THE NITROGEN METABOLISM OF AZOTOBACTER VINELANDII

with special reference to the mechanism of fixation

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by

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ABSTRACT

A method for growing Azotobacter vinelandii under conditions of optimal aeration has been devised such that the culture could be sampled frequently so allowing growth and metabolism to be followed continuously.

At medium culture densities a discontinuity was observed in the culture properties of both strains used for comparison throughout this work. This discontinuity was attributed to oxygen starvation of the cells and the different properties of the exponentially-growing cultures before and after this change have been noted.

The intra- and extra-cellular pools of nitrogen compounds were examined. The presence of 3,4 dihydropyridazinone-5-carboxylic acid could not be confirmed but paper chromatography on its own was clearly inadequate to settle the status of hydrazine derivatives in fixation.

The effect of artificial extracellular nitrogen pools on growth and fixation was examined using cultures in various states of training with respect to the substrate. The results were confirmed and expanded using isotopically labelled ammonium and gaseous nitrogen.

Fixation and ammonium uptake occur simultaneously in trained cultures: the implications of this are discussed and it is proposed that free ammonium can no longer be regarded as an obligatory intermediate in the fixation process. Urea is metabolised by the same routes as ammonium though its hydrolysis is not effected by a simple exogenous urease.

Though L-asparagine and, to a lesser extent, L-glutamine are used as partial nitrogen sources, these effects are of minor importance. The amides, in common with L-aspartic acid, L-glutamic acid and L-alanine, stimulate fixation (per unit cellular material synthesised) by quantities of up to 70% of its normal value. The excess nitrogen thus fixed is excreted either as peptides or in the form of more of the added amino acid itself. These results are interpreted in terms of specific transport mechanisms and interference with structural peptide synthesis, which is assumed to take place in the cell envelope.

The results are fitted into an overall scheme proposed to explain the fate of gaseous nitrogen in the metabolism of Azotobacter vinelandii.

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The natural fertility of the soil is largely determined by the activities of its microflora and years of extensive research are just beginning to shed light on the complexities of their metabolism. Biological nitrogen fixation, the process whereby atmospheric nitrogen is converted into bound forms by the living cell, has long been of interest both to the agronomist and to the microbial physiologist. When fixing nitrogen, the humble bacterial cell lying in the soil performs a reaction which, on an industrial scale, requires chemical plant working at high temperatures and extreme pressures. The reductive fission of gaseous nitrogen is an exergonic reaction and high temperatures must be used in the Haber Process simply because otherwise, given the catalysts now available, the reaction would be infinitely slow. This fact has kindled the interest of the chemist also but, in spite of such a concerted attack, nitrogen fixation remains one of the most difficult problems in biochemistry (143).

The beneficial effect of legume crops on soil fertility had been known by farmers for hundreds of years when, in 1887, Hellriegel and Willfarth suggested its cause: nitrogen fixation resulting from the symbiotic association of bacteria with the roots of plants. Though the discovery of free-living fixing agents soon followed - Clostridium, an obligate anaerobe, by Winogradsky in 1893 and Azotobacter, vigorously aerobic, by Beijerinck in 1901 - most of the

work in subsequent decades was doomed to futility by the use of inadequate cultural conditions. It was not until the middle 'thirties that Dean Burk at Wisonsin established the trace element requirements of Azotobacter and so made reproducible growth experiments possible for the first time (4). His work was carried on by R.H. Burris and P.W. Wilson and, to this day, Wisonsin has remained the largest centre for research into this subject. Their most notable contribution was the introduction of isotopic nitrogen as a research tool, both for detecting more fixing agents and for studying the mechanism of the process (8).

In spite of many and varied attempts, for years it was not found possible to achieve consistent fixation by cell-free extracts (51, 65). This obstacle to further progress was finally overcome in 1960 in the Du Pont laboratories at Wilmington. Their extracts were obtained from Clostridium pasteurianum but other organisms have been used in subsequent work and this advance now embraces most classes of fixing agents (205). With the exception of Clostridium itself, little of importance has come to light yet concerning the detailed mechanism of fixation; many laboratories are engaged on work with other organisms so results may be expected in the near future.

Since 1948 a team in London under E.R. Roberts has investigated the mechanism of fixation by Azotobacter vinelandii with a wide range of physico-chemical techniques centred on the use of isotopic nitrogen. The work reported in the following pages is part of this team approach

and was conducted in parallel with reseach into the large scale production of cell extracts for the purpose of examining the enzyme systems involved in fixation. In contrast, the author has studied actively growing intact cells in an attempt to locate the site of fixation and settle conflicting reports as to the nature of its first stable product.

SECTION II

COMPARATIVE BIOCHEMISTRY OF NITROGEN FIXATION

It was originally thought that the power to fix nitrogen was of rare occurrence and all early researches were performed on the Azotobacteriaceae, Clostridia or the legume symbionts Rhizobia in their root nodules. With the development of the sensitive isotopic method for detecting fixation (8) (as opposed to often equivocal total nitrogen estimations) it has been realized that this faculty, though restricted to micro-organisms, is very widespread: it has been found in most bacterial families (particularly in the photosynthetic ones) as well as some species of yeast and many of blue green algae. A comparison of the reviews of Wilson and Burris in 1953 (39) and Yocum in 1960 (178) will give an indication of the steady increase in the list of known fixing agents during those years, an increase which continues unabated in 1962.

Though the majority of cases thus recorded refer only to the incorporation of isotopic nitrogen, the properties of the fixing system have been examined in detail for a number of organisms: the more important conclusions are summarised and compared with those of Azotobacter vinelandii in Table 1. Properties unique to the organism when fixing nitrogen are normally determined by comparison with cultures using ammonium ion as nitrogen source. In all but one case (192), ammonium nitrogen is the preferred form, bypassing the fixation mechanism and so suppressing it.

It is immediately apparent from Table 1 that behaviour, where

TABLE 1 COMPARATIVE BIOCHEMISTRY OF FIXATION

PROPERTIES PECULIAR TO ORGANISM WHEN FIXING NITROGEN	AZOTOBACTER VINELANDII	CLOSTRIDIUM PASTEURIANUM	LEGUME ROOT NODULES	BACILLUS POLYMYXA	AEROBACTER AEROGENES	NON-LEGUME ROOT NODULES	BLUE-GREEN ALGAE	RHODOSPIRILLUM RUBRUM
EFFECT OF OXYGEN (i) FIXATION ANAEROBIC ONLY (ii) FIXATION AEROBIC, INHIBITION BY OXYGEN	X	X	X	X	X	X		
INHIBITION BY HYDROGEN	X	X	X	X	X	X	X	X
INHIBITION BY CARBON MONOXIDE	X	X	X	X	X	X	X	
PRESENCE OF HYDROGENASE	X	X	X	X	X			X
MICHAELIS CONSTANT IN AIR (in atmospheres)	0.023	0.03	0.025	0.03			c.0.02	
MOLYBDENUM REQUIREMENT	X	X	X	X	X	X	X	
IRON REQUIREMENT	X	X		X	X		X	
REFERENCES	See Section IV	205 67 20 101 149	164 64 65 5 14	202 106 90	139 113 176	179 185 156	17 3 101	30 140

known, is remarkably uniform and the common properties of the fixing agents may be summarised:

(i) Inhibition by oxygen, carbon monoxide and hydrogen.

(ii) Trace metal requirements of molybdenum and iron.

(iii) Presence of the enzyme hydrogenase.

(iv) Michaelis Constant for nitrogen in air 0.025 $\pm$ 0.005 atm.

Though fixing cell free extracts have been obtained from five of the organisms listed (ie. excluding symbionts and Aerobacter), so far the only significant advances towards the mechanism of fixation by them have been made with Clostridium (172, 205). Valuable information about fixation can be obtained, however, from work done with intact cells of two of the other organisms and the results are summarised below:

RHODOSPIRILLUM RUBRUM. The influence of light generates reducing power at a high energy level which, if not needed, can be evolved as gaseous hydrogen (66). This evolution is completely inhibited by nitrogen gas, a fact easily explained if the reducing potential is then required for fixation and assimilation of the product into amino groups. Another photosynthetic bacterium, Chromatium, confirms this theory since extracts from it will fix nitrogen in the dark if supplied with molecular hydrogen (150).

RHIZOBIUM. Once consistent fixation could be obtained using excised root nodules (65), much attention has been paid to leghaemoglobin, a pigment found with the bacteroids and always associated in their fixing activity. Bauer found that it interacts weakly with nitrogen

gas (152) and elaborated a theory of reductive fixation based on this fact (151). Appleby points out that fixation could equally be oxidative, being brought about by reactive oxonium ion or hyperoxyl radical, both of which have been proposed as products of auto-oxidation of oxygenated leghaemoglobin (194). In contrast, Bergersen found that sonic extracts effect oxidation of leghaemoglobin by nitrogen gas, and a stable nitrogen/hydrogen difference spectrum can be obtained (123). Bacteroid 'cells' reduce the pigment back to its original form. Oxygen itself oxygenates leghaemoglobin, oxidising it very slowly and, anyway, Bergersen has detected a barrier which does not permit passage of oxygen into the cells below a partial pressure of 0.4-0.5 atm. (196). Further work by the same author using isotopic nitrogen led him to propose a mechanism (154) which, for its relevance to the problem in Azotobacter, is worth quoting verbatim:

"The primary reactions of the activation of molecular nitrogen and its reduction to ammonia occur in the membrane envelope (which contains the bacteroids); the activated nitrogen is the ultimate acceptor in an electron transport chain which begins in the bacteroids. One of the links in this chain is hemoglobin which lies in solution within the membrane envelope. The host supplies as products of photosynthesis, carbon compounds which are partially oxidized by the bacteroids and serve as the source of electrons for the reduction of activated nitrogen. Products of incomplete oxidation serve as acceptors of ammonia in the production of amino acids by the bacteroids. These amino acids diffuse away and become available to the host

plant."

After examining the two hydrogenase activities (irreversible evolution and deuterium exchange) of nodules and the effect on these of nitrogen, nitrous oxide and carbon monoxide, Hoch, Schneider and Burris (164) postulate the same enzyme site for nitrogen adsorption and hydrogen evolution. The latter is prevented when nitrogen occupies the site while both are in competition with nitrous oxide for the same location. Reduction of the chemisorbed nitrogen is achieved by endogenous hydrogen sources and, the hydrogen atoms on the semireduced species being labile, exchange is then possible with exogenous deuterium gas.

CLOSTRIDIUM PASTEURIANUM (205). The main conclusions reached after investigation of fixation by the cell free extracts obtained either by disruption in a Hughes press or autolysis are:

(i) The fixing extract is soluble, remaining in the supernatant after centrifugation at 144,000 g for one hour.

(ii) By suppressing reactions which tend to convert it into amides or amino groups, virtually all the fixed nitrogen can be obtained as free ammonia which is therefore the first product of fixation.

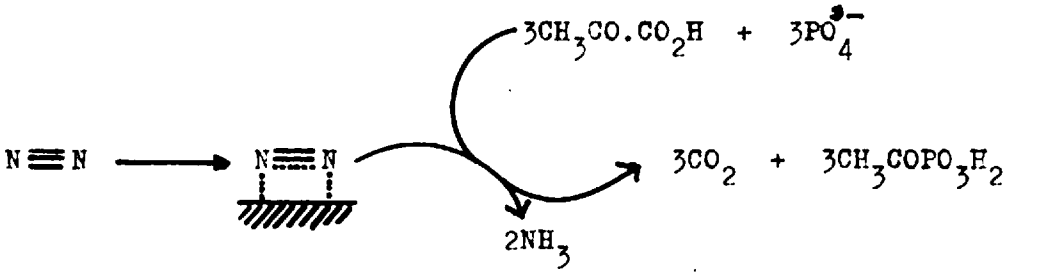
(iii) By fractional precipitation the extracts can be separated into two complementary soluble fractions:

Nitrogen Activating System, which is assayed by a spectral response to nitrogen gas (c 300 mμ). This is an inducible enzyme(s) and is inhibited by carbon monoxide due to the slow formation of an irreversible complex.

Hydrogen Donating System is less stable and contains hydrogenase

activity and the clostridial phosphoroclastic system. It is a constitutive enzyme system and the effect of carbon monoxide is only to cause a long induction period.

The conclusions from this work may be summed up diagrammatically:



SECTION III

PHYSIOLOGICAL BACKGROUND TO FIXATION BY AZOTOBACTER VINELANDII

Until very recently workers on nitrogen fixation had perforce to treat it in isolation from other cellular processes. This fact, forced on them by the undeveloped state of bacterial physiology at the time, meant that many experiments were performed which present-day knowledge shows to have been futile. A pertinent example of this was the use of uptake by whole cells to test whether the substrate concerned was a metabolite in some cellular process. It is now realised that such work can be interpreted only in terms of the specific transport mechanisms in the cytoplasmic membrane. For this reason it is highly unlikely that the unravelling of the fixation sequence <u>in vitro</u>, which can be expected soon, will fully explain the extraordinary ease with which Azotobacter cells transform and assimilate a major metabolite like nitrogen. The nitrogen fixing system can be fully understood only when the following questions are all answered:

(i) where is the mechanism located within the cell?

(ii) what steps between gaseous nitrogen and protein require membrane transport?

(iii) what supplies the electrons required for reductive fission of nitrogen?

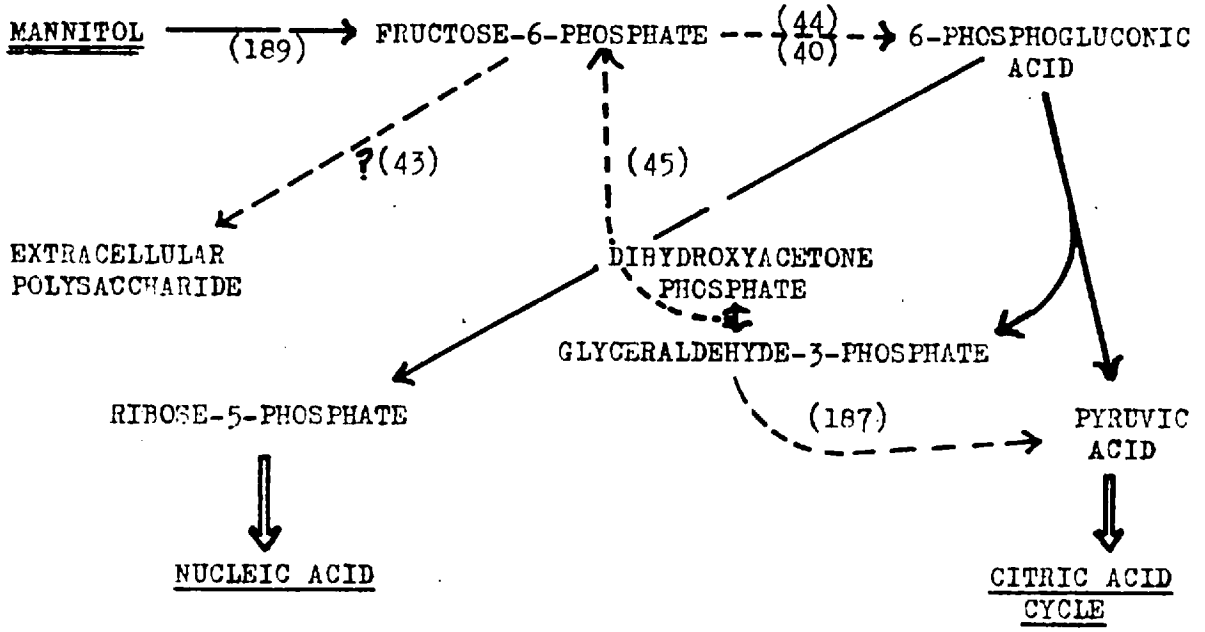
(iv) how are these won in competition with oxygen in such a violently aerobic organism?

(v) what carbon skeleton receives the newly fixed nitrogen?

It is fortunate that there has been, of late, an explosion of

interest in microbiol physiology and, in particular, that Azotobacter has been chosen as the subject in researches covering a wide variety of fields. This is due to its many favourable properties - large cellular size, very high respiratory activity, simple cultural requirements, fast growth rate. As a result it is possible to build a reasonably accurate picture of the main metabolic pathways in Azotobacter vinelandii; the brief survey which follows makes use of results obtained with other organisms only when indicated.

CARBOHYDRATE METABOLISM



(Dashed arrows indicate that steps have been omitted for clarity)

In common with Pseudomonas species (135), degradation of hexose to pyruvate is effected in Azotobacter by the Entner-Doudoroff pathway, a direct route in which 6-phosphogluconic acid is split yielding one molecule each of pyruvate and of glyceraldehyde-3-

phosphate (58). Part of the latter is converted directly to pyruvic acid and the rest is presumably recycled via a reversed aldolase sequence. The net effect is

$$\text{Mannitol} \longrightarrow 2\ \text{Pyruvate} + 6(H)$$

and the hydrogen atoms removed are available at the level of reduced nicotinamide nucleotides ($NADH_2$ and $NADPH_2$).

LOW MOLECULAR WEIGHT CARBOHYDRATE METABOLISM

When growing under favourable conditions Azotobacter oxidizes approximately 98% of the carbohydrate supplied to carbon dioxide (169). It is firmly established (31, 38) that this is done by means of the citric acid cycle (133) which, for growth on acetate, seems to work in conjunction with the 'Glyoxalate Shunt' (61). Ignoring the components removed for use as amino acid skeletons, lipid etc. the net effect is:

$$\text{Pyruvate} \longrightarrow 3CO_2 + 10\ (H)$$

and the hydrogen atoms removed are mostly available at the level of reduced nicotinamide nucleotides. (Though these produced at the decarboxylation of pyruvate are of higher energy and are probably transferred initially to combined lipoic acid (153, 135).) Pyruvate oxidase activity in Azotobacter is inhibited by oxygen (similarly alpha-ketoglutarate which is usually analogous) (201).

TERMINAL RESPIRATION

Azotobacter vinelandii is notable for having the highest respiratory activity known for any organism ($Q_{O_2} \sim 4{,}000$ µl/mg) (1). For this reason its respiratory chain has been studied in great

detail and both Cytochrome c (4 and 5) (138) and Coenzyme Q (134, 211) have been identified as major carriers. Though several sequences have been detected (114, 109, 57) the main pathway between nicotinamide nucleotides and oxygen has yet to be determined. Somewhere in this stepwise degradation three phosphate bonds should be esterified in order that the electron transport sequence performs useful work. Cell free extracts have been obtained which effect oxidative phosphorylation on $NADH_2$, hydrogen and Krebs acids as substrates (129, 60) but the preparations are very sensitive and, when estimated, the P:O ratio is low and extremely variable (62).

AMINO ACID SYNTHESIS

There is overwhelming evidence that in Azotobacter, as in the majority of micro-organisms, glutamic dehydrogenase is the primary means by which ammonia is converted into amino groups (184). Aspartase has been detected in the cells (126) but aspartic acid receives its nitrogen by glutamic/aspartic transaminase activity (16, 182). Fourteen other amino acids received their nitrogen by transamination from glutamic acid in cell free extracts of the organism (87). Common amino acids which usually receive their nitrogen by direct transamination onto the corresponding keto-acid include leucine, isoleucine, valine and alanine. Others obtain their nitrogen by rearrangement of the skeleton of one of the two 'Family Heads' - aspartic acid (25) (threonine, methionine, lysine, diaminopimelic acid etc.) and glutamic acid (arginine, citrulline, ornithine and proline etc.) - while histidine is unique in that it

receives part of its nitrogen from a degraded purine ring (133, 177).

PROTEIN SYNTHESIS

It is now realised that proteins are built from amino acids as units and not peptides, the synthesis of which is a separate matter (145). Protein synthesis in micro-organisms follows a sequence of three steps (168):

(i) Activation of each amino acid by reaction with a specific enzyme (coupled to splitting of one molecule of ATP).

(ii) Transfer to specific soluble ribonucleic acid (sRNA).

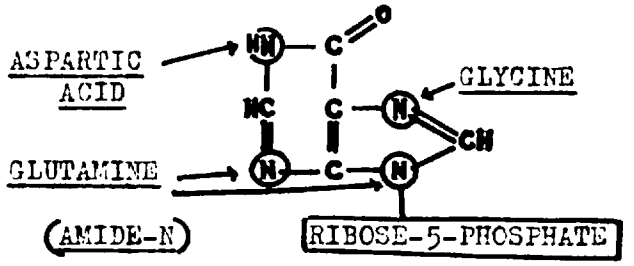
(iii) Transfer to ribonucleoprotein (RNP), sequence determination, condensation and release of protein.

Azotobacter extracts have been obtained which activate glycine (98) while incorporation of several amino acids into protein has been observed, usually with particulate cell fractions (99, 125, 87). The effect of chloramphenicol (which specifically inhibits step (iii) (180)) on fixation and nitrogen metabolism of Azotobacter has been investigated in several laboratories (157, 99, 198, 160).

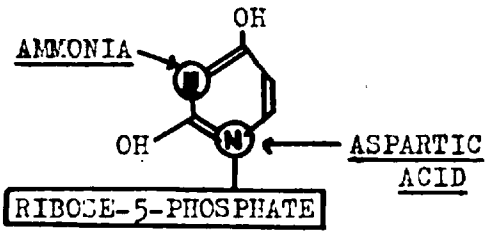
Of special interest in this context is the incorporation of glutamic acid into protein. It has always been assumed, without direct proof, that separate sRNA carriers exist for glutamic acid and glutamine since, in the resulting proteins, they occupy distinct positions as if they were different amino acids. Very recent work on E. coli extracts, however, indicate that probably only one sRNA species exists and that is specific for glutamine: any glutamic acid being incorporated has to do so via glutamine (212).

PURINE AND PYRIMIDINE BIOSYNTHESIS

The biosynthetic pathways leading to purines and pyrimidines have been elucidated for avian liver cells but are regarded to be of quite general application. The examples of nucleotides depicted here indicate the source of nitrogen atoms to be found in typical purine and pyrimidine rings (128, 142).



(INOSINIC ACID)



(URIDINE MONOPHOSPHATE)

Interconversions among nucleotides usually involve transfer of ammonia or amide-nitrogen: in effect these forms are equivalent and may be classed together as 'free and bound ammonia'.

CYTOLOGY

When grown under favourable conditions Azotobacter vinelandii (Azotobacter agilis var. vinelandii) produces large ovoid cells (c. 2 x 4 μ) not unlike those of yeast, though of course on a smaller scale. Reproduction is by simple binary fission and the majority of cells in a vigorous culture are waisted and in the process of division. When in stagnant cultures a fluorescent yellow-green pigment is excreted (131, 76, 110) and the capsule becomes heavier. This slime layer which consists of a protein-free hemicellulose (43) also varies in quantity from strain to strain and can act as carbon

and energy source for the bacteria (141). The cells are motile with lateral flagellae.

Azotobacter are gram-negative. Though the structure of the cell wall is still largely a matter for conjecture, it has been proposed that in gram-negative organisms it consists of mucocomplex (which comprises glucosamine, muramic acid, alanine, lysine and glycine) as in gram-positives but surrounded by a plastic layer of lipoprotein (136). Even as this thesis was being written, a report appeared which, based chiefly on work with E. coli, led the authors to propose a new general structure for the envelope of gram-negative bacteria. It consists essentially of a layer of mucocomplex sandwiched between two membranes of lipoprotein with polysaccharide attached on the inner side of both (200).

However complex it may be, the cell wall is a rigid porous structure which acts as a crude molecular sieve, excluding only large macromolecules. It also supports the pressure of the cytoplasmic membrane which in gram-negative organisms (again in contrast to the gram-positives) is too unstable to be distinguished readily from the surrounding cell wall (191). For this reason the cell wall and cytoplasmic membrane may be considered together as the 'Cell Envelope'. The fragility of the cytoplasmic membrane is such in Azotobacter that much confusion was caused when small fragments of the cell envelope were wrongly identified as intracellular particles ('mitochondria', 'electron transporting particle', enzymically active RNP etc.) (136).

Subsequent investigation has shown that there are only two distinct particles to be found in the cytoplasm of Azotobacter vinelandii: polymetaphosphate granules (which are assumed to act as energy reservoirs) (52) and RNP particles of about 30 mμ diameter (116, 102, 136). It is still not clear whether these latter are the seat of protein synthesis for appreciable amino acid incorporation has only been observed with envelope fragments (165, 136). The cytoplasm is compartmented by internal membranes (207) and contains the nuclear region which is a tightly coiled mass of DNA without a surrounding membrane (136).

The cell envelope, though not homogenous, is flat and contains no granules (165). Work with gram-positive bacteria proves that the transport mechanisms and the bulk of enzymic activity of the envelope reside in the cytoplasmic membrane but, as explained above, it is more accurate to treat it together with the cell wall. The distribution of enzyme activity between the cytoplasm (soluble) and the envelope (particulate) is much the same for Azotobacter as for other micro-organisms and may be summarised (92, 136, 165):

ENVELOPE	CYTOPLASM
Part of Citric Acid Cycle (Malic & Succinic Enzymes)	Bulk of Citric Acid Cycle Enzymes
Oxidative Phosphorylation & Electron Transport Systems	Carbohydrate Enzymes
Metals Fe Ca (107)	Catalase, Phosphatases (130, 52)
Hydrogenase (132), Cytochrome c	Nucleotide Enzymes (173, 56, 93)
Amino Acid Incorporating System	RNP Particles
	Polymetaphosphate Granules
	? Molybdenum (& Tungsten) (77)

SECTION IV

PREVIOUS STUDIES ON NITROGEN FIXATION BY AZOTOBACTER VINELANDII

Cell-Free Extracts

Though almost two years have elapsed since consistent fixation by cell-free extracts of Azotobacter vinelandii was first reported by D.J. Nicholas (166), no positive evidence as to mechanism or requirements has yet been forthcoming. Extracts were obtained by lysozyme treatment or ultrasonic shock but the cells had to be disrupted in their original culture medium. The initial results have been confirmed in Imperial College (209) and Nicholas has extended the scope of his work by using the radioactive $^{13}N_2$ as tracer. This located the fixing sequence in a soluble fraction which, however, required cell particles for sustained activity (190). Hints as to cobalt and vitamin B_{12} requirements were recently reported in a magazine article (199) but the work referred to has not yet been published.

Metabolism of Fixed Nitrogen

The earliest and most obvious means of detecting intermediates in the fixation process was based on the assumption that, if any such compound were in the medium it would be used preferentially by the cells and so inhibit fixation. Though the extent and specificity of the transport mechanisms of the cell membrane, as now understood, make such a conclusion untenable much valuable work has been done based on this assumption.

Horner and Allison (13) in 1944 reviewed all previous work in

which compounds detected as capable of acting as alternative nitrogen sources to the atmosphere were ammonium salts, nitrate, nitrite, urea and asparagine. Long term (6 days +) growth trials of stand cultures under a nitrogen-free atmosphere of oxygen and hydrogen confirmed this list and added glutamic and aspartic acids which, together with asparagine, took a much longer time to attain full growth. Under such very favourable conditions any nitrogen containing substance capable of acting as a source should have been detected so the very extensive list that failed may be eliminated altogether (amino acids, purines, pyrimidines, amides, amines etc.). Equivocal results with some pyrimidines were attributable to extracellular deamidation yielding free ammonia.

