	0.39	43
(4) Supernatant (5ml)*	3.03	0.41	46
(5) Original culture** (5ml)	1.19	0.93	54
(6) Disruptate (5ml)	1.40	0.79	54

* Placed in water at 100°C for 10 min.
** Culture contained 10^9 cells/ml.

160 hr. shake culture.

Disruption : 15 min.

Centrifugation : 1 hr. 30,000 rpm (59,310g $r=r_{av}$)

Exposure : 3 hr. (1)--(4) 70.3cm.pressure.
(5)--(6) 72 cm. pressure.

Atmosphere : (1)--(4) 26.4% ^{15}N

(5)--(6) 18.4% ^{15}N

Plates : (3) +ve. (4) +ve (5) +ve (6) +ve.

EXPERIMENT (12)	Atom% ^{15}N Excess	Total Nitrogen mg.	Increase in Nitrogen µg.
(1) Supernatant (5ml) Pre-reduced Zn/Fe	0.70	-	-
(2) Supernatant (5ml) Pre-reduced HI/sucrose	0.52	0.79	13
(3) Supernatant (5ml)	1.21	0.88	35
(4) Supernatant (5ml)*	0.24	0.74	6
(5) Supernatant (5ml)**	0.65	0.56	12.
(6) Original culture (5ml)	0.97	1.70	53
(7) Disruptate (5ml)	0.64	3.94	81

* Placed in water at 100°C for 10 min.

** Placed in water at 60°C before centrifugation.

3 gm. stored bacteria 40 ml. 20 hr. culture (10^7 cells/ml)

Disruption : 15 min.

Centrifugation : 1 hr. 30,000 r.p.m. (59,310g $r=r_{av}$) approx.

Exposure : 3 hr. pressure 73 cm. (1)-(4)
72 cm. (5)-(7)

Atmosphere : 32% ^{15}N (40% N)

Plates : (3) +ve, (6)+ve

EXPERIMENT (13)	Atom% ^{15}N Excess	Total Nitrogen mg	Increase in Nitrogen µg
(1) Supernatant (5ml)	0.00(5)	1.20	(2)
(2) Supernatant (5ml)*	0.43	1.20	20
(3) Supernatant (5ml) Pre-reduction Zn/Fe.	0.48	0.92	17
(4) Supernatant (5ml)**	2.18	0.12	10

* Placed in water at 100°C for 10 min.

** Placed in water at 70°C for 10 min.

3 gm."freshly" grown continuous culture bacteria.
45 ml. 0.05M phosphate.

Disruption: 15 min.

Centrifugation : 1 hr 30,000 r.p.m.

Exposure : 3 hr 72 cm. pressure

Atmosphere : 25.7% ^{15}N

Plates :

EXPERIMENT (14)	Atom% ^{15}N Excess	Total Nitrogen mg.	Increase in Nitrogen µg.
(1) Supernatant (5ml)	0.00	1.66	0.0
(2) Supernatant (5ml)	0.07	1.82	5
(3) Supernatant (5ml)*	0.12(4)	1.15	6
(4) Supernatant (5ml)**	0.16	1.04	7
(5) Supernatant (5ml)**	0.12	0.83	4
(6) Disruptate (5ml)	2.40	2.50	250

* Placed in water at 100°C for 10 min.

** Placed in water at 70°C for 10 min.

(1) and (2) contained grease.

Continuous culture ("freshly grown") bacteria that had been stored in its original medium.

Disruption : 15 min.

Centrifugation : 1 hr. 30,000 r.p.m.

Exposure : 3 hr. 70.2 cm. pressure

Atmosphere : 22.7% ^{15}N.

Plates : (3) +ve (4) +ve.

EXPERIMENT (15)	Atom% ^{15}N Excess	Total Nitrogen mg	Increase in Nitrogen μg
(1) Supernatant (5ml) Pre-reduced HI/sucrose	0.52	0.79	13.
(2) Supernatant (5ml) Pre-reduced Zn/Fe	0.89	2.40	69
(3) Supernatant (5ml)	0.53	2.00	34
(4) Supernatant (5ml)*	0.34	2.19	24
(5) Supernatant (5ml)	0.58	3.10	58
(6) Supernatant (5ml)	0.25	3.26	26
(7) Original culture (5ml)	0.66	1.20	26
(8) Disruptate (5ml)	0.48	3.92	61

* Placed in water at 100°C for 10 min.

3 gm stored bacteria 40ml. 17 hr culture (10^7 cells/ml)

Disruption : (a) 15 min.
(b) 10 min.

Centrifugation : 1 hr. (1)-(4) 30,000 r.p.m.
(5)-(6) 10,000 r.p.m.

Exposure : 3 hr pressure 73.6 cm.

Atmosphere : 32% ^{15}N (39% N)

Plates : (7) +ve

EXPERIMENT (16)	Atom% ^{15}N Excess	Total Nitrogen mg. .	Increase in Nitrogen µg.
(1) Supernatant (4 ml) Acetyl phosphate (1ml) ADP, NADH .	1.06	0.49	18
(2) Supernatant (4ml) Acetyl phosphate (1ml) ADP, NAD, $NaBH_4$.	0.70	0.53	13
(3) Supernatant (4ml) Acetyl phosphate (1 ml) ADP , $NaBH_4$	0.45	0.53	8
(4) Supernatant (4ml) 0.05M phosphate (1ml) $NaBH_4$.	2.12	0.31	23
(5) Supernatant (4ml) 0.05M phosphate (1ml)	2.41	0.34	28
(6) Supernatant (4ml)** 0.05M phosphate (1 ml)	0.99	0.43	15

** Placed in water at 100°C for 10 min.

190 hr. shake culture 45 ml. **Disruption : 15min.**

Centrifugation : 1 hr. 30,000 r.p.m. (59,380g $r=r_{av}$)

Exposure : 3 hr. pressure (1)-(4)74cm. (5)-(6)72 cm.

Atmosphere : 28.9% ^{15}N

Plates : (5) + **ve.**

Acetyl phosphate (added) 57 µmole, (present in (5) 3.6 µmole)

ADP 4.6µmole, NAD 1µmole, NADH 1 µmole.

Sodim borohydride ($NaBH_4$) c. 05 mg.

EXPERIMENT (17)	Atom% ^{15}N Excess	Total Nitrogen mg.	Increase in Nitrogen µg
(1) Supernatant (4ml) 0.05M phosphate (1 ml)	0.60	1.95	44
(2) Supernatant (4ml) Acetyl phosphate (1ml) ADP, NADH.	0.42	1.87	30
(3) Supernatant (4ml) Acetyl phosphate (1ml) ADP , $NaBH_4$.	0.40	2.24	34
(4) Supernatant (4ml) Acetyl phosphate (1ml) ADP , NAD, $NaBH_4$	0.31	2.50	29
(5) Supernatant (4ml) 0.05M phosphate (1 ml)	0.60	-	-
(6) Supernatant (5ml)* 0.05M phosphate (1ml)	0.33	2.55	31

* Placed in water at 100°C for 10 min.

3 gm. stored bacteria 40 ml. 20 hr. culture (10^8 cells/ml)

Disruption : 15 min.

Centrifugation : 1 hr 20,000 r.p.m. (26,300gg $r=r_{av}$)

Exposure : 3 hr.

Atmosphere : 25.7% ^{15}N

Plates: (3)?, (6) +ve.

Acetyl phosphate 50 µmole. ADP 4.6 µmole.

NADH 1.3 µmole, NAD 1.3 µmole, Sodium borohydride ($NaBH_4$) c.0.5 mg.

EXPERIMENT (18)	Atom% ^{15}N Excess	Total Nitrogen mg	Increase in Nitrogen µg
(1) Supernatant (4ml) Acetyl phosphate (1ml) NADH , ADP .	0.68	2.04	45
(2) Supernatant (4ml) Acetyl phosphate (1ml) ADP , NAD , $NaBH_4$.	0.62	2.16	43
(3) Supernatant (4ml) Acetyl phosphate (1ml) ADP , $NaBH_4$.	0.70	0.96	22
(4) Supernatant (4ml) Acetyl phosphate (1ml) ADP .	0.99	1.17	37
(5) Supernatant (4ml)** Acetyl phosphate (1ml) ADP .	0.43	1.90	26
(6) Supernatant (4ml)*** Acetyl phosphate (1ml) ADP	0.38	1.78	22

*** Placed in water at 100°C for 10 min.

3 gm stored bacteria 45 ml. 0.05M Phosphate (pH 7.6)

Disruption : 15 min.

Centrifugation : 1 hr 20,000 r.p.m. (26,300g $r=r_{av}$)

Exposure : 3 hr (**(5) 1 hr. only)

Atmosphere : 74 cm. pressure
32% ^{15}N (40%N)

Plates:

Acetyl phosphate c. 50 µmole, ADP . 4.6. µmole
NAD 1.2µmole, NAD H 1.2 µmole, sodium borohydride c. 0.5 mg.

EXPERIMENT (19)	%15N Excess	Total Nitrogen mg	Increase in Nitrogen µg.
(1) Disruptate (4ml) 0.05M phosphate (1ml)	0.24	4.79	42
(2) Disruptate (4ml) Acetyl phosphate (1ml) ADP , NADH .	0.38	4.91	71
(3) Disruptate (4ml) Acetyl phosphate (1ml) ADP , $NaBH_4$.	0.60	4.41	101
(4) Disruptate (4ml) 0.05M phosphate (1ml) $NaBH_4$	0.43	4.90	81
(5) Disruptate (4ml) 0.05M phosphate (1ml)	0.24	4.26	37
(6) Disruptate (4ml) Acetyl phosphate (1ml) ADP , NAD , $NaBH_4$.	0,43	4.40	66
(7) Disruptate (5 ml) $NaBH_4$	0.44	5.65	90

3 gm stored bacteria 40 ml 20 hr culture

Disruption : 15 min.

Exposure : 3 hr. pressure (1)-(4)71cm
(5)-(7)72cm.

Atmosphere : 25.3% ^{15}N (1)-(4) 27.0% ^{15}N (5)-(7)

Acetyl phosphate 64 µmole ADP 9.0 µmole
NADH 1 µmole, NAD 1 µmole.
Sodium borohydride ($NaBH_4$) 0.5 - 1.0 mg.

EXPERIMENT (20)	Atom% ^{15}N Excess	Total Nitrogen mε	Increase in Nitrogen με
(1) Disruptate (5ml) Sodium pyruvate, Cocarboxylase DL-Thioctic acid	0.11	5.28	21
(2) Disruptate (5ml)* ADP, α-ketoglutarate NADH, pyridoxal phosphate	0.17(5)	5.14	34
(3) Disruptate (5ml)	0.12	5.40	24
(4) Disruptate (5ml) Sodium borohydride	0.09(7)	5.30	19
(5) Disruptate (4ml) Acetyl phosphate ADP, NAD.	0.09	4.63	15
(6) Disruptate (4ml)** 0.05M phosphate (1ml)	0.00(6)	4.48	(1)
(7) Disruptate (4ml) Acetyl phosphate(1ml) ADP, NADH	0.05	4.55	8
(8) Disruptate (5ml) Sodium pyryvate, Cocarboxylase DL-Thioctic acid	0.03	5.85	8
(9) Disruptate (5ml) * α-ketoglutarate ADP NADH	0.01	5.46	2
(10) Disruptate (5ml)	0.00(4)	5.88	(1)

* After additions disruptate had a distinctive yellow colour.
** Boiled 10 min.
3.5 gm stored bacteria 50 ml phosphate (0.05M. pH7.6)
Disruption 15 min
Exposure 3 hr, 73 cm pressure (1)-(6) (25.3% ^{15}N)
70 cm pressure (7)-(10), exposed to $^{15}N_2$ after storage at 0°C for 36 hr. (26.9% ^{15}N)
Acetyl phosphate 32 μmole (5); 11 μmole (7)
ADP 2 μmole (2) & (9); 9 μmole (5) & (7); NAD 1 μmole
NADH 1 μmole; cocarboylase 2 μmole; DL-Thioctic acid 1 μmole
Pyridoxal phosphate 10 μmole; Sodium pyruvate 140 μmole
α-keto-glutaric acid 140 μmole., sodium borohydride c.1mg.

(VII) DISCUSSION

In the preparation of cell-free enzymes, the choice of method of disruption may be of prime importance. The extent of 'solubilisation' of the required enzyme(s) is an unpredictable quantity and will depend on the particular experimental conditions. A simple relationship between enzyme activity in whole cells and in extracts is not always observed since there is a considerable degree of uncertainty about the effect of exposing the contents of the cell to a new environment.

Certainly in attempting to assess the location of a particular enzyme serious consideration must be given to the method of disruption, particularly as cells of different micro-organismsare not equally easily disintegrated.(119).

[A] METHOD OF DISRUPTION.

Azotobacter cells have been disrupted by a number of methods. Their hydrogenase activities have been studied in cell-free preparations obtained by the following methods:-

(a) by grinding with alumina,(alumina method) (82) (86) (208) or with glass particles (208).

(b) by means of a bacterial mill (209).

(c) by means of a Hughes press (78) (210).

(d) by lysis with lysozyme (178).

(e) by osmotic shock (115).

(f) by ultrasonic disruption (115) (116)

Summaries of the principal work (in which hydrogenase activities are referred to either methylene blue or potassium

ferricyanide as acceptors appear in TABLES XVI, XVII, XVIII.

TABLE XVI

		μl/ml/hr.	Nmg/ml.	$Q_{H_2(N)}$	%
Green, 1953, (86).	Original extract	15,200	3.70	4,100	100
36 hr. culture Alumina method Acceptor 25 μM methylene blue	Supernatant	1,410	2.76	510	9
Hyndman, 1953, (82)	Alumina ground cells 900 g, 15 mins.			10,700	100
24-36 hr. culture.	Supernatant above 20,000g, 20 mins.			5,400	22
Alumina method Acceptor 150 μM potassium ferricyanide	Cell debris from above			14,800	70
	Supernatant above 16,500g 20 min.			4,080	16
	Particles from above.			19,800	4
	Supernatant above 29,00g, 20min.			1,500	5
	Particles from above			26,000	4
Cota-Robles 1957, (116)	Extract 2,000g, 20 mins.	16,100	6.72	2,400	100
24-36 hr. culture.	Supernatant 100,000g 2hr.	2,600	4.02	640	16
Sonic disruption Acceptor 8 μM methylene blue	Particles from above.	13,100	2.47	5,300	81
Jose 1959, (178).	Cells			8,450	
14-16 hr. culture.	Protoplasts			3,280	
Lysozyme method. Acceptor	Lysed Protoplasts			1,300	
	Protoplast membrane			600	
25 μM methylene blue	Protoplast soluble fraction			0	

Distribution of Hydrogenase in Azotobacter

TABLE XVII (117)

30 hr. culture		%
Acceptor 15μM methylene blue		
(a) Sonic disruption in Tris-Cl buffer pH7.5		
Total units*	3.12	
Large particles sedimented 10,000g 15 min.		30
Small particles sedimented 100,000g 2 hr.		60
Supernatant from above.		0
(b) Osmotic shock in Tris-Cl buffer pH7.5		
Total units.	3.75	
Large particles sedimented 10,000g 15 min.		69
Small particles sedimented 100,000g 2 hr.		3
Supernatant from above.		0

* One unit of hydrogenase was taken to be that amount required to oxidise 1 mole of H_2 per min at 30°C. (78)

TABLE XVIII (78)

48 hr. culture Hughes press	Q_{H_2}/ml/hr.	Q_{H_2}/mgN/hr.	%
Acceptor 15μM methylene blue			
(a) 0.1M phosphate pH7.5			
Whole organisms.	35,900	17,950	
Homogenate	10,900		
Particles sedimented 23,000g 1 hr.	22,000		
Supernatant from above.	3,000	1,500	28
(b) 0.2M Tris-Cl buffer pH8			
Whole organisms	35,900	17,950	
Homogenate	16,200		
Particles sedimented 23,000g, 1hr.	1,000		
Supernatant from above	10,400	5,200	63

Despite its relatively high initial cost the ultrasonic method has been widely used in bacterial disruption. Although it is considered a harsh method of disruption, it is rapid and no additions have to be made to the bacterial suspension. It also permits some standardisation of technique and in practice provides a reasonable degree of reproducibility.

The ideal method of disruption would have a high efficiency of disruption, i.e. would give rise to a maximum yield of 'soluble' protein with minimum denaturation. In practice a measure of the efficiency of disruption is not easily obtained and in previous work on ultrasonic disruption it has been expressed in terms of the % of the whole cells remaining, % decrease in turbidity and as % increase in soluble nitrogen..

Hughes (211) had preferred the latter in his investigations on the ultrasonic disruption of yeast and he measured the extent of cell disintegration in terms of the % release of nitrogenous material under standard conditions. With Rodgers (212) he found that the release of about 80% of the cell nitrogen into solution corresponded to 100% disruption. Marr (115) estimated the disruption in terms of % decrease in direct count and viable count. He also observed that although the decrease in turbidity was exponential this reflected the rate of release of

particulate material rather than the rate of disruption of whole cells.

With the present equipment Stewart (8) found that the efficiency of disruption of suspensions of Azotobacter containing 10^9 cells/ml varied with the volume of suspension. For a disruption time of 15 minutes he found that 2% of whole cells remained when 20ml of suspension was disrupted, whereas with a 40ml sample 15% whole cells remained. The latter corresponded to a 66% increase in soluble nitrogen.

In the present work much thicker suspensions of Azotobacter were disrupted (x10), with total (Kjeldahl) nitrogen varying from 0.59 - 6.43 mg/ml. As the efficiency of disruption is of particular importance when assessing errors in cell-free nitrogen fixation, it is worthwhile to examine the method of disruption.

The actual disruption is thought to result from streams of microscopic bubbles impinging on the bacterial cells. This causes pressure differences in the suspensions, "cavitation" (213). The important considerations other than those of cell type are:-

(i) the shape of the disruption vessel, the volume of disruptate, its viscosity and the efficiency of mixing (214).

(ii) the prevention of oxidative denaturation.

Oxidative denaturation may be enhanced if the tip of the ultrasonic probe is not completely immersed, Hughes (211) recommended that the tip should be immersed about 1.5 mm in the bacterial suspension.

(iii) the influence of pH and ionic strength on protein 'solubilisation'.

(iv) the prevention of local heating. An adequate means of cooling is necessary since an appreciable amount of heat is generated by cavitation, by loss of energy from the probe and by absorption of sound energy (213).

The ultrasonic method of disruption has recently been reviewed by Davidson (215). In the work described in this thesis the ultrasonic disruption of 'thick' suspensions of Azotobacter with particular reference to the hydrogenase activity and nitrogen fixing ability of the preparations has been studied. The bacterial suspensions contained between 0.58 - 6.43 mgN/ml and gave rise to supernatants containing 50-70% nitrogen (on total nitrogen basis). It is not possible to assess the extent of disruption when thick suspension of stored cells are used although the % soluble nitrogen does give some guide to the reproducibility of the method. The centrifugation procedure was based on an empirical approach resulting from experimental convenience rather than a rigorous analytical approach.

[B] DISTRIBUTION OF HYDROGENASE.

The hydrogenase in the extracts was measured with potassium ferricyanide as acceptor, the acceptor used by previous workers in this laboratory. In general two principal acceptors have been used to assay the hydrogenase of Azotobacter, methylene blue E_o^1 = 0.011v pH 7, 30°C, and potassium ferricyanide E_o^1 = 0.36v, pH 7, 30°C. It is convenient to note the principal work with these acceptors. (TABLE XIX). Some comment on the differences in values will be made at a later stage with reference to possible mechanisms of hydrogenase activity. In general, the methylene blue values are somewhat higher, although it may be noted that there have been reports that dyestuffs, such as methylene blue, may inhibit 'soluble' hydrogenases if they are not sufficiently pure (216).

It is also evident that there is some variation of hydrogenase activity with age of culture (86). This is an important consideration in the growth of Azotobacter cells for hydrogenase and 'nitrogenase' studies particularly by continuous culture.

In the preliminary studies on the hydrogenase of Azotobacter, grown by continuous culture, the bacteria were disrupted, centrifuged down at 15,000 r.p.m. for 1 hr and the hydrogenase activity of the characteristic 'red 'cytochrome' layer measured. After centrifugation the

TABLE XIX

	Q_{H_2} µl./ml/hr.	Acceptor	Conditions of assay
Nicholas 1960,(78)	6,700	15µM Methylene blue.	48 hr. culture. 0.2M phosphate pH7.5
	9,400		+ 0.5ml. 10^{-2} sodium dithionite
Jose 1959,(178)	8,450	25µM Methylene blue	14hr-16hr culture. 300µM phosphate pH8
Green 1953,(86)	24,000	25µM Methylene blue	18hr. culture.
	18,500		24hr. culture.
	12,400		41hr. culture
			0.2M phosphate pH8
	14,300	25µM Methylene blue	24 hr. culture.
Nicholas 1960,(78)	4,750	150µM potassium ferrycyanide	48 hr. culture.
	5,350		+0.5ml. 10^{-2}M sodium dithionite
Green 1953,(86)	3,200	150µM potassium ferricyanide	12hr. culture.
	3,100		18hr. culture.
	4,300		24hr. culture.
	3,900		30hr. culture.
	4,000		36hr. culture.
	2,100		48hr, culture.
Nicholas 1960,(78)	50	15µM Benzyl viologen	48hr. culture.
	50		+0.5ml. 10^{-2}M sodium dithionite.
	10		48hr. culture.
	10	Methyl viologen	+0.5ml. 10^{-2}M sodium dithionite

sediment consisted of two components, a lower dark brown sediment and an upper red layer. The hydrogenase activity of the pale yellow supernatant and that of the 'red layer', after buffer extraction, have been recorded.

Before discussing this and subsequent results it is necessary to outline some of the earlier work on cell-free hydrogenase preparations, in particular that of Stewart.

The earlier published work on the hydrogenase of Azotobacter (TABLES XVI, XVII, XVIII) has established that it is associated primarily with particulate fractions. It is difficult, owing to the varying experimental conditions, to decide which research has achieved the greatest extent of 'solubilisation' of this hydrogenase. Hyndman et alia (82) reported that the fraction (29,000g, 20 mins) had a high specific activity, 22,000-28,000 microlitres of hydrogen transferred to ferricyanide per hr per mg nitrogen and contained 10% of the total activity. Nicholas (78) has reported that, after disruption by the Hughes press method and centrifugation at 23,000g (1hr), 63% of the activity remained in the supernatant($QH_{2(N)}$ 5,200), whereas Cota-Robles (116) reported that, after ultrasonic disruption and centrifugation at 100,000g, 2hr), 16% of the activity remained in the supernatant($QH_{2(N)}$ 640). The most recent publication from Marr et alia (117), at the University of California, did not detect any 'soluble' hydrogenase

(in supernatant after centrifugation at 100,000g for 2 hr) but found that the activity in extracts prepared by osmotic shock was primarily associated with the large particles, whereas in extracts prepared by ultrasonic disruption it was associated principally with the small particles. The work from this laboratory indicated that hydrogenase was part of the cell envelope and that the 'small particle' fraction (10,000g, 10 min. - 100,000g, 2 hr) consisted of two types of particle containing ,

(i) RNA (from cytoplasm)

(ii) cytochrome and hydrogenase (from cell envelope).

Similarly, the hydrogenase of Proteus vulgaris has been reported as particulate and was not 'solubilised' by any of the methods(including sonic disruption) tried (217). In contrast, the hydrogenase of Clostridium pasteurianum was not sedimented by centrifugation at 144,000g for 30 minutes and had a specific activity of 0.75×10^6 microlitres of hydrogen per mg protein nitrogen per hour (acceptor methylene blue) (96).

The hydrogenase of Desulphovibrio desulphuricans has been extensively studied. Purified hydrogenase preparations with activities of 26×10^6 - 11×10^6 microlitres of hydrogen per protein nitrogen per hr (acceptor methylene blue) have been reported (218)(109). More recently, purified hydrogenase with Q_{H_2} (exchange) 54×10^6 microlitres of

hydrogen per mg protein nitrogen per hour has been obtained (98). Riklis and Rittenberg reported that the hydrogenase appeared to be bound to large molecules within the cell but could be released by treatment with trypsin in glycine solution. This was a modification of their earlier method, (of extraction of glycine (84)) and was necessary since the hydrogenase in some cases appeared to be in a different state of aggregation. It was found that sonic vibration did not facilitate release from this aggregate.

In this laboratory Stewart (8) had carried out the initial experiments on hydrogenase activity of preparations obtained by the ultrasonic disruption of Azotobacter, grown by continuous culture. He disrupted stored bacteria, grown by continuous culture and suspended in 40 ml. of 0.1M pyrophosphate buffer (pH 7.6), for 15 mins and after high speed centrifugation assayed the activity of the supernatant, using potassium ferrycyanide as acceptor. His findings are summarised below:-

(1) Preparations with high hydrogenase activity could be obtained by disrupting Azotobacter cells that had been stored at -10°C for periods of over a year.

(2) The hydrogenase in these preparations was principally associated with the particulate fraction sedimented below 35,000g for 1hr.

(3) A measureable amount of hydrogenase activity remained in the supernatant after centrifugation at 105,000g for 2 hrs and a small portion of this persisted in the supernatant after centrifugation at 173,000g for 3 hrs.

(4) Hydrogenase activity was not significantly enhanced by sodium dithionite or cysteine but these were able to restore much of activity lost on storage.

(5) The fraction, (10,000 - 20,000g, 1hr) was found to contain the highest specific activity of hydrogenase. Electron microscopic examination of this fraction showed the presence of two particles ($\frac{1}{4}\mu - \frac{1}{2}\mu$ diam). The electron microscope studies also showed that slight lysis had occurred in stored bacteria.

In the present work these studies were continued on the stored bacteria. The stored bacteria were a dark brown colour, although a light brown stringy layer tended to form on the outside. In all experiments with stored cells reported in this thesis, only bacteria from the interior were used, the outside layers being trimmed quickly away by means of a razor blade while the cells were frozen. In this way it was hoped that some standardisation of source of the cells was achieved.

There was some evidence of autolysis in stored cells from the earlier electron microscope studies and it was noted that shake cultures of stored bacteria exhibited low

hydrogenase and respiratory activities (e.g. 24 hr shake culture had a $QH_{2(N)}$ 270).

The approach adopted in the present work differed somewhat from that of Stewart. Stewart had attempted to obtain a "purified" hydrogenase fraction by differential centrifugation but had concluded that other means were necessary for any extensive purification of the hydrogenase of Azotobacter. The present work attempted to establish some basis for the subsequent cell-free nitrogen fixation studies in particular it was necessary to determine the hydrogenase activity in selected fractions. The concentration of bacteria in the disruptates was also varied in an attempt to establish an optimum level of hydrogenase. The results, however, are not easily interpreted in this respect.

It is interesting to note that the hydrogenase activities of the disruptates from freshly prepared and stored bacteria (Table X) are comparable. This was advantageous since it permitted the work to be concentrated on the stored bacteria. An examination of the supernatants showed that 30 - 70% of the nitrogen of the disruptates remained after centrifugation. Although, as has been noted in the experimental section these results are not easily interpreted in terms of efficiency of disruption, one can make some comparison to the earlier work. In the alumina method 10 - 20% nitrogen (0.5 - 1.5 mgN/ml) appeared in the

supernatant whereas in ultrasonic disruption of thick cell suspensions 90 - 95% of the nitrogen (3 - 4 mgN/ml) remained in the supernatant). (82) (86).

A summary of the activity of the disruptates (Table X) shows that the specific activities ($\mu l H_2$/mgN/hr) did not vary appreciably over the range of cell densities (corresponding to 0.58 - 3.88 mgN/ml). At the two extremes the hydrogenase activities of the 10 - 20,000 r.p.m. layer, Table XI , (an enlargement of the optimum fraction found by Stewart) did show high specific activities comparable with previous literature values. The % of activity remaining in the supernatant was 30% (3.88 mgN/ml) and 27% for the lower nitrogen concentration.

Differential centrifugation (Table XII) confirmed the particulate nature of hydrogenase. Relatively low nitrogen concentrations (0.59 - 1.90 mg N/ml) were used and the % remaining in the supernatant after centrifugation at 30,000 r.p.m. (1hr) was between 6 and 9% of the total activity of the disruptate.

It will be noted that in the subsequent nitrogen fixing experiments the nitrogen concentrations are about 0.78 - 1.2 mgN/ml. It was felt advisable on the basis of these results, both in the interests of economy of material and efficiency of disruption, to operate at

this level. Disruptates of this range of nitrogen 'concentrations' could be conveniently prepared by weighing out 3 gm of stored bacteria and disrupting these as a suspension in 40 mls of 'medium'.

The most interesting point raised in this work was that of the stability of the hydrogenase of Azotobacter. Table XIII shows that after 15 months storage at below 0°C the disruptate retained a significant fraction of its original hydrogenase activity, which was further restored by the addition of sodium dithionite. Hyndman et alia(82)reported some years ago that their hydrogenase preparationsfrom Azotobacter were more stable than hydrogenase preparations from other bacteria and, in washed particles, hydrogenase only lost its activity gradually over a period of weeks. This stability to storage is illustrated by the activity remaining in stored fraction after two years. This is particularly interesting when one notes the reported labile nature of nitrogen fixing systems. In extracts of Clostridium, it was reported that nitrogen fixing ability was lost on storage for 24 hrs (154) although hydrogenase activity was stable to heat treatment at 60° for 10 mins (5).

It is evident that if hydrogenase is the site of fixation its further 'solubilisation' is necessary although it is possible that part of the lack of success in cell-free

nitrogen fixation by extracts of Azotobacter may result from the absence of part of the electron transport system or of a suitable acceptor of nitrogen.

The absorption spectra of some centrifugates, prepared from stored bacteria in buffer are shown, Figures (10)(11), for comparison with earlier studies. The spectrum of the 20,000 r.p.m. (1hr) supernatant, after flushing with hydrogen shows high absorption at around 320 mμ and maxima at 416 mμ and at about 540 mμ. A similar maximum, at 414 mμ, was observed with the 10,000 - 20,000 r.p.m. fraction.

These spectra are similar to those obtained by Hyndman et alia (82), of washed particles of Azotobacter in phosphate buffer pH6,, These had maxima at 410 mμ and (a less well defined one) at 555 mμ.

Maxima at 419 mμ and 555 mμ, on reduction with hydrogen, have been reported in hydrogenase preparations of Desulfovibrio desulfuricans (218). However, in subsequent work on this organism, hydrogenase preparations were obtained free from bacterial cytochrome and were colourless (108). It was reported that cytochrome was not a factor of the hydrogen activating enzyme and that no absorption bands in the range 200-800 mμ were present which related to hydrogenase activity in the purified hydrogenase preparations (98).

C EXPRESSION OF RESULTS IN ^{15}N STUDIES ON NITROGEN FIXATION

Since the report by Nicholas (78) of consistent cell-free fixation by extracts of Azotobacter appeared some four years ago, the progress on this topic has been extremely slow.

In reviewing previous attempts at cell-free fixation in Azotobacter it was noticeable that little correlation between the results could be made.

Although the particular method of disruption is important, it is unlikely that any one method will give rise to an ideal nitrogen fixing extract. It is probable that both the method of disruption and the experimental conditions will need development so that maximum nitrogen fixation can be obtained, by reducing protein denaturation and other difficulties in the ^{15}N procedure to a minimum. In earlier studies Nicholas and Smith had reported some success in extracts of Azotobacter. Both had prepared extracts by ultrasonic disruption, but whereas the results of Nicholas were confined to extracts prepared by disrupting thin suspensions of Azotobacter, the results of Smith, on more concentrated suspensions, lacked consistency.

In the present work it was hoped that by combining the approach of Nicholas and Smith, namely the disruption

of thick suspensions of stored bacteria in young culture (medium), some advance would be made.

It soon became clear, however, that the results expressed as atom ^{15}N excess were not satisfactory since the important quantity was the amount of nitrogen fixed by an extract. In order to enable comparisons to be made with other work some reference to the amount of enzyme present, either in terms of protein, protein nitrogen or total (Kjeldahl) nitrogen, was necessary but there was little merit in expressing results solely in terms of relative concentrations either as atom% ^{15}N excess or as increases in nitrogen/mg protein/hr.

It was essential to express the results on a quantitative basis, i.e. increase in nitrogen, nitrogen fixed, to enable some estimation to be made of possible sources of error, e.g. contribution from small quantities of whole cells, soluble nitrogen etc.

In the present work no lack of 'nitrogen fixation' was found when the extracts were examined by the $\left[^{15}N\right]$ technique. It was necessary to ascertain to what extent this 'activity' was enzymatic activity.

It became evident that technique was more important than earlier work had suggested and that further refinements were necessary. In this respect it was

regrettable that no critical review had appeared in which the problems of nitrogen fixation were analysed in terms of experimental technique.

It is regrettable that in much of the published work details of the [^{15}N] technique have been reduced to a minimum. This is partly a result of 'publisher pressure' which results in the appearance only of successful experiments. For example it is noticeable that in the papers of Nicholas only two or three experimental results have been given in support of the claim of consistent cell-free nitrogen fixation. This work suggested that the only requirement for cell-free fixation, unfulfilled in previous work, was the culture medium (medium factor). If cell-free preparations from more concentrated suspensions were investigated, it is regrettable that no reference to the problems involved and the differences to the published work on thin suspensions has appeared.

Burris et alia (219) have repeatedly stated that 0.015 atom% ^{15}N excess represented a conservative level for establishing fixation by the [^{15}N] technique. Although this does represent a significant atom%^{15}N excess over the reproducibility of the mass spectrometer this statement is only valid if adequate consideration has been given to other factors relating to the overall

efficiency of technique. Table XX shows that a quantitative approach was essential

Wilson (13) (14) and subsequently Nicholas (7) have emphasised that the [^{15}N] technique is much superior to the Kjeldahl technique in the measurement of nitrogen fixation and suggested that a quantitative Kjeldahl in the former was not essential. In this respect it has been stated that the [^{15}N] technique was independent of the initial nitrogen of the extract and of sampling errors. [This emphasis on atom% ^{15}N excess appears to originate from the paper of Burris et alia (289).]