It should be noted that glutamine was not tried and, in fact, has been reported only once and that was as substrate for a non-fixing mutant (137). This auxotroph was deficient in a step allowing access of pyruvate to the fixing site. Tests for utilization were performed by making a small inoculum into sterile medium in a flask which was shaken in air while turbidity changes were followed over a period of up to fifty hours. Glutamine was found to be an excellent nitrogen source, aspartic and glutamic acids and asparagine were used less readily while others (glycine, valine, citrulline and ornithine) were not used at all.

Though they have long been considered as possible intermediates resulting from an initial oxidative step, it is clear that nitrate and nitrite are metabolised via ammonia. The assimilatory nitrate

reductase system is well established in other organisms (206) and its presence in Azotobacter has been indicated by studies in three different laboratories (55, 69, 175). Much attention has likewise been paid to nitrite reductase (148) but Spencer, Takahashi and Nason (97), having separated and characterised the enzyme, examined its adaptive properties and concluded that it is not involved in fixation by Azotobacter. Working at Lahore, Azim and co-workers have tried to detect utilization of a very wide range of nitrogen compounds by stagnant cultures (80, 81, 82).

The most reliable work of this type was performed in Wisconsin where actively growing cultures were supplied fixed nitrogen in various forms and simultaneously exposed to $^{15}N_2$ (9, 18). Under these conditions urea and ammonium completely suppress fixation, nitrate, nitrite and asparagine do so partially, while glutamic and aspartic acids have no effect at all. This work established ammonium firmly as an intermediate since

(i) both nitrate and nitrite require adaptation whereas ammonium was utilized at full level immediately,

(ii) when supplied ammonium nitrate the cultures used all the ammonium first before metabolising the anion,

(iii) urea is assimilated as ammonium after hydrolysis by cellular urease.

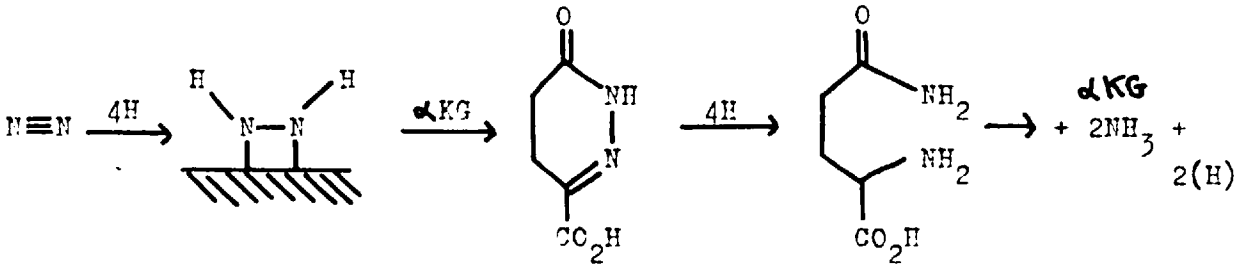
Most interesting of these utilization experiments were those involving inorganic nitrogen compounds suspected as being intermediates:

HYPONITROUS ACID (41), NITRAMIDE (59) and NITROUS OXIDE (50) are not utilized as shown by negligible incorporation of ^{15}N from the labelled compounds (though results are somewhat contradictory in the case of nitrous oxide, uptake, even if positive, is slight (46)). HYDROXYLAMINE inhibits respiration and fixation at low concentrations where it is not actually toxic (47). When the reactive group is masked in oxime formation there is still no evidence of utilization (22,23). Hydroxylamine reductase activity was observed by Suzuki using whole cells (48) and the enzyme itself has been isolated from extracts of the organism on two occasions (72, 97). Such results may be safely discounted, however, since the discovery that a well characterised hydroxylamine reductase from E. coli was equally specific for sulphite reduction, which function was the more likely in vivo (184). It is now recognized that the excretion of 'bound hydroxylamine' once claimed as a specific property of fixing cultures, also occurs with ammonium as nitrogen source (96). HYDRAZINE is the only inorganic compound which has experimental backing for its claim as a possible precursor to ammonia in the fixation sequence. As is the case with hydroxylamine it is very reactive and would react instantly with countless compounds found in abundance in a living cell. For instance, hydrazine can displace ammonia as substrate for glutamine synthetase (122) while it completely inhibits the glyceraldehyde phosphate dehydrogenase of Azotobacter vinelandii (187). It also reacts instantly with any keto-acid. Accordingly Roberts and co-workers found that growth is arrested while the cells detoxify the

molecule and finally excrete it quantitatively as its monacetyl derivative (68, 162, 203). This work provides a simple alternative to the claim by Japanese workers that hydrazine is oxidized by whole cells and extracts of the organism (48, 174). This 'oxidation' was stimulated by respiratory substrates and they apparently did not check whether the 'disappearance' was merely due to its becoming bound by the products of glycolysis.

Bach, working in Wisconsin, confirmed the general conclusions reached by the London group (83); being in heavy resting suspensions the cells could call only on endogenous carbon sources and the bulk of the hydrazine was found to be sequestrated by reaction with α-ketoglutaric acid. He then went on to examine the organic acids giving a positive Ehrlich reaction in the soluble nitrogen fraction from cells grown on (a) ordinary nitrogen, (b) labelled nitrogen, (c) labelled ammonium.

Both with Azotobacter vinelandii and field grown legume nodules chromatographic bands were obtained which were identified with the products of the reaction between hydrazine and alpha-ketoglutaric acid. These bands were labelled much more heavily when $^{15}N_2$ was used as nitrogen source as opposed to $^{15}NH_4^+$. Basing it on these results, Bach proposed a reaction scheme whereby the semi-reduced nitrogen is removed as hydrazine by reaction with α-ketoglutarate and then reduced directly to glutamic acid and ammonia:



'Nitrogenase' 'P.C.A.' Glutamine

Yocum considered the thermodynamics of this 'nitrogen respiration' sequence and estimated that it could effect oxidative phosphorylation with a P/E ratio of 0.17. This, while small for an aerobic organism, is approximately twice the value for anaerobic glycolysis (178). Bulen and Doughty have since independently confirmed Bach's main finding: the presence of 3,4-dihydropyridazinone-5-carboxylic acid (P.C.A.) in extracts of the organism (100). Not surprisingly, they found that P.C.A. is not utilised as a carbon or nitrogen source by intact cells. Cell-free extracts do however react with it forming α-ketoglutarate and succinate. Other forms of bound hydrazine found not to be utilised by Azotobacter are the diacetyl (174) and pyridoxal (65) derivatives.

Hydrazine is not utilised by Clostridium pasteurianum, being toxic to the whole cell (27), while extracts neither accumulate nor metabolise hydrazine or its derivatives (159).

Trace Element Requirements

In common with many other fixing agents Azotobacter vinelandii requires molybdenum and extra iron when fixing nitrogen (73). The

molybdenum may be recovered from fragmented cells largely in a small particulate fraction (77). It is not involved in a silico-molybdate complex (108) and cannot be replaced by vanadium or tungsten, though the latter competes with it in uptake (91, 181). Calcium was once included in this list but recent work shows that some strains have no such requirement (94, 158) and, where necessary, it functions in an indirect manner (being required in smaller quantities for growth on fixed nitrogen also (186)). Two explanations have been proposed by Esposito and Wilson: (i) since calcium is necessary for synthesis of polymetaphosphate this latter could be required as an energy source for fixation (74); (ii) alternatively, calcium being required for synthesis of acetate or some related compound, perhaps its role is in providing an acceptor for juvenile nitrogen (104). The second theory has received support from the recent discovery that fluoracetate is a specific inhibitor of fixation (197).

If, as preliminary reports indicate, cobalt is implicated in cell-free fixation by Azotobacter extracts (199) this may well be another general property of the nitrogen fixing system, since trace requirements for this metal are already established for fixing rhizobia and blue-green algae (171).

Gaseous Inhibitors

The Michaelis constant for nitrogen fixation in air as obtained in Wisconsin (7) and confirmed by the London (49) and Australian (169) groups, is 0.023 atmospheres. Wilson and Roberts also determined it in 30% oxygen by an accurate isotopic method and obtained the much

higher value of 0.066 atm. This was the first quantitative evidence for the competitive inhibition of fixation by oxygen, a fact established later by Parker and Scutt (169). Both hydrogen (6) and nitrous oxide (32, 50) are competitive inhibitors and have about one sixth of the affinity of nitrogen for the enzyme site. Though this effect is superimposed on extensive non-competitive inhibition carbon monoxide is yet another competitive inhibitor (12).

Hydrogenase

As is the case with all other fixing agents, nitrogen fixation by Azotobacter is intimately involved with hydrogenase activity, this being much lower in cultures which have been grown on ammonium nitrogen (10, 35). Hydrogenase is located entirely in the cell envelope (132) and may be centrifuged off without stopping fixation by cell free extracts (190). The hydrogenase of Azotobacter has been separated and found to contain iron (36, 167); it transfers hydrogen to a number of acceptors (oxygen, methylene blue etc.) but not to nitrogen itself (210).

Miscellanea

Pre-irradiation of the nitrogen used by ultra-violet light enhances fixation by up to 23% (193) and, since no isotope effect could be detected, Hoering and Ford concluded that the rate determining step in fixation by Azotobacter cultures does not involve a change in bonding to nitrogen (163). This result was echoed in quite different experiments on the fixing extracts of Clostridium which were found not to effect isotope exchange with a labelled atmosphere (159).

Nitrogen fixation by Azotobacter vinelandii is specifically stimulated by low concentrations of hydrazine and nitrite (143) and specifically inhibited by orthophosphite (88), chloramphenicol (157), trimethylamine oxide (110), azide (95) and fluoracetate (197). Except, perhaps, for trimethylamine oxide and azide, it seems likely that these act indirectly in various ways; by affecting supplies of reducing power or the reactions involved in removing the products of fixation etc.

Thermochemistry

It is a practical impossibility to make accurate estimations of the energy changes involved in fixation for, even if the nature and in vivo concentrations of the components were known there would be insufficient thermochemical data to fill in the equations.

Wilson and Burris estimated the free energy change for the hypothetical reduction of nitrogen by glucose and found it to be slightly endergonic (19). Bayliss criticised this approach for using unrealistic concentrations (70) and estimating likely physiological values himself, constructed the following equation:

$$C_6H_{12}O_6(aq) + 4N_2(g) + 6H_2O(l) + 8H^+(aq) \longrightarrow 6CO_2(g) + 8NH_4^+(aq)$$

$$\Delta G = -112 \text{ k cal.}$$

Since, during glycolysis and the citric acid cycle, large quantities of $NADH_2$ (and $NADPH_2$) are produced by dehydrogenase reactions, these may be taken as typical of the more energetic reducing agents available in the active cell. A good idea of the thermodynamics of fixation may be obtained by assuming the following

reaction under physiologically plausible conditions (data from Bayliss (70) and Burton and Krebs (34)):

$$N_2 + 3NADH_2 + 2H^+ \longrightarrow 2NH_4^+(aq) + 3NAD$$

$$\Delta G = -9.9 \text{ k cal.}$$

Conditions: pH7, substrates 0.01 M., 30°C, nitrogen at 0.8 atm. For simplicity, it is assumed in the above equation that the six hydrogen atoms added to the nitrogen molecule during reductive fission are all at the level of nicotinamide adenine dinucleotide (Eo,pH7: -0.32 v.): it is more probable that each successive pair have different energies which could range from pyruvate (Eo, pH7: -0.63 v.) through hydrogen (Eo, pH7: -0.42 v.) down to flavin nucleotides (Eo, pH7: circa 0.22 v.).

It is clear that, though nitrogen cannot hope to compete directly with oxygen, there are plenty of routes whereby the reduction could be usefully exergonic allowing (as was demonstrated by Yocum (178)) coupling to phosphorylation on an appreciable level.

Isotopic Tracing Experiments

Between 1941 and 1957, Burris and co-workers at Wisconsin have performed a series of experiments on Azotobacter vinelandii using labelled ammonium and nitrogen as substrates. Their intention was to detect the hierarchy of labelling among the nitrogen compounds of the cells and so deduce the pathway travelled by newly fixed nitrogen. Earlier work was greatly handicapped by the crude, non-quantitative methods available at the time for fractionation of the amino acids. Nevertheless these experiments - the results of which

were confirmed using a host of other fixing agents - showed that the lable became distributed similarly whether the cells were grown on $^{15}N_2$ or $^{15}NH_4^+$ (39); the order of enrichment inevitably found in these moderately long-term (20 to 90 mins.) experiments was:

Glutamic > Aspartic > Neutral Amino Acids > Basic Amino Acids

In 1953 it was found that heavy resting suspensions would fix nitrogen and assimilate ammonium simultaneously (37). Glutamic acid again attained the heaviest label but a notable discovery was the fact that the newly fixed nitrogen became randomised with added ammonium in the artificial extracellular pool. Though obtained in a metabolically constrained experiment, this result corresponded to the normal situation with cultures and cell-free extracts of Clostridium pasteurianum (159).

The final refinement came in 1957 when Allison and Burris exposed actively growing cultures of Azotobacter to $^{15}N_2$ for short times (1, 2 and 5 minutes) after which the cultures were killed with acid and separated into two fractions - cells and supernatant (79). These were then analysed by ion exchange chromatography and the label on each nitrogen compound was determined. Steady state conditions were assumed allowing precursor/product relationships to be deduced from the extent and the movements of the label. The results may be summarised as follows:

Total Culture: In all the short duration of these runs the supernatant fraction was enriched to a much greater extent than the cells. After 5 minutes a % Total Activity plot gave a characteristic $NH_3 \rightarrow$ Glutamic

precursor/product relationship with glutamic present in thirteen times the quantity of its precursor.

Cellular Nitrogen (Analysed after total hydrolysis): Amide nitrogen (which was virtually all accounted for as glutamine and asparagine residues) when plotted in a % Total Activity graph was clearly indicated as being precursor to the bulk of cellular bound nitrogen. Likewise the sequence Amide → Glutamic Acid → Aspartic Acid was indicated. All of the other nitrogen compounds (amino acids, purines and pyrimidines) showed steadily increasing enrichment as would be expected from metabolic 'dead-ends'. The exceptions were adenine and guanine whose labels fell off slightly between two and five minutes. This is easily understood since, apart from being nitrogen donors to histidine, purines can act as 'bound ammonia' for instance through the action of cytoplasmic adenase. The order of enrichment after five minutes was:

	Amide N	> Glutamic, Aspartic	> Neutral Amino Acids	> Basic Amino Acids	> Purines and Pyrimidines
% N Excess	0.13	0.09	0.07-0.02	0.04-0.02	0.02-0.01

Supernatant Nitrogen: Approximately 25% of this fraction was peptide nitrogen - mainly two peptides composed mostly of glutamic acid residues. An odd fact was that amide nitrogen had a lower enrichment than its corresponding amino groups. The order of enrichment in the supernatant after 5 minutes was:

	Free Glutamic Acid & Peptide Glutamic Residues	>	Free NH_3	>	Glutamine Amino-N	>	Other Amino Acids	>	Glutamine Amide-N
Atom % Excess N	6.0		1.7		1.0		0.6		0.4

Of these glutamic acid (free and peptide), glutamine amino-N and neutral amino acids became enriched steadily throughout but glutamine amide-N hardly increased after the start while ammonia-N first increased then became less heavily labelled.

This last result is, of course, incompatible with any mechanism in which ammonia is precursor to glutamic acid. Though it was established that acid killing mixed an unlabelled pool of 'old' ammonia with the highly labelled product of fixation the same puzzling result was obtained when the culture was cooled quickly and separated by centrifugation. The authors were forced to ignore this difficulty and assume that, in <u>both</u> cases, the real 'juvenile' ammonia pool had been diluted somehow on killing. Indeed, excellent though this research was, its great weakness lay in the drastic method employed to stop metabolism abruptly. The early, long-exposure, runs were stopped by cooling in ice but it was demonstrated that, before the cells could be centrifuged off, they managed to assimilate considerable quantities of the nitrogen in the medium. In order that fixation be stopped instantaneously in these short-term experiments the cells were speedily mixed with acid to a final pH of 2. Unfortunately this mode of killing extracts nitrogen compounds preferentially from the cells and the supernatant (examined after centrifugal removal of the

cells) contained almost four times its original quantity of nitrogen. The label on the free ammonia was halved by dilution with metabolically 'old' fractions which had been loosely bound in the cell while the enrichment of the rest was increased over sevenfold. Thus the acid extractable portions of Azotobacter cells contain many highly labelled compounds.

This experiment was repeated by Burma and Burris using labelled ammonium rather than nitrogen as the substrate (86). Again the enrichment of the supernatant was higher and appeared instantaneously whereas there was a short delay before the cells became labelled. The ratio of excess label in supernatant to that in cells dropped from 50 (after 1 min.) to 15 (5 mins.). The labelling of cellular constituents took place in the same order as with fixing cultures except for the fact that purines and pyrimidines had an extraordinarily high enrichment - they were second only to glutamic acid after five minutes exposure. Since part of the nitrogen going to make up these heterocyclic rings does not have to go through the glutamic acid 'bottleneck', this result is not surprising - it does however constitute a major difference between cells that are fixing nitrogen and those that are metabolising ammonia.

Scope of This Work

As will have become obvious in the preceding section, our understanding of the mechanism of nitrogen fixation by Azotobacter vinelandii - even though extensive - contains a number of outstanding obscurities and contradictions. Many will doubtless be resolved when

work on cell free extracts is successfully completed (eg. nature of the reactive site, source of reductive power) but there remain others which can only be fully understood by recourse to whole-cell studies. It is these, listed below, that have provided the incentive for the work reported in this thesis:

(i) the status of hydrazine derivatives in fixation: the work of Bach is in direct contradiction to all other reports from Wisconsin.

(ii) the precursor/product relationship of glutamic acid/ammonia: the contradictory nature of Allison and Burris' and Burma and Burris' findings on this point should be resolved.

(iii) the location of the nitrogen fixing system: Burris (79) claims that "rapid excretion suggests that fixation may occur on or near the surface of the cells" yet fixing extracts all seem to be soluble (cytoplasmic) and the system must be protected from competition with oxygen.

SECTION V

EXPERIMENTAL METHODS

(a) BACTERIOLOGICAL

Organism

The organism used throughout this work was Azotobacter vinelandii (A. agilis var. vinelandii) a vigorous aerobe which, though commonly observed in the form of large gram-negative ovoids, frequently exhibits marked plecmorphism (43). In order to distinguish behaviour of fundamental importance from variation due to strain differences, two strains were used in parallel during the main part of this work:

S.K. Originally obtained from Holland, this has been the subject of most previous work at Imperial College. It is heavily encapsulated and gives smooth mucoid colonies on nitrogen-free agar (with no pigment formation in the modified Burk's medium used here).

O Kindly supplied by Professor Burris, this is the strain used at Wisconsin and in many other laboratories. The cells are only slightly encapsulated and colonies on nitrogen-free agar tend to look rough. When fully grown they excrete a yellow-green fluorescent pigment.

Medium

The medium used was Burk's Mineral Salts (37) with 1.5% mannitol as carbon source. To reduce the turbidity of uninoculated medium, and suppress pigment formation, disodium citrate was added at a concentration of 0.5×10^{-3} M. This complexed the iron and prevented it forming insoluble phosphate. As noted by Diamantis (127), this citrate concentration was insufficient to affect growth through

sequestration of magnesium. Previous workers in this laboratory had used a medium of slightly different composition but the one described here was adopted because its lower salt concentration made analysis of the supernatant much easier. It was also more economical and comparative growth runs had shown that, once trained, culture properties were the same on both media. Mannitol, the traditional carbon source, was retained since, having no tendency to char, it could be added to the medium before sterilization. The medium used had the following composition per litre:

Mannitol	15 g	$MgSO_4.7H_2O$	0.2 g	$FeSO_4.7H_2O$	0.015 g
K_2HPO_4	0.64 g	NaCl	0.2 g	Na_2MoO_4	0.00215 g
KH_2PO_4	0.16 g	$CaSO_4.2H_2O$	0.05 g	$Na_2H[C_6H_5O_7].1\frac{1}{2}H_2O$	0.13 g

it had a pH of 7.2 and cultures were never found to drop below pH 7.05 even after growth in the presence of substrates.

For convenience, two stock solutions at one hundred times the above concentrations were used:

(A) Containing phosphates, chloride and molybdate.

(B) Containing magnesium, calcium, iron and sodium citrate.

The latter was a suspension containing finely divided calcium sulphate and had to be well shaken before use.

To make one litre of clear medium suitable for turbidimetric work, 10 ml each of solution (A) and (B) and 15 g of mannitol were dissolved and made up to the mark with distilled water. The mixture was then brought to boiling point and cooled back to room temperature. Filtration at the pump removed excess calcium salts producing a clear,

colourless medium containing well above the minimum requirement of calcium in solution. Solid nitrogen-free medium was prepared as above with the addition of 2% agar.

Sterilization

All media and culture apparatus were sterilized by autoclaving at 15 lbs for 20 minutes. For shake cultures, 65 ml portions were dispensed in 300 ml Kjeldahl flasks and closed with cotton wool plugs. For storage in bulk, quantities of 200 to 500 ml were dispensed in screw-cap round bottles. When substrates were to be used, the medium was made up in 80% the correct volume of water and 80 ml portions were autoclaved in screw-cap bottles. The substrate was made up in water at five times the desired concentration, sterilized by filtration through a 'Sterimat' (Fords 7GS Grade) and added to the medium by pipette (using the usual precautions to preserve sterility). The same procedure was adopted on a smaller scale when shake cultures were required to train the organism to grow in presence of a substrate - 8 ml of concentrated medium were made up to 10 ml with substrate solution at five times the desired concentration.

Stock Cultures

At the outset, nitrogen-free slopes were prepared from rigorously purified strains and stored at +4°C in sealed MacConkey tubes. These master slopes were used in turn, each being subcultured to prepare six slopes which sufficed for a period of one to two months before desiccation became serious. In these subcultures the cells became

heavily encapsulated after 4 to 6 weeks and the growth rate slowed to about 60% of its optimal value. Full activity was easily restored by two subcultures with vigorous shaking at 30°C. For this reason, when a master slope was being subcultured its maximal growth rate was first regained by successive transfers, dilution and plating. After such treatment the properties of a strain were found to return to their original values even over a period of two years. Towards the end of this research it was decided to avoid the hazards of desiccation and master slopes for future work were preserved by freeze-drying.

Purity

Except when heavily encapsulated (which seems to reduce the cells' sensitivity) both strains grow slowly on nutrient agar at 30°C, producing small colourless colonies of about 1 mm diameter after 24 hours incubation. The following was adopted as a routine purity test and applied to every run for which results are quoted in Section VI:

(i) Two loopfuls of test culture were spread on a nutrient plate.

(ii) This was examined after 12, 24 and 48 hours incubation at 30°C.

(iii) A smear was taken from the surface of the agar and examined by grams stain.

When active cultures of 'O' strain are being examined, about 50% of the resulting cells are grossly distorted, many being large 'balloon' forms of random shape and size (up to 12 μ in length). 'SK' is more resistant and about 10% are pleomorphic forms. The rest are neat

ovoids (of up to 2 x 3 μ in size), which stain with blotches and have an easily recognizable 'puffed wheat' appearance.

After growth on nitrogen-free agar, both strains exhibit a yeast-like morphology being large ovoids (typically 2 x 4 μ). The capsule, in SK and old cultures of O, can be readily picked out using nigrosine as a negative stain.

Inoculum

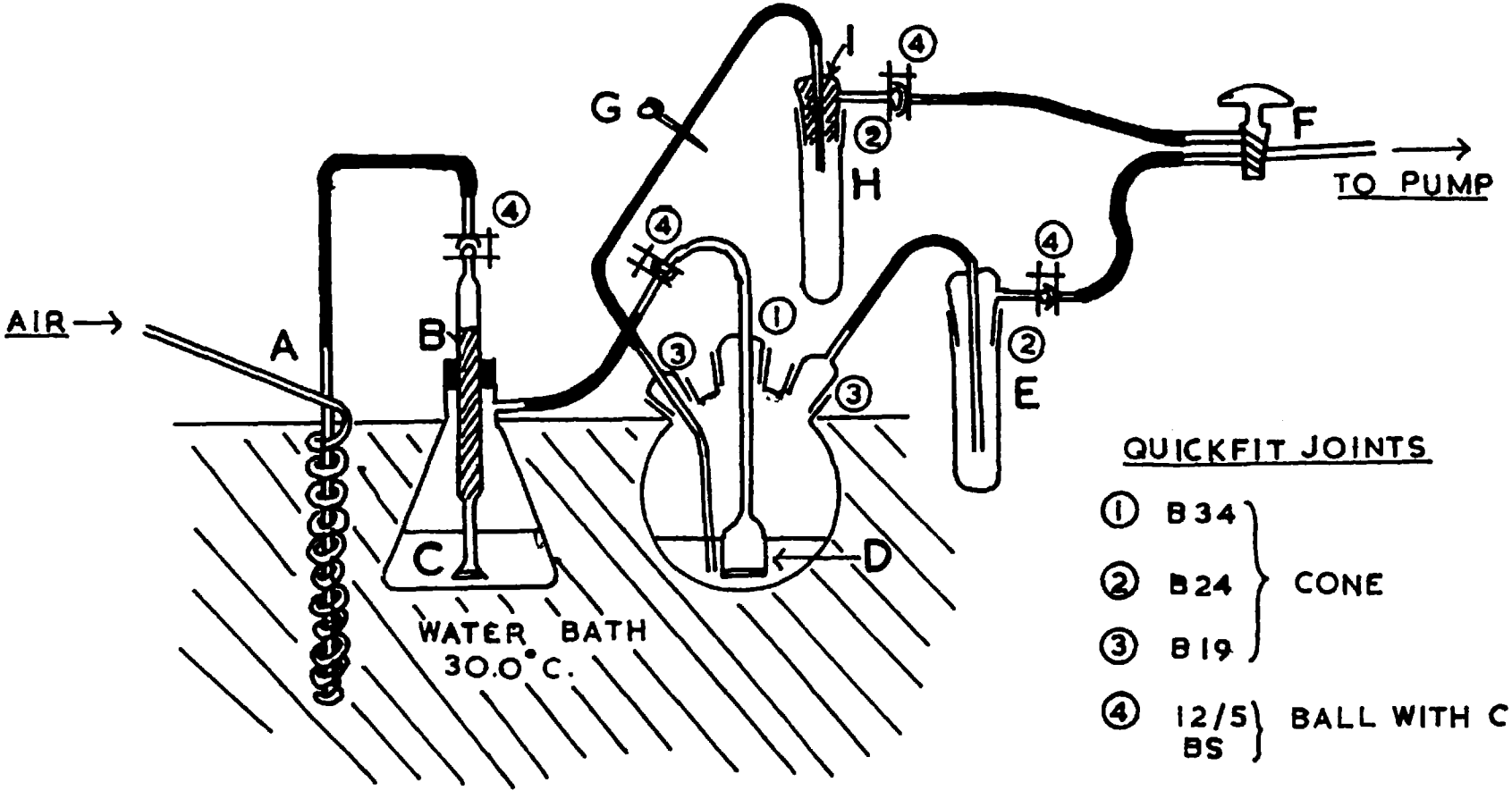
A culture in middle logarithmic growth could be obtained by 24 hours vigorous shaking of an inoculated 65 ml portion of medium. When ready before it was needed, a culture could be stored at +4°C for several days before any effect could be noticed in its subsequent performance.