However, it is evident that results from [^{15}N] experiments should be expressed on a quantitative basis and thus Kjeldahl nitrogen and atom%^{15}N excess are mutually dependent. Direct comparison between atom%^{15}N excess is only valid when the Kjeldahl nitrogen of samples are identical or differ only by a small amount. A quantitative Kjeldahl is essential since as well as being necessary in the calculation of nitrogen fixed, it ensures that all the newly fixed nitrogen is recovered. Since [^{15}N] is usually present in small quantities failure to recover this quantitatively would give misleading results.

The theoretical basis for calculating the increase in nitrogen (nitrogen fixed) is given on Page 136.

TABLE XX SIGNIFICANCE OF ATOM ^{15}N EXCESS

Atom% ^{15}N Excess (1) Atmosphere	Atom% ^{15}N Excess (2) sample	Total Nitrogen of sample (3)	Increase (4) of Nitrogen
5.0	0.015	0.1	0.3.
		1.0	3.0
		10.0	30.0
10.0	0.015	0.1	0.15
		1.0	1.50
		10.0.	15.00
30.0	0.015	0.1	0.05
		1.0	0.50
		10.0	5.00
60.0	0.015	0.1	0.025
		1.0	0.250
		10.0	2.500
98.0	0.015	0.1	0.015
		1.0	0.150
		10.0	1.500

(1) Atom% ^{15}N excess of "exposing" atmosphere.
(2) Atom% ^{15}N excess of sample.
(3) Total nitrogen (mg) of sample.
(4) Calculated increase of nitrogen (ug)

$$\text{INCREASE} = \frac{\text{atom\%}^{15}\text{N excess sample (2)x total nitrogen (3)}}{\text{atom \%}^{15}\text{N excess atmosphere (1)}}$$

This equation for calculating the quantity of nitrogen fixed is identical to the equations used by Neess et alia (290) and Stewart (291).

Calculation of nitrogen fixed from ^{15}N measurements.

Let N = nitrogen at the end

i = increase in nitrogen (nitrogen fixed)

D = atom%^{15}N in exposing atmosphere.

x = " " " " of sample

Initially there is (N-i) mg nitrogen consisting of

$\frac{0.38}{100}$ (N-i) ^{15}N and $\frac{99.62}{100}$. (N-i) ^{14}N

There is an increase consisting of

$\frac{iD}{100}$ ^{15}N and $\frac{(100-D)i}{100}$ ^{14}N, $\frac{x}{100} = \frac{^{15}N \text{ Nitrogen}}{\text{Total Nitrogen}} = \frac{0.38(N-i)+iD}{100N}$

$$\text{i.e. } \frac{i}{N} = \frac{x - 0.38}{D - 0.38}$$

Increase in nitrogen =

$$= \frac{\text{Atom\% } ^{15}N \text{ excess sample}}{\text{Atom\% } ^{15}N \text{ excess atmosphere.}} \times \text{Total nitrogen} \quad \text{.....}$$

This calculation has made the approximation that

$$\frac{^{15}N}{^{14}N + ^{15}N} = \frac{^{15}N}{\text{Total N.}} \quad \text{(by weight)}$$

It is possible to convert atom%^{15}N to a weight ratio, as is shown by the equation below (220) .

Let $\frac{^{15}N}{^{14}N + ^{15}N} \cdot 100 = x = 100Z$

$\frac{^{14}N}{^{15}N} = \frac{14}{15} \left(\frac{1-Z}{Z}\right)$ (proportion by weight)

$$\frac{^{15}N}{^{14}N + ^{15}N} = \frac{1}{\frac{^{14}N}{^{15}N} + 1} = \frac{x}{100}$$

$$X\,(\text{Atom\%}^{15}\text{N by weight}) = \frac{1}{\frac{14\,(1-Z)}{15\,Z} + 1}\,.100$$

$$\frac{100\,.15Z}{14(1-Z)+15Z} = \frac{15 \times 100}{14(100-x)+15x} = \frac{15 \times 100}{1400+x}$$

Thus as $x \longrightarrow 100$ (i.e. $\%^{15}N$ increases to 100) X, atom $\%N$ by weight $\longrightarrow$ atom$\%^{15}N$ i.e. the ratios eventually become identical. By utilising this equation it is possible to correct both atom$\%^{15}N$ sample and atom$\%^{15}N$ atmosphere. e.g.

$$x \simeq \frac{0.38\,(N-i) + i\,D}{N} \qquad \text{(uncorrected)}$$

$$\text{Thus } \frac{15 \times 100}{1400+x} \simeq \frac{15.0.38\;100}{1400+0.38}\left(\frac{N-i}{N}\right) + \left(\frac{15.D100}{1400+D}\right)\frac{i}{N}$$

$$\text{i.e. } \frac{x}{1400+x} \simeq \frac{0.38}{1400.38}\cdot\left(\frac{N-i}{N}\right) + \left(\frac{D}{1400+D}\right)\cdot\frac{i}{N}$$

now when x is very small c.1%

$$1400 + x = 1400.38 = 1400$$

$$(1400 + D)x = 0.38 \cdot (1400 + D).\left(\frac{N-i}{N}\right) + \frac{1400.D.i.}{N}$$

$$\therefore (1400 + D)\,(x-0.38)N = 1400\,i\,(D-0.38) - 0.38\,D.i.$$

$$\text{R.H.S.} \simeq 1400\,i\,(D-0.38)$$

$$\text{i.e. } \frac{i}{N} = \left(\frac{1400+D}{1400}\right)\left(\frac{x-0.38}{D-0.38}\right)$$

e.g. when exposing atmosphere is 32 atom$\%^{15}N$

$$\frac{1400+D}{1400} = \frac{1432}{1400} = 1.02$$

The above calculation is only tenable when the exposing atmosphere is equilibrated. If an unequilibrated atmosphere (resulting from leakage of atmospheric nitrogen) is used, calculation of the atom$\%^{15}N$ from the

$\frac{28}{29}$ ratio will give a low value. An estimate of the actual atom%^{15}N may be made provided that the original equilibrated atom%^{15}N value, before leakage, is known.

In the present work unequilibrated atmospheres were used on a number of occasions, partly from economic considerations (high pN_2) and partly from convenience. The increases in nitrogen were calculated from the corrected atom%^{15}N which was determined as is shown below.

Consider an atmosphere, originally containing nitrogen of 32 atom%^{15}N into which atmospheric nitrogen has leaked and resulted in an atom%^{15}N value of 18.4. Let α be the fraction of ^{15}N, then in an equilibrated atmosphere the ratio of

$(^{14}N - ^{14}N)$, $(^{15}N - ^{14}N)$, $(^{15}N - ^{15}N)$ molecules

is $(1-\alpha)^2 : 2\alpha(1-\alpha) : \alpha^2$

thus for 32% atom%^{15}N, the ratio of

$(^{14}N - ^{14}N)$, $(^{15}N - ^{14}N)$, $(^{15}N - ^{14}N)$ molecules is

0.4624 : 0.4352 : 0.1024

For an equilibrated atmosphere of 18.4 atom%^{15}N, the ratio of $(^{14}N - ^{14}N)$, $(^{15}N - ^{14}N)$, $(^{15}N - ^{15}N)$ molecules is 0.6658 : 0.300 : 0.0358.

Since an air leak results principally in the appearance of $(^{14}N - ^{14}N)$ molecules, the ratio of $(^{14}N - ^{14}N)$, $(^{15}N - ^{14}N)$, $(^{15}N - ^{15}N)$ molecules is 992.4 : 7.57 : 0.046 in air, one can calculate the ratio of

$(^{14}N - {}^{14}N)$ $(^{15}N - {}^{14}N)$ $(^{15}N - {}^{15}N)$ molecules.

Before leakage there were 435.2. molecules of $(^{15}N - {}^{14}N)$ for every 102.4 molecules of $(^{15}N - {}^{15}N)$. After leakage, assuming no significant addition to $(^{15}N - {}^{14}N)$ and $(^{15}N - {}^{15}N)$ molecules, for every 300 molecules of $(^{15}N - {}^{14}N)$ there are not 33.58 molecules of $(^{15}N - {}^{15}N)$ but

$\frac{102.4}{435.2} \times 300 = 70.6$ molecules of $(^{15}N - {}^{15}N)$

Thus the proportion of $(^{14}N - {}^{14}N)$, $(^{15}N - {}^{14}N)$, $(^{15}N - {}^{15}N)$ molecules is 665.8 : 300 : 70.6

and this leads to an atom% ^{15}N $\left(\frac{^{15}N}{^{15}N + {}^{14}N}\right)$ of 21.32

This calculation was carried out when unequilibrated atmospheres were used. This does reduce the accuracy of the calculated increases in nitrogen although a check of the validity of using these corrected atom% ^{15}N values can be made from calculation of atom% ^{15}N value from $\frac{28}{30}$ and $\frac{29}{30}$ ratios. It must be pointed out that with both equilibrated and unequilibrated atmospheres, it is assumed that $(^{14}N - {}^{14}N)$, $(^{15}N - {}^{14}N)$, and $(^{15}N - {}^{15}N)$ molecules are identical with respect to nitrogen fixation, i.e. no preferrential fixation of one of these species occurs. Hoering and Ford found no isotopic effect with Azotobacter (292).

Selective fixation was not found by Burris and Miller (221) or by Carnahan and Mumford (230) with Azotobacter. In studies with cell-free fixing extracts of Clostridium it was concluded that the disruption of nitrogen did not

occur initially at the site of fixation since no exchange was detected (151). This also would support the assumption that the three molecules may be considered identical with respect to nitrogen fixation.

In conclusion, when using the [^{15}N] technique it is necessary to give some consideration to the type of result required, i.e. whether

(i) a comparison between atom%^{15}N excesses (identical sample nitrogen) is adequate for the purpose of the experiment.

(ii) a quantitative approach, with a low degree of accuracy (i.e. without the weight correction) is necessary.

(iii) a quantitative approach, with a high degree of accuracy (for direct comparison to other work e.g. to ammonia determinations) is necessary.

The latter is obviously required by the American workers since they have developed an alternative method for determining nitrogen fixation, namely by measuring the amount of ammonia produced. Some comment on this work appears at a later stage but one may note that the following are essential.

(i) high accuracy of measurement of atom%^{15}N.

(ii) adequate corrections in calculation of increase of nitrogen

(iii) high accuracy of measurement of ammonia.
The difference between the atom%^{15}N and the weight ratio is more significant at low atom%^{15}N (TABLE XXI)

TABLE XXI

x	X	% difference
5	5.33	6.6.
1.0	1.07	7.0
0.1	0.108	8.0

The accuracy of the Kjeldahl procedure and the measurement of atom%^{15}N have been discussed in the experimental section. In the present work the accuracy of measurement of increases in nitrogen was not better than c.4% since the accuracy of the Kjeldahl was c.2% and that of atom%^{15}N measurement was c.3%, although unlike the American workers a high degree of accuracy was not essential.

In his initial paper on cell-free fixation by Azotobacter, Bulen (222) has claimed an accuracy of about 0.5% on small increases of nitrogen, although no details on the calculation of results were given. In his latest paper he has also expressed his results in $\mu g.$ of nitrogen and indicated that the accuracy is 2 parts 1000(223).

It is convenient to consider here the previous work on Azotobacter with reference to pN_2 (and hence dissolved nitrogen) TABLE XXII. The relevant data for Clostridium

appears subsequently in TABLE XXXIII.

In general low pN_2 have been used since $[^{15}N]$ nitrogen is expensive. Burris and Wilson (197) have pointed out that the advantage of low pN_2 was that there was no need (from the expense point of view) to recover the exposing atmosphere.

(b) The Michaelis constant of 'nitrogenase' of whole cells of Azotobacter is pN_2 0.02 - 0.067 atm (29), (112).

It is worth noting that the solubility of oxygen (pO_2=1atm) is greater than that of nitrogen (pN_2=1atm) although under normal atmospheric conditions somewhat less oxygen is present in solution. Thus a low pN_2 pressure (with pO_2=0.2 atm) may not represent the optimum differential concentration over all experimental conditions.

Parker and Dilworth have suggested that the effect of hydrogen on nitrogen fixation in young Azotobacter cultures at different pN_2 may result from differential oxygen concentrations (111).

(c) It has been pointed out (29) that the Michaelis constant, defined as the concentration of substrate at half maximal velocity, could only apply to nitrogen fixation under whole cell conditions provided that fixation is rate limiting and that no restraint exists on the reaction from other growth requirement. The calculation

TABLE XXII PREVIOUS [^{15}N] STUDIES ON AZOTOBACTER

Reference	Total Pressure (atm)	pN_2 (atm)	Calculated Solubility of nitrogen mg /100 gm of water at 30° CC	Atom % ^{15}N	Composition atm.
(224)	1.0	1	1.62		
BURRIS, 1942 (225)	1.0	0.6	0.96	35	0.40xygen
WILSON, 1943 (45)	0.40	0.2.	0.32	25.84 2.58	0.2 Oxygen
NEWTON, 1953 (66)	1.0	0.15	0.24	32	0.85 Oxygen
MAGEE. 1956 (176)	1.0	0.1	0.16	30.36	(0.2 Oxygen (0.7 Helium
ALLISON, 1957 (68)	1.0	0.2	0.32	52	(0.2 Oxygen (0.6.Helium
NICHOLAS, 1960 (136)	1.0	0.3	0.48	30	(0.3 Oxygen (0.4 Helium
NICHOLAS, 1960 (179)	1.0	0.3	0.48	30	(0.3 Oxygen (0.4 Helium
BULEN , 1963 (227)	1.0	0.1	0.16	98	(0.2 Oxygen (0.7 Helium
NICHOLAS, 1963 (181)	1.2	0.2	0.32	33.4	(0.2 Oxygen (0.8 Helium
BULEN , 1964 (222)	1.0	0.2	0.32	95	(0.3 Hydro- (gen (0.5 Helium

of the Michaelis constant assumes that growth is exponential. These workers also observed that during growth in the Warburg vessels there was a tendency for the cells to agglutinate.

Thus it may be inappropriate to place too much "weight" on the Michaelis constant particularly with reference to cell-free extracts prepared from thick suspensions of Azotobacter cells.

It is worthwhile considering the solubility of nitrogen in recent work, particularly with respect to possible limitation of fixation by the rate of diffusion of gas. The calculated solubilities in μg /ml is given in TABLE XXIII.

TABLE XXIII

	pN_2	Solubility μg./ml at 30°C
AIR (224)	0.80	13.8.
PRESENT WORK	0.40	6.9
NICHOLAS(179)	0.30	4.8
BULEN (227)	0.10	1.6
MICHAELIS (29) constant	0.020	0.32
" (112)	0.067	1.07

The paper of Bulen (227) dealing with short term ^{15}N experiments on whole cells, is the most useful paper with respect to assessment of possible whole cell

contributions in cell-free fixation. Bulen reported that 43 µg ^{15}N/mgN were fixed in 30 minutes and that 127 µg ^{15}N/mgN were fixed in 60 minutes. The units are misleading since the exposure (Warburg) flasks contained 0.14 mgN/3ml and thus the amounts fixed are 6 µg ^{15}N/30 minutes and 17.8 µg ^{15}N/60 minutes respectively. Since the calculated soluble nitrogen (in 3ml) is 4.8 µg N, it is clear that a higher pN_2 would have been more desirable.

[D] INITIAL COMMENTS ON THE PRESENT WORK

The nitrogen content of the disruptates in these experiments vary between 0.78 - 1.20 mgN/ml, and a summary of their nitrogen fixing activity appears in TABLE XXIV.

It is clear that a valid correlation between experiments is dependent on the reproducibility of the method of disruption under the conditions adhered to in this work. The variation of the total nitrogen of the disruptates may give an indication of this although a greater variation is evident when a comparison between % soluble nitrogen in centrifugates is made. It was found that there was some variation in the total nitrogen values between supernatants from the same disruptate. Although this was due in some cases to dilution resulting from the "topping" up of centrifuge tubes it was also a result of

the heterogeneous nature of the disruptate. This is evident from the final two experiments. The heterogeneous nature of disruptates and supernatants prepared by sonic disruption has been noted earlier (110) (116) (217). Carr (110) has reported that electron microscopic examination of sonicates of Azotobacter showed these to be highly heterogeneous.

The variation of Kjeldahl nitrogen in the supernatants was further enhanced by the method of transfer. It was essential to make a careful transference of about 5 ml of supernatant without disturbing the sediment rather than an exact 5 ml quantity from the 13.5 ml centrifuge tube.

A primary consideration of the present work is to determine whether it has been advantageous to disrupt (old) bacteria in young culture (medium).

The "nitrogen fixing activity" of the disruptates is summarised in Table XXIV. This shows that in the 6 hr exposures the 'nitrogen fixed' is about 50 µg. The disruptates in experiments (3) (4), prepared in buffer have a higher value than those in experiments (5) (7) prepared in culture medium. It is possible that this reflects the reproducibility of the method of disruption or of the source of Azotobacter cells, but may also result from

'Order' refers to the order in which the disruptates were exposed i.e. whether in the initial or subsequent batches of 4 Thunberg tubes.

TABLE XXIV ACTIVITY OF DISRUPTATES

Expt. No.	Exposure time	*Order	Activity µg	MEDIUM AND ADDITIONS
(3)	6 hr	(1)	52	0.05M phosphate buffer pH 7.6.
(4)	6 hr	(1)	49	0.1M pyrophosphate buffer pH7.6.
(5)	6 hr	(1)	39	19hr culture medium (membrane filtered)
(7)	6 hr	(3)	24	18hr culture medium (15,000 r.p.m., 25 min)
(8)	6 hr	(2)	72	17 hr culture medium (20,000 r.p.m., 30 min)
(9)	3 hr	(2)	77	18 hr culture medium (10,000 r.p.m.)
(10)	3 hr	(2)	24	0.05M phosphate buffer.
(11)	3 hr	(2)	54	160 hr "shake" culture
(12)	3 hr	(2)	81	20 hr culture (10^7 cell /ml)
(14)	3 hr	(2)	250	"freshly" stored bacteria (some contamination)
(15),	3 hr	(2)	61	17 hr. culture (10^7 cells/ml)
(19)	3 hr	(1)	42	20hr CULTURE
		(1)	71	Acetyl phosphate ADP NADH
		(1)	101	Acetyl phosphate ADP $NaBH_4$
		(1)	81	$NaBH_4$
		(2)	37	
		(2)	66	Acetyl phosphate ADP, NAD
		(2)	90	$NaBH_4$ $NaBH_4$
(20)	3 hr	(1)	21	0.05M PHOSPHATE sodium pyruvate etc.
		(1)	34	α-ketoglutarate etc.
		(1)	24	
		(1)	19	$NaBH_4$
		(2)	15	Acetyl phosphate NAD etc
		(2)	(1)	Boiled
		(3)	8	Acetyl phosphate NADH etc
		(3)	8	Sodium pyruvate etc.
		(3)	2	α-keto glurarate etc.
		(3)	(1)	

the 'degassing' technique at this stage.

From the 3 hr exposures it can be seen that the disruptate of experiment (9) is particularly high for a disruptate prepared in culture medium, but may reflect a lower efficiency of disruption, i.e. more whole cells remaining after disruption. The other noteworthy point is the high value for 'freshly' stored bacteria (experiment 14). This is some 3x as great as most of the other values and would imply that the stored cells contain less whole cells (i.e. some autolysis had occurred).

The results of experiments (19) and (20) will receive attention at a later stage although one may note the improved degassing procedures in experiment (20) and the low activities of disruptates stored for 36 hr.

It is evident from TABLE XXIV that, in general, disruptates prepared in buffer are less active than those prepared in culture medium. This is easily explained on the basis that the culture (medium) provides oxidisable substrates and essential electrolytes to sustain the remaining whole cells. It was hoped that some indication as to whether culture medium provided either special requirements for nitrogen fixation or a suitable environment for cell-free enzyme, might be

obtained from a consideration of the activities of the centrifugates. In event the picture was complicated by the requirement for reproducible [^{15}N] procedure.

In the initial experiments (1) and (2) centrifugation was carried out in a M.S.E. 'Minor' centrifuge. After long exposure times, e.g. 18 hr, it was observed that the supernatants were diffuse and plates inoculated from these became heavily coated with whole cells after 36 hr incubation. It is noteworthy that the increases in nitrogen are not so much greater than those in subsequent experiments with shorter exposure times, e.g. experiments (3) and (4).

In experiments (3) and (4) and in subsequent experiments centrifugation was carried out in a refrigerated Spince Model L preparative ultra-centrifuge. In (3) and (4) significant 'blanks' were found. These blanks, were prepared by placing the disruptate in water at 100°C for 10 mins before centrifugation and their values could have resulted from a number of possibilities, e.g. fixation by whole cells, failure of the experimental technique to eliminate soluble or adsorbed nitrogen, or from abnormally high atom% ^{15}N excess resulting from the presence of methylamine in the mass spectrometer.

The possibility that whole cells, soluble or adsorbed nitrogen were responsible for these blanks led to a reappraisal of technique. In subsequent experiments, in order to have a comparable amount of protein in the blank, 5 ml of supernatant in a Thunberg tube was placed in water at 100°C for 10 minutes. It was hoped that this heat treatment would destroy any nitrogen fixing enzymes but would permit a direct comparison with untreated centrifugate of contributions from whole cells and soluble or adsorbed nitrogen.

The possible causes of high blanks are discussed below and it will be noted that examination of the literature did not resolve these problems.

The possible contributions to blanks are,

[E] contribution from small quantities of whole cells in centrifugates.

[F] contribution resulting from inadequate 'degassing' of dissolved nitrogen by the experimental technique.

[G] contributions resulting from the failure to 'degass' adsorbed nitrogen by the experimental technique.

[H] contribution resulting from the particular Kjeldahl procedure, either from fixation or exchange

of nitrogen or from formation of methylamine.

[E] POSSIBLE CONTRIBUTION FROM WHOLE CELLS

It is evident that the blanks reflect the overall efficiency of technique, i.e. the efficiency of disruption, of centrifugation, of transfer to the exposure vessels and of [^{15}N] degassing procedures. When concentrated suspensions of bacteria are disrupted it is likely that, in the absence of sensible precautions, whole cells could make significant contributions to the results. After the centrifugation and transfer of a supernatant has been carried out it is necessary to be able to detect any whole cells remaining and also to assess their contribution to the result.

Previous published work does not provide a specific correlation between whole cell concentration and nitrogen fixed. [Jensen (22)(293) had concluded that the production of 10^9 cells was associated with the fixation of 0·1mg of nitrogen and this correlation has been considered recently (294).]

In their review article on nitrogen fixation (197) Burris and Wilson confined themselves to stating that it required a readily detectable number of contaminating organisms to fix an appreciable amount of [^{15}N]. They suggested that 10^4 contaminants /ml should be detectable but did not enlarge on the suitable methods for estimating either small quantities of organisms or their contribution to nitrogen fixed.

Magee and Burris (176) claimed cell-free fixation in extracts of Azotobacter on the basis that, over the 1 hr exposure period employed, the presence of several million cells was necessary to produce comparable isotopic enrichment to that obtained in the cell-free extract. Burris et alia (177) subsequently reported that sonicates of Azotobacter catalysed nitrogen fixation at 25-100% of whole cells. They also claimed that phosphate stimulated nitrogen fixation 2-4 fold in sonicates and whole cell suspensions and that the calculated relative nitrogen fixation per remaining intact cell of the sonicate averaged twice that of a control of whole cells. Unfortunately no details of these claims and in particular the relevant calculations were given.

Jose and Burris (178) reported that controls containing 10^5 Azotobacter cells/ml did not take up $[^{15}N]$ in any measurable quantity during a 2 hr exposure to $^{15}N_2$(pN2 = 0.1 atm). They reported that 0.3 µg.at. cell nitrogen corresponded to 10^5 cells/ml but their paper can be criticised on the ground that it lacks much important detail. The detail on the $[^{15}N]$ technique is particularly sparse and it would be interesting to know the accuracy of measurement of atom% ^{15}N excess in the mass spectrometer when only small quantities

of nitrogen were available.

Although the bulk of the results have been expressed only as atom%^{15}N excesses it is possible to make one useful calculation on the uptake of $[^{15}N]$ by whole cells.

The following figures were presented TABLE XXV (TABLE 3 (178), partly reproduced).

TABLE XXV

THE EFFECT OF REAGENTS USED TO PRODUCE THE PROTOPLAST ON $^{15}N_2$ UPTAKE

	N µg./ ml	Total Count	Viable Count	Atom%^{15}N Excess
II CELLS	208	16×10^8	5×10^8	0.949
CELLS Lysozyme Treated	200	8×10^8	4×10^8	0.950

The cell suspensions were exposed to 0.1-0.2 atom N_2 (32 atom%^{15}N) presumably in a Warburg vessel, volume 3.2 ml., exposure time 1-2 hr.

$$\text{INCREASE IN NITROGEN} = \frac{0.949}{32-0.38} \times 3.2 \times 208 = 19.7 \mu g.$$

i.e. 5×10^8 viable cells will give rise to 20 µg. nitrogen after 2 hr exposure.

A similar calculation can be made from the paper of Williams and Wilson (31). They reported that cells of Azotobacter adapted to citric acid cycle substrates with negligible fixation of nitrogen.

TABLE XXVI From TABLE 1 (31)

TEST OF FIXATION BY AZOTOBACTER VINELANDII DURING OXIDATION OF SUCCINATE

0.096 mg	LAG min.	$QO_2(N)$	^{15}N ATOM% EXCESS
No CO	110	14,500	0.012 0.005
5% CO	110	14,600	0.005 0.005 0.001

The cell suspensions (0.06-0.10 mgN) were exposed for 2½ hr in Warburg vessels to 10% $^{15}N_2$ (30 atom% ^{15}N excess) and 8-10 'replicate' Warburg vessels were pooled for Kjeldahl digestion and subsequent [^{15}N] analysis.

The calculated increase in nitrogen (by low concentration of cells, of high QO_2) = $\frac{0.012 \times 10^3 \times 0.096 \times 10^3}{32}$

= .36 µg . per 10 'replicates'

The most useful calculations on nitrogen fixation by whole cells can be made from the papers of Bulen (227), (228).

Bulen (228) in his paper on fixation and the uptake of molybdate and tungstate by whole cell suspensions of Azotobacter exposed the cells for 3 hrs in Warburg flasks to 10% nitrogen containing 95 atom% ^{15}N. The respiratory activity of the suspensionswas between 14,000 and 16,000 microlitres oxygen per mgN per hr. He found

that in the presence of 10^{-5}M molybdate and 10^{-3}M tungstate 141 µg. of $[^{15}N]$ per mgN were fixed and in the presence of 10^{-5}M molybdate and 10^{-3} tungstate 29 µg. $[^{15}N]$. This leads to the actual increases in nitrogen fixed as

(i) Warburg vessel contained 0.15 mgN,

$$\text{Increase} = 141 \times 0.15 \times \frac{100}{95} = 22.2 \ \mu g \ N$$

(ii) Warburg vessel contains 0.13 mgN,

$$\text{Increase} = 29 \times \frac{0.13 \times 100}{95} = 3.9 \ \mu gN$$

From the succeeding paper by Bulen (227) on short term $[^{15}N]$ incorporation by Azotobacter it is possible to calculate the growth constant

after 30 mins, Total nitrogen present =

$0.14 \times 10^3 + 0.14 \times 43 = 146 \mu g$

after 60 mins, Total nitrogen present =

$0.14 \times 10^3 + 0.14 \times 127 = 158 \mu g$

$$k_{(30-60)} = 4.606 \log_{10} \frac{158}{146} = 15.7 \times 10^{-2} \ hr^{-1}$$

This corresponds to a mean generation time of 4.4 hrs and relates to growth in Warburg flasks. For shake flasks, a k value of 0.30 hr^{-1} has been reported (26); TABLE XXVI summarised previous values of k for Azotobacter.

If we accept the value of k calculated from the experiments of Bulen, in the present work it is possible to calculate the amount of 'viable nitrogen' that would

TABLE XXVII

PREVIOUSLY REPORTED VALUES OF K, SPECIFIC RATE CONSTANT OF GROWTH

	K hr^{-1}	Mean Generation Time (hr)	Method
Wilson, Lynd (1943) (226)	0.30	2.3	Microrespiration
Alexander, Wilson (1954) (27)	0.264	3.1	Kjeldahl
Wilson, Roberts (1954) (49)	0.25	2.8	Isotopic
Allison, Burris (1957) (68)	0.06	11.6	Isotopic
Parker, Scutt (1960) (29)	0.41 0.25	1.7 2.5	Kjeldahl
Wursteman (1962) (28)	0.41	1.7	Kjeldahl
Bulen (227)	0.15	4.4	Kjeldahl Isotopic

give rise over a 3 hr exposure period to the increases reported herein (c50 μg.)

Thus for an increase of 50 μg., if x = viable nitrogen

$$3 \times \frac{2.3}{4.4} \log_{10} 2 = 2.3 \log_{10} \frac{(x + 50)}{x}$$

$$x = 82 \ \mu g.$$

i.e. if viable cells are present in quantity equivalent to 82 μg of Kjeldahl nitrogen, then after a 3 hr exposure the increase in nitrogen (nitrogen fixed) will be 50 μg.

In the present work the total nitrogen of the centrifugate is about 3 mg.

i.e. % viable cells (in terms of total nitrogen)

$$= \frac{82}{3} \times \frac{100}{1000} = 2.7\%$$

Thus, as well as the efficiency of disruption, the centrifugation and subsequent transfer to the sterile exposure vessels, are steps of some importance, since the overall viable cell population (total nitrogen basis) must be reduced to below 2% of the supernatant.

It is evident that errors resulting from whole cells could be reduced by shortening the exposure time. It was with this in mind that the 6 hr exposure time was reduced to 3 hr but before discussing the results it is convenient here to make a brief comment on technique. In contrast to Nicholas, who examined his extracts after high speed

centrifugation and was not able to detect whole cells by microscopic examination or plating experiments, it became standard practice to examine the centrifugates at the end of the exposure to $[^{15}N_2]$. The supernatants were examined both by microscopic and by plating out procedures but although plating out procedure did sometimes detect whole cells it was usually impossible to make an accurate estimate of them (the Hawskley Thoma counting chamber requiring at least 10^5 cells/ml). Brown et alia have examined the techniques for counting Azotobacter(295).

It was also impossible to obtain an estimate of the efficiency of disruption in terms of whole cell population since the cell debris in the disruptates did not allow an accurate estimate. It is evident that further effort is still required on these two points. An attempt to assess the contributions from whole cells was made by exposing the young cultures (medium), used in the preparation of the disruptate, under identical conditions. These results are summarised in TABLE XXVIII

In experiment (7) the uptake of $[^{15}N]$ by the medium is of the same order as the increases for the centrifugates. The activity of the disruptate is low for 6 hr exposure, particularly if medium is only required to supply oxidisable substrate. In experiment (5) the culture medium has a particularly high value, although somewhat lower than that of the supernatants. Plating

TABLE XXVIII

Experiment No.	Disruptate µg	Original culture medium µg	Original culture µg	Exposure time.
(7)	24	21 18*		6 hr
(8)	72	37		6 hr
(9)	77		56	3 hr
(11)	54		54	3 hr
(12)	81		53	3 hr
(15)	61		26	3 hr

* Ammonium chloride added before exposure to $^{15}N_2$.

out procedure detected whole cells and it is probable that poor transfer technique was responsible for this high result.

An interesting feature of experiment (7) is that No. 9, culture medium with ammonium chloride added before exposure to $[^{15}N]$ and No. 8, with no added ammonium chloride, have about the same values. This would suggest that the efficiency of removal of 'free' $[^{15}N]$ (as opposed to fixed ^{15}N), i.e. efficiency of 'degassing', is an important consideration, since ammonia is considered a powerful inhibitor of fixation in Azotobacter. The possible contribution of soluble and absorbed nitrogen will now be considered.

(F) POSSIBLE CONTRIBUTIONS FROM DISSOLVED NITROGEN

The calculated solubility of nitrogen in this work has already been compared to that in other work (TABLES XXII, XXIII). It is possible to calculate the possible error that would arise if the experimental techniques fail to remove effectively all soluble nitrogen.

In experiment (3), exposure pressure = 71.7 cm

pN_2 = 28.7 cm

the Henry's Law constant (at 30°C) for nitrogen = 6.68×10^{7} (224)

i.e. mole fraction of nitrogen in solution

$= \frac{287}{6.68} \times 10^{-7} = 4.3 \times 10^{-6}$

∴ solubility of nitrogen in 5 ml of water at 30°C

$= \frac{28}{18} \times 4.3 \times 10^{-6} \times 5 = 33 \mu g.$

TABLE XXIX

Conditions	Procedure	Atom% ^{15}N Excess	Total nitrogen mgm.	Increase in nitrogen µg.
5ml. 0.05M phosphate buffer in Thunberg tube, shaken for 3 hr. Atmosphere 18.71% ^{15}N (corrected)	(1) Usual, NH_4Cl added to Thunberg tube before exposure.	0.05	2.77	8
	(2) As in (1) refluxed with sulphuric acid before adding "mercury" catalyst.	0.04	1.54	3
	(3) Buffer refluxed with sulphuric acid before adding NH_4Cl and "mercury" catalyst.	0.05	2.61	7
	THEORETICAL DISSOLVED NITROGEN			c.38

If, after the degassing techniques, $\frac{1}{5}$ of this nitrogen remains and reaches the mass spectrometer, then

$[^{15}N]$ from this source = $\frac{31.8}{100} \times 6.7 = 2\ \mu g$

(atmosphere contained 31.8% ^{15}N)

Initial quantity of $[^{15}N] = 1.81 \times 10^{3} \times \frac{0.38}{100} = 6.6\ \mu g$

(centrifugate contained 1.81 mg of nitrogen)

$\therefore$ Total $[^{15}N] = 8.7 \mu g$

atom% $^{16}N = \frac{8.7}{1.81} \times \frac{100}{1000} = 0.48$

i.e. Atom% ^{15}N excess = 0.10.

An experiment was carried out to assess the contribution arising from soluble nitrogen and this is summarised in TABLE XXIX. 5 mls of phosphate buffer were exposed by the usual procedure in Thunberg tubes. Ammonium chloride was added both before exposure and after preliminary degassing in an attempt to determine the possible sources of error. It can be seen that although some increases of nitrogen were found, they were variable but small. The atom% ^{15}N excesses were low and just outside the reproducibility of the mass spectrometer. One may thus conclude that with proper degassing techniques soluble nitrogen can not make a great contribution to the experimental results.

(9) POSSIBLE CONTRIBUTIONS FROM ADSORBED NITROGEN

In view of the large surface areas of protein it is feasible that extensive adsorption of nitrogen might occur.

If this nitrogen is not removed and became fixed or exchanged during the experimental procedures prior to mass spectrometric analysis then this could result in a significant contribution to the measured atom%^{15}N. There is also the possibility that the glass apparatus used in the [^{15}N] technique could become 'contaminated' and make a contribution to the atom%^{15}N excess of the succeeding samples. Thus the following possibilities require consideration.

(1) Possible contribution from adsorption on glass apparatus. Adsorption of [^{15}N] can occur on:

(i) Exposure vessel (Thunberg tubes).

(ii) Kjeldahl apparatus.

(iii) Gas storage bulb in mass spectrometer (Toepler pump)

(2) Possible contribution from adsorption on protein.

(1) Adsorption on glass.

(i) It is interesting to compare a blank value with the amount of nitrogen present in the exposing atmosphere. If the values for [illegible] (3), experiment (3), are taken e.g. 5 ml of centrifugate (blank) containing 1.81 mg N exposed to $^{15}N_2$, 31.8 atom%^{15}N, pN_2 = 28.7 cm (Total pressure 71.7 cm) in a 25 ml Thunberg tube, gas phase 20 ml approx, i.e. 20 ml of 'atmosphere' (at 30°C and 28.7 cm) contain $\frac{28}{22.4} \times 10^3 \times 20 \times \frac{287}{700} \times \frac{273}{303}$ = 8.5 mg N i.e. 2.7 mg^{15}N

thus for a blank of 33 µg ,

$$\text{blank} = \frac{33}{8.5} \times 10^{-3} \times 10^{2} = 0.4\%$$

(as % of total nitrogen in atmosphere).

Prior to fixation it is unlikely that any $[^{15}N]$ adsorbed on the Thunberg vessel could contribute to the blank, particularly in view of the thorough cleaning procedures employed.

(ii) Contamination of the Kjeldahl apparatus by $[^{15}N]$ or $[^{15}NH_3]$ is a more serious consideration, since there is the possibility that samples of high atom% ^{15}N excesses could cause enrichment in succeeding samples, if appreciable amounts of adsorbed nitrogen or adsorbed enriched ammonia remained.

Huser (201) in his researches on soil microbiology has examined the problem of ammonia adsorption on glass and part of his data on contamination in the Parnas apparatus is reproduced below.

TABLE XXX (TABELLE 2 (201))

2. CONTAMINATION IN DER PARNAS APPARATUR ^{15}N, ADSORPTION AM DER GLAS DER APPARATUR.

A	Dest. 3 mg Atom% ^{15}N	im Destillat Atom% ^{15}N
1	0.3620	0.3636
2	0.3620	0.3617
3	48.20	48.164
4	0.3620	0.7042
5	0.3620	0.4007
6	0.3620	0.3715

The contamination, resulting from the 48.2 atom% ^{15}N

ammonia, could be reduced by extended steam distillation. It is possible to put this contamination on a quantitative basis.

Total $[^{15}N]$ in Parnas apparatus = $\frac{48.2}{100} \times 3 \times 10^3 = 1,446$ µgm

In subsequent samples, Total $[^{15}N] = \frac{0.704}{100} \times 3 \times 10^3 = 21.1$ µgm

$[^{15}N]$ originally present = 10.8 µgm

Gain in $[^{15}N]$ from Parnas apparatus = 10.3 µgm

(i.e. 21 µgm. N as NH_3 adsorbed, assuming no isotope effect was operative.)

i.e. % Remaining in Parnas apparatus = $\frac{10.3}{1,446} \times 100 = 0.7\%$.

It is not likely that significant error arose from this source since the atom%^{15}N excesses of the sample did not differ greatly enough for the order of Kjeldahl analysis to be an important factor.

(iii) Contamination of the storage bulb, (Toepler pump), in vacuum line of the mass spectrometer is the most serious consideration since the atom%^{15}N values of atmospheres (.32 atom%^{15}N) were analysed in the mass spectrometer.

It has been shown that residual nitrogen in a gas storage bulb, which contained 250 ml. of 30%^{15}N nitrogen before evacuation, can make a significant contribution to the atom%^{15}N excess of the subsequent samples (187).

The capacity of the storage bulb in the mass

spectrometer was c100 ml and it was usual practice to have 20 ml of atmosphere at 5 cms pressure for analysis. i.e. 20 ml at 303°K and 5 cm pressure contain

$$\frac{28}{22.4} \times 10^{-3} \times 20 \times \frac{50}{760} \times \frac{273}{303} = 1.5 \text{ mgN}$$

at 1 mm pressure this volume would contain 30.2 µg N

i.e. 10 µg [^{15}N]

Thus it is evident that the 'degassing' of the Toeppler storage bulb is extremely important, particularly if further adsorption of nitrogen on 'dirty mercury' can occur. It is evident that when atom% ^{15}N value of 'normal' nitrogen, nitrogen generated from ammonium chloride, is measured the pressures and volumes should correspond to those of the nitrogen generated from the samples. It is evident that proper 'degassing' procedure and sensible order of mass spectrometric analysis should reduce such 'contamination' to a negligible value.

Huser did report some contamination of suceeding samples in the vessel used to generate nitrogen from ammonium chloride, after a sample with a high atom% ^{15}N value had been generated. It is unlikely that any significant contamination of the Rittenberg tubes occurred in the present work.

(2) Adsorption on protein:

It is possible that a significant amount of nitrogen

adsorbed on protein could be retained on the Kjeldahl catalyst and eventually affect the atom%^{15}N value. It is interesting to compare this possible adsorption of nitrogen on protein to adsorption of nitrogen on carbon black on a tentative quantitative basis. The adsorption of nitrogen on carbon black has been studied (229) and calculations on some relevant data from these studies have been made below.

5 ml of nitrogen were adsorbed on 1 gm of carbon black at pN_2 55.5 cm and 25°C (229)

5 ml of nitrogen at 25°C and 55.5 cm pressure contain

$$\frac{28}{22.4} \times 10^{-3} \times 5 \times \frac{555}{760} \times \frac{273}{298} = 4.17 \text{ mgN}$$

i.e. 4 µg. of nitrogen adsorbed per mg carbon black. If the value for No. 3 experiment (3) is again taken, since total nitrogen is about 14% of the protein

Amount of protein present = $1.81 \times \frac{100}{14}$ = 13 mg

assuming that the protein surface behaves identically as carbon black.

Amount adsorbed = $13 \times 4 \times \frac{28.7}{55.5}$ = 28 µg

This value is very close to the experimental value of the highest blank, but the relevant point is that if adsorbed nitrogen is the major contributor then the protein, must adsorb nitrogen on a similar scale to carbon black. In view of the subsequent degassing procedure, a greater amount would need to be initially adsorbed on

protein

It is interesting to consider the $[^{13}N]$ work of Nicholas (70) (71) in which blanks were reported. These blanks apparently resulted from dissolved(soluble) nitrogen since they were considerably reduced by further 'degassing' procedure, by flushing with oxygen, carbon dioxide or nitrogen for 10 mins. It is possible to attempt some calculation from this work although it must be pointed out that this work is open to some criticism. It is possible that the activity (β emission) attributed to fixed nitrogen may result from other sources, soluble or adsorbed species $[^{13}N\text{-}^{13}C]$, $[^{13}N]$

$$^{13}N_2 \rightarrow {}^{13}N - {}^{13}C + \beta^+$$

$$^{13}N - {}^{13}C \rightarrow {}^{13}N + {}^{13}C$$

$$^{13}N \rightarrow {}^{13}C + \beta^+$$

Thus the measured activity may result from a combination of the above. A calculation on the initial $[^{13}N]$ results of Nicholas (71) is made below.

Calculation:

1 micro-curie = 3.7×10^4 atoms disintegrated per sec

Activity of gas = 20 μc/litre

$$= 20 \times \frac{37\,000}{1000} \text{ disintegrat/sec/ml of gas}$$

$$= \frac{20 \times 37{,}000 \times 2}{1000} \quad " \quad / \ " \ /2\text{ml}$$

Since α_{30^oC} = o.01342 (pN_2 = 1atmosphere) (224) the activity in 2ml. water = 2 x 20 x 37 x 0.01342 =19.86disint/sec.

It would have been more helpful if Nicholas had specified the standard time to which his results were referred and the accuracy of measurement of the c/s,particularly in view of the short-half life of ^{13}N(10.05min.). He merely reported that each sample was counted for 30sec. and that the observed counting rates were corrected for the dead-time of the counter, where appropriate, and for decay from a'suitable'standard time, usually taken as 10-15min. after the end of the exposure.

It one takes the standard time as 11min. after the end of the exposure the maximum activity that could arise from dissolved (soluble) nitrogen can be obtained by substituting 19.86c/s in $C_t = C_o e^{-kt}$. This gives a value($C_{11min.}$) of 9.27c/s.

Thus it would appear that soluble nitrogen cannot give rise to the'blank' of 110c/s particularly as the ^{13}N was swept into the 50ml.exposure flask by pure argon. It must be noted that a better comparison between experiments, with dissolved nitroggn and with nitrogen fixation in other studies,would be obtained by referring the results to the end of the exposure period. On this basis it would appear that the activity of the 'dissolved' nitrogen,by subtraction of the'degassed' blank from its initial value(234-21 =213c/s),is $\frac{213}{19.86}$= 10.7 times the activity estimated for dissolved nitrogen and is $\frac{213 \times 100}{20 \times 37 \times 2}$ = 14.5% of the activity in an equal volume of exposing gas.

A comparison of experiments is also complicated by the variation in the activity of the (^{13}N) gas since the method of preparation gave a gas with activities of between 30 and

60 μc/litre.

In his original paper on [^{13}N] experiments Nicholas did record relatively high activities in boiled cells (60c/s) in comparison to normal cells (430 c/s) even after 10 minutes degassing. Similar values (44 c/s) were observed with cells treated with potassium cyanide, Nicholas apparently considered dissolved nitrogen as the only source of error and did not examine the possibility that appreciable activity could result from adsorbed nitrogen or the presence of other β^+ emanating species,

In his review article, however, the values for boiled cells and cyanide cells appear to have been reinvestigated since these are much lower. It is also reported that when cells or extracts from micro-organisms that did not fix nitrogen were used as controls, there was no incorporation of tracer, although no details were given of the cell densities (amount of protein), particularly with reference to dissolved nitrogen.

Nicholas also reported that a substantial part of the fixing activity of the extract could be recovered in ammonia isolated by a Conway microdiffusion procedure. This report would have been more impressive if the experimental conditions had been enlarged upon, in particular , the standard time to which the results were referred ; and the time allowed for the microdiffusion

ammonia before ~~assaying~~ its [^{13}N] activity was assayed.

If significant errors have arisen in the [^{15}N] technique from dissolved or adsorbed nitrogen, it would suggest either extremely poor 'degassing' procedures or that nitrogen can be either fixed or exchanged during the Kjeldahl procedure. Since the 'degassing' procedure should have eliminated any significant contribution from dissolved nitrogen (originally present in solution or released from the protein surface) the latter possibilities will be discussed in the following comment on the Kjeldahl procedure. It is interesting to recall that Mumford and Carnahan et alia had some difficulty in explaining their [^{15}N] results on a mutant of Azotobacter (230). These results appear in TABLE XXXI.

TABLE XXXI (TABLE 1 (230))

MOLECULAR NITROGEN UTILISATION BY AUXOTROPH AVI

INCUBATION hr	Excess ^{15}N in culture atom %	Kjeldahl nitrogen in culture µg./ml	Optical density
0	0.00	61	0.01
24	0.00	59	0.21
36	0.56	54	0.23
48	18.34	63	0.27

The incubation was carried out in Burk's medium containing 60 µg/ml of urea nitrogen under an ^{15}N atmosphere ($He:O_2:N_2$ in the ratio 6:2:2). The nitrogen gas was 60% atom ^{15}N excess.

They attempted to explain the significant increase of atom% ^{15}N excess in the 36 hr and 48 hr results on the basis of nitrogen exchange but could find no evidence for this. The calculated increase in nitrogen is $\frac{18.36}{60} \times 630 =$ 193 µg, although no increase in Kjeldahl nitrogen was detected. This increase is too large to be attributed to adsorbed nitrogen and perhaps a more likely explanation is that the atom% ^{15} excesses after 24 hr and 36 hr respectively are low as a result of the the dilution of the $[^{15}N]$ atmosphere by atmospheric nitrogen.

In conclusion if adsorbed nitrogen is a major contributor to the blanks (and hence to other results) then in view of the degassing techniques employed it would have to become 'fixed' or 'exchanged', reactions which have few precedents, presumably during the Kjeldahl procedure. In the following section further consideration is given to the Kjeldahl procedure.

[A review of the Kjeldahl method will appear shortly (296).]

(H) THE KJELDAHL PROCEDURE

The Kjeldahl procedure consists essentially of the slow digestion of organic material in concentrated sulphuric acid in the presence of a suitable catalyst. The process results in the oxidation of carbon compounds to carbon dioxide and the reduction of many nitrogen compounds to ammonia. The balance between oxidation and reduction is dependent on the conditions of digestion

and the particular catalyst but conversion to ammonia is not universal in all nitrogen compounds since many require special procedures to ensure the conversion to ammonia. If the oxidation during the digestion is too vigorous then losses of nitrogen and organic amines may occur, losses of nitrogen at the rate of 0.01 mgN/hr/mgN have been reported (192).

After the digestion, the ammonia is normally liberated by the addition of a sodium hydroxide/sodium thiosulphate solution and isolated by steam distillation. Thus the Kjeldahl procedure may be represented as

$$\text{ORGANIC NITROGEN} \longrightarrow CO_2 + (NH_4)HSO_4$$

$$(NH_4)HSO_4 + 2\ NaOH \longrightarrow NH_3 + Na_2\ SO_4 + 2H_2O$$

although in the digest the ammonia is probably present in a mercury complex, $Hg(NH_3)_2SO_4$ (231). Its release from this complex may be represented as

$$Hg\begin{matrix}NH_3\\NH_3\end{matrix}SO_4 + Na_2S_2O_3 \xrightarrow{H_2O} HgS + Na_2SO_4 + (NH_4)_2SO_4$$

$$(NH_4)_2SO_4 + 2NaOH \longrightarrow 2NH_3 + Na_2SO_4 + 2H_2O$$

It is not too remote from possibility that adsorbed nitrogen could become fixed or exchanged during the Kjeldahl procedure. It is unlikely that free $[^{15}N]$ liberated by the acid digestion would remain in significant quantities in view of the experimental technique and thus one must consider the feasibility of Kjeldahl nitrogen

fixation or exchange during the digestion.

It has been reported that nitrogen is dissociatively adsorbed on iron (232) (233) and if a similar adsorption could occur on the mercury catalyst, the reducing conditions of the Kjeldahl procedure might give rise to ammonia.

An exchange reation, which would obviate the need for nitrogen fixation, can be visualised as illustrated in Figure (12).

On addition of alkaline sodium thiosulphate the excess nitrogen would be released, concomitant with randomisation, and some exchange of $[^{15}N]$ with $[^{14}N]$ would result. Huser (201) has reported that an exchange reaction does not occur during the Kjeldahl procedure. He found that the nitrogen content of the Kjeldahl reagents affected the atom%^{15}N values of the samples but as he detected no significant changes in atom%^{15}N values of identical samples which had been subjected to different digestion times, 1 hr, 3 hr, 10 hr, he concluded that the exchange reaction did not occur. However, it remains open to speculation whether similar results would be observed if significant quantities of adsorbed nitrogen ($^{15}N_2$ or $^{14}N_2$) had been present at the beginning of the digestion period.

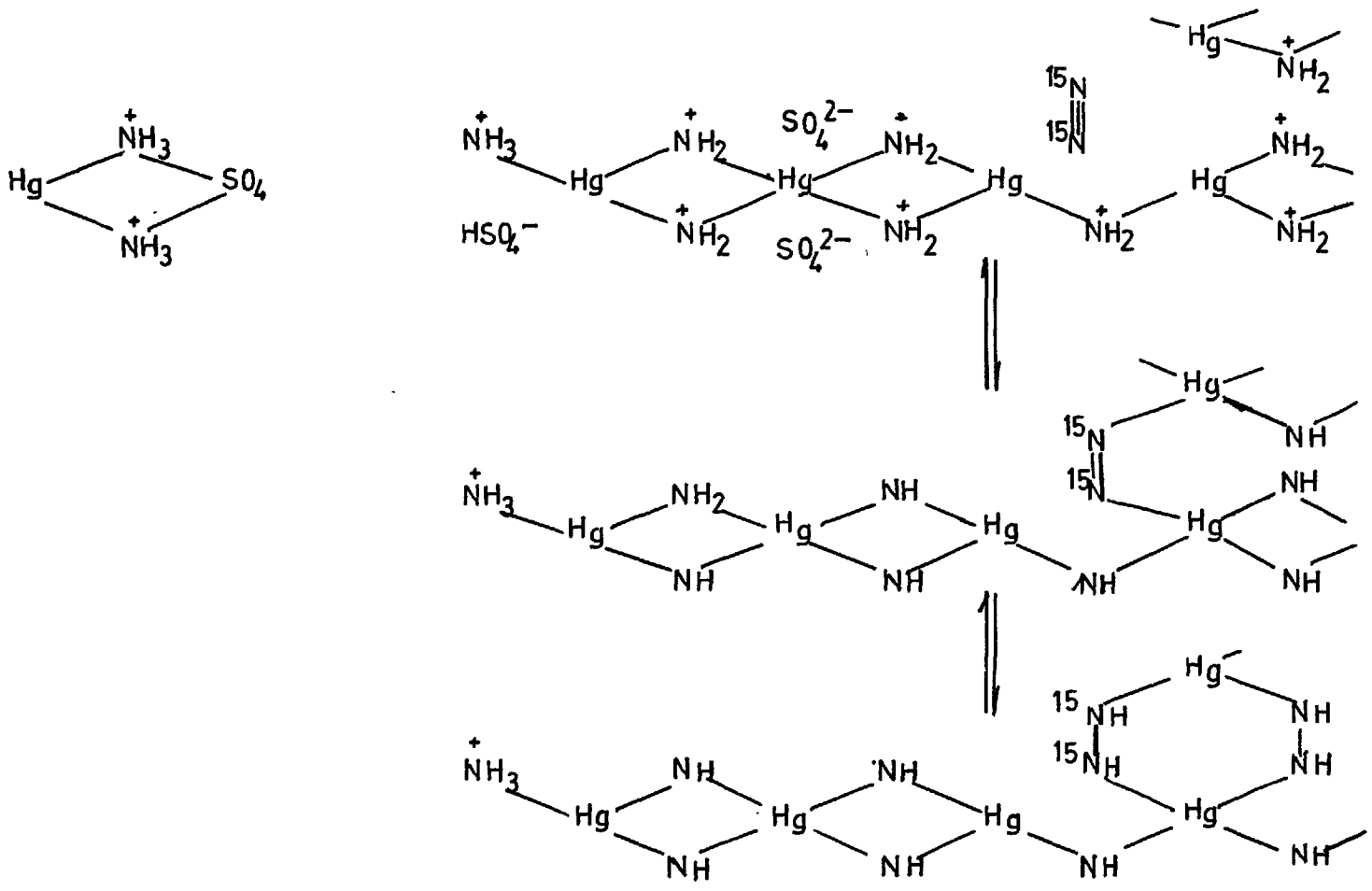


FIGURE12

In the present work, the Kjeldahl procedure was essentially that of Willit and Ogg (191), employing a mercury catalyst, although with a longer digestion period. Hillier et alia (234) have reported that mercury catalysts are most effective and less likely to lead to losses of nitrogen during digestion than other catalysts with digestion periods up to 8 hrs. In studies on the atom % ^{15}N in amino acids, Gaebler et alia (202) used a mercury catalyst with 18 hr digestion periods. The absence of methylamine from the ammonia sample for mass spectrometric analysis is essential since as has been noted methylamine would cause an abnormally high peak at mass number 29 (from species CH_3N^+) and high atom%^{15}N. It is possible to check atom%^{15}N values from $\frac{28}{29}$ ratio by measurement of $\frac{28}{30}$ and $\frac{29}{30}$ ratios but this is not always feasible with low atom% ^{15}N values (c 5%) and thus it is essential to ensure complete "Kjeldahlisation."

It is not likely that methyamine has affected the results reported in this thesis since

(i) In experiment (4) No. 5 was not exposed to $[^{15}N]$ and did not give an atom%^{15}N excess on mass spectrometric analysis.

(ii) Although methylamine has been found when copper catalysts have been employed in the Kjeldahl procedure it has not been found

either with long digestion periods (18 hrs) (196) or with mercuric oxide - potassium sulphate catalysts (197).

(iii) It has been reported that the mass spectrometer requires 'baking' out to remove contamination by methylamine (197). In the present work although the mass spectrometer was regularly checked against nitrogen generated from ammonium chloride no persistent or significant atom% ^{15}N excesses were found.

The catalystsused by Nicholas in his nitrogen fixing studies were mercuric oxide - potassium sulphate (Long Ashton) (179) and copper selenate (Wisconsin) (181).

(I) SUMMARY OF TECHNIQUE

(1) Sterile apparatus was used throughout, particular care being taken in the cleaning of apparatus.

(2) Centrifugation was carried out in a Spinco Model L ultracentrifuge. The disruptate was centrifuged down in 13.5 ml portions.

(3) Particular care was taken in the transfer of the supernatant to the exposure vessel, Thunberg tube. A sterile syringe, (5 ml) was clamped in position over the centrifuge tube and lowered carefully into the supernatant to a suitable depth. The supernatant was gently withdrawn and deposited in a Thunberg tube.

(4) The contents of the Thunberg were quickly frozen in a cardice/methanol mixture, the air pumped out of the Thunberg tube and replaced by the atmosphere containing $[^{15}N]$ at the required pressure.

(5) The Thunberg tubes were shaken 3 hr.

(6) The contents of the Thunberg tube were again frozen and the atmosphere returned to the storage bulb. The supernatant was allowed to warm to room temperature under partial vacuum, to aid degassing.

(7) The supernatant was examined for whole cells by microscopic examination and plating out.

(8) The Kjeldahl procedure was modified during the final stages of this work. The supernatant was transferred to the Kjeldahl flask, 1.5 ml concentrated sulphuric acid (analar) added and refluxed gently 15-20 minutes before the addition of mercury catalyst. Digestion was carried out overnight and contents refluxed a further 1 hr. The ammonia was collected and determined by the method described in the experimental section.

(9) The ammonium chloride solution was reduced to about 3 ml by careful evaporation and placed in one arm of a Rittenberg tube. 1 ml of hypobromite solution was added to the second arm and the air removed via the vacuum line of the mass

spectrometer. After degassing the contents a second time they were allowed to warm up, the nitrogen was generated into the Toepler storage bulb and adjusted to a suitable pressure for leaking into the mass spectrometer.

[J] THE [^{15}N] STUDIES ON CELL-FREE EXTRACTS OF CLOSTRIDIUM.

The principal information on technique is found in paper of Carnahan et alia (151). Although the later results have been expressed on a quantitative basis, nitrogen fixed, in µg., the initial conclusions appear to have been deduced primarily from a consideration of atom ^{15}N excesses.

In all experiments the results are referred to protein nitrogen in the extract rather than Kjeldahl nitrogen. It is useful, particularly as a guide to the accuracy of results, to calculate the total nitrogen values and compare these with protein nitrogen in this and subsequent papers on nitrogen fixation by extracts of Clostridium.

Tentative calculations appear below.

(1) Carnahan et alia (151)

These reported a gain of 247 µg nitrogen, determined from an [^{15}N] enrichment of 0.94% after exposure of an extract (16.8 mg protein N) to [$^{15}N_2$] (60 atom% ^{15}N excess) for 1 hr.

∴ (Calculated) Total nitrogen in extract

$$= \frac{247 \times 60}{0.94 \times 10^3} = 15.8 \text{ mgN}$$

i.e. 15.8 mgN (final) ≡ 16.8 mg proteinN(initial)

There is no reference to added ammonia in this experiment.

(2) Mortenson (175)

In TABLE 1 (175) 210 µg N were fixed which corresponded to an atom%^{15}N excess of 0.64 on the extract (20mg protein N) after exposure to 61.5 atom%^{15}N for 1 hr.

(Calculated) Total nitrogen = $\frac{210 \times 61.1}{0.64 \times 10^3}$ = 20.05 mg N

i.e. 20 mg protein N ≡ 20.05 mg N, for 61.1 atom%^{15}N excess

Total nitrogen = $\frac{210 \times 61.5}{0.64}$ = 20.2 mg

i.e. 20.2 mgN (final) = 20 protein N (initial), for 61.5 atom%^{15}N excess

Since Mortenson added between 50 and 150 µg nitrogen initially, this is more feasible.

i.e. 20 + 50 + 210 = 20.26 mg (final)

(3) D'Eustachio (172)

It was reported that an increase of 158 µg (maximum value) corresponded to 0.11 atom%^{15}N excess, when an extract (44 mg extract protein) was exposed to $[^{15}N_2]$ (7.6 atom%^{15}N excess).

(Calculated) Total nitrogen = $\frac{158 \times 7.6}{0.11 \times 10^3}$ = 10.9 mg

No correlation is apparent particularly since protein nitrogen is usually some 14% of the protein.

$\frac{14}{100} \times 44 = 6.16$ mg

It is evident that whereas the protein nitrogen refers to the initial value, total nitrogen (calculated) will correspond to the final total nitrogen on which the atom% ^{15}N value was measured. The amount of nitrogen fixed can be calculated from initial nitrogen, atom%^{15}N excess of the sample and the 'exposing' atmosphere by substituting these values in the equation

$$n = \left(\frac{x - 0.38}{D - x}\right) N$$

where n = nitrogen fixed, N = initial nitrogen
x = atom%^{15}N sample, D = atom%^{15}N atmosphere.
Reference to initial nitrogen is desirable since as purification of an extract proceeds and pure enzyme is approached, initial nitrogen becomes more and more a measure of enzyme content. Thus $\frac{n}{N}$ eventually represents a rate of enzyme action; this derivation assumes however that no 'turnover' occurs.
Substituting the value in calculation (1) in the above equation,

$$\text{(Calculated) Total nitrogen} = \frac{247 \times 59.4}{0.94 \times 10^3} = 15.6 \text{ mgN}$$

i.e. 15.6 mgN (initial) = 16.8 mg proteinN (initial)

Although it is desirable to state clearly the basis of any calculation of nitrogen fixed, it is evident that virtually all the published work lacks important detail on this point. Nevertheless, on the basis of such calculation, the measurement of nitrogen fixation has been

enthusiastically replaced by a Conway microdiffusion procedure, in which an accuracy of 3.5 µg. on 200 µg. of nitrogen fixed is claimed. This method, the Conway microdiffusion procedure, in which an increase of ammonia is measured, does not appear to have been completely verified by checking the atom% ^{15}N excess of the ammonia formed by nitrogen fixation. This point will be considered at a later stage.

The principal experimental method, that Carnahan et alia, used for preparing cell-free nitrogen fixing extracts was autolysis of dried cells.

In this method 10 gm of cells were disrupted in 100 ml buffer and then centrifuged in 50 ml centrifuge cups. Nitrogen fixation was attributed to cell-free enzyme on the following grounds

(a) Although after disruption 2-5% of the original cell population remained, after centrifugation microscopic examination of the supernatant did not reveal whole cells.

(b) It was reported that the supernatant retained full activity after centrifugation at 27,000g 15 min and did not lose activity (presumably in terms of atom% ^{15}N excesses) in subsequent centrifugations (ultimately at 144,000 g for 2-4 hr).

These centrifugations were carried out in a Spinco Model L preparative ultracentrifuge (volume of centrifugate was not given) and care was taken not to disturb sediment by pipetting out only the upper portion of the supernatant.

From these results, centrifugation at 27,000 g and 0°C for 15 mins in a "Severall" refrigerated centrifuge was adopted as routine procedure to sediment whole cells and debris. The TABLE XXXII given below is taken from Carnahan's paper (151).

TABLE XXXII (TABLE II (151))

NON DEPENDENCE OF ACTIVITY ON INTACT CELLS IN EXTRACTS

SYSTEM AND TREATMENT	CONTENT intact cells	$\%^{15}N$ enrichment
(1) Resting cells	100%	0.10
(2) Broken cells	8%	0.64
(3) Extract 27,000 g/15 min	None	0.54
(4) Extract 144,000 g/90 min	None	0.78

(1) and (2) were from the same experiment, (3) and (4) were from the same experiment.

It is questionable whether a direct comparison, on an atom$\%^{15}N$ basis, between resting cells and centrifugates prepared by the autolysis of dried cells is valid. A direct comparison between (1) and (2) would be more

acceptable if the total nitrogen values or cell density had been given, particularly as the 'broken cells' may actually represent whole and broken cells in a higher state of dispersion. A comparison between (3) and (4) should also be referred to total nitrogen basis since centrifugation at 144,000 g/90 mins will reduce the total nitrogen value of (3).

In this paper, which is the critical paper, so far as cell-free nitrogen fixation in Clostridum is concerned, there is no mention of blank experiments, e.g. results from boiled enzyme preparations, although it is reported that extracts without added pyruvate, did not incorporate $[^{15}N_2]$. It is noteworthy that exposure to $[^{15}N_2]$ in this work was carried out in 25 ml round bottom flask under partial vaccum, 0.5 atm. labelled nitrogen. The Kjeldahl procedure employed was that of Burris and Wilson (197), with a mercuric sulphate-potassium sulphate catalyst. It was reported that the Friedrich modification of the Kjeldahl procedure gave identical results (presumably identical atom% ^{15}N excesses) but that Dumas combustion of the lyophilized extracts produced erratic results. These erratic results were thought to result from difficulties in combustion but may result from incomplete recovery of nitrogen from alkali. Pethica pointed out that the nitrogen is collected over alkali which can not easily be

be degassed. Thus the the alkali may exhibit a memory (235).

In Table XXXIII some relevant features on the work with Clostridum, the amount of 'enzyme', method of preparation etc. is summarised.

TABLE XXXIII

	pN_2 atm.	pX	atom% ^{15}N Excess atmosphere	EXPERIMENT CONDITIONS
Carnahan (151)	0.5		60	Autolysis. 27,000g 15min 10-20 mg protein N/8 ml 100-200 mg. pyruvate.
Schneider (174)	0.03 0.15	Helium to 1 atm	18.96	Autolysis. 20,000g 15min 20,000g, 15 min. 2mg Kjeldahl N/2.5 ml. 20 mg. pyruvate.
Mortenson (175)	0.5		61.1	Autolysis 27,000g, 15 min 10-15mg protein N/8ml 100 mg pyruvate
Dass Dua (154)	0.05 0.1	Argon to latm	64	Autolysis 20,000g, 15 min. 20-25 mg protein/2.5 ml 25-40 mg.pyruvate.
Hardy (172)	0.8 atm		7.6	Autolysis 44mg. extract protein/ 4ml. Fyruvate Acetyl phosphate "reducing agent"

(K) SIGNIFICANCE OF THE PRESENT RESULTS.

The most striking feature of the present work was the appearance and magnitude of the 'blanks'. In earlier [^{15}N] studies there had been no indication that such blanks could occur although it must be pointed out that

in these experiments low pN_2 of $[^{15}N_2]$ were used (c.0.1atm) and exposure times were shorter. It is particularly noticeable that no comparable finding was observed in the work on Clostridium and the work of Bulen in which cell-free extract of comparable or even higher total nitrogen contents were exposed to $[^{15}N]$.

Since longer exposure times were used in the present work (3hr v 1 hr) it is possible that some contribution resulted from the presence of small concentrations of whole cells but the increases appear too great for this to be the major contributor.

In order to make a fair comparison between the activities of the blanks in view of the variation of total nitrogen in the disruptates, it is necessary to make a comparison of the activity of the supernatants from the same centrifuge tube. In TABLE XXXIV a summary in these terms is shown. It is evident that no cell-free fixation can be concluded in the experiments involving ultrasonic disruption of thick suspensions of 'shake' cells.

In the experiments with stored bacteria it is evident that, despite higher values in other centrifugates, the relative activity of the blank is higher than is desirable. It must be noted that the blanks were prepared from the second 5 ml of the 13.5 ml centrifugate and thus the transfer

technique and assessment of possible whole cell contributions are particularly important.

TABLE XXXIV SUMMARY OF BLANK EXPERIMENTS

Experiment No	Blank µg	*%Activity (from same centrifuge tube)	Exposure time	Comment
(6)	23	100	6 hr	Shake culture
(7)	14	60	6 hr	
	22	100	6 hr	
(8)	34	70	6 hr	
(9)	33	40	3 hr	
(10)	8	50	3 hr	Phosphate medium
(11)	46	110	3 hr	Shake culture
(12)	6	40	3 hr	
(15)	24	70	3 hr	
	26	47		
(16)	15	80	3 hr	Shake culture
(17)	31	100	3 hr	

* In this Column the activity (calculated increase in nitrogen) of the heat treated supernatant is recorded as % of the activity of untreated supernatant from the same centrifuge tube.

When considering the increase in nitrogen detected in these experiments two points require further attention.

(a) the varying concentration of protein (and thus surface area) in the centrifugates.

(b) the reproducibility of the 'degassing' procedure.