(b) CULTURE TECHNIQUES

Special apparatus (Figure 1) was developed so that an optimally aerated culture could be sampled at intervals with negligible effect on growth conditions or sterility. The whole apparatus shown was immersed in a water bath at 30.0°C ± 0.01 and at least an hour elapsed before the start of each run to allow everything to attain this temperature. Air was sucked through the train by means of a simple vacuum pump and compressor (W. Edwards 5 lbs/sq.in.). Before meeting the shallow layer of culture it was passed through a copper spiral A, a cotton wool filter B, and sterile water C. In this way equilibrium was ensured between the incoming air and the culture.

The culture vessel was a three-necked flask of one litre capacity fitted with a central inverted bubbler D in the form of a fritted

FIG. 1. CULTURE APPARATUS



glass plate (Pore Size 0) located at less than one centimetre from the bottom. Growth rate experiments showed that aeration was constant until the liquid volume exceeded 190 ml when it became progressively less efficient as the vessel filled up.

Normal aeration was achieved by drawing the air off via a trap E but, in order to sample the culture, the suction was diverted by rotating tap F. On opening clip G culture samples of any desired volume could be siphoned off via the narrow bore tube leading into the culture near the bubbler. It was collected in a clean flask fitted onto the sampling unit H. Just before sampling, the tubing could be flushed by opening the clip G, possible contamination being prevented by a cotton wool filter I.

The apparatus was made entirely of pyrex glass with Quickfit joints except flexible parts which were of heat-resistant polymer tubing (Escorubber). Before use all new glass apparatus was boiled for 30 minutes in 5% nitric acid to remove any trace metals, arsenate etc. which could act as growth inhibitors (169). At the end of a run, after a thorough wash, the apparatus was entirely rinsed with distilled water. It was sterilized in two parts (water saturator and culture vessel + siphon and traps) then clipped into one piece before use. Since evaporation altered the volume of any liquid sterilized in the culture vessel, media were always prepared separately and poured in just before the run was commenced.

Once the procedure described above had been perfected, about two hundred growth runs were performed each lasting from three to five

hours. In all these, only three cases of contamination occurred and each time the cause was traced back to the medium: not once could it be attributed to deficiencies in the apparatus.

(c) ANALYTICAL

Turbidity

The turbidity of culture samples freshly removed from the vessel was read off against a water blank in a Unicam SP 300 Colorimeter using a Tricolour Green filter (which transmits from 4,800 Å to 6,100 Å with a maximum at 5,300 Å). A small correction was subtracted to allow for the turbidity of uninoculated medium. Both strains used form notably stable and uniform aqueous suspensions and, provided readings were taken within 30 seconds of removal from the growing culture, the results were reproducible to $\pm$ 2%.

Dry Weight

10 ml culture samples were pipetted into accurately weighed 15 ml centrifuge tubes. The cells were centrifuged for 20 minutes, the supernatant poured off and the residue was agitated with an equal volume of water. After a second centrifugation the tube containing the washed cells was placed in an oven at 105°C for 24 hours. The tube was then cooled in a desiccator and weighed to the nearest 50γ on an accurate balance. With careful washing a reproducibility of $\pm$ 3% could be obtained.

Isotopic Nitrogen

The sample was prepared and admitted to the mass spectrometer tube as described in Section IV(g) Once a steady flow of gas was

visible through the leak - as observed with a Tesla discharge - the peaks corresponding to ions of mass number 28 and 29 were tracked and their heights measured. After corrections for zero and peak residuals the percentage of ^{15}N in the sample was readily determined by application of the equation:

$$\%\,{}^{15}N = \frac{100}{1 + 2\,\frac{\text{Peak Ht. } 28}{\text{Peak Ht. } 29} \times S}$$

S is the shunt ratio required to bring peak heights to the same order of magnitude. Percentage enrichment was obtained by deducting natural abundance of ^{15}N (0.38%) from each value.

In order to reduce errors due to fluctuations in magnet current, each value was determined at least three times. This fluctuation was the only serious source of error and, due to the nature of the above equation, varies with the actual enrichment being measured. To estimate its effect, some readings were deliberately repeated several times and the following may be taken as indications of maximum probable errors:

Atom % Excess	15.0	7.00	0.80
Error	± 1%	± 2%	± 1%

Nitrogen Determinations

All forms of nitrogen were converted ultimately to ammonia which was distilled from a 50 ml ball-jointed flask into water using a one-piece semi-micro Kjeldahl apparatus. The ammonia thus collected was titrated with 0.02N hydrochloric acid using brom-cresol green as indicator. Blank values were always deducted since the end-point

of yellow-green was not exactly at equivalence (pH 3.6 - 5.2) and, anyway, the pH of the distilled water used fluctuated slightly from day to day.

These titrations were easily reproducible to within ± 0.02 ml.

Cellular Nitrogen (Acid Killed)

This was determined using a modification of the total nitrogen method of Willits and Ogg (26). These analyses were performed in duplicate on 25 ml portions of culture yielding results accurate to ± 0.5%. The sample was acidified to a strength of 0.1N by addition of 5N sulphuric acid (0.5 ml) with vigorous agitation. After 20 minutes centrifugation the supernatant was poured off, the residue agitated with an equal volume of distilled water and the centrifugation was repeated. The washed residue was then transferred quantitatively to a Kjeldahl flask containing sulphuric acid (1.5 ml) and a mercury catalyst tablet (B.D.H., containing 1 g sodium sulphate and 0.1 g mercury). A chip of porous tile was added and the mixture was evaporated vigorously until it had become brown. Heating was continued cautiously until pure acid refluxed from the brown mixture. A further chip of porous tile was then added since the danger of loss by bumping was always greatest at this stage. The mixture was refluxed vigorously (to one-third of the way up the neck of the flask) until colourless and then for one hour. On cooling, the residue was taken up in distilled water and the ammonia was released using 10 ml of a mixed solution containing sodium hydroxide (35%) and hydrated sodium thiosulphate (6%).

Cellular Nitrogen (Filtered)

25 ml. portions of culture were analysed in exactly the same way as for Cellular Nitrogen (Acid Killed) except that the cells were separated from the fresh sample by filtration through a cellulose ester membrane (Oxoid Division, Oxo Ltd., 6 cm. diam.). This was fitted in an all glass holder to which gentle suction could be applied by means of a conventional Buchner and water pump unit. After washing the filtered cells with an equal volume of cold distilled water the membrane was removed carefully and the residue was transferred to a Kjeldahl flask. This was done using generous amounts of wash-water followed by scraping with a small spatula so ensuring quantitative recovery.

Total Nitrogen

This technique had to be developed specially to analyse 10 ml culture samples which contained up to 0.15 g of carbohydrate. They were pipetted directly into the Kjeldahl flask and the routine was the same as that described for cellular nitrogen except that an extra 2 ml of sulphuric acid was added to aid destruction of the organic matter without concommitant loss of nitrogen. Extra caution was necessary at the roasting stage and, at the end, the excess acid was neutralised by addition of 10 ml of 8N sodium hydroxide before the alkali/thiosulphate mixture.

With the semi-micro apparatus available this method could not be scaled up fully for 20 ml portions of culture medium so recovery was rarely quantitative when more than 10 ml was being analysed.

Ammonia Nitrogen

10 ml culture samples were distilled in the normal way after addition of 7 ml of a pH 8 buffer (0.2N sodium hydroxide in N boric acid) and one drop of antifoam (Silicone MS Antifoam Emulsion; Hopkin and Williams).

This method gives unreliable results in the presence of 0.01N glutamine because a portion of the amide is hydrolysed under these conditions.

Amide Nitrogen

10 ml culture samples were pipetted into the Kjeldahl flask containing 2 ml of dilute (50% v/v) sulphuric acid and the mixture was boiled gently for 30 minutes. During this time the volume diminished to about two-thirds of its original amount. The ammonia was released by addition of 7 ml of 8N sodium hydroxide and one drop of antifoam.

This process hydrolyses glutamine and asparagine quantitatively but decomposes urea only partially so it could not be used in presence of the latter.

Urea Nitrogen

For this analysis the Kjeldahl flasks had first to be freed from traces of mercury by 30 minutes exposure to boiling dilute nitric acid (50%). After 10 ml of the culture sample had been pipetted in, the bacteria were killed by quickly heating to boiling point. On cooling back to room temperature four drops of urease solution (saturated aqueous extract of Jack Bean Meal) were added and the

mixture was allowed to stand for 30 minutes. During this time the urea present was hydrolysed quantitatively yielding ammonia which was then estimated in the usual way.

Pre-Reduction Schemes

In case any compounds were present, in samples, which were resistant to a mercury-catalysed Kjeldahl analysis, two methods of pre-reduction were tried preliminary to normal Total Nitrogen analyses:

(a) Friedrich Method (84): Involving reduction in constant boiling hydriodic acid, this was specially useful for hydroxylamine compounds and hydrazides.

(b) Zinc/Iron Couple in the presence of dilute acid (115). This reduced nitrate, nitrite and hydrazones quantitatively.

(d) PAPER CHROMATOGRAPHY

Paper

The paper used throughout this work was Whatmans No. 1.

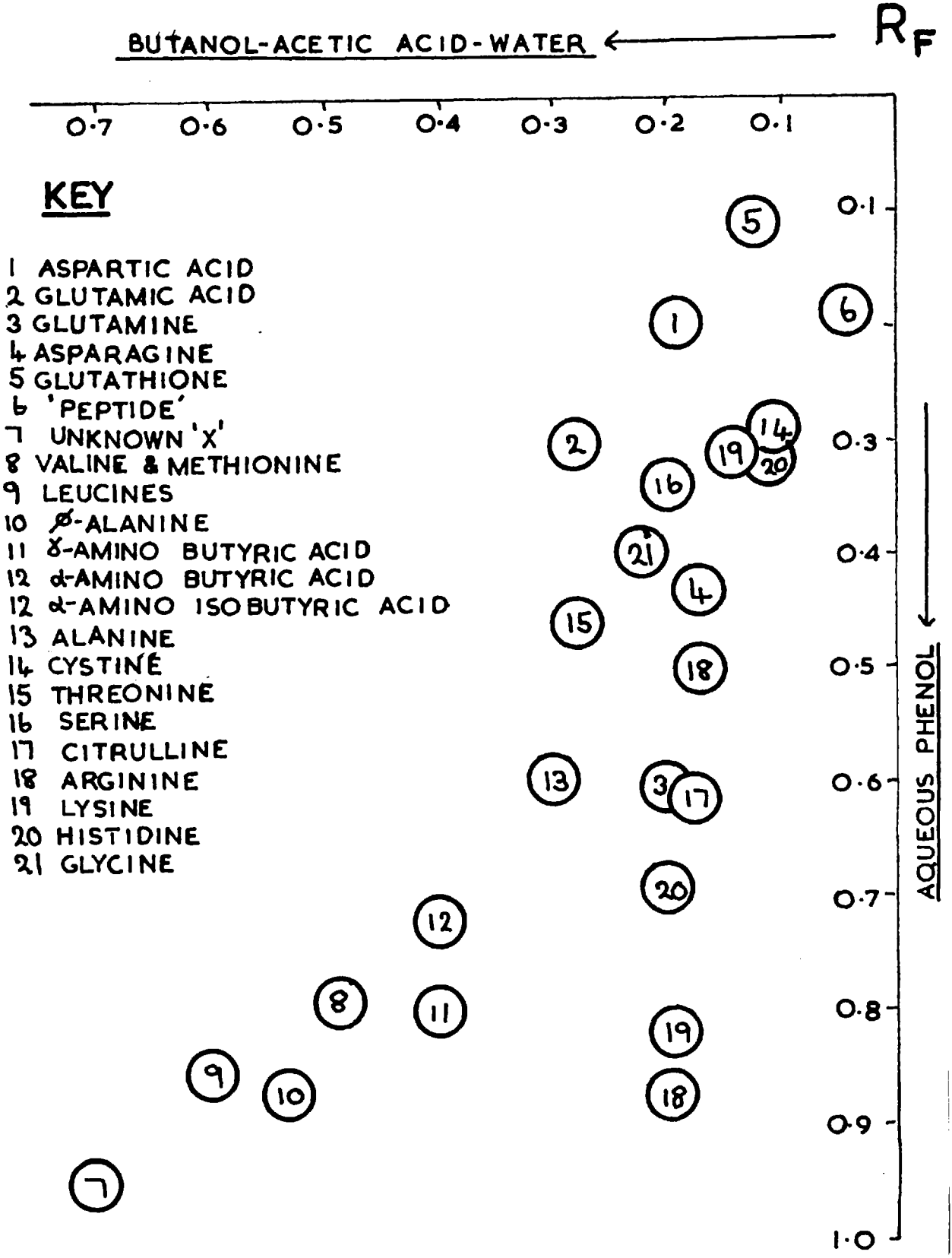
Solvents

(A) n Butanol-Acetic Acid-Water (100/22/50, v/v).
A single phase mixture which was prepared just before use.

(B) Aqueous Phenol (39 ml/100 g).
A single phase mixture which was used in the presence of hydrocyanic acid vapour (potassium cyanide + water) in order to immobilise metal impurities.

(C) Methyl Ethyl Ketone-Acetone-Water-Formic Acid (30/10/10/1, v/v).
A quick running non toxic mixture which yields spots with good definition and is economical in use.

FIG. 2 CHROMATOGRAPHIC MAP



Though several other mixtures were tried at various times the above three proved entirely satisfactory for all routine work. Where quick, convenient separations were needed, particularly for linear work, (C) was used. For two-dimensional analysis of amino acid mixtures the 'traditional' combination of (A) followed by (B) was adopted. Figure 2 illustrates a reference map of all common amino acids obtained using synthetic mextures. They are in good agreement with the R_F values, when recorded, in Stepka (85) and Block, Durrum and Zweig (53) for the same solvent combination.

Difficulty was experienced as a result of the tendency for R_F values to alter after acid hydrolysis. For instance, glutamic and aspartic acids move towards the origin while the basic amino acids move almost to the front in the phenol dimension only. In such cases, Stepka recommends that the paper be saturated with ammonia after the spot has been applied but this precaution did not always work. Careful co-chromatography with standard mixtures was required before such errant spots could be identified with a fair degree of certainty.

Detection of Spots

Spots were detected on the dried chromatograms by spraying the appropriate reagent from an all-glass atomiser (Shandon Scientific Co.) and allowing to dry. This process was often speeded up by careful warming in a current of hot air. Two reagents were used:

(i) NINHYDRIN. Applied in a 0.3% solution in acetone. This reagent gives a purple (brown or yellow) colour with amino acids and most small peptides.

(ii) EHRLICH'S REAGENT. Freshly prepared by dissolving para-dimethyl aminobenzaldehyde (1 g) in a mixture of concentrated hydrochloric acid (1 ml) and ethanol (100 ml). This reagent forms yellow to red spots with hydrazine derivatives (75), urea, urea derivatives and a host of other compounds (eg. indoles) (54). They often take a long time to appear and sometimes undergo colour changes after becoming visible.

Chromatographic Procedures

CIRCULAR gave very quick, crude separations. A specially designed vessel was used (Monax) which was fitted by filter paper discs (14.5 cm. diameter) divided by slots into six segments.

LINEAR DESCENDING was performed in a glass tank accommodating four sheets of 57 x 46 cm. A pencil line was drawn 12 cm. from the short side and the solutions to be examined were spotted at 2.5 cm. intervals along this, so allowing eighteen analyses per sheet.

TWO DIMENSIONAL DESCENDING used the same paper as linear with the sample applied at the intersection of the first line with a second drawn at 12 cm. distance from the other side.

Preparation of Samples

As obtained from the ion exchange purification (Section V (f)) the solid was taken up in 0.2 ml of aqueous 10% isopropanol which acts as a preservative. When the presence of peptides was suspected, the remaining extract was diluted with 1.25 volumes of concentrated hydrochloric acid and transferred to a clean pyrex tube (c. 12 x 0.4 cm. internal size; one end closed) which was then sealed. After 24 hours

in an oven at 105°C the tube was opened and its contents were washed out. The hydrolysate was evaporated on a water-bath and could then be made up and chromatographed exactly as before the treatment. After allowance had been made for some amino acids which are destroyed by acid hydrolysis (cystine and hydroxyacids) direct comparison of the chromatograms led to detection of peptide components in the original extract.

Application of Samples

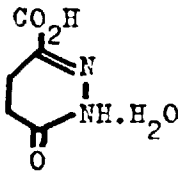
The normal aliquot was 5 γ applied in two successive portions by micropipette. When higher loading was desirable three to four times this quantity could be used. To avoid variable R_F values resulting from application of samples sometimes in salt form and other times as the free acid, all mixtures were spotted as ammonium salts. Where necessary the chromatogram was exposed to ammonia fumes after application of the 'spot'.

(e) PREPARATION OF REAGENTS

Amino Acids, Urea and Ammonium Acetate were all A.R. grade and obtained commercially (Hopkin and Williams, B.D.H.).

Labelled Ammonium Acetate was prepared by distilling a weighed quantity of $^{15}NH_4NO_3$ with pH 8 buffer (Section V (c)) into decinormal acetic acid. The exact amount of the latter was determined in a blank run using an equivalent amount of unlabelled ammonium nitrate. The resulting solution was made up to the mark in a graduated flask and its accurate nitrogen content and enrichment was determined by analysis of small aliquots.

Pyridoxalazine was the gift of M.C. Stewart.



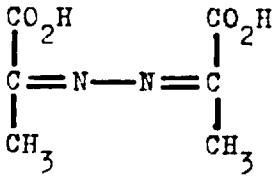
3,4-dihydropyridazinone-5-carboxylic acid (P.C.A.) Alpha-ketoglutaric acid (3 g) dissolved in water (15 ml) was mixed with hydrazine hydrate (1 ml). The solution became pale yellow and a fine crystalline precipitate appeared immediately. Yield 91% Theory. Recrystallized (charcoal) from water to M.Pt. 196°C (Lit. M.Pt. 198°C).

Monacetylhydrazine. $CH_3CONH.NH_2$. Hydrazine hydrate (20 ml) and ethyl acetate (26 ml) were refluxed together (for c. 30 minutes) until they formed one phase. After distilling off excess solvents up to 90°C the liquor was cooled overnight at 0°C. The solid thus obtained was recrystallized from ethanol as flaky plates M.Pt. 65 - 67°C (Lit. Beilstein 67°C). It was extremely hygroscopic and had to be stored in vacuo.

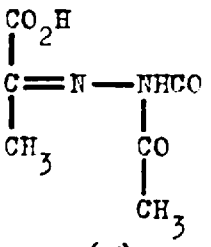
Diacetylhydrazine. $CH_3CONH.NH.COCH_3$. Hydrazine hydrate (10 ml) and ethyl acetate (15 ml) were refluxed together until the mixture became homogenous and, after cooling in ice, acetic anhydride (10 ml) was added slowly in drops. Intense heat was evolved and, on standing overnight a crystalline mass was formed. Recrystallization from ethanol/ether yielded an anhydrous microcrystalline, colourless product. M.Pt. 138 - 139°C (Lit. 138°, 139°, 140°).

Pyruvic Acid Azine was prepared by A.B. Blake as an unstable yellowish powder which, from physical evidence (155), he deduced was the diazide form (a). In solution it behaves identically with a mixture of equimolar quantities of aqueous sodium pyruvate and hydrazine. This

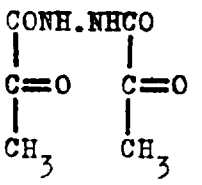
yields three distinct spots on chromatography: with Ehrlich's reagent they give salmon pink, lemon yellow and orange. The situation is analogous to that found by Bach with alpha-ketoglutaric acid and hydrazine though, in that case, one component (PCA) could be separated on account of its sparing solubility. Though there is insufficient evidence to identify them they must be the three theoretical products of such a reaction, ie. the diazide (a), the diazine (b) and the mixed azide/azine (c):



(a)



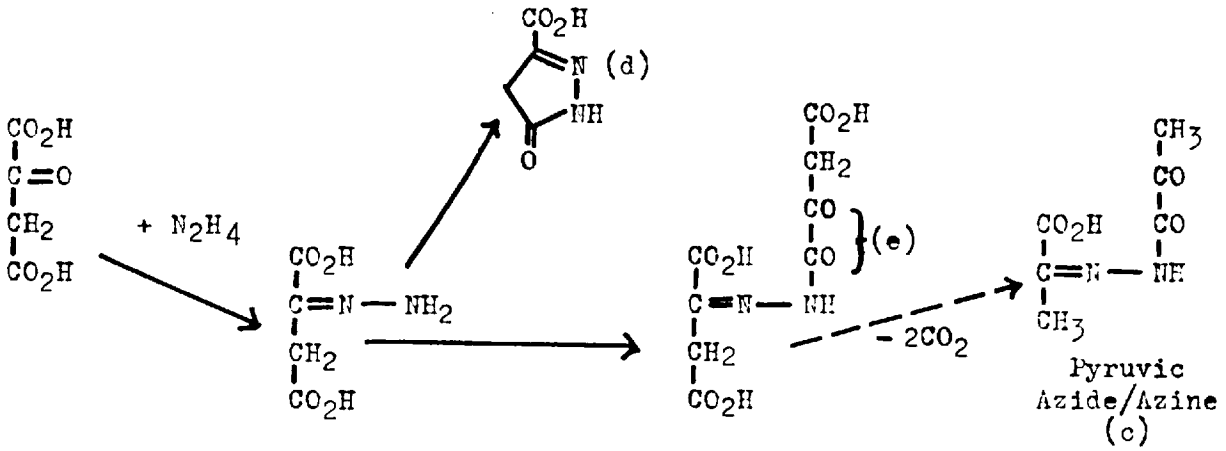
(c)



(b)

Oxaloacetic Acid Azine. On mixing equimolar quantities of solutions of hydrazine and oxaloacetic acid - even at low temperatures - there was effervescence, the solution became yellowish and a pale yellow precipitate was obtained. The latter could not be conveniently recrystallized, had a high melting point and gave a cherry-red spot with Ehrlich's reagent after 48 hours. The reaction mixture gave two main spots on paper chromatography. They were the cherry-red one mentioned above and an orange one which, on co-chromatography in five different solvent mixtures, was identified as one of the pyruvic acid azine components. The insoluble product may be tentatively identified as the lower homologue of P.C.A. (d) and, if the mixed hydrazide/hydrazone were formed inter - rather than intra - molecularly,

it is easy to see that decarboxylation would then be favoured by the alpha-diketo group (e) acting as a powerful electron sink.



(f) ION EXCHANGE CHROMATOGRAPHY

Small ion exchange columns were used, preliminary to paper chromatography, in order to remove interfering solutes (salts, sugars) and to concentrate the desired components (amino acids, weak organic acids, small peptides). The resins used were 8% crosslinked but this could only hold up the larger peptides. All were packed in columns of 1 cm. diameter closed with a fritted glass plate and fitted with a siphon wash to prevent draining of the resin bed. The procedure adopted as a routine was as follows:

(i) the solution to be analysed (usually 50 ml) was run onto the resin (which had been prepared beforehand as in the table and washed with distilled water).

(ii) this was followed by three bed volumes of water.

(iii) it was eluted with the relevant solvent, rejecting the first bed volume and collecting the next three.

(iv) the eluate thus collected was then evaporated on a water bath. Since the eluants were all volatile the extract was obtained in solid form which could be taken up in small volumes of warm water and transferred to a squat 'polytop' bottle of c. 5 ml capacity. Evaporation to dryness in a vacuum desiccator produced an extract suitable for paper chromatography.

The properties of the resins (all from Permutit Ltd.) used may be summarised as follows:

Resin	Particle Size (mesh)	Total Bed Capacity (milli equivs)	Wash Liquids for Storage	Use	Elution
Zeo-Karb 225	< 200	5	2N.HCl	2N.HCl	1.5N.NH_3
DeAcidite FF	< 200	7.5	N.HCl	N.NaOH	N Acetic Acid
DeAcidite E	+16- +50	15	N.HCl	0.5N.NH_3	sat.aq.SO_2

Zeo-Karb 225 consists of sulphonated polystyrene beads and was used (following Mandelstam,(111))to remove amino acids - particularly basic ones - from aqueous extracts of filtered cells.
De-Acidite E, a weakly basic aliphatic amine resin, was used (after the method of Bach (83)) to detect organic acids in cell extracts.
De-Acidite FF contains trimethylammonium groups and is strongly basic. It was adopted (as with Dreze, Moore and Bigwood (42)) for use with cell-free culture medium since it allowed quantitative separation of ampholytes and weaker organic acids from metal ions and the anions of stronger acids (citrate, chloride, phosphate etc.). The former passed straight through the column while the latter were left on the

resin when it was eluted with N. acetic acid. The recoveries are less than quantitative for some basic amino acids but these were not important components anyway. The size of the resin bed was chosen such that it could separate the amino acids and small peptides from a 50 ml sample of culture medium containing 0.84 milli equivs. of strong acid anions as well as 0.5 m eq. of amino acid when that had been added as substrate. A fivefold excess of resin was packed to allow for inefficient equilibration and, at times, up to 80% was seen to be in use (by observing the lighter colour of the acidified resin).

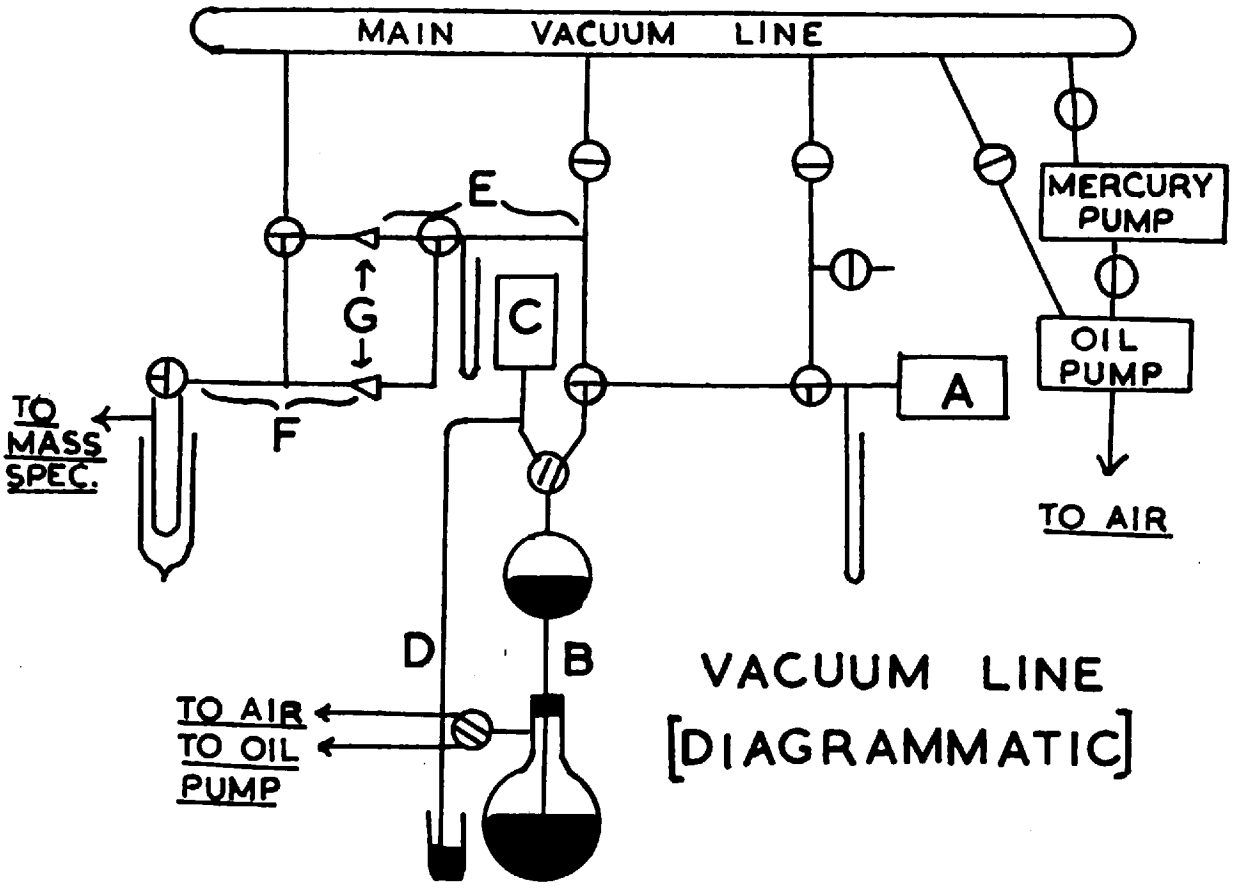
The column's efficiency was impaired if bubbles of carbon dioxide were formed during elution so all materials used in converting the column to its hydroxide form had to be carbon dioxide-free. Boiled distilled water was therefore used for such washes and to prepare the N. sodium hydroxide. This solution was stored in a flask fitted with soda-lime trap and outlet to allow it to be poured while preventing entry of carbon dioxide. For the same reason it was found necessary to acidify the medium filtrate with two drops of concentrated hydrochloric acid and boil it briefly before running onto the column.

(g) HANDLING OF ISOTOPIC NITROGEN

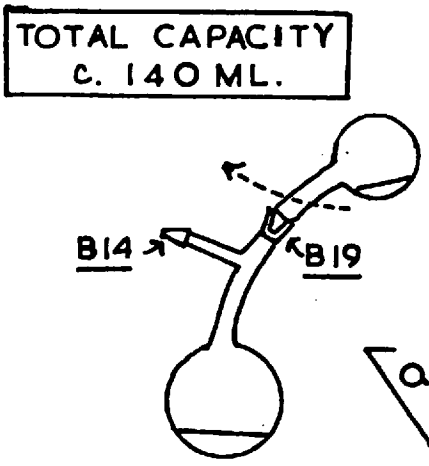
The vacuum line attached to the mass spectrometer (Figure 3) was used for preparation and transfer of all gas mixtures (188).

Labelled Gas Mixture. This was stored in a large bulb of capacity 1.15 litres and had the following composition:

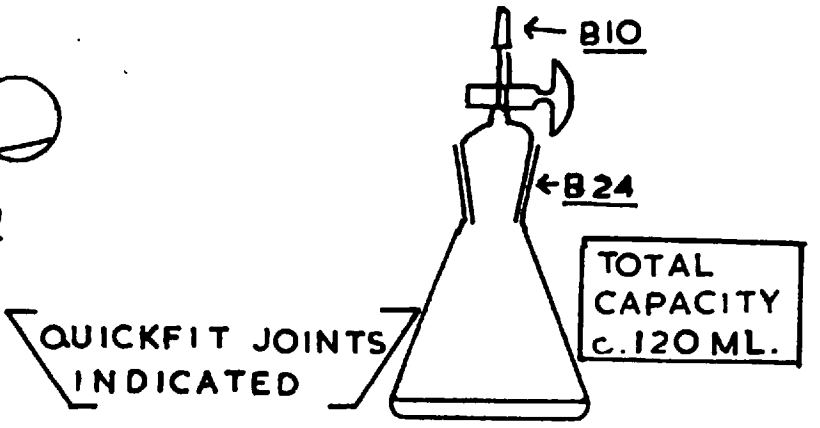
FIG. 3. ISOTOPIC NITROGEN APPARATUS



VACUUM LINE [DIAGRAMMATIC]



NITROGEN GENERATOR



VESSEL FOR $^{15}N_2$ RUNS

	v/v	cm.Hg.	
Nitrogen (31.3% Excess ^{15}N)	10%	7.6	at 76.2 cm. total pressure.
Argon	70%	55.2	
Oxygen	20%	13.4	

The exact amount of labelled ammonium sulphate was weighed out, dissolved in water and transferred quantitatively to one bulb of a nitrogen generation unit (see Figure 3). In the other was placed 10 ml of freshly prepared alkaline hypobromite (40 ml of air-free water containing 10 g of sodium hydroxide, cooled, and shaken with 4 ml of liquid bromine until dissolved). Both component bulbs were cooled in methylated spirits/cardice mixture until their contents were frozen solid. The gas generation unit was then assembled, with joints well greased, fitted to the line at inlet A and evacuated to a hard vacuum. The bulbs were then isolated from the line, warmed in a stream of hot air and their contents were mixed by rotating one of them. The nitrogen produced was transferred quantitatively by means of the Toepler pump B to the storage bulb, fitted at position C, which had previously been evacuated. Using a mercury bubbler to prevent build-up of pressure, pure oxygen (from a cylinder) was then introduced via inlet A and transferred in portions until the requisite pressure rise had been recorded on the manometer D. In the same way sufficient argon was admitted to bring the contents of the bulb to atmospheric pressure.

Nitrogen Enrichment in Samples. Quantities of upward of 0.2 mg of ammonium nitrogen were converted into nitrogen gas by the same

procedure as described above except that a smaller, one-piece generator fitted with a Thunberg stopper was used. After a full Kjeldahl analysis a slight excess of acid was added to the resulting ammonium chloride solution. The indicator used (Brom-cresol green) was nitrogen-free and so could not contaminate the sample. This was then evaporated with the aid of a bumping stick to about 3 ml. The solution was transferred to the nitrogen generator where 3 ml of hypobromite solution was more than adequate to decompose all of the ammonium present. The liberated gas was transferred by the Toepler pump into the portion of the vacuum line behind the leaks E. When the pressure in this section was at a suitable level (1 to 3 cm. of mercury) and the rest of the line was under hard vacuum, that portion F between the leak and the mass spectrometer tube was isolated. The leak was then opened and the sample was released slowly into F and thence into the mass spectrometer tube. Isotope abundance measurements were taken as described in Section V (c).

Labelled Atmosphere Runs were conducted in 100 ml conical flasks with a Quickfit joint allowing an inlet with tap to be fitted making a vacuum tight seal. After the culture had been pipetted in, the top was sealed firmly in position with vacuum grease and the unit was stored at +4°C for 30 minutes. This was done to lessen evaporation when, subsequently, the flasks were quickly evacuated first with a water pump then an oil pump. The latter was allowed to act for about one minute so ensuring that the vessel was free of gaseous nitrogen. The evacuated flask, which contained only water vapour, was then fitted

on the Toepler pump outlet C and filled with gas mixture from the storage bulb at A until atmospheric pressure was reached. The culture flask was closed, transferred to a Warburg bath at 30.0°C and there shaken laterally for the duration of the run. Exposure to ^{15}N was assumed as having started approximately 5 minutes after immersion since equilibrium with the temperature of the bath takes a little over this time.

A) GROWTH OF AZOTOBACTER VINELANDII IN NITROGEN-FREE MEDIUM

Optimal Growth of Cultures in Air

The initial task of this research was to establish a reliable, reproducible pattern for growth in air on nitrogen-free medium. It was only by comparison with such data that experiments involving variation from normal growth conditions could be assessed usefully. The growth requirements of Azotobacter have been well-established for many years so Burks Nitrogen Free Salts are unlikely to be deficient in any component. Likewise the partial pressure of nitrogen in air is many times the concentration required to saturate the fixing system. It was evident at the outset that the extraordinarily high oxygen requirement of Azotobacter would readily become the limiting factor for growth. For this reason it was important to work with cultures as light as could be conveniently estimated using normal analytical techniques. The result was a growth range from total nitrogen of 0.02 to 0.07 mg. per ml. The culture had to be sampled while growing so that it was necessary that its volume could be reduced by up to 50% without affecting aeration: this requirement was obviously fulfilled if the liquid were saturated with oxygen throughout the range of volume used.

After several designs of culture flask had been tried and found wanting the arrangement illustrated in Figure 1 was devised. Though quite simple it was found to be ideal for the purpose. By growing

light cultures and gradually reducing the volume while simultaneously measuring the growth rate it was found that this became constant below a culture volume of c. 190 ml. A range between this value and 80 ml was adopted for all subsequent work. Aeration of such a shallow layer of culture maintained the available oxygen at its maximal level (ie. approaching the saturation pressure for 0.2 atmospheres) while removing carbon dioxide as soon as it was formed.

Calibration of Growth

In order to follow cell proliferation with several samplings over a short period of time, a quick and reliable method was needed for measuring the concentration of cells in an aliquot of culture. The obvious choice was turbidity; such measurements being quick and convenient, particularly so because of the stability of Azotobacter suspensions. Since they were measured in a simple colorimeter using a filter of comparatively broad waveband, the turbidity values were an entirely empirical measure of cell density. They were therefore correlated with the following quantities (see Figures 4 and 5):

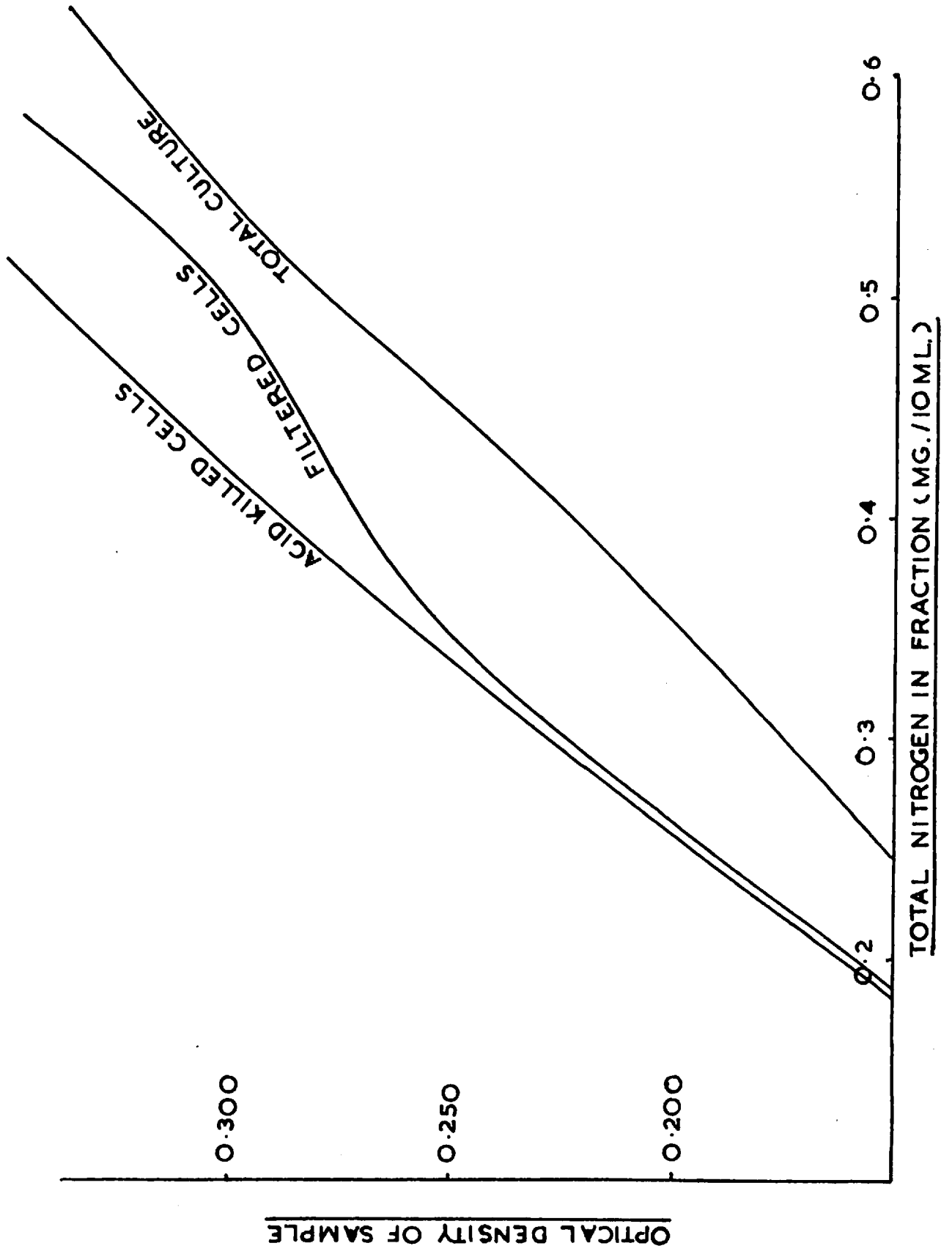
(a) Acid-Killed Cellular Nitrogen - precipitated from a culture made 0.1N with dilute sulphuric acid.

(b) Filtered Cellular Nitrogen - washed cells collected on a membrane filter. This has surface pores of 0.5 to 1.0 micron diameter and anything larger is collected, with negligible penetration, on the surface.

(c) Total Nitrogen - measured using a Kjeldahl technique modified

FIG. 4. TURBIDITY CALIBRATION: STRAIN 'S.K.'

FIG. 5 TURBIDITY CALIBRATION: STRAIN 'O'



to allow quantitative recovery of nitrogen in presence of large quantities of carbohydrate.

(d) Dry Weight of washed cells.

In all of these calibrations, the values obtained for strain 'SK' were found to be highly reproducible, even when subsequent determinations were made after the lapse of a year. Unfortunately strain 'O' was more sensitive to slight variations in growth conditions such that reproducible correlations of (b) or (c) with turbidity were possible only after careful training. Acid-killed cellular nitrogen and dry weight were not susceptible to such influences and mean values for the latter showed that cultures of a given turbidity of either strain had the same dry weight. Unfortunately, individual measurements of dry weight were too inaccurate (being performed on 10 ml aliquots) to be of value as a calibration. This result did demonstrate however that turbidity was a measure of something which transcended the effects of strain variation. Luckily the same could be said for acid-killed cellular nitrogen since if these values for 'O' strain were multiplied by a factor of 1.03 the result, when plotted against turbidity, was superimposable on the same graph for 'SK' strain. For this reason acid-killed cellular nitrogen was adopted as the parameter by which culture density was estimated throughout all subsequent work.

Properties of Cultures on Nitrogen-Free Media (Tables 12 & 13)

TABLE 12 COMPARISON OF THE TWO STRAINS: GENERAL PROPERTIES

	STRAIN 'SK'	STRAIN 'O'
GROWTH ON SOLID MEDIA:		
Capsule Formation	Very Heavy	Medium
Pigment Formation (Nitrogen-Free Agar)	None	Extensive (on old slopes)
Colonies (Nutrient Agar) 48 hrs	2 mm diam	1 mm diam
Distorted Cells (Nutrient Agar)	<10%	circa 50%
GROWTH IN NITROGEN-FREE LIQUID MEDIUM:		
Optimal Mean Generation Time (hrs)	1.7	1.7
Mean Dry Weight - as a multiple of acid-killed cellular nitrogen	8.3	8.1
Turbidity - relative to the same amount of acid-killed cellular nitrogen	0.97	1.00
Extracellular nitrogen - as fraction of total culture nitrogen	3%	11-25%
Fraction of Filtered Cell Nitrogen extracted by Acid Killing:		
Light Culture (0.26 mg Cell N/10 ml)	7.4%	1.6%
Heavy Culture (0.43 mg Cell N/10 ml)	8.4%	15.4%
Nitrogen normally present in Medium - as fraction of Filtered Cell Nitrogen:		
Light Culture (0.26 mg)	2.8%	36%
Heavy Culture (0.43 mg)	2.9%	9.2%

TABLE 13 PROPERTIES OF NITROGEN-FREE CULTURES: FREE AMINO ACIDS AND SMALL PEPTIDES DETECTABLE IN VARIOUS EXTRACTS

Aliquots examined derived from c. 0.05 mg of cellular nitrogen obtained in late log phase.

The following abbreviations are used: Lysine, Histidine, Arginine BASICS; Glutamic Acid GLU; Aspartic Acid ASP; Alanine ALA; Valine VAL; Leucine and IsoLeucine LEU; Cystine CYST; Glycine GLY; Serine SER; Peptide PEP; Gamma amino butyric acid GABA.

SOURCE OF FRACTION	STRAIN 'SK'	STRAIN 'C'
(1) Membrane Filtrate:		
Major Components	GLU	GLU
Abundant	ALA, GLY, SER, BASICS	GLY, BASICS
Trace	VAL, LEU, GABA	VAL, LEU, GABA, ALA, SER, ASP, PEP
(2) Extracted Filtered Cells (Water):		
Trace	GLU	GLU
(3) Extracted Filtered Cells (0.1N Acid):		
Abundant	BASICS, CYST, PEP	BASICS, PEP
Trace		VAL
(4) After hydrolysis of (3):		
Abundant	BASICS, CYST, ALA, GLY	BASICS, ALA, GLY
Trace	GLUT, VAL, LEU	GLOT, VAL, LEU, ASP, SER

(A) Extracellular Nitrogen Compounds (see Table 13)

These membrane filtrates comprised about 3% of the total nitrogen of 'SK' cultures and 11 - 25% of 'O' (the latter dropping considerably as the culture becomes more dense). By visual estimation of 2-D chromatograms it was clear that glutamic acid was by far the most abundant amino acid component of the mixture. Nine other amino acids were observed in filtrates of both strains and 'O' had two more: aspartic acid and an unidentified slow moving spot. Since the latter disappeared on acid hydrolysis it was presumably a peptide or mixture of peptides.

(B) Intracellular Free Amino Acids and Peptides (see Table 13)

Extraction of filtered cells of either strain with water yielded only one faint chromatographic spot - that of glutamic acid.

Extraction by shaking with 0.1N hydrochloric acid yielded the three basic amino acids and the peptide mentioned before together with cystine in strain 'SK' and traces of valine in 'O'. Hydrolysis of these extracts from both strains yielded glutamic acid, and four neutral amino acids together with aspartic acid and serine from 'O' strain only.

(C) Compounds Giving Ehrlich's Reaction

For purposes of comparison and, if possible, identification a range of compounds giving such a reaction were synthesised or otherwise obtained. Those used may be classed in three groups:

(i) hydrazine derivatives of ketones and organic acids abundant in a growing cell (pyruvic, acetic, oxaloacetic and alpha-ketoglutaric acids and pyridoxal).

(ii) urea and its derivative citrulline.

(iii) tryptophan (as representative of indole derivatives) and histidine. After trials in a variety of solvent mixtures (many of which made the spots from the hydrazine derivatives 'tail' badly) mixture C was adopted. R_F values for the compounds and details of their colour reactions are recorded in Table 2, together with the R_F values in aqueous phenol for comparison. Extracts were prepared from whole cells - of 'O' strain only - using boiling aqueous ethanol which was then separated and concentrated using two procedures:

Extract A - 80% ethanol used which was then simply evaporated such that the chromatographic aliquot was equivalent to 0.33 mg cellular nitrogen.

Extract B - 50% ethanol used. After evaporation of the alcohol organic acids present were separated and purified by adsorption on Deacidite E. The resulting 'spot' was equivalent to 0.037 mg cellular nitrogen.

Co-chromatography in mixture C with the synthetic compounds gave the following results:

Extract	Time for Colour Reaction	Initial Colour	R_F	Identification
A (i)	12 hrs	Red (fading soon)	0.56	?
(ii)	12 hrs	Yellow	0.54	?
(iii)	24 hrs	Pale Yellow	0.42	Citrulline
B (i)	12 hrs	Pale Yellow	0.21	Peptides containing Citrulline, Histidine or Tryptophan?
(ii)	12 hrs	Pale Yellow	0.25	
(iii)	24 hrs	Pale Yellow	0.41	Citrulline

TABLE 2 CHROMATOGRAPHIC PROPERTIES OF COMPOUNDS GIVING A COLOUR REACTION WITH EHRLICH'S REAGENT

R_F values compared in solvent (B) - Aqueous Phenol
and solvent (C) - Formic Acid/Ketones Mixture

Substrate	Approx. Time for Spot to Appear	Initial Colour	R_F Values Solvent B	Solvent C
Monacetylhydrazine	12 hrs	Pale Yellow	0.85	0.82
Diacetylhydrazine	24 hrs	Yellow	0.87	0.66
P.C.A.	12 hrs	Orange Yellow	0.60	0.70
Pyruvic acid azine (i)	nil	Orange Yellow	0.24	0.56
(ii)	nil	Lemon Yellow	0.66*	0.85*
(iii)	nil	Orange	0.88	0.65-0.95*
Oxaloacetic acid azine	24 hrs	Cherry-red	0.17	0.81
Pyridoxalazine	nil	Orange	-	0.81
Urea	nil	Lemon Yellow	0.76	0.58
Citrulline	nil	Lemon Yellow	0.62	0.42
Histidine	48 hrs	Pale Yellow	0.65	0.32
Tryptophan	48 hrs	Pale Yellow	0.73	0.80

* indicates a badly diffuse spot

These results are sadly inconclusive though free citrulline can be identified in both extracts. Also the slow-moving components held on Deacidite and yielding pale yellow spots seem likely to be small peptides containing one of the amino acids which give such a colour (citrulline, histidine or tryptophan). The fact that these latter were not detectable in extracts taken with a higher ethanol concentration supports this theory. The other two spots were no longer found after Extract A had been stored at room temperature for a week; neither could they be identified with any of the synthetic compounds used. Though no component recognizable as P.C.A. was detected, it is possible that the two unknowns were hydrazine derivatives of some cell constituents. Even if this were so, they were not present in appreciable quantities since total nitrogen analyses on replicate samples of culture were not enhanced by either of two pre-reduction techniques.

(D) Free and Bound Ammonia

These fractions were very small and, since their quantities did not alter over the usual range of cell density, both seem to be functions of culture volume rather than obligatory cell components. For either strain the values were (per 10 ml) Free Ammonia: 0.01 mg Acid Labile + Free Ammonia: 0.02 mg. The acid-labile nitrogen was equally distributed between cell envelope and cytoplasm since repeated extraction of air dried cells ('O' strain) with 80% ethanol left its proportion virtually unchanged - 6.3% cellular nitrogen before extraction, 6.2% after.

(E) Capsule and Dry Weight

The mean dry weight of centrifuged cells after two washes with water was 8.1 times acid-killed cellular nitrogen for strain 'O' and 8.3 times for strain 'SK'. These values are the mean from nine estimations spread uniformly over the whole growth range for not only did the ratio vary with culture density but it also fluctuated considerably due to the inaccuracy inherent in gravimetric analysis on such small quantities. Together these led to a mean deviation of $\pm$ 7%.

To see if the wash was having any effect it was performed only once, and not at all, on several replicate samples of a culture of 'SK'. The mean factors were then 8.6 (one wash) and 16.6 (no wash). This is clearly due to the capsule and slime layer which, being soluble, are removed almost entirely by one agitation with water. It was noticeable that cultures of strain 'SK' took much longer times than 'O' during membrane filtration. On occasion this was so marked that the volume used had to be reduced to 35 ml in order that the separation be finished within 20 minutes - a time easily achieved by 50 ml of 'O' culture.

(F) Growth Rate

By sampling growing cultures at half-hourly intervals the change of cellular density (x) with time (t) was obtained. From these the exponential growth constant (k) for each interval could be estimated using the equation $k = \left\{\frac{\log_{10}x_2 - \log_{10}x_1}{t_2 - t_1}\right\} 2.303$ (24).

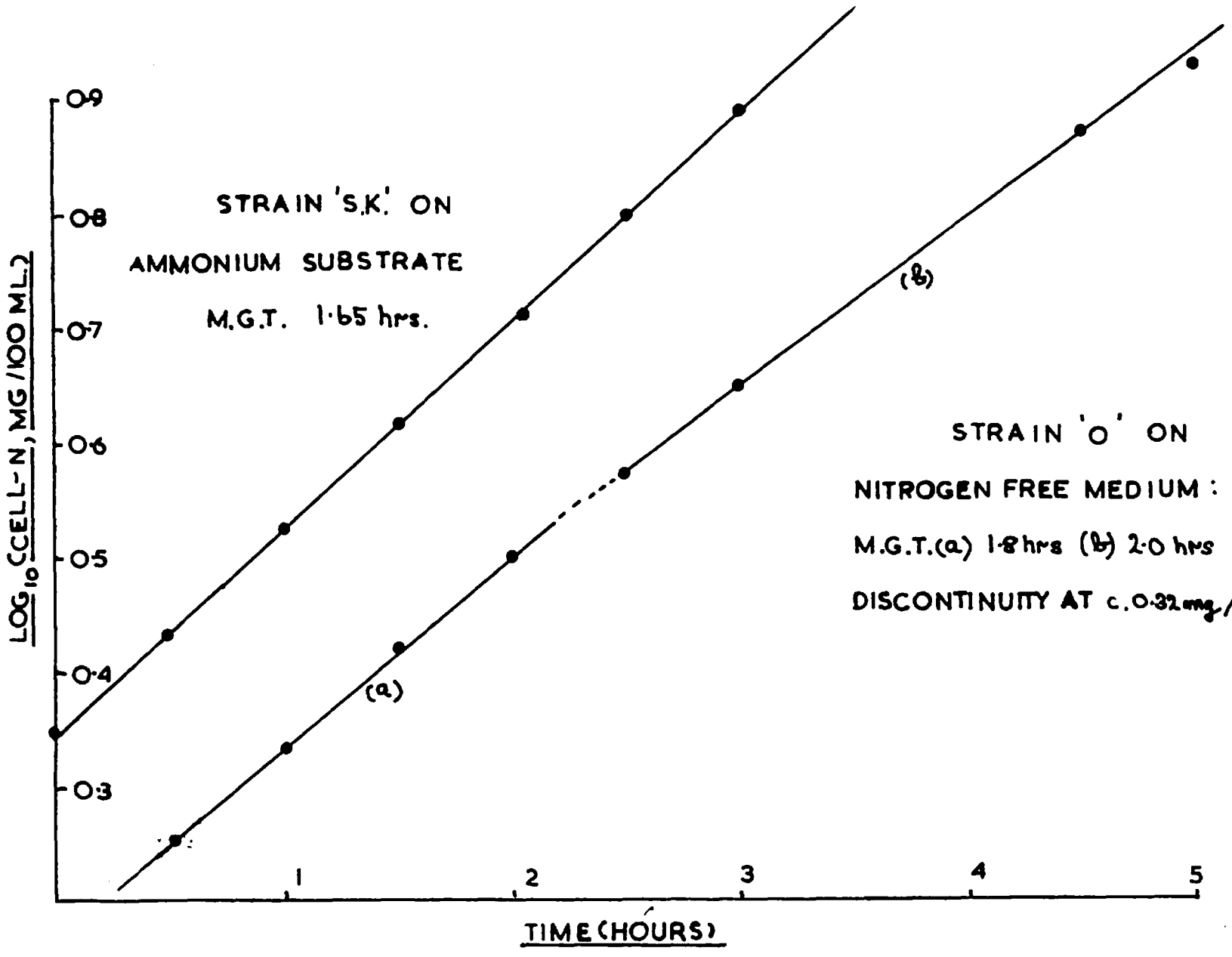


FIG. 6. TYPICAL GROWTH RATE PLOTS

In practice a more convenient measure of growth rate was the Mean Generation Time (M.G.T.). This is the time taken for the fraction being examined to double in quantity and equals $\frac{0.693}{k}$. Though plots of $\log_{10}$ (cellular nitrogen) against time were smooth curves for newly inoculated cultures it became clear that, as they grew, they all approached a maximum slope which, in the case of active cultures, could be sustained over periods of up to 1½ hours (Figure 6). The optimal mean generation time for growth in air was found to be the same for both strains and to equal 1.7 hours. Cultures freshly inoculated from an old slope or heavy ones just diluted would grow at a much slower rate (M.G.T. 2.5 to 3.0 hrs) but subculturing once or twice always restored the value to 1.7 hours. The mean generation time inevitably rose toward the end of the normal growth range (cellular nitrogen circa 0.06 mg/ml) when the whole culture started to decline through oxygen starvation.