It is reasonable, on the basis that adsorption of nitrogen is the primary cause of the 'blanks', to expect that the heat treatment of the supernatants, which could cause protein denaturation and hence a reduction in surface area, would result in a lower 'activity' than untreated supernatant. It is evident that such consideration must also be linked to the reproducibility of the degassing technique.

In the experiments employing the stored bacteria the blank values range between 1 and 34 µgN (an average of 20 µg in the 3 hr exposures) with particularly low values in experiment (10) and experiment (12).

In experiment (10) the bacteria were disrupted in phosphate buffer whereas in experiment (12) the nitrogen values of the centrifugates were particularly low. The latter was a result of the irregular behaviour of the Spince Model L ultra centrifuge since over a short period in 1964 the centrifuge developed a fault which prevented it from operating at r.p.m. greater than 25,000 r.p.m. for any length of time. Thus the centrifugation in this experiment was not continuous because the centrifuge cut out several times and had to be restarted.

It is evident that a quantitative Kjeldahl procedure is essential since if this is incomplete it is difficult even to make a relative assessment of the results.

One " pre-reductive procedure", the HI/sucrose pre-reductive treatment, did not give quantitative results on a number of occasions, experiments (9), (11), (15)) and in considering these atom%^{15}N values the following possibilities arise.

(i) The loss of nitrogen was a result of incomplete digestion, but in spite of this all the freshly fixed nitrogen, [^{15}N], was recovered in the distillate of ammonia. Thus a calculation would still give a quantitative result. (This may be the case in experiments (11) centrifugate No.1 .

(ii) Loss of nitrogen(and ^{15}N) occurred after randomisation in the Kjeldahl digestion. Thus the atom%^{15}N would be correct, but owing to the loss of nitrogen the calculated result for nitrogen fixed would be substantially reduced.

(iii) Loss of nitrogen occurred before randomisation. In this case it is likely that the atom%^{15}N excess or the calculated quantitative result would bear little relation to those obtained

in a properly digested sample.

It is probable that the losses experienced in the HI/sucrose pre-reductive scheme resulted during the removal of iodine, i.e. after adding concentrated sulphuric acid to the centrifuge in excess hydriodic acid. This distillation is particularly unpleasant and required attention.

It is difficult to assess the effect of the pre-reductive treatment since in general no reproducible increases in nitrogen fixed are observed. The results from these pre-reductive techniques, similar to the untreated centrifugates, are interesting when one attempts to assess whether a similar or lower contribution could arise from adsorbed nitrogen than was observed in the 'blanks'. For example, in the pre-reductive treatment with HI/sucrose the supernatant is refluxed several times (to steam distill off iodine) before digestion, thus this treatment would be expected to remove more adsorbed nitrogen and hence lead to a lower result. This, of course, assumes that the pre-reductive treatment was unable to fix adsorbed nitrogen.

It is apparent that in experiment (14) the first two samples showed negligible activity. This was not unexpected, since grease droplets were observed in the Thunberg tubes after removing the tubes from the shaker

and it must be assumed that this grease, from the ground glass joint, either inactivated the cells or enzyme preparation or reduced the effective surface area of the protein.

There was another factor, resulting from the experimental technique employed, that could have caused variation in the results. It was standard practice to freeze the supernatants to enable the air to be replaced by theselected atmosphere.

It is difficult to assess the effect this would have on the nitrogen fixing activity of whole cells and enzymes. It is recognised that the killing of cells by freezing is principally due to high internal and external concentrations of electrolyte in small amounts of unfrozen water rather than rupture by ice crystals. (236). It has been reported that the damage caused by increased electrolyte concentration is maximal between $-5^{o}C$ and $-10^{o}C$ and the rate of protein denaturation increases as the concentration rises. A cooling rate of $1^{o}C$/min has been recommended and, to reduce similar effects during thawing, rapid thawing is necessary.

In the present work the rate of cooling may have exceeded the recommended rate, but it is impossible to make an assessment of damage that this procedure could cause.

In extreme conditions, the ultra-rapid freezing and thawing of Azotobacter vinelandii was found to markedly injure or kill the cells (237). Doebler and Rinfret cooled the cells rapidly by spraying the culture through a hypodermic syringe on to liquid nitrogen and reported that only 28% of the original cell population of 9×10^7 cells survived. The effect of cold storage on the nitrogen fixing system of Clostridum pastourianum has been examined (154). It was reported that 95% of the activity was lost on storage at $0^{\circ}C$ for 24 hr although the loss was reduced when stored at lower temperatures. Unfortunately, long term storage rather than the immediate effect of freezing and thawing were studied in this paper and the activity did not appear to drop too rapidly with short term storage. The paper will be discussed further in the final section of the discussion.

The stability of hydrogenase to storage has been noted and it has been reported that the hydrogenase from Desulfovibrio desulfuricans was stable to freezing and thawing a number of times (84). On further purification, adsorption of hydrogenase on calcium phosphate gel and subsequent elution, the enzyme was less stable to storage.

In experiment (10) sample No.1 was not frozen prior to exposure to $[^{15}N]$ and has a slight increase in nitrogen fixed. This, however, is not significant since, as well

as appearance the high blanks in this expts, whole cells had been detected. From the final experiments with disruptates, cold storage does appear to have an effect on the results. The hydrogenase activities of the blank were also compared with those of untreated supernatant, TABLE (XV). It can be seen that the activity of the supernatant (30,000 r.p.m.) does show some increase over the corresponding supernatant in buffer. After the heat treatment, 100°C for 10 minutes, between 4-7°/o of the hydrogenase activity remained. In this respect Hyndman (82) has reported that when a hydrogenase preparation from Azotobacter, in 0.1M phosphate buffer pH6, was heated to 70°C for 3 minutes it retained about 70% of its original activity.

The behaviour of the hydrogenase of Clostridium was somewhat less sensitive to heat treatment. Carnahan et alia separated a hydrogen donating system from extracts of Clostridium, by heating to 60°C for 10 minutes. All the hydrogenase activity of the original extract was retained in solution.

In summarising these experiments (1)-(15) it is evident that when stored bacteria are disrupted in young cultures, centrifuged and exposed to $[^{15}N]$, significant increases in nitrogen are observed. It is probable that the culture does supply carbon substrate for whole cells but the

increase in nitrogen in the supernatants are not entirely accountable on a whole cell basis.

It is probable that the high blank values result from a combination of factors, from

(a) whole cells or cell-free enzymes not destroyed by the heat treatment

(b) a failure to remove 'excess' ^{15}N, i.e. $[^{15}N]$ that is present though not fixed by bacterial cells or enzymes.

(c) errors in the mass spectrometric analysis.

The evidence suggests that (b) is a contributor. It may be noted that the present work has employed higher pN_2 values, longer exposure times with dense disruptates and longer Kjeldahl digestion than much of the earlier work. The small but significant values for dissolved nitrogen (8 µg), low values for medium after a 6 hr exposure (20 µg) and the improvement in the blank values obtained by modifying the Kjeldahl procedure, by refluxing for 20 minutes in the absence of the mercuric oxide - potassium sulphate catalyst, suggest that a contribution results from adsorbed nitrogen.

In the 3 hr experiments on stored bacteria, the highest blank is 34 µg . If 8 µg is allowed for 'dissolved nitrogen', 15 µg for whole cell contribution and mass spectrometric errors, then about 10 µg must be attributed

to adsorbed nitrogen. This is a small but significant quantity to be artificially fixed, a reaction with virtually no ~~few~~ precedents, or exchanged. It is evident that more experimental work in this direction is both worthwhile and necessary.

It is possible that the contribution of adsorbed nitrogen in untreated supernatant is somewhat higher than observed in the blanks. If this is the case then a simple relationship between increase in nitrogen and protein present (surface area) might be expected. In fact there is no uniformity of values of the disruptates in buffer or young cultures or significant increases over the values of the corresponding supernatants. As has already been pointed out the pre-reduction technique employing HI/sucrose, which involves refluxing several times before digestion, might be expected to result in lower activities, if adsorbed nitrogen constituted the major part of the 'activity'.

Experiment (16)-(20), investigations on the effect of acetyl phosphate and other cofactors on both centrifugates and disruptates will now be considered. These studies were a continuation of the work of Smith, who examined the effect of sodium pyruvate and cofactors on nitrogen fixation in extracts of Azotobacter. It was originlly intended to link the present work more closely with that

of Smith by measuring both the disappearance of acetyl phosphate and pyruvate from extracts of Azotobacter, but owing to difficulties with apparatus this was not possible.

During the experimental work several publications appeared on the effect of or requirement for ATP, acetyl phosphate and ferredoxin in cell-free nitrogen fixing extracts of Clostridum. The later ones claimed that pyruvate could be adequately replaced by an ATP generator and a reducing agent (171)(172). A report on similar success in extracts of Azotobacter appeared in November 1964 (222) and during the final stages of this thesis this work has been enlarged upon. Bulen reported (223) requirements for a hydrogenase preparation from clostridium (containing ferredoxin), ATP and sodium dithionite for cell-free fixation by extracts of Azotobacter. This paper will be discussed at a later stage but it must be noted that in the absence of 'blanks' the increases of nitrogen over 1 hr are impressive. It is possible to make one serious criticism of this work, namely that the earlier studies particularly on Clostridum have not established the role of hydrogenase in nitrogen fixation. It is possible that hydrogenase is the site of fixation and thus in Bulen's work the Azotobacter extract would merely provide necessary cofactors to sustain fixation by the hydrogenase preparation of Clostridum.

This possibility has received some attention in the final section of this thesis, in which mechanisms for hydrogenase activity and nitrogen fixation have been proposed.

In the present work the acetyl phosphate was prepared from the disilver monoacetyl phosphate. Unlike previous work in which acetyl phosphate was added in the form of dilithium acetyl phosphate, di-sodium acetyl phosphate was added to the contents of the Thunberg tube. The disodium acetyl phosphate was prepared by adding a weighed excess of sodium chloride to a suspension of disilver acetyl phosphate and removing the precipilate by centrifugation. The concentration of acetyl phosphate was noted and the solution immediately used. This method did have the disadvantage in that the concentration of the sodium chloride was increased. Calculations showed that an error of 3% in the weight of sodium chloride could result in a 40% increase in the sodium chloride concentration of the supernatant. This may be of significance when one recalls the report of Postgate that the inhibition of hydrogenase by dyestuffs was increased by the addition of sodium chloride (238).

In the work on Clostridium both acetyl phosphate and creatine phosphate have been used as ATP generators. It was reported that 200 μmole of acetyl phosphate and 38.7 μmole

potassium borohydride could replace 455 μmole of pyruvate. It was also reported that hydrogen and NADH could act as reductants in nitrogen fixation by extracts of Clostridium and that the acetyl phosphate-borohydride system was ADP dependent.

The potassium borohydride was added in two portions. "Pre addition" KBH_4, was added to the incubation flask prior to flushing and covering with $^{15}N_2$ and "substrate" KBH_4 was placed in the side arm with the acetylphosphate. It was reported that the potassium borohydride had disappeared after 5 minutes, whereas nitrogen fixation, as measured by ammonia synthesis, continued for about 45 minutes.

In the present work the sodium borohydride was placed in the centre arm of the Thunberg tube. It was found that providing this was dry it was possible to add the sodium borohydride in small quantities by suitably adjusting the shaking rate, i.e. by increasing the shaking rate momentarily. The acetyl phosphate concentrations were somewhat lower but a higher relative concentration of ADP was used. In this respect a weakness of these experiments is evident, namely that no evidence is presented to confirm the formation of ATP under these conditions, particularly as myo-kinase has been reported in the soluble fraction from extracts of Azotobacter.

It can be seen that, in experiment (16) , there was an apparent concentration of acetyl phosphate of 0.72 μmole/ml before the addition of acetyl phosphate. There is a suggestion that some inhibition of fixation by acetyl phosphate has occurred but conclusion is complicated by the high relative blank and by the detection of whole cells in sample No. (5).

Experiment (17) appears to support this but the high blank (100%) renders conclusion impracticable. In experiment (18), disruption in phosphate buffer, it can be seen that the increase in nitrogen are about 40 μgs in the presence of acetyl phosphate and a reducing agent. Although the blank is 50% of its pair, it is only somewhat less than that for the 1 hr exposure.

In the final experiment, the effect of acetyl phosphate on disruptate, prepared in buffer and young culture was studied.

The heterogeneity of the disruptatesis evident from the variation in total nitrogen. From experiment (19), disruption in young culture, it can be seen that:

(a) the presence of a reducing agent increases the values significantly.

(b) short term storage of (5)-(7), while (1)-(4) were being exposed to $[^{15}N_2]$ has not

appreciably affected the values.

In experiment (20), in phosphate buffer, it can be seen that despite the greater nitrogen values of the disruptate the increases in nitrogen do not match those in the previous experiment. It is evident that No (2), the disruptate with added ∝— keto-glutarate, NADH , pyridoxal phosphate and ADP exhibits the greatest increase. The value for the disruptate with acetyl phosphate is somewhat less than the disruptate with no additions, but the concentration of acetyl phosphate is perhaps low for a disruptate. The blank is particularly encouraging since it would suggest that the technique is effective in removing adsorbed nitrogen and does suggest that whole cell contribution in phosphate buffer is negligible.

It is evident that the extracts after storage at $-10^{o}C$ in the Thunberg tubes show significantly less increases in nitrogen than their 'twin'. This drop in activity contrasts strongly with the stability of hydrogenase preparation to storage at $-10^{o}C$.

It is convenient to summarise the principal finding of this work before commenting on the recent paper of Bulen (228).

(1) The ultrasonic disruption of stored bacteria in young culture, has given rise to supernatants which

show increases in fixed nitrogen over blanks.

(2) The results have been complicated by the appearance of high blanks and interpretation has been rendered more difficult by a variation of total nitrogen in centrifugates from the same disruptate. The technique would be improved by the centrifugation of disruptate, in a larger volume than 13.5 ml.

(3) It is evident that the experimental technique, with particular reference to transfer, degassing and Kjeldahl procedures, is more important than earlier work has suggested and can only be properly analysed when the results are expressed on a quantitative basis.

(4) The activity of supernatant, prepared by the disruption of stored bacteria in buffer, shows an increase in fixed nitrogen over the blank when acetyl phosphate and reducing agent (sodium borohydride, NADH) are present. In this respect it is evident that further consideration of the time of exposure (3hr v 1 hr) with respect to stability of additives is necessary.

(5) The increased activity of disruptates prepared in young cultures over those prepared in buffer is a result of the presence of young cells and/or old cells and nitrogen fixing enzymes. A stimulation of activity is observed in the

presence of a reducing agent but this stimulation is not found in disruptatesprepared in buffer. It is evident that the presence of a reducing agent prior to disruption or centrifugation may be worthwhile.

(6) The stored cells are a useful source of hydrogenase preparations although little improvement in the solubilisation of hydrogenase is found.

(7) Unlike hydrogenase activity, nitrogen fixation in disruptates appears to be reduced significantly by storage at -10°C for 36 hr.

The outstanding points of the papers of Bulen are summarised below.

(L) COMMENT ON THE RECENT WORK OF BULEN (223)

(i) Nitrogen fixation in extracts of Azotobacter could only be achieved in the presence of a hydrogenase preparation from Clostridium pasteurianum. This preparation contained Ferredoxin.

(ii) The presence of sodium dithionite and an ATP generator was required. It was reported that the hydrogenase was ATP dependent since evolution of hydrogen was detected only in the presence of ATP.

(iii) Oxygen was not required and hydrogen could

not supply electrons to nitrogen fixation.

(iv) Ammonia was reported to be the product of the fixing reaction.

(v) In the preliminary report there was some suggestion that molybdenum was important.

Bulen disrupted the Azotobacter cells in a French pressure cell and maintained a source of ATP from creatine phosphate in the presence of creatine kinase. The highest value for nitrogen fixed per hour was 1028 μg, in the presence of 10 μmole ATP.

The claim that ammonia was the principal product of the fixing reaction was made on the basis of two results, in which the increase of ammonia after 40 minute exposure, measured by 'Nesslerisation' after separation of the ammonia, corresponded to the calculated amount fixed (from atom%^{15}N measurements). The agreement of these two results was good (within 2%) but no details of the calculations from atom%^{15}N excesses were given. In this experiment a high pN_2 was used (0.75 atmosphere, 97 atom%^{15}N nitrogen) in the exposure in the Warburg flask. It is perhaps surprising that in view of the reported ammonia incorporating capacity of extracts of Azotobacter that such a quantitative result was obtained. This result was not confirmed by measuring the atom%^{15}N of the ammonia and this point will receive further comment with

respect to the work on Clostridum.

Although high protein concentrations were used (15 mg protein) no blank experiments appear to have been carried out. It was reported that some fractions with acetaldehyde, hypoxanthine and other additives resulted only in very low atom%^{15}N excesses, about 0.01 atom%^{15}N. Whole cell contributions to the results were ruled out in view of the high centrifugal speeds employed.

It may be significant that:

(a) After centifugation for 6 hr at 144,000g all the activity was recovered in the particles.

(b) Dialysis against 0.01M phosphate, pH7 for 8 hr, caused little loss of activity.

(c) Preparations kept at 0°C were stable up to 48 hr, after which time a gradual decrease in activity occurred.

This stability is perhaps surprising in view of the difficulties in preparing a cell-free fixing extract from Azotobacter, which presumably reflected the instability of the electron transport particle in the aerobe in comparison to that in the anaerobe. Certainly, further experiments on the role of the HECP with added cofactors that might be contributed by extract of

Azotobacter is necessary.

It is worthwhile to examine the previous [^{15}N] work on Clostridum and Azotobacter.

(M) COMMENT ON SOME RELEVANT [^{15}N] WORK.

Serious criticism can be levelled at much of the earlier [^{15}N] work on Azotobacter and other nitrogen fixing organism for the failure to express the results on a quantitative basis. For a recent example, it has been reported that cell-free extracts from Bacillus polymyxa fix "considerable amounts of nitrogen when pyruvate is supplied as substrate." (239). On examination of this paper it is apparent that the results are expressed as atom%^{15}N values without sufficient detail to permit quantitative calculations. It is thus not possible to compare the activity in the supernatant and sediment, in the absence of total nitrogen values, with other work. This is also the case with the recent paper by Grau and Wilson (240) in which nitrogen fixation by an organism tentatively designated Achromobacter N.4 is examined. In this case also atom%^{15}N excesses are quoted, without reference to total nitrogen, when discussing the nitrogen fixation by intact cells, sediment and supernatant. [Other studies employing [^{15}N] nitrogen have appeared (297)(298).]

It is probable that the nitrogen fixation in cell-free extracts of Azotobacter originally reported by

Nicholas actually represents increases in nitrogen of a few µg , since the disruptates (and hence supernatants) were prepared from young (thin) suspensions of Azotobacter. These were exposed (in 2 ml portions) in the Warburg apparatus.

When the results of Schneider et alia (174) are examined it is evident that the fixation of nitrogen claimed for some of the organisms is of the order of a few µg .

e.g. Rhodospirullum rubrum

in expt 1 Added pyruvate in light

Calculated Increase = $\frac{1.231}{96.2.} \times 1.2 \times 10^3 = 15.3$ µg

expt 2. Light only.

Calculated increase = $\frac{1.15 \times 0.2 \times 10^3}{96.2} = 2.5$ µg

The latter result (0.2 mgN/vessel) is more typical of the results presented.

Similar calculations can be made with the results on Mastigocladus Laminosus.

Light - no addition

Calculated increase = $\frac{0.143 \times 0.37 \times 10^3}{96.2} = 0.5$ µg

Dark - no addition

Calculated increase = $\frac{0.137}{96.2} \times 0.402 \times 10^3 = 0.5$ µg

It is evident that all claims relating to nitrogen

fixation should be expressed on a quantitative basis and when these are on such a small scale some reference to the efficiency of experimental technique is essential.

The paper by Schneider et alia (174) appeared shortly after the report of consistent cell-free fixation in extracts of Clostridum by Carnahan et alia. (151).

Schneider et alia prepared their extracts by the autolytic method of Carnahan et alia (151) although the nitrogen fixing activity was measured over longer periods, up to 4 hrs. They reported that the nitrogen fixation after 60 minutes exposure was greater than that after 120 minutes and 240minute exposure (TABLE XXXV). They suggested that these discrepancies may have resulted from air leakage but when the results are expressed on a quantitative basis it is evident that this variation may not be significant.

The most interesting data in this paper is on ammonia. The atom% ^{15}N of ammonia isolated from the extract was extremely high ($\frac{1}{2}$ that of the exposing atmosphere). These results are reproduced in TABLE XXXVI with the calculated increases in nitrogen. It is evident that the amounts of nitrogen fixed are larger in this experiment. The results also differ from those of Carnahan et alia (151) since the latter

TABLE XXXV (TABLE 2 (174))

TIME COURSE OF NITROGEN FIXATION BY CELL-FREE EXTRACTS FROM CLOSTRIDIUM PASTEURIANUM

Time in minutes	mg.N per vessel	atom% ^{15}N excess	*CALCULATED µg.
30	1.96	0.720	14.6
	1.99	0.978	20.2.
	1.52	0.862	13.6
60	1.81	1.466	27.5.
	1.91	1.322	26.2.
	1.96	1.576	32
120	2.17	1.008	22.5.
	2.11	1.129	25
	1.35	1.289	19
240	1.94	1.190	24
	1.94	1.312	26.5
	2.24	0.938	22

* The increases of nitrogen given in this column have been calculated from the published results.

Exposing atmosphere

0.04 atm nitrogen (96.2 atom% ^{15}N excess)

0.96 atm helium

TABLE XXXVI (TABLE 3 (174))

ISOLATION OF FREE AMMONIA, GLUTAMINE AMIDE N, AND ASPARAGINE N FROM REACTION MIXTURES AFTER CELL-FREE FIXATION BY EXTRACTS OF CLOSTRIDIUM PASTEURIANUM

TIME	N-Fraction	mg.N	Atom% ^{15}N Excess	*Calculated increase µg
60 min	Ammonia	0.070	42.32	30.6
	Glutamine amide N	0.120	14.2	17.7.
	Asparagine amide N	0.185	0.81	1.55
180min	Ammonia	0.191	52.06	103
	Glutamine amide N	0.122	8.66	10.9
	Asparagine amide N	0.345	0.569	2.0

* In this column the increases in nitrogen have been calculated from the published results.

Exposing atmosphere 0.10 atm
nitrogen (96.2 atom% ^{15}N)
0.90 atm helium

reported that when cell-free fixation was allowed to continue longer than 10-15 minutes the $[^{15}N]$ was incorporated in a form no longer liberated by alkaline distillation. There is some similarity in the atom%^{15}N excesses of glutamic acid; Carnahan et alia found 21.7. atom%^{15}N excess, Schneider et alia 14.2 atom%^{15}N excess. It is necessary to consider the evidence on which ammonia has been deduced the first product of fixation.

Carnahan et alia reported that after 12 minutes 100% of fixed $[^{15}N]$ on extract was recovered by alkaline distillation. In the presence of an ammonium pool (120 µmoles/ml) all the fixed nitrogen after 1 hr exposure could be recovered as ammonia. The presence of glutamine (120 µmoles/ml) was equally effective. It was reported that differential distillation showed that over 90% of the fixed nitrogen was present as ammonia.

At face value this evidence appears overwhelming support for ammonia as the initial and sole product of the nitrogen fixing reaction. There are, however, several points which can be raised.

(a) Differential distillation.

Although this procedure is probably effective to separate ammonia from the more common amides,

it is unwise to rule out the possibility that ammonia could under these experimental conditions arise from other labile amides or amino compounds. (b) The paper is reticent on the method of calculating the amount of nitrogen fixed as ammonia. In some cases it is not clear whether the increase in ammonia has been calculated from an atom%^{15}N value or merely from the titration of the alkaline distillate. In the presence of the ammonia pool it is not apparent whether any appreciable ammonia utilisation occurred during the 1 hr exposure. The initial ammonia concentration is high (1.4 mg) in comparison to the amount of nitrogen fixed. Assuming no significant change in the 'distillable' ammonia concentration it is possible to calculate its atom%^{15}N, after 1 hr exposure to 61% atom%^{15}N . Since in the presence of 1.4 mgN 182 µg of nitrogen are fixed then

$$\text{atom\%}^{15}\text{N (ammonia)} = \frac{182}{1582} \times \frac{61}{100} \times 100 = 7$$

although this value will be dependent on the extent of randomisation . This point will be enlarged upon with reference to the method developed for measuring nitrogen fixation by Mortenson and to the recent work of Bulen. (23).

Mortenson (175) reported that nitrogen fixation in the cell-free extracts can be simply and rapidly measured by measuring the increase in ammonia, compared with the value for a blank (incubated under Argon). For this determination it was usual to add 50-150 µg N as ammonia and its effect of nitrogen fixation was assumed negligible. The main support, other than the comparison of increase in ammonia with total nitrogen fixed which will be discussed below, is the reproducibility of the distillation-titration method (a modified Conway procedure) for the determination of ammonia in extracts exposed to nitrogen and argon respectively. It must be noted that the partial pressure of nitrogen and argon are much less than atmospheric being 0.5 atm.

The validity of this method depends on whether the increase in ammonia does correspond to the amount of nitrogen fixed. The results from Mortenson paper (Table 1) are considered below.

Experiment (1)

Under argon the procedure gave 462 µg of ammonia N

Under nitrogen " " 672 µg " " "

∴ Increase in nitrogen = 210 µg.

the atom% excess of the total extract = 0.64 % after exposure to 61.5 atom% $^{15}N_2$.

i.e. if X = total nitrogen (Kjeldahl) in extract and the increase is 210 µg.

$$\frac{0.64 \times 10^{-3}}{61.1} X = 210$$

$$X = 20 \text{ mg}$$

Some confirmation for this method should be obtained by measuring the atom%^{15}N excess of the liberated ammonia which, even with some randomisation, should have a high label.

Assuming no randomisation then $\left[{}^{15}N\right]$ in ammonia $= \frac{61}{100} \times 210$ µg

$\therefore$ Atom%^{15}N of liberated ammonia $= \frac{61.1}{100} \times \frac{210 \times 100}{672} = 19.1$

Bulen (223) has also concluded that ammonia is the product of fixation since the calculated increase in nitrogen of the extract (from atom%^{15}N) corresponded with the measured increase in ammonia. It is evident that the appearance of blanks would seriously complicate this correlation.

Similar criticism may be applied to the work of Hardy and D'Eustachio (172). They support their results in terms of ammonia formed per mg protein per minute by reference to atom%^{15}N excess on the extract. There has been no attempt to put these on a quantitative basis or to confirm that $\left[{}^{15}N\right]$ is produced in the ammonia.

The recent communication on the stability of nitrogen fixing enzymes in Clostridum pasteurianum (154)

can also be criticised for the failure to express the results on a quantitative basis. It is evident that a comparison between different experiments can only be valid if the method of disruption is absolutely reproducible with respect to amount of total nitrogen in the extracts. It must be noted that there is a high concentration of protein (20-27 mg protein per ml) and that no precautions appear to have been taken to ensure the atom% ^{15}N excesses after heat treatment and adsorption on calcium phosphate gel treatment are comparable. A comparison is only possible if the total nitrogens are of a comparable order. Particular exception can be taken to the conclusions drawn from the activity on storage. An extract initially 'fixed' 0.736 atom% ^{15}N excess, after storage under hydrogen at 30°C for 12 hr it 'fixed' 0.718 atom% ^{15}N excess and after storage 36 hr 0.666 atom% ^{15}N excess. Since the extracts were centrifuged after storage and before testing it is evident that the atom% ^{15}N excesses were taken on different Kjeldahl nitrogen concentrations and thus this renders conclusion on an atom% ^{15}N excess basis impracticable. [A further paper has been published (299).]

Before considering this point with respect to the mechanisms previously proposed for nitrogen fixation (SECTION VIII) it is convenient to refer to the inhibition of fixation in Azotobacter by ammonia.

The paper of Newton is one of the few papers which have sufficient details to permit calculation to be made on a quantitative basis. Newton investigated the nitrogen fixation and ammonia assimilation using the $[^{15}N]$ technique. (66).

The effect on ammonia concentrations on fixation of $[^{15}N_2]$ is reproduced below. In this series of experiments the flask contained 158.5 mg cell N, and gas phase was 0.1 atm $^{15}N_2$ (30 atom% ^{15}N excess) and 0.9 atm oxygen. In all the experiments high oxygen concentrations (inhibitory effects?) have been used. Quantitative calculations have been included in TABLE XXXVI.

TABLE XXXVII (TABLE III (66))

Effect of concentration of ammonia incorporation of $^{15}N_2$ into cells and supernatant ammonia by Azotobacter vinelandii

Flask No.	NH_4^+ added mg	NH_4^+N consumed mg	Atom% ^{15}N excess		*CALCULATED	
			Cells	NH_4^+ from medium	^{15}N Cells µg	$^{15}NH_4^+$ µg
1	30	10.00	0.0	0.15		30
2	20	5.00	0.01	0.33	16.3	50
3	10	4.25	0.03	0.97	49	55
4	5	3.75	0.09	1.68	145	21
5	0		0.18	0.04	285	0.8

+ 2 mg of normal NH_4^+N added as carrier
* The results in these columns have been calculated from the published results.

The $[^{15}N]$ content cells have been calculated by assuming that the final total nitrogen = initial total nitrogen + ammonia consumed and that fixation does not exceed a few mgs.

It can be seen that there is a progressive inhibition by ammonia e.g. 158.5 mg N of culture in the presence of 20mg added NH_4N, fixation = $16.3 \times \frac{100}{31.9} = 49 \mu g.N$

(NH_4^+N) concentration = $\frac{20}{100} \times 100 = 13\%$
(relative to total nitrogen)

Under these conditions 5 mg of ammonia are utilised whilst 200 µg. nitrogen are fixed, a ratio of 25:1. A comparison to the amount fixed in the absence of ammonia gives

in cells, $\frac{16.3}{285} \times 100 = 6\%$

Total, $\frac{66}{285} \times 100 = 23\%$

It is interesting to note that from TABLE II experiment II the calculated uptake of $[^{15}N]$ in Azotobacter culture (similar total nitrogen) in the presence of 10 mg NH_4^+N after 45 mins = $\frac{0.15}{100} \times 150 \times 10^3 = 225$ µg.

This compares with the value of 49 µg in No.3 TABLE III. It is unfortunate that the nitrogen values of the samples submitted for mass spectrometric analysis are not given, particularly in TABLE I. This would have permitted a quantitative comparison in this work.

It is evident that nitrogen fixation is inhibited by ammonia concentrations but it is not evident to what extent cell growth is effected by high concentrations of ammonia. It is probable that the results have been influenced by other factors, rates of diffusion, the high pO_2, (pO_2 = 0.9 atm), possible effects of blanks etc. and consequently cannot support the complete reduction of one molecule of nitrogen to two molecules of ammonia.

In the final section a mechanism for nitrogen fixation has been proposed.

(VIII) MECHANISM OF NITROGEN FIXATION

(A) FIXATION OF GASEOUS NITROGEN BY CLOSTRIDIUM AND AZOTOBACTER

The following comment represents an attempt to correlate the currently held views on the mechanism of nitrogen fixation, in particular with the role of hydrogenase.

Although some success has been achieved with Clostridium the experimental evidence has not established any comprehensive mechanism for nitrogen fixation. There is strong support for ammonia (only) as the first 'free' product of fixation, but the manner in which nitrogen is disruptively reduced remains open to speculation.

The evidence in support of ammonia as the sole initial intermediate in nitrogen fixation by Azotobacter is much less impressive than that on Clostridium, and it is advisable to note the chief differences between the aerobe, Azotobacter vinelandii and the anaerobe, Clostridium pasteurianum.

(a) The successful level of nitrogen fixation, reported in extracts of Clostridium, has not yet been achieved in extracts of Azotobacter.

(b) Added pyruvate was essential for fixation by extracts of Clostridium, although recent work has indicated that replacement by an ATP generator

and a reducing agent is possible. Earlier work had already suggested a high energy phosphate requirement for fixation.

(c) It has been reported that the nitrogen fixing system from Clostridium has been separated into two components, a "nitrogen activating system(NAS) and a 'hydrogen donating system' (HDS). Fixation was apparently obtained by their combination in the presence of pyruvate and it was reported that hydrogenase activity was confined to hydrogen activation. It was implied that nitrogen adsorbtion was a property of the 'NAS' and that hydrogenase was not involved in this.

(d) Ferredoxin, an electron transport particle that could couple the phosphoclastic reaction to hydrogenase, was isolated from extracts of Clostridium. It contained non-haem iron.

(e) The hydrogenase of Azotobacter, unlike that of Clostridium, has not been purified or 'solubilised' to any great extent and is essentially particulate.

In this respect it has been suggested that there are two types of hydrogenases (83):

(i) anaerobic hydrogenases(e.g. in Clostridium pasteurianum and Desulphovibrio desulphuricans) and

(ii) aerobic hydrogenases (e.g. in Azotobacter and Acetobacter.)

The latter group (ii) are able to catalyse the reduction of 'two electron dyes' but cannot evolve hydrogen from reduced methyl viologen or effect isotopic exchange with deuterium labelled water at a measurable rate. The hydrogenase of Azotobacter has been shown to be reversible (241) but hydrogenase activity can only be assayed conveniently with a 'two electron dye' (methylene blue) or potassium ferricyanide as acceptor. It was suggested that both types of hydrogenase contain an iron flavoprotein but that group (ii) lacked a second metal (Mo) necessary for 'one electron' transfer. This classification is not a rigid one and fractionation procedures may convert group (i) hydrogenase to group (ii) hydrogenase.

It must be pointed out, however, that the most recent communications on nitrogen fixation in extracts of Azotobacter claim great similarity between fixation in Azotobacter and Clostridium. It reported that consistent fixation in Azotobacter may be achieved provided that a crude hydrogenase preparation containing ferredoxin, and an 'ATP generator' was added. This would imply that an election transport factor in Azotobacter, similar to ferredoxin, was markedly less stable in the aerobe. It was also reported that no oxygen was required, but until the role of

hydrogenase in nitrogen fixation is clearly recognised the validity of these results must be questioned.