Discontinuity in Growth Curves

Even in those cases when logarithmic growth could be maintained for long periods, a point was always found in the middle of the graph which fell appreciably off the line. At first dismissed as random errors, discontinuities of this kind continued to appear until it became obvious that some sort of change always occurs in cultures between a cellular density of 0.025 and 0.040 mg acid-killed nitrogen/10 ml. Almost every property of Azotobacter vinelandii is affected by this change and, though exhibited by both strains, it was often more accentuated with 'O' (as usual the more sensitive of the two).

Between the limits stated above the actual location and extent of this discontinuity varied unpredictably. It was, however, always detectable in the following properties:

(i) Growth Rate. Though constant for a considerable time (1 to 1½ hours) after the break this was lower by anything from 5% to 20% of its earlier value. Typical behaviour is shown below and in Figure 6:

	MGT (a)	MGT (b)	Position of Break
Strain 'SK'	1.70	1.84	0.35 mg cell N/10 ml
Strain 'O'	1.84	2.14	0.31 mg cell N/10 ml

(ii) The Ratio of Dry Weight to Acid-Killed Cellular Nitrogen (again the mean of many determinations) reached a minimum just before the range when the discontinuity is normally observed. This may be taken to indicate a high protein, or conversely, low carbohydrate content at this point. The average factors for the range when they become minimum can be compared with the mean values for all culture densities:

	Overall Factor	Minimum Factor	Range Where Determined
Strain 'SK'	8.3	8.1	0.30-0.39 mg cellular N/10 ml
Strain 'O'	8.1	7.6	0.22-0.29 mg cellular N/10 ml

(iii) The Fraction of Filtered Cell Nitrogen extractable by Acid. This increases for both strains after the discontinuity; by as much as a factor of 10 with 'O'.

(iv) Extracellular Nitrogen Fraction: with 'O' strain only

this is reduced (to approximately ¼ of its previous value).

(v) Fixation: close examination of growth rate plots reveals that fixation actually stops for a short time; this varies in duration from 2 to 10 minutes. No such break is detectable in the culture density though its rate of increase is slower once the change has been effected.

Immediately after dilution of a heavy culture to low density it would continue to behave in a manner characteristic of its previous state. Vigorous aeration for an hour or so gradually restored the properties of a young culture thus proving that the effects of the change were only slowly reversible.

It is clear that this change is not merely due to exhaustion of a metabolite or accumulation of a waste product - these would cause a gradual falling off as is indeed found with very heavy cultures which cannot be aerated sufficiently. Rather, it is a profound re-organization of the cellular economy brought about by some such symptom of increasing culture density. Growth of 'O' strain in medium with the following alterations was studied to see if the discontinuity in growth rate plots could be removed or altered appreciably:

(i) Lowering the pH of fresh medium from 7.26 to 7.03. The pH of fully grown cultures lies always between 7.10 and 7.20.

(ii) Doubling mannitol concentration to 3%.

(iii) Doubling the trace amounts of iron and molybdenum.

(iv) Increasing available calcium (by means of a sterile marble chip).

(v) Doubling salt concentration.

None of these had any effect so the incoming air for a culture was enriched with oxygen and a more sensitive test applied - the fraction of filtered-cell nitrogen extracted by acid was measured at stages when the culture was normally well into the second phase of logarithmic growth. This yielded the following results:

Cellular Nitrogen	Normal Fraction (in air)	Fraction in Air + 15% O_2
0.37 mg/10 ml	8.1%	9.2%
0.44 mg/10 ml	16.3%	10.6%

Thus increased oxygen supply offsets one of the most marked symptoms of this change.

B) EFFECT OF EXTRACELLULAR NITROGEN SOURCES ON GROWTH AND FIXATION

One of the primary aims of this research was to establish whether the fixing mechanism is directly accessible from outside the growing cell. To this end the effect of artificial extracellular pools of known intermediates (NH_4^+, Glutamic Acid) and possible ones (Glutamine and Asparagine) on growth and fixation by optimally growing cultures was measured. In order that, if utilised, there would be sufficient substrate present to act as sole nitrogen source over the range of culture densities studied, a standard concentration of 0.01N in terms of available nitrogen atoms was adopted. Three compounds which are unlikely to act as intermediates (Urea, Alanine and Aspartic Acid) were also studied in order to provide controls for the above runs.

Training

It was thought that the cultures might take varying periods to react to the changed environment; for instance, even if a normal metabolic pool were suddenly increased a hundred-fold, time would be required before transport mechanisms could be adapted to deal with the increased traffic or before its inhibitory effect could be felt on fixation. For this reason three different periods of training were allowed for each substrate. In common, all experiments started with the mixing of an active culture with an equal volume of medium containing substrate at twice the desired concentration. In the three different types of run the measurements were started (a) 15 minutes, (b) 1½ - 2 hours, (c) 1½ - 2 hours, after mixing. The inoculum for (c) had been trained by three previous subcultures on medium containing the substrate at the same concentration. Additional information obtained from runs of type (a) (TRAINING: NIL) was the final composition of intra- and extra-cellular nitrogen pools, the relationship of dry weight to acid-killed cellular nitrogen (this on unmixed substrates only) and the acid-labile ammonia to be found in filtered cells. The runs of type (b) (TRAINING: ONE M.G.T. DELAY) and (c) (TRAINING: FULL) were followed in much greater detail so that continuous curves for growth, assimilation and fixation could be plotted for each run.

Mode of Comparison

Perhaps the most difficult problem in the whole research was selection of a suitable means of 'processing' the results from over

seventy growth runs such that significant trends could be distinguished from the results of 'biological' fluctuations in behaviour. In particular, the confusing effects of the discontinuity noted in Section III(A) had to be minimised. It was finally decided to straddle the difficult period and base all comparisons on the growth during a standard interval of approximately one generation. The range adopted was the increase of 0.25 mg acid-killed cellular nitrogen/10 ml between 0.18 and 0.43 mg/10 ml. All values quoted in the results that follow (Tables 3 - 8) are net changes for that interval per increase of 1.00 mg cellular nitrogen. The error due to analysis in these ratios is $\pm$ 0.04 but random fluctuations are greatly in excess of these limits. When performing runs of type (b) and (c) samples were taken for various analyses at half-hourly intervals. The results were plotted (examples of typical graphs Figures 7 - 13) as total nitrogen in the given fraction (ie. Total Culture Nitrogen, Free NH_3, Acid Labile + Free NH_3, Urea + Free NH_3) versus acid-killed cellular nitrogen. From the smooth curves thus obtained the net changes over the stated interval could be read off. In some cases the culture density was rather high when starting so, being more convenient, a slightly different interval was chosen. These results were corrected by applying a factor obtained by the same treatment applied to fixation by nitrogen-free cultures grown over a wide range of cell-densities.

Concentration Range (mg cellular N/10 ml)	Ratio: Net Fixation / Cellular Nitrogen		
	'SK'	'O'	
0.18 - 0.43	1.20	1.20	'Standard Range'
0.25 - 0.50	1.20	1.24	
0.30 - 0.55	1.20	1.26	
0.35 - 0.60	1.24	1.30	

KEY TO TABLES 3 - 8: RESULTS OF GROWTH RUNS

I The pH of all SUBSTRATE SOLUTIONS was adjusted to 7.1-7.2. After mixing with the nitrogen-free culture the TOTAL POTENTIALLY AVAILABLE NITROGEN was 0.01N. This meant that half molecular quantities were used for urea, glutamine and asparagine and that the two components in a mixed solution were at half their normal values.

II All results are recorded as NET TURNOVER per UNIT INCREASE in CELLULAR NITROGEN (acid-killed) over the standard range from 0.028 to 0.043 mg N/ml. When the results available did not allow this treatment they were adjusted with the aid of total assimilation values obtained with pure cultures. Such results are marked with an asterisk *.

III The degree of TRAINING was determined by the time allowed for the culture to become accustomed to the substrate before the measurements quoted here were taken. They are

NIL: measurements commenced 15 minutes after mixing. Before dilution with substrate-medium the culture was of medium density, about 0.4 mg cellular N/10 ml.

DELAYED: measurements commenced, on average, one generation after mixing. Before dilution the culture was light, having between 0.2 and 0.3 mg cellular N/10 ml.

FULL: the time allowed was the same as in 'DELAYED' except that the inoculum had been trained beforehand by three successive subcultures through media containing the stated substrate at the usual concentration (0.01N).

IV TOTAL ASSIMILATION is equivalent to NET FIXATION in the cases of amino acids and amides but, whenever ammonium acetate or urea are present, the net ammonia used must be added to the latter. Maximum errors on all values quoted are $\pm$0.04 units. Whenever detected ASPIRATION LOSSES have been corrected for by deducting them from apparent ammonia utilization and adding to apparent net fixation - which is usually a negative quantity in such cases.

TABLE 3 EFFECT OF AMMONIUM ACETATE ON GROWTH AND FIXATION BY AZOTOBACTER VINELANDII

TRAINING	NIL	DELAY(i)	FULL (NH_4)	FULL (UREA)
STRAIN 'SK'				
Total Assimilation	1.39	1.32	1.36	1.20
Net Fixation	0.62	0.40	0.32	0.16
Ammonium Used	0.77	0.92	1.04	1.04
Amide Formed	0.12	0.06	0.08	0.08
STRAIN 'O'				
Total Assimilation	1.18	1.57	1.92	1.92
Net Fixation	0.12	0	0.48	0.16
Ammonium Used	1.06	1.57	1.44	1.76
Amide Formed	0.16	0.22	0.32	0.20
Aspiration Losses	0	0.54	0.20	1.00
	*	*	*	

(i) These results, for both strains, are the mean of two separate 'DELAY' growth runs.

TABLE 4 EFFECT OF MIXED SUBSTRATES ON GROWTH AND FIXATION BY AZOTOBACTER VINELANDII

AMMONIUM ACETATE + L-GLUTAMINE

TRAINING: Both 'DELAY'

	STRAIN 'SK'	STRAIN 'O'
Total Assimilation	1.16	1.41
Net Fixation	0.36	0.46
Utilization of Amide + Free NH_3	0.80	0.95
Aspiration Losses	0.12	0.04 *

UREA + AMMONIUM ACETATE

	STRAIN 'SK'		STRAIN 'O'	
TRAINING	NIL	DELAY	NIL	DELAY
Total Assimilation	1.35	1.48	1.47	1.60
Net Fixation	0.44	0.44	0.22	0
x Utilization of NH_3	0.91	1.04	1.25	1.60
Urea Hydrolysed	0.83	0.76	0.82	0.56
Aspiration Losses	0	0.08	0.22	0.28

x NOTE: NH_3 utilized is that derived from urea as well as that already added. 'Urea hydrolysed' indicates the former portion.

TABLE 5 EFFECT OF AMINO ACIDS ON FIXATION BY AZOTOBACTER VINELANDII

TRAINING:	NIL	DELAY	FULL
STRAIN 'SK'			
L-Alanine	1.56	1.72	1.36
L-Aspartic Acid	1.26	1.48	1.36
L-Glutamic Acid	1.50	1.16	1.52
STRAIN 'O'			
L-Alanine	1.73*	1.66*	1.48
L-Aspartic Acid	1.62	1.92	1.48
L-Glutamic Acid	1.46	1.32*	1.43*

(The key to these tables may be found on P. 71.)

TABLE 6 EFFECT OF AMINO ACID AMIDES ON FIXATION BY AZOTOBACTER VINELANDII

TRAINING:		NIL	DELAY	FULL
STRAIN 'SK'				
L-Asparagine:	Fixation	1.56	1.00	1.72
	Extra Amide	0.42	0.20	0.36
L-Glutamine:	Fixation	1.68	1.80	2.44
	Extra Amide	0.32	0.33	0.24
STRAIN 'O'				
L-Asparagine:	Fixation	1.64	1.55	1.40
	Extra Amide	(-0.05)	0.50	0.28
L-Glutamine:	Fixation	2.00	2.32*	1.79*
	Extra Amide	0.52	0.38	0.61

Extra Run: 'SK', L-Asparagine	Fixation		1.96
Fully Trained on Glutamine	Extra Amide		0.28

TABLE 7 EFFECT OF UREA ON GROWTH AND FIXATION BY AZOTOBACTER VINELANDII

	UREA			
TRAINING:	NIL	DELAY	FULL (UREA)	FULL (NH_4)
STRAIN 'SK'				
Total Assimilation	1.03	1.40	1.20	1.00
Net Fixation	0.71	0.32	0.64	0.56
Utilization of NH_3	0.32	1.08	0.56	0.44
Urea Hydrolysed	0.32	1.00	0.36	0.24
STRAIN 'O'				
Total Assimilation	1.35*	1.52	1.43*	1.41*
Net Fixation	0.33	1.20	0.46	0.30
Utilization of NH_3	1.02	0.32	0.97	1.11
Urea Hydrolysed	1.87	0.32	0.77	0.81

TABLE 8 EFFECT OF MIXED SUBSTRATES ON GROWTH AND FIXATION BY AZOTOBACTER VINELANDII

AMMONIUM ACETATE + L-GLUTAMIC ACID

TRAINING:	NIL	DELAY	FULL (GLUTAMINE)
STRAIN 'SK'			
Total Assimilation	1.73	1.60	2.00
Net Fixation	0.65	1.04	0.32
Utilization of NH_3	1.08	0.56	1.68
Amide Formed	0.16	0.32	0.56
STRAIN 'O'			
Total Assimilation	1.74	1.72	1.80
Net Fixation	0.24	0.52	0.32
Utilization of NH_3	1.50	1.20	1.48
Amide Formed	0.12	0.24	0.08

FIG.7. GROWTH IN MEDIUM CONTAINING AMMONIUM ACETATE

STRAIN: O TRAINING: FULL

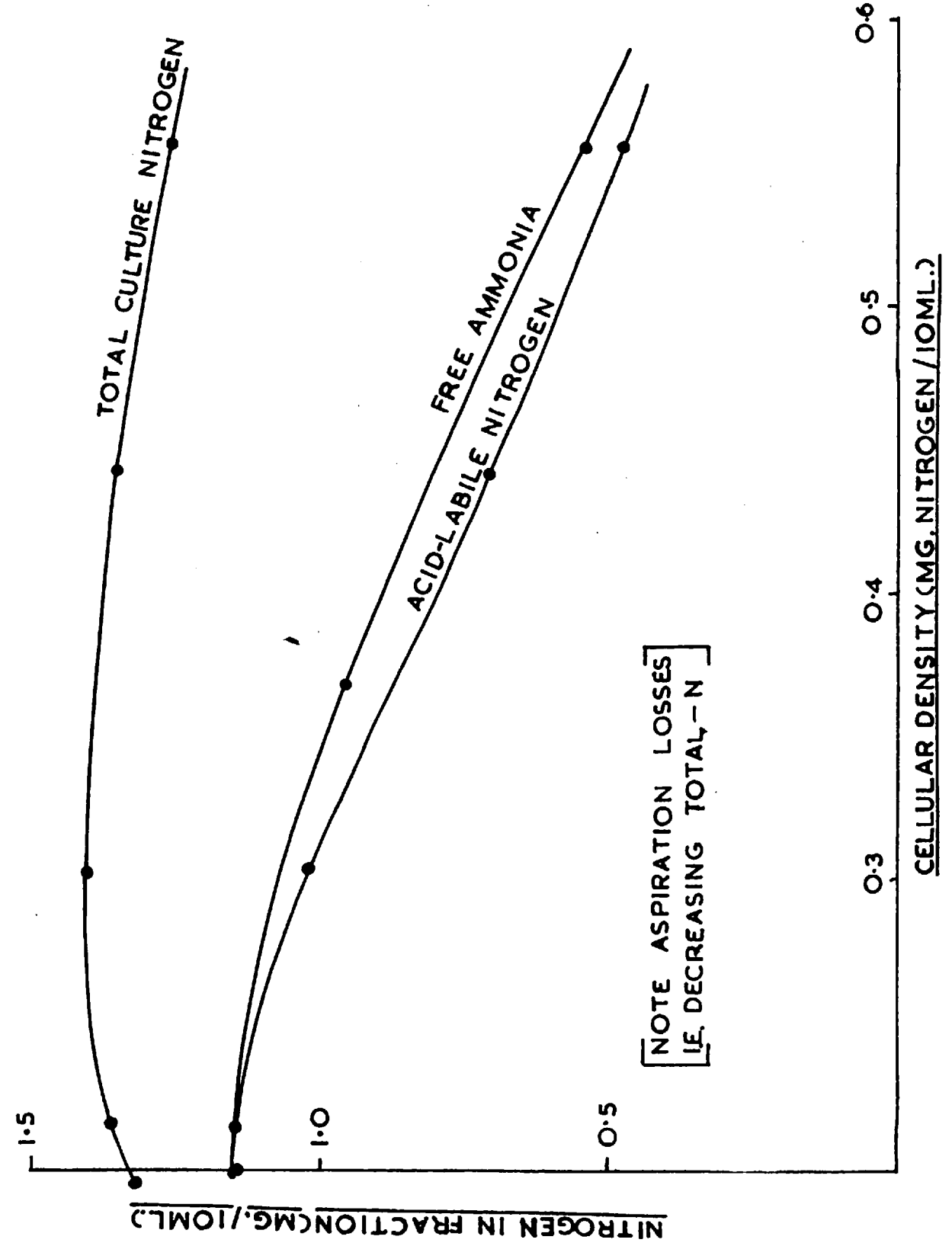


FIG. 8. GROWTH IN MEDIUM CONTAINING UREA

STRAIN: 'O' TRAINING: ONE M.G. DELAY

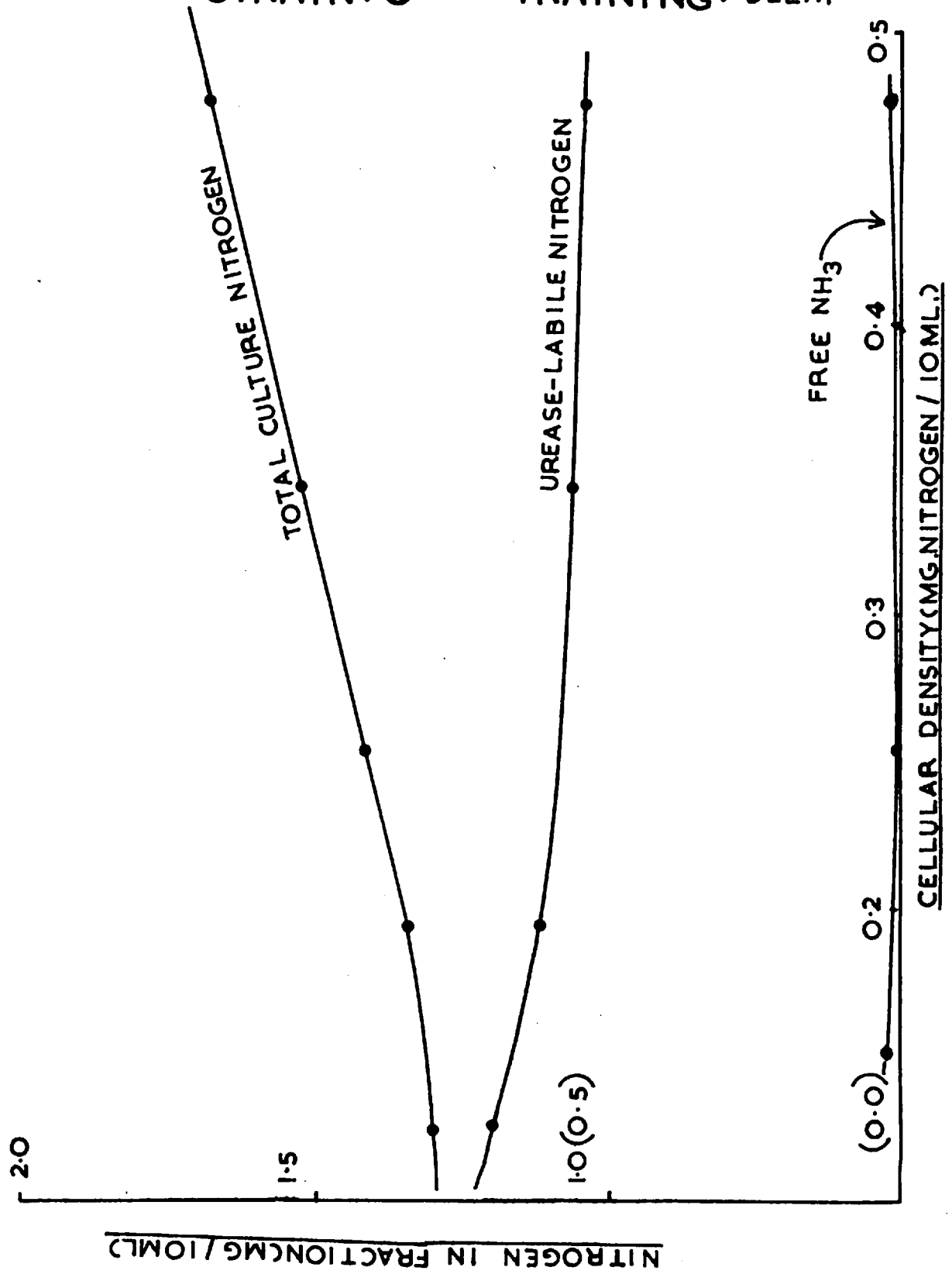


FIG.9. GROWTH IN MEDIUM CONTAINING L-ASPARAGINE
STRAIN: S.K. TRAINING: FULL

FIG. 10. GROWTH IN MEDIUM CONTAINING L-GLUTAMINE

STRAIN: SK TRAINING: ONE M.G. DELAY

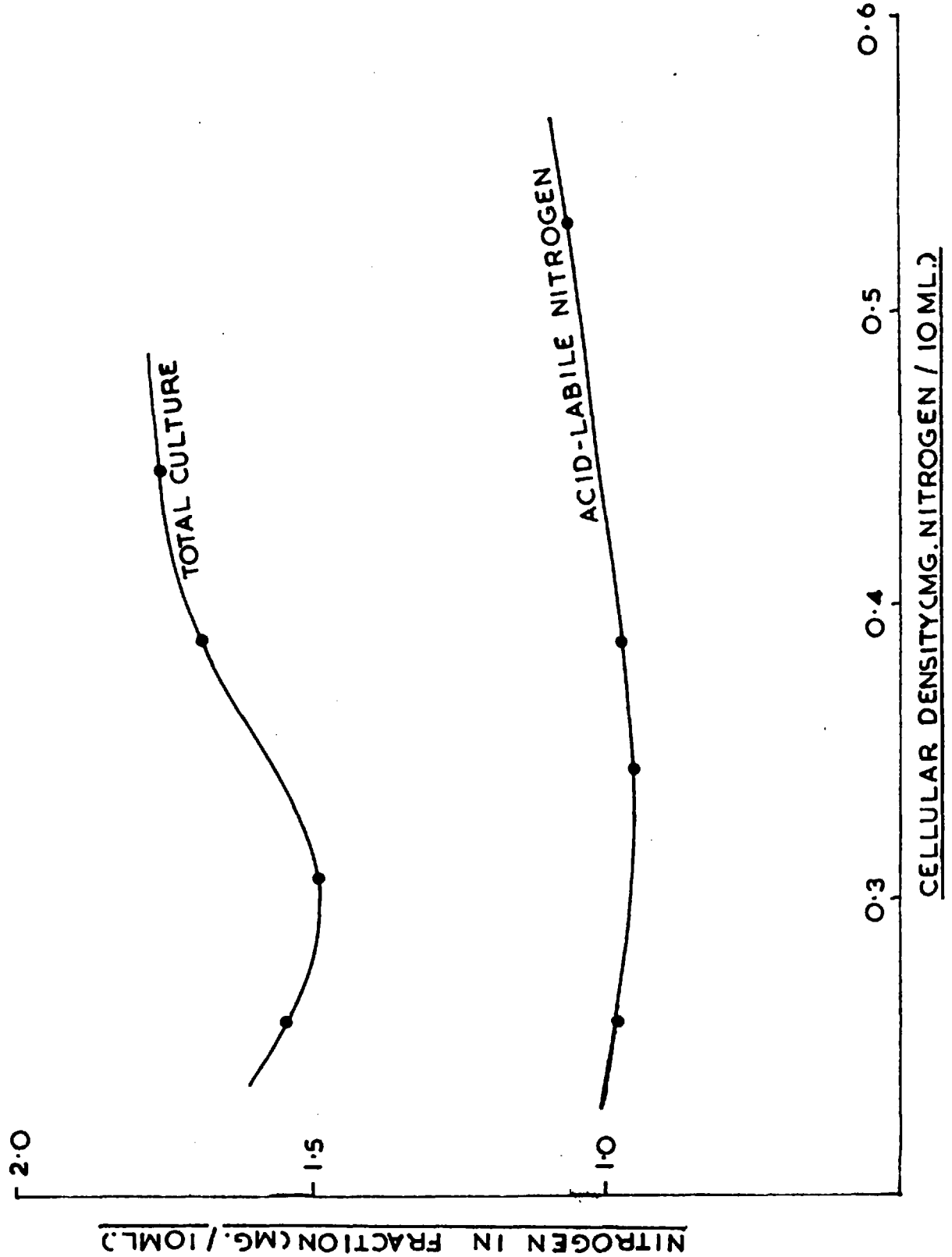


FIG. 11. GROWTH IN MEDIUM CONTAINING L-GLUTAMIC ACID + AMMONIA STRAIN: 'O' TRAINING: ONE M.G. DELAY

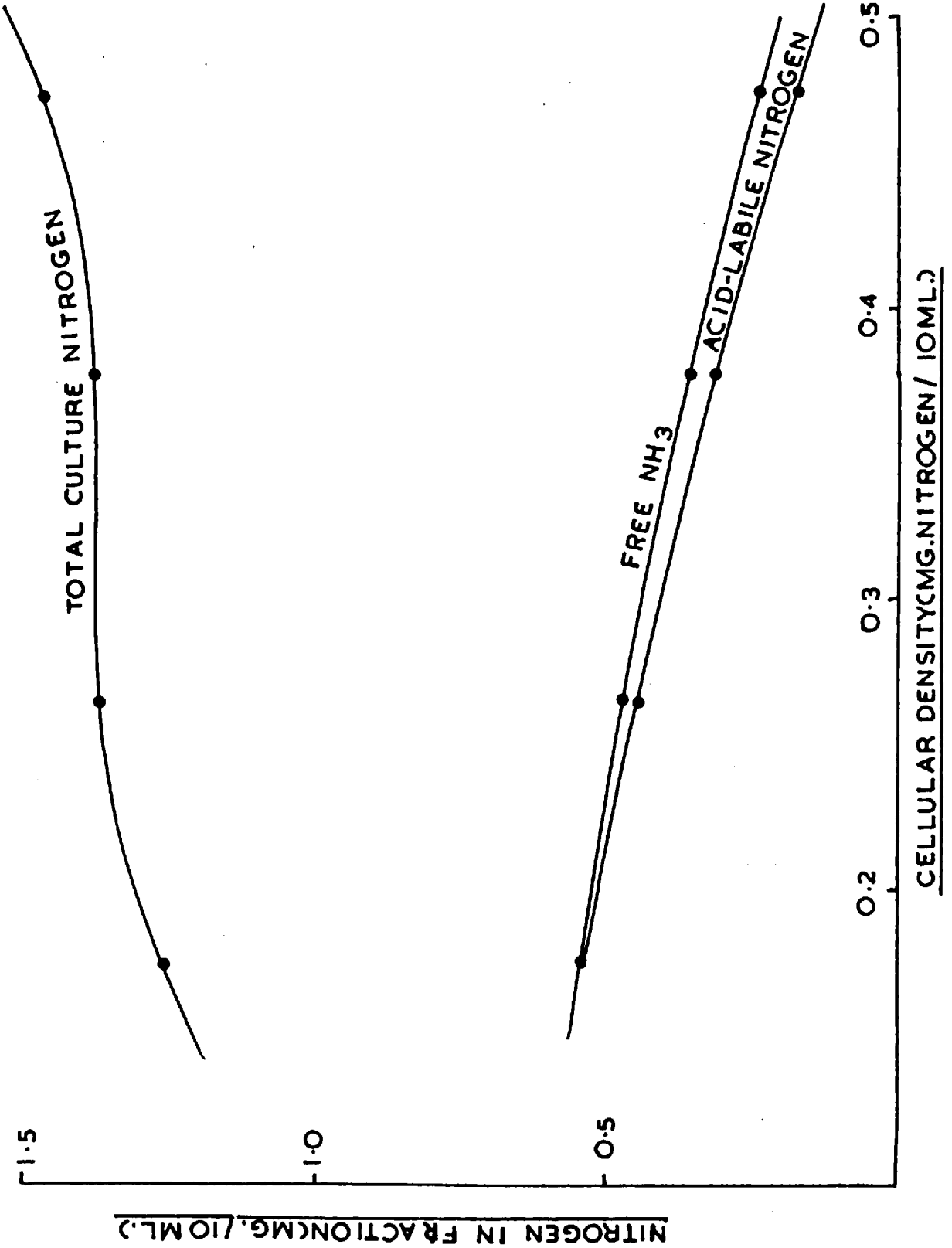


FIG. 12. GROWTH IN MEDIUM CONTAINING AMMONIUM ACETATE

STRAIN: SK TRAINING: FULL

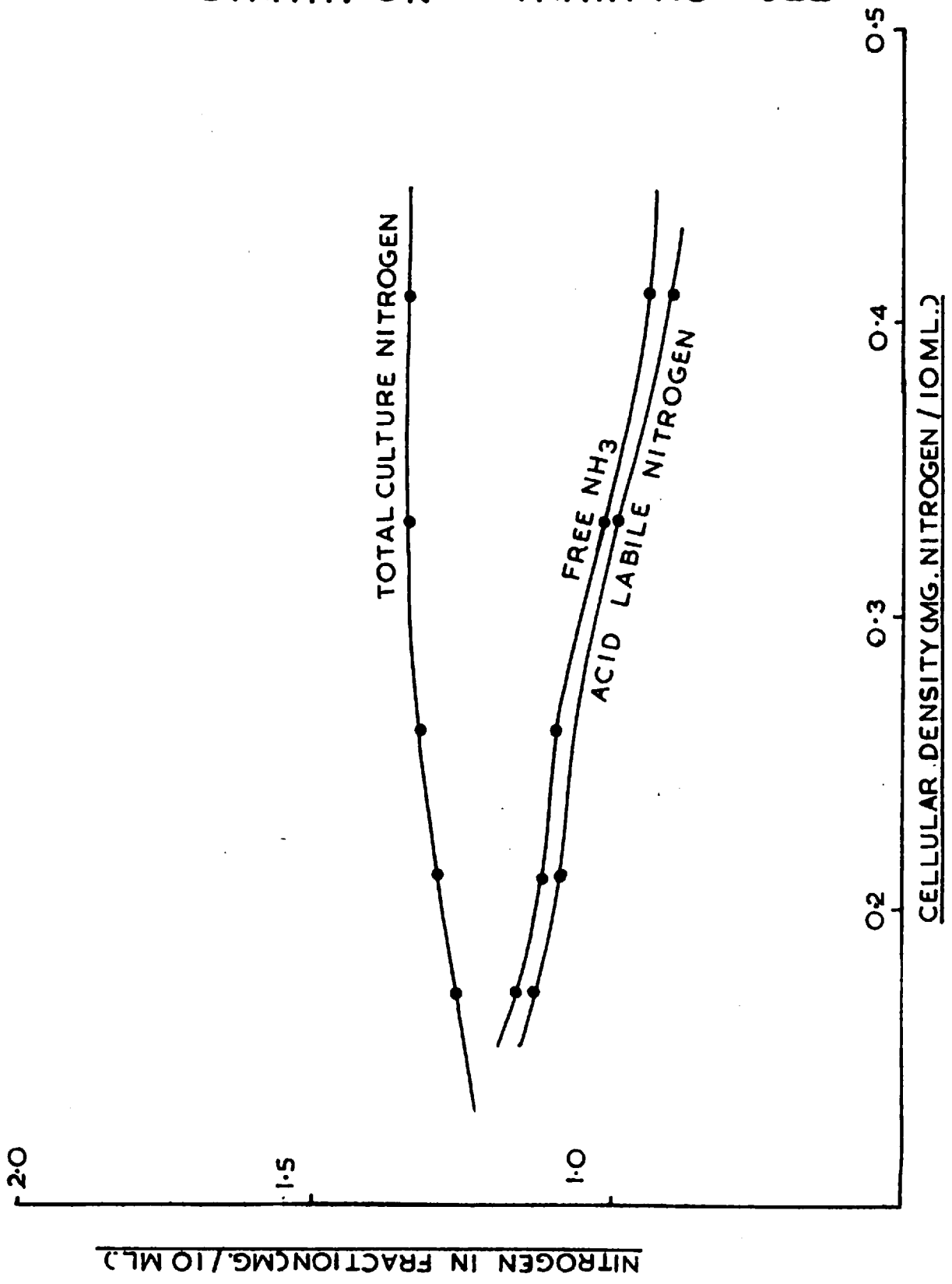
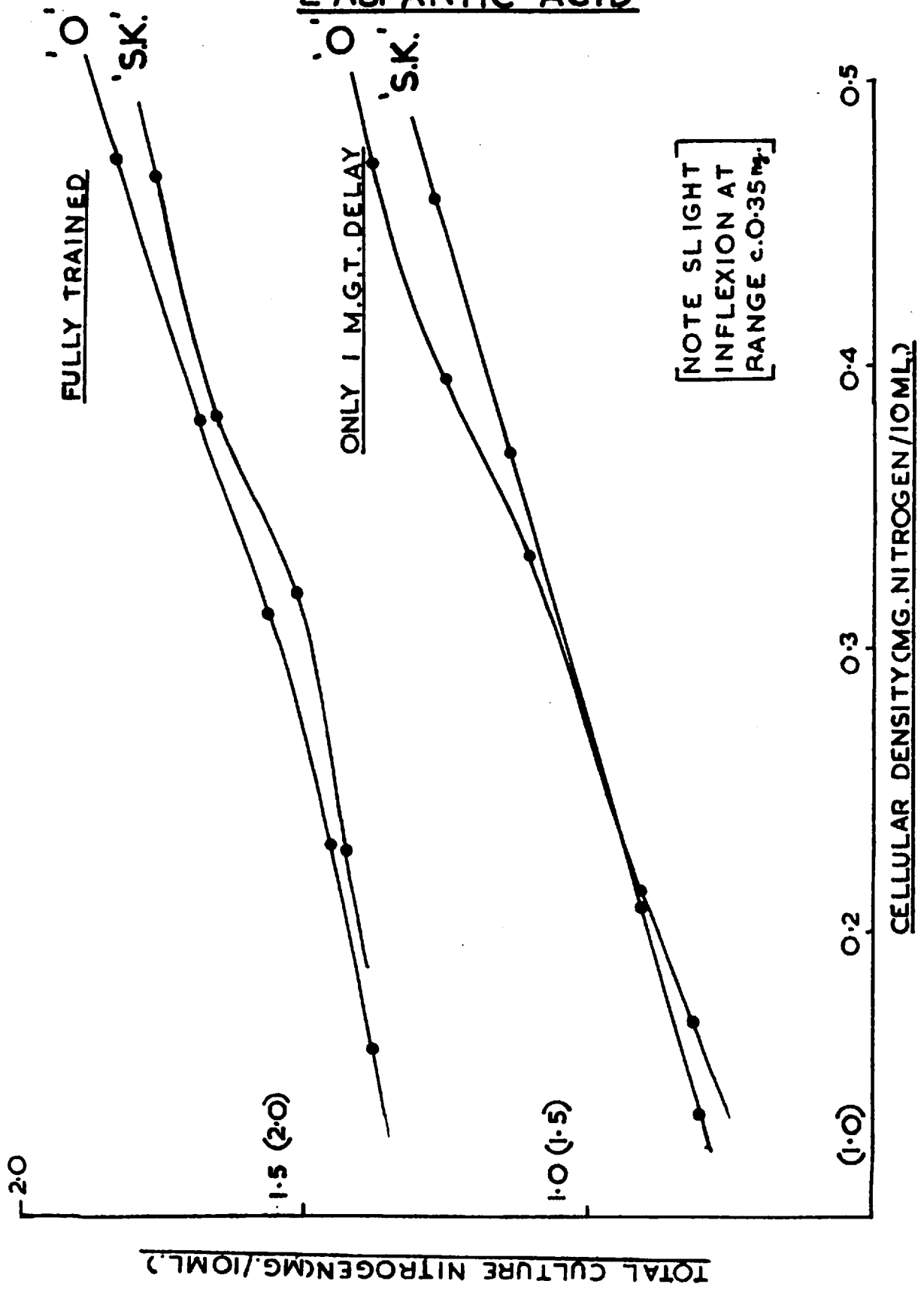


FIG. 13. GROWTH IN MEDIUM CONTAINING L-ASPARTIC ACID



Apparent Nitrogen Losses

When growing strain '0' in presence of ammonium acetate it was usually found that total nitrogen decreased steadily concurrent with a notably high disappearance of ammonium ion (see Figure 7). It was first thought that the extra nitrogen taken up was being converted into some form which is not detectable by a straightforward Kjeldahl analysis, but two pre-reduction techniques were attempted and failed to substantiate this. By inserting a water trap on the outlet side of the culture vessel all of the 'lost' nitrogen could be recovered as ammonia. Surprisingly enough the pH of the culture never rose above 7.2 during this time so, if it were a result of alkaline conditions, this would have to be highly localised. Though these aspiration losses were extensive only for '0' strain growing on ammonium acetate, they were detected with both strains at or around the transition point when growing on mixed substrates containing ammonium. These losses are corrected for in the results with requisite adjustments being made to total nitrogen and ammonium nitrogen values.

Properties of Cultures Grown in the Presence of Fixed Nitrogen

(A) Extracellular Nitrogen Compounds

Details will be discussed later but all substrates caused an increase in the proportion of non-cellular nitrogen in a culture (not counting added nitrogen, of course). At the end of runs of type (a), 50 ml portions of culture were fractionated by membrane filtration and the filtrate was examined, after purification on an ion exchange resin, by two-dimensional paper chromatography for free amino acids

and peptides. Only one spot identifiable as a peptide was ever found - the same as was present in the filtrate of '0' cultures in nitrogen-free medium - but hydrolysis on three different occasions led to three different amino acid components being enhanced. Thus it probably represented a mixture of similar peptides, which from its position near the origin, may be assumed to contain many acidic residues. The chromatographic technique used was, however, not quantitiative and glutamic acid - always a strong component - could never be definitely said to have been reinforced by hydrolysis. This applied to any amino acid which had been added deliberately. The strength of such components was such that adjacent spots were swamped and, though several dilutions were applied for each sample to overcome this, the results can only be interpreted as indicating major changes. Luckily the effects being investigated were on a relatively large scale. Another limitation, inherent in the use of Deacidite FF, was the fact that the amides glutamine and asparagine were hydrolysed and could not be distinguished from their parent acids. Large scale extracellular amidation or deamidation were tested for by examining filtrate samples directly. When the substrates were glutamic acid + ammonium acetate, glutamine or asparagine no such conversions were ever detected. The spots which were detectably reinforced after peptide hydrolysis were leucine, valine and an unknown which moved further than any recorded amino acid in both solvents. It has been found impossible to identify this component and it is hereinafter called X.

Since there were rarely any notable differences between the

strains, filtrate components are listed for both 'SK' and 'O' under the substrate and any exceptions are specially noted.

AMMONIUM led to the excretion of a comprehensive range of amino acids; glutamic acid (major component), alanine, gamma-aminobutyric acid and serine (abundant) with varying smaller quantities of valine, leucine, glycine, aspartic acid, the peptide component and the three basic amino acids.

UREA + AMMONIUM caused a greatly diminished range. Only glutamic acid was abundant together with small quantities of alanine, glycine and aspartic acid.

UREA alone also resulted in a smaller selection of amino acids notable for the absence of alanine. Glutamic acid was again the major component together with varying amounts of aspartic acid, gamma-aminobutyric acid, serine and glycine.

GLUTAMIC ACID (with and without AMMONIUM) caused excretion of appreciable quantities only of gamma-aminobutyric acid. A notable strain difference was observed here: with strain 'O' only traces of valine and leucine were detected. This was the only occasion on which these were found free after growth on fixed-nitrogen substrates apart from ammonium. Peptides formed in the presence of glutamic acid yielded amino acid X_1 which was together with leucine for strain 'O' only.

GLUTAMINE. 'O' strain yielded no detectable quantities of any amino acid while 'SK' gave only small quantities of aspartic acid and glycine. A peptide component was present giving valine and amino acid X on

hydrolysis.

ASPARTIC ACID and ASPARAGINE gave similar patterns characteristic for each strain. With 'SK' the full range of amino acids was found excluding only valine and leucine. The latter was present in abundance in peptide form. In contrast 'O' contained glutamic acid only in abundance, with faint traces of the basic amino acids and a few others. The peptide was again present and again gave leucine on hydrolysis (not found with asparagine but this turned out to be an anomalous case with uptake of amide definitely proved).

ALANINE caused a restricted spread with glutamic acid and serine the only abundant amino acids together with traces of gamma-aminobutyric acid and glycine. No peptide components were detectable (though, of course, one composed of glutamyl and alanyl residues could not have been recognised with the technique used).

To sum up:

(i) The full range of twelve amino acids - as in filtrates of strain 'O' on nitrogen-free medium - was only detected with ammonium as nitrogen source.

(ii) Glutamic acid was always the major component.

(iii) The unusual amino acid gamma-aminobutyric acid was detected free in all filtrates except those with glutamine or urea + ammonium substrates.

(iv) Peptides are detectable and consist of the added amino acid presumably together with

(A) leucine and perhaps glutamic with aspartic acid and

asparagine.

(B) amino acid X with glutamic acid and glutamine (together with leucine (glutamic acid) and valine (glutamine)).

(B) Intracellular Free Amino Acids and Peptides

Ammonium as nitrogen source caused a striking increase in the variety of water-extractable amino acids. Glutamic and aspartic acids, serine, glycine and glutathione were present in both strains while 'SK' also contained detectable alanine, peptide and basic amino acids. The other substrates - including urea - caused much the same accumulation as was found on nitrogen-free medium; the only appreciable component was glutamic acid usually accompanied by traces of aspartic acid, occasionally by serine and glycine.

(C) Free and Bound Ammonia

No increase in free ammonia over the small quantities present in aliquots of nitrogen-free culture was ever detected with glutamic or aspartic acids, alanine or asparagine (the method of analysis precluded such a test being applied with glutamine). Likewise the total bound ammonia in cultures was unaffected by the presence of glutamic acid, aspartic acid or alanine. Extra quantities of amide were formed when ammonium or amides were substrates. Washed cells which had been centrifuged from 25 ml portions of cultures were analysed for total bound ammonia. Small increases (0.01 to 0.02 mg/10 ml) were detected when the substrates were glutamine, urea, urea + ammonium and glutamic acid + ammonium but not when it was ammonium, asparagine, aspartic or glutamic acids, or alanine. These increases in cellular bound ammonia,

even when detected, were never very large (at most their ratio to the increase in acid-killed cellular nitrogen was 0.08) so the bulk, if not all of excess amide detected in cultures was present in the medium.

(D) Capsule and Dry Weight

Direct determinations were not made on the capsule but the speed of membrane filtration indicated little variation from substrate to substrate except in the case of ammonium. On this strain 'SK' became as easily filterable as 'O' implying that production of extracellular polysaccharide had been suppressed. As mentioned before the accuracy of individual dry-weight determinations was much too low for them to be of much use. Three or four measurements were taken - spread over the whole range of cell density - for each substrate. The overall mean ratios of these to acid-killed cellular nitrogen are of use, however, since sheer weight of numbers gives the results an appreciable degree of accuracy:

	N_2-Free Medium		All Substrates	
	Mean Ratio	(Readings)	Mean Ratio	(Readings)
Strain 'SK'	8.3	(9)	8.1	(29)
Strain 'O'	8.1	(9)	8.1	(30)

These are needed to back up the reliability of turbidity readings in presence of substrates containing fixed nitrogen. In spite of the fact that microscopic examination failed to reveal any such effect, it could be argued that the apparent stimulation of fixation was due to the turbidity calibration becoming invalid as a result of changes

in cell shape or composition. This was rendered unlikely by the fact that all recorded results are compared over a common interval so that turbidity need be only a relative measure anyway. The unchanging ratios recorded above provide unequivocal proof since, corresponding to mean increases in total nitrogen uptake of between 20% and 25%, they show that during these changes, turbidity remains a faithful measure of acid-killed cellular nitrogen.

(E) Growth Rate

No appreciable changes occurred in the M.G.T. on any substrates except those consisting of, or producing, ammonium. Ammonium acetate itself allows growth to accelerate to a M.G.T. of 1.5 hours. This was the same with '0' strain on urea but 'SK' once accelerated on this substrate down as low as 1.3 hours.

(F) Transition Point

The effects of the transition on growth rate and fixation were very marked on all substrates but ammonium itself where it was completely absent (see Figure 6). Striking changes were observed between a cellular nitrogen of 0.28 and 0.40 mg/10 ml in plots of total nitrogen versus acid-killed cellular nitrogen. Unfortunately, shortage of space prevents all but a representative few being reproduced here (Figures 7 - 13) but, since these effects fall into a limited number of categories, they may be summarised as follows:

DISCONTINUITIES OBSERVED IN FIXATION PER UNIT INCREASE IN ACID-KILLED CELLULAR NITROGEN: A SUMMARY

No Effect	:	Ammonium & Glutamic Acid
Fixation Enhanced (by c. 25% over average) eg. Figure 13	:	Aspartic Acid, Alanine & Glutamine[a]
Fixation Stopped or Diminished: eg. Figure 11		Asparagine[b], Urea[c], Glutamic Acid + Ammonium
Aspiration Losses (Temporary) eg. Figure 10	:	Ammonium + Urea, Ammonium + Glutamine

Notes: (a) With fully trained '0' strain the acceleration took place much later starting at about 0.39 mg/10 ml as opposed to 0.32 as before. There is strong evidence for utilization in this case.

(b) 'SK' fully trained is an exception. This culture (Figure 9) showed positive evidence of assimilation and fixation was enhanced during the discontinuity.

(c) This effect is detectable only on fully trained cultures.

The actual extent and position of these discontinuities varies between wide limits but most occur between 0.30 and 0.40 mg acid-killed cellular nitrogen/10 ml (typically it was 0.32 - 0.39 mg/10 ml). The only notable exceptions are urea and glutamine in '0' strain only (both when fully trained) whose ranges occur at higher cell density (eg. 0.38 - 0.44 mg/10 ml).

Summary of Results

It must be confessed that, even as presented in Tables 3 - 9, the results of growth runs are difficult to interpret readily. For this reason summarised tables have been prepared (Tables 14 - 16) in which arithmetic mean values have been derived for growth by both strains on each substrate. By inspection the effect of training on fixation, or

TABLE 14 COMPARISON OF THE TWO STRAINS: SUMMARISED RESULTS FROM GROWTH RUNS IN PRESENCE OF FIXED NITROGEN

All figures quoted are mean values derived from the experiments reported more fully in Tables 3 - 8.

	STRAIN 'SK'	STRAIN 'O'
AMMONIUM		
Total Nitrogen Assimilated	1.32	1.63
Fraction obtained by Fixation	28%	9%
Amide Produced	0.08	0.23
Effect of Training	Fixation Drops to 24%	Fixation Rises to 25%
UREA		
Total Nitrogen Assimilated	1.16	1.43
Fraction obtained by Fixation	48%	39%
Net Urease Activity (in excess of utilization)	-0.12	+0.09
Effect of Training	Nil	Nil
UREA/AMMONIUM		
Total Nitrogen Assimilated	1.42	1.54
Fraction obtained by Fixation	31%	7%
Net Urease Activity	-0.18	-0.74
Effect of Training	Nil	Fixation Drops to Zero

TABLE 15 COMPARISON OF THE TWO STRAINS: SUMMARISED RESULTS FROM GROWTH RUNS IN PRESENCE OF FIXED NITROGEN

All figures quoted are mean values derived from the experiments reported more fully in Tables 3 - 8.

	STRAIN 'SK'	STRAIN 'O'
GLUTAMIC/AMMONIUM		
Total Nitrogen Assimilated	1.78	1.75
Fraction obtained by Fixation	37%	21%
Amide Produced	0.35	0.15
Effect of Training*: on Fixation	A	A
on Total Assimilation	B	B
Evidence of Utilization of Glutamic	+ve after training	-ve
GLUTAMINE/AMMONIUM		
Total Nitrogen Assimilated	1.16	1.41
Fraction obtained by Fixation	30%	33%

*NOTE: EFFECT OF TRAINING

Characteristic types of behaviour on training are described by the following shorthand:

A Behaviour stimulated by delay then falling off after full training

B Behaviour stimulated with training

C Behaviour suppressed with training

D Behaviour depressed by delay followed by stimulation on full training

TABLE 16 COMPARISON OF THE TWO STRAINS: GROWTH RUNS IN PRESENCE OF FIXED NITROGEN

	STRAIN 'SK'	STRAIN 'O'
GLUTAMINE		
Total Nitrogen Fixed	1.97	2.04
Amide Formed	0.30	0.50
Effect of Training* on Fixation	B	A
Evidence for Utilization	Slight	+ve after full training
ASPARAGINE		
Total Nitrogen Fixed	1.43	1.53
Amide Formed	0.33	0.24
Effect of Training* on Fixation	D	C
Evidence for Utilization	Definite	Definite
ALANINE		
Total Nitrogen Fixed	1.55	1.62
Effect of Training* on Fixation	A	C
ASPARTIC ACID		
Total Nitrogen Fixed	1.37	1.67
Effect of Training* on Fixation	A	A
GLUTAMIC ACID		
Total Nitrogen Fixed	1.39	1.40
Effect of Training* on Fixation	D	D

*For Key, see Table 15

total nitrogen assimilation, or both, has been found and recorded. Since training can effect these activities in four different ways they have been represented by letters as shown on Table 15.

Growth on ammonium acetate leads to a stimulation of total nitrogen assimilation per increase in cellular nitrogen but also to production of extracellular amide. If, as seems likely, this is carried by the abundant glutamic acid molecules and residues detected in the filtrate one can deduct twice the amount of nitrogen found in extra amide from the total assimilation. The resulting ratios (1.16 for 'SK', 1.17 for 'O') are sufficiently near those for nitrogen-free cultures (1.20) to justify this approach. If such a procedure is repeated with the full results from asparagine and glutamine runs the discrepancy is often too great to be explained away even by assuming that all the normally occurring carboxylic acid residues in the filtrate have been amidated. In these cases the only explanation is that the substrate - in particular its amino nitrogen - has been utilized for cell synthesis. Such conclusions are noted in Table 16.

The effect of alanine, glutamic acid and aspartic acid on fixation is, quite simply, stimulation. The percentage excess fixation over that in nitrogen-free cultures growing over the same range of cell densities is recorded below:

MEAN PERCENT STIMULATION OF FIXATION BY SUBSTRATES

	Strain 'SK'	Strain 'O'
Glutamine	64 (39*)	70 (29*)
Asparagine	19 (-8*)	29 (8*)
Alanine	29	35
Aspartic Acid	14	39
Glutamic Acid	16	17

*after deduction of the extra amide produced

C) EXPOSURE OF GROWING CULTURES TO LABELLED SUBSTRATES

The final part of this work entailed the use of isotopic nitrogen to test whether the extracellular amino acids are intermediates in the fixing process or merely the result of side reactions. For this purpose membrane filtration was used in order to make a quantitative separation between cells and culture medium without the confusions resulting from acid-killing. Since the heavy capsule on 'SK' strain made filtration slow and separations difficult, 'O' strain was used in all these runs. As originally suspected, and confirmed in Section B of these results, the only possible candidates as intermediates were ammonium, glutamic acid and the two amides. Each of these was added to form an artificial extracellular pool in a growing culture which was then exposed to labelled atmosphere for 40 minutes (Table 9). If any of these compounds was a normal extracellular intermediate it would have diluted the label on the newly fixed nitrogen. The bulk of the newly assimilated ^{15}N would then be found in the filtrate with only secondary quantities in the cells. In case the oxygen supply affected uptake of extracellular amino acids the experiment was performed on both light and heavy cultures. The anticipated

TABLE 9 EXPOSURE OF CULTURES (GROWING IN THE PRESENCE OF AN ARTIFICIAL EXTRACELLULAR POOL) TO A LABELLED ATMOSPHERE

20 ml of (a) heavy culture 0.62 mg cellular N
(b) light culture 0.39 mg cellular N
containing substrate at a concentration of 0.0025 N, exposed to a labelled atmosphere (31.3% Excess ^{15} N) for 45 minutes, with shaking, at 30°C.

On removal, separated into cell and residue fractions by membrane filtration: ammonia or amide analysed also where indicated.

SUBSTRATE	RUN	FINAL ATOM % EXCESS IN CELLS	RESIDUE	LABILE NH_3
AMMONIUM ACETATE	a	0.43	-	2.18
L-GLUTAMIC ACID	a	1.78	0.23	-
	b	4.95	0.45	
L-GLUTAMINE	a	1.55	0.27	0.07
	b	2.96	0.28	
L-ASPARAGINE	a	2.07	-	0.15
	b	2.88	0.26	

randomisation of the label took place only with the extra ammonium pool clearly indicating that this (and not L-Glutamic Acid, L-Glutamine or L-Asparagine) is an intermediate in the major metabolic pathway for assimilation of newly-fixed nitrogen.