From the overall knowledge on nitrogen fixation the following components appear to be necessary.

(a) a site of fixation,

(b) a reducing power,

(c) an ATP requirement.

In Clostridium the 'NAS' is considered the site of fixation, the initial adsorption or activation of nitrogen, but the experimental evidence, particularly the spectral evidence (which may merely be a reflection of the purity of exposing gases), is not too convincing. If, on the other hand, hydrogenase is the active site two factors would need explanation. Firstly, what component is present in the 'NAS', but is absent from the 'HDS'? Secondly, why is the nitrogen fixing system so labile whereas hydrogenase activity is reasonably stable to storage? It is possible that a labile nitrogen acceptor is required, a component that is labile to heat treatment and storage, and that ATP is necessary to maintain a source of this acceptor. Stanier (242) has already pointed out that all photosyntheses have a common step, the excitation of chlorophyll molecule by light leading to the expulsion of an electron. The subsequent electron transfer,

concomitant with ATP generation, is dependent on the particular organism, in purple bacteria resulting in NADH ,in photosynthetic bacteria, resulting in the evolution of hydrogen or nitrogen fixation.

On this basis it is possible to atribute the limited success with cell-free fixation in extracts of Azotobacter to one or more factors:

(i) the particulate nature of the oxidative phosphorylating system, which has resulted in failure to 'solubilise' the ATP generating system and has hence restricted the formation of an 'acceptor' of nitrogen.

(ii) the instability of an electron transport particle corresponding to ferredoxin.

(iii) the lack of solubilisation of hydrogenase (site of fixation?).

From a chemical viewpoint the intriguing questions remain;

(i) How is the nitrogen adsorbed? Does physical adsorption or chemisorption occur?

(ii) How is the nitrogen disrupted? Does disruption occur prior to or subsequent to reduction?

(iii) How is the nitrogen reduced? What is the reducing power?

In view of the high bond energy of nitrogen, the initial step is of particular importance. The overall

energy changes for the fixation of a molecule of nitrogen, have been calculated from thermodynamic data. The original estimate of Wilson and Burris (16) for the reduction of nitrogen by glucose was subsequently modified by Bayliss (243).

For the equation,

$$C_6H_{12}O_6\ (aq) + 4N_2 + 6H_2O + 8H^+ \longrightarrow 6CO_2 + 8NH_4^+$$

the energy change was undoubtedly favourable ($\Delta G = -119$ kcal/mole) Bayliss, by consideration of the respective half reactions, deduced that for the glucose $\rightarrow CO_2$ couple, $N_2 \rightarrow NH_4^+(aq)$ was favourable, $N_2 \rightarrow N_2H_5^+(aq)$ unfavourable and $N_2 \rightarrow NH_2OH(aq)$ highly unfavourable.

Wusteman (28) has recently suggested that a better estimate of the energy changes in fixation could be obtained from

$$N_2 + 3\ NADH_2 + 2H^+ \longrightarrow 2NH_4^+aq + 3\ NAD$$

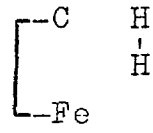
However, these thermodynamic considerations can only give some feasibility of nitrogen fixation because of the lack of accurate estimates of the activities of the species involved and cannot throw light on the mechanism involved.

Winfield (77) attempted to put hydrogenase activity and nitrogen fixation on a chemical basis. He examined the reaction of hydrogen gas in solution and suggested sites for both hydrogenase and nitrogenase.

The uptake of hydrogen by certain metal complexes was compared with the adsorption of hydrogen gas on metal surfaces. Some resemblances were observed; viz chemisorption of hydrogen, by homolytic dissociation of hydrogen with bond formation to a metal 'atom' which had a suitable unpaired electron in an outer orbital.

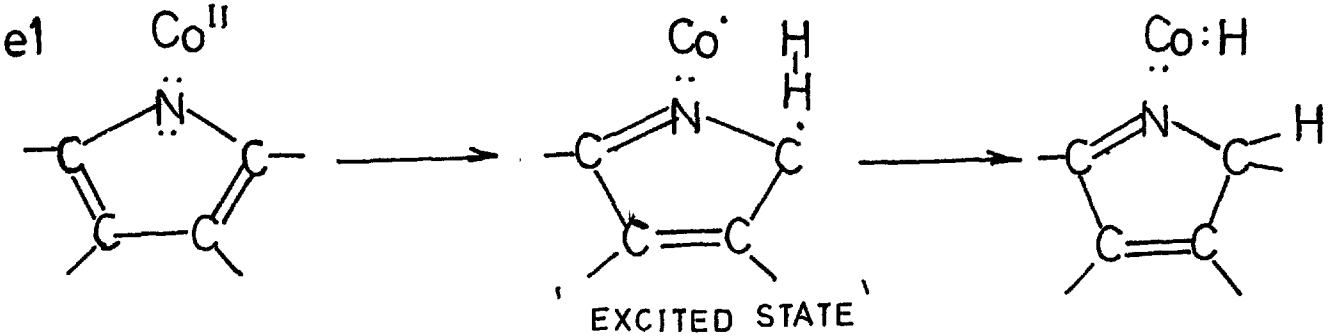
Although both a mononuclear complex and a dinuclear compex were considered as possible key intermediates in hydrogenase action, from consideration of the mode of reaction of oxygen with hydrogenase and cobalt-histidine, a mononuclear complex was favoured.
It was proposed that hydrogenase activity was essentially similar to the mononuclear reaction proposed between cobalt di-histidine and hydrogen (scheme 1, Figure 13) in which Co^{II} required initial excitation before reaction with hydrogen. It was proposed that Fe^{II} could, with suitable co-ordination, behave in a similar manner, and that either a carbon or nitrogen atom could function as the second 'receptor' of a hydrogen.

e.g.



Nitrogen fixation was visualised as requiring two such hydrogenase units, held 3Å apart by protein as illustrated in scheme 2 (Figure 13). Winfield suggested that the reduction of the adsorbed nitrogen was mediated by the

scheme1



scheme 2

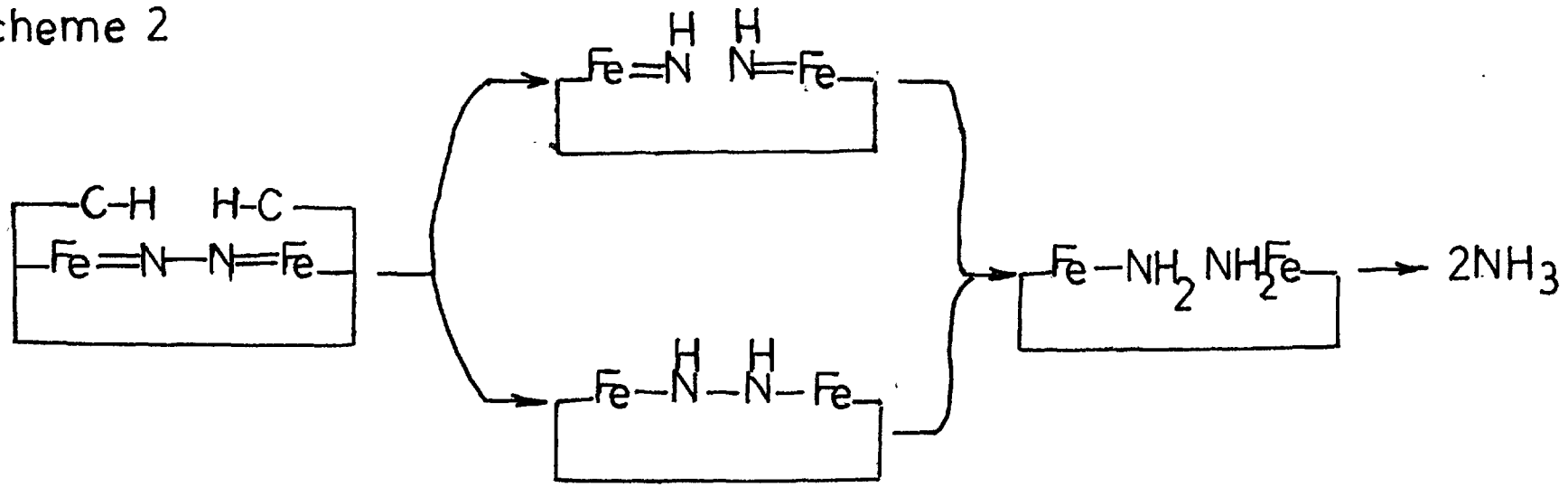


FIGURE 13

224A

carbon atom holding a hydrogen atom in a suitable position. The reduction of nitrogen via the upper pathway was favoured from considerations of heats adsorption etc. In essence, Winfield proposed that when a nitrogen molecule finds itself between two iron 'atoms' (3Å apart) then chemisorption by bond formation with an outer orbital electron of the metal 'atom' occurs. He offered, however, little explanation either of the mode of reduction of the adsorbed nitrogen by the suitably placed hydrogen or of the subsequent electron transfers.

Gundry (244) has pointed out that chemisorption can only occur at a metal surface when the strength of the bond to the metal atom is greater than the bond dissociation energy of the 'adsorbed' molecule. In this respect data on nitrogen, hydrogen and oxygen appear in TABLE XXXVIII

TABLE XXXVIII

	Electo-negativity	Inter-atomic distance Å	Bond dissociation energy Kcal /mole
NITROGEN	3.0	1.10	226
N_2^+		1.12	206
HYDROGEN	2.1	0.74	108
H_2^+		1.06	64
OXYGEN	3.5	1.20	119
O_2^+		1.12	
O_2^-		1.26	
O_2^{2-}		1.49	

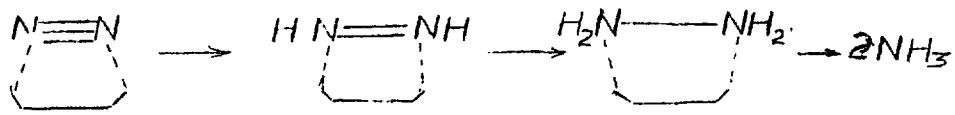
It can be seen that although the bond energy of nitrogen is appreciably higher than that hydrogen it is not lowered to the same extent in the ionised molecule (H_2^+ v N_2^+). This could be interpreted to mean that the initial step in molecular disruption is more likely to involve a free radical in the case of hydrogen than nitrogen.

The scheme for nitrogen fixation postulated by Winfield (scheme 2) implies a radical mechanism and takes little account of transport factors. Roberts has pointed out (17) that no comparable uptake (to that of hydrogen) of nitrogen by complexes in solution has yet been reported. The overall credibility of any mechanism postulated for enzymatic catalysis must depend on how effectively it justifies the high specific rates characteristic of enzyme catalysis. In this particular case it is questionable whether a valid case has been made for the rapid adsorption, reduction and subsequent desorption of ammonia.

The scheme of Winfield does suggest that the fixation of the nitrogen molecule leads to a formation of a type of nitride. Carnahan (245) has also suggested that a nitride ion (of iron) is an intermediate in fixation and suggested that its formation was mediated by molybdenum. The suggestion of a nitride intermediate

has received some support from the communication by Chatt (246). Chatt obtained a brick red complex from the reduction of rhenium heptoxide by hydrazine hydrochloride in the presence of phosphine. The complex was reasonably stable and the evidence suggested that a nitride ion was present. However, it is unlikely that the bacterial fixation of nitrogen involves such unusual conditions, although the principal criticism against a mechanism involving a nitride ion must be that no explanation of the efficiency and specifity of enzymatic catalysis is apparent.

The currently accepted scheme for the fixation of nitrogen does not attempt to specify the nature of the 'bound' nitrogen but merely postulates the stepwise reduction of nitrogen to ammonia, as shown below.



In symbiotic fixation it has been suggested that the initial adsorbtion of nitrogen may occur on the haem group of legogloblin. It was reported that a 'secondary' attachment of nitrogen to the haem group of legoglobin occurred (247) and subsequently, in 1963, it was claimed that a protein had been isolated, in which the haem group of ferrole globin preferentially complexed with nitrogen, rather than oxygen, in an irreversible

manner (248). As a general model for nitrogen fixation this is in conflict with earlier work on Clostridium since it has been reported that the existence of soluble haemprotein in Clostridium had not been detected (249). In the scheme, shown above, it is implied that a symmetrical reduction of nitrogen occurs. If such a route is operative, the stepwise reduction via 'bound' diimide and 'bound' hydrazine, one would expect that added hydrazine would be reduced (and then incorporated).

The work on the uptake of hydrazine by cultures of Azotobacter has been discussed earlier. It appears that hydrazine is merely sequestrated, the 'bound' form being dependent on the particular cultural conditions, with no convincing evidence in support of its disruption or utilisation.

The role of hydrazine in nitrogen fixation by Clostridium has not been established. A preliminary communication did appear which reported that hydrazine was reduced by cell-free fixing extract of Clostridium (250). These extracts were reported to reduce nitrite, hydrazine and hydroxylamine to ammonia, with hydrogen as reductant at a comparable rate to nitrogen fixation, with the exception of hydrazine which reacted slowly. However, hydrazine was not detected either in the initial experiments on successful cell-free fixation or in the

subsequent [^{15}N] work of Garcia Rivera and Burris (251). These workers found that no exchange reaction occurred between 'enzymatically held' hydrazine and added hydrazine. Hydrazine was added to an extract which was fixing [^{15}N] labelled nitrogen gas and was subsequently recovered as its benzaldazine derivative. No [^{15}N] was detected in the recovered hydrazine.

There has been a report that an uptake of hydrogen occurred when hydrazine was incubated with cells of Micrococcus Lactylicus under an atmosphere of hydrogen (252). However, no evidence was put forward to confirm that an increase in ammonia had taken place.

It would thus appear that hydrazine is not directly implicated in the fixation mechanism. This point should be considered a factor against mechanism involving a symmetrically held nitrogen intermediate. It is possible that a tightly bound form of hydrazine occurs on the enzyme but this would make a facile reductive removal difficult to understand. In symbiotic nitrogen fixation a free radical mechanism involving hydrazine as intermediate has been justified on thermodynamic grounds (253) (254). It was proposed that N_2^- was formed by the interaction of nitrogen with legoglobin and then, by free radical reactions, this was progressively converted to hydrazine and finally ammonia.

However, the recent work of Zacharias (255) (256) (257) (258) (259) has suggested that positive nitrogen ions (N_2^+) affect fixation in Azotobacter whereas negative ions are without effect. It must be pointed out that Zacharias measured nitrogen fixation in terms of mg nitrogen fixed per gm glucose consumed. He did not observe an increase in the amount of nitrogen fixed but an increase in mgN fixed/gm glucose consumed which may merely reflect a reduction in the amount of glucose consumed. This may mean that the transport of nitrogen to the site of fixation is facilitated by the formation of the N_2^+ cluster ions. The existence of N_2^+ has been observed in photo-electron spectroscopy (260) and Virtanen had previously suggested that this ionisation of nitrogen may be the initial step in nitrogen fixation. (15). It is interesting to note that the (N-N) bond is weakened by this ionisation ($N_2 \rightarrow N_2^+$) see TABLE XXXVIII.

It can be seen that there is little experimental backing for the symmetrical stepwise reduction of nitrogen to ammonia. Such a scheme has arisen from the (incomplete) evidence in support of ammonia being the sole product of fixation in extracts of Clostridium. Even if ammonia is the sole (initial) product of fixation, it does not rule out the possibility that ammonia formation may be accomplished in an unsymmetrical manner.

It has been mentioned earlier that Nason gave an indication of this (21) but it was Pratt who boldly proposed, from energy considerations, that the heterolysis of the nitrogen molecule could occur. (20). It may well be that the initial adsorption holds the key to the solution and that more consideration should be given to the type of mechanism involved, i.e. ionic v free radical mechanism.

In this respect, Virtanen (15), several years ago, did suggest that an ionic mechanism was possible and one can set out the stepwise reduction of the nitrogen molecule on an ionic basis. Such a mechanism is proposed below.

[B] PROPOSED MECHANISM OF NITROGEN FIXATION

This is shown in Figure 14 and consists of three steps:

(I) ADSORPTION OF NITROGEN AND ITS CONCURRENT STABILISATION.

(II) REDUCTION.

(III) DISPLACEMENT AND REDUCTION.

M represents the metal ion, P the suitably positioned nucleophile.

(I) INITIAL STEP

It is proposed that this is a concerted step (push-pull mechanism). The nitrogen, already polarised in solution to $\overset{\delta+}{N}\equiv\overset{\delta-}{N}$, has its electron density further

scheme 3

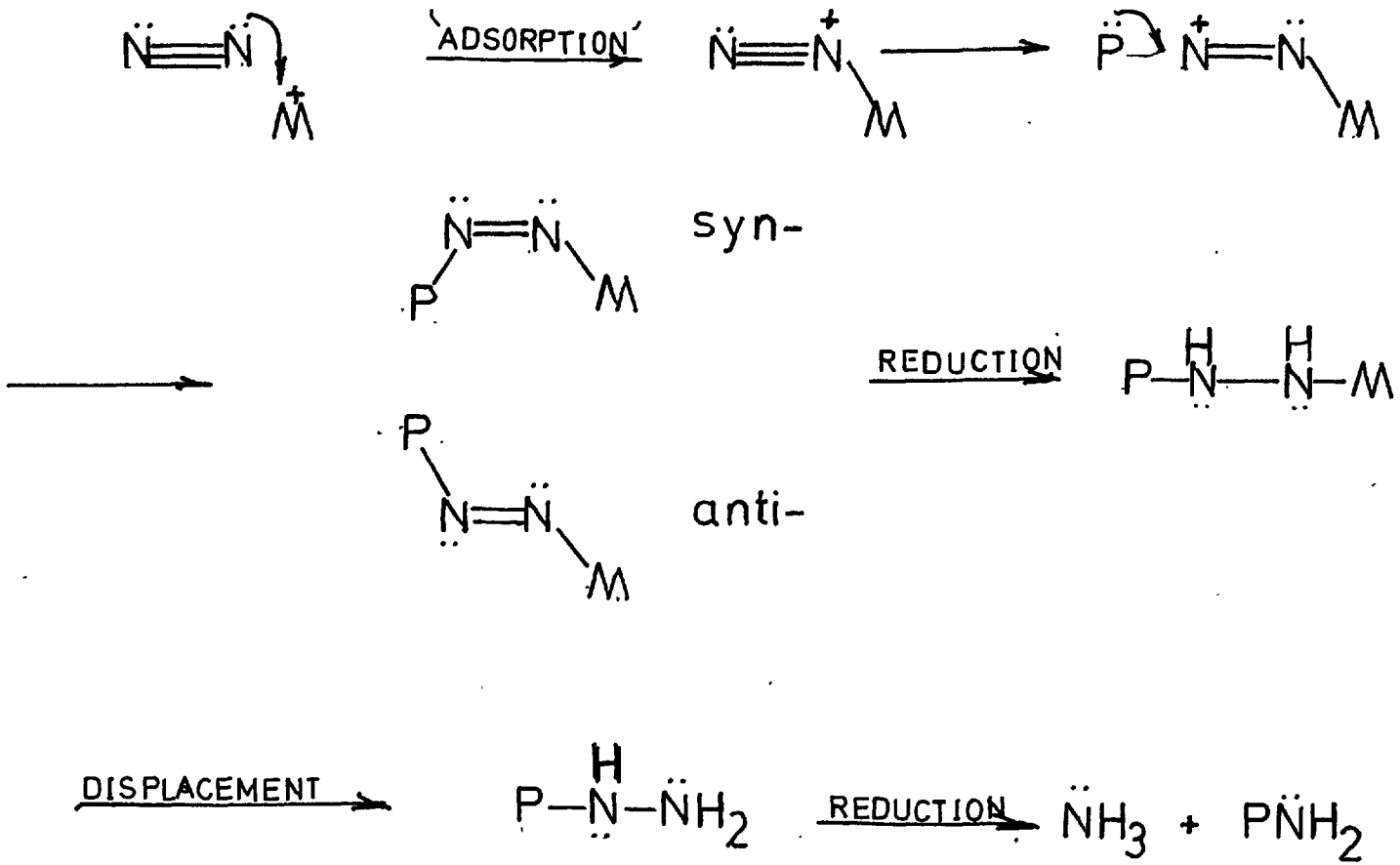


FIGURE 14

distorted by a positive ion. The intermediate is stabilised by reaction with a suitably-positioned nucleoplile.

Ingraham (261) has summarised the function of metal ions in biochemistry. He states that they may;

(a) aid gathering of reactants and thus increase entropy of activation,

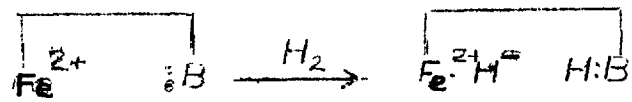
(b) act as intermediates between one or two electron oxidation-reductions,

(c) stabilise free radicals,

(d) act as general acid catalysts.

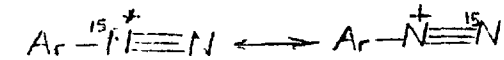
In general the effectiveness of a metal ion will increase with increasing charge and decreasing radius and will depend on the extent of shielding of (d) electrons. Eichhorn (262) has pointed out that the actual effectiveness of a metal ion in enzymatic catalysis will depend both on its effectiveness in the desired function and its tendency to engage in undesirable side reactions. Thus, although in the catalysis of non-enzymatic reactions by metal ions the order of activation follows the order of complex stability, it is impossible to make similar predictions in enzymatic reactions. Certainly the stereochemical requirements of the particular reaction may be critical as is illustrated by the mechanism of aconitase action, catalysed by Fe^{2+} ion (262).

The initial adsorption of nitrogen is more feasible if it is regarded as a concerted reaction. Harris (61) had originally made a similar suggestion, in which he proposed that B was a sulphydryl group. A mechanism of hydrogenase action on these lines had also been suggested by Rittenberg (85).

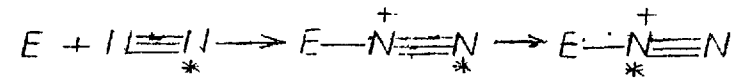


It was postulated that a distance of 2.5A between the metal ion and nucleophile was necessary and it was suggested that B might be an imidazolyl group or a sulphydryl group. A mechanism for hydrogenase activity on the same site as nitrogen fixation, will be proposed at a later stage.

There is some resemblance of the proposed initial step for the adsorption of nitrogen to the reported rearrangement of aryl diazonium salts, that accompanied their hydrolysis (263).



The possibility that this rearrangement resulted from the reaction of molecular nitrogen with a highly energetic and unselective electrophilic species (E). e.g. an aryl cation, was considered



This reaction, a possible model for nitrogen fixation,

was rejected on the grounds that CO, an iso-electronic but more powerful nucleophile than nitrogen, did not, even under a high pressure of CO, react with diazonuim fluoro-borate to form benzoic acid.

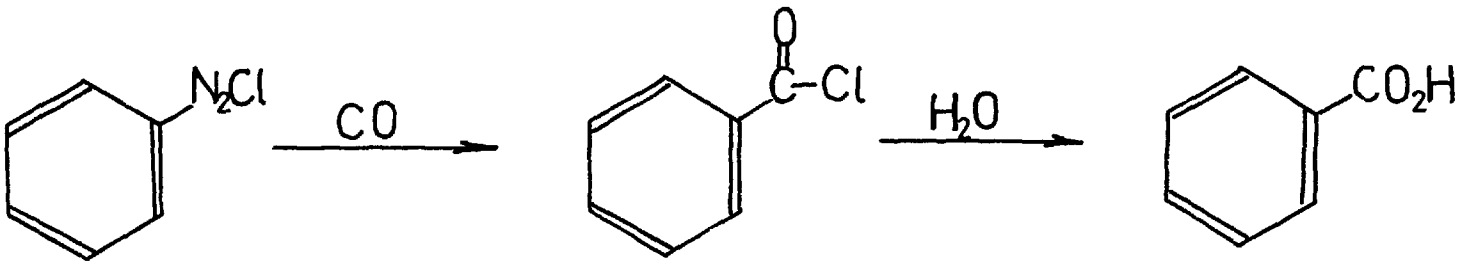
i.e. the reaction $Ar+ CO \rightarrow Ar^{\oplus}C \equiv O \rightarrow ArCO_2H$ did not occur.

A reaction, however, has been found to occur between benzene diazonium chloride and nickel tetracarbonyl (264), scheme 4 (Figure 15).

The existence of a 'di-imide derivative' in a metal complex has already been postulated (265). Clezy and Morell, from spectroscopic data, concluded that when haemin chloride reacted with diazomethane in ethereal solution, a complex, $Fe^{3+} \leftarrow N \equiv N-CH_2Cl$, was formed. They proposed that its breakdown in aqueous solution could be represented as shown in Scheme 5 (Figure 15). However, the experimental evidence does not support such a detailed mechanism.

Before considering the subsequent reduction of the adsorbed nitrogen it must be recorded that a recent paper, (266), has suggested that molybdenum occupied a key role in nitrogen fixation. The cathodic reduction of nitrogen, using platinum or porous graphite electrodes in solutions of molybdic acid, was reported. Although the absolute quantities of nitrogen fixed were not given,

scheme 4



scheme 5

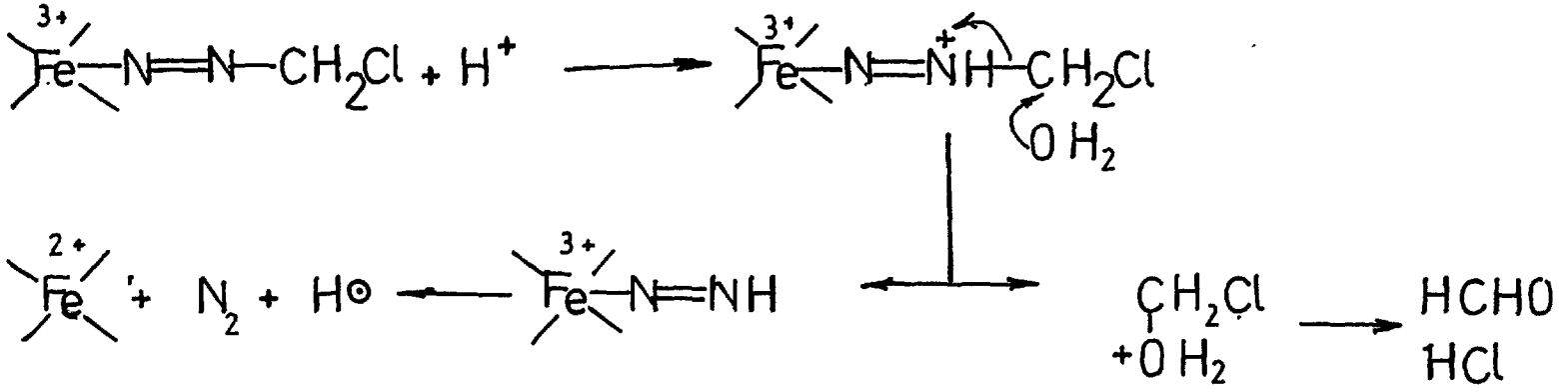


FIGURE 15

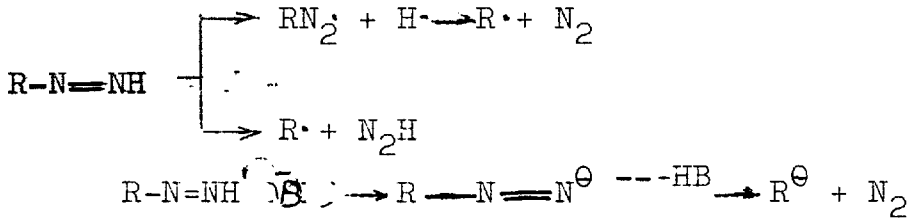
2344

the paper emphasised the importance of Mo^{4+} and Mo^{5+} in this fixation.

(II) REDUCTION

The proposed mechanism suggests that after the initial adsorption two forms, the syn-and anti-, are possible. These may be important from a reductive point of view.

Aylward (267) in his investigations into the reduction of ethylene bonds by hydrazine in the presence of oxygen, concluded that only the Syn-isomer of diimide reduced the double bond. The decomposition of substituted diimides, generated in situ, has also been studied (268). It was found that nitrogen was eliminated either by a homolytic reaction or by a base catalysed elimination, as shown.



Certainly diimide and related compounds have received more attention in recent years, but there still exists considerable scope for further work related to the fixation of nitrogen. Callander (269) in this laboratory, did investigate the reduction of certain azo-compounds by hydrogen in the presence of hydrogenase. Of the compounds studied only the azopyridines were reduced, although not completely to amino-compounds. It is

interesting to note that the trans 2,2' azopyridine was reduced more rapidly than the cis-isomer. Callander concluded that reduction was feasible in -N=N- linkages if the electron density between the nitrogen atoms could be decreased in some way.

(III) DISPLACEMENT AND SUBSEQUENT REDUCTION.

During the reduction of nitrogen, the bond length between the nitrogen atoms increases (58), (N-N) distance in nitrogen 1.07Å, (N-N) distance in hydrazine 1.47Å, and it is proposed that this increase results in a strain factor. This strain factor, aided by lone pair - lone pair repulsion of the hydrazino-nitrogen atoms causes the facile displacement of the unsymmetrical hydrazino-derivative, which is subsequently reduced to ammonia and a labile amino-derivative.

[C] PROPOSED ACTIVE SITE AND MECHANISMS OF HYDROGENASE ACTIVITY

If hydrogenase is the site of fixation then one must offer some explanation of nitrogen fixation and of the variation of hydrogenase activity with the particular acceptor.

Gest (83) postulated a generalised scheme for the mechanism of hydrogenase action, Scheme 6 (Figure 16). This attempted to correlate the previously reported components of hydrogenase, namely iron, molybdenum and

scheme 6

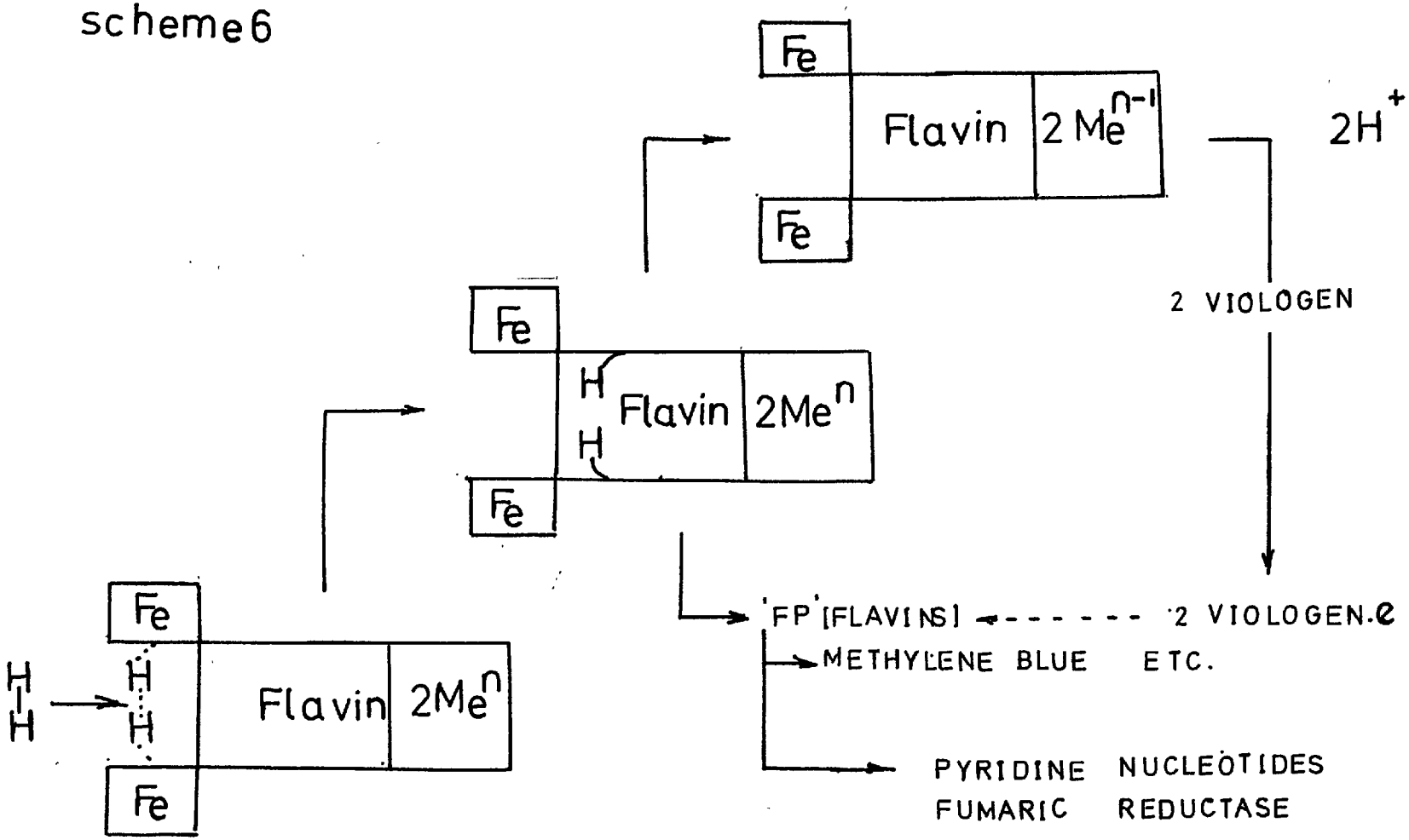


FIGURE 16

flavin, but did not specify the actual site of hydrogenase or the electron transfers involved.

In this thesis it is proposed that the site of both hydrogenase and nitrogenase is essentially an iron flavoprotein, as illustrated in Figure 17. It is suggested that the hydrogenase activities, measured with different acceptors, result from different mechanisms at this site. Thus, both homolytic dissociation (cf. Winfield, Gest) and heterolytic dissociation of hydrogen (cf. Rittenberg) may occur but the actual mechanism is determined by a number of factors, e.g. the particular organism (aerobic v anaerobic), pH, role of molybdenum and the particular acceptor. It is proposed that hydrogen dissociation and nitrogen 'adsorption' on this site may be accounted for by consideration of the flavin semiquinones, illustrated in Figure 18. The proposed mechanisms of hydrogenase activity will now be discussed.