The experiments were repeated using labelled ammonium acetate as nitrogen source and, by shaking in air, concurrent fixation was allowed (Table 10). Unfortunately it was impossible to recover filtrate nitrogen quantitatively - due to the great excess of salts and carbohydrate present - but, since determinations were performed in parallel, the losses were all of the same order. The residual ammonia was determined accurately however and the ^{15}N used from this source could be calculated allowing the relative amounts ending up in cells and residue to be compared (Table 10). In spite of inefficient recoveries the results were all remarkably similar indicating that the presence of extracellular glutamic acid and amides had not affected the metabolism of ammonium to any detectable extent.

As was confirmed in Table 9 all newly fixed nitrogen first randomises with the extracellular pool. The extent of fixation during these runs could therefore be estimated as the sum of unlabelled ammonia assimilated plus that left in the extracellular pool (Table 11A). To obtain the former it had to be assumed, as an approximation, that the average label on ammonia taken up by the culture was the arithmetic mean of that at the beginning and at the end. Since the quantities were small anyway, this procedure has little effect on the accuracy of the result. Expressed as percentage of total uptake

TABLE 10 EXPOSURE OF CULTURES TO LABELLED AMMONIUM ACETATE WHEN GROWING IN THE PRESENCE OF AN ARTIFICIAL EXTRACELLULAR POOL

20 ml of culture (a) 0.67 mg cellular N
(b) 0.62 mg cellular N
mixed with concentrated solutions of labelled ammonium acetate (31.8% Excess ^{15}N) and substrate (1 ml each) such that their final concentrations are 0.0020N and 0.0025N respectively. Shaken in air at 30°C for 45 minutes then fractionated by membrane filtration followed by recovery of unused ammonia from the filtrate.

SUBSTRATE	RUN	FINAL ATOM % EXCESS			RECOVERED ^{15}N (mg.)		
		CELLS	NH_3	RESIDUE	CELLS	NH_3	RESIDUE
BLANK	(a)	7.08	13.7	3.92	65.1	34.3	31.4
L-GLUTAMINE	(a)	6.66	14.6	3.42	60.7	36.5	28.0
L-ASPARAGINE	(a)	7.20	11.0	4.46	69.8	22.0	35.7
L-GLUTAMIC	(b)	7.01	13.8	3.86	67.3	41.4	29.7
	(b)	DUPLICATE WHOLE CULTURE			176 mg.		

ESTIMATED FATE OF EXTRACELLULAR NH_3 POOL
MOVEMENT OF ^{15}N-EXCESS (TOTAL ADDED INITIALLY 172 mg.)

SUBSTRATE	LOST mg FROM NH_3	% FOUND IN CELLS	% FOUND IN RESIDUE	% RECOVERY
BLANK	138	47	23	70
L-GLUTAMINE	135.5	45	21	66
L-ASPARAGINE	150	47	24	71
L-GLUTAMIC	131	51	23	74

TABLE 11 (A) ESTIMATED FIXATION IN PRESENCE OF ^{15}AMMONIUM ACETATE (TABLE 12) BY CALCULATING TURNOVER FROM THE EXTRACELLULAR POOL

ORIGINAL LABEL ON AMMONIA - 31.8% EXCESS ^{15}N 0.54 mg.

SUBSTRATE	MEAN LABEL %	NH_3 USED AT THIS %	UPTAKE OF UNLAB. NH_3	RESIDUE NH_3 31.8% LEFT	RESIDUE NH_3 0% LEFT	TOTAL UNLAB. NH_3	% TOTAL UPTAKE
Blank	22.5	0.61	0.07	0.11	0.14	0.21	34
Glutamine	23.0	0.59	0.05	0.11	0.14	0.19	32
Asparagine	21.2	0.71	0.17	0.07	0.13	0.30	42
Glutamic	22.6	0.58	0.04	0.13	0.17	0.21	36

(B) SHORT TERM UPTAKE OF $^{15}N_2$ BY PURE CULTURES: ATTEMPTED DETECTION OF FRACTION TO BE LABELLED FIRST

20 ml of culture (0.62 mg Cellular N) was exposed to a labelled atmosphere (31.3% EXCESS ^{15}N) with shaking for (a) 7 mins.; (b) 15 mins. - the culture attaining 30°C after approx. 6 minutes. After this short exposure the cells were fractionated by membrane filtration. Cells were extracted with 0.1N sulphuric acid at 30°C for 15 minutes, then separated again by filtration.

	NITROGEN mg. Cells	Filtrate	Extract	% Total Activity Cells	Filtrate	Extract	Total Enrich.
(a)	0.59	0.21	0.07	18	72	9.6	1.78
(b)	0.61	0.22	0.07	26	65	8.7	2.80
			Ratio:	1.4	0.9	0.9	

of ammonium, it is striking that the asparagine run has a higher value (42%) than that of glutamic acid or glutamine which both had virtually the same percentage as the control where the substrate was added at the end (34 ± 2%). These latter are obviously due entirely to concurrent fixation and though it could be that asparagine stimulates fixation, it is more likely that some utilization occurred allowing unlabelled nitrogen derived from asparagine to find its way into the extracellular ammonium pool.

A final short-term experiment was performed to see which culture fraction (filtrate or acid extract from cells) is involved earlier in fixation. As shown in Table 11 (B), % Total Activity diminishes in both these fractions while that of the cells rose steadily. This indicates a precursor/product relationship vis a vis the cells but, within the accuracy of the experiment, it was impossible to distinguish between the two. Clearly they must be fractionated into individual components before such comparisons can yield positive results.

In a recent and very penetrating review of biological nitrogen fixation, Yocum (178) pointed out the need for studies under optimal conditions of growth so that the various systems concerned could attain genetically controlled levels. It was to this end that special culture techniques were evolved and the conclusions arrived at checked by comparison of two strains. Having thus reduced the effect of biological imponderables to a minimum, the results - together with those of previous workers - allow the status of extracellular nitrogen compounds in the fixation sequence of Azotobacter to be established with some certainty.

The success of the culture technique may be measured by the optimal mean generation time achieved, 1.7 hours (which corresponds to a k value of 0.41 hrs.$^{-1}$). This is equal to the best values cited by Werkman and Wilson (28) and is higher than those obtained from runs reported by Wilson and Lind (k = 0.30) (11), Wilson and Roberts (k = 0.25) (50), or, more recently, by Australian workers (k = 0.28 - 0.41) (169). By comparison, the cultures used for the important labelling experiments of Allison and Burris (79) grew with a k of only 0.05 - 0.06.

At the light culture densities used throughout the work there was clearly only one rate-limiting factor - the supply of oxygen. This indeed could be the only possible explanation for the fact that two strains, having many incidental dissimilarities and whose places

of origin lie thousands of miles apart, should react identically in certain situations. For instance, strain 'SK' had a heavy capsule and, perhaps for reasons associated with this, was less permeable to solutes and gave more easily reproducible results. Yet its trained growth rate and behaviour at the 'discontinuity' were identical to that of strain 'O'. The latter phenomenon was detectable only because samples were removed continuously from the growing culture; its existence should help to explain many contradictory results obtained under apparently identical conditions. In summary, it can be said that the active growth of a culture of Azotobacter vinelandii in air falls into three distinct phases:

	Approximate Extent (mg. cellular N/10 ml.)
Early Log Phase	? to 0.30
Discontinuity	0.30 to 0.40
Late Log Phase	0.40 to 0.70

These are, of course, preceded by a lag phase and followed by a general decline due to exhaustion of nutrients or accumulation of toxic by-products. Though the lowering of acid extractable nitrogen in the late log-phase by supplementing the oxygen supply is not conclusive evidence, it may be inferred that the uniquely high respiration rate of the organism is the cause of this discontinuous log-phase. As the culture becomes denser there must come a time when the dissolved oxygen is removed from the medium at a faster rate than it can be replaced. (The respiration of an Azotobacter culture of medium density is such that the dissolved oxygen has to be replaced from the air approximately four times a minute.) In certain conditions

oxygen supply becomes growth-limiting even with E. coli, a much less demanding aerobe (208).

When this happens synthesis of unessential polysaccharide is stopped and the protein content, as percent of the dry weight, rises. This economy is not enough and a profound readjustment of the metabolism is forced on the culture which switches over to a less extravagant means of respiration. Logarithmic growth can continue for a considerable time (at a slower rate) before the cells again become starved and the whole culture begins to decline. The situation just described is exactly analogous to that found when bacterial cultures exhaust one carbon source and then have to adapt to a second whose uptake had been inhibited by the presence of the first (21). So what may be termed a diauxic growth curve is obtained when a culture of Azotobacter vinelandii is forced to adapt itself to a low oxygen supply after growth with abundant aeration. One of the major users of the products of respiration is the fixation mechanism. It is not surprising therefore that, when this is suppressed almost entirely by ammonium, 'diauxie' does not occur until the culture is very heavy while, with substrates used as part nitrogen sources ('SK' & 'O' urea and 'O' glutamine - all fully trained), it happens at a detectably higher cell density. Asparagine is also used as a partial nitrogen source but does not postpone diauxie, a fact probably related to the requirements of transport for this molecule or its products which (in contrast to glutamine and urea) are not already catered for on a large scale.

When adapting for a new enzyme or transport system, micro-organisms

need extra energy and nitrogen sources - the raw materials for building new specific protein. Fixation demands large quantities of energetic reducing agents so it is not surprising that a clash of interests is apparent at diauxie whenever the normal situation is upset. Thus (in terms of cellular material synthesised) fixation is enhanced when the substrates present are unavailable as nitrogen sources (aspartic acid, alanine and most instances for glutamine) while it is diminished or stops completely when assimilation can occur (asparagine, urea and glutamic acid + ammonium). Glutamic acid is abundant in normal supernatants so its addition as substrate leads to the same behaviour as on nitrogen-free medium where no definite effect on fixation is observed one way or another. The (acid-extractable) intracellular nitrogen pools are, however, augmented and - with strain 'O' only - this is accompanied by a drastic reduction in the amount of nitrogen excreted.

The increased tendency to lose ammonia by aspiration is readily explained by the demands of the adaptation process. When growing the organism on ammonium acetate Wilson and Lind (11) found that the pH of the medium dropped slightly indicating that the cation was removed at a slightly faster rate than its anion. The author's own work has confirmed this and aspiration can only be explained by a localised condition of slightly elevated pH due to accelerated metabolism of acetate.

It may be protested that the phenomenon described above is of academic interest only and, while a fascinating subject for research

by a microbial physiologist, is strictly irrelevant to nitrogen fixation. On the contrary, much of the most significant work in recent years has been based on the untested assumption that cultures of Azotobacter exhibit steady logarithmic growth with no substantial changes in properties between cellular densities ranging from 0.03 (Parker and Scutt,(169))to 1.00 mg. N/10 ml (Allison and Burris, (79)). It is obvious that, to avoid needless confusion in the future, all results obtained must be considered in terms of the growth phase in which the culture was examined.

For many years, it was assumed that uniform growth rate implied uniform cellular composition - that is, constant relative concentrations of all metabolites and enzymes (24). Perret (170) has developed an improved model for the bacterial biophase and demonstrated that this could be true only when the environment remains constant. This fact has been confirmed in practice during studies on many bacteria, for instance Salmonella (144) and E. coli (183). Since the environment could in no way be described as constant during logarithmic growth by Azotobacter, one may question the significance of results based, as these all are, on turbidity measurements. Though long used by the Wisconsin school as a measure of growth for trace requirement and inhibition studies (73), turbidity was dismissed by Parker and Scutt (169) because they found that the apparent cell-density of a resting culture increased after three hours standing. The use of turbidity measurements throughout this work as a valid measure of cell-substance

is based on three facts:

(i) Measurements were reproducible and made within minutes of removal from cultures which, as shown by their mean generation times, were always growing at or near the optimal rate.

(ii) All results were compared over a common turbidity range so that only a relative measure of cell density was needed anyway.

(iii) The relationship between turbidity and dry weight remained substantially unaltered even when fixation in the given interval was stimulated by up to 70% of its normal value.

The failure to detect PCA - as found by Bach (83) and Bulen and Doughty (100) - is puzzling. It is worth noting, however, that Bach did not find the 'indole bodies' reported by Bukatsch and Schlutter (71) which may possibly be identified as the 'unknowns' of this work. In the author's experience, and that of Bach, the R_F values of compounds giving colours with Ehrlich's reagent were very sensitive to salt concentration. In addition, hydrazine derivatives had a tendency to 'tail'. This must be due to exchange of hydrazine molecules with reactive components in the paper but it meant that the only satisfactory solvent mixture was that which moved at the fastest rate (Mixture C). Examination of Bach's results shows that his 'PCA' fraction was labelled less - on both a relative and absolute basis - than the others he designated Bands 1 and 3. Band 1 is probably identical with the slow moving spots attributed here to peptides containing citrulline or histidine. Band 3 was identified as PCA by co-chromatography in

three different solvents (aqueous phenol, ethanol and benzyl alcohol) but it is worth noting that, in aqueous phenol, PCA and citrulline have virtually identical R_F values. They are quite distinct in the mixture used in this work where, in contrast to that reported by Bach, the amino acid citrulline was identified. Another's results cannot be dismissed on such flimsy evidence but it is just possible that the two compounds in question have similar R_F values in alcohol solvents also.

The situation is very confused but clearly paper chromatography on its own will not settle unequivocally whether hydrazine derivatives are present in Azotobacter cells. A final decision on this subject must wait until each compound yielding the colour reaction has been separated and characterised.

The most important conclusion to be drawn from this work is that ammonium can no longer be regarded as an obligatory intermediate in the fixation process in Azotobacter vinelandii. Contrary to reports from Wisconsin (18) ammonium does not suppress fixation completely. Though this is indeed observed in short-term experiments with 'O' strain, the fixing mechanism has been swamped only temporarily and, on training, both strains tended towards a balanced state in which 25% of the total nitrogen uptake is obtained by fixation. The reasons for this disagreement are easy to see: 'O' strain was used at Wisconsin for untrained runs only and, in the words of Professor Burris 'Calculations show that.....(ammonia) is used to the virtual exclusion of nitrogen fixation' (65). Under such conditions total nitrogen uptake has increased so that fixation could be easily overlooked when it was found that utilization of ammonium equalled net fixation in its absence. The evidence pointing to the fact that extracellular ammonium, while closely linked metabolically, is not an intermediate in the assimilation of newly-fixed nitrogen may be summarised:

(i) (Burma and Burris, (86)). Growth on ammonium as nitrogen source leads to very heavy labelling of purines and pyrimidines which does not occur when using atmospheric nitrogen.

(ii) (Allison and Burris,(79)). In the extracellular fraction glutamic acid becomes labelled before ammonium in the manner characteristic of a precursor. This result has recently been echoed in work with

non-legume symbionts (Bond, (117)) and was explained away in the same unconvincing manner as the above.

(iii) (Bach, (83)). The label in organic acid fractions is profoundly different in cells grown on isotopic ammonium from those fixing gaseous nitrogen.

The following have been detected in the course of these researches:

(iv) With trained cultures ammonium uptake and fixation take place side by side.

(v) Growth on ammonium only produces large quantities of extra-cellular amide, presumably attached to glutamic acid or peptide glutamyl residues.

(vi) Extracellular ammonium as nitrogen source resulted in a considerable increase in the number of amino acids which were detectable in the intracellular pools.

Though it may be argued that many of these effects could be the consequence of swamping a normal metabolic pool, points (ii) and (iv) can be explained only if there is a metabolic step between extracellular ammonium and the product of fixation. Free ammonia has been actually established as the product of fixation by cell-free Clostridium extracts though this need not be the case <u>in vivo</u> and the process in Azotobacter is not necessarily analogous to that in a strict anaerobe. Transport of ammonium into the cell would require an energy source, as is the case with Pseudomonas aeruginosa (112).

It is clear that urea is metabolised via ammonium and not assimilated

as a unit, for instance via a reversed ornithine cycle (121). This is indicated by the fact that the mixed substrate with ammonium gives results very similar to those when urea was not present at all. Slight differences in behaviour - *eg.* the 'diauxic' effect is present - show that the urease activity is not simply exogenous and either urea or ammonium must be transported in the process. Urea, a small uncharged molecule, is one of the few regarded as not necessarily requiring a specific transport mechanism (147) and, with strain 'SK' one may deduce that no extracellular amide has been formed in contrast to the situation when free ammonium is nitrogen source. It seems likely therefore that urease activity is located somewhere within the envelope from which the ammonium produced must be transported in order to equilibriate slowly with the extracellular pool. Net urease activity is always highest at the beginning of a run thereafter proceeding at a slower rate than uptake such that free ammonium in the culture drops steadily. Since the ammonium is not present in such overwhelming quantities fixation is more pronounced (c. 40% of uptake) and the initial burst of urease activity may be an illusion, simply reflecting a slower inhibition of fixation.

The extracellular amino acids in cultures of Azotobacter on nitrogen-free medium were studied by Schlutter and Bukatsch (146) who found aspartic acid, glutamic acid and alanine abundant with most species and noted that excretion of amino acids in cultures using mannitol as carbon source was very low. Even so, an extensive range was detected during this work both with nitrogen-free cultures and those growing on ammonium. The presence of other substrates reduced the variety of amino acids found but led to substantial increases in the quantities of peptide and amide components. A small portion of the ammonium utilised could be recovered as extracellular amide which was attached to glutamyl residues either free or in peptides. (The identity of the amide-forming carboxyl group can only be inferred indirectly but the stoichimetry of amide formation, the abundance of glutamic acid, and its strong stimulation of this 'binding' of ammonium when in mixed substrate all point to such a conclusion.) Almost all of the ammonium taken up by a Leuconostoc species and a coliform organism became bound in a similar manner - though in this case to cellular amino acid residues - while they assimilated preformed amino acids and peptides preferentially (78). Blue-green algae normally excrete a portion of the nitrogen they have newly fixed and it is also in the form of amino acids and peptides (29).

Those amino acids extractable with boiling water from filtered and washed cells have been regarded as being derived from internal

pools. Work with gram-positive organisms - for instance Staphylococci (105) - showed that actively growing cells accumulate amino acids in appreciable quantities. It was first thought that gram-negative organisms did not do this but Mandelstam (111) showed that it was merely on a much smaller scale and he identified fifteen different amino acids in Escherichia coli extracts. He considered that these accumulations were due to specific adsorption sites inside the cell but it is now generally agreed that access mechanisms in the cell envelope are mainly responsible for the striking concentrations sometimes achieved. On nitrogen-free medium, as on all substrates except ammonium, glutamic acid is the only free amino acid found in any quantity in Azotobacter cells. The only 'free amino acid pool' in the organism under such conditions lies in the extracellular fraction. To be honest it cannot even be called that since, contrary to all expectations, the vigorously growing cells assimilated only one added amino acid consistently and that (asparagine) made but a minor contribution to total nitrogen uptake. In fact, addition of L-alanine, L-aspartic acid, L-glutamic acid, L-asparagine and L-glutamine stimulated fixation by amounts varying from 14 to 70%, with virtually all of the extra nitrogen appearing in the medium.

The normal components of the extracellular pool are early products of fixation, as demonstrated by their rapid uptake of label, so means must exist for the excretion of at least twelve different amino acids. The answer lies, of course, in the highly specific mechanisms for transporting solutes across biological membranes, a vast subject of which the first principles are just becoming understood.

With due allowance for various electrical effects, inorganic ions generally require a specific carrier and a source of metabolic energy. This enables them to be moved across a membrane against the concentration gradient in a step which can be, to all intents and purposes, irreversible (147). The situation is similar for amino acids and sugars except that the carrier is inevitably a protein. This led Cohen and Monod (89) to emphasise the analogy to enzyme action and so term the transport agent a 'permease'. This was defined as a protein y capable of forming stereospecific, reversible complexes with a substrate G and depositing it on either side of an osmotic barrier:

$$\underset{\text{Phase 1}}{G + y} \rightleftharpoons \underset{\text{Membrane}}{Gy} \rightleftharpoons \underset{\text{Phase 2}}{y + G}$$

Mitchell (136) and Christiansen (161) regard this sort of treatment as being rather 'exclusive' and 'parochial'. Mitchell on the other hand points out that, provided the active centre of the normal metabolic enzymes were anisotropic, the many accumulations and migrations taking place in the cell could be explained without invoking any special permeation systems. The apparently useless side-chains of such enzymes would determine the location of the active site and several instances are quoted of bound enzymes acting as 'group translocases' (136). Christiansen after considering all possible mechanisms stresses that an oscillating or moving carrier must always be involved. He finally proposes a theory in which he sees the cell membrane as a complex three-dimensional network with hundreds of bonding points capable of interacting with external solutes. Only a few of these are

metabolically significant and, though the gradient from one such interaction to the next is low, a succession of such steps could make interphase movements possible. Energy is supplied by phosphate ester bonds in a manner that is not yet understood (161).

Whatever the actual mechanisms are that effect transport, Azotobacter is unusual among micro-organisms in that, while possessing specific carriers for so many amino acids, many of these act in one direction only. Whereas, for instance, free amino acids and even peptides are taken up in preference to ammonium by representatives of both gram-positive and gram-negative bacteria (78), glycine and leucine are toxic to Azotobacter (43) and those amino acids tested here cause wasteful excretion of large quantities of the newly fixed nitrogen. Extracellular peptides behave very differently from their component amino acids (184) and it is possible that, if the right one were chanced upon, it could act as an alternative nitrogen source for the organism. As it is, assimilation of amino-nitrogen is of minor importance and can best be described as incidental.

A species of yeast has been found to assimilate asparagine by first deamidating it at the cell surface (120) and, though neither aspartic acid nor ammonium were ever present in detectable amounts, this seems to happen with Azotobacter also. Since assimilation took place simultaneously with large-scale excretion of amide (presumably asparagine, either free or in peptide form) a second access mechanism must be involved, transporting the dicarboxylic acid after its amide group had been transferred to acid residues remaining in the extracellular fraction. The

much greater ease with which asparagine nitrogen was assimilated (as opposed to glutamine) is probably a reflection of the lower concentration gradient to be traversed by an amino acid which is comparatively rare in the free state inside the cell. All this implies that the amides are of greater importance in the cells' economy than the free dicarboxylic acids, a fact underlined by the more pronounced disturbances caused by asparagine and glutamine as opposed to aspartic acid and glutamic acid respectively. A striking example, in which confusion due to simultaneous uptake is at a minimum, is afforded by the stimulatory effect of glutamic acid and its amide on fixation; with both strains glutamine causes four times as much excess fixation as glutamic acid. It is quite likely that the major access mechanisms deal only with these amides since they are closely analogous to the bulk of amino acids, which are simple dipolar ions; it would parallel a similar restriction noted for species of soluble RNA in the second stage of protein synthesis (212).

Unless the extracellular amino acids were of the D-series, the negligible effect of large quantities of added L-glutamic acid or L-glutamine on the distribution of newly fixed ^{15}N in comparison with that in a nitrogen-free culture can only mean that the extracellular pool is, metabolically speaking, a dead end. Though produced very quickly from newly fixed nitrogen (as Fogg found with blue-green algae (29)) this fraction has no apparent function. It is still linked in some way to the metabolism since, when individual components are augmented as in this work, excretion of nitrogen compounds is stimulated on a massive scale. Since time did not allow separation and characterisation of

each of these compounds their identification must be part speculation but they certainly fall into two general categories:

(a) more of the added amino acid itself (alanine and the amides).

(b) peptide or peptides specific to the dicarboxylic acid residue concerned (glutamyl residue + mostly amino acid X; aspartyl residue + leucine and perhaps glutamic acid).

In addition to these two it is possible that large polypeptides were excreted since these would be held on a resin with 8% cross-linking and so escape detection. Such an eventuality was not allowed for when designing the runs since the results obtained were completely unexpected. Though replication of the added substrate is the more extraordinary of the two effects there can be only one possible explanation for it: the presence of the amino acid in the medium induces formation of the specific carrier and thus makes extensive transport possible. Under conditions of active cell growth, the effective concentration gradient favours excretion (even though the extracellular pool has been augmented) and so the access mechanism thus induced acts in one direction only.

The added substrates are clearly interfering with a metabolic activity involving major quantities of amino acids. Protein synthesis springs to mind at once but, apart from the fact that this probably takes place on cytoplasmic RNP particles, we are concerned here with something which takes place in the outer metabolic regions of the cell. It is now recognised that synthesis of peptides is a completely separate matter from production of soluble protein and enzymes for which the

three step sequence is well established (145). The major function of cellular peptides is as components in the cell envelope (144) and, though Mitchell considers that cell-wall precursors are prefabricated in the cytoplasm and transported as units through its membrane (136), it is quite likely that in Azotobacter these structural peptides are assembled at a site in the envelope with fairly easy access from both the cytoplasm and the supernatant medium. Such an arrangement would conform neatly to the sandwich structure recently proposed for the envelope of gram-negative organisms (200) and would explain why appreciable incorporation of amino acids by cell-free extracts of Azotobacter has been obtained only with particulate - that is envelope - fractions. The bulk protein synthesis mechanism would have escaped detection because of the quick turnover of juvenile protein from ribosomes (144).

The sudden access of large quantities of one amino acid throws such a system out of gear and by an obvious feed-back reaction induces synthesis of large quantities of peptide. These contain the substrate in question together with its complementary amino acids which have to be summoned in large quantities from their sites of synthesis in the cytoplasm. The bulk of this peptide is not needed and so is excreted. It is worth noting that cell-walls often contain unusual amino acids (191) so the presence of the unknown X is not too difficult to explain. Another apparently unrelated fact - the tendency to form distorted cells when grown on nutrient medium - also points to the sites of cell-wall synthesis, in Azotobacter, being unusually vulnerable from outside.

Since amino acids and peptides are normally used as preferred nitrogen sources for bacteria, their effect on excretion is rarely noted. Steiner (120) found that a yeast, while assimilating amino acids, excreted other 'secondary' ones in what may easily have been a similar disturbance to the one examined here. The cell would, given time, adjust itself to prevent such a wasteful state of affairs. It is not surprising, therefore, that alanine and aspartic acid cause very much the same behaviour: the short term stimulation increases over a few generations' growth but falls back to a more moderate value on training. The stimulation, still positive, then has a value characteristic of the strain ('SK' 13%, 'O' 23%). Being ubiquitous in the cell and its extracts, cultures on glutamic acid are unaffected by full training and stimulation remains at approximately 23% for both strains; there is an inexplicable decrease after only one generation's training. Training stimulates uptake of amides; which effect obscures any alterations in fixation that may have resulted.

Using the conclusions derived from work described in this thesis together with those from previous studies on the mechanism of nitrogen fixation it is possible to describe, with a fair degree of certainty, the fate of gaseous nitrogen in the metabolism of Azotobacter vinelandii. The actual 'fixation' sequence involves the concerted action of an enzyme system located in the cytoplasm (or on the inner side of its membrane) to which the nitrogen gas comes by unfacilitated diffusion. In this location competition from oxygen is avoided by the shield of electron carriers in the envelope while adequate supplies of both metabolic hydrogen and αketoglutaric acid are available from the glycolytic and citric acid cycle enzymes. The reducing hydrogen atoms are supplied in pairs at more than one energy level; those derived directly from pyruvate are probably transferred via hydrogenase and the rest by molybdoflavoproteins such as the aldehyde oxidase of Azotobacter (79). It is the supply of these hydrogen atoms that is the rate-determining step in fixation (2, 7) and, of course, is where competitive inhibition by oxygen is felt.