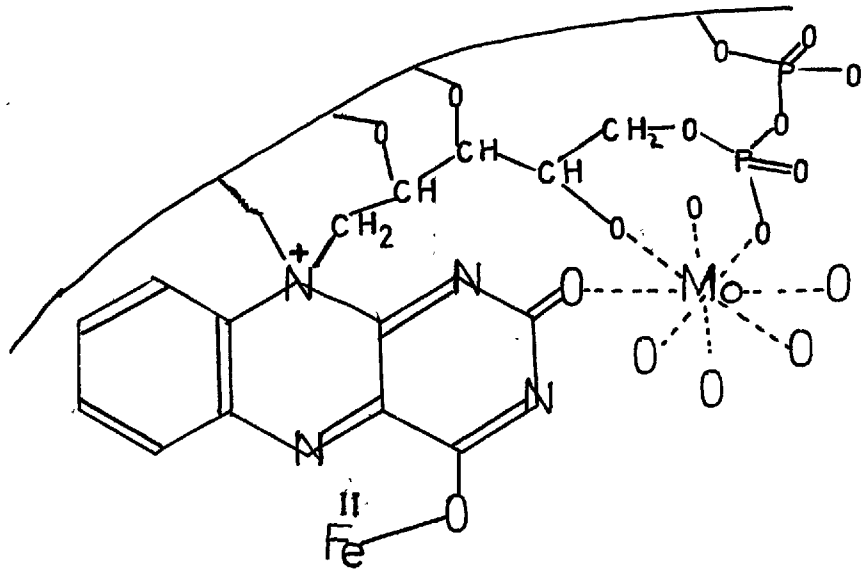
Three basic mechanisms have been proposed. These are:

(1) Hydrogenase activity with molybdenum participation.

(2) Hydrogenase activity without molybdenum participation.

(3) Hydrogenase activity with potassium ferricyanide as acceptor.

PROPOSED ACTIVE SITE FOR HYDROGENASE AND NITROGENASE


CH
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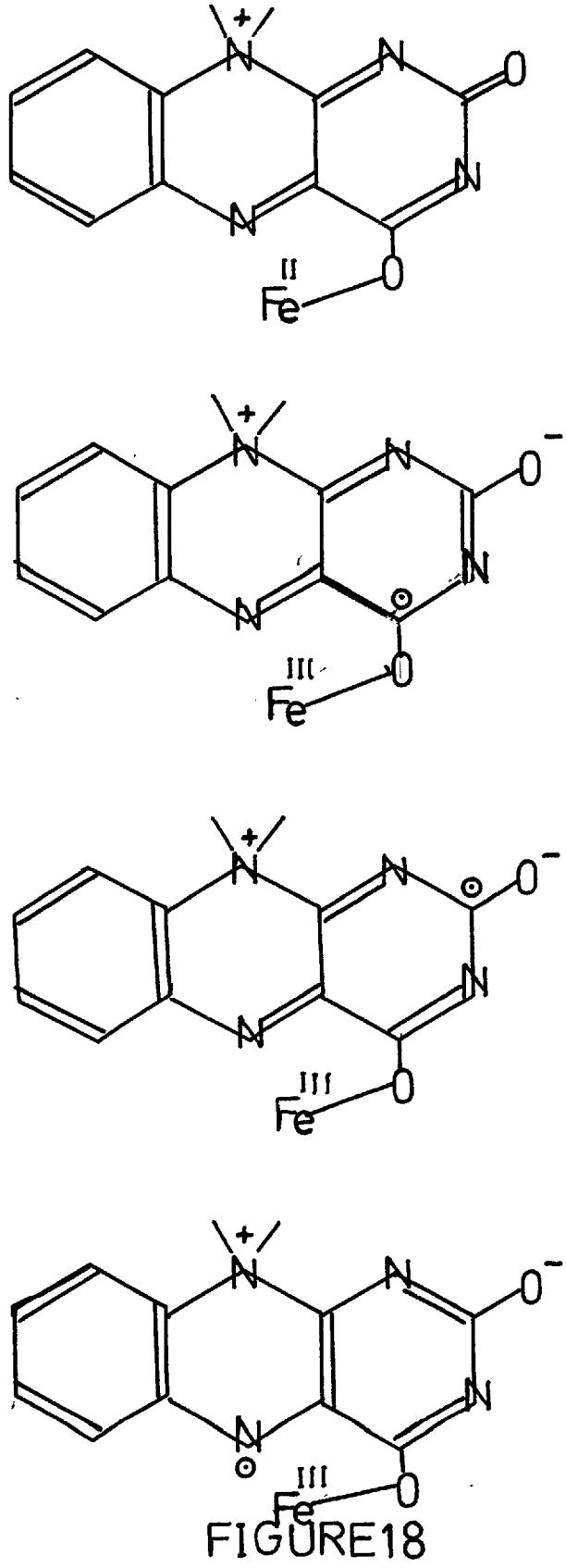


FIGURE18

(1) Hydrogenase activity with molybdenum participation. This is illustrated by Schemes, 7, 8 Figures 19, 20 and consists of:

(i) the homolytic dissociation of hydrogen,

(ii) the intervention of Mo^{V} to aid expulsion of $H^{\bullet}$ to a suitable acceptor.

(iii) The reformation of the original site, scheme 8, Figure 20. This can occur either by the upper or lower sequence of reaction.

In the upper sequence, a hydrogen ion is held on the nitrogen atom and expelled as a hydrogen radical by further intervention of molybdenum. In the lower sequence the original site is reformed either by oxidation of Fe^{II} or Mo^{IV} by the acceptor,

i.e. overall reaction

$$2\,\dot{x} + H_2 \longrightarrow 2\,\ddot{x} + 2H^{+}$$

scheme7

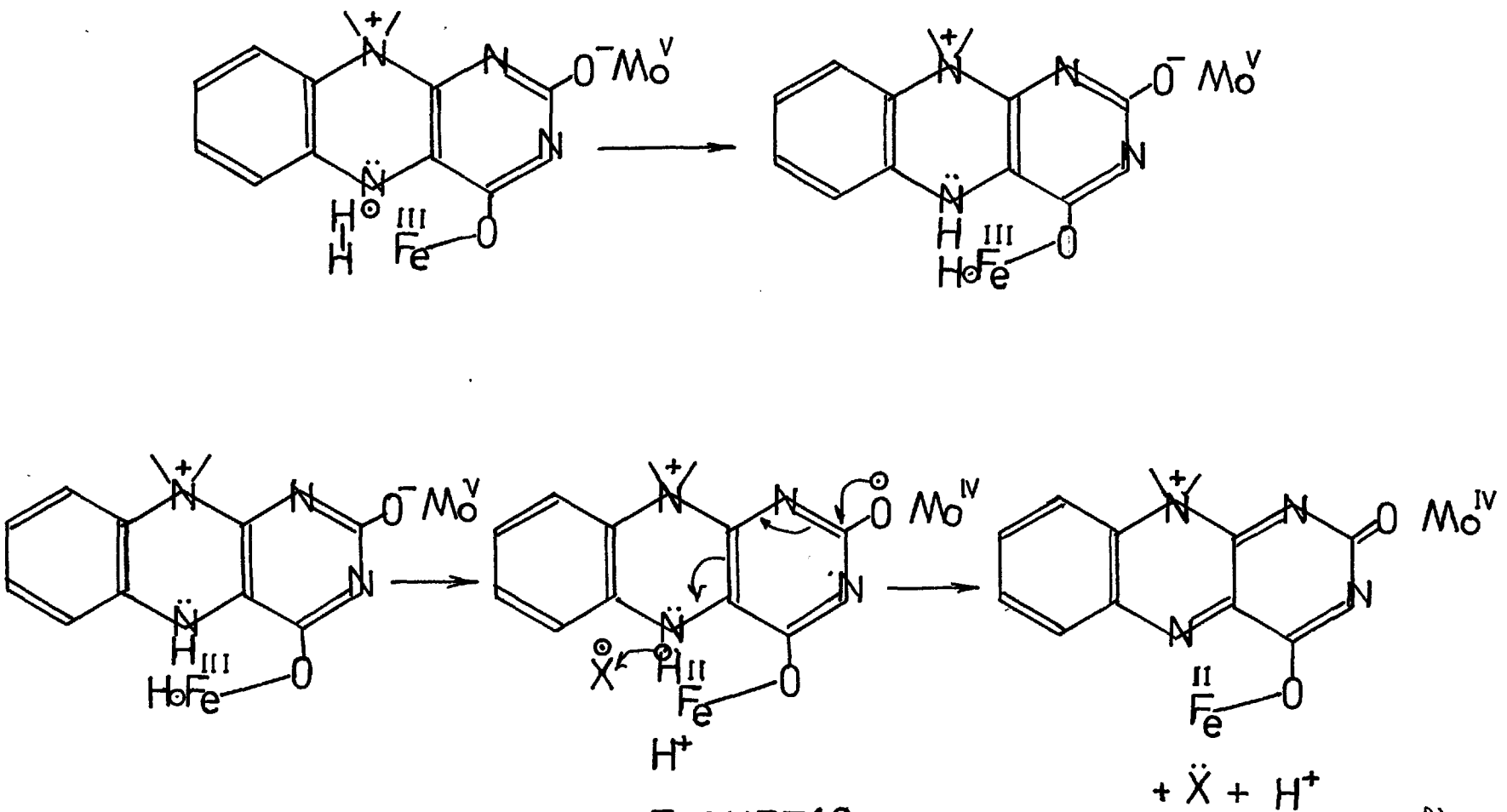


FIGURE19

scheme 8

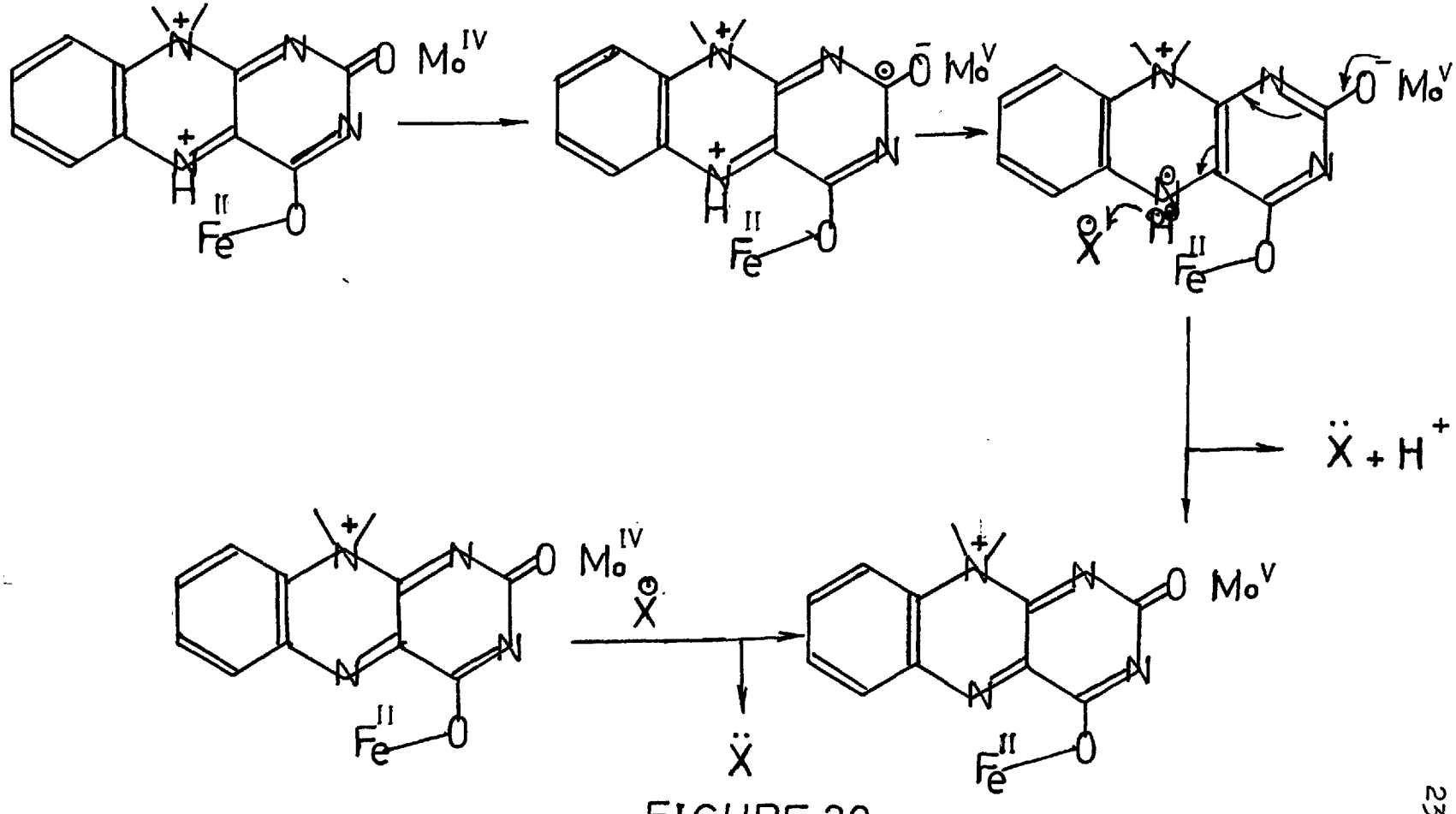


FIGURE 20

(2) Hydrogenase activity without molybdenum participation. This is illustrated in Figure 21 and is essentially,

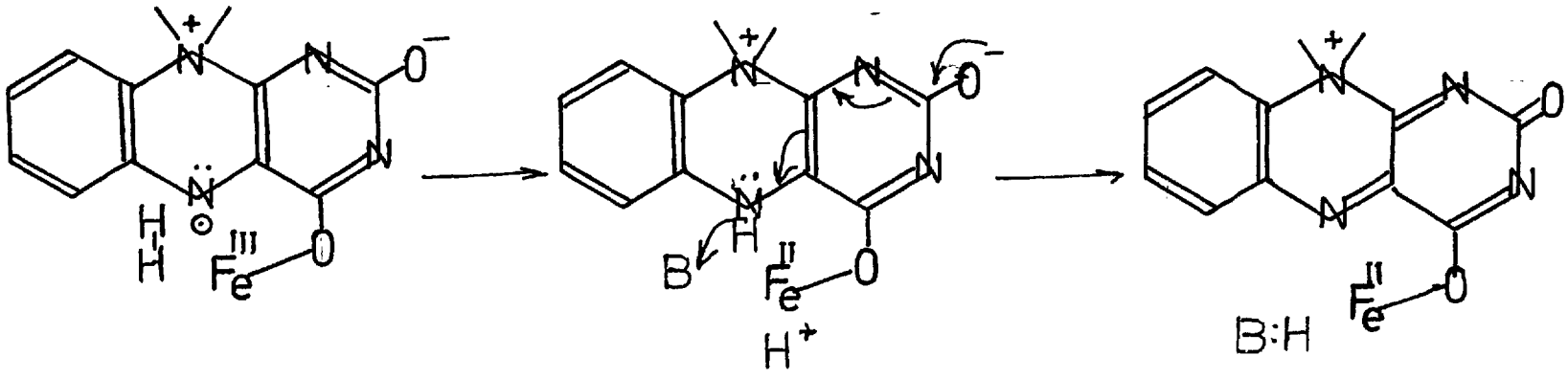
(i) the homolytic dissociation of hydrogen,

(ii) the explusion of hydride ion to an acceptor.

The reaction with methlene blue is shown.

i.e. MB + H_2 → reduced MB + H^+

scheme 9



B = METHYLENE BLUE, R = CH_3

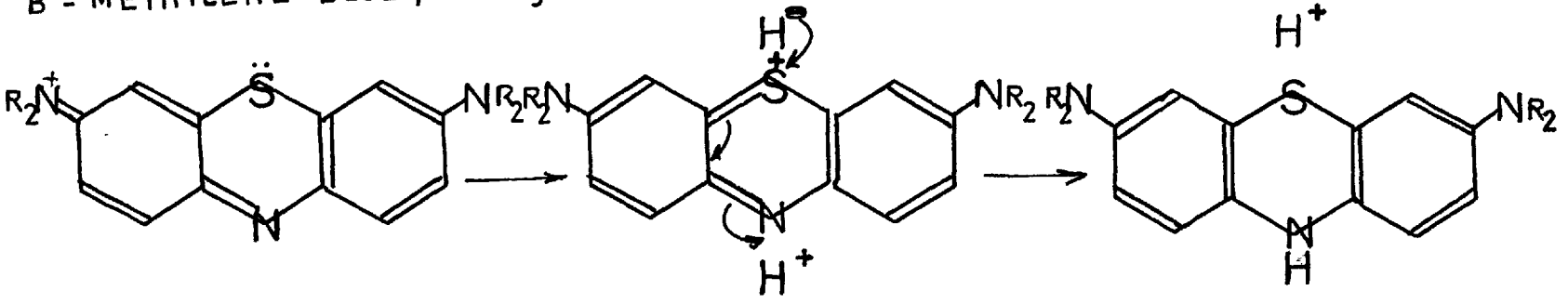


FIGURE 21

(3) Hydrogenase activity with potassium ferricyanide as acceptor.

This is illustrated in Figure 22, and differs from the other mechanisms in that a heterolytic dissociation of hydrogen is postulated. The principal steps are:

(i) The oxidation of Fe^{II} to Fe^{III} which prevents the formation of flavin semiquinones.

(ii) The heterolytic dissociation of hydrogen.

(iii) The breakdown of Fe : H by collision with ferricyanide to reform the original site.

Overall, $2\left[Fe(CN)_6\right]^{3-} + H_2 \rightarrow 2\left[Fe(CN)_6\right]^{4-} + 2H^+$

scheme 10

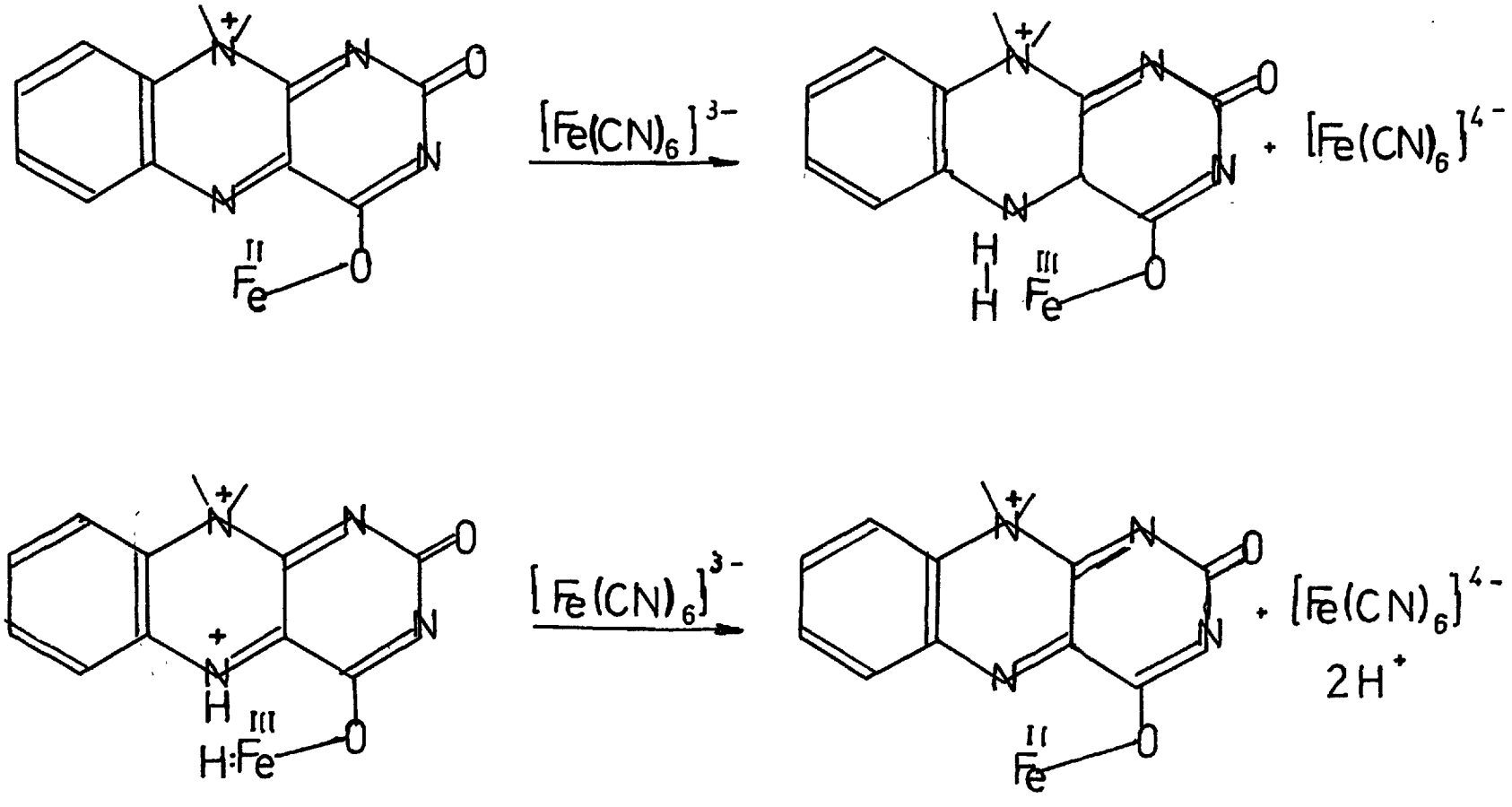
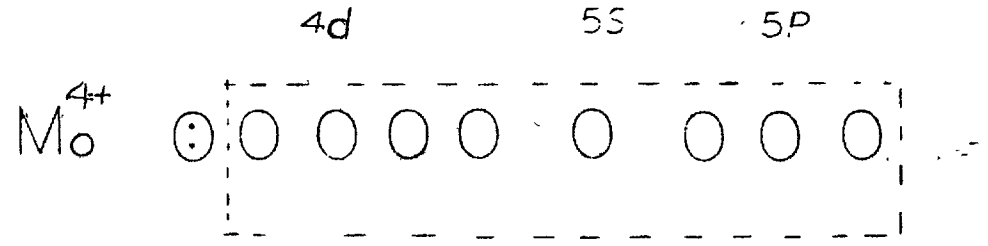


FIGURE 22

It is evident that several mechanisms for hydrogenase activity can be proposed on the basis of a common site. There appears little doubt that iron is necessary for hydrogenase activity. Nicholas has reported an iron requirement for the hydrogenase of Azotobacter (78). Sadana et alia (108) (218) have reported that iron was necessary for their purified hydrogenase from Desulphovibrio Desulphuricans. They were not able to detect a molybdenum requirement for hydrogenase (assayed by one or two 'electron dyes') although low hydrogenase activity was obtained when the method of Gest was used (80). Riklis and Rittenberg (98) have reported that iron (but no molybdenum) was detected by X Ray fluorescence spectroscopy in purified hydrogenase from Desulphovibrio desulphuricans (hydrogenase activity assayed by the exchange reaction). This was in agreement with the finding on the hydrogenase of Proteus vulgaris (217).

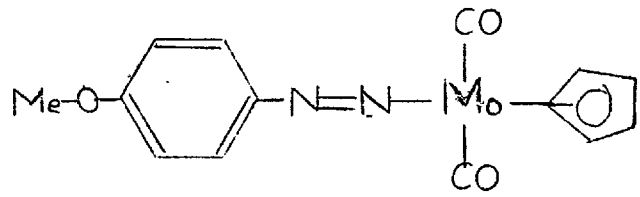
The presence of flavin semiquinones and Mo^{5+} in extracts of Azotobacter was indicated by the ESP studies of Nicholas et alia (106). It was observed that the signal associated with Mo^{5+} disappeared on oxidation and reduction. The changes in the oxidation states of molybdenum in the mechanisms have been arbitrarily represented as Mo^{4+} and Mo^{5+}, but the molybdenum requirement of nitrogen fixation (and hydrogenase activity) needs further investigation to ascertain the valence states and changes involved.

Nicholas and Stevens (270) have reported the isolation and separation of three oxidation states of molybdenum, Mo^{3+}, Mo^{5+}, Mo^{6+}, from the nitrate reductase of Neurospora. They also reported that Mo^{5+} was as effective as TPNH as electron donor for the reduction of nitrate and implied that the valence change was $Mo^{6+} \leftrightarrow Mo^{5+}$. It must be emphasised that the valence state and oxidation changes will depend on the type of molybdenum complex. It has been pointed out that in eight co-ordination complexes, Mo^{IV} valence state has been characterised, involving d^4 sp^3 orbitals.



The cyanide complex can be readily oxidised to paramagnetic Mo^{V}, $Mo(CN)_8^{3-}$, and it has been pointed out(271) that no complex $[Mo(CN)_8]^{2-}$, corresponding to Mo^{VI}, has yet been found since it would involve a vacant d-orbital, whereas normally Mo^{V} compounds are readily oxidised to Mo^{VI}.

The recent report of molybdate and tungstate catalysed fixation of nitrogen suggested that Mo^{IV} was important and it was pointed out that both Mo^{IV} and Mo^{V} form complexes with $(CN)^{-}$, (isolectronic with nitrogen) (266). Mo^{IV} has been postulated to be more catalytically active than Mo^{V} and Mo^{VI} in oxy-ion reduction and it was reported that fixation of nitrogen ceased when the molybdenum was reduced to Mo^{III} (272) (266). It is, however, advisable to limit speculation on the possible valence state of molybdenum in extracts of Azotobacter, particularly as the latter work implied that molybdenum might be the site of fixation. In this latter respect a recent communication has reported the isolation of the compound



the first compound in which an azo group is bound to a transition metal (273).

It is clear that more information is required on the type of Mo complexes present in extracts of Azotobacter.

[A review on the role of molybdenum in biology has appeared (300)]

It is worth noting that the $[^{99}Mo]$ taken up by particulate preparations from Azotobacter was stable to dialysis, both by phosphate and by cyanide (99)(100).

An important aspect of the proposed mechanisms is that they attempt to explain hydrogenase activity on a common site and suggest that environmental factors (which are dependent on the particular organism, aerobic V anaerobic, and include potential at site, the pH., co-ordination arrangement of iron and the potential of the acceptor) may be responsible for the mechanism in a particular organism.

If hydrogenase has a common site some basis must be presented for the exchange reaction and evolution of hydrogen from reduced viologen exhibited by some hydrogenases (but not the hydrogenase of Azotobacter). The principal workers on hydrogenase, Krasna and Rittenberg,(85) (217) (274) deduced from exchange studies, that the heterolytic disruption of hydrogen occurred in hydrogenase action.

They presented the mechanism of hydrogenase action by $Enz{:}OH + H_2 = Enz{:}H + HOH$ and the exchange reaction by $Enz{:}H + D_2O = Enz{:}OD + HD$ since in 100% D_2O, HD and not D_2 was the predominant species. They assumed that the hydride was not exchangeable with H^+ (eg $Enz{:}\ H + D^+ \rightleftharpoons Enz{:}\ D + H^+$) and concluded that only one atom from hydrogen was

exchangeable (i.e. heterolytic dissociation).

$$H_2 \text{ (gas)} \longrightarrow H^+_{aq} + H^-_{aq} \quad \Delta H = 42.8 \text{ kcal}$$

Rittenberg (85) suggested that this reaction could be catalysed by a metal ion,

$$\text{e.g. } Me^{++} + H_2 \longrightarrow Me^{++}\,\overline{H} + H^+aq$$

and proposed that the site of hydrogenase was

$$\left[Fe^{++} \qquad\qquad :B\right]$$

where Fe^{++} and nucleophile B were 2.5Å apart.

In the most recent paper Hartman and Krasna (274) have summarised the variation of the $\frac{DD}{HD}$ ratios of hydrogenase in different organisms TABLE XXXIX

TABLE XXXIX

HYDROGENASE ORGANISM	$\frac{DD}{HD}$
Proteus Vulgaris	0.2
Desulphovibrio desulphuricas	1
Scenedesmus	0.5.

Although some variation between hydrogenases of different organisms is evident, HD is predominant and it is argued that this eliminated the possibility of homolytic dissociation of hydrogen since DD would then predominate, as was found in platinum catalysis.

The proposed mechanisms suggest that both homolytic

and heterolytic fission of hydrogen may occur although even in the case of homolytic fission the hydrogen atoms become non-equivalent. Thus it would be difficult to distinguish between this type of homolytic and heterolytic fissions of hydrogen from exchange studies.

The exchange reaction, the appearance of HD, can be explained without difficulty by the second mechanism (without molybdenum participation). In this B becomes D^+

$$H^{\bullet} + D^+ \longrightarrow HD.$$

The possible mechanisms for the exchange reaction in the first case (with participation by molybdenum) are showin in Figure 23.

In this case one may postulate either a stepwise reformation of hydrogen after dissociation or merely the reversal of the initial dissociation.

The reversal of the initial step, after equilibration of the hydrogen ion at 'iron' site with solvent deuterium ion, is feasible. The reformation of hydrogen may be facilitated by the molybdenum if this could aid the expulsion of hydrogen radical from the iron.

In the stepwise procedure the exchange reaction must be represented by the reactions.

$$H\cdot + D^{\oplus} \longrightarrow HD^{\odot} + H^{\odot} \longrightarrow HD + H^+$$

the second hydrogen radical arising from the subsequent

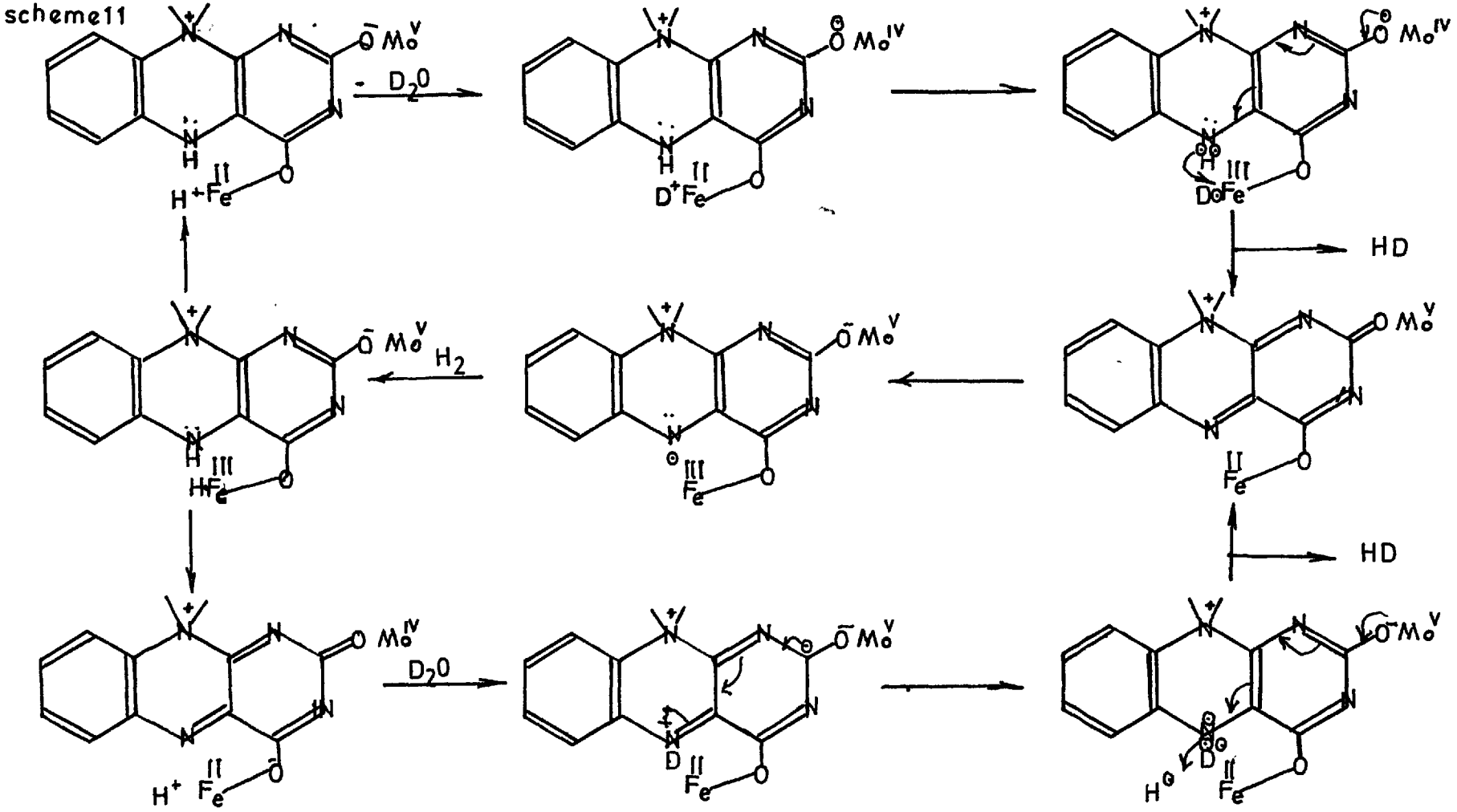


FIGURE 23

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expulsion of hydrogen radical at the 'iron' or 'N_{10}' sites, aided by molybdenum. If this were the case a greater degree of randomisation might well be expected.

The evolution of hydrogen from reduced viologen (method of Gest) can be represented by mechanisms involving hydride or hydrogen radical (Figure 24). In the first mechanism (Scheme 12) the reduced viologen initiates the free radical site for adsorption of H^+ and then restores the original site after the expulsion of a hydrogen radical.

In the second mechanism (Scheme 13) reduced viologen is required to furnish electrons, which become paired, before expulsion of hydride ion.

On the basis of these mechanisms, the mechanism of hydrogenase action of Azotobacter is more easily explained by the third mechanism, the heterolytic dissociation of hydrogen. This is almost identical to the mechanism of Rittenberg, although a fundamental difference is that the hydrogenase of Azotobacter cannot be assayed by the evolution and exchange method. Thus one would have to postulate that in the aerobic hydrogenase the valence state of iron is III, i.e. a heterolytic fission of hydrogen occurs and that the breakdown of the hydride ion at the iron site is irreversible and requires collison with a

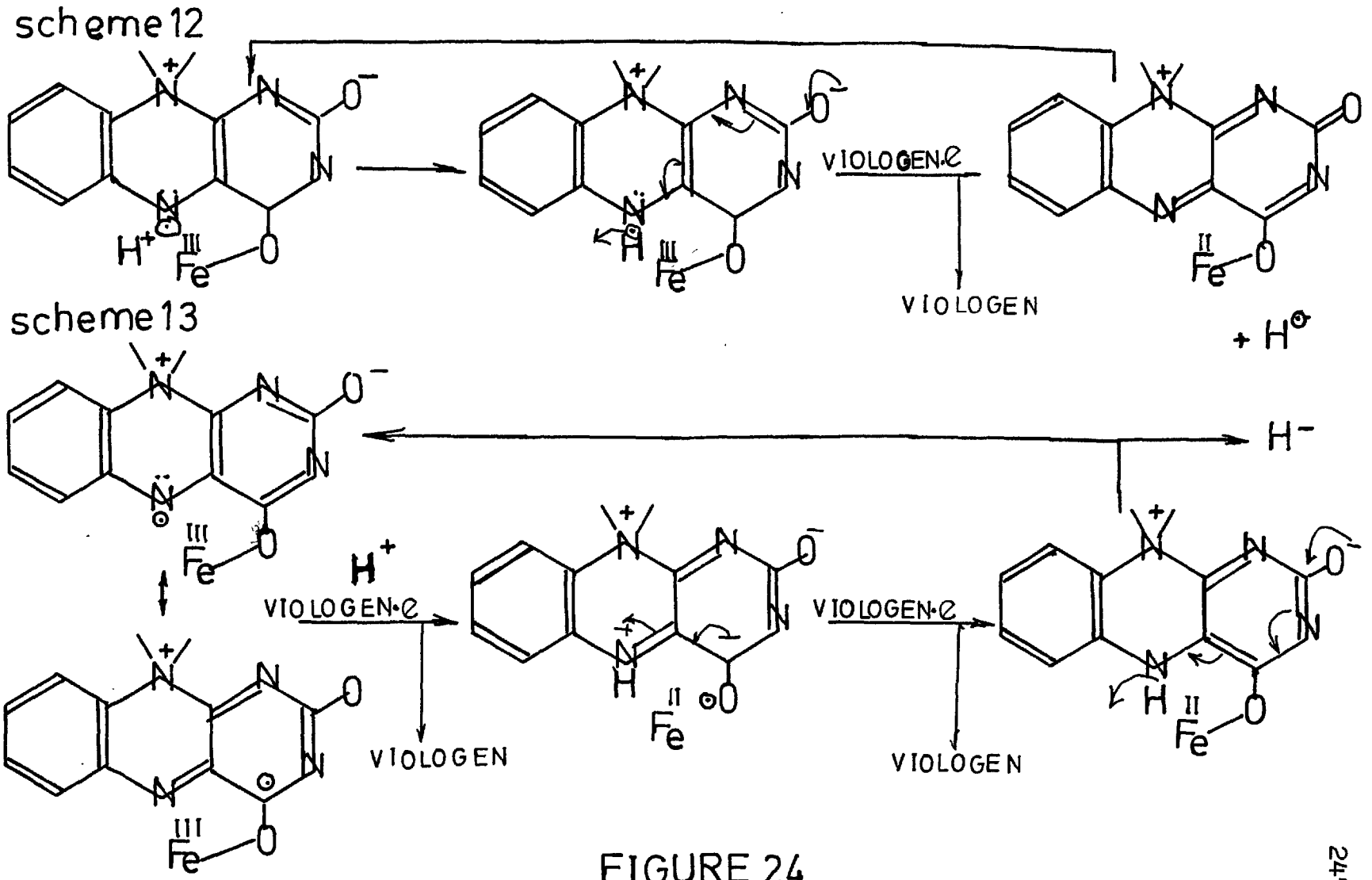
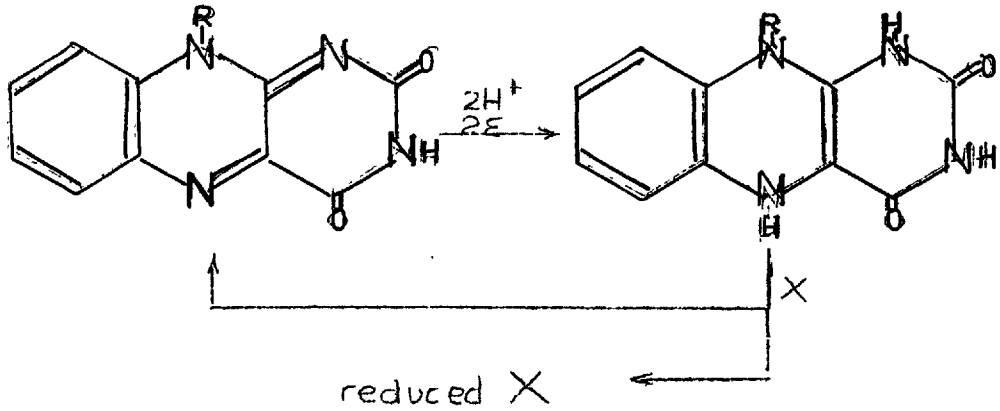

scheme 12
VIOLOGEN·e
VIOLOGEN
+ H⊙
scheme 13
H⁻
H⁺
VIOLOGEN·e
VIOLOGEN
VIOLOGEN·e
VIOLOGEN

FIGURE 24

suitable acceptor. With methylene blue as acceptor a two step reduction would have to be postulated.

It is possible that the oxidation/reductions mediated by flavins and represented by

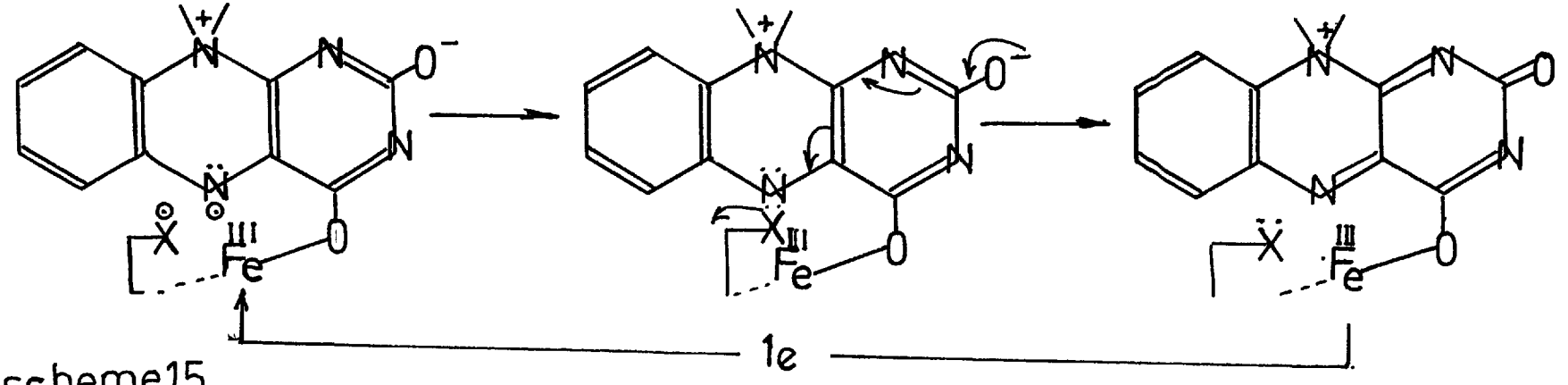


may operate either by the one electron transfer (scheme 14) or the two electron transfer (Scheme 15), shown in Figure 25. The one electron transfer involves the interaction of the 'one electron acceptor' with the flavin semiquinone. The reaction would be facilitated if the acceptor were held by co-ordination in a suitable position.

A two electron transfer is possible (Scheme 15) although this involves 'pairing' of unpaired electrons. Although iron is shown as the source of single electrons, molybdenum mediation is equally feasible.

The electron transfers postulated in this thesis are comparable to the reported transmission of electrons from Cr^{II} to co-ordinated Co^{III} (275). This is illustrated

scheme14



scheme15

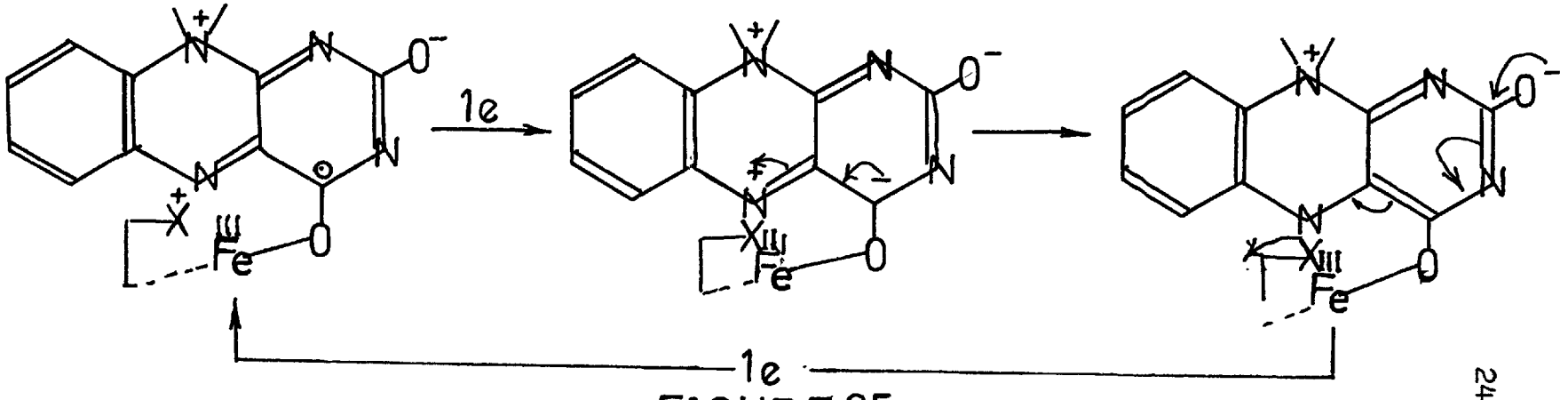
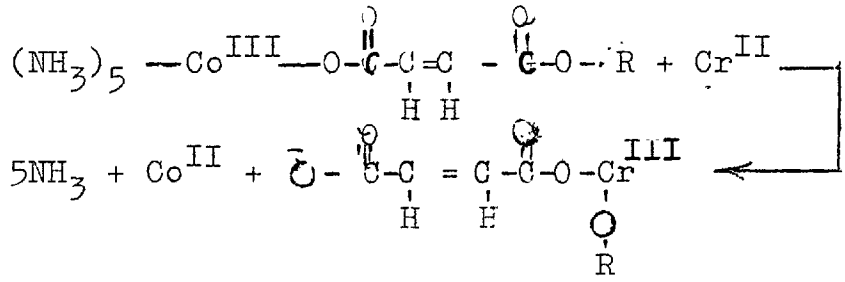


FIGURE 25

below and results in the hydrolysis of the half ester of fumarate.



It may be noted that in the proposed mechanism a quaternary nitrogen at N_9 is postulated to prevent any interaction of its lone pair with the reaction sequence. Calculations show that the key distance in the active site, $Fe\text{-}N_{10}$, is about 1.8Å (as compared to 2.5Å, (Rittenberg) and 3-4Å (Winfield). In the Section D consideration is given to this site of hydrogenase as the site of nitrogen fixation.

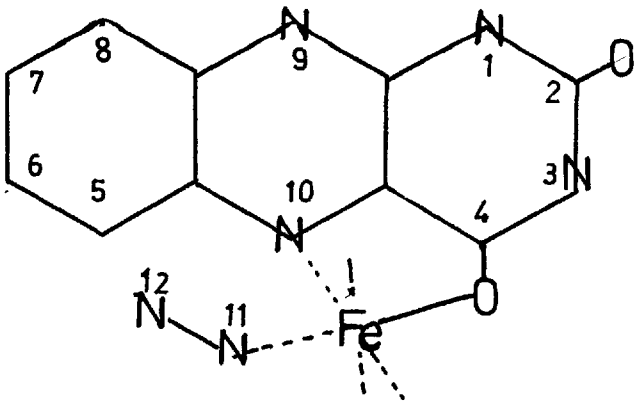
[D] A SPECIFIC MECHANISM FOR NITROGEN FIXATION.

The prime requirement of the active site in nitrogen fixation by the proposed mechanism is an ability to distort the electron density of the stable nitrogen molecule to permit further reaction. In any proposed mechanism for nitrogen fixation serious thought is required about the 'electron density gradient' of the site (i.e. consideration of the polarisability of the metal ion, Fajans rules, the availability of suitable orbitals and interatomic distances involved). It is possible to make some estimate of the interatomic distances involved when one molecule of nitrogen is 'adsorbed' on an iron flavin and

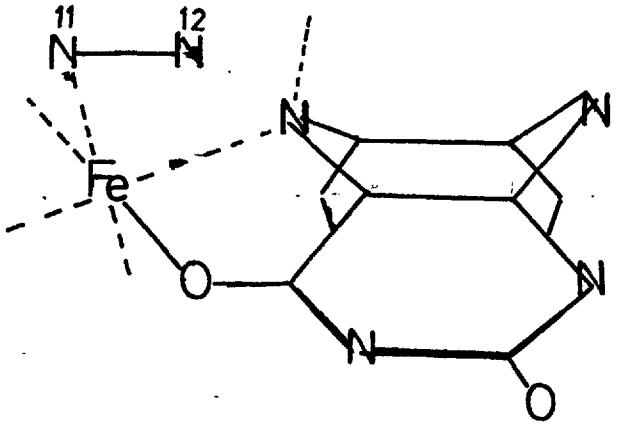
TABLE XC

SOME RELEVANT BOND DATA (276) (277)

BOND	BOND LENGTH in Å	BOND ANGLE	MEASURED IN
C——C	1.40		Guanidine hydrochloride monohydrate
C——N	1.41		"
C—O	1.20	∠OCN 116°	"
O——O	1.47		H_2O_2
O——H	0.96	∠OOH 105°	H_2O_2, H_2O, NH_2OH
Fe—O	2.08		$FeCl_5$, H_2O.
	1.95	∠FeOC 132°C	Triacetyl acetonato iron' III
Fe——N	1.77		$Fe(NO)_2(CO)_2$
N——N	1.10		
N——N	1.25	∠ NNF 116°	N_2F_2
	1.24	∠ CNN 110°	(trans-azomethane)
	1.23	∠ CNN 121 ∠ NCC 116°	cis-azobenzene
	1.23	∠ CNN 120° ∠ NCC 120°	trans-azobenzene
C——N	1.46		cis-azobenzene
	1.41		trans-azobenzene
N——N	1.46	∠ CNC 110°	1,1 dimethyl hydrazine
	1.41	∠ CNN 110°	(N_2H_6)
C——N	1.47		1,1 dimethyl hydrazine
N——H	1.00 1.03		NH_3 + (NH_4)
H——H	0.74		



[I]



[II]

FIGURE 26

some relevant bond data is summarised in TABLE XL. Calculations with this data on the planar structure, (I)Figure 26, show that Fe-N_{10} is about 1.8Å. This would suggest that the lone pair of N_{10} could occupy one of the octahedral positions of the central iron atom. In the azo-compound N_{11}-N_{10} and N_{12}- N_{10} distances are about 2.4Å.

It has recently been pointed out that flavins may not be planar in the reduced forms. Pullman (278) has considered some free radicals of biological interest and has attempted to assess the relative electron densities in these molecules. In the case of the reduced flavins (iso-alloxazines) the four folded structures shown were considered, Figure 27. In these circumstances (II) (Figure 26)may be a more likely representation of the iron flavin free radical. This point has been considered with respect to the proposed mechanism of nitrogen fixation. The essential features of this mechanism, shown in Scheme 16, are discussed below.

In the folded Structure (II) Figure 26, the Fe-N_{10} interatomic distance is somewhat greater than in the planar structure. It is also evident from Figure 27,(IIA)(IIB), that electron density at N_{10} is not symmetrical. It is proposed that this particular structure (IIB), in which a +ve charge resides on.

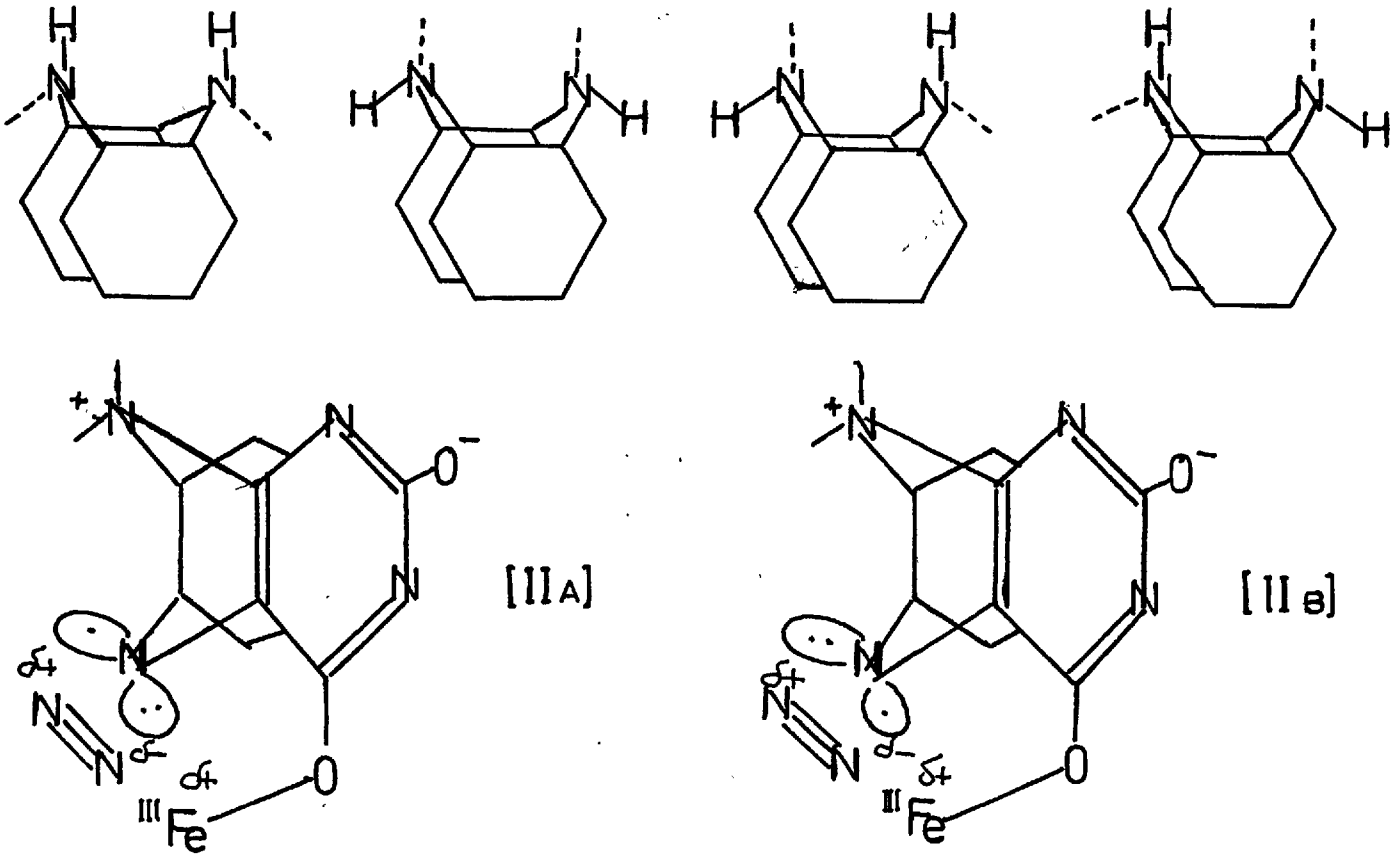


FIGURE 27

the central iron atom, resulting from the reduced donation by the N_{10} radical, together with the effect of the lone pair at N_{10} is capable of distorting the symmetrical electron density of the nitrogen molecule, $\overset{\delta+}{N}\equiv\overset{\delta-}{N}$ It is proposed that this results when a nitrogen molecule approaches along one particular octahedral position, perpendicular to the plane of the cis ring and the Fe-O bond. This initial distortion aided by concurrent stabilisation by the suitably placed nucleophile, P, renders the nitrogen molecule susceptible to reduction. The approach of P is liable to result in a trans-azo- compound.

It is suggested that lone pair interaction on nitrogen atoms, N_{11},N_{10}, would cause conversion of the trans-azo compound to the cis-azo-compound although this may also need consideration from a reductive viewpoint.

There are several pathways for the release of the site together with reduced nitrogen (as P-$NHNH_2$) as is illustrated in Figure 28, although the pathway involving V, VI and VIII is more favoured, since it is proposed that by reversion to the planar form, and Fe^{III} to Fe^{II}, the displacement of the hydrazino-compound is facilitated, Scheme 18 (Figure 29). This might be expected since in the planar form the central atom loses its slight +ve charge and experiences an increase in electron density from N_{10} along the relevant octahedral position.

scheme 16

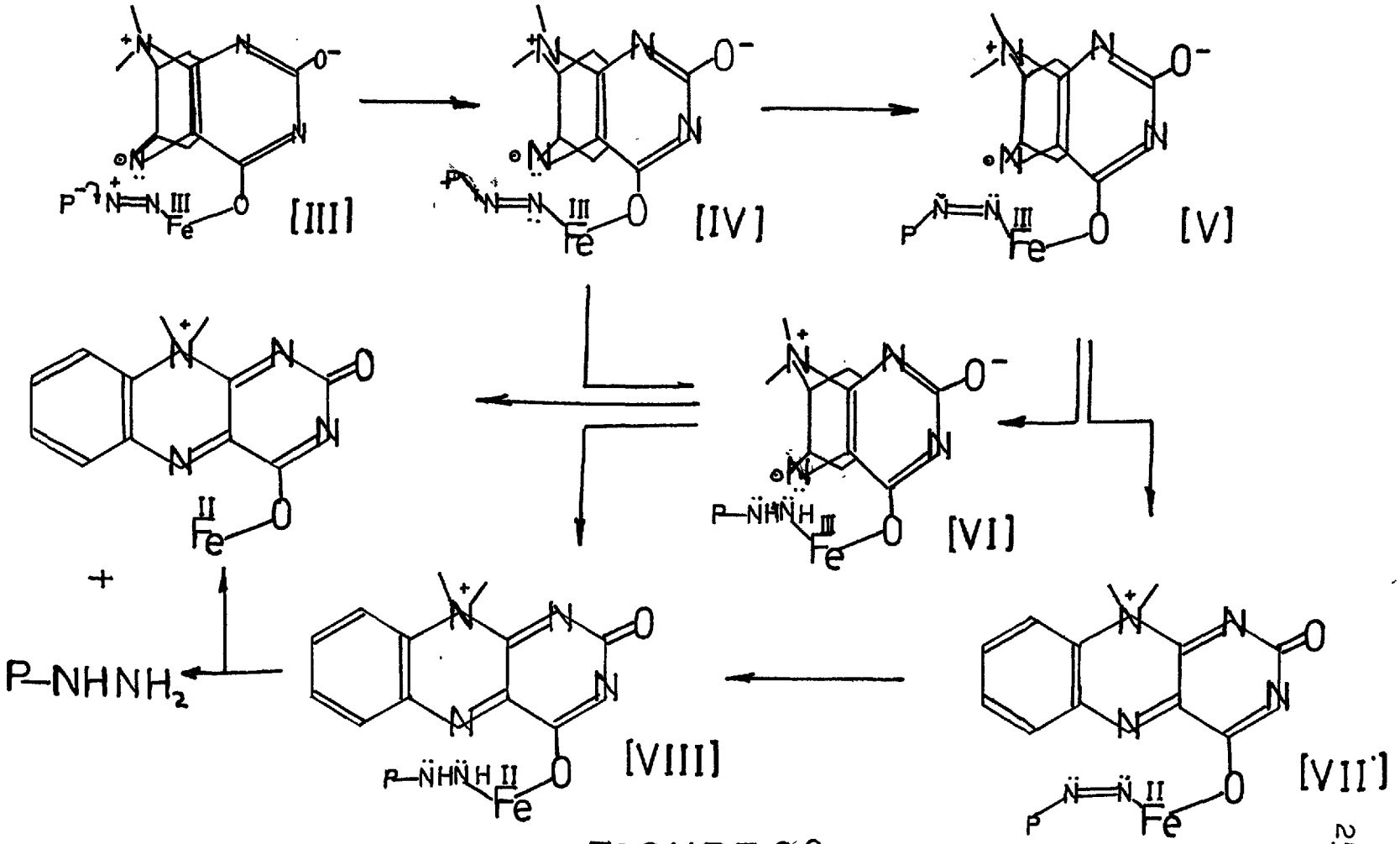
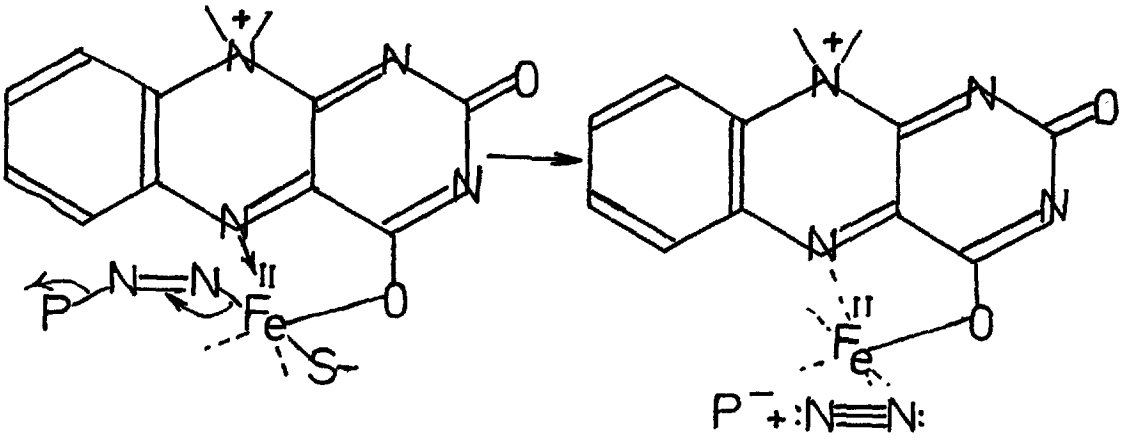


FIGURE 28

scheme17



scheme18

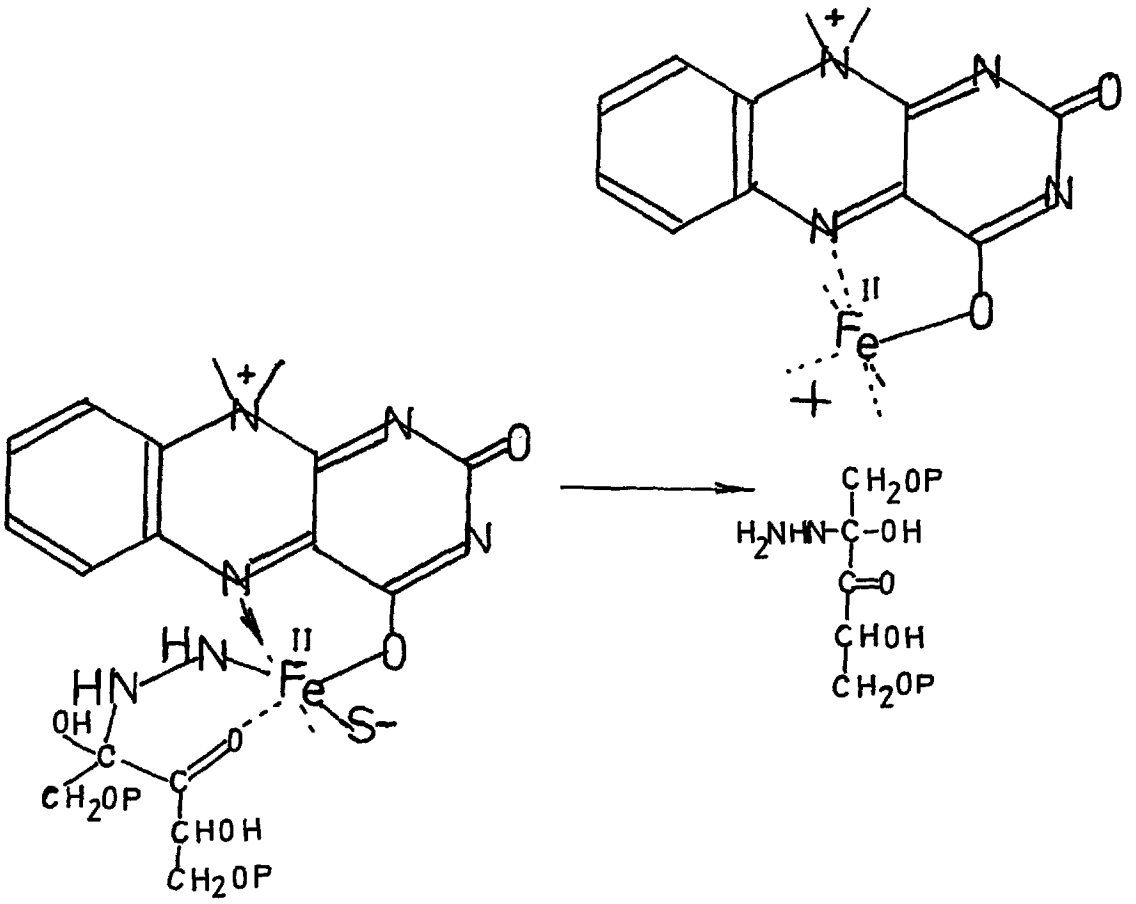


FIGURE 29

The repulsion between electron density at N_{10} and N_{11} would also aid this.

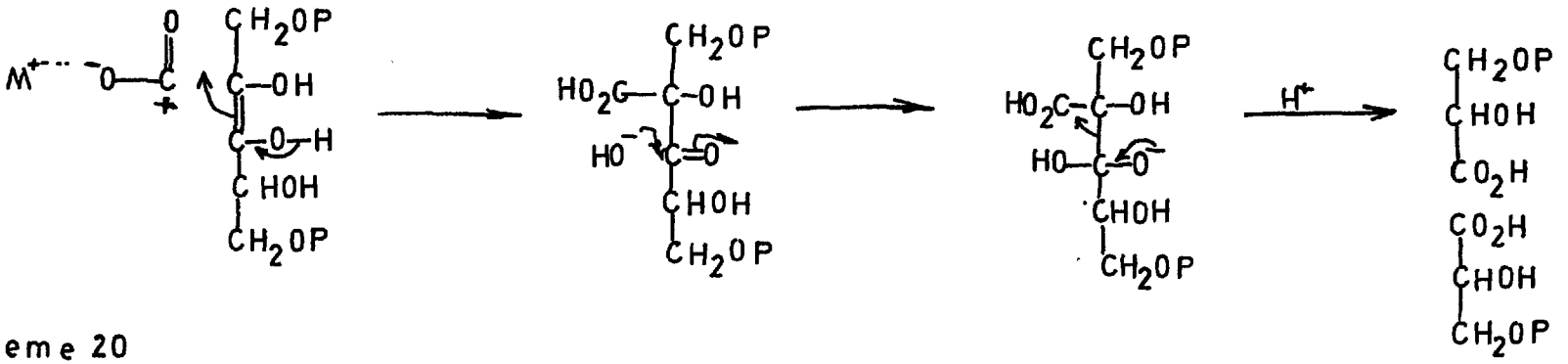
It is interesting to note that the possible reversion of the azo-compound to the planar form could result in loss of nitrogen. This is illustrated in scheme 17.

In Figures 26,27,28,29 the steric nature of the proposed active site is illustrated. In Figure 29 it is suggested, quite arbitrarily, that the central iron atom is held in position by a thiol link. The proposed active site is essentially similar to that proposed by Mahler and Elowe for the active site of DPNH - cytochrome reductase. (279).

Possible nature of P

It is possible that a sulphydryl-or an imidazolyl-group could function adequately as P. However, when a comparison is made to the mechanism of fixation of carbon dioxide, in which ribulose 1,5 diphosphate is the nucleophile, it is not unreasonable to postulate this or a closely related nucleophile. The two mechanisms are compared in Figure 30. In carbon dioxide fixation carboxydismutase is responsible for the fixation of carbon dioxide and the subsequent formation of 3 phospho-glyceric acid (280). It is interesting to note that a reductive fission of the intermediate has been considered and may participate to a limited extent (281).