NITROGEN ACTIVASE $N_2 \longleftrightarrow (N_2)E$

It has long been considered likely that fixation is initiated by chemisorption of the nitrogen molecule on a metalloprotein and both Roberts (118) and Winfield (63) have suggested mechanisms for the first step. The metal could be either molybdenum or iron and it catalyses the reversible activation of the nitrogen molecule so making it liable

to attack by a suitable hydrogen donor. This change is detectable by spectral effects in the UV (67) and is not very profound, leaving the nitrogen atoms firmly bound to one another (163) and is even capable of being bypassed by ultraviolet activation of the nitrogen gas (193). The reactive centre can be blocked by hydrogen, nitrous oxide, carbon monoxide (azide ion & trimethylamine oxide?) so that these act as competitive inhibitors of fixation.

NITROGEN REDUCTASE $(N_2)E \longrightarrow (N_2H_2)E \longrightarrow (N_2H_4)E$

Addition of four hydrogen atoms leads to hydrazine via the transient intermediate di-imide, both being bound enzymatically. The latter is now established as the intermediate in the liquid-phase hydrogenation of ethylenic linkages by hydrazine from which it is readily produced by oxidation (195). Though the great bulk of the hydrazine remains bound and is further reduced to ammonia, a portion could be removed by non-enzymatic reaction with any one of several carbonyl compounds present in an actively respiring cell. Most such products would be highly reactive themselves except that from α-ketoglutaric acid. This, of course, is present in abundance at the site of fixation and so the semi-reduced nitrogen so diverted would tend to accumulate in the stable six-membered ring of PCA.

HYDRAZO REDUCTASE & REMOVAL OF PRODUCT

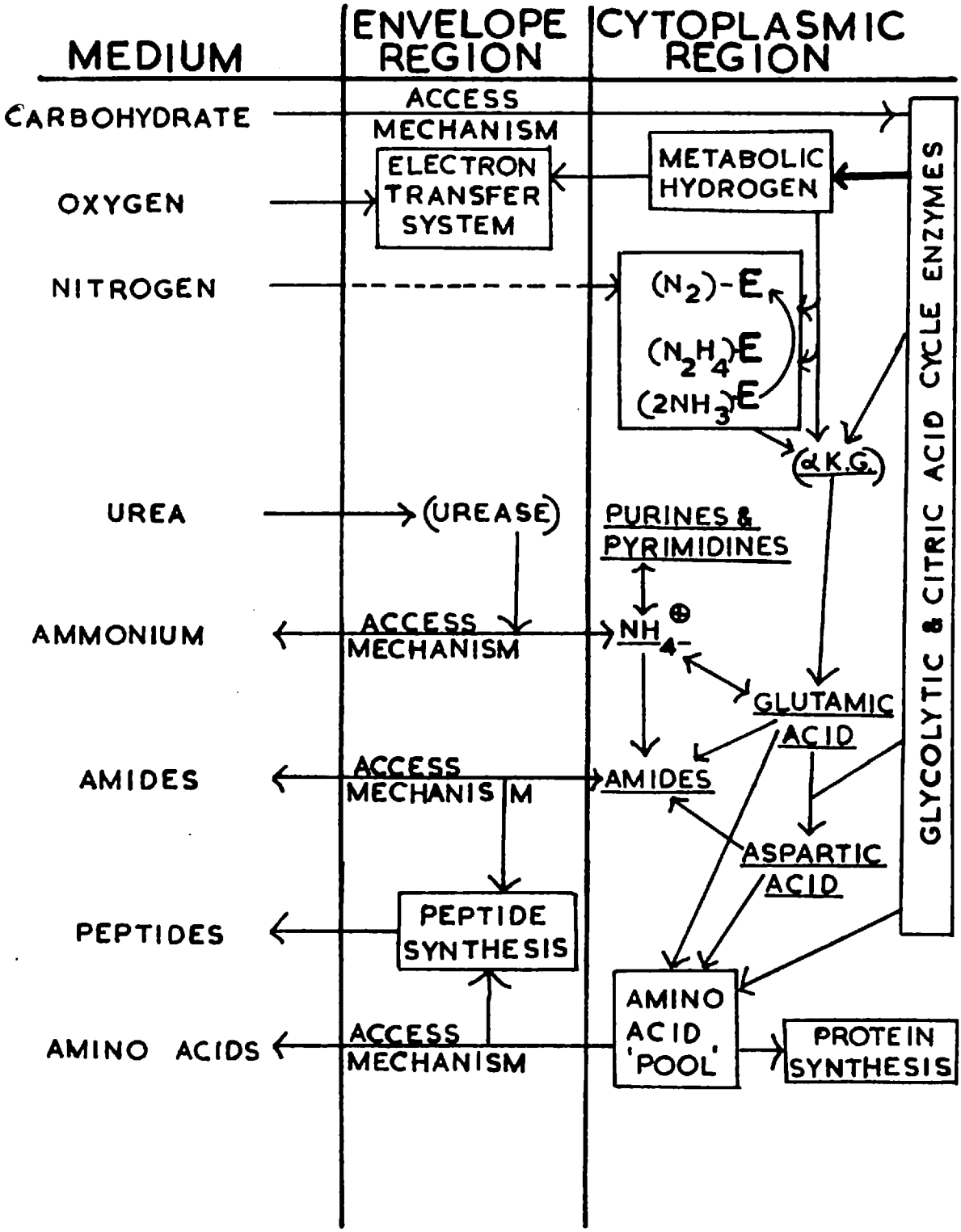
$$(N_2H_4)E \longrightarrow (2NH_3)E + 2\alpha KG \xrightarrow[2\ NADPH_2]{} \underset{+\ 2\ NADP}{2\ \text{Glutamic Acid}}$$

Reduction continues to a bound-ammonia stage which is not however normally liberated to form a 'juvenile' ammonia pool. Instead it is

removed from combination with the enzyme by reductive amination of α-ketoglutaric acid forming glutamic acid. This latter forms the first true nitrogen pool from which ammonia is formed by way of a reversible glutamic dehydrogenase system which could be located anisotropically and so effect 'metabolic' transport into the outer regions of the cell. All the various equilibria leading to amino acids occur in the cytoplasm and the products of these reactions reach the envelope and extracellular regions via specific access mechanisms which are effectively irreversible - possibly because of steep concentration gradients maintained by intracellular compartmentalization. These are then either assembled into structural peptides or are excreted into the medium. Synthesis of the bulk of enzyme- and soluble-protein takes place in the cytoplasm.

The above plan is illustrated symbolically in Figure 14. Though largely based on speculation, this diagram reflects accurately the confusing trends apparent in the results of recent studies and is considerably more complex than the picture of fixation imagined by early workers. It is, however, only by facing up to the difficulties of microbial physiology that the mechanism of nitrogen fixation will ever be elucidated. It is hoped that, paradoxically, by thus complicating the picture, the author has paved the way to a full understanding, sometime in the future, of this, one of the most difficult yet fascinating topics in modern biochemistry.

FIG. 14. PROPOSED MECHANISM FOR ASSIMILATION OF NITROGEN BY AZOTOBACTER (SIMPLIFIED & DIAGRAMMATIC)



1933

(1) H. Lineweaver, J.Biol.Chem., 99, 575.

1938

(2) H. Lineweaver, J.Biol.Chem., 122, 549.

1940

(3) H. Bortels, Arch.Mikrobiol., 11, 155.

1941

(4) D. Burk & R.E. Burris, Ann.Rev.Biochem., 10, 587.

(5) C.J. Lind & P.W. Wilson, J.Amer.Chem.Soc., 63, 3511.

(6) O. Wyss, C.J. Lind, J.B. Wilson & P.W. Wilson, Biochem.J., 35, 845.

1942

(7) P.W. Wilson, R.H. Burris & C.J. Lind, Proc.Natl.Acad.Sci.U.S., 28, 243.

1943

(8) R.H. Burris, F.J. Eppling, H.B. Wahlin & P.W. Wilson, J.Biol.Chem., 148, 349.

(9) P.W. Wilson, J.F. Hull & R.H. Burris, Proc.Natl.Acad.Sci.U.S., 29, 289.

(10) S.B. Lee & P.W. Wilson, J.Biol.Chem., 151, 377.

(11) P.W. Wilson & C.J. Lind, J.Bacteriol., 45, 219.

1944

(12) E.R. Ebersole, C. Guttentag & P.W. Wilson, Arch.Biochem., 3, 399.

(13) C.K. Horner & F.E. Allison, J.Bacteriol., 47, 1.

(14) P.W. Wilson & R.H. Burris, J.Bacteriol., 47, 410.

1945

(15) R.H. Burris & P.W. Wilson, Ann.Rev.Biochem., 14, 685.

(16) H.C. Lichstein & P.P. Cohen, J.Biol.Chem., 157, 85.

1946

(17) R.H. Burris & P.W. Wilson, Botan.Gaz., 108, 254.

(18) R.H. Burris & P.W. Wilson, J.Bacteriol., 52, 505.

1947

(19) P.W. Wilson & R.H. Burris, Bacteriol.Revs., 11, 41.

(20) H.L. Jensen & D. Spencer, Linnean Soc.N.S.W., 72, 73.

1948

(21) E.F. Gale, "The Chemical Activities of Bacteria", University Tutorial Press, London.

(22) J.G. Wood, M.R. Hone, M.E. Mattner & C.P. Symons, Austral.J.Sci. Res., B.1., 38.

(23) R. Novak & P.W. Wilson, J.Bacteriol., 55, 517.

1949

(23) W. Segal & P.W. Wilson, J.Bacteriol., 57, 55.

(24) J. Monod, Ann.Rev.Microbiol., 3, 371.

1950

(25) W.E. David & H. Lichstein, Proc.Soc.Exptl.Biol.Med., 73, 216.

(26) C.C. Willits & C.L. Ogg, J.Assoc.of Official Agric.Chemists, 33, 179.

1951

(27) E.D. Rosenblum & P.W. Wilson, J.Bacteriol., 61, 475.

(28) C.H. Werkman & P.W. Wilson, "Bacterial Physiology", P.467, Academic Press, New York.

1952

(29) G.E. Fogg, Proc.Roy.Soc., Ser.B, 139, 372.

(30) H. Gest, J.Bacteriol., 63, 111.

(31) R.W. Stone & P.W. Wilson, J.Biol.Chem., 196, 221.

(32) R. Repaske & P.W. Wilson, J.Amer.Chem.Soc., 74, 3101.

(33) T.G.G. Wilson, Ph.D. Thesis, London University.

1953

(34) K. Burton & H.A. Krebs, Biochem.J., 54, 94.

(35) H. Green, M. Alexander & P. Wilson, Proc.Soc.Exptl.Biol.Med., 82, 361.

(36) L.A. Hyndman, R.H. Burris & P.W. Wilson, J.Bacteriol., 65, 522.

(37) J.W. Newton, P.W. Wilson & R.H. Burris, J.Biol.Chem., 204, 445.

(38) R. Repaske & P.W. Wilson, Proc.Natl.Acad.Sci.U.S., 39, 225.

(39) P.W. Wilson & R.H. Burris, Ann.Rev.Microbiol., 7, 415.

1954

(40) A.F. Brodie & F. Lipmann, Bacteriol.Proc., 107.

(41) M.T. Chaudhary, T.G.G. Wilson & E.R. Roberts, Biochim.Biophys.Acta, 14, 507.

(42) A. Dreze, S. Moore & E.J. Bigwood, Anal.Chim.Acta, 11, 554.

(43) H.L. Jensen, Bacteriol.Revs., 18, 195.

(44) L.E. Mortenson & P.W. Wilson, Arch.Biochem.Biophys., 53, 425.

(45) L.E. Mortenson & P.W. Wilson, Bacteriol.Proc., 108.

(46) M.M. Mozen & R.H. Burris, Biochim.Biophys.Acta, 14, 577.

(47) B.A. Pethica, E.R. Roberts & E.R.S. Winter, Biochim.Biophys.Acta, 14. 85.

(48) N. Suzuki & S. Suzuki, Sci.Repts.Tohoku Univ., Ser.4, 20, 195.

(49) T.G.G. Wilson & E.R. Roberts, Biochim.Biophys.Acta, 15, 390.

(50) T.G.G. Wilson & E.R. Roberts, Biochim.Biophys.Acta, 15, 568.

(51) T.G.G. Wilson & E.R. Roberts, Nature, 174, 795.

1955

(52) M.A. Alexander & P.W. Wilson, Proc.Natl,Acad.Sci.U.S., 41, 843.

(53) R.J. Block, E.L. Durrum & G. Zweig, "Paper Chromatography and Paper Electrophoresis", P.75, Academic Press, New York.

(54) Idem, P.250.

(55) M.A. Azim & E.R. Roberts, Biochim.Biophys.Acta, 18, 363.

(56) M. Grunberg-Manago & S. Ochoa, J.Amer.Chem.Soc., 77, 3165.

(57) H.M. Lenhoff & N.O. Kaplan, J.Biol.Chem., 220, 967.

(58) L.E. Mortenson, P.B. Hamilton & P.W. Wilson, Biochim.Biophys.Acta, 16, 238.

1955 (contd)

(59) M.M. Mozen & R.H. Burris, J.Bacteriol., 70, 127.

(60) I.A. Rose & S. Ochoa, J.Biol.Chem., 220, 307.

(61) R.A. Smith & I.C. Gunsalus, Nature, 175, 774.

(62) A. Tissieres & E.C. Slater, Nature, 176, 736.

(63) M.E. Winfield, Revs.Pure & Appl.Chem.Austral., 5, 217.

1956

(64) A.J. Anderson, in "Symposium on Inorganic Nitrogen Metabolism", P.3, John Hopkins University Press, Baltimore.

(65) R.H. Burris, Idem, P.316.

(66) H. Gest, J. Judis & H.D. Peck, Idem, P.299.

(67) A.L. Shug, P.B. Hamilton & P.W. Wilson, Idem, P.344.

(68) M.A. Azim & E.R. Roberts, Biochim.Biophys.Acta, 21, 562.

(69) M.A. Azim & S.D. Saraf, Biochim.Biophys.Acta, 21, 321.

(70) N.S. Bayliss, Austral.J.Biol.Sci., 9, 364.

(71) F. Bukatsch, K. Burger & M . Schlutter, Zentr.Bakt.Parasitenk.II, 109, 226.

(72) W.A. Bulen, Plant Physiol., 31, Suppl.xxix.

(73) R.G. Esposito & P.W. Wilson, Proc.Soc.Exptl.Biol.Med., 93, 564.

(74) R.G. Esposito & P.W. Wilson, Biochim.Biophys.Acta, 22, 186.

(75) R.L. Hinman, Anal.Chim.Acta, 15, 125.

(76) D.B. Johnstone & J.R. Fishbein, J.Gen.Microbiol., 14, 330.

(77) R.E. Keeler, W.A. Bulen & J.E. Varner, J.Bacteriol., 72, 394.

(78) A.C.I. Warner, Biochem.J., 64, 1.

1957

(79) R.M. Allison & R.H. Burris, J.Biol.Chem., 224, 351.

(80) M.A. Azim & F. Mohyuddin, J.Ind.Chem.Soc., 34, 344.

(81) M.A. Azim & S.D. Saraf, J.Ind.Chem.Soc., 34, 551.

(82) M.A. Azim & M.Shafi, J.Ind.Chem.Soc., 34, 780.

(83) M.K. Bach, Biochim.Biophys.Acta, 26, 104.

(84) R. Ballentine in "Methods in Enzymology", 145, P.984, Academic Press, New York.

(85) W. Stepka, Idem, 79, P.504.

1957 (contd)

(86) D.P. Burma & R.H. Burris, J.Biol.Chem., 225, 287.

(87) D.P. Burma & R.H. Burris, J.Biol.Chem., 225, 723.

(88) W.A. Bulen & D.S. Frear, Arch.Biochem.Biophys., 66, 502.

(89) G.N. Cohen & J. Monod, Bacteriol.Revs., 21, 169.

(90) S. Hiai, T. Mori, S. Hino & T. Mori, J.Biochem.(Jap.)., 44, 839.

(91) R.F. Keeler & J.E. Varner, Arch.Biochem.Biophys., 70, 585.

(92) A.G. Marr & E.H. Cota-Robles, J.Bacteriol., 74, 79.

(93) M.M. Mozen & P.S. Lavik, U.S. Atomic Energy Comm., N.Y., 02055, 12.

(94) J.R. Norris & H.L. Jensen, Nature, 180, 1493.

(95) J.A. Rakestraw & E.R. Roberts, Biochim.Biophys.Acta, 24, 555.

(96) N.E. Saris & A.I. Virtanen, Acta Chem.Scand., 11, 1438, 40.

(97) D. Spencer, A. Takahashi & A. Nason, J.Bacteriol., 73, 553.

1958

(98) R.W. Bernlohr & G.C. Webster, Nature, 182, 531.

(99) R.W. Bernlohr & G.C. Webster, J.Bacteriol., 76, 233.

(100) W.A. Bulen & C.C. Doughty, Plant Physiol., 33, Suppl.xi.

(101) J.E. Carnahan & J.E. Castle, J.Bacteriol., 75, 121.

(102) L.B Carr, Exptl.Cell.Res., 15, 455.

(103) E.M. Cota-Robles, A.G. Marr & E.H. Nilson, J.Bacteriol., 75, 243.

(104) R.G. Esposito & P.W. Wilson, Proc.Natl.Acad.Sci.U.S., 44, 472.

(105) R. Hancock, Biochim.Biophys.Acta, 28, 402.

(106) S. Hino & P.W. Wilson, Proc.Natl.Acad.Sci.U.S., 44, 472.

(107) R.F. Keeler, L.B. Carr & J.E. Varner, Exptl.Cell Res., 15, 80.

(108) R.F. Keeler & J.E. Varner, Nature, 181, 127.

(109) E.C. Layne & A. Nason, J.Biol.Chem., 231, 889.

(110) P.A. Lemin, Ph.D. Thesis, London University.

(111) J. Mandelstam, Biochem.J., 69, 103, 110.

(112) A. Nason & H. Takahashi, Ann.Rev.Microbiol. 12, 203.

(113) R.M. Pengra & P.W. Wilson, J.Bacteriol., 75, 21.

(114) R. Repaske & J.J. Josten, J.Biol.Chem., 233, 466.

(115) A. Steyermark, B.E. McGee, E.A. Bass & R.R. Kaup, Anal.Chem., 30, 1561.

1958 (contd)

(116) A. Tissieres & J.D. Watson, Nature, 182, 778.

1959

(117) C. Bond, Symp.Soc.Exptl.Biol. XIII, P.59.

(118) E.R. Roberts, Idem, P.24.

(119) B.F. Folkes, Idem, P.126.

(120) M. Steiner, Idem, P.177.

(121) E.G. Bollard, Idem, P.304.

(122) G.C. Webster, Idem, P.330.

(123) F.J. Bergersen & P.W. Wilson, Proc.Natl.Acad.Sci.U.S., 45, 1641.

(124) M. Chakravorty, Trans.Bose Res.Institute, 23, 62.

(125) G.E. Connell, P. Lengyel & R.C. Warner, Biochim.Biophys.Acta, 31, 391.

(126) G. Delhumeau-Arrecillas & R.H. Burris, J.Bacteriol., 78, 740.

(127) A.A. Diamantis, Ph.D. Thesis, London University.

(128) S.C. Hartman & J.M. Buchanan, Ann.Rev.Biochem., 28, 365.

(129) H.G. Hovenkamp, Biochim.Biophys.Acta, 34, 485.

(130) E.J. Johnson & M.K. Johnson, J.Bacteriol., 78, 792.

(131) D.B. Johnstone, M. Pfeffer & G.C. Blanchard, Can.J.Microbiol., 5, 299.

(132) A.G. Jose & P.W. Wilson, Proc.Natl.Acad.Sci.U.S., 45, 692.

(133) H. Kornberg, Ann.Rev.Microbiol., 13, 49.

(134) R.L. Leston & F.L. Crane, Biochim.Biophys.Acta, 32, 492.

(135) H. Holzer, Ann.Rev.Biochem., 28, 171.

(136) P. Mitchell, Ann.Rev.Microbiol., 13, 407.

(137) F.E. Mumford, J.E. Carnahan & J.E. Castle, J.Bacteriol., 77, 86.

(138) N.P. Neumann & R.H. Burris, J.Biol.Chem., 234, 3286.

(139) R.M. Pengra & P.W. Wilson, Proc.Soc.Exptl.Biol.Med., 100, 436.

(140) D.C. Pratt & A.W. Frenkel, Plant Physiol., 34, 333.

(141) M.M. Proctor, Nature, 184, 1934.

(142) P. Reichard, IVth Intern.Congr.Biochem.(Vienna), Vol.VIII, P.119.

(143) E.R. Roberts, Proc.Fertiliser Society, 54.

(144) R.B. Roberts, K. McQuillen & I.Z. Roberts, Ann.Rev.Microbiol., 13.

1959 (contd)

(145) J.L. Simkin, Ann.Rev.Biochem., 28, 145.

(146) M. Schlutter & F. Bukatsch, Zentr.Bakteriol.Parasitenk. II, 112, 509.

(147) A. Rothenstein, Bacteriol.Revs., 23, 175.

(148) N. Suzuki, Sci.Repts.Tohoku Univ., Ser.4, 25, 111.

(149) D.W.S. Westlake & P.W. Wilson, Can.J.Microbiol., 5, 617.

1960

(150) D.I. Arnon, M. Losada, M. Nozaki & K. Tagawa, Biochem.J., 77, 23P.

(151) N. Bauer, Nature, 188, 471.

(152) N. Bauer & R.G. Mortimer, Biochim.Biophys.Acta, 40, 170.

(153) A. Beloff-Chain & F. Pocchiari, Ann.Rev.Biochem., 29, 295.

(154) F.J. Bergersen, Bacteriol.Revs., 24, 246.

(155) A.B. Blake, Ph.D. Thesis, London University.

(156) G. Bond, J.Exper.Bot., 11, 91.

(157) J.H. Bruemmer & A.P. Rinfret, Biochim.Biophys.Acta, 37, 154.

(158) G.L. Bullock, J.A. Bush & P.W. Wilson, Proc.Soc.Exptl.Biol.Med., 105, 26.

(159) J.E. Carnahan, L.E. Mortenson, H.F. Mower & J.E. Castle, Biochim.Biophys.Acta, 44, 520.

(160) M. Chakravorty, Nature, 187, 239.

(161) H.N. Christiansen, Advances Prot.Chem., 15, 239.

(162) A.A. Diamantis & E.R. Roberts, Biochim.Biophys.Acta, 42, 76.

(163) T.C. Hoering & H.T. Ford, J.Amer.Chem.Soc., 82, 376.

(164) G.E. Hoch, K.C. Schneider & R.H. Burris, Biochim.Biophys.Acta, 37, 273.

(165) A.G. Marr, Ann.Rev.Microbiol., 14.

(166) D.J.D. Nicholas & D.J. Fisher, J.Sci.Food Agric., 11, 603.

(167) D.J.D. Nicholas, D.J. Fisher, W.J. Redmond & M.A. Wright, J.Gen.Microbiol., 22, 191.

(168) G.D. Novelli, Ann.Rev.Microbiol., 14, 65.

(169) C.A. Parker & P.B. Scutt, Biochim.Biophys.Acta, 38, 230.

(170) C.J. Perret, J.Gen.Microbiol., 22, 589.

(171) H.M. Reisenauer, Nature, 186, 375.

1960 (contd)

(172) K.C. Schneider, C. Bradbeer, R.N. Singh, Li Chuan Wang, P.W. Wilson & R.H. Burris, Proc.Natl.Acad.Sci.U.S., 46, 726.

(173) A. Stevens & R.J. Hilmoe, J.Biol.Chem., 235, 3016, 3023.

(174) N. Suzuki, Sci.Repts.Tohoku Univ., Ser.4, 26, 167.

(175) S. Taniguchi & K. Omachi, J.Biochem.(Jap.)., 48, 50.

(176) A. Temperli, R.M. Pengra & P.W. Wilson, Biochim.Biophys.Acta, 38, 557.

(177) S. Udenfriend, H. Weissbach & C. Mitoma, Ann.Rev.Biochem., 29, 207.

(178) C.S. Yocum, Ann.Rev.Plant Physiol., 11, 25.

1961

(179) G. Bond, Zeitschr.f.Allg.Mikrobiol., 12, 93.

(180) T.D. Brock, Bacteriol.Revs., 25, 32.

(181) W.A. Bulen, J.Bacteriol., 82, 130.

(182) S.C. Chakravarti, Ind.J.Microbiol., 1, 143.

(183) R.E. Ecker & W.R. Lockhart, J.Bacteriol., 82, 511.

(184) C. Gilvarg, Ann.Rev.Biochem., 30, 239.

(185) E.J. Hewitt & G. Bond, Plant & Soil, 14, 159.

(186) A. Jakobsons, E.A. Zell & P.W. Wilson, Arch.Mikrobiol., 41, 1.

(187) E.J. Johnson & M.K. Johnson, Proc.Soc.Exptl.Biol.Med., 108, 728.

(188) J.E. Leveson, Ph.D. Thesis, London University.

(189) L. Marcus & A.G. Marr, J.Bacteriol., 82, 224.

(190) D.J. D. Nicholas, D.J. Silvester & J.F. Fowler, Nature, 189, 634.

(191) M.R.J. Salton, Bacteriol.Revs., 25, 77.

(192) W.D.P. Stewart & G. Bond, Plant & Soil, 14, 347.

(193) B. Zacharias & C.G.Heden, Nature, 190, 817.

1962

(194) C.A. Appleby, Biochim.Biophys.Acta, 60, 226.

(195) F. Aylward & M. Sawistowska, Chem. & Ind., 484.

(196) F.J. Bergersen, Nature, 194, 1059.

(197) J.H. Bruemmer, Biochim.Biophys.Res.Communs., 7, 53.

(198) M. Chakravorty & D.P. Burma, Biochim.Biophys.Acta, 55, 110, 120.

(199) Chemical & Engineering News, May 7, 43.

1962 (contd)

(200) P.H. Clarke & M.D. Lilly, Nature, 195, 516.

(201) M.J. Dilworth, Biochim.Biophys.Acta., 56, 127.

(202) F.H. Grau & P.W. Wilson, J.Bacteriol., 83, 490.

(203) P.Harris, Private Communication.

(204) R.K. Mandal, Biochim.Biophys.Acta, 55, 238.

(205) L.E. Mortenson, H.F. Mower & J.E. Carnahan, Bacteriol.Revs., 26, 42.

(206) A. Nason, Bacteriol.Revs., 26, 16.

(207) S.A. Robrish & A.G. Marr, J.Bacteriol., 83, 158.

(208) N.A. Sinclair & J.L. Stokes, J.Bacteriol., 83, 1147.

(209) M.J. Smith, Private Communication.

(210) M.C. Stewart, Private Communication.

(211) A. Temperli & P.W.Wilson, Nature, 193, 171.

(212) G. Zubay, Proc.Natl.Acad.Sci.U.S., 48, 894.