Scheme 19



Scheme 20

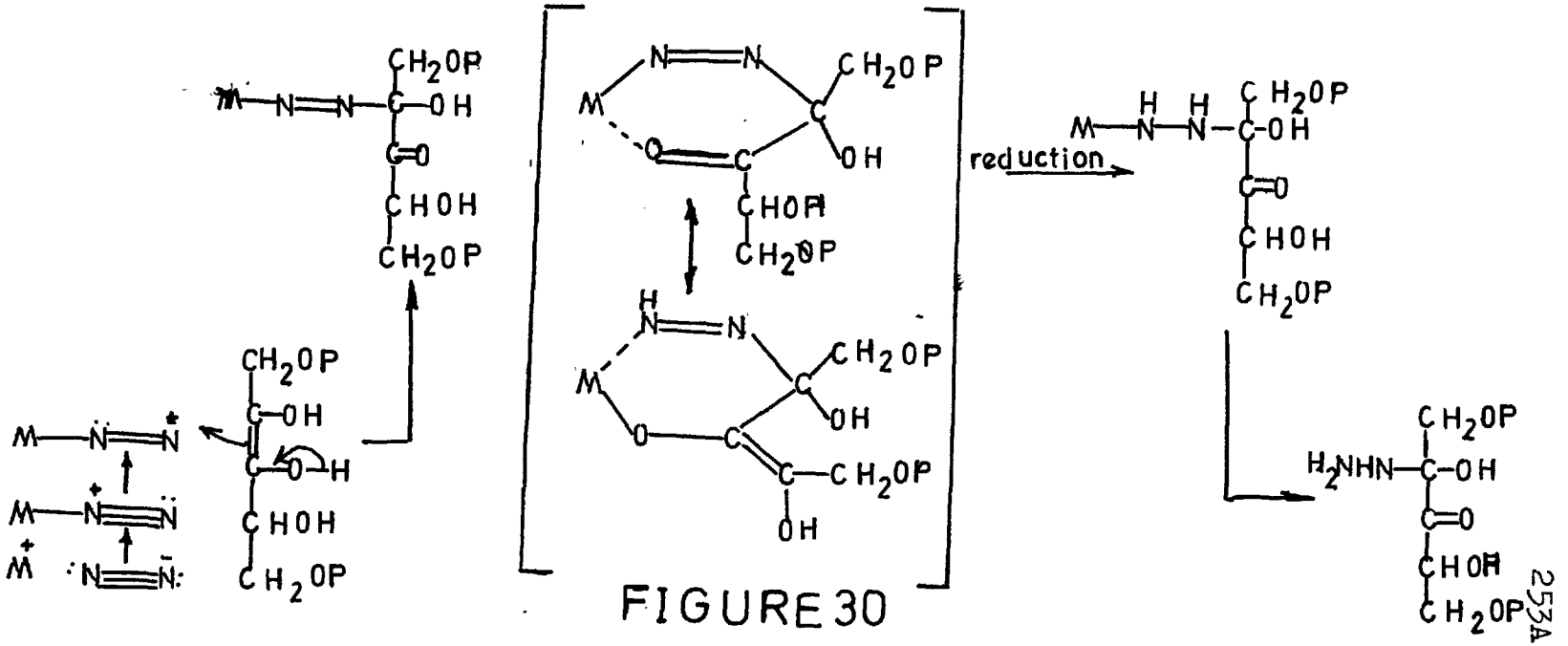


FIGURE 30

scheme 21

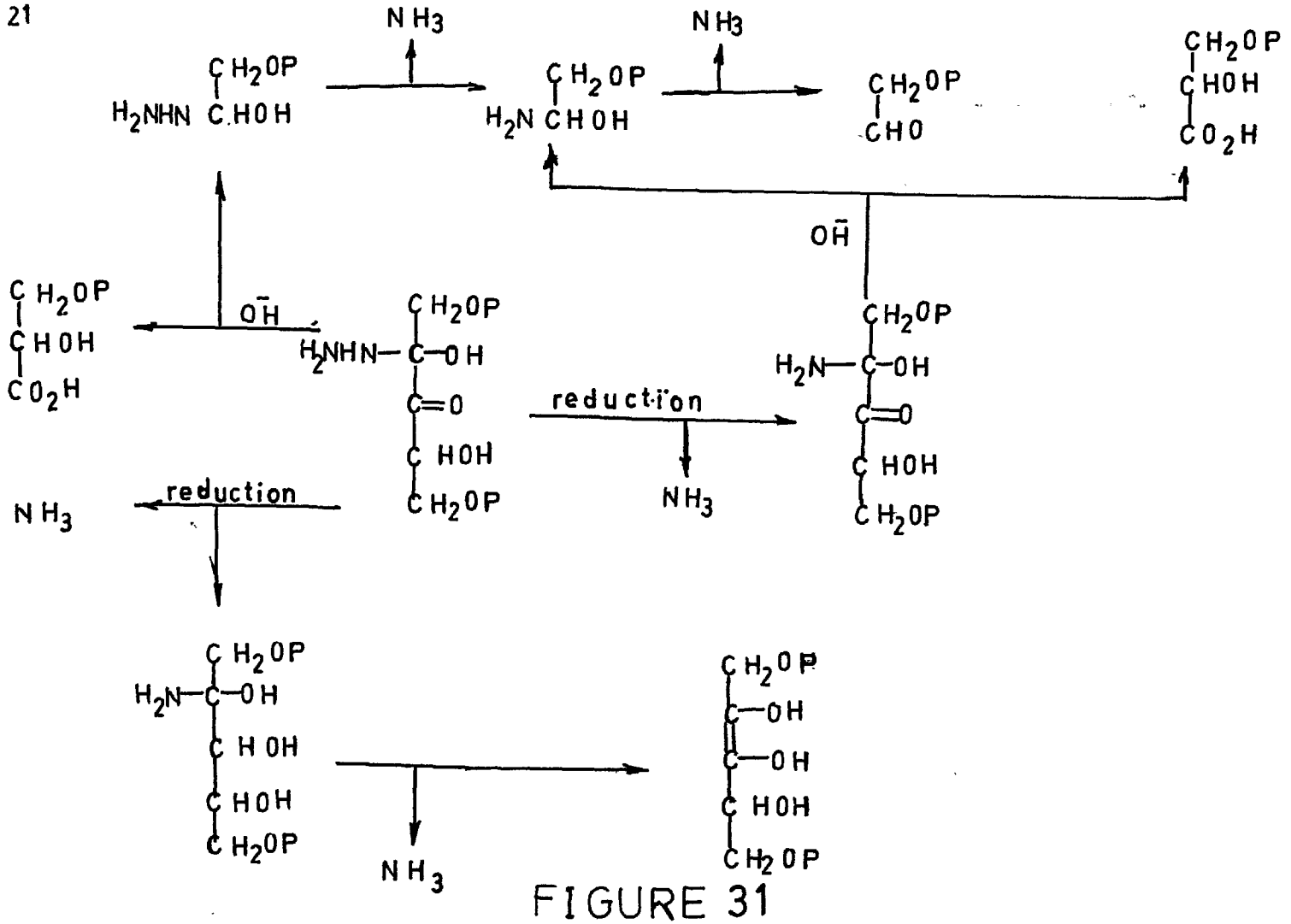


FIGURE 31

scheme 22

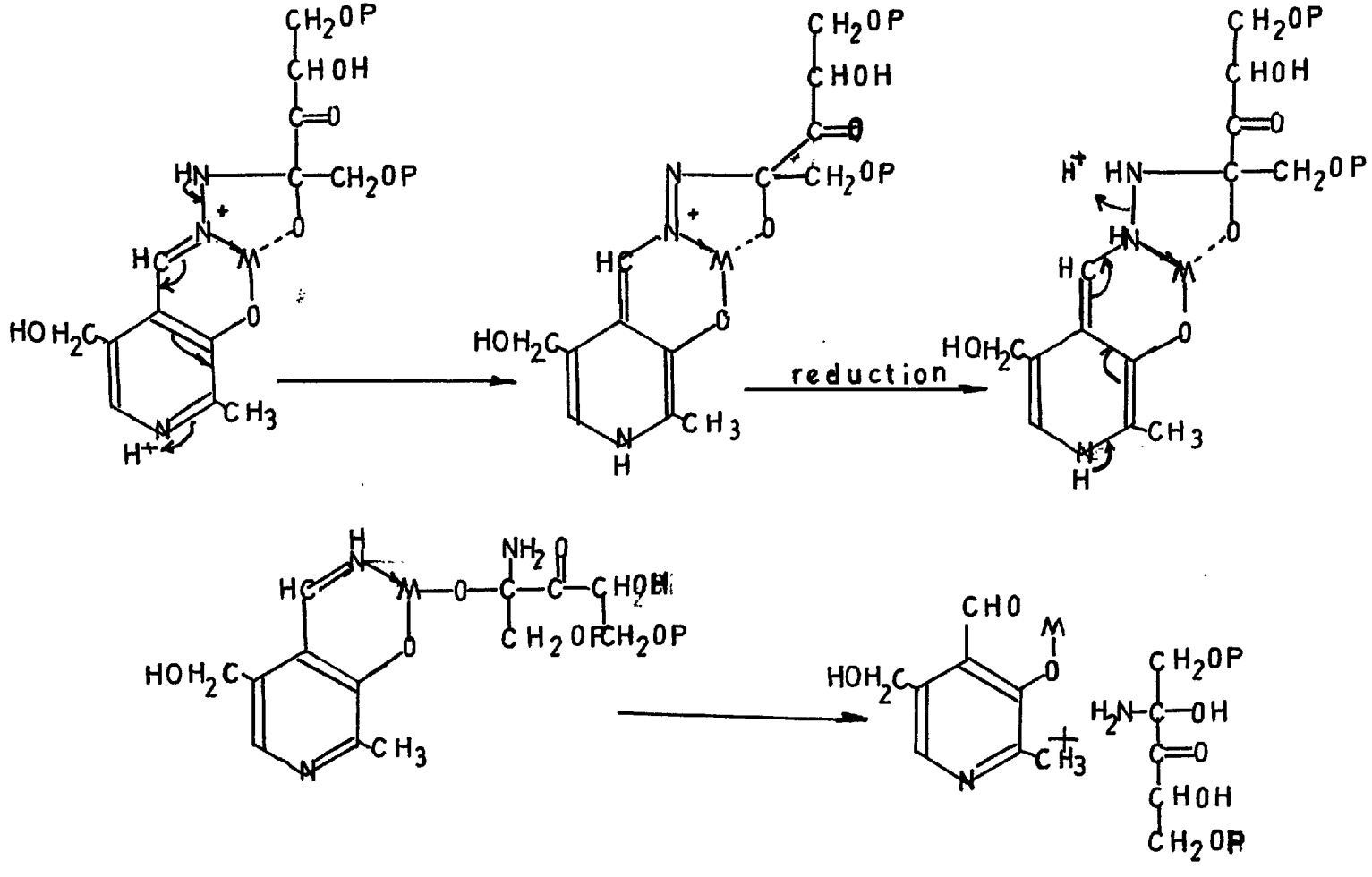
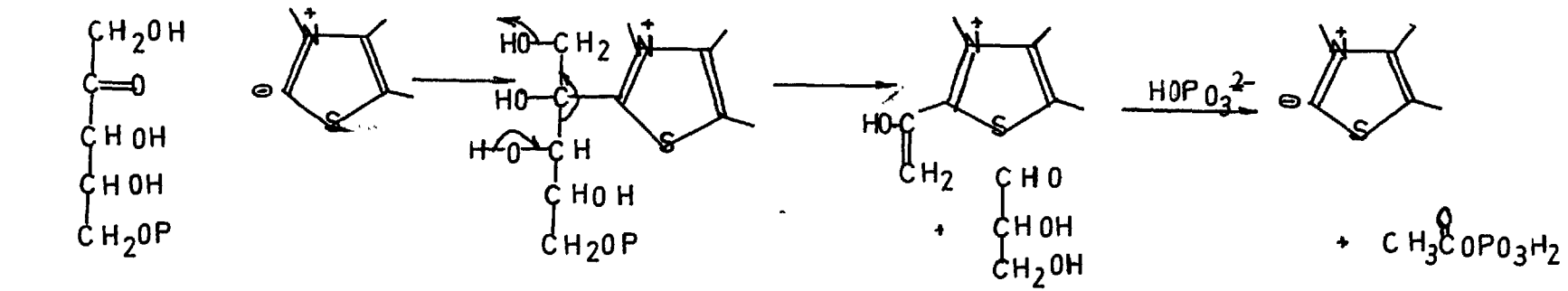


FIGURE 32

scheme 23



scheme 24

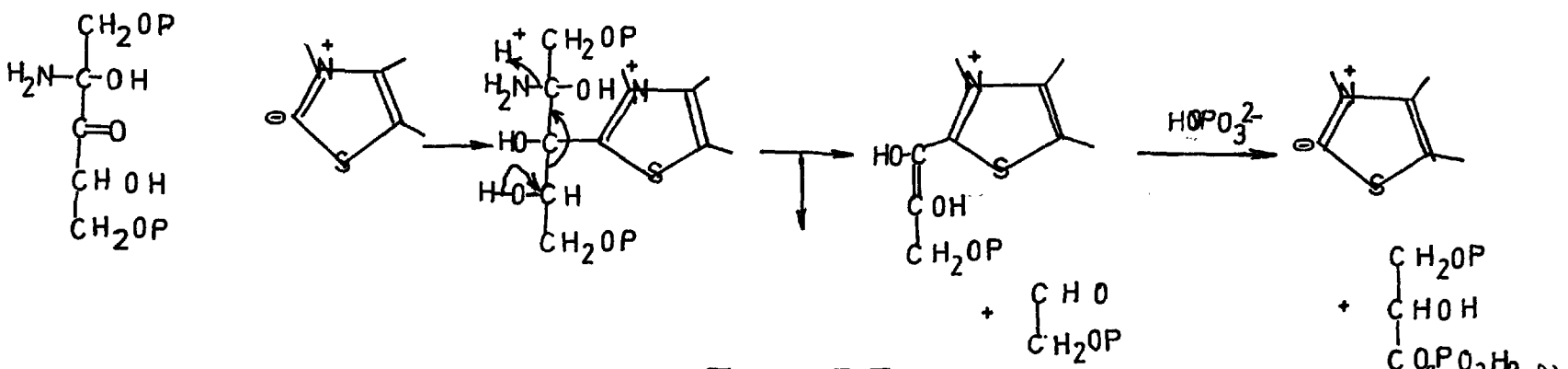


FIGURE 33

This type of fission is of interest when considering the possible routes for the breakdown of the intermediate proposed in nitrogen fixation. A reductive fission could result in 3-phosphoglyceraldehyde (cf. reductive fission in fixation of CO_2) ammonia and amino-derivative of phosphoglyoxylic acid. Possible schemes for the breakdown of the intermediate are shown in Figure 31.

It is not difficult to postulate a reduction of the hydrazino compound involving a pyridoxal derivative, as is shown in Figure 32.

The hydrolysis of the 'amino sugar' would result in ammonia as the sole product of the fixation of nitrogen. This hydrolysis might be expected to be facile although it is possible to postulate a thiamine pyrophosphate catalysed reaction (cf. TPP a requirement in nitrogen fixation by extracts of Clostridium). This is somewhat similar to the thiamine pyrophosphate catalysed breakdown of xylulose 5-phosphate, schemes 23, 24 in Figure 33, (282).

[E] RELEVANCE TO CURRENT EXPERIMENTAL KNOWLEDGE.

Enzymatic reactions are characterised by their high efficiency relative to non-enzymatic reactions and any mechanism proposed for enzymatic catalysis must attempt some explanation of these facile reactions. A specific mechanism for such reactions is normally difficult to

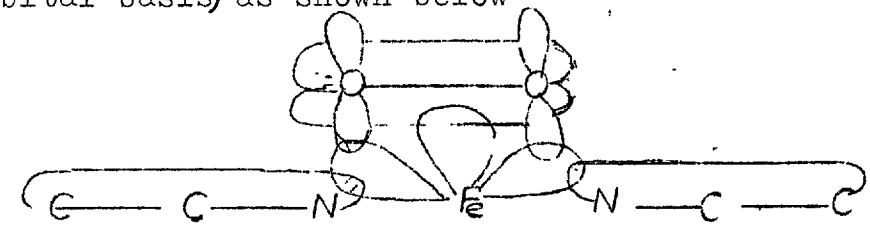
establish because of the difficulties experienced in biochemistry in devising critical experiments to test a particular mechanism and to assess the relevance of suggested model compounds. This often results in a greater level of speculation than is desirable and in this respect the proposed mechanism of nitrogen fixation is no exception. It is hoped, however, that this mechanism does unify some of the relevant experimental data and the salient features are reconsidered below.

It is proposed that the site of fixation, an iron flavo-protein, in conjunction with a nucleophile is able to cause the chemisorption of nitrogen by a concerted initial step.

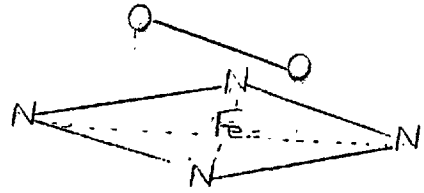
It is evident that the 'electron density gradient' at this proposed site is of prime importance and that further consideration of the orbitals involved is necessary. Some consideration of the types of organism that are able to fix molecular nitrogen is also necessary. In view of the fairly widespread occurrence of flavo-proteins in biological chemistry some explanation of the widespread lack of nitrogen fixation ability and the completely different oxygen requirements of the two principal asymbiotic fixers is called for. It is possible that some explanation could be made from consideration of pH, ionic strength and potential at a suitable site in particular organisms, and the particular acceptor of nitrogen,

but further work on the type of bonding in the iron-protein-nitrogen complex and in subsequent steps is necessary, particularly as in this mechanism essentially ionic linkages have been proposed.

With reference to orbital requirements, it is interesting to note the representation of oxy-haemglobin (284). Griffiths (283) suggested the orbital-overlap (on a molecular orbital basis) as shown below



The stereochemical arrangement can be seen below



A spectroscopic interpretation of the peroxidase-peroxide complex has been proposed both in molecular orbital and valence bond formulations (284). This is shown in Figure 34.

The proposal that the site of hydrogenase and nitrogen fixation are identical is not supported by cell-free investigations on Clostridium. This work suggested that hydrogenase was only involved in the hydrogen donating

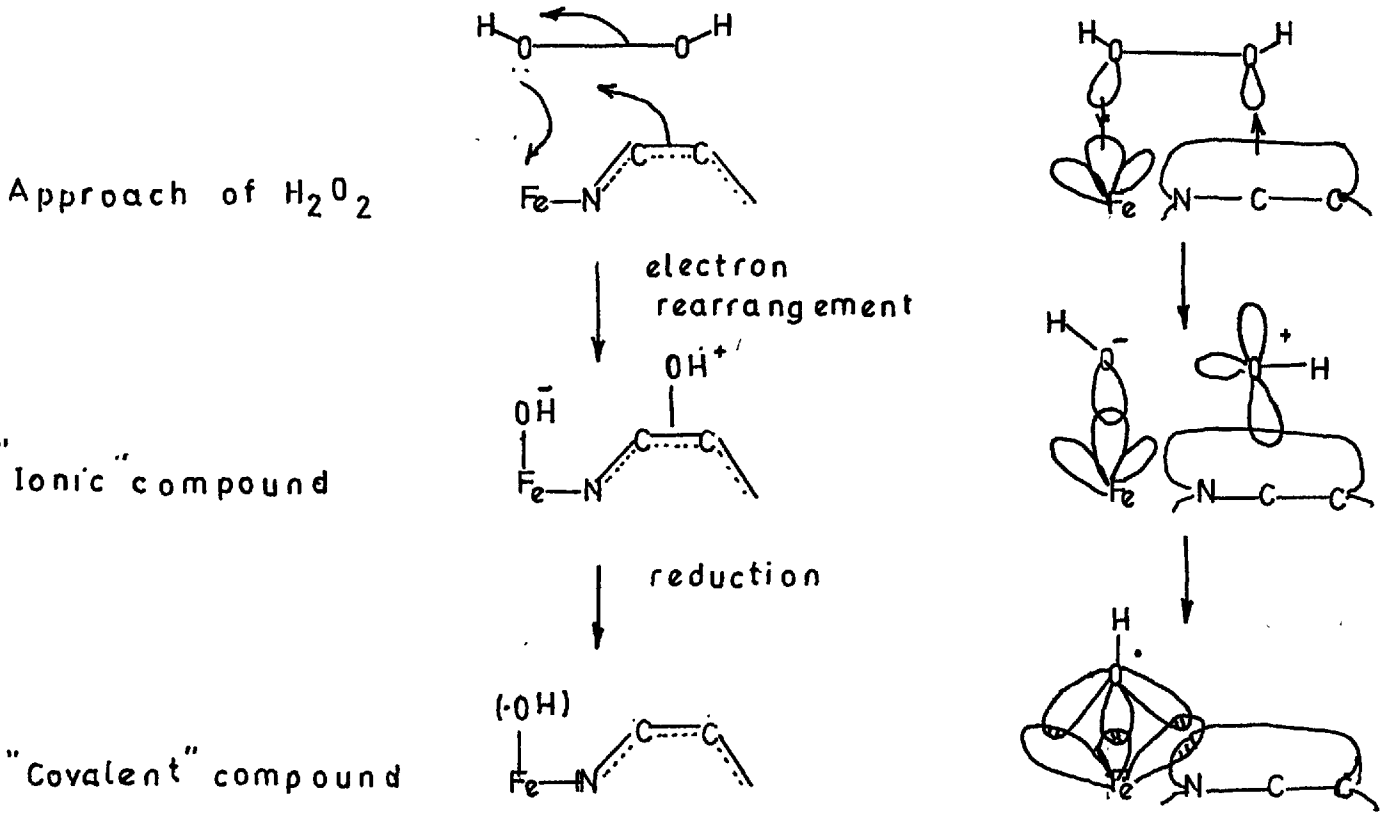

Valence bond representation
Molecular orbital representation
Approach of H_2O_2
electron rearrangement
"Ionic" compound
reduction
"Covalent" compound

FIGURE 34

system (HDS) and not in the nitrogen activating system. (NAS).

However the evidence in support of the nitrogen activation is unimpressive. Much of the spectral evidence appears to lack consistency and it is noticeable that irreversible inhibition of the 'NAS' by CO was reported. An alternative explanation of the requirement of both 'NAS' and 'HDS' for a fixing extract is possible in terms of the need for an acceptor (nucleophile) which is absent from the 'HDS.' This is enlarged upon subsequently.

It is interesting to note one feature of this work on Clostridium, namely that the combination of the 'NAS' from cells grown on nitrogen with the 'HDS' from cells grown on ammonia resulted in a fixing extract, whereas the reverse did not. It has also been reported that in Azotobacter grown on ammonia the hydrogenase activity is markedly lower than that in cells grown on molecular nitrogen.

The explanation of Pratt for the role of hydrogenase in nitrogen fixation is not satisfactory. He represented nitrogen fixation in aerobes as $BH_2 + \frac{1}{2}O2 + N_2 \longrightarrow BN_2 + H_2O$,

and in anaerobes as $BO + 2H + N_2 \longrightarrow BN_2 + H_2O$,

BH_2, BO being methylene and α-keto acids respectively. He proposed that the function of hydrogenase in aerobes was to permit the oxidation of organic substrate by

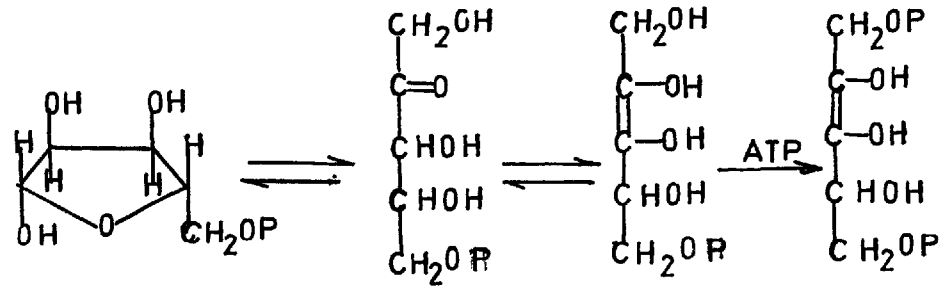
oxygen without the formation of ATP. This would mean that, contrary to some evidence (the most recent publication reports an ATP requirement in nitrogen fixation by Clostridium (285)), ATP is not required in fixation. It is also apparent that Pratt makes no reference to the recent series of papers by Parker and associates (29) (113) (114) on the oxygen inhibition (of fixation) in Azotobacter vinelandii.

It is also difficult to visualise an oxidative incorporation of the type suggested for aerobes particularly as incorporation involving a metal complex is not favoured. Pratt merely states that the scheme represents a termolecular collison "but in reality each must consist of a number of steps". This was not enlarged upon.

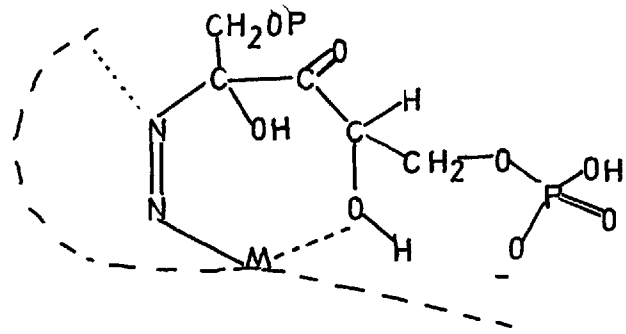
In the proposed mechanism the acceptor of carbon dioxide ribulose 1, .5 diphosphate, rather than the organic acids of Pratt, has been proposed as the 'acceptor' of nitrogen. This is an essentially speculative proposal although this acceptor or a similar compound would provide an explanation for "high energy" phosphate in fixation by Azotobacter and Clostridium.

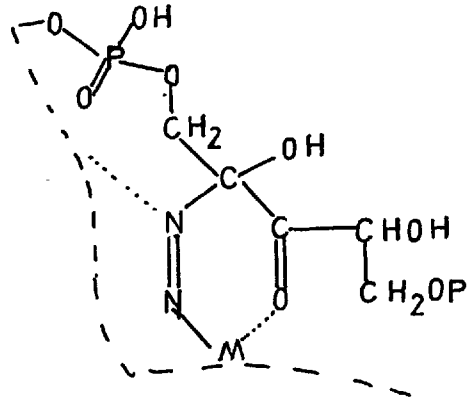
In order to maintain a suitable concentration of this acceptor, a source of ATP would be desirable so as to prevent the locking up of the acceptor by furanoside

scheme 25



[IX]





[X]

FIGURE 35

formation. This is illustrated in Scheme 25, Figure 35.

An ATP requirement for fixation would also imply that a principal difference in aerobic and anaerobic fixation was the source of ATP, i.e. in anaerobic fixation ATP being provided by pyruvate metabolisn and in aerobic fixation by oxidative phosphorylation.

The need for the 'NAS' in cell-free fixation of Clostridium can be adequately explained on the basis that this contains the required acceptor whereas the 'HDS' lacks this acceptor. This would merely mean that during the preparation of the 'HDS' the acceptor is destroyed. It would be worthwhile to consider the type of compounds that are able to survive protamine sulphate precipitation and calcium phosphate gel treatment.

It is possible to explain the lack of nitrogen fixing activity when the 'NAS' from ammonia grown cells is combined with the 'HDS' from nitrogen grown cells. This would result if(in ammonia grown cells) the cell metabolism was altered such as to severely limit the concentration of the required acceptor.

It should be noted that a mutant of Azotobacter has been reported which can assimilate ammonia but can

only fix nitrogen when supplied with certain carbon sources, e.g. lactate and pyruvate. (230).

The lability of the nitrogen fixing system in extracts to prolonged storage as opposed to the more stable hydrogenase activity can be explained if the actual site is relatively unaffected whereas the acceptor is much more sensitive, particularly to freezing, and thawing.

The proposed mechanism takes little accountof the reported molybdenum requirement of nitrogen fixation, although it is possible to explain this on the basis of molybdenum being required in a subsidiary role rather than as the site of adsorption of nitrogen. A function for molybdenum could be to hold the acceptor in position by means of a phospho-molybdate complex; possible stabilised forms (IX), (X) are shown in Figure 35. If this were the case it is possible that the inhibition of nitrogen fixation by arsenate (which had been attributed to the disruption of the ATP supply through formation of acetyl arsenate) may result from inhibition of such a complex. This could occur by formation of an arseno-molybdate.

After the initial trapping of nitrogen, the reduction of the azo-compound and its displacement to free the active site for further reaction are important steps.

There is little point in making speculations about possible reducing agents but the subsequent facile displacement may be attributed to two factors.

(i) A strain factor resulting from increased interatomic distance in the nitrogen molecule on reduction. This will be of particular significance if the acceptor is held in position by a phospho-molybdate complex or in similar manner.

(ii) A more powerful aid to displacement of the hydrazine derivativeis the lone pair - lone pair repulsion at N_{10} and N_{11} enhanced by the change in configuration of the flavin. The latter, which involves the conversion of Fe^{III} to Fe^{II} resulting in one octahedral position being fully taken up by electron donation from N_{10} in the planar form of flavin, would provide a powerful aid to displacement. In this case, excess charge would be built up on the central iron atom and be suitably relieved by displacement of the hydrazine group.

The mechanisms of disruption of the hydrazino-compounds were postulated after consideration of pyridoxal and thiamine pyrophosphate catalysed reactions. The former is of interest because of its role in transaminations and the latter of interest because of a thiamine pyrophosphate. requirement in extracts of Clostridium, although this is assumed merely to relate to the phospoclastic reaction.

The overall products of nitrogen fixation by the proposed mechanism are ammonia and an amino derivative (an aldehyde-ammonia). It is likely that the amino-derivative would be hydrolysed to ammonia and thus it would be experimentally difficult to distinguish the amino derivative from ammonia. It is possible to explain the experimental data in support of ammonia as sole intermediate in extracts of Clostridium on this basis. It was reported that in the presence of an 'ammonium pool' or glutamine after one hour exposure to $[^{15}N_2]$, 95% of the $[^{15}N]$ was recovered in ammonia by differential distillation. This result is equally explicable on the basis of an 'alkaline-labile' amino-derivative but only further refined experimental work can resolve this point.

In conclusion it must be accepted that the present experimental evidence on nitrogen fixation by Azotobacter vinelandii and Clostridium pasteurianum still leaves considerable scope for speculation. It is evident that the intriguing question of the extent to which fixation by Azotobacter and by Clostridium are similar must be considered. In making comparison between the anaerobic Clostridium and the aerobic Azotobacter, it is advisable to examine their differences, but it is possible that the principal difference may result from the location of their hydrogenase and in their mode of generation of ATP

However, before concrete proposals on the fate of gaseous nitrogen can be made there is a need for further work on hydrogenase, the ATP requirement of fixation, the initial product of fixation and the source of reduction. The present work has merely attempted to correlate some relevant chemical and biochemical knowledge on fixation. It is evident that any proposed mechanism for nitrogen fixation or hydrogenase activity must remain answerable to current experimental data and it is hoped that this work may make some contribution to the complex and difficult problem of bacterial nitrogen fixation.

(IX) SUMMARY.

(1) The preparation of cell-free extracts from stored Azotobacter cells has been studied with respect to hydrogenase and 'nitrogen fixing' activities.

(2) The stored bacteria, originally grown by continuous culture, proved to be a useful source of hydrogenase preparations and by disruption in young cultures an attempt was made to establish suitable environmental conditions for hydrogenase and 'nitrogen fixing' activities. The method of disruption has given hydrogenase preparations comparable to other published work but the principal disadvantage has been the difficulty of assessing the efficiency of disruption with suspensions of stored bacteria.

(3) The $[^{15}N]$ technique has been used to measure nitrogen fixation by disruptates and centrifugates (supernatants) and this procedure, as a valid method for determining nitrogen fixation by cell-free extracts, has been discussed.

The results have shown that enzymatic activity must be related to efficiency of technique before cell-free nitrogen fixation can be established. In this respect it was essential to express the results on a quantitative basis and not merely as atom% ^{15}N excesses particularly as heat-

treated supernatants also gave rise to increases in nitrogen when exposed to $[^{15}N]$.

(4) The increase of nitrogen, the amount of nitrogen 'fixed', was calculated from the atom%^{15}N sample : (disruptate or supernatant), the atom%^{15}N of the 'exposing' atmosphere and the total Kjeldahl nitrogen of the sample by substitution in the equation;

$$\text{Increase in nitrogen } (\mu g) = \frac{(X - 0.38)}{(D - 0.38)} \times N$$

where X = atom%^{15}N of sample

D = atom%^{15}N of atmosphere

N = total (Kjeldahl) nitrogen after exposure to ^{15}N in μg.

The calculation of the amount of nitrogen fixed by $[^{15}N]$ exposure has been discussed and it has been pointed out that the $[^{15}N]$ technique assumes that no discrimination occurs between ($^{15}N\equiv{}^{15}N$), ($^{15}N\equiv{}^{14}N$) and ($^{14}N\equiv{}^{14}N$) molecules.

(5) An analysis of 'enzymatic nitrogen fixation' relative to the experimental conditions of assay has been made. It was evident that when nitrogen fixation is determined by the $[^{15}N]$ technique serious consideration of the following is required;

(i) the method of disruption and the environmental conditions of disruption e.g. the medium employed and possible

requirement for pre-reduction or reducing conditions.

(ii) centrifugation, suitable fractionation of the disruptate to eliminate or substantially reduce the whole cell concentration.

(iii) the pN_2 of the 'exposing' atmosphere, the composition of this atmosphere and the method employed to expose the disruptates (and supernatants).

(iv) the exposure time (a suitable exposure time, with respect to stability of cell-free enzymes, is required).

(v) the Kjeldahl procedure and the need for pre-reduction (a quantitative Kjeldahl is essential).

(vi) mass spectrometric analysis, the techniques used in generating and analysing small quantities of nitrogen.

(6) The establishment of cell-free nitrogen fixation must be dependent on the careful analysis of the above factors, particularly on,

(i) the detection and estimation of whole cells and their contribution to the nitrogen fixed. In this respect further refinements of the counting and plating techniques are desirable although some assessment of the contribution from small quantities of whole cells can be made by exposure of thin suspensions of Azotobacter under identical conditions.

(ii) the effectiveness of the experimental technique in removing ('degassing') dissolved or adsorbed nitrogen, particularly relative to the specific Kjeldahl technique.

(7) The increasesin nitrogen found in the present work were not easily correlated to other published [^{15}N] studies, particularly with respect to the blanks, although the differences might be ascribed to the higher pN_2's, the longer exposure times (3 hr v 1 hr) and the particular Kjeldahl procedure employed. The present work has indicated that the experimental conditions are of greater importance than the earlier published work has suggested.

(8) Although the present work has not offered further support for the suggestion that 'nitrogenase' and hydrogenase are identical, mechanisms for nitrogen fixation and hydrogenase activity on a common site have been proposed.

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